BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DRUGS AND METABOLITES BY LC-MS/MS WITH A SPECIAL EMPHASIS ON BIOEQUIVALENCE STUDY

Thesis Submitted By

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Preface

The present thesis work entitled ***"Bioanalytical Method Development and Validation of Drugs and Metabolites by LC-MS/MS with a Special Emphasis on Bioequivalence Study"*** deals with the bioanalytical study, i.e., method development, validation and the complete investigation of pharmacokinetic profile of some model drugs (Aspirin or Acetylsalicylic Acid, Enalapril and Nimesulide) with their active metabolites (Salicylic Acid, Enalaprilat and 4-Hydroxynimesulide) in healthy human subjects. It is required to develop and validate the bioanalytical methods to assess these pharmacokinetic profiles in human plasma by LC- MS/MS.

In recent times, bioanalysis of the active metabolites along with its parent or prodrug is very necessary to establish a complete pharmacokinetic profile for the drug development process. It has been understood from the literature survey that there are some limitations in the previous methods of above mentioned drugs and active metabolites and also the comprehensive clinical studies concerning its dose dependent therapeutic efficacy are required. The main objective of the research will be focusing on the development and validation of reliable, unique and high throughput LC-MS/MS methods and also to be applied these methods to evaluate pharmacokinetic as well as bioavailability or bioequivalence study with a concern to describing its therapeutic effects. The outcome of this study will be more advantageous for the drug development process and also will be of immense helpful for carrying out pharmacokinetic as well as bioequivalence studies of respective drugs with their active metabolites in such laboratories those having LC-MS/MS.

Aims of the present investigation to develop and validate simple and sensitive methods for simultaneous analysis of some model drugs and their active metabolites in plasma and finally establish their pharmacokinetic profile in healthy human subjects to correlate the therapeutic efficacy. The outcome of this thesis will be very much helpful to the researchers, clinicians and medical practitioners who are engaged in the treatment of patients. Further research in the field of pharmacology will indicate which molecule, the parent drug or its active metabolite is responsible for its therapeutic efficacy.

The introduction part of this thesis is Chapter – I where the main focus on to determine drugs and metabolites levels in biological fluids which were essentially required by employing various chromatographic techniques. Here also discussed different types of chromatography, specifically LC-MS/MS instruments and its components – different ion sources and mass analyzers, applications of LC-MS/MS, method development procedure, various parameters of method validation and its application to bioavailability and bioequivalence studies of drugs and active metabolites. In addition to this a brief discussion on the biological effects of drug molecules, metabolic modification and active form, significance of active metabolites in bioequivalence and present scenarios of bioanalysis,

regulation and regulatory guidances for bioanalysis of active metabolites and also its main challenges in pharmacokinetic assessment etc.

The literature survey is Chapter – II where discussed relevant published research works, i.e., described regarding all aspects of this research work and reviews in the area related to present thesis. This part also deals with available literature on background of chromatography with emphasis on LC-MS/MS methods, bioanalytical method development procedure and optimization, matrix effect, method validation, physicochemical characterization, absorption, drug distribution, significance of drug metabolism, elimination, analysis of pre-pharmacokinetic properties, evaluation of pharmacokinetics of drugs and active metabolites etc. Finally, a complete discussion on the physical properties, the chemical properties, synthesis, functions, pharmacology, i.e., indication, pharmacodynamics (mechanism of drug action), pharmacokinetics and toxicology of above mentioned drugs and active metabolites. Lastly bioanalytical assay methods are also given.

The main aims and objectives of the present thesis work describes in Chapter – III.

The methodology section, i.e., materials and methods segment (Chapter – IV) where the list of materials and instruments are described – primarily LC-ESI-MS/MS methods and its optimized parameters both chromatographic and mass spectrometric. Also it discusses the method development strategies, pre-validation studies, method validation procedure and parameters and application to bioequivalence study and evaluation of pharmacokinetic parameters.

The results and discussions part was presented in Chapter – V where all investigations and description of the work were expressed elaborately, i.e., method development, pre-validation study, method validation, application to bioequivalence study and evaluation of pharmacokinetic parameters in healthy human subjects for above mentioned drugs and active metabolites by dividing in three main sections, such as Section–A, Section–B and Section–C, respectively.

The summary and the conclusion of the present thesis work were described in Chapter – VI.

Next, future research scope is also demonstrated in the Chapter – VI.

An extensive list of bibliography, i.e., references cited in the text of the thesis gives in Chapter – VII.

Abbreviations

AAS	Atomic absorption spectroscopy
AC	Alternating current
ACE	Angiotensin-converting enzyme
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photoionization
AR	Analytical reagent
ASA	Acetylsalicylic acid
ATP	Adenosine triphosphate
BA	Bioavailability
BE	Bioequivalence
^{o}C	Degree Celsius
CC	Calibration concentration
CDSCO	Central Drugs Standard Control Organization
CEMA	Channel electron multiplier array
CFFAB	Continuous flow fast atom/ion bombardment
CPS	Counts per second
CSF	Cerebrospinal fluid
C18	Saturated carbon chain of 18 carbon atoms
CID	Collision-induced dissociation

CI	Chemical Ionization
CMD	Column management device
CN	Cyano
CNS	Central nervous system
CoA	Certificate of analysis
COX	Cyclooxygenase
CZE	Capillary zone electrophoresis
Da	Dalton
DC	Direct current
DCGI	Drugs Controller General of India
DIOS	Desorption/ionization on silicon
DLI	Direct Liquid Introduction
DLPME	Single drop-liquid phase micro extraction
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra acetic acid
EI	Electron ionization
EM	Electron multiplier
EMA	European Medicines Agency
EPL	Enalapril
EPLT	Enalaprilat
ESI	Electrospray ionization
eV	electro volt or electronvolt
FAB	Fast atom/ion bombardment
FIA	Flow injection analysis
FT-ICR	Fourier transform-ion cyclotron resonance

GC	Gas chromatography
GCMS	Gas chromatography mass spectrometry
GFP	Green fluorescent protein
GI	Gastrointestinal
GLP	Good Laboratory Practice
HILIC	Hydrophilic-interaction liquid chromatography
HPLC	High performance liquid chromatography
HQC	High quality control
ICH	International Conference on Harmonisation
IEC	Institutional Ethics Committee
IIEC	Institutional Independent Ethics Committee
ID	Internal diameter
IS	Internal standard
ISO	International Standard Organisation
IUPAC	International Union of Pure and Applied Chemistry
IV	Intravenous
kV	Kilovolt
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LPME	Liquid phase microextraction
LQC	Low quality control
MALDI	Matrix-assisted laser desorption ionization
ME	Matrix effect
MPa	Megapascal

MQC	Medium quality control
MRM	Multiple reaction monitoring
mM	millimolar
MS	Mass spectrometry
ms	millisecond
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
NDA	New drug application
ng	nanogram
NP	Normal phase
NSAID	Non-steroidal anti-inflammatory drug
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate-buffered saline
pg	picogram
PK	Pharmacokinetics
PP	Protein precipitation
ppb	Parts per billion
ppm	Parts per million
PSI	Pounds per Square Inch (pressure)
Q	Quadrupole mass analyzer
QC	Quality control
QQQ	Triple quadrupole mass analyser
RAM	Restricted access materials
RF	Radio frequency
RP	Reversed phase
rpm	Revolutions per minute
RS	Reference standard
Rt	Retention time

SA	Salicylic acid
SFC	Supercritical fluid chromatography
SIM	Selected - ion monitoring
SIMS	Secondary ion mass spectrometry
SME	Supported membrane extraction
S/N	Signal-to-Noise ratio
SOP	Standard Operating Procedure
SPE	Solid phase extraction
SRM	Selected reaction monitoring
SST	System suitability test
TDM	Therapeutic drug monitoring
TEA	Triethylamine
TFC	Turbulent flow chromatography
TNT	Trinitrotoluene
TOF	Time-of-flight
ULOQ	Upper limit of quantification
USFDA	United States Food and Drug Administration
UV	Ultraviolet
V	Volt
V/V	Volume by volume
W/V	Weight by volume

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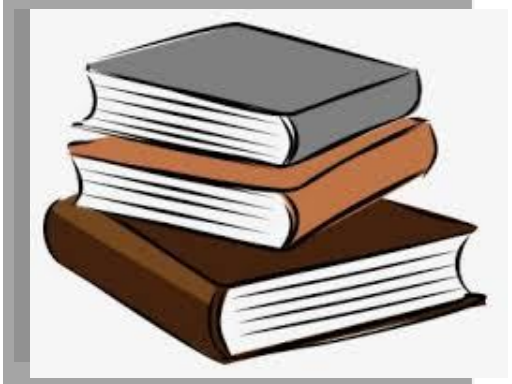
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Chapter – 1: Introduction



- Chromatography
- Method development
- Method validation
- Application of bio-analytical method
- Significance of active metabolites
- Significance of LC-MS/MS method
- Regulation and regulatory guidance's

Development and validation of a bioanalytical method is important to understand the pharmacokinetics of any drug and its *metabolites*. Bio-analytics is the application of the analytical techniques for the determination, quantification and characterization of drug or metabolite concentrations in biological matrix samples mostly in plasma, serum or urine samples related to Bioavailability (BA) and Bioequivalence (BE) studies. It plays a key role throughout the drug development from discovery to drug approval. The field of bio-analytics covers a wide cross-section of modern analytical techniques and advanced equipments. It is very important to decide whether we require to estimate only analyte(s) or metabolite(s) or both before starting method development (Snyder *et al.*, 1997; Lunn *et al.*, 1997 and Meyer 2004).

Many of these bioanalytical techniques and technologies can also be used to measure biologicals in biological fluid, which adds to the confusion. The distinctions between drug analysis for pharmacokinetics, forensic science, biomarkers, clinical chemistry, and therapeutic drug monitoring are gradually blurring. Cross-fertilization of ideas between these distinct ‘disciplines,' while also comprehending the scientific and regulatory forces behind them, is critical to removing the silo mentality; one of the key goals of this research is to catalyse this process. Once upon a time, an analytical approach was seen as an art form, and the ability to be the person who made it work was regarded as a rare skill, but that is no longer the case. Methods must be reliable and capable of being implemented by anyone with the necessary skills using highly automated systems. This research should help bioanalyst’s troubleshoot problems by encouraging and sponsoring discourse on "how to" and stimulating the creation of best practises at both the technical and regulatory levels. In order to generate fit for purpose criteria related to the technology and its intended use, it is critical to develop an understanding between chemistry-based sciences and biologically-based sciences (Campbell *et al*., 2011).

In the early days, as part of the new science of forensic medicine/toxicology, the initial motivation for measuring the presence of drugs in bodily fluids was to evaluate possible overdosing. The development of pharmacokinetics as a science in the 1930s fueled the need to detect drug levels in biological fluids even further. Many of the assays for drugs in biological fluids used in drug development were nonspecific and did not distinguish between the drug and its metabolites; for example, aspirin (discovered in 1889 and became available over-the-counter circa 1900) and colorimetric techniques were used to quantify sulphonamides (discovered in the 1930s), whilst antibiotics' capacity to inhibit bacterial

growth was used to quantify antibiotics. Pharmacokinetics, a driving force for more specific assays, rose to prominence in the 1930s.

As the identification of metabolites for pharmaceuticals in biological fluids accelerated, the lack of specificity became a serious problem. Because metabolites might be copyrighted as "new" medicinal substances, their development as standalone medications made identification a commercial concern. Understanding the influence of drugs metabolism on the body became essential for identifying the therapeutically active moiety as well as any potentially “toxic” metabolites. In the 1940s, chromatographic techniques like paper chromatography were developed, allowing for the separation of the drug from its metabolites. Thin-layer chromatography was developed later in the 1950s and used to measure drugs in biological fluids, however its primary application was the separation of radiolabeled metabolites. Unfortunately, the sensitivity of these technologies was insufficient to detect new compounds introduced in the 1950s, such as “tricyclies” which had levels in the ng/mL range. Since the 1970s, high-performance liquid chromatography (HPLC) has been the preferred laboratory instrument for separating, analysing and purifying organic chemical mixtures.

There are several chromatography instrument can be used for the bioanalysis were HPLC (high performance liquid chromatography) and in biological matrixes, liquid chromatography mass spectrometry (LC-MS), liquid chromatography tandem mass spectrometry (LC-MS/MS), and gas chromatography mass spectrometry (GC-MS) are used. Each instrument has its own set of advantages and disadvantages. Many compounds can be estimated using HPLC plus a UV, PDA, or fluorescence detector, but it lacks the high sensitivity and selectivity necessary by some of the potent, low-dose drugs. Because of its selectivity and specificity, LC-MS/MS has been chosen over HPLC and GC for experiments that require less time to perform. Shorter run durations, in turn, aid in high throughput sample analysis, which is the need for accelerating the development of molecules through clinical phases. Low detection limits, the capacity to obtain structural information, the need for minimal sample preparation, and the ability to cover a large range of analytes with different polarity are all advantages of LC-MS/MS.

The technology of liquid chromatography-mass spectrometry (LC-MS/MS) combines liquid chromatography (or HPLC) with mass spectrometry. LC-MS/MS is a method for qualitative and quantitative analysis of pharmacological substances, drug products and biological

samples that is extensively used in laboratories. Various extraction procedures, such as liquid–liquid extraction, solid phase extraction, and protein precipitation, are used in sample preparation and LC-MS/MS detection. All techniques that demonstrate that a method used for quantitative measurement of analytes in a biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use are included in bioanalytical method validation (Murugan *et al*., 2013).

So, now for quantitative bioanalysis of medicines and active metabolites in bio fluids such as plasma and serum to assist clinical development and bioequivalence of new chemical entities and generic medications, LC-MS/MS has become the technology of choice for research laboratories and the pharmaceutical industry. High-throughput bioanalysis has been made possible by the introduction of automated sample preparation procedures such as solid phase extraction, liquid-liquid extraction, and protein precipitation. For the successful execution of preclinical, biopharmaceutics, and clinical pharmacology investigations, selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are essential. All techniques that demonstrate the usefulness of a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, in a reliable and reproducible manner for the intended use are included in bioanalytical method validation. The following are the basic parameters for this validation : (a) accuracy, (b) precision, (c) selectivity, (d) sensitivity, (e) reproducibility, (f) matrix effect, and (g) stability.

Consequently for need of the work, here I want to emphasize some important points regarding drugs and its metabolites –

- Some drugs are chemically altered by the body (metabolized) when the drugs pass through the liver, which is the primary site for drug metabolism. The molecules produced by metabolism (metabolites) may be inactive, or they may have therapeutic action or toxicity that is comparable to or distinct from the original drug.

- The drugs are converted into metabolite and reaches into plasma. After reaching into plasma the drugs and its metabolite shows therapeutic activity. The therapeutic activity of the drug and its metabolite depends on the bioavailability of the drug metabolite that can be depicted by using dug concentration time profile.

- The analysis of the drug into plasma concentration is an important tool to get a dug concentration time profile plot which will give us different important pharmacokinetic parameters like Cmax, Tmax, AUCo-t, AUCo-infinity.

- To understand the biological action of the drug and its metabolite the importance of the pk parameters are beyond any doubt.

- The pk parameters are important tool for health profession to understand therapeutic drug monitoring. In addition the pharmacokinetic parameters are also very useful for various health profession including clinical pharmacologist, toxicologist and pharmacist.

- The bioanalytical method development and its successful application to analyses the drug metabolite is a difficult task which needs an elaborate critical research operations where human volunteers are involved with necessary permission of Ethical Committee.

- The metabolites of the drugs which I decided to analyses into human plasma are not only costly but also these are not easily available in the market. The analysis of the drug into plasma is also a cumbersome process which requires the sophisticated instruments like LC-MS/MS.

- The execution of the entire research work is somehow impossible for small companies and hospitals present in developing country like India.

- As a result successful completion of my project will be hugely beneficial for millions of health profession. The outcome of the project will have positive impact in our society.

1.1 Chromatography

Chromatography is an analytical technique used for the separation of complex chemical or compound mixtures into individual components. Chromatography (in Greek: "*chroma"* means color and "*graphein"* means to write) is a phrase that refers to a group of laboratory techniques for separating mixtures. It includes passing a mixture dissolved in a "mobile phase" through a "stationary phase," which, based on differential partitioning between the mobile and stationary phases, separates the analyte(s) to be measured from other molecules in the mixture. Differences in the partition coefficient of the compounds cause unequal retention

on the stationary phase and, as a result, separation. Chromatography can be used for either preparative or analytical purposes. The aim of preparative chromatography is to separate the components of a mixture such that they can be used separately (and is thus a form of purification). Analytical chromatography is used to measure the relative quantities of analytes in a mixture and is usually done with smaller volumes of material and also the two are not mutually exclusive.

1.1.1 Types of Chromatography

We can divide chromatography into liquid chromatography (LC) and gas chromatography (GC) depending on whether the mobile phase is a liquid or a gas (GC). Apart from these approaches, there are two more that employ a liquid mobile phase but move it through the porous stationary phase using (a) capillary forces, as in planar chromatography (also known as thin-layer chromatography, TLC), or (b) electroosmotic flow, as in capillary electro chromatography (CEC).

The nature of the stationary phase determines the following classification step. It could be a liquid or a solid in gas chromatography; as a result, we distinguish gas-liquid chromatography (long capillary coated with a thin film of relatively viscous liquid or liquid-like polymer; in older systems, liquid-coated porous particles were used) from gas-solid chromatography (long capillary coated with a thin film of relatively viscous liquid or liquid-like polymer; in older systems, liquid-coated porous particles were used) and (capillary with thin porous layer on the walls or packed columns with porous particles). In Suprcritical Fluid Chromatography (SFC), a supercritical fluid (usually carbon dioxide) transports the sample through a separating column, where the mixture is split into distinct bands based on the amount of interaction between the various analytes and the stationary phase in the column. The identities and amounts of these bands are determined by a detector when they leave the column (O'Leary. 1997).

The earliest type of HPLC developed by chemists was normal phase chromatography (NP), which separates analytes depending on polarity. Because it has a polar stationary phase and a non-polar mobile phase, NP performs well with moderately polar analytes. Reversed phase HPLC (RP) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. Silica that has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}, is a common stationary phase. Non-polar molecules have a longer retention time with these stationary phases, whereas polar molecules elute more quickly.

Ion-exchange chromatography is the third mode of liquid chromatography, and it is based on the analyte's ionic interactions with the stationary phase (IEX). The difference in affinity of the ionic analyte for the counter ions on the stationary phase surface is the basis for separation in this technique. Size-exclusion chromatography (SEC) is a specific and essentially stand-alone mode of liquid chromatography that is connected with the absence or suppression of any analyte interactions with the stationary phase. In SEC, the eluent is chosen so that any possible analyte interactions with the surface are suppressed, and the separation of the analyte molecules is primarily based on their physical dimensions (size). The greater the size of the analyte molecules, the less likely they are to penetrate the porous region of the column packing material, and hence the faster they will pass through the column.

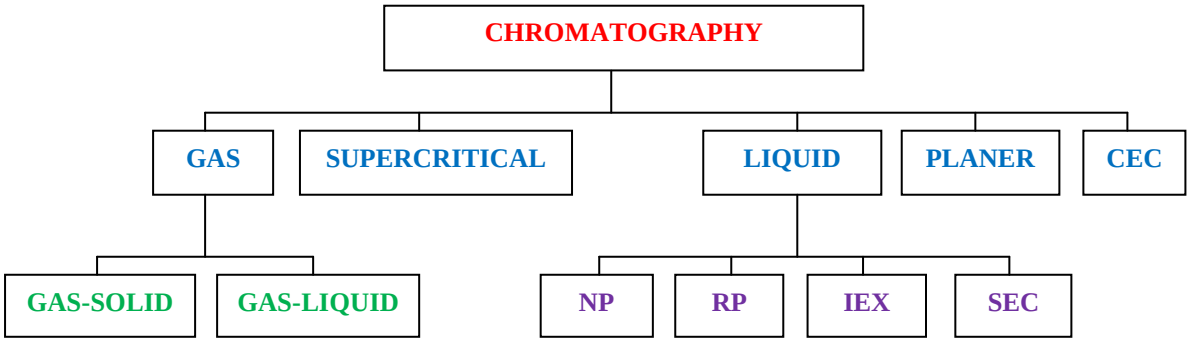


Figure.1.1 Types of Chromatography

1.1.2 Liquid chromatography mass spectrometry (LC-MS)

In the last two decades, mass spectrometry (MS) has made its way into the research and clinical laboratories, where it is currently employed for a variety of purposes. In combination with either gas chromatography (GC) or liquid chromatography, the approach can be regarded crucial for the measurement of several therapeutically relevant analytes (LC). Research and clinical laboratories around the world realise the power of MS, especially when combined with LC, and the expanding adaptability of these systems puts clinical laboratories in a position to respond quickly to changing clinical needs. In the laboratory, rather than waiting for a manufacturer to reply, much-needed assays can be produced, even though with some effort. Furthermore, it is undeniable that, in many circumstances, these procedures give a better level of sensitivity and specificity than conventional analytical techniques, and that their adoption has benefitted patient care. Apart from specificity and sensitivity, the capacity

of these techniques to detect many analytes at the same time is a huge advantage of LC-MS approaches, as many other techniques can only determine one analyte at a time. When it comes to the normal use of MS in clinical laboratories, the scope of testing has increased and is no longer centred on toxicological purposes (Garg and Hammett-Stabler, 2010).

Because MS is more sensitive and specific than other chromatographic detectors, it has always been desirable to combine it with chromatographic techniques. Gas chromatography and mass spectrometry (GC-MS) were first coupled in the 1950s, with commercial instruments becoming available in the 1970s. Many clinical biochemistry laboratories now have GC-MS systems, which are essential in several areas where complex mixtures must be analysed and unambiguous identification is required, such as screening urine samples for inborn metabolic errors or drugs. The combination of MS and LC (LC-MS) was an obvious step forward, but progress was hampered for many years due to the relative incompatibility of conventional MS ion sources with a continuous liquid stream. Several interfaces were developed, but because they were difficult to use and unreliable, clinical laboratories were slow to adopt them. This situation altered in the 1980s when John Bennett Fenn developed the electrospray ion source (Fenn *et al.*, 1989). Instruments with electrospray sources were rapidly developed, which had a significant impact on protein and peptide biochemistry. In 2002, Fenn and Koichi Tanaka were jointly awarded the Nobel Prize for their work on matrix aided laser desorption ionisation, another incredibly valuable MS ionisation technology for the study of biological molecules. (http://nobelprize.org/nobel_prizes/chemistry/ laureates/2002).

Mass spectrometers work by charging (ionising) analyte molecules, then analysing the ions and any fragment ions formed during the ionisation process based on their mass-to-charge *(m/z)* ratio. For both ionisation and ion analysis, several different technologies are available, resulting in a variety of mass spectrometers with various combinations of these two processes. In practice, some configurations are significantly more adaptable than others, thus the descriptions below focus on the main types of ion sources and mass analysers that are likely to be employed in clinical or research laboratories utilising LC-MS systems (Pitt. 2009).

A single quadrupole mass analyzer (LC-MS) is made up of four rods that are connected by direct current (DC) and radio frequency (RF) voltages. Only when a precise DC/RF voltage combination is supplied will an ion with a specific mass-to-charge *(m/z)* ratio be stable and

pass through the quadrupole. As a result, quadrupoles are known as mass filters. There is only one mass filtering quadrupole in single quadrupole systems. It has two modes of operation: scanning and filtering. The DC and RF voltages are increased during a mass scan, resulting in the capture of full scan mass spectra. These kinds of spectra are commonly used in qualitative data analysis. Scanning a quadrupole, on the other hand, has a low sensitivity and a slow scan speed. Quantitative research are thus carried out with quadrupoles in filtering mode. (Figure.1.2). Selected Ion Monitoring (SIM) is the most selective mode when using a single quadrupole MS. A fixed set of DC and RF voltages is delivered to the quadrupole in this way, allowing just one m/z to pass through. Different *m/z* ions are filtered out (Schreiber. 2010).

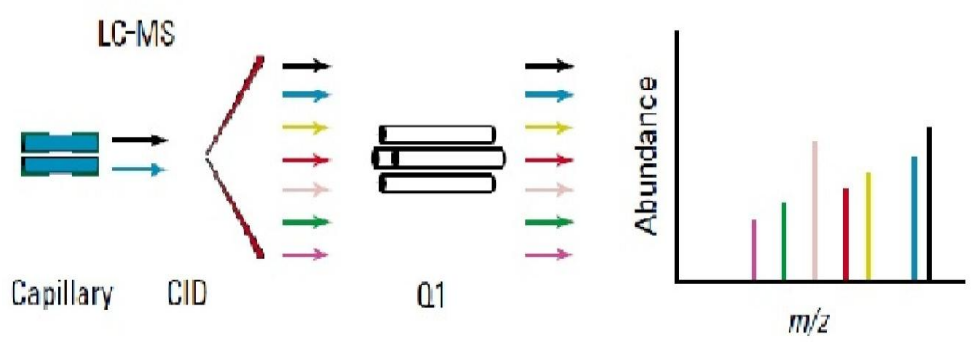


Figure.1.2 In-source collision-induced dissociation (CID) with a single quadrupole mass analyzer

1.1.2.1 Instrumentation

Ionizing molecules and sorting and identifying ions based on their mass-to-charge *(m/z)* ratios are how mass spectrometers function. The ion source, which generates the ions, and the mass analyzer, which sorts the ions, are two important components in this process. For LC/MS, several distinct types of ion sources are routinely utilised. Each is appropriate for different classes of compounds. Mass analyzers of various types are also used. Depending on the sort of information required, each offers benefits and drawbacks.

1.1.2.1.1 Ion Sources

The development of ion sources and procedures to ionise analyte molecules and separate the resultant ions from the mobile phase has accounted for much of the advancement in LC-MS over the previous two decades. Previously, LC/MS systems used interfaces that either did not separate the mobile phase molecules from the analyte molecules (direct liquid inlet,

thermospray) or did so before ionisation (particle beam). The analyte molecules were then ionised in a mass spectrometer under vacuum, most commonly by electron ionisation. Only a small number of compounds were successful using these methods.

The development of atmosheric pressure ionisation (API) techniques considerably increased the number of compounds that may be examined satisfactorily by LC/MS. The analyte molecules are initially ionised at atmospheric pressure in atmospheric pressure ionisation. The analyte ions are subsequently separated from neutral molecules mechanically and electrostatically. The following are some of the most common atmospheric pressure ionisation techniques :

- *Electrospray ionization (ESI)*
- *Atmospheric pressure chemical ionization (APCI)*
- *Atmospheric pressure photoionization (APPI)*

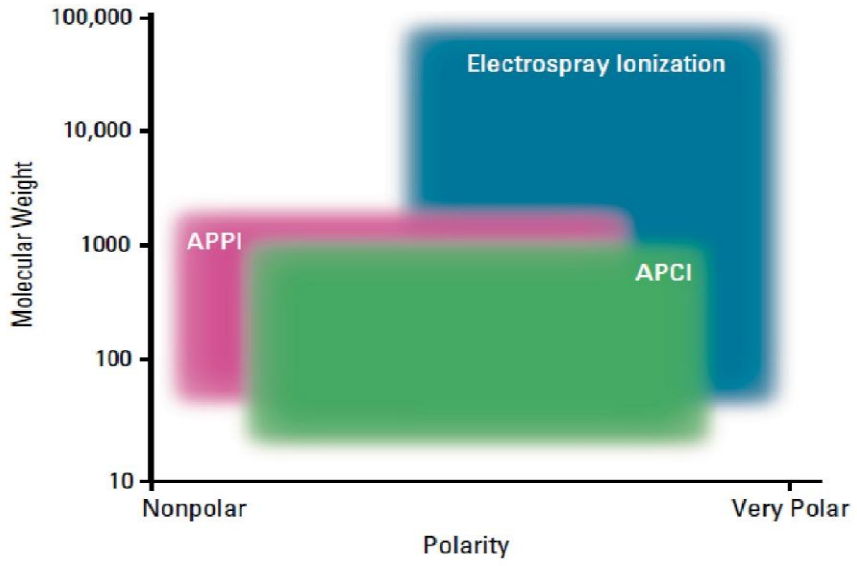


Figure.1.3 Applications of various LC-MS ionization techniques

1.1.2.1.1.1 Electrospray ionization (ESI)

Before the analyte reaches the mass spectrometer, electrospray relies on chemistry to produce analyte ions in solution. At atmospheric pressure, the LC eluent is sprayed (nebulized) into a chamber in the presence of a strong electrostatic field and heated drying gas. The analyte

molecules are further dissociated by the electrostatic field. The solvent in the droplets evaporates due to the heated drying gas. The charge concentration in the droplets increases as the droplets shrink. The repulsive force between ions with similar charges eventually overcomes the cohesive forces, causing ions to be ejected (desorbed) into the gas phase (see Figure.1.4). These ions are attracted to a capillary sampling orifice and travel through it into the mass analyzer. Between the time ions are discharged from the droplets and the time they reach the mass analyzer, several gas-phase processes, namely proton transfer and charge exchange, can occur.

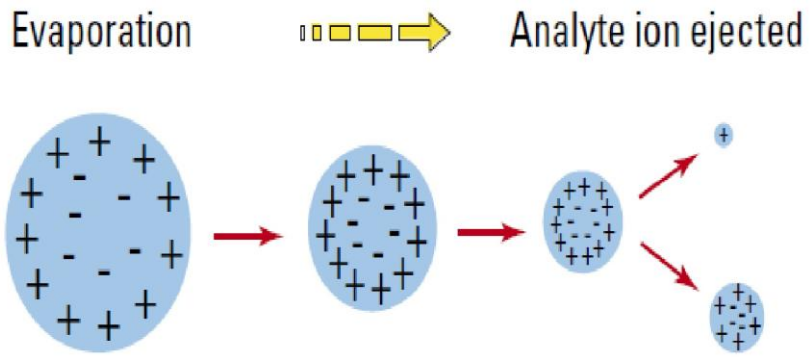


Figure.1.4 Desorption of ions from solution

Electrospray is best for analyzing large biomolecules like proteins, peptides, and oligonucleotides, but it may also be used to analyze smaller compounds like benzodiazepines and sulphated conjugates. Multiple charges are frequently acquired by large molecules. Even though the mass range (or, more precisely, mass-to-charge range) for a standard LC/MS equipment is roughly 3000 *m/z*, electrospray can be utilised to examine molecules as large as 150,000 u due to this multiple charging. For instance, 100,000 u/10 z = 1,000 m/z. When a big molecule accumulates multiple charges, a mathematical procedure known as deconvolution is frequently employed to calculate the analyte's real molecular weight (Figure.1.4).

Singly charged ions are produced by small molecules (less than 500 Da) containing a single functional group capable of transporting electrical charge. When the ion source is in positive ion mode, a proton is added to the analyte ($M+H^+$), and when it is in negative ion mode, a proton is lost ($M-H^-$). When salts are present, adduction of cations (e.g. $M+NH_4^+$, $M+Na^+$, $M+K^+$) and anions (e.g. M+formate-, M+acetate-) can occur. Multiple charging can occur in larger molecules and molecules with multiple charge-carrying functional groups, such as

proteins and peptides, resulting in ions such as $M+2H^+$, $M+3H^+$, and so on. This results in an envelope of ions with various charge states for proteins. On mass spectrometers that scan up to 4000 *m/z*, this feature can be used to correctly detect analytes with high molecular weights, such as proteins up to 100 kDa. It is unusual to find ions with *m/z* values greater than this. While ESI is the most extensively utilised ion source for biological molecules, it may not be effective in ionising neutral and low polarity molecules like lipids.

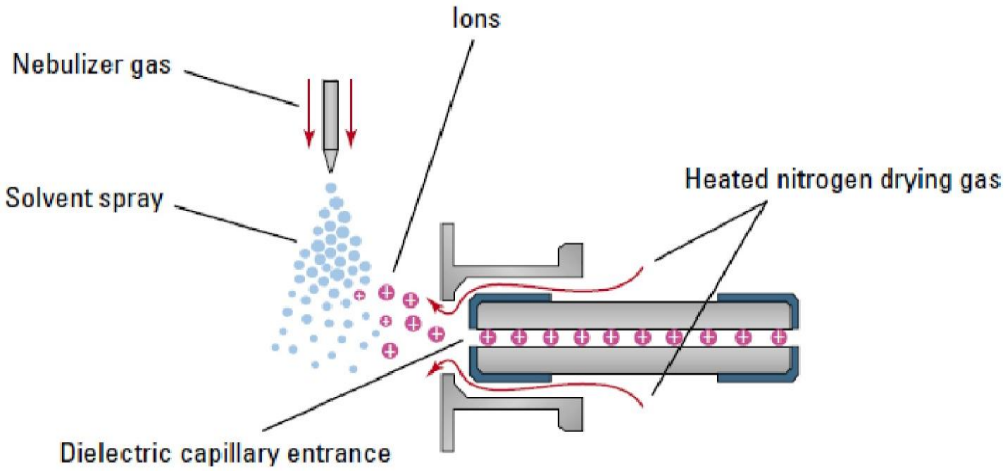


Figure.1.5 Electrospray ion (ESI) source

1.1.2.1.1.2 Atmospheric pressure chemical ionization (APCI)

The LC eluent is sprayed through a heated (usually 250°C – 400°C) vaporizer at atmospheric pressure in atmospheric pressure chemical ionisation (APCI). The heat causes the liquid to evaporate. Electrons emitted from a corona needle ionise the gas-phase solvent molecules. Chemical processes then allow the solvent ions to transfer charge to the analyte molecules (chemical ionization). The analyte ions enter the mass analyzer through a capillary sampling orifice.

A wide range of polar and nonpolar compounds can be analysed with APCI. It is primarily utilised for molecules less than 1,500 u because it rarely results in multiple charging. APCI is less well-suited than electrospray for the investigation of large biomolecules that may be thermally unstable because of this, and because it involves high temperatures. Because the analytes are usually nonpolar, APCI is employed more frequently with normal-phase chromatography than electrospray.

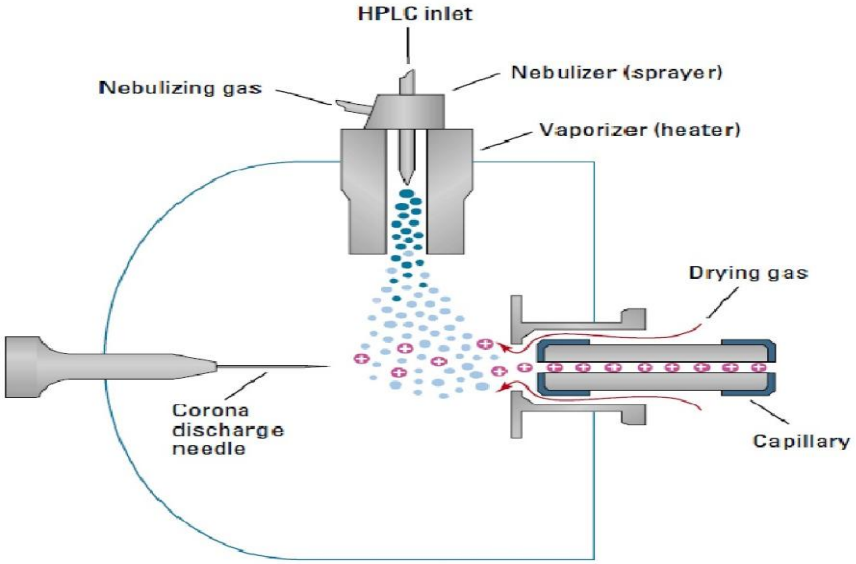


Figure.1.6 Atmospheric pressure chemical ionization (APCI) ion source

1.1.2.1.1.3 Atmospheric pressure photoionization (APPI)

The technique of atmospheric pressure photoionization (APPI) for LC-MS is relatively new. A vaporizer, like in APCI, transfers the LC eluent to the gas phase. Photons with a narrow range of ionisation energies are generated by a discharge lamp. The energy range has been carefully selected to ionise as many analyte molecules as feasible while reducing ionisation of solvent molecules. The ions are then injected into the mass analyzer through a capillary sampling orifice.

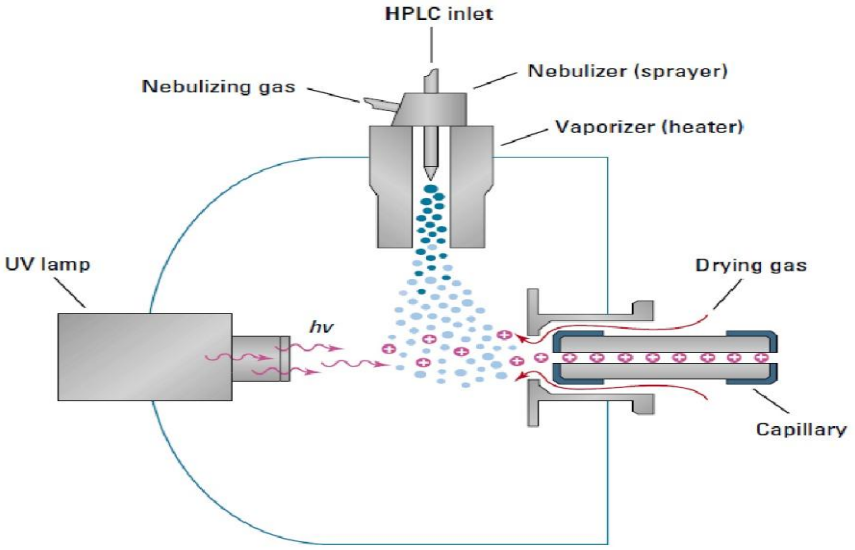


Figure.1.7 Atmospheric pressure photoionization (APPI) ion source

Many of the same compounds that APCI analyses are also applicable to APPI. It has special potential in two applications: highly nonpolar compounds and low flow rates (less than 100 µL/min), where APCI sensitivity can be lowered.

Nanoelectrospray ionisation (nanoESI), matrix-assisted laser desorption ionisation (MALDI), desorption/ionization on silicon (DIOS), fast atom/ion bombardment (FAB), electron ionization (EI), and chemical ionization (CI) are some of the other types of ion sources.

Table.1.1 Summary of event according to ionization sources

Ionization Source	Event
Electrosray ionization (ESI)	Evaporation of charged droplets
Atmospheric pressure chemical ionization (APCI)	Corona discharge and proton transfer
Atmospheric pressure photoionization (APPI)	Discharge lamp generates photons in a narrow range of ionization energies
Nanoelectrospray ionization (nanoESI)	Evaporation of charged droplets
Matrix-assisted laser desorption ionization (MALDI)	Photon absorption/proton transfer
Desorption/ionization on silicon (DIOS)	Photon absorption/proton transfer
Fast atom/ion bormbardment (FAB)	Ion desorption/proton transfer
Electron ionization (EI)	Electron beam/electron transfer
Chemical Ionization (CI)	Proton transfer

Ion losses occur between major components of a mass spectrometer and occur between the source and the analyzer, within the analyzer, and between the analyzer and the detector. Atmospheric pressure ion sources, such as those used in ESI, APCI, APPI, and other systems, present a unique difficulty in terms of effective ion transfer from the ion source to the *m/z* analyzer. Ionization techniques have been crucial in identifying what types of samples can be analyzed by MS (see Table.1.1). For gases and vapours, EI and chemical ionization (CI) are

used (Kang. 2012). ESI and MALDI are two techniques that are frequently used with liquid and solid biological samples, and they have obviously evolved to be the methods of choice when it comes to biomolecular analysis. While the concept of ESI is not new, its application to biomolecules has given it fresh life (Chiu and Muddiman. 2008).

So, in all circumstances, the nature of the analyte(s) and separation conditions have a significant impact on whether ionisation approach will produce the best results : electrospray, APCI, or APPI. It's not always straightforward to determine which strategy will be the most effective.

1.1.2.1.2 Mass Analyzers

Although any sort of mass analyzer might theoretically be used for LC-MS, there are seven different types :

- Sector
- Quadrupole
- Time-of-flight
- Ion trap
- Orbitrap
- Hybrid analysers
- Fourier transform-ion cyclotron resonance (FT-ICR or FT-MS)

are the most frequently used. Depending on the requirements of a specific analysis, each offers advantages and disadvantages.

1.1.2.1.2.1 Sector

The route and/or velocity of charged particles are affected in some way by an electric and/or magnetic field in a sector field mass analyzer. According to their *m/z* ratios, the sector instruments bend the trajectories of the ions as they travel through the mass analyzer, deflecting the more charged and faster-moving, lighter ions more. The analyzer can be used to narrow down a *m/z* range or to sweep through a range to catalogue the ions present (Paul and Steinwedel. 1953; Kang. 2012).

1.1.2.1.2.2 Quadrupole

Four parallel rods are positioned in a square in a quadrupole mass analyser (Figure.1.8). The ions of the analyte are guided down the square's center. Electromagnetic fields are created when voltages are applied to the rods. These fields control which ion mass-to-charge (*m/z*) ratios can flow through the filter at any particular time. Quadrupole mass analyzers are the simplest and least expensive.

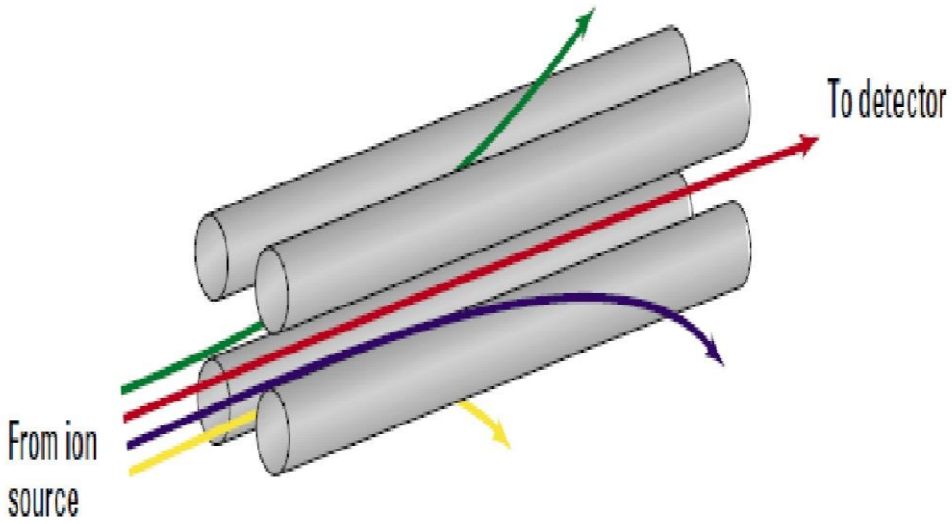


Figure.1.8 Quadrupole mass analyzer

Quadrupole mass analyzers have two modes of operation.:

- Scanning (scan) mode
- Selected ion monitoring (SIM) mode

The mass analyzer analyses a number of different mass-to-charge (*m/z*) ratios in scan mode. Only a few mass-to-charge (*m/z*) ratios are monitored in SIM mode. SIM mode is much more sensitive than scan mode, however it only delivers information on a small number of ions. When all analyte masses are unknown in advance, scan mode is often used for qualitative analyses or quantification. For the quantification and monitoring of target molecules, SIM mode is used (Figure.1.9).

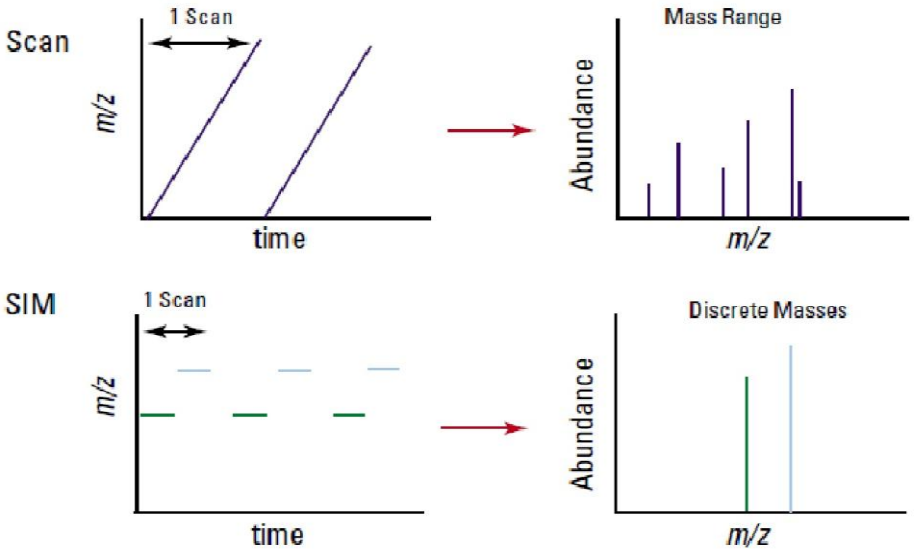


Figure.1.9 The quadrupole mass analyzer can scan over a range of mass-to-charge (*m/z*) ratios or alternate between just a few

1.1.2.1.2.3 Time-of-flight

A consistent electromagnetic force is delivered to all ions at the same time in a time-of-flight mass analyzer, forcing them to accelerate down a flight tube. The mass-to-charge *(m/z)* ratios of the ions are determined by their arrival times since lighter ions travel faster and arrive at the detector first. The time-of-flight mass analyzers have a wide mass range and can be extremely precise when measuring mass (Figure.1.10).

The time-of-flight (TOF) analyser operates by accelerating ions through a high voltage (Williamson and Bartlett. 2007). The *m/z* values of the ions determine their velocity and thus the time it takes for them to travel down a flight tube to reach the detector. The output of the detector as a function of time can be turned into a mass spectrum if the initial accelerating voltage is pulsed. The TOF analyser is capable of acquiring spectra at a high rate and with high sensitivity. It also has a high mass accuracy, allowing for the determination of molecular formulas for small molecules (Bristow. 2006).

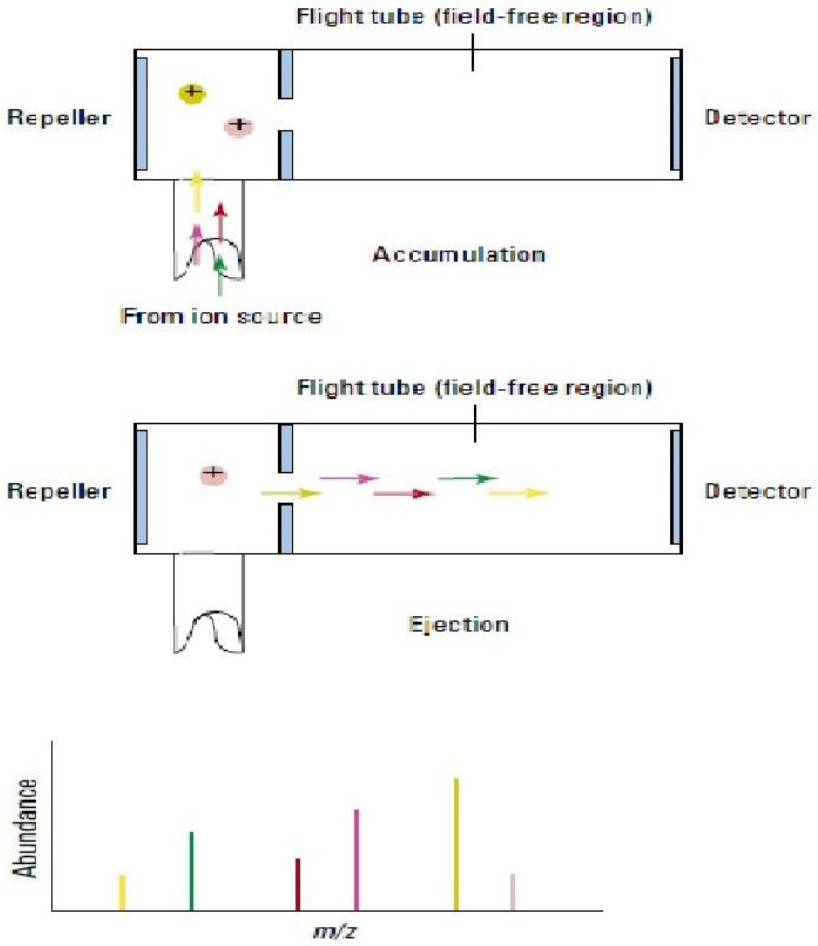


Figure.1.10 Time-of-flight mass analyzer

1.1.2.1.2.4 Ion trap

The chamber of an ion trap mass analyzer is made up of a circular ring electrode and two end caps. Electromagnetic fields "trap" the ions as they enter the chamber. To selectively eject ions from the trap, another field can be applied. Ion traps offer the advantage of allowing many stages of mass spectrometry to be performed without the use of additional mass analyzers (Figure.1.11).

Ion trap analysers use three hyperbolic electrodes to trap ions in a three-dimensional space using static and radio frequency voltages (Payne and Glish. 2005). Ions are subsequently released from the trap in order of their m/z values, resulting in a mass spectrum. Alternatively, an exciting voltage can be applied to isolate a specific ion in the trap while other ions are evacuated. To induce fragmentation, an inert gas can also be put into the trap. The capacity of these ion trap analysers to fragment and isolate ions several times in

succession before obtaining the final mass spectrum, resulting in so-called MS^n capabilities, is an interesting feature (Tozuka *et al*., 2003).

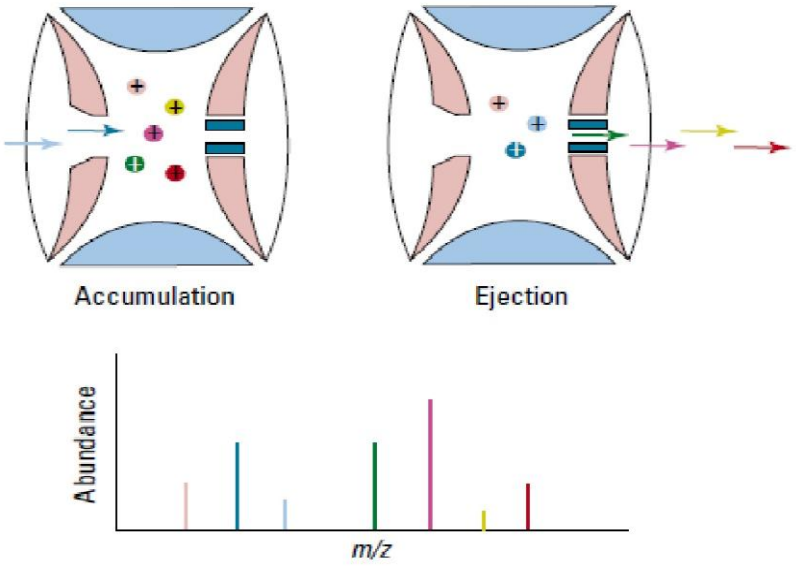


Figure.1.11 Ion trap mass analyzer

1.1.2.1.2.5 Orbitrap

The orbitrap mass spectrometer is the most recent advancement in *m/z* analyzer trapping devices. The orbitrap is a new mass analyzer (Hardman and Makarov. 2003; Hu *et al*., 2005; Makarov *et al*., 2006); However, the orbitrap can be thought of as a modified Knightstyle Kingdon trap with specifically shaped inner spindle-like (axial) and outer barrel-like (coaxial) electrodes that create an electrostatic field with a quadro-logarithmic potential distribution (Makarov. 2000; Makarov *et al*., 2006a; Hu *et al*., 2005). The frequency of these harmonic oscillations is inversely related to the square root of the mass-to-charge ratio (m/z or m/q) and is independent of ion velocity (Figure.1.12). The trap can be utilised as a mass analyzer by monitoring the ion oscillation in a way similar to that employed in Fourier transform ion cyclotron resonance (FTICR)-MS. Orbitraps have great mass precision (1–2 ppm), resolving power (up to 200,000), and dynamic range (around 5000) (Makarov *et al*., 2006a; Makarov *et al*., 2006b). Ions are electrostatically trapped in an orbit around a central, spindle-shaped electrode in a nonmagnetic FTMS that is very similar. The acquired image currents are transformed into mass spectra using a Fourier transformation. Orbitraps have a high mass accuracy, high sensitivity, and a good dynamic range, similar to Fourier transform ion cyclotron resonance mass spectrometers (Hu *et al*., 2005 and Kang. 2012).

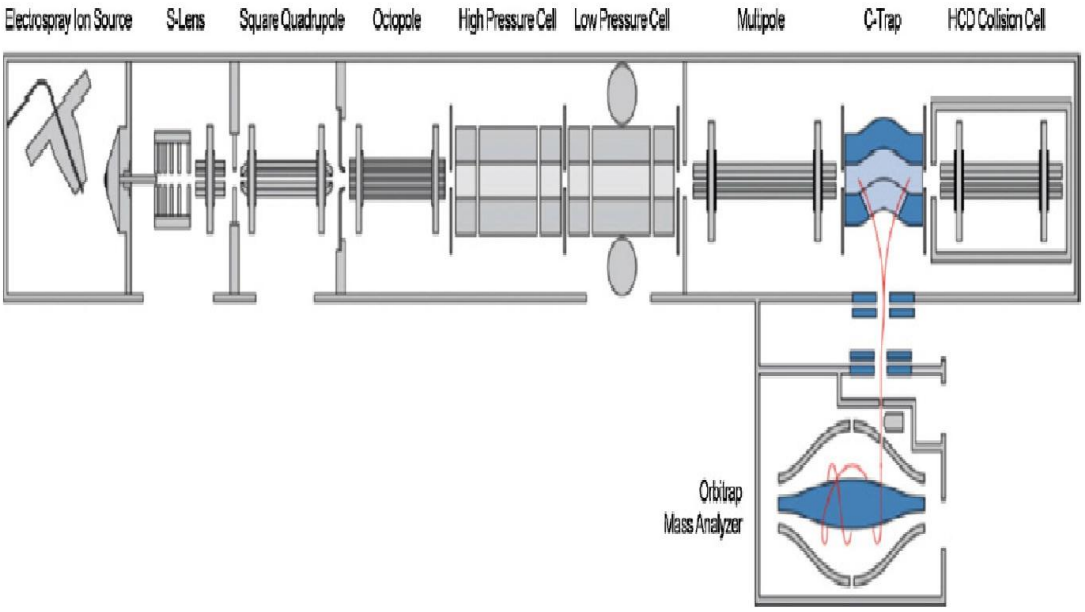


Figure.1.12 Schematic layout of the LTQ Orbitrap Velos mass spectrometer

1.1.2.1.2.6 Hybrid analysers

Tandem mass spectrometers have also been developed, which combine multiple mass analysers. Two configurations that are especially useful for LC-MS are described below. A TOF analyser can be used to replace the third quadrupole of a triple quadrupole MS to create a hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer (Chernushevich *et al*., 2001, Ens and Standing. 2005). Q-TOF instruments have been widely used in the proteomics area, but their scanning capabilities are limited compared to triple quadrupole instruments. It is also possible to design instruments with the third quadrupole of a triple quadrupole MS operating in a different mode, in which ions are trapped and then ejected sequentially based on their *m/z* values (Figure.1.13). This is referred to as a linear ion trap, and the entire setup is referred to as a Q-Trap instrument (Hopfgartner *et al*., 2004). The instrument's end quadrupole can be switched between ion trap and conventional quadrupole modes, giving it the advantages of both triple quadrupole and ion trap analysers. When employed in ion trap mode, product ion scanning sensitivity is significantly improved, and extra fragmentation can be triggered within the ion trap, allowing for an additional stage of fragmentation and mass analysis (MS^3).

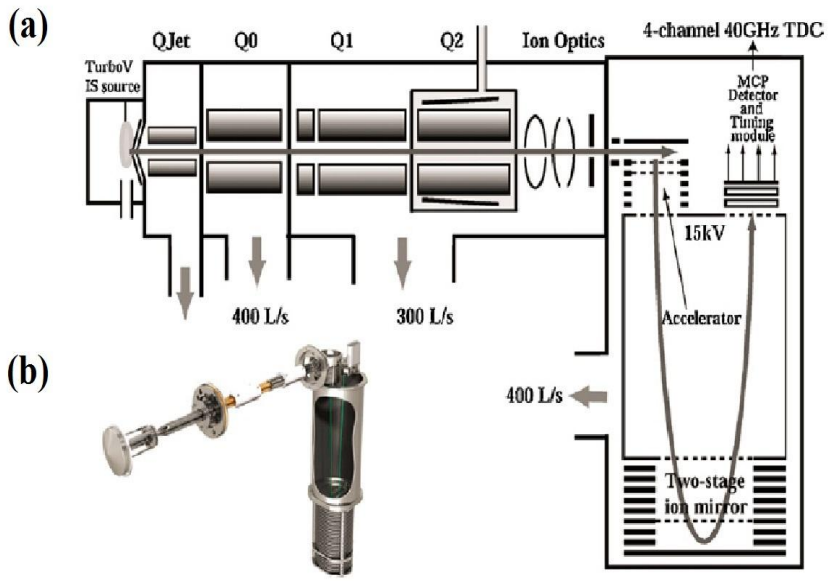


Figure.1.13 The Triple TOF MS technology features diagrammed (a) A detailed illustration of the major platform features (b) An image of the machined Triple TOF MS instrument platform

1.1.2.1.2.7 Fourier transform-ion cyclotron resonance (FT-ICR)

Another type of trapping analyzer is the fourier transform-ion cyclotron resonance (FT-ICR) mass analyzer (also known as FT-MS). Ions are confined in circular orbits by intense electrical and magnetic fields as they enter a chamber. The ions generate a time-dependent current when activated by a radio-frequency (RF) electrical field. The Fourier transform converts this current into ion orbital frequencies, which correspond to their mass-to-charge (*m/z*) ratios (Figure.1.14). FT-ICR mass analyzers, like ion traps, can perform many stages of mass spectrometry without the need for additional mass analyzers. They also have a wide mass range and high mass resolution. However, they are the most expensive mass analyzers.

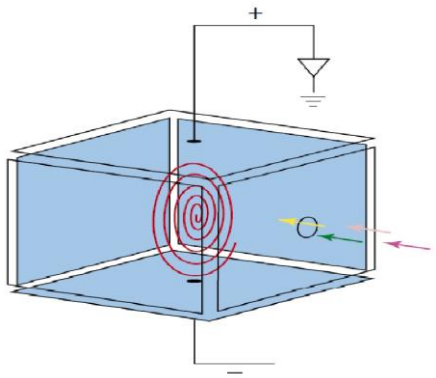


Figure.1.14 Fourier transform-ion cyclotron resonance (FT-ICR) mass analyzer

1.1.2.1.3 Collision-Induced Dissociation and Multiple-Stage MS

The strategies for atmospheric pressure ionisation that have been presented are all considered "soft" techniques. They mostly produce :

a. Molecular ions M^+ or M^-
b. Protonated molecules $[M + H]^+$
c. Simple adduct ions $[M + Na]^+$
d. Ions representing simple losses such as the loss of a water $[M + H – H2O]^+$

Although the resulting molecular weight information is extremely useful, further structural information is frequently required. Analyte ions are fragmented by colliding with neutral molecules to obtain structural information, a process known as collision-induced dissociation (CID) or collisionally activated dissociation (CAD). The analyte ions are given voltages to add energy to the collisions and increase fragmentation.

1.1.2.1.3.1 CID in single-stage MS

CID is most commonly linked with multistage mass spectrometers, where it occurs between each stage of MS filtering, but it can also occur in single-stage quadrupole or time-of-flight mass spectrometers. CID occurs in the ion source in single-stage mass spectrometers, and is thus referred to as source CID or in-source CID. Analyte (precursor) ions are accelerated and collide with residual neutral molecules, producing fragments called product ions. The simplicity and inexpensive cost of performing CID in singlestage instruments are two advantages. The drawback is that all of the ions are fragmented. Because there is no method to choose a specific precursor ion, it is impossible to know which product ions came from which precursor ion with certainty. The mass peaks from background ions or co-eluting compounds, as well as those from the analyte of interest, may appear in the resulting spectra. When analysing relatively pure samples, this determination may be satisfactory, but it may not produce good results if chromatographic peaks are not well resolved or background levels are high.

1.1.2.1.3.2 CID and multiple-stage MS

Multiple-stage MS (also known as tandem MS, MS/MS, or MS^n) is a highly effective method for obtaining structural data. The first quadrupole in triple-quadrupole or quadrupole/quadrupole/time-of-flight instruments is employed to select the precursor ion.

CID takes place in the collision cell, which is found in the second stage (quadrupole or octopole). The ability to use the first stage of MS to eliminate nonanalyte ions is a major advantage of multiple-stage MS. Cleaning the sample and chromatographic separation are no longer as important. When working with relatively pure samples, it's usual to skip chromatographic separation entirely and immediately inject samples into a mass spectrometer to acquire product mass spectra for characterization or confirmation.

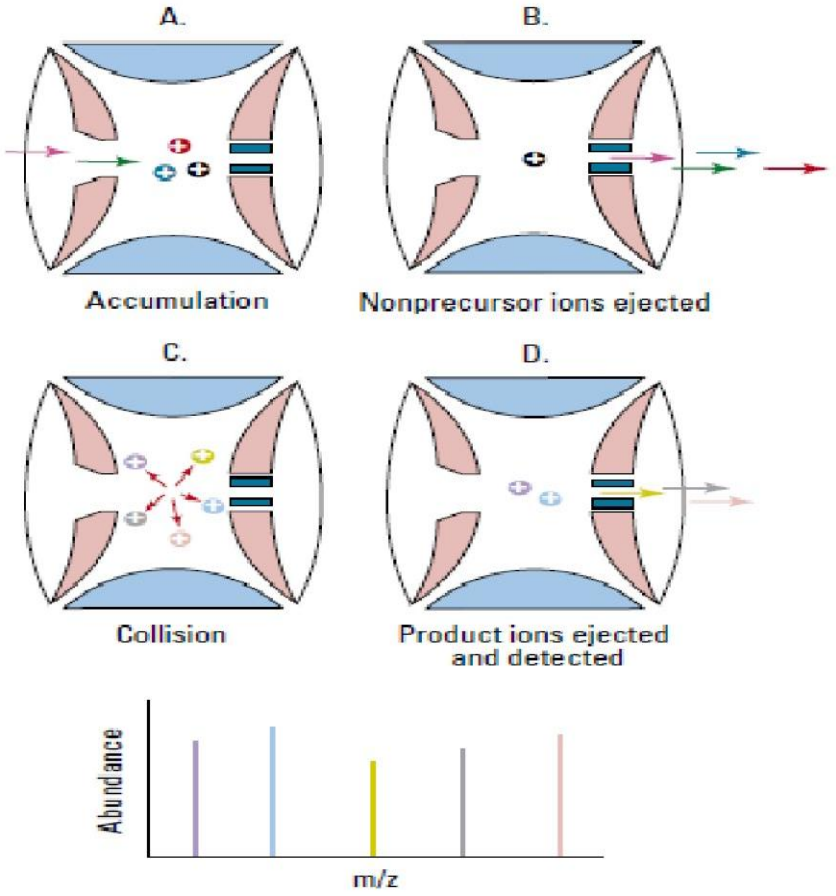


Figure.1.15 MS/MS in an ion trap mass spectrometer

After that, the third stage (quadrupole or TOF) generates a spectrum of the product ions. When quantitating target compounds, it can also perform selected ion monitoring of only a few product ions. All ions except the desired precursor ion are ejected from the trap in ion trap and FT-ICR mass spectrometers. To form product ions, the precursor ion is energised and collided. The product ions can be ejected to form a mass spectrum, or they can be kept and collided to produce another set of product ions. This process can be automated in stages, retaining and colliding the most abundant ions from each stage of MS (see Figure.1.15). For

determining the structure of molecules, this is a very powerful approach. It is also a great way to get peptide mass information that is related to the amino acid sequence in a peptide.

1.1.2.2 Applications

When liquid chromatography is an essential step in an analytical procedure, such as the determination of highly polar, chemically or thermally labile compounds, LC-MS is always the method of choice. It is also the ideal method when the detector has to be more sensitive, specific, or identify unknowns. The development of a method does not require any compromises for adaption to mass spectrometry, at least for some of the LC-MS procedures; disadvantages include increased instrumentation complexity and the necessity for internal standards in investigations requiring precise quantitative work.

LC-MS is suitable for many applications, from pharmaceutical development to environmental analysis (Linscheid And Westmoreland. 1994; Ju-Seop Kang. 2012). It is widely used in a variety of fields due to its ability to detect a wide range of compounds with high sensitivity and specificity. For example,

- in molecular weight and structural determination of different compounds
- in pharmacokinetics studies of drugs and their metabolites
- in proteomics and metabolomics studies
- in drug development
- in biochemical screening for genetic disorders
- in therapeutic drug monitoring and toxicology
- in vitamins and related metabolites studies
- in steroid hormones studies
- in forensic science etc.

1.1.3 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Since LC–MS/MS technology is widely used for quantification in clinical science and other sectors of science, there is a need for continual technological advances. In routine and high-throughput quantitative clinical analysis, a triple quadrupole instrument in SRM mode is the instrument of choice (Himmelsbach. 2012). Atmospheric pressure ionization (API) sources are frequently employed in commercial triple quadrupole MS today. Bennett identified the most generally sought enhancements for triple quadrupole instruments as better sensitivity,

dynamic linear range, mass resolution, wider mass range, faster acquisition cycle time, and lower cost of ownership (Bennett. 2011). Advances in triple quadrupole technology are challenging, and the focus is still on improving ruggedness and reducing matrix effects in the source and interface regions. Minor improvements in quadrupole manufacturing processes and RF power supply stability allowed the commercialization of a system with improved mass resolution and no substantial ion transmission losses (Yang *et al*., 2002 and Paul *et al*., 2003).

Basically, LC-MS/MS is a triple quadrupole (Q1, Q2 and Q3) mass spectrometer consists of a set of four parallel metal rods as mass analyser (see Figure.1.26). A narrow band of *m/z* values can be transmitted along the axis of the rods using a combination of constant and variable RF (radio frequency) voltages. A mass spectrum can be produced by adjusting the voltages over time and scanning across a range of *m/z* values. The majority of quadrupole analysers run at a frequency of 4000 *m/z*, with scan speeds of 1000 *m/z* per second or more being common. They usually work with a unit mass resolution, which means mass accuracy is rarely better than 0.1 *m/z*. As an alternative to scanning, the quadrupoles can be programmed to monitor a specific *m/z* value, then another, and so on. The voltages are stepped to do this. Because more detector time may be allocated to detecting specific ions rather than scanning through ions that are not produced by the analyte, this approach helps to improve the detection limits of targeted analytes. Stepping takes only a few milliseconds, and a panel of *m/z* values can be stepped through for the detection of multiple analytes.

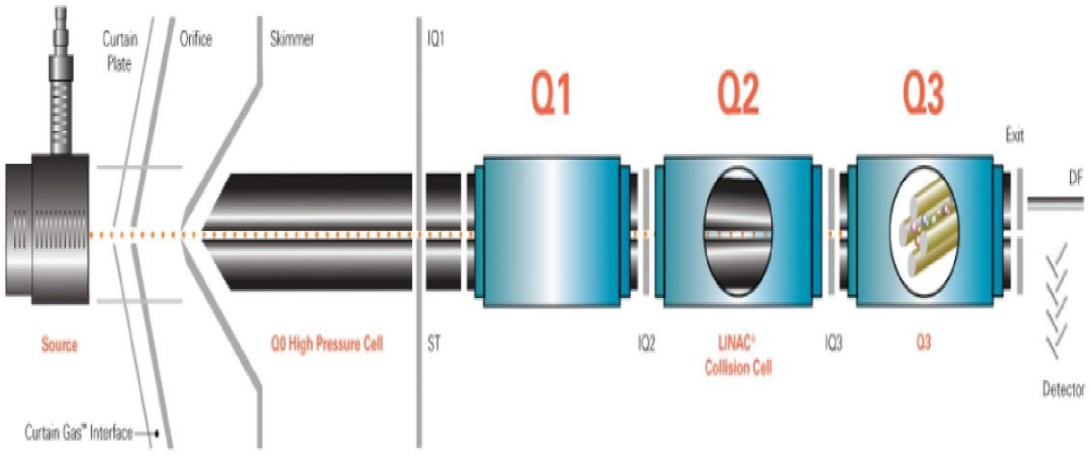


Figure.1.26 Schematic of triple quadrupole configuration

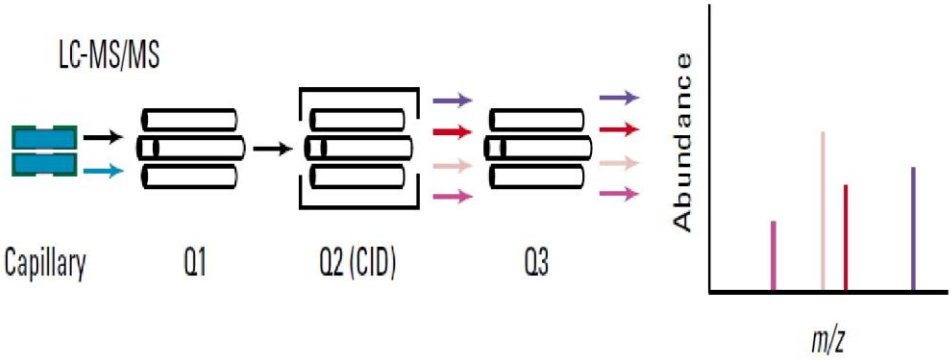


Figure.1.27 MS/MS in a triple-quadrupole mass spectrometer

Collisions with an inert gas such as nitrogen or argon can cause ions to fragment, a process known as collision induced dissociation (CID). A quadrupole collision cell is one type of collision cell that has been engineered to maintain the low pressure of the collision gas required for dissociation while also transmitting the majority of the fragment ions produced. Placing a collision cell between two quadrupole mass analysers produces a particularly effective mass spectrometer setup. A triple quadrupole mass spectrometer is an example of tandem MS, in which two or more stages of mass analysis are applied independently (Figure.1.27).

The advantage of tandem MS over single stage mass analysis is the greater specificity of the analysis. For example, 25-hydroxy vitamin D3 produces a major $[M+H]^+$ ion with a mass of 401 *m/z* during ESI, which loses water during collision induced dissociation to produce a significant 383 *m/z* product ion (Chen *et al*., 2008). When the first and third quadrupoles are set to transmit only 401 and 383 *m/z* ions, very precise detection of 25-hydroxy vitamin D3 is achieved. The fragmentation is denoted 401>383 in this technique, which is also known as single reaction monitoring. Only analytes containing this precursor/product ion pair will be detected. Other components in a complex biological sample may form a 401 *m/z* precursor ion during ESI, but they are unlikely to fragment to a 383 *m/z* product ion. The first and third quadrupoles can be stepped to different *m/z* values at the same time, and panels of precursor/product ion pairs can be constructed to detect a large number of targeted analytes. In LC-MS/MS assays, this technique is known as multiple reaction monitoring (MRM). For triple quadrupole mass spectrometers, there are a number of other relevant modes of operation, which are given in the Table. 1.2. Most instruments are capable of data-dependent

acquisition, which means they can switch between multiple modes of operation throughout a run based on the data being collected. For example, if a particular MRM signal is detected over a certain threshold value, a product ion scan can be obtained to confirm the identity (Pitt. 2009). Product ion scans not only provide structural information about the analyte, but they also serve as a "fingerprint" that can be utilised to validate identity with high certainty, such as when confirming the presence of an illicit drug.

Table.1.2 Operational modes of triple quadrupole mass spectrometers

Name	First Quadrupole	Third Quadrupole	Applications
Product scan	Fixed	Scanned	Structural studies, identification of unknowns, confirmation by spectral Matching with standard
Precursor scan	Scanned	Fixed	Detection of structurally related analytes that produce a common fragment ion
Neutral loss (or gain) scan	Scanned	Scanned with fixed offset relative to first quadrupole	Detection of structurally related analytes that eliminate (or gain) a common neutral molecule on collision
Multiple reaction monitoring	Fixed	Fixed	High sensitivity detection of a panel of targeted analytes

1.1.3.1 Triple quadrupole (MS/MS) systems provide in comparison to single quadrupole (MS) systems

- Greater selectivity means less interference from co-eluting compounds and matrix, requiring less HPLC separation
- A better signal-to-noise ratio (S/N) allows for quantitation with lower limits of quantification (LLOQ)
- In comparison to Selected Ion Monitoring (SIM), Multiple Reaction Monitoring (MRM) provides a more reliable identification of detected analytes
- Wider linear range of quantification
- Better accuracy and reproducibility particularly at low concentrations

1.1.4 Components of LC-MS/MS

Liquid chromatography tandem mass spectrometers (LC-MS/MS instruments) couple sample fractionation and analysis by combining two components: a liquid-phase separation system (LC) and a mass spectrometer (MS).

1.1.4.1 Autosampler

The autosampler improves sample injection speed, accuracy, and processing capacity, resulting in enhanced analysis efficiency. Even with high-sensitivity LC-MS/MS detection, revolutionary cross-contamination countermeasures allow for analysis without carryover.

1.1.4.1.1 SIL-30AC Autosampler

The total-volume injection SIL-30AC autosampler has a 130 MPa pressure tolerance and the world's fastest sample injection (10 seconds), reducing total cycle time significantly. It comes with auto pretreatment and overlapping functions as standard, as well as the possibility of configuring the loop-injection mechanism to reduce delay volume. The SIL-30AC achieves a new level of low carryover performance with reduced needle contact area, special coatings, surface treatments, and a new needle seal, which is especially helpful for LC-MS/MS analysis. Additionally, the sample cooler includes a dehumidifier function for storing samples at a constant temperature of 4°C to 40°C.

1.1.4.1.2 SIL-20AXR/SIL-20ACXR Autosampler

The SIL-20AXR is a complete volume injection type (direct injection) autosampler with a high-speed injection function and a high-precision injection function for injecting trace quantities, as well as an enhanced high-pressure valve that increases pressure resistance (max. allowable pressure : 66 MPa). The internal volume of the high-pressure valve was lowered to reduce sample diffusion, making it ideal for high separation analysis. The sample cooler on the 20ACXR keeps samples in sample racks at a constant temperature of 4ºC to 40ºC.

1.1.4.1.3 SIL-20A/20AC Autosampler

The SIL-20A is a complete volume injection (direct injection) autosampler with high-speed injection and multi-analyte processing capabilities. Materials used in needle, valve, and other flow lines were carefully evaluated, and a needle rinse feature was added to help reduce cross-contamination (carryover), which is particularly important for high-precision LC-MSMS analysis. The SIL-20AC has a sample cooler with a dehumidifier function for holding samples at a constant temperature of 4ºC to 40ºC.

1.1.4.1.4 SIL-10AF/P Autosampler

This is a multi-purpose model with a large injection volume capability. It is especially well-suited to automatic pretreatment applications like dilution and mixing with auto injectors. The SIL-10AF has faster sample injection speeds than the preceding SIL-10A model (which has been lowered to 1/3 of its prior speed). It can accommodate two 96-well microplates. Large volumes of up to 5 mL can be injected with the standard SIL-10AP. It is used in applications ranging from normal analysis to semi-preparative.

The amount of sample constituents adsorbing has been decreased to an absolute minimum. Enhancements include the following :

- Special needle treatment (patent pending) – masks active sites and minimizes ionic interactions
- One piece injection port/needle seal at valve center – eliminates sample transfer tubing
- Redesigned needle seal to minimize surface area
- Low-adsorption valve materials

Even with challenging basic and hydrophobic compounds, these advancements combined to eliminate cross contamination. Dual-solvent rinsing is available for particularly difficult samples.

High Speed/Throughput - 10s for a 10 µL injection - With the SIL-20A/20AC, ultra-fast sample processing - 10 seconds for a 10 µL injection - is now possible. When combined with a high-speed separation column, an analysis cycle time of under one minute is possible.

Precise Sample Injection - A high-performance precision metering pump was used to achieve greater injection volume accuracy. It is feasible to inject volumes of less than 1 litre, and the direct injection approach ensures that valuable samples are never wasted.

Optional Rack Changer - Increases sample capacity significantly as the speed of the machine increases, capacity becomes a limiting factor for unattended operation. Microplates are automatically loaded into the SIL-20A/AC sample compartment along the optional rack changer. Over 1000 samples can be processed quickly with the 12-plate capacity. The "C" model changer has the same plate capacity as the SIL-20AC and includes a 4°C to 40°C cooling function.

1.1.4.2 LC Pumps

The pump is required in practically all modern LC systems to deliver mobile phase through the column at a constant flow rate. Modern LC pumps must function reliably and precisely at pressures of 10,000 psi or at least 6,000 psi due to the small particles used in modern LC instruments. LC pumps normally contain sapphire pistons, stainless steel cylinders, and return valves with sapphire balls and stainless steel seats to work at high pressures while being sensibly inert to the wide variety of solvents used. LC pumps should have flow rates ranging from 0 to 0.5 mL/min. for analytical purposes, however flow rates raise upto 1 mL/min. may be required in special cases. Two types of pumps mainly used.

1.1.4.2.1 Isocratic pump

Isocratic pump is both simple and inexpensive the most simple. It consist one pump that supply constant mobile phase composition (premixing).

1.1.4.2.2 Gradient pump

There are two basic types of gradient pump. The solvents are mixed at high pressure in the first, and then premixed at low pressure before being supplied to the pump in the second.

1.1.4.2.2.1 High pressure gradient

Because each solvent requires its own pump, the high pressure programmer is the simplest but most expensive. Although a mobile phase programme can theoretically have any number of solvents, most LC analyses only require two solvents. However, up to four solvents can be accommodated.

1.1.4.2.2.2 Low pressure gradient

The solvent from each reservoir flows to an oscillating valve, whose output is coupled to a mixing manifold, where it is mixed at a low pressure gradient. The manifold receives and mixes solvents from each of the programmed valves. The valves are electrically controlled and are programmed to open and close for specific periods of time.

1.1.4.3 Column oven

The temperature around the column is accurately controlled in the column oven, allowing for stable analysis that is unaffected by ambient temperature. A manual injector, gradient mixer, high-pressure flow-line selection valves (2-position/6-port valves or 6-position/7-port valves, total of two), a conductivity-detection cell block, and a reaction coil, among other parts and units, can be accommodated in addition to the column.

1.1.4.3.1 CTO-20A column oven

The CTO-20A is a type of column oven that uses forced-air circulation. It has a temperature range of 10°C above room temperature to 85°C that it can regulate. It also enables for the setting of complex temperature programmes that include linear or step-wise temperature increases and decreases, for example.

1.1.4.3.2 CTO-20AC column oven

The CTO-20AC can heat and cool the column compartment, with sub-ambient conditions provided by a Peltier cooling device. Temperature is controlled over a wide range of temperatures, from 10°C below room temperature to 85°C, to improve reproducibility and separation performance.

A high-performance thermistor regulates the temperature of the interior of the oven very precisely. To ensure a high level of temperature accuracy, the temperature is calibrated at two different temperatures. The column management device (CMD) is supported by both the CTO-20A and the 20-AC. The CMD provides - recorded information about column usage, the amount of mobile phase delivered, the composition of the previous mobile phase used, and the number of samples injected.

1.1.4.4 LC columns

Because the separation of the sample components is achieved when those components pass through the column, the LC columns are one of the most significant components of the LC chromatograph. In the past few years, the ideal column size for method development has changed dramatically. To reduce solvent use and achieve compatibility with MS detectors, smaller 3.0 mm ID or 2.1 mm ID columns are being employed more than 4.6 mm ID columns. Length of the column become shorter, ranging from 50 to 100 mm (50, 75 or 100 mm) with 3 to 5 mm diameter and 3 to 5 µm particle size. The columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to achieve the low dispersion that allows modern LC systems to achieve plate counts. With modern columns, plate counts in excess of thousands of plates per column are possible. Normally, silica gel is used to fill columns because its particle shape, surface properties, and pore structure aid in a good separation. Silica is wetted by almost all potential mobile phases, is inert to most compounds, and has a high surface activity that can be easily modified with water and other agents. Silica can be used to separate a wide variety of chemical compounds, and its chromatographic behaviour is usually predictable and reproducible.

1.1.4.5 Mass spectrometer

A mass spectrometer can ionize the separated peak solution and determine the molecular weight of each peak component. An LC-MS/MS system can fragment the precursor ion into a distinctive fragmentation pattern and can separate the product ions for identification and quantification. By comparing fragmentation patterns provided by standard computerised databases, the characteristic fragmentation pattern from each precursor ion can be determined (McMaster. 2005). As a result, the mass spectrometer is a particularly powerful scientific tool since it can be successfully employed even if you only have a small quantity of material to analyse - as little as 10-12 gm, 10-15 moles for a compound with a mass of 1000 Daltons (Da). Compounds in chemically complex mixtures can be identified by mass spectrometry at

very low concentrations (one part in 1012) (Watson and Sparkman. 2007). The fundamental mass spectrometry instrumentation processes are as follows :

a) Introduction of sample; a sample which can be a solid, liquid, or vapour is loaded onto a mass spectrometry device and is vaporized
b) Ionization; sample components are ionized by one of several available methods to create ions
c) Analyzer sorting; the ions are sorted in an analyzer according to their *m/z* ratios through the use of electromagnetic fields
d) Detector; the ions then pass through a detector where the ion flux is converted into a proportional electrical current
e) Data conversion; the magnitude of the ion/electrical signals is converted into a mass spectrum

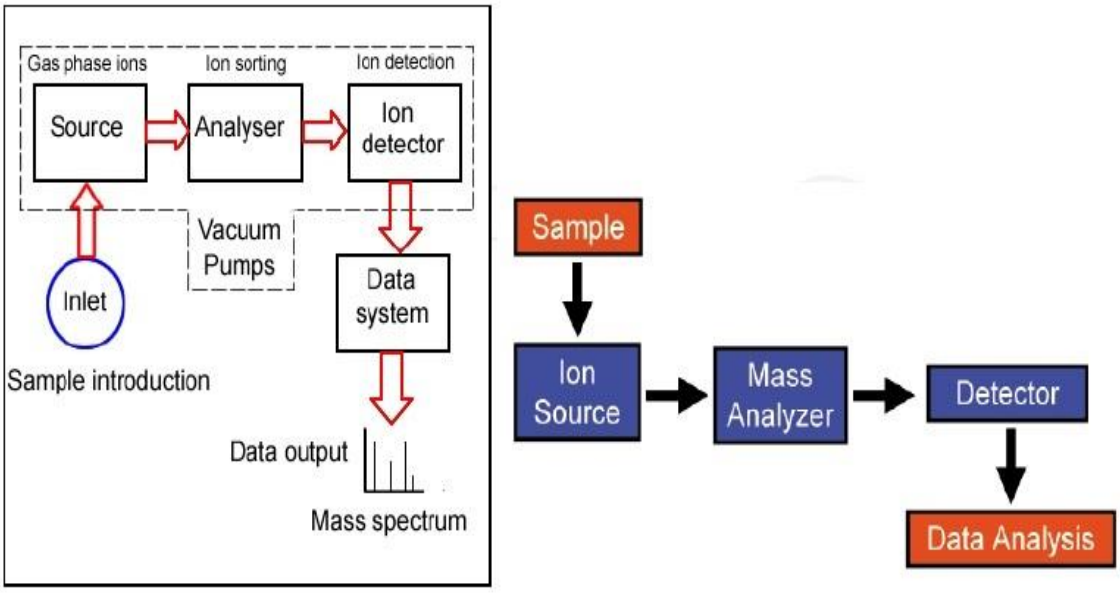


Figure.1.28 Simplified diagram and function of instrumentation of typical mass spectrometer

MS instruments are made up of three parts : an ion source that can convert gas phase sample molecules into ions (or, in the case of ESI, APCI or APPI, move ions that exist in solution into the gas phase); a mass analyzer that uses electromagnetic fields to sort the ions by their masses; and a detector that measures the value of an indicator quantity and so provides data for calculating the abundances of each ion present (see Figure.1.28). The technique can be used for both qualitative and quantitative purposes. Unknown compounds can be identified,

the isotopic composition of elements in a molecule can be determined, and the structure of a compound can be determined by observing its fragmentation (Kang. 2012). Quantifying the amount of a molecule in a sample or studying the fundamentals of gas phase ion chemistry are some of the other applications (the chemistry of ions and neutrals in a vacuum). MS is now commonly used in analytical laboratories for determining the physical, chemical, or biological properties of a wide range of molecules. About ion sources and mass analyzers has already discussed earlier in this thesis (1.1.2.1.1 and 1.1.2.1.2).

A vacuum is required in all mass spectrometers in order for ions to reach the detector without interacting or colliding with other gaseous molecules or atoms. Resolution and sensitivity of the instrument would be reduced if some molecules collided. In *m/z* analyzers, low pressure (i.e. infrequent ion/molecule or molecule/molecule collisions) is also necessary for preserving resolving power. Any interaction with other matter can cause an ion's path to change direction once it has been established (Watson and Sparkman. 2007). Coupling the sample source to a mass spectrometer was one of the initial challenges faced by the inventors of the mass spectrometer. The sample is initially at atmospheric pressure (760 torr) before being transferred to the vacuum of the mass spectrometer (~10^{-6} torr), which represents approximately a billion-fold difference in pressure. One approach is to introduce the sample through a capillary column (in GC) or through a small orifice directly into the instrument. Another approach is to use a vacuum lock (MALDI) to evacuate the sample chamber, and then present the sample to the primary vacuum chamber (< 10^{-5} torr) once an reasonable vacuum has been obtained (< 10^{-2} torr). For most MS, the mechanical pump serves as a general workhorse, allowing for an initial vacuum of around 10^{-3} torr to be obtained (see Figure.1.29). After achieving a 10^{-3} torr vacuum, the other pumping systems, such as diffusion, cryogenic, and turbomolecular, can be used to achieve pressures as low as 10^{-11} torr (Chiu and Muddiman. 2008).

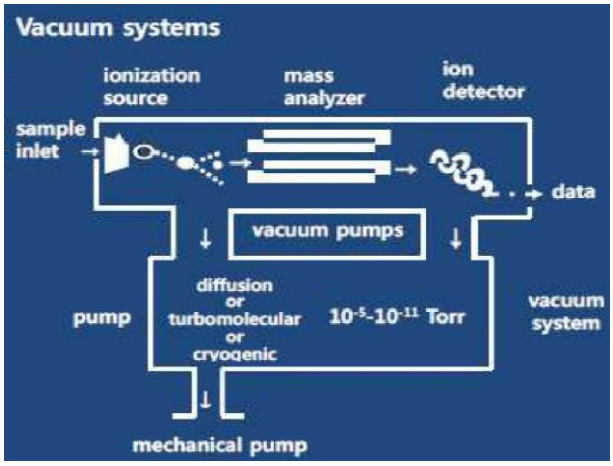


Figure.1.29 A well-maintained vacuum is essential to the function of a mass spectrometer

The detector, which records either the charge generated or the current produced when an ion passes by or collides with a surface, is the final component of the mass spectrometer. The signal produced in the detector during the scan versus where the instrument is in the scan (at what *m/z* or *m/q*) can produce a mass spectrum, a record of ions as a function of m/z or m/q, in a scanning instrument (Kang. 2012). Some type of electron multipliers are commonly used, however other detectors such as Faraday cups and ion-to-photon detectors are also used. Because the number of ions exiting the mass analyzer at a particular instant is usually quite small, considerable amplification is frequently required to obtain a signal. In modern commercial instruments, microchannel plate detectors are commonly used (Dubois *et al*., 1999). The detector in FT-MS and Orbitraps is made up of a pair of metal surfaces within the mass analyzer/ion trap region that the ions only pass near as they oscillate. In a circuit between the electrodes, no DC current is produced, only a weak AC image current is produced. Other types of inductive detectors have been used as well (Park *et al*., 1994).

Sensitivity, accuracy, and response time are all key parameters that distinguish different ion detection system (Koppenaal *et al*., 2005). Because high accuracy and rapid response times are frequently mutually exclusive features, the fast-response electron multiplier required to follow rapid scans (e.g. *m/z* 50 to 500 in 0.1 sec.) is unlikely to offer the accuracy desired (better than ±0.1%) in isotope ratio measurements. The Faraday cup detector is commonly used to measure isotope ratios like this (Nygren *et al*., 2006; Harris *et al*., 1984; Mckinney *et al*., 1950). A sensitivity statement should ideally define the ion current received by the detector for a specified rate of sample consumption. The definition should include :

- The name of the calibration compound
- The m/z value of the ion current that is measured
- The resolving power of the instrument
- The quantity of calibration compound consumed per second
- The intensity of the electron beam and pressure in the ion source for CI
- The ion current arriving at the detector (e.g., that impinging the conversion dynode of an electron multiplier)

Different types of detectors are used in MS, such as electron multiplier (EM), Faraday cup, negative-ion detection, post-acceleration detector, channel electron multiplier array (CEMA), photomultiplier conversion dynode, the daly detector, array detector etc.

1.2 Method development

The process of developing an analytical method for identifying and quantifying a compound of interest in a matrix is known as method development. A compound can often be measured by different methods and there are many factors to consider when choosing an analytical method, such as : chemical properties of the analyte, concentration levels, sample matrix, cost of the analysis, speed of the analysis, quantitative or qualitative measurement, precision required, and necessary equipment (Murugan *et al*., 2013). A well-organized development of bionalytical methods are important components in the development of pharmaceuticals (Breaux *et al*., 2003). The legitimate experimental techniques that result from these developmental procedures are utilized by quality control laboratories to guarantee the identity, purity, potency and bioavailability of drug products (Breaux *et al*., 2003; Singh and Shah. 2011). Methods for LC-MS/MS analysis can be developed if the nature of the sample is known, such as its molecular weight, polarity, ionic character, pKa values, and solubility parameter. Method development cannot be generalised or standardized across the board because it is unique and specific for each drug and metabolite. It also depends on the nature of the sample, the level of sensitivity required, and other factors.

A well-developed method should be easy to validate. A method has to be developed with the objective to be able to instantly check clinical samples, formulation prototypes and commercial samples. By advances in method development procedures, the evidence congregated is taken into consideration for the design and proceeding for development of

method. A suitable protocol should be made for method development technique. In spite of advances in chromatographic theory, method development is still based mainly on "trial and error". So, to develop a relevant bioanalytical method, specialists must first comprehend the rationale of the trial in order to determine which analytical information will be used, the method's prerequisites, and the outcome of the results once the investigation is over. As soon as these aspects are well-defined, a suitable method can be established (Kamalraj *et al*., 2012). Method development consists of five essential interrelated parts: sampling, sample preparation, chromatographic separation, MS detection and evaluation of the results (Murugan *et al*., 2013 and Chandramouli *et al*., 2010).

1.2.1 Basic conditions for LC-MS/MS method development

LC-MS/MS method associates the physical separation performance of liquid chromatography with mass analysis capabilities of mass spectrometry (Kamalraj *et al*., 2012). Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) has appeared as the supreme approach and authoritative method for quantifying drugs and their metabolites in biological matrix because of its great sensitivity, specificity, rapid rate of analysis and quick data acquisition and processing (Savoie *et al*., 2010). A full-scan LC-MS examination can suggest probable oxidative or conjugative metabolic conversion by considering ionic species observed. Tandem mass spectrometry (MS/MS) is a technique that involves performing one mass selective operation after another. The goal of first ionization is to separate an ion species known as a precursor ion or molecular ion, whereas the goal of second ionization is to determine the mass to charge ratio (*m/z*) of the fragment or product ion formed by collision induced dissociation (CID) of the precursor ions (Link. 2011).

The instrument can detect positive or negative ions in full scan mode or in the more sensitive selected-ion monitoring mode (SIM). Four different operating combinations are possible for MS/MS analysis: precursor - ion scanning (scan mode in the first analyser, SIM in the second analyser), product - ion scanning (SIM in the first analyser, scan mode in the second analyser), constant neutral - loss scanning (CNLS; scan mode in both analysers), and selected - reaction monitoring (SRM; SIM in both analysers). SRM is also called as multiple reactions monitoring (MRM). When identifying drugs and/or their metabolites in complex matrices, product - ion scanning is preferred. Separation takes place on the LC and in the first mass analyser, whereas fragmentation in the second analyser provides structural information (Link. 2011).

1.2.2 Sample preparation

In a bioanalytical study, sample preparation is a crucial step in analysing drugs and metabolites. Biological samples contain various complexes like proteins, endogenous and exogenous substances, together called as "unwanted substances" (Kamalraj *et al*., 2012). These can interfere with the drug in plasma which is present in very small quantities like picogram/mL to nanogram/mL range. During analysis, the "unwanted substances" can interfere with small concentrations of the drug. The primary goal of sample preparation is to remove all potentially unwanted contaminants while minimising analyte loss (Kamalraj *et al*., 2012). Solid phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation (PP) are some of the techniques now available for extracting analytes from biological matrices.

1.2.2.1 Solid phase extraction (SPE)

The SPE principle is based on the separation or partitioning of the analytes into two phases. The analyte of interest must have a higher affinity for the solid phase than the matrix components. Nowadays, SPE sorbent is packed between two fritted discs in a polypropylene cartridge. As a result, the liquid phase can pass through the cartridge. Silica-base with chemically bonded functional groups or highly cross-linked polymers such as styrene divinylbenzene and polymethacrylate are generally used materials for preparation of solid phase cartridges. To avoid any interference and, in most cases, to eliminate carry-over effects, these cartridges can only be used once (Rahman *et al*., 2012).

Non-polar, polar, and ion exchange sorbents are among the various types of SPE sorbents used. The stationary phase in reverse phase SPE is commonly silica bonded with alkyl and/or aryl functional groups. The most commonly used sorbents are C18 and C8. The packing materials required for SPE and liquid chromatography are comparable; the only difference is that the particle size in SPE is larger than in liquid chromatography. Solid phase microextraction modifies the SPE technique (Said. 2010 and Gad. 2008).

Advantages

- Low concentration of drug or metabolite can be detected.
- Effective in selective removal of interferences.
- Different types of adsorbents can be used.

- Extending the analytical column life, reduced system maintenance, minimizing ion suppression.

Limitations

- Extraction is difficult for high-density materials.
- In extraction processes a number of steps are to be carried out making it a time consuming process.

1.2.2.2 Liquid-liquid extraction (LLE)

The principle of liquid-liquid extraction is the distribution of analyte between two immiscible liquids (aqueous and organic). Extraction of analyte occurs from aqueous phase into organic phase. The organic layer is separated from the aqueous phase after analyte extraction and evaporated in the presence of nitrogen gas or any inert gas (e.g. helium, neon, argon, krypton, xenon, and radon) in order to obtain the dry form of the sample. In recent years, advanced and improved techniques such as liquid phase microextraction (LPME), single drop-liquid phase micro extraction (DLPME), and supported membrane extraction (SME) have replaced traditional LLE (Said. 2010).

Advantages

- Clean extracts obtained.
- Greater sample capacity.
- The technique is simple, rapid and has relatively less cost per sample.

Limitations

- Sometimes pH control of the sample necessary for extraction.
- During evaporation, since the temperature is increased, the method cannot be used for thermolabile substances.

1.2.2.3 Protein precipitation (PP)

Protein precipitation is a technique that can be used to extract plasma and blood samples. The principle of PP is to precipitate (denaturate) proteins using various reagents such as acids (trichloroacetic acid, trifluoroacetic acid, and perchloric acid), organic solvents (methanol, acetone, and acetonitrile), or salts (ammonium sulphate). The sample is centrifuged after denaturation, which results in analyte extraction in the precipitating solvent (Rahman *et al*.,

2012). Methanol is the most commonly used organic solvent because it produces a clear supernatant that can be directly injected into an LC-MS/MS. Other alternatives to acid and organic solvent precipitation include salts. This technique is known as salt-induced precipitation. Proteins aggregate and precipitate from the solution when the salt concentration of a solution is increased (Said. 2010 and Gad. 2008).

Advantages

- This method can be applied for extraction of both hydrophobic and hydrophilic substances.

Limitations

- Protein precipitation may clog the column.

1.2.3 Chromatographic separation

Improved chromatography makes it simple to separate interfering compounds from analytes. Gradient elution can help in washing the column after injection and preventing late eluting compounds from the previous injections from interfering. If the analytes are ionizable, the pH of the mobile phase can have a major impact on the retention, selectivity, and sensitivity of the separation (Chamber *et al*., 2007). Further according to the findings, fast gradient LC promotes matrix effects by reducing chromatographic separation between analytes and endogenous compounds. Because chromatographic conditions are unable to reduce matrix effects, effective sample pretreatment becomes crucial when high throughput is required. Another possibility is to utilise a stationary phase with a different selectivity, such as hydrophilic-interaction liquid chromatography (HILIC). HILIC, which combines the use of bare silica or polar bonded stationary and mobile phases with a high amount of organic solvents, has proven to be a valuable technique for the analysis of polar compounds in biological samples (Ji *et al*., 2008). HPLC uses columns with small diameter (1.7 µm) particles and high pressures to increase the speed, resolution, and sensitivity of the method while retaining the practicality and principles of classical LC. The higher resolution could help with matrix effects by improving separation from endogenous components, and the HPLC technology was used to achieve a statistically significant reduction in matrix effects under a variety of chromatographic conditions and with multiple basic analytes (Kang. 2012).

1.2.4 MS detection

MS detection is known as the world's smallest scale, not because of its size, but because of what it weighs: a molecule and a microanalytical technique that can be used selectively to detect and determine the amount of a specific analyte (Watson and Sparkman. 2007; Chiu and Muddiman. 2008). The data collected during mass detection is termed mass spectra, and it can be displayed in many different ways to extract easily the desired information about the analyte (Watson and Sparkman. 2007). A mass spectrometer (MS) is an instrument that generates a beam of gaseous ions from a sample, sorts the resulting mixture of ions based on their mass-to-charge ratios, and gives output signals that indicate the relative abundance of each ionic species present. MS are usually classified on the basis of how the mass separation is accomplished, but they are all ion optical devices that use electric and/or magnetic force fields to separate ions according to their mass-to-charge (*m/z*) ratios. The concept of MS is to generate ions from a sample, separation of the ions based on their *m/z* ratio (which can be considered the same as the mass because the ion has just a single charge in most cases), and to measure the abundance of the ions. During MS detection, all processes such as ionization separation of the ions, rate of data acquisition, detection of the ions, and storage of the data are controlled by the computer. Molecules are ionized at the ion source to form molecular ions, some of which will fragment, and ions with different *m/z* values transit through the mass analyzer one at a time to reach the detector by several processes. When the ions strike the detector, they are turned to an electrical signal, which is then converted to a digital response that can be stored by the computer (Sparkman. 2000).

The mass of a molecule is determined by detecting the *m/z* of its ion in a mass spectrometer (MS), rather than directly. Ions are charged atoms, groups of atoms, or molecules that are positively or negatively charged. Ionization is the process by which an electrically neutral atom or molecule becomes electrically charged by losing or gaining one or more of its additional nuclear electrons. Although MS can analyze both positive and negative ions, the majority of instruments are used to investigate positive ions because they are produced in larger number than negative ions in most ion sources (Chiu and Muddiman. 2008). In order for ion formation to occur, a minimum amount of energy, referred to as the "ionization potential," must be delivered. The energy input required to remove (to an infinite distance) a valence electron from the highest occupied atomic or molecular orbital of the neutral particle to form the corresponding atomic or molecular ion, also in its ground state, is defined as the first ionization potential of an atom or molecule. When only one electron is removed the ion,

it is called an atomic or molecular ion; the term "precursor ion" is frequently used. Ionization without cleavage can be defined as the formation of precursor ions. The charge on the nucleus, the atomic or molecular radius, the shielding effect of the inner electronic shells, and the extent to which the most loosely bound electrons penetrate the cloud of electric charge of the inner shells all influence the numerical magnitude of the ionization potential. Only ions are detected in MS, and any non-ionic (molecules or radicals) particles with no charge are eliminated from the MS by the continuous pumping that maintains the vacuum. When only individual ions are present, they can be grouped according to their unique properties (mass and number of charges) and freely moved from one point to another. Individual ions must be analysed in a vacuum, which implies that the ions must be in the gas phase, in order to be free of any other forms of matter. It is a fundamental requirement of MS that ions be in the gas phase before they can be separated and detected based on their respective *m/z* values (Kang. 2012).

1.3 Method validation

Method validation is a procedure for ensuring that an analytic method is acceptable for its intended purpose and that it provides reliable and valid results or data for a specific or given analyte(s). Bioanalytical methods can be developed in the laboratory that is performing the validation, or they can be obtained from another laboratory or from the literature. Method validation results can only be as good as the quality of the method that was developed. Validation process demonstrates the method will successfully meet or exceed the minimum standards recommended in the United States Food and Drug Administration (USFDA) guidance for selectivity, sensitivity, accuracy, precision, recovery, reproducibility, linearity and range, lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), matrix effect and stability evaluation (stock solution, freeze/thaw, short term, long term, and post-processed). Like FDA also International Conference on Harmonization (ICH) and European Medicines Agency (EMA) guidelines are standard for validation parameters, evaluation and requirements (Chandramouli *et al*., 2010). The approach employed to demonstrate that the analytical method used to assess an analyte in a biological matrix is reliable and reproducible is known as bioanalytical method validation (Savoie *et al*., 2009). This process is performed using a control matrix spiked with the compounds to be quantified. Other parameters are determined during validation while obtaining these parameters (e.g. extraction efficiency, calibration range and response function positional differences within an

analytical run and dilution integrity for analysing above limit of quantitation samples). For quantitative bioanalytcal procedures, there is a general agreement, that at least the following validation parameters should be evaluated :

1.3.1 Selectivity

The selectivity test is used to evaluate whether the bioanalytical method can differentiate and quantify the analyte(s) in the presence of other components in the sample. Analyses of blank samples of appropriate biological matrix (plasma, urine, or other matrix) acquired from atleast six sources should be carried out to ensure selectivity. Interference should be assessed on each blank sample, and selectivity should be ensured at the lower limit of quantification (LLOQ).

1.3.2 Sensitivity

The lowest standard concentration of an analyte on the calibration curve in a sample that can be quantitatively determined the lower limit of quantitation (LLOQ) with an acceptable precision and accuracy. The LLOQ is the lowest assessed concentration that can reproducibly provide an analyte response that is both accurate (100 ± 20% recovery) and precise (≤ 20% RSD). According to the bioanalytical guidance, the LLOQ should be at least 5 times the response as compared to the blank response. The EMA guidance states that "the LLOQ should be adapted to expected concentrations and to the aim of the study". For pre-clinical bioanalysis, ng/mL levels are commonly used; for example, the 1-1000 ng/mL range. Clinical bioanalysis methods frequently require lower LLOQ, down to the pg/mL level, limiting the sample preparation techniques and instrument platforms available.

1.3.3 Accuracy

The accuracy of an analytical method refers to how close the mean test results obtained by the method to the true value (concentration) of the analyte. Replicate analysis of samples containing known concentrations of the analyte are used to determine accuracy. A minimum of six determinations per concentration should be used to assess accuracy. A minimum of three concentrations in the range of expected concentrations is recommended. Except at LLOQ, where it should not deviate by more than 20%, the mean value should be within 15% of the actual value The deviation of the mean from the true value serves as the measure of accuracy. So, it is typically measured as relative error (%RE).

1.3.4 Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix. A minimum of six determinations per concentration should be used to determine precision. It is typically measured as coefficient of variation (%CV). A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, interbatch precision or repeatability, which measures precision over time and may involve different analysts, equipment, reagents, and laboratories.

1.3.5 Standard curve or calibration curve

The relationship between the instrument or experimental response value and the known analytical concentration. Fresh standard or calibration curves must be used for method validation. Frozen calibration curves may only be used after stability has been determined. There should be two curves in each run, one at the beginning and one at the end of the injection sequence. Standard or calibration curves should be prepared in the same biological matrix as the intended study samples; however, if the study matrix is rare, they might be prepared in a surrogate matrix.

1.3.6 Linearity and range

The ability of an analytical procedure to produce test results which are directly proportional to the concentration (amount) of analyte in a sample (within a specific range) is known as linearity. The range of the method is the concentration interval where accuracy, precision and linearity are validated. The simplest model that appropriately explains the concentration-response relationship should be used as the calibration curve. The deviation from the nominal concentration of the LLOQ should not exceed more than 20%, and the variation from the other standards in the curve should not be more than 15%. A linear regression approach based on the equation $y = mx + c$, where y- is the peak area ratio of analyte to IS, x- is the plasma concentration of analyte, and m and c are the constants respectively with $1/x$ or $1/x^2$ weighting is usually sufficient. The use of a quadratic fit is allowed but should be limited, and the cause for non-linearity should be determined. The selection of final regression model and

weighting factor should be based on all of the validation data, and evaluation should be documented.

1.3.7 Limit of detection (LOD) and lower limit of quantification (LLOQ)

The limit of detection (LOD) is a specific characteristic of the limit test. Under the provided experimental conditions, it is the lowest amount of analyte in a sample that can be detected but not necessarily quantified. The detection is commonly represented as the concentration of the analyte in the sample, for example, percentage, parts per million (ppm), or parts per billion (ppb). The lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy is known as the lower limit of quantification (LLOQ). The most practical approach is to determine LLOQ on the basis of precision and accuracy. The LLOQ is defined as the lowest concentration of the sample that can still be quantified with acceptable precision and accuracy. When there is baseline noise, such as in chromatographic methods, LLOQ based on signal to noise ratio (S/N) can only be applied. The maximum analyte concentration of a sample that can be measured with acceptable precision and accuracy is known as the upper limit of quantification (ULOQ). The concentration of the highest calibration standards is the same as the ULOQ.

1.3.8 Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. The extraction efficiency of an analytical method within the limits of variability is referred to as recovery. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be carried out by comparing analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

1.3.9 Reproducibility

The reproducibilty of the method is evaluated by replicate measurements using the assay, including quality controls and possibly incurred samples. The reproducibility of reinjection is assessed to determine if an analytical run could be reanalyzed in the case of instrument interruptions.

1.3.10 Matrix effect (Ion suppression, ion enhancement)

The matrix effect is investigated to ensure that the precision, selectivity and sensitivity are not compromised within the screened matrix. The presence of matrix components in biological samples suppresses or enhances the ionization of analytes, which is known as the matrix effect. In the validation of MS-based bioanalytical methods, quantitative evaluation of matrix effect gives useful information. Matrix Factor (MF) is a quantitative measure of matrix effect, defined as a ratio of analyte peak response in the presence of matrix ions to analyte peak response in the absence of matrix ions. So, Matrix Factor (MF) can be calculated as,

$$\text{Matrix Factor (MF)} = \frac{\text{Peak response in presence of matrix ions}}{\text{Peak response in the absence of matrix ions}}$$

Stable isotope labelled internal standard (IS) can compensate for matrix effects on quantification of the target analyte.

1.3.11 Carry-over effect

The carry-over effect can adversely affect accuracy and precision. Carry-over is evaluated during validation by injecting blank extracts after the highest calibration standard or the QC high sample. Carry-over is considered significant when the peak response of the carry-over blank is greater than 20% of the LLOQ response. Carry-over should be noted in the method when it is unavoidable. The method should also contain recommendations for reducing the influence of the carry-over effect during analysis of study samples, such as using extra blanks or injecting plasma samples in a certain order. Unexpected carry-over must be investigated. Adsorption of the analyte to instrument components is a major source of carry-over effect. Improper sample diluent, inappropriate mobile phases, and adsorption of the analyte to instrument components are all major drivers of carry-over effect.

1.3.12 Dilution integrity

This test is performed to determine the ability of the method to quantify the analyte and produce accurate and precise results after sample dilution. When subject sample concentrations exceed the range of the validated calibration curve, a dilution integrity test can be used. Six sets of quality control samples are prepared for dilution integrity by diluting them twice and four times prior to extraction with screened interference free blank matrix.

1.3.13 Robustness

The robustness of an analytical procedure, according to ICH guidelines, is a measure of its ability to remain unaffected by small but deliberate variations in method parameters, and it indicates its reliability during normal use. Robustness is defined as the ability to reproduce the (analytical) method in different laboratories or under different conditions without the occurrence of unexpected differences in the acquired result(s), and a robustness test as an experimental set-up to evaluate the robustness of a method.

1.3.14 Ruggedness

This involves various analysts, laboratories, columns, instruments, sources of reagents, chemicals and solvents. Ruggedness of a bioanalytical method is the degree of reproducibility of test findings achieved by analysing the same samples under a variety of normal test condition. The ruggedness of the method is considered by changing the experimental condition like,

- Changing to another column of the similar type
- Different operation in the same laboratory

1.3.15 Stability

The stability (chemical or physical) of the analyte under various conditions are also be studied during method validation for given time intervals. Stability experiments are conducted in conditions that are similar to those that might be encountered during actual sample handling and analysis (Murugan *et al*., 2013). The following stability conditions are required by USFDA and EMA and are advisable to investigate;

1.3.15.1 Stock solution stability

The stability of the analyte and IS stock and working solutions should be assessed using the lowest and highest concentrations of the solutions under the storage conditions used during the analysis of study samples. The stability of the stock solution is evaluated at room temperature for at least 6 hours.

1.3.15.2 Short-term matrix stability

The short-term matrix stability experiments should be designed and conducted to cover the laboratory handling conditions for the study samples. The stability of the analyte in biological matrix at ambient temperature is determined. Three aliquots of low, medium, and high concentrations (QCs) are stored for at least 6 to 24 hours before being analysed.

1.3.15.3 Long-term matrix stability

The long-term stability of the analyte in matrix stored in the freezer should be assessed. Low and high stability QCs should be kept in the freezer under the same storage conditions and at least for the same duration of time as the study samples i.e., the stability of the analyte in the matrix should exceed the time period from sample collection to analysis completion.

1.3.15.4 Freeze and thaw matrix stability

The stability of the analyte should be evaluated after multiple cycles of freezing and thawing to assess the impact of repeatedly removing samples from frozen storage. The QCs with low and high stability should be thawed and analysed using the same procedures as the study samples. Three aliquots of low, medium, and high concentrations (QCs) are frozen for 12 to 24 hours and then thawed at ambient temperature. The stability of the analyte is assessed, however at least three cycles should be carried out.

1.3.15.5 Post-preparative stability

The stability of the analyte during stages of the analysis process is evaluated. Post-preparative stability is established through "reinjection reproducibility" and "extract stability". Reinjection reproducibility is carried out to define the period of time in which an entire run can be reinjected and produce the same reportable results. After storage at autosampler conditions, the originally extracted standards and QC samples are reinjected in this experiment. Extraction stability defines the period of time in which the extracted samples are representative of fresh extracts. Extract stability can be determined by comparing the results from injection of stored extracted QCs versus freshly extracted curves and QC. Quantifying reinjected QC extracts against the results of the initial injection of the curves associated with the reinjected QCs is an alternative approach. During validation, it is recommended that both types of post-preparative stability be performed.

1.3.16 Purity of working standard

Purity of working standard exercise is performed to evaluate the interference due to internal standard (deuteriated) at retention time or m/e ratio of analyte. For evaluating purity of working standard, recovery comparison samples containing internal standard are prepared and injected in triplicate. Also recovery comparison samples of lower limit of quantitation (LLOQ) concentration of analyte are prepared (in absence of internal standard) and injected in triplicate.

1.3.17 Exchange Reaction

Exchange reaction exercise is performed to evaluate conversion of deuteriated internal standard into analyte during sample preparation. For exchange reaction plasma samples containing an anticoagulant are spiked with internal standard, processed and injected in triplicate. Also analyte containing lower limit of quantitation (LLOQ) (in absence of internal standard) is prepared in plasma, processed and injected in triplicate.

1.4 Application of a validated bioanalytical method to drug and active metabolite analysis

It should be reminded that the effort of a method validation is undertaken to guarantee during the analysis a quality of the measurement data as needed for the application of bioavailability, bioequivalence or pharmacokinetic studies such as preclinical pharmacokinetic studies, preclinical toxicokinetic study, clinical toxicokinetic study, regulatory toxicokinetic study etc. The different pharmacokinetic and bioequivalence studies require such validated bioanalytical methods, which meet the international rules and the selective and specific determination of the compound, metabolites and the internal standard. Pharmacokinetics describes the absorption, distribution, metabolism and elimination of drugs. Pharmacokinetic studies are important in the generic drug and its active metabolite development, when the mentioned safety studies are not necessary to execute. Substitutability of the generic and original formulations is proved bioequivalence studies. The biological equivalence is investigated by studying the statistical accordance of pharmacokinetic parameters.

1.4.1 Bioavailability and bioequivalence study

A multisource drug product is one that is marketed by more than one pharmaceutical manufacturer and includes the same active drug substance in the same dosage form. Single-

source drug products are products for which the patent has not yet expired or for which there are certain exclusivities that allow only one manufacturer to make it. Brand-name (innovator) drug products are usually single-source drug products. A pharmaceutical company may manufacture a generic drug product that can be substituted for the branded drug product after the patent and other exclusivities for the brand-name drug expire. The generic drug manufacturer must establish that the generic drug product is bioequivalent and therapeutically equivalent to the brand-name drug product, because the formulation and technique of manufacture of the drug product can affect the bioavailability and stability of the drug. The rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action is known as bioavailability. Bioavailability may be measured to represent the rate and extent to which the active ingredient or active moiety becomes available at the site of action for drug formulations that are not intended to be absorbed into the bloodstream.

1.4.2 Purpose of bioavailability or bioequivalence studies

Both approved active drug ingredients and therapeutic moieties not yet approved for marketing by the drug approving regulatory authority are subjected to bioavailability or bioequivalence studies. Before being marketed, new formulations of active drug ingredients must be approved by that authorities. The regulatory authority ensures that a drug product is safe and effective for the labelled indications for use before approving it for marketing. In addition, the drug product must meet all applicable standards of identity, strength, quality and purity. The regulatory authority requires bioavailability or pharmacokinetic studies, where necessary, bioequivalence studies for all drug products to ensure that these standards are met (USFDA Guidance for Industry, 2003). Bioavailability may be considered as one aspect of drug product quality that links *in-vivo* performance of the drug product used in clinical trials to studies showing evidence of safety and efficacy.

In-vitro and/or *in-vivo* bioequivalence studies must be carried out on the drug formulation proposed for marketing as a generic drug product for un-marketed drugs that do not have full NDA (new drug application) approval by the USFDA. Furthermore, the essential pharmacokinetics of the active drug ingredient or therapeutic moiety must be characterized. After single- and multi-dose administration, essential pharmacokinetic parameters such as rate and extent of systemic absorption, elimination half-life and rates of excretion and

metabolism should be determined. These *in-vivo* bioavailability studies provide crucial information (data) for determining optimal dosage regimens and to support drug labeling.

1.4.3 Drug and metabolite concentration in plasma

The most direct and objective method of determining systemic drug bioavailability is to measure drug and metabolite concentrations in blood, plasma, or serum after drug administration. A validated drug and metabolite assay method can be used to get an accurate description of the plasma drug or metabolite concentration-time profile of the therapeutically active drug substance(s) by appropriate blood sampling.

1.4.4 Pharmacokinetic and statistical evaluation of the data

The pharmacokinetic analyses for single-dose studies, such as a fasting study or a food intervention study, include the calculation of the area under the curve to the last quantifiable concentration (AUC_{0-t}) and to infinity ($AUC_{0-\infty}$), maximum concentration in plasma (C_{max}) and time of maximum concentration in plasma (T_{max}) for each subject. Additionally, the elimination rate constant (k), the elimination half-life ($t_{1/2}$) and other parameters may be estimated.

A comparison of population averages of a bioequivalence parameter, such as AUC and C_{max}, is commonly used to determine bioequivalence. The 90% confidence limits for the mean pharmacokinetic parameters of the test product were within 0.80-1.25 (80-125%) of the reference product means based on log transformation of the data. To establish bioequivalence, the calculated confidence interval should be within a prescribed bioequivalence limit, which is usually 80-125% for the product averages ratio.

1.5 Drug molecules in living body

When a foreign (xenobiotic) substance, such as a drug, enters a living body or an organism, different kind of enzyme systems work to eliminate it through various metabolic activities. As a result, metabolism is defined as the conversion or biotransformation of one chemical entity to another, i.e., a change of characteristics that makes it easier for a foreign or xenobiotic substance to be eliminated as it obtains more hydrophilic properties (Dyson. 1996; Kanu and Hill. 2008).

1.6 Drug molecules with their biological effects

Biological effect and activity of a drug in the body depends on its interaction with the target structures and which in turn depends on its characteristics. Some properties, including as lipophilicity, hydrophilicity, acid-base ionization, and chemical reactivity, influence the characteristics, and thus the concentration of the drug at the site of action in the body through absorption, distribution, metabolism, and elimination (Dyson. 1996).

1.7 Metabolic modification and active form of drug molecules

Phase I and phase II reactions are occurred due to metabolic modifications. A part of the molecule is modified or removed, or a functional group is added during phase I reaction, e.g., oxidation, reduction or hydrolysis. In a phase II reaction, this conversion is often a preparation of the drug which involves conjugation with a more water-soluble molecule. Glutathione conjugation, glucuronidation, and sulfation are the three examples of phase II reactions. In the urine, conjugated drug metabolites can be easily excreted and thus be eliminated from the body (Dyson. 1996; Kanu and Hill. 2008).

Generally, activity of a drug involves deactivation during metabolism. However, some investigations have shown that a drug metabolite is more active than the drug itself, such as in the case of the antihypertensive drug enalapril [also a vasodialator and an angiotensin-converting enzyme (ACE) inhibitor] and its phase I metabolite enalaprilat, or the drug acetyl-salicylic acid (used for treatment of headache, transient musculoskeletal pain, dysmenorrheal and gastric irritation) and its more active phase I metabolite Salicylic acid (Portole's *et al*., 2004 and Jain *et al*., 2012). As a result, both of these latter forms could be used in medicinal treatment. Nowadays, in development of a drug, a so called pro-drug can be purposefully created. A pro-drug is a pharmacologically inactive drug in the form in which it is administered, but which is converted or metabolized to a pharmacologically active substance in the body. Prontosil was first used as an antibiotic in the 1930s and also is a classic example of a pro-drug because it is transformed to its active metabolite sulfanilamide by azo reduction (Kanu and Hill. 2008).

1.8 Significance of active metabolites in bioequivalence study

Xenobiotics are metabolized to form active metabolites which are approximately 40-70 % in humans. Therefore, it is necessary to investigate active metabolite to evaluate bioavailability

and pharmacokinetic study which directly involves with narrow therapeutic index etc. Also, these active metabolites have shown to exert indirect effects on the pharmacokinetics of the parent drug in some cases.

1.9 Present scenarios of bioanalysis of active metabolites

Nowadays, the application of combinatorial chemistry and modern high-throughput bioassays has resulted in a significant growth in the number of new chemical entities and potential drugs. Because of this, there is an increasing demand for novel bioanalytical techniques and systems that allow for the rapid determination the metabolites of these substances (Prakash *et al*., 2007).

1.10 Regulation and regulatory guidances for bioanalysis of active metabolites

There are a number of regulatory bodies those have emphasized the need to understand the metabolism of drugs. During drug development, the Food and Drug Administration (FDA) is responsible for regulating drug safety testing, recommends that the metabolic pathway of the drug should been identified using *in vivo* or *in vitro* methods (Whitmire *et al*., 2011). Furthermore, any metabolites formed to a greater extent than 10% of the parent drug (systemic exposure at steady state) should be of safety concern. Because of the safety concerns, a non-clinical study of the drug metabolite is being conducted ("Guidelines for Bioavailability & bioequivalence studies" CDSCO. 2005).

1.11 Significance of LC-MS/MS method for the analysis of active form of drug molecules

These regulatory guidances and recommendations suggest that studies of active metabolites of drug molecule under the purview of bioequivalence Study of pro-drug. In combination with liquid chromatography (LC) counting detector system along with electron spray mass spectrometry methods, there are a number of techniques used for assessment of active metabolites in human plasma (Prakash *et al*., 2007). These methods are complementary and often used in combination with the dominant techniques of mass spectrometry (MS). MS has played an invaluable role as a detection method for biopharmaceutical characterization and quantification of a substance in human plasma. Over the last ten years, the use of MS in combination with LC has enormously increased and and it is now a well-known approach for

qualitative characterization of drugs and their metabolites, due to its selectivity and high sensitivity (Gelpi. 2003; Hoffmann and Stroobant. 2007).

However, HPLC is still the most commonly used technique for determining drug molecules and their metabolites, but MS is the superior method for analysis of biological samples as it requires smaller sample amounts, shorter run time, more sensitive, selective than HPLC.

1.12 Active metabolites in bioequivalence study

Bioequivalence studies (BE) are performed to demonstrate that different drug (active metabolite) formulations or regimens are similar or equivalent to each other in terms of efficacy (therapeutic benefit) and safety (nontherapeutic side effects). In the drug development process, they play a key and pivotal role to ensuring that when a patient switches to a new formulation in the marketplace, safety and efficacy will be maintained. Particularly, these studies are applied in the study of solid oral dosage forms (i.e., drugs administered as a tablet or capsule when ingested). To demonstrate equivalence in plasma, the rate and extent of bioavailability of the drug substance in plasma concentration profiles must be sufficiently similar to meet the regulatory standard for showing that exposure of the body to the drug substance is the same between formulations (Benet. 1999).

1.13 Primary challenges in pharmacokinetic evaluation of active metabolites

After oral administration, bioequivalence study of drugs which got active metabolites, are being fully metabolized and difficult to analyse. There are limited scopes for these types of bioequivalence study in India due to the following reasons :

- In therapeutic human doses, there are number of active metabolites of drug molecules having low exposure to the central compartment and are detectable only in picogram to nanogram levels. So therefore, a highly sensitive liquid chromatography-mass spectrometry (LC-MS/MS) is required to perform the bioequivalence study of such active drug metabolite, which is very expensive.

- Only by using HPLC (high performance liquid chromatography) or GC (gas chromatography), it is very difficult to perform as their exposure to plasma is very low in therapeutic doses.

- The pharmaceutical industry has invested in technologies to increase throughput, decrease costs and provide access to scientific data in last two decades. But for drug discovery activities, this has not led too much by the domestic companies. When compared to drug discovery, technical innovation is relatively slow in pharmaceutical manufacturing. Some of the major challenges faced by the domestic industry are in terms of lack of experience. A precisely integrated drug discovery effort requires investment and expertise in preclinical development of pharmacokinetics, drug metabolism and formulation science etc.

- Often the various metabolic pathways yield a mixture of metabolites, which can be very different from each other on a quantitative as well as on a qualitative point of view (molecular weight, pKa, lipophilicity etc.). Moreover, the drug molecule and its metabolites may be detectable in various biological fluids and body compartments at very low concentration levels (pictogram or nanogram). So due to this reason, the clarification of drug metabolism necessitates highly specific and sensitive analytical devices. These two features are gathered by modern mass spectrometry for owing to the developments made in this field, mass spectrometer design have had in this arena, with presentations that reflect the substantial increases in information content available for qualitative characterization of metabolic steps, and the greatly increased capacity for generating high quality quantitative data.

In this thesis, new approaches for optimization of LC-ESI-MS/MS unified methods are introduced. Also, a step-by-step optimization procedure is established that probably will facilitate to optimize the performance of such an experimental set-up. In this framework, the thesis constitutes a number of precise applications of LC-ESI-MS/MS, including analyses of some model pro-drugs which will form their active metabolite after single oral administration in healthy human subjects and finally based on developed and validated LC-ESI-MS/MS methods to access their dose dependent comparative bioavailability.

Chapter – 2:

Literature Review



- Background of chromatography
- Bio-analytical method development
- Recent development in bio-analysis
- Bio-analysis by LC-MS/MS
- Basic approaches in method development
- Bio-analytical method performance
- Information of drugs and metabolites

2.1 Background of chromatography

Mikhail Tswett, a Russian botanist developed a colourful separation of plant pigments such as chlorophyll, carotenes and xanthophylls through calcium carbonate column in year 1903. For the separation of mixtures, chromatography (from Greek : chroma "color" and graphein "to write") is the common term for a set of laboratory techniques. Through a stationary phase (column), it includes passing a mixture dissolved in a "mobile phase" after separation of the analyte(s) to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. In differential retention on the stationary phase, narrow differences in a compound's partition coefficient result and consequently separation become changed. During the 1930s and 1940s, new types of chromatography were developed, making the technology applicable for a wide range of separation processes. During the 1940s and 1950s, Archer John Porter Martin and Richard Laurence Millington Synge contributed significantly to the development of chromatography technique (Martin and Synge. 1941, http://www.umich.edu/~orgolab/chroma/chromahis.html and from Wikipedia, the free encyclopedia).

In the 1970's, a variety of techniques containing open-column chromatography, paper chromatography and thin-layer chromatography were used to perform the majority of chemical separations. Some valid chromatographic methods were commercially available to the laboratory scientist. For quantification of compounds and resolution between related compounds, these chromatographic techniques were not enough. So, pressure liquid chromatography began to be used to minimize flow through time, therefore decreasing purification times of compounds being separated by column chromatography during this time. Although, flow rates were unstable and the question of whether it was better to have a constant flow rate or constant pressure was deliberated (http://www.Kerouac.pharm. uky.edu/asrg/hplc/history.html). In any laboratory HPLC was an approach to requiring better separations often used for the separation of chemical compounds in the late 1970s (Brown. 1973 and Snyder *et al.*, 1979). For the above previous techniques, new approaches improved separation, purification, identification and quantification. The benefit of HPLC is added by computers and automation. Improvements in type of columns and therefore reproducibility were made as such terms as affinity columns, micro-columns and fast HPLC began to come out.

Nowedays, bioanalytical functions are constantly under pressure to reduce development times in the pharmaceutical industry. This is often accompanied with an increase in the number of biological samples requiring pharmacokinetic analysis and a decrease in the desired quantitation levels. Hyphenated techniques are examples of new tools that pharmaceutical industry adopted for developing fast and cost-effective analytical methods. One of the most prevalent hyphenated techniques, liquid chromatography tandem mass spectrometry (LC–MS/MS), has led to major breakthroughs in the field of quantitative bioanalysis since the 1990s due to its inherent specificity, sensitivity and speed. It is now widely acknowledged as the preferred technique for quantitating small-molecule drugs, metabolites, and other xenobiotic biomolecules in biological matrices (plasma, blood, serum, urine and tissue) (Xu *et al*., 2007). To reduce run times, fast gradients and short columns were first used in early applications of high-throughput LC–MS/MS assays (Ackermann *et al*., 2002, Hsieh and Korfmacher. 2006, Zhou *et al*., 2005). The need for adequate chromatographic separation of analytes from endogenous biological components in quantitative bioanalysis using LC–MS/MS analysis has been reemphasized as a result of a better understanding of how matrix effects can compromise the integrity of bioanalytical methods (Jemal and Xia. 2006; Bakhtiar *et al*., 2002; Naidong. 2003 and Veuthey *et al*., 2004).

However, LC-MS/MS is broadly reviewed to be an approach mainly for bioanalytical, biochemical, biomedical and biotechnological research as well as for the pharmaceutical industry. Without sensitivity gains, many of the commonly used techniques such as the "dilute and shoot" would not be practical. So, now LC-MS/MS is used in different fields including forensic science, food, energy, environmental and cosmetics industries etc.

2.2 Bio-analytical method development

Bio-analytical method development activities are not required to be performed in compliance with good laboratory practices (GLPs) but should be adequately documented to support a reproducible method document for validation. The objective of method development is to define the method and provide good scientific evidence for method design and suitability for its intended purpose. The resultant method design should be considered suitable to proceed with validation if the method development experiments outlined in Table.2.1 are incorporated into the method development plan and properly executed (Whitmire *et al*., 2011; Lausecker *et al*., 2009; Bansal *et al*., 2010 and Van Amsterdam *et al*., 2010). A bioanalytical method is a set of all of the procedures involved in the collection, processing, storing and analysis of a

biological matrix for an analyte (Shah *et al*., 1992). Analytical methods employed for quantitative determination of drugs and their metabolites in biological fluids are the key determinants in generating reproducible and reliable data, which are then used in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetics (Shah *et al*., 2000).

The following aspects should also be considered during method development :

- "Carryover should be addressed and minimized during method development" (European Medicines Agency, EMEA/CHMP/EWP/192217/2009). If carryover is inevitable or unavoidable, it should be noted in the method and extra blanks should be inserted after the high calibration standard and high quality control (QC) injections in a non-randomized sequence.

- Precautions should be taken to eliminate any possibility of contamination of the mobile phases, diluents, wash buffers, stocks, and working stocks. Use of disposable glassware, pipet tips with filters, and automation, whenever possible, is highly recommended.

- Analyte stability (freeze/thaw and use of stabilizer agents) should be evaluated during method development to help avoid delays in validation activities due to QC (analyte in matrix) stability concerns.

- The method development scientist should select the anticoagulant, and it should be used consistently throughout the toxicological study (nonclinical and clinical). If the anticoagulant or counter ion is changed for a pharmacokinetic or toxicokinetic (PK/TK) study, at a minimum an equivalency experiment must be done to demonstrate that the existing method can be used for the new anticoagulant. Additional proof of stability may also be necessary.

- Whole blood stability and collection process stability are not often performed as a method development activity; however, assessment may provide valuable information for both nonclinical and clinical studies. During the time period between sample collection and sample processing, the analyte may adsorb to cellular or proteinaceous components.

- The FDA Bioanalytical Guidance does not require an impact assessment on quantitation of analyte interference in matrix containing lipids and hemolysis; however, this assessment should be considered if the desired submission will target an EMA country or others (Food and Drug Administration of the United States. 2001 https://www.fda.gov/cder/guidance/index.html and European Medicines Agency, EMEA/CHMP/EWP/192217/2009).

- Depending on the dose route, the effect of the dosing vehicle on the matrix should also be evaluated during method development, particularly for nonclinical studies. Because the dosing vehicle may be present at relatively high levels in nonclinical samples, it can produce potential interference or matrix ionization effects. The dosage vehicle is typically present at low levels in clinical study samples, therefore this may not be an issue for the clinical approach. Depending on the excipient, this is a requirement in the EMA draft guidance (European Medicines Agency, EMEA/CHMP/EWP/192217/2009).

- If metabolites are known and standards are available, then an evaluation of metabolite impact on quantification of analyte is required. These are not always known in nonclinical studies, and if they are, there may be no reference standards available or characterized.

- During method development, specific instrument parameters must be clearly defined to ensure a consistent parameter approach can be set and remain fixed during validation: mobile phase composition, gradient profile, ionization mode, LC column identity. Minor parameters may be optimized to account for instrument to instrument variability and improve response (i.e., voltage and gas settings, precursor and product ion mass adjustment to optimize mass center).

- The sample preparation parameters must be fixed during validation once they have been refined. Changes in aliquot volume or sample preparation parameters necessitate at least partial revalidation. The type of sample preparation procedure utilized must be fit for purpose, and is dependent upon the analytical range, study size (i.e., small nonclinical study versus large clinical study), and matrix type. Several types of sample preparations may be evaluated during method development: Simple dilution in diluent, protein precipitation (maximum 100 µL sample volume for efficient

precipitant), liquid-liquid extraction, supported-liquid extraction or solid phase extraction.

- During method development, phospholipid removal should be assessed and the impact on the assay reduced by using different sample preparation techniques, chromatographic gradients, or a diversion valve to shunt the phospholipids to waste before they enter the LC column.
- After the method development experiments have been completed satisfactorily, a "mock validation run" should be carried out to test the draft method design prior to proceeding with validation.
- Before going to method validation, it is also recommended to conduct a pilot study or dose range finding samples to evaluate vehicle effects, selectivity, curve range, and other factors.

Table.2.1 An overview of method development exercises

Analyte Information	Structure and Properties
Method Optimization **QC Preparation Appropriate to Range** **Evaluation of Extraction Strategies (Protein Precipitation, LLE/SLE, SPE)**	Phospholipid impact evaluation, Recovery, Matrix Stability, Extract Stability, Initial Carryover Evaluation
Post-column infusion experiment **Mock Validation Run**	Accuracy and Precision, Carryover, Freeze / Thaw Stability, Bench Top (pre-processed) Stability, Long Term Stability, Recovery, Matrix Effect, Selectivity
Length of run should mimic longest expected sample set, padded with blanks	----------

So therefore, evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantification are involved in method development. To begin this work with a thorough review of the literature, reading work done on the same or similar analyte, and also summarizing main starting points for future work is of primary importance. During initial phase of method development, a set of initial conditions (ion source or interface, column, mobile phase etc.) are selected to obtain the first

"searching" chromatograms of the sample. These are based on reversed-phase separations on a C18 or a cyano (CN) column with MS/MS detection in most cases.

2.3 Recent development in high-throughput quantitative bioanalysis by liquid chromatography (LC) coupled with mass spectrometry (MS or MS/MS)

The development of standard analytical detection techniques in clinical chemistry is based on indirect properties of an analyte, such as its absorption of light, chemical reactivity or physical interaction with macro-molecules (Vogeser and Kirchhoff. 2011). LC-MS/MS technologies have significantly expanded the methodologic armamentarium of clinical laboratories over the last decade and it has become one of the essential basic technologies in laboratory medicine (Vogeser and Seger. 2008). In the MS method, in contrast, analytes are detected directly from the molecular characteristics as molecular mass and molecular disintegration patterns. Thus, MS techniques are very attractive for quantification of biomarkers or xenobiotics in the context of diagnostic procedures, since those techniques can enable analyses of much higher specificity compared to standard technologies such as photometry or ligand binding tests. Introduction of API techniques about 20 years ago made practically all potential bio-medical analytes amenable for MS. Furthermore, powerful new technologies of ion-analyses (tandem MS, TOF-MS, ion-trap MS) substantially increased the capabilities of MS analyzers with respect to specificity and to the extent of data read-out in the 1990s. These developments suggested a widespread use of MS methods in routine laboratory medicine. In particular tandem MS instruments hyphenated to LC systems used for sample introduction and prefractionation have been implemented in a constantly growing number of clinical laboratories worldwide now (Kang. 2012; Vogeser and Seger. 2008).

LC-MS/MS is attractive for laboratory medicine for three main reasons. (1) The development of new methods is in general straightforward and independent from the diagnostic industry, without the need e.g. to develop analytical antibodies. (2) The highly multiplexed analyses are feasible with very low current costs; the range of potential analytes is practically unlimited; individual "metabolomic analyses" addressing hundreds of analytes from different biochemical pathways and from different chemical classes are possible, as well as a comprehensive and individual description of xenobiotics ("xenobiom"). (3) When applying the principle of isotope dilution internal standardisation, analyses on a reference method-level of accuracy can be performed in a routine laboratory setting (Vogeser and Kirchhoff. 2011).

LC-MS/MS today holds enormous potentials for improvements in the pharmaceutical fields and laboratory medicine mainly including therapeutic drug monitoring (TDM), endocrinology, toxicology and metabolomic analyses, therefore, hyphenated techniques are examples of new tools that adoped for developing fast and cost-effective analytical methods. One of the most prevalent hyphenated techniques, LC-MS/MS, has led to major breakthroughs in the field of quantitative bioanalysis since the 1990s due to its inherent specificity, sensitivity, and speed. It is now generally accepted as the preferred technology for quantitating small molecule drugs, metabolites, and other xenobiotic biomolecules in biological matrices as like plasma, blood, serum, urine, and tissue (Xu *et al*., 2007; Yang and Wang. 2008).

Fast gradients and short columns were first utilized in early applications of high-throughput LC-MS/MS assays to reduce run times. Better understanding of how matrix effects can compromise the integrity of bioanalytical methods has reemphasized the need for adequate chromatographic separation of analytes from endogenous biological components in quantitative bioanalysis using LC-MS/MS method (Chiu and Muddiman. 2008; Xu *et al*., 2007). New developments from chromatographic techniques such as ultra-performance LC with sub-2 mm particles and monolithic chromatography are showing promise in delivering higher speed, better resolution and sensitivity for high-throughput analysis while minimizing matrix effects. On the other hand, automation in LC-MS/MS is great advance in instrumentation field and has to address a number of different processes in laboratory medicine application of LC-MS/MS that includes management of primary samples, assayspecific work-up of samples prior to actual MS analysis, integrated control of the subunits of the LC-MS/MS systems, processing of primary read-outs, and further handling of result data (Kang. 2012; Zeng *et al*., 2003 and Romanyshyn. 2001).

2.4 Quantitative bioanalysis of the analytes by liquid chromatography tandem mass spectrometry with ESI source

2.4.1 Liquid chromatography (LC)

For analysis of drugs in biological samples, liquid chromatography (LC) is one of the most widely used separation techniques. Modern days, a simple LC system include solvent reservoirs, solvent degassers, autosampler, mobile phase high-pressure dual pumps, solvent mixer, sample injector (switching) valve and a column oven with analytical column (having

temperature controller). The separation takes place under high pressure in the column containing a stationary phase, and separation of the analytes is achieved by means of their different distribution between the mobile and stationary phases (Snyder. 2009 and Meyer. 2010). Almost all regular stationary phase is a non-polar material, usually silica with covalently bonded carbon chains e.g., C18 column or cyano-bonded silica endcapped spherically (cyanopropylsilane) e.g., CN column. This kind of stationary phase is mentioned to as a reversed phase, and when in combination with a mobile phase (supplied as a gradient or isocratically) it is used mainly containing water, usually as a buffer such as ammonium formate or ammonium acetate or with a small quantity of an acid such as formic acid or acetic acid, the technique is known as reversed-phase chromatography (Molnár and Horváth. 1976; Benet. 1999) (Figure.2.1).

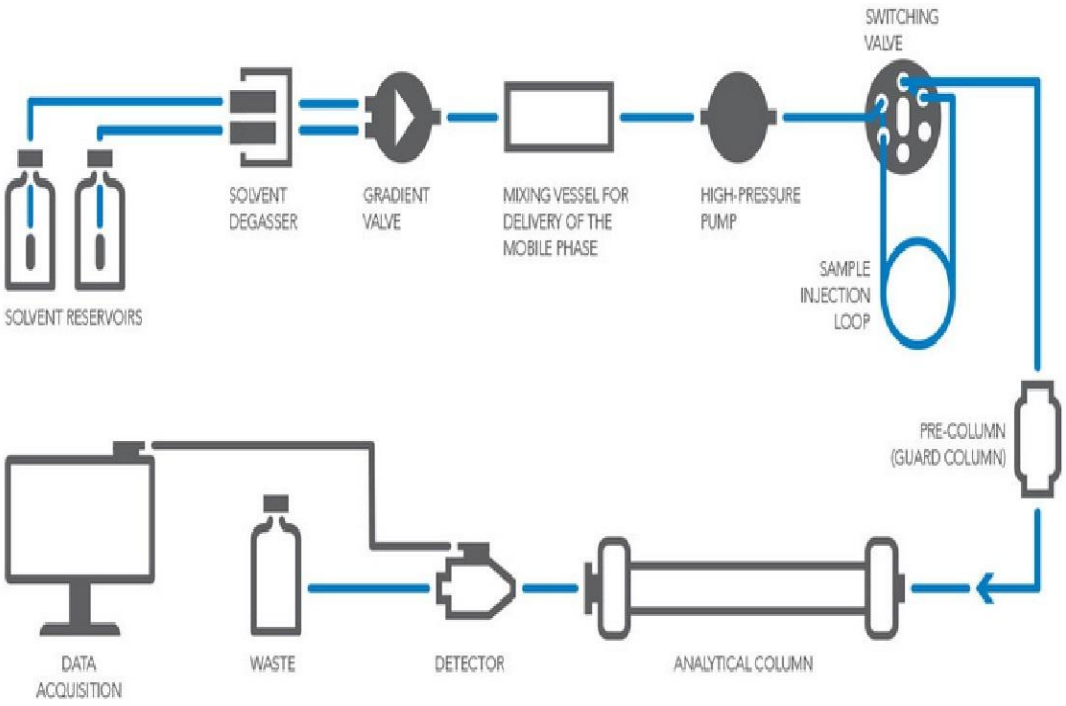


Figure.2.1 Schematic diagram of a modern HPLC system

Now in this thesis, LC separation technique was applied the entire works using a reversed-phase system with a C18 Agilent eclipse plus and C18 Phenomenex Kinetex column and a mobile phase containing methanol or 0.1% formic acid in methanol or acetonitrile in combination water containing 5 or 10mM ammonium formate and 0.1% formic acid. The separation system was coupled to mass spectrometer with an electrospray ionization (ESI) source for analysis of drugs and their active metabolites.

2.4.2 Mass spectrometry (MS)

Mass spectrometry is a technique that can be used for large samples such as biomolecules; their molecular mass can be measured with an accuracy of 0.01% of the total molecular mass of the sample. Structural information can also be generated by using certain type of mass spectrometers usually those which are employed with multiple analyzers which are also known as tandem mass spectrometers. This may be achieved by fragmenting the sample inside the instrument and analyzing the products generated (Nair *et al*., 2010; www.sitemaker.umich.edu/Massspectrometry/sample preparation).

A mass spectrometer is a device that measures the mass-to-charge ratio of molecules or charged particles (*m/z* or *m/q* or *m/Q*) (Kenakin. 1993). In its incarnation for use in bio-specimen analysis it consists of four fundamental components :

1. a sample inlet device that mediates the transition of a solid or liquid bio-specimen into the gaseous phase, a fundamental requirement for all subsequent steps of mass analysis

2. an ionisation device that ionises vaporised bio-samples

3. an ion path that transitions ions from the nearatmospheric pressure of the source into the high vacuum of the actual mass analyser and moves them towards a detector while separating them from each other based on their *m/z*

4. an ion detector to detect and quantify ions

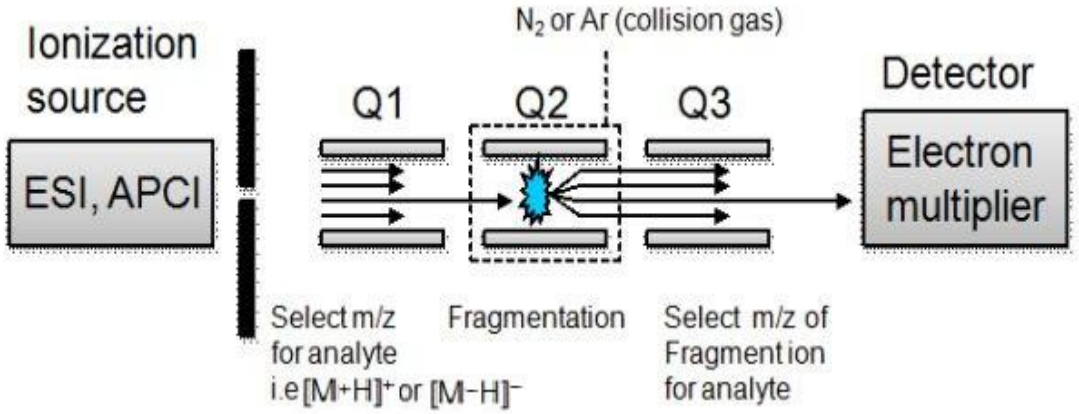


Figure.2.2 Schematic diagram of a triple quadrupole mass spectrometer

Tandem mass spectrometry (MS/MS) gives structural information of a compound by fragmenting specific sample ions inside the mass spectrometer in the Q2 quadrupole which act as a collision cell and identifying the resulting fragment ions that are produced (see Figure.2.2). The analyzers can be of the same or of different types, the most common combinations being quadrupole – quadrupole and quadrupole - time-of-flight (Xu *et al*., 2007; Hoffmann and Stroobant. 2007; Nair *et al*., 2010).

2.4.2.1 Background

Mass spectrometry (MS) is now around one hundred years old. Its basic principles were first described by Nobel laureate Sir Joseph John ('J. J.') Thomson during a lecture he gave to the Cambridge Philosophical Society in 1897 (Thomson. 1897), followed in 1913 by experimental validation, when he separated a stream of ionised neon gas into two isotopic components by applying a magnetic and an electric field to it (Thomson. 1913; Grebe and Singh. 2011). So due to this in the year 1906, Thomson was awarded the Nobel Prize in Physics (Kenakin. 1993). Cameron and Eggers were demonstrated a design as well as mass spectra for a linear time-of-flight (TOF) mass analyzer in 1948; however two decades later, the first commercial instrument was instigated (Kenakin. 1993). In 1953, Paul *et al*. described the first quadrupole mass analyzer and some years later who also explained the ion trap in a patent (Paul *et al*., 1958). The description and development by Paul of these instruments were rewarded many years later (in 1989) with the Nobel Prize in Physics. During 1967-1968, McLafferty *et al*. and Jennings were introduced the collision-induced dissociation (CID) procedure and shortly after the first commercial quadrupole mass spectrometer became available (Kenakin. 1993). Also, Dole *et al*. proposed the initial electrospray based mass analysis system in 1968. In the book "Metastable Ions", Cooks made a remarkable approach in MS/MS (Cooks. 1973) and after one year, first LC-MS system was presented by Arpino, Baldwin, and McLafferty. In late 1980s, the first triple quadrupole instrument was built by Yost *et al*. and four years later, the first commercial implementation became available (Kenakin. 1993).

During the following decades, MS underwent rapid technical development and became a widely used analytical technique in the physical and chemical sciences. However, use for biological specimens remained limited, principally because the ionisation techniques available were only suitable for fairly low molecular weight compounds (~200 Da or less), and because no good methods existed for easy introduction of biospecimens into the high-

vacuum of the mass spectrometer (Grebe and Singh. 2011). This situation changed dramatically in the 1980s when John Bennett Fenn perfected soft ionisation of large bio-molecules by electrospray ionisation (ESI) and developed some of the key technologies that allowed easy sample introduction into the mass spectrometer (Fenn et al., 1989). In 1984, Yamasita, Fenn and Aleksandrov were demonstrated the development of the electrospray to electrospray ionization (ESI) and integration of ESI with MS for small molecule analysis (Yamashita and Fenn. 1984a & 1984b; Aleksandrov *et al*., 1984). Also in this period, the technique was applied for analysis of multiply charged ions from proteins. In 2002, Fenn was awarded the Nobel Prize in Chemistry for his work (http://nobelprize. org/nobel prizes/chemistry/laureates/2002/). Thereafter, the use of MS for biospecimen analysis grew swiftly, initially within research and the pharmaceutical industry, followed by clinical laboratories. The growth of MS use for biospecimen analysis accelerated further during the mid and late 1990s, which saw a shift from gas-chromatography (GC) as a mass spectrometry front-end technology to liquid-chromatography (LC), a technique that allowed much simpler work flows and significantly faster analytical turnaround times (Grebe and Singh. 2011; Vogeser and Seger. 2010). Currently, LC-ESI-MS/MS is one of the most frequently used methods for biochemical, bioanalytical and chemical analysis of different kinds of molecules.

2.4.2.2 Ionization method in MS

Ionization method refers to the mechanism of ionization while the ion source is the mechanical device that allows ionization to occur. The ionization method work by either ionizing a neutral molecule through protonation, deprotonation or cationization by transferring a charged molecule from a liquid phase to the gas phase (Watson and Sparkman. 2007; Kang. 2012). Protonation is a method of ionization by which a proton is added to a molecule, producing a net positive charge of 1^+ for every proton added. Positive charges tend to reside on the more basic residues of the molecule, such as amines, to form stable cations. Peptides are often ionized via protonation. Deprotonation is an ionization method by which the net negative charge of 1^- is achieved through the removal of a proton from a molecule. This mechanism of ionization, commonly achieved via ESI and APCI is very useful for acidic species including phenols, carboxylic acids and sulfonic acids (Kang. 2012).

Cationization is a method of ionization that produces a charged complex by non-covalently adding a positively charged ion to a neutral molecule. While protonation could fall under this same definition, cationization is distinct for its addition of a cation adducts other than a

proton (e.g. alkali, ammonium). Moreover, it is known to be useful with molecules unstable to protonation. The binding of cations other than protons to a molecule is naturally less covalent, therefore, the charge remains localized on the cation. This minimizes delocalization of the charge and fragmentation of the molecule. Carbohydrates are excellent candidates for this ionization mechanism, with Na^+ a common cation adduct. For transform molecules in a liquid phase to ions in a gas phase, separation from LC to a mass spectrometer is required. When LC is used as a separation technique, the main ionization methods used are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), these are atmospheric pressure ionization (API) methods (Kenakin. 1993).

ESI uses electrical energy to assist the transfer of ions from solution into the gaseous phase before they are subjected to mass spectrometric analysis. Ionic species in solution can thus be analysed by ESI-MS with increased sensitivity. Neutral compounds can also be converted to ionic form in solution or in gaseous phase by protonation or cationisation (e.g. metal cationisation) (Ho *et al.*, 2003). The transfer of ionic species from solution into the gas phase by ESI involves three steps: (a) dispersal of a fine spray of charge droplets, followed by (b) solvent evaporation and (c) ion ejection from the highly charged droplets. Within an ESI source, a continuous stream of sample solution is passed through a stainless steel or quartz silica capillary tube, which is maintained at a high voltage (e.g. 2.0 - 6.0 kV) relative to the wall of the surrounding chamber (Figure.2.3). A mist of highly charged droplets with the same polarity as the capillary voltage is generated. The application of a nebulising gas (e.g. nitrogen), which shears around the eluted sample solution, enhances a higher sample flow rate. The charged droplets, generated at the exit of the electrospray tip, pass down a pressure gradient and potential gradient toward the analyser region of the mass spectrometer. With the aid of an elevated ESI-source temperature and/or another stream of nitrogen drying gas, the charged droplets are continuously reduced in size by evaporation of the solvent, leading to an increase of surface charge density and a decrease of the droplet radius (Cole. 1997; Kebarle and Ho. 1997; Ho *et al*., 2003).

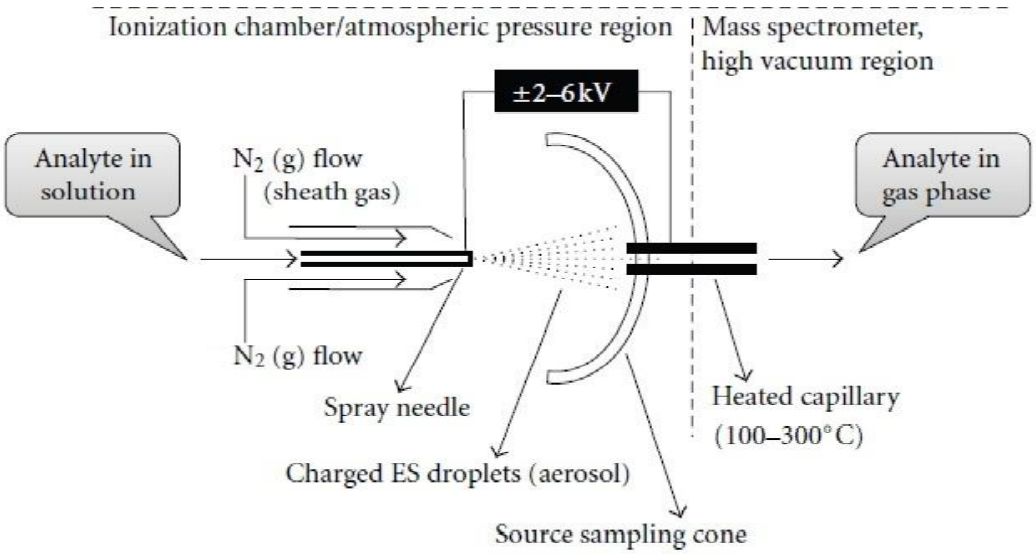


Figure.2.3 A schematic representation of the ESI-ion source

Finally, the electric field strength within the charged droplet reaches a critical point at which it is kinetically and energetically possible for ions at the surface of the droplets to be ejected into the gaseous phase (see Figure.2.4). The emitted ions are sampled by a sampling skimmer cone and are then accelerated into the mass analyser for subsequent analysis of molecular mass and measurement of ion intensity (Bruins. 1998). In this thesis, the whole work was done by only ESI ionization technique.

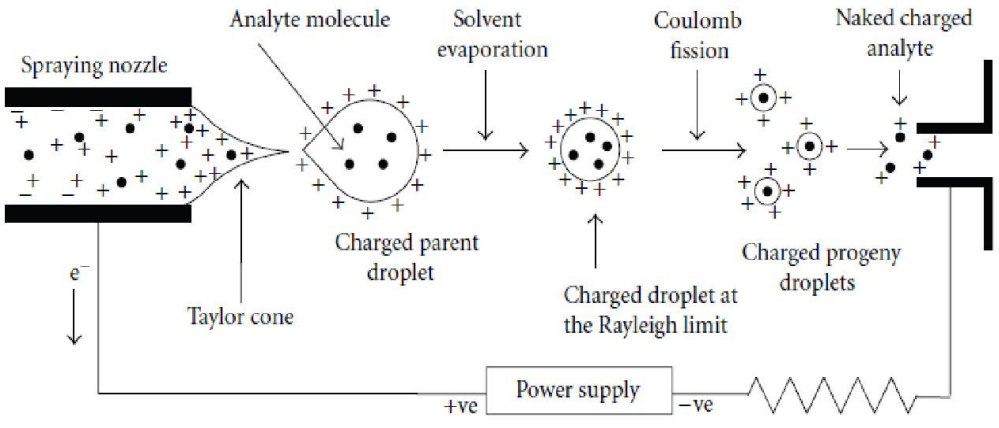


Figure.2.4 Schematic representation of the electrospray ionization (ESI) process

2.4.2.3 Mass Analyzer of MS

The mass analyzer is the heart of the mass spectrometer. The mass analyzer can be compared with the prism. The component wavelengths of a light are separated by a prism, and then they are detected by an optical receptor. Similarly in the mass analyzer, the different types of ions (*m/z*) of an ion beam are separated, and then they are passed to the detector. There are many types of mass analyzers (Hoffmann and Stroobant. 2001; Gross. 2004), for example, magnetic or electric sector mass analyzer, linear quadrupole ion trap (LIT), three-dimensional quadrupole ion trap (QIT) (March. 1997), orbitrap, time-of-flight mass analyzer (TOF), and ion cyclotron resonance mass analyzer (ICR), all of these which use the static or dynamic magnetic/electric field, and all operate according to two fundamental laws of physics, for example, Lorentz force law and Newton's second law of motion. Proper selection of the mass analyzer depends on the resolution (Price. 1991), mass range, scan rate, and detection limit required for an application (Kenakin. 1993). Each analyzer has very different operating characteristics and the selection of an instrument involves important tradeoffs (Shelke *et al*., 2012).

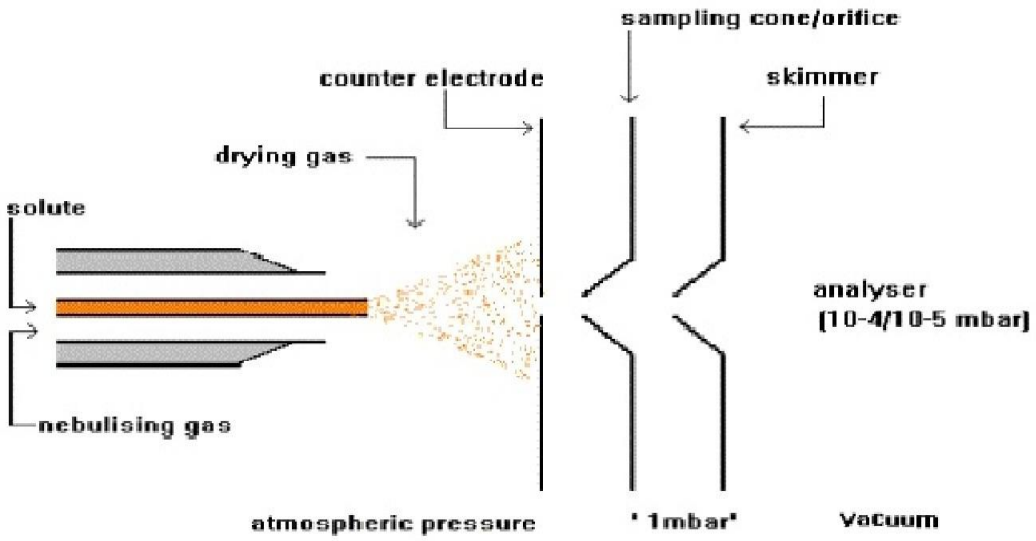


Figure.2.5 The ions enter in the vacuum systems

2.4.2.4 Quadrupole of MS

When ions travel through a magnetic or electrical field, their movement is affected by their *m/z* ratio and this is the main principle of separating ions in MS. A quadrupole mass analyser is most commonly used in both the clinical and the research laboratory. In such system, an assembly of 4 parallel metal rods is kept at equal distance. Each pair of opposite rods is connected electrically. An equal but opposite direct current (DC) voltage superimposed with a radio frequency (RF) voltage is applied to the diagonally placed pair of rods. The resulting electrical field causes the ions to travel forward in the z direction with oscillatory motion in the x-y plane (Figure.2.6). The amplitude of oscillation bears a unique relationship with the *m/z* ratio and can be controlled by changing the DC and RF voltages simultaneously in a pre-fixed ratio. These DC and RF voltages can be set so that amplitudes of oscillation for desirable m/z ratios are "stable" with the ions travelling along the z-axis without hitting the quadrupole rods, and finally reaching the detector. On the other hand, the oscillatory amplitudes of undesirable ions are large and "unstable"; they hit the metal rods, get neutralised, and fail to reach the detector. Quadrupole mass analysers are robust, economical, physically small, and more readily interfaced with a wide variety of inlet systems when compared with other conventional mass analysers like the magnetic sector (Ho *et al*., 2003; Shelke *et al*., 2012; Steel. 1998; Kenakin. 1993).

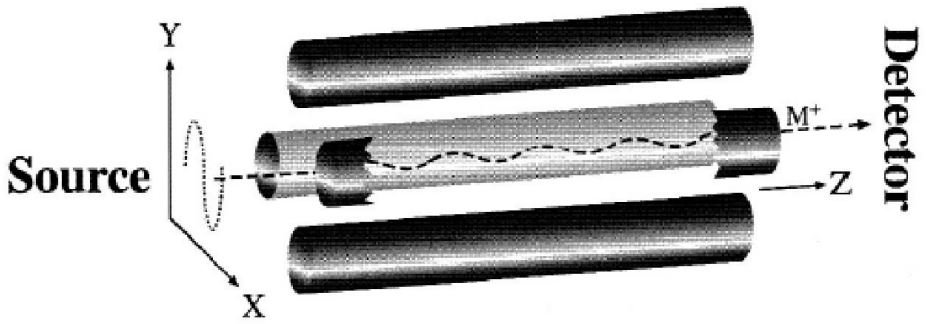


Figure.2.6 Operation of a quadrupole mass analyser. The ion (M^+) travels from the source, through the 4 metal rods arrangement in the unique oscillating pattern, and reaches the detector

In a typical tandem quadrupole system (QQQ) there are three quadrupoles set up in a linear fashion, often called "triplequad" (Figure.2.7). The analyte ion of interest (usually called the precursor ion) is mass-selected by the first quadrupole (Q1) and allowed to collide with a

collision gas (usually nitrogen or may be argon) in a second RF-only quadrupole collision cell (Q2), where the precursor ions are activated by collision and undergo further fragmentation. This process is known as collision-induced dissociation (CID). The product ions resulting from CID are related to the molecular structure of the ions and can be monitored by a third quadrupole mass analyser (Q3) providing structural information of the molecular ions. This tandem system is commonly denoted as MS/MS in the literature. When Q1 is set to select only one specific *m/z* ratio, it filters out other molecular ions having different *m/z* ratios. This is a "purification" step inside the MS system, eliminating complicated and time-consuming sample purification procedures prior to MS analysis (Ho *et al*., 2003 and Rashed *et al*., 1995).

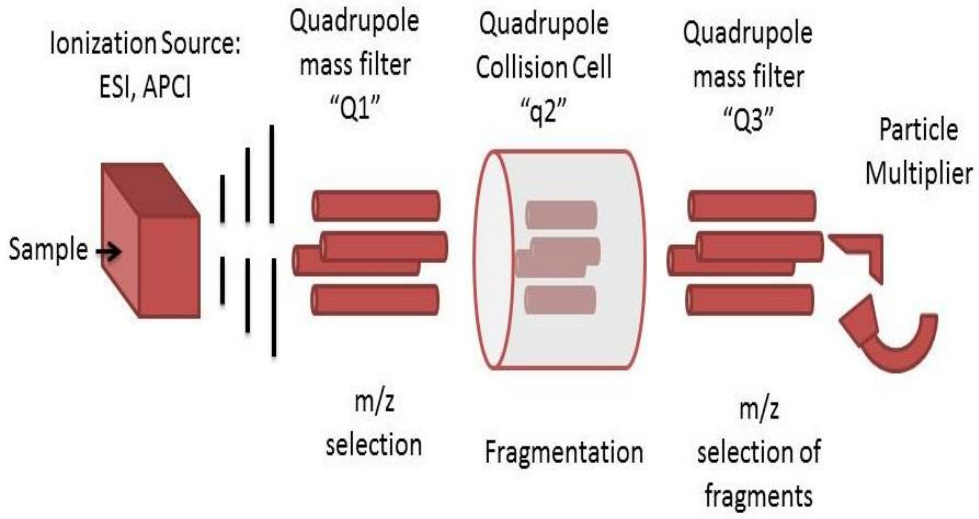


Figure.2.7 Schematic diagram of a triple quadrupole (QQQ) system

The following modes of data acquisition are commonly used in a triple or tandem quadrupole (QQQ) system:

1. **Full Scan:** Scan across the entire (or part of the) mass range of both mass filters (Q1 and Q3) while Q2 does not contain any collision gas – this experiment allows the user to see all ions contained in a sample (Figure.2.8A).

2. **Product Ion Scan:** Select one specific *m/z* in Q1, fill Q2 with collision gas to fragment the selected *m/z*, and then scan across the entire (or part of the) mass range of Q3 – this experiment allows the user to see all fragment/ product ions of the selected precursor ion (Figure.2.8B).

3. **Precursor Ion Scan:** Scan across the entire (or part of the) mass range of Q1, fill Q2 with collision gas to fragment all ions in the scan range, and then select one specific *m/z* in Q3 – this experiment allows the user, by temporal correlation of detection of the product ion and the *m/z* that just preceded its detection, to determine which *m/z* precursor ion(s) might have given rise to the selected product ion (Figure.2.8C).

4. **Neutral Loss Scan:** Scan across the entire (or part of the) mass range of Q1, fill Q2 with collision gas to fragment all ions in the scan range, and then scan Q3 across a predetermined range that corresponds to a fragmentation-induced loss of one specific mass having occurred for every potential ion in the precursor scan range. This experiment allows identification of all precursors that have lost a selected common chemical group, e.g. all precursors that have lost the mass corresponding to a methyl-group (Figure.2.8D).

5. **Selective (or Multiple) Reaction Monitoring (SRM or MRM):** Select one specific *m/z* in Q1, fill Q2 with collision gas to fragment the selected *m/z*, and then select one specific *m/z* of these fragments in Q3 – this experiment allows highly specific detection of an analyte with the *m/z* selected in Q1 that is known to fragment specifically into the product ion selected into Q3. For this specific ion-pair the detection sensitivity is also vastly increased, since the detector now processes primarily (or exclusively) this single analyte-specific ion-pair, and can do so repeatedly over several cycles (Figure.2.8E).

During method development the first three of these experiments are frequently used, (i) to identify the precursor *m/z* of a molecule of interest (Full Scan), (ii) to determine the *m/z* it fragments into (Product Ion Scan), and (iii) to confirm that in a biological matrix only the molecule of interest gives rise to the *m/z* product ion (Precursor Ion Scan) that is selected for the final method. This final method would be MRM (or SRM) in most situations. For select applications, when looking for common metabolites or a family of related compounds, Neutral Loss Scans might be used instead, but well over 90% of final LCMS/ MS methods in research or in clinical use are MRM (or SRM) methods (Grebe and Singh. 2011; Ho *et al*., 2003; Kenakin. 1993).

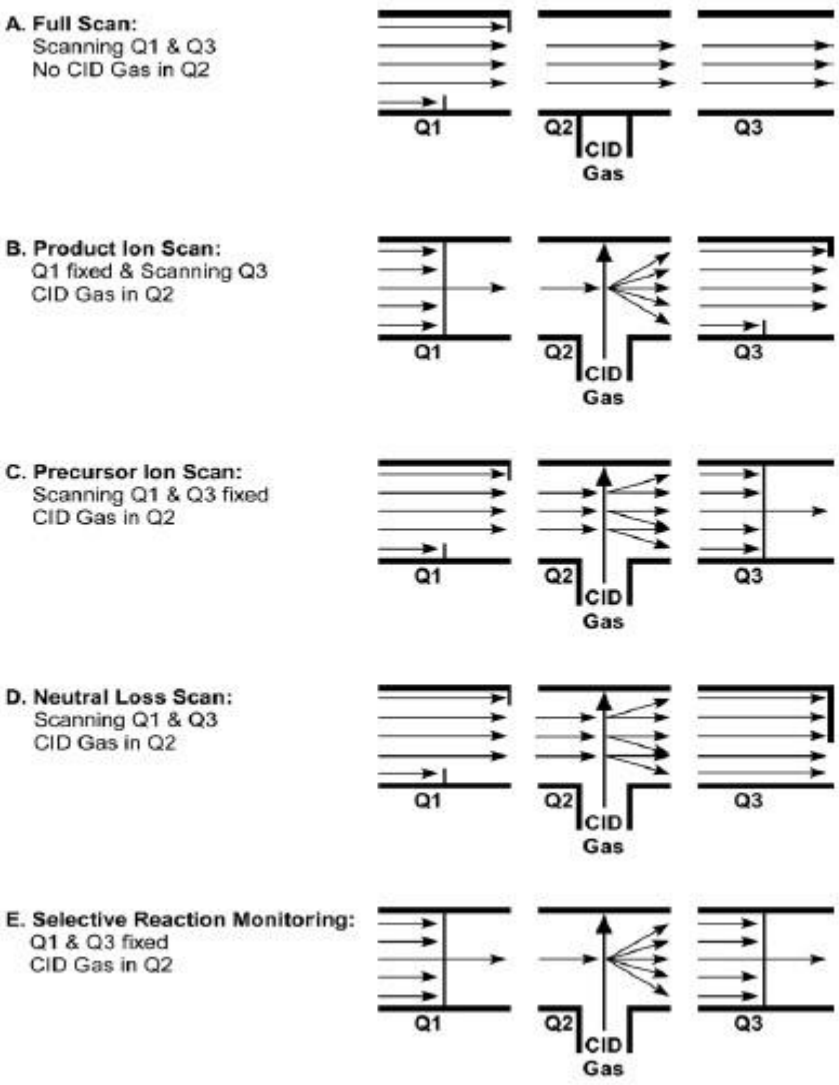


Figure.2.8 The five principal experiments that can be performed with mass filtering tandem mass spectrometers: Full Scan (A), Product Ion Scan (B), Precursor Ion Scan (C), Neutral Loss Scan (D) and Selective (Multiple) Reaction Monitoring (E). CID, collision-induced dissociation

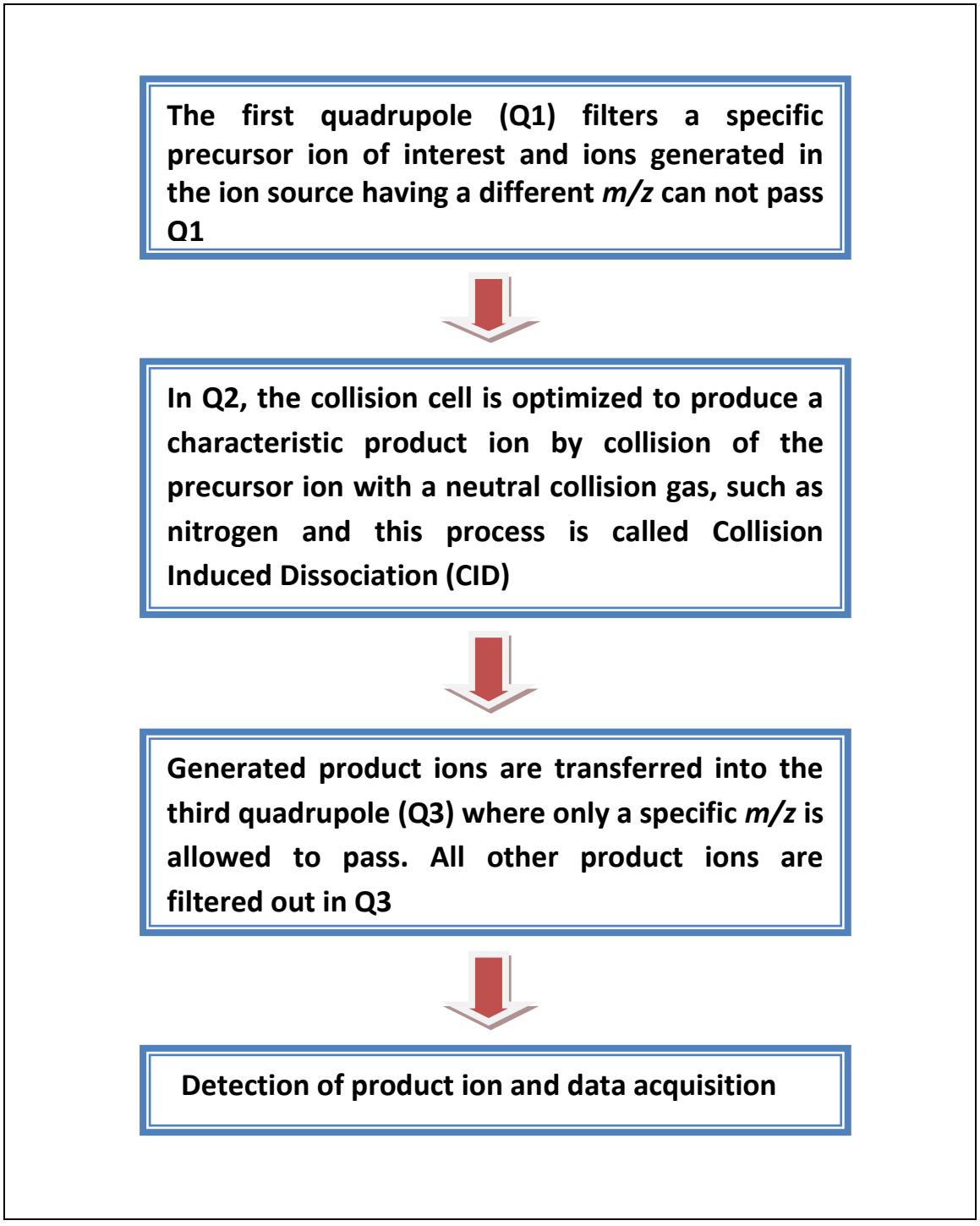


Figure.2.9: Working principles of LC-MS/MS in MRM mode

2.5 Basic approaches in method development

2.5.1 Quantitative analysis by LC-ESI-MS/MS method of drugs and its metabolites

Currently, LC-ESI-MS/MS is considered as the method of choice for quantitative analysis of drugs and their metabolites. The advantages of using this technique in MRM mode due to high sensitivity, selectivity and speed allow developing high throughput methods with little or no sample preparation and minimal chromatographic retention (Roškar and Lušin. 2012; Eeckhaut et al., 2009; Taylor. 2005). During analysis, selection of the right experimental conditions are very essential due to obtaining the proper knowledge about different areas of science like chemistry, biochemistry, physics etc. In this way there are various parameters which affects the analysis and that has been already reported. Basically the LC system, the ESI ion source and the mass spectrometer, these three major portions are considered for this interpretation (see Figure.2.10).

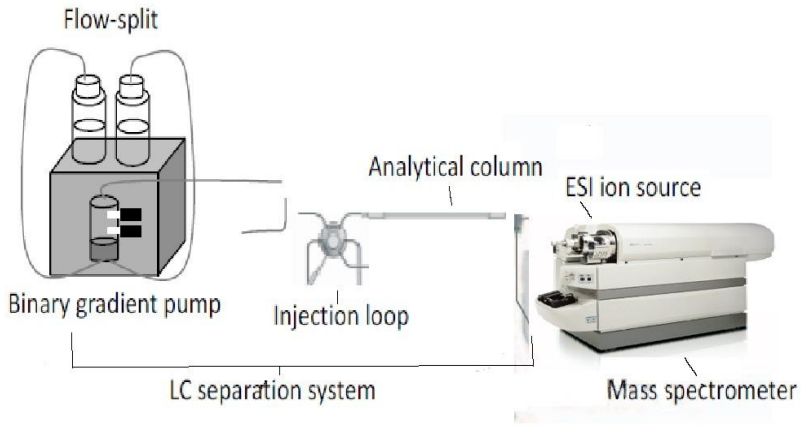


Figure.2.10 A schematic presentation of LC-ESI-MS/MS showing the LC separation system, the ESI ion source and the mass spectrometer

The retention of analyte(s) is completely affected during the chromatographic run by some crucial parameters such as the mobile phase composition, the flow rate, the type and dimension of the separation column with the applied temperature in the column oven or not (Rosés. 2004 and Subirats *et al*., 2004). Precisely, at ambient temperature, the selectivity of the mobile phase is determined usually from the pH and the amount and type of organic modifier in isocratic elution reverse phase LC (RPLC) and also its affected by the type and ionic strength of the buffer (Muinasmaa *et al*., 1997; Rosés. 2004; Subirats *et al*., 2004).

Table.2.2 Some experimental parameters for the analyte(s) during LC-ESI-MS/MS analysis

Component	Impact	Experimental Parameters
LC system	LC separation	Organic solvent type and concentration, gradient elution, flow rate, buffer type, pH, packing material and column dimension, column oven temperature
ESI ion source	Ionization efficiency	Organic solvent type and concentration, applied voltage, positioning of the ESI, flow rate, buffer type, pH and concentration

2.5.2 Mobile phase selection

Typical solvents used in reverse phase LC (e.g. water, acetonitrile, methanol, ethanol, chloroform) are compatible with ESI. It should noted that a grade of solvent (including water) that is suitable for a conventional LC separation may not always be suitable for an LC-MS/MS based separation e.g. the solvent may be UV transparent to 210 nm but may contain traces of compounds that affect the quality of LC-MS/MS measurements. For example, one study found large differences between commercial grades of methanol (Annesley. 2007 and Pitt. 2009). There is a solvent-selectivity triangle which comes to choosing amongst different organic solvents. This triangle offers excellent means of identifying solvents with different selectivity (Canals *et al*., 2001). In the triangle, frequently used solvents are placed according to their a) acidity, b) basicity and c) dipolar properties. Also, from the solvent-selectivity triangle it is easily realized that methanol, acetonitrile and tetrahydrofuran display different solvent features. In reverse phase chromatography, water is typically the most abundant solvent for mobile phase. So therefore, for any LC-MS/MS analysis ultra pure water is an essential ingredient. Commercial water system produces 18.2 $m\Omega cm^{-1}$ quality water that indicates there is no ion present in the water. But, often the quality of trace organic material is

unknown. Within the purification system, the growth of bacteria can affect the final quantity of the water due to its contamination with trace organics.

During LC method development, a reasonable approach is to try all these three solvents (methanol, acetonitrile and tetrahydrofuran) or mixtures and in order to promote the formation of small droplets and aid evaporation during the ESI process. In this way, the organic solvent(s) used in the mobile phase(s) should have relatively low surface tension and heat of vaporization. Higher organic content of the mobile phase can result in improvements in ionisation efficiency in ESI, and this may affect the choice of separation mode. But, very high amount of organic solvent (more than 80 %) might actually result in a decreased ESI response (Bosch *et al.*, 1996). For example, polar molecules are poorly retained on reverse phase columns and elute at the beginning of the chromatogram with a low organic content. This will result in relatively poor ionisation efficiency, and polar interferences are more likely to co-elute in this region causing ion suppression. One way this can be overcome is to use hydrophilic interaction chromatography (HILIC) in which polar analytes are retained and elute with higher organic content of the mobile phase (Martens-Lobenhoffer and Bode-Böger. 2006; Oertel *et al.*, 2004). By force the analyte ions are distributed to the droplet surface and also the low solvent dielectric constant is desirable. Further, the proton affinity another chemical properties of the organic solvent will affect the production of ions in the gas phase (Canals *et al.*, 2000). So, methanol and acetonitrile are the most commonly used organic solvents for LC-ESI-MS/MS method and also have advantageous chemical properties for ESI.

2.5.3 Selection of additives and buffers for mobile phase

The additives and buffers can serve to protonate basic molecules when operating in positive ion mode and vice versa for acidic molecules in LC-ESI-MS/MS method. Buffer solution reduces the change of pH for addition of small amounts of acid or base, or any organic, inorganic and biological compounds. Normally, volatile buffers and additives (e.g. ammonium acetate, formate or hydroxide, formic or acetic acid or trifluoroacetic acid) are preferred to the non-volatile ones (e.g. phosphates, sulphates, borates and citrates). In reality the non-volatile eluent components can be deposited on the ion surface and leading to contamination of the electrodes inside the ion source. At the electrode surface, this type of contamination reduces the effective charge density emulating a lower voltage for the electrode. So, in the signal intensity a reduction will be observed over time. While, volatile

buffer and additive must be used at a very low concentration and consistently with chromatographic resolution (Mannur *et al*., 2011; Billiet and Galan. 1989; Vailaya and Horváth. 1998). On the electrosprayed droplets, buffer and additive species compete for surface site and an increase in their concentration leads to decrease in the analyte signal. So, the competition for surface sites of the droplets favours the higher concentration species and, in this way, analyte species become exhausted. Due to this compound dependent effect, some analytes show only a small decrease of signal while for others the response can be decreased much more (Mannur *et al*., 2011; Muinasmaa *et al*., 1997 and Espinosa et al., 2002). In 2004, Mallet *et al*. demonstrated an interesting study on the ion suppression or enhancement due to additives and the buffer of mobile phase.

But, the use of inorganic ions buffers should be avoided such as phosphate and sodium acetate. Because, these cause significant ion suppression, can create MS adducts of sodium and potassium and also can quickly contaminate the ion source. Substitution of buffers that are more suitable and compatible with MS such as ammonium acetate, ammonium formate or ammonium bicarbonate is generally possible (Pitt. 2009). However, these buffers also cause ion suppression so the concentration of buffer used should be the low or minimum required to produce satisfactory chromatography. Trifluoroacetic acid and other fluorinated carboxylic acids, such ion pairing reagents can occur ion suppression so their concentrations should also be minimised as much as is practical. Volatile buffers can create some adducts during ionization in the source in different ion modes. At higher buffer concentrations, ammonium adducts can be observed in positive ion mode and formate or acetate adducts in negative mode. For the analyte, formation of adduct is detrimental only in that it can cause greater variability and loss of detection sensitivity. Sometimes by increasing the source temperature, voltage, or both, these adducts can be destroyed (Cappiello *et al*., 1997; Mannur *et al*., 2011; Mallet *et al*., 2004).

When preparing low pH mobile phases, formic or acetic acid concentrations of 0.1–1% (v/v) are recommended to enhance ionization in electrospray. For protein and peptide separations, trifluoroacetic acid is preferred but careful and should be avoided when negative ion mode is utilized. For high pH mobile phases, ammonium hydroxide or triethylamine (in few cases) are recommended. 0.1% acid should be mixed with the organic component for basic compounds, while water or neutral buffer should be used for neutral or acidic species. Ammonium acetate or formate, ammonium bicarbonate or carbonate buffers can be used with concentrations ranging from 2 to 50 mM, whereas a maximum concentration of 10–20 mM is

recommended due to ion suppression. The acceptable buffer concentration that does not adversely affect ionization has been observed to be highly compound dependent. So, a useful rule of thumb is to use as low a concentration of buffers as possible to give reasonable chromatographic performance (Mannur *et al*., 2011; Temesi and Law. 1999).

Table.2.3 pKa values of common mobile phase additives

Compound	**pKa**
Trifluoroacetic acid	0.3
Phosphoric acid	2.15, 7.20 & 12.33
Citric acid	3.13, 4.76 & 6.40
Formic acid	3.75
Acetic acid	4.76
Propionic acid	4.86
Carbonic acid	6.35 & 10.33
Tris	8.06
Boric acid	9.23
Ammonia	9.25
Glycine	9.78
Triethylamine	10.72
Pyrrolidine	11.27

2.5.4 Selecting the right pH for mobile phase

When preparing mobile phases slight variations in pH can have a dramatic impact on separation and retention and also consistent certain techniques should be employed to ensure good reproducibility. Usually, due to the presence of ionisable impurities it is equally

important to control pH when working with field samples of non-ionisable compounds. Samples with ionisable compounds are strongly affected by pH of the mobile phase. pH values of the mobile phase are usually between 2 and 7.5 in reversed-phase chromatography. The sample solution is outside this pH range or when the analyte is ionisable under reversed phase conditions because buffers are required (Mannur *et al*., 2011; Rosés 2004 and Subirats *et al*., 2004). Generally under reverse phase conditions, analytes ionisable have amine or acid functional groups with pKa's between 1 and 11. The ionisable function group is in a single form, whether ionic or neutral due to correct pH value of the buffer. The buffer will quickly bring the pH of the injected solution to a less harmful pH if the sample solution is at pH damaging to the column (Bosch *et al*., 1998 and Mannur *et al*., 2011).

If required, the pH of the mobile phase should be adjusted by adding reagent before diluting to final volume and prior to blending of any organic solvents. For example, blending 25% methanol will raise the apparent, measure pH of the combined mobile phase by about 0.5 pH units. Alternatively, equimolar solutions of different ionic forms of the same buffer can be blended to reach the desired pH. It is desirable to select a mobile phase with a final pH at least one pH unit away from any analyte's pKa value to cause ionization or suppression of the analytes when developing a rugged method (Mannur *et al*., 2011). To evaluate the change in retention due to the degree of ionization of the solute, a common recommendation is to try two different pH values (Yamashita and Fenn. 1984a). Generally, there is some hypothesis in this because the effect of type and concentration of organic solvent on either mobile phase pH of solute pKa values is absolutely unknown. Mobile phase pH is very crucial and should be selected at least ± 1.5 pH units from the analyte's pKa, i.e. one at pH = pKa + 1.5 of the solute and one at pH = pKa – 1.5. This assures that the analytes are either 100% ionized or 100% non-ionized. Acidic compounds are ionized and are much more hydrophilic than under ion suppression conditions at high pH level. These conditions actually for fast analysis and low retention (Aleksandrov *et al*., 1984; Mannur *et al*., 2011; Billiet and Galan. 1989; Snyder *et al*., 1997; Vailaya and Horváth. 1998; Rosés. 2004).

Table.2.4 Common buffers with their pH range and LC-MS/MS compatibility

Buffer	pH range	LC-MS/MS compatible
Phosphate	1.1 – 3.1 6.2 – 8.2 11.3 – 13.3	No
Ammonium acetate	3.8 – 5.8	Yes
Citrate	2.1 – 4.1 3.7 – 5.7 4.4 – 6.4	No
Trifluoroacetic acid (0.1%)	2.0	Yes
Phosphoric acid (0.1%)	2.0	No
Formic acid (0.1%)	2.7	Yes
Ammonium formate	2.7 – 4.7	Yes
Ammonium bicarbonate	6.6 – 8.6	Yes
Borate	8.3 -10.3	Yes

2.5.5 Selection of gradient elution

Mobile phase composition is programmed and applied in different experimental parameters that increase in gradient elution. The gradient shape and the composition for the slope or multiple slopes (in segmented gradients) or with some pumps and possible curvature are measured. There are different collective approaches have been mentioned in some literatures that to facilitate the selection of the mobile phase composition (Beynon and Easterby. 1996; Mallet *et al*., 2004; Petritis *et al*., 2002). During LC method development, the use of retention models to demonstrate retention characteristic of the various solutes using different

chromatographic conditions. Development of retention models for the determination of solute elution characteristic utilizing in different modes of LC, for example in normal-phase, reversed-phase and micellar liquid chromatography (García. 2005; Snyder *et al*., 1993; Cech and Enke. 2001; Amad *et al*., 2000; Quarry *et al*., 1986).

2.5.6 Stationary phase or column selection

It is necessary to understand the properties of column packing material for the selection of stationary phase (i.e. column). Above pH 8, silica tends to dissolve and cross-linked polymeric particles, such as polystyrene or polymethacrylates are used for separation of bases that can resist strongly basic mobile phase. Silica particles have surface silanol (-SiOH) groups, which are used for chemical bonding of stationary phases by silanization reactions with chlorosilanes. Almost half of the silanol groups are chemically bonded and the rest are capped with tri-methyl silyl groups to render them inert. In reversed phase chromatography, the most commonly used non-polar bonded phases are C18 and C8. C18 is also known as ODS for octadecylsilane being the most popular with 18-carbon aliphatic chain and in hydrophobicity, C8 is intermediate where C18 is non polar. Phenyl groups are useful [R= $(CH_2)_3$ C_6H_5] to increase in polarity leads to decrease in retention (Nair *et al*., 2010; Schoenmakers *et al*., 1978; Dolan *et al*., 1979; Chandrul and Srivastava. 2010). For separation of non-polar analyte, C18 (Octaldecylsilane – ODS) bonded ligand is actually very useful. In otherhand, C8 bonded ligand provides similar selectivity and shorter run times. As compared to longer chain C18 and C8 counterparts, C3/C4 bonded ligand is not so stable, but they provide good separations for a variety of protein and polypeptide analytes. CN (Cyano) bonded ligand interacts with the polar functional groups and is used in both reversed-phase and normal phase chromatography (Snyder *et al*., 1997 and Nair *et al*., 2010).

Silica or polymeric particles with a coating of long chain saturated hydrocarbons or other non-polar functional groups are in reversed phase columns. Water, methanol and acetonitrile are used as most common solvents. Between the mobile and stationary phase, the partitioning of an analyte depends upon hydrophobic interactions between the sample and the mobile phase. Small polar molecules elute very fast than large ones (Hoffmann and Stroobant. 2007; Nair *et al*., 2010). Classical alkyl bonded silica chemistry (typically C18) with the addition of a polar function group for specialization of phases which are modified, such as an amide or a carbamate group, within the alkyl chain or with a polar functional group. Compared with the use of the classical alkyl bond phases, these modifications provide increased retention for

polar analytes, give good peak shapes, and allow higher sample throughput because of faster mobile phase re-equilibration (Ghrist and Snyder. 1988; Snyder and Dolan. 1996; Jandera. 1989).

2.5.7 Selection of internal standard (IS)

The selection of a suitable internal standard (IS) is one of the key parameters for establishing a successful LC-MS/MS method. At the preliminary stage in the bioanalytical process, the internal standard (IS) compound is added to the sample to act as a measure of recovery in the isolation of components, generation of ions and mass selection. Actually, it is used to reduce the analytical variations. Care should be taken to achieve equilibration with the analyte throughout the sample. First of all an internal standard (IS) should be added at the beginning of the sample work-up, typically before the plasma crash or solid phase extraction and also at the same level in every sample including the standards. An IS should give a reliable MS response (Kang. 2012 and Honour. 2011). The amount of the IS should be well above the limit of quantitation but not so high as to suppress the ionization of the analyte. So, during method development it should be checked that no interference with the performance of the developed method and the intensity of molecular ion peak in mass spectrometric analysis remained unaffected as compared to others.

Generally, as IS either a stable isotope labeled-IS or a structural analogue can be applied. Although, the analyte and the ionization of the analogue IS may be differently affected by the matrix. This can be solved by using a stable isotope labeled-IS which co-elutes with the drug or metabolite, because matrix effects should not affect the relative efficiency of ionization of the drug (or metabolite) and its stable isotope labeled-IS. The stable isotope labeled-IS are compounds in which several atoms in the analyte are replaced by their stable isotopes, for example ^{2}H (D-deuterium), ^{13}C, ^{15}N or ^{17}O with ^{2}H being the most frequently used isotope. It is crucial that the mass difference between the analyte and the stable isotope labeled-IS is at least 3 mass units (Stokvis *et al*., 2005 and Honour. 2011) and to avoid signal contribution of the abundance of the natural isotopes to the signal of the IS. If the compound and IS are not separated adequately by mass then this will result in quadratic standard curves (http://www.ionsource.com/tutorial/msquan). The IS should be pure (ideally > 95% but can be as low as > 85% with appropriate verification) and must have minimal contamination with the target analyte. In all aspects of sample treatment and mass spectrometric analysis, the analyte and IS fundamentally act in a similar manner chemically. A compound is structurally

related to the analyte may have to be used as IS, which also may elute separately from the compound of interest, accordingly losing any carrier effect of a true IS in few cases. In this way, an isomer, homologue or derivative of the analyte may be used. A demethylated or hydroxylated analogue should be avoided as IS because these are common transitions of metabolites of the analyte. Though, some issues like isotopic purity of compounds, cross-contamination and cross-talk between MS/MS channels, isotopic integrity of the label in biological fluid and during sample processing, etc. should be carefully mentioned (Matuszewski *et al*., 2003). The ^{13}C, ^{15}N or ^{17}O-labeled IS may be more perfect than the ^{2}H-labeled ones, because deutrium and hydrogen have greater differences in their physical properties than ^{12}C and ^{13}C (Wang *et al*., 2007). During method development, it is basic importance to select an appropriate IS and to closely monitor the method performance in routine use, because only limited lots of biological matrix are tested during method validation (Kang. 2012 and Wang *et al*., 2007). The signal to noise may decrease to a point where accuracy and precision may be negatively affected when ion suppression significantly reduces the signal of analyte and/or of the IS (Annesley. 2003). However, this is not always reasonable that a number of labeled IS identical to the number of compounds to be analyzed would be needed (Hernandez *et al*., 2005).

Now, "How much internal standard should be added?", this is an important question. It pays to know roughly how much compound is in the sample. This can be accomplished by making trial analyses of an early, middle and late time point with perhaps one or two standard points. This information will be very valuable when building an appropriate standard or calibration curve and in knowing how much internal standard to add. So, the concentration of IS used for sample preparation is also important and should be approximately at the middle of standard or calibration curve. Optimization of IS concentration is critical to avoid ion suppression by co-eluting analyte leading to standard curve non-linearity (Roškar and Lušin. 2012; Bakhtiar and Majumdar. 2007). For example, if trying to quantify samples in the range of 1 ng to 100 ng and the limit of detection is 1 ng it might add 20 to 25 ng of internal standard to every sample. A good rule of thumb is to target the internal standard to the lower 1/3 of the working standard curve. This is a range that will give a comfortable response without interfering with the ionization of the analyte.

2.5.8 Optimisation principles for hyphenated methods

A significant number of conditions and parameters need to be taken into account and optimised in setting up an LC-MS/MS method. On the nature of the analyte, the actual conditions are highly dependent and making it difficult to give generic conditions for the LC separation. So, individual optimisation of each analyte is required (Morgan and Deming. 1975; Massart *et al*., 1997; Siouffi and Phan-Tan-Luu. 2000). However, reported methods are a valuable starting point, assay performance and optimal conditions can vary significantly between different instruments and sample matrices (Pitt. 2009). Sensitivity is highly dependent on the instrument used and the method conditions. Manufacturers of the instrument are frequently improving the sensitivity of their mass spectrometers and normally offer a range of models with different sensitivities. Therefore, it is vital to assess if an instrument has the required sensitivity to achieve the precise limits of detection. The nature of the analyte will primarily determine the optimal ionisation method and polarity used. By derivatisation, detection of some analytes can be enhanced (Gao *et al*., 2005; Zaikin and Halket. 2006; Johnson. 2007). So, free steroids give comparatively weak responses using ESI since they lack a functional group capable of carrying charge. In 2005, Johnson reported a method on derivatisation of the keto group of keto-steroids like 17α-hydroxyprogesterone that give a much higher electrospray response in lower detection limits.

With a dilute solution of the analyte, ion source parameters and collision energy can be optimised during continuous infusion and ideally in the same mobile phase used for the LC separation. Proper monitoring must be required during the selection of fragment ion because the most abundant fragment may not always be the best choice. It is desirable if other significant fragment ions occur then evaluate them as they may actually provide cleaner chromatograms with superior signal-to-noise ratio (S/N) and also to monitor a second fragment ion to check for possible interferences. Now, the ratio of the two fragment ions is different to that of the standard if any interference is there. Literally, in tandem MS (MS/MS) absolute signal can be decreased due to losses in the collision cell and so the signal may be propagate across several fragments when only one or two are being detected. The advantage of tandem MS is its very high specificity, that means chemical noise is significantly reduced and the signal-to-noise ratio (S/N) is basically superior to single MS despite the decrease in signal (Pitt. 2009).

2.5.9 A step-by-step optimization procedure

The practical procedure is not so desirable during development of a bioanalytical method followed by several optimizing approaches. In MS operation, the optimization strategy preferred aims at better chromatographic quality as well as high sensitivity concerning high signal or high signal-to-noise ratio (S/N). Screening experiments are performed for the identification of precursor ions and product ions by applying different mass parameters during MS/MS method development in the initial stage. The initial sets of conditions are evolved during the method development which improved and maximized in terms of resolution and peak shape, plate counts asymmetry, capacity, elution time, detection limits, limit of quantitation and overall potential to quantify the specific analyte of interest. In optimization, a method can follow either of two general approaches such as manual or computer driven (Nair *et al*., 2010).

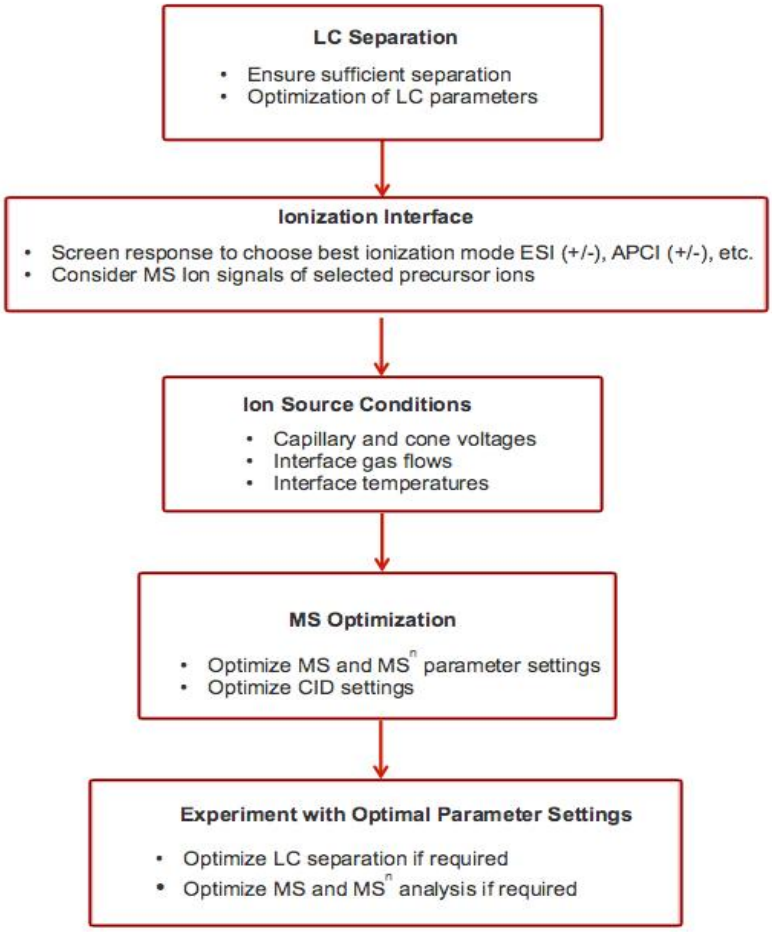


Figure.2.11 Flowchart of the step-by-step optimization procedure

At a time, the manual approach includes varying one experimental variable while belongings all others constant and recording the changes in response. These variables are flow rates, mobile phase and its composition, stationary phase or column, temperature etc (Kimanani. 1998). In this approach, system optimization is slow, time consuming and potentially expensive. In otherhand, computer driven automated methods development provides a much better understanding of the principles and theory involved of interactions of the variables. Efficiency of this approach is optimized but experimental input is minimized. Also, it can be applied to many applications like capable of significantly reducing the time, energy and cost of virtually all-instrumental method developed (Sethi. 2001). During method development, the various parameters that essential to be optimized such as mode of separation, selection of stationary phase or column and selection of mobile phase.

2.5.10 Sample preparation techniques

Detection and quantification of drug or its metabolite in biological matrices are usually complicated by the matrix effects. Due to this, various types of cleanup procedures involving solvent extraction and chromatography are employed to effectively separate the drug components from endogenous biologic material. This cleanup process is also known as sample preparation (Nair *et al*., 2010; Roškar and Lušin. 2012). Sample preparation with adequate volume is a crucial aspect of quantitative bioanalysis and normally it is the most time consuming part of analysis. It deliberate to prepare a reproducible and homogeneous solution that is suitable for injection onto the column (Rosing *et al*., 2000). So, the aim of sample preparation is to get a sample aliquot which is free of interferences because of matrix effect and should not produce any damage to the column and need to be compatible with the method. Interfering matrix compounds like proteins, lipids, salts, other endogenous and background compounds should be removed in sample as pretreatment. Not only to avoid column clogging and instrument soiling, but also to improve the sensitivity, selectivity and reliability of analysis by this process (Roškar and Lušin. 2012). A sample treatment procedure should produce quantitative recovery of analytes, include a minimum number of steps and be easily automated. For analyte quantification, selection of an appropriate sample preparation procedure depends upon drug or metabolite characteristics, their expected concentrations, the sample size and matrix, and the availability of analytical techniques. When using spectroscopic detection techniques like UV-absorbance or fluorescence, inadequately treated samples may cause interfering peaks. But, analysis by LC-MS/MS methods are less susceptible to sample matrix and so generally require less affected sample

clean up (Kang. 2012; Honour. 2011; Naidong *et al*., 2005; Roškar and Lušin. 2012). Frequently and conventionally applied sample preparation techniques are protein precipitation (PP), liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Manual operations combined with sample treatment may be very labor intensive and also time consuming process. So, this could be avoided with automation in 96-well plate format or direct sample injection followed by on-line extraction methods (Roškar and Lušin. 2012; Kang. 2012; Naidong *et al*., 2005).

2.5.10.1 Protein precipitation or denaturation technique

Protein precipitation technique (PPT) is very simple and uncomplicated method most commonly used in bioanalysis of plasma samples for the processing of biomolecules or biological products such as proteins. This technique is normally induced by addition of a neutral salt like ammonium sulfate which compresses the salvation layer and increases the protein-protein interaction or by changing the pH to alter the nature of the solution. Generally, it is done by using some organic solvents like acetonitrile or methanol or acetone or some acids like perchloric acid or trichloroacetic acid or may be trifluroacetic acid and salt such as zinc sulphate or by proteolysis enzymes etc (Nair *et al*., 2010; Xu *et al*., 2007; Nováková and Vlcková. 2009). In this process an exact volume of denaturating solvent (often two parts of denaturant to one part of sample) is added to a sample containing protein and cellular components. Protein denaturation is facilitated by vortexing, and centrifugation, allowing collection of the denatured protein into a pellet at the bottom of the vessel and a supernatant is formed (Polson *et al*., 2003). This process is typically known as phase separation. So, denaturation is significant due to the presence of proteins, lipids, salts, and other endogenous material in the samples that cause rapid deterioration of LC-MS/MS columns and also interfere with the method (Ahuja and Rasmussen. 2007).

Analytes are usually released from the proteins and remain in the supernatant liquid. This supernatant can be directly injected or pre-concentrated after evaporation and reconstitution. While only proteins are removed and other endogenous compounds remain which can still cause interferences like matrix effect in MS analysis. PPT can be easily automated because of very fast and rapid sample preparation. This method has been also applicable for quantification of drugs and metabolites from whole blood (Koseki *et al*., 2006). PPT is also suitable for other biological samples such as urine. Furthermore, these matrices allow direct injection without sample pretreatment due to the absence of proteins. However, it is desirable

to dilute and filter or centrifuge the samples to reduce matrix effect and to remove ultimately present particles (Ketola and Hakala. 2010). PPT is also the most suitable technique for less complex biological matrices in pharmacokinetic studies like hepatocytes or microsomes (Hakala *et al*., 2009 and Lušin *et al*., 2012). In the same way protein precipitation by ice-cold methanol (triple volume) at the same time terminates the incubation reaction and introduces IS to the final sample.

2.5.10.2 Liquid-liquid extraction technique

Liquid-liquid extraction (LLE) technique is based on the principles of differential solubility and partitioning equilibrium of analyte molecules between the organic phases and aqueous i.e. the original sample. Basically, this process requires the extraction of a substance from one liquid phase to another liquid phase (Kazakevich and Lobrutto. 2007; Steinborner and Henion. 1999). Usually for a sensitive analysis with a complex biological media such as plasma or urine, LLE is needed instead of PPT. In comparison with solid phase extraction (SPE), sometimes it gives exceptional sample clean up showing less matrix effect (Bakhtiar and Majumdar. 2007). In this technique using a separating funnel or tube with addition of an immiscible organic extraction solvent pursued by agitation (handmixing or vortexing) to facilitate equilibrium portioning of analyte molecules between phases. Then the phases are separated and the aqueous component is discarded. Next the organic phase is completely evaporated to dryness and re-suspended or reconstituted with mobile phase or a similar solvent system. Then collected in autosampler vials and injected into the system.

Moreover, LLE technique is generally simple procedure and may be applicable in the laboratories using different variety of organic solvents. Also, LLE is very economical or cost-effective and adaptable as several samples may be prepared in a uniform way. It should be considered for emulsion formation, mutual solubility of analytes in both phases or large volumes of flammable and toxic solvents. Plenis and Baczek demonstrated some methods for measurement of antidepressants and their metabolites in biofluids by different extraction procedures including LLE (Plenis and Baczek. 2011). But, LLE is very monotonous and time consuming and also the risk of sample loss and contamination is high. On the other hand lack of automation possibilities is an another important drawback of LLE. Though, different research groups have developed different strategies to solve mixing and phase separation problems that typically seen in a 96-well plate LLE method (Xu *et al*., 2007; Steinborner and

Henion. 1999; Shen *et al*., 2002). Li *et al*. (2007) reported a semi automated LLE procedure by using 96-well plates.

2.5.10.3 Solid-phase extraction technique

Today, solid-phase extraction (SPE) technique has become very well known and is considered as a basic approach for sample preparation of drugs and their metabolites from biological matrices in many laboratories. From a wide variety of matrices, SPE is used to isolate analytes of interest, such as blood, plasma, urine, water samples, beverages, soil, animal tissue and consumer products. SPE has various advantages over LLE, like higher recoveries, without any problems in emulsions, little solvent consumption and a smaller sample volume demand. The separation is occurred due to the affinity of desired analyte towards stationary phase. After retained on the stationary phase, the analyte is eluted from the solid phase extraction cartridge with the proper solvent (Nair *et al*., 2010; Roškar and Lušin. 2012; Naidong *et al*., 2005). A commercial solid phase extraction cartridge is short inert plastic tube packed with an adsorbent which is basically a reversed phase or an ion exchange resin. To ensure a rational permeability, the particle size of the packing is often significantly larger than that used in LC columns (Xu *et al*., 2007).

The standard procedure is loading of a sample onto a SPE cartridge after conditioning with water and methanol. Then washing of the sample solution containing the analyte or sample matrix (commonly used solvent is HPLC grade water or Milli-Q water) for removing any unwanted components and eluting out the actual analyte into a test tube. In SPE there is the same type of stationary phase as it is used in LC columns. The stationary phase is in a glass or plastic column above a frit or glass wool. The column might have a frit on top of the stationary phase and also have a stopcock to control the flow of solvent through the column. Normally, SPE cartridges are in the range of 1-10 ml in volume and are discarded after use. The concept of SPE is similar to that of liquid chromatography, but different in applications. This technique involves partitioning between a liquid phase which is a sample matrix or a solvent with an analyte and a solid phase i.e. packing bed or sorbent. By this technique enables the concentration and purification of analyte from the sample matrix due to adsorption on the packing bed then purification of extract after extraction is finished (Nair *et al*., 2010; Kang. 2012 and Honour. 2011).

Additionally, automation of sample treatment with high speed and feasibility for treatment of many samples at a time is possible. SPE has some demerits or drawbacks such as high dead

volume that can lead to loss of sample and may cause dilution of applied samples, clot formation variability in column or blockage due to sample viscosity or precipitation may also occur. For manual use, columns can be supplied as individual units and also in 96-well plate format for use with robotic sample processors. A novel 96- well SPE plate has been designed to minimize elution volume (not more than 25μL) and so the column dead volume has been overcome. Because of the concentration ability of the sorbent the evaporation and reconstitution steps are avoided in SPE (Xu *et al*., 2007). Different types of cartridge and solvents make SPE procedure suitable for many polar or nonpolar analytes. Except classical reverse phase (e.g. C18 or C8) also polymer reverse phase (e.g. divinylbenzene, N-vinylbenzene) and polymer ion-exchange (e.g. weak or strong anion/cation-exchange) or mixed mode ion-exchange sorbents are available. Polymeric reverse phase materials have both hydrophilic and lipophilic properties which are competent of capturing polar analytes like drug metabolites (Nakamura. 2011). Another advantage of silica based phase is the simplicity of use, because there is no need to keep those phases dampened to maintain interaction. Mixed mode ion-exchange and ion-exchange sorbents are even more appropriate because strongly retained ionic metabolites allow rigorous washing of cartridge (e.g. 100 % methanol) achieving cleaner sample without any matrix interferences (Chambers *et al*., 2007).

2.5.10.4 On-line solid-phase extraction technique

The on-line SPE technique provides speed, high sensitivity due to the pre-concentrating factor and low extraction cost per sample analysis. Yet, normally the use of program switch valves and column re-configurations are involved (Xu *et al*., 2007). In the LC system, biological samples can be directly injected without any sample preparations except for aliquoting samples adding the IS and sometimes sample diluting or centrifugation. Like PPT, on-line SPE is considered as another dilute and injection approach. Though, it gives cleaner extract with reduced or less matrix effect. Different demensions of column can be configured for the fast analysis of drug and their metabolites in biological matrix at the ng/mL level or lower. Commonly used columns for on-line SPE are packed with large particles (usually more than 20μm) of stationary material like polymeric and silica based which worked as similar to reversed phase, ion-exchange or mixed mode of separation. The combination of large particle size in these narrow bore columns (usually 50x1 mm) and fast flow (usually 3-5 mL/min), also Known as turbulent flow chromatography (TFC) which promotes the quick removal of proteins with simultaneous retention of the small-molecular analytes (Xu *et al*.,

2005). The direction of the flow becomes changed after discarded all the proteins and then the analytes are back-flushed onto the analytical column for chromatographic separation and detection. Completely integrated commercial or home-built systems allow eluting analytes from the extraction column onto analytical column in narrow bands. For better sensitivity, that enables multiple injections onto analytical column prior to elution into the instrument detector (Herman. 2005; Roškar and Lušin. 2012; Kang. 2012). Maximum on-line SPE techniques use column-switching to couple with both the analytical column and additional LC pump.

Normally, on-line SPE columns remain undamaged or unaffected after several injections of diluted plasma or urine samples followed by the injection volume and sample matrix (Saunders *et al*., 2009). Other SPE sorbents for turbulent flow chromatography (TFC) are restricted access materials (RAM), monolithic materials and disposable SPE cartridges. RAM works with a small pore size by eliminating the access of large molecules like proteins to the inner surface of the particles, otherwise, small molecules can freely bind to the sorbent in the normal hydrophobic interaction mode. The proteins are not able to to penetrate into the hydrophobic pores and the hydrophilic outer layer of particles. So, these proteins are eluted to waste and the smaller molecules enter into pores and are also retained through the hydrophobic forces (Hsieh and Korfmacher. 2006). RAM columns may be used either as the SPE and analytical column in the single column mode or in combination with another analytical column in column-switching mode (Vintiloiu *et al*., 2005 and Kawano *et al*., 2005). Single column mode approach is very simple but is limited due to chromatographic separation ability (Jemal and Xia. 2006). Monolith phases are favourable as extraction sorbents for sample treatment (Saunders *et al*., 2009). As single column mode, monolith columns are very suitable for high throughput method in LC-MS/MS analysis (Zang *et al*., 2005 and Xu *et al*., 2005).

2.5.10.5 New techniques for sample preparations

Nowadays, SPE in a 96 position array, PPT in an array, and on-line SPE are used in many clinical laboratories and also many advance methods of sample preparation may become useful for clinical LC-MS/MS applications in the future. Few of them are already in environmental and food analysis (Kang. 2012). Automated 96-well plate technology is well established and accepted and has been shown to effectively replace manual tasks. The 96-well instruments can execute automated off-line extraction and sample clean-ups. Automated

solid-phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation technique (PPT) all can be performed in 96-well format. In 96-well SPE, both cartridge and disc have been successfully used. Automated sample preparation saved at least 50% time in comparison to manual operation (Naidong *et al*., 2005). SPE materials are packed into pipetting tips and procedure is executed within these tips by automated pipetting of sample. Washing solution and final elution medium with direct injection of the eluate into an injection port (Erve *et al*., 2009 and Kang. 2012). So, a clear separation between sample preparation and chromatographic fractionation has been occurred.

A quadrupole TOF-MS was coupled with a NanoMate system for immunosuppressant quantification is outlined with automated clean-up of the hemolysed samples by chromatographic zip-tips and direct injection to a 400 nozzle nano flow-ESI chip source without another chromatographic separation (Almeida *et al*., 2008). SPE materials are packed in permanently used syringes of autosamplers and also mentioned to as solid phase microextraction (SPME) or microextraction by packed sorbents (MEPS) (Blomberg. 2009 and Vuckovic *et al*., 2010). Valid methods can be applied in modern high-end autosampler devices (e.g., CTC PAL) and also used a substitute to on-line SPE. Ferromagnetic micro-particles with modified surfaces (e.g., C-18 material) are in the automation of sample preparation for LC methods (Vogeser *et al*., 2008). These kind of particles represent a solid phase. But, these can be handled in a suspension as a liquid. In MS, the use of such particles might parallel the achievements in the automation of heterogonous immunoassays which also need a proper handling of solid phases. Another approach of sample preparation for MS involves miniaturized “lab-on-the-chip” solutions for microfluidic applications (Koster and Verpoorte. 2007).

2.5.11 Matrix effect

2.5.11.1 What is the matrix effect?

Matrix effect (ME) is defined as any changes in the MS response of analyte that can lead to either a reduced response (ion suppression) or an increased response (ion enhancement) of the LC-MS/MS system. ME is occurred due to molecules originating from the sample matrix or mobile phase that co-elute with the analyte of interest and consequently interfere with the ionization process in the MS ion source. The ME is likely arises from the competition between an analyte and the coeluting, undetected matrix components (Matuszewski *et al*., 2003; Jessome and Volmer. 2006; Ghosh *et al*., 2010). ME is the outcome of competition

between non-volatile matrix components and analyte ions for access to the droplet surface for transfer to the gas phase (King *et al*., 2000). Better interpretation of how ME can compromise the integrity of bioanalytical methods has re-emphasized the need for proper chromatographic separation of analytes from endogenous biological components in quantitative bioanalysis by LC–MS/MS (Xu *et al*., 2007). Due to inherent specificity of LC–MS/MS methods, do not found any obvious interference in chromatograms while sometimes relatively high concentrations of matrix components are present (Hernández *et al*., 2005).

ME is both compound or component and matrix dependent. The chemical nature of a compound has a remarkable effect on the degree of matrix effect (Bonfiglio *et al*., 1999). It was described that matrix induced ion suppression is mainly significant for early eluting compounds, like polar metabolites. Normally, ME more strongly influences lower analyte concentrations than higher (Xu *et al*., 2007). In LC-MS/MS bioanalysis, the main sources of the commonly observed ME are the endogenous phospholipids and proteins of plasma samples. Generally, the lysophospholipids are elute earlier in reversed phase chromatography and also more likely to cause ME compared to the later eluting phospholipids in spite of the larger concentrations (Xia and Jemal. 2009). Phosphatidylcholines, also a class of phospholipid molecular species, can cause ion suppression in both positive and negative ESI modes and must be resolved or removed chromatographically. In addition to the endogenous components in biological samples, like PEG400, Propylene glycol etc. have also been found to cause ME to varying degrees (Tong *et al*., 2002; Shou and Naidong. 2003). Another potential source of ME is the containers or tubes used for sample processing and storage (Mei *et al*., 2003 and Xia *et al*., 2005). Also, ME can be caused by co-eluting internal standards and including stable isotope labeled analogs (Liang *et al*., 2003 and Sojo *et al*., 2003). This phenomenon is explained by competition between the analyte and internal standard for the droplet surface charge (Enke. 1997; Cech and Enke. 2001). The significance of ME on the reliability of LC–ESI–MS/MS has been exhibited in terms of accuracy and precision (Matuszewski *et al*., 1998). The sensitivity and LLOQ (lower limit of quantification) of a method may be negatively affected during ion suppression (Buhrman *et al*., 1996). Therefore to develop a reliable and valid LC–ESI–MS/MS method, experiments should be performed to find out and understand the ME by careful consideration to evaluate and eliminate ME.

2.5.11.2 Evaluation of matrix effect

The post-extraction addition and the post-column infusion are two main approaches used to assess the degree of ME on a LC–ESI– MS/MS method. The post-extraction addition technique needs sample extracts with the analyte of interest spiked or added post-extraction compared with pure solutions prepared in mobile phase or in diluent containing equivalent amounts of the analyte of interest (Annesley. 2003 and Matuszewski *et al*., 2003). The difference in response between pure solution sample and post-extraction spiked sample is known as the absolute ME, while difference between several lots of post-spiked samples is also known as the relative ME. Primarily, the accuracy can be affected by an absolute ME and the precision can be affected by relative ME of the method. For the evaluation and validation of bioanalytical method in biological fluids, the determination of a relative ME is much more significant than the determination of absolute ME (Matuszewski *et al*., 2003). The relative ME caused by the individual variability in the sample matrix is evaluated based on at least five or six different sources of matrices (Matuszewski *et al*., 2003). Relative ME can be demonstrated as a coefficient of variation at particular concentration level or calculated based on slope lines. The assessed coefficient of variation of determined slopes in different sources of matrices should not exceed 3-4% for the method to be considered reliable and free from the relative ME (Matuszewski. 2006).

The post-column infusion method provides a qualitative estimation of ME and also determining chromatographic regions most likely to experience ME (Bonfiglio *et al*., 1999). This qualitative assessment technique allows the determination of the ME of endogenous components in blank matrix. Each endogenous compound that elutes from the column and causes an inequality in ESI response of the infused analyte which is appeared as either a suppression or enhancement in the response of the infused analyte (Bonfiglio *et al*., 1999; Bakhtiar and Majumdar. 2007). Every compounds should be infused separately to investigate possible ME for each analyte if various compounds are determined in one method. Thus, ME is not investigated for lower than LLOQ (lower limit of quantification). The analyte response is monitored to give information about the chromatographic run interferences between the analyte and matrix compounds during analysis of blank matrix. ME is demonstrated as response deviation in the otherwise flat response time trace of the constantly post-column infused analyte (Bonfiglio *et al*., 1999). During method development, this post-column infusion technique is very convenient because it provides information on the retention times

where ME has to be expected and subsequently that can be avoided for analyte of interest by optimizing chromatographic parameters (Roškar and Lušin. 2012; Kang. 2012).

2.5.11.3 How to overcome matrix effect?

Modification to the sample extraction technique (SPE or LLE instead of PPT) and improved chromatographic separation to reduce or remove ME must be executed. The use of particular sample extraction techniques like LLE and SPE can reduce or minimize the ME. Significance of SPE is removing ME, reduces ion suppression, concentrates analyte of interest and improves analytical system performance. Most of the ME occur in the solvent front of a chromatographic run if the analytes can be retained to some degree and ME can be reduced (Roškar and Lušin. 2012; Nair *et al*., 2010). Proper sample preparation and chromatographic parameters are related and help to develop a prosperous and rugged quantitative LC-MS/MS method (Van Eeckhaut *et al*., 2009 and Taylor. 2005). Selection of ionization interface is an important factor for the estimation of ME. Generally, APCI is considered to be less susceptible to ion suppression compared with ESI (Matuszewski *et al*., 2003; Matuszewski. 2006 and Chambers *et al*., 2007), although, method sensitivity and thermal stability of the analyte should be determined for ultimate APCI application. By post column splitting may reduce or totally remove the ion suppression due to decreasing the flow rate (20 µL/min or less) directed to ESI source (Kloepfer. 2005). Other approaches LC-MS/MS analysis by polymeric mixed-mode SPE and appropriate mobile phase pH may provide significant advantages for minimizing ME (Chambers *et al*., 2007). Few additives of mobile phase like triethylamine and trifluoroacetic acid can also cause ion suppression. In this way some reagents like formic or acetic acid and trifluoroacetic acid in conjunction with 10 mM ammonium acetate or addition of 1% propionic acid to the mobile phase may overcome the ME of trifluoroacetic acid. Also triethylamine may be replaced by hexylamine, an ion paring reagent (Bakhtiar and Majumdar. 2007). Though, the most effective procedure to remove the impact of ME on the accuracy and precision of a quantitative bioanalytical method is the use of stable isotope labeled analogs as internal standards (Matuszewski. 2006).

2.6 Bioanalytical method performance

2.6.1 Method validation

It is very essential to validate a bioanalytical method properly before it is used in day-to-day bioanalysis after the method has been developed. The search for the reliable range of a

method and continuous application of this knowledge is known as validation (Bruce *et al*., 1998). It can also be defined as the process of documenting that the method under consideration is competent for its intended purpose (Hartmann *et al*., 1998). Method validation includes all the procedures required to demonstrate that a particular method for quantitative determination of the concentration of an analyte (or a series of analytes) in a particular biological matrix is reliable for the intended application (Shah *et al*., 1992). Validation is also a confirmation of the specificity, repeatability and suitability of the method. Bioanalytical methods must be validated if the results are used to support the registration of a new drug or a new formulation of an existing one. Validation is involved to express the performance of the method and reliability of analytical results (Wieling *et al*., 1996). It is essential to ensure that validation experiments has been conducted and yields satisfactory results if a bioanalytical method is demanded to be for quantitative biomedical application (Causon. 1997). It is crucial to stress that validation in bioanalysis should not be considered as an isolated field before discussing how to carry out the validation experiment. So, a consensus on common terminology for all analytical fields are needed. The validation terminology is recommended by some important international organisations like the ISO (International Standard Organisation), IUPAC (International Union of Pure and Applied Chemistry) and AOAC (Association of Official Analytical Chemists) (Hartmann *et al*., 1998). Bioanalytical method validation can be found in guidelines for general recommendations the Food and Drug Administration (FDA), the European Medicines Agency (EMA) and the International Conference on Harmonisation (ICH) (https://www.fda.gov/cder/guidance/index.html,https://www.ema.europa.eu/docs/en_GB/...guideline/.../WC500109686.pdf,http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf).

Generally, validations are subdivided into three categories; full validation, partial validation, and cross validation.

2.6.1.1 Full validation

Full validation is carried out when developing and implementing a bioanalytical method for the first time. This validation is mandatory for any new drug entity, that means this should be performed to support pharmacokinetic, bioavailability, bioequivalence and drug interaction studies in a new drug application (NDA) (Shah *et al*., 2000; Shah. 2007 and Rahman *et al*., 2012). If metabolites are introduced to current method of already existing analyte then full

method validation is required and also the method is revised for the determination of all analytes (Shah *et al*., 2000; Shah. 2007).

2.6.1.2 Partial validation

Partial validations are modifications of already validated bioanalytical methods which are not required essentially for the complete revalidations. Partial validation can range from as little as one intra-assay and precision assessment to a nearly full validation. Some of the typical bioanalytical method changes that fall into this category include bioanalytical method transfer between laboratories and analysts, instrument and/or software applications and platform steps. Also, this validation can be performed if there is alteration in species within matrix for example rat plasma to mice plasma, changes in matrix within a species like human plasma to human urine, change in analytical methodology (e.g., change in detection systems), change in sample processing procedure(s) and change in anticoagulant in harvesting biological fluid (Shah *et al*., 2000; Shah. 2007 and Anonymous. May 2001). The resolution of which parameters to be revalidated depend on the logical consideration of the specific validation parameters likely to be influenced by the change made to the bioanalytical method.

2.6.1.3 Cross validation

Cross validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. In this validation, the original validated bioanalytical method considers as the reference and the revised or repeated bioanalytical method is the comparator and vice-versa (Shah *et al*., 2000 and Anonymous, May 2001). Cross validation with spiked plasma or matrix and subject samples should be performed at each site or laboratory to develope inter-laboratory reliability when sample analysis within a single study are performed at more than one site or more than one laboratory. This should be considered during data acquisition by using different analytical approaches (Shah *et al*., 2000; Shah, 2007 and Rahman *et al*., 2012). According to Dadgar *et al*., (1995), cross-validation is used for three different outlines :

a) The method validation is carried out by using a certain type of analytical system or instrument, for instance LC-UV and it is desirable to use another methodology, for instance LC-MS or LC-MS/MS.

b) The method validation is performed in one type of matrix, for instance plasma and it is desirable to use a new matrix in a study, for instance serum.

c) Comparison of two or more laboratories using the same bioanalytical method. The final outline is studied in the thesis to prove the performance of a validated method. For analysis, a set of calibrators and a number of "unknown" samples were further analysed in the Bioanalytical Laboratory-II, Bioequivalence Study Centre, Jadavpur University.

2.6.2 Validation parameters

The validation ensures the performance of the method and includes the fundamental parameters according to USFDA guidelines are accuracy, precision, recovery, selectivity, sensitivity, reproducibility and stability. The EMA guidelines include: selectivity, sensitivity, carry-over effect, lower limit of quantification, calibration curve, accuracy, precision, recovery, dilution integrity, matrix effect and stability. The ICH guidelines include the parameters for a validation are accuracy, precision, specificity, detection limit, quantification limit, linearity and range.

2.6.2.1 Specificity and selectivity

Both specificity and selectivity are two very crucial parameters of a valid bioanalytical method. In this way, a brief discussion of these concepts as defined by various organisations that has been described by Vessman (1996). A method is specific or not, it only determines the analyte without any type of interferences. Selectivity of the method is an ability to produce a response for the analyte of interest distinguishing it from all other interferences (Persson and Vessman. 1998; Aboul-Enein. 2000). Interferences in biological samples originate from a number of endogenous such as analyte metabolite, degradation products, co-administered drugs and chemicals usually arising in biological fluids and exogenous sources like impurities in reagents and dirty lab-ware. So, zero level interference of the analyte is desired, yet it is hardly ever the case. According to Vessman, the selectivity could be measured in grade or in scale whereas the specificity is an absolute characteristic. With respect to interferences from biological fluids, selectivity should be determined by processing blank samples from six different sources of the same matrix (Matuszewski et al., 2003) and also it should be established at the lower limit of quantification (LLOQ). If all the efforts to get free of interferences in the chromatographic process fail, changing to a more selective detector like Mass Spectrometry (MS) or Tandem Mass Spectrometry (MS/MS) may give an outstanding result (Dadgar and Burnett. 1995).

Dadgar and Burnett (1995) have mentioned some practical approach that may be used during method development to evaluate the selectivity of an analytical method.

- Processing blank samples from different sources will help to demonstrate lack of interference from substances native to the biological sample but not from the analyte metabolite.
- If analyte concentration is sufficiently high, and the chromophores differ sufficiently, the use of photodiode array (DAD) or scanning UV detection under the regular condition can give evidence of peak purity.
- Potential metabolites can be produced in vitro by incubation with liver homogenates, and chromatographed to check for potential interference with the analyte of interest.
- Processing of reagent blank in the absence of biological matrix is normally adequate to demonstrate selectivity with regard to exogenous interferences mentioned above.

While it would be better that all tested blanks, if procured under controlled conditions, be rid from interferences, factors such as food and beverage intake and cigarette smoking can affect selectivity (Dadgar *et al*., 1995). Shah *et al*., (1992) have mentioned the Washington conference on 'Analytical Methods Validation' proposed assessment of a minimum of six matrix sources to approve the selectivity of the method.

2.6.2.2 Sensitivity

Generally, the sensitivity of the method is demonstrated as limit of detection (LOD) and lower limit of quantification (LLOQ). According to the US pharmacopoeia (USP) defines the LOD as the lowest concentration of an analyte in a sample that can be detected but not necessarily quantitated and also define the LLOQ as the lowest amount of a sample that can be determined or quantitated with an adequate acceptable precision and accuracy under the stated operational condition of the method (Krull and Swartz. 1998). Usually, these limits are associated with the signal to noise ratio (S/N). For LOD, S/N ratio should be 2:1 or 3:1, whereas for LLOQ, at least five times (5:1) or often 10:1 is considered to be necessary for the response of a blank sample. Also, it should have an accuracy of 80-120% with a coefficient of variance (% CV) or RSD (Relative Standard Deviation) less than 20% (Matuszewski *et al*., 2003). It is essential that the samples used for evaluation of the precision and accuracy at the

LLOQ are not the similar as the standard curve samples. If it is occur, then thay would influence the regression and for that, accuracy would be also affected (Bonfiglio *et al*., 1999).

2.6.2.3 Accuracy

The accuracy of a bioanalytical method expresses the closeness of the determined value obtained by the method to the nominal concentration of the analyte and is evaluated by replicate analysis of samples of a standard amount. Accuracy should be determined on samples spiked with known concentrations of the analyte, the quality control (QC) samples which are spiked separately from the calibration standards. The QC samples are analysed against the calibration curve and the acquired concentrations are compared with the nominal value. The accuracy should be described as percent of the nominal value. Sometimes, accuracy is demonstrated in the terms of a mean value, a standard deviation (SD) and a difference between the mean and the actual value. According to the ICH guidelines, accuracy should be described as percent recovery by the analysis of known added quantity of analyte in the sample or as the difference between the mean and the accepted real value along with the confidence intervals. Therefore, accuracy is best reported as percentage bias which is calculated from the expression (Causon. 1997) :

$$\text{Abso\% Bias} = \frac{\text{measured value} - \text{true value}}{\text{true value}} \text{ X } 100$$

Estimation for the values of the QC samples acquired within a single run or intra-assay (the within-run accuracy) and in different runs or inter-assay (the between-run accuracy) (Kamalraj *et al*., 2012). Intra-assay accuracy should be estimated by analysing in a single run a minimum of three to five or six samples per level at a minimum of four concentration levels which are in the calibration curve range: the LLOQ, low quality control (LQC; three times of the LLOQ), medium quality control (MQC; around 40 - 50% of the calibration curve range), and high quality control (HQC; at least 75 - 80% of the upper calibration curve range) (Matuszewski *et al*., 2003; King *et al*., 2000; Pan and McLuckey. 2003). The mean concentration should be within 15% of the nominal values for all the QC and the calibration samples except for the LLOQ which should be within 20% of the nominal value (Matuszewski *et al*., 2003). Inter-assay accuracy should be determined at LLOQ, low, medium and high QC samples by analysing in different runs a minimum of three samples per

level on at least two different days. Also, the mean concentration value should be within 15% of the nominal values for all the QC and calibration samples except for the LLOQ which should be within 20% of the nominal value. Accuracy was demonstrated in the terms of mean recovery with a 95% confidence interval. This mean recovery was determined as the ratio of estimated concentration divided by actual concentration in percent (Sojo *et al*., 2003).

2.6.2.4 Precision

The precision of the bioanalytical method expresses the closeness of repeated individual measures of analyte between multiple analysis of a single homogenous sample. Precision is demonstrated as the coefficient of variation (% CV) or RSD (Relative Standard Deviation) (Causon. 1997).

$$\% \text{ CV} = \frac{\text{standard deviation}}{\text{mean}} \text{ X } 100$$

Precision should be evaluated at the LLOQ, low, medium and high QC samples by analysing within a single run and between different runs, that means using the same runs and data as for the presentation of accuracy. The precision is subdivided into three categories, such as repeatability or intra-assay (within-run) precision, intermediate precision or inter-assay (between–run) precision and reproducibility (Matuszewski *et al*., 2003; King *et al*., 2000; Pan and McLuckey. 2003).

For the evaluation of repeatability or intra-assay precision, should be determining at LLOQ, low, medium and high QC samples by analysing in a single run a minimum of five or six samples per concentration level. For the CV value of intra-assay precision should not exceed 15% for all the QC samples and calibration samples except for the LLOQ which should be within 20% (King *et al*., 2000; Pan and McLuckey. 2003). Intermediate precision or inter-assay precision should be determined at LLOQ, low, medium and high QC samples by analysing a minimum three runs on at least two different days. Basically, it is done to ensure how the performance of the assay is influenced by day-to-day variation, equipment, analysts etc. Here also the CV value should not exceed 15% for all the QC samples and calibration samples except for the LLOQ which should be within 20%. Reproducibility is related to variation within or between laboratories. It is important to assess the reproducibility if a pharmacokinetic study will be divided into two sets and analysed at two different laboratories.

2.6.2.5 Extraction recovery

Extraction recovery of the analyte and internal standard (IS) should be assess to determine loss during sample preparation and matrix ionization. It can be defined as the detector response from a sample of a given concentration of analyte added to the biological matrix prior to sample preparation compared to the detector response from a sample of the same concentration of analyte added to the biological matrix after sample preparation. It has mentioned in the USFDA guidelines, but is not considered as a basic parameter (Matuszewski *et al*., 2003). In ICH guidelines, it has constituted as one of two probabilities to demonstrate the accuracy (King *et al*., 2000; Pan and McLuckey. 2003). Many authors have declared that the level of recovery should not be so essential as long as the method performs better in terms of sensitivity, accuracy and precision (Liang *et al*., 2003 and Bonfiglio *et al*., 1999). Investigations of additional recovery is required if the method shows low recovery. The recovery should be independent and invariable of different variations in the biological matrix such as different red blood cells and plasma ratios, various protein contents etc. So from various source of blood, this can be examined by comparing recovery from spiked matrices.

Absolute recovery is the measured response of a processed spiked matrix standard demonstrated as a percentage of the response of a pure standard that has not been subjected to sample pre-treatment and expresses if the method gives a response for the total quantity of the analyte which is present in the sample (Bressolle *et al*., 1996).

$$\text{Absolute recovery} = \frac{\text{response of analyte spiked into matrix (processed)}}{\text{response of analyte of pure standard (unprocessed)}} \text{ X } 100$$

Also, a study can be performed by by comparing the response of extracted samples spiked before extraction with the response of the extracted blank matrix sample to which analyte has been added at the same nominal concentration just before injection (Causon. 1997 and Bonfiglio *et al*., 1999). This application requites the proper responses of the analyte which is affected by the matrix. Better precision and accuracy can be acquired from methods with moderate recoveries that give adequate sensitivity. In relative recovery, there is a comparison between responses from extracted samples i.e. spiked matrix with responses from extracted pure standards i.e. spiked aqueous solution. By this application, it can be find out the origin of the losses either because of the matrix or the extraction procedure.

2.6.2.6 Linearity and standard curve

A linearity or standard curve is representing the relation between the analyte concentration in the sample and the detected response. It is required to use an adequate number of standards to define sufficiently the relationship between response and amount (concentration). In this way, the relationship between response and concentration should be described to be continuous and reproducible. Generally, five or six to eight concentrations or calibration standards (spiked biological matrix excluding blank values), a blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS) may define the standard curve as well as should be used to establish the linearity. USFDA, EMA and ICH needs a minimum of six to eight, six and five calibration standards, respectively (King *et al*., 2000; Pan and McLuckey. 2003). It would be better that the calibration standards are prepared by serial dilution not directly or independently. More calibration concentrations may be useful for non-linear relationships than for a linear relationship (Shah *et al*., 1992).

A linear regression equation i.e. $y = mx + c$, can be estimated by the method of least squares to get the y-intercept, slope and correlation coefficient (r). The most suitable weighting factor is the inverse of the variance of the standard, while $1/x$, $1/x^2$,$1/y$ and $1/y^2$ (x = concentration or amount and y = response of the peak area ratio of the analyte and IS) are proper estimations (Lang and Bolton. 1991). It is essential to use a standard curve that should cover the entire range of the concentration of the unknown samples. It is not suggested that the calculation of the unknown by extrapolation of standard curve below the lower standard and above the higher standard. Rather, it is recommended that the standard curve be re-determined or sample re-assayed after dilution (Shah *et al*., 1992). Usually for the response versus concentration plot, acceptability of the linearity data is determined by investigating the correlation coefficient (r) and y-intercept of the linear regression curve. Generally, a correlation coefficient (r) of > 0.99 or > 0.98 is considered as confirmation of an acceptable fit of the data to the regression curve (Green. 1996).

2.6.2.7 Carry-over effect

Carry-over can negatively influence the accuracy and precision of the method. So during method development, carry-over should be marked and reduced. During validation, carry-over should be determined by injecting blank samples (without analyte and IS) after a high concentration sample or calibration standard at the upper limit of quantification (ULOQ) (Hughes *et al*., 2007 and Rahman *et al*., 2012). This effect is used to study the rinsing cycles

or washing process of autosampler needed to clean the injection needle properly so for that not to get any interference from the previous injection. Carry-over in the blank sample following the high concentration standard should not be more than 20% of the lower limit of quantification (LLOQ) and 5% for the internal standard (IS) (https://www.ema. europa.eu/docs/en_GB/...guideline/.../WC500109686.pdf). If carry-over is inevitable then it requires to be recognized in the method (Whitmire *et al*., 2011). Definite evaluations must be considered and proved during the validation and also applied during the analysis of the study samples. So for that reason, it does not influence accuracy and precision. Before going to the study sample, this could involve the injection of blank samples after samples of a high concentration.

2.6.2.8 Matrix effect on method validation

Investigation of matrix effect in quantitative bioanalysis reported that the ion suppression or ion enhancement is repeatedly followed by significantly deterioration of the precision and accuracy of the method. The precision is plotted as a function of the analyte concentration which is represented as relative standard deviation (RSD). Although, the precision is only acceptable for a single plasma lot and unacceptable when five different lots of plasma are taken (Matuszewski *et al*., 2003 and Niessen *et al*., 2006). Besides, an absolute matrix effect will particularly influence the accuracy of the method (Matuszewski *et al*., 2003). Matrix effect can be remunerated by a proper IS and this should be done for the elimination of these co-eluting compounds that reduces method sensitivity. So, this gives a negative result if analysing low concentrated samples. Biological fluids like plasma and urine express highly complex matrices and its composition can be diverse significantly within an individual, between individuals and also in species (Kang *et al*., 2012; Georgi and Boos. 2006). Almost all method validations are performed using calibration standards and QC samples processed from the same blank matrix pool. These homogenous samples are used for validation without any variability in the inter and intra-sample matrix (Taylor. 2005a). Investigating the quality of a method by QC samples which are processed from the same matrix as calibration standards that will report without any matrix effect in the incurred samples (Dewe *et al*., 2007). To determine any concealed analytical effect in the method, repetitive analysis of incurred samples is one of the primary approaches. At the same time for each individual matrix source, it is nonviable to prepare calibration standards and QC standards and some estimation of samples variability should be initiated (Taylor. 2005b).

2.6.2.9 Stability

The stability of the analyte in biological matrix or samples under specific conditions for given time intervals is often critical. Degradation is not unexpected even when all precautions are taken to avoid precisely known stability problems of the analyte, for example light sensitivity. Therefore, it is essential to find out that there is not sample degradation between the time of collection of the sample and their analysis that would compromise the result of the study. So, the aim of stability study is to detect any degradation during the whole period of sample collection, processing, storing, preparing and analysis. The condition under which the stability is evaluated is mainly dependent on the nature of the analyte, the biological matrix, and the anticipated time period of storage (before analysis) (Sonawane *et al*., 2014). Stability assessment is performed to show that the concentration of analyte at the time of analysis corresponds to the concentration of the analyte at the time of sampling (Hartmann *et al*., 1998). Other fundamental aspect of validation is to establish that analyte(s) is/are stable in the biological matrix and in all solvents encountered during the sample processing under the conditions to which study samples will be analysed (Dadgar and Burnett. 1995). Shah *et al*., (1992) reported that at ambient temperature, the stability of the analyte in matrix should be determined over a time that encloses the duration of normal sample preparation, sample handling and analytical run time.

Stability should be determined at least at two concentration levels, using blank biological matrix samples spiked at a low and high concentration (QCs) level. It must be evaluated in each matrix and species where the analyte will be quantified. Under different conditions, the stability of the analyte should be tested in the standard solutions used to the preparation of calibration curves, in any biological matrix stored at -20°C and at room temperature prior to the analysis. The QC samples are evaluated against a calibration curve that obtained from freshly spiked calibration standards. The obtained concentrations are compared to the nominal concentrations and at each level, the mean concentration should be within ±15% of the nominal concentration. So,

$$\% \text{ Stability} = \frac{\text{mean response of stability samples}}{\text{mean response of comparision samples}} \text{ X } 100$$

According to the USFDA and EMA guidelines, stability is classified by the followings ;

2.6.2.9.1 Stock solution stability

The stability test for the standard stock solution must be performed at the same temperature, container and solvent that used for the study. The time period should be at least 6 hours. It can be favourable to continue the stability study if the analyte is considered stable in stock solution at about 2 - 8ºC after 14 days or two weeks. Satisfactory stability in the stock solution for several days or months excludes the requirement of freshly prepared stock solutions for every new analysis. In this way, it is recommended to check its status before preparation of new standards by a spectrophotometer after the analyte has proven to be stable in the stock solution. The stock solution is diluted to a relevant concentration such as, 10µg/mL and wavelength scan is carried out. The molar absorption coefficient value can be estimated at one or many wavelengths (e.g. at the maximum absorptions) by using the Lambert-Beers law. The obtained value can be correlated to a tabulated value or a value from the day of preparation. The stability data of the stock solution should be propagated to rationalize the duration of stock solution storage stability if the stock solution exists in a different state such as, solutions or solid and in a various buffer composition (usually for the macromolecules) from the certified reference standard (Gao *et al*., 2011 and Sonawane *et al*., 2014).

2.6.2.9.2 Short-term stability

This must be determined following the storage under laboratory conditions used for sample processing for a period at least 6 hours to 24 hours and correlated with data from the same samples prepared and analysed without interval. The samples should be maintained at room temperature or sample processing temperature (ambient temperature) (Murugan *et al*., 2013 and Sonawane *et al*., 2014). Stability at 30ºC temperature should be assessed over the time between collection and freezing if there are no facilities for immediate freezing. Everytime, there is a risk of exposure to human immunodeficiency virus (HIV) when handling biological samples. Now, HIV is thermolabile, so its deactivated at 56ºC by heating for 30 minutes (Chiap *et al*., 1999 and Causon. 1997).

2.6.2.9.3 Long-term stability

This is performed to evaluate whether the analyte is stable in the plasma matrix under the sample storage conditions for the time period required for the samples produced in a clinical

study to be analysed. Therefore, this stability of the analyte in the matrix should equal or exceed the time period between the date of first sample collection and the date of last sample analysis (Murugan *et al*., 2013; Sonawane *et al*., 2014 and Dadgar *et al*., 1995). There is conventional approach to prepare quality control (QC) samples at one or two concentration levels and after preparation, these samples are stored under the same conditions just like potential study samples. At day zero, replicates of two or more are analysed simultaneously with a freshly prepared calibration curve and after specified periods of time. Evaluation of the concentrations on each time is correlated to provide stability. But, there is a huge errors that are emerged both from the constituted between-run variation and the various preparation errors.

Dadgar *et al*., (1995) have suggested two different approaches to reduce these types of errors. In the first procedure, prepared batches of QC samples at two concentrations and stored under the recommended conditions. New batches of QC samples at the same concentrations are prepared after the specified period of time. Ten replicates of each of the four batches are analysed together at a single time. The responses are evaluated and for each batch a mean value with confidence interval can be determined. This procedure removes the between-run variation and also bias correlated with the use of various calibration curves. But, because of two different preparations it could not remove errors. In another procedure, begins with same as the first one i.e. the preparation of a batch of QC samples at a proper concentration. The prepared batch is divided into two parts, one part is stored under the recommended conditions whereas the other part is stored at -20ºC or at -70ºC. At these temperatures, this part can be studied as freshly prepared samples because literatures have demonstrated that highly unstable drugs (e.g. nafamostat mesilate, otilonium bromide etc) are stable for several times in biological matrices (Cao *et al*., 2008; Zhao *et al*., 2010; Dadgar and Burnett. 1995).

2.6.2.9.4 Freeze and thaw stability

During freeze and thaw stability assessments, the freezing and thawing of stability samples should be determined the proposed sample handling conditions to be used during sample analysis. Stability should be evaluated for a minimum of three freeze-thaw cycles (Gao *et al*., 2011 and Sonawane *et al*., 2014). This stability test is carried out to ensure that the sample remains stable after it is managed to multiple freeze-thaw cycles in the process of the study. This can be executed by thawing samples at high, medium and low concentrations (QCs) separated and admitting the samples to freeze again for at least 12 hours to 24 hours. The

freeze-thaw cycle is repeated twice and at the end of the third cycle, the sample is processed and with freshly prepared sample its result is compared. Measures should be taken to store adequate amounts of aliquots to permit repeats, without having to freeze and thaw the sample more than once if the analyte is found not stable after three cycles. Acceptable range of this stability is 2% change in standard solution or sample solution response compare to freshly prepared standard. However, acceptable stability is 20% change in response for standard solution and sample solution compare to a freshly prepared sample at the LLOQ (Green. 1996).

2.6.2.9.5 Bench-top stability

Bench top stability studies should be designed and managed to cover the laboratory handling conditions that are expected for study samples (Gao *et al*., 2011 and Sonawane *et al*., 2014). In this study, storage of the thawed sample at the laboratory bench prior to analysis and samples are stored for 4 hours to 24 hours under room temperature (Merbel *et al*., 2014).

2.6.2.9.6 Post-preparative stability

Post preparative stability is assessed to cover the expected run time for the analytical batch and to handle the situations such as system malfunctioning etc. where samples are injected after a certain period of time. At low, medium and high concentrations, processed six replicates of QC samples and placed the samples (after a liquid-liquid or precipitation or solid-phase extraction process) in the autosampler as per the requirements of the bioanalytical method. The same QC samples can be injected along with the calibration standards and another set of freshly extracted QC samples after the stability period. All the QC sample concentrations are back calculated using the calibration curve (Kalakuntla and Kumar. 2009; Merbel *et al*., 2014). These types of stabilities are usually performed during a two-day period that should cover the ultimate resident time for a sample in the process.

2.6.2.10 Quality assurance in clinical studies

During the application of the method for routine drug analysis with their active metabolite, some validation parameters are constantly assessed to ensure satisfactory method performance. Almost, analysis of unknown biological samples can be carried out by single determination. It may be essential to analyse duplicates or even triplicates of each sample to maintain a scientific result when the method has quite high variability. The balance between accuracy and cost must be battered. During each run, at least six standards including a blank

sample should be prepared for a standard curve (Vessman. 1996). However, adequate QC samples should be used to ensure method performance in each run (Matuszewski *et al*., 2003). Usually, accepted approach is to use duplicate QCs at low, intermediate (medium) and high concentration levels within the range. To accept the run, at least four of them and one at each level must be within 20% of their nominal value, respectively (Bonfiglio *et al*., 1999 and Vessman. 1996).

It is very significant that standards and QC samples are processed in the same matrix as the clinical samples, such as plasma, serum, blood, urine etc. By addition of small volumes, the standards and QC samples can be prepared from a stock solution with high concentration. Too much high a percentage of stock solution in the samples which mainly dissolved in organic solvent can lead to variations in the sample matrix and inappropriate results for the clinical samples. In this thesis, all standards and QC samples are processed with the volume of stock solution which has been less than 2% of the total sample volume. If the stock solution is dissolved in an organic solvent then it can be difficult to pipette small volumes with accuracy. A convenient way to conquer this problem is to pipette the volumes on an analytical balance. The weight along with the density can be used to measure the volume for the stock solution. It is desirable to perform a system suitability test to ensure optimum conditions for the liquid chromatography (LC) system before every new run (Matuszewski *et al*., 2003 and Vessman. 1996). This can be executed by injecting a pure standard and determine essential parameters such as resolution, peak efficiency, retention times etc. The complete explanation of the method must be written before a validated method is developed for day-to-day analysis.

The Standard Operating Procedures (SOPs) should contain everything from sample handling, reagent usage and reference materials to batch rejecting, repeat analysis and archiving followed by Good Laboratory Practice (GLP) (Matuszewski *et al*., 2003). These SOPs should be followed precisely without any conversion. It must be documented in written form and this procedure should also be covered by a SOP if an alteration is required. For repeat analysis and rejecting a batch or run, criteria should be established a rational in the SOP. Instrument failure, QC samples outside predefined tolerance, loss or errors during sample preparation etc. are possible reasons for repeat analysis (Bonfiglio *et al*., 1999 and Vessman. 1996). During the analysis of pharmacokinetic studies, an exceptional case emerges. Few results from the analysed samples could deviate from the expected pharmacokinetic profile. So for these “unfitted” samples, many authors suggest attentive during the repeat analysis (Bonfiglio

et al., 1999 and Vessman. 1996). It should be executed in duplicates if a decision is made to reanalyse few samples (Bonfiglio *et al*., 1999). The best typical approach to clarify the results is to use the mean value of the duplicates as the new value. Further, there is a probability to use a median value of the original and the two reanalysed samples (Bonfiglio *et al*., 1999). The quality of the bioanalytical method is very essential as the quality in the preanalytical phase.

During a pharmacokinetic study, some topics that have to be considered are (Persson and Vessman. 1998) :

2.6.2.10.1 Procedure for sampling

It is crucial that the sampling pursues a standardized procedure. In this procedure, points to consider are; the body position, a preceding phase of rest, needle size, tourniquet application time etc. If the tourniquet is kept on during a five minute long sampling procedure then it has been shown that the total protein concentration rises considerably in serum (Persson and Vessman. 1998). It can start to haemolysis if the needle has too small an internal diameter. After skin puncture, the capillary blood is taken and the first drop of blood should be discarded because the drop is expected to be contaminated with tissue fluids. During sampling, squeezing the area around the puncture should be avoided because it can start to dilution of the blood with interstitial fluids. Administration of drugs and collection of samples must be carried out in separate locations and also by separate persons to reduce contaminations of the samples (Aboul-Enein. 2000).

2.6.2.10.2 Food and drinking

The absorption of a drug is significantly influenced by food and drinking. If highly lipophilic drugs are administered after a fatty meal then absorption gets increased. Preceding to each sampling event, it is desirable that diet and drinking are standardized. Regularly a large number of people consume caffeine as hot drinks and so the effect of caffeine should be considered on the concentration of analytes in the blood. Due to the huge intake of caffeine, drugs are bound to albumin and influence the quantification process. Further, the concentration of certain analytes in the blood are influenced by smoking and alcohol consumption.

2.6.2.10.3 Matrix and effects of centrifugation

The initial point to consider is that what type of matrix to be used, such as plasma, urine serum, capillary blood or whole blood. After completion of the platelet and clotting factor coagulation process, serum is collected from whole blood by centrifugation. On the other hand after centrifugation, plasma is acquired from whole blood to which anticoagulant has been added. For certain analytes, there are differences between plasma and serum concentrations. After an uninterrupted rest phase, it is essential that the centrifugation is carried out within one hour from the time of sampling. According to Bergqvist and Domeij-Nyberg (1983) before centrifugation if the whole blood was stored at 23^{o}C (room temperature) more than 1-2 hours then the plasma concentrations of chloroquine were increased. It is likely to get various kinds of plasma by changing the relative centrifugal force. A low relative centrifugal force, such as 150-200 g gives a platelet rich plasma whereas a high relative centrifugal force, such as 2000-3000 g gives plasma without platelets [g = gravitational acceleration or g-force].

2.6.2.10.4 Plasma and serum lipids

Occasionally, plasma and serum samples are vague because of an increased content of lipoproteins. This is principally lead to an increased triglyceride concentration. It is necessary that the handling of these samples are rational and documented in a SOP. There are two approaches to handle these samples. First, it is likely to mix the samples very delicately and pursue a homogenous volume for analysis. Second, to use ultracentrifugation or precipitation and analyse the cleared sample.

2.6.2.10.5 Haemolysis

Usually, haemolysis can be identified visually due to a more reddish presence of both haemolysed plasma and serum samples compared to normal samples. During the haemolysis of blood, the components of blood cell are discharged into the serum or plasma. So by the use of standardised procedures, haemolysis should be reduced as much as possible. If the analyte is present inside the red blood cells haemolytic samples might show incorrect concentrations of the analyte in the serum or plasma. With attention results from haemolytic samples must be explained. During analysis of clinical and pharmacokinetic studies, it is crucial that haemolytic samples are recognized and documented.

2.7 The physicochemical characterization of drug molecules

The organic nature of a naturally occurring molecule is an essential to being able to achieve the approach and combine with the binding site of the endogenous compound. So, many drugs are designed to either mimic or block the effects of these molecules. Because of the different electronegativities, the molecular bonds between heteroatoms (like oxygen, nitrogen, sulphur or phosphorous in addition to carbon and hydrogen) and carbon exhibit unequal sharing of electrons. These bonds have the comprehensive result of shifting the distribution of electrons within the molecule such that areas of positive and negative charge are generated, so therefore polarity is introduced into the molecule. The binding of the endogenous substrate with the receptor due to the presence of charged groups offers the potential for electrostatic interaction with molecules of opposite electrical charge and these interactions are crucial to controlled this binding (Campbell and Blanc. 2011).

2.8 Absorption of drugs

Drug absorption is determined by the drug's physicochemical properties, formulation and route of administration. Dosage forms such as tablets, capsules, solutions consisting of the drug plus other ingredients are formulated to be given by various routes like oral, buccal, sublingual, rectal, parenteral, topical, inhalational. Regardless of the route of administration, drugs must be in solution to be absorbed. Thus, solid forms (e.g. tablets) must be able to disintegrate and deaggregate. Absorption is the transfer of a drug from its site of administration to the bloodstream or the systemic circulation. The rate and efficiency of absorption depend on the route of administration. In case of IV delivery, absorption is complete; i.e. the total dose of drug reaches the systemic circulation. Drug delivery by other routes may result in only partial absorption and thus, lower bioavailability. For example, the oral route requires that a drug dissolves in the gastrointestinal (GI) fluid and then penetrates the epithelial cells of the intestinal mucosa, yet disease states or the presence of food may affect this process.

2.8.1 Absorption from the stomach

In the stomach pH of the gastric juices is strongly acidic with a value of 1 (approximately), whereas the intestinal pH is almost neutral in the range pH 6 to 7 (Kanu and Hill. 2008). The pH of the plasma is 7.4 and hence the large difference in pH between the plasma and the stomach. GI tract widely determines whether or not a weakly ionised compound will be

absorbed into the plasma. It will also alternatively determine whether or not a drug could be released from plasma back into the stomach or intestine. It can be calculated how aspirin, a weak acid of a pKa value 3.5 would distribute across the gastric mucosa between the plasma (pH 7.4) and the gastric acid (pH 1) by applying the Henderson – Hasselbalch equation [shown in equation (i) and (ii)].

$$pka - pH = \log \frac{[\text{un-ionised}]}{[\text{ionised}]} \qquad \text{…….. (i)}$$

after rearrangement it gives,

$$pH - pka = \log \frac{[\text{ionised}]}{[\text{un-ionised}]} \qquad \text{……(ii)}$$

Hence, in plasma, pH – pKa = 7.4 – 3.5 = 4 and in the stomach, pH – pKa = 1 – 3.5 = –2.5 for aspirin. Now, log [ionised]/[un-ionised] = 4 and [ionised]/[un-ionised] is the antilog of this, that is 10,000 in plasma at equilibrium. However, in the stomach, log [ionised]/[un-ionised] = 2.5 and [ionised]/[un-ionised] = 0.003. Because, it is the acid moiety that is un-ionised for a weak acid and so freely able to distribute to equilibrium, the distribution will be as follows :

Stomach (pH 1.0) Plasma (pH 7.4)

$$H^+ + A^- \rightleftharpoons HA \rightleftharpoons HA \rightleftharpoons H^+ + A^-$$

(0.003) (1.0) (1.0) (10,000)

It can be noticed that aspirin is more than 99% in the un-ionised form in the acid environment of the stomach and so, its rapidly absorbed from the stomach. On the other hand, in the plasma, the matter is completely reversed i.e. more than 99% of the drug is in the ionised form. For example, a basic drug like procaine with pKa value of 9.0, the reverse situation will use to that of an acid. With less than one molecule in ten million being un-ionised of the drug will remain almost completely in the ionised form in acidic conditions of the stomach. So, it can be noticed that the most of weakly basic drugs will be ionised in the stomach and thus poorly absorbed. However, in the small intestine, in almost the neutral conditions, ionisation of weakly basic drugs will be suppressed and thus basic drugs likely to be rapidly absorbed. In reality, acidic drugs can also be absorbed because of the large surface area for absorption

offered by the small intestine. Therefore, it has been shown above that aspirin is almost exclusively ionised in the small intestine and in human after oral administration, it has to be well absorbed from this site. So, also it is clear that strong acids or bases like sulphanilamide with pKa value of 10.4 and quaternary ammonium salts like tetraethylammonium will be ionised over the normal range of physiological pH and so, either from the stomach or from the small intestine it will be poorly absorbed.

2.8.2 Absorption from the gastrointestinal (GI) tract

Drugs may be absorbed from the GI tract depending on their chemical properties by either passive diffusion or active transport.

2.8.2.1 Passive diffusion

The drug moves from a region of high concentration to one of lower concentration (according to Fick's low). Passive diffusion does not involve a carrier. The huge majority of drugs gain access to the body by this mechanism. Because of the solubility in the membrane bi-layers, lipid-soluble drugs easily move across most biologic membranes. The cell membrane is penetrated by the water-soluble drugs through aqueous channels or pores. Other agents can enter the cell through specialized transmembrane carrier proteins that facilitate the passage of large molecules. This process is known as facilitated diffusion. This kind of diffusion does not require energy, can be saturated, and may be inhibited.

2.8.2.2 Active transport

This form of drug entry also includes specific carrier proteins that cross the membrane. Active transport is energy-dependent and is driven by the hydrolysis of adenosine tri-phosphate. It is capable of moving drugs against a concentration gradient i.e., from a region of low drug concentration to one of higher drug concentration. Active transport seems to be limited to drugs structurally similar to endogenous substances, such as ions, vitamins, sugars, amino acids. These drugs are normally absorbed from specific sites in the small intestine.

2.8.2.3 Endocytosis, pinocytosis and exocytosis

In endocytosis, drug delivery transports drugs of exceptionally large size across the cell membrane. Endocytosis involves engulfment of a drug molecule by the cell membrane and transport into the cell by pinching off the drug-filled vesicle. For example, vitamin B12 is transported across the gut wall by endocytosis. In pinocytosis, fluid or small particles are

engulfed by a cell. The cell membrane invaginates, encloses the fluid or small particles, then fuses again, forming a small vesicle that later detaches and moves to the cell interior. Energy expenditure is required. Pinocytosis probably plays a small role in drug transport, except for protein drugs. Exocytosis is the reverse of endocytosis and is used by cells to secrete many substances by a similar vesicle formation process. Certain neurotransmitters, such as nor-epinephrine (NE) are stored in membrane-bound vesicles in the nerve terminal and are released by exocytosis.

2.9 Drug distribution to the tissues

After a drug enters the systemic circulation, it is distributed to the body's tissues and fluids. Distribution is generally uneven because of differences in blood perfusion, tissue binding due to lipid content, regional pH, and permeability of cell membranes. The distribution of drugs between the plasma and the tissues are affected, because the pH of the plasma (7.4) is more basic than that of the tissues (7.0). The entry rate of a drug into a tissue depends on the rate of blood flow to the tissue, tissue mass, and partition characteristics between blood and tissue. Distribution equilibrium when entry and exit rates are the same between blood and tissue is reached more rapidly in richly vascularized areas, unless diffusion across cell membranes is the rate-limiting step. After equilibrium, drug concentrations in tissues and in extracellular fluids are reflected by the plasma concentration. Metabolism and excretion occur simultaneously with distribution, making the process dynamic and complex. In comparison, weakly basic drugs are present at least partially in the un-ionised form which can diffuse out of the plasma into the tissues. In tissue, the equilibrium in tissue is shifted back towards the ionised form with the result that the drug tends to remain in the tissue, this is happen due to the pH difference between the tissues and plasma. Primarily, drug distribution to the interstitial fluid is determined by perfusion after a drug has entered tissues. For poorly perfused tissues, such as muscle, fat, distribution is very slow, especially if the tissue has a high affinity for the drug. Also, the intrinsic lipophilicity of a drug can distinctly affect its preferred site of accumulation. Because, almost 15% of the body weight is accounted by fat in normal individuals and can increase to approximately 50% in excessive obese individuals. For highly lipophilic drugs, this can perform as a depot or slow release ability. For example, it has been exhibited that the volume of distribution of biperiden, an anticholinergic drug which correlates with the fat volume per unit mass (Dyson. 1996).

2.9.1 Volume of distribution

The apparent volume of distribution is the theoretical volume of fluid into which the total drug administered would have to be diluted to produce the concentration in plasma. For example, if 1000 mg of a drug is given and the subsequent plasma concentration is 10 mg/L, that 1000 mg seems to be distributed in 100 L.

$$\text{Now,} \quad \frac{\text{dose}}{\text{volume}} = \text{concentration}$$

$$\text{So,} \quad \frac{1000\ \text{mg}}{\text{x L}} = \text{concentration}$$

$$\text{Therefore,} \quad x = \frac{1000\ \text{mg}}{10\ \text{mg/L}} = 100\ \text{L}$$

Volume of distribution has nothing to do with the actual volume of the body or its fluid compartments but rather involves the distribution of the drug within the body. For a drug that is highly tissue-bound, very little drug remains in the circulation. Hence, plasma concentration is low and volume of distribution is high. Drugs that remain in the circulation likely to have a low volume of distribution. Volume of distribution provides a reference for the plasma concentration expected for a given dose but provides little information about the specific pattern of distribution. Each drug is uniquely distributed in the body. Some drugs distribute mostly into fat, others remain in extracellular fluid, and others are bound extensively to specific tissues. Most of the acidic drugs, such as warfarin, aspirin etc. are highly protein-bound and so have a small apparent volume of distribution. Many basic drugs, like amphetamine, meperidine etc. are extensively taken up by tissues and therefore have an apparent volume of distribution larger than the volume of the entire body.

2.9.2 Binding to the plasma proteins and blood cells

The extent of drug distribution into tissues depends on the degree of plasma protein and tissue binding. Drugs are carried partly in solution as free or unbound and partly reversibly bound to blood components such as plasma proteins, blood cells in the bloodstream. Many plasma proteins can interact with drugs, for example serum albumin, alpha-1 acid glycoprotein, and lipoproteins (Prakash *et al*., 2007). The free drug is able to cross membranes and interact with the receptor, the distribution and ultimately, the efficacy of a

drug can be affected by its binding to blood constituents. Generally, acidic drugs are bound more extensively to albumin and basic drugs are usually bound more extensively to alpha-1 acid glycoprotein, lipoproteins, or both (Guidelines for Bioavailability & Bioequivalence Studies. CDSCO, India, 2005). Only unbound drug is available for passive diffusion to extravascular or tissue sites where the pharmacologic effects of the drug occur. Therefore, the unbound drug concentration in systemic circulation typically determines drug concentration at the active site and thus efficacy. The amount of bound drug approaches an upper limit determined by the number of available binding sites at high drug concentrations. Saturation of binding sites is the basis of displacement interactions among drugs. Drugs bind to many substances other than proteins. Binding mostly occurs when a drug associates with a macromolecule in an aqueous environment but may occur when a drug is partitioned into body fat. Because fat is poorly perfused, equilibration time is long, especially if the drug is highly lipophilic.

Accumulation of drugs in tissues or body compartments can prolong drug action due to the tissues release the accumulated drug as plasma drug concentration decreases. For example, thiopental is highly lipid soluble, rapidly enters the brain after a single IV injection and has a marked and rapid anesthetic effect; the effect ends within a few minutes as the drug is redistributed to more slowly perfused fatty tissues. Then, thiopental is slowly released from fat storage and maintaining subanesthetic plasma levels. These levels may become significant if doses of thiopental are repeated and causing large amounts to be stored in fat. So, storage in fat initially shortens the drug's effect but then prolongs it. Due to the binding with proteins, phospholipids or nucleic acids, some drugs accumulate within cells. For example, chloroquine concentrations in liver cells and WBCs can be thousands of times higher than those in plasma. Drug in cells is in equilibrium with drug in plasma and moves into plasma as the drug is eliminated from the body.

2.9.3 Blood-brain barrier

Drugs reach the central nervous system (CNS) via brain capillaries and cerebrospinal fluid (CSF). Although the brain receives about one sixth of cardiac output and because of the brain's permeability characteristics, drug penetration is restricted. However, some lipid-soluble drugs like thiopental, enter the brain readily but not the polar compounds. The reason is the blood-brain barrier and it consists of the endothelium of brain capillaries and the astrocytic sheath (Ballabh *et al*., 2004). The endothelial cell of brain capillaries appears to be

more tightly joined to one another than those of most capillaries and slow the diffusion of water-soluble drugs. The astrocytic sheath consists of a layer of glial connective tissue cells, i.e. astrocytes which close to the basement membrane of the capillary endothelium. With aging, the blood-brain barrier may become less potent and allowing increased passage of compounds into the brain. Drugs may enter ventricular CSF directly via the choroid plexus, then passively diffuse into brain tissue from CSF (Abbott *et al*., 2010). Also, the organic acids such as penicillin, are actively transported from CSF to blood in the choroid plexus. The drug penetration rate into CSF is similar to other tissue cells and determined primarily by the extent of protein binding, degree of ionization and lipid-water partition coefficient of the drug. For highly protein-bound drugs, the penetration rate into the brain is slow and almost nonexistent for the ionized form of weak acids and bases. Because the CNS is so well perfused and the drug distribution rate is mainly determined by permeability.

2.10 The significance of drug metabolism

The principal site of drug metabolism is liver. Although metabolism typically inactivates drugs and some drug metabolites are pharmacologically active, sometimes even more so than the parent compound. A weakly or inactive active substance that has an active metabolite is called a prodrug, especially if designed to deliver the active moiety more effectively. Metabolism is a process of biotransformation when drugs are transformed by enzymatic reactions into a different chemical form. Primarily, metabolism increases drug hydrophilicity and activity of most drugs and decreases the toxicity. In addition, the biotransformation reactions could lead to bioactivation of drugs in which case the metabolite is more active and/or more toxic than the parent drug (reactive metabolite formation) (Walsh and Miwa. 2011; Roškar and Lušin. 2012). The main purpose of metabolism is to convert a lipophilic drug molecule which is effortlessly able to cross membranes and hence access its site of action, into a molecule which has a lower lipophilicity, a lower affinity for the tissues and a greater propensity for removal from the body through the urine. The intrinsic lipophilicity of a molecule is a key determinant of its metabolism. Lipophilicity is essential, not only in terms of facilitating crossing of membranes to reach the drug-metabolising enzymes, but is also a crucial parameter controlling binding of the drug to the active site of the drug-metabolising enzymes.

Drugs can be metabolized by oxidation, reduction, hydrolysis, hydration, conjugation, condensation, or isomerization; whatever the process, the goal is to make the drug easier to excrete. Generally, the enzymes are more concentrated in the liver and involved in metabolism and also are present in many tissues. Drug metabolism rates differ among patients. Some patients metabolize a drug so rapidly that therapeutically effective blood and tissue concentrations are not reached. On the other hand, metabolism may be so slow that usual doses have toxic effects. Individual drug metabolism rates are affected by various factors like physiologic, pharmacodynamics, environmental and genetic factors, coexisting disorders such as chronic liver disorders and advanced heart failure and drug interactions, especially those involving induction or inhibition of metabolism. Several physiological factors such as age, sex, disease state, pregnancy, exercise, circadian rhythm and starvation lead to the impaired metabolism among subjects and should be taken into consideration when evaluating the drug metabolism. Dose, frequency, route of administration, tissue distribution and protein binding of the drug influence its metabolism. Moreover, environmental factors like environmental chemicals, co-administered drugs, tobacco, smoking, alcohol drinking and dietary constituents may change both the kinetics of enzyme reaction and the whole pattern of metabolism, so thereby altering the bioavailability, pharmacokinetics, pharmacologic activity or the toxicity of the drug (Williams. 2002; Ma and Lu. 2011).

Metabolism occurs in two phases for many drugs. Phase I reactions include formation of a new or modified functional group or cleavage, such as oxidation, reduction, hydrolysis and these reactions are nonsynthetic. Phase II reactions include conjugation with an endogenous substance like glucuronic acid, sulfate, glycine and these reactions are synthetic. In synthetic reactions, metabolites are formed which are more polar and so more readily excreted by the kidneys (in urine) and the liver (in bile) than those formed in nonsynthetic reactions. Some drugs undergo only phase I or phase II reactions and thus, phase numbers reflect functional rather than sequential classification. Some enzymes such as CYP2D6, CYP2C19, CYP2C9, CYP3A4, CYP3A5 are responsible for metabolism of many drugs and are also highly polymorphic (Williams. 2002). Many non-cytochrome P-450 drug metabolizing enzymes also play key role in the metabolism of a variety of drugs. Among them polymorphisms of thiopurine methyltransferase (TPMT), butyrylcholinesterase, N-acetyltransferase (NAT) and UDP-glucuronosyltransferase (UGT) influence the metabolism of drugs (Ma and Lu. 2011).

2.10.1 Metabolism rate

For almost all drugs, the metabolism rate in any given pathway has an upper limit i.e., capacity limitation. However, only a small fraction of the metabolizing enzyme's sites are occupied at therapeutic concentrations of most drugs and the metabolism rate increases with drug concentration. This is called first-order elimination or kinetics and the metabolism rate of the drug is a constant fraction of the drug remaining in the body i.e., the drug has a specific half-life. For example, if 500 mg is present in the body at time zero and after metabolism, 250 mg may be present at 1 h and 125 mg at 2 h (illustrating a half-life of 1 h). However, metabolism occurs at its maximal rate when most of the enzyme sites are occupied and does not change in proportion to drug concentration. Rather, a fixed amount of drug is metabolized per unit time (zero-order kinetics). In this case, if 500 mg is present in the body at time zero and after metabolism, 450 mg may be present at 1 h and 400 mg at 2 h (illustrating a maximal clearance of 50 mg/h and no specific half-life). Metabolism shifts from first-order to zero-order kinetics when the drug concentration is increased.

2.10.2 Cytochrome P-450

The cytochrome P-450 (CYP450) is an isoenzyme of the microsomal superfamily that catalyzes the oxidation of many drugs and also most vital enzyme system of phase I metabolism. The NADPH–CYP450 reductase is a flavoprotein which are supplied electrons i.e., transfers electrons from NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate) to CYP450. Many drugs are induced or inhibited the CYP450 enzymes and substances resulting in drug interactions in which one drug enhances the toxicity or reduces the therapeutic effect of another drug (drugs interact with specific enzymes). The main isoforms of cytochrome P-450 involved in the metabolism of xenobiotics in human are CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. The CYP450 is responsible for the oxidative clearance of the majority of lipophilic drugs i.e. with a log D above 0 (Gelpi. 2003). The mechanism of catalysis of this CYP450 is thought to be constant across all the families and to be determined by the ability of a high valent formal $(FeO)3^{+}$ species to carry out the one electron oxidations through abstraction of hydrogen atoms, abstraction of electrons in n or * orbitals or the addition to * bonds (Hoffmann and Stroobant. 2007). Due to the decrease of hepatic volume and blood flow, the capacity of the liver for metabolism through the CYP450 enzyme system is reduced (≥ 30%) with aging. Thus, drugs that are metabolized through this system reach higher levels and have prolonged half-lives in the elderly. Because neonates have partially developed hepatic microsomal enzyme systems

and also they have difficulty metabolizing many drugs.

Table.2.5 Common substances that interact with cytochrome P-450 enzymes

Enzyme	Substrates	Inhibitors	Inducers
CYP1A2	Caffeine Clarithromycin Estradiol Haloperidol Lidocaine	Cimetidine Ciprofloxacin Erythromycin Fluvoxamine Ticlopidine	Cigarette smoke Omeprazole Phenobarbital Phenytoin Rifampin
CYP2C9	Celecoxib Diclofenac Fluoxetine Glipizide Glyburide	Amiodarone Cimetidine Fluconazole Lovastatin Ritonavir	Dexamethasone Phenobarbital Other barbiturates Phenytoin Rifampin
CYP2C19	Diazepam (S)-Mephenytoin Omeprazole Pentamidine	Cimetidine Fluoxetine Fluvoxamine Ketoconazole	Carbamazepine Phenobarbital Prednisone Rifampin
CYP2D6	Beta blockers Codeine Dextromethorphan Flecainide Haloperidol	Amiodarone Bupropion Celecoxib Cimetidine Fluoxetine	Carbamazepine Dexamethasone Phenobarbital Phenytoin Rifampin
CYP2E1	Acetaminophen Alcohol	Disulfiram	Alcohol Isoniazid Tobacco use
CYP3A4	Amiodarone Aprepitant Azole antifungals Benzodiazepines Ca channel blockers Caffeine Carbamazepine	Amiodarone Amprenavir Atazanavir Azole antifungals Cimetidine Ciprofloxacin Clarithromycin	Carbamazepine Dexamethasone Isoniazid Phenobarbital Phenytoin Prednisone Rifampin

Table.2.6 The nominal mass changes in biotransformation of drugs by common metabolic reactions

Biotransformation	Change in molecular formula	Change in mass (Da)
Dehydration	- H_2O	-18
Demethylation	- CH_2	-14
Dehydrogenation	- H_2	-2
Hydrogenation	+ H_2	+2
Methylation	+ CH_2	+14
Hydroxylation	+ O	+16
Epoxidation	+ O	+16
S/N-oxidation	+ O	+16
Hydration	+ H_2O	+18
Dihydroxylation	+ O_2	+32
Acetylation	+C_2H_2O	+42
Sulfation	+SO_3	+80
Glucuronidation	+$C_6H_8O_6$	+176
Glutathione conjugation	+$C_{10}H_{15}O_6N_3S$	+305

2.10.3 Conjugation

The most common phase II reaction is glucuronidation and is the only one that occurs in the liver microsomal enzyme system. In bile, glucuronides are secreted and eliminated in urine. Thus, conjugation makes most drugs more soluble and easily excreted by the kidneys. With glutamine or glycine, amino acid conjugation produces conjugates that are readily excreted in urine but not extensively secreted in bile. Aging does not affect glucuronidation. However, conversion to glucuronide is slow in neonates and potentially resulting in serious effects (eg, as with chloramphenicol). Also, conjugation may occur through acetylation or sulfoconjugation. Sulfate esters are polar and readily excreted in urine. Aging does not affect these processes (Kostiainen *et al*., 2003 and Holcapek *et al*., 2008).

2.11 Elimination of drug through excretion

The principal organs for excreting water-soluble substances are the kidneys. The biliary system contributes to excretion to the degree that drug is not reabsorbed from the GI tract.

Usually, the contribution of intestine, saliva, sweat, breast milk and lungs to excretion is small, except for exhalation of volatile anesthetics. Excretion via breast milk may affect the breastfeeding infant. Often, hepatic metabolism increases drug polarity and water solubility. Then the resulting metabolites are more readily excreted.

2.11.1 Renal excretion

Renal filtration occurs for most drug excretion. Around one fifth of the plasma reaching the glomerulus is filtered through pores in the glomerular endothelium. Almost all water and many electrolytes are actively and passively reabsorbed from the renal tubules back into the circulation. Non-volatile drugs those have a low molecular weight (less than 500), are highly soluble or lowly metabolised will be eliminated from the body by renal excretion. However, polar compounds are accounted for most drug metabolites which cannot diffuse back into the circulation and are excreted unless a specific transport mechanism exists for their reabsorption, such as glucose, ascorbic acid and B vitamins. Renal drug excretion decreases with aging, for example at age 80, clearance is typically reduced to half of what it was at age 30. The principles of transmembrane passage regulate renal handling of drugs. Glomerular filtration is a unidirectional process that occurs for most low molecular weight compounds (less than 500) irrespective of their degree of ionisation. Drugs bound to plasma proteins remain in the circulation and only unbound drug is included in the glomerular filtrate. Un-ionized forms of drugs and their metabolites tend to be reabsorbed readily from tubular fluids.

pH of the urine varies from 4.5 to 8.0 and may greatly affect drug reabsorption and excretion because it determines the ionization state of a weak acid or base. Urine increases reabsorption due to acidification and decreases excretion of weak acids and also decreases reabsorption of weak bases. In alkalinisation, urine has the opposite effect. Often in overdose, these fundamentals are used to enhance the excretion of weak acids or bases; for example, enhancement of the excretion of acetylsalicylic acid due to the alkalinization of urine. Urinary pH change the rate of drug elimination that depends on the contribution of the renal route to total elimination, the polarity of the un-ionized form and degree of ionization of the molecules. In the elimination of many drugs, active tubular secretion in the proximal tubule is crucial. This energy dependent process may be blocked by metabolic inhibitors. Secretory transport can reach an upper limit (transport maximum) when drug concentration is high and each substance has a characteristic transport maximum. Anions and cations are controlled by

separate transport mechanisms. Usually, the anion secretory system eliminates metabolites those conjugated with glycine, sulfate and glucuronic acid. For secretion, anions compete with each other. This competition can be used therapeutically; for example, probenecid blocks the normally rapid tubular secretion of penicillin and results of higher penicillin concentrations in plasma for a longer time. Cations or organic bases, such as pramipexole, dofetilide are secreted by the renal tubules in the cation transport system and cimetidine, trimethoprim, prochlorperazine, megestrol or ketoconazole are inhibited this process.

2.11.2 Biliary excretion

In bile, few drugs and their metabolites are widely excreted. Active secretory transport is needed because they are transported across the biliary epithelium with a concentration gradient., This secretory transport may approach an upper limit, i.e. transport maximum when drug concentrations are high in plasma. For excretion, substances having similar physicochemical properties may compete. In bile, drugs with a molecular weight of greater than 300 gram per mole and with both lipophilic and polar groups are more likely to be excreted. Normally, smaller particles are excreted only in negligible amounts. In biliary excretion, conjugation especially with glucuronic acid is facilitated. In bile, a secreted drug is reabsorbed into the circulation from the intestine in the enterohepatic cycle. Elimination of the substances from the body only to the extent that enterohepatic cycling is incomplete in the biliary excretion and when from the intestine, some of the secreted drug is not reabsorbed.

2.12 The analysis of active metabolites with its prodrug by the pre-pharmacokinetic properties

The competence to selectively analyse for the active metabolite with its prodrug of interest in different biological matrices is an essential pre-requisite to acquiring authentic pharmacokinetic data. By excellence of the selectivity of the detector a simple protein precipitation preceding to injection may be adequate unless the mechanism of detection is mass spectrometry. Usually, this needs some degree of clean up or concentration followed by chromatographic separation preceding to detection. Because of the sample clean up and the chromatographic separation are the phases at which knowledge of the physicochemical properties of the drug or metabolite can be applied to optimise recovery and separation. The solvents are mixed just before an equilibrium is achieved and the drug (or metabolite) to be isolated is located mainly in one of the two immiscible layers. Normally the organic to

facilitate speed of evaporation and reconstitution in a suitable mobile phase. Mostly, liquid–liquid extraction has been replaced by solid phase extraction, where extraction of the analyte is dependent upon its partitioning between a solid stationary phase (like C18 bonded silica) and solvent(s) running over this phase. This is expressed diagrammatically in Figure.2.12

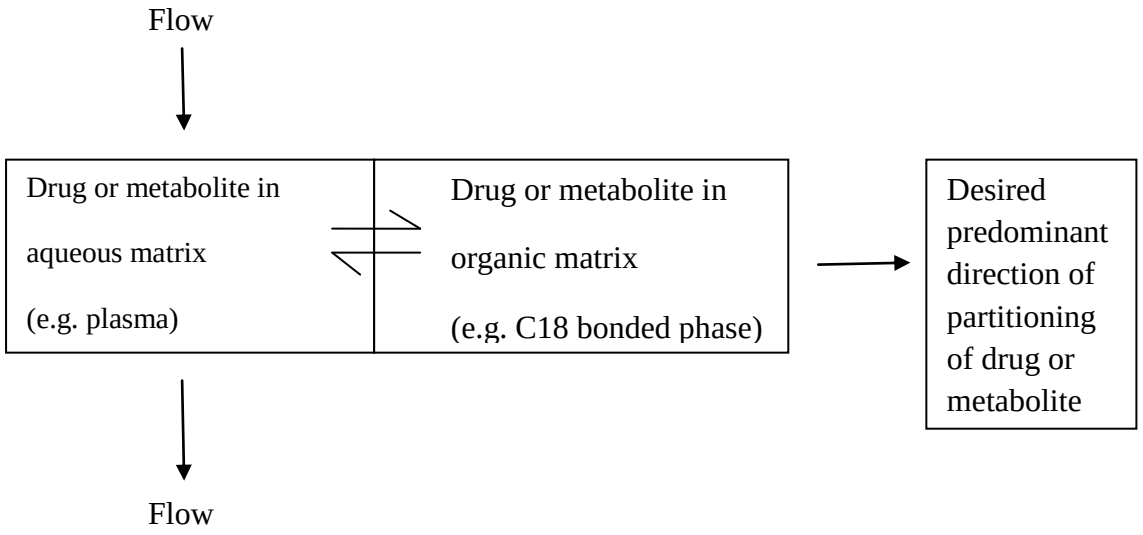


Figure.2.12 Diagrammatic explanation of the potent interactions involved in the extraction of an analyte using a solid phase extraction cartridge from a biological matrix

The process is rapid and so there may be inadequate time for the drug and metabolite to partition into the stationary phase. From the intrusive elements, the drug (with metabolite) of interest may pass through uncertained. Also, similar progressive considerations will refer to the chromatographic position in which there is a range of flow rates within which optimum chromatography will be accomplished. Due to high flow rates above this optimum, column capability will be decreased resulting in disintegration of the peak shape and a corresponding decline in sensitivity. With immense value to the extraction of a compound either by liquid–liquid or solid phase extraction and the optimal chromatographic separation is executed on the basis of pKa value of the compound. Depending upon the pH value, the competence of a drug and metabolite to partition between an organic phase and an aqueous phase for its efficiency to cross a membrane. According to the above consideration, acids distribute into an organic phase and are well retained on chromatography columns when they are extensively unionised at a pH below their pKa value and are poorly retained when they are completely ionised at pH above their pKa value. In case of bases, the situation is opposite where they are ionised and so poorly retained at a pH below their pKa value, but well retained when ionisation is suppressed at a pH above their pKa value.

Further, the chromatographic separation of weakly ionisable acids and bases can be optimised effortlessly because the pH of the medium can be easily manipulated. Therefore, it can be noticed that, by including a recognition of the simple physicochemical properties of a compound, predictions as to its feasible role in a given chromatographic condition can be formed and the effort of acquiring a convenient sample clean up and chromatographic separation can be made much simpler.

2.13 The assessment of pharmacokinetics of active metabolites and their prodrugs

Usually, pharmacokinetics assessment or evaluation of active metabolites with their prodrugs has focused on distinguishing between products or placebo to provide advanced and better medications for the benefit of public health (Spire *et al*., 1985). However, this is primarily not so economic and time consuming and over time steps have been taken to minimize costs and to increase quantity of pharmaceutical products while sustaining the potential for novelty. Bioequivalence is the one such instance that to be relevant.

2.13.1 What is pharmacokinetics ?

Generally, pharmacokinetics (PK) is broadly defined as "what the body does to a drug" i.e., the movements of drugs within biological systems, as affected by absorption (uptake), distribution, binding, metabolism (biotransformation) and elimination; specifically the rates of similar movements (Martin *et al*., 1985). Normally, when a tablet (pharmaceutical dosage form containing drug) is taken orally, it extents to the stomach and starts to disintegrate followed by absorption (A). The drug is moved on to the small intestine when dissolved into the solution of acid in the stomach (Muirhead and Smart. 2000). Several things can occur in the small intestine. Some of the drug will be take up and distributed (D) through the portal circulation into the body. Some will be metabolised (M) into a various substance right in the intestine and some drug substances passes through the liver first where it is frequently metabolized. Some of the drug will move right on through and be eliminated (E) by urine from the body. The residue moves through the liver and reaches the bloodstream where it is distributed throughout the body after oral administration. The entire process where the drug is retained and go through four 'phases' preceding to being totally excluded from the body, recognized as ADME, i.e. Absorption (crossing the gut wall : uptake through the mouth, throat, stomach and small or large intestine), Distribution (movement between compartments

: the drug substance is transported through the blood to its site of action), Metabolism (mechanisms of metabolism : the drug substance converts into by-products) and Elimination (excretion : disperses the drug product from the body). So therefore, pharmacokinetics is an investigation of ADME. For pharmacokinetics also use the acronym ABCD, existing for administration, bioavailability, clearance and distribution (Shah *et al*., 1992; Doogue and Polasek. 2013).

Though, this process is quite difficult to measure. Advance technology gives several preferences, like tag a radio-label marker in a molecule and pursue the movement of the molecule by using X-ray imaging and similar techniques. Now, the best natural factor is to measure the dose (i.e., how much drug substance enter into the body) and by means of blood sampling, how much drug extents the systemic circulation. A typical plasma concentration profile versus time has been shown in Figure.2.13. After absorption and distribution of the drug, the plasma concentration increases and extents at the highest level, i.e. called maximum concentration or the Cmax. Afterwards, plasma concentrations decrease until the drug is fully eliminated from the body. The complete exposure of the drug is measured the area under the plasma concentration curve (AUC) by a computing process.

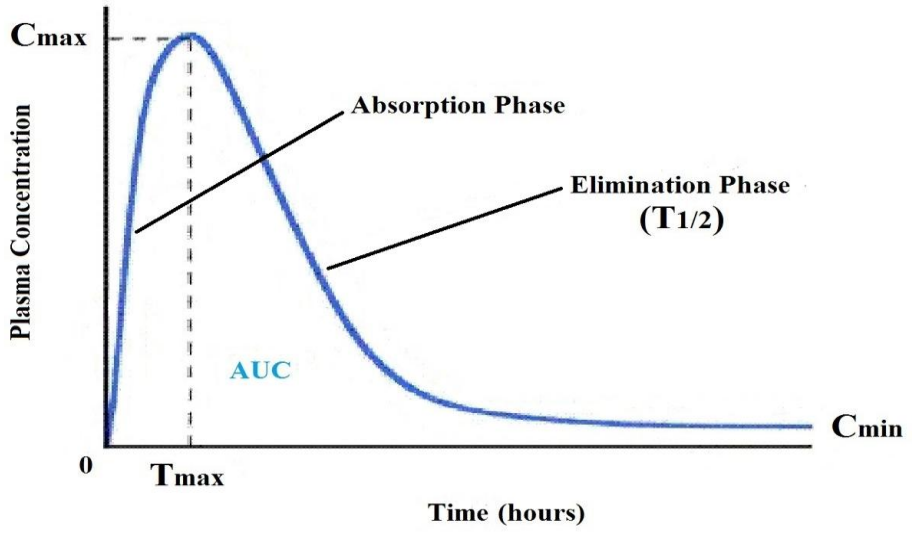


Figure.2.13 Plasma concentration versus time profile of a single dose of a drug ingested orally

Commonly, area under the curve (AUC) is derived of the area under each time interval and accumulating them by a computing process. Concise measures (Muirhead and Smart. 2000) for the plasma concentration versus time curve are obtained as :

- C_{max} [i.e., maximum drug concentration],
- T_{max} [i.e., time of maximum concentration],
- $T_{1/2}$ [i.e., terminal elimination half-life of drug substance],
- $AUC_{(0-t)}$ [i.e., area under the plasma concentration-time curve from zero (0) hours to time (t) where time (t) is the time of last measured concentration],
- $AUC_{(0-\infty)}$ [i.e., Area under the plasma concentration-time curve from zero (0) hours to infinity (∞)] and $AUC_{(0-\infty)} = AUC_{(0-t)} + C_t/\lambda$

Where, C_t is the concentration at time (t) and λ is terminal elimination rate constant, i.e. -2.303 times the slope of the terminal phase of the $\log_e$ -concentration time curve (Guder *et al*., 2001). Likewise in this thesis, competent a model to summarize a plasma concentration curve.

2.13.2 Bioequivalence (BE) studies

The definition of bioequivalence expressed in terms of rate and extent of absorption of the active ingredient or moiety to the site of action, emphasize the use of pharmacokinetic measures in an accessible biological matrix such as blood, plasma, serum, urine etc. to indicate the release of the drug substance from the drug product into the systemic circulation. This approach resets on the understanding that measuring the active moiety or ingredient at the site of action is not usually possible. Moreover, that some relationship exists between the efficacy or safety and concentration of the active moiety and its important metabolite(s) in the systemic circulation. Bioequivalence studies are designed to compare the *in vivo* performance of a test pharmaceutical product (multi-source) compared to a reference pharmaceutical product (Anroop Nair *et al*., 2010). This study involves administration of the test and reference products on two occasions to volunteer subjects, each occasion is called phase (i.e. phase I or phase II) and its separated by a washout period. The washout period is preferred to ensure that drug given in one treatment is entirely eliminated preceding to administration of the next treatment. Subsequently preceding to administration, blood or urine samples are collected and determined for the concentration of the drug substance and its active metabolite(s). The increase and decrease of these concentrations over time in each subject in

the study provide an assessment of how the drug substance is released from the test and reference products and absorbed into the body. These blood i.e., plasma or serum and, or urine concentration time curves are used to calculate certain bioequivalence metrics of interest to concede comparisons between the two products. These metrics are determined for each subject in the study and the resulting values are correlated statistically.

There are some primary approaches those are considered during a bioequivalence study. Firstly in regulatory and public-health considerations, the process used must protect public health and at a predetermined rate, the risk of a false positive (i.e., Type 1 error) market approach should be controlled. Second approach is the sponsor attentions and that should be competent to demonstrate the principles have been met with a quantified chance of success by employing a well-designed, controlled, and rationally sized study or set of studies. Finally in the statistical interpretation, it must be quantifiable, accurate, precise, well understood and should be transparent.

2.13.3 Significance of the BE studies

Bioequivalence (BE) studies are carried out to establish that various formulations or regimens of drug product are related to each other in terms of their efficacy i.e., therapeutic benefit and safety i.e., non-therapeutic adverse effects. In the drug development process, they play an important and crucial role by ensuring that the efficacy and safety will be sustained when a patient switchover to a new formulation in the dispensary. Mainly, these studies are used in the investigation of a tablet or capsule i.e., a solid oral dosage forms after ingestion. In this chapter, this kind of drug product will restrain itself to consideration. Now, the old and recent formulations are pharmaceutically equivalent i.e., use specifically the same entity [Multilateral Initiative on Malaria (MIM)/UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR), 2002]. So, what is the rationale for these studies ? The rate and extent of absorption of the drug into the bloodstream is known as bioavailability (BA). It is accessible at the site of action after dosing and it can be influenced by very minor modifications in formulation. Some of the factors that change the bioavailability are the content of composition of the formulation, little modifications to the interlining of the formulation and by condensation into tablet versus administration as a capsule (Bergqvist and Domeij-Nyberg. 1983; Senn. 1997).

By the pharmaceutical sponsors, bioequivalence studies are mainly performed for new drug entities and also they have managed essential confirmatory trials with a definite formulation

of a drug analysis. However, there is required retail approach for a commercially more convenient formulation that can be selected to a great extent. These studies are contributed fundamental and satisfactory support to regulators that the formulation to be marketed which are applied in the clinical confirmatory trials. So, repetition of the development program is not required or to carry out a therapeutic equivalence study with clinical endpoints in patients (William and Wilkins. 1990). Apparently with a new formulation, repetition of a drug development program become absurd when it is familiar to last over a decade and also the expense is almost a billion dollars. This kind of exercise is unsuitable for the present industrial potentiality. Likewise, BE studies must be carried out following massive postmarketing formulation modification. Further, they are applied by the pharmaceutical industry (i.e., denoted as the 'generic') to achieve market access for formulations of conventional drug treatments when the patent of formulation is expired of the initial sponsor. Sometimes the original sponsors execute a formulation alteration (for example, switch the site of manufacture) following recommendation and usually they again must perform a bioequivalence study to assure regulators that the new formulation is potent and safe to public health. Now, various companies may produce and retail similar formulations to the actual marketed product following patent completion and they can also establish bioequivalence to the original product. Hence, not required any repetition of the whole clinical pharmacology development batch when the patent is expired because the generic replacement has contributed a way of distributing to the market with effective, safe and low-priced drug products. Therefore, BE studies are regulated to fit cross-over study designs, documented and legislated regulatory standards are generally used (Rowland and Tozer. 1980: Atkinson *et al*., 2001).

2.13.4 Design and application of bioequivalence study

Commonly, bioequivalence studies are conducted in both male and female healthy individuals or sometimes in patients (Nair *et al*., 2010). Two products, such as reference (R) and test (T) are administered in each individual subject in one of two sequences of regimen or medications, i.e., RT and TR(see Table.2.7). Where, R is the 'standard' and T is the 'new' formulation.

Table.2.7 Representational design of a 2 × 2 cross-over study

Sequence Group	Period/Phase			Number of Subjects
	1	Washout	2	
1 (RT)	R	__	T	n/2
2 (TR)	T	__	R	n/2
'R' = Reference, 'T' = Test and 'n' = Total no. of volunteers in the study				

In the study, each administration is segregated by a washout period relevant to the drug (see Table.2.8). Between administrations, this washout period comprises the five half-lives. Now, what is half-life of a drug molecule ? The concentration of a drug in plasma is halved after one elimination half-life. Accordingly, less drug is eliminated in each consecutive half-life. The extent of drug remaining in the body is 50% after one half-life and after two half-lives it's become 25%. Subsequently, three and four half-lives it's become 12.5% and 6.25%, respectively. Finally, after five half-lives the amount of drug become 3.125% which is considered to be insignificant concerning its therapeutic effects. The half-life of a drug is controlled by its clearance and volume of distribution. The elimination half-life is considered to be independent of the quantity of drug in the body. Half-life regulates the length of the drug effect. It also indicates whether accumulation of the drug will happen under a multiple dosage regimen and it is necessary to set on the applicable dosing interval.

Generally in BE study, this kind of form or pattern i.e., 2 × 2 cross-over design is used (Rowland and Tozer. 1980). Now, 2 × 2 cross-over design or pattern with order RT and TR is the most frequent among various convenient list of designs for application in bioequivalence studies. This design has a potential complication and the potency of a formulation given in both the period-I and II (or phase-I and II) is same with an inadequate washout period. Because of incomplete washout period, the potency of a formulation given in the first period may carry forward into the second period. So, this is a feasible complexity of this design. The statistical data analysis from such studies is so much complicated due to the presence of these carry-over effects (Steinijans *et al*., 1995 and Benet. 1999). Carry-over effects are usually considered to be negligible after a sufficient washout period is included (Levy. 1995; Balthasar. 1999; Huque *et al*., 1989 and Benet. 1999).

Table.2.8 Illustration of randomization of sequences in a 2 × 2 cross-over study to healthy human subjects

Subject No.	Mode of Treatment	Period/Phase-I	After five half-lives (washout period)	Period/Phase-II
1	TR	T	——	R
2	RT	R	——	T
3	RT	R	——	T
-	----	----	----	----
-	----	----	----	----
-	----	----	----	----
n	TR	T	——	R
'R' = Reference, 'T' = Test and 'n' = Total no. of volunteers in the study				

In each formulation, the dose of drug entity is pharmaceutically equivalent and normally the formulations are stable i.e., recognizable to both the investigator and the patient. Randomization of volunteer subject to sequence is executed to establish that time-related consequences (such as period to period variations in blood sampling timings or laboratory handling of the sample) can be considered in the analysis and are not confused with the assessment for the variation between formulations. In this instance, the process of random allocation is one of the approach that implemented to assure bias does not creep into the study. Blood samples will be obtained before the analysis, routine intervals preceding to and subsequently each dose of formulation to generate the concentration curves against time have been demonstrated in the results and discussion sections of this thesis. For the manifestation of equivalence in plasma concentration profiles, rate and extent of bioavailability of the drug entity must be competently equivalent. Therefore according to the regulatory standard, the exposure of drug entity to the body is the similar between formulations [Multilateral Initiative on Malaria (MIM)/UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR), 2002].

In this way to get regulatory approval, Cmax i.e., rate and AUC i.e., extent are usually used as concise evaluations for the plasma concentration curves and are needed to be established as equivalent under predecided regulations. For the clinical effectiveness and safety, Cmax and AUC are considered as surrogate markers at this circumstances (Jones and Kenward. 2003). For instance, unwanted adverse effects coluld arise when Cmax increases excessively with the new formulation. Moreover, when it decreases too much, the drug can not be potent in treating the disease. Also for AUC, similar assessments can be used. Now, the quantity or amount of the drug material in the new formulation must be assayed delicately during the quality assurance and improvement programme. With distressed at the pharmacokinetic endpoints, typically Cmax and AUC are pretended more strictly and are assigned as a log-normal distribution. The normal distribution is a numerical or statistical explanation of the state of attribute from which specific considerations derive, such as Cmax and AUC data are obtained from the BE studies. What pursues is a non-scientific explanation of distribution theory describing to the bioequivalence. This should be reviewed for those concerned in the details of distributional theory [Multilateral Initiative on Malaria (MIM)/UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR), 2002]. An illustration of a normal distribution is projected in Figure.2.14. The density of the distribution is on the perpendicular axis and the correlating AUC values are on the plane axis. The area under the curve is the probability of observing the AUC values for a given interval on the plane axis. This is also feasible to recognize the values in the specific interval is the greater area of the density. By such kind of distribution, the numbers of instance of a plenty of data in nature are competently expressed.

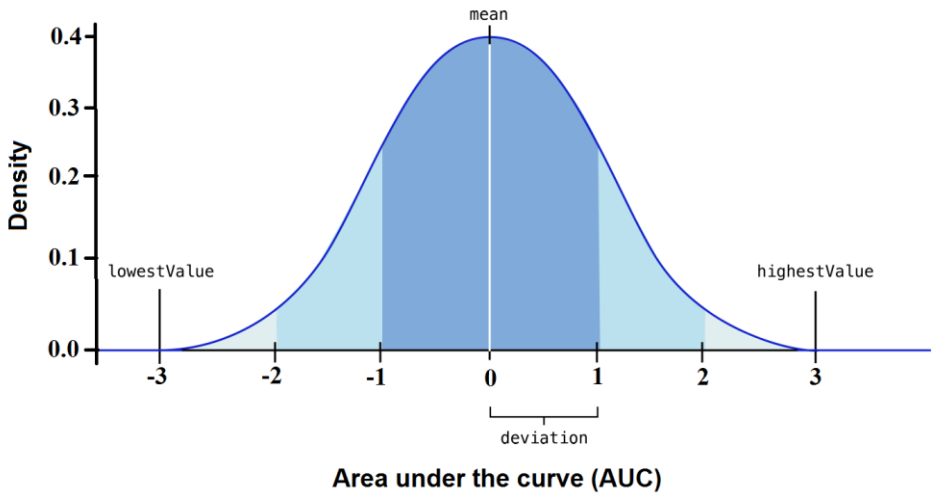


Figure.2.14 A normal distribution curve

The size (or, volume) of the distribution is consolidated throughout a parameter termed as the mean (μ), i.e., the determination of centrality and is diversified to a certain measure expressed by the standard deviation (σ), i.e., a determination of expansion. 50% of the distribution exists above the mean (μ) and rest 50% exists below. Certainly, this is unknown that what is the significance of the values of both μ and σ are, therefore obtained data are evaluated by applying statistics with a log-normal distribution (Senn. 2002; Zariffa *et al*., 2000; Zariffa and Patterson. 2001). Thus, this is effective and truly appropriate in mathematical contexts. By taking the natural-logarithm of Cmax and AUC (just like a mathematical conversion) the following log-transformed Cmax and AUC are typically distributed when AUC and Cmax are log-normal in distribution. So, the following log-variable is normal in distribution when the log of a log-normal variable such as Cmax or AUC is taken. For the explanation, AUC has been taken and as demonstrated in Figure.2.15 and observe that the distribution is altered and log-normally distributed.

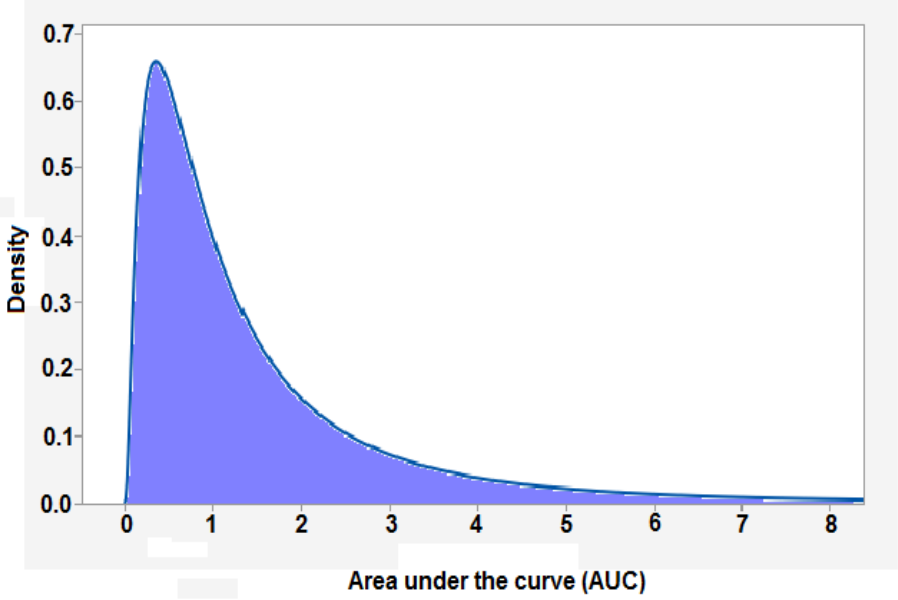


Figure.2.15 A Log-Normal Distribution curve

In Figure.2.16, the natural logarithm of the AUC values and it has been obtained the distribution of logAUC is plotted. In this Figure, the plane axis stands for the natural-logarithm of AUC (it is expressed as ln-or log-transformed AUC) and it has been mentioned as logAUC. The data are consistently greater than or equal to zero (i.e., ≥ 0) when it is acceptable for logAUC or logCmax data with an unfavorable value that usually transform the

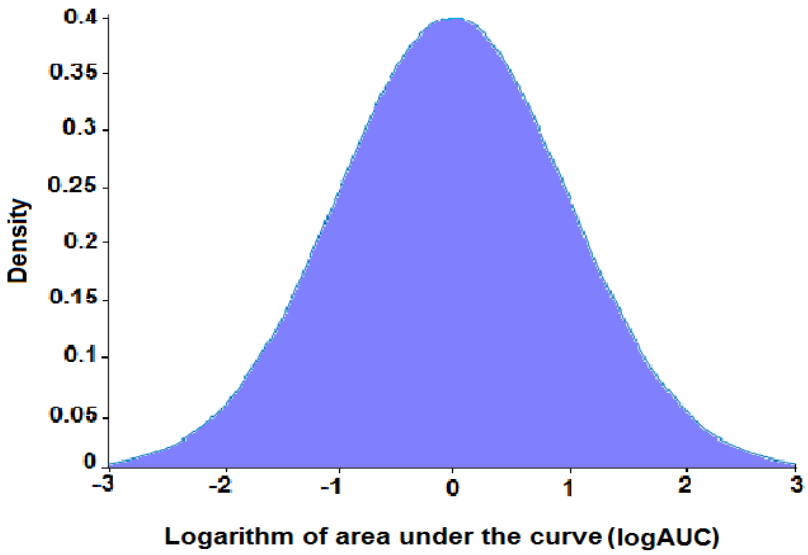


Figure.2.16 The normal distribution originating from log-transformation for AUC data

value by exponentiating to following their initial distribution. Some controversies have been consolidated about Cmax and AUC, the major endpoints which are required for the evaluation of bioequivalence. Some discoveries reveal that Cmax and AUC are not consistently enough to perfectly exhibit bioequivalence (Guder *et al*., 2001; D'Angelo *et al*., 2001 and Senn *et al*., 2004). Yet since the early 1990s, international regulatory authorities have considered these endpoints and for some drug substances, the pharmacodynamic data and safety data might be needed (Biomarker Definition Working Group. 2001). Remember that AUC is controlled by international regulators to be a standard assessment for extent of bioavailability (Midha *et al*., 1993; Lacey *et al*., 1997; Julious and Debarnot. 2000). In reviews, the Cmax as an assessment of rate of bioavailability has been established to be confused with extent of bioavailability and in few cases it is recognized to not define the rate of bioavailability exceptionally competent (Rescigno and Powers. 1998; Midha *et al*., 1993).

Apparently, the Cmax is dependent on the best selection of blood sampling scheme. It is noted to be usually more irregular than AUC and sometimes it's become so complicated during the evaluation of pharmacokinetics of the bioequivalence study (Lacey *et al*., 1995 and Marzo *et al*., 2000). Nevertheless, rathar than various options the Cmax has been retained to be more dependable for the administrators (Cartwright. 1991). Alternative calculations for the rate of absorption have been suggested in some literatures like direct curve metrics (Herchuelz. 1996) and Cmax/AUC (El-Tahtawy *et al*., 1998), and indirect metrics (Basson *et*

al., 1998), respectively. Although, propagation of the evaluation of alternatives have expressed these calculations to be less advantageous than the value of Cmax (Tsang *et al*., 1996 and Buice *et al*., 1996). Current performance in alternative calculations of absorption rate, for example partial AUCs is continuing in consideration to research laboratory and regulatory authorities (Bois *et al*., 1994; Marston and Polli. 1997; Endrenyi *et al*., 1991). However, this is recognized as effective calculations for the evaluation of bioequivalence studies (Ring *et al*., 2000). Therefore for the rate of bioavailability, the Cmax appears to be controlled as the minimum unacceptable measure usable at nowadays (Julious and Debarnot. 2000).

2.13.5 The rationale of BE studies

Progresses in technology raised the potentiality to generate cost-effective copies of patented drug products that known as generics since from the late sixties and seventies. These advanced formulations could possibly be marketed resulting patent completion (Tozer *et al*., 1996). For the public health concern, this thing was rational of the governmental point of view. This kind of approach could be assumed to increase the supply of the drug products in the dispensaries for requirement and so decrease the expenses for users. Due to reduced in the prices this provide essential benefit to public health. While in the United States, declarations of therapeutic failure obtained a huge concern of public consideration after the production of few copies of drug products which were pharmaceutically equivalent or similar. Such failures comprised absence of proper efficacy or necessary response, for example Carbamazepine, Oxytetracycline, Amitriptyline, Glibenclamide and intoxication, unwanted adverse effects, such as Phenytoin, Carbamazepine, Digoxin. For entry into the market, establishment of a specified regulated norms were essential (Tozer and Hauck. 1997; Endrenyi *et al*., 1998). The USFDA has recommended an approval procedure for generic drug products that was accredited by "*The Drug Price Competition and Patent Term Restoration Act, 1984"*. The following reported about uprising approaches for entry into the market of generic products over the years (Tozer *et al*., 1996). So, the USFDA has assured to need a bioequivalence study with pre-stated specified regulations for acceptability that established on the data acquisition for the authorization to market access. Likewise, these kind of studies were necessary for expansion of the patent stability for the inventors seeking to sustain market uniqueness (Shah *et al*., 1996).

2.13.6 Present international guidelines

For the completion of bioequivalence study, the prospective topics with concern to the sponsor attentions, analytical, statistical and regulatory. However, the major likely issue was the regulatory considerations. The process was agitated by examination of the formulation and without includes any definite criteria belonging to individual subjects. Also, it was noticed that the addition of criteria relating to difference might express those aspects. For low variability products with a narrow therapeutic index, the regulatory limits of 20% were uncertain or questionable as they perhaps more extensive. Because of the huge sample sizes required to certify a great possibility of success for high variability products, the 20% acceptance limits evolved a real complication for the sponsors. In alternative designs, the FDA exhibited such topics given by low variability drugs by constricting the domain in few cases, such as vaccines (Patnaik *et al*., 1997) and for highly variable drug products, mixed modelling techniques might be applied to establish standard bioequivalence to express attentions of the sponsor (Barrett *et al*., 2000). Yet some strategies like the statistical and regulatory concerns were not specifically described. For the determination of such matters, the FDA extended the consideration with the publishing of the 1997 preliminary draft guidance and an important global discussion pursued (Strom. 1987). Finally in 2003 the controversy was elucidated with an ultimate resolution to sustain following the standard bioequivalence (Rheinstein. 1990). Therefore, nowadays bioequivalence study has been adapted in practice globally to evaluate the variation in measure between formulations in a common approach.

2.14 Bioanalytical investigation of the three active metabolites with their prodrugs (parent drugs)

In the present thesis, three active metabolites and their prodrugs have quantitatively determined in the human plasma and carried out the pharmacokinetic as well as the bioequivalence study.

2.14.1 Aspirin (acetylsalicylic acid)

Aspirin i.e., 2-acetoxy benzoic acid is typically known as acetylsalicylic acid (ASA) (Figure.2.17) which is a non-steroidal analgesics (classified as a non-steroidal anti-inflammatory drug or NSAID) having with antipyretic, anti-inflammatory and anti-thrombic properties. It is one of the most extensively used anionic drugs worldwide (Jain *et al*., 2012).

It is particularly used for the treatment of transient musculoskeletal pain, dysmenorrheal, gastric irritation and headache (Ebuehi *et al*., 2007). Also, aspirin has an effect of anti-clotting or antiplatelet. In low doses, it is used as an antithrombotic agent to prevent the formation of a blood clot in a blood vessel, long-term heart attacks and strokes at high risk in human (Lewis *et al*., 1983 and Hennekens *et al*., 1989). Various investigations have proposed that aspirin may be a potent chemopreventive agent against cancer, especially colorectal cancer (Baron and Sandler. 2000; Kuehl *et al*., 2006). ASA inhibits the cyclooxygenase (both COX-1 and COX-2), an enzyme which is involved with the production of prostaglandins and thromoxanes. These hormones are performed various functions in the body, including pain transmission to the brain. Accordingly, due to the inhibition of cyclooxygenase, production of both prostaglandins and thromoxanes are reduced and so that relief from pain (Ebuehi *et al*., 2007).

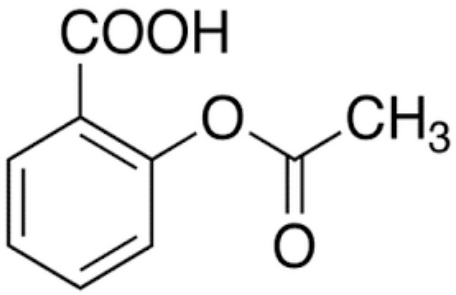


Figure.2.17 Molecular structure of acetylsalicylic acid (aspirin)

The chemical structure of ASA is quite simple. There are three different types of atoms such as, carbon, hydrogen and oxygen. These atoms are either single bonded or double bonded composed to form the complete structure of ASA. Another aspect is the various functional groups that help to construct the structure of ASA. Generally, there are three functional groups and those are carboxylic acid (R–COOH), aromatic and ester (RCOOR') (see Figure.2.17). Actually, ASA is found in the form of leaves from the willow tree which contained the active ingredient called “salicin”. It is a starting material used to make ASA. Likewise, willow bark is used to form ASA. ASA is readily hydrolyzed in the body to produce salicylic acid (SA), an active metabolite. SA is further metabolized by hydroxylation to form gentisic acid (GA) and by conjugation to form salicyluric acid (SUA) and other conjugates (Mays *et al*., 1984). Formation of the conjugates are catalysed by UDP-glucuronosyltransferases (UGTs) (Kuehl *et al*., 2006). However, the metabolism process is occurred for SA by conjugation, with either glycine or glucuronic acid, or by oxidation. SUA

is the glycine conjugate and the most abundant metabolite in the urine. Salicylic acid phenolic glucuronide (SAPG) and salicylic acid acyl glucuronide (SAAG) are the glucuronide conjugates of SA and the excretion of these salicyl glucuronides is extremely irregular, varying from 0.8 to 42% of the administered ASA dose (Kuehl *et al*., 2006). GA is either conjugated with glycine to form gentisuric acid (GUA) or is glucuronidated by glucuronic acid to give gentisic acid glucuronide (GAG) (Mullangi *et al*., 2012).

2.14.1.1 Salicylic acid

Salicylic acid (SA) (Figure.2.18) is a principle, active and major metabolite of acetylsalicylic acid (ASA). SA is also known as monohydroxybenzoic acid which is a class of phenolic acid and a beta hydroxy acid (BHA). It is a colorless crystalline organic acid with the chemical formula $C_7H_6O_3$ and the molar mass is 138.12 grams $mole^{-1}$.

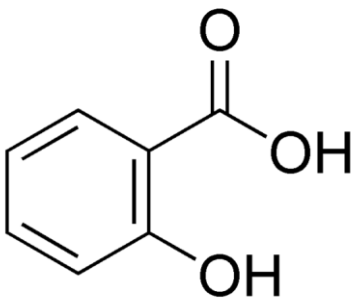


Figure.2.18 Molecular structure of salicylic acid

SA is generally used in organic synthesis and functions just like a plant hormone. It is derived from the metabolism of salicin (Ghosh *et al*., 2011). This compound i.e., SA is not similar to the active component of ASA but chemically equivalent. It is likely well known for its use as an essential element in topical anti-acne products and its treatments also. The salts and esters of SA are called salicylates. SA is found in the form of the inner bark (the medicinal part) from the willow tree. After administration into the body, ASA is rapidly hydrolysed (or deacetylated) to form SA by ubiquitous esterases in the gastrointestinal tract (mainly in the gut wall), in the liver (catalysed by Cytochrome P450 2C19 i.e., CYP2C19 and perhaps Cytochrome P450 3A i.e., CYP3A) and other tissues (Brayfield. 2014). The compound SA is mainly responsible for the pharmacological activity of ASA (Kuehl *et al*., 2006; Coudray *et al*., 1996 and Mullangi *et al*., 2012). After oral intake, it extents its maximum concentration peak in plasma from about 0.5 hr. to 2.0 hrs. It remains unchanged about 60% and can

undergo °0H (free hydroxyl radical) intervention to produce three products such as, 2,3-dihydroxybenzoate (2,3-DHBA), 2,5-dihydroxybenzoate (2,5-DHBA), and catechol (with a minor quantity). Though, the stability of SA is comparatively longer in the blood circulation with a half-life of 2.5 hrs.to 4.0 hrs. (Coudray *et al*., 1996 and Mullangi *et al*., 2012). It has the molecular formula $C_6H_4(OH)COOH$, where the OH group is ortho to the carboxyl group which is also recognized as 2-hydroxybenzoic acid (see Figure.2.18). It is poorly soluble in water i.e., 2 gm/L or 0.2 gm/100 mL of H_2O at 20 ^oC (Salicilyc Acid. *International Programme on Chemical Safety and the Commission of the European Communities, Great Britain*, 2001). ASA can be formed by the esterification of the phenolic hydroxyl group of salicylic acid with the acetyl group from acetic anhydride or acetyl chloride. By employing the Kolbe-Schmitt reaction, SA can also be produced.

SA produces the anti-inflammatory effects by suppressing the activity of cyclooxygenase (COX) enzyme which is responsible for the production of the prostaglandins (the pro-inflammatory mediators) ("Anti-Inflammatory Activity of Aspirin – It's All About Salicylic Acid". *American Chemical Society*). It triggers adenosine monophosphate-activated protein kinase (AMPK) that perform a pivotal role in the anticancer effects just like its prodrugs ASA and salsalate. The antidiabetic effects of SA are likely mediated by AMPK stimulation mainly through allosteric conformational change that raises the levels of phosphorylation (Hawley *et al*., 2012). Furthermore, it dissociates the oxidative phosphorylation which leads to increased ADP : ATP and AMP : ATP ratios in the cell (Lisa Raffensperger, "Clues to aspirin's anti-cancer effects revealed". *New Scientist*, 2012). At transcriptional and post-transcription levels, SA regulates c-Myc level. Hence, ASA exhibits an anti-cancer effect due to the inhibition of c-Myc (a significant pathway) and so as a result diminishing the occurrence of cancer in epithelial tissues (Ai *et al*., 2016).

2.14.1.2 Categories

Non-steroidal analgesic, non-steroidal anti-inflammatory drug (NSAID), antipyretic and anti-thrombic agent.

2.14.1.3 IUPAC name

2-(acetyloxy)benzoic acid

2.14.1.4 Chemical (or Molecular) formula

$C_9H_8O_4$ or $CH_3COOC_6H_4COOH$

2.14.1.5 Molecular Weight (Da, Average)

180.157 grams per mole

2.14.1.6 Physical state

Odorless white crystals or crystalline weakly acidic substance or powder with a slightly bitter taste.

2.14.1.7 Melting point

135 °C (275 °F)

2.14.1.8 Boiling point

140 °C (284 °F at 760 mm Hg)

2.14.1.9 Flash point

250 °C or 482 °F

2.14.1.10 Solubility

It is freely soluble in alcohol, soluble in chloroform, ethyl acetate and ether, slightly soluble in water. Its octanol / water partition coefficient is 13.49. Less than 1 mg/mL at 73° F. In water, 4,600 mg/L at 25 °C or 3 mg/mL at 20 °C [gm/100 mL at 15°C: 0.25 i.e., poor]. 1 gm of ASA is soluble in 300 mL of water at 25 °C, 100 mL of water at 37 °C, 5 mL of alcohol, 17 mL of chloroform, 10 to 15 mL of ether, but less soluble in anhydrous ether.

2.14.1.11 Stability

ASA is stable in dry air and it is gradually hydrolyzed into salicylic and acetic acids in moist air. It is most stable at a pH of 2 to 3, less stable at a pH of 4 to 8 and least stable at a pH less than 2 or more than 8 in aqueous solutions. At a pH of 5 to 7, ASA is almost completely hydrolyzed within one week at 25 °C in a saturated aqueous solution.

2.14.1.12 Decomposition

It is decomposed by heating around 140 °C or 284 °F (i.e., thermal decomposition). During decomposition it releases pungent smoke and fumes.

2.14.1.13 Dissociation constants or pka value

Acid dissociation constant (pKa) of ASA is 3.49 (≈ 3.5) at 25 °C (77 °F).

2.14.1.14 Biological half-life ($t_{1/2}$)

Biological half-life i.e., $t_{1/2}$ of ASA is literally dose-dependent. For low doses, it is 2 to 3 hours and for high doses, it may be 15 to 30 hours.

2.14.1.15 Storage condition (Temperature)

Store at 2 to 8 °C for longer duration in a well closed container.

2.14.1.16 Pharmacology

2.14.1.16.1 Indication

ASA is on the World Health Organization's list of essential medicines, the most effective and safe medicines required in a health system (*"WHO Model List of Essential Medicines, 19th List", World Health Organization, 2015*). It is accessible as a generic medication. It is used in the treatment of pain, fever, rheumatic fever and inflammatory diseases, including Kawasaki disease, pericarditis and rheumatoid arthritis (Ebuehi *et al*., 2007; Kotani *et al*., 2010; Jain *et al*., 2012; Mohammad *et al*., 2012 and *"Aspirin". The American Society of Health-System Pharmacists*). After a heart attack, it given shortly to reduces the risk of death. Moreover, it is used long-term basis to avoid heart attacks, strokes and blood clots in people at high risk. In some cases lower doses of ASA have been exhibited to decrease the risk of death from a heart attack or the risk of stroke (*"Aspirin". Drugs.com. American Society of Health-System Pharmacists, 2016*; *"Aspirin for reducing your risk of heart attack and stroke: know the facts". U.S. Food and Drug Administration*; *"Aspirin for the prevention of cardiovascular disease". U.S. Preventive Services Task Force*; Seshasai *et al*., 2012). In USA, low dose is considered to rational in 50 to70 years old people who have a more than 10% risk of cardiovascular disease and are not at an expanded risk of bleeding who are otherwise healthy (Bibbins-Domingo. 2016). There is some proof that it is also competent to inhibit colorectal cancer. Yet, the mechanisms of this potency are not clear (Algra and Rothwell. 2012;

Patrignani and Patrono. 2016). Its effects usually commence within 30 minutes for pain or fever. ASA works just like to other NSAID drugs though it is also an antiplatelet and therefore restrains the typical functioning of platelets. High doses of ASA may occured in ringing in the ears. Normally, it is not prescribed in the final stage of pregnancy and also not suggested in children with infections due to the risk of Reye's syndrome (*"Aspirin". Drugs.com. American Society of Health-System Pharmacists, 2016*).

2.14.1.16.2 Pharmacodynamics (Mechanism of drug action)

ASA restricts platelet aggregation by irreversible inhibition of cyclooxygenase (COX) enzyme and consequently suppresses the production of thromboxane A2 (TXA2) which is an inducer of platelet aggregation and vasoconstriction (Nagelschmitz *et al*., 2014 and Postula *et al*., 2013). This effect persists for the life of the platelet. ASA has competence to inhibit the generation and release of prostaglandins and thromboxanes is because of its irreversible inactivation of the cyclooxygenase (COX) enzyme. This COX enzyme is needed for the synthesis of prostaglandin and thromboxane (Hennekens *et al*., 1989). ASA emerges to generate analgesia by integrity of both peripheral and CNS effect. In the active site of the COX enzyme, it performs as an acetylating agent where an acetyl group is covalently coupled to a serine residue (Tóth *et al*., 2013). So due to this, ASA is different from other NSAID drugs like diclofenac and ibuprofen which are reversible inhibitors. Though, its other effects are the uncoupling oxidative phosphorylation in mitochondria and the modulation of signaling through a transcription factor complex i.e., NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) are also being studied. It performs a key role in various biological processes including inflammation. There are two different cyclooxygenase isozymes, COX-1 (PTGS1) and COX-2 (PTGS2). Now, ASA is non-selective and irreversibly inhibits both forms yet it is weakly more selective for COX-1 (Sharma and Sharma. 1997; Kerola *et al*., 2009). Hence, it occurs by acetylating the hydroxyl of a serine residue (Vane and Botting 2003). Usually COX produces prostaglandins, most of which are pro-inflammatory and thromboxanes, which stimulate clotting. ASA-modified COX-2 generates lipoxins and most of which are anti-inflammatory. Recent NSAID drugs are known as COX-2 selective inhibitors that have been developed for the inhibition of COX-2 only with the desire for depletion of gastrointestinal side-effects (Warner and Mitchell. 2002). Although after confirmation expressed that COX-2 inhibitors enhance the risk of heart attack, various COX-2 selective inhibitors have been withdrawn; afterwards. The unrevealed mechanism for the detrimental effect proposes that endothelial cells lining the microvasculature in the body

express COX-2 and its selective inhibition consequences in levels of prostaglandin I2 (PGI2, prostacyclin) down-regulated comparative to thromboxane, because COX-1 is unchanged in platelets. Hence, the protective anti-coagulative effect of PGI2 is reduced, increasing the risk of thrombus and related heart attacks and other circulatory complications. The principal difference between ASA and the reversible inhibitors is that the platelets are incompetent to produced new COX before ASA has irreversibly suppressed the enzyme, because platelets have no DNA.

Accordingly, the pharmacological profile of ASA as a cyclooxygenase (COX) inhibitor (both COX-1 and COX-2) has been considerably confirmed.

- It is active in most conventional models of inflammation, irrespective of their several mechanisms.
- Likewise, it has been exhibited to be a peripheral analgesic and antipyretic.
- The anti-thrombotic activity of ASA depends on both a specific anti-platelet activity and a fibrinolytic activity due to selective acetylation of thrombocyte COX-1 and N-acetylation of fibrinogen, respectively.

Prostaglandins are local hormones (paracrine) synthesize in the body and have various functions including yet not limited to transmission of pain information to the brain, inflammation and modulation of the hypothalamic thermostat. Thromboxanes are the potent activators for the aggregation of platelets that form blood clots. Low-dose of ASA in long-term use irreversibly restricts the production of thromboxane A2 (TXA2) in platelets and thus triggering an inhibitory effect on platelet aggregation. This antiplatelet characteristic makes it effective for minimizing the occurrence of heart attacks which are mainly caused by blood clots and their reduction with the introduction of small amounts of ASA has been recognized to be a convenient therapeutic intervention. A dose of 40 mg per day is competent to prevent a large quantity of highest TXA2 release stimulated intensely with the PGI2 synthesis being slightly influenced. While, higher doses of ASA are needed to obtain more inhibition (Tohgi *et al*., 1992). Often excessive bleeding is occurred due to the potentiality of the blood clot is decreased for the use of ASA.

2.14.1.16.3 Pharmacokinetics

- **Absorption** After oral administration of ASA, it has established to follow first-order kinetics. Several factors influence the rate of absorption like the rate of gastric emptying, blood volume, simultaneous administration of other drugs, exercise, posture and pH of stomach content (Zeitoun *et al*., 2014; Lopez-Farre *et al*., 1995 and Ridker et al., 1997). ASA is rapidly absorbed to provide detectable concentrations in plasma even after 30 minutes before a gradual decrease with transformation of ASA to salicylate after oral administration. It is a weak acid and after administration, very small of it is ionized in the stomach. In the acidic conditions, ASA is quickly absorbed through the cell membrane of the stomach. Because of the increased pH (i.e., alkaline medium) and larger surface area, it is absorbed more slowly in the small intestine (mainly from the upper small intestine) as more of it is ionised. Accordingly, the absorption is delayed by 3 to 6 hours and the time to reach the mean peak of ASA concentration is 4 to 6 hours. Hence, the corresponding times to obtain peak SA concentrations are 8 to 14 hours. (Ridker *et al*., 1997). After 5 to 7 days of therapy, it is achieved the steady-state levels (Lopez-Farre *et al*., 1995). During overdose, aspirin is absorbed much more slowly and after ingestion, plasma concentrations can sustain to increase for up to 24 hours (Ferguson and Boutros. 1970; Kaufman and Dubansky. 1970; Levy and Tsuchiya. 1972).

- **Distribution** Both ASA and salicylate are rapidly and extensively distributed in body fluids. ASA is determinable in the cerebral, spinal, and synovial fluid after the administration of high doses. It crosses the placenta (i.e., placental barrier) and passes into breast milk (Zeitoun *et al*., 2014). They are confined in liver and kidney, i.e., the excretory organs and glandular stomach. Bioavailability of ASA is about 80% to 100% and protein binding is apparently 80% to 90% (Brayfield. 2014). ASA actively binds to plasma proteins and can replace particular compounds including other acidic nonsteroidal anti-inflammatory drugs (NSAID) or coumarins. In the blood, around 50% to 80% of salicylate is bound to albumin protein. Whereas, the rest remains in the active and ionized condition. Basically plasma protein binding is concentration-dependent, in case of SA its approximately 66% to 98%. In adults, volume of distribution ranges from 9.6 L/kg to 12 L/kg (Clissold *et al*., 1986). Saturation of binding sites creates more free salicylate and enhanced toxicity. The volume of

distribution is 0.1 L/kg to 0.2 L/kg. Due to the increase of tissue penetration of salicylates, acidosis raises the volume of distribution (Levy and Tsuchiya. 1972).

- **Metabolism** During and after absorption, ASA is converted i.e., rapidly hydrolysed or deacetylated into its main active metabolite salicylic acid (SA) with a half-life of 15 to 20 minutes. Hence, from this phase onward the pharmacokinetics of ASA are principally dependent on the salicylate moiety. The acetyl group initiates to divide hydrolytically even during passage through the gastrointestinal mucosa, but mostly this process occurs in the liver (almost 80% of therapeutic doses of SA is metabolized) (Zeitoun *et al*., 2014). Now, the biotransformation (i.e., metabolism) of ASA accomplishes in two steps. The first step is dose-independent and comprises transformation to salicylate by pre-absorptive hydrolysis in the gut mucosa and a hepatic first-pass (or presystemic) effect. The second step is dose-dependent and the metabolic transformation of salicylate and following formation of conjugates and their renal excretion. Conjugation with glycine forms salicyluric acid (SUA) and with glucuronic acid to form two different glucuronide esters. The conjugate with the acetyl group complete is mentioned to as the acyl glucuronide and the deacetylated conjugate is the phenolic glucuronide (Kuehl *et al*., 2006). These metabolic pathways have exclusively a limited capacity. Moreover, small extents of SA are hydroxylated to gentisic acid (GA). The kinetics shift from first-order to zero-order with extensive salicylate doses, as metabolic pathways become saturated and renal excretion becomes more and more essential (Levy and Tsuchiya. 1972).

- **Excretion or Elimination** ASA is excreted from the body through the urine (around 80% to 100%), sweat, saliva, feces. The metabolism is restricted by the capacity of liver enzymes, so as a result the elimination kinetics of ASA is dose-dependent after Michales-Menten kinetics. Subsequently low doses to about 12 hours after normal analgesic doses, the elimination half-life differs between 2 to 3 hours (Zeitoun *et al*., 2014). Salicylates are excreted (eliminated) mostly by the kidneys as SUA (75%), free SA (10%), salicylic phenol (10%) and acyl glucuronides (5%), GA (less than 1%) and 2,3-dihydroxybenzoic acid (Grootveld and Halliwell. 1988). All pathways proceed by first-order kinetics with an elimination half-life of about 2.0 hours to 4.5 hours when small doses (< 250 mg) are ingested in an adult (Hartwig. 1983 and Done. 1960). The half-life becomes much longer (around 15 hours to 30 hours) after higher doses (more than 4 gm) of salicylate are ingested (Chyka *et al*., 2007). Because the metabolic

pathways concerned with the formation of SUA (salicyluric acid) and SPG (salicyl phenolic glucuronide) become saturated (Prescott *et al*., 1982). As the biotransformation pathways become saturated, renal excretion of SA becomes increasingly significant because it is highly sensitive to variations in urinary pH. When urine pH is increased from 5 to 8 then a 10 to 20 fold rise in renal clearance take places. The use of urinary alkalinization accomplishes the specific aspect of salicylate elimination (Dargan *et al*., 2002).

2.14.1.17 Toxicology

ASA is the most common agent associated with SA poisoning. Different forms of salicylic acid are found in various over the counter products comprising methyl salicylate (oil of wintergreen), bismuth subsalicylate (Pepto-Bismol), enteric-coated aspirin (Ecotrin). It elucidate a significant number of deaths from over the counter drug overdoses. Salicylates irritate gastric mucosa, depress the CNS, activate medullary centers (increased respiratory rate and vomiting), increase tissue glycolysis, inhibit Krebs cycle enzymes and uncouple oxidative phosphorylation, stimulate lipid metabolism and suppress amino acid metabolism. Metabolic toxicity results in mixed metabolic acidosis or respiratory alkalosis, altered glucose metabolism i.e., both hypoglycemia and hyperglycemia, hyperthermia, electrolyte imbalances and fluid depletion. Acidosis triggers salicylate penetration of CNS which exacerbates toxicity. Elimination half-life increases from 3 to 4 hours with therapeutic dosing to as long as 24 hours after acute overdose or chronic intoxication.

Table.2.9 Estimated toxic doses

Ingested Dose (mg/kg)	Estimated Toxicity
<150	No toxicity reaction expected
150 to 300	Mild to moderate toxicity
300 to 500	Severe or serious toxicity
> 500	Potentially lethal toxicity

Usually, toxicity is more severe with chronic overdose in patients, the aged who underlying medical complications and (PEDS) infants. Commencement of clinical toxicity and peak

serum levels can be delayed in patients with ingestion of sustained release or enteric-coated ASA, or if pylorospasm or pharmacobezoar develops. Patients must be observed until serial serum salicylate levels are decreasing and clinical symptoms are enhancing.

- **Gastrointestinal** - Epigastric pain, nausea and vomiting generally arise shortly following acute ASA ingestion. Hematemesis or haematemesis is infrequent.

- **Central Nervous System** - Common symptoms of mild CNS toxicity include nausea and vomiting, lethargy, tachypnea and tinnitus. Patients may be irritable and disoriented, indicating moderate CNS toxicity. Patients with serious or severe CNS toxicity develop asterixis, hallucinations, seizures and coma. In addition to express salicylate-induced toxicity, the presence of seizures exhibits a serious prognosis that can be referred to an electrolyte and/or metabolic disorder.

- **Respiratory -** Tachypnea and hyperpnea are secondary to the stimulation of the respiratory center in the midbrain, and also reparation for metabolic acidosis. There are some risk factors of non-cardiogenic pulmonary edema, and those are - age above 40 years, smoking, metabolic acidosis, chronic salicylate ingestion, salicylate levels more than 60 mg/dL, neurologic symptoms etc.

- **Cardiovascular -** Tachycardia is common but is not generally hemodynamically significant. Hypotension, dysrhythmias and shock are not usual but can develop in patients with severe or serious toxicity.

The most common side effects of daily ASA intake include an upset stomach or indigestion, i.e., its action on the stomach resulting in dyspepsia, ulcers, gastric and duodenal erosions, occult bleeding, haemorrhage and worsening asthma (Zeitoun *et al*., 2014). Bleeding risk is higher among those who are older, drink alcohol, take other NSAIDs, or are on blood thinners. Dyspepsia is a relatively common disorder in patients but does not correlate with gastric bleeding. To diminish dyspepsia, ASA was given with meals or antacids with the concern that by buffering the acid in the stomach, gastric irritation and blood loss will be decreased (Zeitoun *et al*., 2014). Although, employing an antacid with ASA has a significant effect on plasma levels of salicylate. The rise in the urinary pH induced an increase in the renal clearance of salicylate and therefore produces reduced plasma level of the salicylate level. Likewise, dyspepsia, if not prevented, would reduce the gastric emptying which would change the level of ASA absorbed. Enteric-coated ASA was reported to reduce this

complication (Zeitoun *et al*., 2014; Awtry and Loscalzo. 2000; Clissold. 1986; Lopez-Farre *et al*., 1995; Husain *et al*., 1998).

2.14.1.18 Bioanalytical assay methods overview of active metabolite SA with prodrug ASA

Different methods have been published for the bioanalysis of active metabolite SA and its prodrug ASA in biological fluids. These includes both HPLC and LC-MS/MS methods employing various sample preparation and extraction techniques. A summary of these methods are given below.

- An extensive overview of various bioanalytical methods, both in HPLC and LC-MS/MS which have been described for direct quantitation of aspirin along with its metabolite(s). This review also contributes general information on sample collection, sample processing, internal standard selection, conditions for chromatographic separation, concise validation data and relevant conclusions for reported analysis (Mullangi *et al*., 2012).

- Ghosh, Upadhayay, Singh *et al*., (2011) have developed and validated a simple, rapid, sensitive, selective, precise and accurate liquid chromatography (UPLC) method with UV detection for simultaneous estimation of aspirin (ASA) and salicylic acid (SA) in human plasma using benzoic acid (BA) as internal standard (IS) were extracted by protein precipitation technique (PPT) with perchloric acid. The entire method was performed by C18 column, 100 x 2.1 mm i.d., particle size 1.7 µm with the mobile phase composed of 20 mM potassium dihydrogen phosphate (pH 2.1 ± 0.05) in Milli-Q water : methanol : acetonitrile (70:15:15, v/v/v), at a flow-rate of 0.350 mL min^{-1}. Total run time of single injection and injection volume were 3 min and 30 µL, respectively. The lower limit of quantification (LLOQ) was found 15 ng mL^{-1}.

- Kumar, Jamadar, Bhat *et al*., (2010) have developed and validated a sensitive, specific, precise and cost effective high performance liquid chromatographic (HPLC-UV) method of analysis for aspirin in presence of its degradation products by employing Hypersil BDS C18, 100 x 4.6 mm, 5 µ column as stationary phase with sodium perchlorate buffer (pH 2.5): acetonitrile: isopropyl alcohol (85:14:1%, v/v) as eluent i.e., mobile phase, at a flow rate of 1.5 mL min^{-1} at 275 nm of UV detection. Column run for 10 min at temperature of 50ºC.

- A liquid chromatography–tandem mass spectrometry (LC-MS/MS) method has been developed and validated for determination of acetylsalicylic acid (ASA) and its major metabolite salicylic acid (SA) in human plasma using simvastatin as an internal standard (IS) by method was using a reversed-phase column C18, 50 x 2.0 mm, 3 µm particle size with an isocratic mobile phase consisting of acetonitrile and water containing 0.1% formic acid (8:2, v/v), at a flow rate of 0.2 mL/min with 5 µL of injection volume. Plasma sample containing potassium fluoride was extracted applying a mixture of ethyl acetate and diethyl ether in the presence of 0.5% formic acid for ASA and protein precipitation with acetonitrile for SA. Total chromatographic run time was 3.0 min. The linearity for ASA and SA were in the ranges of 5 to 500 ng/mL and 50 to 5000 ng/mL, respectively. This bioanalytical method was successfully applied for the determination of pharmacokinetics of ASA and SA after single oral administration of Astrix 100 mg (entero-coated pellet) to 10 Korean healthy male volunteers (Bae *et al*., 2008).

- Xu , Koetzner , Boulet *et al*., (2009) have developed and validated a simple, rapid and sensitive analytical method by liquid chromatography–tandem mass spectrometry (LC-MS/MS) for the evaluation of acetylsalicylic acid (ASA) and its major metabolite salicylic acid (SA) in animal plasma using 6-methoxysalicylic acid as the internal standard (IS). The compounds were separated on a reversed-phase C18 column (100 × 2.0 mm i.d., 3 µm particle size) with an isocratic mobile phase comprises a mixture of acetonitrile and water with 0.1% formic acid (63:37, v/v), at a flow rate of 0.3 mL/min. The lower limits of quantification (LLOQ) were found 3 ng/mL and 30 ng/mL for ASA and SA, respectively.

- Pirola, Bareggi, Benedittis (1998) reported a HPLC method to assess the concentrations of acetylsalicylic acid (ASA) and salicylic acid (SA) in human stratum corneum and in plasma. ASA and SA were separated on a LiChrospher 100 RP-18 column at flow rate of 1 mL/min using a water, phosphate buffer (200 mM potassium dihydrogenphosphate) adjusted to pH 2.5 with phosphoric acid and acetonitrile (35:40:25, v/v/v) as the mobile phase. The method has been developed to determine the concentrations in human stratum corneum employing the stripping method and direct injection in plasma using solid-phase extraction (SPE) technique.

- A column-switching liquid chromatographic (HPLC) method is described for the simultaneous determination of aspirin and salicylic acid in human plasma without using an internal standard (IS). The complete method was carried out by an on-line solid-phase extraction (SPE) technique using a Hypersil C-8 guard column (10 mm x 4.0 mm, 30 µm) which was fitted prior to the Nucleosil C-8 analytical column (250 mm x 4.6 mm, 5 µm) with a mixture of water, methanol, acetonitrile and orthophosphoric acid (650:200:150:1, v/v/v/v) as the mobile phase at a flow rate of 1 mL/min. The λ (lamda) max was at 225 nm (McMahon and Kelly. 1998).

- Kees, Jehnich and Grobecker. (1996) have expressed a HPLC method for the simultaneous estimation of acetylsalicylic acid (ASA) and its main metabolite salicylic acid (SA) in human plasma using 2-methylbenzoic acid as internal standard (IS). The compounds were separated on a reversed-phase C18 column (column oven temperature was at 30ºC), 150 x 4 mm, 4 µm silica with a mobile phase consisted of 740 mL water, 900 µL orthophosphoric acid (85%) and 180 mL acetonitrile with the resulting pH 2.5 at a flow rate of 1 mL/min. and photometric detection was at 237 nm. By adding sodium chloride, acidified plasma was separated from the aqueous layer which was deproteinized by acetonitrile. ASA and SA were extracted into the acetonitrile layer with a large amount.

- Coudray, Mangournet, Bouhadjeb *et al*., (1996) have developed a simple, rapid and convenient HPLC assay for salicylic acid (SA) in human plasma without organic solvent extraction to investigate the in vivo free radical production, i.e., emphasizes the investigation of the in vivo oxidative stress in humans following the administration of 1000 mg aspirin in 20 healthy subjects.

2.14.2 Enalapril

Enalapril maleate is a salt of enalapril and maleic acid (Figure.2.19). It is a derivative of two amino acids, L-alanine and L-proline and also is a synthetic peptidic derivative (Yoon *et al*., 2004; Saeed *et al*., 2013; Nagarajan *et al*., 2013; Pawar *et al*., 2011; Spanakis and Niopas 2010). Enalapril (EPL) belongs to the series of substituted N-carboxymethyl dipeptides (Lu *et al*., 2009) (Figure.2.20). It is a potent and effective antihypertensive and a vasodialator in congestive heart failure. This agent is competent to reduce cardiovascular mortality and morbidity in patients with cardiac failure (Pawar *et al*., 2011 and Santos *et al*., 2009). EPL is basically a potent long-acting oral inhibitor of angiotensin-converting enzyme (ACE). It is

widely used for the treatment of high blood pressure i.e., essential or renovascular hypertension in adults and children, diabetic nephropathy or diabetic kidney disease and heart failure ("Enalaprilat/Enalapril Maleate". *The American Society of Health-System Pharmacists.*; Rao *et al*., 2014; Santos *et al*., 2009; Rahman and Haque 2008).

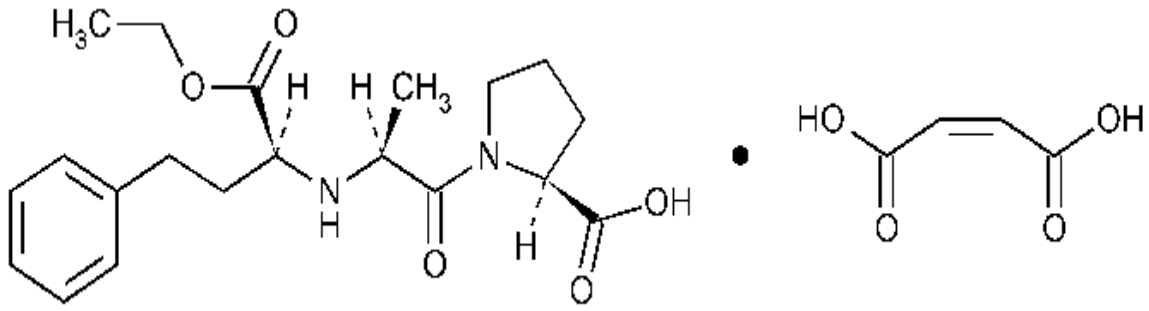


Figure.2.19 Molecular structure of enalapril maleate

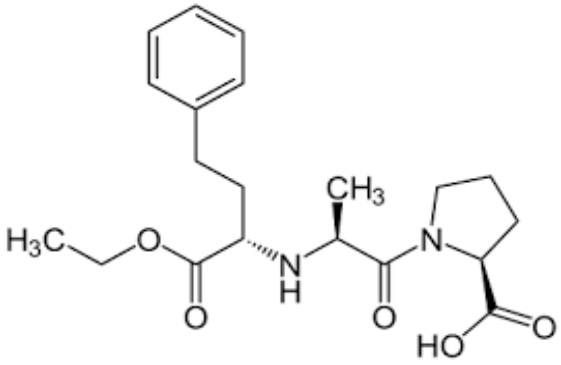


Figure.2.20 Molecular structure of enalapril

Likewise, treatment of symptomatic congestive heart failure and prevention of symptomatic cardiac failure in patients with asymptomatic left ventricular dysfunction (ejection fraction less than 35%) (Nagarajan *et al*., 2013; Spanakis and Niopas 2010; Kang *et al*., 2014; Lee *et al*., 2003). It decreases the plasma concentration of angiotensin II due to the inhibition of the conversion of angiotensin I to angiotensin II, leads to a depletion in vasopressin activity and also increased prostaglandin synthesis which is involved in the antihypertensive action. EPL diminishes peripheral vascular resistance without causing an increase in the heart rate. So it is an ideal drug for hypersensitive patients those who are intolerant to beta-blockers (Nagarajan *et al*., 2013; Rahman and Haque 2008; Kang *et al*., 2014). EPL is a dipeptide with a proline peptide bond and can exist as rotamers and a monovalent anion (Lee *et al*., 2003 and Loftsson *et al*., 2010). There are four different types of atoms like, carbon, hydrogen, oxygen and nitrogen. These atoms are either single bonded or double bonded composed to construct the

complete structure. There are two types of functional groups such as the carboxyl group (-COOH) and the amine group (-NH2) (see Figure.2.20). In aqueous solutions, EPL has maximum stability at pH about 3 (shelf-life [t_{90}] 262 days at pH 2 and 114 days at pH 5 and room temperature) and the major degradation product is EPLT. Whereas in solid state, EPL is chemically stable when protected from humidity and high temperatures (Loftsson *et al*., 2010). EPL is a pro-drug without direct biological activity. It is bioactivated by hydrolysis of its ethyl ester, i.e., it is de-esterified in vivo (i.e., in the body) after absorbtion from the gastrointestinal (GI) tract to its active metabolite enalaprilat (EPLT) by hepatic metabolism in the liver. EPL exerts its activity (ACE inhibition) in vivo after it is converted to its active diacid metabolite EPLT (Loftsson *et al*., 2010; Yoon *et al*., 2004; Makwana *et al*., 2011; Mohan *et al*., 2011 and Martindale The Extra Pharmacopoeia, 31st ed.). EPL is rapidly absorbed after oral administration and maximum plasma concentrations are arised within one hour approximately. The extent of absorption is about 60% from oral EPL tablet (Nagarajan *et al*., 2013).

2.14.2.1 Enalaprilat

Enalaprilat (EPLT) (Figure.2.21) i.e., (S)-1-N-1-(carboxy)-3-phenylpropyl-L-alanyl-L-proline (II) or (2S)-1-[(2S)-2-[[(1S)-1-carboxy-3-phenylpropyl]amino]propanoyl]pyrrolidine-2-carboxylic acid is a main active diacid metabolite of enalapril (EPL) and responsible for the therapeutic action (Pisarev *et al*., 2005; Spanakis and Niopas. 2010; Lee *et al*., 2003; Ghosh *et al*., 2012). It is the only metabolite of EPL because there is no evidence of metabolites of EPL, other than EPLT (Ghosh *et al*., 2012). It is also known as enalapril acid, enalapril diacid, enalaprilic acid, enalaprilat anhydrous and enalaprilat dihydrate (Loftsson *et al*., 2010). It is a white to off-white anhydrous crystalline odourless powder, slightly photosensitive compound with the chemical formula $C_{18}H_{24}N_2O_5$ and the molar mass is 348.399 grams $mole^{-1}$ (Pisarev *et al*., 2005 and Sultana *et al*., 2013). EPLT is both the biologically and the pharmacologically active form that has two carboxylic acid moieties (see Figure.2.21) with pKa values of 2.3 and 3.4, respectively and a secondary amide moiety with a pKa value of 8.0. The adjacent carboxylic acid reduces the basicity of the secondary amine due to the esterification (i.e. formation of EPL) and lowering its pKa from 8.0 to 5.5 (Loftsson *et al*., 2010). Accordingly at neutral pH, EPLT carries two anions and a cation. This considerably enhances the lipophilicity of the drug and increasing its calculated log P (octanol / water) value from - 0.94 to 2.45 (which is a 2500-fold increase in the octanol / water partition coefficient).

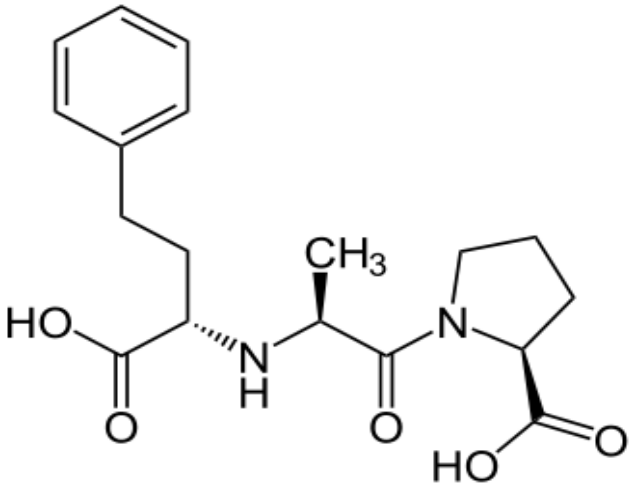


Figure.2.21 Molecular structure of enalaprilat

Therefore, as a result enhances its absorption after oral administration (Loftsson *et al*., 2010). However, EPLT is more hydrophilic than EPL, it is rather less soluble in water and organic solvents. Also, it is much more stable than EPL and when stored at less than 30^{o}C, the parenteral solution is reported to be stable and protected from light (Trissel. 2003 and Loftsson *et al*., 2010). EPLT is a diketopiperazine derivative (DKP) and it is formed in the body by instant and extensive hydrolysis of its ethyl ester i.e., de-esterified through hepatic metabolism by different esterases after rapidly absorbed from the GI tract following oral administration of EPL (Mohan *et al*., 2011; Spanakis and Niopas. 2010; Ghosh *et al*., 2012; Wang *et al*., 2007; Kang *et al*., 2014). EPLT is an intravenously (IV) administered angiotensin-converting enzyme (ACE) inhibitor and also is a more potent and effective ACE inhibitor than EPL. It is poorly absorbed when administered orally. Peak plasma concentrations occur 2 to 4 hours after an oral dose administration of EPL (Pisarev *et al*., 2005 and Ghosh *et al*., 2012). EPLT is an effective agent for the treatment of essential hypertension and has beneficial hemodynamic effects in heart failure (i.e., congestive heart failure) by dilating peripheral vascular resistance without causing significant changes in heart rate or cardiac output and also without any significant side effects (Lu *et al*., 2009; Kang *et al*., 2014; Gu *et al*., 2004 and Wang *et al*., 2007). It is the first dicarboxylate-containing ACE inhibitor or a dicarbocyl-containing peptide and was developed partially to overcome these limitations of captopril. The sulfhydryl-moiety was substituted by a carboxylate-moiety, yet further modifications were needed in its structure-based design to obtain an effectiveness similar to captopril. The outcome of the structural modifications was to provide it ionisation characteristics that do not permit satisfactory GI absorption for oral administration in tablets.

Therefore, EPLT was more convenient for intravenous (IV) administration. This was overcome to produce EPL by the esterification of EPLT with ethanol (Jones *et al*., 2012). EPLT competitively binds to and inhibits ACE, so that blocking the conversion of angiotensin I to angiotensin II. This prevents the potent vasoconstrictive actions of angiotensin II and results in vasodilation. It also reduces angiotensin II-induced aldosterone secretion by the adrenal cortex, which leads to an increase in sodium excretion and subsequently increases water outflow (Yoon *et al*., 2004 and Saeed *et al*., 2013).

2.14.2.2 Categories

Antihypertensive drug and vasodialator, an angiotensin-converting enzyme (ACE) inhibitor.

2.14.2.3 IUPAC name

(2S)-1-[(2S)-2-[[(2S)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]pyrrolidine-2-carboxylic acid (for enalapril)

Synonyms: (S)-1-[N-[1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl]-L-proline maleate; 1-[N-[(S)-1-carboxy-3-phenylpropyl]-L-alanyl]-L-proline 1'-ethyl ester maleate (for enalapril maleate salt)

2.14.2.4 Chemical (or Molecular) formula

$C_{20}H_{28}N_2O_5$ for enalapril and $C_{20}H_{28}N_2O_5 \bullet C_4H_4O_4$ for enalapril maleate salt

2.14.2.5 Molecular Weight (Da, Average)

376.453 grams per mole (enalapril) and 492.5 grams per mole (enalapril maleate salt)

2.14.2.6 Physical state

White to off-white crystalline odourless powder.

2.14.2.7 Melting point

143 °C to 144.5 °C (289.4 °F to 292.1 °F) and 148 °C to 151 °C for EPLT.

2.14.2.8 Boiling point

0 °C

2.14.2.9 Flash point

765.3 °C at 760 mmHg

2.14.2.10 Solubility

In water, it is 16400 mg/L i.e., 0.025 gm/mL or 25 mg/mL at 25 °C (for EPLT it is 5 mg/mL). In methanol, it is 200 mg/mL or 0.20 gm/mL and in chloroform, 0.6 mg/mL or 0.0006 gm/mL (for EPLT, 15 mg/mL and < 0.1 mg/mL, respectively). In alcohol, it is 0.00008 mg/mL or 0.08 gm/mL.

2.14.2.11 Stability

It suggested that enalapril maleate tablets must be stored in tight containers at a temperature below 30 °C and that transient exposure to temperatures warmer than 50 °C should be avoided. The tablets should be protected from moisture and also when stored at under 30 °C, the tablets have an expiration date of 30 mos following the date of manufacture. Due to the drug is not adequately stable in solution, the manufacturer states that extemporaneous preparation of oral solutions must be avoided. Commercially available EPLT injection should be stored at a temperature less than 30 °C. Following dilution of EPLT injection in 5% dextrose, 0.9% sodium chloride, 5% dextrose and 0.9% sodium chloride, 5% dextrose in lactated Ringer's, or Isolyte(R) E, solutions of the drug are stable at room temperature for 24 hrs.

2.14.2.12 Decomposition

It is decomposed through the thermal degradation process i.e., by heating upto 150 °C – 340 °C.

2.14.2.13 pH

pH 2.6 (1% water)

2.14.2.14 Dissociation constants or pka value

pKa 1 = 2.97 (≈ 3.0) (for the carboxyl group) and pKa 2 = 5.35 (≈ 5.4) (for the amine group) at 25 °C.

2.14.2.15 Biological half-life ($t_{1/2}$)

The effective biological half-life ($t_{½}$) of EPL is less than 2 hours i.e., approximately 1.3 hours. Whereas for EPLT, following multiple doses is approximately 11 to 14 hours in healthy individuals with normal renal function (determined from urinary recovery). The average terminal half life of EPL and EPLT are about 2 hours and 35 to 38 hours, respectively. Following oral administration, the half-life of unchanged EPL occurs to be less than 2 hours in healthy individuals and in patients with normal hepatic and renal functions, however it can be increased in patients with congestive heart failure. After oral administration of a single 5 or 10 mg dose of enalapril maleate in patients with congestive heart failure, the half-life of EPL is 3.4 hours or 5.8 hours, respectively.

2.14.2.16 Storage conditions (Temperatures)

Store at below 40 to 50 °C (104 to 122 °F), preferably between 15 and 30 °C (59 and 86 °F), in a well closed container.

2.14.2.17 Pharmacology

2.14.2.17.1 Indication

EPL is used for the treatment of high blood pressure i.e., essential or renovascular hypertension, symptomatic congestive heart failure and asymptomatic left ventricular dysfunction ("Enalaprilat/Enalapril Maleate". The American Society of Health-System Pharmacists.; Nagarajan *et al*., 2013; Rao *et al*., 2014; Rahman and Haque. 2008; Spanakis and Niopas. 2010 and Lee *et al*., 2003). It can be used alone or in combination with thiazide diuretics (Spanakis and Niopas. 2010). It has been demonstrate to protect the function of the kidneys in hypertension, heart failure, and diabetes (diabetic nephropathy or diabetic kidney disease), and can be used in the absence of hypertension for its kidney protective effects (Santos *et al*., 2009). So that it is extensively used in chronic kidney failure (He *et al*., 2013). Moreover, EPL is an emerging treatment for psychogenic polydipsia. A double-blind, placebo-controlled trial exhibited that when used for this purpose, EPL lead to reduced water consumption (determined by urine output and osmality) in 60% of patients (Greendyke *et al*., 1998).

2.14.2.17.2 Pharmacodynamics (Mechanism of drug action)

Typically, angiotensin I is converted to angiotensin II by an angiotensin-converting enzyme (ACE) [ACE cleaves the terminal two peptides from decapeptide (angiotensin I) to form octapeptide (angiotensin II)] (Yoon *et al*., 2004; Sultana *et al*., 2013; Nagarajan *et al*., 2013 and Ghosh *et al*., 2012). Angiotensin II is a potent vasoconstrictor substance or agent which constricts blood vessels and enhances blood pressure (Saeed *et al*., 2013 and Ghosh *et al*., 2012). EPL, after hydrolysis to EPLT (an active metabolite), inhibits ACE in human subjects and animals. As a result reduces levels of angiotensin II leading to less vasoconstriction and decreased blood pressure. ACE is a peptidyl dipeptidase that catalyzes the conversion of angiotensin I to angiotensin II. This angiotensin II stimulates aldosterone secretion by the adrenal cortex (Yoon *et al*., 2004; Saeed *et al*., 2013 and Nagarajan *et al*., 2013). Consequently, there is an increase in blood pressure and cardiac failure. ACE has two functionally active domains, N and C, which arise from tandem gene duplication. Although the two domains have high sequence similarity, they play distinct physiological roles. The C-domain is predominantly involved in blood pressure regulation while the N-domain plays a role in hematopoietic stem cell differentiation and proliferation. ACE inhibitors bind to and inhibit the activity of both domains, but have much greater affinity for and inhibitory activity against the C-domain. The advantageous effects of EPL in hypertension and heart failure occur to result mainly from suppression of the renin-angiotensinaldosterone system (Yoon *et al*., 2004 and Saeed *et al*., 2013). Renin is synthesized by the kidneys and it is released into the circulation where it performs on a plasma precursor to produce angiotensin I (Ghosh *et al*., 2012). EPL blocks the conversion of angiotensin I to angiotensin II by the inhibition of ACE. So due to this reason decreases vasopressor activity i.e., reduces blood pressure (by reducing peripheral vascular resistance without increasing cardiac output rate and contractility), decreases aldosterone secretion and increases endogenous bradykinin concentrations, slightly increasing serum potassium levels, and causing sodium and fluid loss. Furthermore, increases prostaglandin synthesis that can be implicated in the antihypertensive activity (Yoon *et al*., 2004; Foda *et al*., 2010; Saeed *et al*., 2013; Sultana *et al*., 2013; Nagarajan *et al*., 2013; Spanakis and Niopas. 2010; Ghosh *et al*., 2012).

2.14.2.17.3 Pharmacokinetics

- **Absorption** EPL is well and rapidly absorbed from the GI tract in healthy subjects following oral administration (Kang *et al*., 2014; Gu *et al*., 2004 and Ghosh *et al*.,

2012). While, EPLT is poorly absorbed from the GI tract due to its high polarity (clinically administered IV) and only about 3% to 12% of an orally administered dose being absorbed (Ghosh *et al*., 2012). Around 60% to 70% (i.e., bioavailability) of an oral dose of EPL is rapidly absorbed from the GI tract in both healthy individuals and hypertensive patients (Nagarajan *et al*., 2013; Kang *et al*., 2014 and Ghosh *et al*., 2012). Food in the GI tract does not occur to substantially affect the rate or extent of absorption of EPL (Nagarajan *et al*., 2013 and Ghosh *et al*., 2012). It appears to undergo first pass metabolism i.e., hydrolyzed to EPLT, mainly in the liver after oral administration. The hypotensive effect of a single oral dose is normally observable within 1 hour and maximal in 4 to 6 hours. This effect of normal doses of the drug usually persists for 12 to 24 hours but may decline toward the end of the dosing interval in few cases. Generally, the hypotensive effect is noticeable within 5 to 15 minutes with maximal effect occurring within 1 to 4 hours following IV administration of EPLT. Duration of the effect is dose related and the duration of action is around 6 hours for the recommended doses in most cases. The therapeutic daily dose of EPL is 10 to 20 mg and the time for maximum plasma concentration is reached in the blood is about 1 hour i.e., T_{max} (Pisarev *et al*., 2005 and Kang *et al*., 2014). It disappears from the circulation below level of 10 ng/mL after 4 hours of drug dose. The terminal half-life of EPL is approximately 2 hours after a single oral dose, whereas for EPLT it is about 30 to 35 hours. So therefore EPLT is detectable and measurable for up to 72 hours. Moreover, T_{max} of EPLT is about 3 to 4 hours after administration (Pisarev *et al*., 2005; Kang *et al*., 2014 and Gu *et al*., 2004).

- **Distribution** Distribution of EPL into human body tissues and fluids has not been completely characterized. Around 50 to 60% of EPLT is bound to plasma proteins (Loftsson *et al*., 2010). Two binding sites have been recognized, a low affinity, high capacity site and a high affinity, low capacity site. Drug bound to the second site may represent EPLT bound to circulating serum ACE, perhaps accounting for the extended terminal elimination of the drug. Information on distribution into the CNS is limited, though EPL exhibits to cross the blood brain barrier poorly and EPLT does not appear to distribute into the CNS. The drug did not accumulate in any tissue subsequent multiple dose administration in animals. EPL crosses the placenta and in a premature neonate i.e., 35 weeks gestation whose mother received EPL 20 mg daily for 17 days

prior to delivery, plasma EPLT concentration was 28 ng/mL shortly after birth in the neonate. EPL and EPLT are distributed into milk in trace amounts.

- **Metabolism** Almost 60% of an absorbed dose of EPL from the GI tract is extensively hydrolyzed of its ethyl ester i.e., it is de-esterified to EPLT, principally in the liver by various esterases (Makwana *et al*., 2011; Mohan *et al*., 2011; Pisarev *et al*., 2005; Spanakis and Niopas 2010; Lee *et al*., 2003). Around 20% occurs to be hydrolyzed on first pass through the liver and this hydrolysis does not exhibit to occur in plasma in humans. EPLT is a more potent ACE inhibitor than EPL. There is no confirmation of other metabolites of EPL in humans, rats, or dogs (Ghosh *et al*., 2012). While in rhesus monkeys, a despropyl metabolite of EPLT was identified in urine, accounting for 13% of an oral dose of EPL. Hydrolysis of EPL to EPLT may be delayed and/or impaired in patients with severe hepatic impairment, although the pharmacodynamic effects of the drug do not appear to be significantly changed.

- **Excretion or Elimination** Excretion of EPL is primarily renal. About 94% of the dose is recovered in the urine and faeces as EPL or EPLT. The major components in urine are EPLT, accounting for almost 40% of the dose and complete EPL (Ghosh *et al*., 2012). Subsequently elimination is biphasic, the initial phase which reflects renal filtration (elimination half-life 2 to 6 hours) and the later prolonged phase exhibits equilibration of drug from tissue distribution sites (elimination half-life 36 hours) (Kang *et al*., 2014). In mediating drug effects, the prolonged phase does not contribute to drug accumulation on repeated administration yet the concept has pharmacological significance. Renal impairment effects in significant accumulation of EPLT (specially creatinine clearance < 20-30 mL/min or < 1.2 L/h) and requires dosage reduction. Due to the depletion in urinary excretion, EPL can increase the extent of hydrolysis of EPL to EPLT or increase extrarenal elimination of the drug, e.g. through the biliary excretion. An average of 60 to 78% (an average of 43 to 56% as EPLT and the rest as unchanged drug) of a 10 mg oral dose of EPL is excreted in urine within 24 to 48 hours after administration in healthy subjects and about 33% (approximately 27% as EPLT and 6% as unchanged drug) is excreted in faeces within 24 to 48 hours. In a multiple dose study (e.g. 10 mg daily) in healthy individuals with normal renal function, urinary excretion of EPLT and entire drug increased during the first 4 days of therapy and later stabilized. Urinary excretion of the metabolite averaged 45% of the cumulative dose and that of overall drug averaged 62%. In adults

with normal renal function, renal clearance of EPL and EPLT are described to be nearly 300 mL/min and 100 to 158 mL/min, respectively. The higher renal clearance of EPL is compared with that of the metabolite which may express some degree of active tubular secretion of unchanged drug. In hypertensive patients, renal clearance may be reduced and also renal clearance and/or volume of distribution may decline in geriatric i.e., elderly individuals.

2.14.2.18 Toxicology

2.14.2.18.1 Hepatotoxicity

In most cases, clinically recognizable acute liver damage have been described with EPL therapy. This is occurred generally within 2 to 12 weeks of starting the therapy and the serum enzyme pattern is usually cholestatic. In few occurrences, cholestasis is extended and deteriorating and related with constant increases in serum alkaline phosphatase, signifying of disappearing bile duct syndrome. Immunoallergic symptoms like rash, fever, eosinophilia etc. are rare and autoantibodies are not generated in most patients. Moreover, there have been noticeable instances of EPL liver damage with a hepatocellular motif. These cases usually have a long interval (one or more years) and some of which have been severe and fatal. The manifestation of an acute liver damage more than a year after starting a treatment is precisely exceptional, although has been reported for various ACE inhibitors.

2.14.2.18.2 Interactions

The hypotensive effect of EPL is increased when it is administered with diuretics or other hypotensive drugs. The effect is normally used to therapeutic benefit, however conscientious modification of dosage is required when these drugs are used simultaneously or unitedly. EPL and diuretics appear to have additive hypotensive effects. Although, severe hypotension and reversible renal insufficiency may sometimes appear, particularly in volume and/or sodium depleted patients. The hypotensive effect of EPL is increased due to release of renin (e.g., diuretics).

2.14.2.18.3 Genotoxicity

In the Ames microbial mutagen test with or without metabolic activation, neither EPL nor the active diacid is mutagenic. Moreover, EPL is negative in the following genotoxicity studies : rec-assay, reverse mutation assay with E. coli, sister chromatid exchange with cultured

mammalian cells and the micronucleus test with mice, likewise in an in vivo cytogenic study using mouse bone marrow.

Apparent hypotension and stupor i.e., unconsciousness may appear due to overdosage. Renal dysfunction, including acute renal failure, hyperkalemia and hyponatremia may also occur. However, the most common adverse effects or side effects are hypotension, headache, tiredness, dizziness i.e., lightheadedness with standing, fatigue and cough ("Enalaprilat/Enalapril Maleate". *The American Society of Health-System Pharmacists.*). Also, EPL include increased serum creatinine (20%), dizziness (2 to 8%), low blood pressure (1 to 7%), syncope (2%) and dry cough (1 to 2%). The most serious or severe adverse events are angioedema (swelling, 0.68%) and low blood pressure. Angioedema usually affects the face and lips, occurring airway obstruction of the patient. During treatment with EPL, it can happen at any time yet is most common after the initial few doses. Thus, angioedema and fatality are reportedly higher among black people (FDA Label: Enalapril Maleate Tablet. *The Wayback Machine*). Use during pregnancy is supposed to effect in harm to the baby ("Enalaprilat/Enalapril Maleate". *The American Society of Health-System Pharmacists.*).

2.14.2.19 Bioanalytical assay methods overview of active metabolite EPLT with prodrug EPL

Various methods have been reported for the bioanalysis of active metabolite EPLT and its prodrug EPL in biological fluids. These includes both GC-MS and LC-MS/MS methods using different sample preparation and extraction techniques. An outline of these methods are given below.

- Spanakis and Niopas (2010) have developed and validated a rapid, simple, sensitive, accurate and precise SIM GC–MS method for the quantitative determination of enalapril (EPL) and enalaprilat (EPLT) in human plasma using perindopril as internal standard (IS) were extracted by solid-phase extraction (SPE) and derivatization with methyl iodide. The separation was carried out on an HP-5MS (5% phenyl)-methylpolysiloxane non-polar capillary column, 30 m x 0.25 mm i.d., 0.25 µm film thickness. The carrier gas was ultrahigh purity helium at a flow rate of 0.8 mL min^{-1} and a split ratio 1:5. The injection volume and the total run time were 1 µL and 14.5 min., respectively. The lower limit of quantification (LLOQ) was 2 ng mL^{-1} for both analytes.

- A liquid chromatography–tandem mass spectrometry (LC-MS/MS) method has been developed and validated for evaluation of enalapril (EPL), enalaprilat (EPLT) and hydrochlorothiazide in human serum using solid phase extraction (SPE) technique. The entire method was performed with reverse phase (RP) C18 column, 100 x 3.0 mm, and composition of the mobile phase was a isocratic mixture of 0.01% ammonia in water : acetonitrile (30:70%, v/v). The flow rate was set at 0.7 mL/min (Makwana *et al*., 2011).

- A bioanalytical method has been developed and validated for the determination of enalapril (EPL) in human serum by LC-MS/MS detection. Serum samples were extracted by a typical liquid-liquid extraction (LLE) procedure for the isolation of drug. The separation was done by Phenomenex C18 column, 50 x 4.6 mm i.d., 5µm particle size in atmospheric pressure electrospray ionization (ESI) with an isocratic mobile phase comprises a mixture of methanol, acetonitrile, water and formic acid (70:20:10:0.01%, v/v) at a flow rate of 1.0 mL/min (Makwana *et al*., 2013).

- Kang, Kim, Park *et al*., (2014) reported a simple and rapid liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the estimation of enalapril (EPL) in human plasma using glibenclamide as an internal standard (IS). The extraction procedure was carried out by a simple protein precipitation technique (PPT) with acetonitrile and the separation was performed on YMC hydrosphere C8 column (2.0 x 50 mm i.d., 3 µm particle size) using methanol and 10 mM ammonium formate (80:20, v/v) as the mobile phase at a flow rate of 0.2 mL/min. The injection volume and the chromatographic run time were 5 µL and 2.0 min., respectively. The method was successfully applied to pharmacokinetic (PK) and bioequivalence (BE) studies in 22 healthy korean subjects under fasting state.

- An LC-MS/MS method has been developed for the quantification of enalapril (EPL) and enalaprilat (EPLT) from human plasma. The extraction was achieved by solid phase extraction (SPE) using Thermo Scientific SOLA cartridges and the separation of the analytes was done on a Thermo Scientific Hypersil GOLD column (50 x 2.1 mm, 1.9 µm particle size). The mobile phase composition was water with 0.1% formic acid and acetonitrile with 0.1% formic acid in gradient elution (10-100% B in 1 min.) at a flow rate of 0.6 mL/min. The linearity for EPL and EPLT were in the

range of 1 to 100 ng/mL with a r^2 of 0.9986 (for EPL) and 0.9974 (for EPLT) (Jones *et al*., 2012).

- Ghosh, Jain, Shinde *et al*., (2012) described a rapid and sensitive ESI-LC-MS/MS for simultaneous determination of enalapril (EPL) and enalaprilat (EPLT) in human plasma using ramipril as an internal standard (IS). The extraction was done by a simple solid-phase extraction (SPE) followed by evaporation and the chromatographic separation was performed on a reversed phase (RP) Hypurity C18 column, 50 x 4.6 mm i.d., 5 µm particle size with an isocratic mobile phase comprises a mixture of 0.1% ammonia solution in water (pH 2.88; adjusted with acetic acid) and methanol (15:85, v/v) at a flow rate of 0.30 mL/min. Total run time of single injection and injection volume were 2.0 min and 15 µL, respectively. The method was successfully applied for the evaluation of the pharmacokinetics and bioequivalence study of EPL and EPLT in 36 healthy male Indian volunteers after an oral dose of 20 mg enalapril maleate tablet.

- A rapid, selective and sensitive high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method has reported to simultaneously quantify enalapril (EPL) and its active metabolite enalaprilat (EPLT) in human plasma with benazepril as internal standard (IS). The sample extraction was done by a one-step protein precipitation (PPT) with methanol and the entire analysis was carried out on an Ultimate™ XB-C18 column (50 x 2.1 mm, i.d., particle size 3µm) with the mobile phase consisting of methanol : water : formic acid (62:38:0.2, v/v/v) at a flow rate of 0.20 mL/min. The injection volume and the chromatographic run time were 5 µL and 2.5 min, respectively. The method was successfully applied to the pharmacokinetic study of enalapril maleate capsules (50 mg) in 20 healthy male Chinese subjects after oral administration (Lu *et al*., 2009).

- Ramusovic, Thielking and Läer (2012) have established a HPLC-tandem mass spectrometry (MS) method for the determination of enalapril (EPL) and enalaprilat (EPLT) in human serum using benazepril as internal standard (IS). The samples were extracted by solid-phase extraction (SPE) procedure and the chromatographic separation was achieved on a Luna® RP-C18 (2) column, 50 x 2 mm, 3 µm particle size, 100 A, in combination with a Security-Guard® RP-C18 guard column, 4 x 2 mm, with methanol: water: formic acid (65:35:1, v/v/v) as mobile phase at a flow rate of

0.4 mL/min. The total run time was set to 5 min and the injection volume was 20 µL. The lower limits of quantification (LLOQ) were found 1.61 ng/mL and 1.84 ng/mL for EPL and EPLT, respectively. The bioanalytical method was used to evaluate the human serum concentrations after oral administration of 20 mg of enalapril maleat in a healthy male volunteer.

2.14.3 Nimesulide

Nimesulide (NSD) is an effective and potent non-steroidal anti-inflammatory drug (NSAID) or a nonsteroidal arylsulfonamide with novel chemical aspects belonging to the sulphonanilide class (Figure.2.22) and also it is the primary cyclooxygenase-2 (COX-2) preferential inhibitor to be considered worldwide. It has anti-inflammatory, analgesic and antipyretic properties (Sora *et al*., 2007; Parija *et al*., 2012; Macpherson *et al*., 2013; Bianchi *et al*., 2006; Barrientos-Astigarraga *et al*., 2001; Toutain *et al*., 2001; Rao *et al*., 2007; Giachetti and Tenconi. 1998). NSD differs from other NSAIDs as it does not have a carboxylic acid moiety. Due to the presence of suifonanilide moiety, it exhibits weakly acidic feature and this facilitates its binding to the active site of COX-2 (Kucukguzel *et al*., 2005). Also, due to the rapid anti-inflammatory and analgesic action, it is the most prescribed NSAID worldwide (Parija *et al*., 2012). It has an extensive clinical use for the treatment of acute pain, musculoskeletal disorder, the symptomatic treatment of osteoarthritis or chronic rheumatoid arthritis, dyemenorrhoea, thrombophlebitis and dental pain, inflammation, soft tissue and oral cavity lesions, respiratory tract infections and fever (Reddy. 2009; Bianchi *et al*., 2006; Giachetti and Tenconi. 1998; Kucukguzel *et al*., 2005).

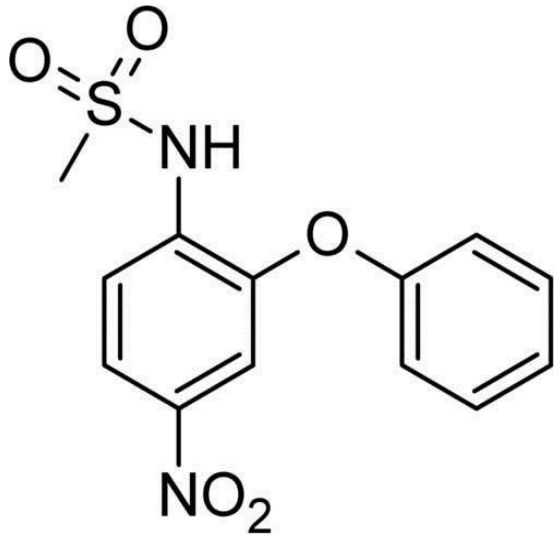


Figure.2.22 Molecular structure of nimesulide

Classical NSAIDs are performed by inhibiting the cyclooxygenase (COX) enzyme which is responsible for the formation of prostaglandins, the key mediators of pain and inflammation (Sora *et al*., 2007). The conventional NSAIDs inhibit both cyclooxygenase COX-1 and COX-2 isoforms, whereas NSD is a selective inhibitor of COX-2 which is induced in inflammatory conditions. So for that reason it is widely used for the treatment of several inflammatory processes (Parija *et al*., 2012; Toutain *et al*., 2001 and Reddy. 2009). NSD shows better gastrointestinal tolerability compared to classical NSAIDs and this correlates to its preferential COX-2 inhibitory potency (Kucukguzel *et al*., 2005; Rao *et al*., 2007 and Singla *et al*., 2000). Decrease the progression of dementia in Alzheimer's disease by the selective COX-2 inhibitors including NSD. It can also interact with macromolecular targets apart from COX-2 and inhibits phosphodiesterase (PDE4) which is the main enzyme involved in cAMP degradation. Moreover, it has been described to control inflammatory occurrences at various stages besides COX-2 inhibition, like competetive inhibition of histamine release from human basophils and tissue mast cells, platelet-activating factor synthesis in phagocyte-stimulated human neutrophils, metalloproteinase synthesis such as collagenase and stromelysin, depletion of superoxide anion formation and direct scavenging action of different forms of free radicals which are principally involved in inflammatory disease (Kucukguzel *et al*., 2005 and Kim *et al*., 2012).

In the structure there are five different types of atoms such as, carbon, hydrogen, oxygen, nitrogen and sulphur. These atoms are either single bonded or double bonded composed to form the complete structure of NSD. There are three types of functional groups : the sulfonamide group ($–SO_2NH_2$), the phenoxy group ($C_6H_5O–$) and the nitro group ($−NO_2$) and the structure is stabilized by N-H···O hydrogen bond (see Figure.2.22). It is an aromatic homomonocyclic compound with C-nitro compound, aromatic ether, sulphonamide i.e., an aromatic ether having phenyl and 2-methylsulfonamido-5-nitrophenyl as the two aryl groups. Basically, belongs to the class of organic compounds known as diphenylethers i.e., the aromatic compounds containing two benzene rings linked to each other by an ether group. It consists of a sulphonanilide moiety which gives rise to a weakly acidic nature and the compound pKa value is 6.5. This is a very significant advantage of the drug because it shows less irritative and toxic effects on the gastrointestinal mucosa and the kidney (Sora *et al*., 2007; Bernareggi. 2001; Sanphui *et al*., 2011 and Auda. 2014). NSD has five metabolites, such as 2-(4-hydroxyphenoxy)-4-nitromethanesulfonanilide (M1), 2-phenoxy-4-amino-methanesulfonanilide (M2), 2-(4-hydroxyphenoxy)-4-aminomethanesulfonanilide (M3), 2-

phenoxy-4-N-acetylamino-methanesulfonanilide (M4) and 2-(4-hydroxyphenoxy)-4-N-acetylamino-methanesulfonanilide (M5). All of these are found in urine, but only M1 is detected in plasma as free compound i.e. in unconjugated form. M1 and M5 are present almost completely in conjugated form in urine but M5 is unconjugated in faeces. Other three i.e. M2, M3 and M4 are minor metabolites and present as free compound in urine. Whereas M2 and M3 are also found in faeces as uncojugated form (Bernareggi. 2001). The major and biologically active metabolite is 4-hydroxynimesulide (M1) and the metabolism is occurred in the liver by an aromatic hydroxylation process which is catalyzed by enzymes from cytochrome P-450 family (Sora *et al*., 2007 and Carini *et al*., 1998). NSD is rapidly and adequately absorbed from the gastrointestinal tract and maximum plasma concentrations are reached within 2 hours after oral administration. The plasma concentrations of almost 50% of the highest (i.e., C_{max}) appear 30 minutes after administration. Pharmacological potency is normally shown earlier than time to C_{max}, from 30 to 60 minutes following the administration (Bernareggi. 2001).

2.14.3.1 4-hydroxynimesulide

4-hydroxynimesulide (M1) (Figure.2.23) i.e., 4-Nitro-2-(4'-hydroxyphenoxy) methanesulfonanilide or N-[2-(4-hydroxyphenoxy)-4-nitrophenyl]methanesulfonamide is a major and main active metabolite of NSD (Sora *et al*., 2007; Bianchi *et al*., 2006; Bernareggi. 2001; Giachetti and Tenconi. 1998). It is a yellowish or light-yellowish crystalline powder with the molecular formula $C_{13}H_{12}N_2O_6S$ (see Figure.2.23) and the molar mass is 324.307 grams $mole^{-1}$. Some physical properties are, density : 1.538 gm/cm^3, boiling point : 496.7 ºC at 760 mmHg, flash point : 254.2 ºC, LogP : 4.14120, vapour pressure : 1.72E-10 mmHg at 25 °C etc. It sustains anti-inflammatory and analgesic properties, but its potency is lesser i.e., to a minor extent compared to the parent drug (Bianchi *et al*., 2006 and Bernareggi. 2001). Biotransformation of NSD is occurred through the hepatic metabolism to form M1 by a cytochrome P_{450}-mediated oxidative reaction, an aromatic hydroxylation process (Sora *et al*., 2007; Rainsford. 1999 and Carini *et al*., 1998). In children, the extent of NSD biotransformation to M1 is similar to that in adults (Bernareggi. 2001).

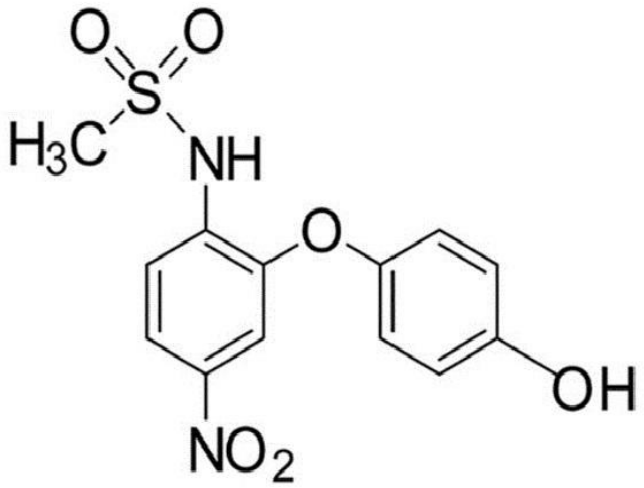


Figure.2.23 Molecular structure of 4-hydroxynimesulide

M1 is detected in human plasma samples in the same time interval as recommended for plasma concentration profiles for NSD. Because its biological activity is similar to the parent drug, during bioavailability / bioequivalence studies (BA/BE) its concentration is also determined in plasma samples from volunteers (Sora *et al*., 2007). The effect of M1 on prostaglandin synthesis shows similar to NSD (Rainsford. 1999). It appears a peak level after 3 to 8 hours (on an average) during therapy and an elimination half-life is approximately 5 hours (Giachetti and Tenconi. 1998). After a single oral administration of 100 mg dose, the distinct terminal phase of M1 plasma concentration-time curve is characterized by $t_{1/2,z}$ values 1.5 to 2-fold higher than the elimination half-life evaluates for unchanged NSD (Bernareggi. 2001). After oral administration around 70% of the dose is excreted in faeces and the remaining part in urine as M1 metabolite (Giachetti and Tenconi. 1998).

2.14.3.2 Categories

Non-steroidal anti-inflammatory drug (NSAID); anti-inflammatory, analgesic, antirheumatic and antipyretic agent.

2.14.3.3 IUPAC name

N-(4-nitro-2-phenoxyphenyl)methanesulfonamide

2.14.3.4 Chemical (or Molecular) formula

$C_{13}H_{12}N_2O_5S$

2.14.3.5 Molecular Weight (Da, Average)

308.311 grams per mole

2.14.3.6 Physical state

Yellowish or light-yellowish crystalline powder.

2.14.3.7 Melting point

143 °C to 144.5 °C

2.14.3.8 Boiling point

442.03 °C at 760 mmHg

2.14.3.9 Flash point

221 °C to 230 °C

2.14.3.10 Solubility

NSD is soluble in acetone, chloroform and ethyl acetate, slightly soluble in ethanol and practically insoluble in aqueous systems (solubility 0.01 mg/mL). Soluble in water (< 50 µg/mL) i.e., solubility in water 5.5 to 11.4, 1:10 DMSO : PBS (pH 7.2) (< 200 µg/mL), ethanol (1 mg/mL), DMSO (15 mg/mL), DMF (15 mg/mL), chloroform, dichloromethane, acetone (freely soluble) and 1N NaOH (sodium hydroxide).

2.14.3.11 Stability

NSD is stable at normal temperatures and pressures. Some solutions can be difficult to acquire and can be promoted by rapid stirring, sonication or gentle warming in a 45 °C to 60 °C water bath. For solid forms, storage is stated on the product label and the vial is kept tightly sealed, the product can be stored for up to 6 months from date of receipt. It is suggested that stock solutions, once prepared, are stored aliquoted in tightly sealed vials at -20 °C or below and used within one month. Wherever possible solutions should be made up and used on the same day. Because, it can not recommend storing the aqueous solution for more than 24 hours or one day.

2.14.3.12 Decomposition

It is decomposed by heating i.e., thermal decomposition approximately 300 °C.

2.14.3.13 LogP

2.6 or 2.5 (log $P_{Octanol/Water}$)

2.14.3.14 Dissociation constants or pka value

pKa is 6.5 (weakly acidic) or with a range of 6.4 to 6.8.

2.14.3.15 Biological half-life ($t_{1/2}$)

The biological half-life ($t_{1/2}$) of NSD is varies from about 1.80 to 4.73 hours (or ≈ 2 to 5 hours) and for M1 its value about 5 hours (or ≈ 2.9 to 4.8 hours).

2.14.3.16 Storage conditions (Temperatures)

Store at temperatures not exceeding 25 °C. So, NSD is normally stored at 4 °C, but for the long term storage it should be stable for at least one year at -20 °C. Keep out of the reach of children.

2.14.3.17 Pharmacology

2.14.3.17.1 Indication

NSD is used for the treatment of acute (short term) pain (because it has pain medication and fever reducing properties), the symptomatic treatment of osteoarthritis (chronic arthritis), respiratory tract infections, otorhinolaryngological diseases, soft tissues and oral cavity inflammation, phlebitis / thrombosis, urogenital disease, postoperative pain states, sports injuries and primary dysmenorrhoea (period pains) in adolescents and adults above 12 years old. Because, it is contraindicated in children below 12 years and also in patients those who are suffering from liver problems (Bianchi *et al*., 2006; Kucukguzel *et al*., 2005; Reddy. 2009; Giachetti and Tenconi. 1998). A specific warning is for patients who develop fever and /or flu-like symptoms, which should stop the treatment. It has a multifactorial mode of action and is characterized by a rapid onset of action. NSD is not accessible in the United States, but is used widely in other countries in the medication of acute pain. It is similar in gastrointestinal safety to other NSAIDs (Bernareggi. 2001; Sora *et al*., 2007; Auda. 2014 and Sanphui *et al*., 2011). It should never be taken long-term as for chronic conditions like

arthritis. This is because of its relation with an increased risk of liver toxicity including fulminant hepatic failure. It has been associated to a low rate of transient serum enzyme elevations during therapy. But besides several occurrences of clinically manifest acute liver injury that can be severe and can result in acute liver failure. In that case its require an emergency liver transplantation, otherwise death.

2.14.3.17.2 Pharmacodynamics (Mechanism of drug action)

NSD inhibits the cyclooxygenase-mediated conversion of arachidonic acid to pro-inflammatory prostaglandins. Basically, it is selective for COX-2 and therefore it binds to the enzyme, so that inactivation is occurred (Sora *et al*., 2007; Parija *et al*., 2012; Toutain *et al*., 2001 and Kim *et al*., 2012). The therapeutic effects are the consequence of its complete mode of action which targets a number of key mediators of the inflammatory process like COX-2 mediated prostaglandins, free radicals, proteolytic enzymes and histamine (Kucukguzel *et al*., 2005 and Kim *et al*., 2012). NSD can also inhibit some carcinogenic COX-2 related carcinogenic effects on xenobiotic metabolism, apoptosis, immune surveillance and angiogenesis i.e., overexpressed COX-2 in tumor epithelial cells enhances production of vascular growth factors and the formation of capillary-like networks. It has an unique chemical features belonging to the sulphonanilide group and with a preferential inhibition on cyclooxygenase (COX)-2 activity and less effect on COX-1. According to selectivity for COX-2, NSD can be classified in the group of NSAIDs indicating 5 to 50-fold selectivity for COX-2 over COX-1 (Bianchi *et al*., 2007). In vivo, it has been exhibited not to affect prostaglandin production in the bronchial tree or in the gastric mucosa. By comparison, NSD distinctly affects prostaglandin synthesis in inflammatory exudate where prostaglandin synthesis is COX-2 mediated (Bernareggi. 2001). A significant number of studies have revealed that this is an effective drug for the symptomatic treatment of osteoarthritis (OA) with a proper dosing schedule of 100 mg twice a day (Bianchi *et al*., 2007). New findings expressed the competency of NSD to inhibit prostaglandin E_2 (PGE_2) at joint level in patients with arthritis (Bianchi *et al*., 2006).

NSD shows its therapeutics effects through a variety of mechanisms, such as :

- Selective cyclooxygenase-2 inhibitor.
- Reduced generation of superoxide anions by stimulated polymorphonuclear leucocytes.

- Inhibition of platelet aggregation factor synthesis by activated cells.
- Scavenger of inactivation of alpha1-protease inhibitor.
- Inhibition of histamine release.
- Inhibition of protein kinase C through inhibition of phosphodiesterase type IV.
- Reduced degradation of cartilage matrix through inhibition of metalloprotease synthesis.
- Potent inhibition of induced platelet aggregation.
- Prevention of bradykinin or cytokine induced hyperalgesia of nerves (inhibiting release of TNF- alpha).

2.14.3.17.3 Pharmacokinetics

- **Absorption** NSD is absorbed rapidly and extensively from the gastrointestinal tract following oral administration. It has a comparatively fast onset of action with significant reductions in pain and inflammation observed within 15 minutes from drug intake (Bernareggi. 1998; Rainsford. 2006; Bianchi and Broggini. 2003). After oral administration of a 100 mg dose to healthy fasting subjects, mean maximum plasma concentration i.e., C_{max} values of 2.86 to 4.58 mg/L is achieved within 1.22 to 3.83 hours (≈ 2 to 4 hours) i.e., t_{max} and for 200 mg dose it is about 1 to 6 hours. For M1 C_{max} is about 0.8 to 2.3 µg/mL (100 mg dose of NSD) and 2.0 to 4.4 µg/mL (200 mg dose of NSD) and t_{max} is approximately 3 to 8 hours (Bernareggi. 1993 and 1998; Giachetti and Tenconi. 1998). After dosing, the area under the plasma concentration-time curve (AUC) values ranged from about 17.3 to 30.9 mg.hr/L. NSD concentrations of almost 50% of the C_{max} show 30 minutes after administration and pharmacological effectiveness is normally shown earlier than time to C_{max} from 30 to 60 minutes after the administration (Bernareggi. 2001). Oral absorption is assumed to be complete from mass balance studies of the drug and accordingly by mass balance, it can be concluded that at least 80% of the administered dose was absorbed by the gastrointestinal tract. Bioequivalence studies revealed that the rate and the extent of NSD absorption are similar whether the drug is administered in tablet, suspension or granular form (Bernareggi. 1993 and 2001). Food, gender and advanced age have negligible effects on pharmacokinetics. However, the presence of food has a limited

influence on the rate and extent of absorption. Oral administration of 100 mg tablets after a standard meal results in a C_{max} value almost 20% lower than that acquired under fasting conditions. While, neither t_{max} nor AUC are considerably changed by food intake (Bernareggi. 1998 and 2001). Estimation of the pharmacokinetic parameters in males and females are same after single and multiple oral administration and also the ratios between mean parameter values establish in the different studies for C_{max}, t_{max}, AUC, $t_{1/2,z}$, CL/F and V_z/F are randomly distributed throughout integrity to express that gender does not significantly affect NSD pharmacokinetics. Hence, no remarkable effect of age on the pharmacokinetic profile of it has been evaluated in paediatric patients after single oral administration of 50 mg and in the elderly after single and multiple oral administration of 100 mg (Bernareggi. 2001).

- **Distribution** NSD exhibits a small volume of distribution in the post distribution phase ranges from 0.18 to 0.39 L/kg (Brouwers and de Smet. 1994; Bernareggi. 2001), showing that the drug is principally distributed in the extracellular fluids. Literally with the exception of salicylates, NSAIDs are largely bound to human serum albumin and below 1% of the total plasma concentration is in the unbound form, accessible to distribute to extravascular tissues. Over a drug concentration range of 0.5 to 10 mg/L, the unbound fraction for NSD varies from 0.007 to 0.04 which is expressive of its extensive plasma protein binding (more than 97.5%). 2.5 mg/kg oral (^{14}C) NSD is treated in rats and radioactivity is widely distributed throughout the body to almost all the organs. The maximum concentrations are achieved between 1 and 4 hours after the administration in the liver, heart, kidneys, lungs, adrenals, fat tissue and gut, whereas the brain is slightly involved in the drug distribution with the minimum observed concentrations. The tissue-to-plasma concentration ratios for total radioactivity are usually lower than unity during the whole observation interval i.e., up to 48 hours. This finding demonstrated a low affinity of the drug for tissue components and no accumulation phenomena in tissue compartments. Specific distribution studies have been carried out in humans with oral NSD in female genital tissues (Pulkkinen *et al*., 1991 and Bernareggi. 2001) and in the synovial fluid of patients with rheumatoid arthritis. The tissue-to-serum concentration ratios and synovial fluid-to-plasma concentration ratios are generally lower than unity, probably due to a high plasma protein binding, as for other NSAIDs, which retains the drug

mostly in the plasma. Over a wide concentration range i.e., 0.77 to 20 mg/L which covers therapeutic NSD levels, the unbound fraction of NSD in serum is 1%. Binding is predominantly because of the extensive interaction with albumin (about 99%) and a weak binding is identified to α_1-acid glycoprotein and lipoproteins, whereas no binding is formed with γ-globulin (Singla *et al*., 2000; Bree *et al*., 1993 and Bernareggi. 2001).

- **Metabolism** NSD is almost completely and oxidatively metabolized in the liver and its extensive biotransformation undergoes principally to M1, which is biologically active (Sora *et al*., 2007; Rainsford. 1999 and Carini *et al*., 1998). Metabolic biotransformation of NSD is hepatic and can take place at both the phenoxy ring moiety and the aromatic nitro group. The major oxidative metabolite found in the plasma is para-hydroxy or 4-hydroxynimesulide i.e., M1 in both free and conjugated forms and it also appears to contribute to the anti-inflammatory activity of the compound (Rainsford. 2006). The primary biological pathway for metabolizing NSD involves an aromatic hydroxylation and this reaction is catalyzed by enzymes from cytochrome P-450 family, mainly by CYP‑2C9, CYP‑2C19 and perhaps CYP‑1A2 (Sora *et al*., 2007; Rainsford. 2006 and Carini *et al*., 1998). The metabolites of NSD emerge from hydroxylation to the phenoxy nucleus (M1), reduction of the nitro group to an amino derivative (M2), concomitant hydroxylation and reduction (M3), N-acetylation of the M2 (M4) and of the M3 (M5) metabolites (Carini *et al*., 1998).

- **Excretion or Elimination** NSD is primarily excreted or eliminated by the renal route i.e., renal is 50% and fecal is 29%. However, the excretion or elimination of the parent drug in urine and faeces is negligible and it is principally eliminated through the metabolic transformation (Bernareggi. 2001). Elimination is progressive with a half-life ($t_{1/2}$) of about 2 to 5 hours for NSD and about 3 to 9 hours for M1 in plasma after oral administration of 100 mg twice-daily dosage without any confirmation of accumulation (Rainsford. 1999 and 2006; Bernareggi. 2001; Kim *et al*., 2012; Macpherson *et al*., 2013; Giachetti and Tenconi. 1998; Singla *et al*., 2000). The elimination of NSD is not influenced by age, gender or moderate renal impairment, such as might be seen in the elderly. The use of drug is contraindicated in patients with hepatic impairment (Bernareggi. 1998 and Rainsford. 2006). Whereas, the elimination of M1 is decreased marginally in elderly subjects. Moderate renal

impairment is accompanied by approximately 30% lower clearance and a 50% increase in the plasma elimination half-life ($t_{1/2}$) for the M1 from 4 hours to 6 hours. This is within the range of variability observed in the $t_{1/2}$ for normal subjects (Rainsford. 1999). Other metabolites of NSD, such as M2, M3, M4 and M5 are excreted through urine and faeces including M1 either conjugated or unconjugated (i.e., free) forms. The extent of the metabolites is in conjugated form and accounted for approximately 40% of the administered dose. The percentage excretion of the unchanged drug and the metabolite M4 are below 0.5%. In the urine, the lesser recovery is referred to partial gastrointestinal absorption and to uniform faecal excretion of the metabolites (Singla *et al*., 2000). Total plasma clearance of NSD varies from 35.2 to 90.9 mL/hours/kg and is almost completely attributed to metabolic clearance. In humans, NSD has a comparatively short plasma half-life. This possibly one of the factors that report for the relatively some studies of severe gastrointestinal (GI) and renal toxicity with NSD (Rainsford. 1999).

2.14.3.18 Toxicology

2.14.3.18.1 Hepatotoxicity

Proposed studies reveal that up to 15% of patients taking NSAIDs experience at least transient serum aminotransferase elevations and a lower rate has been reported with NSD. These elevations are usually transient, mild and asymptomatic and can determine even with drug continuation. Distinct aminotransferase elevations (more than 3 fold elevated) appear in less than 1% of patients. However, NSD has been repeatedly associated to occurrences of clinically manifest liver damage with jaundice and more than 100 instances outlined in the literature. The pattern of enzyme elevations is typically hepatocellular and while cholestatic forms have been expressed also (Rainsford. 1999). Immunoallergic features are normally absent and if present then not prominent. Autoimmune features are rare and in most cases starting to determine a few days after stopping therapy. However, several instances of acute liver failure with death or require an emergency liver transplantation (Walker *et al*., 2008 and Singla *et al*., 2000). The mortality rate is between 10% and 20% of NSD related acute hepatitis with jaundice. The overall incidence of hepatotoxicity is unknown, but it is generally indicated in large case series on drug induced liver injury and acute liver failure (Bernareggi. 2001), and a reasonable assessment is one in 50,000 users. Liver injury might be

unusual when the duration of therapy is limited to 15 days. Although, severe cases of NSD liver injury have been reported after courses of therapy as short as 3 to 5 days.

2.14.3.18.2 Gastrointestinal toxicity

In GI system, the adverse reactions are of low overall severity and most are symptomatic such as, epigastric pain, nausea etc and are reversible upon termination of the drug (Rainsford. 1999; Bjarnason. 1999 and Wober. 1999). There appears to be a lesser tendency for development of upper GI ulceration detected endoscopically (Rainsford. 1999 and Bjarnason. 1999). NSD is involved in production of gastroprotective prostaglandins could be implied to consider for the relatively low gastric mucosal irritancy. In dyspeptic patients, double-blind gastroduodenal endoscopic determination has exhibited that NSD 100 or 200 mg per day for 7 days produced only slightly more mucosal injury than placebo. Relatively some mucosal changes are observed endoscopically in osteoarthritis patients above 50 years of age after treatment for one month with NSD 200 mg per day. There are slightly fewer erosions and ulcers in the NSD compared with the diclofenac group, but apparently this is not clinically significant (Rainsford. 1999). Some gastric erosion are observed endoscopically in patients with arthritic conditions who has taken NSD 200 mg per day than in those who has taken an equivalent dose of indomethacin for 12 to 15 days. A recent endoscopic study in normal human subjects has revealed that NSD shows significantly lower GI toxicity than naproxen (Bjarnason. 1999). Moreover, the considerably decreased gastric irritancy correlates with the reduced inhibiting effects on prostaglandin production both in the gastric mucosa and in blood ex vivo. These investigations appear to establish the ‘proof of principle’ that preferential inhibition of COX-2 acts supplementary to the mucosa in the GI tract from injury because of the inhibition of prostaglandin production from NSAIDs. Although, it is feasible that the inhibitory effects of NSD on oxy-radical and leukotriene generation and histamine discharge from mast cells can provide extra safety from the mucosal injury (Rainsford. 1999).

The most common side effects are diarrhea, vomiting, dizziness, skin rash, itchiness, bitterness in mouth etc. for repeated use of NSD i.e., more than 15 days. During lactation, women should use the drug with caution and also it is contraindicated throughout pregnancy.

2.14.3.19 Bioanalytical assay methods overview of active metabolite M1 with prodrug NSD

Several methods have been described for the bioanalysis of active metabolite M1 and its prodrug or parent drug NSD in biological fluids. These include both HPLC and LC-MS/MS

methods applying various sample preparation and extraction techniques. A summary of these methods are given below.

- A sensitive, selective and accurate HPLC method is described for the assessment of nimesulide (NSD) and its major metabolite 4-hydroxynimesulide (M1) in human plasma samples using nitrazepam as an internal standard (IS). The entire method was carried out by simple protein precipitation technique (PPT) with acetonitrile by an UV detection of the analytes at 300 nm and the separation was performed on a Chromolith Performance RP 18e column (10 mm x 4.6 mm) using a mobile phase consisting of aqueous 0.2% triethylamine at pH 3.0 with H_3PO_4 and methanol (1:1, v/v), pumped at a flow rate of 1.5 mL/min. The lower limits of quantification (LLOQ) for NSD and M1 were 64 ng/mL and 41 ng/mL, respectively. The method was applied for a bioavailability (BA) study to determine both NSD and M1 in 22 Romanian subjects after administration of a single oral dose of NSD on fasting conditions (Sora *et al*., 2007).

- Kim, Park, Kim *et al*., (2012) have developed and validated a simple, robust and sensitive isocratic HPLC-UV method for quantification of nimesulide (NSD) in human plasma using zaltoprofen as internal standard (IS) by applying Capsell Pak C18 UG120, 150 x 4.6 mm, 5 µm column as stationary phase with methanol and 50 mM sodium phosphate (57:43, v/v, pH 6.03) as mobile phase, run at a flow rate of 1.0 mL/min at 334 nm of UV detection. Total chromatographic run time was set to 15 min and the column temperature was 35^{o}C. The lower limits of quantification (LLOQ) was found 0.2 µg/mL. This method was employed to pharmacokinetic studies of NSD in 28 healthy Korean volunteers after single oral doses of 2 tablets (100 mg each).

- Macpherson, Best, Gedik *et al*., (2013) have described a study for the biotransformation and pharmacokinetics of ^{14}C-Nimesulide in humans following a single dose oral administration to 4 healthy male volunteers at a dose of 100 mg per subject. Nimesulide (NSD) and 4-hydroxynimesulide (M1) both were measured in plasma to allow the comparison of their concentrations with total radioactivity. These samples were extracted and partially purified prior to analysis by LC-MS/MS.

- A reversed-phase high performance liquid chromatographic (RP-HPLC) method was developed for the determination of nimesulide in both pharmaceutical formulations as well as in human serum samples. The chromatographic separation was achieved

isocratically on an ACE 5 C18 column (250 mm x 4.0 mm, 4μm) using a mobile phase of acetonitrile : triethylamine (TEA) : water (50:0.5:49.5, v/v/v), adjusted to pH 5.0 with formic acid, at a flow rate of 1.0 mL/min with UV detection at 230 nm and the retention time was 7.545 min. The method was validated concerning accuracy (99.15 to 101.85%), precision (intra-day variation 0.13 to 1.60% and inter-day variation 0.30 to 1.70%) and linearity within range of 0.1 to 30 μg/mL (r^2 = 0.999) (Reddy. 2009).

- Bianchi, Ferrario, Balzarini *et al*., (2006) have demonstrated a study that evaluate plasma and synovial fluid concentrations of the non-steroidal anti-inflammatory drug nimesulide (NSD) and its major metabolite 4-hydroxynimesulide (M1) after a single 100 mg dose of NSD and a repeated administration for 14 days of 100 mg twice per day in patients with osteoarthritis of the knee and joint effusion. Plasma and synovial fluid concentrations were measured by high performance liquid chromatography (HPLC) with UV detection employing a validated method and the lower limit of quantification (LLOQ) was 25 ng/mL for both NSD and M1.

- A high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for the quantification of nimesulide (NSD) using celecoxib as internal standard (IS). The chromatographic separation was done by an X-Terra TM-MS C18 column, 150 x 4.6 mm i.d., 3.5 μm particle size fitted with a guard column of the same material (during analysis both column and pre-column temperatures were kept at 40°C) utilizing a isocratic mobile phase of acetonitrile and water containing 5 mM ammonia solution (80:20, v/v) at a flow rate of 0.5 mL min^{-1} with 10 μL of injection volume. The chromatographic run time was 4.5 min. Plasma samples were extracted by a liquid liquid extraction procedure using an organic mixture of diethyl ether and dichloromethane (70:30, v/v). The linearity was in the range of 10 to 2000 ng mL^{-1}. The method was applied in a bioequivalence study of one NSD drop formulation (NSD 50 mg mL^{-1} drop, Medley S/A Ind ústria Farmacêutica, Brazil) against one standard NSD drop formulation (Nisulid, 50 mg mL^{-1} drop, Astra Médica, Brazil) (Barrientos-Astigarraga *et al*., 2001).

- Carini, Aldini, Stefani *et al*., (1998) have proposed a study on the biotransformation pathways of NSD in man after single oral acute administration 200 mg of the drug, following isolation by TLC separation and mass spectrometric characterization by

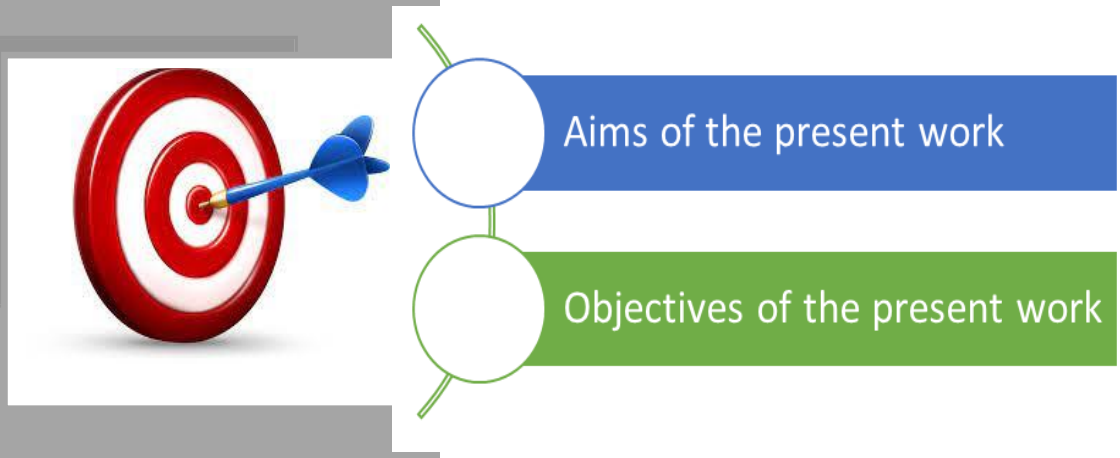
electron impact ionization of its main urinary metabolites. In addition, a HPLC assay was developed for the simultaneous quantitative determination of NSD and of the identified metabolites in urine from treated healthy subjects.

- Two specific methods have developed and validated for the simultaneous determination of nimesulide (NSD) and its hydroxylated metabolite (M1) in human plasma using tolbutamide as internal standard (IS). Established a high performance liquid chromatographic (HPLC) method with UV detection of 230 nm and the compounds were extracted from plasma in acidic medium, also were separated on ODS columns as stationary phase under gradient conditions employing a phosphate buffer solution and methanol as mobile phase. The lower limits of quantification (LLOQ) was 25 ng/mL for both NSD and M1 and the methods were applied in pharmacokinetic studies in healthy volunteers (Giachetti and Tenconi. 1998).

- Kucukguzel, Kucukguzel, Oral *et al*., (2005) have illustrated a study to characterize the metabolism of NSD in rat plasma at particular time intervals. In vitro studies were also prepared to investigate if nitro reduction occur in vitro using rat hepatic subcellular fractions (microsomal and S9 fraction) as well as aromatic hydroxylation. This explanation outlines detection and characterization of NSD metabolites which are isolated from plasma and hepatic subcellular post-incubates by the application of HPLC-UV/ DAD (diode array) and LC-MS/MS.

Chapter – 3

Aims and Objectives



Rapid progress in the application of LC-MS/MS specially for the bioanalysis of drugs and their active metabolites in biological matrices has been constrained by the requirement for timely and high-quality data at various stages in drug discovery and development process (Sparkman. 2000). Precise and rational method development, validation, and transfer perform a significant role in achieving the goals of "faster, better and economical" for bioequivalence studies. The present scenario is more concerned and focused on bioanalytical techniques that emphasizes four primary figures of merit such as sensitivity, selectivity, speed and high throughput. Mass spectrometry (MS) has been benchmarked as an effective solution for both drug and metabolite analysis at each stage of drug development because it provides each of these key attributes. The ultimate goal of any study sample analysis is to ensure that bioanalytical methods are proven potent through development, validation (as per a protocol, study plan, or standard operating procedure [SOP]), and applied as written in order to robustly quantify the analyte in the presence of a specific matrix. The bioanalytical method employed for the quantitative determination drugs and their active metabolites in biological fluids, plays a pivotal role in the evaluation and interpretation of bioequivalence and drug exposure studies in animals. Depending on the sensitivity, selectivity and cost effectiveness of the method an option needs to be made between HPLC and LC-MS/MS.

Mass spectrometry has undergone massive technological improvements in the last two decades, allowing for it application to proteins, peptides, carbohydrates, DNA, drugs, metabolites and a wide range of other biologically relevant molecules. MS is one of the most sensitive methods for molecular analysis and has the possibility to offer insights to the structure of the analyte. The MS instrument also provides high selectivity in the analysis. When complex samples are to be analysed, the possibility to couple separation techniques, such as chromatography, to MS further enhances the potentiality of the technique. Over the past decade, the combination of high-performance liquid chromatography and mass spectrometry has had a significant impact on drug development. Steady advancements in LC-MS/MS interface technologies combined with robust characteristics for structure analysis, qualitative and quantitative, have resulted in a wide range of applications. Therefore, new paradigms for the analysis of drugs, metabolites and related substances have been developed. The progress in LC-MS/MS applications has been extensive, with retention time and molecular weight emerging as important analytical aspects from drug target to product. An iterative phase of what is it ? and how much is there ? continues to boost the immense progress of LC-MS/MS in the drug development industry. So now a days LC-MS/MS has

become widely accepted as an integral part of the drug development process. In the twentieth century, it is likely that no other type of complex instrument has been as important for various fields of science. Bioequivalence is a term in drug kinetics used to assess the expected *in vivo* biological equivalence of two proprietary preparations of a drug. If two products are said to be bioequivalent it means that they would be expected to be, for all intents and purposes, the same. Birkett (2003) defined bioequivalence by stating that, "two pharmaceutical products are bioequivalent if they are pharmaceutically equivalent and their bioavailabilities (rate and extent of availability) after administration in the same molar dose are similar to such a degree that their effects, with respect to both efficacy and safety, can be expected to be essentially the same. Pharmaceutical equivalence implies the same amount of the same active substance(s), in the same dosage form, for the same route of administration and meeting the same or comparable standards."

Now, pharmacokinetics study or bioequivalence study of drugs which got active metabolites, are being fully metabolized after oral administration are difficult, inconvenient and challenging to analyse. There are limited possibilities for these types of in vivo pharmacokinetics study or bioequivalence study in India because of the following reasons :

- There are number of active metabolites of drug molecules that possesses low exposure to the central compartment in therapeutic human doses and are exclusively detectable in picogram to nanogram levels.
- It is very difficult to carry out by using HPLC or GC only as their exposure to plasma is extremely low in therapeutic doses.

So therefore, a highly sensitive Liquid chromatography-mass spectrometry (LC-MS/MS) is required to accomplish the pharmacokinetics as well as bioequivalence study of such active drug metabolites.

Moreover, due to the important aspect that drug metabolism plays in determining in vivo drug action, several pharmaceutical companies are concerned in investigation of drug metabolism properties as part of the screening process in their drug development efforts. In earlier, some matters were not examined when different drugs were developed and marketed that need to be confirmed for the purpose of both safety and efficacy. Major concern is about metabolism is that it can significantly affect the safety and efficacy of the drug because of the formation of therapeutically active or toxic metabolites. To get additional information,

metabolism studies are very essential and should also be subjected for those drugs which are already marketed. The regulatory organisations are concerned about the metabolism of a compound to know whether the metabolites are potentially more toxic than the drug itself or not. So before the drugs are administered to the subjects, this kind of information is essential to acquire. But, the problem is that those who are taking multiple drugs and the drug levels are either very low or very high in patients with serious chronic diseases like arthritis, asthma, diabetes, heart disease, alzheimer's disease and dementia, epilepsy etc., the results can be life-threatening for them. Presently, identification of metabolites and metabolic pathways in drug metabolism research is predominantly experimental and established on the data acquired from in vitro incubation systems and in vivo animal and human trials. A number of metabolites are produced in the several metabolic pathways and which can be very dissimilar from each other both quantitatively and qualitatively, such as molecular structure, molecular weight, lipophilicity, solubility, pka value, absorption, polar surface area etc. Besides, some of the drug molecules and its metabolites may be detectable at very low concentration levels like pictogram or nanogram in different biological fluids and body compartments. So that's why highly sensitive and specific analytical tools and methods are needed for the clarification of drug metabolism. Recent advance mass spectrometry facilitates these two distinctive characteristics attributable to the progress made in this domain, development of MS have had in this field, with performances that consider the significant increases in data content accessible for qualitative characterization of metabolic pathways and the largely increased capability for generating high standard quantitative data.

The primary concern and main focus of the present thesis was to develop simple, sensitive, reproducible, selective, robust, rapid, high-throughput and specified newly bioanalytical methods to analyze some active metabolites with their prodrugs or parent drugs in plasma, employed these methods to quantify large number of plasma samples during bioequivalence study and finally established their pharmacokinetic profile in healthy human subjects to correlate the therapeutic efficacy. Previous published methods for the analysis of Acetylsalicylic Acid (Aspirin), Enalapril and Nimesulide with their active metabolites such as Salicylic Acid, Enalaprilat and 4-Hydroxynimesulide, respectively have some limitations and disadvantages in some specified areas such as sensitivity, selectivity, extraction recovery, extraction procedure, method throughput, chromatographic run time, application of the method in human subjects etc. Present validated ESI-LC-MS/MS methods in this thesis have been improved and developed of such areas very precisely and delicately.

3.1 Aims of the present work

3.1.1 To develop simple, sensitive, reproducible, selective, robust, rapid and high throughput LC-MS/MS methods for the simultaneous determinations of novel drugs and its active metabolites in human plasma.

3.1.2 Developed bioanalytical methods were validated as per USFDA and EMA guidelines.

3.1.3 Application of the validated LC-MS/MS methods in Bioequivalence study to evaluate the Pharmacokinetic parameters and Bioavailability after single oral administration of either Test and Reference preparations of the subject drugs.

3.2 Objectives of the present work

3.2.1 Selection of the drugs and its active metabolites (Aspirin or Acetylsalicylic Acid and Salicylic Acid; Enalapril and Enalaprilat; Nimesulide and 4-Hydroxynimesulide).

3.2.2 Obtain the above mentioned drugs and its active metabolites.

3.2.3 Method development for all the above mentioned drugs and metabolites in human plasma - consideration of both mass parameters and chromatographic parameters for LC-MS/MS method development of Acetylsalicylic Acid (Aspirin), Enalapril and Nimesulide with their active metabolites Salicylic Acid, Enalaprilat and 4-Hydroxynimesulide, respectively to be followed :

- MS-MS method development (scanning and manual tuning of mass, optimization of mass parameters etc.).
- LC-MS/MS method development (optimization of chromatographic parameters such as mobile phase composition, pH, buffer concentration, stationary phase i.e., column selection, flow rate, internal standard selection, selection of extraction procedure etc.).
- Study of extraction recovery.

3.2.4 Method validation study i.e., validation of developed LC-MS/MS methods by different parameters such as a) Selectivity, b) Sensitivity, c) Accuracy and Precision, d) Reproducibility, e) Stability (such as Stock Solution Stability, Freeze and Thaw Stability,

Bench Top Stability, Post-Preparative or Autosampler Stability, Dry State Stability, Short Term and Long Term Stability), f) System Suitability, g) Carry Over, h) Extraction Recovery, i) Matrix Effect, j) Calibration Range and Response Function, k) Dilution Integrity or Parallelisms etc.

3.2.5 Ethical Considerations - permission from Institutional Ethics Committee (Nimesulide and 4-Hydroxynimesulide) and HURIP Independent Bioethics Committee (Aspirin or Acetylsalicylic Acid and Salicylic Acid and Enalapril and Enalaprilat).

3.2.6 To conduct clinical studies for above mentioned drugs and its active metabolites - performing the bioequivalence study -

- To carry out pre-study screening of healthy subjects.
- Administration of drug in volunteers.
- Sample collection i.e., blood sampling and plasma separation according to SOPs approved by DCGI (Drugs Controller General of India).
- Analysis of plasma samples by developed and validated LC-MS/MS methods (Data were collected experimentally and processed by using Analyst 1.4.2 and Analyst 1.5 softwares of LC-MS/MS) and also to evaluate the plasma – concentration profile.
- Compilation of the data and reporting -
    - Determination of Bioequivalence parameters by WinNonlin software.
    - To assess Pharmacokinetics parameters such as C_{max}, T_{max}, AUC_{0-t}, $AUC_{0-\infty}$, K_{el}, $t_{1/2}$ and Relative Bioavailability (%).
    - Accomplishing statistical interpretation i.e., ANOVA test and confidence intervals for Pharmacokinetic parameters like C_{max}, AUC_{0-t} & $AUC_{0-\infty}$ on their un-transformed data and ln transformed data or log-transferred values.
    - Establishing Bioequivalence of Test formulations with Reference formulations.

Chapter – 4: Methodology

(Materials and Methods)



- Procurement of chemicals and reagents
- Bio-analytical method development
- Pre-validation study
- Method validation
- Application of bio-analytical methods

4.1 Chemicals, reagents and solvents

4.1.1 Determination of acetylsalicylic acid (ASA) and its active metabolite salicylic acid (SA) in human plasma by LC-ESI-MS/MS method

- The active pharmaceutical ingredient (API) of ASA (purity 99.55%) ($C_9H_8O_4$) and SA (purity 98.48%) ($C_7H_6O_3$) were gifted from TAAB Biostudy Services, Jadavpur, Kolkata, India. Hydrochlorothiazide (HCTZ) (purity 99.99%) ($C_7H_8ClN_3O_4S_2$) as internal standard (IS) was obtained from Bioequivalence Study Center, Jadavpur University, Kolkata, India.

- Analytical grade (AR grade) acetonitrile (C_2H_3N) and methanol (CH_3OH) were obtained from Merck Specialties Pvt. Ltd., (Mumbai, India) and guaranteed analytical reagent grade ammonium formate (CH_5NO_2), ethyl acetate ($C_4H_8O_2$), methyl tert-butyl ether ($C_5H_{12}O$) and EDTA ($C_{10}H_{16}N_2O_8$) were also purchased from Merck Specialties Pvt. Ltd., (Mumbai, India).

- In the entire bioanalysis, water (H_2O) was purified using Milli-Q water purification system until a resistivity of 18.2 MΩ.cm (megaohms x centimeter), conductivity < 0.055 µS/cm (microsiemens per centimeter) was reached that supplied by Millipore (Elix, Milli-Q A10 Academic, Bedford, MA, USA) and this water (H_2O) was obtained from the analytical laboratory of Bioequivalence Study Centre, Jadavpur University.

- The blank plasma samples for calibration standards (CC) and quality control (QC) samples and human plasma samples both with EDTA-K3 anticoagulant were collected from Clinical Pharmacology Unit (CPU) of Bioequivalence Study Center, Jadavpur University, Kolkata, India and TAAB Biostudy Services, Jadavpur, Kolkata, India. These plasma samples were stored at -20 °C until use.

4.1.2 Determination of enalapril (EPL) and its active metabolite enalaprilat (EPLT) in human plasma by LC-ESI-MS/MS method

- The active pharmaceutical ingredient (API) of EPL (purity 99.45%) ($C_{20}H_{28}N_2O_5$), EPLT (purity 99.95%) ($C_{18}H_{24}N_2O_5$) and tolbutamide (TBM) (IS, internal standard) (purity 99.62%) ($C_{12}H_{18}N_2O_3S$) were gifted from TAAB Biostudy Services, Jadavpur, Kolkata, India.

- HPLC grade methanol (CH_3OH) and acetonitrile (C_2H_3N) were obtained from Merck Specialties Pvt. Ltd., (Mumbai, India) and guaranteed analytical reagent grade (AR grade) formic acid (HCOOH), chloroform ($CHCl_3$), isopropyl alcohol (C_3H_8O) were also procured from Merck Specialties Pvt. Ltd., (Mumbai, India).

- Water used in the entire analysis was obtained from Milli-Q water purification system in the analytical laboratory of Bioequivalence Study Centre, Jadavpur University and that procured from Millipore (Elix, Milli-Q A10 Academic, MA, USA) until a resistivity of 18.2 MΩ.cm, conductivity < 0.055 µS/cm was achieved.

- The blank plasma samples for calibration standards (CC) and quality control (QC) samples and human plasma samples both with EDTA-K3 anticoagulant were collected from Clinical Pharmacology Unit (CPU) of Bioequivalence Study Center, Jadavpur University, Kolkata, India and TAAB Biostudy Services, Jadavpur, Kolkata, India and was stored at -20 °C until use.

4.1.3 Determination of nimesulide (NSD) and its active metabolite 4-hydroxy-nimesulide (M1) in human plasma by LC-ESI-MS/MS method

- The active pharmaceutical ingredient (API) of NSD (purity>99%) ($C_{13}H_{12}N_2O_5S$) and M1 (purity>99%) ($C_{13}H_{12}N_2O_6S$), the metabolite and Celecoxib (CXB) (purity>99%) ($C_{17}H_{14}F_3N_3O_2S$) as internal standard (IS) were obtained from Bioequivalence Study Center, Jadavpur University, Kolkata, India.

- Ammonium formate (CH_5NO_2), dichloromethane (CH_2Cl_2) (analytical-reagent grade), methanol (CH_3OH) (HPLC grade) and acetonitrile (C_2H_3N) (HPLC grade) were purchased from Merck Specialties Pvt. Ltd., Mumbai, India.

- Water (H_2O) was purified by Milli-Q gradient system of Millipore (Elix, Milli-Q A10 Academic, Bedford, MA, USA) until a resistivity of 18.2 MΩ.cm, conductivity < 0.055 µS/cm was achieved and obtained from the analytical laboratory of Bioequivalence Study Centre, Jadavpur University.

- The blank plasma samples for calibration standards (CC) and quality control (QC) samples and human plasma samples both with EDTA-K3 anticoagulant were collected from Clinical Pharmacology Unit (CPU) of Bioequivalence Study Center,

Jadavpur University, Kolkata, India and TAAB Biostudy Services, Jadavpur, Kolkata, India and also stored at -20 °C until use.

4.2 Preparation of stock solutions and working standard solutions

4.2.1 For ASA and SA

Stock solutions (1.0 mg/mL, w/v) of ASA, SA and HCTZ (IS) were prepared separately. In 10 mL of acetonitrile, 10 mg of both drug and the metabolite were added and stock solutions (1.0 mg/mL, w/v) of IS was prepared by methanol. To obtain a stock solution of 200 µg/mL for both ASA and SA and 10 µg/mL for HCTZ (IS) were diluted by mobile phase i.e., acetonitrile and water containing 10 mM ammonium formate :: 9:1 (v/v). Likewise, to obtain working standard solutions for calibration concentration (CC) and quality control (QC) samples for both ASA and SA from serial dilution of the stock solutions. All stock solutions and QC solutions were stored at -20 °C and all working standard solutions were stored at 2 to 8 °C.

4.2.2 For EPL and EPLT

Stock solutions of EPL and EPLT and TBM (IS) were prepared separately by dissolving accurately weighed samples in the methanol i.e., 10 mg of all analytes were dissolved in 10 mL of methanol to obtain concentration of 1.0 mg/mL (w/v). The stock solutions were then gradually diluted with diluent i.e., methanol : water :: 50 : 50 (v/v) to obtain working standard solutions for calibration concentration (CC) and quality control (QC) samples for both EPL and EPLT. All stock solutions and QC solutions were stored at -20 °C and working standard solutions were stored at 4 °C.

4.2.3 For NSD and M1

In order to prepare stock solutions (1.0 mg/mL, w/v) of NSD, M1 and CXB (IS) separately, 10 mg of all analytes were dissolved in 10 mL of methanol (w/v). These solutions were further diluted with the diluent was mobile phase i.e., acetonitrile and water containing 5 mM ammonium formate (9:1 v/v) to obtain a stock solution of 100 µg/mL for both NSD and M1 and 10 µg/mL for CXB (IS). These stock solutions were diluted appropriately with diluent to obtain working standard solutions for calibration concentration (CC) and quality control (QC) samples for both NSD and M1. All stock solutions and QC solutions were stored at -20 ºC and working standard solutions were stored at 2 to 8 ºC.

4.3 Instrument used for the bioanalysis

The complete work i.e., the entire bioanalysis such as, method development (both MS/MS development and LC-MS/MS development), method validation and simultaneous quantification of ASA and SA, EPL and EPLT, NSD and M1 in human plasma were achieved by the liquid chromatography tandem mass spectrometry (LC-MS/MS) system (Figure.4.1). This system has two major parts/components like the liquid chromatography (LC) and the mass spectrometer (MS).

4.3.1 Liquid chromatography (LC)

This is nothing but a high performance or high pressure liquid chromatography (HPLC). The liquid chromatographic (LC) system (Shimadzu series, prominence, Japan) has been equipped with an auto sampler, temperature set at 15 °C (SIL-20AC) and CBM-20A Lite System Control Compartment, an online solvent degasser (DGU- 20A3), binary pump i.e., pump A and pump B (LC-20AD) and a column oven (CTO-10 AS VP) were used for the sample injection, sample run and separation of ASA and SA, EPL and EPLT, NSD and M1, respectively.

4.3.2 Mass spectrometer (MS)

The mass spectrometer (MS) is an API 2000 triple quadrupole tandem mass spectrometer i.e., MS/MS system (Applied Biosystems/MDS SCIEX, Toronto, Canada) (Figure.4.1) coupled with electrospray ionization (ESI) source and used for both detection and quantitative determination of ASA and SA, EPL and EPLT, NSD and M1 in human plasma samples. Data acquisition was accomplished and processed by using Analyst 1.4.2 and Analyst 1.5 softwares of the LC-MS/MS system.



Figure.4.1 Liquid chromatography mass spectrometry system (LC-MS/MS)

4.3.3 Other equipments related to the bioanalysis

Top loading balance or weighing balance/microbalance (Sartorius, Germany), pH meter (Sartorius, India), Milli-Q gradient system for water purification of Millipore (Elix, Milli-Q A10 Academic, Bedford, MA, USA), centrifuge (REMI group, India), refrigerated or cold centrifuge (maintain 4 °C temperature) (REMI group, India), evaporator (Takahe Analytical Instruments, India) connected with nitrogen (N_2) gas cylinder, deep freezer (-20 ºC) (Celfrost, India), micropipette both 100 µL (0.1 mL) and 1000 µL (1 mL) etc.

4.4 Chromatographic conditions for the bioanalysis

4.4.1 Chromatorgaphic parameters for both ASA and SA

4.4.1.1 Mobile phase

The mobile phase was eluted isocratically with composition of acetonitrile and 10 mM ammonium formate in water (Milli Q, resistivity 18.2 MΩ.cm, conductivity < 0.055 µS/cm), total pH 5.9 (90:10, v/v).

4.4.1.2 Stationary phase or column

The chromatographic separation was performed on a Phenomenex Kinetex (USA) C18 column, 50 mm x 3 mm (length x inner diameter), with a particle size of 5 µm utilized under isocratic conditions. Also, the pressure of the LC system with this column was not more than 600 psi.

4.4.1.3 Temperature of column oven and autosampler

The column oven temperature was maintained at 60 °C whereas the autosampler temperature was set at 15 °C.

4.4.1.4 Flow rate, sample injection volume and total chromatographic run time

0.5 mL/min. of flow rate of the mobile phase with 20 µL of sample injection volume and 1.8 minutes of total chromatographic run time were continued in the method.

4.4.2 Chromatorgaphic parameters for both EPL and EPLT

4.4.2.1 Mobile phase

The elution were achieved by gradient system using 0.1% formic acid in Milli Q water, pH

2.45 (resistivity 18.2 MΩ.cm, conductivity < 0.055 µS/cm) at pump A and 0.1% formic acid in methanol at pump B as the mobile phase. The analytes and IS were separated by the following gradient (minutes, % mobile phase B or pump B conc.): 0.01, 10; 0.50, 10; 0.70, 90; 2.00, 90; 2.10, 10; 3.00.

4.4.2.2 Stationary phase or column

The chromatographic separation was carried out on a C18 analytical column (50 mm × 3 mm, 5 µm particle size, Phenomenex Kinetex, USA) under gradient conditions.

4.4.2.3 Temperature of column oven and autosampler

The column oven temperature was disabled i.e., not required for this particular method and it was maintained at ambient temperature or room temperature (22 °C) while the autosampler temperature was set at 15 °C.

4.4.2.4 Flow rate, sample injection volume and total chromatographic run time

The flow rate was set at 0.5 mL/min. with 10 µL of sample injection volume and the total chromatographic run time was 3 minutes.

4.4.3 Chromatorgaphic parameters for both NSD and M1

4.4.3.1 Mobile phase

An isocratic mobile phase was used and the composition of the mobile was acetronitrile and water (Milli Q, resistivity 18.2 MΩ.cm, conductivity < 0.055 µS/cm) containing 5 mM ammonium formate (pH 3.2) in the ratio of 9:1, v/v.

4.4.3.2 Stationary phase or column

The chromatographic separation was done on an Agilent ZORBAX Eclipse Plus C18 column (USA), 75 mm x 4.6 mm, particle size 3.5 µm under isocratic conditions.

4.4.3.3 Temperature of column oven and autosampler

The column oven temperature was not applicable in this way and it was continued at ambient temperature or room temperature (20 to 22 °C) whereas the autosampler temperature was set at 15 °C.

4.4.3.4 Flow rate, sample injection volume and total chromatographic run time

The flow rate was set at 0.4 mL/min. with 10 µL of sample injection volume and the total chromatographic run time was 3 minutes.

4.5 Mass spectrometric conditions for the bioanalysis

Mass spectrometric conditions are one of the most important aspect for the bioanalysis of both drugs and its active metabolites by LC-MS/MS. So that is why the mass spectrometric parameters have to be optimized during method development process. In this way two MS parameters are there, one is the source and gas parameters and other is the compound-dependent parameters that have to be optimized. Now, rest two parameters are the resolution and detector parameters which are fixed parameters and also not to be optimized during method development. The source and gas parameters and compound-dependent parameters are stored with the method. On the other hand, the resolution and detector parameters are instrument-dependent and are stored as instrument data. To build the method in the tune mode, at first it was manually tuned the working parameters to optimize for best performance in MS/MS development. Accordingly, ramp each parameter, one at a time, while cycling the experiment.

Source and Gas Parameters : These working parameters are source-dependent parameters and vary depending on the instrument, source and scan type. Source and gas parameters are as follow –

- **Curtain Gas (CUR) :** This is the gas between the curtain plate and the orifice plate. It prevents the contamination of the ion optics.
- **Collision Activated Dissociation Gas (CAD) :** This is the gas in the collision cell or quadrupole 2 (Q2). During Q3 MS and MS/MS-type scans, the CAD parameter controls the pressure of collision gas in the collision cell. The collision gas helps to focus the ions as they pass through the collision cell (Q2) in Q3 MS scans; by default, this parameter is in fixed mode. The collision gas acts as a target to fragment the precursor ions in MS/MS-type scans. When the parent ions collide with the collision gas, they can dissociate to fragment ions, i.e., product ions.
- **Ion Spray Voltage (ISV) :** this is the voltage applied to the needle that ionizes and nebulizes the sample in an ion spray or turbo ion spray source.

- **Nebulizer (or Needle) Current (NC) :** This is the current applied to the corona discharge needle to charge the spray coming from a heated nebulizer. (only applicable to APCI source)

- **Turbo Ion Spray Source Temperature or Turbo Gas Temperature (TEM) :** This is the temperature of the heater gas in a turbo ion spray source or the probe in a heated-nebulizer source.

- **Nebulizer Gas (GS1) :** This is the gas that focuses the spray in the nebulizer. The GS1 parameter controls the nebulizer gas for the turbo ion spray probe and the auxiliary gas for the APCI probe.

- **Turbo Ionspray Gas or Auxiliary Gas or Desolvation Gas (GS2) :** For the turbo ion spray probe, the GS2 parameter controls the auxiliary or turbo gas. It helps in the evaporation of the spray droplets. GS2 works in conjunction with the TEM parameter.

- **Interface Heater (ihe) :** The ihe parameter controls whether the interface heater is turned on or off. Heating the interface helps maximize the ion signal and prevents contamination of the ion optics. Unless the compound which are analysing is extremely fragile and it is recommended to heat the interface.

Compound-Dependent Parameters : The compound-dependent parameters consist mostly of lens elements in the ion path. The optimal values for compound-dependent parameters vary depending on the compound being analysed. The parameters are as follow –

- **Declustering Potential (DP) :** The DP parameter controls the voltage on the orifice, which regulates the ability to decluster ions between the orifice and the skimmer. It is used to minimize the solvent clusters that may remain on the sample ions once they enter the vacuum chamber and if required to fragment ions. The higher the voltage, the higher the energy imparted to the ions. Unwanted fragmentation may occur if the DP parameter is too high.

- **Focusing Potential (FP) :** The voltage applied to the focusing ring lens is controlled by the FP parameter. The focusing potential helps focus the ions through the skimmer region of the mass spectrometer interface. Similar to the declustering potential, it can induce fragmentation in the interface area.

- **Entrance Potential (EP) :** The EP parameter regulates the potential difference between the voltage on Q0 and ground. The ions are guided and focused by the entrance potential as they pass through the high-pressure Q0 region.

- **Collision Cell Entrance Potential (CEP) :** The CEP parameter controls the collision cell entrance potential which is the potential difference between Q0 and IQ2 (i.e., focusing lens 2). It focuses ions into Q2 (collision cell). In Q1 and MS/MS-type scans, CEP is used. This voltage is known as IQ2 in Q3 scans and it is present to fixed-mode.

- **Collision Energy (CE) :** The CE parameter controls the potential difference between Q0 and Q2 (collision cell). It is used only in MS/MS-type scans. This is the amount of energy that the precursor ions receive as they are accelerated into the collision cell where they collide with gas molecules and fragment. The voltage applied to the collision cell in the Q1 and Q3 scans is RO2 (collision cell rod offset).

- **Collision Cell Exit Potential (CXP) :** Depending on the instrument, the CXP parameter controls the potential difference between RO2 and IQ3 (focusing lens 3) or between RO2 and ST3 (prefilter 3). It is only used in Q3 and MS/MS scans where the ions are transmitted into Q3. The voltage applied to IQ3/ST3 in Q1 scans is accessed through the IQ3 or ST3 parameters.

Resolution Parameters : Resolution parameters are the calibration parameters and also the instrument-dependent parameters that affect the resolution, sensitivity and peak shape and depend on the type of scan.

Q1 Scans

- **Ion energy 1 (IE1) :** Increasing this value increases the sensitivity but broadens peaks for Q1 scans.

- **Q1 resolution :** This is the resolution for Q1 or precursor ion scans.

Q3 Scans

- **Ion energy 3 (IE3) :** Increasing this value increases the sensitivity but broadens peaks for Q3 and MS/MS scans.

- **Q3 resolution :** This is the resolution for Q3 or product ion scans.

Detector Parameters : These parameters are associated with the detection of ions.

- **Channel Electron Multipliers (CEM) :** The CEM parameter controls the voltage applied to the detector. It is used to detect ions by amplifying ion signals by producing a cascade of electrons.
- **Deflector (DF) :** The voltage applied to the deflector is controlled by the DF parameter. Its purpose is to guide ions into the detector. It is preset to fixed mode.

Source and Gas Parameter Range	CUR Gas (psi) : 0 to 50
	CAD Gas (psi) : 0 to 12
	Ion Spray Voltage (ISV) (V) : Positive Polarity/Mode : Upto 5500 Negative Polarity/Mode : Upto 4500
	Temperature (°C) : Upto 450
	GS1 (psi) : 0 to 100
	GS2 (psi) : 0 to 100
Compound-Dependent Parameter Range	DP (V) : 0 to 200
	FP (V) : 50 to 400
	EP (V) : 1 to 12
	CEP (V) : 0 to 188
	CE (eV) : 5 to 130
	CXP (V) : 0 to 58

4.5.1 Mass spectrometric parameters for both ASA and SA

The mass spectra scanning or detection i.e., the identification of the ions and manual tuning process for the analytes (ASA and SA) and also IS (HCTZ) and finally for the quantification of both ASA and SA in human plasma were performed by using API 2000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, Canada) (Figure.4.2) equipped with a turbo ion spray interface i.e., electrospray ionization (ESI) source to generate the negatively charged ions. Because, intensity of the mass spectra of the anlytes (ASA and SA) and IS (HCTZ) were more and better in negative polarity or negative ionization mode $[M - H]^-$ than positive $[M + H]^+$.



Figure.4.2 API 2000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, Canada) coupled with electrospray ionization (ESI) source

In the Harvard syringe pump method with an external syringe pump (syringe diameter 4.6 mm and volume capacity 1000 µL or 1 mL) directly connected to the mass spectrometer with a high voltage of 5.5 kV was applied to the spray needle i.e., turbo ion spray probe for optimization of the tuning parameters by injecting 100 ng/mL of standard solution containing ASA, SA and HCTZ (IS) separately at 10 µL/min flow. The dwell time of 200 ms (ms = millisecond) was programmed for a scan in the MS/MS system. The turbo ion spray source temperature or turbo gas temperature and turbo ion spray voltage were set at 400 °C and -4500 Volts (V), respectively. After manual tuning or optimization, the operating conditions or parameters were also optimized i.e., both for the source and gas parameters and the compound-dependent parameters by flow injection analysis (FIA) with the method of compound optimization in tune mode in the negative polarity by the software (Analyst 1.5) with the mobile phase but not with column and the injecting concentration was 100 ng/mL of a mixture of both analytes and IS.

The optimized source and gas parameters were as follows : curtain (CUR) gas flow was 20 psi (1 p.s.i. = 6894.76 Pa) and collision activated dissociation (CAD) or collision-induced dissociation (CID) gas flow was 4 psi (arbitary scale). Turbo ion spray voltage (ISV) and turbo ion spray source temperature or turbo gas temperature (TEM) were -4500 V and 400 ^{o}C, respectively. Both nebulizer gas (GS1) and turbo ionspray gas or auxiliary gas or desolvation gas (GS2) (i.e., heater gas) were applied at 50 psi each. The compound-dependent parameters were as follows : for ASA, declustering potential (DP), focusing potential (FP) and entrance potential (EP) were -10.00 V, -400.00 V and -10.00 V, respectively. Collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP) were -13.00 V, -10.00 eV and -6.00 V, respectively with dwell times of 200 ms. For SA, declustering potential (DP), focusing potential (FP) and entrance potential (EP) were -19.00 V, -400.00 V and -10.00 V, respectively. Afterwards, collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP) were -11.95 V -27.00 eV and -10.00 V, respectively with dwell times of 200 ms. Lastly for IS (HCTZ), declustering potential (DP), focusing potential (FP) and entrance potential (EP) were -20.00 V, -400.00 V and -10.00 V, respectively. Subsequently, collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP) were -15.92 V, -25.00 eV and -15.00 V, respectively with dwell times of 200 ms. In multiple reactions monitoring (MRM) mode, quantitation was carried out by monitoring deprotonated precursor ion $[M - H]^-$ to product ion transitions *m/z* 179.0 → 137.0 for ASA, *m/z* 137.0 → 93.0 for SA and *m/z* 295.9 → 268.9 for HCTZ (IS), respectively. Quadrupoles, Q1 and Q3 were set to unit resolution. Instrument control, automated data acquisition, data processing and analysis were performed by Analyst software version 1.5.

4.5.2 Mass spectrometric parameters for both EPL and EPLT

The mass spectra detection and manual tuning process for the analytes (EPL and EPLT) and IS (TBM) and finally for the quantification of both EPL and EPLT in human plasma were performed by using API 2000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, Canada) equipped with a turbo ion spray interface i.e., electrospray ionization (ESI) source to produce the positively charged ions. Because, intensity of the mass spectra of the anlytes (EPL and EPLT) and IS (TBM) were more and better in positive polarity or positive ionization mode $[M + H]^+$ than negative $[M - H]^-$.



Figure.4.3 Syringe pump method used in MS/MS method development

By the syringe pump method (Figure.4.3), direct infusion of 100 ng/mL standard solution of each analyte (EPL, EPLT and IS separately) in methanol at 10 µL/min flow to optimize parameters to detect the most vivid signals of transitions from precursor to product ions. The turbo ion spray source temperature or turbo gas temperature was 400 °C and turbo ion spray voltage was set at 5000 V. After completion of manual tuning or optimization, the operating conditions (parameters) were optimized for both the source and gas parameters and the compound-dependent parameters by flow injection analysis (FIA) technique of a mixture 100 ng/mL, the injecting concentration of both analytes and IS.

The optimized source and gas parameters were as follows: curtain (CUR) gas flow and collision activated dissociation (CAD) or collision-induced dissociation (CID) gas flow were 30 psi and 6 psi, respectively. Turbo ion spray voltage (ISV) was 5000 V and turbo ion spray source temperature or turbo gas temperature (TEM) was 400 ^{o}C. Nebulizer gas (GS1) gas was set at 55 psi and turbo ionspray gas or auxiliary gas or desolvation gas (GS2) (i.e., heater gas) was set at 45 psi. The compound-dependent parameters were as follows : for EPL, declustering potential (DP), focusing potential (FP) and entrance potential (EP) were 30.00 V, 400.00 V and 11.00 V, respectively. Collision cell entrance potential (CEP), collision

energy (CE) and collision cell exit potential (CXP) were 29.73 V, 30.00 eV and 4.00 V, respectively with dwell times of 200 ms. For EPLT, declustering potential (DP) was 30.00 V, focusing potential (FP) was 400.00 V and entrance potential (EP) was set at 11.00 V. Likewise, collision cell entrance potential (CEP) was 29.08 V, collision energy (CE) was 30 eV and collision cell exit potential (CXP) was set at 4.00 V with dwell times of 200 ms. Finally for IS (TBM), declustering potential (DP), focusing potential (FP) and entrance potential (EP) were 30.00 V, 400.00 V and 11.00 V, respectively. Similarly, collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP) were 27.29 V, 30.00 eV and 4.00 V, respectively with dwell times of 200 ms. The quantification was performed using multiple reaction monitoring (MRM) mode, based on protonated precursor ion $[M + H]^+ \rightarrow$ product ion transitions *m/z* 377.2 → 234.2 for EPL, *m/z* 349.1 → 206.1 for EPLT and *m/z* 271.1 → 155.0 for TBM (IS), respectively. Quadrupole Q1 and quadrupole Q3 were maintained at unit resolution and dwell time was set at 200 ms. Analyst software (version 1.5) was used for instrument control, data acquisition and data processing.

4.5.3 Mass spectrometric parameters for both NSD and M1

The mass spectra detection and manual tuning process for the analytes (NSD and M1) and IS (CXB) and finally for the quantification of both NSD and M1 in human plasma were carried out by using API 2000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, Canada) equipped with a turbo ion spray interface i.e., electrospray ionization (ESI) source. Ions were measured in negative ionization mode i.e., in negative polarity $[M - H]^-$. Because, intensity of the mass spectra of the anlytes (NSD and M1) and IS (CXB) were more and better in negative polarity or negative ionization mode $[M - H]^-$ than positive $[M + H]^+$. The tuning parameters were optimized by injecting 100 ng/mL of standard solution containing NSD, M1 and CXB (IS) separately at 10 µL/min flow by means of an external syringe pump (i.e., Harvard syringe pump method) directly connected to the mass spectrometer. A high voltage of 5.5 kV was applied to the spray needle (i.e., turbo ion spray probe) and the instrument was programmed for a scan dwell time of 200 ms. The turbo ion spray source temperature or turbo gas temperature was set at 400 ºC and turbo ion spray voltage was -4500 V. After manual tuning or optimization, the operating conditions i.e., parameters were optimized for both the source and gas parameters and the compound-dependent parameters by flow injection analysis (FIA) technique of a mixture 100 ng/mL, the injecting concentration of both analytes and IS.

The optimized values of the source and gas parameters were: curtain (CUR) gas and collision activated dissociation (CAD) or collision-induced dissociation (CID) gas flow at instrument were 30 psi and 6 psi (arbitary scale), respectively. Turbo ion spray voltage (ISV) and turbo ion spray source temperature or turbo gas temperature (TEM) were -4500 V and 400 ^{o}C, respectively. Nebulizer gas (GS1) gas was set at 55 psi and turbo ionspray gas or auxiliary gas or desolvation gas (GS2) (i.e., heater gas) was set at 50 psi. Moreover, the optimized values of the compound-dependent parameters were : for NSD, declustering potential (DP), focusing potential (FP) and entrance potential (EP) were -20.00 V, -400.00 V and -7.00 V, respectively. Collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP) were -24.61 V, -25.00 eV and -4.00 V, respectively with dwell times of 200 ms. For M1, declustering potential (DP) was -25.00 V, focusing potential (FP) was -400.00 V and entrance potential (EP) was set at -10.00 V. Whereas, collision cell entrance potential (CEP) was -25.07 V, collision energy (CE) was -20.00 eV and collision cell exit potential (CXP) was set at -20.00 V with dwell times of 200 ms. Lastly for IS (CXB), declustering potential (DP), focusing potential (FP) and entrance potential (EP) were -45.00 V, -400.00 V and -10.00 V, respectively. While, collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP) were -26.73 V, -32.00 eV, and -20.00 V, respectively with dwell times of 200 ms. The multiple reaction monitoring (MRM) transition of *m/z* 307.2 → 229.2, *m/z* 323.0 → 245.0 and *m/z* 380.2 → 316.2 were used to measure NSD, M1 and CXB (IS), respectively. Quadrupoles, Q1 and Q3 were set to unit resolution. Instrument control, automated data acquisition, data processing and quantitation were performed by using Analyst 1.5 software.

4.6 Preparation of mobile phase

Mobile phase is a very crucial part of the bioanalysis. So on the basis of the trial and error method, ratio of the buffer and organic modifier (i.e., methanol and acetonitrile) was optimized. Each of the buffer systems was separately or mixed with either methanol or acetonitrile at particular ratios to prepare the mobile phase using either isocratic or gradient elution system. The chromatographic run time was set very short to avoid the unnecessary wastage of the mobile phase. Ultimately selection of the best and suitable mobile phase was based on peak shape, resolution, retention time, asymmetry factor, theoretical plate count etc.

4.6.1 For the method of ASA and SA

4.6.1.1 Preparation of the buffer : water containing 10 mM ammonium formate

1000 mL of Milli-Q grade water with a resistivity of 18.2 MΩ.cm, conductivity < 0.055 µS/cm was measured by graduated measuring cylinder and also was taken in a 1000 mL solvent reservoir bottle or reagent bottle. After that accurately weighed 0.6306 gm i.e., 630.6 mg of ammonium formate was added in this 1000 mL water (w/v). This solution was sonicated for 1 minute and then filtered by Nylon filter membrane (pore size 0.45 µm and diameter 47 mm). Lastly, the solution was sonicated for 5 minutes. This solution was used within 3 days from the date of preparation.

4.6.1.2 Preparation of the organic modifier : acetonitrile

1000 mL of analytical grade (AR grade) acetonitrile was measured by graduated measuring cylinder and poured into a 1000 mL solvent reservoir bottle or reagent bottle. It was filtered by Nylon filter membrane (pore size 0.45 µm and diameter 47 mm) and sonication was done for 5 minutes. Also, it was used within 3 days from the date of preparation.

In the method, this mobile phase was eluted isocratically in the ratio of 90:10 (v/v) and the total pH of 5.9 was maintained throughout the bioanalysis.

4.6.2 For the method of EPL and EPLT

4.6.2.1 Preparation of the buffer : water containing 0.1% formic acid

Firstly, 999 mL of Milli-Q grade water with a resistivity of 18.2 MΩ.cm, conductivity < 0.055 µS/cm was measured by graduated measuring cylinders and poured into a 1000 mL solvent reservoir bottle or reagent bottle. Then 1 mL of formic acid (AR grade) was added and sonicated for 1 minute. pH of the solution was 2.45. After that the solution was filtered by Nylon filter membrane (pore size 0.45 µm and diameter 47 mm) and sonicated for 5 minutes. This solution was used within 3 days from the date of preparation.

4.6.2.2 Preparation of the organic modifier : methanol containing 0.1% formic acid

999 mL of analytical grade (AR grade) methanol was measured by graduated measuring cylinders and poured into a 1000 mL solvent reservoir bottle or reagent bottle. Afterwards, 1 mL of formic acid (AR grade) was added and sonicated for 1 minute. This solution was filtered by Nylon filter membrane (pore size 0.45 µm and diameter 47 mm) and after

sonication was done for 5 minutes. This solution was also used within 3 days from the date of preparation.

This mobile phase was eluted under gradient conditions in the method, i.e., the analytes (EPL and EPLT) and IS (TBM) were separated by the following gradient (minutes, % mobile phase B or pump B conc.): 0.01, 10; 0.50, 10; 0.70, 90; 2.00, 90; 2.10, 10; 3.00 and throughout the analysis.

4.6.3 For the method of NSD and M1

4.6.3.1 Preparation of the buffer : water containing 5 mM ammonium formate

1000 mL of Milli-Q grade water with a resistivity of 18.2 MΩ.cm, conductivity < 0.055 µS/cm was measured by graduated measuring cylinder and taken in a 1000 mL solvent reservoir bottle or reagent bottle. Then 0.3153 gm i.e., 315.3 mg of ammonium formate was accurately weighed and added in this 1000 mL water (w/v). This solution was sonicated for 1 minute and filtered by Nylon filter membrane (pore size 0.45 µm and diameter 47 mm). Finally, the solution was sonicated for 5 minutes and used within 3 days from the date of preparation.

4.6.3.2 Preparation of the organic modifier : acetronitrile

1000 mL of acetonitrile (AR grade) was measured by graduated measuring cylinder and poured into a 1000 mL solvent reservoir bottle or reagent bottle. Then it was filtered by Nylon filter membrane (pore size 0.45 µm and diameter 47 mm) and sonication was done for 5 minutes. This was used within 3 days from the date of preparation.

The mobile phase was eluted under isocratic conditions with the ratio of 9:1 (v/v) in the method and throughout the analysis.

4.7 Stationary phase or column

The objective of using a column (stationary phase) in chromatography i.e., in a bioanalytical method was to separate the molecules or the analytes during analysis. During method development, a column was selected for the bioanalysis again by trial and error method. Bioanalytical method differs from simple LC-MS/MS method signified for analysis of raw drug or aqueous. The matrix used in bioanalysis arises the problem to the analyst as the matrix compounds mainly coelute with analyte. Different stationary phases such as, C18

Phenomenex Kinetex, C18 Agilent ZORBAX Eclipse Plus, Cyano (CN) Agilent ZORBAX etc were tried to separate the interfering peak from the analyte. During the entire procedure, all other chromatographic conditions except the column were remained unchanged.

4.8 Internal standard (IS)

Internal standard (IS) is one of the most important factor in bioanalytical method. Generally, a compound with structural similarity with the analyte or with significant intensity, response and stability is considered as IS in the bioanalytical method. Better extraction recovery, chromatographic properties and ionization properties have similar to the analyte would be added an advantage. The main purpose of using an IS in a bioanalytical method is to reduced or minimized the analytical variations.

4.8.1 IS for the method of ASA and SA

A stable compound is the ideal IS for the LC-MS/MS method, because the presence of matrix effect can influence the analytical results. Usually, analogs of the compounds act as the IS material because of similar properties during the sample conduction, chromatographic elution and mass spectrometric detection. So during method development, different compound were tested for the selection of IS, such as celecoxib, hydrochlorothiazide, letrozole, diclofenac, ibuprofen and prothionamide under the present conditions. Finally, hydrochlorothiazide (HCTZ) was used as the IS throughout the bioanalysis due to its better response and rapid elution rate under the same LC-MS/MS conditions as ASA and SA. Moreover, the extraction recovery of HCTZ was higher than the other tested compounds that already mentioned.

4.8.2 IS for the method of EPL and EPLT

Initially, different compounds were investigated for the selection of IS, such as metformin, losartan, tolbutamide, propranolol, metoprolol and rosiglitazone. Finally tolbutamide (TBM) was determined to be the best internal standard (IS) because it was shown that there was no interference with the performance of the developed method and the intensity of the molecular ion peak in mass spectrometric analysis remained unaffected or uninfluenced as related with both the analytes EPL and EPLT. On basis of the retention time of analytes, the flow rate was optimized at 0.5 mL/min and faster separation was done due to the gradient elution with the chromatographic conditions.

4.8.3 IS for the method of NSD and M1

In the beginning various compounds were examined for the selection of IS, like letrozole, celecoxib, hydrochlorothiazide, prothionamide, diclofenac and ibuprofen. But, a stable compound has to be used as an IS to deal with sample matrix effects. So therefore, celecoxib (CXB) was chosen as IS due to its similar recovery, chromatographic properties and ionization properties with the analytes NSD and M1. It provided the intention of method development and also was shown high recovery potentials as the results reveal that the component of IS did not influence or deteriorate the performance of the proposed method. Similarly, the intensity of both NSD and M1 molecular ion peaks in mass spectrometric analysis remained unaffected as coeluted with the IS (CXB). Another reason for the selection of this IS was good chromatographic separation.

4.9 Plasma sample preparation and extraction

4.9.1 Plasma sample preparation and extraction procedure for ASA and SA

A simple liquid-liquid extraction (LLE) procedure was developed for plasma extraction of both the drug (ASA) and its active metabolite (SA) from the human plasma samples. Firstly, all human plasma samples were retrieved from the deep freezer (temperature -20 ºC). Then at ambient temperature or room temperature, all these frozen human plasma samples were thawed. After thawed, 300 µL volume of human plasma samples were taken in 15 mL centrifuge tubes and 30 µL of IS solution (from 4 µg/mL of HCTZ) was added in each and every tubes [in case of calibration concentration (CC) and quality control (QC) samples it was 270 µL of human blank plasma, 30 µL of working standard solution i.e., drug with metabolite concentration and 30 µL of IS solution]. Then it mixed by a vortex/cyclomixture for 30 seconds. After that, up to 4 mL of an organic solvent mixture i.e., ethyl acetate and methyl tert-butyl ether was added in 50:50 (v/v) ratio in all tubes and then hand mixing was done for 10 minutes. Next, centrifugation was performed for 10 minutes at 5000 rpm. Due to this a clear supernatant i.e., organic layer was formed and this organic layer was contained the analytes ASA, SA and IS (HCTZ). So, the organic layer was separated and transferred to another clean centrifuge tubes. This organic layer was evaporated in water bath i.e., in evaporator to dryness under a stream of nitrogen (N_2) at 40 to 45 ºC. Dry residues were collected and reconstituted with the 200 µL of mobile phase followed by vortex mixed for 3 to 4 minutes. Finally, the reconstituted samples were transferred to autosampler vials, which

were then loaded into an autosampler cabinet, and a 20 µL aliquot of each extracted sample was injected into the liquid chromatography mass spectrometry (LC-MS/MS) system. The blank plasma samples and human plasma samples both with EDTA-K3 anticoagulant were obtained from Clinical Pharmacology Unit (CPU) of Bioequivalence Study Center, Jadavpur University, Kolkata and TAAB Biostudy Services, Jadavpur, Kolkata and these plasma samples were stored at -20 °C in the deep freezer until use.

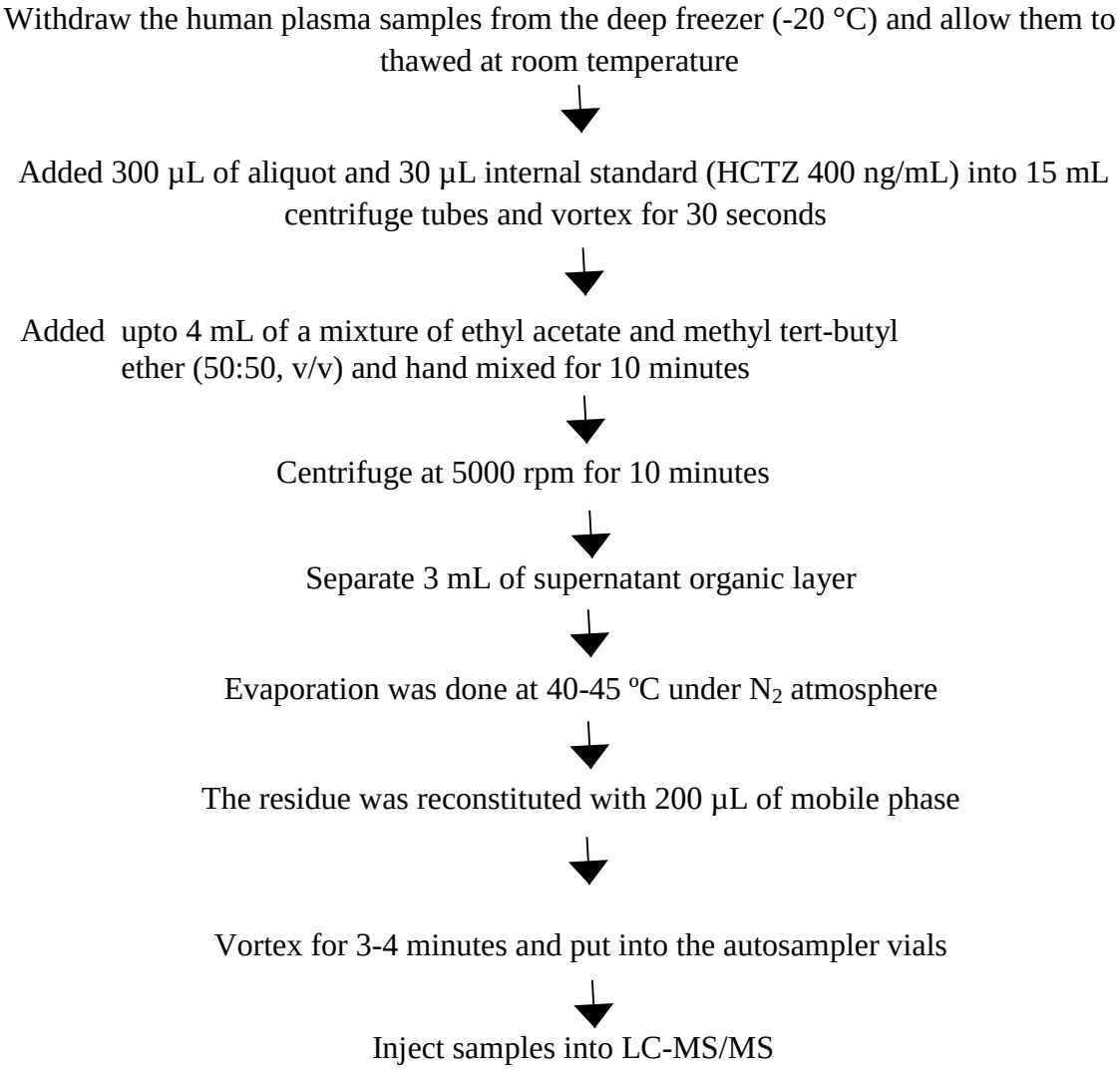


Figure.4.4 A schematic presentation of plasma sample extraction procedure of ASA and SA

Now during method development, 270 µL blank human plasma was taken and spiked with mixed concentration of ASA, SA and IS (HCTZ) of 10 ng/mL and 100 ng/mL. Extraction was done in various organic solvents such as, ethyl acetate, dichloromethane, chloroform, diethyl ether, methyl tert-butyl ether and n-hexane. These organic solvents were tried and tested according to the extraction procedure that already mentioned earlier. Finally, an

organic mixture of ethyl acetate and methyl tert-butyl ether (50:50, v/v) was confirmed as extracting solvent due to the better reproducibility, good peak shape and higher level of recovery.

4.9.2 Plasma sample preparation and extraction procedure for EPL and EPLT

Plasma sample preparation and extraction were performed by simple one step protein precipitation (PPT) method for both the drug (EPL) and its active metabolite (EPLT) from the human plasma samples. Initially, all volunteer plasma samples were retrieved from the -20 ºC deep freezer and these frozen plasma samples were thawed at room temperature or ambient temperature. Then 300 µL of plasma was taken in 2 mL microcentrifuge tubes (eppendorf tubes) and 900 µL of ice cold acetonitrile containing 1 µg/mL TBM (IS) solution was added. Next, vortex mixed was done properly and thoroughly for 10 minutes. After that, centrifugation was performed for 15 min at 10,000 rpm and due to this a clear supernatant was formed. Then the supernatant was separated and loaded into autosampler vials for the LC–MS/MS run. Furthermore, calibration concentration (CC) and quality control (QC) samples were crashed and processed following the same procedure. The blank plasma samples and human plasma samples with EDTA-K3 anticoagulant were both collected from Clinical Pharmacology Unit (CPU) of Bioequivalence Study Center, Jadavpur University, Kolkata and TAAB Biostudy Services, Jadavpur, Kolkata and also these plasma samples were stored in the deep freezer (at -20 °C) until use.

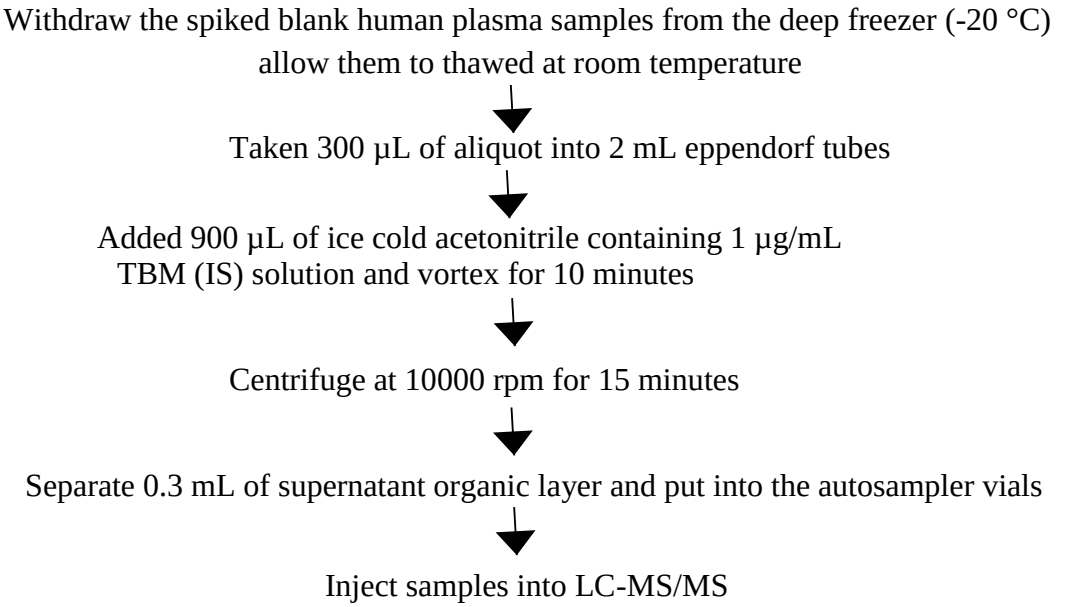


Figure.4.5 A schematic presentation of plasma sample extraction procedure of EPL and EPLT

Some organic solvents were tried and tested for the extraction by protein precipitation technique (PPT), like methanol, cold acetonitrile and acetone with mixed concentration of EPL, EPLT and IS (TBM) of 10 ng/mL and 100 ng/mL during method development process. Finally, cold acetoniltrile was selected for this method because of the better reproducibility, good peak shape and higher level of recovery.

4.9.3 Plasma sample preparation and extraction procedure for NSD and M1

Liquid-liquid extraction procedure was employed for the extraction of the drug NSD and its active metabolite M1 from the plasma. All frozen human plasma samples were retrieved from the -20 ºC deep freezer and thawed at ambient temperature or room temperature. After thawed, 100 µL volume of human plasma samples were placed in 15 mL centrifuge tubes and 10 µL of IS solution (from 50 µg/mL of CXB) was added in each and every tubes [in case of calibration concentration (CC) and quality control (QC) samples it was 90 µL of human blank plasma, 10 µL of working standard solution i.e., drug with metabolite concentration and 10 µL of IS solution]. Then vortex mixed was performed for 30 seconds. After that, upto 3 mL of dichloromethane was added to all tubes and then hand mixing was done for 10 minutes followed by centrifugation for another 10 minutes at 5000 rpm. After centrifugation, a clear supernatant i.e., an organic layer was formed having with the analytes. Therefore, 2 mL of organic layer was separated and transferred to clean centrifuge tubes. The organic layer was evaporated in water bath/ evaporator to dryness under a stream of nitrogen (N2) at 40 to 45 ºC. Dry residues were collected and reconstituted with the 400 µL of mobile phase that was acetronitrile and water containing 5 mM ammonium formate (9:1, v/v) followed by vortex mixed for 3 to 4 minutes. Finally, the reconstituted samples were transferred to autosampler vials, which were then loaded into an autosampler cabinet, and a 10 µL aliquot of each extracted sample was injected into the liquid chromatography mass spectrometry (LC-MS/MS) system. Both the blank plasma and human plasma samples with EDTA-K3 anticoagulant were obtained from Clinical Pharmacology Unit (CPU) of Bioequivalence Study Center, Jadavpur University, Kolkata and TAAB Biostudy Services, Jadavpur, Kolkata and these plasma samples were stored at -20 °C in the deep freezer until use.

During method development, 90 µL blank human plasma was taken and spiked with mixed concentration of NSD, M1 and IS (CXB) of 10 ng/mL and 100 ng/mL. Extraction was carried out in different organic solvents such as, ethyl acetate, dichloromethane, chloroform, diethyl ether, methyl tert-butyl ether and n-hexane. These organic solvents were tried and tested

according to the extraction procedure that already mentioned above. Finally, dichloromethane was selected as extracting solvent due to the better reproducibility, good peak shape and higher level of recovery.

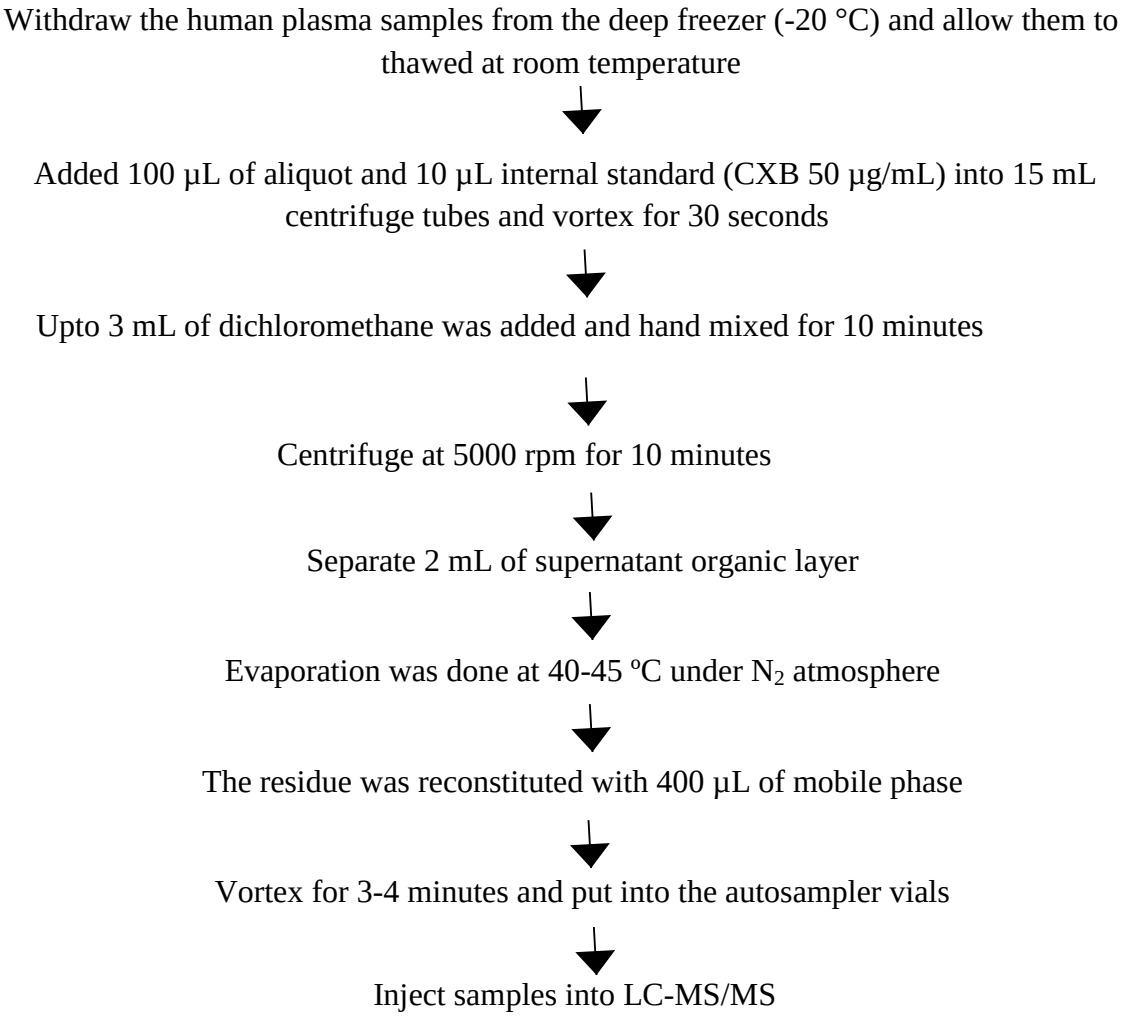


Figure.4.6 A schematic presentation of plasma sample extraction procedure of NSD and M1

4.10 Estimation of limit of detection (LOD) and lower limit of quantification (LLOQ)

The signal-to-noise ratio, throughout the analytes retention times were measured by applying the signal-to-noise method. Afterwards, the concentration of the analytes that would capitulate a signal equal to definite value of noise ratio was assessed. The noise value was determined according to the peak height of the blank plasma throughout the retention time of the analytes. The noise magnitude was measured either manually on the chromatogram printout or by the help of the analyst software. Usually the analyte quantity or amount for

which the signal-to-noise ratio was equal or more than 3 times was recognized as limit of detection i.e., LOD and lower limit of quantification (LLOQ) was determined by the analyte quantity or amount for which the signal-to-noise ratio was equal or more than 5 times. Now for the method of ASA and SA, LOD was 5 ng/mL and LLOQ was 10 ng/mL; in case of EPL and EPLT, LODs were 0.25 ng/mL and 0.80 ng/mL, respectively whereas LLOQ was 1 ng/mL for both EPL and EPLT. Lastly for the method of NSD and M1, LOD was 3 ng/mL and LLOQ was 10 ng/mL.

4.11 Pre-validation study of bioanalytical methods

A pre-validation study was carried out before starting the formal or final validation study or process, because it was necessary to establish that all procedures as set up capitulate the predicted outcomes.

4.11.1 System suitability test (SST)

System suitability test (SST) is a practice to show whether the instrument in use is working properly or not i.e., the system is ok for sample run. Also this test is more precisely applied for the chromatographic methods to ensure that the system is adequately sensitive, specific and reproducible for the ongoing analytical run throughout its use during a particular study. System suitability samples were run at the beginning of each batch of samples, sometimes it could be included at the middle and end of the batch. The concentration of the system suitability samples for the method of ASA and SA were 100 ng/mL and 500 ng/mL, respectively. For EPL and EPLT, it was 500 ng/mL and also 500 ng/mL for NSD and M1. Relative standard deviation (RSD) percentage of analyte peak area and retention time for six consecutive injections were checked to see whether they were below 2% and 5%, respectively or not (i.e., the acceptance criterion was not more than 2% for RSD). For checking the suitability of the systems with the samples were well within the acceptable % RSD.

4.11.2 Reproducibility test

Reproducibility refers to the variation in measurements made on a subject under changing conditions i.e., to the degree of agreement between the results of experiments carried out by different individuals, at different locations, using different instruments. It measures the ability to replicate the findings of others, that means whether an entire study or experiment can be

reproduced in its entirety. Reproducibility of the method assayed quality controls satisfies existing acceptance criteria. Therefore, the reproducibility of the method was assessed according to the precision value i.e., the coefficient of variance (CV%) of the area ratio of analyte and IS for replicate assays with respective concentrations and also the low CV% value establish the reproducibility of the method to be satisfactory and acceptable.

In the reproducibility test for the method of ASA and SA, 270 µL of blank plasma was taken and 60 µL of mixed solution of drug, metabolite and IS were spiked in two concentrations from 100 ng/mL (resulting or final concentration 10 ng/mL in plasma) and 1000 ng/mL (resulting or final concentration 100 ng/mL in plasma) solutions, respectively. For the method of EPL and EPLT, 200 µL of blank plasma was taken and 100 µL of mixed solution of drug, metabolite and IS were spiked in two concentrations from 2.5 µg/mL (resulting or final concentration 250 ng/mL in plasma) and 5 µg/mL (resulting or final concentration 500 ng/mL in plasma) solutions, respectively. In case of NSD and M1, 90 µL of blank plasma was taken and 20 µL of of mixed solution of drug, metabolite and IS were spiked in two concentrations from 1 µg/mL (resulting or final concentration 100 ng/mL in plasma) and 10 µg/mL (resulting or final concentration 1000 ng/mL in plasma) solutions, respectively. Three samples of each concentration were prepared in all cases.

4.11.3 Construction of standard curve or calibration curve for the bioanalytical methods

During pre-validation process, range of the standard curve or calibration curve must be determined, because, the first parameter was the range of the calibration standards.

4.11.3.1 Construction of standard or calibration curve for LC-MS/MS method of ASA and SA

According to Bae *et al*., (2008), the maximum plasma concentration i.e., C_{max} of both ASA and SA were found 170 (±96.7) ng/mL and 3780 (±865) ng/mL, respectively in a pharmacokinetic study which was accomplished after administration of 100 mg dose in healthy human subjects. Now to the best of our knowledge, no pharmacokinetic study or data have been reported in the literature till date after oral administration of 150 mg film coated tablet. The purpose of a bioanalytical method is to be competent to analysis plasma samples over a period of about 2 to 3 hours half-lives after dosing in pharmacokinetic studies. So because of the C_{max} were about 170 ng/mL and 3780 ng/mL, the quantitative method should have an LLOQ in the region of near about 10 to 15 ng/mL. Consequently during the method

development process, the LLOQ observed for samples assayed at 10 ng/mL. It was confirmed and selected 15000 ng/mL as the highest calibration standard and hypothesized that plasma sample will not have any concentration beyond that higher level. Thus, it was determined to prepare calibration standards ranging from 10 ng/mL to 15000 ng/mL for both ASA and SA.

4.11.3.2 Construction of standard or calibration curve for LC-MS/MS method of EPL and EPLT

According to Lu *et al*., (2009), the maximum plasma concentration (C_{max}) of EPL was 90.5 (±28.4) ng/mL and EPLT was 47.5 (±12.4) ng/mL, respectively in a pharmacokinetic study which was carried out after administration of 10 mg dose in healthy male volunteers. The purpose of a bioanalytical method is to be competent to analysis plasma samples over a period of about 1.3 to 2 hours half-lives after dosing in pharmacokinetic studies. So therefore if the C_{max} of EPL and EPLT around 250 ng/mL and 120 ng/mL are expected for 20 mg single oral dose of EPL maleate tablet, then the quantitative method should have an LLOQ in the region of near about 5 to 10 ng/mL. Now during the method development process, the LLOQ observed for samples assayed at 1 ng/mL which was found more sensitive than the prediction. It was confirmed and selected 500 ng/mL as the highest calibration standard and hypothesized that plasma sample will not have any concentration beyond that higher level. Thus, it was determined to prepare calibration standards ranging from 1 ng/mL to 500 ng/mL for both EPL and EPLT.

4.11.3.3 Construction of standard or calibration curve for LC-MS/MS method of NSD and M1

According to some literatures (Bernareggi. 1993 and 1998; Giachetti and Tenconi. 1998), the maximum plasma concentration (C_{max}) of both NSD and M1 were found 2.86 to 4.58 mg/L and 0.8 to 2.3 µg/mL for 100 mg dose of NSD. Therefore, it was expected maximum concentration (C_{max}) of NSD and M1 found as per literature. The calibration or standard curve was developed based on the C_{max} value. The purpose of a bioanalytical method is to be competent to analysis plasma samples over a period of about 2 to 4 hours half-lives after dosing in pharmacokinetic studies. Accordingly if the Cmax of NSD and M1 around 2800 ng/mL and 1190 ng/mL are expected for 100 mg single oral dose of NSD tablet, subsequently the quantitative method should have an LLOQ in the region of near about 10 to 15 ng/mL. The LLOQ observed for samples assayed at 10 ng/mL during the method development process. It was confirmed and selected 6000 ng/mL as the highest calibration standard and

hypothesized that plasma sample will not have any concentration beyond that higher level. Therefore, it was determined to prepare calibration standards ranging from 10 ng/mL to 6000 ng/mL for both NSD and M1.

4.11.4 Quality control (QC) standards

The aim of quality control (QC) samples or standards is to determine or evaluate the performance of the assay procedure and also it must cover the entire range of calibration points. The following QC samples were considered for LC-MS/MS method validation :

- Low quality control (LQC), i.e., three times of the LLOQ (lower limit of quantification)
- Medium quality control (MQC), i.e., 40 to 50% or near about of the highest calibration point
- High quality control (HQC), i.e., 75 to 80% or near about of the highest calibration point

All the QC samples were selected such a way that those samples were not included into the calibration points. For the method of ASA and SA, LQC, MQC and HQC were 30 ng/mL, 7500 ng/mL and 12000 ng/mL, respectively. For the method of EPL and EPLT, LQC, MQC and HQC were 3 ng/mL, 200 ng/mL and 400 ng/mL, respectively. For the method of NSD and M1, LQC, MQC and HQC were 30 ng/mL, 2500 ng/mL and 5000 ng/mL, respectively.

4.11.5 Preparation of working stock solutions of ASA and SA for calibration concentration (CC) and quality control (QC) standard

In order to prepare stock solutions i.e., 1.0 mg/mL of ASA, SA and HCTZ (IS), 10 mg of both drug and metabolite were dissolved in 10 mL of acetonitrile separately and IS was dissolved in methanol. These solutions were diluted with the mobile phase to obtain the stock solution (i.e., intermediate concentration) of 200 µg/mL for both ASA and SA and 10 µg/mL for HCTZ (IS). This intermediate concentration or solution was further diluted with the mobile phase to obtain working standard solutions for calibration and also IS was diluted to prepared the final concentration before spiking in the plasma.

4.11.5.1 Preparation of intermediate concentration

Firstly, 2 mL of each ASA and SA were taken from 1 mg/mL (i.e., 1000 µg/mL) stock solutions and then 6 mL of diluent i.e., the mobile phase was added to get the concentration of 200 µg/mL (see Table.4.1).

Table.4.1 Preparation of intermediate concentration

Stock Conc. (µg /mL)	Stock Aliquot (mL)	Diluent Added (mL)	Final Volume (mL)	Final Conc. (µg/mL)
1000	4	6	10	200

4.11.5.2 Preparation of stock dilutions for standard or calibration curve

Stock dilutions were prepared for both ASA and SA in the concentration of 150 µg/mL to 0.1 µg/mL using intermediate starting concentration 200 µg/mL and further serial dilutions (see Table.4.2).

4.11.5.3 Preparation of stock dilutions for quality control (QC) samples

Stock dilutions for QC samples were prepared for both ASA and SA in a concentration using diluents as described in the Table.4.3.

4.11.5.4 Drug spiking in blank human plasma for standard or calibration curve

Eight non-zero calibration standards with concentration ranging from 10 to 15000 ng/mL i.e., standard or calibration curves were prepared by spiking 30 µL of each of the corresponding concentrations of the stock dilutions (see Table.4.2) of ASA and SA and also 30 µL of IS solution (from 4 µg/mL of HCTZ) into 270 µL blank human plasma in separate 15 mL centrifuge tubes (i.e., polypropylene-capped tubes) to achieve calibration concentration (CC) points as described in the Table.4.4. Calibration curves of both ASA and SA have shown correlation coefficients (r^2) of 0.99 or better. These calibration curves were plotted with peak area ratio of the analytes (i.e., drug and metabolite) and IS on Y – axis and concentration on X- axis.

Table.4.2 Preparation of ASA and SA stock dilutions

Stock Conc. (µg/mL)	Stock Volume Taken (mL)	Solvent/Diluent Added (mL)	Final Volume (mL)	Final Conc. (µg/mL)
200	3	1	4	150
150	2	1	3	100
100	2	2	4	50
50	1	4	5	10
10	2	2	4	5
5	1	4	5	1
1	2	2	4	0.5
0.5	1	4	5	0.1

Table.4.3 Preparation of quality control (QC) stock dilutions

Stock Conc. (µg/mL)	Stock Aliquot (mL)	Diluent Added (mL)	Final Volume (mL)	Final Conc. (µg/mL)
1	3	7	10	0.3
100	3	1	4	75
150	4	1	5	120

Table.4.4 Preparation of ASA and SA standard or calibration concentrations (CC) in spiked plasma

Stock Concentrations (or Related Raw Concentrations) (µg/mL)	Final Concentrations in Spiked Plasma (µg/mL)	Spiked Plasma Concentrations ID
0.1	0.01	CC1
0.5	0.05	CC2
1.0	0.1	CC3
5.0	0.5	CC4
10.0	1.0	CC5
50	5.0	CC6
100	10.0	CC7
150	15.0	CC8

4.11.5.5 Spiking of plasma for quality control (QC) samples

Quality control (QC) samples were prepared by spiking 30 µL of each of the corresponding concentrations of the stock dilutions (see Table.4.3) of ASA and SA and also 30 µL of IS solution (from 4 µg/mL of HCTZ) into 270 µL blank human plasma plasma in separate 15 mL centrifuge tubes (i.e., polypropylene-capped tubes) to achieve LQC, MQC and HQC, respectively as described in the Table.4.5.

Internal standard (IS) i.e., hydrochlorothiazide (HCTZ) was spiked from 4 µg/mL of working solution and so the final concentration in plasma was 400 ng/mL. All stock solutions (i.e., 1mg/mL of the analytes and IS) and QC solutions were stored at −20 °C and working standard solutions were stored at 2 to 8 °C for 30 days. The complete study was conducted in accordance with USFDA and EMA guidelines.

Table.4.5 Preparation of quality control (QC) concentrations in spiked plasma

Stock Concentrations (or Related Raw Concentrations) (µg/mL)	Final Concentrations in Spiked Plasma (µg/mL)	Spiked Plasma Concentrations ID
0.3	0.03	LQC
75	7.5	MQC
120	12.0	HQC

4.11.6 Preparation of working stock solutions of EPL and EPLT for calibration concentration (CC) and quality control (QC) standard

In order to prepare stock solutions, 10 mg of EPL, EPLT and TBM (IS) were dissolved in 10 mL of methanol separately to obtain concentration of 1 mg/mL. After that, these stock solutions were diluted with diluent i.e., methanol : water :: 50 : 50 (v/v) to obtain the intermediate concentration of 10µg/mL for both EPL and EPLT. This intermediate concentration or solution was further diluted with the diluent to obtain working standard solutions for calibration.

4.11.6.1 Preparation of solution of intermediate concentration

Initially, 0.1 mL (i.e., 100 µL) of each EPL and EPLT were taken from 1 mg/mL (i.e., 1000 µg/mL) stock solutions and then 9.8 mL of diluent i.e., methanol: water: 50:50 (v/v) was added to get the concentration of 10 µg/mL (see Table.4.6).

Table.4.6 Preparation of solution of intermediate concentration

Stock Conc. (µg /mL)	Stock Aliquot (mL)	Diluent Added (mL)	Final Volume (mL)	Final Conc. (µg/mL)
1000	0.2	9.8	10	10

4.11.6.2 Preparation of stock dilutions for standard or calibration curve

Stock dilutions were prepared for both EPL and EPLT in the concentration of 5 µg/mL to 0.01 µg/mL using intermediate starting concentration 10 µg/mL and further serial dilutions (see Table.4.7).

4.11.6.3 Preparation of stock dilutions for quality control (QC) samples

Stock dilutions for QC samples were prepared for both EPL and EPLT in a concentration using diluents as described in the Table.4.8.

Table.4.7 Preparation of EPL and EPLT stock dilutions

Stock Conc. (µg/mL)	Stock Volume Taken (mL)	Solvent/Diluent Added (mL)	Final Volume (mL)	Final Conc. (µg/mL)
10	2	2	4	5
5	2	2	4	2.5
2.5	2	3	5	1
1	2	2	4	0.5
0.5	2	2	4	0.25
0.25	2	3	5	0.1
0.1	2	2	4	0.05
0.05	1	4	5	0.01

Table.4.8 Preparation of quality control (QC) stock dilutions

Stock Conc. (µg/mL)	Stock Aliquot (mL)	Diluent Added (mL)	Final Volume (mL)	Final Conc. (µg/mL)
1	0.3	9.7	10	0.03
10	1	4	5	2
10	2	3	5	4

4.11.6.4 Drug spiking in blank human plasma for standard or calibration curve

Eight non-zero calibration standards with concentration ranging from 1 to 500 ng/mL i.e., standard or calibration curves were prepared by spiking 100 µL of each of the corresponding concentrations of the stock dilutions (see Table.4.7) of both EPL and EPLT into 200 µL of drug free human blank plasma in 2 mL eppendorf tubes (i.e., polypropylene-capped tubes) to achieve calibration concentration (CC) points as described in the Table.4.9. A calibration or standard curve was prepared also with a blank plasma sample i.e., plasma sample processed without drug and IS, blank plasma with IS and eight non-zero samples covering the concentration range with the LLOQ. Calibration curves of both EPL and EPLT have shown correlation coefficients (r^2) of 0.99 or better. These calibration curves were plotted with peak area ratio of the analytes (i.e., drug and metabolite) and IS on Y – axis and concentration on X- axis.

4.11.6.5 Spiking of plasma for quality control (QC) samples

Quality control (QC) samples were prepared by spiking 100 µL of each of the corresponding concentrations of the stock dilutions (see Table.4.8) of EPL and EPLT into 200 µL blank human plasma plasma in separate 2 mL eppendorf tubes (i.e., polypropylene-capped tubes) to achieve LQC, MQC and HQC, respectively as described in the Table.4.10.

Spiking concentration of internal standard i.e., tolbutamide (TBM) in plasma was 1 µg/mL and prepared in ice cold acetonitrile. All stock solutions (i.e., 1 mg/mL of the analytes and IS) and QC solutions were stored at -20 °C and working standard solutions were stored at 4 °C

for 30 days. The whole study was conducted in accordance with USFDA and EMA guidelines.

Table.4.9 Preparation of EPL and EPLT standard or calibration concentrations (CC) in spiked plasma

Stock Concentrations (or Related Raw Concentrations) (µg/mL)	**Final Concentrations in Spiked Plasma (ng/mL)**	**Spiked Plasma Concentrations ID**
0.01	1	CC1
0.05	5	CC2
0.1	10	CC3
0.25	25	CC4
0.5	50	CC5
1	100	CC6
2.5	250	CC7
5	500	CC8

Table.4.10 Preparation of quality control (QC) concentrations in spiked plasma

Stock Concentrations (or Related Raw Concentrations) (µg/mL)	**Final Concentrations in Spiked Plasma (ng/mL)**	**Spiked Plasma Concentrations ID**
0.03	3	LQC
2	200	MQC
4	400	HQC

4.11.7 Preparation of working stock solutions of NSD and M1 for calibration concentration (CC) and quality control (QC) standard

10 mg of each analyte and IS were dissolved in 10 mL of methanol to prepare stock solutions (1.0 mg/mL) of NSD, M1 and CXB (IS), respectively. These solutions were diluted further with the mobile phase, acetonitrile : water containing 5 mM ammonium formate (9:1 v/v), to obtain a stock solution of 100 µg/mL (intermediate concentration) for both NSD and M1 and 10 µg/mL for CXB (IS). These stock solutions were diluted properly with diluent (mobile phase) to achieve working standard solutions for calibration and also IS was diluted to prepared the final concentration before spiking in the plasma.

4.11.7.1 Preparation of solution of intermediate concentration

Firstly, 1 mL of each NSD and M1 were taken from 1 mg/mL (i.e., 1000 µg/mL) stock solutions and then 8 mL of diluent i.e., the mobile phase was added to get the concentration of 100 µg/mL (see Table.4.11).

Table.4.11 Preparation of intermediate concentration

Stock Conc. (µg /mL)	Stock Aliquot (mL)	Diluent Added (mL)	Final Volume (mL)	Final Conc. (µg/mL)
1000	2	8	10	100

4.11.7.2 Preparation of stock dilutions for standard or calibration curve

Stock dilutions were prepared for both NSD and M1 in the concentration of 60 µg/mL to 0.1 µg/mL using intermediate starting concentration 100 µg/mL and further serial dilutions (see Table.4.12).

4.11.7.3 Preparation of stock dilutions for quality control (QC) samples

Stock dilutions for QC samples were prepared for both NSD and M1 in a concentration using diluents as described in the Table.4.13.

Table.4.12 Preparation of NSD and M1 stock dilutions

Stock Conc. (µg/mL)	Stock Volume Taken (mL)	Solvent/Diluent Added (mL)	Final Volume (mL)	Final Conc. (µg/mL)
100	3	2	5	60
60	2	1	3	40
40	2	2	4	20
20	2	2	4	10
10	2	2	4	5
5	1	4	5	1
1	2	2	4	0.5
0.5	1	4	5	0.1

Table.4.13 Preparation of quality control (QC) stock dilutions

Stock Conc. (µg/mL)	Stock Aliquot (mL)	Diluent Added (mL)	Final Volume (mL)	Final Conc. (µg/mL)
1	3	7	10	0.3
100	1	3	4	25
100	2	2	4	50

4.11.7.4 Drug spiking in blank human plasma for standard or calibration curve

There were eight non-zero calibration standards, ranging in concentration from 10 to 6000 ng/mL for both NSD and M1 were prepared by adding 10 µL of each of the corresponding concentrations or the working solutions of the stock dilutions (see Table.4.12) and 10 µL of IS solution (from 50 µg/mL of CXB) into 90 µL of blank (drug free) human plasma in

separate 15 mL centrifuge tubes (i.e., polypropylene-capped tubes) to achieve calibration concentration (CC) points as described in the Table.4.14. A calibration or standard curve was prepared also with a blank plasma sample i.e., plasma sample processed without drug and IS, blank plasma with IS and eight non-zero samples covering the analytes concentration range with the LLOQ. The mean correlation coefficient of calibration curves were (r^2) of 0.9992 for NSD and 0.9993 for M1. These calibration curves were plotted with peak area ratio of analytes (i.e., drug and metabolite) and IS on Y - axis and concentration on X - axis.

Table.4.14 Preparation of NSD and M1 standard or calibration concentrations (CC) in spiked plasma

Stock Concentrations (or Related Raw Concentrations) (µg/mL)	**Final Concentrations in Spiked Plasma (µg/mL)**	**Spiked Plasma Concentrations ID**
0.1	0.01	CC1
0.5	0.05	CC2
1	0.1	CC3
5	0.5	CC4
10	1	CC5
20	2	CC6
40	4	CC7
60	6	CC8

4.11.7.5 Spiking of plasma for quality control (QC) samples

Quality control (QC) samples were prepared by spiking 10 µL of each of the corresponding concentrations or the working solutions of the above mentioned stock dilutions (see Table.4.13) of NSD and M1 into 90 µL blank human plasma plasma in separate 15 mL centrifuge tubes (i.e., polypropylene-capped tubes) to obtain LQC, MQC and HQC, respectively as described in the Table.4.15.

Table.4.15 Preparation of quality control (QC) concentrations in spiked plasma

Stock Concentrations (or Related Raw Concentrations) (µg/mL)	Final Concentrations in Spiked Plasma (µg/mL)	Spiked Plasma Concentrations ID
0.3	0.03	LQC
25	2.5	MQC
50	5	HQC

Internal standard (IS) i.e., celecoxib (CXB) was spiked from 50 µg/mL of working solution and so the final concentration in plasma was 5 µg/mL. All stock solutions (i.e., 1 mg/mL of the analytes and IS) and QC solutions were stored at −20 ºC and working standard solutions were stored at 2 to 8 ºC for one month. The entire study was conducted in accordance with USFDA and EMA guidelines.

4.11.8 Collection of blank plasma used for bioanalytical LC-MS/MS methods

For the method development, pre-validation and finally validation process, human blank plasma was collected from healthy human volunteers participating in bioequivalence study. The blank plasma was obtained from each and every human volunteers in 5 mL RIA vials during study and then it was pooled into one 50 mL polypropylene centrifuge tube. The relatively clot–free plasma was stored at −20 ºC after removal of clotted fibrin by centrifugation. These blank pooled plasma samples were collected from Clinical Pharmacology Unit (CPU) of Bioequivalence Study Center, Jadavpur University, Kolkata and TAAB Biostudy Services, Jadavpur, Kolkata and was stored at -20 °C until use.

4.12 Validation of developed bioanalytical LC-MS/MS methods

Method validation is a process that used to ensure and establish the developed bioanalytical method is convenient for its proposed objective that contributes reliable and valid data for a particular analyte(s). The outcomes of the pre-validation determined that the formal or final method validation could be carried out. The validation was performed by the fundamental parameters to satisfy the acceptance criteria according to United States Food and Drug

Administration (USFDA, 2001), European Medicines Agency (EMA, 2011) and International Conference on Harmonization (ICH, 1996) guidelines.

4.12.1 Specificity and selectivity

This was performed by determining the blank plasma samples from six different sources to detect for any chromatographic interference at the retention times of the analytes (both drug and metabolite) and IS. A minimum of six matrix i.e., plasma lots were used and screened that which one was heamolytic and which one was lipemic. These lots were spiked with known concentration of analytes along with IS at low, medium and high concentrations. From each lot of matrix, a blank and lower limit of quantification (LLOQ) sample was spiked and processed according to the method procedure. The processed samples were injected and analyzed. The interference in the blank matrix i.e., plasma was evaluated by comparing the response at the retention time of analytes and IS against the response of analytes and IS in the extracted LLOQ samples. Generally, this was performed to find out or to confirm lack of interferences and absence of several variations of the present bioanalytical methods. Now, response of the interfering peak at the retention time of analytes should be ≤ 20% of mean response of analytes in LLOQ concentration. For internal standard (IS), the response of the interfering peak at the retention time of IS should be ≤ 5% of mean response of IS in LLOQ sample.

4.12.2 Sensitivity

Limit of detections (LODs) and lower limit of quantifications (LLOQs) of the present bioanalytical methods were satisfactory as per the bioanalytical guidelines (USFDA, 2001 and EMA, 2011). LOD was found with detector response two to three times greater than the blank plasma i.e., the signal to noise (S/N) ratio should be 2:1 or 3:1, whereas in case of LLOQ it was found five times greater than the blank plasma i.e., the S/N ratio should be 5:1. The analytes (both drug and metabolite) response or peaks in LLOQ samples were found identifiable, reproducible and discrete and within the acceptance criteria i.e., accuracy should be 100 ± 20% or within 80 to 120% with precision i.e., coefficient of variance (%CV) or RSD (relative standard deviation) less than 20% (≤ 20%).

4.12.3 Linearity and standard or calibration curve

A series of calibration standards, eight-point standard or calibration curves were prepared by spiking appropriate amounts of working solutions i.e., by adding known concentrations of the

analytes (such as ASA and SA; EPL and EPLT; NSD and M1) with the internal standards (such as HCTZ, TBM and CXB) into the blank plasma (drug free) to obtain the final concentrations (see Table.4.4, Table.4.9 and Table.4.14). The standard or calibration curves were prepared by plotting the peak area ratio of the analytes (both drug and metabolite) to that of IS against the nominal concentrations of the calibration standards. The results were found better and fitted to linear regression analysis by the equation, $y = mx + c$ and the values of correlation coefficient or regression coefficient (r or r^2). 75% of the calibration standards were within the acceptance range including the lowest and highest calibration standard as per guidelines. The acceptance criterion for each back-calculated standard concentration was within ± 15% standard deviation (SD) compare to the nominal value, except for LLOQ, which was within ± 20% of the nominal value (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

4.12.4 Precision and accuracy

Intra-day or intra-assay i.e., within run or within day precision was assessed by analyzing six replicates (n = 6) of each LLOQ, LQC, MQC and HQC samples on same day or within same day. While in case of inter-day or inter-assay i.e., between run or between days precision, reproducibility (day-to-day variation) of the method was validated using six replicates (n = 6) of each LLOQ, LQC, MQC and HQC samples on three different or separate days. Each batch comprised of one set of calibration standards (n = 1) and six replicates of both LLOQ and quality control (QC) samples at low, medium and high concentrations (LQC, MQC and HQC), respectively. Intra and inter-day assay precision were measured as percentage (%) of the coefficient of variation (%CV) or RSD (relative standard deviation), i.e., the ratios of standard deviation (SD) to the mean and expressed as percentage.

$$\text{Precision (\% CV) or RSD} = \frac{\text{Standard deviation (SD)}}{\text{Mean}} \times 100$$

Intra-day and inter-day assay accuracy was evaluated by analysing six replicates (n = 6) of each LLOQ and at three QC levels, i.e., LQC, MQC and HQC on the same day or within same day and on three different or separate days respectively. The accuracy (i.e. % nominal) or absolute percent bias (%) was calculated by the ratio of estimated concentration and actual concentration multiplied by 100.

$$\text{Accuracy (\%) or \% Nominal} = \frac{\text{Mean concentration}}{\text{Actual concentration}} \times 100$$

The acceptance criteria of the data were included accuracy within ± 15% standard deviation (SD) from the nominal values and precision of within ± 15% relative standard deviation (RSD), i.e., all the standard or calibration curve points, LQC, MQC and HQC samples were within ± 15% of the specified concentrations except for LLOQ, where it should not exceed ± 20% of standard deviation (SD) (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

4.12.5 Extraction recovery

The extraction recovery of the analytes (i.e., ASA and SA; EPL and EPLT; NSD and M1) from the plasma was determined by comparing the mean detector responses of six replicates of processed QC samples at low, medium, and high concentrations (LQC, MQC and HQC) to the detector responses of standard solutions of same concentrations. As a result, recovery experiments were carried out by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery. The percentage recoveries were calculated by measuring the peak areas of the analytes (both drug and metabolite) from the prepared plasma at six replicates of LQC, MQC, and HQC samples. The absolute peak area of the unextracted standards containing the same concentrations of both the drug and the metabolite was compared to the peak areas of these QC Samples.

$$\text{Rrecovery (\%)} = \frac{\text{Mean area of extracted analyte spiked into plasma}}{\text{Mean area of unextracted analyte of aqueous standard}} \times 100$$

$$\text{Total Mean Recovery (\%)} = \frac{\text{\% Recovery of (LQC+MQC+HQC)}}{3} \times 100$$

Therefore, six replicates of extracted LQC, MQC and HQC are prepared according to the validation procedure. Freshly prepared aqueous QC samples based on recovery along with

the extracted QC samples were injected. Now, the mean peak area response of the extracted samples were compared with the mean peak area response of the aqueous samples. The mean of percentage (%) recovery, standard deviation (SD) and %CV were calculated for the analytes at each level and also IS were calculated. As per the guidelines percentage (%) recovery should not be more than 115% and %CV of area at three different concentrations i.e., LQC, MQC and HQC should be less than 15%.

4.12.6 Evaluation of matrix effect

Matrix effect study was performed to ensure the suppression or enhancement of ionization of analytes (ASA and SA; EPL and EPLT; NSD and M1) by the presence of matrix i.e., plasma components in the biological samples. Six different lots of plasma i.e, six different source of human plasma were processed (without spiking analytes and IS) according to the extraction procedure and before reconstitution step (specially for the LLE), the aqueous LQC, MQC and HQC samples (n = 6) were spiked with IS along with one set of calibration standards (n = 1) and analyzed.

$$\% \text{ of Matrix Effect (ME\%)} = \frac{\text{Extracted area value of post spiked analyte}}{\text{Mean area of unextracted analyte of aqueous standard}} \text{ X } 100$$

Whereas, matrix factor (MF) was calculated by the following formula :

$$\text{Matrix Factor (MF)} = \frac{\text{Peak response in presence of matrix ions}}{\text{Peak response in the absence of matrix ions}}$$

The aqueous samples were prepared as per recovery for both analytes (drug and metabolite) and IS at LQC, MQC and HQC levels and injected along with the post spiked samples and analyzed to assess the effect of matrix with the analytes or the IS. Matrix effect for both the analytes and IS should be within ± 15%, i.e., within 85 to 115% and 75% of lots used should pass the matrix effect (Matuszewski *et al*., 2003; United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

4.12.7 Stability study

Stability study or experiments was performed according to the method validation procedures or guidelines (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011). The obtained values were compared to the nominal values and at each level, the mean value should be within ± 15% of the nominal value. Therefore, percentage (%) stability was determined by the following formula :

$$\text{Stability (\%)} = \frac{\text{Mean response of stability samples}}{\text{Mean response of comparison samples}} \text{ X } 100$$

To determine the degradation and decomposition of the analytes which may occur because of the various reasons, the following stability parameters were evaluated.

4.12.7.1 Stability of the stock solutions

The standard stock solutions or aqueous solutions containing 1 mg/mL of both drugs and metabolites, i.e., ASA and SA; EPL and EPLT; NSD and M1 and also 1 mg/mL of internal standards, i.e., HCTZ, TBM and CXB were prepared separately and stored at 2 to 8 °C for 30 days. The response obtained from the three drugs and their active metabolites were calculated and compared with that of the freshly prepared solutions of the same concentration with an acceptable limit of ± 2%. The study of stock solution stability was carried out during the validation process. The stock solution was divided into three parts. The first part was stored at room temperature, the second part was stored at normal deep freezer and the third or last part was stored at −20 ºC deep freezer. The solutions of analytes and IS from each storage conditions were taken out at predetermined time intervals, such as 0, 12, 24, 48, 96, 192, 360 and 720 hrs (30 days). These solutions were diluted by diluent or mobile phase to 10 µg/mL and then futher diluted to 1 µg/mL and introduced into the LC-MS/MS system. The peak area of the analyte from each sample was compared with that of freshly prepared samples. Stock solution stability was calculated by the the following formula :

$$\text{Percentage (\%) Stability} = \frac{\text{Average area of n hrs.}}{\text{Average area of 0 hr.}} \text{ X } 100$$

Where ‘n’ = number of hours

4.12.7.2 Freeze and thaw stability

A standard or calibration curve (n = 1) was processed along with six replicates of stability samples at low, medium and high QC levels (LQC, MQC and HQC) (n = 6) which were subjected to three freeze thaw cycles. In first cycle samples were frozen at -20 °C for 24 hours, then it was thawed to room temperature until it gets completely thawed. The second cycle and the subsequent cycles were further frozen at -20 °C for minimum 12 hours to 24 hours and thawed to room temperature. After the 3rd cycle the samples were analyzed in a single run. The mean concentration obtained for stability QC samples should be 85 to 115% of nominal concentration of freshly prepared QC samples and the % CV should be within ± 15%.

4.12.7.3 Bench top stability

In bench top stability study, the analytes (both drug and metabolite) and IS were spiked in the human blank plasma and left for 8 hours in room temperature (almost at 24 °C). After 8 hours these samples were processed and analyzed. The bench top was assessed at three concentrations of low, medium and high QC levels (LQC, MQC and HQC) in six replicates (n = 6) along with standard or calibration (n = 1) samples. The acquired mean value for the QC samples should be within 85 to 115% of nominal value of freshly prepared QC samples and also the % CV should be within the acceptance criteria, i.e., ± 15%.

4.12.7.4 Post-preparative (autosampler) stability

Post-preparative stability was on-instrument or autosampler stability of the processed sample at injector or autosampler temperature. Each QC sample at low, medium and high concentrations (LQC, MQC and HQC) were processed in six replicates and placed the samples after extraction in the autosampler cabinet at 10 to 15 °C for 24 hours. After that the samples were analyzed and the obtained concentrations were compared with the actual values. The prepared QC samples were injected along with the calibration standards and another batch of freshly extracted QC samples after this stability period. All the QC sample concentrations were back calculated using the calibration curve. The obtained results were within the acceptable limit 85 to 115% and % CV or RSD was within ±15% of the actual value.

4.12.7.5 Dry state stability

Six replicates of each QC level at low, medium and high concentrations (LQC, MQC and HQC) were prepared and stored at -20°C in dry state i.e., without reconstitution after extraction. This dry state stability was performed for ASA with SA and NSD with M1, because these analytes were extracted by liquid-liquid extraction (LLE) procedure. But in case of EPL with EPLT, the extraction was protein precipitation technique (PPT). The samples were analyzed after 24 hours and a deviation of ± 15% was in acceptable limit.

4.12.7.6 Short term stability

Short term stability was performed by analyzing at three QC levels, i.e., LQC, MQC and HQC samples in six replicates (n = 6) stored or kept in deep freezer at -20 °C ± 2 °C for 7 days. After 7 days, all samples were retrieved from the deep freezer (-20 °C) and thawed at room temperature. Then the samples were processed and analysed. The obtained concentrations were compared with the actual values of QC samples and also with the freshly prepared samples of three QC levels (LQC, MQC and HQC) in six replicates. The samples were assessed to be stable if the results were within the acceptable limits i.e., accuracy ± 15% SD and precision (% CV) ± 15% RSD of the actual value (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

4.12.7.7 Long term stability

In long term stability study, spiked plasma samples at three QC levels, i.e, LQC, MQC and HQC in six replicates (n = 6) were stored or kept in deep freezer at -20 °C ± 2 °C for 30 days. After 30 days, all spiked plasma samples were retrieved from the deep freezer (-20 °C) and thawed at room temperature. Then the samples were processed and analysed. The acquired concentrations were compared with the actual values of QC samples and the freshly prepared samples of three QC levels (LQC, MQC and HQC) in six replicates. The samples were evaluated and considered to be stable if the assay values were within the acceptable limits of accuracy i.e., ± 15% SD and precision (% CV) i.e., ± 15% RSD of the actual value (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

4.12.7.8 Dilution integrity

The study of dilution integrity was performed to confirm that the final concentration did not affect or influenced by the samples which were diluted with blank matrix. However, dilution

integrity was achieved by preparing the concentration four times of ULOQ i.e., upper limit of quantification, which was further diluted five and ten times (5 fold and 10 fold) with drug free plasma in six replicates (n = 6). The five and ten times diluted sample were run against calibration standards and accuracy and precision were evaluated. Now, dilute samples should be within the acceptable limits of accuracy i.e., ± 15% SD and precision (% CV) i.e., ± 15% RSD.

4.13 Application to bioequivalence study and assessment of pharmacokinetic parameters

The validated bioanalytical methods were successfully applied to determine the concentrations as well as pharmacokinetic parameters of ASA and SA; EPL and EPLT; NSD and M1 in human plasma obtained after conducting bioequivalence studies to compare the bioequivalence (comparative bioavailability) of two preparations, i.e., test and reference in healthy human subjects.

4.13.1 Ethical considerations and consent procedure

The clinical study i.e., the bioequivalence study was started after the receipt of the study written consent of the Bio-Ethics Committee of Bioequivalence Study Centre, Jadavpur University, Kolkata, India, HURIP Independent Bioethics Committee of TAAB Biostudy Services, Jadavpur, Kolkata, India, certificate of analysis (CoA) and approval from the Drugs Controller General of India (DCGI) with Central Drugs Standard Control Organization (CDSCO), New Delhi, India. The protocol and related materials were approved by this Bio-Ethics Committee (Reg. No. ECR/93/Indt/WB/2013) and HURIP Independent Bioethics Committee (Reg. No. ECR/103/Indt/WB/2013) in order to conduct BA/BE study in human subjects. The protocol, informed consent form and other material were submitted to the Institutional Ethics Committee (IEC) or Institutional Independent Ethics Committee (IIEC) prior to the initiation of the study. The approval of the IEC was taken prior to the commencement of the study. Volunteers who signed the written informed consent form for the participation in the study were allowed to the screening trial phase. The clinical study (BE study) was carried out under ICH-GCP [Technical code of common practice (TCCP) 184-2009 (02040) "Good Clinical Practice"] and World Medical Association Declaration of Helsinki (Tokyo, 2004 and Brazil, 2013).

4.13.2 Informed consent

Prior to screening activity, the screening consent was obtained from each of the volunteers. Before recruitment and enrolment of the volunteers into the study, a detailed description or information to the volunteers about purpose of the study i.e., the objective, the procedures to be carried out i.e., methodology, study period, the potential hazards or possible risks, benefits and rights of the volunteer in a language that the subject understand. Ample time and opportunity were provided to volunteer for deciding to or not to participate in the study. The subjects were required to read and sign the informed consent form summarizing the discussion prior to enrolment.

4.13.3 Selection criteria of subject and restriction

4.13.3.1 Subjects

The study was included healthy adult human male subjects within age of 18 to 45 years, normal body mass index (BMI) was 18.5 to 24.99 kg/m^2 (± 20% deviation of BMI) and with a minimum body weight of 50 kg.

4.13.3.2 Subject screening tests

Medical histories and demographic data including initial, sex, age, race, weight (kg), height (cm), BMI (kg/m^2), occupation, alcohol consumption and smoking habits were recorded. Each subject was undergo a complete physical and clinical examination and also laboratory tests (different biochemical and haematological tests) of haematopoietic, hepatic and renal functions as mentioned later. Only medically healthy adult human subjects with clinically acceptable or clinically not significant laboratory profiles were enrolled into the study. The selected volunteers were screened (the subjects have to undergo physical and laboratory tests) for inclusion in the study, i.e., should fulfill or satisfy all the clauses of inclusion and none of the exclusion criteria within 21 days before the commencement of the study.

Following laboratory tests were performed during screening visit:

- Complete physical and clinical examination including personal and family history.
- Complete haemogram i.e., haematology such as haemoglobin, WBC count, neutrophils, lymphocytes, monocytes, eosinophils and basophils.

- Serum chemistry such as cholesterol, random blood sugar, blood urea and creatinine, sodium, potassium, uric acid, S. Proteins, virological tests like HIV Antibody and HBsAg, LFT i.e., Liver Function Tests such as alkaline phosphatase, SGPT, SGOT, total proteins, albumin, globulin and A/G ratio, total and direct bilirubin, RFT i.e., Renal Function Tests like BUN (blood urea nitrogen) and S. Creatinine.

4.13.3.3 Inclusion criteria

The following aspects were considered as basic eligibility criteria for the subjects to participate in the study –

- Healthy (eligible after physical, clinical, haematological and biochemical examinations) adult human male subjects within the age range of 18 to 45 years.
- Body weight not less than 50 kg (also not more than 130% of BMI) and subjects should be within limit of 15% of their ideal weight as of Life Insurance Company's table.
- Normal BMI should be within 18.5 to 24.99 kg/m^2.
- Willingness to provide written Informed Consent to participate in the study.
- Normal blood pressure (BP) and heart rate as measured after resting supine for three minutes. Normal BP is taken to be 100 to 150 mm Hg systolic and 50 to 90 mm Hg diastolic supine. Normal heart rate is taken to be 50 to 90 beats per minute.
- Free of significant diseases or clinically significant abnormal findings during screening, medical history, physical examination, laboratory evaluations.
- Absence of disease markers of HIV 1 and 2, Hepatitis B and Syphilis.
- No subject should have received any medication (including over-the-counter products) for 14 days preceding the start of the study.
- For the last one month, no history of drug abuse (benzodiazepines and barbiturates) and for the last six months, no history of other illicit drug addiction.
- To be a non-smoker and non-alcoholic.
- The volunteer should be able to communicate well with the investigator.

- Availability of subject for the entire study period and willingness to adhere to protocol requirements (such as diet and drug consumption) as evidence by written informed consent.

4.13.3.4 Exclusion criteria

The following aspects were considered as basic criteria for the exclusion of subjects from the study –

- History or presence of significant glaucoma, cardiovascular, pulmonary, hepatic, renal, hematological, gastrointestinal, endocrine, immunologic, dermatologic, neurological, psychiatric disease, or any other conditions known to interfere with the Absorption, distribution, metabolism or excretion of common medications.

- History or presence of significant :

    - Alcohol dependence, alcohol abuse or drug abuse during past one year.

    - Asthma, chronic bronchitis or other bronchospastic condition, urticaria or other allergic type reactions (hypersensitivity) after taking aspirin or any other drug or related products.

    - Ulceration or a history of gastric and / or duodenal ulcers.

    - Jaundice in the past six months.

- Bleeding disorder.
- Any clinically significant illness during the 4 weeks prior to day 1 of the study.
- Maintenance therapy with any drug.
- Poor mental development or impaired cerebral function i.e., inability to communicate or cooperate with the investigator due to language problem.
- Allergy to the test drug, any medicine that is chemically similar to it, or to the excipients of the products under investigation.
- Participation in a clinical trial with an investigation drug within 30 days prior to day 1 of the study.
- HIV and Australian antigen positive subjects.
- Clinically relevant abnormal physical and / or clinical findings at the screening.
- 1 unit or 350 ml blood loss within 56 days or in the period 0 to 12 weeks prior to the

start of study (before entry to the study) or during the study.

- Subjects who have previously participated in another clinical study in the past 3 months prior to commencement of this study.
- Any difficulty in accessibility of forearm veins for cannulation or blood sampling.
- Refuse to abstain from food for at least 10 hrs prior to drug administration and for at least 4 prior to drug administration and for at least 4 hrs after drug administration in each period.
- Refuse to abstain from fluid for at least 1 hr prior to drug administration and until 1 hr after drug administration.
- Found positive in breath alcohol test on the day of the study.
- History of difficulty in swallowing tablet.
- Use of enzyme modifying drugs within 30 days prior to receiving the first dose of study medication.
- Hypersensitivity to any drug or other related class of drugs.

4.13.3.5 Criteria for subject withdrawal

Subjects would be free to withdraw at any time without stating any reason. The investigator may withdraw a subject from the study in any of the following conditions :

- During the course of the study, the subject suffers from significant intercurrent illness or undergoes surgery.
- The subject experiences serious adverse events (SAEs), when withdrawal would be in the best interest of the subjects.
- If the subject requires concomitant medications which may interfere with the pharmacokinetics of study drug.
- If the investigator thinks it was necessary to further protect the health of the subject or the integrity of the study.
- If subject vomits at or before the time equivalent to 2 median T_{max} after administration of investigational product.
- The subject fails to comply with the requirements of the protocol i.e., major violation of the protocol.

4.13.3.6 Prohibitions and concomitant medication

No subject should have received any medication (including over-the-counter products) for at least 14 days preceding the study. This prohibition includes cold preparations, antacid preparations and vitamins taken as nutritional supplements for non-therapeutic indication as considered by the investigator or doctor. If concomitant medication was required during the time of sample collection or during the washout period between drug administrations, a decision to continue or discontinue the subjects would be made based on the time the medication was administered and its pharmacology and pharmacokinetics by the investigator. No subject should have smoked at least 24 hours before the study and smoking was prohibited throughout the study period. No subject should have taken alcoholic beverages and any other stimulant drinks at least 48 hours before the study. No subject should have taken xanthine containing beverages and food or grape fruit juice at least 48 hours before the study and the same was prohibited throughout the study period. No strenuous exercises during the whole study period was permitted.

4.13.4 Study design

4.13.4.1 Study design for aspirin (ASA)

This was a single oral dose, open label, randomized, two period, two treatment and two-way cross over study in 30 healthy Indian male volunteers under fasting conditions with a washout period of 7 days between the two dosing sessions (i.e., Phase-I and Phase-II). Volunteers received either of the test or the reference preparation of acetylsalicylic acid 150 mg film coated tablets only on the study day at a fixed time in each dosing session.

4.13.4.2 Study design for enalapril (EPL)

This was a single oral dose, open label, randomized, two period, two treatment and two-way cross over study in 30 healthy Indian male volunteers under fasting conditions with a washout period of 14 days between the two dosing sessions (i.e., Phase-I and Phase-II). In each dosing session, volunteers received either of the test or the reference preparation of enalapril maleate 20 mg only on the study day at a fixed time.

4.13.4.3 Study design for nimesulide (NSD)

This was a single oral dose, open label, randomized, two period, two treatment and two-way cross over study 24 (total 30 with 6 backup volunteers) healthy Indian male volunteers under

fasting conditions with a washout period of 7 days between the two dosing sessions (i.e., Phase-I and Phase-II). Volunteers received either of the test or the reference preparation of nimesulide 100 mg only on the study day at a fixed time in each dosing session.

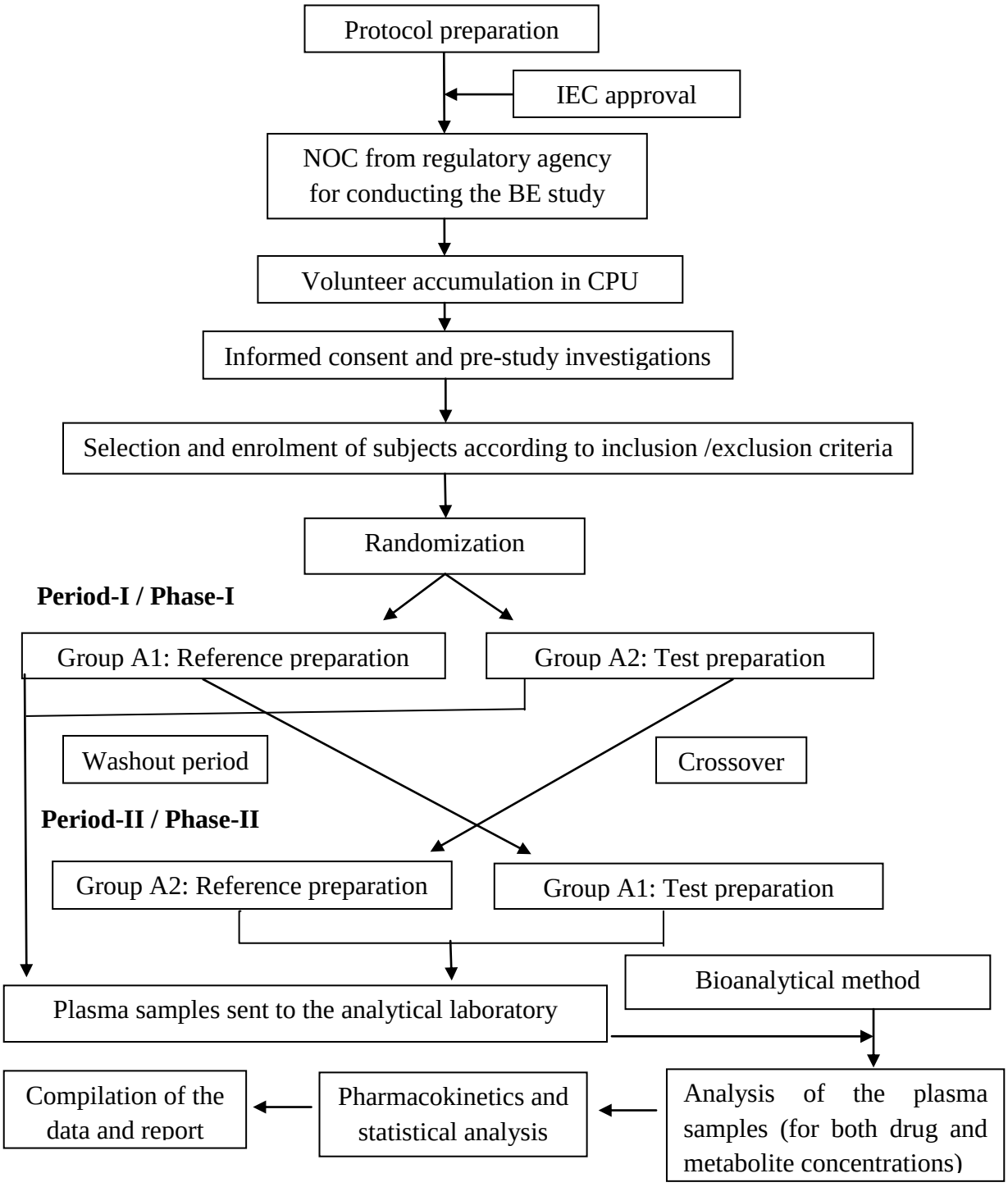


Figure.4.7 A flow chart of the bioequivalence study

Volunteers were given code numbers based on allocated treatment A / B (reference / test preparation) in accordance with the randomization code. The analyst was blind for that i.e.,

not known the sequence of administration. The investigator was the only person who knew the dosing method due to preparing the drugs for administration.

4.13.5 Product information

4.13.5.1 Reference and test preparation for aspirin (ASA)

Reference Preparation (A) :

CARDIOMAGNYL (Acetylsalicylic Acid 150 mg film coated tablet)

Mfg. by : NYCOMED DANMARK APS, DENMARK

Batch No. : 10926915

Mfg. Date : 07/2013

Exp. Date : 06/2018

Test Preparation (B) :

CARDIOMAGNUM (Acetylsalicylic Acid 150 mg film coated tablet)

Mfg. by : PHARMLAND JV LLC, OFFICE 3, 124 LENINSKAYA STR., 222603 NESVIZH, MINSK REGION, REPUBLIC OF BELARUS

Batch No. : 010213

Mfg. Date : 02/2013

Exp. Date : 02/2015

4.13.5.2 Reference and test preparation for enalapril (EPL)

Reference Preparation (A) :

RENITEC 20 mg tablet (containing Enalapril Maleate 20 mg)

Mfg. by : MERCK SHARP & DOHME B.V., THE NETHERLANDS

Batch No. : 334139

Mfg. Date : July, 2013

Exp. Date : January, 2016

Test Preparation (B) :

ENALAPRIL 20 mg tablet (containing Enalapril Maleate 20 mg)

Mfg. by : PHARMLAND JV LLC, OFFICE 3, 124 LENINSKAYA STR., 222603 NESVIZH, MINSK REGION, REPUBLIC OF BELARUS

Batch No. : 011214

Mfg. Date : December, 2014

Exp. Date : January, 2017

4.13.5.3 Reference and test preparation for nimesulide (NSD)

Reference Preparation (A) :

Tablet NISE (containing Nimesulide 100 mg)

Mfg. by : DR. REDDY'S LABORATORIES LTD., VILLAGE – MAUJA THANA, BADDI – 173 205 DIST. SOLAN, (HP), INDIA

Batch No. : E300161

Mfg. Date : April, 2013

Exp.Date : March, 2016

Test Preparation (B) :

Tablet containing Nimesulide 100 mg

Mfg. by : PHARMLAND JV LLC, OFFICE 3, 124 LENINSKAYA STR., 222603 NESVIZH, MINSK REGION, REPUBLIC OF BELARUS

Batch No. : NMS/12120948

Mfg. Date : December, 2012

Exp. Date : November, 2017

4.13.6 Dose regimen and mode of administration

4.13.6.1 For aspirin (ASA)

Administration of one tablet of either the test or the reference product containing acetylsalicylic acid 150 mg once daily before meal. The test or reference medicinal product was administered to each volunteer with 240 ± 2 mL of drinking water. The last meal was taken not later than 10 hours before the study initiation. The medicinal product administration was verified by the oral cavity examination. The volunteers were prohibited to lie down or to sleep for 2 hours after the medicinal product administration. The randomization was done for the volunteers on the previous day of study, i.e., Period-I or Phase-I. In Phase-I, each volunteer was received either the test preparation or the reference preparation as a single dose on the study at a fixed time. According to the randomization in Period-II or Phase-II, this order was reversed. The volunteers were allocated to the treatment A / B (reference / test) in accordance with the randomization code.

4.13.6.2 For enalapril (EPL)

The test or reference medicinal product was administered to the volunteers without chewing with 240 ± 2 mL of drinking water without sweeteners, caffeine or flavouring agents. The fact of administration of medicinal product was evidenced via oral cavity examination. The volunteers are prohibited to lie down or to sleep within 3 hours after medicine intake. During the first period i.e., in Phase-I, single-dose oral intake on an empty stomach of one tablet of investigational i.e., test medicinal product (B) Enalapril 20 mg tablets containing Enalapril Meleate 20 mg and one tablet of reference medicinal product (A) Renitec 20 mg tablets containing Enalapril Meleate 20 mg were administered. During the second period i.e., in Phase-II, the volunteers taken the test (investigational) medicinal product (B) take one tablet of reference medicinal product (A) Renitec 20 mg tablets containing Enalapril Meleate 20 mg. Whereas the volunteers taken reference medicinal product (A) take one tablet of test (investigational) medicinal product (B) Enalapril 20 mg tablets containing Enalapril Meleate 20 mg.

4.13.6.3 For nimesulide (NSD)

One tablet of either the test medicinal product or the reference medicinal product containing 100 mg of nimesulide was administered. The test or reference medicinal product was administered to each volunteer with 240 ± 2 mL of drinking water. The last meal was taken

not later than 10 hours before the study initiation. The medicinal product administration was verified by the oral cavity examination. The volunteers prohibited to sleep for 3 hours after the medicinal product administration. The volunteers were randomized on the earlier day of Period-I or Phase-I. In Phase-I, each volunteer was taken either the test preparation or the reference preparation as a single dose on the study at a fixed time. This sequence was reversed as per the randomization in Period-II or Phase-II. The volunteers were allocated to the treatment A / B (reference / test) in accordance with the randomization code.

4.13.7 Collection of blood and plasma separation

4.13.7.1 For aspirin (ASA)

All the volunteers were assembled in Clinical Pharmacology Unit (CPU) ward at 6.00 a.m. on the study day of each session (Phase-I or Phase-II), after overnight fasting of 10 hours. Their TPR i.e., temperature, pulse rate and respiration and blood pressure (BP) were recorded and an indwelling intravenous catheter or cannula was introduced with strict aseptic precautions in the suitable i.e., antecubital vein for blood collection. The volunteers were received either of the reference preparation (A) or the test preparation (B) according to their randomization code nos. A total of 20 blood samples were collected at 0 hour (before drug administration i.e., the first blood sample or pre-dose sample) and 0.167, 0.33, 0.5, 0.67, 0.83, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 12.0 and 24.0 hours (after drug administration) in the centrifuge tubes with EDTA-K3 anticoagulant at each time point. After drug ingestion, breakfast, lunch and dinner were provided after 4 hours, 8 hours and 14 hours respectively. On the study days, volunteers were allowed to engage in normal activities, with the exception of intense or strenuous exercise. The collected blood samples were centrifuged immediately, and the plasma was separated in polypropylene tubes and stored frozen i.e., in a deep freezer at –20 ^{o}C with appropriate labeling of volunteer code no., study date and collection or sampling time. The whole study was carried out with the cooperation of the principal investigator, doctors (i.e., clinical pharmacologists), pharmacists, nursing staffs, phlebotomists etc.

4.13.7.2 For enalapril (EPL)

All the volunteers assembled in CPU ward at 5.00 a.m. on the study day of each session (Phase-I or Phase-II), after overnight fasting of 10 hours. Their TPR (temperature, pusle rate and respiration), and BP (blood pressure) were recorded and an indwelling intravenous

catheter or cannula was introduced with strict aseptic precautions in the suitable vein (antecubital vein) for blood collection. The volunteers were received either of the reference preparation (A) or the test preparation (B) according to their randomization code nos. A total of 23 blood samples were collected at 0 hour (before drug administration or pre-dose sample) and 0.17, 0.33, 0.5, 0.75, 1.0, 1.42, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 8.0, 10.0, 12.0, 24.0, 36.0, 48.0 and 72.0 hours (after drug administration) in the centrifuge tubes containing EDTA-K3 anticoagulant at each time point. Lunch, snacks and dinner were provided after 4 hours, 8 hours and 14 hours respectively after drug ingestion. On the study days, volunteers were permitted normal activities, excluding intense or strenuous exercise. The collected blood samples were centrifuged immediately, and the plasma was separated in polypropylene tubes and stored frozen at –20 ^{o}C with proper labeling of volunteer code no., study date and collection or sampling time. The complete study was performed by the participation of the principal investigator, doctors (i.e., clinical pharmacologists), pharmacists, nursing staffs, phlebotomists etc.

4.13.7.3 For nimesulide (NSD)

All the volunteers assembled in CPU ward at 6.00 a.m. on the study day of each session (Phase-I or Phase-II), after overnight fasting of 10 hours. Their TPR (temperature, pusle rate and respiration), BP (blood pressure) were all recorded and an indwelling intravenous catheter or cannula was placed in the suitable vein (antecubital vein) for blood collection with strict aseptic precautions. The volunteers were received either of the reference preparation (A) or the test preparation (B) according to their randomization code nos. A total of 15 blood samples of each volunteer, were collected at 0 hour (before drug administration i.e., pre-dose sample) and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 12.0, 18.0 and 24.0 hours (after drug administration) in the centrifuge tubes containing EDTA-K3 anticoagulant at each time point. Lunch, Snacks and dinner were provided after 4 hours, 8 hours and 14 hours respectively after drug ingestion. Volunteers were allowed to engage in normal activities during the study days, with the exception of intense or strenuous exercise. The collected blood samples were immediately centrifuged, and the plasma was separated in polypropylene tubes and stored in a deep freezer at –20 ^{o}C with appropriate labeling of volunteer code no., study date and collection or sampling time. The entire study was performed with the cooperation of the principal investigator, doctors (i.e., clinical pharmacologists), pharmacists, nursing staffs, phlebotomists etc.

4.13.8 Adverse events

Abnormal signs or symptoms (adverse reactions) were monitored (if any) during the study period and for one week after the study period and if noticed, their details were included in the case report sheets and tabulated at the end of the study. Medical (doctor or clinical pharmacologist) and nursing personnel were supervised all critical phases of the study. The actual cause for such adverse reactions were determined and recorded for future investigations.

4.13.9 Emergency procedures

Emergency equipment and medicines were preserved and maintained in the CPU (Clinical Pharmacology Unit) for precautionary measures.

4.13.10 Sample preparation

The details of sample preparation are already described in section 4.9 (4.9.1 for ASA and SA; 4.9.2 for EPL and EPLT; 4.9.3 for NSD and M1).

4.13.11 Quantification of drugs and metabolites by LC-MS/MS

The concentration of both drug and metabolite in plasma samples were analyzed by using developed and validated LC-ESI-MS/MS method in the bioanalytical laboratory of Bioequivalence Study Centre, Jadavpur University. The bioanalytical methods were validated prior to the start of study (LC-MS/MS method details in Chapter 5). The blood plasma concentration of the volunteers for both test and reference preparation are tabulated in results and discussions section (Chapter 5).

4.13.12 Evaluation of pharmacokinetic parameters

The plasma levels of both drug and metabolite (ASA and SA; EPL and EPLT; NSD and M1) for every volunteer at each time point were plotted to obtain time-plasma concentration curves for the study preparations. Generally, this produced by the administration of the studied drugs (aspirin, enalapril maleate and nimesulide) in each volunteer and corrected for the measured content of the dosage form was used to establish the pharmacokinetic profile of test and reference preparations. The plasma drug level profile was presented in both tabular and graphical forms. The following variables i.e., the pharmacokinetic parameters of both test and reference preparations were determined for each subject.

- C_{max} [Maximum or peak plasma concentration]
- t_{max} [Time to maximum plasma concentration]
- $AUC_{(0-t)}$ [The area under plasma concentration time curve zero (0) hour to time (t)]
- $AUC_{(0-\infty)}$ [The area under plasma concentration time curve zero (0) hour to infinity (∞)]
- $t_{1/2}$ [Elimination half-life]
- K_{el} [Elimination rate constant]

The C_{max} and t_{max} were observed values. Kel was the elimination rate constant calculated by a linear least-squares regression analysis of the individual concentrations observed as a function of time during the elimination phase. The elimination $t_{1/2}$ was obtained using the following relationship :

$$t_{1/2} = \frac{0.693}{K_{el}}$$

4.13.13 Statistical analysis of pharmacokinetic parameters

All the pharmacokinetic parameters for drug and metabolite were calculated or determined by using descriptive statistics methods and registered thoroughly i.e., properly recorded. Standard descriptive analysis including the mean and standard deviation (SD) were used for variables like the height, weight and age. These statistical parameters including the coefficient of variance (CV) were also used to describe plasma concentrations at each individual time point as well as the pharmacokinetic parameters. The analysis of variance (ANOVA), double single-tail tests for the bioequivalence assessment, 90% confidential interval (CI) for ratios of T/R (test medicinal product/reference medicinal product) mean square deviation, strength and ratio were assessed in respect of the C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ untransformed and logarithmically transformed i.e., log-transformed or ln-transformed variables for the analytes (both drug and metabolite). The double single-tail tests for the bioequivalence assessment, 90% confidential interval (CI) for ratios of standard deviation T/R was calculated for the C_t [the concentration at time (t)], k_{el} and $t_{1/2}$ untransformed variables for the analytes.

Following statistical test were applied on untransformed [C_{max}, t_{max}, $AUC_{(o-t)}$, $AUC_{(o-\infty)}$] and log-transformed pharmacokinetic data [C_{max}, $AUC_{(0-t)}$, $AUC_{(0-\infty)}$]. The statistical evaluation was done by using WinNonlin version 5.3 (Pharsight Corporation, Mountain View, CA, USA) software. Plasma concentration-time data of each subject was determined by non-compartmental method.

- ANOVA of C_{max}, t_{max}, $AUC_{(0-t)}$, $AUC_{(0-\infty)}$ were subjected to a two-way ANOVA accounting for subjects, periods and treatment.
- For untransformed and log-transformed (ln-transformed) parameters, the 90% confidence interval (CI) consistent with a two-one sided t-test with a significance level of 5% [C_{max}, t_{max}, $AUC_{(o-t)}$, $AUC_{(o-\infty)}$].

Bioequivalence was considered and confirmed when the corresponding 90% confidential intervals (CI) for ratios of mean geometric values standard deviation of the mean values of the C_{max} and $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ log-transformed variables for the analytes were within 80% to 125%, i.e., within the acceptable range of Log (0.8) to Log (1.25). Relative bioavailability of the analytes in test preparation were calculated taking bioavailability of the analytes in reference preparation as 100%.

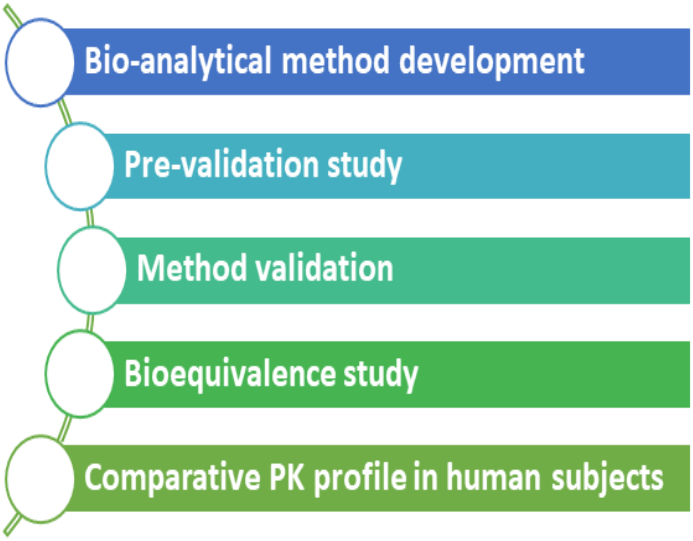
4.13.14 Quality control and quality assurance : Good Laboratory Practice and Good Clinical Practice

The entire clinical study was accomplished as per the regulations of DCGI (drugs controller general of India) guidelines for 'bioavailability and bioequivalence studies'- CDSCO, guidelines of good clinical research practice of ICH (international conference on harmonisation) and GCP (good clinical practice) guidelines by Latin square crossover design.

Chapter – 5:

Results and Discussions





SECTION–A

5.1 Development of method for acetylsalicylic acid (ASA) and salicylic acid (SA) by liquid chromatography tandem-mass spectrometry (LC-MS/MS)

5.1.1 Determination of precursor ion and product ion for ASA, SA (active metabolite) and IS (HCTZ)

During MS/MS development, it has been shown that the electrospray ionization (ESI) source provided better results as compared to the atmospheric chemical ionization (APCI) source for proper ionization of both the analytes, i.e., ASA and SA and IS (Hydrochlorothiazide, HCTZ). The precursor ion was determined in quadrupole 1 (Q1), i.e., QIMS scan and the product ion was determined in quadrupole 3 (Q3), i.e., MS2 scan, and measured by the mass-to-charge ratio (*m/z*). The transition pairs were monitored precisely and compared in both positive $[M + H]^+$ and negative mode or polarity $[M - H]^-$, i.e., protonated and deprotonated. It has been observed that more intense response in negative ion mode / polarity $[M - H]^-$ than positive $[M + H]^+$ for both analytes (ASA and SA) and IS (HCTZ).

At the time of mass scanning of the analytes and IS, the compound-dependent parameters were manually tuned, i.e., declustering potential (DP), focusing potential (FP) and entrance potential (EP) were tuned in Q1 scan i.e., Q1MI or multiple ions scan and collision energy (CE) and collision cell exit potential (CXP) were tuned in Q3 scan or in MS2 scan. Whereas, collision cell entrance potential (CEP) was setting up as by default in the software. Molar mass or molecular weight of ASA, SA and HCTZ (IS) were 180.157 g/mol, 138.12 g/mol and 297.74 g/mol, respectively. In multiple reaction monitoring (MRM) mode for ion transitions of all compounds deprotonated precursor $[M - H]^-$ to product ion spectra were observed at *m/z* 179.0 → 137.0 for ASA (Figure.5.1), *m/z* 137.0 → 93.0 for SA (Figure.5.2) and *m/z* 295.9 → 268.9 for HCTZ (IS) (Figure.5.3), respectively. The fragments or product ions were most abundant stable spectrum with better response as compared to others product ions. So, the following conditions were selected for optimization of the fragmentation conditions. Automated data acquisition, data processing, analysis and instrument control were performed by Analyst software version 1.5.

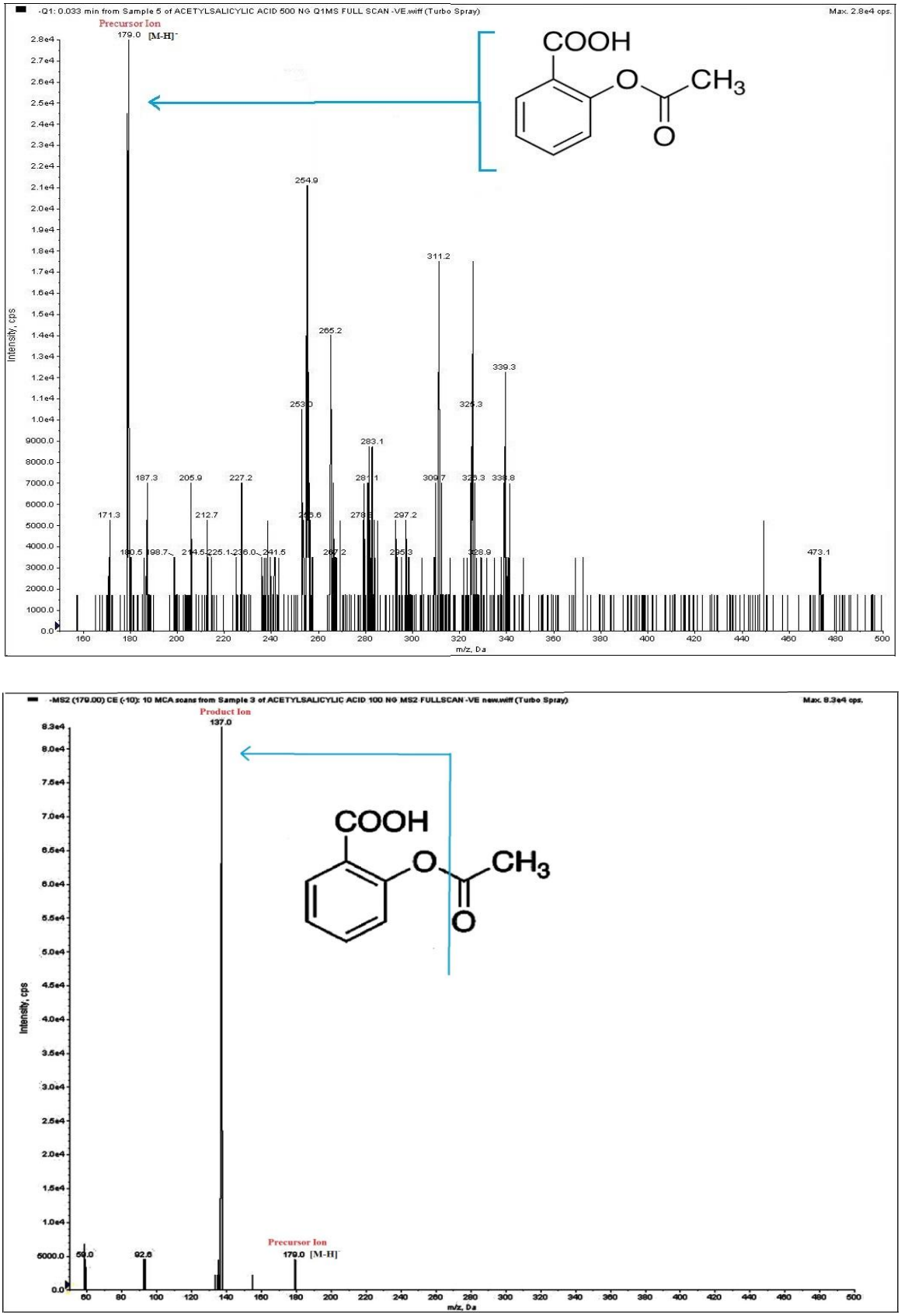


Figure.5.1 Mass spectra (Q1 and MS2) of the negative ion $[M-H]^-$ of acetylsalicylic acid (ASA) with structural interpretation

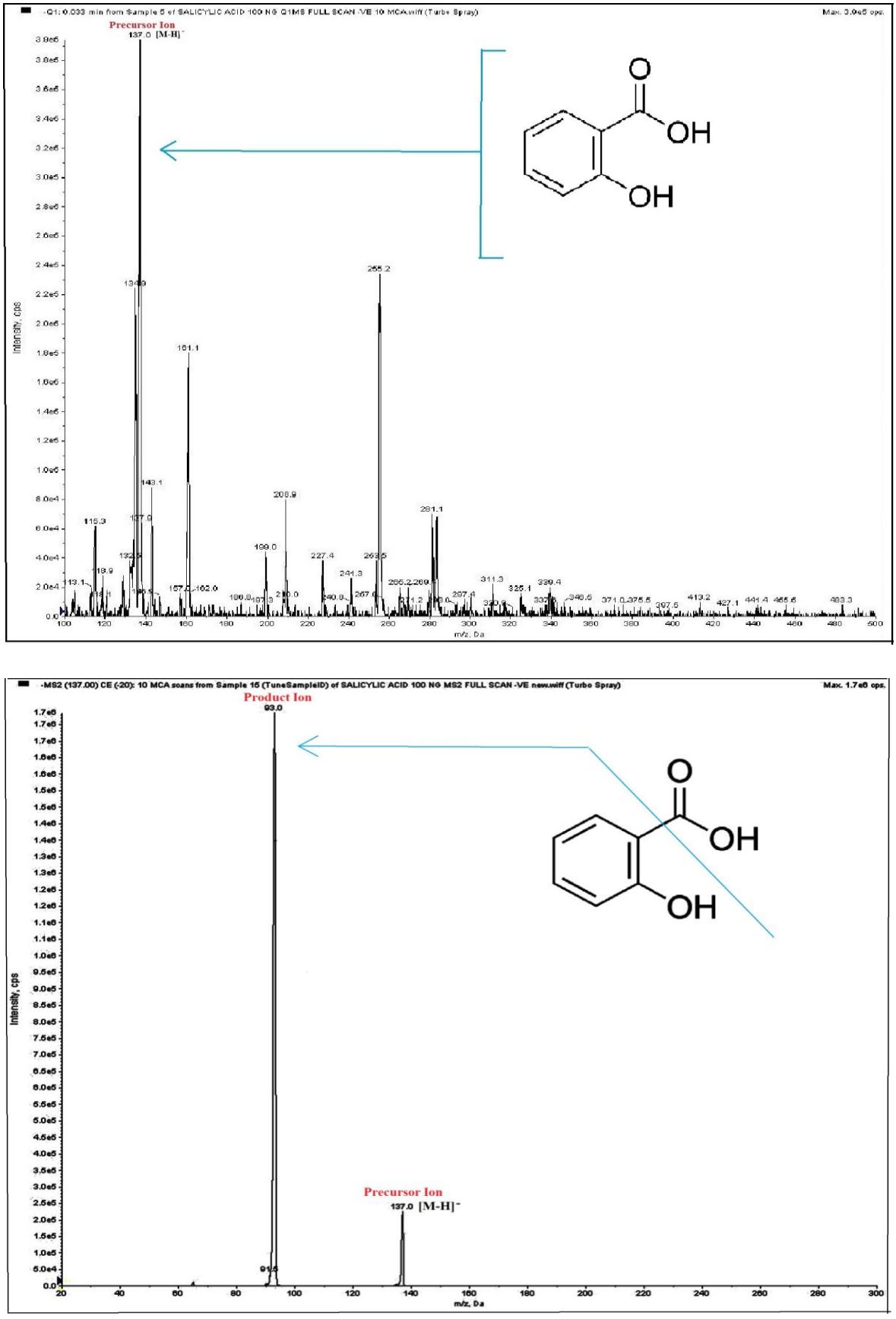


Figure.5.2 Mass spectra (Q1 and MS2) of the negative ion $[M-H]^-$ of salicylic acid (SA) with structural interpretation

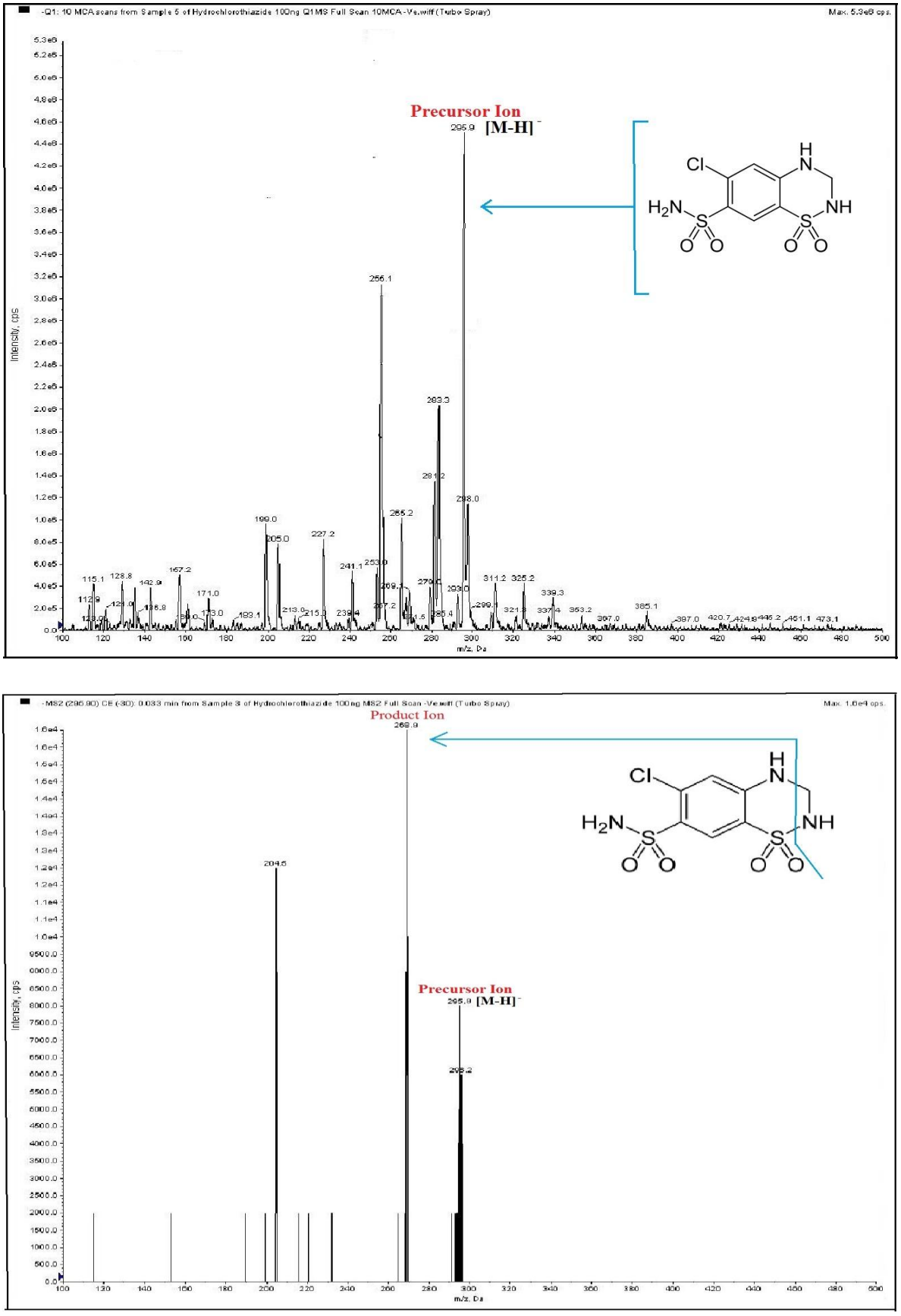


Figure.5.3 Mass spectra (Q1 and MS2) of the negative ion $[M-H]^-$ of hydrochlorothiazide (HCTZ, IS) with structural interpretation

5.1.2 Selection of the mobile phase for ASA and SA

Different mobile phase were tried for the elution of both ASA and SA properly. Various solvents, such as acetonitrile, methanol, and Milli-Q water (with a resistivity of 18.2 MΩ.cm and a conductivity of < 0.055 µS/cm) in different ratios consisting of either water : acetonitrile or water : methanol were tried to enhance response, sensitivity and efficiency of both the analytes and IS, as well as to improve the peak shape. Usually acetonitrile and methanol both were used as organic modifier in the mobile phases. For buffers, MS suitable reagents or salts like ammonium acetate and ammonium formate were used in different strengths (2 mM, 5 mM and 10 mM), whereas MS suitable acids such as formic acid and acetic acid also in different strengths (i.e., 0.1% to 0.5%) were dissolved in Milli-Q water and used. During selection of the mobile phase, several trials were achieved with various mobile phases, such as methanol : 0.1% of formic acid in Milli-Q water :: 90:10 (v/v), methanol : 0.2% formic acid in Milli-Q water :: 90:10 (v/v), acetonitrile : 0.1% formic acid in Milli-Q water :: 90:10 (v/v), acetonitrile : 0.2% formic acid in Milli-Q water, acetonitrile : 10 mM ammonium acetate in Milli-Q water and acetonitrile : 10 mM ammonium formate in Milli-Q water. But finally acetonitrile : water containing 10 mM ammonium formate :: 90:10 (v/v) at a flow rate of 0.5 mL/min was selected for suitable mobile phase as it has got better separation, high resolution and higher intensity, symmetric peak shapes compared to other mobile phases and also the best signal for the analytes and IS were achieved (see Figure.5.4).

5.1.3 Selection of the column (stationary phase) for ASA and SA

Different types of columns were tried such as with the selected mobile phase i.e., acetonitrile : water containing 10 mM ammonium formate :: 90:10 (v/v) to check the separation as well as resolution with good selectivity and sensitivity. During selection of columns, Phenomenex Kinetex C18 [50 mm (length) x 3 mm (ID) with 5 µm particle size], Agilent-Eclispse Plus C18 [75 mm (length) x 4.6 mm (ID) with 3.5 µm particle size] and Agilent ZORBAX CN [50 mm (length) x 4.6 mm (ID) with 3.5 µm particle size] were used to confirm the significant effects on separation, peak shape and response of the analytes as well as the IS also. Finally Phenomenex Kinetex C18 column was selected as stationary phase for the bioanalysis of both ASA and SA with IS (HCTZ), it was shown that Phenomenex Kinetex C18 column has more efficient with the selected mobile phase than both Agilent-Eclispse Plus C18 and Agilent ZORBAX CN column as it has got more response, sensitivity, better resolution and

symmetric peak shapes (Figure.5.4). Therefore, Phenomenex Kinetex C18 column had been selected for the entire bioanalysis.

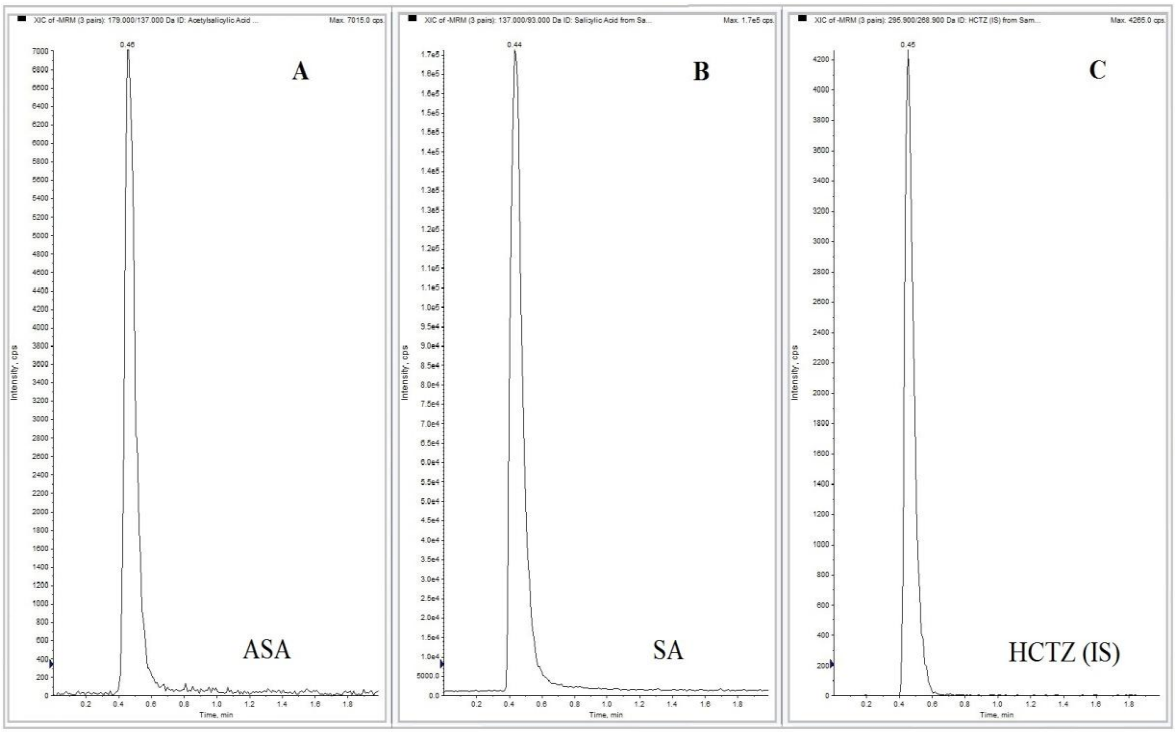


Figure.5.4 Representative chromatograms of A) ASA, B) SA and C) HCTZ (IS) after selection of mobile phase and column

5.1.4 Selection of the internal standard (IS) for ASA and SA

Preferably, structurally similar with same pharmacological classes or isotopically labelled compound should be used as internal standard (IS) for all analytes during bioanalysis. But the problem is that these compounds are not available commercially everytime. Different compound were examined for the selection of IS during method development, such as celecoxib, hydrochlorothiazide, letrozole, diclofenac, ibuprofen and prothionamide. Finally, hydrochlorothiazide (HCTZ) was selected as an appropriate IS for the present method of both ASA and SA due to its good response, rapid elution rate, better chromatographic separation and similar ionization properties with the analytes. HCTZ was coeluted properly in the selected mobile phase (acetonitrile : water containing 10 mM ammonium formate :: 90:10, v/v) and column (Phenomenex Kinetex C18) and also unaffected the analytes. Another major aspect was the extraction recovery of HCTZ in plasma and that was higher than the other tested compounds (see Table.5.1 and Figure.5.5) along with the analytes in plasma during method development.

Table.5.1 Extraction recovery (%) of different drug molecules in plasma

Compound	Extraction recovery (%)
Celecoxib	87.56
Hydrochlorothiazide	99.75
Letrozole	92.18
Diclofenac	76.33
Ibuprofen	94.05
Prothionamide	90.86

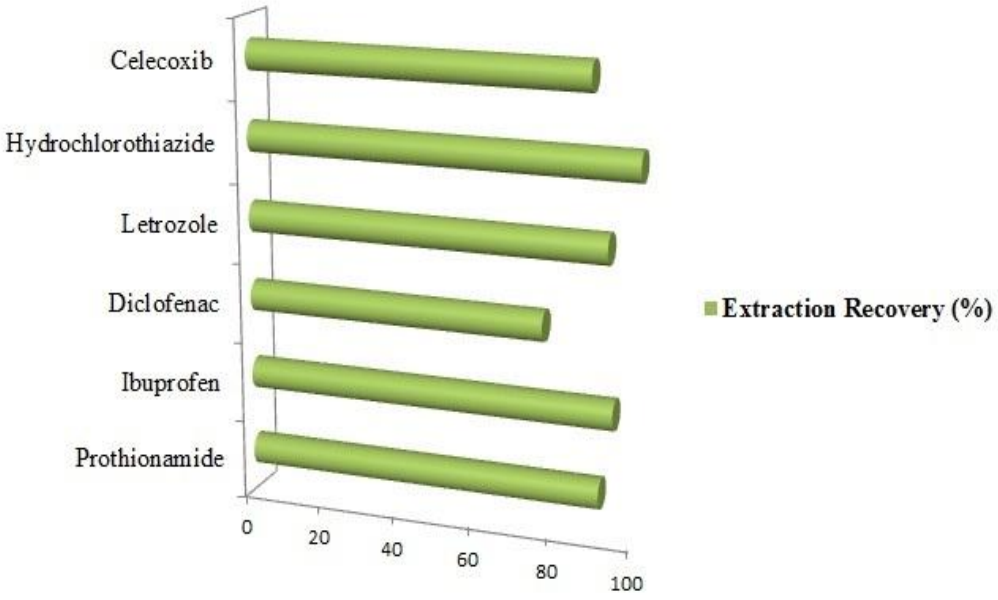


Figure.5.5 Extraction recoveries of different drug molecules in plasma

5.1.5 Selection of the diluent for ASA and SA

A suitable diluent was selected for both ASA and SA after the confirmation of mobile phase, column and IS in method development process. Methanol, acetonitrile, methanol : acetonitrile :: 50:50 (v/v) and the mobile phase, i.e., acetonitrile : water containing 10 mM ammonium formate :: 90:10, (v/v) were tried as diluents to check the response, sensitivity and peak

shape. The mobile phase was shown much better response, sensitivity and peak shape than the other used solvents. All results are tabulated in Table.5.2.

Table.5.2 Intensity (cps) of ASA, SA and HCTZ (IS) in different diluents

Diluent	Intensity (cps)		
	ASA (conc. 100 ng/mL)	**SA (conc. 100 ng/mL)**	**HCTZ (IS) (conc. 100 ng/mL)**
Methanol	200	150000 (1.50 e^5)	5700
Acetonitrile	7895	150000 (1.50 e^5)	24000 (2.40 e^4)
Methanol : Acetonitrile :: 50:50 (v/v)	7690	140000 (1.40 e^5)	1905
Mobile Phase (acetonitrile : water containing 10 mM ammonium formate :: 90:10, v/v)	10000 (1.00 e^4)	150000 (1.50 e^5)	6000

5.1.6 Optimization of the final mobile phase with selected buffer and organic modifier for ASA and SA

Optimization of the selected mobile phase was performed after the selection of column and IS. Composition and strength of the buffer i.e., ammonium formate, percentage (%) of acetonitrile (the organic modifier) and total pH of the mobile phase were tuned to improve chromatographic results. Various compositions and strengths of the buffer ammonium formate in Milli-Q water mentioned in Table.5.3 were tried and differences in the intensity of ASA, SA and IS (HCTZ) were noticed.

It has been observed in graphical representation, i.e., in Figure.5.6 that the intensity (cps) of ASA, SA and IS (HCTZ) increases with gradually increasing the buffer (ammonium formate) strengths 2 mM, 5 mM and 10 mM. Finally, 10 mM ammonium formate in Milli-Q water was selected and optimized as the most suitable buffer for the mobile phase, because the use of 10 mM ammonium formate buffer was found to be optimum to maintain the pH throughout the bioanalysis for getting uniform peak shape along with consistency in ionization and

fragmentation of the analytes and IS which can only be achieved if the molecules are solubilized to the desired concentration in the mobile phase.

Table.5.3 Effect of buffer composition and strength of the mobile phase on the intensity (cps) of the analytes and IS

Buffer composition (Strength of ammonium formate in Milli-Q water)	**Intensity (cps)**		
	ASA (conc. 100 ng/mL)	**SA (conc. 100 ng/mL)**	**HCTZ (IS) (conc. 100 ng/mL)**
2 mM	6875	113000 (1.13 e^5)	3650
5 mM	8540	120000 (1.20 e^5)	4860
10 mM	10000 (1.00 e^4)	150000 (1.50 e^5)	6000

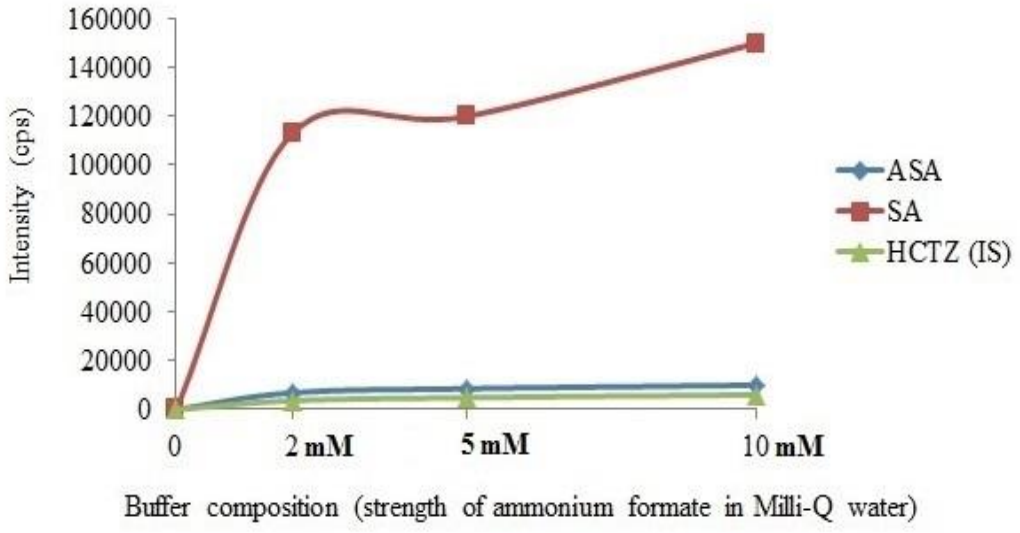


Figure.5.6 Effect of buffer composition on the intensity (cps) of ASA, SA and HCTZ (IS)

Organic modifier is an important part of the mobile phase, so after selection of the buffer in the mobile phase, percentage (%) of acetonitrile i.e., the organic modifier was optimized to improve the peak shape as well as enhanced the response or intensity (cps) of the analytes and IS also. The different percentage strength of acetonitrile were tried in the mobile phase

and which are given in Table.5.4. It is realized from the graph (Figure.5.7) that the response i.e., the intensity (cps) of both the analytes and IS increases by the increase of acetonitrile percentage (%) in the mobile phase. Now, the percentage (%) of acetonitrile in the mobile phase were tried from 65% to 90%. The maximum intensity (response), resolution and better peak shape were found in 90% of acetonitrile. Mainly in bioanalytical LC-MS/MS method, it required that both the peak of analytes and IS were in good shape and resolution and also the retention time (RT) as well as total chromatographic run time were short compared to any HPLC method. So, organic modifier i.e., acetonitrile concentration or percentage (%) was high in the mobile phase to improve these aspects. The high proportion of organic solvent eluted ASA, SA and IS (HCTZ) at retention times of 0.46, 0.44, and 0.45 min., respectively with a flow rate of 0.5 mL/min generated better peak shapes and a total chromatographic run time of 1.8 minutes.

Table.5.4 Effect of acetonitrile composition of the mobile phase on the intensity (cps) of the analytes and IS

Percentage (%) of acetonitrile as the organic modifier	**Intensity (cps)**		
	ASA (conc. 100 ng/mL)	**SA (conc. 100 ng/mL)**	**HCTZ (IS) (conc. 100 ng/mL)**
65	5320	78000 (7.80 e^4)	3657
70	7560	93000 (9.30 e^4)	4590
80	9800	110000 (1.10 e^5)	5435
90	10000 (1.00 e^4)	150000 (1.50 e^5)	6000

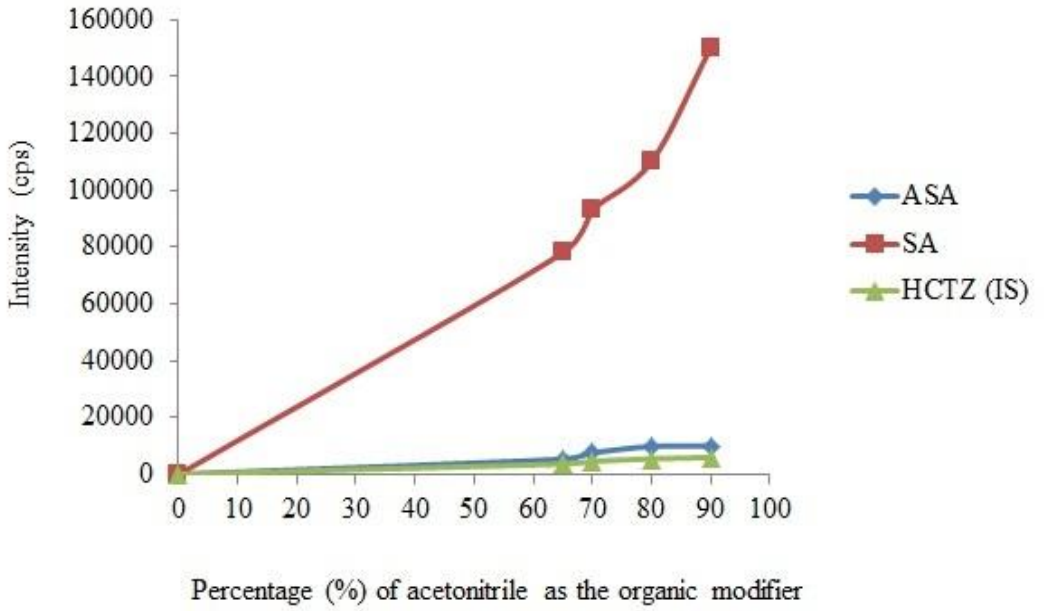


Figure.5.7 Effect of acetonitrile composition on the intensity (cps) of ASA, SA and HCTZ (IS)

After that, total pH of the mobile phase was optimized. Now, pKa value of ASA, SA and IS (HCTZ) are 3.45 (≈ 3.5), 2.98 (≈ 3.0) and 7.9 (≈ 8), respectively. The mobile phase was prepared by premixed of both the buffer and the organic modifier in the ratio of 90 : 10 (v/v) at isocratc conditions. The optimum total pH value of the mobile phase, acetonitrile : water containing 10 mM ammonium formate :: 90:10, (v/v) was found 5.9 because in this pH the response (intensity) and the peak shape were better than others (see Table.5.5 and Figure.5.8). By trial and error method, pH was decreased by formic acid (conjugate acid) and increased by ammonia solution or ammonium hydroxide (conjugate base) to check the optimum total pH value of the mobile phase. Finally after the optimization process, acetonitrile and water containing 10 mM ammonium formate with total pH 5.9 (90:10, v/v) was selected an appropriate mobile phase of the LC-ESI-MS/MS method for ASA and SA.

Table.5.5 Effect of total pH of the mobile phase on the intensity (cps) of the analytes and IS

Total pH of the mobile phase	Intensity (cps)		
	ASA (conc. 100 ng/mL)	SA (conc. 100 ng/mL)	HCTZ (IS) (conc. 100 ng/mL)
3.62	395	60000 (6.00 e^4)	4785
5.90	10000 (1.00 e^4)	150000 (1.50 e^5)	6000
7.98	5430	76000 (7.60 e^4)	2896

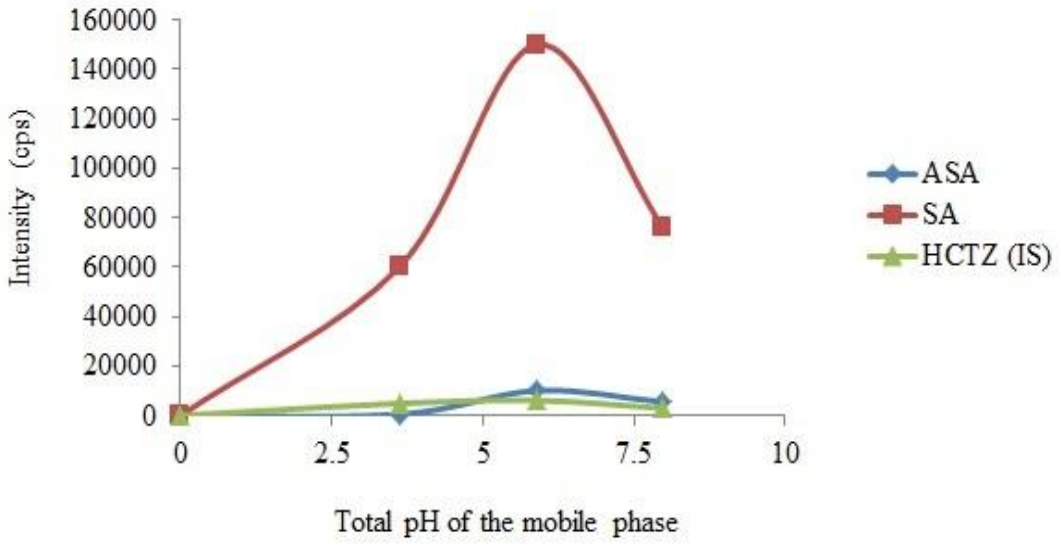


Figure.5.8 Effect of the total pH on the intensity (cps) of ASA, SA and HCTZ (IS)

5.1.7 Optimization of the flow rate for ASA and SA

By trial and error method, different flow rates of the mobile phase were tried to see which one was the suitable for present method. Because, it was one of the key factor to get better result, proper ionization in ESI, more sensitivity, proper retention time (RT) as well as total chromatographic run time. It was observed that on increasing the flow rate the response (intensity) was increased, whereas RT and total chromatographic run time were decreased

and short. In MS/MS development, i.e., in mass tuning flow rate was 10 µL/min (in Harvard syringe pump method). Next in LC-MS/MS development, i.e., the MS was coupled with the LC at a flow rate of 0.5 mL/min (500 µL/min) found optimum response and proper RT with shorter total run time. Graphical illustration had been established to understand that how the flow rate influenced on sensitivity or response (intensity) of ASA, SA and IS (HCTZ) molecules (see Figure.5.9 and Figure.5.10). Finally, after investigations an ideal flow rate 0.5 mL/min was continued throughout the entire bioanalysis.

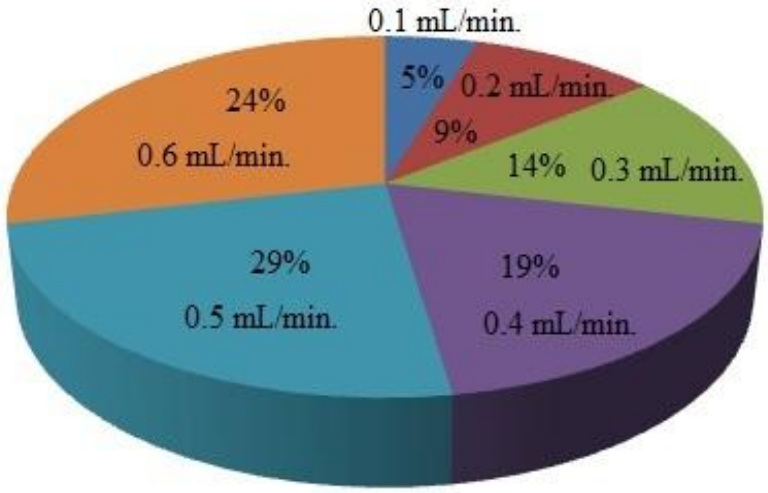


Figure.5.9 An impact of different flow rate on the intensity (cps) of ASA, SA and HCTZ (IS)

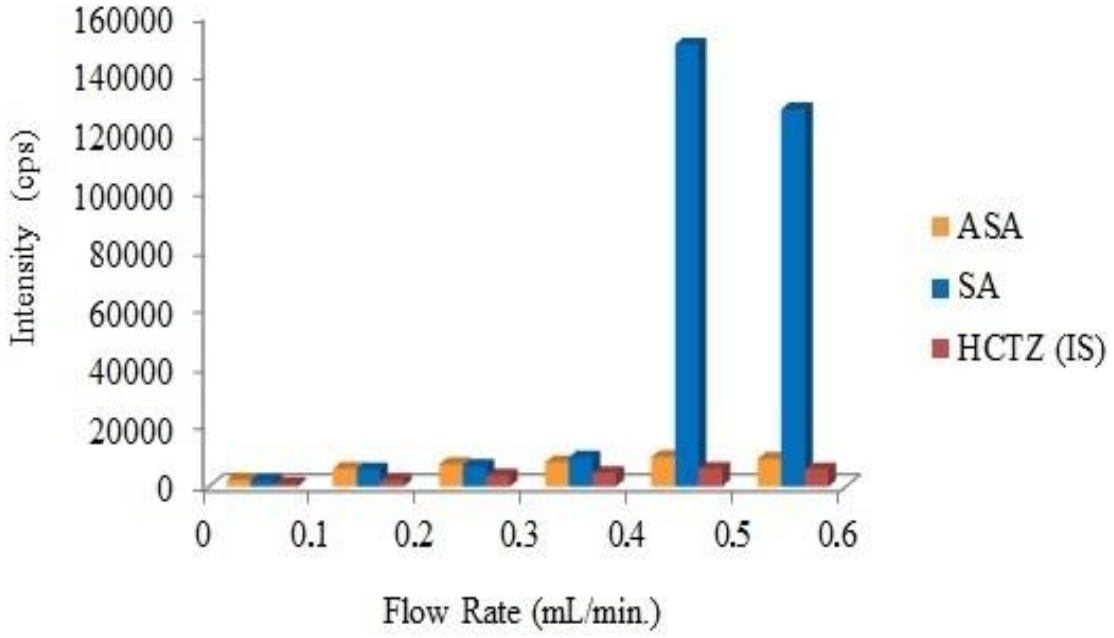


Figure.5.10 Effect of different flow rate on the intensity (cps) of ASA, SA and HCTZ (IS)

5.1.8 Optimization of the column oven temperature for ASA and SA

Various temperature were used in the column oven to see the intensity and peak shape of the analytes and IS during method development. In optimized conditions, the intensity, i.e., the response of ASA, SA and IS (HCTZ) was increased. It was observed that the high intensity (response) and symmetric peak shape with better resolution of the analytes and IS at 60 ^{o}C of the column oven temperature and therefore it was selected as the optimum temperature for the column oven throughout the bioanalysis. Now, in different temperature the response of the analytes and IS were different and it was described in both Table.5.6 and Figure.5.11

Table.5.6 Effect of different column oven temperature on the intensity (cps) of the analytes and IS

Column oven temperature (^{o}C)	Intensity (cps)		
	ASA (conc. 100 ng/mL)	SA (conc. 100 ng/mL)	HCTZ (IS) (conc. 100 ng/mL)
20*	6965	122000 (1.22 e^5)	4000
35	7795	130000 (1.30 e^5)	4610
50	7420	150000 (1.50 e^5)	5000
60	10000 (1.00 e^4)	150000 (1.50 e^5)	6000
70	7015	150000 (1.50 e^5)	4000

*Without column oven temperature (column oven was disabled during the sample run), i.e., in room temperature.

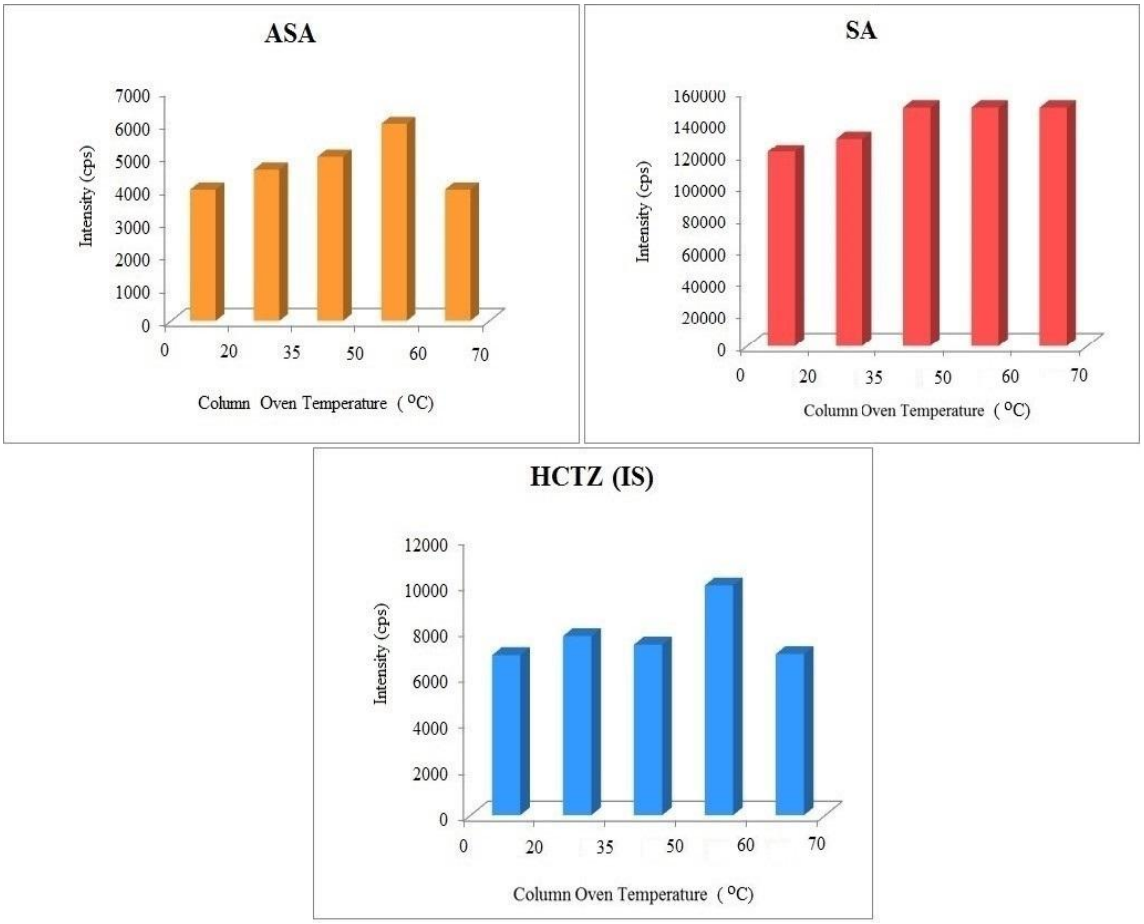


Figure.5.11 Effect of various column oven temperature (^{o}C) on the intensity (cps) of ASA, SA and HCTZ (IS)

5.1.9 Optimization of the mass spectrometric (MS) parameters for ASA, SA and HCTZ (IS)

An experimental setup was processed after selection and optimization of the final mobile phase to determine the optimal MS parameters, i.e., compound dependent parameters as well as source and gas parameters by flow injection analysis (FIA) with the method of compound optimization in tune mode in the negative polarity of the software (Analyst 1.5). In FIA, the range of all desired parameters were manually inputted and got the final optimized results for the analytes (ASA and SA) and IS (HCTZ) which have been described in Figure.5.12 to Figure.5.19 and Table.5.7 and Table.5.8.

FIA Compound Optimization Results -

Compound-Dependent Parameters :

Declustering Potential (DP)

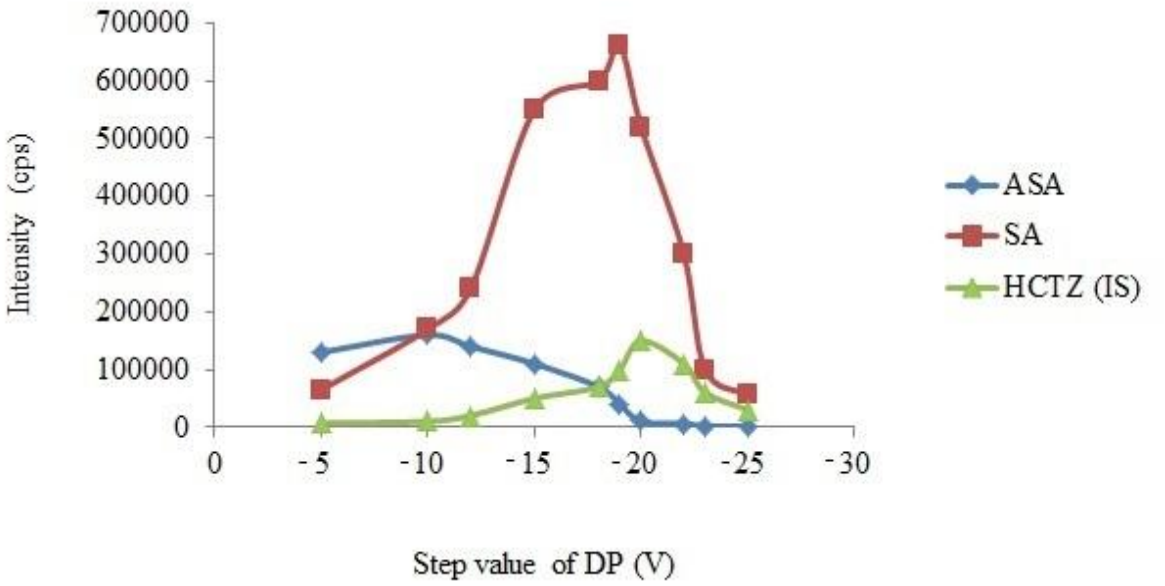


Figure.5.12 Optimization of declustering potential (DP) value for ASA, SA and HCTZ (IS)

Focusing Potential (FP)

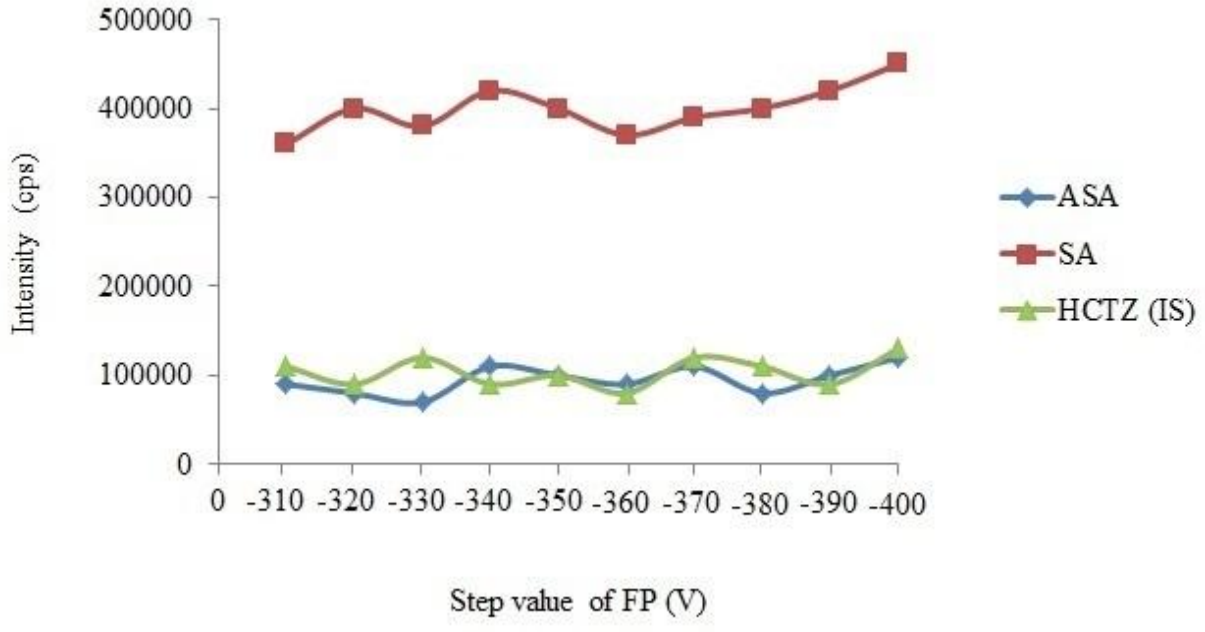


Figure.5.13 Optimization of focusing potential (FP) value for ASA, SA and HCTZ (IS)

Entrance Potential (EP)

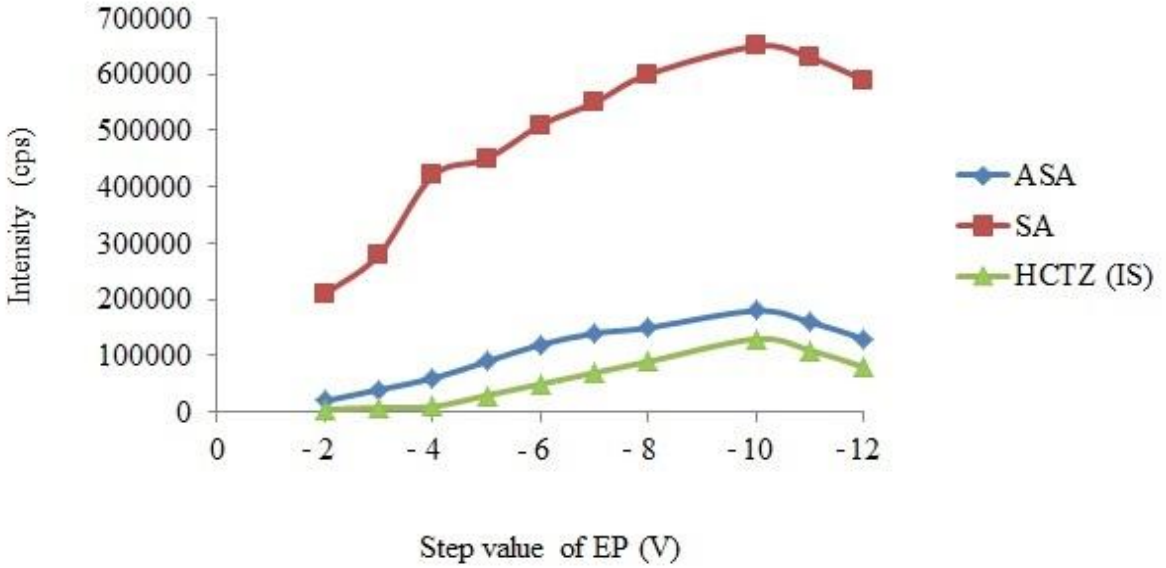


Figure.5.14 Optimization of entrance potential (EP) value for ASA, SA and HCTZ (IS)

Collision Cell Entrance Potential (CEP)

Usually, collision cell entrance potential (CEP) was setting up as by default in the software. So, during manual tuning it was not necessary to optimize. But in FIA it could be optimized and therefore it was performed successfully.

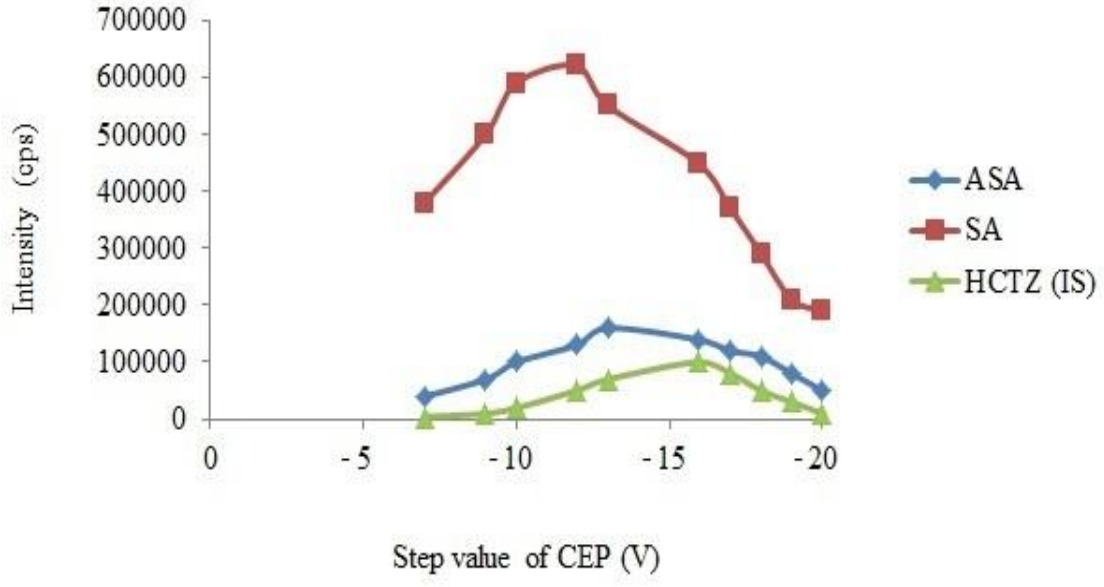


Figure.5.15 Optimization of collision cell entrance potential (CEP) value for ASA, SA and HCTZ (IS)

Collision Energy (CE)

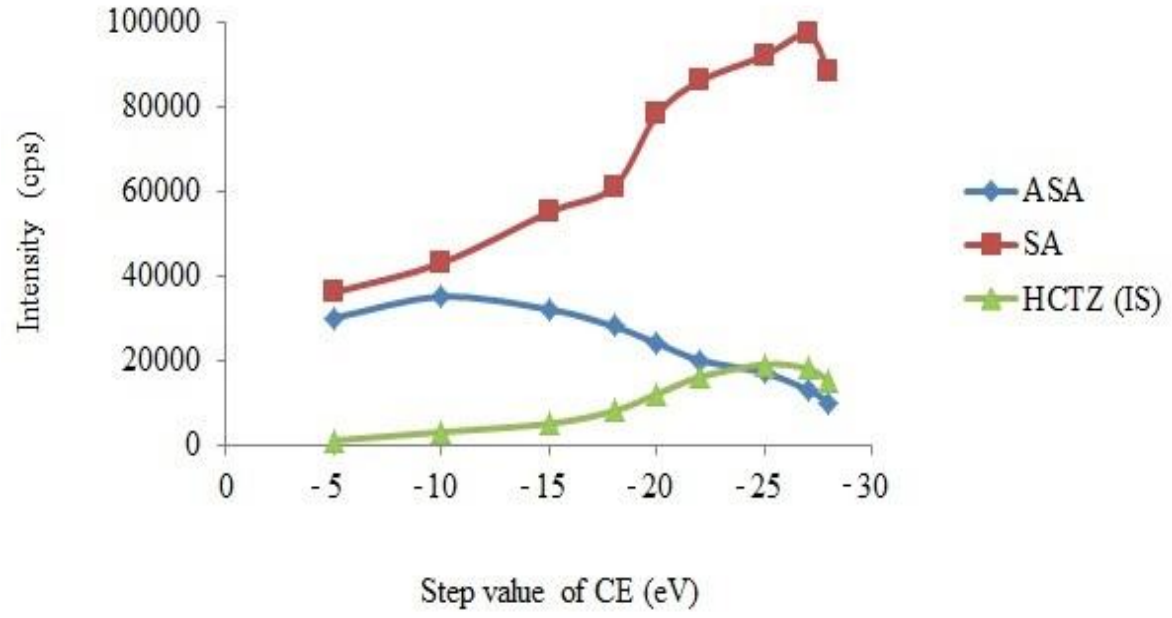


Figure.5.16 Optimization of collision energy (CE) value for ASA, SA and HCTZ (IS)

Collision Cell Exit Potential (CXP)

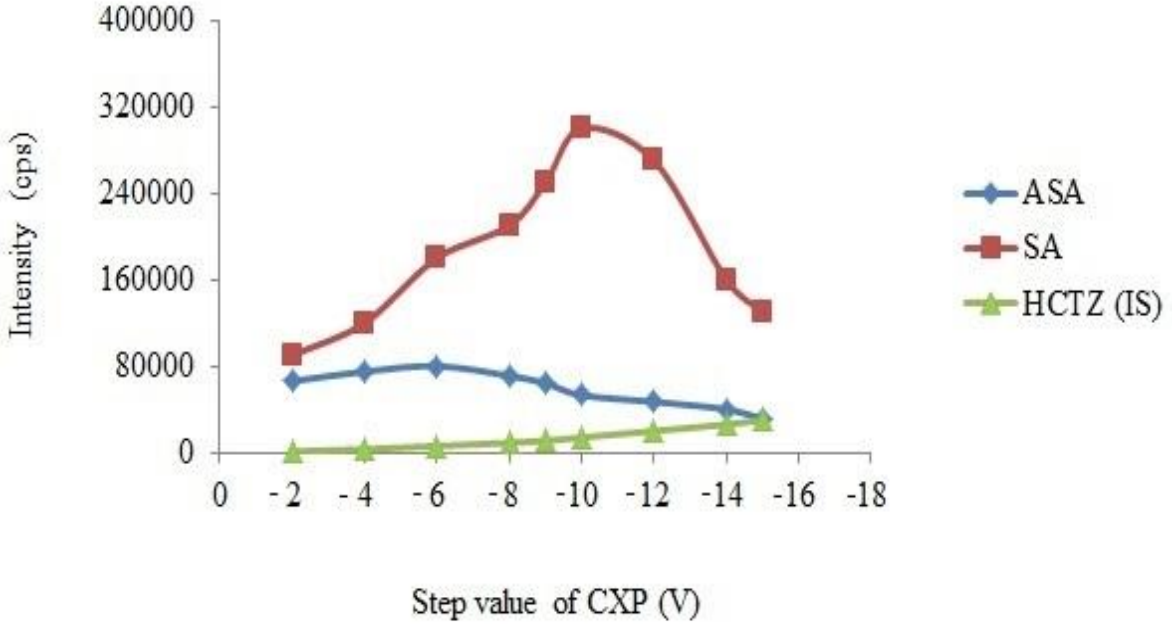


Figure.5.17 Optimization of collision cell exit potential (CXP) value for ASA, SA and HCTZ (IS)

Table.5.7 Optimized compound-dependent parameters of ASA, SA and HCTZ (IS)

Compound-dependent parameters	ASA	SA	HCTZ (IS)
DP (V)	-10.00	-19.00	-20.00
FP (V)	-400.00	-400.00	-400.00
EP (V)	-10.00	-10.00	-10.00
CEP (V)	-13.00	-11.95	-15.92
CE (eV)	-10.00	-27.00	-25.00
CXP (V)	-6.00	-10.00	-15.00

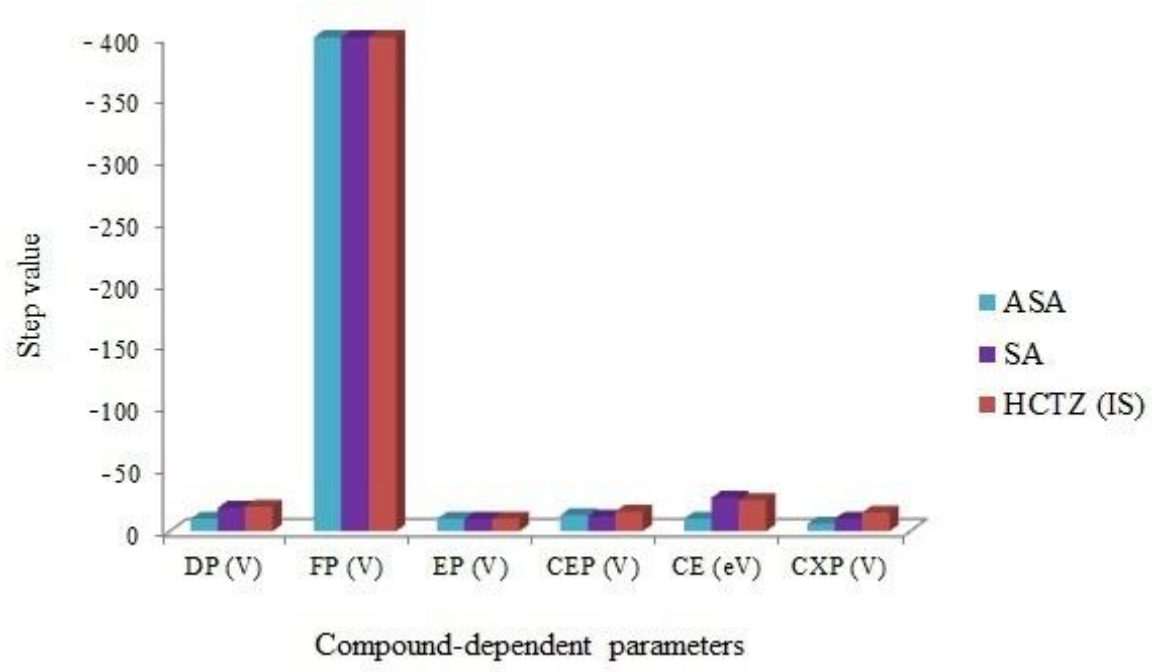


Figure.5.18 Optimized compound-dependent parameters for ASA, SA and HCTZ (IS)

Source and Gas Parameters :

The source and gas parameters were curtain (CUR) gas, collision activated dissociation (CAD), turbo ion spray voltage (ISV), turbo ion spray source temperature or turbo gas temperature (TEM), nebulizer gas (GS1) and turbo ionspray gas or auxiliary gas or desolvation gas (GS2) (i.e., heater gas).

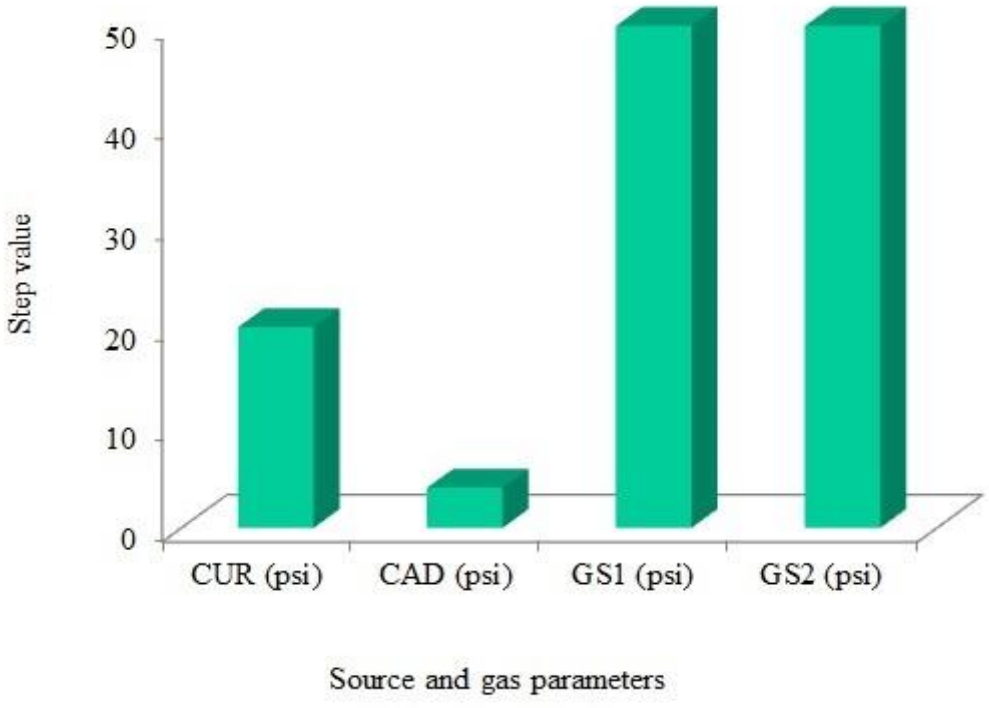


Figure.5.19 Optimized source and gas parameters of ASA, SA and HCTZ (IS)

Table.5.8 Optimized source and gas parameters of ASA, SA and HCTZ (IS)

Source and gas parameters	
CUR (psi)	20.0
CAD (psi)	4.0
ISV (V)	-4500.0
TEM (^{o}C)	400.0
GS1 (psi)	50.0
GS2 (psi)	50.0

5.1.10 Selection of extraction solvent and procedure for ASA and SA from human plasma

A liquid-liquid extraction (LLE) technique was selected or chosen as the sample preparation and extraction of both ASA and SA from the human plasma due to the increase of sensitivity or response (intensity) of the analytes as well as the IS (HCTZ). So, the main purpose was to get simple process, more and better extraction recovery, cost effective or low cost, easily

available etc. On the other hand it also raises the column life because of the extracted samples are cleaner, i.e., the supernatant or organic layer than that of the protein precipitation technique (PPT). Different organic solvents were used as trial and error such as ethyl acetate, dichloromethane, chloroform, diethyl ether, methyl tert-butyl ether and n-Hexane to find out the proper solvent for optimum response, higher and consistent recovery as well as better peak shape of the analytes (ASA and SA) along with IS (HCTZ). The sample preparation procedure were demonstrated earlier in the section 4.9.1 and were determined the extraction yields of the analytes and IS (Table.5.9). As per extraction recovery (%) except ethyl acetate and methyl tert-butyl ether, others were very poor in recovery (%), peak shape, resolution and reproducibility (see Table.5.9 and Figure.5.20). In ethyl acetate, % recovery of ASA, SA and HCTZ (IS) were 72.85%, 50% and 41.67% respectively. Whereas in methyl tert-butyl ether, recovery of ASA and SA were very very poor (1.5% and 6.67%) but for IS it was 83.33% that much better than ethyl acetate (41.67%).

Table.5.9 Extraction recovery (%) of ASA, SA and HCTZ (IS) from plasma by various organic solvents

Extracting solvents	Extraction recovery (%)		
	ASA	**SA**	**HCTZ (IS)**
Ethyl acetate (EA)	72.85	50	41.67
Dichloromethane (DCM)	0.9	2.43	25
Chloroform (CHL)	0.7	2	1.58
Diethyl ether (DEE)	0.75	6.67	77.67
Methyl tert-butyl ether (MTBE)	1.5	6.67	83.33
n-Hexane	1.4	7.73	2.17

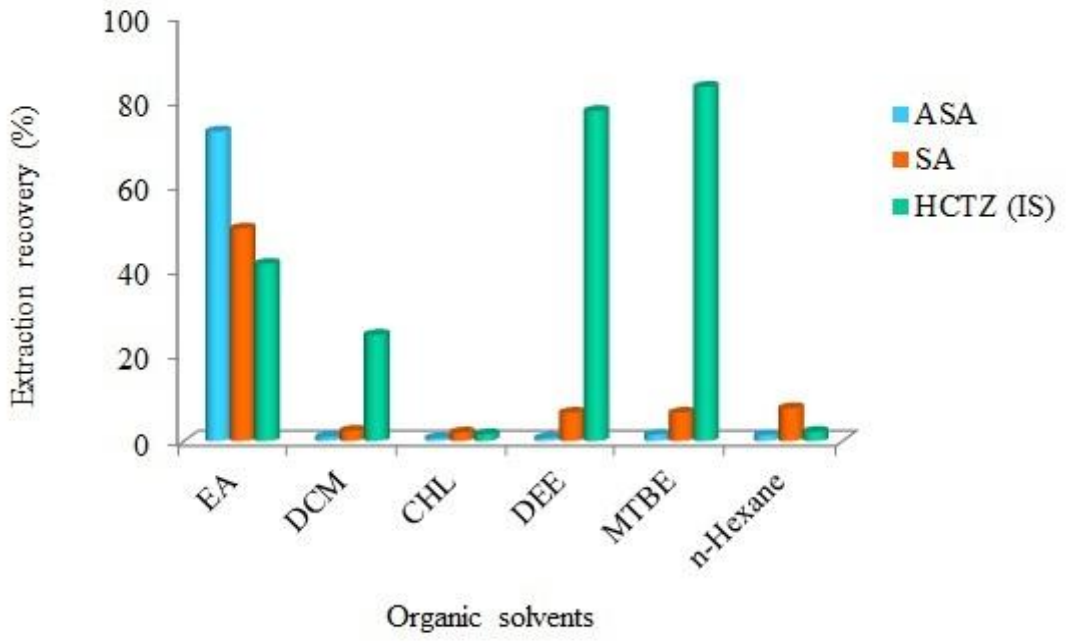


Figure.5.20 The extraction recovery (%) of ASA, SA and HCTZ (IS) after treating with different organic solvents

Therefore, it was confirmed that the mixed solvent, ethyl acetate and methyl tert-butyl ether was tried to further improve in the recovery as well as peak shape, resolution and reproducibility. So, three combinations were tried like ethyl acetate (EA) with 0.2% formic acid (this composition was prepared in Milli-Q water separately), ethyl acetate : methyl tert-butyl ether :: 1:1 v/v (EA : MTBE :: 1:1 v/v) and ethyl acetate : methyl tert-butyl ether :: 1:1 v/v with 0.2% formic acid (EA : MTBE :: 1:1 v/v with 0.2% formic acid). Ethyl acetae : methyl tert-butyl ether :: 1:1 v/v (EA : MTBE :: 1:1 v/v) was found suitable mixtures for extraction because this resulted better reproducibility and higher recovery of the analytes and IS than other two, 78% for ASA, 65.33% for SA and 113.08% for IS (HCTZ), respectively as well as better intensity (cps) and peak shape than the other two mixtures (see Table.5.10 and Figure.5.21 and 5.22). Also, the chromatograms were very clean, i.e., the endogenous peaks were not seen in this combination (EA : MTBE :: 1:1 v/v). So therefore the final extraction solvent was selected as ethyl acetae : methyl tert-butyl ether :: 1:1 v/v (EA : MTBE :: 1:1 v/v).

Table.5.10 Extraction recovery (%) of ASA, SA and HCTZ (IS) from plasma by different organic solvent mixtures

Mixed solvents (combinations)	Extraction recovery (%)		
	ASA	SA	HCTZ (IS)
Ethyl acetate (EA) with 0.2% formic acid	60	53.33	66
Ethyl acetate : methyl tert-butyl ether :: 1:1 v/v (EA : MTBE :: 1:1 v/v)	78	65.33	113.08
Ethyl acetate : methyl tert-butyl ether :: 1:1 v/v with 0.2% formic acid (EA : MTBE :: 1:1 v/v with 0.2% formic acid)	22.5	46	83.33

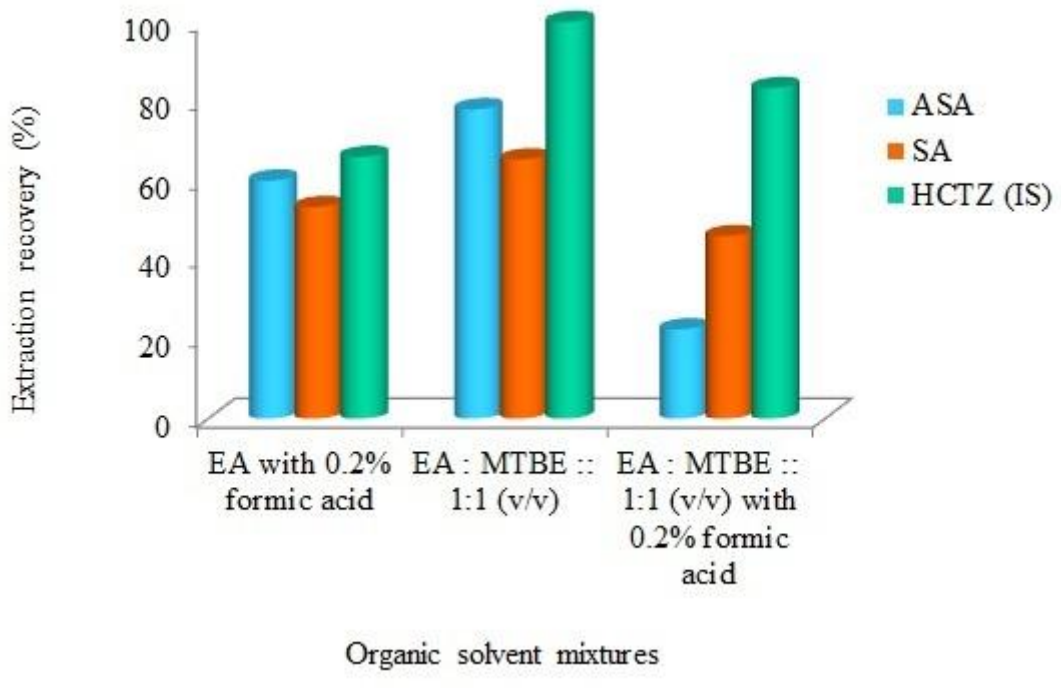


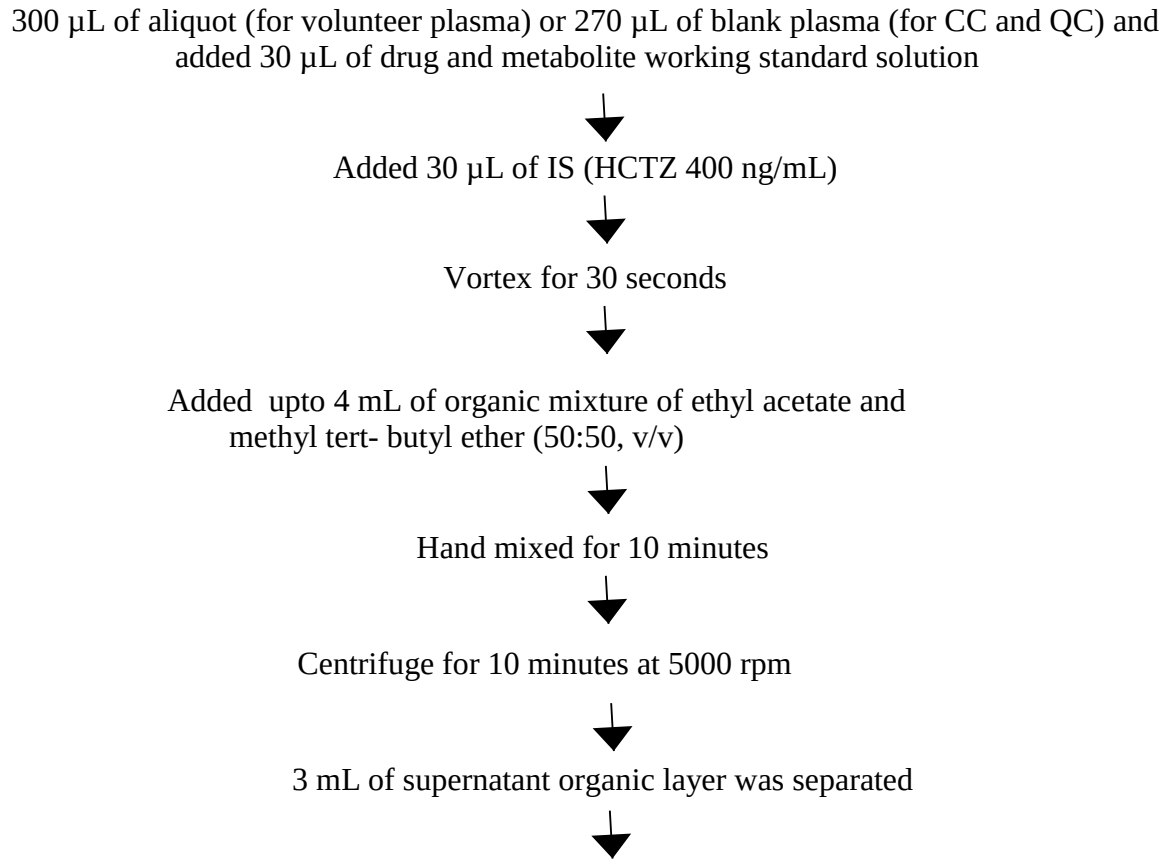
Figure.5.21 The extraction recovery (%) of ASA, SA and HCTZ (IS) after treatment with different organic solvent mixtures



Figure.5.22 The intensity (cps) and peak shape of ASA, SA and HCTZ (IS) using A) EA with 0.2% formic acid, B) EA : MTBE :: 1:1 v/v and C) EA : MTBE :: 1:1 v/v with 0.2% formic acid

5.1.11 Final optimized plasma extraction procedure for ASA and SA

First, 300 µL volume of human plasma was taken in 15 mL centrifuge tubes and 30 µL of IS was added from 4 µg/mL of HCTZ working standard solution in each and every tubes. Whereas, for calibration concentration (CC) and quality control (QC) samples it was 270 µL of human blank plasma (i.e., containing no drug or pre-dose plasma), 30 µL of working standard solution of both drug and metabolite concentration and 30 µL of IS solution. Then vortex mixed was done for 30 seconds. Next upto 4 mL of an organic solvents mixture of ethyl acetate and methyl tert-butyl ether was added in 50:50 i.e., 1:1 (v/v) ratio in all tubes and hand mixing was performed for 10 minutes. After that, centrifugation was done at 5000 rpm for 10 minutes. After the centrifugation, upper 3 mL of the organic layer was separated and then the layer was pipetted out and transferred to the clean centrifuge tubes. The organic layer was totally evaporated under nitrogen (N2) atmosphere at 40 to 45 ºC temperature and the remaining dry residues were collected and then reconstituted with the 200 µL of mobile phase followed by vortex mixed for 3 to 4 minutes. Lastly, the reconstituted samples were transferred to autosampler vials and then the prepared samples were ready for run in the LC-MS/MS system.



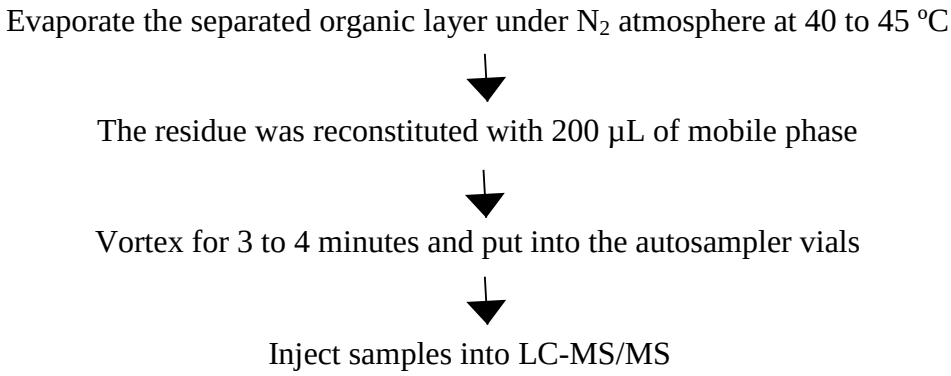


Figure.5.23 A schematic presentation of final optimized plasma extraction procedure of ASA and SA

5.1.12 Final optimized chromatographic conditions for bioanalysis of ASA and SA in human plasma

5.1.12.1 Chromatographic parameters

Integrated System : Shimadzu Controller prominence, autosampler SIL-20AC, CBM-20A Lite System Control Compartment, binary pump : pump A and pump B LC-20AD, online solvent degasser DGU- 20A3, column oven CTO-10 AS VP and liquid chromatography.

Column	Phenomenex Kinetex C18, 50 mm x 3 mm (length x inner diameter), 5 µm particle size
Mobile Phase	Acetonitrile : 10 mM ammonium formate in Milli-Q water (total pH 5.9) :: 9 : 1 (v/v)
Flow Rate	0.5 mL/min.
Injection Volume	20 µL
Column Oven Temperature	60 °C
Autosampler Temperature	15 °C
Total Chromatographic Run Time	1.8 minutes

Shimadzu LC Method Properties

Shimadzu LC system Equlibration time = 0.00 min

Shimadzu LC system Injection Volume = 20.00 µL

Shimadzu LC Method Parameters

Pumps

Pump A Model: LC-20AD

Pump B Model: LC-20AD

Pumping Mode: Binary Flow

Total Flow: 0.5000 mL/min.

Pump B Pct: 50.0 %

B Curve: 0

Pressure Range (Pump A/B): 0.0 – 40.0 MPa

Autosampler

Model: SIL-20A

Rinsing Volume: 200 µL

Needle Stroke: 52 mm.

Rinsing Speed: 35 µL/sec.

Sampling Speed: 15.0 µL/sec. Sampling Speed: 15.0 µL/sec.

Purge Time: 25.0 min.

Rinse Dip Time: 0 sec.

Rinse Mode: Before and after aspiration

Cooler Enabled: Yes

Cooler Temperature: 15 deg. C

Control Vial Needle Stroke: 52 mm.

Oven

Model: CTO-10ASvp

Temperature Control: Enabled

Temperature: 60 deg. C

Max. Temperature: 85 deg. C

System Controller

Model: CBM-20A Lite

Power: On

Event 1: Off

Event 2: Off

Event 3: Off

Event 4: Off

Time Program

Time	Module	Events	Parameter
0.01	Pumps	Pump B Conc.	50
1.00	Pumps	Pump B Conc.	50
1.80	Pumps	Pump B Conc.	50
1.81	System Controller	Stop	

5.1.12.2 Mass spectrometric parameters

Quantitation Information:

Sample Type: Standard

Dilution Factor: 1.000000

Custom Data:

Quantitation Table:

Period 1:

Scans in Period: 176

Relative Start Time: 0.00 msec

Experiments in Period: 1

Period 1 Experiment 1:

Scan Type: MRM (MRM)

Scheduled MRM: No

Polarity: Negative

Scan Mode: N/A

Ion Source: Turbo Spray

Resolution Q1: Low

Resolution Q3: Low

Intensity Thres.: 0.00 cps

Settling Time: 0.0000 msec

MR Pause: 5.0070 msec

MCA: No

Step Size: 0.00 Da

Acetylsalicylic Acid (ASA)

Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Parameter	
Volt		ID		
179.00	137.00	200.00	DP	-10.00
			CEP	-13.00
			CE	-10.00
			CXP	-6.00

Salicylic Acid (SA)

Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Parameter	Volt
137.00	93.00	200.00	DP	-19.00
			CEP	-11.95
			CE	-27.00
			CXP	-10.00

Hydrochlorothiazide (HCTZ, IS)

Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Parameter	Volt
295.90	268.90	200.00	DP	-20.00
			CEP	-15.92
			CE	-25.00
			CXP	-15.00

Parameter Table (Period 1 Experiment 1)

CUR:	20.00
CAD:	4.00
IS:	-4500.00
TEM:	400.00
GS1:	50.00
GS2:	50.00
ihe:	ON
FP	-400.00
EP	-10.00

Instrument Parameters:

Detector Parameters (Negative):

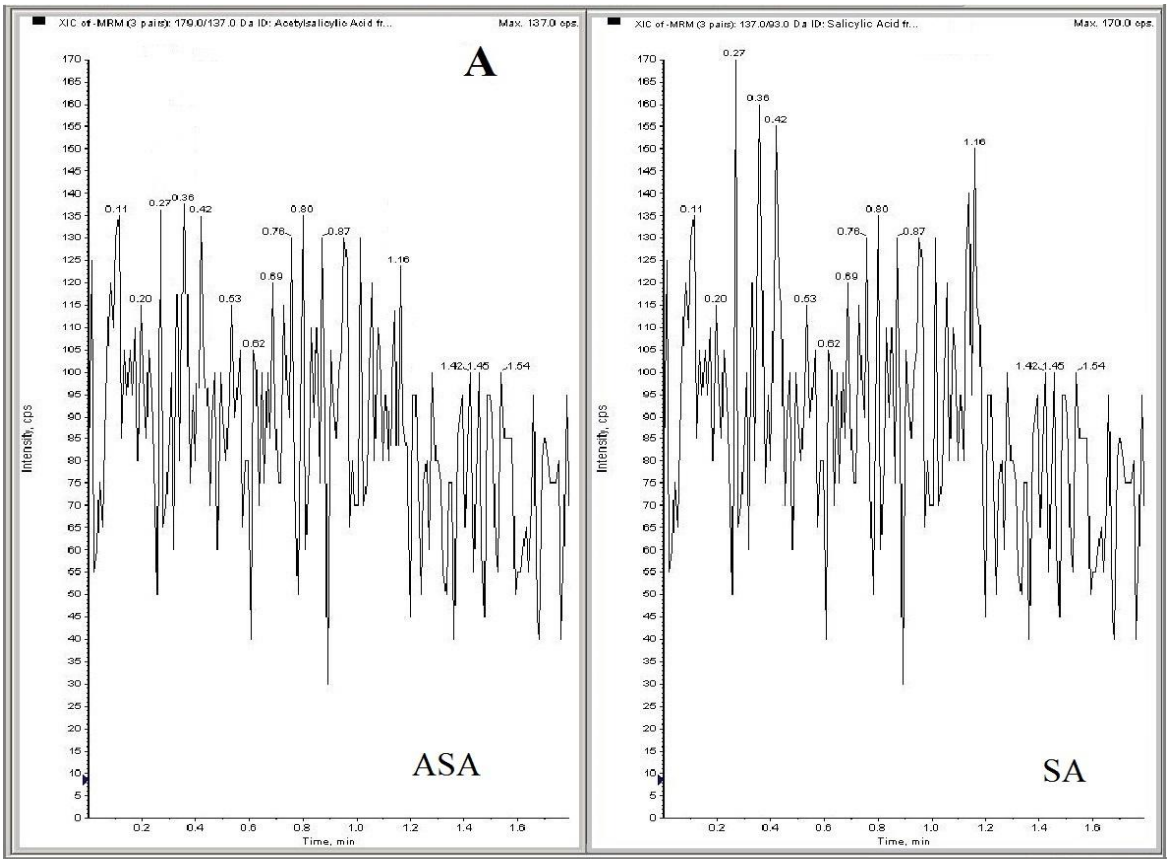
DF	200.0
CEM	2000.0

Keyed Text:

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5.1.13 Estimation of limit of detection (LOD) and lower limit of quantification (LLOQ) for ASA and SA

The LOD of both ASA and SA was found 5 ng/mL where signal-to-noise ratio was 3 times and the LLOQ was 10 ng/mL where signal-to-noise ratio was 5 times (Figure.5.24).



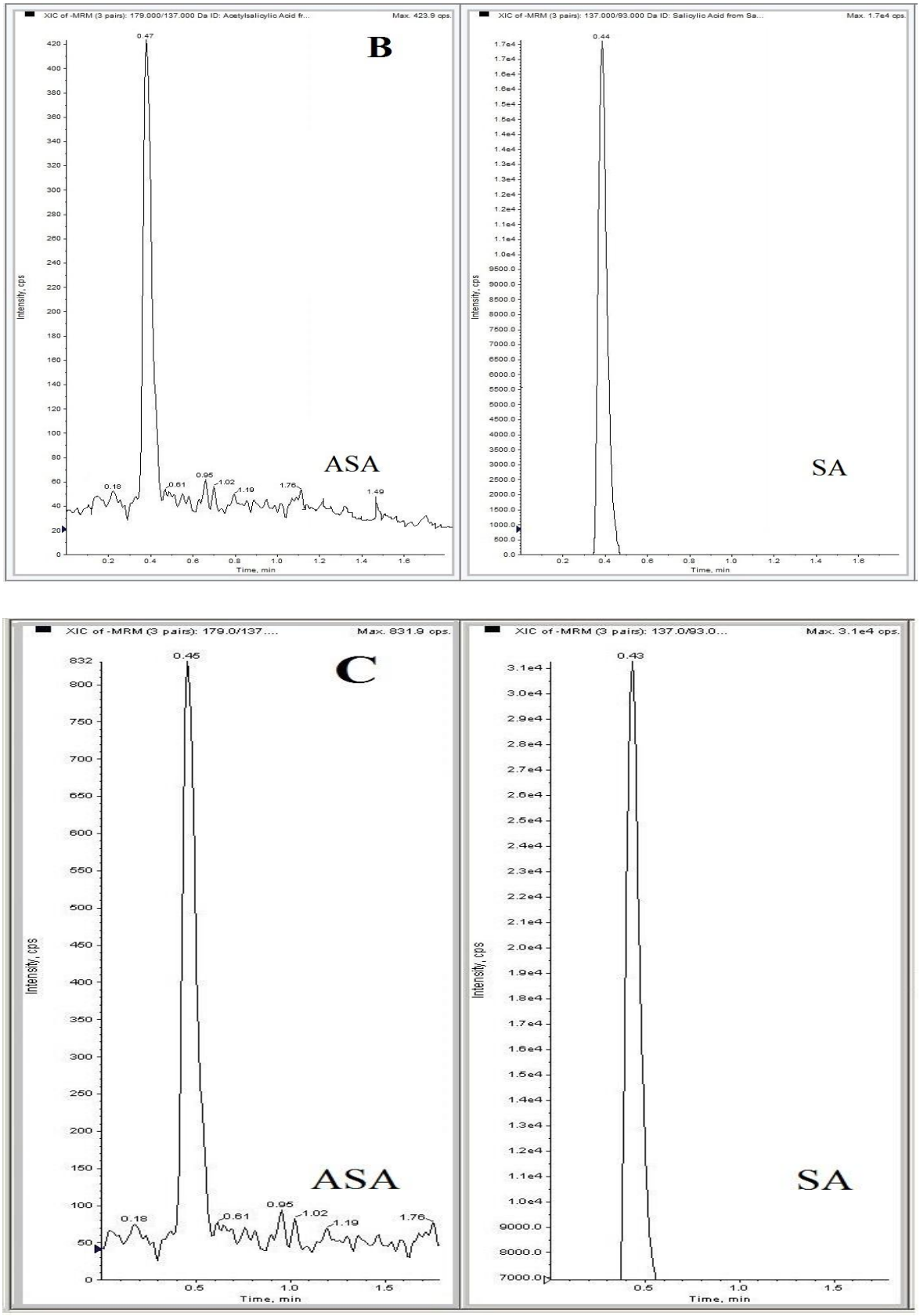


Figure.5.24 Chromatograms for determination of LOD and LLOQ of ASA and SA : A) chromatogram of blank plasma, B) chromatogram of LOD and C) chromatogram of LLOQ

5.2 Pre-validation study of bioanalytical method for ASA and SA

5.2.1 System suitability test (SST) for ASA and SA LC-MS/MS method

The system suitability test (SST) was assessed by replicate (n=6) injection of the same reference standard (RS) solution containing ASA, SA and IS (HCTZ) at 500 ng/mL. At the beginning of bioanalysis, i.e., during sample run, this mixed solution was injected (i.e., RS 500 ng/mL). Now, it was confirmed from the Table.5.11 that the low RSD% (CV%) of the analytes (ASA and SA) area as well as IS area and retention time (RT) were within or below 2% as per the guidelines. Therefore, it was ensured that the system was reproducible for the present analytical run and working or functioning properly.

Table.5.11 System suitability test (n=6) of ASA, SA and HCTZ (IS) during sample run

No. of injection	ASA (conc. 500 ng/mL)		SA (conc. 500 ng/mL)		HCTZ (IS, conc. 500ng/mL)	
	Peak area	RT (min.)	Peak area	RT (min.)	Peak area	RT (min.)
1	1.38E+05	0.46	4.21E+06	0.43	2.15E+05	0.45
2	1.38E+05	0.47	4.28E+06	0.42	2.15E+05	0.45
3	1.37E+05	0.45	4.28E+06	0.42	2.13E+05	0.45
4	1.36E+05	0.46	4.29E+06	0.43	2.13E+05	0.46
5	1.38E+05	0.45	4.25E+06	0.42	2.15E+05	0.46
6	1.37E+05	0.45	4.25E+06	0.44	2.12E+05	0.46
Mean	**1.37E+05**	**0.4567**	**4.26E+06**	**0.4267**	**2.14E+05**	**0.4550**
SD	**816.4966**	**0.0082**	**29664.7939**	**0.0082**	**1329.1601**	**0.0055**
RSD% (CV%)	**0.5945**	**1.7879**	**0.6964**	**1.9137**	**0.6216**	**1.2038**

RSD% (or CV%), Relative Standard Deviation [(SD/Mean) x 100]

5.2.2 Reproducibility test for ASA and SA LC-MS/MS method

The reproducibility of the bioanalytical method was determined on the basis of the coefficient of variance (CV%) i.e., the relative standard deviation (RSD) of the area ratio of both ASA/ HCTZ (IS) and SA/HCTZ (IS) for replicate (n=3) assay with conc. 10 ng/mL and conc. 100 ng/mL.

Table.5.12 Reproducibility test of the plasma spiked with 10 ng/mL of ASA, SA and HCTZ (IS)

No.	Peak area of ASA (conc. 10 ng/mL)	Peak area of SA (conc. 10 ng/mL)	Peak area of HCTZ (IS) (conc. 10 ng/mL)	Area ratio (ASA)	Area ratio (SA)
1	2.06E+03	5.33E+04	1.62E+03	1.27E+00	3.28E+01
2	2.05E+03	5.31E+04	1.64E+03	1.25E+00	3.25E+01
3	2.06E+03	5.30E+04	1.63E+03	1.27E+00	3.25E+01
Mean	**2.06E+03**	**5.31E+04**	**1.63E+03**	**1.26E+00**	**3.26E+01**
SD	**5.9713**	**160.9979**	**5.4788**	**0.0071**	**0.1839**
CV% (RSD%)	**0.2903**	**0.3031**	**0.3362**	**0.5647**	**0.5642**

Table.5.13 Reproducibility test of the plasma spiked with 100 ng/mL of ASA, SA and HCTZ (IS)

No.	Peak area of ASA (conc. 100 ng/mL)	Peak area of SA (conc. 100 ng/mL)	Peak area of HCTZ (IS) (conc. 100 ng/mL)	Area ratio (ASA)	Area ratio (SA)
1	2.84E+04	4.59E+05	2.82E+04	1.01E+00	1.63E+01
2	2.86E+04	4.50E+05	2.85E+04	1.00E+00	1.58E+01
3	2.80E+04	4.57E+05	2.83E+04	9.88E-01	1.61E+01
Mean	**2.83E+04**	**4.55E+05**	**2.83E+04**	**9.99E-01**	**1.61E+01**
SD	**304.8546**	**4930.6626**	**156.5067**	**0.0100**	**0.2594**
CV% (RSD%)	**1.0768**	**1.0825**	**0.5522**	**0.9969**	**1.6141**

In Table.5.12 and Table.5.13, results of reproducibility test are described for 10 ng/mL and 100 ng/mL concentrations respectively. The low CV% (RSD%) value proves the reproducibility of the method to be satisfactory and so CV% (RSD%) of both concentrations for ASA and IS; SA and IS were found to be within acceptable limit.

5.3 Validation of method for bioanalytical study of acetylsalicylic acid (ASA) and salicylic acid (SA) by liquid chromatography tandem-mass spectrometry (LC-MS/MS)

The method of ASA and SA was validated to meet the acceptance criteria of industrial guidance for the validation of bioanalytical method.

5.3.1 Specificity and selectivity

According to the representative chromatograms of the double blank plasma sample (see Figure.5.25), blank plasma sample spiked with IS (see Figure.5.26.) and blank plasma spiked with the analytes (ASA and SA) at 10 ng/mL and IS (HCTZ), i.e., at LLOQ (see Figure.5.27), the total chromatographic run time was 1.8 minutes and the retention time of ASA, SA and HCTZ (IS) were about 0.45, 0.43 and 0.46 min., respectively. The retention time never shifted beyond 10 to 15 seconds on repeated injection of the samples. In Figure.5.27, the chromatogram shown that there was no significant interference from the endogenous plasma matrix was observed at the retention times of analytes and IS. Similar things were observed in case of the chromatogram of blank plasma spiked with IS (single blank, Figure.5.26) and also in the chromatogram of blank plasma (double blank, Figure.5.25).

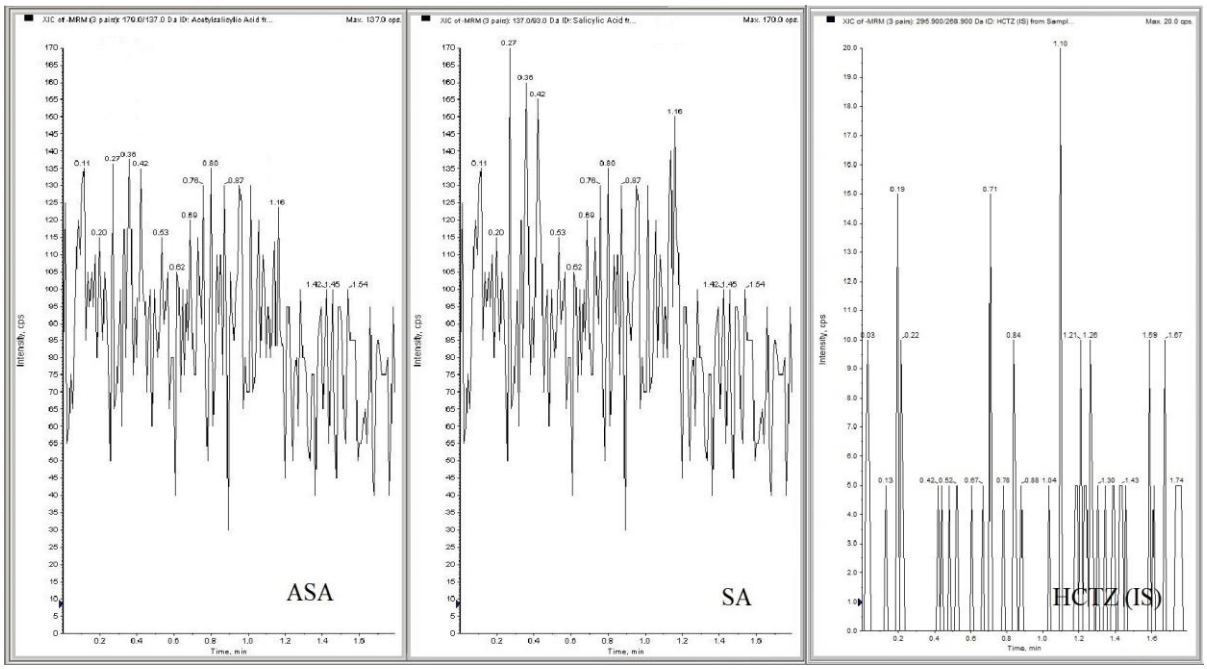


Figure.5.25 Chromatogram of blank human plasma or double blank plasma [free of ASA, SA and HCTZ (IS)]

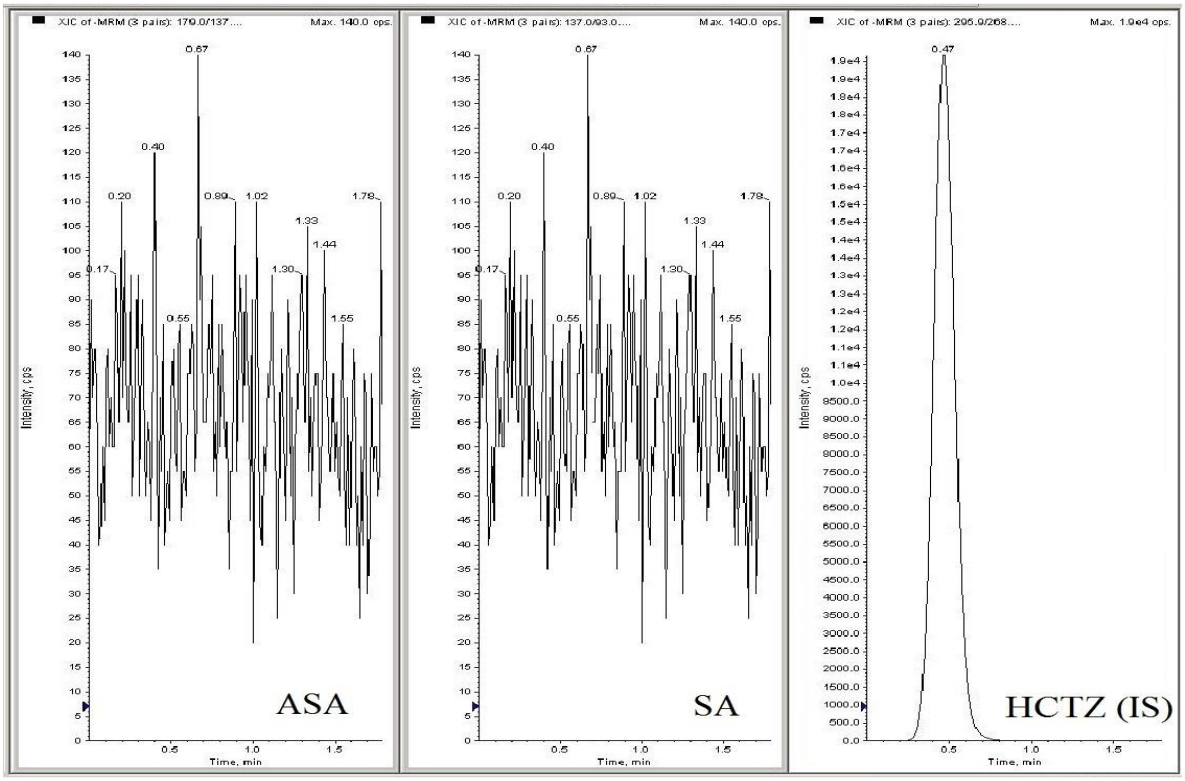


Figure.5.26 Chromatogram of single blank plasma spiked with IS (HCTZ)

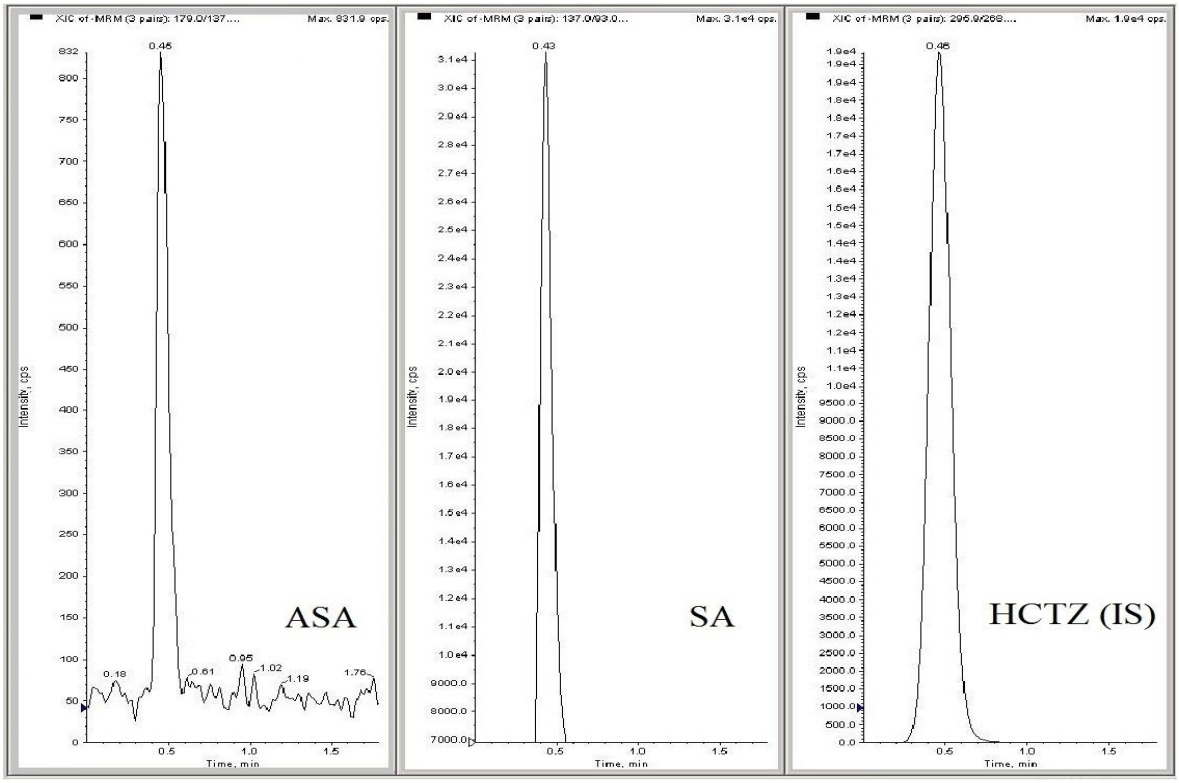


Figure.5.27 Chromatogram of blank human plasma spiked with analytes (ASA and SA) at LLOQ 10 ng/mL and IS (HCTZ)

5.3.2 Sensitivity

The method of ASA and SA was found very sensitive as per the result. Now, the limit of detection (LOD) and the lower limit of quantification (LLOQ) were found at 5 ng/mL and 10 ng/mL, respectively (see Figure.5.28). The signal to noise (S/N) ratio was maintained 3:1 for LOD and 5:1 for LLOQ. All LLOQ samples were found within the acceptance criteria as per the guidelines.

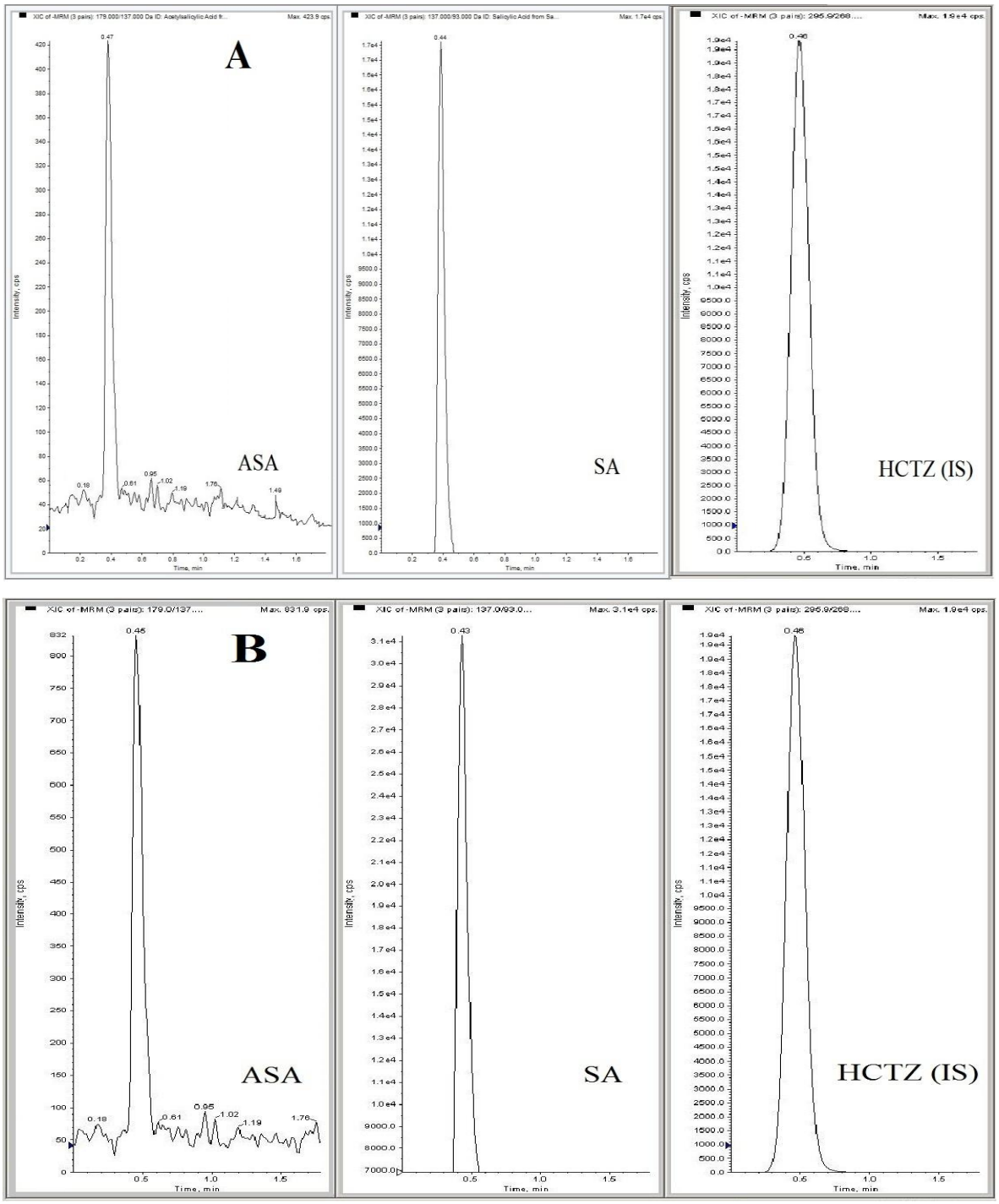


Figure.5.28 Chromatograms showing A) LOD and B) LLOQ of ASA and SA

5.3.3 Linearity and calibration (standard) curve of ASA and SA in human plasma

The validation was carried out to determine the calibration, precision and accuracy of both ASA and SA in human blank plasma. The standard or calibration curves were constructed by plotting the peak area ratio of the analytes (ASA and SA) to IS (HCTZ) versus the nominal calibration concentrations (CC) of the analytes. The standard curves have consistently good reproducibility over the standard concentrations across the calibration range and the representative calibration curves (LIN I) for ASA and SA are given in Figure.5.29 and Figure.5.30. The linearity of the calibration curve was determined by an unweighted ($1/x^2$) least square or linear regression analysis. Later the regression equation of the calibration curve was used to calculate the concentrations of both ASA and SA in human plasma. All these procedures were done by the Analyst 1.5 software in quantitate mode during analysis.

The back calculated values of the concentrations were evaluated statistically. These back calculated concentrations from the representative calibration standards by LC-MS/MS determination for ASA and SA and corresponding regression equations are mentioned in Table.5.14 and Table.5.16. The assay result was shown better linear response over the linearity range of 10 ng/mL to 15,000 ng/mL for both ASA and SA in human plasma. The regression equations were $y = 0.0009x + 0.0097$ ($r = 0.9996$) for ASA and $y = 0.0161x + 0.4147$ ($r = 0.9992$) for SA (see Table.5.15 and Table.5.17), where 'y' is the peak area ratio of analyte to IS and 'x' is the nominal plasma concentration of the analyte. In Table.5.14 and Table.5.16 were observed the CV% and accuracy (%) values ranging from 0.76% to 7.59% and 97.37% to 110.00% for ASA and from 1.52% to 7.88% and 97.57% to 108.00% for SA, respectively.

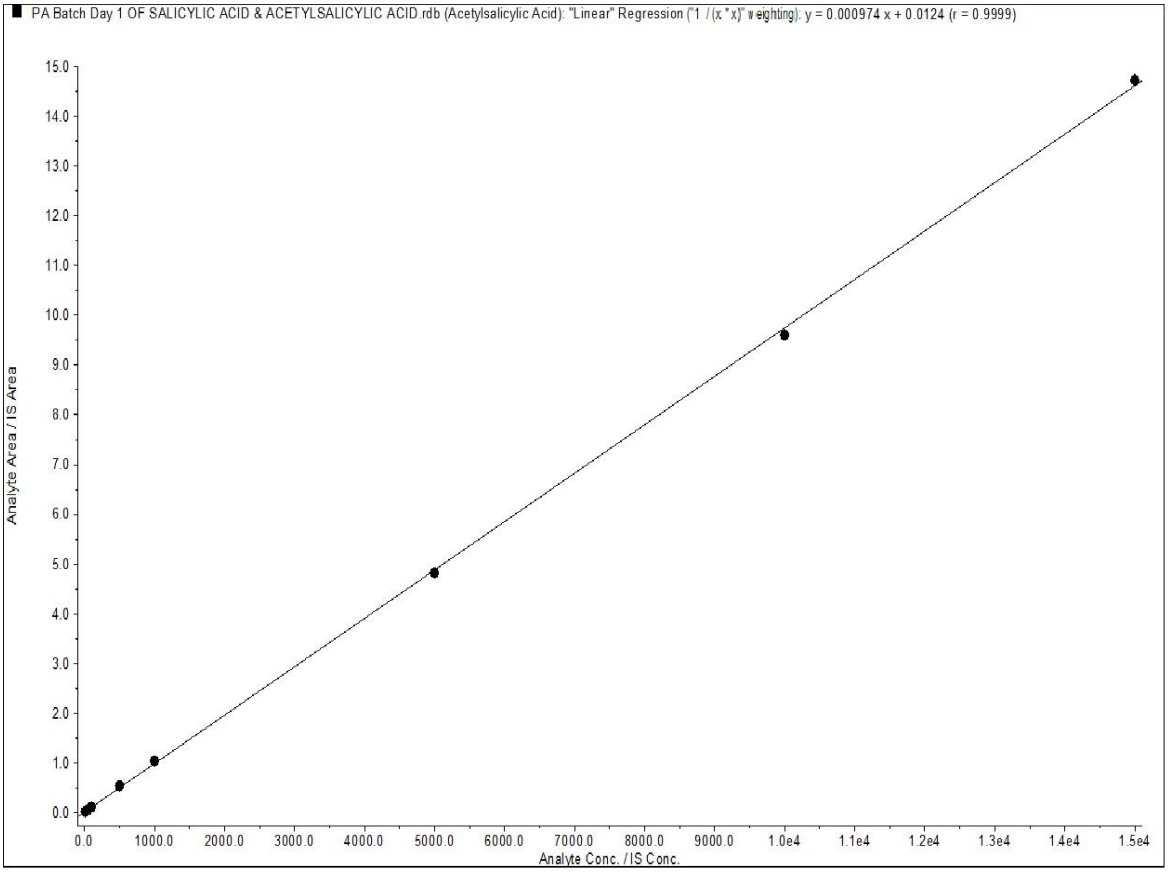


Figure.5.29 Plasma calibration curve of ASA

Table.5.14 Linear regression data for the determination of ASA in human plasma

Linearity	Plasma calibration concentration (ng/mL) of ASA							
	10 (CC1)	**50 (CC2)**	**100 (CC3)**	**500 (CC4)**	**1000 (CC5)**	**5000 (CC6)**	**10000 (CC7)**	**15000 (CC8)**
LIN 1	9.45	50.70	101.00	539.00	1050.00	4940.00	9850.00	15100.00
LIN 2	10.20	55.10	103.00	562.00	1040.00	5140.00	9410.00	15300.00
LIN 3	11.00	56.70	108.00	549.00	1090.00	4810.00	9950.00	15100.00
Mean	**10.22**	**54.17**	**104.00**	**550.00**	**1060.00**	**4963.33**	**9736.67**	**15166.67**
S.D.	**0.78**	**3.11**	**3.61**	**11.53**	**26.46**	**166.23**	**287.29**	**115.47**
CV%	**7.59**	**5.74**	**3.47**	**2.10**	**2.50**	**3.35**	**2.95**	**0.76**
Accuracy (%)	**102.17**	**108.33**	**104.00**	**110.00**	**106.00**	**99.27**	**97.37**	**101.11**

Table.5.15 Pre study linearity of detector response of ASA

Linearity	Statistics		
	Slope (M)	**Intercept-C**	**R square**
LIN 1	0.0010	0.0124	0.9999
LIN 2	0.0009	0.0121	0.9989
LIN 3	0.0009	0.00467	0.9999
Mean	**0.0009**	**0.0097**	**0.9996**
S.D.	**0.0001**	**0.0044**	**0.0006**
CV%	**5.5262**	**45.0348**	**0.0578**

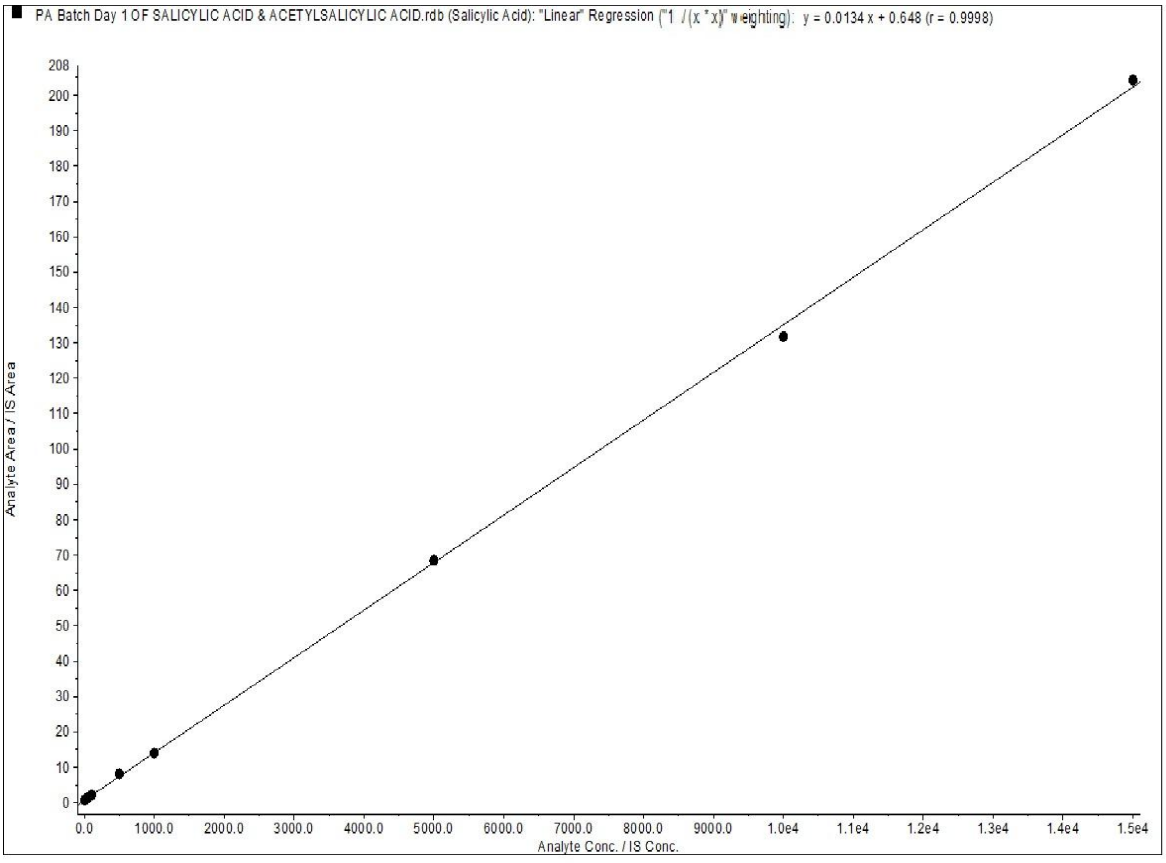


Figure.5.30 Plasma calibration curve of SA

Table.5.16 Linear regression data for the determination of SA in human plasma

Linearity	Plasma calibration concentration (ng/mL) of SA							
	10 (CC1)	50 (CC2)	100 (CC3)	500 (CC4)	1000 (CC5)	5000 (CC6)	10000 (CC7)	15000 (CC8)
LIN 1	9.88	52.20	104.00	557.00	990.00	5050.00	9750.00	15100.00
LIN 2	10.30	54.90	101.00	527.00	1020.00	5310.00	9220.00	15400.00
LIN 3	9.53	54.90	111.00	476.00	1010.00	4840.00	10300.00	14800.00
Mean	**9.90**	**54.00**	**105.33**	**520.00**	**1006.67**	**5066.67**	**9756.67**	**15100.00**
S.D.	**0.39**	**1.56**	**5.13**	**40.95**	**15.28**	**235.44**	**540.03**	**300.00**
CV%	**3.89**	**2.89**	**4.87**	**7.88**	**1.52**	**4.65**	**5.53**	**1.99**
Accuracy (%)	**99.03**	**108.00**	**105.33**	**104.00**	**100.67**	**101.33**	**97.57**	**100.67**

Table.5.17 Pre study linearity of detector response of SA

Linearity	Statistics		
	Slope (M)	Intercept-C	R square
LIN 1	0.0134	0.6480	0.9998
LIN 2	0.0180	0.3080	0.9981
LIN 3	0.0170	0.2880	0.9996
Mean	**0.0161**	**0.4147**	**0.9992**
S.D.	**0.0024**	**0.2023**	**0.0009**
CV%	**14.9961**	**48.7910**	**0.0930**

5.3.4 Precision and accuracy of ASA and SA

The intra-day and inter-day assay precision, i.e., CV% or RSD% values for different concentrations ranged from 2.75% to 5.17% and 2.77% to 5.97% for ASA and 3.63% to 4.99% and 2.75% to 5.38% for SA, respectively. Whereas for the same concentrations, the percentage of accuracy (accuracy %) was in the range of 98.25% to 104.83% and 96.39% to

103.19% for ASA and 94.95% to 104.16% and 95.21% to 103.97% for SA, respectively. Both the precision and accuracy data were in the acceptable range as per USFDA and EMA guidelines. The assay method expressed high degree of precision and accuracy. The precision and accuracy data for both intra-day and inter-day assays of LLOQ and three QC samples (LQC, MQC and HQC) are given in Table.5.18 and Table.5.19.

Table.5.18 Intra-day and inter-day precision and accuracy data of ASA in human plasma (n = 6)

Intra-day (Within run)					
Quality control (QC) sample	**Spiked/Nominal Concentration (ng/mL)**	**Mean concentration (ng/mL)**	**SD**	**CV% (RSD%)**	**Accuracy (%)**
LLOQ	10	9.8250	0.3543	3.6057	98.2500
LQC	30	31.4500	1.3398	4.2600	104.8333
MQC	7500	7628.3333	210.4677	2.7590	101.7111
HQC	12000	12183.3333	630.6082	5.1760	101.5278
Inter-day (Between run)					
LLOQ	10	9.6396	0.5762	5.9769	96.3958
LQC	30	30.9583	1.1271	3.6407	103.1944
MQC	7500	7633.3333	211.4889	2.7706	101.7778
HQC	12000	12250.0000	604.3322	4.9333	102.0833

SD = Standard deviation; CV (%) coefficient of variation or RSD (%) relative standard deviation = [(SD / mean) x 100]; Accuracy (%) = [(measured concentration / spiked or nominal concentration) x 100]; LLOQ = Lower limit of quantification; LQC = Low quality control; MQC = Middle quality control; HQC = High quality control; n = number of samples.

Table.5.19 Intra-day and inter-day precision and accuracy data of SA in human plasma (n = 6)

Intra-day (Within run)					
Quality control (QC) sample	**Spiked/Nominal Concentration (ng/mL)**	**Mean concentration (ng/mL)**	**SD**	**CV% (RSD%)**	**Accuracy (%)**
LLOQ	10	9.4950	0.4742	4.9946	94.9500
LQC	30	30.7833	1.1197	3.6373	102.6111
MQC	7500	7611.6667	287.2920	3.7744	101.4889
HQC	12000	12500.0000	529.1503	4.2332	104.1667
Inter-day (Between run)					
LLOQ	10	9.5217	0.5124	5.3811	95.2167
LQC	30	31.1917	1.2000	3.8471	103.9722
MQC	7500	7573.7500	208.4271	2.7520	100.9833
HQC	12000	12229.1667	546.5140	4.4689	101.9097

SD = Standard deviation; CV (%) coefficient of variation or RSD (%) relative standard deviation = [(SD / mean) x 100]; Accuracy (%) = [(measured concentration / spiked or nominal concentration) x 100]; LLOQ = Lower limit of quantification; LQC = Low quality control; MQC = Medium quality control; HQC = High quality control; n = number of samples.

5.3.5 Extraction recovery of ASA and SA

The percentage (%) of extraction recoveries were calculated by measuring the peak areas of the analytes at low, medium and high quality control samples. The peak areas of the extracted plasma at low, medium, and high quality control samples were compared to the peak areas of the unextracted standards containing the same concentrations of the ASA and SA. Recovery results revealed that the maximum recovery was found at low (30 ng/mL), medium (7500 ng/mL) and high (12000 ng/mL) quality control (QC) samples ranged from 98.18% to

100.81% for ASA and 96.72% to 99.41% for SA, respectively (see Table.5.20). Also, recovery of the internal standard (IS, HCTZ) was found 100.07% (see Table.5.20). The recovery of both the ananlytes was found to be satisfactory as well as sufficiently high and also data indicated better sensitivity as it was precise, consistent and reproducible.

Table.5.20 Absolute recovery of ASA and SA in human plasma

Analyte	Spiked/Nominal Concentration (ng/mL)	Mean Concentration (ng/mL)	Recovery (%) (Mean ± S.D.)
ASA	30	30.2667	98.1884 ± 2.4480
	7500	7480.0000	99.1407 ± 3.7465
	12000	12150.0000	100.8108 ± 2.2290
SA	30	30.1167	96.7219 ± 6.8534
	7500	7570.0000	99.4120 ± 2.5935
	12000	12083.3333	97.7474 ± 3.4000
HCTZ (IS)	400	415.0000	100.0769 ± 3.6613

5.3.6 Matrix effect of ASA and SA

No significant matrix effect was found for ASA, SA and HCTZ (IS) by comparing the area under the curve (AUC) ratios of the extracted QCs (LQC, MQC and HQC) with the AUC of unextracted QCs (LQC, MQC and HQC) obtained from the injecting raw or aqueous solution prepared at similar concentration levels (n = 6) as shown in Table.5.21, Table.5.22, Table.5.23 and Table.5.24.

Table.5.21 Evaluation of matrix effect for ASA

Sample name	Analyte peak area		% Matrix effect	Matrix factor
	Spiking in aqueous sample	Spiking in extracted blank matrix (plasma)		
LQC-1	4120.0000	4194.4031	101.8059	1.0181
LQC-2	4000.0000	3870.3080	96.7577	0.9676
LQC-3	4370.0000	4924.7365	112.6942	1.1269
LQC-4	3900.0000	3669.3930	94.0870	0.9409
LQC-5	4300.0000	4109.5143	95.5701	0.9557
LQC-6	4210.0000	4505.8199	107.0266	1.0703
MQC-1	778000.0000	741863.4560	95.3552	0.9536
MQC-2	734000.0000	691610.0320	94.2248	0.9422
MQC-3	715000.0000	690366.1050	96.5547	0.9655
MQC-4	870000.0000	911177.9700	104.7331	1.0473
MQC-5	889000.0000	908610.4510	102.2059	1.0221
MQC-6	856000.0000	847295.3360	98.9831	0.9898
HQC-1	1200000.0000	1190318.4000	99.1932	0.9919
HQC-2	1200000.0000	1217360.4000	101.4467	1.0145
HQC-3	1190000.0000	1132650.3300	95.1807	0.9518
HQC-4	1250000.0000	1353330.0000	108.2664	1.0827
HQC-5	1360000.0000	1316290.9600	96.7861	0.9679
HQC-6	1280000.0000	1432074.2400	111.8808	1.1188

Table.5.22 Evaluation of matrix effect for SA

Sample name	Analyte peak area		% Matrix effect	Matrix factor
	Spiking in aqueous sample	Spiking in extracted blank matrix (plasma)		
LQC-1	101000.0000	100183.1120	99.1912	0.9919
LQC-2	95600.0000	90363.7968	94.5228	0.9452
LQC-3	107000.0000	105192.2350	98.3105	0.9831
LQC-4	93800.0000	96029.6260	102.3770	1.0238
LQC-5	105000.0000	108739.0500	103.5610	1.0356
LQC-6	98200.0000	96548.6688	98.3184	0.9832
MQC-1	13500000.0000	14442826.5000	106.9839	1.0698
MQC-2	13900000.0000	13406605.6000	96.4504	0.9645
MQC-3	12800000.0000	12630220.8000	98.6736	0.9867
MQC-4	15500000.0000	15757362.0000	101.6604	1.0166
MQC-5	17300000.0000	17014584.6000	98.3502	0.9835
MQC-6	16200000.0000	16659253.8000	102.8349	1.0283
HQC-1	22400000.0000	22072982.4000	98.5401	0.9854
HQC-2	22200000.0000	24269839.2000	109.3236	1.0932
HQC-3	23600000.0000	23346087.6000	98.9241	0.9892
HQC-4	22100000.0000	23973461.2000	108.4772	1.0848
HQC-5	24000000.0000	23350440.0000	97.2935	0.9729
HQC-6	23900000.0000	23081472.8000	96.5752	0.9658

Table.5.23 Evaluation of matrix effect for HCTZ (IS)

Sample name	IS peak area		% Matrix effect	Matrix factor
	Spiking in aqueous sample	Spiking in extracted blank matrix (plasma)		
LQC-1	123000.0000	121573.2000	98.84	0.9884
LQC-2	123000.0000	118030.8000	95.96	0.9596
LQC-3	129000.0000	127013.4000	98.46	0.9846
LQC-4	120000.0000	121836.0000	101.53	1.0153
LQC-5	125000.0000	130400.0000	104.32	1.0432
LQC-6	106000.0000	100933.2000	95.22	0.9522
MQC-1	107000.0000	112339.3000	104.99	1.0499
MQC-2	96000.0000	98073.6000	102.16	1.0216
MQC-3	123000.0000	120195.6000	97.72	0.9772
MQC-4	128000.0000	130572.8000	102.01	1.0201
MQC-5	109000.0000	108204.3000	99.27	0.9927
MQC-6	107000.0000	105159.6000	98.28	0.9828
HQC-1	108000.0000	109933.2000	101.79	1.0179
HQC-2	106000.0000	111554.4000	105.24	1.0524
HQC-3	120000.0000	116316.0000	96.93	0.9693
HQC-4	119000.0000	121748.9000	102.31	1.0231
HQC-5	122000.0000	130857.2000	107.26	1.0726
HQC-6	109000.0000	112956.7000	103.63	1.0363

Table.5.24 Matrix effect of ASA and SA calculated from six different source of human plasma

Analytes	Matrix Effect (ME %) [a]						Mean	SD	CV%
	SET 1*	SET 2	SET 3	SET 4	SET 5	SET 6			
ASA									
LQC (30 ng/mL)	101.8059	96.7577	112.6942	94.0870	95.5701	107.0266	101.3236	7.3262	7.2305
MQC (7500 ng/mL)	95.3552	94.2248	96.5547	104.7331	102.2059	98.9831	98.6761	4.1130	4.1682
HQC (12000 ng/mL)	99.1932	101.4467	95.1807	108.2664	96.7861	111.8808	102.1257	6.6132	6.4756
SA									
LQC (30 ng/mL)	99.1912	94.5228	98.3105	102.3770	103.5610	98.3184	99.3802	3.2361	3.2563
MQC (7500 ng/mL)	106.9839	96.4504	98.6736	101.6604	98.3502	102.8349	100.8256	3.8095	3.7783
HQC (12000 ng/mL)	98.5401	109.3236	98.9241	108.4772	97.2935	96.5752	101.5223	5.7833	5.6966

[a] ME% expressed as the ratio of the mean peak area of an analyte spiked postextraction to the mean peak area of the same analyte standards multiplied by 100. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression.

*SET 1-6 represent the ME% from six different source of human plasma.

5.3.7 Stability study of ASA and SA

5.3.7.1 Stability of ASA, SA and HCTZ (IS) stock solutions

The stock solutions of both the analytes (ASA and SA) and IS (HCTZ) were kept in three different storage conditions and peak area was evaluated of each condition. It has demonstrated in the Figure.5.31, Figure.5.32 and Figure.5.33, where ASA, SA and HCTZ (IS) exhibits significant decrease in peak area with time, under the condition of room temperature and normal deep freezer. The stock solution was stable at −20 ºC for 30 days, i.e., 720 hours and stable at normal deep freezer about a week.

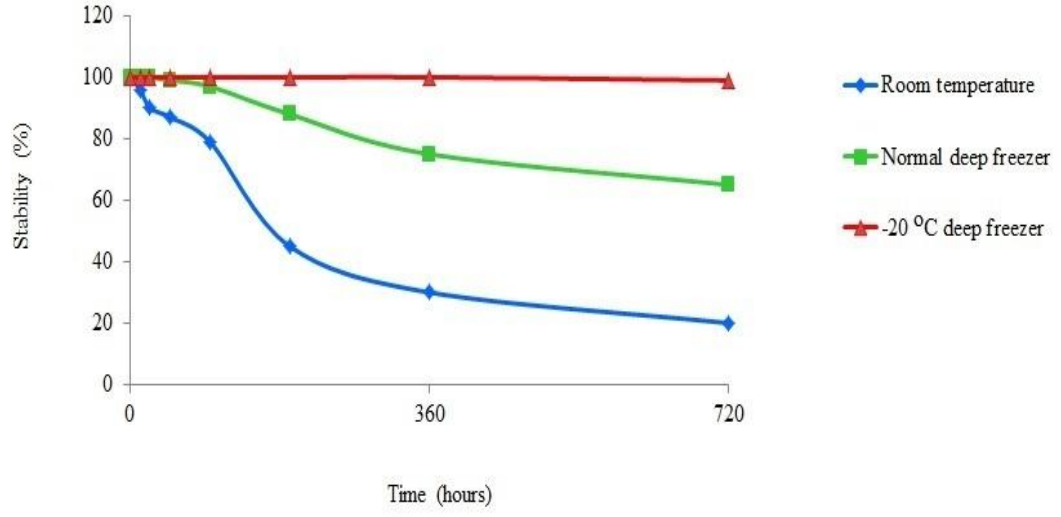


Figure.5.31 Stability of stock solution of ASA in three different conditions

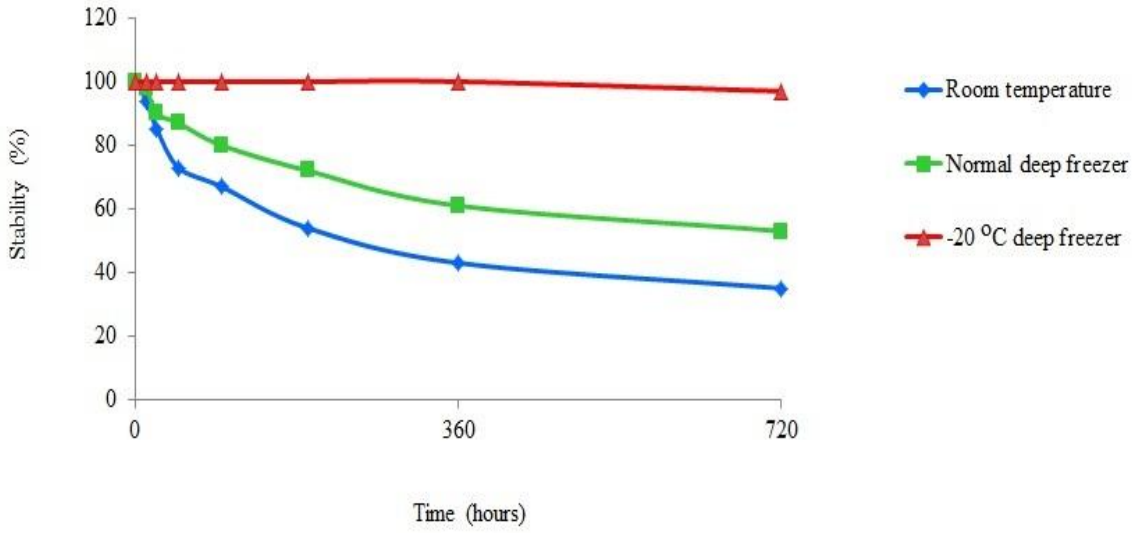


Figure.5.32 Stability of stock solution of SA in three different conditions

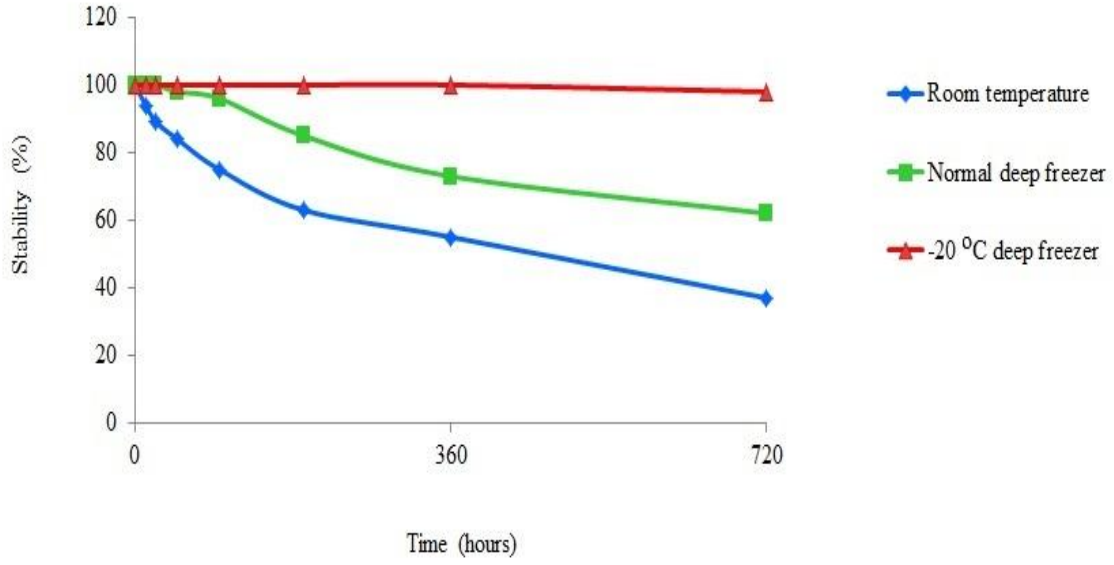


Figure.5.33 Stability of stock solution of HCTZ (IS) in three different conditions

5.3.7.2 Freeze and thaw stability study of ASA and SA

This stability study or test included six replicates (n = 6) of three levels of QC samples (LQC, MQC and HQC) along with a standard or calibration curve (n = 1), the linear regression correlation coefficient (r or r^2) were assessed after three freeze thaw cycles comparing against freshly thaw samples of the same concentration. The freeze thaw stability of ASA and SA ranged between 93.05% to 99.29% and 91.79% to 97.64%, respectively (Table.5.25 and Table.5.26). Results indicate that estimated concentrations for the analytes at LQC, MQC and HQC samples varied within the assay acceptance limits (±15%) of the nominal concentrations as per the guidelines (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.25 Freeze thaw stability study of ASA

	Freshly thaw (Day - 0)			After three freeze thaw cycles		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.5000	7520.0000	12600.0000	30.8000	7690.0000	11500.0000
2	35.2000	7710.0000	11700.0000	29.2000	7700.0000	11900.0000
3	32.8000	7480.0000	11800.0000	30.3000	7250.0000	12300.0000
4	31.9000	7660.0000	13600.0000	28.6000	7570.0000	11800.0000
5	30.1000	7580.0000	11900.0000	30.0000	7340.0000	12400.0000
6	32.8000	7470.0000	13800.0000	31.9000	7550.0000	12700.0000
Mean	32.3833	7570.0000	12566.6667	30.1333	7516.6667	12100.0000
SD	1.7034	98.3870	935.2362	1.1690	184.3547	442.7189
CV%	5.2602	1.2997	7.4422	3.8796	2.4526	3.6588
% Freeze thaw stability				**93.0520**	**99.2955**	**96.2865**

Table.5.26 Freeze thaw stability study of SA

	Freshly thaw (Day - 0)			After three freeze thaw cycles		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.6000	7660.0000	11900.0000	30.6000	7750.0000	11500.0000
2	32.3000	7560.0000	13700.0000	29.2000	7450.0000	12200.0000
3	33.3000	7730.0000	12400.0000	30.3000	7600.0000	11700.0000
4	34.4000	7810.0000	14400.0000	29.1000	7290.0000	11800.0000
5	32.2000	7470.0000	11800.0000	28.7000	7160.0000	12400.0000
6	32.5000	7620.0000	12900.0000	32.3000	7520.0000	13100.0000
Mean	32.7167	7641.6667	12850.0000	30.0333	7461.6667	12116.6667
SD	0.9908	120.8994	1032.9569	1.3322	212.7361	584.5226
CV%	3.0284	1.5821	8.0386	4.4356	2.8511	4.8241
% Freeze thaw stability				**91.7983**	**97.6445**	**94.2931**

5.3.7.3 Bench top stability study of ASA and SA

The bench top stability was performed for 8 hours in room temperature, around at 24 °C and evaluated at three concentrations of low, medium and high QC levels (LQC, MQC and HQC) in six replicates (n = 6) along with a standard or calibration curve (n = 1). According to Table.5.27 and Table.5.28, the bench top stability ranged from 98.25% to 101.59% for ASA and 97.53% to 102.64% for SA, respectively and also all results were found to be within the acceptable limit.

Table.5.27 Bench top stability study of ASA

	Day - 0			**Bench top stability for 8 hours**		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.5000	7520.0000	12600.0000	32.4000	7590.0000	12700.0000
2	35.2000	7710.0000	11700.0000	31.1000	7470.0000	11900.0000
3	32.8000	7480.0000	11800.0000	30.7000	7460.0000	13400.0000
4	31.9000	7660.0000	13600.0000	33.2000	7580.0000	11800.0000
5	30.1000	7580.0000	11900.0000	30.6000	7720.0000	13300.0000
6	32.8000	7470.0000	13800.0000	32.9000	7680.0000	13500.0000
Mean	32.3833	7570.0000	12566.6667	31.8167	7583.3333	12766.6667
SD	1.7034	98.3870	935.2362	1.1548	105.9560	763.3261
CV%	5.2602	1.2997	7.4422	3.6297	1.3972	5.9791
% Bench top stability				**98.2501**	**100.1761**	**101.5915**

Table.5.28 Bench top stability study of SA

	Day - 0			Bench top stability for 8 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.6000	7660.0000	11900.0000	32.8000	7740.0000	11700.0000
2	32.3000	7560.0000	13700.0000	34.5000	7490.0000	14100.0000
3	33.3000	7730.0000	12400.0000	32.1000	7670.0000	12400.0000
4	34.4000	7810.0000	14400.0000	35.7000	7680.0000	13200.0000
5	32.2000	7470.0000	11800.0000	32.9000	7580.0000	11600.0000
6	32.5000	7620.0000	12900.0000	33.5000	7630.0000	12200.0000
Mean	32.7167	7641.6667	12850.0000	33.5833	7631.6667	12533.3333
SD	0.9908	120.8994	1032.9569	1.3121	87.5024	958.4710
CV%	3.0284	1.5821	8.0386	3.9071	1.1466	7.6474
% Bench top stability				**102.6490**	**99.8691**	**97.5357**

5.3.7.4 Post-preparative or autosampler stability study of ASA and SA

The post-preparative or autosampler stability study was done at three concentrations of low, medium and high QC levels (LQC, MQC and HQC) in six replicates (n = 6) along with the calibration standards (n = 1). After extraction, the samples were placed in the autosampler cabinet at 10 to 15 °C for 24 hours and then analyzed and the obtained concentrations were compared with the actual values. Now, the percentage (%) of post-preparative or autosampler stability was varied from 95.41% to 101.15% for ASA and 94.44% to 102.02% for SA, respectively (see Table.5.29 and Table.5.30). Results show that predicted concentrations for the analytes at LQC, MQC and HQC samples deviated within the acceptance criteria (±15%) of the nominal concentrations according to the guidelines (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.29 Post-preparative or autosampler stability study of ASA

	Day - 0			Autosampler stability for 24 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.5000	7520.0000	12600.0000	31.8000	7490.0000	12500.0000
2	35.2000	7710.0000	11700.0000	33.5000	7570.0000	12300.0000
3	32.8000	7480.0000	11800.0000	29.7000	7690.0000	11700.0000
4	31.9000	7660.0000	13600.0000	30.6000	7730.0000	14100.0000
5	30.1000	7580.0000	11900.0000	31.7000	7480.0000	12200.0000
6	32.8000	7470.0000	13800.0000	30.4000	7560.0000	11900.0000
Mean	32.3833	7570.0000	12566.6667	30.9000	7586.667	12450.0000
SD	1.7034	98.3870	935.2362	1.34969	102.8915	857.3214
CV%	5.2602	1.2997	7.4422	4.3679	1.3562	6.8861
% Autosampler stability				**95.4195**	**101.1556**	**99.0716**

Table.5.30 Post-preparative or autosampler stability study of SA

	Day - 0			Autosampler stability for 24 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.6000	7660.0000	11900.0000	32.7000	7580.0000	13800.0000
2	32.3000	7560.0000	13700.0000	33.5000	7670.0000	12300.0000
3	33.3000	7730.0000	12400.0000	34.6000	7680.0000	14100.0000
4	34.4000	7810.0000	14400.0000	32.1000	7790.0000	11800.0000
5	32.2000	7470.0000	11800.0000	33.9000	7740.0000	11900.0000
6	32.5000	7620.0000	12900.0000	31.7000	7450.0000	12400.0000
Mean	32.7167	7641.6667	12850.0000	30.9000	7651.667	12716.6667
SD	0.9908	120.8994	1032.9569	1.11071	121.5593	986.7455
CV%	3.0284	1.5821	8.0386	3.5945	1.5887	7.7595
% Autosampler stability				**94.4473**	**102.0222**	**98.9624**

5.3.7.5 Dry state stability study of ASA and SA

Table.5.31 Dry state stability study of ASA

	Day - 0			Dry state stability for 24 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.5000	7520.0000	12600.0000	30.9000	7515.0000	12400.0000
2	35.2000	7710.0000	11700.0000	34.5000	7700.0000	11500.0000
3	32.8000	7480.0000	11800.0000	33.7000	7530.0000	12100.0000
4	31.9000	7660.0000	13600.0000	32.9000	7720.0000	13200.0000
5	30.1000	7580.0000	11900.0000	30.2000	7480.0000	12700.0000
6	32.8000	7470.0000	13800.0000	31.7000	7495.0000	13600.0000
Mean	32.3833	7570.0000	12566.6667	32.3167	7573.3333	12583.3333
SD	1.7034	98.3870	935.2362	1.6666	107.4089	757.4079
CV%	5.2602	1.2997	7.4422	5.1572	1.4183	6.0191
% Dry state stability				**99.7941**	**100.0440**	**100.1326**

Table.5.32 Dry state stability study of SA

	Day - 0			Dry state stability for 24 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.6000	7660.0000	11900.0000	31.5000	7680.0000	12500.0000
2	32.3000	7560.0000	13700.0000	33.2000	7530.0000	13800.0000
3	33.3000	7730.0000	12400.0000	32.9000	7710.0000	11800.0000
4	34.4000	7810.0000	14400.0000	34.3000	7590.0000	15600.0000
5	32.2000	7470.0000	11800.0000	33.4000	7540.0000	12700.0000
6	32.5000	7620.0000	12900.0000	33.7000	7640.0000	12600.0000
Mean	32.7167	7641.6667	12850.0000	33.1667	7615.0000	13166.6667
SD	0.9908	120.8994	1032.9569	0.9459	73.9594	1354.4987
CV%	3.0284	1.5821	8.0386	2.8519	0.9712	10.2873
% Dry state stability				**101.3754**	**99.6510**	**102.4643**

5.3.7.6 Short term stability study of ASA and SA

The short term stability study was performed at three QC levels; LQC, MQC and HQC samples in six replicates (n = 6) along with a standard or calibration curve (n = 1) and were stored in deep freezer at -20 ºC for 7 days. After 7 days, the samples were analysed and the obtained concentrations were compared with the actual values of QC samples containing the same concentrations of the ASA and SA. The short term stability of ASA and SA ranged between 96.02% to 99.40% and 99.13% to 100.02%, respectively (Table.5.33 and Table.5.34). The results of calculated concentrations for the analytes at LQC, MQC and HQC samples were within the assay acceptance criteria, i.e., ±15% of the nominal concentrations according to the guidelines (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.33 Short term stability study of ASA

	Day - 0			**Short term stability for 7 days**		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.5000	7520.0000	12600.0000	30.2000	7480.0000	12200.0000
2	35.2000	7710.0000	11700.0000	33.5000	7690.0000	11400.0000
3	32.8000	7480.0000	11800.0000	31.7000	7450.0000	11300.0000
4	31.9000	7660.0000	13600.0000	30.5000	7610.0000	12800.0000
5	30.1000	7580.0000	11900.0000	29.8000	7530.0000	11300.0000
6	32.8000	7470.0000	13800.0000	32.6000	7390.0000	13400.0000
Mean	32.3833	7570.0000	12566.6667	31.3833	7525.0000	12066.6667
SD	1.7034	98.3870	935.2362	1.4662	109.8636	889.1944
CV%	5.2602	1.2997	7.4422	4.6718	1.4600	7.3690
% Short term stability				**96.9120**	**99.4055**	**96.0212**

Table.5.34 Short term stability study of SA

	Day - 0			Short term stability for 7 days		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.6000	7660.0000	11900.0000	31.2000	7640.0000	12300.0000
2	32.3000	7560.0000	13700.0000	31.8000	7550.0000	13500.0000
3	33.3000	7730.0000	12400.0000	33.1000	7750.0000	12100.0000
4	34.4000	7810.0000	14400.0000	34.3000	7790.0000	14200.0000
5	32.2000	7470.0000	11800.0000	31.9000	7450.0000	12200.0000
6	32.5000	7620.0000	12900.0000	32.3000	7680.0000	12500.0000
Mean	32.7167	7641.6667	12850.0000	32.4333	7643.3333	12800.0000
SD	0.9908	120.8994	1032.9569	1.1094	126.7544	853.2292
CV%	3.0284	1.5821	8.0386	3.4204	1.6584	6.6659
% Short term stability				**99.1340**	**100.0218**	**99.6109**

5.3.7.7 Long term stability study of ASA and SA

The long term stability study was done at LQC, MQC and HQC in six replicates (n = 6) along with a standard or calibration curve (n = 1) and were stored in deep freezer at -20 ºC for 30 days. The samples were analysed after 30 days and the obtained concentrations were compared with the actual values of QC samples containing the same concentrations of the ASA and SA. The long term stability was varied from 94.80% to 99.20% for ASA and 97.01% to 99.17% for SA, respectively (see Table.5.35 and Table.5.36). The results of calculated concentrations for the analytes at LQC, MQC and HQC samples were within the assay acceptance limits (±15%) of the nominal concentrations as per the guidelines (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.35 Long term stability study of ASA

	Day - 0			Long term stability for 30 days		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.5000	7520.0000	12600.0000	29.8000	7440.0000	11800.0000
2	35.2000	7710.0000	11700.0000	32.0000	7680.0000	11300.0000
3	32.8000	7480.0000	11800.0000	31.8000	7420.0000	11400.0000
4	31.9000	7660.0000	13600.0000	30.2000	7570.0000	12900.0000
5	30.1000	7580.0000	11900.0000	29.5000	7510.0000	11500.0000
6	32.8000	7470.0000	13800.0000	30.9000	7440.0000	12800.0000
Mean	32.3833	7570.0000	12566.6667	30.7000	7510.0000	11950.0000
SD	1.7034	98.3870	935.2362	1.0431	100.3992	717.6350
CV%	5.2602	1.2997	7.4422	3.3976	1.3369	6.0053
% Long term stability				**94.8019**	**99.2074**	**95.0928**

Table.5.36 Long term stability study of SA

	Day - 0			Long term stability for 30 days		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	31.6000	7660.0000	11900.0000	31.8000	7610.0000	11600.0000
2	32.3000	7560.0000	13700.0000	30.9000	7580.0000	13100.0000
3	33.3000	7730.0000	12400.0000	31.7000	7530.0000	12200.0000
4	34.4000	7810.0000	14400.0000	32.6000	7670.0000	14100.0000
5	32.2000	7470.0000	11800.0000	33.4000	7460.0000	11500.0000
6	32.5000	7620.0000	12900.0000	32.1000	7620.0000	12300.0000
Mean	32.7167	7641.6667	12850.0000	32.0833	7578.3333	12466.6667
SD	0.9908	120.8994	1032.9569	0.8519	74.1395	985.2242
CV%	3.0284	1.5821	8.0386	2.6552	0.9783	7.9029
% Long term stability				**98.0642**	**99.1712**	**97.0169**

5.3.7.8 Dilution integrity of ASA and SA

The dilution integrity was performed by preparing the concentration four times of ULOQ (upper limit of quantification) i.e., 60000 ng/mL (60 µg/mL), which was further diluted five and ten times (5 fold and 10 fold) with drug free plasma (human blank plasma) in six replicates (n = 6) and these diluted samples were run against calibration standards. The dilution integrity data of ASA and SA are summarized in Table.5.37, where the precision (%RSD) values were 5.49% and 4.33% for ASA and 3.51% and 4.15% for SA and also with the accuracy of 99.44% and 99.72% for ASA and 98.05% and 97.13% for SA, respectively. These results support sample dilution up to 10 fold for bioanalysis.

Table.5.37 Dilution integrity of ASA and SA in human plasma

	Dilution for ASA		**Dilution for SA**	
	5 fold	**10 fold**	**5 fold**	**10 fold**
	Concentration		**Concentration**	
Serial No.	**12000 ng/mL**	**6000 ng/mL**	**12000 ng/mL**	**6000 ng/mL**
1	11600.0000	5950.0000	11600.0000	5820.0000
2	12400.0000	6230.0000	11400.0000	5730.0000
3	11300.0000	5780.0000	12300.0000	5610.0000
4	12800.0000	5660.0000	11800.0000	6240.0000
5	11200.0000	6340.0000	12200.0000	5740.0000
6	12300.0000	5940.0000	11300.0000	6430.0000
Mean	11933.3333	5983.3333	11766.6667	5828.0000
SD	656.2520	259.5894	413.1182	242.2189
Accuracy	99.4444	99.7222	98.0556	97.1333
Precision (%RSD)	5.4993	4.3385	3.5109	4.1561

SD, Standard deviation; RSD (%), Relative standard deviation [(SD / mean) x 100]

5.4 Application to bioequivalence study and assessment of pharmacokinetic parameters in healthy human subjects : a single oral dose, open label, randomized, two period, two treatment and two-way cross over study

The validated bioanalytical method was successfully applied to determine the concentration of both ASA and SA in human plasma for a bioequivalence (BE) study in 30 healthy Indian male volunteers under fasting conditions after an oral administration of Aspirin (acetylsalicylic acid) 150 mg film coated tablets. The BE study was approved by the Bio-Ethics Committee (HURIP Independent Bioethics Committee, Reg. No. ECR/103/Indt/WB/2013) of Bioequivalence Study Centre, Jadavpur University, Kolkata, India.

Thirty healthy adult male volunteers were participated in this comparative, i.e., bioequivalence study at Clinical Pharmacology Unit (CPU) of Bioequivalence Study Center, Jadavpur University and the whole work was supported by TAAB Biostudy Services, Jadavpur, Kolkata. The mean age of the selected volunteers was 26.27 ± 6.19 years with a range of 18 to 40 years; the mean body weight was 64.87 ± 6.12 kg with a range of 53 to 76 kg; the mean body height was 171.23 ± 5.35 cm with a range of 158 to 181 cm and finally, the BMI was 22.10 ± 1.49 Kg/m^2 with range of 19.15 to 23.94 Kg/m^2 (see Table.5.38).

5.4.1 Reference and Test preparation for Aspirin (acetylsalicylic acid, ASA)

- **Reference Preparation (A) :**

  CARDIOMAGNYL (Acetylsalicylic Acid 150 mg film coated tablet)

  Mfg. by : NYCOMED DANMARK APS, DENMARK

  Batch No. : 10926915

  Mfg. Date : 07/2013 Exp. Date : 06/2018

- **Test Preparation (B) :**

  CARDIOMAGNUM (Acetylsalicylic Acid 150 mg film coated tablet)

  Mfg. by : PHARMLAND JV LLC, OFFICE 3, 124 LENINSKAYA STR., 222603 NESVIZH, MINSK REGION, REPUBLIC OF BELARUS

  Batch No. : 010213

  Mfg. Date : 02/2013 Exp. Date : 02/2015

Table.5.38 Demographic data of 30 volunteers

Vol. No.	Sex	Age (yr)	Height (cm)	Weight (kg.)	BMI (Kg/m^2)
1	M	32	166	64	23.23
2	M	28	169	60	21.01
3	M	23	171	56	19.15
4	M	34	167	58	20.80
5	M	21	173	64	21.38
6	M	32	176	67	21.63
7	M	20	181	70	21.37
8	M	29	169	65	22.76
9	M	18	172	69	23.32
10	M	20	174	72	23.78
11	M	23	168	66	23.38
12	M	22	170	63	21.80
13	M	18	180	70	21.60
14	M	22	177	75	23.94
15	M	32	168	62	21.97
16	M	30	164	53	19.71
17	M	37	169	62	21.71
18	M	40	171	69	23.60
19	M	21	172	70	23.66
20	M	24	172	62	20.96
21	M	23	176	73	23.57
22	M	37	180	76	23.46
23	M	29	169	61	21.36
24	M	22	171	70	23.94
25	M	18	167	65	23.31
26	M	25	174	70	23.12
27	M	30	158	59	23.63
28	M	29	166	53	19.23
29	M	20	166	57	20.69
30	M	29	181	65	19.84
Mean		**26.27**	**171.23**	**64.87**	**22.10**
S.D.		**6.19**	**5.35**	**6.12**	**1.49**

Table.5.39 Mode of treatment

Subject No.	PERIOD I	PERIOD II
1	A	B
2	A	B
3	A	B
4	B	A
5	B	A
6	A	B
7	B	A
8	B	A
9	B	A
10	B	A
11	A	B
12	A	B
13	A	B
14	A	B
15	A	B
16	B	A
17	B	A
18	A	B
19	B	A
20	B	A
21	B	A
22	B	A
23	A	B
24	A	B
25	A	B
26	A	B
27	A	B
28	B	A
29	B	A
30	A	B

A – Reference Preparation B – Test Preparation

Table.5.40 Bioequivalence of CARDIOMAGNUM (ACETYLSALICYLIC ACID 150 MG FILM COATED TABLET)
Plasma concentration of Acetylsalicylic Acid (ng/mL) of the Reference Preparation (A) in (1 to 15) volunteers

Vol. No.	Time (in hours)																			
	0	**0.167**	**0.33**	**0.5**	**0.67**	**0.83**	**1.0**	**1.25**	**1.5**	**1.75**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**8.0**	**12.0**	**24.0**
1	0	524.4	1264.0	908.0	725.4	608.4	587.0	524.1	443.5	326.1	288.0	252.7	243.9	189.4	105.6	74.6	40.8	20.8	12.8	ND
2	0	638.7	1021.0	809.6	705.6	634.5	548.5	469.7	382.5	305.3	278.9	224.6	186.2	110.8	76.5	49.3	30.8	19.9	14.5	ND
3	0	598.3	1265.0	1076.8	825.6	735.3	659.0	487.2	408.9	352.4	302.4	248.2	191.3	147.1	100.6	74.4	50.1	34.2	21.4	ND
4	0	569.3	1050.0	1660.5	1025.8	745.2	569.3	512.8	463.8	395.4	384.9	303.8	275.4	200.1	146.4	105.7	60.5	30.9	19.9	ND
5	0	689.3	1291.0	1120.7	869.3	745.2	604.0	524.0	412.8	354.2	294.4	257.0	179.0	145.8	103.0	76.4	55.5	29.9	15.8	ND
6	0	498.8	864.1	1280.0	945.9	693.6	512.4	441.3	390.7	305.2	234.9	180.6	127.4	80.8	55.5	43.0	31.7	20.9	14.3	ND
7	0	401.3	957.6	741.3	662.6	520.6	485.8	401.3	356.4	290.5	269.3	203.3	169.8	125.1	91.5	62.3	38.3	23.7	15.6	ND
8	0	509.5	846.9	724.4	689.3	627.9	603.2	503.0	453.7	366.9	227.1	198.7	142.6	110.6	82.9	66.5	47.2	27.5	20.5	ND
9	0	700.3	1437.0	1032.1	824.8	693.8	583.4	412.8	342.9	310.5	289.5	201.8	149.8	107.8	85.9	72.5	60.2	28.8	21.6	ND
10	0	305.9	1201.0	965.3	774.2	561.9	456.8	401.2	378.0	319.6	301.3	256.4	156.4	99.6	75.5	53.3	37.9	26.9	16.3	ND
11	0	402.6	1448.0	1005.6	789.6	600.3	569.5	452.3	369.3	300.2	274.6	200.5	138.8	95.7	78.6	50.3	27.9	20.4	14.8	ND
12	0	369.9	630.5	929.7	742.6	617.9	570.6	501.5	412.6	360.9	307.8	240.4	169.4	108.6	87.9	50.3	30.9	18.6	12.6	ND
13	0	520.5	1065.6	823.7	702.6	612.6	503.2	452.2	401.0	378.6	302.4	269.1	175.1	121.3	78.9	60.3	42.2	28.5	18.5	ND
14	0	516.5	1245.0	992.9	891.5	834.8	769.0	654.2	542.9	508.2	458.4	305.7	243.9	202.2	152.8	101.8	67.5	42.1	22.5	ND
15	0	629.1	1480.3	1270.3	1102.3	1091.9	989.4	936.9	738.3	538.9	421.8	392.5	319.9	234.9	181.9	120.4	56.0	27.5	14.3	ND

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.40 (continued) for 16 to 30 volunteers

Vol. No.	Time (in hours)																			
	0	**0.167**	**0.33**	**0.5**	**0.67**	**0.83**	**1.0**	**1.25**	**1.5**	**1.75**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**8.0**	**12.0**	**24.0**
16	0	757.8	1038.5	1503.9	1222.0	950.8	718.1	670.9	540.7	484.8	445.6	384.4	275.0	243.4	191.6	124.5	74.0	52.4	28.9	ND
17	0	560.8	834.3	1215.6	810.4	637.0	560.8	505.1	456.8	369.5	329.1	219.3	185.3	162.1	144.2	104.1	59.6	24.4	19.6	ND
18	0	679.0	1271.6	1003.9	856.3	734.0	604.9	536.1	406.6	348.9	289.9	223.1	166.3	143.6	101.4	75.2	52.6	29.4	15.6	ND
19	0	491.3	851.1	1260.8	1031.7	783.2	614.7	530.6	445.8	300.7	231.3	177.9	122.5	79.6	54.7	42.3	31.2	19.6	14.1	ND
20	0	395.3	643.2	930.2	652.6	512.8	478.5	395.3	351.0	280.1	265.3	200.3	167.3	129.2	90.1	61.4	35.7	23.3	16.4	ND
21	0	501.9	834.2	703.5	679.0	608.5	594.1	495.4	426.9	361.4	223.7	192.7	140.4	109.0	81.6	65.5	40.5	27.0	20.2	ND
22	0	689.8	1415.5	956.6	745.4	683.4	574.7	406.6	317.8	305.8	280.2	198.8	147.6	100.2	84.6	71.4	59.3	28.3	21.3	ND
23	0	301.3	683.0	950.8	762.6	553.4	410.0	355.2	342.3	304.8	296.8	242.5	174.0	128.1	74.4	52.5	37.3	24.5	16.1	ND
24	0	396.6	1426.3	990.5	777.8	591.3	561.0	445.5	363.8	295.7	270.5	197.5	136.7	94.3	77.4	49.5	27.5	20.1	14.6	ND
25	0	364.3	621.0	915.8	731.4	608.6	542.0	494.0	426.4	355.5	303.2	236.8	166.9	126.9	96.6	49.5	30.5	18.3	12.4	ND
26	0	512.7	749.6	811.3	692.0	603.4	495.7	442.4	395.0	372.9	277.9	265.1	172.5	109.5	77.7	50.4	41.6	23.1	17.2	ND
27	0	490.7	1182.8	943.2	866.0	850.0	749.5	621.5	536.7	492.3	475.4	309.5	249.8	201.6	173.7	115.7	81.2	47.6	21.4	ND
28	0	597.7	783.9	1206.7	1080.5	1037.3	940.0	861.6	720.3	463.0	429.7	375.9	283.9	220.2	172.8	118.2	53.2	27.0	13.6	ND
29	0	719.9	1651.6	1428.7	1160.9	903.3	682.2	643.1	513.6	470.1	423.3	365.2	263.2	231.2	187.7	118.2	69.4	52.6	27.5	ND
30	0	432.7	782.5	1053.8	759.9	697.3	532.7	479.9	434.0	370.0	364.2	264.3	207.7	167.3	137.0	98.9	56.6	28.9	18.6	ND
Mean	**0**	**525.53**	**1061.21**	**1040.54**	**836.85**	**702.61**	**602.32**	**518.58**	**439.16**	**366.27**	**318.05**	**252.95**	**190.93**	**144.20**	**108.30**	**75.27**	**47.58**	**28.23**	**17.75**	**ND**
S. D.	**0**	**126.29**	**294.51**	**229.37**	**155.81**	**145.94**	**127.61**	**129.54**	**98.41**	**72.03**	**72.36**	**61.88**	**53.12**	**49.14**	**41.07**	**26.64**	**14.78**	**9.24**	**4.16**	**ND**
C.V.%	**0**	**24.03**	**27.75**	**22.04**	**18.62**	**20.77**	**21.19**	**24.98**	**22.41**	**19.67**	**22.75**	**24.46**	**27.82**	**34.08**	**37.92**	**35.39**	**31.06**	**32.72**	**23.46**	**ND**

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.41 Bioequivalence of CARDIOMAGNUM (ACETYLSALICYLIC ACID 150 MG FILM COATED TABLET)
Plasma concentration of Salicylic Acid (ng/mL) of the Reference Preparation (A) in (1 to 15) volunteers

Vol. No.	Time (in hours)																			
	0	**0.167**	**0.33**	**0.5**	**0.67**	**0.83**	**1.0**	**1.25**	**1.5**	**1.75**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**8.0**	**12.0**	**24.0**
1	0	2520.1	2870.0	3070.0	3450.3	5160.1	6670.0	7230.3	7873.5	8258.3	8990.0	8300.0	7800.0	6360.0	5040.0	3258.9	3010.0	2450.5	1022.8	ND
2	0	4638.7	9850.0	10600.0	10254.6	9858.5	9385.3	9042.7	8769.8	7526.8	6900.0	6600.0	5925.8	5470.0	5070.0	4626.3	4310.0	3540.3	2015.4	ND
3	0	3769.3	9230.0	10600.0	10695.0	10775.8	10900.0	10810.5	10785.6	10750.3	10700.0	9710.0	8130.0	6910.0	6420.0	5020.1	3250.0	2960.4	1590.8	ND
4	0	6052.3	7200.0	8560.0	8998.2	9245.3	9680.3	9220.5	9000.5	8750.3	8430.0	8180.0	7830.0	7510.0	7380.0	6650.3	5810.0	4025.6	2150.9	ND
5	0	5090.5	9390.0	10800.0	10705.3	10655.3	10600.0	10450.3	10100.5	9980.6	9640.0	9110.0	8810.0	8050.0	6720.0	5050.6	3650.0	2150.8	1590.2	ND
6	0	6050.3	8990.0	11500.0	11280.5	10965.3	10650.0	10400.3	10290.9	9960.5	9590.0	8780.0	8020.0	6810.0	5190.0	4420.3	3270.0	2500.6	2000.5	ND
7	0	2510.3	3720.0	4550.0	5005.8	5980.6	6570.0	7050.6	7650.5	8250.3	8795.0	8540.0	6490.0	4860.0	3540.0	3000.5	2620.0	2140.5	1568.7	ND
8	0	2050.6	3580.0	4660.0	5980.8	7750.5	8820.0	8750.6	8490.3	8370.5	7540.0	6840.0	5400.0	4590.0	3900.0	3190.3	2850.0	2250.4	1850.6	ND
9	0	4700.3	5990.0	8940.0	8245.8	8030.8	7490.0	7150.3	6895.5	6650.3	6420.0	3790.0	3340.0	3010.0	2740.0	2450.5	1830.0	1460.3	1025.5	ND
10	0	3005.8	4720.0	6270.0	7740.3	9261.9	10400.0	9960.5	9025.5	8690.3	8230.0	7160.0	6850.0	6140.0	5380.0	4500.5	3610.0	2480.3	1690.3	ND
11	0	5560.3	6580.0	7360.0	7895.6	8600.3	9660.0	9250.5	8900.2	8740.5	8550.0	6660.0	6010.0	5410.0	4660.0	3240.3	2260.0	1890.6	1460.5	ND
12	0	2400.5	3010.0	5620.0	5990.5	6500.4	7710.0	7960.6	8240.5	8590.2	8780.0	7590.0	6330.0	5780.0	4450.0	3560.5	2800.0	2005.5	1470.8	ND
13	0	2150.5	2870.0	5500.0	6500.3	7860.5	9640.0	10200.5	10450.3	10750.3	10900.0	8410.0	7330.0	6120.0	3510.0	3010.4	2580.0	2015.6	1475.3	ND
14	0	2516.5	4510.0	4920.0	5291.5	5534.8	5960.0	6654.2	7042.9	7408.2	7840.0	9950.0	8920.0	5540.0	4680.0	3501.8	2770.0	2005.5	1022.5	ND
15	0	5425.6	7830.0	8570.0	8750.6	9050.3	10560.0	10105.5	8738.3	7538.9	6200.0	5020.0	4460.0	3950.0	3310.0	2680.3	2100.0	1690.3	1240.8	ND

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.41 (continued) for 16 to 30 volunteers

Vol. No.	Time (in hours)																			
	0	**0.167**	**0.33**	**0.5**	**0.67**	**0.83**	**1.0**	**1.25**	**1.5**	**1.75**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**8.0**	**12.0**	**24.0**
16	0	2560.8	3770.0	5810.0	6780.5	7520.8	8430.0	8870.9	9250.7	9650.8	10200.0	10800.0	8640.0	7590.0	6460.0	5025.5	3520.0	2690.3	1528.9	ND
17	0	5560.8	9550.0	11400.0	11250.4	10500.5	9850.5	9505.1	9056.8	8369.5	8020.0	7410.0	6930.0	6320.0	5860.0	5425.2	4130.0	3150.3	1429.6	ND
18	0	6079.0	8810.0	10100.0	10290.3	10525.6	10700.0	10480.3	10240.6	9750.2	9560.0	9110.0	8180.0	7230.0	6720.0	5075.2	3320.0	2780.5	1590.6	ND
19	0	3591.3	6395.0	7750.0	9031.7	9525.6	11600.0	9240.5	8060.3	6900.8	6560.3	5289.6	4340.0	4070.0	3690.0	3081.0	2870.0	2405.6	1810.4	ND
20	0	3675.3	4260.2	5800.0	7080.3	8250.5	10700.0	8890.3	7980.5	6975.3	6070.0	5590.0	4870.0	4080.0	3530.0	2825.3	2090.0	1825.6	1540.9	ND
21	0	4025.9	5210.0	6805.0	8200.3	9075.6	9960.0	9550.4	9285.3	8650.3	8400.0	7800.0	7580.0	7070.0	6800.0	6230.3	3570.0	2050.5	1280.9	ND
22	0	3089.8	6640.0	8270.0	8550.3	8848.7	9010.0	9350.2	9865.1	10100.8	10808.0	9430.0	8840.0	7720.0	6740.0	5030.3	4420.0	2896.3	2087.5	ND
23	0	1001.3	1530.0	4080.0	6978.8	7542.6	8200.0	8564.9	8930.3	9245.6	9580.0	6860.0	5370.0	4810.0	3740.0	3150.3	2860.0	2075.6	1660.4	ND
24	0	4350.8	6100.0	8500.0	8156.3	7740.6	7540.0	7290.3	7050.8	6760.9	6550.0	4020.0	3550.0	3060.0	2720.0	2145.6	1575.5	1260.9	1024.7	ND
25	0	1050.3	4460.0	5740.0	7731.4	8608.6	10100.0	9494.0	8426.4	8055.5	7990.0	7010.0	6830.0	6320.0	6060.0	5485.2	3320.0	2740.5	1845.6	ND
26	0	5012.7	8290.0	8500.0	8780.3	9130.7	9350.0	9024.5	8798.3	8540.3	8080.0	7450.0	6980.0	6250.0	5640.0	3450.7	2070.0	1645.2	1230.4	ND
27	0	1480.7	2860.0	5200.0	6366.0	6850.0	7520.0	7850.5	8010.7	8150.3	8600.0	7370.0	6530.0	5758.0	4530.0	3520.7	2750.0	1687.5	1421.3	ND
28	0	2597.7	3090.0	5930.0	6590.3	8125.7	9630.0	9240.8	8720.3	8463.0	8020.0	7610.0	7110.0	6880.0	3510.0	2945.8	2530.0	1690.9	1003.5	ND
29	0	2719.9	4460.0	4730.0	5002.7	5360.9	5710.0	6643.1	7513.6	8470.1	9250.0	8510.0	7840.0	4370.0	3820.0	2470.8	2198.5	1830.7	1505.6	ND
30	0	3068.6	7260.0	8910.0	8545.9	8125.6	7895.6	7760.4	7494.0	7270.0	7010.0	6850.0	6150.0	4680.0	3700.0	2890.4	2136.9	1890.4	1465.4	ND
Mean	**0**	**3610.20**	**5767.51**	**7301.50**	**7870.67**	**8365.40**	**9029.72**	**8866.45**	**8697.94**	**8518.97**	**8406.78**	**7524.99**	**6712.86**	**5757.27**	**4850.33**	**3897.11**	**3002.70**	**2272.92**	**1520.04**	**ND**
S. D.	**0**	**1524.90**	**2436.93**	**2381.33**	**1984.66**	**1613.81**	**1581.28**	**1207.62**	**1047.42**	**1129.81**	**1371.17**	**1662.71**	**1544.60**	**1374.61**	**1364.79**	**1204.77**	**894.96**	**622.06**	**324.94**	**ND**
C.V.%	**0**	**42.24**	**42.25**	**32.61**	**25.22**	**19.29**	**17.51**	**13.62**	**12.04**	**13.26**	**16.31**	**22.10**	**23.01**	**23.88**	**28.14**	**30.91**	**29.81**	**27.37**	**21.38**	**ND**

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.42 Bioequivalence of CARDIOMAGNUM (ACETYLSALICYLIC ACID 150 MG FILM COATED TABLET)
Plasma concentration of Acetylsalicylic Acid (ng/mL) of the Test Preparation (B) in (1 to 15) volunteers

Vol. No.	Time (in hours)																			
	0	**0.167**	**0.33**	**0.5**	**0.67**	**0.83**	**1.0**	**1.25**	**1.5**	**1.75**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**8.0**	**12.0**	**24.0**
1	0	548.60	1000.00	939.00	834.00	803.40	745.00	687.40	589.60	524.10	416.00	356.00	250.00	134.60	89.80	70.40	43.50	25.80	17.60	ND
2	0	742.50	1280.00	2270.00	1698.40	1405.60	1270.00	1004.70	863.90	500.26	329.00	285.30	146.00	96.10	73.50	65.80	52.70	21.80	16.80	ND
3	0	668.90	1780.00	1250.00	1025.60	885.60	698.25	614.20	578.40	412.60	315.00	260.30	145.87	82.56	63.98	52.14	40.26	29.80	15.87	ND
4	0	478.50	892.00	817.00	769.50	624.80	581.00	502.46	449.63	412.60	377.00	312.80	250.70	116.39	90.25	62.87	39.47	24.78	16.90	ND
5	0	687.95	1150.00	2150.00	1690.25	1369.45	1100.00	951.24	569.37	448.50	302.00	214.89	137.56	101.12	82.36	65.39	42.35	29.30	18.74	ND
6	0	1025.60	1600.00	1140.00	875.60	500.90	412.60	345.91	300.00	256.24	201.00	166.10	119.20	73.30	59.70	40.19	28.27	20.69	14.98	ND
7	0	480.23	756.00	889.00	741.69	669.30	502.98	401.69	329.60	290.14	245.87	120.00	94.40	53.20	38.20	29.63	21.22	16.38	12.78	ND
8	0	642.87	1010.00	2020.00	1580.56	1284.60	1080.00	789.60	663.45	400.25	328.00	184.10	124.70	99.90	57.20	40.15	29.65	20.12	14.68	ND
9	0	805.74	1640.00	1060.00	900.85	838.74	735.00	684.27	542.69	425.80	357.00	254.10	147.80	91.50	69.40	52.39	40.12	28.87	16.90	ND
10	0	301.48	449.00	807.00	701.29	658.97	614.00	560.39	489.61	412.50	377.00	271.00	156.90	135.60	98.20	52.39	34.80	25.69	20.30	ND
11	0	500.27	847.00	717.00	642.30	549.80	505.00	476.30	450.80	401.29	378.69	279.00	149.90	87.20	82.50	61.58	40.96	18.50	12.63	ND
12	0	375.48	560.78	1025.50	741.29	632.58	560.00	426.39	378.45	320.48	280.95	148.00	90.28	78.50	53.60	42.69	30.89	24.89	15.69	ND
13	0	304.80	736.00	606.25	520.68	465.85	409.00	365.48	340.18	287.45	212.47	128.00	84.10	62.10	39.80	24.89	19.48	16.58	14.56	ND
14	0	523.36	954.00	895.81	795.64	766.44	710.73	655.78	562.48	500.99	396.86	319.62	238.50	128.41	90.67	72.16	51.50	24.61	16.79	ND
15	0	758.35	1021.12	1465.58	1120.27	940.94	803.58	728.48	624.16	477.25	313.87	272.18	139.28	91.68	70.12	62.77	50.28	20.80	16.03	ND

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.42 (continued) for 16 to 30 volunteers

Vol. No.	Time (in hours)																			
	0	**0.167**	**0.33**	**0.5**	**0.67**	**0.83**	**1.0**	**1.25**	**1.5**	**1.75**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**8.0**	**12.0**	**24.0**
16	0	638.13	1598.12	1192.50	978.42	844.86	666.13	585.95	551.79	393.62	300.51	248.33	139.16	78.76	61.04	49.74	38.41	28.43	15.14	ND
17	0	456.49	850.97	779.42	734.10	596.06	554.27	479.35	428.95	393.62	359.66	298.41	239.17	111.04	86.10	59.98	37.65	23.64	16.12	ND
18	0	656.30	1097.10	1451.10	1012.50	902.46	809.40	707.48	543.18	427.87	288.11	205.01	131.23	96.47	78.57	62.38	40.40	27.95	17.88	ND
19	0	678.42	1226.40	1087.56	835.32	477.86	393.62	330.00	286.20	244.45	191.75	158.46	113.72	69.93	56.95	38.34	26.97	19.74	14.29	ND
20	0	458.14	721.22	848.11	707.57	638.51	479.84	383.21	314.44	276.79	234.56	114.48	90.06	50.75	36.44	28.27	20.24	15.63	12.19	ND
21	0	613.30	963.54	1427.08	1107.85	925.51	830.32	703.28	632.93	381.84	312.91	175.63	118.96	95.30	54.57	38.30	28.29	19.19	14.00	ND
22	0	768.68	1164.56	1011.24	859.41	800.16	701.19	652.79	517.73	436.21	340.58	242.41	141.00	87.29	66.21	40.98	38.27	24.54	16.12	ND
23	0	287.61	628.35	1069.88	669.03	628.66	585.76	534.61	467.09	393.53	359.66	258.53	149.68	129.36	93.68	49.98	33.20	24.51	19.37	ND
24	0	477.26	808.04	1274.02	912.75	624.51	581.77	454.39	430.06	382.83	361.27	266.17	143.00	83.19	78.71	58.75	39.08	17.65	12.05	ND
25	0	358.21	534.98	978.33	707.19	603.48	534.24	406.78	361.04	305.74	268.03	141.19	86.13	74.89	51.13	40.73	29.47	23.75	14.97	ND
26	0	290.78	702.14	1078.36	796.73	544.42	490.19	398.67	324.53	252.23	202.70	122.11	80.23	59.24	37.97	23.75	18.58	15.82	13.89	ND
27	0	481.50	657.68	1124.14	831.99	725.13	653.87	603.32	517.48	459.99	365.11	312.45	219.42	118.14	78.82	61.79	38.18	22.64	15.45	ND
28	0	451.68	823.43	1292.33	1090.65	933.67	820.65	761.81	658.23	439.07	288.76	250.40	128.14	84.35	64.51	57.75	46.25	19.13	14.75	ND
29	0	587.08	1562.27	1097.10	900.15	777.27	612.84	539.07	507.65	362.13	276.47	228.46	128.03	72.46	56.15	45.76	35.34	26.15	13.93	ND
30	0	419.97	782.89	717.06	675.37	548.37	509.93	441.00	394.63	362.13	330.89	274.54	220.03	102.15	79.21	55.18	34.64	21.75	14.83	ND
Mean	**0**	**548.94**	**993.25**	**1149.35**	**915.23**	**765.60**	**665.04**	**572.53**	**488.94**	**386.08**	**310.39**	**228.93**	**146.77**	**91.52**	**67.98**	**50.24**	**35.68**	**22.64**	**15.54**	**ND**
S. D.	**0**	**175.64**	**356.19**	**403.52**	**290.10**	**244.29**	**207.17**	**173.07**	**132.65**	**77.00**	**61.28**	**68.19**	**50.74**	**23.25**	**17.70**	**13.57**	**9.11**	**4.20**	**2.01**	**ND**
C.V.%	**0**	**32.00**	**35.86**	**35.11**	**31.70**	**31.91**	**31.15**	**30.23**	**27.13**	**19.94**	**19.74**	**29.79**	**34.57**	**25.41**	**26.04**	**27.01**	**25.52**	**18.56**	**12.95**	**ND**

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.43 Bioequivalence of CARDIOMAGNUM (ACETYLSALICYLIC ACID 150 MG FILM COATED TABLET)
Plasma concentration of Salicylic Acid (ng/mL) of the Test Preparation (B) in (1 to 15) volunteers

Vol. No.	Time (in hours)																			
	0	**0.167**	**0.33**	**0.5**	**0.67**	**0.83**	**1.0**	**1.25**	**1.5**	**1.75**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**8.0**	**12.0**	**24.0**
1	0	1407.5	2198.2	2856.6	3511.8	4299.0	5092.6	5534.9	6957.6	7430.0	8074.8	9050.4	9486.4	8382.9	7782.7	6058.6	3494.5	3146.4	990.1	ND
2	0	4058.3	6454.4	9685.8	9200.9	8702.2	8069.0	7534.1	7089.2	6285.9	5679.2	5088.8	4436.2	3895.0	3007.8	2478.3	1772.1	1227.0	1050.9	ND
3	0	1648.7	2934.6	3160.8	4052.8	4631.0	5951.2	8164.6	9240.5	9806.2	10300.6	9560.3	8869.8	7688.9	6214.6	4859.5	3146.0	2865.6	1539.9	ND
4	0	5858.6	9873.6	10260.8	10355.9	10498.2	10551.2	10474.2	10164.5	9777.1	9612.2	9079.8	8547.4	7850.5	7143.8	6437.4	5624.1	3896.8	2082.1	ND
5	0	3227.6	4089.5	5654.4	7862.7	9070.3	9960.8	9115.8	8207.3	7661.2	6931.5	6218.5	5728.1	5092.4	4505.0	4089.0	3532.2	2082.0	1539.3	ND
6	0	5856.6	7402.3	9100.0	8419.5	7614.4	7209.2	6567.4	5961.5	5601.8	4283.1	3899.0	3063.4	2592.1	2023.9	1678.8	1465.4	1220.6	1036.5	ND
7	0	3882.0	4537.0	5040.4	6281.6	8125.2	9045.8	8861.0	8541.7	8108.6	7876.8	7466.7	5682.3	4704.5	3426.7	2904.5	2536.2	2072.0	1518.5	ND
8	0	1985.0	3465.4	4510.9	5789.4	7502.5	8537.8	8470.6	8218.6	8102.6	7298.7	6621.1	5227.2	4443.1	3775.2	3088.2	2758.8	2178.4	1791.4	ND
9	0	5049.8	7698.3	8653.9	8081.9	7873.8	7250.3	6921.4	6674.8	6437.4	6214.6	3668.7	3233.1	2913.7	2652.3	2372.1	1771.4	1413.5	992.7	ND
10	0	2909.6	4569.0	6069.4	6492.6	6865.5	7407.2	8041.8	8536.7	9212.2	9906.6	9030.9	8330.8	6943.5	5207.8	4356.5	3494.5	2400.9	1636.2	ND
11	0	5382.3	6369.4	7124.5	7643.0	8325.1	9350.9	8954.5	8615.4	8460.8	8276.4	6446.9	5817.7	5236.9	4510.9	3136.6	2187.7	1830.1	1413.8	ND
12	0	2323.7	2913.7	5440.2	5798.8	6292.4	7563.3	8005.9	8576.8	9015.3	9499.0	8347.1	7127.4	6595.0	5207.6	3446.6	2710.4	1941.3	1423.7	ND
13	0	2081.7	4078.2	7624.0	8292.2	9609.0	11200.5	10874.1	10100.8	9806.2	9551.2	8640.9	7095.4	5924.2	3397.7	2914.1	2497.4	1951.1	1428.0	ND
14	0	2436.0	4365.7	4762.6	5122.2	5357.7	5769.3	6041.2	7417.6	8571.1	9889.1	9631.6	8634.6	5362.7	4530.2	3389.7	2681.4	1941.3	989.8	ND
15	0	2252.0	3609.4	4095.8	4670.6	5260.6	5855.2	6282.1	7158.6	8097.7	8981.6	9659.4	9317.3	7823.6	6204.1	4594.5	3032.8	2236.2	1601.1	ND

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.43 (continued) for 16 to 30 volunteers

Vol. No.	Time (in hours)																			
	0	**0.167**	**0.33**	**0.5**	**0.67**	**0.83**	**1.0**	**1.25**	**1.5**	**1.75**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**8.0**	**12.0**	**24.0**
16	0	2478.8	3649.4	5624.1	6563.5	7280.2	8160.2	8587.0	8954.6	9342.0	9873.6	10454.4	8363.5	7347.1	6253.3	4864.6	3407.4	2604.2	1480.0	ND
17	0	5382.8	8544.4	9535.2	9890.3	10164.5	11205.3	10525.9	9667.0	8101.6	7763.4	7172.9	6708.2	6117.8	5672.5	5251.6	3997.8	3049.4	1383.8	ND
18	0	5884.5	8528.1	9776.8	9561.0	9188.8	8857.6	8444.9	8012.9	7638.2	7254.1	6818.5	6318.2	6098.6	5505.0	4912.8	3213.8	2691.5	1539.7	ND
19	0	4444.3	8905.6	9486.4	9710.7	10188.8	11228.8	10880.8	10706.4	10552.0	10222.4	9680.0	9525.1	8973.4	8411.9	6564.0	4840.0	3291.8	1946.1	ND
20	0	3019.2	3554.3	4264.0	4919.3	5884.1	6625.6	7022.6	7445.3	8024.0	9260.8	9083.1	8583.0	7827.8	6914.6	5251.7	4346.3	2928.8	2072.4	ND
21	0	3897.1	6979.3	9486.4	9777.0	10430.8	11035.2	10841.9	10440.1	10309.4	10067.2	9873.6	9563.8	8199.0	6072.8	5418.1	3391.8	2446.5	1401.5	ND
22	0	2990.9	6427.5	8489.4	8060.6	7552.7	7299.3	6931.8	6528.5	6013.0	5767.2	5360.2	5021.1	4621.0	4056.3	3669.3	3078.6	2103.6	2020.7	ND
23	0	969.2	1481.0	3949.4	6755.5	7301.2	7937.6	8290.8	8644.5	8949.7	9273.4	6640.5	5198.2	4656.1	3620.3	3049.5	2768.5	2009.2	1607.2	ND
24	0	4211.6	5904.8	8228.0	9095.3	9492.9	10100.7	9657.0	9225.2	8544.5	8040.4	6891.4	5436.4	4962.1	3633.0	2776.9	2025.1	1620.6	1091.9	ND
25	0	1016.7	4317.3	5556.3	7484.0	8333.1	9776.8	9190.2	8156.8	7797.7	7234.3	6785.7	6411.4	6117.8	5866.1	5309.7	3213.8	2652.8	1786.5	ND
26	0	4852.3	7524.7	9028.0	8799.3	8538.5	8250.8	7835.7	7516.8	7267.0	6821.4	6511.6	6056.6	5150.0	4459.5	3640.3	2003.8	1592.5	1191.1	ND
27	0	1433.3	2768.5	4433.6	5062.2	5630.8	6279.4	6999.2	7554.3	8089.4	8524.8	8134.2	7321.0	6073.7	5385.0	4408.0	3062.0	2033.5	1375.8	ND
28	0	2514.5	7091.1	10040.2	9879.4	9565.6	9321.8	8945.1	8441.3	8192.1	7763.4	7366.5	6882.5	6659.8	3397.7	2851.5	2449.0	1636.7	971.4	ND
29	0	2632.8	4317.3	4578.6	4842.6	5189.3	5527.3	6430.5	7273.2	8199.0	8954.0	8237.7	7589.1	4230.2	3697.8	2391.7	2128.1	1772.1	1457.4	ND
30	0	2970.4	5427.7	6224.9	8072.4	10065.6	11501.0	11212.0	10054.2	9837.4	8785.7	7630.8	6953.2	5530.2	4581.6	3797.9	2068.5	1829.9	1418.5	ND
Mean	**0**	**3301.93**	**5332.65**	**6758.04**	**7334.96**	**7827.79**	**8397.38**	**8387.97**	**8336.09**	**8307.72**	**8266.05**	**7635.04**	**6884.29**	**5933.78**	**4903.92**	**3998.72**	**2956.64**	**2222.21**	**1459.26**	**ND**
S. D.	**0**	**1507.98**	**2204.15**	**2360.14**	**1979.46**	**1849.43**	**1919.02**	**1593.69**	**1235.47**	**1238.15**	**1532.15**	**1756.76**	**1808.39**	**1619.44**	**1550.16**	**1288.68**	**926.80**	**634.19**	**326.99**	**ND**
C.V.%	**0**	**45.67**	**41.33**	**34.92**	**26.99**	**23.63**	**22.85**	**19.00**	**14.82**	**14.90**	**18.54**	**23.01**	**26.27**	**27.29**	**31.61**	**32.23**	**31.35**	**28.54**	**22.41**	**ND**

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.44 Pharmacokinetic parameters

C_{max} (ng/mL), t_{max} (hr.), AUC_{0-t} (ng. hr./mL), $AUC_{0-\infty}$, $t_{1/2}$, K_{el}

in 30 volunteers with the Test and Reference Preparation of Acetylsalicylic Acid (ASA)

Volunteer	C_{max}		t_{max}		AUC_{0-t}		$AUC_{0-\infty}$		$t_{1/2}$		K_{el}	
no.	A	B	A	B	A	B	A	B	A	B	A	B
1	1264.00	1000.00	0.33	0.33	1872.26	2135.45	1903.45	2178.48	1.68	1.69	0.41	0.41
2	1021.00	2270.00	0.33	0.50	1645.66	2778.66	1681.23	2814.85	1.70	1.49	0.41	0.46
3	1265.00	1780.00	0.33	0.33	1977.46	2126.66	2034.81	2163.59	1.86	1.61	0.37	0.43
4	1660.50	892.00	0.50	0.33	2278.34	1838.83	2331.19	1881.86	1.84	1.76	0.38	0.39
5	1291.00	2150.00	0.33	0.50	1994.46	2554.71	2034.62	2597.48	1.76	1.58	0.39	0.44
6	1280.00	1600.00	0.50	0.33	1611.99	1642.81	1646.83	1679.47	1.69	1.70	0.41	0.41
7	957.60	889.00	0.33	0.50	1551.67	1340.85	1592.97	1371.43	1.83	1.66	0.38	0.42
8	846.90	2020.00	0.33	0.50	1656.98	2325.11	1713.28	2356.21	1.91	1.47	0.36	0.47
9	1437.00	1640.00	0.33	0.33	1864.83	2106.38	1923.45	2146.45	1.88	1.64	0.37	0.42
10	1201.00	807.00	0.33	0.50	1654.77	1699.74	1696.60	1753.28	1.78	1.83	0.39	0.38
11	1448.00	847.00	0.33	0.33	1670.05	1646.02	1705.53	1677.04	1.66	1.70	0.42	0.41
12	929.70	1025.50	0.50	0.50	1605.14	1454.30	1635.46	1494.61	1.67	1.78	0.41	0.39
13	1065.60	736.00	0.33	0.33	1735.23	1152.44	1784.27	1188.89	1.84	1.73	0.38	0.40
14	1245.04	954.00	0.33	0.33	2370.53	2051.20	2431.47	2092.80	1.88	1.72	0.37	0.40
15	1480.34	1465.58	0.33	0.50	2773.16	2165.13	2805.93	2202.42	1.59	1.61	0.44	0.43
16	1503.90	1598.12	0.50	0.33	2699.76	2012.19	2782.42	2047.52	1.98	1.62	0.35	0.43
17	1215.59	850.97	0.50	0.33	1948.21	1754.23	2001.61	1795.28	1.89	1.76	0.37	0.39
18	1271.64	1451.10	0.33	0.50	1931.64	2069.13	1971.27	2112.85	1.76	1.69	0.39	0.41
19	1260.80	1226.40	0.50	0.33	1678.68	1467.79	1711.91	1503.11	1.64	1.71	0.42	0.40
20	930.15	848.11	0.50	0.50	1512.95	1279.17	1557.43	1308.34	1.88	1.66	0.37	0.42
21	834.20	1427.08	0.33	0.50	1613.27	1963.13	1668.50	1994.15	1.90	1.54	0.37	0.45
22	1415.45	1164.56	0.33	0.33	1805.73	1932.37	1863.97	1970.48	1.90	1.64	0.37	0.42
23	950.84	1069.88	0.50	0.50	1532.31	1705.86	1575.38	1756.11	1.86	1.80	0.37	0.39
24	1426.28	1274.02	0.33	0.50	1644.99	1757.62	1679.94	1785.93	1.66	1.63	0.42	0.43
25	915.75	978.33	0.50	0.50	1599.36	1387.42	1629.27	1425.89	1.67	1.78	0.41	0.39
26	811.33	1078.36	0.50	0.50	1619.98	1278.43	1665.74	1311.81	1.84	1.67	0.38	0.42
27	1182.79	1124.14	0.33	0.50	2403.22	1908.41	2462.66	1945.79	1.93	1.68	0.36	0.41
28	1206.74	1292.33	0.50	0.50	2529.24	2004.58	2560.93	2038.36	1.62	1.59	0.43	0.44
29	1651.60	1562.27	0.33	0.33	2691.64	1866.52	2767.77	1898.94	1.92	1.61	0.36	0.43
30	1053.81	782.89	0.50	0.33	1920.65	1613.89	1971.79	1651.66	1.91	1.76	0.36	0.39
N	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**
Mean	**1200.785**	**1260.155**	**0.398**	**0.421**	**1913.138**	**1833.968**	**1959.722**	**1871.502**	**1.798**	**1.671**	**0.387**	**0.416**
S.D.	**239.627**	**419.985**	**0.085**	**0.086**	**383.810**	**379.589**	**390.308**	**380.564**	**0.111**	**0.088**	**0.025**	**0.022**
C.V.%	**19.956**	**33.328**	**21.283**	**20.506**	**20.062**	**20.698**	**19.916**	**20.335**	**6.178**	**5.274**	**6.336**	**5.409**

A – Reference Preparation B – Test Preparation

Table.5.45 Pharmacokinetic parameters

C_{max} (ng/mL), t_{max} (hr.), AUC_{0-t} (ng. hr./mL), $AUC_{0-\infty}$, $t_{1/2}$, K_{el}

in 30 volunteers with the Test and Reference Preparation of Salicylic Acid (SA)

Volunteer	C_{max}		t_{max}		AUC_{0-t}		$AUC_{0-\infty}$		$t_{1/2}$		K_{el}	
no.	A	B	A	B	A	B	A	B	A	B	A	B
1	8990.00	9486.40	2.0	3.0	45629.70	53467.49	50285.76	57564.49	3.15	2.87	0.22	0.24
2	10600.00	9685.80	0.5	0.5	56962.56	35281.23	71148.32	40049.34	4.88	3.14	0.14	0.22
3	10900.00	10300.60	1.0	2.0	61039.25	53621.62	69274.20	61350.36	3.59	3.48	0.19	0.20
4	9680.25	10551.20	1.0	1.0	67638.58	70044.99	84124.38	84077.93	5.31	4.67	0.13	0.15
5	10800.00	9960.80	0.5	1.0	59421.09	46515.85	67819.84	55396.59	3.66	4.00	0.19	0.17
6	11500.00	9100.00	0.5	0.5	58141.88	29950.95	69364.54	34790.96	3.89	3.24	0.18	0.21
7	8795.00	9045.76	2.0	1.0	43124.89	43319.69	52097.02	51490.78	3.96	3.73	0.17	0.19
8	8820.00	8537.76	1.0	1.0	44332.46	42913.82	55595.48	53816.42	4.22	4.22	0.16	0.16
9	8940.00	8653.92	0.5	0.5	33884.96	33232.49	38797.82	37978.01	3.32	3.31	0.21	0.21
10	10400.00	9906.64	1.0	2.0	52189.15	52326.49	61907.01	60950.66	3.98	3.65	0.17	0.19
11	9660.00	9350.88	1.0	1.0	45701.74	44239.29	53071.99	51373.69	3.50	3.50	0.20	0.20
12	8780.00	9499.04	2.0	2.0	44929.17	46462.44	52915.05	53500.60	3.76	3.43	0.18	0.20
13	10900.00	11200.52	2.0	1.0	47539.56	47649.49	55003.10	54243.77	3.51	3.20	0.20	0.22
14	9950.00	9889.12	2.5	2.0	44690.03	44522.45	49102.90	48676.53	2.99	2.91	0.23	0.24
15	10560.00	9659.36	1.0	2.5	40311.32	50397.31	46374.97	58251.34	3.39	3.40	0.20	0.20
16	10800.00	10454.40	2.5	2.5	56730.25	54914.88	64254.33	62198.19	3.41	3.41	0.20	0.20
17	11400.00	11205.28	0.5	1.0	58498.54	56996.99	66723.43	64712.04	3.99	3.86	0.17	0.18
18	10700.00	9776.80	1.0	0.5	59949.22	52384.84	68390.95	61735.95	3.68	4.21	0.19	0.16
19	11600.00	11228.80	1.0	1.0	44824.14	69328.04	56174.72	80851.58	4.34	4.10	0.16	0.17
20	10700.00	9260.80	1.0	2.0	39752.46	56719.77	48166.95	69267.85	3.78	4.20	0.18	0.17
21	9960.00	11035.20	1.0	1.0	54275.72	59763.51	60766.11	66541.69	3.51	3.35	0.20	0.21
22	10808.00	8489.36	2.0	0.5	61740.37	43329.74	73880.94	57984.51	4.03	5.03	0.17	0.14
23	9580.00	9273.44	2.0	2.0	43822.10	42419.80	54064.52	52334.45	4.27	4.27	0.16	0.16
24	8500.00	10100.72	0.5	1.0	32812.56	42366.48	37526.77	47183.07	3.19	3.06	0.22	0.23
25	10100.00	9776.80	1.0	1.0	53201.91	51211.93	64799.74	62533.57	4.35	4.39	0.16	0.16
26	9350.00	9028.00	1.0	0.5	46713.92	42403.85	52428.09	48338.61	3.22	3.45	0.22	0.20
27	8600.00	8524.80	2.0	2.0	43369.43	46048.11	50748.16	53028.43	3.60	3.52	0.19	0.20
28	9630.00	10040.24	1.0	0.5	43267.99	44155.07	47675.02	48438.16	3.04	3.06	0.23	0.23
29	9250.00	8954.00	2.0	2.0	41526.69	40197.84	49402.42	47821.54	3.63	3.63	0.19	0.19
30	8910.00	11500.95	0.5	1.0	42210.98	47651.01	50197.34	54158.35	3.78	3.18	0.18	0.22
N	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**
Mean	**9972.108**	**9782.580**	**1.250**	**1.317**	**48941.087**	**48127.915**	**57402.729**	**56021.315**	**3.764**	**3.648**	**0.187**	**0.194**
SD	**938.046**	**859.788**	**0.653**	**0.725**	**8747.326**	**9057.221**	**10738.070**	**10784.167**	**0.522**	**0.538**	**0.024**	**0.027**
CV%	**9.407**	**8.789**	**52.259**	**55.061**	**17.873**	**18.819**	**18.707**	**19.250**	**13.859**	**14.742**	**12.794**	**13.826**

A – Reference Preparation B–Test Preparation

Table.5.46 Pharmacokinetic parameters in 30 volunteers with the Test and Reference Preparation of Acetylsalicylic Acid (Aspirin) 150 mg film coated tablet

Pharmacokinetic parameters		Acetylsalicylic Acid (ASA)		Salicylic Acid (SA)	
		Reference Preparation (A)	Test Preparation (B)	Reference Preparation (A)	Test Preparation (B)
C_{max} (ng./mL.)	Mean	1200.785	1260.155	9972.108	9782.580
	± S.D.	239.627	419.985	938.046	859.788
t_{max} (hr.)	Mean	0.398	0.421	1.250	1.317
	± S.D.	0.085	0.086	0.653	0.725
AUC_{0-t} (ng. hr./mL.)	Mean	1913.138	1833.968	48941.087	48127.915
	± S.D.	383.810	379.589	8747.326	9057.221
$AUC_{0-\infty}$ (ng. hr./mL.)	Mean	1959.722	1871.502	57402.729	56021.315
	± S.D.	390.308	380.564	10738.070	10784.167
k_{el} ($hr.^{-1}$)	Mean	0.387	0.416	0.187	0.194
	± S.D.	0.025	0.022	0.024	0.027
$t_{1/2}$ (hr.)	Mean	1.798	1.671	3.764	3.648
	± S.D.	0.111	0.088	0.522	0.538
Relative Bioavailability (%)		**100%**	**95.86%**	**100%**	**98.34%**

Table.5.47 90% confidence interval (CI) with the Test and Reference Preparation

Confidence Interval (CI)		Acetylsalicylic Acid (ASA)	Salicylic Acid (SA)
C_{max}	Untransformed Data	0.845561 - 1.223323	0.928144 - 1.033844
	Ln transformed Data	0.975983 - 1.029123	0.992211 - 1.003753
AUC_{0-t}	Untransformed Data	0.839301 - 1.077934	0.871854 - 1.094915
	Ln transformed Data	0.978980 - 1.008908	0.987642- 1.008971
$AUC_{0-\infty}$	Untransformed Data	0.837186 - 1.072781	0.868135 - 1.083734
	Ln transformed Data	0.978782 - 1.008270	0.987451 - 1.007932

Table.5.48 ANOVA Summary for C_{max} Values untransformed data

		Acetylsalicylic Acid (ASA)				Salicylic Acid (SA)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	3699248.96705	127560.30921	1.16208	N.S.	32374771.97194	1116371.44731	2.19461	N.S.
Treatment	1	52871.35980	52871.35980	0.48166	N.S.	538816.73233	538816.73233	1.05923	N.S.
Period	1	7686.95928	7686.95928	0.07003	N.S.	337740.04267	337740.04267	0.66394	N.S.
Error	28	3073522.72297	109768.66868	-	-	14243292.09710	508689.00347	-	-
Total	59	6833330.00910	-	-	-	47494620.84404	-	-	-

Table.5.49 ANOVA Summary for in Ln C_{max} Values Ln transformed data

		Acetylsalicylic Acid (ASA)				Salicylic Acid (SA)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	2.31425	0.07980	1.23448	N.S.	0.33056	0.01140	2.20632	N.S.
Treatment	1	0.00489	0.00489	0.07562	N.S.	0.00517	0.00517	1.00105	N.S.
Period	1	0.00628	0.00628	0.09718	N.S.	0.00298	0.00298	0.57594	N.S.
Error	28	1.81004	0.06464	-	-	0.14466	0.00517	-	-
Total	59	4.13546	-	-	-	0.48336	-	-	-

Table.5.50 ANOVA Summary for AUC_{0-t} Values untransformed data

		Acetylsalicylic Acid (ASA)				Salicylic Acid (SA)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	5776882.51245	199202.84526	2.08740	N.S.	3039029820.53098	104794131.74245	1.92047	N.S.
Treatment	1	94020.70861	94020.70861	0.98522	N.S.	9918730.52377	9918730.52377	0.18177	N.S.
Period	1	1583.42888	1583.42888	0.01659	N.S.	31017257.36133	31017257.36133	0.56843	N.S.
Error	28	2672064.36675	95430.87024	-	-	1527873718.60898	54566918.52175	-	-
Total	59	8544551.01670	-	-	-	4607839527.02505	-	-	-

D.F. – Degree of Freedom

S.S. – Sum of Squares

M.S.S. – Mean Sum of Squares

F. – M.S.S. / Error M.S.S.

P - Probability

N.S. – Non significant

S – Significant

Table.5.51 ANOVA Summary for Ln AUC_{0-t} Values Ln transformed data

		Acetylsalicylic Acid (ASA)				Salicylic Acid (SA)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	1.63348	0.05633	2.41671	N.S.	1.27130	0.04384	1.81008	N.S.
Treatment	1	0.03126	0.03126	1.34141	N.S.	0.00500	0.00500	0.20656	N.S.
Period	1	0.00161	0.00161	0.06893	N.S.	0.01396	0.01396	0.57635	N.S.
Error	28	0.65260	0.02331	-	-	0.67812	0.02422	-	-
Total	59	2.31896	-	-	-	1.96838	-	-	-

Table.5.52 ANOVA Summary for AUC_{0-inf} Values untransformed data

		Acetylsalicylic Acid (ASA)				Salicylic Acid (SA)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	5884445.05627	202911.89849	2.07900	N.S.	4686343709.21951	161598058.93860	2.30432	N.S.
Treatment	1	116741.52600	116741.52600	1.19611	N.S.	28624555.77679	28624555.77679	0.40817	N.S.
Period	1	624.16651	624.16651	0.00640	N.S.	66587710.69080	66587710.69080	0.94951	N.S.
Error	28	2732816.66879	97600.59531	-	-	1963596445.13193	70128444.46900	-	-
Total	59	8734627.41757	-	-	-	6745152420.81903	-	-	-

Table.5.53 ANOVA Summary for Ln AUC_{0-inf} VALUES Ln transformed data

		Acetylsalicylic Acid (ASA)				Salicylic Acid (SA)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	1.59219	0.05490	2.41073	N.S.	1.42324	0.04908	2.13461	N.S.
Treatment	1	0.03596	0.03596	1.57899	N.S.	0.00957	0.00957	0.41625	N.S.
Period	1	0.00089	0.00089	0.03912	N.S.	0.02169	0.02169	0.94349	N.S.
Error	28	0.63769	0.02277	-	-	0.64375	0.02299	-	-
Total	59	2.26673	-	-	-	2.09825	-	-	-

D.F. – Degree of Freedom

S.S. – Sum of Squares

M.S.S. – Mean Sum of Squares

F. – M.S.S./Error M.S.S.

P - Probability

N.S. – Non significant

S – Significant

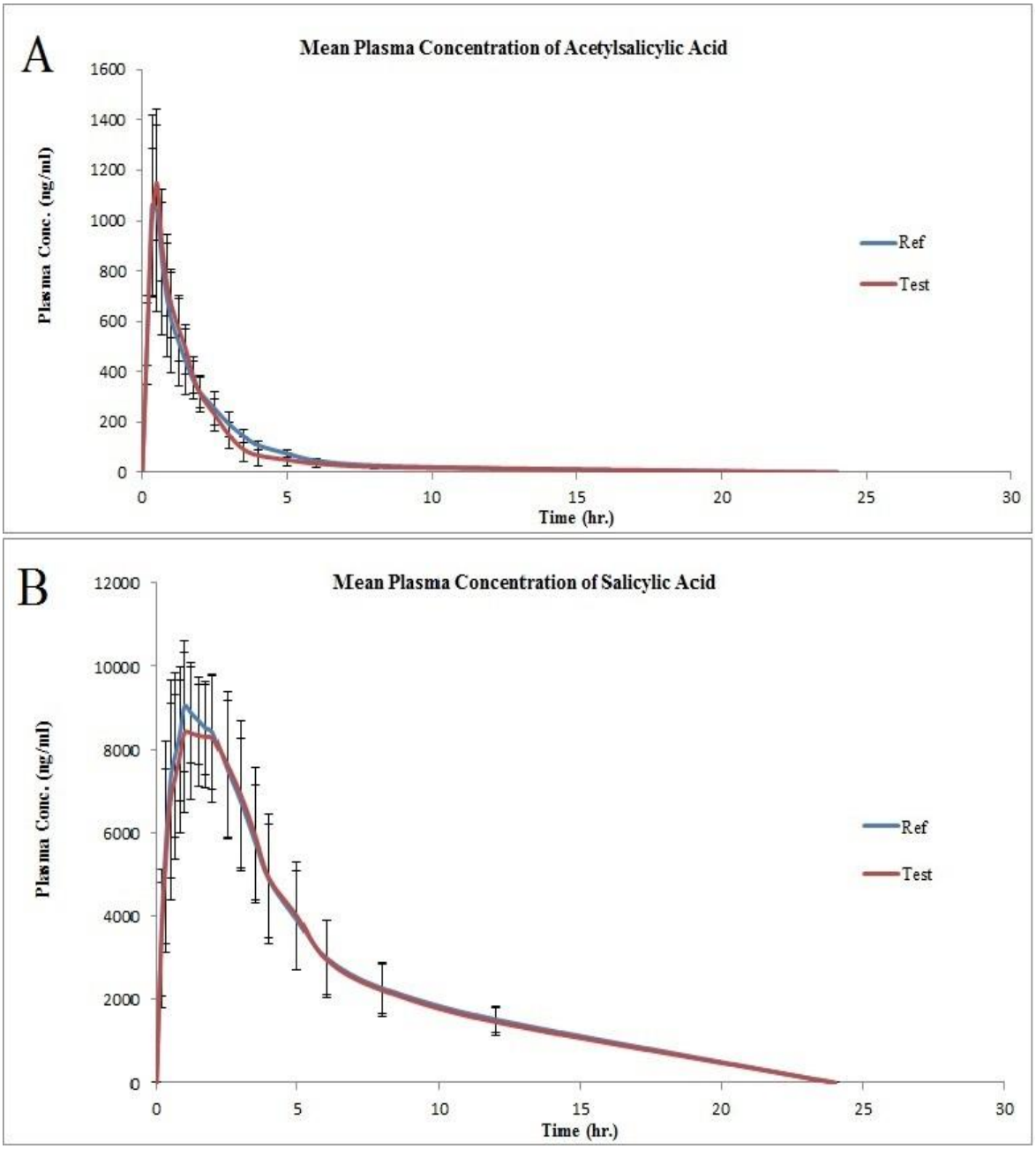


Figure.5.34 Plasma concentration versus time profile : (A) Mean ± SD plasma concentration - time profile of ASA following 150 mg single oral dose of reference and test preparation to 30 healthy human subjects and (B) Mean ± SD plasma concentration - time profile of SA following 150 mg single oral dose of reference and test preparation to 30 healthy human subjects

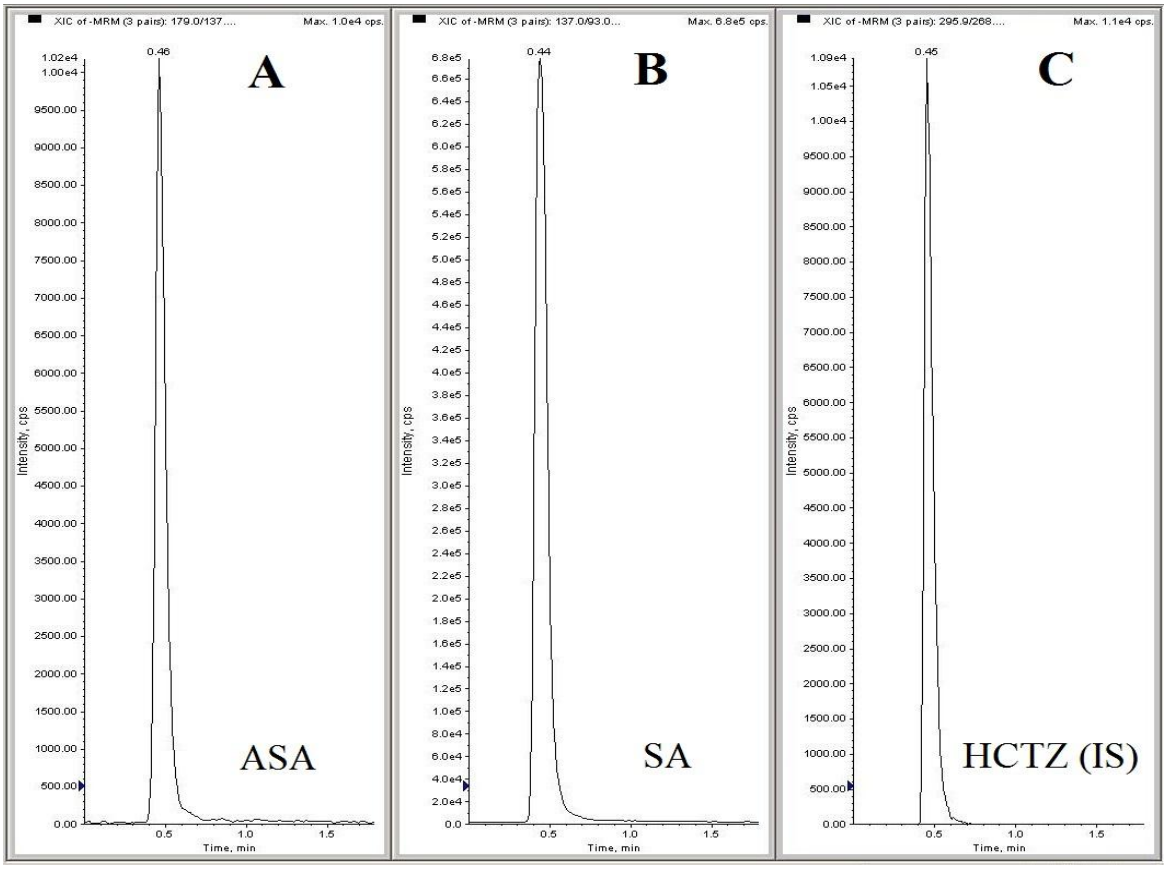


Figure.5.35 Representative MRM chromatograms of volunteer plasma samples : (A) *in vivo* plasma sample showing ASA peak obtained following single oral dose of 150 mg tablets to healthy humans, (B) *in vivo* plasma sample showing SA peak obtained following single oral dose of 150 mg tablets to healthy humans and (C) HCTZ (IS) spiked in human volunteer plasma at 400 ng/mL

5.4.2 Pharmacokinetics and statistical analysis

The volunteer plasma samples were analysed by the proposed bioanalytical method as already mentioned in the section 5.1.11 and 5.1.12, respectively and also the plasma concentrations and mean plasma concentrations were determined and described in Table.5.40 to Table.5.43. The mean plasma concentration-time profile graphs for both Test and Reference preparation and also representative chromatograms of volunteer plasma samples of both ASA and SA with IS (HCTZ) were shown in Figure.5.34 and Figure.5.35. The reference tablet as a single oral dose in the fasting state produced the maximum plasma concentration of 1200.785 ± 239.627 ng/mL (C_{max}) at the time of 0.398 ± 0.085 hr. (t_{max}) for ASA and 9972.108 ± 938.046 ng/mL (C_{max}) at the time of 1.250 ± 0.653 hr. (t_{max}) for SA whereas the test product in the fasting state produced the maximum plasma concentration of 1260.155 ±

419.985 ng/mL (C_{max}) at the time of 0.421 ± 0.086 hr. (t_{max}) for ASA and 9782.580 ± 859.788 ng/mL (C_{max}) at the time of 1.317 ± 0.725 hr. (t_{max}) for SA, respectively (see Table.5.46).

After administration of the reference tablet produced the area under plasma concentration time curve (AUC_{0-t}) 1913.138 ± 383.810 ng. hr./mL for ASA and 48941.087 ± 8747.326 ng. hr./mL for SA, whereas in case of the test tablet produced the area under plasma concentration curve (AUC_{0-t}) 1833.968 ± 379.589 ng. hr./mL for ASA and 48127.915 ± 9057.221 ng. hr./mL for SA, respectively (Table.5.46).

When administered as a single oral dose, in the fasting state, the reference product of Aspirin or Acetylsalicylic Acid (ASA) produced the area under plasma concentration time curve upto infinity ($AUC_{0-\infty}$) 1959.722 ± 390.308 ng. hr./mL (ASA) and 57402.729 ± 10738.070 ng. hr./mL (SA), and for the test product ($AUC_{0-\infty}$) 1871.502 ± 380.564 ng. hr./mL (ASA) and 56021.315 ± 10784.167 ng. hr./mL (SA) (Table.5.46).

Administration of Aspirin (Acetylsalicylic Acid, ASA) produced the plasma elimination half-life, i.e., $t_{1/2}$ 1.798 ± 0.111 hr. (ASA) and 3.764 ± 0.522 hr. (SA) for the reference and 1.671 ± 0.088 hr. (ASA) and 3.648 ± 0.538 (SA) for the test, respectively (Table.5.46). While administration of the reference and test preparation produced the plasma elimination rate constant, i.e., k_{el} 0.387 ± 0.025 $hr.^{-1}$ and 0.187 ± 0.024 $hr.^{-1}$ for ASA and 0.416 ± 0.022 $hr.^{-1}$ and 0.194 ± 0.027 $hr.^{-1}$ for SA, respectively (Table.5.46).

As a result of comparison of the AUC_{0-t} for Reference and Test after single oral dose administration, the relative bioavailability (%) of the test product of Aspirin (Acetylsalicylic Acid, ASA) tablet was 95.86% for ASA and 98.34% for SA (Table.5.46). The 90% confidence interval (CI) for Untransformed data of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values of the test product were 0.845561 - 1.223323; 0.839301 - 1.077934 and 0.837186 - 1.072781 for ASA and 0.928144 - 1.033844; 0.871854 - 1.094915 and 0.868135 - 1.083734 for SA, respectively of that the reference product. Whereas Ln transformed data of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values of the test product were 0.975983 - 1.029123; 0.978980 - 1.008908 and 0.978782 - 1.008270 for ASA and 0.992211 - 1.003753; 0.987642- 1.008971 and 0.987451 - 1.007932 for SA, respectively of that the reference product (see Table.5.47).

5.4.3 Statistical inference

The 90% CI, i.e., confidence interval for C_{max}, Ln C_{max}, AUC_{0-t}, Ln AUC_{0-t}, $AUC_{0-\infty}$ and Ln $AUC_{0-\infty}$ values of the Test preparation of Aspirin (Acetylsalicylic Acid, ASA) tablet were within the accepted limit of that of the Reference preparation, i.e., CI = 0.8 to 1.2. ANOVA (Subjects, Treatment, Period) was applied to the C_{max}, Ln C_{max}, AUC_{0-t}, Ln AUC_{0-t}, $AUC_{0-\infty}$ and Ln $AUC_{0-\infty}$ values. Finally the results were found statistically nonsignificant, i.e., there was no statistically significant difference for the analysis values of the C_{max}, Ln C_{max}, AUC_{0-t}, Ln AUC_{0-t}, $AUC_{0-\infty}$ and Ln $AUC_{0-\infty}$ (see Table.5.48 to Table.5.53).

5.4.4 Discussion

The present ESI-LC-MS/MS bioanalytical method has been described for the analysis of both Acetylsalicylic Acid (ASA) and its active metabolite Salicylic Acid (SA) in human plasma which is precise, accurate, reproducible, stable, and robust enough to establish pharmacokinetic, bioavailability or bioequivalence studies on a long-term basis. This method is highly sensitive, specific, selective with better extraction recovery and shorter chromatographic run time and also very simple utilizing liquid-liquid extraction (LLE) procedure that make the method high-throughput for the bioanalysis. Now, the limit of detection (LOD) is 5 ng/mL and the lower limit of quantification (LLOQ) is 10 ng/mL of both ASA and SA which is more sensitive than some published HPLC and LC-MS/MS methods (Ebuehi *et al*., 2007; Ghosh *et al*., 2011; Polagani *et al*., 2012; Xu *et al*., 2009; Abu-Qare and Abou-Donia. 2001; McMahon and Kelly. 1998). Extraction recovery of ASA and SA in the present method is 98% to 100% and that is far better than other methods (Gajula *et al*., 2012; Polagani *et al*., 2012; Ghosh *et al*., 2011; Bae *et al*., 2008; Xu *et al*., 2009; Abu-Qare and Abou-Donia. 2001; Coudray *et al*., 1996). The plasma extraction procedure is very simple and employing one step liquid-liquid extraction (LLE) using just 300 µL of plasma and this procedure is also very economic or cost-effective. Whereas in the other published methods (such as Gajula *et al*., 2012; Kotani *et al*., 2010; Franeta *et al*., 2002; Pirola et al., 1998; Kees *et al*., 1996; McMahon and Kelly. 1998), plasma extraction procedures were very much complicated, using many solvents, time consuming and not so economic or cost-effective. The total chromatographic run time of the present method is 1.8 min. which is comparatively shorter than other methods like Kotani *et al*., 2010; Abu-Qare and Abou-Donia. 2001; Kees *et al*., 1996. So, due to the shorter run time a large number of plasma

samples can be analysed per day. This bioanalytical method was successfully validated by the USFDA and EMA guidelines and all the validation data were within the acceptance limits.

The major implication of the method is the simultaneous pharmacokinetic study as well as bioequivalence study of ASA and its active metabolite SA after single oral dose administration of Aspirin (Acetylsalicylic Acid) 150 mg film coated tablets in 30 healthy human subjects under fasting conditions. Because to date, to best our knowledge, no pharmacokinetic data and bioequivalence study on both ASA and SA after a single oral dose administration of 150 mg film coated tablet in healthy subjects have been reported in the literature. The published methods have already described the pharmacokinetic and bioequivalence study of different doses except 150 mg dose of Aspirin or Acetylsalicylic Acid, such as 75 mg, 81 mg, 100 mg, 200 mg, 250 mg, 300 mg, 500 mg, 600mg and 1000 mg, respectively (Nagelschmitz *et al*., 2014; Zeitoun *et al*., 2014; Postula *et al*., 2013; Gajula *et al*., 2012; Polagani *et al*., 2012; Ghosh *et al*., 2011; Bae *et al*., 2008; McMahon and Kelly. 1998; Kees *et al*., 1996; Coudray *et al*., 1996). Therefore simple, rapid, specific, highly sensitive and fully validated ESI-LC-MS/MS bioanalytical method was successfully applied for the simultaneous determination of ASA and SA in human plasma without any interference or matrix effect, i.e., for the pharmacokinetic and bioequivalence study and also the resulted pharmacokinetic parameters would be helpful to provide some guidance to clinical application and investigation.

SECTION–B

5.5 Development of method for enalapril (EPL) and enalaprilat (EPLT) by liquid chromatography tandem-mass spectrometry (LC-MS/MS)

5.5.1 Determination of precursor ion and product ion for EPL, EPLT (active metabolite) and IS (TBM)

It has been found during MS/MS development that the electrospray ionization (ESI) source provided better results as compared to the atmospheric chemical ionization (APCI) source for convenient ionization of both the analytes EPL and EPLT and IS (Tolbutamide, TBM). The precursor ion was determined in quadrupole 1 (Q1), i.e., QIMS scan and the product ion was determined in quadrupole 3 (Q3), i.e., MS2 scan, and measured by the mass-to-charge ratio (*m/z*). The transition pairs were monitored definitely and compared in both positive $[M + H]^+$ and negative mode or polarity $[M - H]^-$, i.e., protonated and deprotonated. Now, it has been noticed that more intense response in positive ion mode / polarity $[M + H]^+$ than negative $[M - H]^-$ for both analytes (EPL and EPLT) and IS (TBM).

During mass scanning, the compound-dependent parameters of the analytes and IS were manually tuned, i.e., declustering potential (DP), focusing potential (FP) and entrance potential (EP) were tuned in Q1 scan i.e., Q1MI or multiple ions scan and collision energy (CE) and collision cell exit potential (CXP) were tuned in Q3 scan or in MS2 scan. Whereas, collision cell entrance potential (CEP) was setting up as by default in the software. Molar mass or molecular weight of EPL, EPLT and TBM (IS) were 376.453 g/mol, 348.399 g/mol and 270.35 g/mol, respectively. In multiple reaction monitoring (MRM) mode for ion transitions of all compounds protonated precursor $[M + H]^+$ to product ion spectra were found at *m/z* 377.2 → 234.2 for EPL (see Figure.5.36), *m/z* 349.1 → 206.1 for EPLT (see Figure.5.37) and *m/z* 271.1 → 155.0 for TBM (IS) (see Figure.5.38), respectively. The fragments or product ions were most abundant stable spectrum with better response as compared to others product ions. Therefore, the following conditions were selected for optimization of the fragmentation conditions. Automated data acquisition, data processing, analysis and instrument control were achieved by Analyst software (version 1.5).

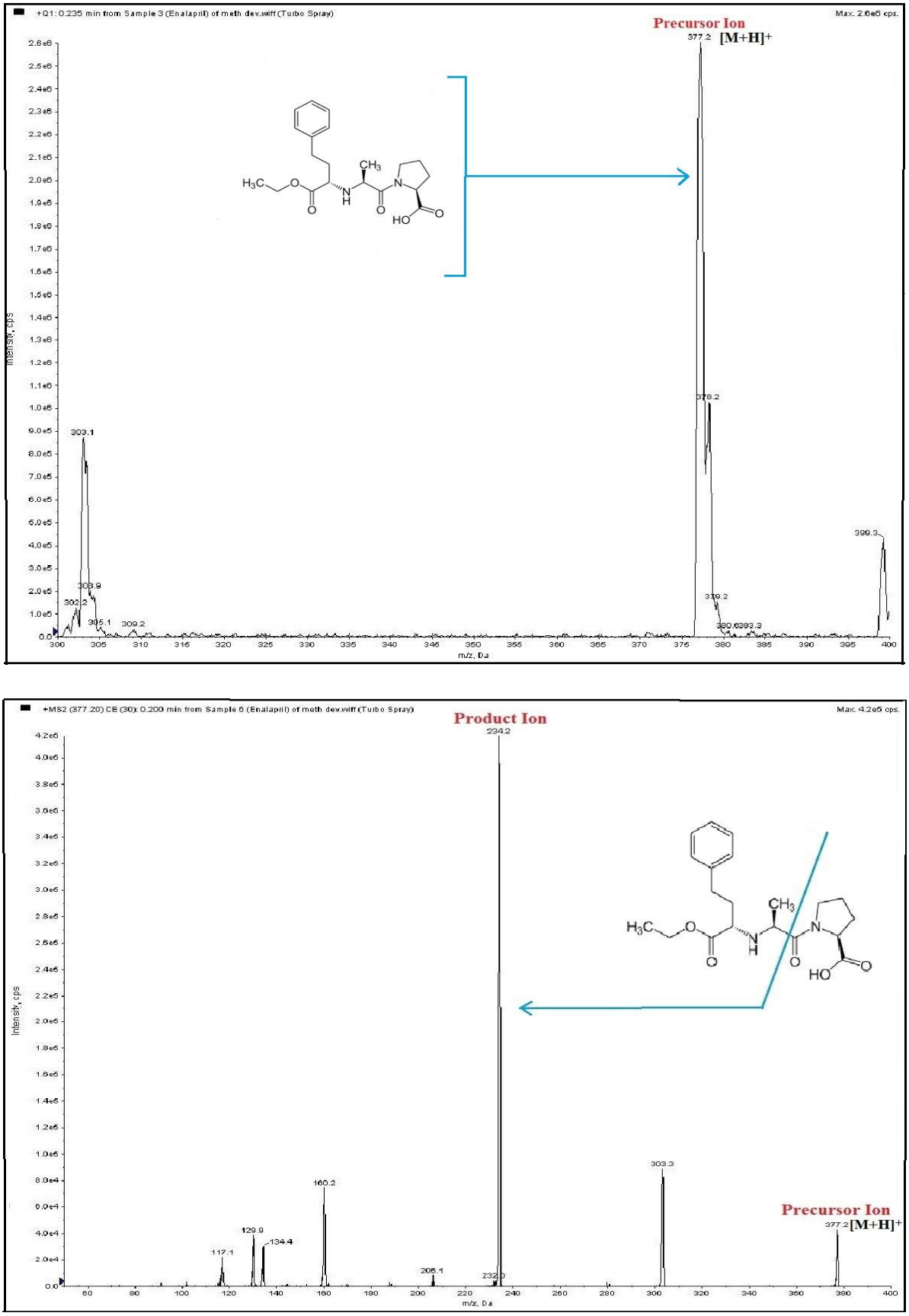


Figure.5.36 Mass spectra (Q1 and MS2) of the positive ion $[M+H]^+$ of enalapril (EPL) with structural interpretation

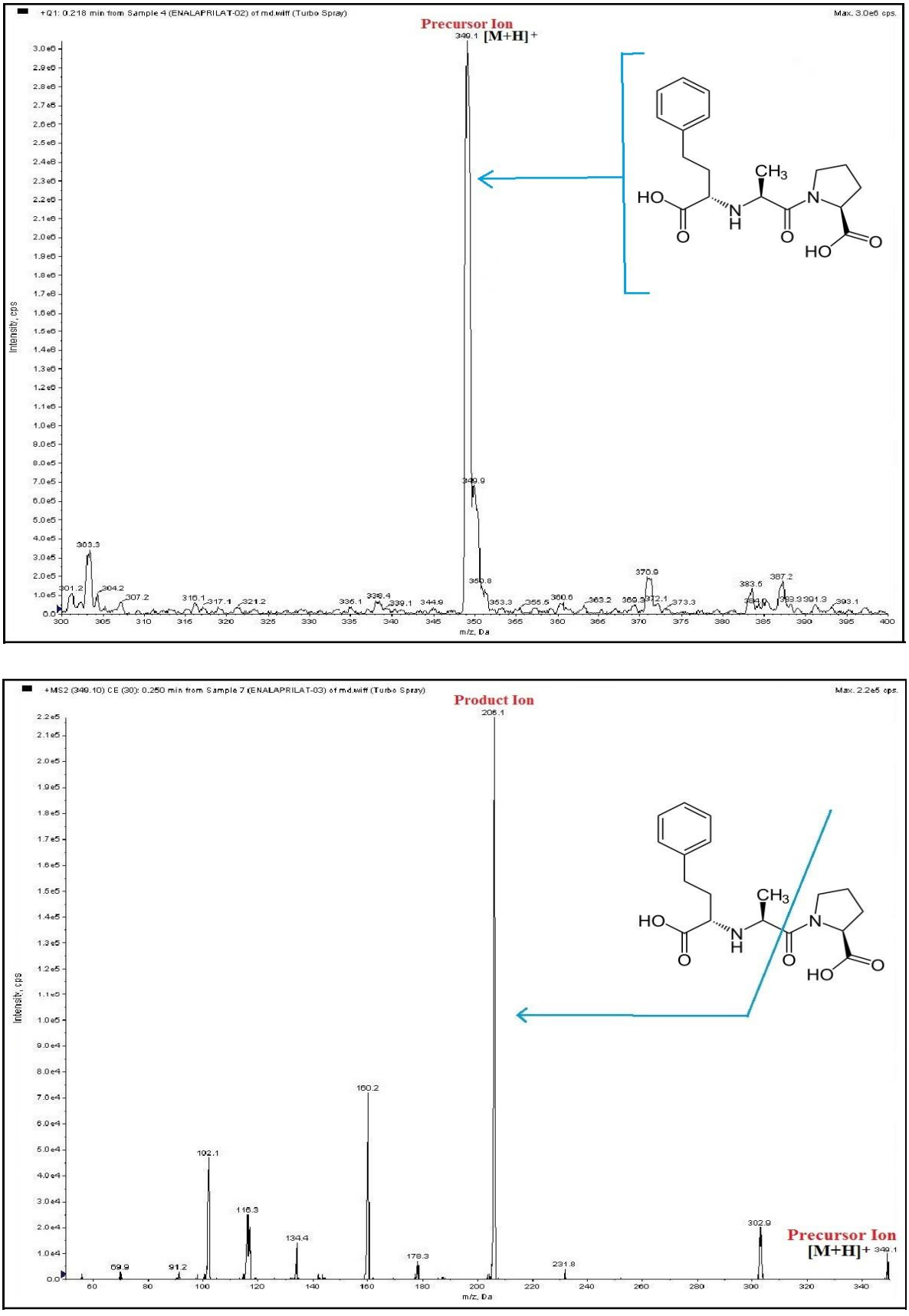


Figure.5.37 Mass spectra (Q1 and MS2) of the positive ion $[M+H]^+$ of enalaprilat (EPLT) with structural interpretation

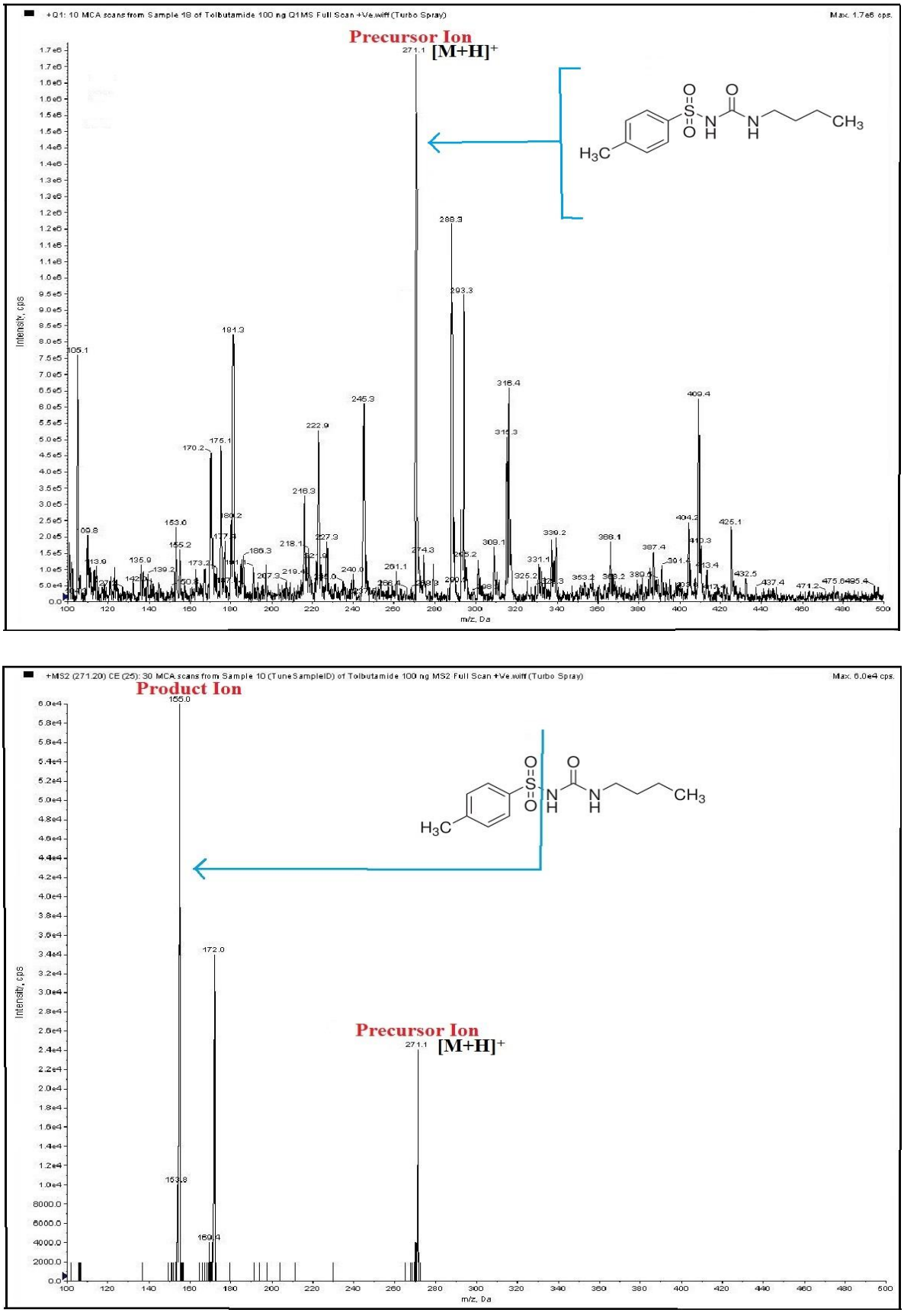


Figure.5.38 Mass spectra (Q1 and MS2) of the positive ion $[M+H]^+$ of tolbutamide (TBM, IS) with structural interpretation

5.5.2 Selection of the mobile phase for EPL and EPLT

EPL and EPLT have less structural variations with pka (strongest acidic) values of 3.0 and 2.97, polar surface area of 95.9 and 106.9, respectively. Therefore, different mobile phase were tried for the proper elution of both EPL and EPLT. Various solvents such as acetonitrile, methanol, and Milli-Q water (with 18.2 MΩ.cm resistivity and conductivity of < 0.055 µS/cm) were used in different ratios with buffers such as ammonium acetate and ammonium formate, as well as acid additives like formic acid and acetic acid as enhancer in varying strengths were tried to optimise response, sensitivity, efficiency and also to improve the peak shape of both the analytes and IS. Generally acetonitrile and methanol both were used as organic modifier in the mobile phases during method development. For buffers, MS suitable reagents or salts like ammonium acetate and ammonium formate were used in different strengths like 2 mM, 5 mM and 10 mM, whereas MS suitable acids such as formic acid and acetic acid also in different strengths i.e., 0.1% to 0.5% were dissolved in Milli-Q water and used.

At the time of selection of the mobile phase, different combinations under gradient conditions were tried, such as 0.1% of formic acid in Milli-Q water at pump A and 0.1% formic acid in methanol at pump B, 0.2% formic acid in Milli-Q water at pump A and 0.2% formic acid in methanol at pump B, 0.1% formic acid in Milli-Q water at pump A and 0.1% formic acid in acetonitrile at pump B, 0.2% formic acid in Milli-Q water at pump A and 0.2% formic acid in acetonitrile at pump B, 10 mM ammonium acetate in Milli-Q water at pump A and 0.1% formic acid in methanol at pump B and 10 mM ammonium formate in Milli-Q water at pump A and 0.1% formic acid in methanol at pump B. Finally, it was observed that under gradient conditions using 0.1% formic acid in Milli-Q water at pump A and 0.1% formic acid in methanol at pump B by the following gradient (minutes, % mobile phase B or pump B conc.): 0.01, 10; 0.50, 10; 0.70, 90; 2.00, 90; 2.10, 10; 3.00 at a flow rate of 0.5 mL/min was most appropriate for EPL, EPLT and IS (TBM) to give best sensitivity, efficiency and better peak shape compared to other mobile phases (Figure.5.39).

5.5.3 Selection of the column (stationary phase) for EPL and EPLT

Different kinds of columns or stationary phases such as Phenomenex Kinetex C18 [50 mm (length) x 3 mm (ID) 100 Å with 5 µm particle size], Agilent-Eclispse Plus C18 [75 mm (length) x 4.6 mm (ID) with 3.5 µm particle size] and Agilent ZORBAX CN [50 mm (length) x 4.6 mm (ID) with 3.5 µm particle size] were tried for checking the separation, peak shape

and response, i.e., better selectivity and sensitivity for both the analytes and IS during method development. The use of a short chromatographic column, such as Phenomenex Kinetex C18, and an optimized total flow rate of 0.5 mL/min resulted in the better separation and elution of the analytes and IS of interest in a very short time with good peak shape and response (see Figure.5.39), whereas the use of Agilent-Eclispse Plus C18 and Agilent ZORBAX CN resulted in poor peak shape and in response also. So finally this Phenomenex Kinetex C18 column was chosen for the entire bioanalysis.

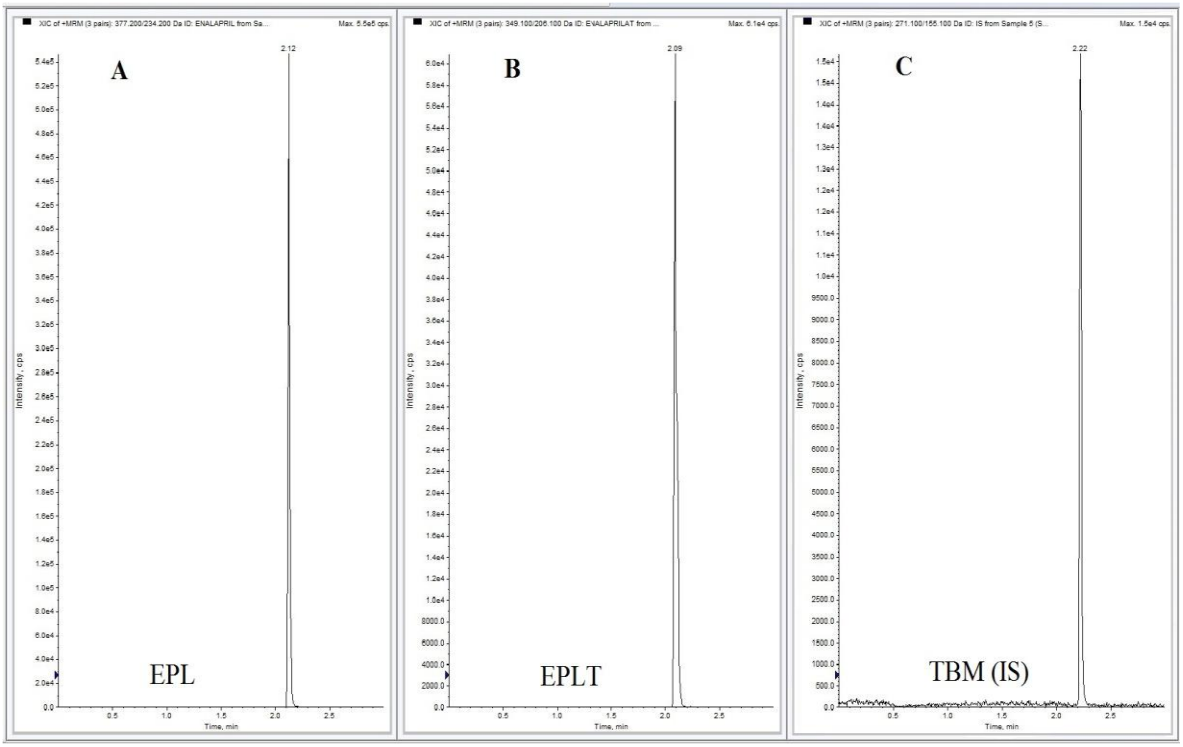


Figure.5.39 Representative chromatograms of A) EPL, B) EPLT and C) TBM (IS) after selection of mobile phase and column

5.5.4 Selection of the internal standard (IS) for EPL and EPLT

In the initial phase, several compounds such as metformin, losartan, tolbutamide, propranolol, metoprolol and rosiglitazone were investigated and finally tolbutamide (TBM) was concluded as a best choice of internal standard (IS). It has shown no interference with the analytes during analysis and the intensity of molecular ion peak in mass spectrometric analysis remained unaffected as compared with others and also similar ionization properties with the analytes. TBM was coeluted properly with EPL and EPLT in the selected mobile phase (0.1% formic acid in Milli-Q water at pump A and 0.1% formic acid in methanol at pump B under gradient conditions) and column (Phenomenex Kinetex C18). The flow rate was 0.5 mL/min

that optimized on the basis of the retention time of the analytes and gradient elution provides faster separation with the developed chromatographic conditions. Now, the extraction recovery of TBM in plasma was much higher than the other tested compounds along with the analytes in plasma during method development (see Table.5.54 and Figure.5.40).

Table.5.54 Extraction recovery (%) of different drug molecules in plasma

Compound	Extraction recovery (%)
Metformin	56.71
Losartan	72.63
Tolbutamide	97.44
Propranolol	96.83
Metoprolol	96.15
Rosiglitazone	95.30

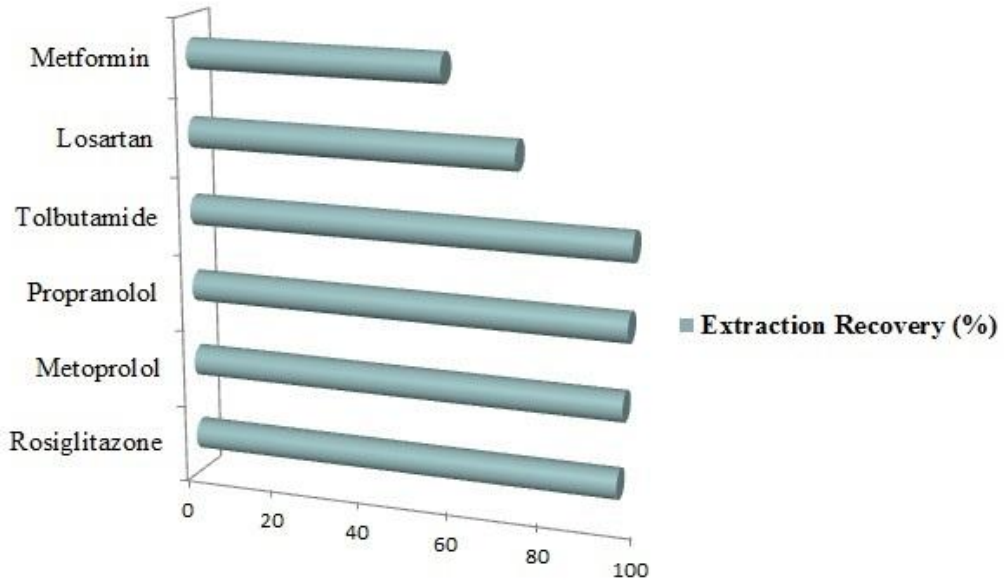


Figure.5.40 Extraction recoveries of various compounds in plasma

5.5.5 Selection of the diluent for EPL and EPLT

An appropriate diluent was selected for both EPL and EPLT after the selection of mobile phase, column and IS in method development process. Methanol, acetonitrile, methanol :

H_2O :: 50:50 (v/v) and acetonitrile : H_2O :: 50:50 (v/v) were tested as diluents to confirm the response, sensitivity as well as peak shape. Hence, methanol : H_2O :: 50:50 (v/v) was found much better response, sensitivity and peak shape than the other used solvents (Table.5.55).

Table.5.55 Intensity (cps) of EPL, EPLT and TBM (IS) in different diluents

Diluent	Intensity (cps)		
	EPL (conc. 500 ng/mL)	**EPLT (conc. 500 ng/mL)**	**TBM (IS) (conc. 500 ng/mL)**
Methanol	270000 (2.7 e^5)	34000 (3.4 e^4)	11000 (1.1 e^4)
Acetonitrile	75000	12000	7600
Methanol : H_2O :: 50:50 (v/v)	550000 (5.5 e^5)	60000 (6.0 e^4)	38000 (3.8 e^4)
Acetonitrile : H_2O :: 50:50 (v/v)	93000	27000	8400

5.5.6 Optimization of the final mobile phase with selected buffer and organic modifier for EPL and EPLT

The selected mobile phase was optimized after the selection of both column and IS. Composition and concentration of the buffer i.e., formic acid and also its percentage (%) in methanol, the organic modifier and pH were adjusted to improve chromatographic results. Different compositions and concentrations of the buffer formic acid in Milli-Q water described in Table.5.56 were checked and differences in the intensity of EPL, EPLT and IS (TBM) were also observed. It has been seen in Figure.5.41, a graphical illustration that the intensity (cps) of EPL, EPLT and IS (TBM) increases with gradually decreasing the concentration, i.e., percentage (%) of formic acid like 0.5%, 0.4%, 0.3%, 0.2% and 0.1% respectively. Finally, 0.1% formic acid in Milli-Q water was found most suitable buffer for the mobile phase due to properly maintained the pH throughout the bioanalysis for getting uniform peak shape along with consistency in ionization and fragmentation in the MS for both the analytes and IS.

Table.5.56 Effect of buffer composition and concentration of the mobile phase on the intensity (cps) of the analytes and IS

Buffer composition (Concentration or percentage of formic acid in Milli-Q water)	Intensity (cps)		
	EPL (conc. 500 ng/mL)	EPLT (conc. 500 ng/mL)	TBM (IS) (conc. 500 ng/mL)
0.1%	550000 (5.5 e^5)	60000 (6.0 e^4)	38000 (3.8 e^4)
0.2%	420000 (4.2 e^5)	56000 (5.6 e^4)	35000 (3.5 e^4)
0.3%	370000 (3.7 e^5)	32000 (3.2 e^4)	29000 (2.9 e^4)
0.4%	98000 (9.8 e^4)	13000 (1.3 e^4)	10000 (1.0 e^4)
0.5%	83000 (8.3 e^4)	8700	7450

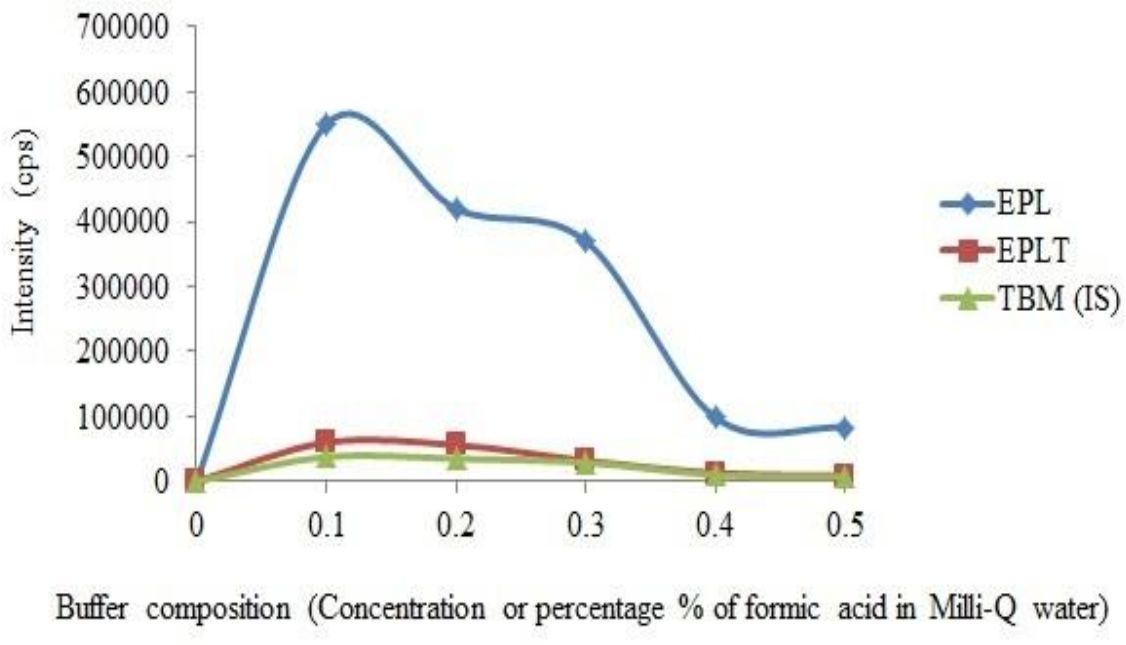


Figure.5.41 Effect of buffer composition on the intensity (cps) of EPL, EPLT and TBM (IS)

After optimization of the buffer organic modifier was also optimized, because it is a key part of the mobile phase. So concentration or percentage (%) of formic acid in methanol (the organic modifier) was optimized to improved the peak shape and also enhanced the response or intensity (cps) of both the analytes and IS. The different percentage of formic acid in methanol were tried as similar to the percentage of formic acid in Milli-Q water i.e., the buffer such as 0.1%, 0.2%, 0.3%, 0.4% and 0.5% respectively and also the response or intensity (cps) increases by the decrease in percentage of formic acid in the methanol just like the buffer optimization. Now, the optimum intensity (response), resolution and good peak shape were seen in 0.1% formic acid in methanol and this condition was maintained throughout the bioanalysis. Therefore, the mobile phase, i.e., the buffer and the organic modifier was run under gradient conditions at a flow rate of 0.5 mL/min and a total chromatographic run time of 3 minutes where at retention times of EPL, EPLT and IS (TBM) 2.13, 2.10, and 2.23 minutes, respectively.

Lastly, pH of the buffer, i.e., 0.1% formic acid in Milli-Q water was optimized. Different pH of the buffer was checked during optimization process. So finally it was found that at pH 2.45 of the buffer gives more response (intensity), better resolution and peak shape than other pH (see Figure.5.42 and Table.5.57). Therefore, 0.1% formic acid in Milli-Q water (pH 2.45) at pump A and 0.1% formic acid in methanol at pump B by the following gradient (minutes, % mobile phase B or pump B conc.): 0.01, 10; 0.50, 10; 0.70, 90; 2.00, 90; 2.10, 10; 3.00 was considered as a suitable mobile phase of the present LC-ESI-MS/MS method for EPL and EPLT.

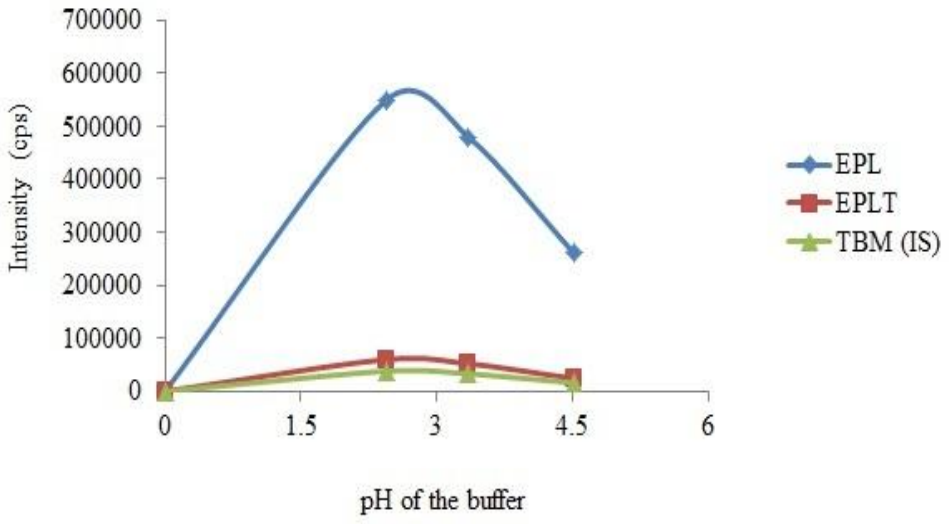


Figure.5.42 Effect of pH of the buffer on the intensity (cps) of EPL, EPLT and TBM (IS)

Table.5.57 Effect of pH of the buffer on the intensity (cps) of the analytes and IS

pH of the buffer	Intensity (cps)		
	EPL (conc. 500 ng/mL)	**EPLT (conc. 500 ng/mL)**	**TBM (IS) (conc. 500 ng/mL)**
2.45	550000 (5.5 e^5)	60000 (6.0 e^4)	38000 (3.8 e^4)
3.35	480000 (4.8 e^5)	52000 (5.2 e^4)	33000 (3.3 e^4)
4.52	260000 (2.6 e^5)	24000 (2.4 e^4)	16000 (1.6 e^4)

5.5.7 Optimization of the flow rate for EPL and EPLT

Different flow rates of the mobile phase were tested to find out the suitable one for present method. Flow rate was one of the important aspect for good result, proper ionization in ESI, better sensitivity, proper retention time (RT) and also total chromatographic run time. It was seen that on increasing the flow rate the response or intensity was increased, whereas RT and total run time were decreased and short. Mass tuning flow rate was 10 µL/min in Harvard syringe pump method during MS/MS development. In LC-MS/MS development when the MS was coupled with the LC at a flow rate of 0.5 mL/min (500 µL/min) found maximum response and proper RT with shorter run time.

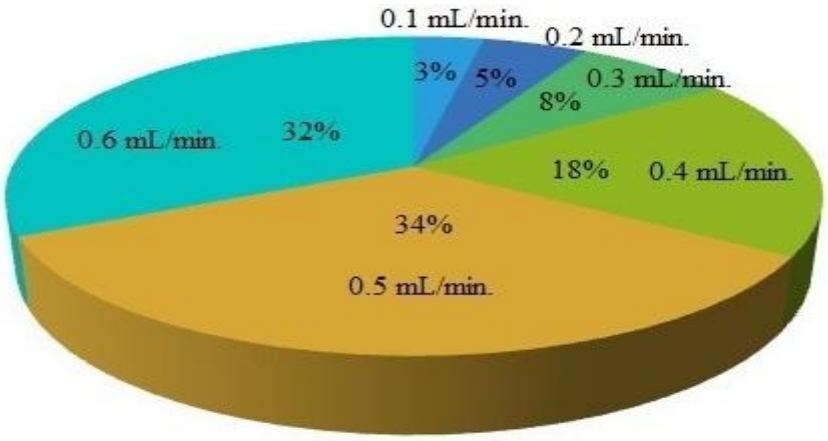


Figure.5.43 An impact of different flow rate on the intensity (cps) of EPL, EPLT and TBM (IS)

Now, it was graphically expressed in Figure.5.43 and Figure.5.44 to realize that how the flow rate affected on sensitivity or response (intensity) of desired molecules such as EPL, EPLT and IS (TBM). Finally after some trial and error, a perfect flow rate was 0.5 mL/min which continued throughout the whole bioanalysis.

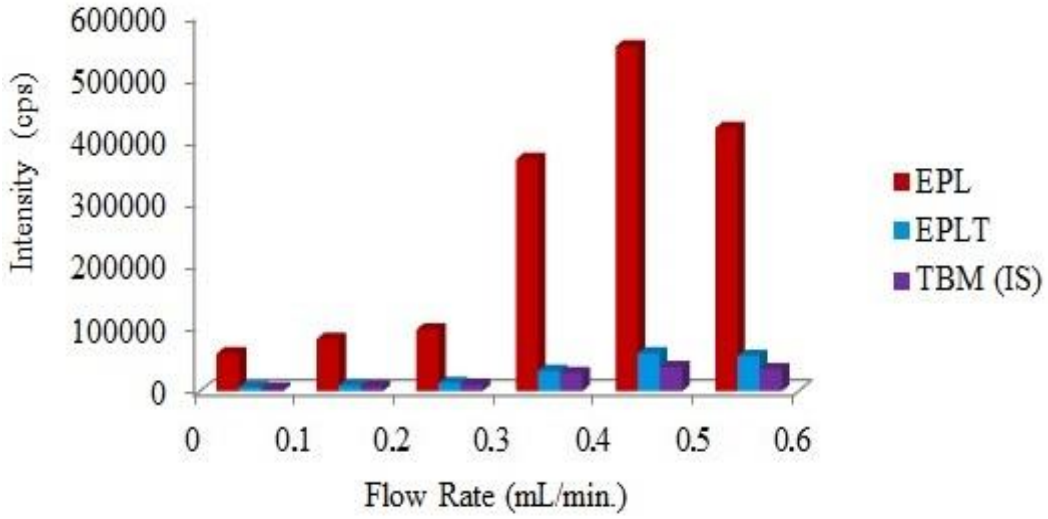


Figure.5.44 Effect of different flow rate on the intensity (cps) of EPL, EPLT and TBM (IS)

5.5.8 Optimization of the mass spectrometric (MS) parameters for EPL, EPLT and TBM (IS)

After selection and optimization of the final mobile phase, an experimental setup was prepared to determine the optimal MS parameters or compound dependent parameters with source and gas parameters also by flow injection analysis (FIA) of the method of compound optimization in tune mode in the positive polarity of the Analyst 1.5 software. In FIA, the range of all desired parameters were manually inputted and got the final optimized results for the analytes (EPL and EPLT) and IS (TBM) which have been expressed in Figure.5.45 to Figure.5.52 and Table.5.58 and Table.5.59.

FIA Compound Optimization Results -

Compound-Dependent Parameters :

Declustering Potential (DP)

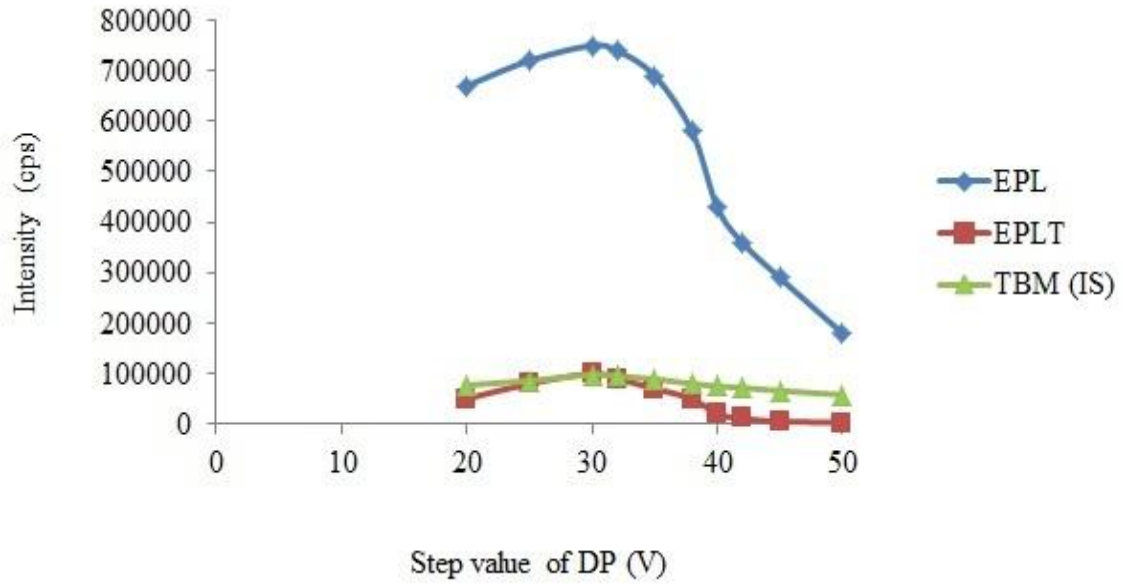


Figure.5.45 Optimization of declustering potential (DP) value for EPL, EPLT and TBM (IS)

Focusing Potential (FP)

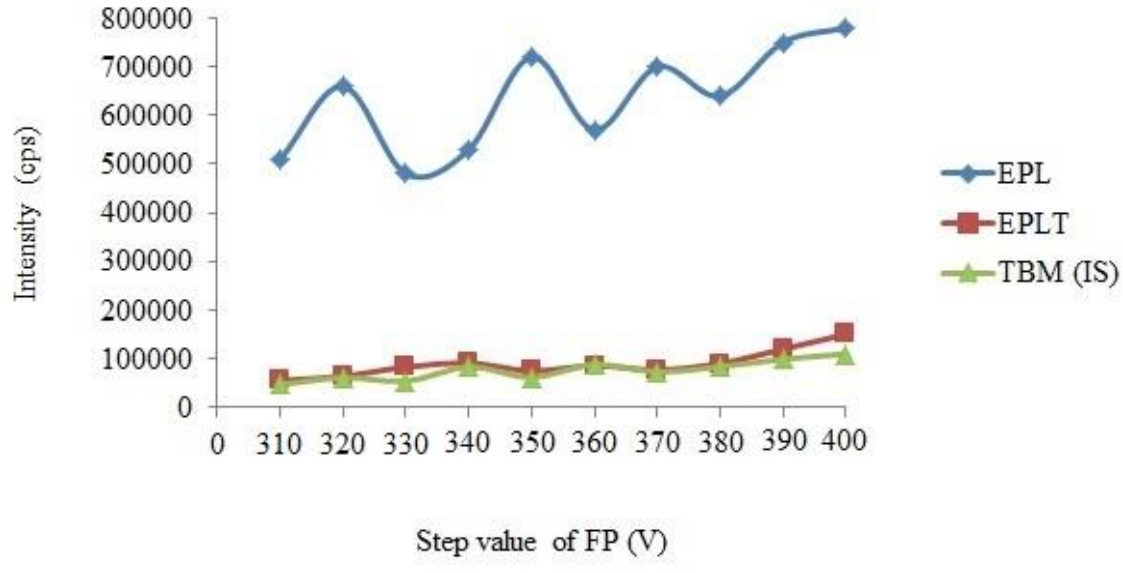


Figure.5.46 Optimization of focusing potential (FP) value for EPL, EPLT and TBM (IS)

Entrance Potential (EP)

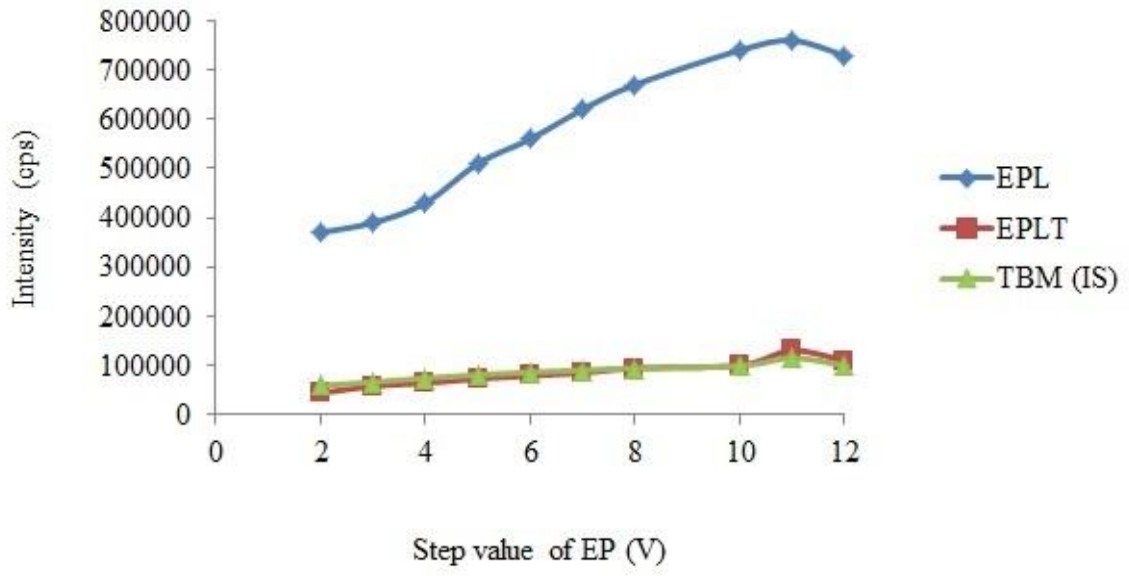


Figure.5.47 Optimization of entrance potential (EP) value for EPL, EPLT and TBM (IS)

Collision Cell Entrance Potential (CEP)

Generally, collision cell entrance potential (CEP) was introduced as by default in the software. Therefore, during manual tuning it was not required to optimize. Whereas in FIA it could be optimized and so it was successfully carried out.

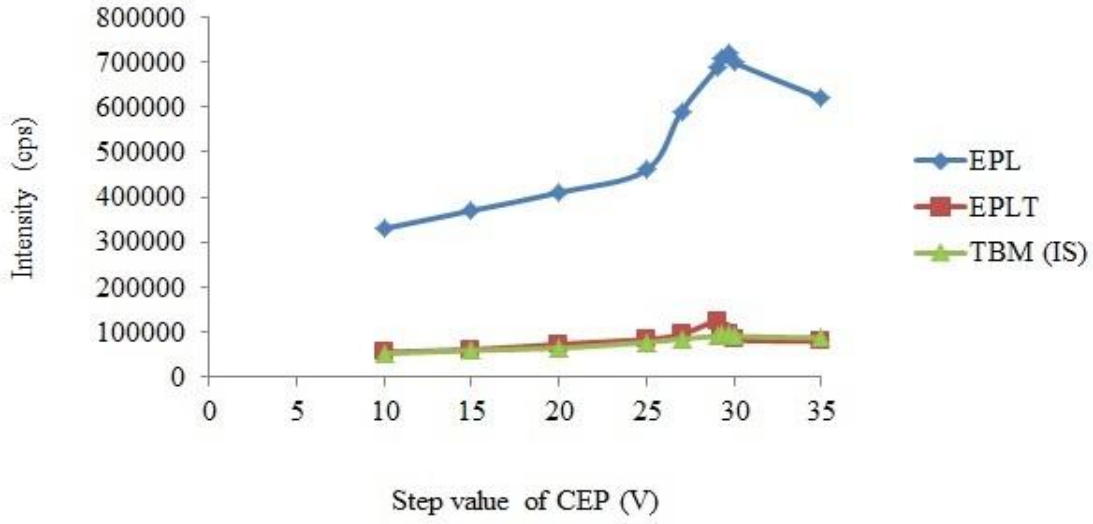


Figure.5.48 Optimization of collision cell entrance potential (CEP) value for EPL, EPLT and TBM (IS)

Collision Energy (CE)

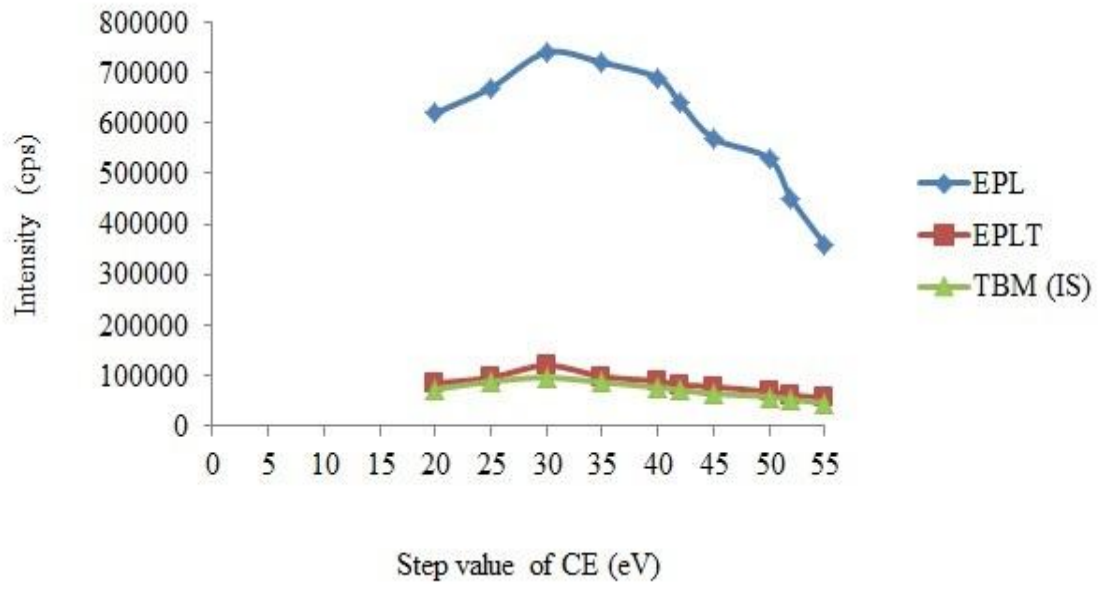


Figure.5.49 Optimization of collision energy (CE) value for EPL, EPLT and TBM (IS)

Collision Cell Exit Potential (CXP)

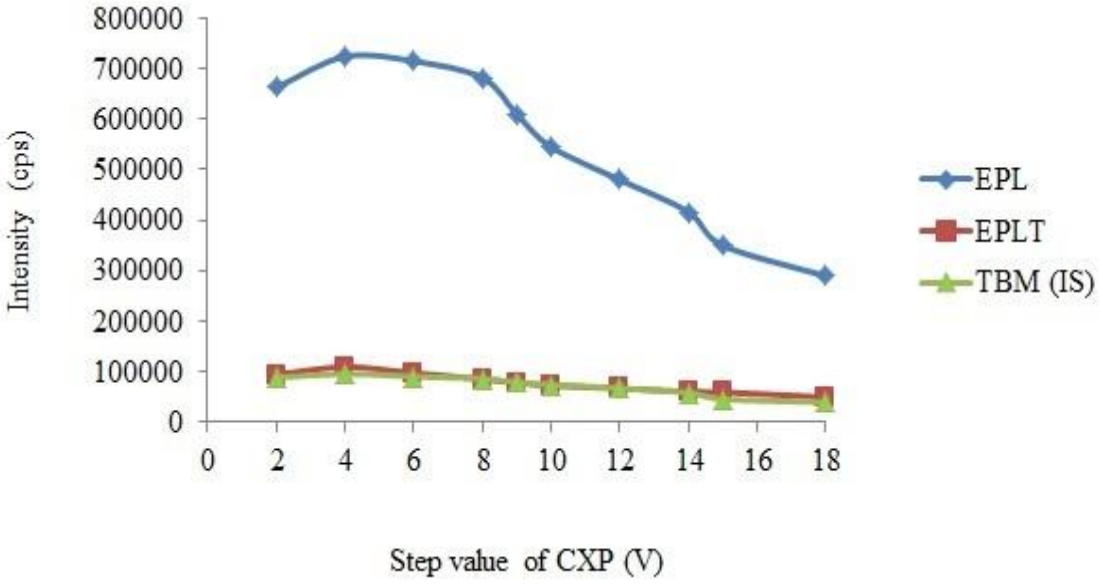


Figure.5.50 Optimization of collision cell exit potential (CXP) value for EPL, EPLT and TBM (IS)

Table.5.58 Optimized compound-dependent parameters of EPL, EPLT and TBM (IS)

Compound-dependent parameters	EPL	EPLT	TBM (IS)
DP (V)	30.00	30.00	30.00
FP (V)	400.00	400.00	400.00
EP (V)	11.00	11.00	11.00
CEP (V)	29.73	29.08	27.29
CE (eV)	30.00	30.00	30.00
CXP (V)	4.00	4.00	4.00

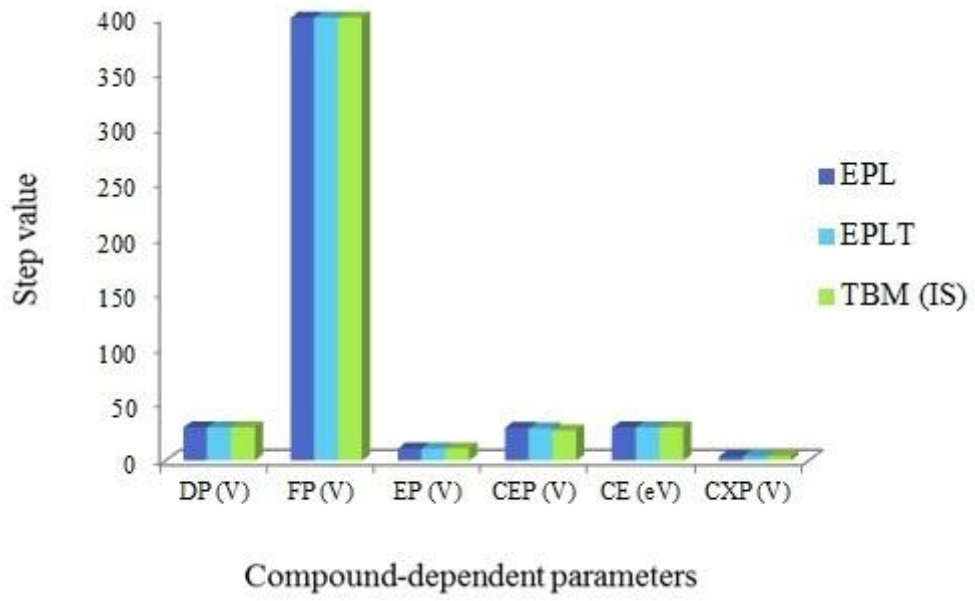


Figure.5.51 Optimized compound-dependent parameters for EPL, EPLT and TBM (IS)

Source and Gas Parameters :

Curtain (CUR) gas, collision activated dissociation (CAD), turbo ion spray voltage (ISV), turbo ion spray source temperature or turbo gas temperature (TEM), nebulizer gas (GS1) and turbo ionspray gas or auxiliary gas or desolvation gas (GS2) (i.e., heater gas) were the source and gas parameters.

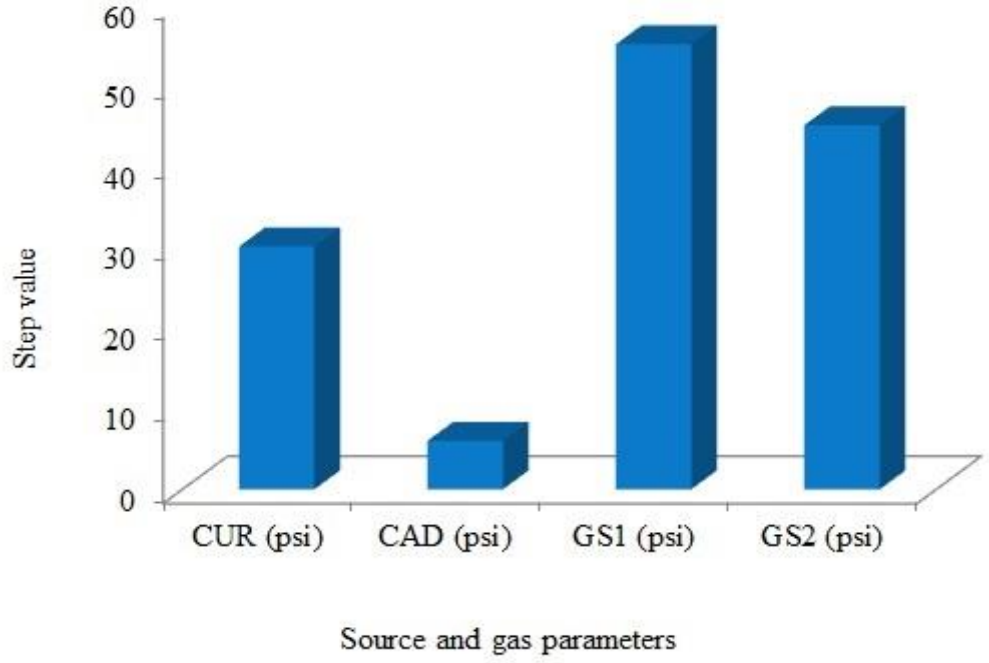


Figure.5.52 Optimized source and gas parameters of EPL, EPLT and TBM (IS)

Table.5.59 Optimized source and gas parameters of EPL, EPLT and TBM (IS)

Source and gas parameters	
CUR (psi)	30.0
CAD (psi)	6.0
ISV (V)	5000.0
TEM (^{o}C)	400.0
GS1 (psi)	55.0
GS2 (psi)	45.0

5.5.9 Selection of extraction solvent and procedure for EPL and EPLT from human plasma

A simple protein precipitation technique (PPT) was selected or considered as the sample preparation and extraction of both the analytes (EPL and EPLT) and IS (TBM) from the human plasma because of the increase of sensitivity or response (intensity) as well as the percentage (%) of the extraction recovery. Therefore, main advantages of the PPT was to

establish a simple extraction procedure, more and better recovery, cost effective, i.e., low cost, simple and direct preparation procedure than both liquid-liquid extraction (LLE) and solid phase extraction (SPE) or 96 well SPE technique. In case of PPT, used just one organic solvent and also it was less time consuming than LLE and SPE. But it has some drawbacks such as the column life was reduced and chances of contamination was quite high. Initially, liquid-liquid extraction (LLE) was tried and so various organic solvents were used as trial and error like ethyl acetate, dichloromethane, chloroform, diethyl ether, methyl tert-butyl ether and n-Hexane to determine the suitable solvent for maximum response, better and consistent recovery with good peak shape of EPL, EPLT and TBM (IS). Now according to the results of extraction recovery (%) in LLE, ethyl acetate (only for EPL not EPLT), diethyl ether and methyl tert-butyl ether have shown a moderate response, but in case of dichloromethane, chloroform and n-Hexane it was very poor or not upto the mark (see Table.5.60 and Figure.5.53). Whereas in case of IS (TBM), recovery was much better than both EPL and EPLT in most of the solvents (Table.5.60).

Table.5.60 Extraction recovery (%) of EPL, EPLT and TBM (IS) from plasma by different organic solvents

Extracting solvents	Extraction recovery (%)		
	EPL	**EPLT**	**TBM (IS)**
Ethyl acetate (EA)	66.43	25.3	86.63
Dichloromethane (DCM)	30.9	26.44	90.91
Chloroform (CHL)	27.81	20.12	95.45
Diethyl ether (DEE)	69.75	65.42	77.27
Methyl tert-butyl ether (MTBE)	74.8	72.6	81.82
n-Hexane	43.25	39.04	63.64

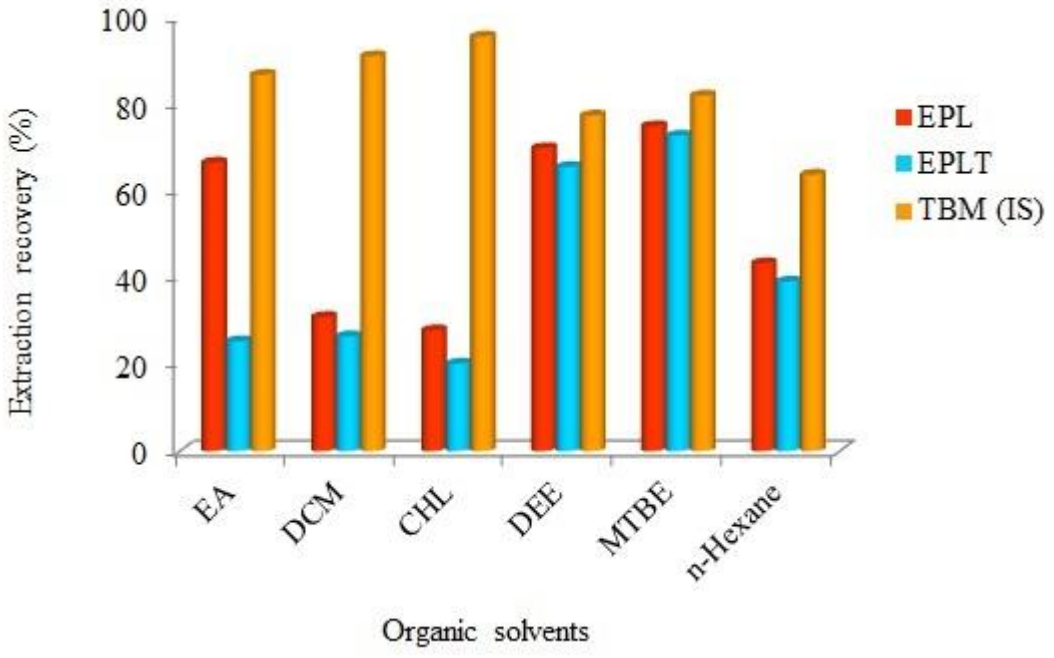


Figure.5.53 The extraction recovery (%) of EPL, EPLT and TBM (IS) after treating with various organic solvents

Therefore, it was realized that LLE was not enough to get better reproducibility and higher recovery for both EPL and EPLT from plasma. So protein precipitation technique (PPT) was tried to adopt for the appropriate sample extraction procedure. Now, five solvents were selected for the protein precipitation and those were methanol, acetonitrile (cold), acetone, 10% perchloric acid in Milli-Q water and 10% aqueous orthophosphoric acid solution. The sample preparation procedure, i.e., PPT were described previously in the section 4.9.2 and were find out the extraction yields of the analytes and IS (Table.5.61). In protein precipitation (PPT), results of the recovery of both analytes and IS were much better than LLE. Now among all the above mentioned precipitants, acetonitrile (cold) has shown better and quite higher recoveries, 97.55% for EPL, 99.43% for EPLT and 99.82% for IS (TBM), respectively also with good intensity (cps) and peak shape (see Table.5.61, Figure.5.54 and Figure.5.55). The chromatograms were found properly without any interfering or endogenous peaks. So finally, cold acetonitrile was confirmed as the precipitant for protein precipitation (PPT).

Table.5.61 Extraction recovery (%) of EPL, EPLT and TBM (IS) from plasma by various solvents in protein precipitation

Solvents for protein precipitation	Extraction recovery (%)		
	EPL	EPLT	TBM (IS)
Methanol	95.34	92.8	96.71
Acetonitrile (cold)	97.55	99.43	99.82
Acetone	86.35	89.6	93.42
10% perchloric acid solution	94.65	91.7	88.63
10% aqueous orthophosphoric acid solution	78.9	75.12	86.33

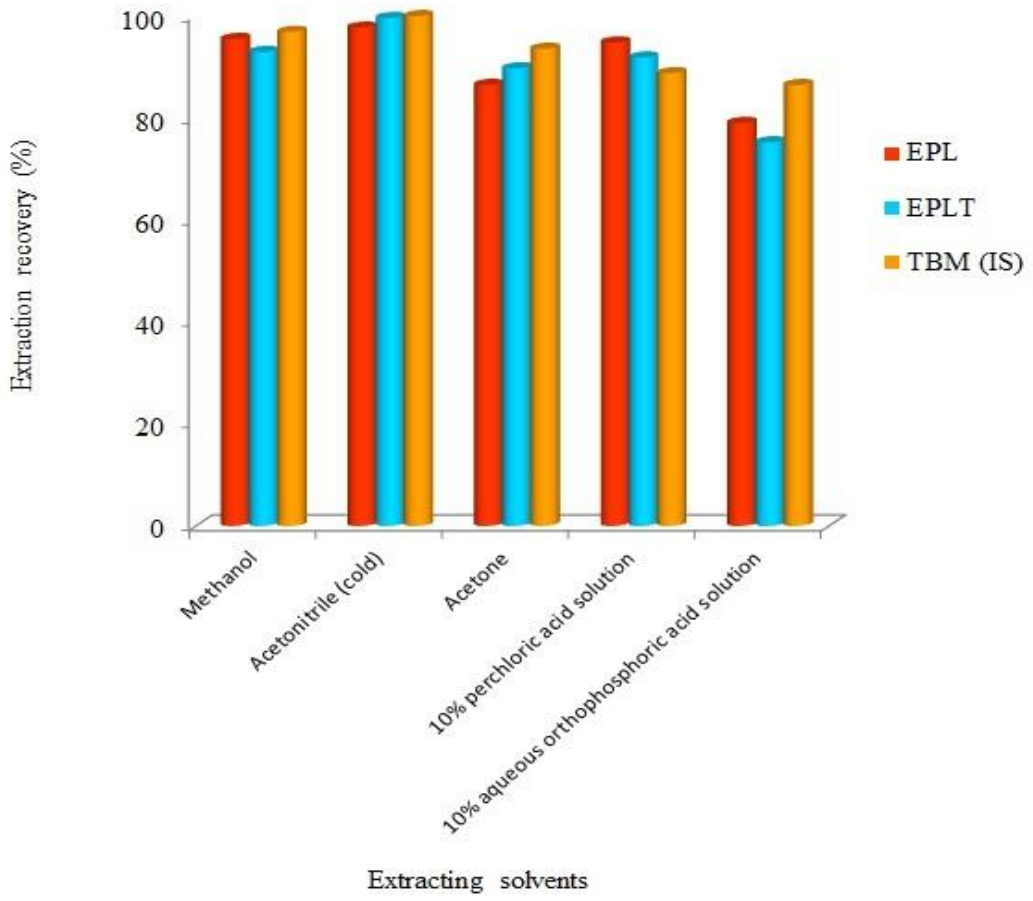
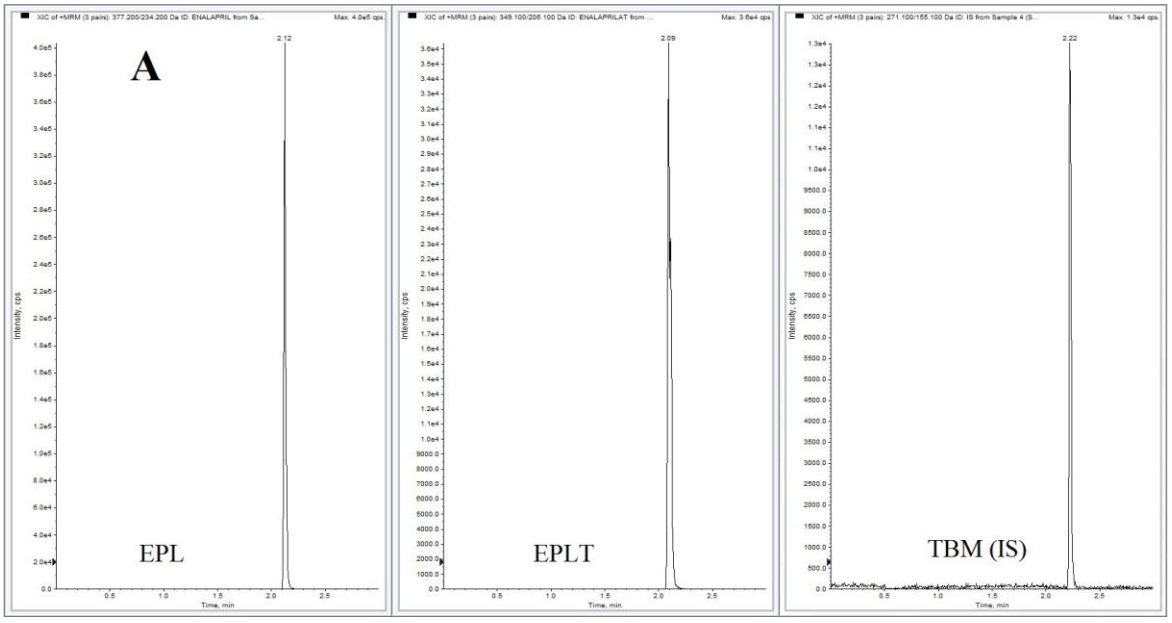
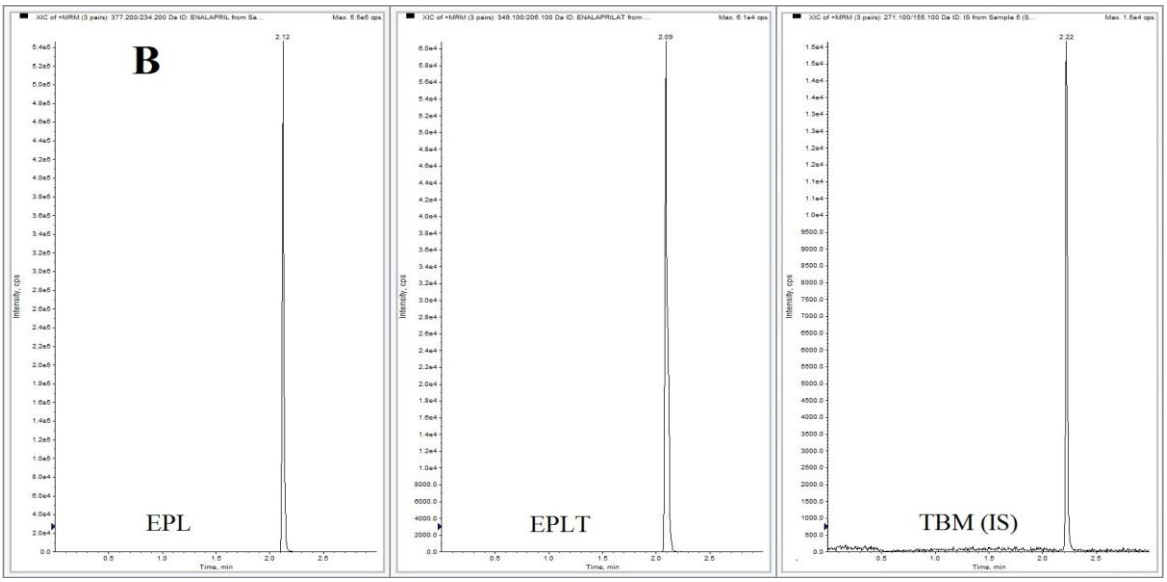
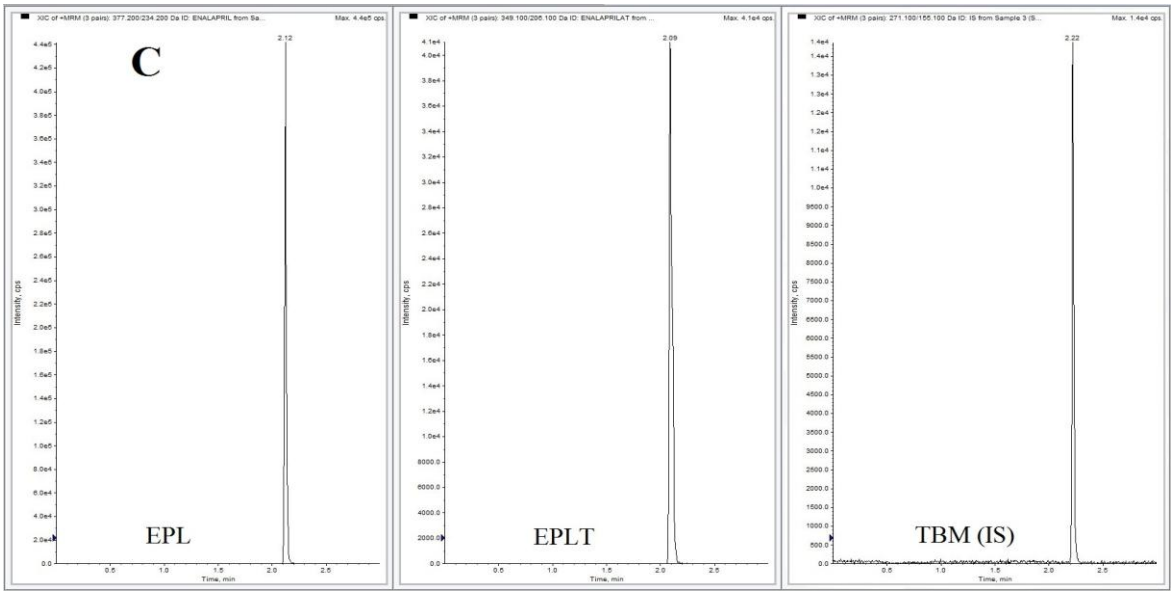


Figure.5.54 The extraction recovery (%) of EPL, EPLT and TBM (IS) after treating with different extracting solvents


A
EPL
EPLT
TBM (IS)
Intensity, cps
Time, min


B
EPL
EPLT
TBM (IS)
Intensity, cps
Time, min


C
EPL
EPLT
TBM (IS)
Intensity, cps
Time, min

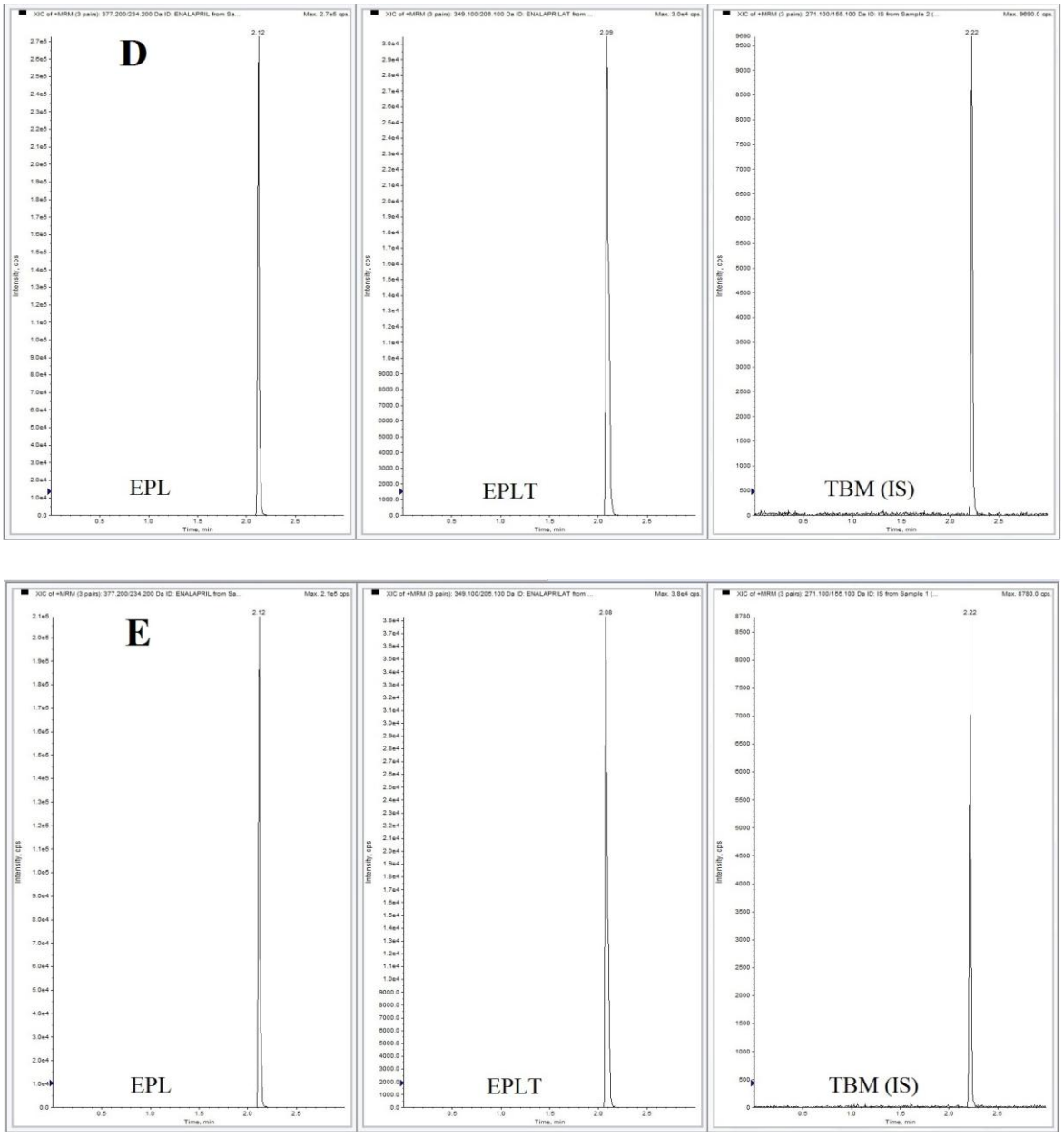


Figure.5.55 The intensity (cps) and peak shape of EPL, EPLT and TBM (IS) using A) Methanol, B) Acetonitrile (cold), C) Acetone, D) 10% perchloric acid solution and E) 10% aqueous orthophosphoric acid solution

5.5.10 Final optimized plasma extraction procedure for EPL and EPLT

At first 300 µL of plasma was taken in 2 mL eppendorf tubes (microcentrifuge tubes) and added 900 µL of ice cold acetonitrile containing 1 µg/mL TBM (IS) solution. After that, vortex mixed were performed accurately and extensively for 10 minutes. Next, centrifugation was done at 10,000 rpm for 15 min and formed a clear supernatant. The supernatant was separated very carefully and filled it into autosampler vials for the LC–MS/MS analysis.

Moreover, calibration concentration (CC) and quality control (QC) samples were prepared following the same procedure.

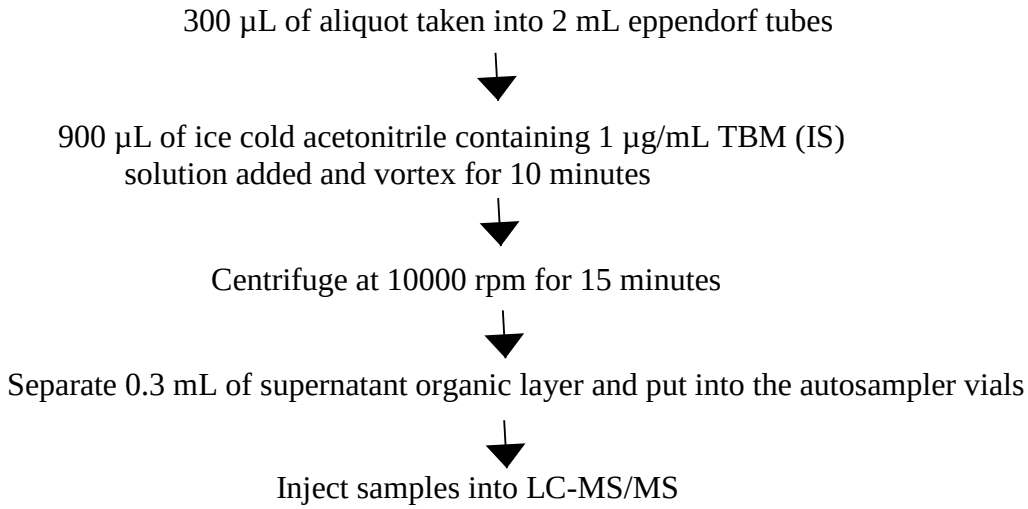


Figure.5.56 A schematic presentation of final optimized plasma extraction procedure of EPL and EPLT

5.5.11 Final optimized chromatographic conditions for bioanalysis of EPL and EPLT in human plasma

5.5.11.1 Chromatographic parameters

Integrated System : Shimadzu Controller prominence, autosampler SIL-20AC, CBM-20A Lite System Control Compartment, binary pump : pump A and pump B LC-20AD, online solvent degasser DGU- 20A3, column oven CTO-10 AS VP and liquid chromatography.

Column	Phenomenex Kinetex C18, 50 mm x 3 mm (length x inner diameter), 5 µm particle size
Mobile Phase	Pump A: 0.1% formic acid in Milli Q water (pH 2.45)
	Pum B: 0.1% formic acid in methanol
Flow Rate	0.5 mL/min.
Injection Volume	10 µL

Column Oven Temperature	Not Applicable
Autosampler Temperature	15 °C
Total Chromatographic Run Time	3.0 minutes

Shimadzu LC Method Properties

Shimadzu LC Equlibration time = 0.00 min

Shimadzu LC system Injection Volume = 10.00 µL

Shimadzu LC Method Parameters

Pumps

Pump A Model: LC-20AD

Pump B Model: LC-20AD

Pumping Mode: Binary Flow

Total Flow: 0.5000 mL/min.

Autosampler

Model: SIL-20AC

Rinsing Volume: 200 µL

Needle Stroke: 52 mm.

Rinsing Speed: 35 µL/sec.

Sampling Speed: 15.0 µL/sec.

Purge Time: 25.0 min.

Rinse Dip Time: 0 sec.

Rinse Mode: Before and after aspiration

Cooler Enabled: Yes

Cooler Temperature: 15 deg. C

Control Vial Needle Stroke: 52 mm

Oven

Model: CTO-10ASvp

Temperature Control: Disabled

System Controller

Model: CBM-20A Lite

Power: On

Event 1: Off

Event 2: Off

Event 3: Off

Event 4: Off

Time Program

Time	Module	Events	Parameter
0.01	Pumps	Pump B Conc.	10
0.50	Pumps	Pump B Conc.	10
0.70	Pumps	Pump B Conc.	90
2.00	Pumps	Pump B Conc.	90
2.10	Pumps	Pump B Conc.	10
3.00	System Controller	Stop	

5.5.11.2 Mass spectrometric parameters

Period 1 Experiment 1:

Scan Type:	MRM (MRM)
Scheduled MRM:	No
Polarity:	Positive
Scan Mode:	N/A
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Unit
Intensity Thres.:	0.00 cps
Settling Time:	0.0000 msec
MR Pause:	5.0070 msec
MCA:	No
Step Size:	0.00 Da

Enalapril (EPL)

Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Parameter	Volt
377.2	234.2	200.00	DP	30.00
			CEP	29.73
			CE	30.00
			CXP	4.00

Enalaprilat (EPLT)

Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Parameter	Volt
349.1	206.1	200.00	DP	30.00
			CEP	29.08
			CE	30.00
			CXP	4.00

Tolbutamide (TBM, IS)

Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Parameter	Volt
271.1	155.0	200.00	DP	30.00
			CEP	27.29
			CE	30.00
			CXP	4.00

Parameter Table (Period 1 Experiment 1)

CUR:	30.00
TEM:	400.00
GS1:	55.00
GS2:	45.00
ihe:	ON
IS:	5000.00
CAD	6.00
FP	400.00
EP	11.00

Instrument Parameters:

Detector Parameters (Positive):

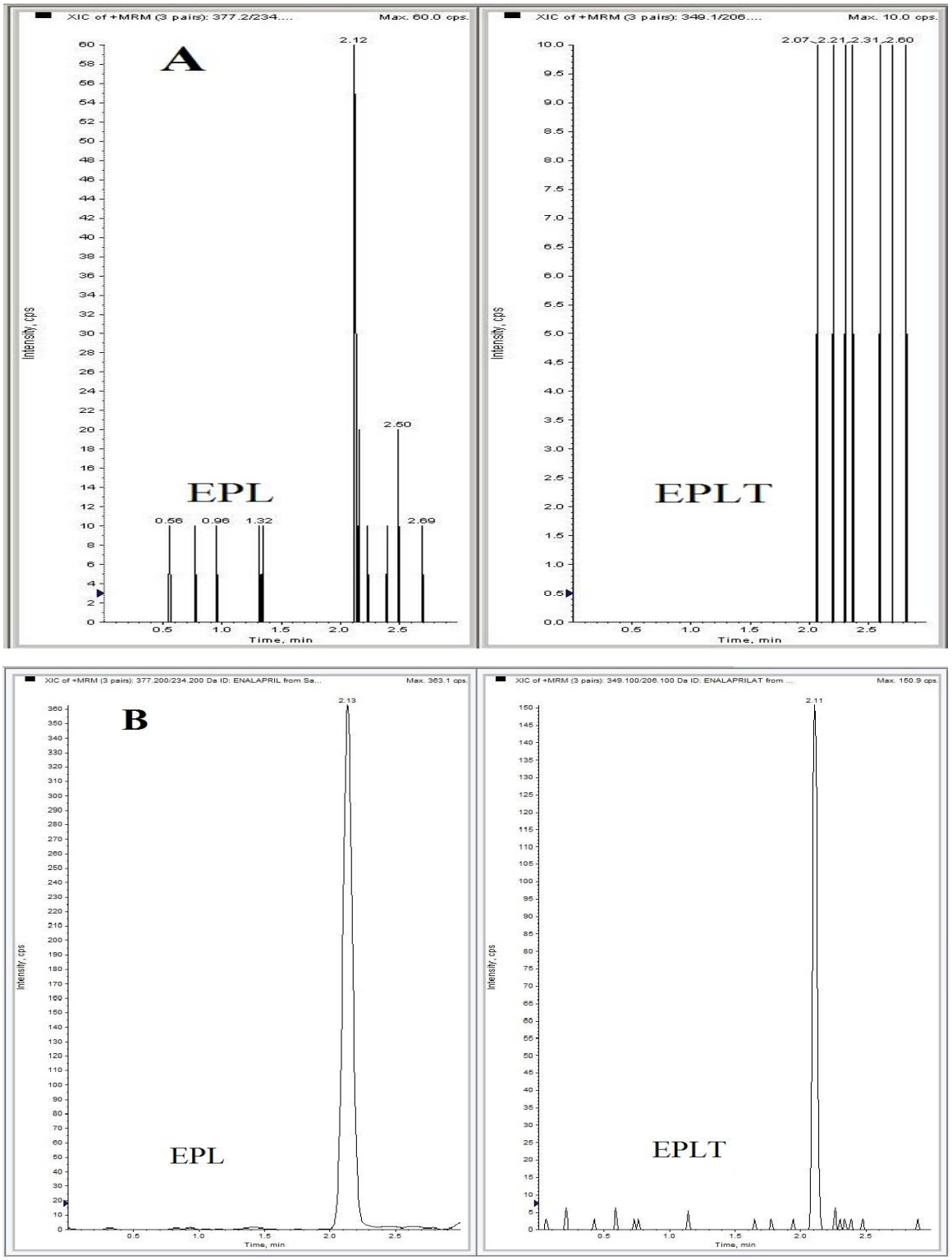
DF	-125.0
CEM	2300.0

Keyed Text:

File was created with the software version: Analyst 1.5

5.5.12 Evaluation of limit of detection (LOD) and lower limit of quantification (LLOQ) for EPL and EPLT

The LOD of EPL was 0.25 ng/mL and EPLT was 0.80 ng/mL, respectively where signal-to-noise ratio was 3 times. Whereas LLOQ of both EPL and EPLT was found 1 ng/mL where signal-to-noise ratio was 5 times (Figure.5.56).



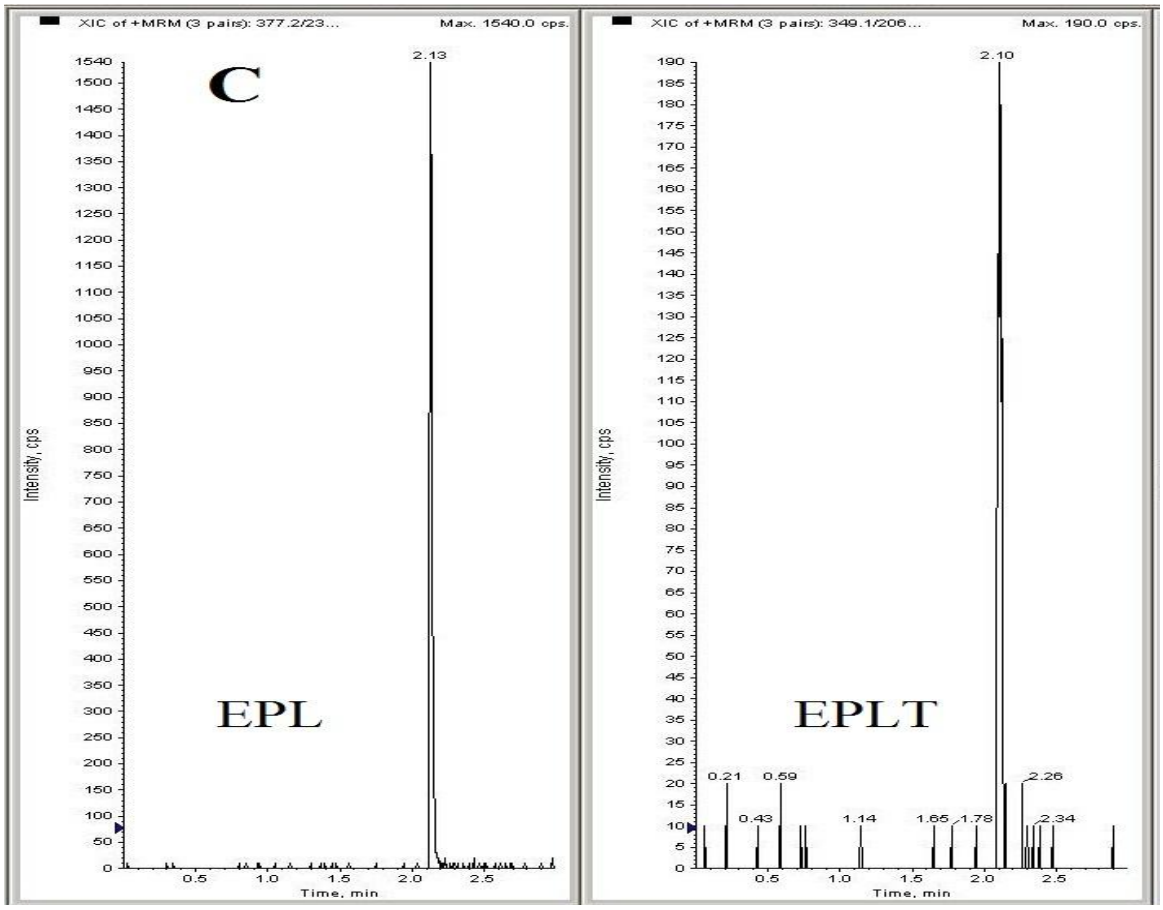


Figure.5.56 Chromatograms for determination of LOD and LLOQ of EPL and EPLT : A) chromatogram of blank plasma, B) chromatogram of LOD and C) chromatogram of LLOQ

5.6 Pre-validation study of bioanalytical method for EPL and EPLT

5.6.1 System suitability test (SST) for EPL and EPLT LC-ESI-MS/MS method

The system suitability test (SST) was determined by replicate (n=6) injection of the same reference standard (RS) solution containing EPL, EPLT and IS (TBM) at 500 ng/mL. This mixed solution, i.e., RS 500 ng/mL was injected at the beginning of bioanalysis or during sample run. Hence, it was established from the Table.5.62 that the low RSD% (CV%) of EPL, EPLT and TBM (IS) peak areas and retention times (RTs) were within or below 2% as per the guidelines. So, it was confirmed that the system was reproducible for the current analytical run and satisfactorily working or functioning.

Table.5.62 System suitability test (n=6) of EPL, EPLT and TBM (IS) during sample run

No. of injection	EPL (conc. 500 ng/mL)		EPLT (conc. 500 ng/mL)		TBM (IS, conc. 500ng/mL)	
	Peak area	RT (min.)	Peak area	RT (min.)	Peak area	RT (min.)
1	6.82E+05	2.12	1.41E+05	2.08	5.02E+04	2.22
2	6.76E+05	2.12	1.44E+05	2.09	5.07E+04	2.22
3	6.74E+05	2.12	1.44E+05	2.09	5.06E+04	2.22
4	6.82E+05	2.12	1.45E+05	2.09	5.15E+04	2.22
5	6.70E+05	2.12	1.42E+05	2.09	5.06E+04	2.22
6	6.86E+05	2.13	1.43E+05	2.10	5.06E+04	2.23
Mean	**6.78E+05**	**2.1217**	**1.43E+05**	**2.0900**	**5.07E+04**	**2.2217**
SD	**5855.7041**	**0.0041**	**1394.1939**	**0.0063**	**425.5268**	**0.0041**
RSD% (CV%)	**0.8632**	**0.1924**	**0.9739**	**0.3026**	**0.8391**	**0.1838**

RSD% (or CV%), Relative Standard Deviation [(SD/Mean) x 100]

5.6.2 Reproducibility test for EPL and EPLT LC-MS/MS method

The reproducibility of the bioanalytical method was determined on the basis of the coefficient of variance (CV%) i.e., the relative standard deviation (RSD) of the area ratio of both EPL/TBM (IS) and EPLT/TBM (IS) for replicate (n=3) assay with conc. 250 ng/mL and conc. 500 ng/mL.

Table.5.63 Reproducibility test of the plasma spiked with 250 ng/mL of EPL, EPLT and TBM (IS)

No.	Peak area of EPL (conc. 250 ng/mL)	Peak area of EPLT (conc. 250 ng/mL)	Peak area of TBM (IS) (conc. 250 ng/mL)	Area ratio (EPL)	Area ratio (EPLT)
1	3.28E+05	7.12E+04	4.92E+04	6.66E+00	1.45E+00
2	3.27E+05	7.14E+04	5.01E+04	6.54E+00	1.42E+00
3	3.31E+05	7.23E+04	4.93E+04	6.72E+00	1.47E+00
Mean	**3.29E+05**	**7.16E+04**	**4.95E+04**	**6.64E+00**	**1.45E+00**
SD	**2253.4640**	**583.6243**	**485.6607**	**0.0927**	**0.0207**
CV% (RSD%)	**0.6852**	**0.8147**	**0.9806**	**1.3960**	**1.4336**

Table.5.64 Reproducibility test of the plasma spiked with 500 ng/mL of EPL, EPLT and TBM (IS)

No.	Peak area of EPL (conc. 500 ng/mL)	Peak area of EPLT (conc. 500 ng/mL)	Peak area of TBM (IS) (conc. 500 ng/mL)	Area ratio (EPL)	Area ratio (EPLT)
1	6.36E+05	1.40E+05	9.68E+04	6.56E+00	1.44E+00
2	6.34E+05	1.39E+05	9.91E+04	6.40E+00	1.40E+00
3	6.42E+05	1.43E+05	9.85E+04	6.52E+00	1.45E+00
Mean	**6.38E+05**	**1.40E+05**	**9.81E+04**	**6.50E+00**	**1.43E+00**
SD	**4327.7515**	**1930.4231**	**1150.5216**	**0.0831**	**0.0256**
CV% (RSD%)	**0.6788**	**1.3744**	**1.1724**	**1.2790**	**1.7886**

Results of reproducibility test are reported in Table.5.63 and Table.5.64 for the concentration 250 ng/mL and 500 ng/mL, respectively. The low CV% (RSD%) value establishes the reproducibility of the method to be acceptable and therefore CV% (RSD%) of both concentrations for EPL and IS; EPLT and IS were found to be within the limit.

5.7 Validation of method for bioanalytical study of enalapril (EPL) and enalaprilat (EPLT) by liquid chromatography tandem-mass spectrometry (LC-MS/MS)

The method of EPL and EPLT was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation.

5.7.1 Specificity and selectivity

As per the representative chromatograms of the double blank plasma sample (Figure.5.57), blank plasma sample spiked with IS (Figure.5.58) and blank plasma spiked with the analytes (EPL and EPLT) at 1 ng/mL and IS (TBM), i.e., at LLOQ (Figure.5.59), the total chromatographic run time was 3.0 minutes and the retention time of EPL, EPLT and TBM (IS) were about 2.13, 2.10 and 2.23 min., respectively. Hence, the retention time never shifted beyond 10 to 15 seconds on repeated injection of the samples. The chromatogram shown in Figure.5.59 that there was no significant interference from the endogenous plasma matrix was noticed at the retention times of analytes and IS. Similar things were noticed in the

chromatogram of blank plasma spiked with IS (single blank, Figure.5.58) and in the chromatogram of blank plasma (double blank, Figure.5.57) also.

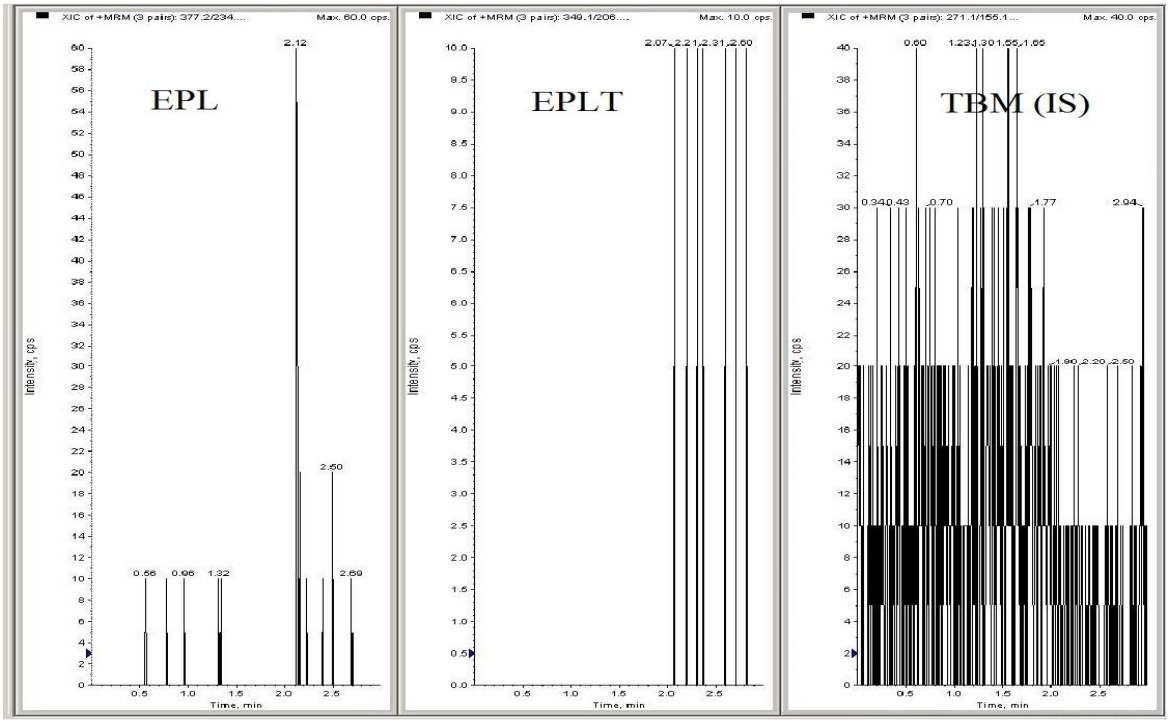


Figure.5.57 Chromatogram of blank human plasma or double blank plasma [free of EPL, EPLT and TBM (IS)]

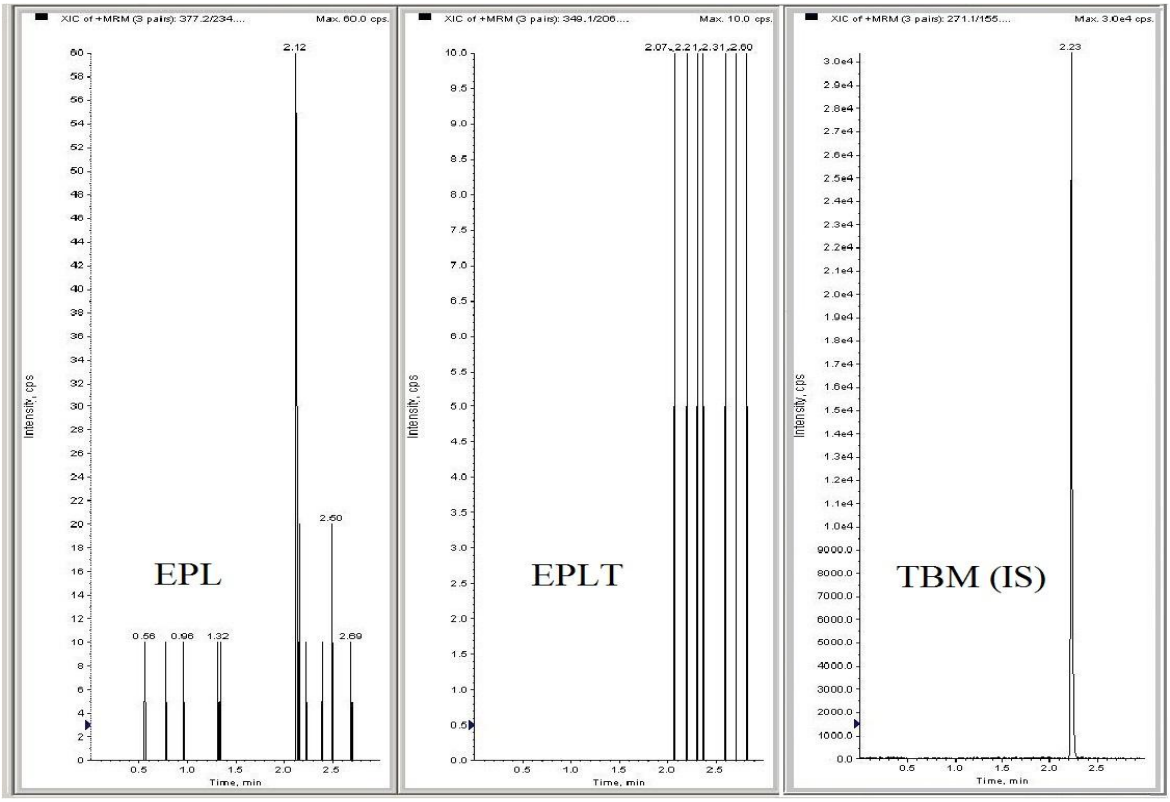


Figure.5.58 Chromatogram of single blank plasma spiked with IS (TBM)

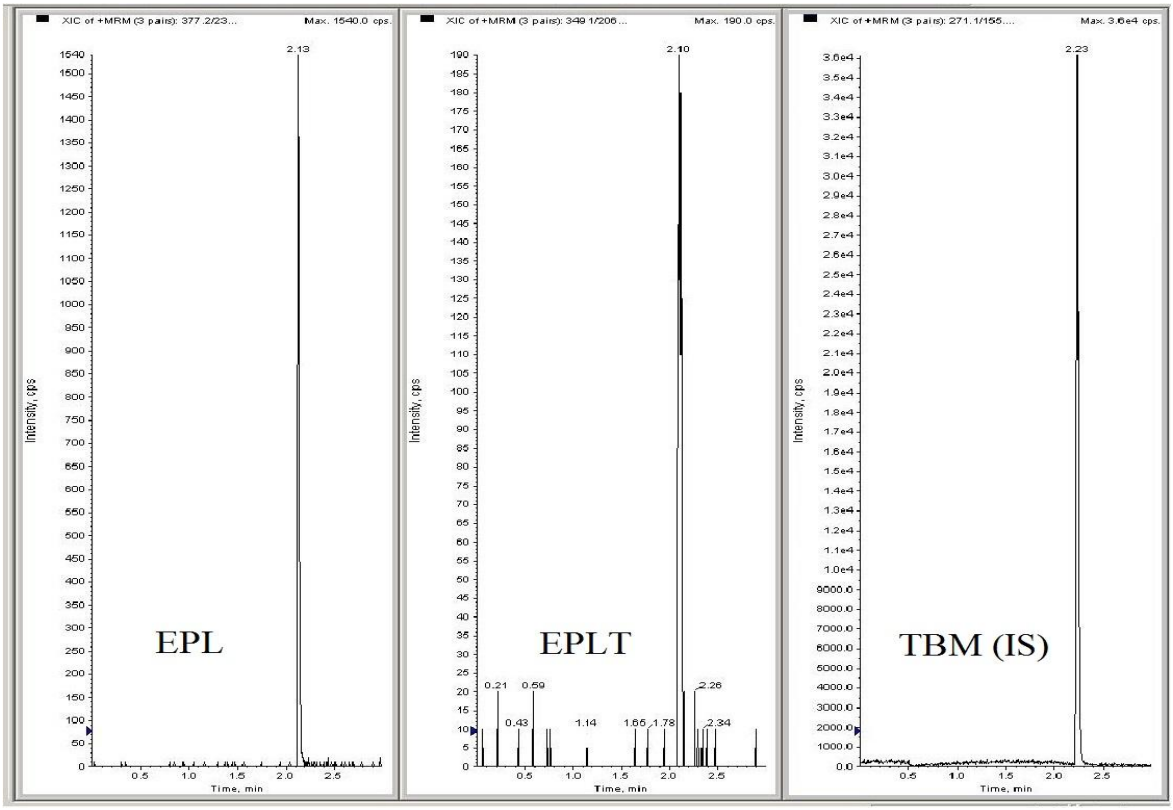


Figure.5.59 Chromatogram of blank human plasma spiked with analytes (EPL and EPLT) at LLOQ 1 ng/mL and IS (TBM)

5.7.2 Sensitivity

According to the result, the method of EPL and EPLT was found very sensitive. Hence, the limit of detection (LOD) was found at 0.25 ng/mL for EPL and at 0.80 ng/mL for EPLT, respectively whereas the lower limit of quantification (LLOQ) was found at 1 ng/mL for both EPL and EPLT (Figure.5.60). The signal to noise (S/N) ratio was maintained 3:1 for LOD and 5:1 for LLOQ. Now according to the guidelines, all LLOQ samples were found within the acceptance range.

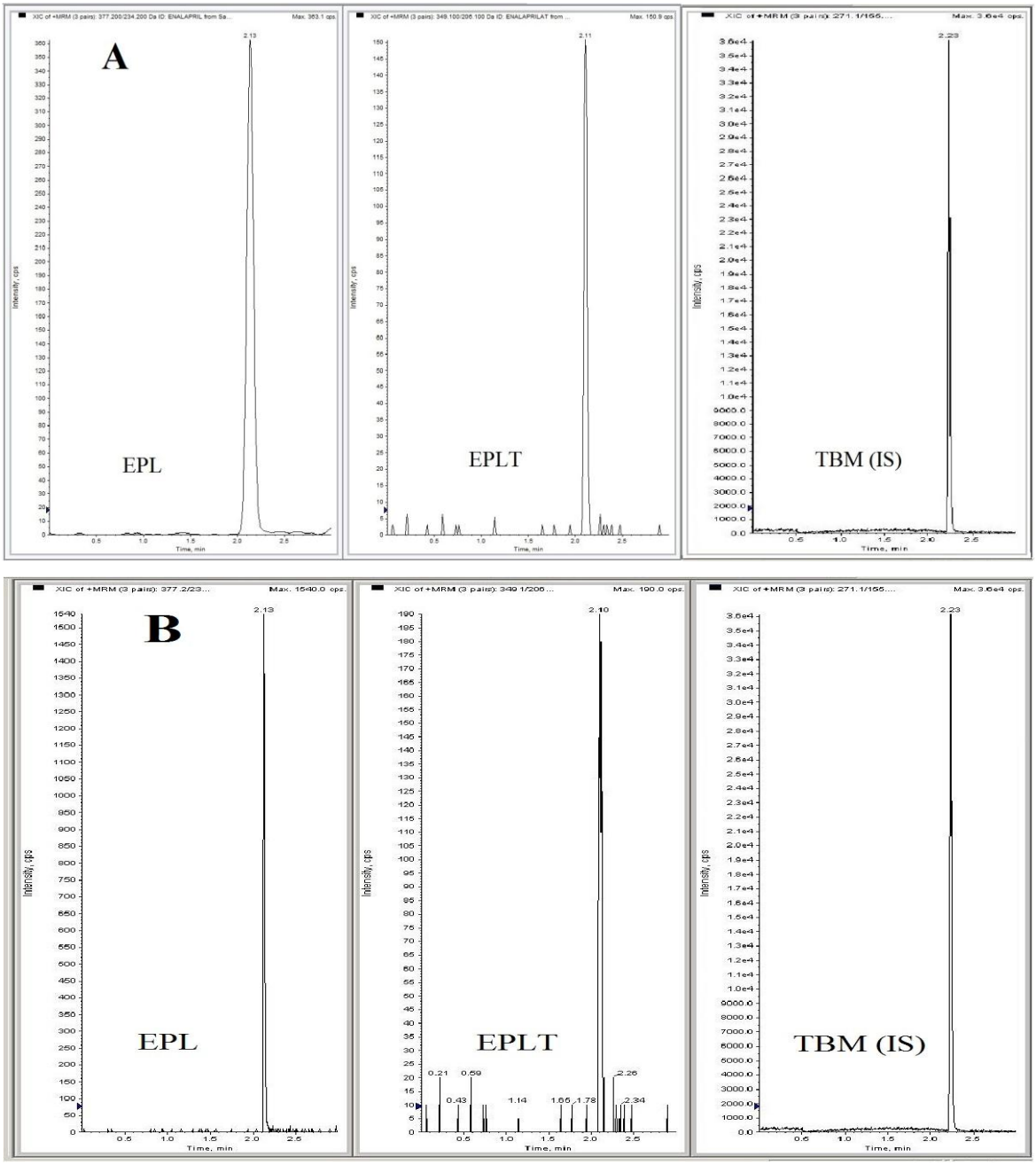


Figure.5.60 Chromatograms showing A) LOD and B) LLOQ of EPL and EPLT

5.7.3 Linearity and calibration (standard) curve of EPL and EPLT in human plasma

The validation was performed to establish the calibration, precision and accuracy of both EPL and EPLT in human blank plasma. The standard or calibration curves were constructed by plotting the peak area ratio of the analytes, i.e., EPL and EPLT to TBM (IS) versus the nominal calibration concentrations (CC) of the analytes. The standard curves have consistently better reproducibility over the standard concentrations across the calibration range and the representative calibration curves (LIN I) for EPL and EPLT are shown in

Figure.5.61 and Figure.5.62. The linearity of the calibration curve was evaluated by an unweighted ($1/x^2$) least square or linear regression analysis. Subsequently the regression equation of the calibration curve was used to determine the concentrations of both EPL and EPLT in human plasma. During analysis, all these processes were performed by the Analyst 1.5 software in quantitate mode.

The back calculated values of the concentrations were assessed statistically. These back calculated concentrations from the representative calibration standards by LC-MS/MS determination for EPL and EPLT and corresponding regression equations are reported in Table.5.65 and Table.5.67. The assay result was demonstrated good linear response over the linearity range of 1 ng/mL to 500 ng/mL for both EPL and EPLT in plasma. The regression equations were y = 0.0270x + 0.0076 (r = 0.9974) for EPL and y = 0.0052x + 0.0014 (r = 0.9973) for EPLT (see Table.5.66 and Table.5.68), where 'y' is the peak area ratio of analyte to IS and 'x' is the nominal plasma concentration of the analyte. In Table.5.65 and Table.5.67 were observed the CV% and accuracy (%) values ranging from 0.51% to 9.99% and 93.39% to 105.53% for EPL and from 2.67% to 16.87% and 96.47% to 108.40% for EPLT, respectively.

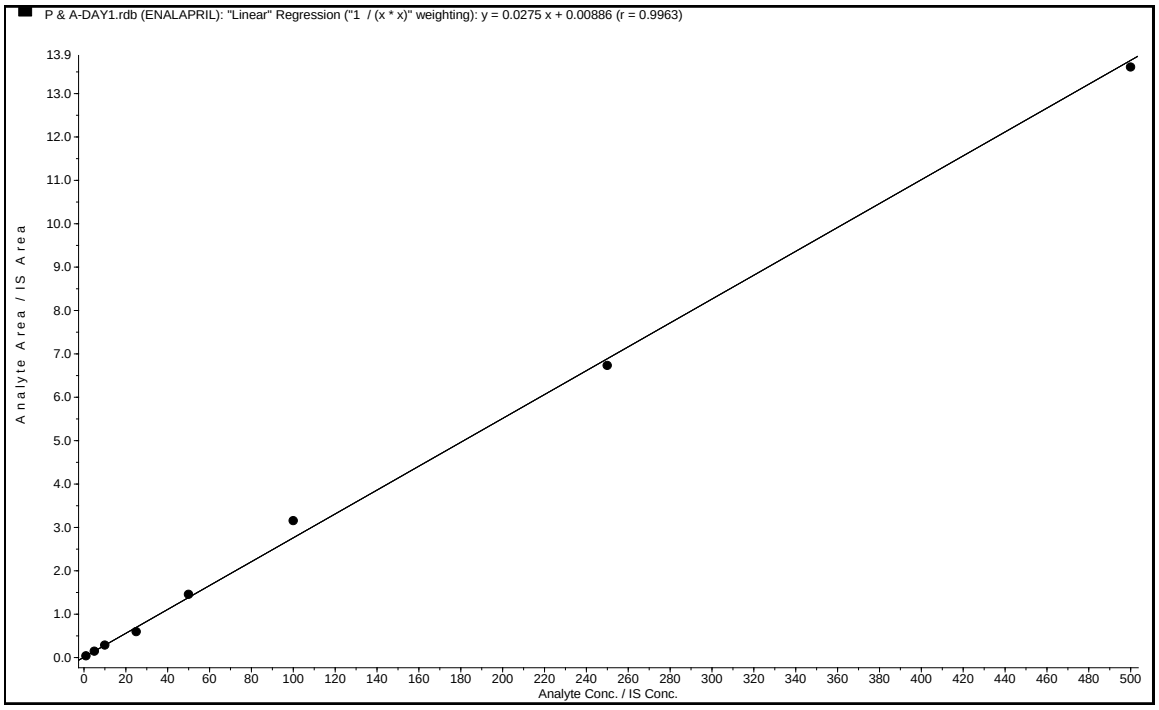


Figure.5.61 Plasma calibration curve of EPL

Table.5.65 Linear regression data for the determination of EPL in human plasma

Linearity	Plasma calibration concentration (ng/mL) of EPL							
	1 (CC1)	5 (CC2)	10 (CC3)	25 (CC4)	50 (CC5)	100 (CC6)	250 (CC7)	500 (CC8)
LIN 1	1.01	4.9	10.02	21.27	52.55	114.26	244.3	494.3
LIN 2	0.99	5.39	9.68	24.03	47.88	105.66	241.97	510.98
LIN 3	0.97	5.54	10.66	24.74	51.61	93.48	243.93	460.26
Mean	**0.99**	**5.28**	**10.12**	**23.35**	**50.68**	**104.47**	**243.40**	**488.51**
S.D.	**0.02**	**0.33**	**0.50**	**1.83**	**2.47**	**10.44**	**1.25**	**25.85**
CV%	**2.02**	**6.34**	**4.92**	**7.85**	**4.87**	**9.99**	**0.51**	**5.29**
Accuracy (%)	**99.00**	**105.53**	**101.20**	**93.39**	**101.36**	**104.47**	**97.36**	**97.70**

Table.5.66 Pre study linearity of detector response of EPL

Linearity	Statistics		
	Slope (M)	Intercept-C	R square
LIN 1	2.75E-02	8.86E-03	0.9963
LIN 2	2.61E-02	6.59E-03	0.9988
LIN 3	2.73E-02	7.32E-03	0.9972
Mean	**0.0270**	**0.0076**	**0.9974**
S.D.	**0.0008**	**0.0012**	**0.0013**
CV%	**2.8079**	**15.2679**	**0.1269**

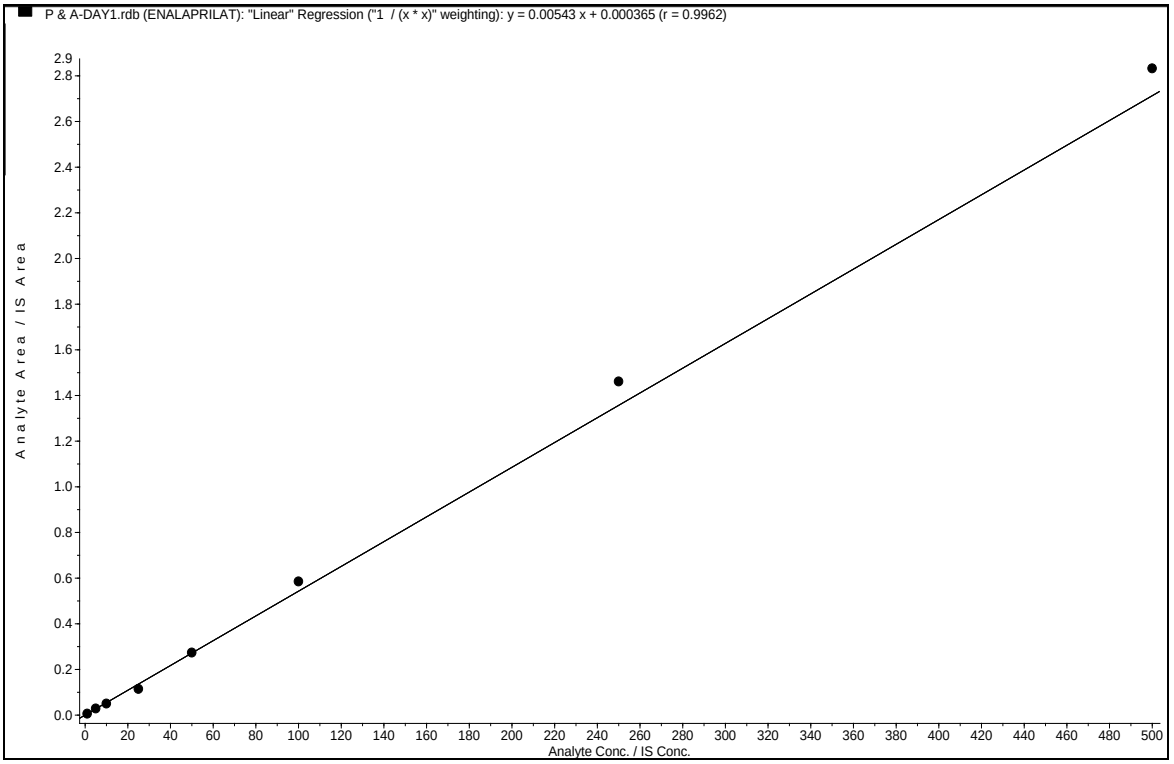


Figure.5.62 Plasma calibration curve of EPLT

Table.5.67 Linear regression data for the determination of EPLT in human plasma

Linearity	Plasma calibration concentration (ng/mL) of EPLT							
	1 (CC1)	**5 (CC2)**	**10 (CC3)**	**25 (CC4)**	**50 (CC5)**	**100 (CC6)**	**250 (CC7)**	**500 (CC8)**
LIN 1	1.01	5.17	9.15	20.99	50.31	107.74	269.2	521.96
LIN 2	1.00	5.15	9.21	23.58	47.64	110.06	253.66	516.29
LIN 3	0.96	5.94	10.58	29.1	55.04	88.95	220.94	409.12
Mean	**0.99**	**5.42**	**9.65**	**24.56**	**51.00**	**102.25**	**247.93**	**482.46**
S.D.	**0.03**	**0.45**	**0.81**	**4.14**	**3.75**	**11.58**	**24.63**	**63.57**
CV%	**2.67**	**8.31**	**8.38**	**16.87**	**7.35**	**11.32**	**9.94**	**13.18**
Accuracy (%)	**99.00**	**108.40**	**96.47**	**98.23**	**101.99**	**102.25**	**99.17**	**96.49**

Table.5.68 Pre study linearity of detector response of EPLT

Linearity	Statistics		
	Slope (M)	**Intercept-C**	**R square**
LIN 1	5.43E-03	3.65E-04	0.9962
LIN 2	5.03E-03	1.41E-03	0.9981
LIN 3	5.25E-03	2.52E-03	0.9975
Mean	**0.0052**	**0.0014**	**0.9973**
S.D.	**0.0002**	**0.0011**	**0.0010**
CV%	**3.8256**	**75.2733**	**0.0974**

5.7.4 Precision and accuracy of EPL and EPLT

The intra-day and inter-day assay precision, i.e., CV% or RSD% values for different concentrations ranged from 3.47% to 11.81% and 7.72% to 10.93% for EPL and 3.10% to 11.61% and 6.78% to 13.76% for EPLT, respectively. Although the percentage of accuracy (accuracy %) for the same concentrations were in the range of 101.00% to 113.43% (intra-day) and 98.37% to 106.28% (inter-day) for EPL and 93.96% to 112.66% (intra-day) and 96.62% to 104.12% (inter-day) for EPLT, respectively. According to the USFDA and EMA guidelines, both the precision and accuracy data were in the acceptance criteria. The assay method demonstrated high degree of precision and accuracy. In Table.5.69 and Table.5.70, the precision and accuracy data for both intra-day and inter-day assays of LLOQ and three QC samples (LQC, MQC and HQC) are described.

Table.5.69 Intra-day and inter-day precision and accuracy data of EPL in human plasma (n = 6)

Intra-day (Within run)					
Quality control (QC) sample	**Spiked/Nominal Concentration (ng/mL)**	**Mean concentration (ng/mL)**	**SD**	**CV% (RSD%)**	**Accuracy (%)**
LLOQ	1	1.0100	0.1055	10.4407	101.0000
LQC	3	3.0933	0.1075	3.4738	103.1111
MQC	200	223.9300	26.4513	11.8123	111.9650
HQC	400	453.7200	35.9618	7.9260	113.4300
Inter-day (Between run)					
LLOQ	1	0.9838	0.0916	9.3108	98.3750
LQC	3	3.0113	0.3226	10.7128	100.3750
MQC	200	207.6733	22.7122	10.9365	103.8367
HQC	400	425.1263	32.8410	7.7250	106.2816

SD = Standard deviation; CV (%) coefficient of variation or RSD (%) relative standard deviation = [(SD / mean) x 100]; Accuracy (%) = [(measured concentration / spiked or nominal concentration) x 100]; LLOQ = Lower limit of quantification; LQC = Low quality control; MQC = Middle quality control; HQC = High quality control; n = number of samples.

Table.5.70 Intra-day and inter-day precision and accuracy data of EPLT in human plasma (n = 6)

Intra-day (Within run)					
Quality control (QC) sample	**Spiked/Nominal Concentration (ng/mL)**	**Mean concentration (ng/mL)**	**SD**	**CV% (RSD%)**	**Accuracy (%)**
LLOQ	1	1.1267	0.1309	11.6167	112.6667
LQC	3	3.1150	0.3038	9.7515	103.8333
MQC	200	187.9250	19.3644	10.3043	93.9625
HQC	400	393.6150	12.2396	3.1095	98.4038
Inter-day (Between run)					
LLOQ	1	1.0413	0.1324	12.7167	104.1250
LQC	3	2.8988	0.3990	13.7661	96.6250
MQC	200	199.1229	23.3814	11.7422	99.5615
HQC	400	399.9475	27.1177	6.7803	99.9869

SD = Standard deviation; CV (%) coefficient of variation or RSD (%) relative standard deviation = [(SD / mean) x 100]; Accuracy (%) = [(measured concentration / spiked or nominal concentration) x 100]; LLOQ = Lower limit of quantification; LQC = Low quality control; MQC = Medium quality control; HQC = High quality control; n = number of samples.

5.7.5 Extraction recovery of EPL and EPLT

Extraction recoveries were determined in the percentage (%) by calculating the peak areas of the analytes at low, medium and high quality control samples. The peak areas of the extracted plasma at low, medium, and high quality control samples were compared to the peak areas of the unextracted standards containing the same concentrations of the EPL and EPLT. Recovery results indicated that the maximum recovery was observed at low (3 ng/mL), medium (200 ng/mL) and high (400 ng/mL) quality control (QC) samples ranged from

96.54% to 99.37% for EPL and 94.20% to 98.83% for EPLT, respectively whereas recovery of the internal standard (IS, TBM) was found 105.70% (see Table.5.71). The recovery of both the ananlytes was shown more convenient and enough high and also data shown good sensitivity as it was reproducible, precise and consistent.

Table.5.71 Absolute recovery of EPL and EPLT in human plasma

Analyte	Spiked/Nominal Concentration (ng/mL)	Mean Concentration (ng/mL)	Recovery (%) (Mean ± S.D.)
EPL	3	2.8043	96.5478 ± 15.6653
	200	204.5113	97.8200 ± 5.7916
	400	400.2418	99.3747 ± 8.2698
EPLT	3	2.9450	98.8382 ± 6.7260
	200	196.4750	94.2010 ± 10.2092
	400	402.6923	98.5074 ± 10.9687
TBM (IS)	100	97.5000	105.7088 ± 21.5145

5.7.6 Matrix effect of EPL and EPLT

No significant matrix effect was found for EPL, EPLT and TBM (IS) by comparing the area under the curve (AUC) ratios of the extracted QCs (LQC, MQC and HQC) with the AUC of unextracted QCs (LQC, MQC and HQC) acquired from the injecting raw or aqueous solution prepared at same concentration levels (n = 6) as mentioned in Table.5.72, Table.5.73, Table.5.74 and Table.5.75.

Table.5.72 Evaluation of matrix effect for EPL

Sample name	Analyte peak area		% Matrix effect	Matrix factor
	Spiking in aqueous sample	Spiking in extracted blank matrix (plasma)		
LQC-1	2400.0000	2300.4250	95.8510	0.9585
LQC-2	2500.0000	2615.9950	104.6398	1.0464
LQC-3	2300.0000	2439.0900	106.0474	1.0605
LQC-4	2250.0000	2091.5802	92.9591	0.9296
LQC-5	2130.0000	2322.6800	109.0460	1.0905
LQC-6	2690.0000	2547.6150	94.7069	0.9471
MQC-1	1.503100000	1.703100000	113.3058	1.1331
MQC-2	1.698700000	1.798700000	105.8869	1.0589
MQC-3	1.989700000	1.809700000	90.9534	0.9095
MQC-4	1.624100000	1.824100000	112.3145	1.1231
MQC-5	2.015100000	1.845100000	91.5637	0.9156
MQC-6	1.900400000	1.833400000	96.4744	0.9647
HQC-1	3.257400000	3.367400000	103.3769	1.0338
HQC-2	3.826400000	3.526400000	92.1597	0.9216
HQC-3	3.400000000	3.370000000	99.1176	0.9912
HQC-4	3.434000000	3.634000000	105.8241	1.0582
HQC-5	3.700000000	3.512600000	94.9351	0.9494
HQC-6	3.850800000	3.750800000	97.4031	0.9740

Table.5.73 Evaluation of matrix effect for EPLT

Sample name	Analyte peak area		% Matrix effect	Matrix factor
	Spiking in aqueous sample	Spiking in extracted blank matrix (plasma)		
LQC-1	380.0000	406.5950	106.9987	1.0700
LQC-2	320.5500	308.9050	96.3672	0.9637
LQC-3	300.2300	330.9650	110.2372	1.1024
LQC-4	340.1000	321.4650	94.5207	0.9452
LQC-5	350.3400	334.0750	95.3574	0.9536
LQC-6	286.3700	302.6400	105.6815	1.0568
MQC-1	2.75000000	2.61010000	94.9127	0.9491
MQC-2	2.48000000	2.71740000	109.5726	1.0957
MQC-3	2.62000000	2.66190000	101.5992	1.0160
MQC-4	2.70000000	2.65790000	98.4407	0.9844
MQC-5	2.54510000	2.67910000	105.2650	1.0527
MQC-6	2.75400000	2.68840000	97.6180	0.9762
HQC-1	5.34200000	5.09380000	95.3538	0.9535
HQC-2	4.79320000	5.42060000	113.0894	1.1309
HQC-3	5.74500000	5.23280000	91.0844	0.9108
HQC-4	4.96400000	5.29630000	106.6942	1.0669
HQC-5	5.71200000	5.18330000	90.7440	0.9074
HQC-6	5.75800000	5.48940000	95.3352	0.9534

Table.5.74 Evaluation of matrix effect for TBM (IS)

Sample name	IS peak area		% Matrix effect	Matrix factor
	Spiking in aqueous sample	Spiking in extracted blank matrix (plasma)		
LQC-1	1.36460000	1.40750000	103.1438	1.0314
LQC-2	1.51100000	1.39830000	92.5414	0.9254
LQC-3	1.37290000	1.38640000	100.9833	1.0098
LQC-4	1.44450000	1.42260000	98.4839	0.9848
LQC-5	1.38160000	1.34020000	97.0035	0.9700
LQC-6	1.30880000	1.37480000	105.0428	1.0504
MQC-1	1.29560000	1.31110000	101.1964	1.0120
MQC-2	1.43490000	1.32510000	92.3479	0.9235
MQC-3	1.36210000	1.33660000	98.1279	0.9813
MQC-4	1.33860000	1.34210000	100.2615	1.0026
MQC-5	1.33510000	1.41490000	105.9771	1.0598
MQC-6	1.31010000	1.29260000	98.6642	0.9866
HQC-1	1.37480000	1.40880000	102.4731	1.0247
HQC-2	1.44020000	1.38060000	95.8617	0.9586
HQC-3	1.24260000	1.34450000	108.2005	1.0820
HQC-4	1.39640000	1.37190000	98.2455	0.9825
HQC-5	1.37830000	1.31100000	95.1172	0.9512
HQC-6	1.40650000	1.36560000	97.0921	0.9709

Table.5.75 Matrix effect of EPL and EPLT calculated from six different source of human plasma

Analytes	Matrix Effect (ME %) [a]						Mean	SD	CV%
	SET 1*	SET 2	SET 3	SET 4	SET 5	SET 6			
EPL									
LQC (3 ng/mL)	95.8510	104.6398	106.0474	92.9591	109.0460	94.7069	100.5417	6.8260	6.7893
MQC (200 ng/mL)	113.3058	105.8869	90.9534	112.3145	91.5637	96.4744	101.7498	10.1036	9.9299
HQC (400 ng/mL)	103.3769	92.1597	99.1176	105.8241	94.9351	97.4031	98.8028	5.1258	5.1879
EPLT									
LQC (3 ng/mL)	106.9987	96.3672	110.2372	94.5207	95.3574	105.6815	101.5271	6.8825	6.7789
MQC (200 ng/mL)	94.9127	109.5726	101.5992	98.4407	105.2650	97.6180	101.2347	5.4176	5.3515
HQC (400 ng/mL)	95.3538	113.0894	91.0844	106.6942	90.7440	95.3352	98.7168	9.1079	9.2263

[a] ME% expressed as the ratio of the mean peak area of an analyte spiked postextraction to the mean peak area of the same analyte standards multiplied by 100. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression.

*SET 1-6 represent the ME% from six different source of human plasma.

5.7.7 Stability study of EPL and EPLT

5.7.7.1 Stability of EPL, EPLT and TBM (IS) stock solutions

The stock solutions of both the analytes (EPL and EPLT) and IS (TBM) were kept in three different storage conditions and peak area was determined of each condition. In the Figure.5.63, Figure.5.64 and Figure.5.65, where EPL, EPLT and TBM (IS) shows significant decrease in peak area with time, under the condition of room temperature and normal deep freezer. The stock solution was stable at −20 ºC for 30 days, i.e., 720 hours and stable at normal deep freezer for 7 days.

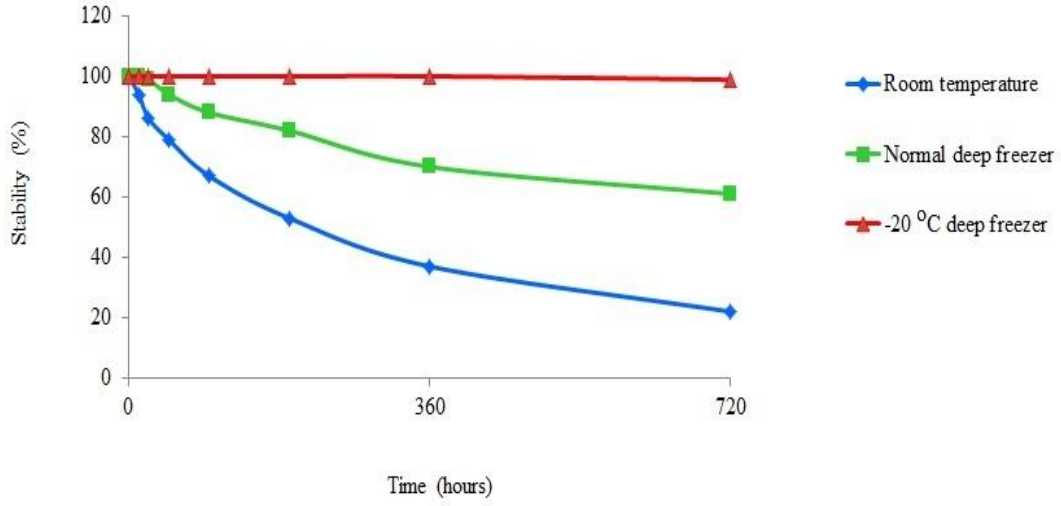


Figure.5.63 Stability of stock solution of EPL in three different conditions

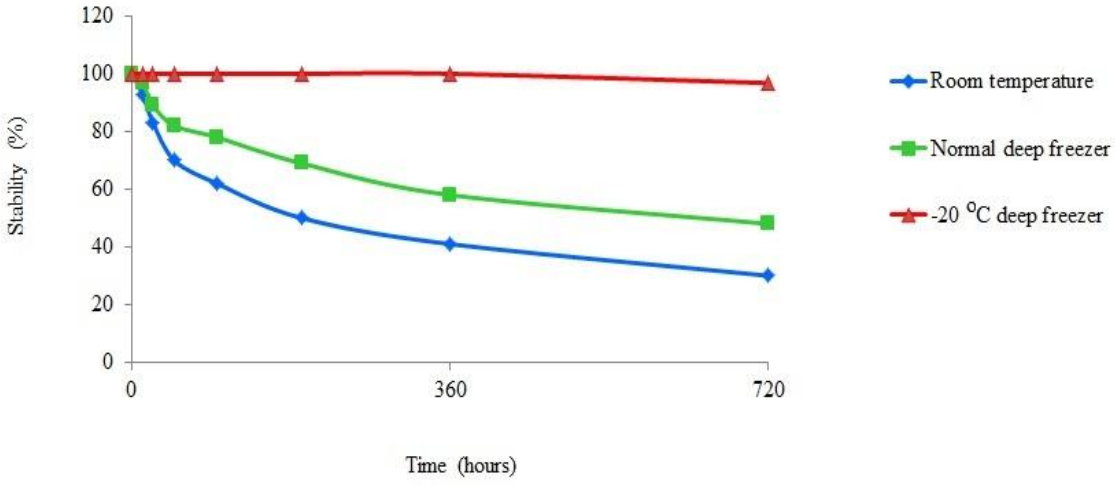


Figure.5.64 Stability of stock solution of EPLT in three different conditions

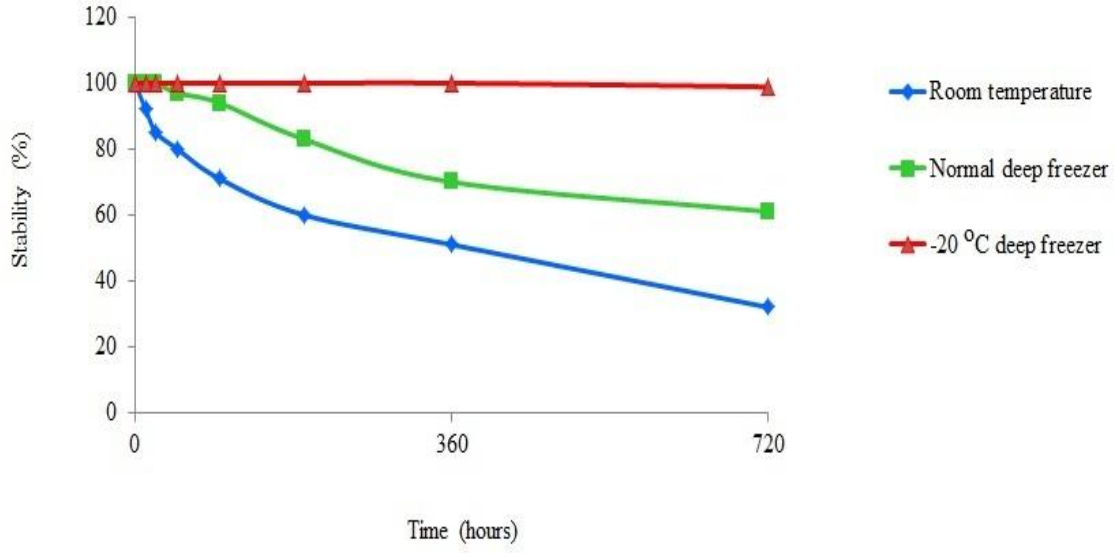


Figure.5.65 Stability of stock solution of TBM (IS) in three different conditions

5.7.7.2 Freeze and thaw stability study of EPL and EPLT

In this stability study or test, six replicates (n = 6) of three levels of QC sample (LQC, MQC and HQC) included along with a standard or calibration curve (n = 1), the linear regression correlation coefficient (r or r^2) were evaluated after three freeze thaw cycles comparing to freshly thaw samples of the similar concentration. The freeze thaw stability ranged between 91.63% to 94.72% for EPL and 87.82% to 95.32% for EPLT, respectively (see Table.5.76 and Table.5.77). According to the guidelines, results show that calculated concentrations for the analytes at LQC, MQC and HQC samples varied within the assay acceptance limits (±15%) of the nominal concentrations (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.76 Freeze thaw stability study of EPL

	Freshly thaw (Day - 0)			After three freeze thaw cycles		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	2.6500	221.1200	400.0500	3.3400	185.3600	358.7200
2	2.7800	212.1500	441.6000	2.4100	175.2600	426.5300
3	2.9300	208.9200	376.5300	2.3600	188.3200	344.5900
4	3.1200	190.8000	438.6400	2.2800	192.5400	402.3100
5	3.0500	198.5300	454.9500	2.7400	176.3300	390.5400
6	3.2800	170.2500	380.4500	3.1900	220.5600	407.5600
Mean	2.9683	200.2950	415.3700	2.7200	189.7283	388.3750
SD	0.2303	18.1361	33.9374	0.4528	16.5433	31.0460
CV%	7.7570	9.0547	8.1704	16.6459	8.7195	7.9938
% Freeze thaw stability				**91.6339**	**94.7244**	**93.5010**

Table.5.77 Freeze thaw stability study of EPLT

	Freshly thaw (Day - 0)			After three freeze thaw cycles		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	2.6100	189.1500	347.6900	1.8800	179.3300	376.4300
2	3.0150	177.5900	401.7600	1.8300	168.4400	286.3000
3	2.2500	187.7600	336.4100	2.6100	178.7800	297.2500
4	2.7450	167.8400	392.9800	2.5800	156.5300	314.4600
5	2.3550	174.5200	400.0100	2.1200	170.9100	365.6200
6	3.2500	220.3400	490.6700	3.2300	210.9500	450.5300
Mean	2.7042	186.2000	394.9200	2.3750	177.4900	348.4317
SD	0.3830	18.5705	54.5868	0.5358	18.3801	61.8666
CV%	14.1616	9.9734	13.8223	22.5595	10.3556	17.7557
% Freeze thaw stability				**87.8274**	**95.3222**	**88.2284**

5.7.7.3 Bench top stability study of EPL and EPLT

The bench top stability was carried out for 8 hours in room temperature, around at 24 °C and determined at three concentrations of low, medium and high QC levels (LQC, MQC and HQC) in six replicates (n = 6) along with a standard or calibration curve (n = 1). According to Table.5.78 and Table.5.79, this stability of EPL and EPLT ranged from 92.13% to 100.33% and 98.07% to 106.62%, respectively and also all results were found to be within the acceptance criteria.

Table.5.78 Bench top stability study of EPL

	Day - 0			**Bench top stability for 8 hours**		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	2.6500	221.1200	400.0500	2.4400	203.8000	423.5600
2	2.7800	212.1500	441.6000	2.5800	219.1100	407.6200
3	2.9300	208.9200	376.5300	2.7700	184.7700	427.8600
4	3.1200	190.8000	438.6400	2.5100	220.8100	382.1200
5	3.0500	198.5300	454.9500	2.8200	196.9000	441.8800
6	3.2800	170.2500	380.4500	3.2900	180.3500	390.4500
Mean	2.9683	200.2950	415.3700	2.7350	200.9567	412.2483
SD	0.2303	18.1361	33.9374	0.3092	16.9404	23.0392
CV%	7.7570	9.0547	8.1704	11.3068	8.4299	5.5887
% Bench top stability				**92.1392**	**100.3303**	**99.2485**

Table.5.79 Bench top stability study of EPLT

	Day - 0			Bench top stability for 8 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	2.6100	189.1500	347.6900	3.1500	180.7400	380.0800
2	3.0150	177.5900	401.7600	2.5350	191.7800	377.4500
3	2.2500	187.7600	336.4100	2.5950	165.0600	383.7300
4	2.7450	167.8400	392.9800	2.5950	193.4800	360.9900
5	2.3550	174.5200	400.0100	3.0750	181.2000	401.1400
6	3.2500	220.3400	490.6700	3.3500	230.4100	420.4300
Mean	2.7042	186.2000	394.9200	2.8833	190.4450	387.3033
SD	0.3830	18.5705	54.5868	0.3502	22.0556	20.7039
CV%	14.1616	9.9734	13.8223	12.1460	11.5811	5.3457
% Bench top stability				**106.6256**	**102.2798**	**98.0713**

5.7.7.4 Post-preparative or autosampler stability study of EPL and EPLT

The post-preparative or autosampler stability study was performed at three concentrations of low, medium and high QC levels (LQC, MQC and HQC) in six replicates (n = 6) along with the calibration standards (n = 1). The samples were placed in the autosampler cabinet at 10 to 15 °C for 24 hours after preparation (extraction). Then the samples were run and analyzed and the acquired concentrations were compared with the actual values. The percentage (%) of this stability was varied from 92.60% to 100.46% for EPL and 92.52% to 98.55% for EPLT, respectively (Table.5.80 and Table.5.81). As per the guidelines, results indicate that expected concentrations for the analytes at LQC, MQC and HQC samples deviated within the acceptable limit (±15%) of the nominal concentrations (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.80 Post-preparative or autosampler stability study of EPL

	Day - 0			Autosampler stability for 24 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	2.6500	221.1200	400.0500	2.7900	213.4800	374.7800
2	2.7800	212.1500	441.6000	2.8800	210.2800	367.7700
3	2.9300	208.9200	376.5300	2.2500	191.7100	393.8100
4	3.1200	190.8000	438.6400	2.1100	208.3400	385.2200
5	3.0500	198.5300	454.9500	3.0500	201.1500	375.6000
6	3.2800	170.2500	380.4500	3.4200	180.6500	410.8600
Mean	2.9683	200.2950	415.3700	2.7500	200.935	384.6733
SD	0.2303	18.1361	33.9374	0.49336	12.6188	15.7191
CV%	7.7570	9.0547	8.1704	17.9402	6.2800	4.0863
% Autosampler stability				**92.6446**	**100.4675**	**92.6098**

Table.5.81 Post-preparative or autosampler stability study of EPLT

	Day - 0			Autosampler stability for 24 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	2.6100	189.1500	347.6900	2.6100	177.0900	355.6500
2	3.0150	177.5900	401.7600	2.8100	173.7700	350.4800
3	2.2500	187.7600	336.4100	2.4400	180.0800	360.4800
4	2.7450	167.8400	392.9800	2.3800	177.6400	358.8800
5	2.3550	174.5200	400.0100	2.5800	167.4300	366.3500
6	3.2500	220.3400	490.6700	3.1700	250.7300	400.5700
Mean	2.7042	186.2000	394.9200	2.6650	187.790	365.4017
SD	0.3830	18.5705	54.5868	0.28919	31.1432	18.0123
CV%	14.1616	9.9734	13.8223	10.8514	16.5840	4.9295
% Autosampler stability				**98.5516**	**93.8950**	**92.5255**

5.7.7.5 Short term stability study of EPL and EPLT

The short term stability study was done at three QC levels; LQC, MQC and HQC samples in six replicates (n = 6) along with a standard or calibration curve (n = 1) and were stored in deep freezer at -20 ºC for 7 days. The samples were analysed after 7 days and the acquired concentrations were compared with the actual values of QC samples containing the similar concentrations of the EPL and EPLT. Now, this stability ranged between 98.50% to 102.24% for EPL and 99.02% to 99.60% for EPLT, respectively (see Table.5.82 and Table.5.83). All the results of measured concentrations for the analytes at LQC, MQC and HQC samples were within the assay acceptable limit (±15%) of the nominal concentrations as per the guidelines (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.82 Short term stability study of EPL

	Day - 0			**Short term stability for 7 days**		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	2.6500	221.1200	400.0500	2.6300	221.0000	397.3000
2	2.7800	212.1500	441.6000	2.7500	212.1100	420.4700
3	2.9300	208.9200	376.5300	3.2100	205.6600	377.4300
4	3.1200	190.8000	438.6400	3.1100	192.3000	431.3300
5	3.0500	198.5300	454.9500	3.2500	196.4400	452.7800
6	3.2800	170.2500	380.4500	3.2600	171.5400	375.6600
Mean	2.9683	200.2950	415.3700	3.0350	199.8417	409.1617
SD	0.2303	18.1361	33.9374	0.2751	17.3290	30.9575
CV%	7.7570	9.0547	8.1704	9.0637	8.6714	7.5661
% Short term stability				**102.2459**	**99.7737**	**98.5053**

Table.5.83 Short term stability study of EPLT

	Day - 0			Short term stability for 7 days		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	2.6100	189.1500	347.6900	2.6000	186.3400	342.8900
2	3.0150	177.5900	401.7600	3.1200	174.3300	397.5500
3	2.2500	187.7600	336.4100	2.2300	188.6500	333.2100
4	2.7450	167.8400	392.9800	2.5800	165.3000	390.6400
5	2.3550	174.5200	400.0100	2.3470	172.8600	402.4400
6	3.2500	220.3400	490.6700	3.1900	221.8800	493.5000
Mean	2.7042	186.2000	394.9200	2.6778	184.8933	393.3717
SD	0.3830	18.5705	54.5868	0.3958	20.1221	57.0976
CV%	14.1616	9.9734	13.8223	14.7822	10.8831	14.5149
% Short term stability				**99.0262**	**99.2982**	**99.6079**

5.7.7.6 Long term stability study of EPL and EPLT

The long term stability study was performed at LQC, MQC and HQC in six replicates (n = 6) along with a standard or calibration curve (n = 1) and were stored in deep freezer at -20 °C for 30 days. 30 days later, the samples were analysed and the acquired concentrations were compared with the actual values of QC samples containing the similar concentrations of the EPL and EPLT. The long term stability of EPL and EPLT were varied from 90.96% to 98.66% and 92.32% to 105.67%, respectively (Table.5.84 and Table.5.85). Now, the results of measured concentrations for the analytes at LQC, MQC and HQC samples were within the assay acceptance criteria, i.e., ±15% of the nominal concentrations according to the guidelines (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.84 Long term stability study of EPL

	Day - 0			Long term stability for 30 days		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	2.6500	221.1200	400.0500	2.3200	208.4800	406.9700
2	2.7800	212.1500	441.6000	2.4600	192.6600	409.6200
3	2.9300	208.9200	376.5300	2.6800	174.2000	379.9900
4	3.1200	190.8000	438.6400	2.3800	181.1700	396.6000
5	3.0500	198.5300	454.9500	2.4700	188.4100	385.5400
6	3.2800	170.2500	380.4500	3.8900	240.7800	380.9400
Mean	2.9683	200.2950	415.3700	2.7000	197.6167	393.2767
SD	0.2303	18.1361	33.9374	0.5956	24.1216	13.0709
CV%	7.7570	9.0547	8.1704	22.0599	12.2062	3.3236
% Long term stability				**90.9601**	**98.6628**	**94.6810**

Table.5.85 Long term stability study of EPLT

	Day - 0			Long term stability for 30 days		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	2.6100	189.1500	347.6900	2.3800	192.9100	373.7200
2	3.0150	177.5900	401.7600	2.1600	184.8900	402.0000
3	2.2500	187.7600	336.4100	2.3400	192.3400	409.1900
4	2.7450	167.8400	392.9800	2.8100	205.8200	398.8000
5	2.3550	174.5200	400.0100	2.0800	199.3200	387.3700
6	3.2500	220.3400	490.6700	3.2100	205.3100	393.1600
Mean	2.7042	186.2000	394.9200	2.4967	196.7650	394.0400
SD	0.3830	18.5705	54.5868	0.4318	8.2097	12.4395
CV%	14.1616	9.9734	13.8223	17.2939	4.1723	3.1569
% Long term stability				**92.3267**	**105.6740**	**99.7772**

5.7.7.7 Dilution integrity of EPL and EPLT

The dilution integrity was achieved by preparing the concentration four times of ULOQ (upper limit of quantification) i.e., 2000 ng/mL (2 µg/mL), which was further diluted five and ten times (5 fold and 10 fold) with human blank plasma (drug free plasma) in six replicates (n = 6) and these diluted samples were run against calibration standards. In Table.5.86, the dilution integrity data of EPL and EPLT have described where the precision (%RSD) values were 2.08% and 4.73% for EPL and 2.18% and 6.54% for EPLT and also with the accuracy of 99.58% and 98.75% for EPL and 99.41% and 99.25% for EPLT, respectively. These results sustain sample dilution up to 10 fold for bioanalysis.

Table.5.86 Dilution integrity of EPL and EPLT in human plasma

	Dilution for EPL		**Dilution for EPLT**	
	5 fold	**10 fold**	**5 fold**	**10 fold**
	Concentration		**Concentration**	
Serial No.	**400 ng/mL**	**200 ng/mL**	**400 ng/mL**	**200 ng/mL**
1	395.0000	210.0000	398.0000	204.0000
2	411.0000	199.0000	386.0000	194.0000
3	388.0000	193.0000	390.0000	186.0000
4	405.0000	185.0000	410.0000	183.0000
5	397.0000	206.0000	403.0000	216.0000
6	394.0000	192.0000	399.0000	208.0000
Mean	398.3333	197.5000	397.6667	198.5000
SD	8.2865	9.3541	8.6872	12.9885
Accuracy	99.5833	98.7500	99.4167	99.2500
Precision (%RSD)	2.0803	4.7363	2.1845	6.5433

SD, Standard deviation; RSD (%), Relative standard deviation [(SD / mean) x 100]

5.8 Application to bioequivalence study and evaluation of pharmacokinetic parameters in healthy human volunteers : a single oral dose, open label, randomized, two period, two-sequence and two-way cross over study

The developed and validated LC−ESI−MS/MS assay method was applied successfully to determine the concentration of both EPL and EPLT in human plasma for a bioequivalence (BE) study in 30 healthy Indian male subjects after an oral administration of Enalapril Maleate 20 mg tablets under fasting conditions. The BE study was approved by the Bio-Ethics Committee (HURIP Independent Bioethics Committee, Reg. No. ECR/103/Indt/WB/2013) of Bioequivalence Study Centre, Jadavpur University, Kolkata, India.

Thirty healthy adult male subjects were participated in this comparative, i.e., bioequivalence study at Clinical Pharmacology Unit (CPU) of Bioequivalence Study Center, Jadavpur University and the whole work was supported by TAAB Biostudy Services, Jadavpur, Kolkata. The mean age of the selected volunteers was 27.53 ± 5.86 years with a range of 19 to 37 years; the mean body weight was 60.23 ± 6.21 kg with a range of 52 to 75 kg; the mean body height was 168.30 ± 5.53 cm with a range of 158 to 180 cm and finally, the BMI was 21.25 ± 1.68 Kg/m^2 with range of 18.50 to 23.94 Kg/m^2 (see Table.5.87).

5.8.1 Reference and Test preparation for Enalapril (EPL)

- **Reference Preparation (A) :**

  RENITEC 20 mg tablet (containing Enalapril Maleate 20 mg)

  Mfg. by : MERCK SHARP & DOHME B.V., THE NETHERLANDS

  Batch No. : 334139

  Mfg. Date : July, 2013 Exp. Date : January, 2016

- **Test Preparation (B) :**

  ENALAPRIL 20 mg tablet (containing Enalapril Maleate 20 mg)

  Mfg. by : JV LLC “PHARMLAND”, THE REPUBLIC OF BELARUS

  Batch No. : 011214

  Mfg. Date : December, 2014 Exp. Date : January, 2017

Table.5.87 Demographic data of 30 volunteers

Vol. No.	Sex	Age (yr)	Height (cm)	Weight (kg.)	BMI (kg/m^2)
1	M	35	168	62	21.97
2	M	25	174	56	18.50
3	M	25	168	65	23.03
4	M	22	161	56	21.60
5	M	22	168	55	19.49
6	M	25	166	56	20.32
7	M	26	164	62	23.05
8	M	26	163	61	22.96
9	M	25	170	56	19.38
10	M	37	169	58	20.31
11	M	36	168	55	19.49
12	M	35	164	62	23.05
13	M	36	158	58	23.23
14	M	21	159	52	20.57
15	M	22	164	54	20.08
16	M	23	161	62	23.92
17	M	24	171	56	19.15
18	M	35	176	67	21.63
19	M	25	176	73	23.57
20	M	19	177	75	23.94
21	M	20	169	61	21.36
22	M	21	172	65	21.97
23	M	21	170	63	21.80
24	M	27	177	70	22.34
25	M	28	180	70	21.60
26	M	29	164	53	19.71
27	M	35	169	53	18.56
28	M	34	165	54	19.83
29	M	32	170	55	19.03
30	M	35	168	62	21.97
Mean		**27.53**	**168.30**	**60.23**	**21.25**
S.D.		**5.86**	**5.53**	**6.21**	**1.68**

Table.5.88 Mode of treatment

Subject No.	PERIOD I	PERIOD II
1	A	B
2	A	B
3	B	A
4	B	A
5	B	A
6	A	B
7	A	B
8	A	B
9	A	B
10	B	A
11	B	A
12	B	A
13	A	B
14	B	A
15	A	B
16	A	B
17	A	B
18	B	A
19	B	A
20	B	A
21	A	B
22	A	B
23	B	A
24	B	A
25	B	A
26	A	B
27	A	B
28	B	A
29	B	A
30	A	B

A – Reference Preparation B – Test Preparation

Table.5.89 Bioequivalence of ENALAPRIL 20 mg TABLET containing ENALAPRIL MALEATE 20 mg
Plasma concentration of Enalapril (ng/mL) of the Reference Preparation (A) in 1 to 15 volunteers

Vol. No.	Time (in hours)																			
	0	**0.17**	**0.33**	**0.50**	**0.75**	**1.00**	**1.42**	**1.50**	**2.00**	**2.50**	**3.00**	**3.50**	**4.00**	**4.50**	**5.00**	**6.00**	**8.00**	**10.00**	**12.00**	**24.00**
1	0	51.22	125.80	261.21	235.55	177.82	105.25	88.31	53.33	35.58	27.06	15.54	4.21	3.51	2.57	1.40	1.30	1.20	1.05	ND
2	0	66.36	117.88	229.50	205.29	162.28	113.87	92.62	63.25	53.53	34.08	20.45	6.42	4.21	3.51	2.01	1.40	1.30	1.20	ND
3	0	32.08	76.08	103.74	154.94	261.82	140.92	83.97	71.57	48.82	30.77	21.27	7.58	5.09	4.12	3.47	1.53	1.21	1.16	ND
4	0	53.23	121.83	241.67	180.83	156.57	111.86	91.21	53.83	38.29	26.26	18.78	6.27	4.20	2.89	1.63	1.43	1.31	1.15	ND
5	0	66.46	135.06	277.05	204.95	155.67	135.19	84.80	57.34	40.27	22.45	15.75	7.02	5.34	4.16	3.25	2.06	1.43	1.16	ND
6	0	44.07	95.63	114.37	176.29	234.95	177.95	97.11	48.54	35.49	20.79	11.43	7.46	5.18	4.06	2.21	1.77	1.24	1.17	ND
7	0	48.61	159.11	240.87	184.00	158.37	92.69	75.48	53.32	30.12	23.66	12.52	7.28	5.25	3.23	2.59	1.63	1.37	1.15	ND
8	0	59.84	174.10	257.74	206.14	169.30	86.55	62.55	56.13	26.64	13.13	10.12	7.92	6.22	4.33	3.81	1.70	1.42	1.16	ND
9	0	44.58	83.67	113.47	154.74	268.13	143.62	70.86	34.48	25.56	20.63	12.50	8.14	5.33	4.12	2.31	1.50	1.29	1.16	ND
10	0	54.23	175.65	263.12	198.25	158.67	111.31	81.29	52.12	34.55	23.66	15.97	7.32	4.23	3.06	1.40	1.32	1.20	1.02	ND
11	0	68.86	124.62	276.15	182.84	151.86	92.86	66.16	57.23	40.09	14.13	10.11	6.92	5.04	4.89	4.31	1.49	1.32	1.28	ND
12	0	56.12	92.91	121.19	158.58	252.19	124.16	74.58	43.46	27.66	10.42	8.25	4.41	4.03	3.89	2.11	1.40	1.23	1.15	ND
13	0	50.72	130.70	242.77	182.58	140.63	109.35	87.47	44.20	26.69	12.33	7.54	7.12	3.65	2.67	1.70	1.23	1.19	1.09	ND
14	0	60.54	122.47	229.74	160.84	128.00	101.26	81.39	53.33	45.59	13.43	9.22	8.32	4.37	3.65	2.44	2.13	1.35	1.25	ND
15	0	48.11	79.04	108.15	160.98	266.73	146.41	77.68	46.11	30.67	10.12	8.07	4.21	3.29	2.71	2.21	1.40	1.26	1.20	ND

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.89 (continued) for 16 to 30 volunteers

Vol. No.	Time (in hours)																			
	0	**0.17**	**0.33**	**0.50**	**0.75**	**1.00**	**1.42**	**1.50**	**2.00**	**2.50**	**3.00**	**3.50**	**4.00**	**4.50**	**5.00**	**6.00**	**8.00**	**10.00**	**12.00**	**24.00**
16	0	49.52	126.57	246.28	187.87	163.69	116.22	87.71	48.51	39.78	15.04	9.49	4.31	4.15	2.61	1.63	1.42	1.38	1.15	ND
17	0	66.66	140.32	234.27	197.94	147.05	120.41	71.72	54.93	41.83	13.53	10.12	5.24	4.78	4.32	3.06	1.66	1.49	1.21	ND
18	0	59.10	99.35	105.25	149.08	171.70	235.00	84.10	66.46	36.88	19.75	11.87	5.21	5.38	4.22	1.90	1.40	1.29	1.22	ND
19	0	47.91	165.31	242.07	191.17	147.35	96.30	81.29	51.02	31.30	26.26	13.01	7.52	5.46	3.35	2.51	2.11	1.43	1.20	ND
20	0	63.85	180.88	262.72	202.15	149.25	89.93	76.38	41.80	27.68	13.23	10.52	7.72	6.46	4.50	4.61	2.11	1.48	1.21	ND
21	0	34.18	86.93	109.56	160.77	280.96	149.21	85.10	52.92	26.56	15.24	12.99	6.11	5.54	4.28	2.01	1.60	1.34	1.21	ND
22	0	45.91	182.50	258.61	205.97	151.16	115.65	73.97	51.72	35.90	26.26	16.59	5.21	4.39	3.18	2.41	2.11	1.25	1.06	ND
23	0	63.15	129.48	266.13	189.96	166.39	96.48	87.51	52.42	41.66	23.36	10.51	7.82	5.24	5.08	4.01	3.11	1.37	1.15	ND
24	0	53.82	96.53	109.36	164.76	232.35	129.00	96.93	62.55	38.76	18.84	8.57	6.32	4.19	4.04	3.41	2.41	1.28	1.20	ND
25	0	46.41	135.79	240.57	183.09	147.35	113.61	85.20	47.91	38.41	23.86	10.76	5.41	3.79	2.77	2.41	1.51	1.30	1.14	ND
26	0	64.75	127.24	271.04	228.15	167.29	122.91	71.37	59.44	27.71	15.04	9.04	7.52	6.55	4.79	4.51	2.83	1.41	1.30	ND
27	0	35.78	82.12	125.90	167.25	284.97	152.11	93.62	49.72	28.64	17.64	10.93	5.71	5.50	3.44	2.44	1.60	1.31	1.25	ND
28	0	47.11	131.50	254.10	195.19	148.35	120.75	79.69	46.61	31.31	23.76	15.26	6.40	4.53	3.12	2.15	1.53	1.42	1.13	ND
29	0	62.45	145.78	266.63	221.23	156.47	145.93	88.11	58.04	43.46	21.25	12.99	7.42	5.77	4.49	2.54	2.03	1.23	1.13	ND
30	0	44.61	83.17	105.95	190.30	250.09	192.08	98.93	62.65	38.31	26.86	11.33	6.66	5.59	4.38	2.01	1.40	1.34	1.12	ND
Mean	**0**	**53.01**	**124.27**	**205.97**	**186.06**	**186.91**	**126.29**	**82.57**	**53.16**	**35.59**	**20.09**	**12.38**	**6.50**	**4.87**	**3.75**	**2.61**	**1.74**	**1.32**	**1.17**	**ND**
S. D.	**0**	**10.04**	**32.08**	**69.00**	**22.45**	**49.97**	**32.55**	**9.21**	**7.75**	**7.19**	**6.28**	**3.61**	**1.25**	**0.86**	**0.74**	**0.91**	**0.45**	**0.08**	**0.06**	**ND**
C.V.%	**0**	**18.95**	**25.82**	**33.50**	**12.07**	**26.74**	**25.78**	**11.15**	**14.57**	**20.19**	**31.25**	**29.19**	**19.14**	**17.64**	**19.80**	**34.93**	**25.99**	**6.38**	**5.37**	**ND**

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.90 Bioequivalence of ENALAPRIL 20 mg TABLET containing ENALAPRIL MALEATE 20 mg

Plasma concentration of Enalaprilat (ng/mL) of the Reference Preparation (A) in 1 to 15 volunteers

Vol. No.	Time (in hours)																						
	0	**0.17**	**0.33**	**0.5**	**0.75**	**1.0**	**1.42**	**1.50**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**4.5**	**5.0**	**6.0**	**8.0**	**10.0**	**12.0**	**24.0**	**36.0**	**48.0**	**72.0**
1	0	0	6.42	17.71	45.15	76.40	115.38	124.70	128.02	120.49	112.98	101.83	81.37	69.43	60.50	51.30	47.69	32.27	24.17	12.44	8.53	3.51	ND
2	0	0	7.73	12.64	38.02	69.73	106.15	112.17	130.93	122.10	119.59	105.84	87.76	62.70	58.79	51.47	41.50	30.67	25.66	10.57	7.32	4.21	ND
3	0	0	10.64	20.47	52.97	73.64	100.83	115.72	128.68	111.06	102.73	90.70	81.77	72.74	65.57	54.03	43.11	32.78	19.55	12.44	6.52	2.91	ND
4	0	0	12.40	19.62	48.96	67.69	83.03	91.08	110.87	98.66	90.12	87.00	75.87	68.47	60.28	54.60	43.47	32.21	25.82	10.17	5.91	2.37	ND
5	0	0	6.72	10.53	18.56	22.61	32.41	64.93	84.18	135.94	126.02	116.12	90.71	82.47	77.88	64.82	53.36	40.77	29.33	13.29	6.26	3.36	ND
6	0	0	7.82	13.54	25.88	42.39	87.08	96.72	124.87	115.96	95.72	81.83	78.96	63.81	50.57	46.05	40.61	31.20	26.10	12.26	5.54	2.28	ND
7	0	0	10.01	18.50	45.45	72.65	78.86	103.30	126.10	112.73	108.91	94.05	88.88	70.90	62.67	54.94	45.52	34.98	22.28	13.53	6.72	3.31	ND
8	0	0	7.07	15.35	22.57	35.52	63.71	85.86	93.12	102.31	116.26	100.90	89.82	75.55	60.25	53.57	48.62	29.06	18.86	12.69	7.38	3.35	ND
9	0	0	9.59	18.96	28.89	33.71	52.97	79.83	108.96	95.78	85.48	77.94	69.54	62.50	58.59	50.21	43.55	35.20	22.32	11.31	7.27	4.26	ND
10	0	0	8.28	18.25	33.91	68.58	85.98	102.28	128.16	115.88	109.95	94.21	88.11	80.51	60.70	55.92	38.54	23.44	18.46	12.34	6.65	4.91	ND
11	0	0	7.40	20.41	42.03	60.05	82.98	91.66	102.34	123.87	134.96	101.37	82.53	75.02	63.73	51.75	40.20	25.67	21.32	10.86	7.83	4.28	ND
12	0	0	8.90	14.57	43.82	80.36	91.34	102.39	120.28	110.72	96.05	81.99	76.77	72.27	67.76	55.22	38.11	24.98	18.35	11.28	8.44	2.85	ND
13	0	0	12.26	23.59	61.05	84.87	96.21	101.00	110.49	98.00	85.15	74.53	70.65	63.83	51.75	47.64	35.14	28.56	19.08	12.31	7.52	3.05	ND
14	0	0	14.29	22.62	45.43	56.02	68.74	73.97	87.77	92.21	105.15	94.10	86.44	78.92	69.47	52.50	40.03	23.29	18.23	11.60	6.81	2.24	ND
15	0	0	7.75	12.14	32.39	46.06	67.35	93.31	114.30	105.68	98.41	87.83	77.79	69.05	58.76	51.23	42.20	30.85	20.21	13.05	7.22	1.87	ND

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.90 (continued) for 16 to 30 volunteers

Vol. No.	Time (in hours)																						
	0	**0.17**	**0.33**	**0.5**	**0.75**	**1.0**	**1.42**	**1.50**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**4.5**	**5.0**	**6.0**	**8.0**	**10.0**	**12.0**	**24.0**	**36.0**	**48.0**	**72.0**
16	0	0	9.01	15.61	29.83	48.85	61.37	85.09	119.32	113.65	98.02	94.31	86.53	73.54	58.28	42.75	35.76	28.96	18.56	10.75	7.38	2.25	ND
17	0	0	11.54	21.32	35.38	48.73	60.89	79.14	85.31	129.92	135.04	108.39	90.84	81.19	63.71	60.97	49.18	28.79	14.15	11.49	7.75	3.31	ND
18	0	0	8.15	17.69	26.02	40.93	73.43	117.94	113.02	100.74	92.83	84.49	71.05	67.07	56.44	49.09	40.26	33.49	21.74	10.52	8.51	4.47	ND
19	0	0	11.05	21.86	33.30	38.85	61.05	109.68	125.90	110.39	92.53	89.83	83.02	72.04	67.53	58.13	34.54	25.96	18.42	12.20	8.38	3.97	ND
20	0	0	9.54	21.03	39.08	47.05	68.10	74.55	85.94	100.55	115.53	108.58	78.61	69.79	56.96	43.63	39.63	27.01	21.28	12.37	7.67	2.88	ND
21	0	0	8.53	23.52	45.97	62.49	71.26	83.54	109.75	100.06	95.71	83.27	76.64	72.22	66.36	52.93	38.12	29.59	17.12	14.11	8.33	3.59	ND
22	0	0	10.26	31.79	50.51	72.62	95.00	102.61	126.74	106.19	100.23	92.60	88.14	83.29	78.10	59.11	42.81	30.79	20.46	12.57	6.73	2.47	ND
23	0	0	14.13	27.19	40.37	57.82	61.94	74.49	81.66	94.53	110.92	102.48	91.76	86.62	71.17	50.78	38.08	31.92	21.99	13.12	7.01	3.10	ND
24	0	0	16.47	26.07	45.04	58.92	66.85	89.98	108.05	96.60	78.13	73.51	68.52	60.96	57.07	43.98	39.48	26.84	21.02	12.25	7.85	2.87	ND
25	0	0	8.93	13.99	24.65	30.04	84.05	112.66	101.38	96.58	84.34	76.25	70.12	65.55	54.46	47.02	42.51	35.56	26.37	15.70	8.32	2.55	ND
26	0	0	10.38	17.99	34.38	56.31	71.68	84.11	95.43	105.03	136.61	98.69	82.88	74.76	67.17	59.63	54.30	41.45	21.39	11.72	6.36	2.37	ND
27	0	0	13.30	24.58	60.37	76.50	94.75	103.79	124.86	104.74	90.59	84.93	72.15	68.89	58.97	50.62	40.65	33.18	24.31	12.35	8.93	3.59	ND
28	0	0	9.40	20.39	39.99	57.18	84.63	113.17	118.12	109.12	95.26	81.92	78.96	70.35	62.03	48.15	32.86	28.60	22.05	12.98	7.81	4.41	ND
29	0	0	12.74	25.19	38.38	44.78	60.37	74.95	95.89	127.23	147.94	90.54	74.82	63.03	57.83	50.20	43.65	32.45	26.99	12.02	9.66	2.77	ND
30	0	0	11.00	24.24	45.05	61.10	74.21	93.45	106.30	100.93	96.24	85.14	74.54	66.95	60.63	57.01	39.40	31.13	24.52	13.37	8.84	3.85	ND
Mean	**0**	**0**	**10.06**	**19.71**	**39.11**	**56.41**	**76.75**	**94.60**	**109.89**	**108.59**	**105.25**	**91.51**	**80.52**	**71.48**	**62.13**	**52.31**	**41.76**	**30.72**	**21.67**	**12.26**	**7.51**	**3.24**	**ND**
S. D.	**0**	**0**	**2.48**	**4.93**	**10.51**	**16.11**	**17.52**	**15.42**	**15.49**	**11.36**	**17.49**	**10.87**	**7.23**	**6.77**	**6.53**	**5.24**	**5.06**	**4.33**	**3.42**	**1.16**	**0.97**	**0.79**	**ND**
C.V.%	**0**	**0**	**24.64**	**25.01**	**26.88**	**28.55**	**22.82**	**16.30**	**14.10**	**10.46**	**16.62**	**11.88**	**8.98**	**9.48**	**10.51**	**10.02**	**12.13**	**14.09**	**15.76**	**9.48**	**12.85**	**24.43**	**ND**

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.91 Bioequivalence of ENALAPRIL 20 mg TABLET containing ENALAPRIL MALEATE 20 mg

Plasma concentration of Enalapril (ng/mL) of the Test Preparation (B) in 1 to 15 volunteers

Vol. No.	Time (in hours)																			
	0	**0.17**	**0.33**	**0.50**	**0.75**	**1.00**	**1.42**	**1.50**	**2.00**	**2.50**	**3.00**	**3.50**	**4.00**	**4.50**	**5.00**	**6.00**	**8.00**	**10.00**	**12.00**	**24.00**
1	0	50.12	83.33	130.30	244.03	182.29	107.04	84.26	66.50	39.98	21.15	15.64	4.30	3.64	2.71	1.95	1.28	1.11	1.02	ND
2	0	62.57	124.93	280.30	236.29	217.00	115.81	92.89	59.00	43.60	24.75	17.21	5.60	4.33	3.60	1.85	1.37	1.25	1.18	ND
3	0	30.25	80.63	110.73	160.52	232.90	143.32	104.30	62.63	54.84	21.48	18.24	7.80	5.23	4.23	2.07	1.50	1.16	1.11	ND
4	0	51.78	71.29	113.80	187.33	240.90	131.77	94.19	53.24	43.02	22.21	16.72	5.84	4.31	2.97	2.52	1.36	1.24	1.12	ND
5	0	64.31	143.14	236.50	212.32	203.70	163.75	85.90	60.50	45.24	19.15	15.86	5.30	3.55	2.27	1.60	2.01	1.36	1.10	ND
6	0	41.94	85.01	105.64	182.64	251.80	180.98	105.00	52.91	39.88	20.42	11.51	7.85	5.32	4.17	2.21	1.64	1.20	1.13	ND
7	0	47.53	76.86	126.40	190.62	237.80	169.43	114.41	48.32	33.84	21.21	12.61	6.64	5.40	3.32	2.00	1.59	1.30	1.14	ND
8	0	57.22	184.52	268.30	213.56	179.95	128.80	81.00	64.90	29.93	17.30	10.19	7.50	6.39	4.45	3.17	1.68	1.38	1.10	ND
9	0	45.1	88.68	117.06	160.31	240.20	146.06	94.30	77.42	28.72	16.80	12.59	9.00	5.48	4.23	2.23	1.46	1.22	1.13	ND
10	0	53.84	138.17	211.82	255.38	196.40	113.21	87.90	42.30	20.39	15.30	10.08	5.36	3.35	3.14	2.20	1.27	1.17	1.11	ND
11	0	65.33	132.08	238.40	198.42	132.50	109.44	97.87	65.80	25.45	16.10	9.10	6.30	5.18	4.03	3.18	1.43	1.25	1.14	ND
12	0	52.42	69.85	115.49	164.29	240.60	126.28	101.90	73.38	31.07	17.50	8.31	5.99	4.14	3.40	2.41	1.35	1.19	1.13	ND
13	0	48.3	78.14	130.60	189.15	249.30	132.61	94.39	52.34	29.99	13.14	7.59	5.38	3.75	2.74	1.90	1.19	1.13	1.10	ND
14	0	55.11	129.80	242.00	166.63	120.29	102.99	92.20	60.53	25.12	15.40	9.28	6.60	5.49	3.75	2.80	2.10	1.37	1.01	ND
15	0	46.04	68.38	105.30	166.77	230.60	148.90	101.90	42.90	20.34	11.67	8.12	7.28	5.13	2.78	2.21	1.33	1.24	1.08	ND

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.91 (continued) for 16 to 30 volunteers

Vol. No.	Time (in hours)																			
	0	**0.17**	**0.33**	**0.50**	**0.75**	**1.00**	**1.42**	**1.50**	**2.00**	**2.50**	**3.00**	**3.50**	**4.00**	**4.50**	**5.00**	**6.00**	**8.00**	**10.00**	**12.00**	**24.00**
16	0	46.25	63.15	115.20	194.63	247.00	118.20	94.60	53.33	24.69	12.00	9.56	8.39	4.26	3.27	2.80	1.35	1.32	1.16	ND
17	0	64.28	148.72	237.40	220.59	162.08	122.46	102.96	65.57	19.47	10.43	7.74	5.30	4.91	4.44	3.16	1.62	1.40	1.00	ND
18	0	55.73	81.05	105.62	124.44	182.80	239.00	138.11	77.46	41.43	24.20	11.95	8.20	5.53	4.33	2.01	1.34	1.21	1.09	ND
19	0	44.11	75.21	146.00	198.05	230.70	143.98	84.58	43.20	35.16	19.21	13.10	7.75	4.66	3.45	2.30	2.07	1.38	1.14	ND
20	0	60.61	191.71	241.80	209.42	163.21	112.46	76.20	42.30	31.10	18.00	10.59	7.00	6.63	4.62	3.02	2.10	1.42	1.17	ND
21	0	36.24	92.13	127.57	166.56	290.40	151.75	108.70	65.90	29.84	14.90	13.08	8.60	5.69	4.40	2.37	1.55	1.30	1.03	ND
22	0	46.29	89.42	191.90	243.38	189.00	117.62	85.47	42.34	21.43	13.10	10.70	6.47	4.52	3.26	2.10	2.03	1.41	1.11	ND
23	0	61.77	137.23	264.60	196.79	135.21	98.12	86.70	55.50	30.68	16.00	9.11	5.90	5.38	5.22	3.22	3.02	1.27	1.04	ND
24	0	50.64	95.10	125.68	170.69	239.60	163.94	93.40	48.70	26.44	17.30	8.63	7.50	4.30	4.15	3.13	2.34	1.37	1.07	ND
25	0	42.13	74.92	120.20	189.68	256.30	171.54	74.29	34.28	19.32	11.00	9.11	4.90	3.89	2.85	2.00	1.47	1.24	1.10	ND
26	0	62.45	134.86	267.20	203.35	142.33	96.25	69.34	30.56	18.11	12.58	7.75	5.30	4.73	3.92	2.60	2.66	1.36	1.21	ND
27	0	33.2	87.04	142.20	173.27	281.20	154.70	93.30	42.90	20.32	16.00	11.01	7.90	5.65	3.54	2.35	1.51	1.18	1.12	ND
28	0	48.1	73.38	142.67	250.21	196.80	122.80	64.98	28.30	15.18	10.30	8.37	6.36	4.66	3.20	2.70	1.46	1.31	1.08	ND
29	0	65.44	154.51	254.70	229.19	186.04	148.41	92.80	36.01	16.49	12.90	9.13	5.60	4.93	4.61	3.17	1.96	1.20	1.11	ND
30	0	40.73	88.15	154.60	261.14	184.00	90.35	50.11	23.00	14.43	10.52	7.11	6.08	5.75	4.50	2.24	1.39	1.26	1.06	ND
Mean	**0**	**50.99**	**104.71**	**172.33**	**198.65**	**208.10**	**135.73**	**91.60**	**52.40**	**29.18**	**16.40**	**11.00**	**6.60**	**4.87**	**3.72**	**2.45**	**1.68**	**1.27**	**1.10**	**ND**
S. D.	**0**	**9.72**	**36.02**	**62.97**	**33.20**	**44.46**	**31.13**	**16.11**	**14.38**	**10.18**	**4.17**	**3.11**	**1.23**	**0.82**	**0.72**	**0.48**	**0.44**	**0.09**	**0.05**	**ND**
C.V.%	**0**	**19.07**	**34.40**	**36.54**	**16.71**	**21.36**	**22.93**	**17.58**	**27.44**	**34.89**	**25.43**	**28.29**	**18.65**	**16.87**	**19.42**	**19.47**	**26.16**	**6.94**	**4.51**	**ND**

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.92 Bioequivalence of ENALAPRIL 20 mg TABLET containing ENALAPRIL MALEATE 20 mg

Plasma concentration of Enalaprilat (ng/mL) of the Test Preparation (B) in 1 to 15 volunteers

Vol. No.	Time (in hours)																						
	0	**0.17**	**0.33**	**0.5**	**0.75**	**1.0**	**1.42**	**1.50**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**4.5**	**5.0**	**6.0**	**8.0**	**10.0**	**12.0**	**24.0**	**36.0**	**48.0**	**72.0**
1	0	0	8.45	19.03	39.66	54.04	76.91	97.17	119.10	102.65	95.77	86.69	78.05	71.44	62.25	54.90	43.90	30.07	23.82	14.70	7.47	2.21	ND
2	0	0	6.96	15.79	24.83	37.70	56.76	72.38	91.71	111.96	126.40	99.96	80.15	64.52	60.50	55.35	37.45	28.39	21.87	15.59	6.70	2.38	ND
3	0	0	6.53	19.50	35.27	50.81	71.11	92.73	107.80	122.69	145.80	115.85	92.80	74.85	67.55	50.90	42.90	27.89	18.63	13.40	7.18	3.50	ND
4	0	0	7.09	12.70	25.86	44.46	63.33	93.99	118.70	103.57	88.56	76.60	70.05	65.64	59.50	56.20	36.30	22.74	17.81	11.45	8.99	4.72	ND
5	0	0	10.03	18.21	20.42	24.88	35.65	57.14	75.48	95.47	112.80	94.69	88.90	78.49	60.14	48.00	32.60	26.63	15.09	10.27	8.86	4.19	ND
6	0	0	8.87	14.39	28.47	46.63	79.58	94.08	118.40	124.82	149.10	106.22	81.30	65.66	52.03	46.90	33.90	25.35	18.12	10.42	8.10	3.04	ND
7	0	0	12.78	18.51	49.99	79.91	86.74	113.63	117.60	105.51	91.48	83.33	75.92	66.27	60.05	55.60	39.40	28.11	21.82	14.96	8.92	3.01	ND
8	0	0	9.57	25.28	32.83	43.07	70.08	94.45	100.20	115.99	124.10	104.48	80.05	72.77	61.99	57.20	44.32	32.70	25.12	14.28	7.94	3.22	ND
9	0	0	11.18	21.63	31.78	40.59	58.27	75.00	87.81	93.08	98.76	103.60	112.51	93.89	76.03	51.00	43.17	35.12	22.86	10.48	7.19	2.37	ND
10	0	0	9.43	24.20	37.30	55.44	79.46	81.15	107.30	100.71	89.11	82.34	75.84	68.85	62.46	46.10	39.40	26.38	20.78	13.89	8.78	2.69	ND
11	0	0	6.44	13.00	25.24	39.86	51.13	79.43	87.30	93.68	118.10	95.58	88.93	77.19	65.57	54.30	43.09	30.29	23.99	12.22	6.71	2.50	ND
12	0	0	10.42	21.05	48.21	58.40	61.00	68.13	79.39	91.75	107.02	81.05	79.96	67.44	56.97	46.11	35.35	27.81	20.65	13.70	9.43	3.79	ND
13	0	0	7.75	20.19	46.16	69.36	75.83	86.11	104.90	88.36	80.64	74.79	68.00	60.68	53.25	45.90	36.70	29.32	21.47	13.85	8.25	4.66	ND
14	0	0	7.77	10.84	49.97	67.72	76.62	82.37	95.89	102.32	114.10	98.48	81.05	76.81	67.15	59.60	42.11	31.26	22.52	12.06	6.01	3.71	ND
15	0	0	8.96	13.93	35.63	50.67	74.08	82.64	97.40	111.48	125.40	102.25	90.20	83.75	76.47	50.50	39.90	30.72	22.74	14.69	7.07	2.45	ND

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.92 (continued) for 16 to 30 volunteers

Vol. No.	Time (in hours)																						
	0	**0.17**	**0.33**	**0.5**	**0.75**	**1.0**	**1.42**	**1.50**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**4.5**	**5.0**	**6.0**	**8.0**	**10.0**	**12.0**	**24.0**	**36.0**	**48.0**	**72.0**
16	0	0	10.67	27.96	32.82	53.74	67.50	93.60	102.90	94.47	85.29	74.48	67.10	61.57	54.00	45.12	36.10	27.33	20.89	12.10	6.89	3.07	ND
17	0	0	14.17	18.77	38.92	53.60	66.98	87.05	102.90	110.89	115.00	94.58	89.80	83.54	65.55	52.20	41.36	30.40	15.93	10.29	9.33	4.07	ND
18	0	0	7.84	20.99	28.62	45.03	80.77	91.73	103.05	111.37	128.20	102.79	81.37	69.02	58.07	42.37	36.30	29.38	19.46	11.08	7.68	4.50	ND
19	0	0	12.04	14.99	36.63	42.74	67.16	92.06	114.90	102.37	96.38	85.58	74.08	70.13	62.49	55.90	39.40	29.22	20.72	13.73	7.03	5.18	ND
20	0	0	9.62	24.26	42.99	58.95	66.91	81.15	92.30	105.74	131.80	111.60	89.26	71.82	58.61	45.82	38.10	30.40	23.94	13.92	8.27	4.52	ND
21	0	0	6.74	12.26	25.57	38.73	51.78	61.89	76.20	91.02	115.90	101.06	94.71	81.74	66.29	58.83	30.30	23.30	19.27	15.88	8.80	3.79	ND
22	0	0	12.30	12.49	55.56	79.88	104.50	112.87	125.00	114.28	97.58	88.34	72.90	68.71	60.36	53.64	41.10	34.65	23.02	14.15	7.11	2.61	ND
23	0	0	7.43	21.06	34.40	53.60	65.13	72.94	80.70	92.49	113.50	102.25	96.00	89.13	73.23	55.40	43.45	35.92	24.75	14.77	6.24	2.50	ND
24	0	0	9.04	16.62	29.54	36.81	53.54	59.98	71.22	98.47	107.30	88.58	71.45	62.73	58.72	50.11	40.40	32.21	23.65	13.79	6.61	3.55	ND
25	0	0	10.05	21.20	37.12	43.04	69.45	82.93	99.90	91.05	86.63	80.00	76.98	67.45	56.04	50.64	45.39	40.02	29.68	17.67	5.85	2.41	ND
26	0	0	8.31	22.48	47.82	56.94	78.85	82.52	90.11	105.28	112.00	92.25	86.50	76.93	69.12	56.90	40.90	36.64	24.07	13.19	10.21	2.93	ND
27	0	0	7.10	20.97	36.41	58.15	64.23	71.14	96.11	111.18	121.40	102.15	93.17	87.09	70.67	64.80	51.33	37.35	27.36	13.90	7.10	3.50	ND
28	0	0	8.93	25.91	30.44	46.90	69.09	81.24	100.40	91.46	86.49	77.71	70.57	62.39	56.83	45.33	38.70	32.18	24.82	14.61	7.80	3.54	ND
29	0	0	11.58	24.93	32.22	49.26	56.40	75.82	87.90	105.00	121.40	102.98	92.20	76.85	65.51	52.00	43.40	36.52	30.37	13.53	7.40	3.27	ND
30	0	0	8.34	26.81	49.55	56.21	74.63	82.80	92.38	111.46	125.90	105.46	88.10	68.89	62.39	56.80	49.34	35.04	23.06	15.05	8.29	3.03	ND
Mean	**0**	**0**	**9.21**	**19.33**	**36.34**	**51.24**	**68.45**	**83.34**	**98.16**	**103.35**	**110.40**	**93.86**	**82.93**	**72.87**	**62.66**	**52.15**	**40.20**	**30.78**	**22.28**	**13.45**	**7.74**	**3.36**	**ND**
S. D.	**0**	**0**	**2.00**	**4.80**	**9.01**	**12.21**	**12.85**	**13.33**	**14.20**	**9.85**	**18.09**	**11.54**	**10.20**	**8.63**	**6.28**	**5.28**	**4.59**	**4.23**	**3.48**	**1.81**	**1.09**	**0.82**	**ND**
C.V.%	**0**	**0**	**21.75**	**24.81**	**24.79**	**23.83**	**18.78**	**15.99**	**14.46**	**9.53**	**16.39**	**12.29**	**12.30**	**11.85**	**10.02**	**10.12**	**11.42**	**13.73**	**15.63**	**13.42**	**14.07**	**24.33**	**ND**

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.93 Pharmacokinetic parameters

C_{max} (ng/mL), t_{max} (hr.), AUC_{0-t} (ng. hr./mL), $AUC_{0-\infty}$, $t_{1/2}$, K_{el}

in 30 volunteers with the Test and Reference Preparation of Enalapril (EPL)

Volunteer	C_{max}		t_{max}		AUC_{0-t}		$AUC_{0-\infty}$		$t_{1/2}$		K_{el}	
no.	A	B	A	B	A	B	A	B	A	B	A	B
1	261.21	244.03	0.50	0.75	334.15	308.91	336.09	311.64	1.28	1.41	0.54	0.49
2	229.50	280.30	0.50	0.50	342.82	365.26	345.10	367.58	1.31	1.31	0.53	0.53
3	261.82	232.90	1.00	1.00	333.15	325.17	335.45	327.53	1.38	1.38	0.50	0.50
4	241.67	240.90	0.50	1.00	316.78	313.03	318.99	315.45	1.33	1.42	0.52	0.49
5	277.05	236.50	0.50	0.50	343.42	354.97	345.71	357.23	1.36	1.31	0.51	0.53
6	234.95	251.80	1.00	1.00	320.50	332.38	322.91	334.84	1.42	1.43	0.49	0.48
7	240.87	237.80	0.50	1.00	308.51	323.26	310.80	325.99	1.38	1.44	0.50	0.48
8	257.74	268.30	0.50	0.50	317.21	349.54	319.59	351.72	1.42	1.34	0.49	0.52
9	268.13	240.20	1.00	1.00	296.57	320.08	299.03	322.52	1.47	1.42	0.47	0.49
10	263.12	255.38	0.50	0.75	326.70	322.78	328.61	324.87	1.30	1.38	0.53	0.50
11	276.15	238.40	0.50	0.50	312.71	311.25	315.32	313.43	1.41	1.37	0.49	0.50
12	252.19	240.60	1.00	1.00	288.45	306.14	290.93	308.57	1.49	1.43	0.46	0.48
13	242.77	249.30	0.50	1.00	287.63	305.73	289.75	308.05	1.34	1.43	0.52	0.48
14	229.74	242.00	0.50	0.50	292.18	295.03	294.74	297.68	1.42	1.43	0.49	0.48
15	266.73	230.60	1.00	1.00	295.52	287.52	298.08	290.20	1.48	1.51	0.47	0.46
16	246.28	247.00	0.50	1.00	308.87	297.07	311.11	299.66	1.34	1.51	0.52	0.46
17	234.27	237.40	0.50	0.50	313.55	325.61	315.99	327.87	1.40	1.39	0.50	0.50
18	235.00	239.0	1.42	1.42	311.57	332.98	314.21	335.56	1.50	1.43	0.46	0.48
19	242.07	230.70	0.50	1.00	312.63	312.78	315.05	315.18	1.40	1.48	0.50	0.47
20	262.72	241.80	0.50	0.50	313.08	324.29	315.61	326.82	1.45	1.42	0.48	0.49
21	280.96	290.40	1.00	1.00	312.28	339.72	314.80	342.01	1.45	1.41	0.48	0.49
22	258.61	243.38	0.50	0.75	328.29	302.68	330.36	304.88	1.35	1.41	0.51	0.49
23	266.13	264.60	0.50	0.50	330.83	311.11	333.17	313.51	1.42	1.42	0.49	0.49
24	232.35	239.60	1.00	1.00	313.48	314.78	316.04	317.47	1.48	1.52	0.47	0.46
25	240.57	256.30	0.50	1.00	306.34	294.86	308.55	297.39	1.35	1.51	0.51	0.46
26	271.04	267.20	0.50	0.50	335.87	286.27	338.56	289.09	1.44	1.47	0.48	0.47
27	284.97	281.20	1.00	1.00	320.73	320.32	323.34	323.05	1.45	1.48	0.48	0.47
28	254.10	250.21	0.50	0.75	311.14	275.56	313.34	278.04	1.36	1.49	0.51	0.47
29	266.63	254.70	0.50	0.50	349.40	333.91	351.57	336.23	1.34	1.39	0.52	0.50
30	250.09	261.14	1.00	0.75	340.00	265.82	342.23	268.35	1.38	1.53	0.50	0.45
N	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**
Mean	**254.313**	**249.788**	**0.681**	**0.806**	**317.479**	**315.294**	**319.834**	**317.747**	**1.396**	**1.429**	**0.497**	**0.486**
S.D.	**16.182**	**15.499**	**0.270**	**0.253**	**16.478**	**22.334**	**16.415**	**22.250**	**0.059**	**0.058**	**0.021**	**0.020**
C.V.%	**6.363**	**6.205**	**39.696**	**31.400**	**5.190**	**7.083**	**5.132**	**7.002**	**4.257**	**4.051**	**4.270**	**4.097**

A – Reference Preparation B – Test Preparation

Table.5.94 Pharmacokinetic parameters

C_{max} (ng/mL), t_{max} (hr.), AUC_{0-t} (ng. hr./mL), $AUC_{0-\infty}$, $t_{1/2}$, K_{el}

in 30 volunteers with the Test and Reference Preparation of Enalaprilat (EPLT)

Volunteer	C_{max}		t_{max}		AUC_{0-t}		$AUC_{0-\infty}$		$t_{1/2}$		K_{el}	
no.	A	B	A	B	A	B	A	B	A	B	A	B
1	128.02	119.10	2.00	2.00	1137.83	1080.69	1184.95	1108.58	9.30	8.73	0.07	0.08
2	130.93	126.40	2.00	3.00	1095.62	1040.71	1152.40	1071.43	9.34	8.96	0.07	0.08
3	128.68	145.80	2.00	3.00	1060.44	1114.54	1097.28	1161.47	8.78	9.30	0.08	0.07
4	110.87	118.70	2.00	2.00	1020.14	975.04	1049.22	1045.96	8.51	10.42	0.08	0.07
5	135.94	112.80	2.50	3.00	1164.10	913.16	1205.88	975.22	8.62	10.26	0.08	0.07
6	124.87	149.10	2.00	3.00	1015.77	992.35	1043.64	1033.88	8.48	9.46	0.08	0.07
7	126.10	117.60	2.00	2.00	1100.92	1090.73	1143.90	1132.21	9.00	9.55	0.08	0.07
8	116.26	124.10	3.00	3.00	1025.66	1121.41	1071.22	1165.41	9.42	9.47	0.07	0.07
9	108.96	112.51	2.00	4.00	992.15	1035.45	1053.60	1065.43	9.99	8.78	0.07	0.08
10	128.16	107.30	2.00	2.00	1036.12	1021.16	1104.25	1057.63	9.62	9.40	0.07	0.07
11	134.96	118.10	3.00	3.00	1049.84	1027.25	1111.02	1058.91	9.91	8.77	0.07	0.08
12	120.28	107.02	2.00	3.00	1016.00	1009.11	1053.51	1067.08	9.12	10.60	0.08	0.07
13	110.49	104.90	2.00	2.00	982.78	1007.23	1024.86	1079.81	9.56	10.79	0.07	0.06
14	105.15	114.10	3.00	3.00	967.65	1049.26	995.85	1099.16	8.72	9.32	0.08	0.07
15	114.30	125.40	2.00	3.00	1010.64	1087.35	1033.37	1118.33	8.43	8.76	0.08	0.08
16	119.32	102.90	2.00	2.00	959.57	948.26	987.43	990.59	8.58	9.55	0.08	0.07
17	135.04	115.00	3.00	3.00	1029.98	1016.74	1073.88	1075.53	9.19	10.01	0.08	0.07
18	117.94	128.20	1.50	3.00	1013.21	999.56	1077.04	1066.13	9.91	10.24	0.07	0.07
19	125.90	114.90	2.00	2.00	1020.26	1073.00	1075.68	1113.28	9.67	10.20	0.07	0.07
20	115.53	131.80	3.00	3.00	997.77	1089.41	1036.93	1156.35	9.42	10.26	0.07	0.07
21	109.75	115.90	2.00	3.00	1024.85	1033.15	1075.31	1088.26	9.74	10.07	0.07	0.07
22	126.74	125.00	2.00	2.00	1081.45	1096.63	1111.60	1130.08	8.46	8.88	0.08	0.08
23	110.92	113.50	3.00	3.00	1042.15	1093.79	1083.11	1124.80	9.16	8.59	0.08	0.08
24	108.05	107.30	2.00	3.00	971.83	1001.40	1011.49	1051.85	9.57	9.85	0.07	0.07
25	112.66	99.90	1.50	2.00	1077.00	1115.44	1111.18	1146.46	9.31	8.94	0.07	0.08
26	136.61	112.00	3.00	3.00	1084.13	1121.37	1112.93	1161.76	8.42	9.56	0.08	0.07
27	124.86	121.40	2.00	3.00	1089.83	1168.44	1140.31	1214.56	9.75	9.14	0.07	0.08
28	118.12	100.40	2.00	2.00	1037.80	1031.83	1101.62	1083.03	10.02	10.02	0.07	0.07
29	147.94	121.40	3.00	3.00	1105.12	1131.28	1143.08	1175.08	9.50	9.28	0.07	0.07
30	106.30	125.90	2.00	3.00	1082.62	1137.38	1138.03	1178.36	9.96	9.36	0.07	0.07
N	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**	**30**
Mean	**121.321**	**117.948**	**2.250**	**2.700**	**1043.107**	**1052.904**	**1086.819**	**1099.888**	**9.249**	**9.551**	**0.075**	**0.073**
S.D.	**10.766**	**11.554**	**0.487**	**0.535**	**50.296**	**60.532**	**53.305**	**56.189**	**0.532**	**0.619**	**0.004**	**0.005**
C.V.%	**8.874**	**9.796**	**21.640**	**19.814**	**4.822**	**5.749**	**4.905**	**5.109**	**5.757**	**6.482**	**5.854**	**6.416**

A – Reference Preparation B–Test Preparation

Table.5.95 Pharmacokinetic parameters in 30 volunteers with the Test and Reference Preparation of Enalapril (EPL) 20 mg Tablet

Pharmacokinetic parameters		Enalapril (EPL)		Enalaprilat (EPLT)	
		Reference Preparation (A)	Test Preparation (B)	Reference Preparation (A)	Test Preparation (B)
C_{max} (ng./mL.)	Mean	254.313	249.788	121.321	117.948
	± S.D.	16.182	15.499	10.766	11.554
t_{max} (hr.)	Mean	0.681	0.806	2.250	2.700
	± S.D.	0.270	0.253	0.487	0.535
AUC_{0-t} (ng. hr./mL.)	Mean	317.479	315.294	1043.107	1052.904
	± S.D.	16.478	22.334	50.296	60.532
$AUC_{0-\infty}$ (ng. hr./mL.)	Mean	319.834	317.747	1086.819	1099.888
	± S.D.	16.415	22.250	53.305	56.189
k_{el} ($hr.^{-1}$)	Mean	0.497	0.486	0.075	0.073
	± S.D.	0.021	0.020	0.004	0.005
$t_{1/2}$ (hr.)	Mean	1.396	1.429	9.249	9.551
	± S.D.	0.059	0.058	0.532	0.619
Relative Bioavailability (%)		**100%**	**99.31%**	**100%**	**100.94%**

Table.5.96 90% confidence interval (CI) with the Test and Reference Preparation

Confidence Interval (CI)		Enalapril (EPL)	Enalaprilat (EPLT)
C_{max}	Untransformed Data	0.942478 - 1.021930	0.911458 - 1.032921
	Ln transformed Data	0.989615 - 1.003958	0.981258 - 1.006678
AUC_{0-t}	Untransformed Data	0.954151 - 1.032086	0.976312 - 1.042472
	Ln transformed Data	0.991730 - 1.005477	0.996544 - 1.006002
$AUC_{0-\infty}$	Untransformed Data	0.954972 - 1.031975	0.979075 - 1.044975
	Ln transformed Data	0.991890 - 1.005454	0.997024 - 1.006365

Table.5.97 ANOVA Summary for C_{max} Values untransformed data

		Enalapril (EPL)				Enalaprilat (EPLT)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	9274.64058	319.81519	1.71089	N.S.	4280.70399	147.61048	1.48464	N.S.
Treatment	1	307.23393	307.23393	1.64359	N.S.	170.75814	170.75814	1.71746	N.S.
Period	1	51.20808	51.20808	0.27394	N.S.	168.53856	168.53856	1.69513	N.S.
Error	28	5234.00552	186.92877	-	-	2783.89700	99.42489	-	-
Total	59	14867.08811	-	-	-	7403.89769	-	-	-

Table.5.98 ANOVA Summary for in Ln C_{max} Values Ln transformed data

		Enalapril (EPL)				Enalaprilat (EPLT)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	0.14008	0.00483	1.67306	N.S.	0.29083	0.01003	1.47458	N.S.
Treatment	1	0.00475	0.00475	1.64456	N.S.	0.01255	0.01255	1.84484	N.S.
Period	1	0.00209	0.00209	0.72517	N.S.	0.00557	0.00557	0.81896	N.S.
Error	28	0.08084	0.00289	-	-	0.19043	0.00680	-	-
Total	59	0.22776	-	-	-	0.49938	-	-	-

Table.5.99 ANOVA Summary for AUC_{0-t} Values untransformed data

		Enalapril (EPL)				Enalaprilat (EPLT)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	14257.59137	491.64108	1.75399	N.S.	118260.50365	4077.94840	1.87007	N.S.
Treatment	1	71.59590	71.59590	0.25543	N.S.	1439.56139	1439.56139	0.66016	N.S.
Period	1	233.72161	233.72161	0.83383	N.S.	303.59702	303.59702	0.13922	N.S.
Error	28	7848.34593	280.29807	-	-	61057.85294	2180.63760	-	-
Total	59	22411.25481	-	-	-	181061.51499	-	-	-

D.F. – Degree of Freedom

S.S. – Sum of Squares

M.S.S. – Mean Sum of Squares

F. – M.S.S. / Error M.S.S.

P - Probability

N.S. – Non significant

S – Significant

Table.5.100 ANOVA Summary for Ln AUC_{0-t} Values Ln transformed data

		Enalapril (EPL)				Enalaprilat (EPLT)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	0.14166	0.00488	1.70187	N.S.	0.10765	0.00371	1.87695	N.S.
Treatment	1	0.00097	0.00097	0.33802	N.S.	0.00117	0.00117	0.59337	N.S.
Period	1	0.00341	0.00341	1.18827	N.S.	0.00077	0.00077	0.39160	N.S.
Error	28	0.08037	0.00287	-	-	0.05537	0.00198	-	-
Total	59	0.22640	-	-	-	0.16497	-	-	-

Table.5.101 ANOVA Summary for AUC_{0-inf} Values untransformed data

		Enalapril (EPL)				Enalaprilat (EPLT)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	14163.52730	488.39749	1.75860	N.S.	108135.17568	3728.79916	1.58764	N.S.
Treatment	1	65.36067	65.36067	0.23535	N.S.	2562.02062	2562.02062	1.09085	N.S.
Period	1	231.63813	231.63813	0.83407	N.S.	62.51175	62.51175	0.02662	N.S.
Error	28	7776.12397	277.71871	-	-	65761.85163	2348.63756	-	-
Total	59	22236.65008	-	-	-	176521.55967	-	-	-

Table.5.102 ANOVA Summary for Ln AUC_{0-inf} VALUES Ln transformed data

		Enalapril (EPL)				Enalaprilat (EPLT)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	29	0.13862	0.00478	1.70671	N.S.	0.09109	0.00314	1.60923	N.S.
Treatment	1	0.00088	0.00088	0.31408	N.S.	0.00210	0.00210	1.07773	N.S.
Period	1	0.00331	0.00331	1.18054	N.S.	0.00031	0.00031	0.15998	N.S.
Error	28	0.07842	0.00280	-	-	0.05466	0.00195	-	-
Total	59	0.22123	-	-	-	0.14817	-	-	-

D.F. – Degree of Freedom

S.S. – Sum of Squares

M.S.S. – Mean Sum of Squares

F. – M.S.S./Error M.S.S.

P - Probability

N.S. – Non significant

S – Significant

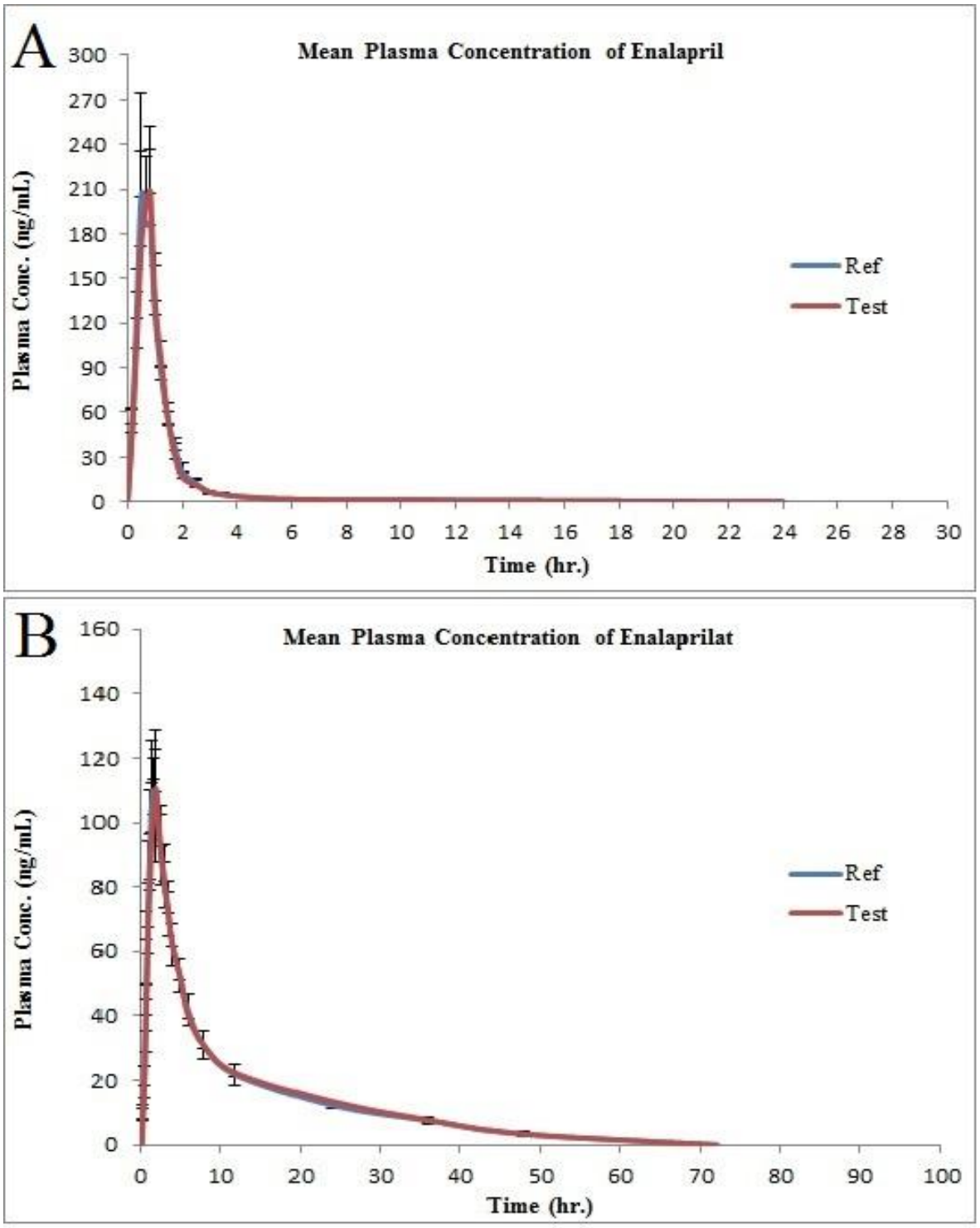


Figure.5.66 Graphical representation of mean plasma concentration versus time of (A) EPL and (B) EPLT following 20 mg single oral dose of test and reference preparation to 30 healthy human volunteers

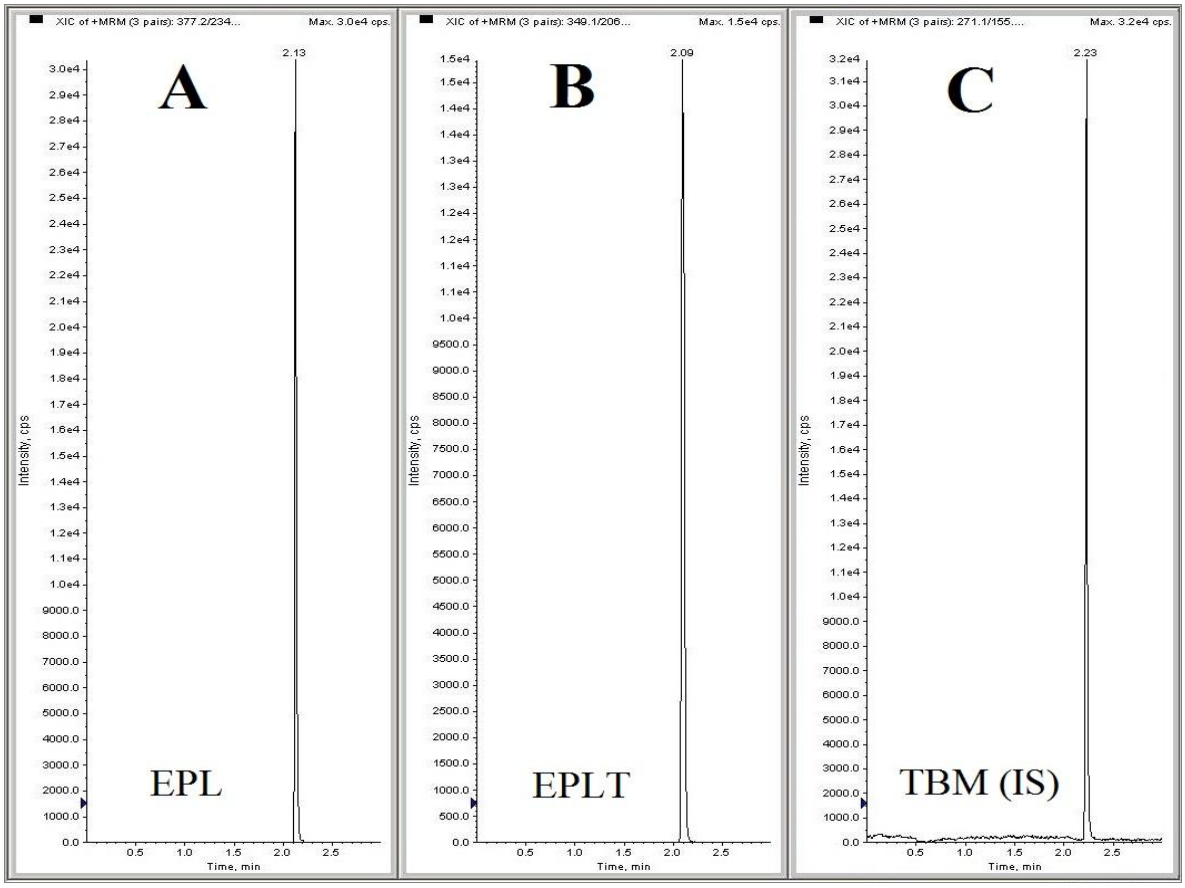


Figure.5.67 Representative MRM chromatograms of volunteer plasma samples : (A) *in vivo* plasma sample showing EPL peak obtained following single oral dose of 20 mg tablets to healthy humans, (B) *in vivo* plasma sample showing EPLT peak obtained following single oral dose of 20 mg tablets to healthy humans and (C) TBM (IS) spiked in human volunteer plasma at 100 ng/mL

5.8.2 Pharmacokinetics and statistical analysis

The volunteer plasma samples were analysed by the developed and validated LC-ESI-MS/MS method which has already described in the section 5.5.10 and 5.5.11, respectively and the plasma concentrations and mean plasma concentrations were evaluated and reported in Table.5.89 to Table.5.92. In Figure.5.66 and Figure.5.67, the mean plasma concentration-time profile graphs of both EPL and EPLT were demonstrated for both Test and Reference preparation and representative chromatograms of volunteer plasma samples of both EPL and EPLT with IS (TBM) were also shown. The reference tablet as a single oral dose in the fasting state produced the maximum plasma concentration of 254.313 ± 16.182 ng/mL (C_{max}) at the time of 0.681 ± 0.270 hr. (t_{max}) for EPL and 121.321 ± 10.766 ng/mL (C_{max}) at the time

of 2.250 ± 0.487 hr. (t_{max}) for EPLT whereas the test product in the fasting state produced the maximum plasma concentration of 249.788 ± 15.499 ng/mL (C_{max}) at the time of 0.806 ± 0.253 hr. (t_{max}) for EPL and 117.948 ± 11.554 ng/mL (C_{max}) at the time of 2.700 ± 0.535 hr. (t_{max}) for EPLT, respectively (see Table.5.95).

After administration of the reference tablet produced the area under plasma concentration time curve (AUC_{0-t}) 317.479 ± 16.478 ng. hr./mL for EPL and 1043.107 ± 50.296 ng. hr./mL for EPLT, whereas in case of the test tablet produced the area under plasma concentration curve (AUC_{0-t}) 315.294 ± 22.334 ng. hr./mL for EPL and 1052.904 ± 60.532 ng. hr./mL for EPLT, respectively (Table.5.95).

When administered as a single oral dose, in the fasting state, the reference product of Enalapril (EPL) produced the area under plasma concentration time curve upto infinity ($AUC_{0-\infty}$) 319.834 ± 16.415 ng. hr./mL (EPL) and 1086.819 ± 53.305 ng. hr./mL (EPLT), and for the test product ($AUC_{0-\infty}$) 317.747 ± 22.250 ng. hr./mL (EPL) and 1099.888 ± 56.189 ng. hr./mL (EPLT) (Table.5.95).

Administration of Enalapril (EPL) produced the plasma elimination half-life, i.e., $t_{1/2}$ 1.396 ± 0.059 hr. (EPL) and 9.249 ± 0.532 hr. (EPLT) for the reference and 1.429 ± 0.058 hr. (EPL) and 9.551 ± 0.619 (EPLT) for the test, respectively (Table.5.95). While administration of the reference and test preparation produced the plasma elimination rate constant, i.e., k_{el} 0.497 ± 0.021 $hr.^{-1}$ and 0.075 ± 0.004 $hr.^{-1}$ for EPL and 0.486 ± 0.020 $hr.^{-1}$ and 0.073 ± 0.005 $hr.^{-1}$ for EPLT, respectively (Table.5.95).

As a result of comparison of the AUC_{0-t} for Reference and Test after single oral dose administration, the relative bioavailability (%) of the test product of Enalapril (EPL) tablet was 99.31% for EPL and 100.94% for EPLT (see Table.5.95). The 90% confidence interval (CI) for Untransformed data of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values of the test product were 0.942478 - 1.021930; 0.954151 - 1.032086 and 0.954972 - 1.031975 for EPL and 0.911458 - 1.032921; 0.976312 - 1.042472 and 0.979075 - 1.044975 for EPLT, respectively of that the reference product. Whereas Ln transformed data of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values of the test product were 0.989615 - 1.003958; 0.991730 - 1.005477 and 0.991890 - 1.005454 for EPL and 0.981258 - 1.006678; 0.996544 - 1.006002 and 0.997024 - 1.006365 for EPLT, respectively of that the reference product (see Table.5.96).

5.8.3 Statistical inference

The 90% CI, i.e., confidence interval for C_{max}, Ln C_{max}, AUC_{0-t}, Ln AUC_{0-t}, $AUC_{0-\infty}$ and Ln $AUC_{0-\infty}$ values of the Test preparation of Enalapril (EPL) tablet were within the acceptance criteria of that of the Reference preparation, i.e., CI = 0.8 to 1.2. ANOVA (Subjects, Treatment, Period) was employed to the C_{max}, Ln C_{max}, AUC_{0-t}, Ln AUC_{0-t}, $AUC_{0-\infty}$ and Ln $AUC_{0-\infty}$ values. Finally the results were found statistically nonsignificant, i.e., there was no statistically significant difference for the analysis values of the C_{max}, Ln C_{max}, AUC_{0-t}, Ln AUC_{0-t}, $AUC_{0-\infty}$ and Ln $AUC_{0-\infty}$ (see Table.5.97 to Table.5.102).

5.8.4 Discussion

The present LC-ESI-MS/MS bioanalytical method has been developed and validated for the simultaneous determination of both Enalapril (EPL) and its active metabolite Enalaprilat (EPLT) in human plasma which is simple, selective, rapid, specific, highly sensitive and high-throughput tandem mass spectrometric method. The proposed bioanalytical method has significant advantages over the previously reported methods (such as Makwana *et al*., 2013; Gu *et al*., 2004; Spanakis and Niopas. 2010; Wang *et al*., 2007; Lee *et al*., 2003; Ramusovic *et al*., 2012; Jones *et al*., 2012). The limit of detection (LOD) is 0.25 ng/mL for EPL and 0.80 ng/mL for EPLT, respectively and the lower limit of quantification (LLOQ) is 1 ng/mL for both EPL and EPLT which is more sensitive than some published HPLC, GC-MS and LC-MS/MS methods (Foda *et al*., 2010; Rao *et al*., 2014; Spanakis and Niopas. 2010; Wang *et al*., 2007; Ramusovic *et al*., 2012). Because of the lower LLOQ of 1 ng/mL for both EPL and EPLT, it allows plasma drug monitoring for at least 72 hours after oral administration. In the present method, extraction recovery of EPL and EPLT is in between 94% to 99% and which is much better than other methods like Makwana *et al*., 2011 and 2013; Spanakis and Niopas. 2010; Gu *et al*., 2004; Jones *et al*., 2012; Ghosh *et al*., 2012; Ramusovic *et al*., 2012. Sample extraction procedure is carried out by a simple one-step protein precipitation technique (PPT) with ice cold acetonitrile using just 300 µL of plasma. This extraction technique provides higher levels of reproducibility, increased sensitivity, less time consuming and reduced excess solvent requirements, hence reduced solvent costs and so that the method has become more economic or cost-effective. Whereas in the other published methods such as Yoon *et al*., 2004; Makwana *et al*., 2011 and 2013; Spanakis and Niopas. 2010; Wang *et al*., 2007; Gu *et al*., 2004; Jones *et al*., 2012; Lee *et al*., 2003; Ghosh *et al*., 2012; Cheregi *et al*., 2013; Ramusovic *et al*., 2012, plasma extractions were found complicated, using many solvents,

time consuming and also expensive, i.e., not so economic. Now, the total chromatographic run time of the present method is 3.0 min. which is comparatively shorter than other methods (Foda *et al*., 2010; Saeed *et al*., 2013; Sultana *et al*., 2013; Pisarev *et al*., 2005; Spanakis and Niopas. 2010; Wang *et al*., 2007; Gu *et al*., 2004; Lee *et al*., 2003; Cheregi *et al*., 2013; Ramusovic *et al*., 2012). So, the simplicity of sample preparation and the short chromatographic run time gives the method capability for high sample throughput. The present method has been successfully validated as per the USFDA and EMA guidelines and all the validation data were within the acceptance limits.

The present method was developed and fully validated and also successfully applied for simultaneous quantification of EPL and EPLT in human plasma after single oral dose administration of Enalapril 20 mg tablets in 30 healthy Indian male volunteers under fasting conditions. In few published methods, only EPL was quantified in human plasma and performed the pharmacokinetic as well as bioequivalence study but not the metabolite EPLT (Yoon *et al*., 2004; Foda *et al*., 2010; Makwana *et al*., 2013 and Kang *et al*., 2014). Now according to the literature review, EPLT is the only metabolite of EPL and its an active metabolite. It is a more potent and effective angiotensin-converting enzyme (ACE) inhibitor than EPL i.e., pharmacological activity of EPLT is more than EPL in the body and it can also effectively used to treat hypertension and congestive heart failure through dilation of peripheral vascular resistance without causing any significant changes in heart rate or cardiac output (Desai *et al*., 2012; Gu *et al*., 2004; Spanakis and Niopas. 2010; Ghosh *et al*., 2012; Lee *et al*., 2003; Tamboli *et al*., 2014; Kang *et al*., 2014). EPLT has low exposure to the central compartment in therapeutic human dose and is detectable in pictogram and quantified in nanogram level in human plasma. Because of exposure to plasma in therapeutic dose in very low strength (20 mg), so it is very difficult to quantify by HPLC method. Therefore, it is very important to quantify both EPL and EPLT by LC-MS/MS in human plasma and also to establish the pharmacokinetic as well as bioequivalence study for the drug development process. So finally it is established that the proposed method has significant advantages over earlier reported methods for simultaneous quantification of two analytes (EPL and EPLT) in human plasma without any interference or matrix effect, shorter run time (3.0 min.), wider linearity range (1 ng/mL to 500 ng/mL) with a more sensitivity (lower limit of quantification 1 ng/mL) and simple reproducible extraction with higher extraction recovery. Accordingly, this kind of approach in the near future will enrich the potentiality of pharmacokinetics or quantitative study of the active metabolites in plasma.

SECTION–C

5.9 Development of method for nimesulide (NSD) and 4-hydroxynimesulide (M1) by liquid chromatography tandem-mass spectrometry (LC-MS/MS)

5.9.1 Determination of precursor ion and product ion for NSD, M1 (active metabolite) and IS (CXB)

During MS/MS development, tuning of mass spectrometer involves optimizing voltages, currents, flow rates and optimization of ion source parameters to achieve the maximum mass spectral sensitivity and proper resolution. It has been shown that the electrospray ionization (ESI) source provided better results as compared to the atmospheric chemical ionization (APCI) source for proper ionization of both the analytes NSD and M1 and IS (Celecoxib, CXB). The precursor ion was determined in quadrupole 1 (Q1), i.e., QIMS scan and the product ion was determined in quadrupole 3 (Q3), i.e., MS2 scan, and measured by the mass-to-charge ratio (*m/z*). The transition pairs were monitored accurately and compared in both positive $[M + H]^+$ and negative mode or polarity $[M - H]^-$, i.e., protonated and deprotonated. It has been observed that more intense response in negative ion mode / polarity $[M - H]^-$ than positive $[M + H]^+$ for both analytes (NSD and M1) and IS (CXB).

During mass scanning, the compound-dependent parameters of the analytes and IS were manually tuned, such as declustering potential (DP), focusing potential (FP) and entrance potential (EP) were tuned in Q1 scan i.e., Q1MI or multiple ions scan and collision energy (CE) and collision cell exit potential (CXP) were tuned in Q3 scan or in MS2 scan. While, collision cell entrance potential (CEP) was setting up as by default in the software. Molar mass or molecular weight of NSD, M1 and CXB (IS) were 308.311 g/mol, 324.307 g/mol and 381.373 g/mol, respectively. In multiple reaction monitoring (MRM) mode for ion transitions of all compounds deprotonated precursor $[M - H]^-$ to product ion spectra were used to measure at m/z 307.20 → 229.20 for NSD (see Figure.5.68), m/z 323.00 → 245.00 for M1 (see Figure.5.69) and m/z 380.20 → 316.20 for CXB (IS) (see Figure.5.70), respectively. The fragments or product ions were most abundant stable spectrum with better response as compared to others product ions. Therefore, the following conditions were selected for optimization of the fragmentation conditions. Automated data acquisition, data processing, analysis and instrument control were performed by Analyst software (version 1.5).

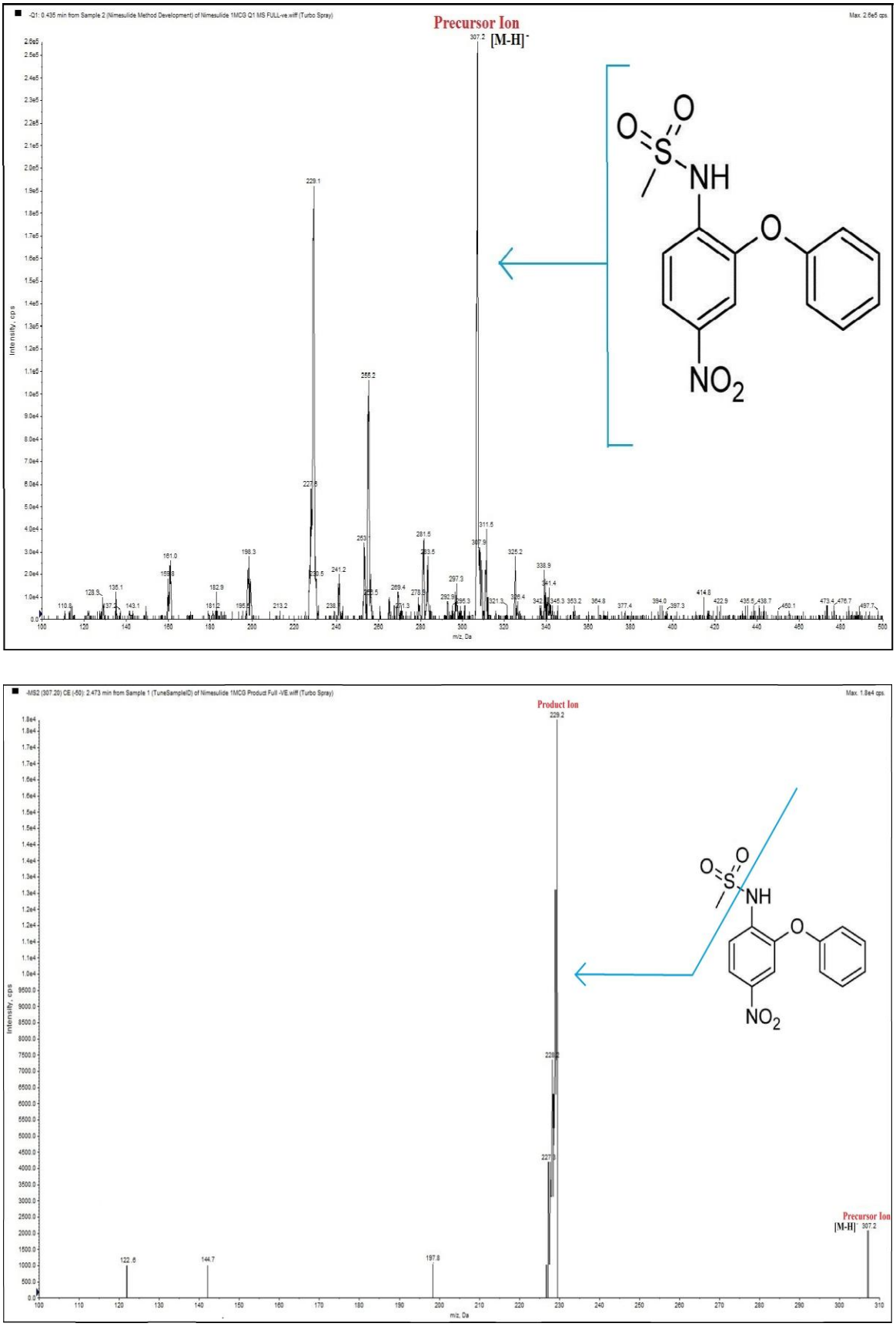


Figure.5.68 Mass spectra (Q1 and MS2) of the negative ion $[M-H]^-$ of nimesulide (NSD) with structural interpretation

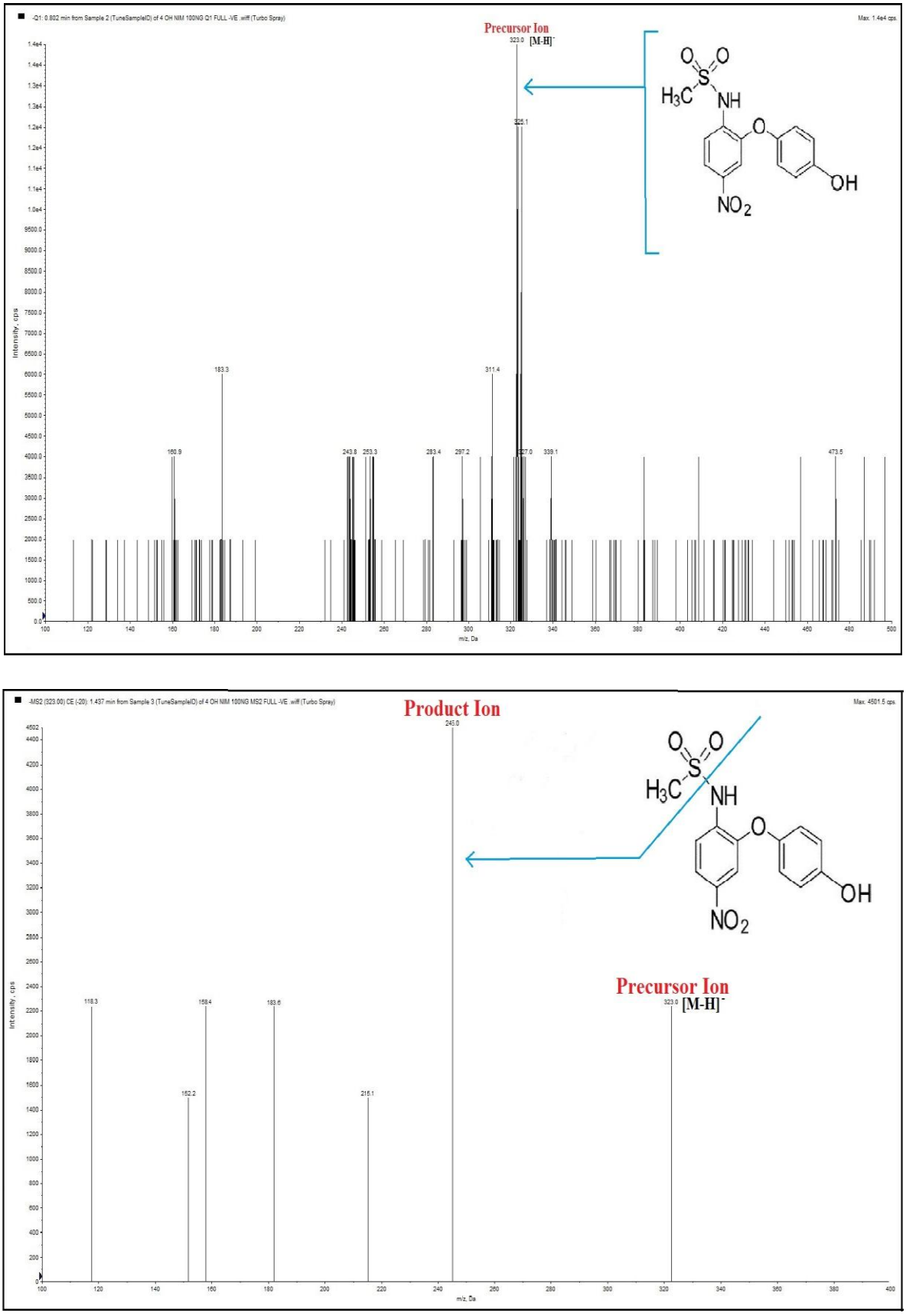
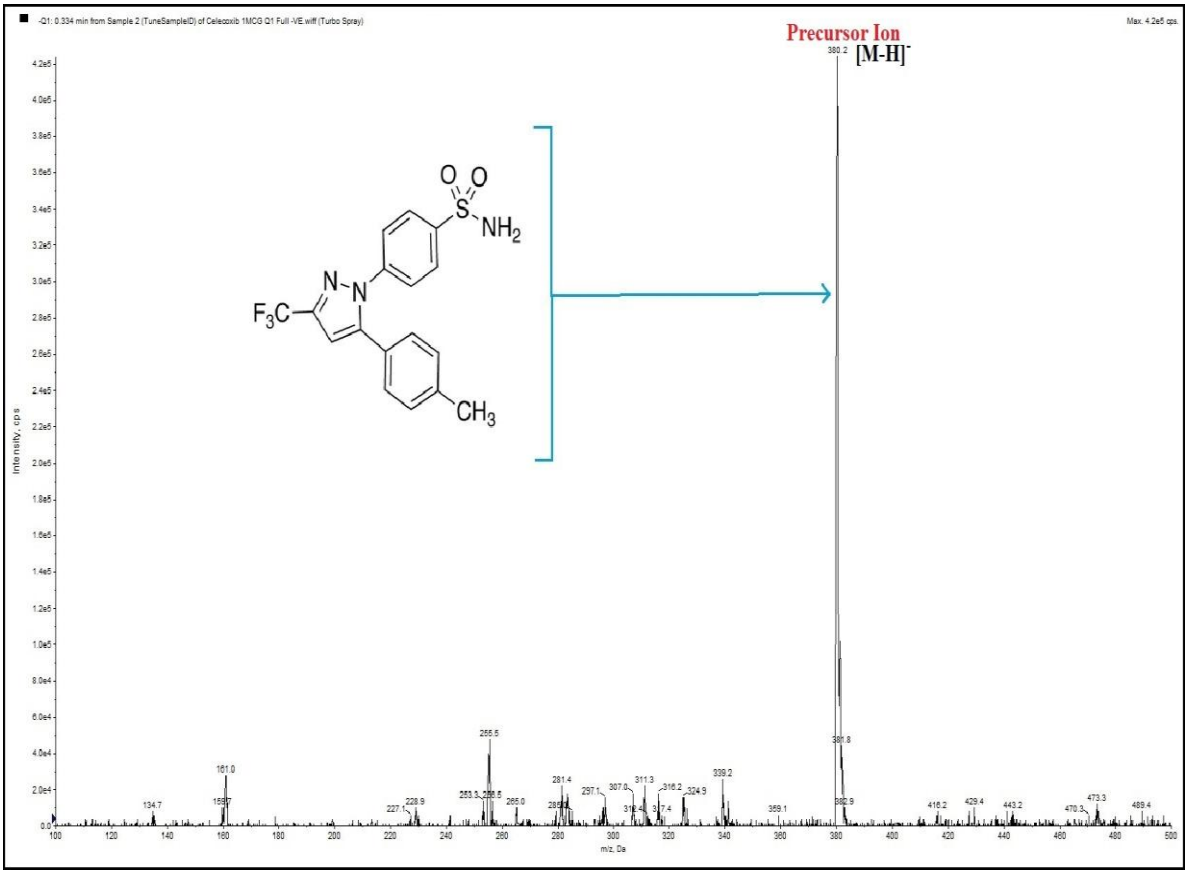


Figure.5.69 Mass spectra (Q1 and MS2) of the negative ion $[M-H]^-$ of 4-hydroxynimesulide (M1) with structural interpretation



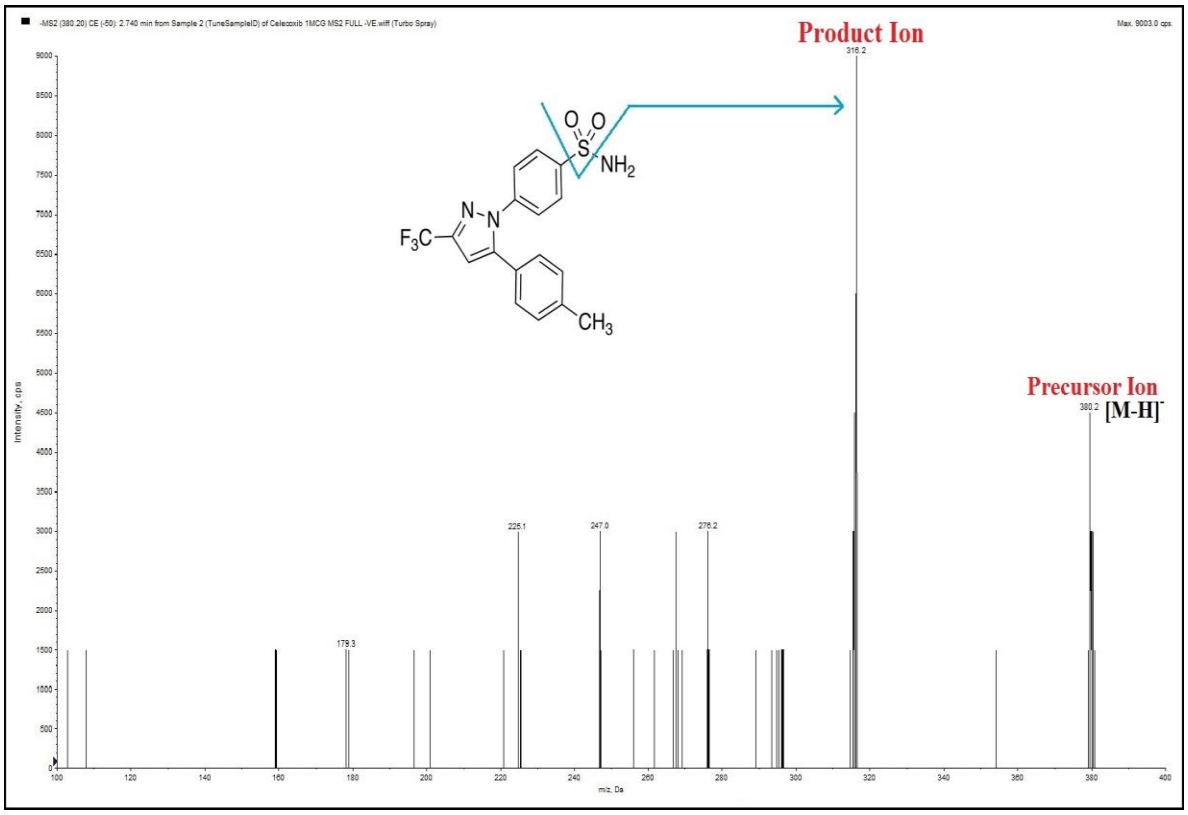


Figure.5.70 Mass spectra (Q1 and MS2) of the negative ion $[M-H]^-$ of celecoxib (CXB, IS) with structural interpretation

5.9.2 Selection of the mobile phase for NSD and M1

The composition of the mobile phase was selected and optimized by several trials to increase the signal of the analytes as well as to minimize running time of the analysis. So, different mobile phases with different solvents like methanol, acetonitrile and Milli-Q water (with 18.2 MΩ.cm resistivity, conductivity < 0.055 µS/cm) in different ratios consisting of either water : methanol or water : acetonitrile not only had been tried to maximize response of the analytes, to improve the peak shape but also for achieving appreciable LC separation. Normally methanol and acetonitrile were used as organic modifier and in case of buffers, MS suitable reagents or salts such as ammonium acetate and ammonium formate were used in different strengths like 2 mM, 5 mM and 10 mM, while MS suitable acids such as formic acid and acetic acid also in different strengths (i.e., 0.1% to 0.5%) were prepared in Milli-Q water and used. During selection of the mobile phase, several trials were performed with different mobile phases, such as methanol : 0.1% of formic acid in Milli-Q water :: 9:1 (v/v), methanol : 0.2% formic acid in Milli-Q water :: 9:1 (v/v), acetonitrile : 0.1% formic acid in Milli-Q water :: 9:1 (v/v), acetonitrile : 0.2% formic acid in Milli-Q water :: 9:1 (v/v), acetonitrile : 10 mM ammonium acetate in Milli-Q water :: 9:1 (v/v), acetonitrile : 5 mM ammonium acetate in Milli-Q water (pH 6.09) :: 9:1 (v/v), acetonitrile : 10 mM ammonium formate in Milli-Q water, acetonitrile : 5 mM ammonium formate in Milli-Q water (pH 3.2) :: 9:1 (v/v), maethanol : 10 mM ammonium acetate in Milli-Q water (pH 5.14) :: 9:1 (v/v), methanol : 5 mM ammonium acetate in Milli-Q water:: 9:1 (v/v), methanol : 10 mM ammonium formate in Milli-Q water and methanol : 5 mM ammonium formate in Milli-Q water :: 9:1 (v/v). Now, the best signal i.e., higher intensity, high resolution, better separation and symmetric peak shapes for the analytes were achieved by using acetonitrile : 5 mM ammonium formate in Milli-Q water (pH 3.2) :: 9:1 (v/v) at a flow rate of 0.4 mL/min (see Figure.5.71) and so this composition was selected as mobile phase for the analytes and IS of the entire bioanalysis.

5.9.3 Selection of the column (stationary phase) for NSD and M1

During method development process, trying out some columns of different polarity such as Agilent ZORBAX C18 [50 mm (length) x 4.6 mm (ID) with 5 µm particle size], Agilent ZORBAX Eclipse Plus C18 [75 mm (length) x 4.6 mm (ID), 3.5 µm particle size] and Agilent ZORBAX CN [50 mm (length) x 4.6 mm (ID) with 3.5 µm particle size] along with numerous combinations of mobile phases that already described in the section 5.9.2. This was performed to check the significant effects on separation, peak shape and response with better

selectivity and sensitivity for both the analytes and IS. It was observed that Agilent ZORBAX Eclipse Plus C18 column has more efficient with the selected mobile phase at a flow rate of 0.4 mL/min and has got more response, sensitivity, better resolution with symmetric peak shapes (Figure.5.71) compared to Agilent ZORBAX C18 and Agilent ZORBAX CN column. So therefore this Agilent ZORBAX Eclipse Plus C18 column was selected for the entire bioanalysis.

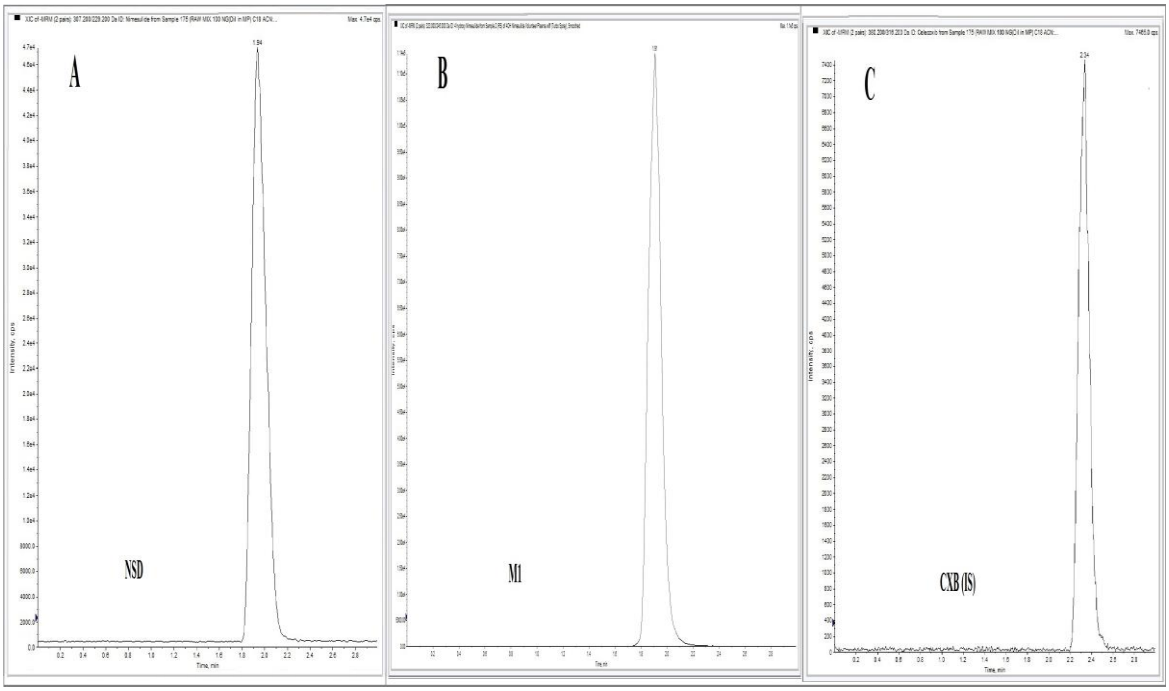


Figure.5.71 Representative chromatograms of A) NSD, B) M1 and C) CXB (IS) after selection of mobile phase and column

5.9.4 Selection of the internal standard (IS) for NSD and M1

At the time of method development, different compound like celecoxib, hydrochlorothiazide, letrozole, diclofenac, ibuprofen and prothionamide were examined for the selection of an appropriate internal standard (IS). Finally, Celecoxib (CXB) was confirmed as IS because of better response, rapid elution rate and good peak shape. It has also similar chromatographic and ionization properties with the analytes. CXB is coeluted properly in the selected mobile phase (acetonitrile : water containing 5 mM ammonium formate (pH 3.2) :: 9:1, v/v) and column (Agilent ZORBAX Eclipse Plus C18) at the flow rate of 0.4 mL/min and no interference was found with the performance of the developed method and the intensity of molecular ion peak in mass spectrometric analysis remained unaffected as compared with others. Also during the method development, extraction recovery of CXB in plasma was

better than the other tested compounds (see Table.5.102 and Figure.5.72) along with the analytes.

Table.5.103 Extraction recovery (%) of different drug molecules in plasma

Compound	Extraction recovery (%)
Celecoxib	83.33
Hydrochlorothiazide	81.64
Letrozole	78.66
Diclofenac	65.31
Ibuprofen	72.50
Prothionamide	79.28

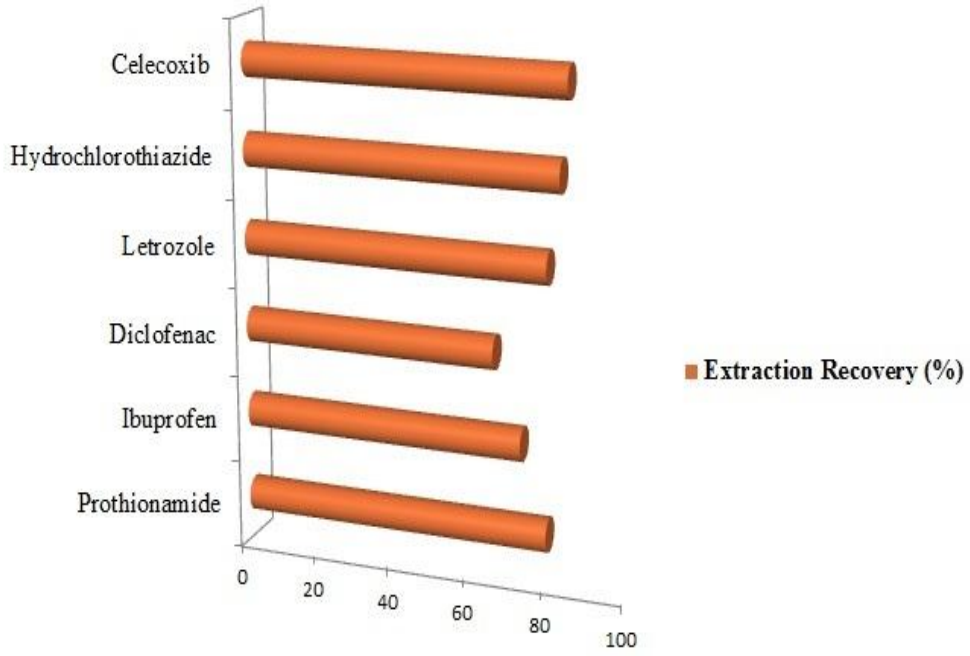


Figure.5.72 Extraction recoveries of different compounds in plasma

5.9.5 Selection of the diluent for NSD and M1

A proper diluent was confirmed for both NSD and M1 after the selection of mobile phase, column and IS during method development process. Methanol, acetonitrile, methanol :

acetonitrile :: 50:50 (v/v) and the mobile phase, i.e., acetonitrile : water containing 5 mM ammonium formate (pH 3.2) :: 9:1, (v/v) were tried as diluents to verify the response, sensitivity and peak shape. Lastly, the mobile phase was found better response, sensitivity and peak shape compared to the other diluents and all results are described in Table.5.104.

Table.5.104 Intensity (cps) of NSD, M1 and CXB (IS) in different diluents

Diluent	Intensity (cps)		
	NSD (conc. 1 µg/mL)	**M1 (conc. 1 µg/mL)**	**CXB (IS) (conc. 1 µg/mL)**
Methanol	35000 (3.5 e^4)	58000 (5.8 e^4)	9800
Acetonitrile	80000 (8.0 e^4)	120000 (1.2 e^5)	40000 (4.0 e^4)
Methanol : Acetonitrile :: 50:50 (v/v)	56000 (5.6 e^4)	72000 (7.2 e^4)	11300 (1.13 e^4)
Mobile Phase [acetonitrile : water containing 5 mM ammonium formate (pH 3.2) :: 9:1, (v/v)]	260000 (2.6 e^5)	140000 (1.4 e^5)	60000 (6.0 e^4)

5.9.6 Optimization of the final mobile phase with selected buffer and organic modifier for NSD and M1

After the selection of column and IS, optimization of the selected mobile phase was carried out. Now, composition and strength of the buffer i.e., ammonium formate, percentage (%) of acetonitrile (the organic modifier) and also pH of the buffer in mobile phase were optimized to enhance the response (cps) as well as to improve the peak shape of both the analytes and IS for better chromatographic results. Different compositions and strengths of the buffer ammonium formate in Milli-Q water were tested and differences in the intensity of NSD, M1 and IS (CXB) were observed (see Table.5.105). In Figure.5.73, a graphical representation express that the intensity (cps) of NSD, M1 and IS (CXB) varies in different buffer (ammonium formate) strengths like 2 mM, 5 mM and 10 mM. Among these strengths 5 mM ammonium formate was found to be optimum to maintain the pH throughout the bioanalysis, resulting in uniform peak shape along with consistency in ionization and fragmentation of the

analytes and IS which can be achieved only on desired solubilization of the molecules in the mobile phase.

Table.5.105 Effect of buffer composition and strength of the mobile phase on the intensity (cps) of the analytes and IS

Buffer composition (Strength of ammonium formate in Milli-Q water)	**Intensity (cps)**		
	NSD (conc. 1 µg/mL)	**M1 (conc. 1 µg/mL)**	**CXB (IS) (conc. 1 µg/mL)**
2 mM	130000 (1.3 e^5)	95000 (9.5 e^4)	36000 (3.6 e^4)
5 mM	260000 (2.6 e^5)	140000 (1.4 e^5)	60000 (6.0 e^4)
10 mM	220000 (2.2 e^5)	110000 (1.1 e^5)	53000 (5.0 e^4)

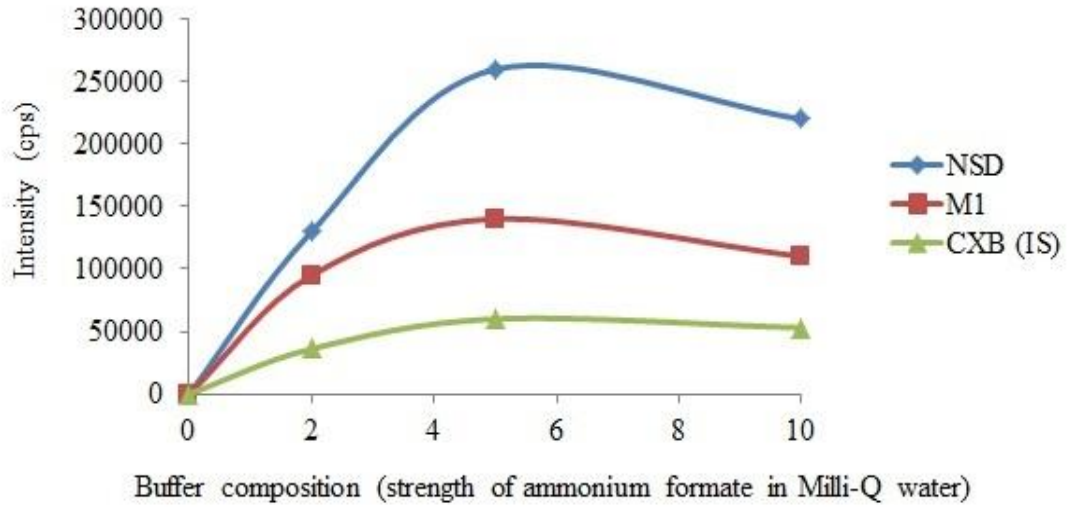


Figure.5.73 Effect of buffer composition on the intensity (cps) of NSD, M1 and CXB (IS)

Next, optimization of the organic modifier i.e., percentage (%) of acetonitrile was done because it is also a key part of the mobile phase. So, the different percentage of acetonitrile were tried in the mobile phase to improve the peak shape and enhanced the response, i.e., intensity (cps) of both the analytes and IS (see Table.5.106). Now, it is clearly understand

from the graph (Figure.5.74) that the intensity (cps) of both the analytes and IS increases by the increase of acetonitrile percentage (%) like 65% to 90% in the mobile phase. The maximum intensity (response), resolution and better peak shape were found in 90% of acetonitrile. Actually, high percentage (%) of organic modifier (acetonitrile) in bioanalytical LC-MS/MS method is required for good sharp peak, better resolution and also to get the shorter retention time (RT) as well as total chromatographic run time which is comparatively much better than HPLC method. So, the high proportion of organic solvent eluted NSD, M1 and IS (CXB) at retention times of 1.97, 1.90 and 2.32 minutes, respectively with a flow rate of 0.4 mL/min generated better peak shapes and a total chromatographic run time of 3 minutes.

Table.5.106 Effect of acetonitrile composition of the mobile phase on the intensity (cps) of the analytes and IS

Percentage (%) of acetonitrile as the organic modifier	**Intensity (cps)**		
	NSD (conc. 1 µg/mL)	**M1 (conc. 1 µg/mL)**	**CXB (IS) (conc. 1 µg/mL)**
65	63000 (6.3 e4)	45000 (4.5 e4)	11000 (1.1 e4)
70	87000 (8.7 e4)	69000 (6.9 e4)	34000 (3.4 e4)
80	120000 (1.2 e5)	98000 (9.8 e4)	51000 (5.1 e4)
90	260000 (2.6 e^5)	140000 (1.4 e^5)	60000 (6.0 e^4)

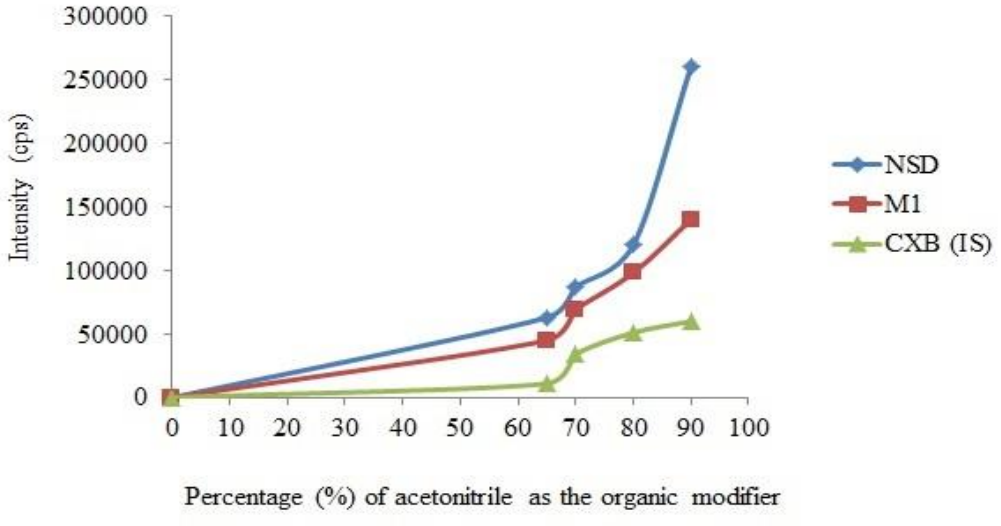


Figure.5.74 Effect of acetonitrile composition on the intensity (cps) of NSD, M1 and CXB (IS)

Lastly, pH of the buffer, i.e., 5 mM ammonium formate in Milli-Q water was optimized. Now, pka value of NSD and IS (CXB) are 6.5 and 11.1, respectively. By trial and error method, pH was decreased by adding formic acid (conjugate acid) and increased by adding ammonia solution or ammonium hydroxide (conjugate base) in the buffer to find out the optimum pH value. It was observed that at pH 3.2, the response (intensity) and the peak shape of the analytes and IS were better than others (see Table.5.107 and Figure.5.75). So therefore, acetonitrile and water containing 5 mM ammonium formate with pH of 3.2 (9:1, v/v) was considered as an appropriate mobile phase of the LC-ESI-MS/MS method for NSD and M1.

Table.5.107 Effect of pH of the buffer on the intensity (cps) of the analytes and IS

pH of the buffer	**Intensity (cps)**		
	NSD (conc. 1 µg/mL)	**M1 (conc. 1 µg/mL)**	**CXB (IS) (conc. 1 µg/mL)**
3.2	260000 (2.6 e^5)	140000 (1.4 e^5)	60000 (6.0 e^4)
4.8	100000 (1.0 e5)	97000 (9.7 e4)	46000 (4.6 e4)
6.7	53000 (5.3 e4)	45000 (4.5 e4)	21000 (2.1 e4)

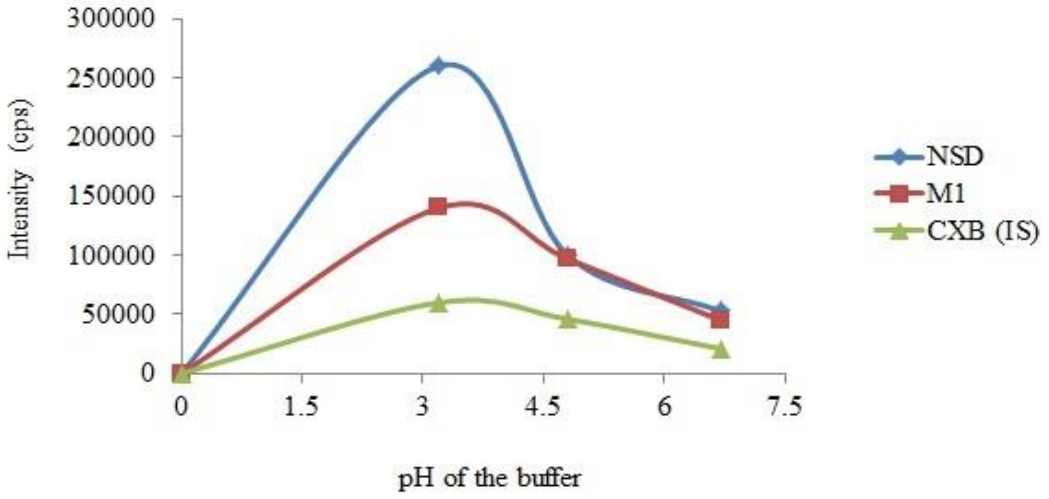


Figure.5.75 Effect of pH of the buffer on the intensity (cps) of NSD, M1 and CXB (IS)

5.9.7 Optimization of the flow rate for NSD and M1

During method development, different flow rates of the mobile phase were tested to confirm the suitable one for present method. Because, it was one of the crucial factor to get better result, proper ionization in ESI, more sensitivity, proper retention time (RT) and also total chromatographic run time. It was noticed that on increasing the flow rate the response (intensity) was increased and reached into the optimum level and then decreased, whereas RT and total chromatographic run time were shorter. Now, mass tuning flow rate was 10 µL/min during MS/MS development in Harvard syringe pump method. In LC-MS/MS development, the MS was coupled with the LC at a flow rate of 0.4 mL/min (400 µL/min) found maximum response and proper RT with shorter run time.

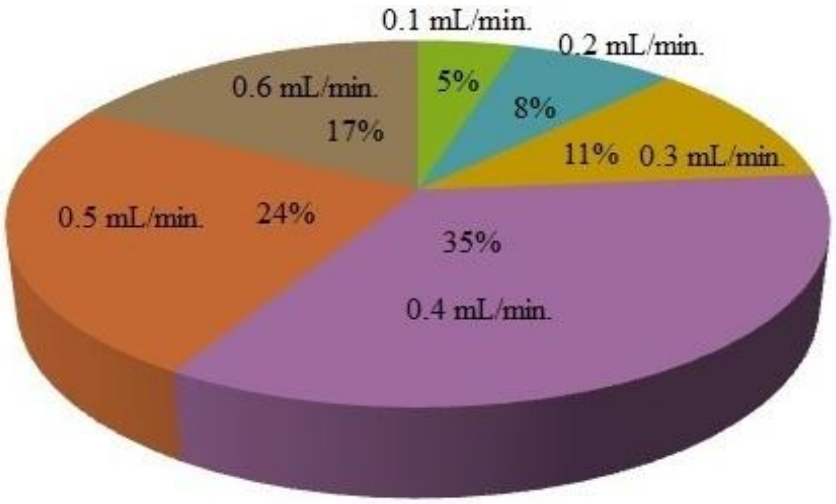


Figure.5.76 An impact of different flow rate on the intensity (cps) of NSD, M1 and CXB (IS)

Graphical presentation had been established to described that how the flow rate influenced on response or intensity of NSD, M1 and IS (CXB) molecules (see Figure.5.76 and Figure.5.77). Finally after some trial and error, a suitable flow rate was 0.4 mL/min which continued throughout the entire bioanalysis.

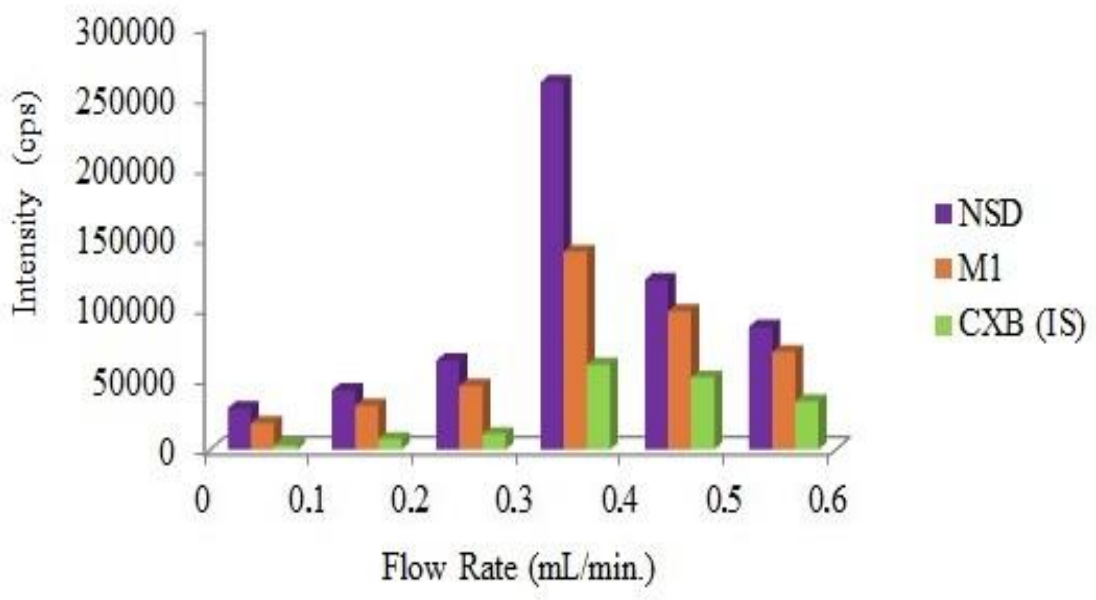


Figure.5.77 Effect of different flow rate on the intensity (cps) of NSD, M1 and CXB (IS)

5.9.8 Optimization of the mass spectrometric (MS) parameters for NSD, M1 and CXB (IS)

An experimental setup was processed after selection and optimization of the final mobile phase to determine the optimal MS parameters, i.e., compound dependent parameters as well as source and gas parameters by flow injection analysis (FIA) with the method of compound optimization in tune mode in the negative polarity of the software, Analyst 1.5. In FIA, the range of all desired parameters were manually inputted and got the final optimized results for the analytes (NSD and M1) and IS (CXB) which have been described in Figure.5.78 to Figure.5.85 and Table.5.108 and Table.5.109.

FIA Compound Optimization Results -

Compound-Dependent Parameters :

Declustering Potential (DP)

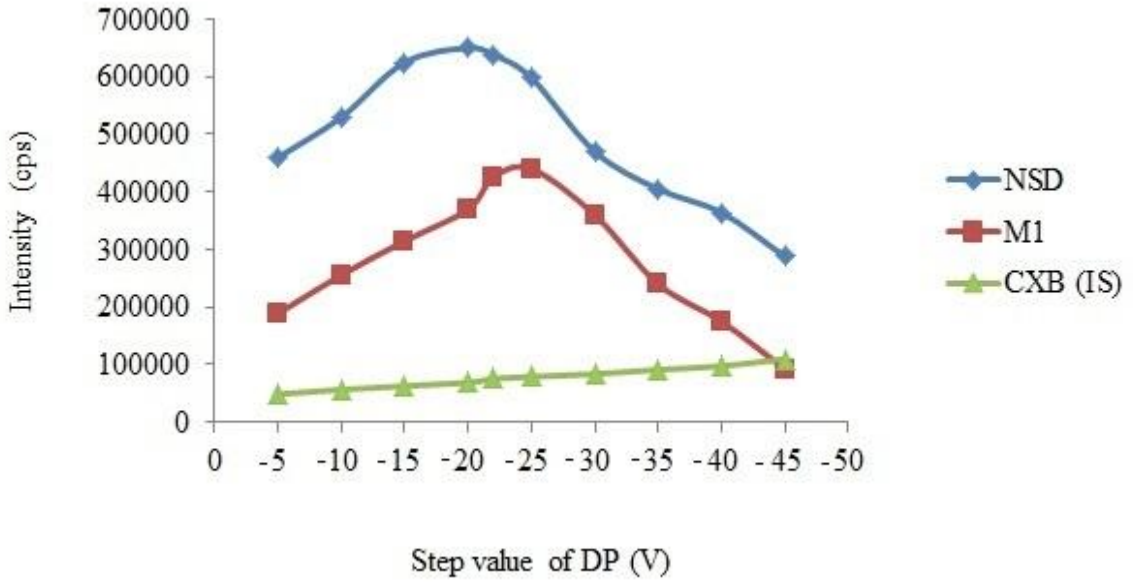


Figure.5.78 Optimization of declustering potential (DP) value for NSD, M1 and CXB (IS)

Focusing Potential (FP)

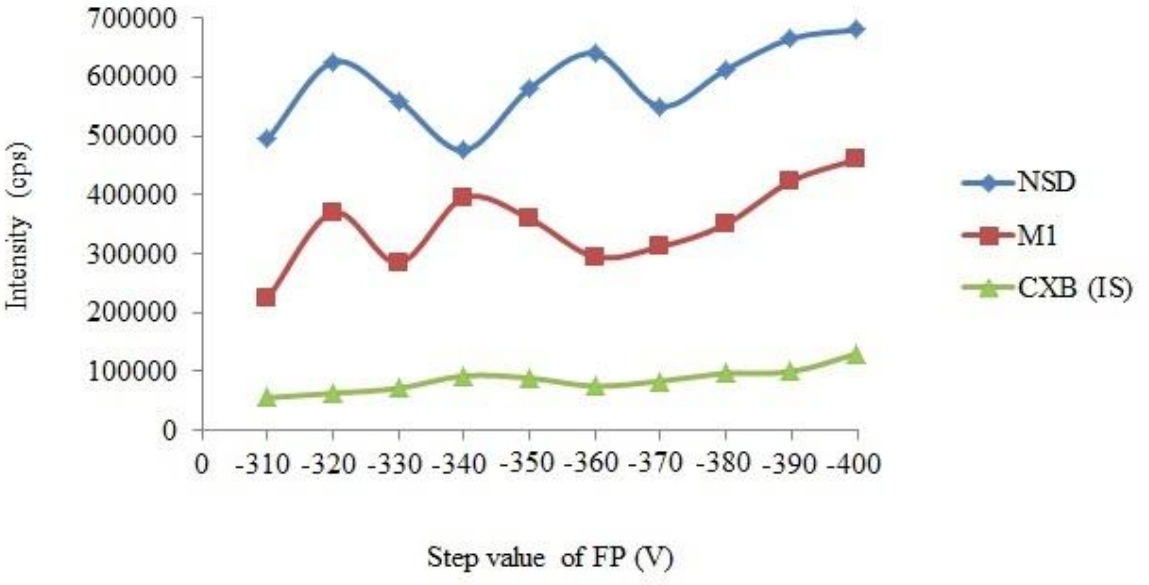


Figure.5.79 Optimization of focusing potential (FP) value for NSD, M1 and CXB (IS)

Entrance Potential (EP)

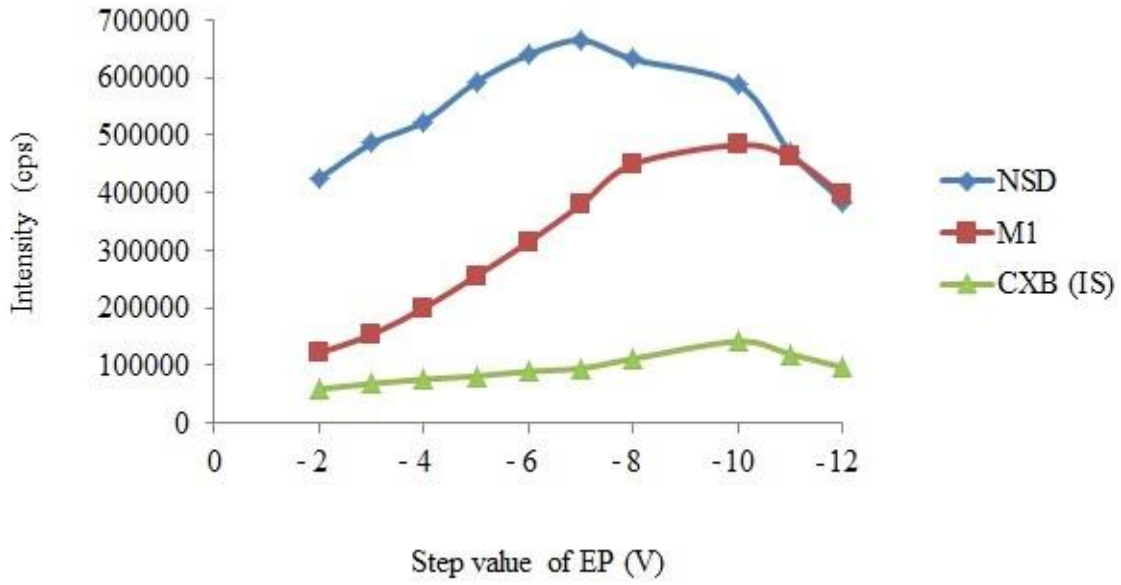


Figure.5.80 Optimization of entrance potential (EP) value for NSD, M1 and CXB (IS)

Collision Cell Entrance Potential (CEP)

Normally, collision cell entrance potential (CEP) was setting up as by default in the software. So, during manual tuning it was not needed to optimize. Whereas in FIA it could be optimized and therefore it was successfully worked out.

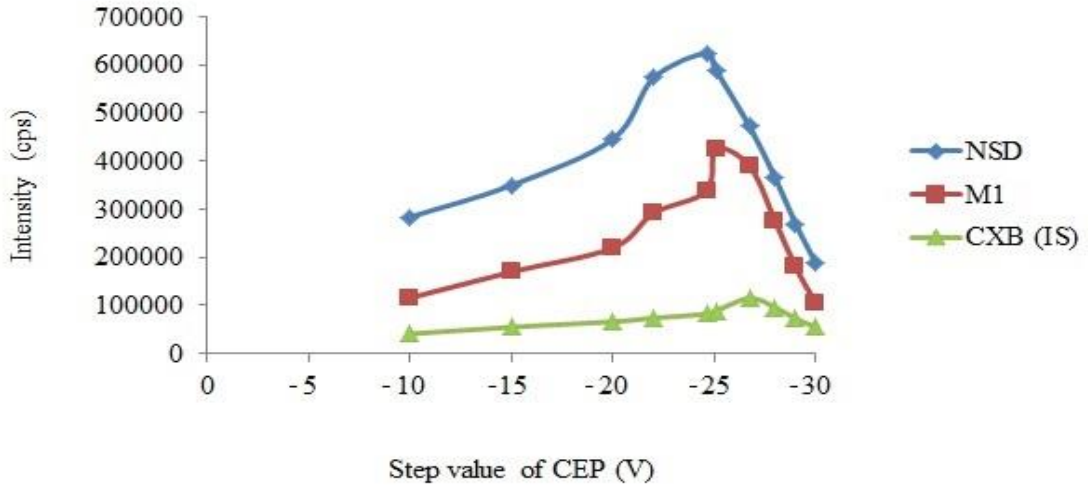


Figure.5.81 Optimization of collision cell entrance potential (CEP) value for NSD, M1 and CXB (IS)

Collision Energy (CE)

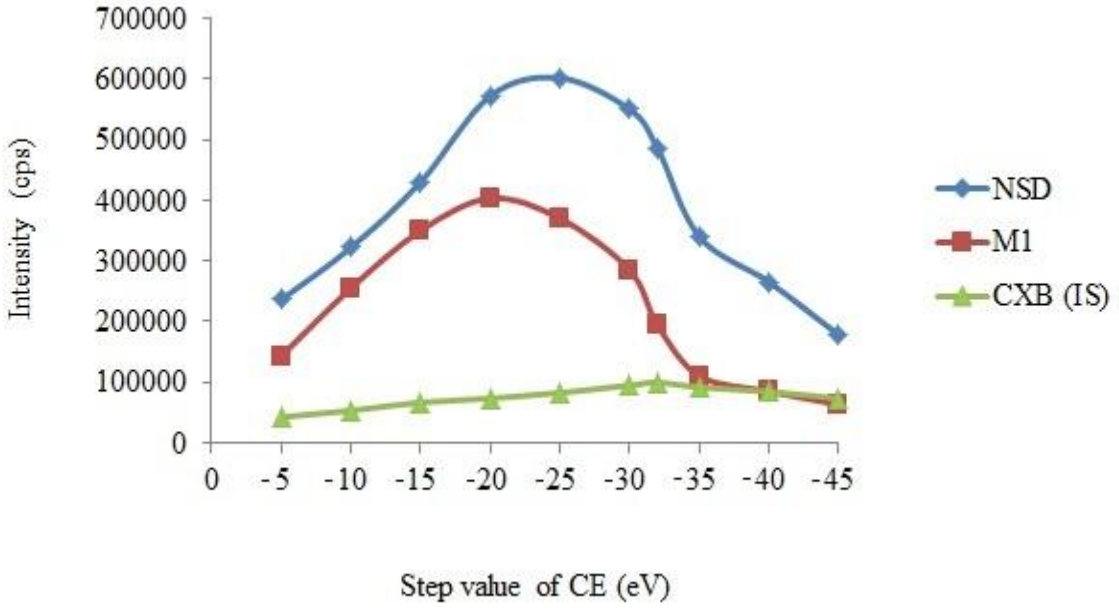


Figure.5.82 Optimization of collision energy (CE) value for NSD, M1 and CXB (IS)

Collision Cell Exit Potential (CXP)

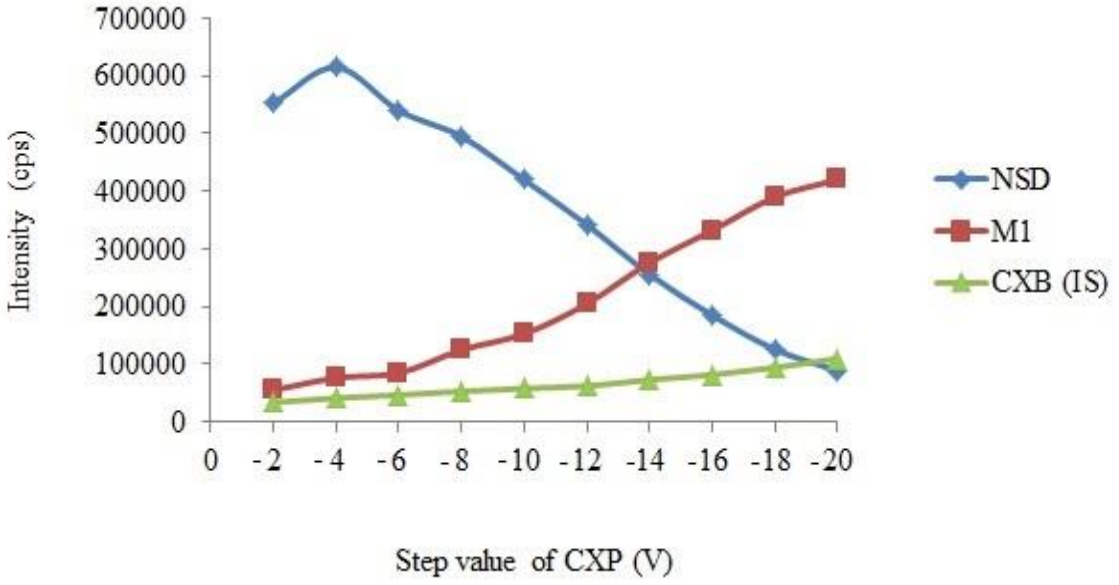


Figure.5.83 Optimization of collision cell exit potential (CXP) value for NSD, M1 and CXB (IS)

Table.5.108 Optimized compound-dependent parameters of NSD, M1 and CXB (IS)

Compound-dependent parameters	NSD	M1	CXB (IS)
DP (V)	-20.00	-25.00	-45.00
FP (V)	-400.00	-400.00	-400.00
EP (V)	-7.00	-10.00	-10.00
CEP (V)	-24.61	-25.07	-26.73
CE (eV)	-25.00	-20.00	-32.00
CXP (V)	-4.00	-20.00	-20.00

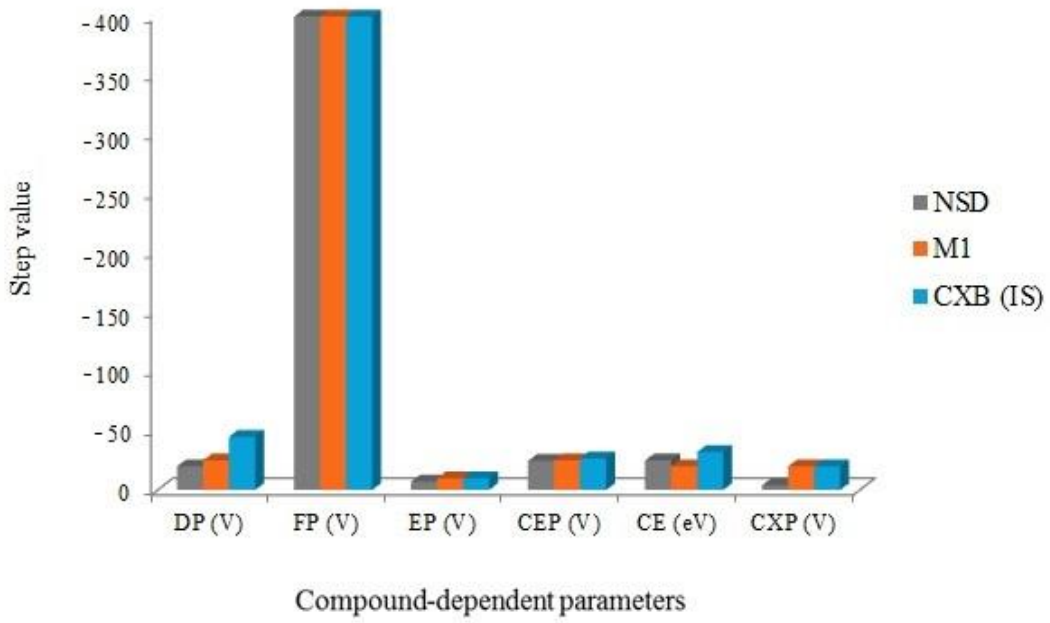


Figure.5.84 Optimized compound-dependent parameters for NSD, M1 and CXB (IS)

Source and Gas Parameters :

The source and gas parameters were curtain (CUR) gas, collision activated dissociation (CAD), turbo ion spray voltage (ISV), turbo ion spray source temperature or turbo gas temperature (TEM), nebulizer gas (GS1) and turbo ionspray gas or auxiliary gas or desolvation gas (GS2) i.e., heater gas (see Figure.5.85 and Table.5.109).

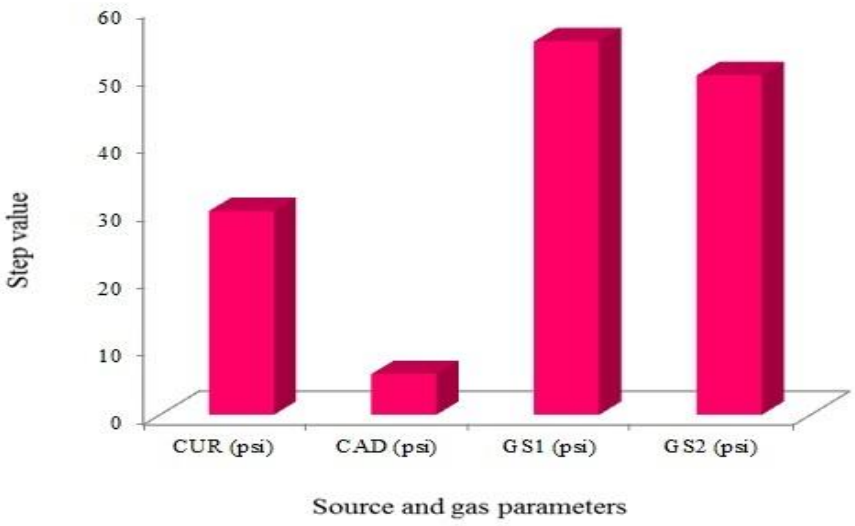


Figure.5.85 Optimized source and gas parameters of NSD, M1 and CXB (IS)

Table.5.109 Optimized source and gas parameters of NSD, M1 and CXB (IS)

Source and gas parameters	
CUR (psi)	30.0
CAD (psi)	6.0
ISV (V)	-4500.0
TEM (^{o}C)	400.0
GS1 (psi)	55.0
GS2 (psi)	50.0

5.9.9 Selection of extraction solvent and procedure for NSD and M1 from human plasma

A liquid-liquid extraction (LLE) technique was selected as the sample preparation and extraction of both NSD and M1 and also IS (CXB) from the human plasma because of the increase of response or intensity and the extraction recovery. Therefore, the main reason was to get simple process, more and better extraction recovery, easily available etc. Also it raises the column life due to the cleanness of extracted samples or the supernatant (organic layer) than that of the protein precipitation technique (PPT). Now, LLE is a cost effective or low cost technique compared to both the solid phase extraction (SPE) and 96 well SPE technique.

During method development, various organic solvents were tried just like trial and error such as ethyl acetate, dichloromethane, chloroform, diethyl ether, methyl tert-butyl ether and n-Hexane to investigate for optimum response, higher and consistent recovery and also for better peak shape of both the analytes (NSD and M1) along with IS (CXB). The process of sample preparation were described previously in the section 4.9.3 and were assessed the extraction yields of the analytes and IS (Table.5.110). Extraction recovery (%) as well as peak shape, resolution and reproducibility of both NSD and M1 were comparatively much better in dichloromethane, chloroform, diethyl ether, methyl tert-butyl ether than ethyl acetate and n-Hexane. Whereas in case of IS (CXB), it was far better in ethyl acetate, dichloromethane and diethyl ether than chloroform, methyl tert-butyl ether and n-Hexane. So according to the overall results (see Table.5.110 and Figure.5.86 and 5.87), it has very clear that dichloromethane was the proper extracting solvent for the present LLE technique to extract NSD, M1 and IS (CXB) from plasma.

Table.5.110 Extraction recovery (%) of NSD, M1 and CXB (IS) from plasma by various organic solvents

Extracting solvents	Extraction recovery (%)		
	NSD	M1	CXB (IS)
Ethyl acetate (EA)	61.02	54.89	82.14
Dichloromethane (DCM)	96.15	92.86	86.67
Chloroform (CHL)	80.77	84.23	36.67
Diethyl ether (DEE)	90.33	92.75	79.56
Methyl tert-butyl ether (MTBE)	88.27	89.44	59.62
n-Hexane	50.78	52.33	47.12

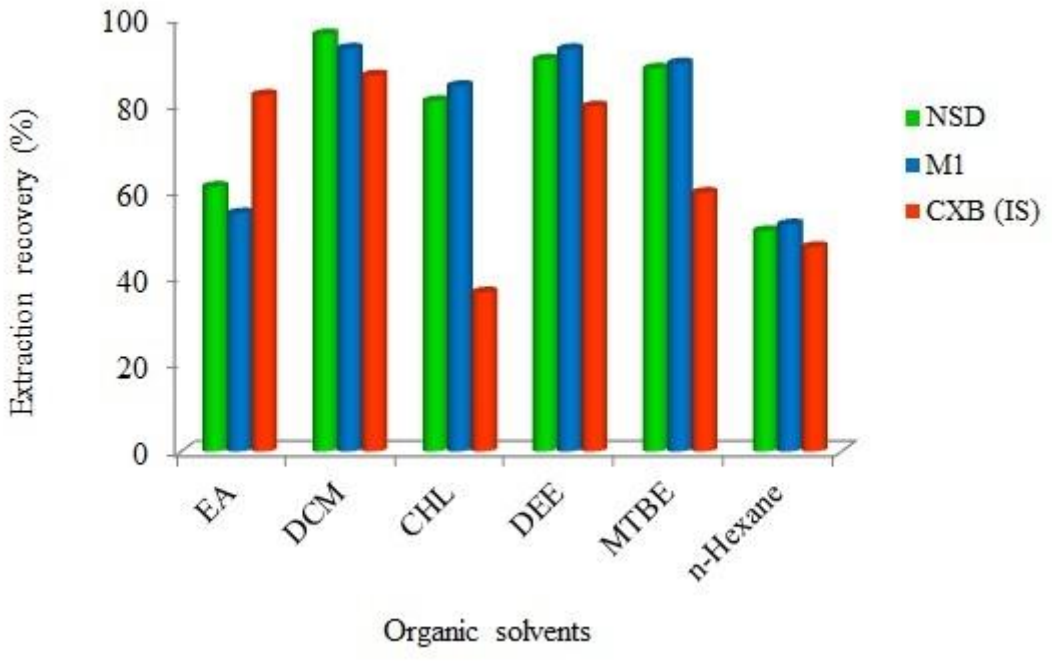
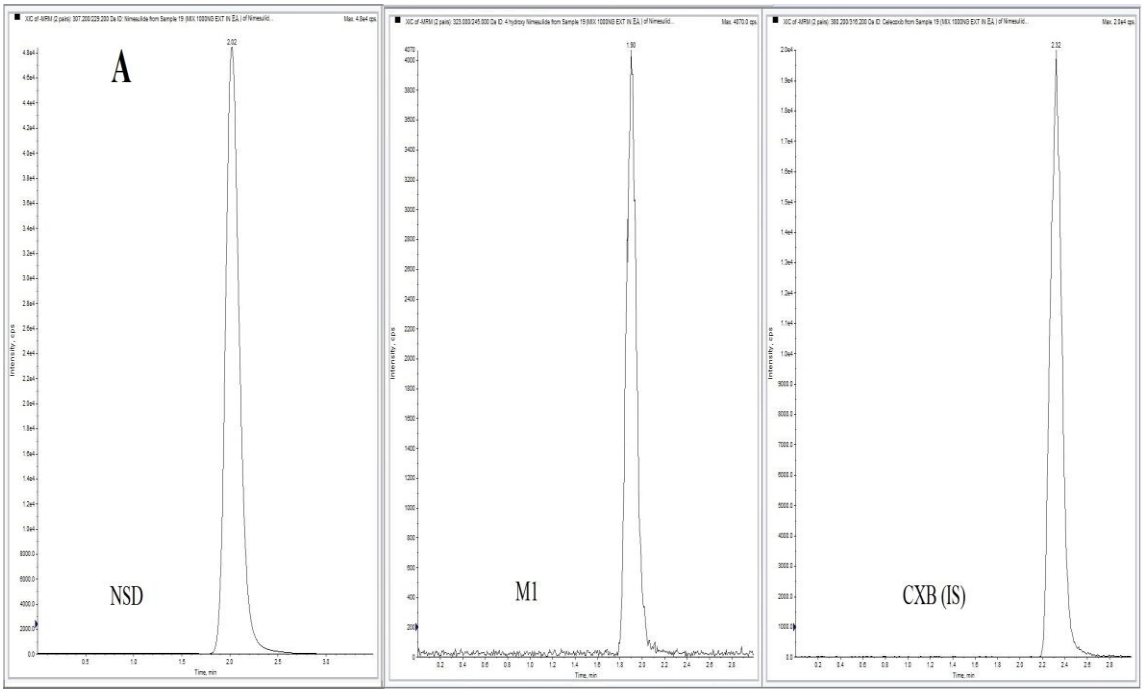
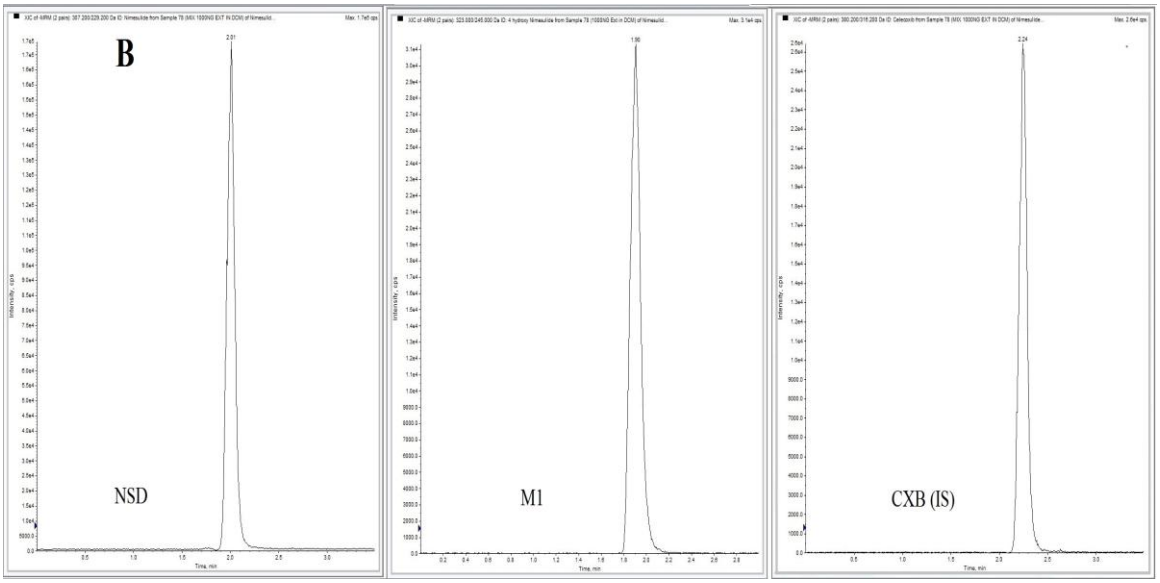
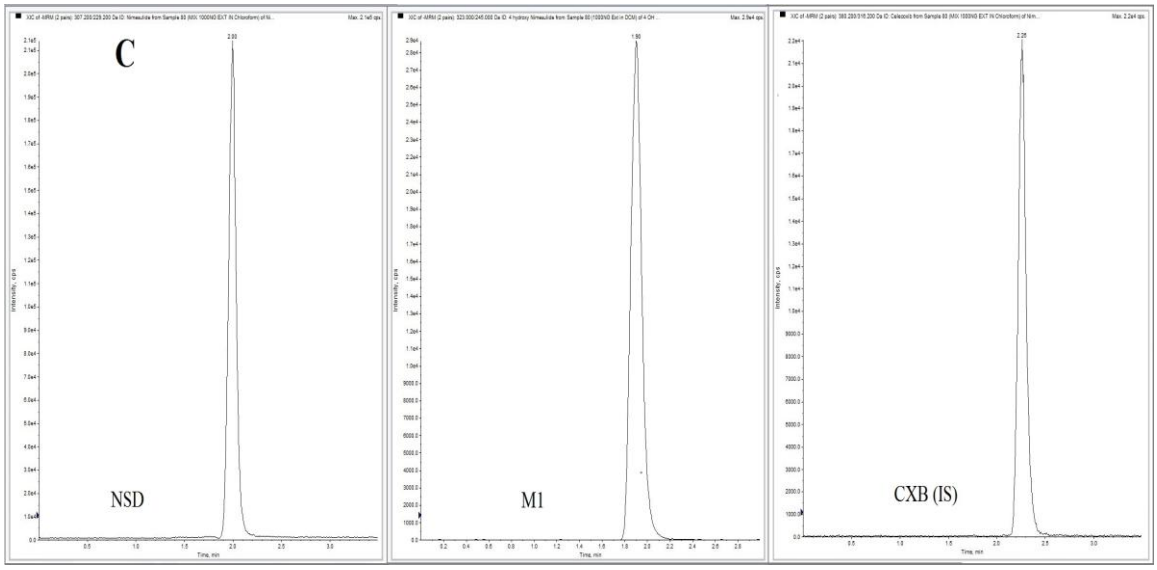
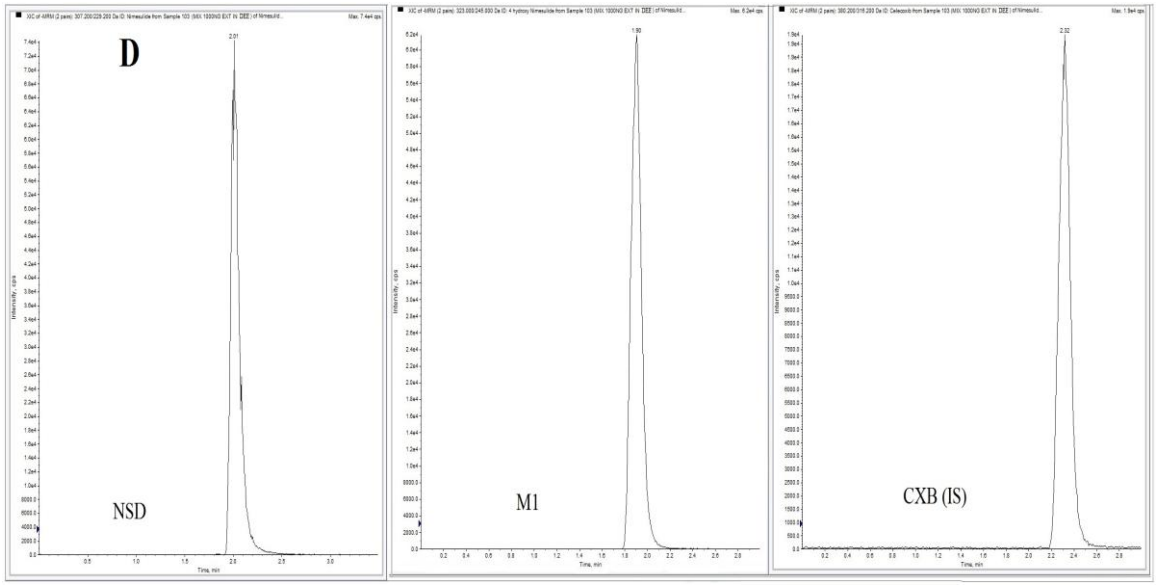


Figure.5.86 The extraction recovery (%) of NSD, M1 and CXB (IS) after treating with different organic solvents




B
NSD
M1
CXB (IS)


C
NSD
M1
CXB (IS)


D
NSD
M1
CXB (IS)

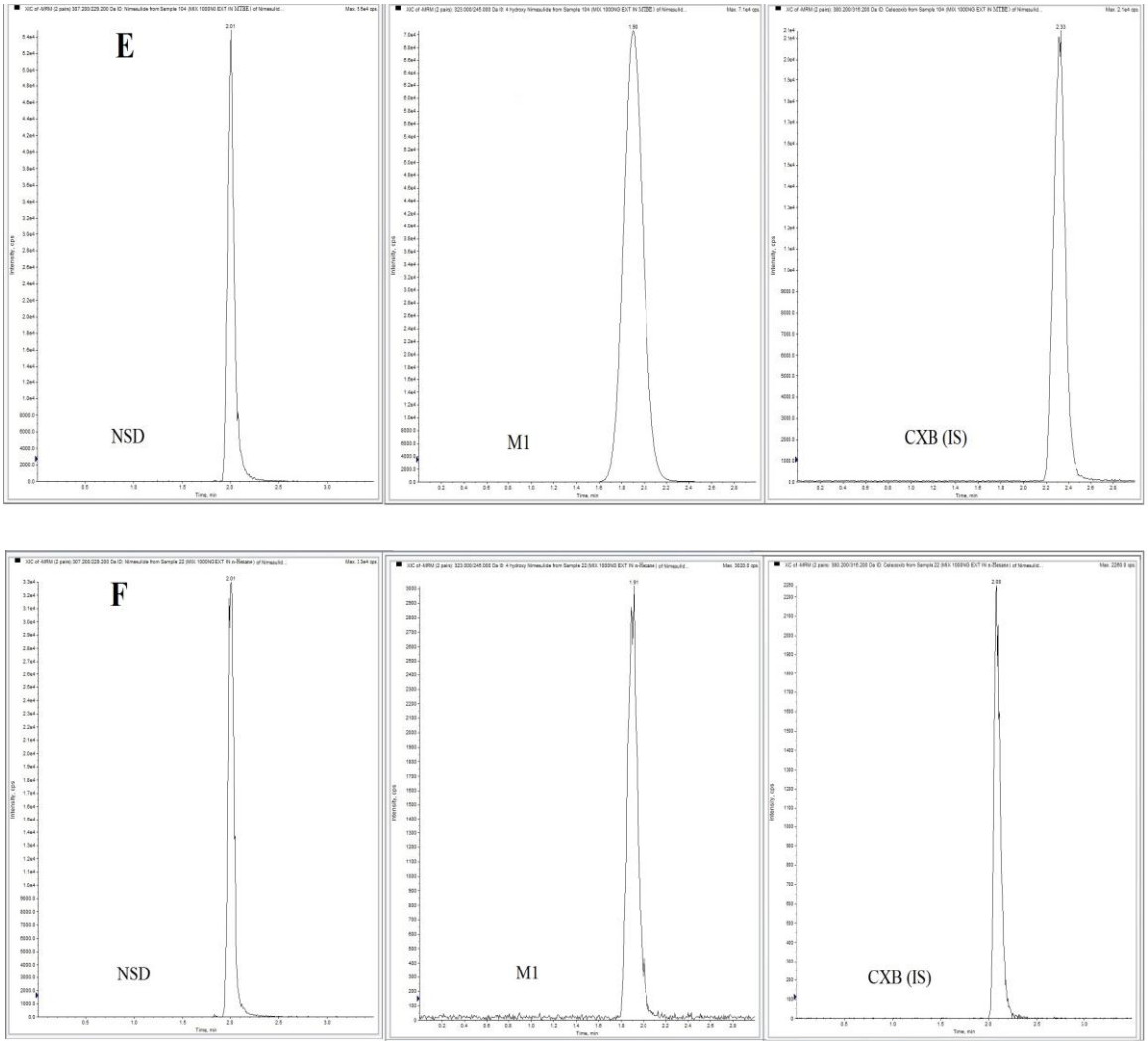


Figure.5.87 The intensity (cps) and peak shape of NSD, M1 and CXB (IS) using A) EA, B) DCM, C) CHL, D) DEE, E) MTBE and F) n-Hexane

5.9.10 Final optimized plasma extraction procedure for NSD and M1

At first, 100 µL volume of human plasma sample was taken in 15 mL centrifuge tubes and 10 µL of IS was added from 50 µg/mL of CXB working standard solution in each and every tubes. Whereas, for calibration concentration (CC) and quality control (QC) samples it was 90 µL of human blank plasma or drug-free plasma (i.e., pre-dose plasma), 10 µL of working standard solution of both drug and metabolite concentration and also 10 µL of IS solution. After that, vortex mixed was done for 30 seconds and then upto 3 mL of dichloromethane was added to all tubes and hand mixing was performed for 10 minutes followed by centrifugation for another 10 minutes at 5000 rpm. Next after centrifugation, a clear supernatant or an organic layer was formed and 2 mL of organic layer was separated by pipetting and transferred to clean centrifuge tubes. The organic layer was completely

evaporated under nitrogen (N2) atmosphere at 40 to 45 ºC temperature and the remaining dry residues were collected and then reconstituted with the 400 µL of mobile phase followed by vortex mixed for 3 to 4 minutes. Lastly, the reconstituted samples were transferred to autosampler vials and then the prepared samples were ready for run in the LC-MS/MS system.

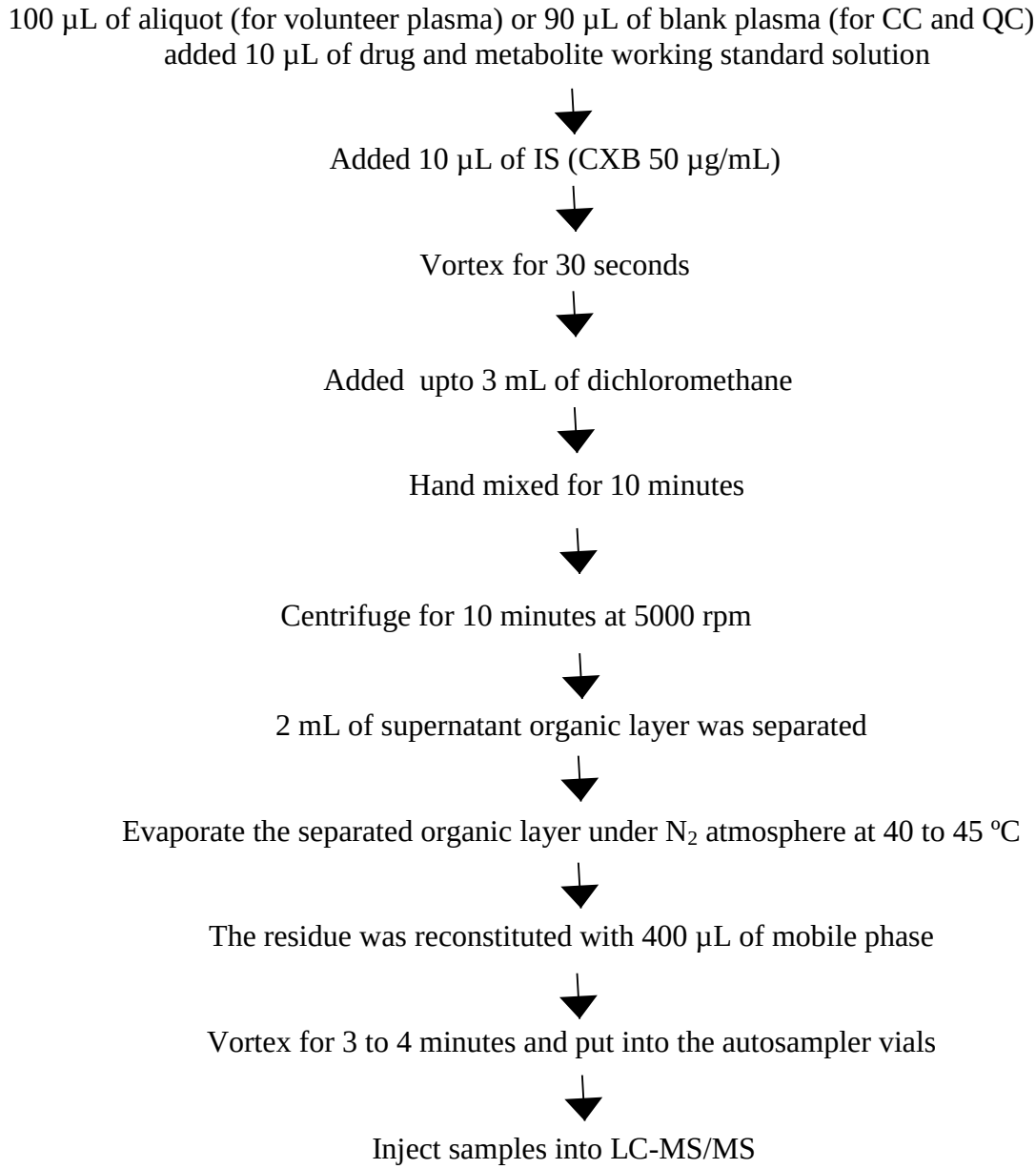


Figure.5.88 A schematic presentation of final optimized plasma extraction procedure of NSD and M1

5.9.11 Final optimized chromatographic conditions for bioanalysis of NSD and M1 in human plasma

5.9.11.1 Chromatographic parameters

Integrated System : Shimadzu Controller prominence, autosampler SIL-20AC, CBM-20A Lite System Control Compartment, binary pump : pump A and pump B LC-20AD, online solvent degasser DGU- 20A3, column oven CTO-10 AS VP and liquid chromatography.

Column	Agilent ZORBAX Eclipse Plus C18, 75 mm x 4.6 mm (length x inner diameter), 3.5 µm particle size
Mobile Phase	Acetonitrile : 5 mM ammonium formate in Milli-Q water (pH 3.2) :: 9 : 1 (v/v)
Flow Rate	0.4 mL/min.
Injection Volume	10 µL
Column Oven Temperature	Not Applicable
Autosampler Temperature	15 °C
Total Chromatographic Run Time	3.0 minutes

Shimadzu LC Method Properties

Shimadzu LC system Equlibration time = 0.00 min

Shimadzu LC system Injection Volume = 10.00 µL

Shimadzu LC Method Parameters

Pumps

Pump A Model: LC-20AD

Pump B Model: LC-20AD

Pumping Mode: Binary Flow

Total Flow: 0.4000 mL/min.

Pump B Pct: 90.0 %

B Curve: 0

Pressure Range (Pump A/B): 0.0 – 40.0 MPa

Autosampler

Model: SIL-20AC

Rinsing Volume: 200 µL

Needle Stroke: 52 mm.

Rinsing Speed: 35 µL/sec.

Sampling Speed: 15.0 µL/sec.

Purge Time: 25.0 min.

Rinse Dip Time: 0 sec.

Rinse Mode: Before and after aspiration

Cooler Enabled: Yes

Cooler Temperature: 15 deg. C

Control Vial Needle Stroke: 52 mm

Oven

Model: CTO-10ASvp

Temperature Control: Disabled

System Controller

Model: CBM-20A Lite

Power: On

Event 1: Off

Event 2: Off

Event 3: Off

Event 4: Off

Time Program

Time	Module	Events	Parameter
0.01	Pumps	Pump B Conc.	90
3.01	System Controller	Stop	

5.9.11.2 Mass spectrometric parameters

Quantitation Information:

Sample Type: Unknown

Dilution Factor: 1.000000

Custom Data:

Quantitation Table:

Period 1:

Scans in Period: 439

Relative Start Time: 0.00 msec

Experiments in Period: 1

Period 1 Experiment 1:

Scan Type: MRM (MRM)

Scheduled MRM: No

Polarity: Negative

Scan Mode: N/A

Ion Source: Turbo Spray

Resolution Q1: Unit

Resolution Q3: Unit

Intensity Thres.: 0.00 cps

Settling Time: 0.0000 msec

MR Pause: 5.0070 msec

MCA: No

Step Size: 0.00 Da

Nimesulide (NSD)

Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Parameter	Volt
307.20	229.20	200.00	DP	-20.00
			EP	-7.00
			CEP	-24.61
			CE	-25.00
			CXP	-4.00

4-hydroxynimesulide (M1)

Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Parameter	Volt
323.00	245.00	200.00	DP	-25.00
			EP	-7.00
			CEP	-24.61
			CE	-25.00
			CXP	-4.00

Celecoxib (CXB, IS)

Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Parameter	Volt
380.20	316.20	200.00	DP	-45.00
			EP	-10.00
			CEP	-26.73
			CE	-32.00
			CXP	-20.00

Parameter Table (Period 1 Experiment 1)

CUR:	30.00
CAD:	6.00
IS:	-4500.00
TEM:	400.00
GS1:	55.00
GS2:	50.00
ihe:	ON
FP	-400.00

Instrument Parameters:

Detector Parameters (Negative):

DF	150.0
CEM	2400.0

Keyed Text:

File was created with the software version: Analyst 1.5

5.9.12 Estimation of limit of detection (LOD) and lower limit of quantification (LLOQ) for NSD and M1

The LOD of both NSD and M1 was found 3 ng/mL where signal-to-noise ratio was 3 times and the LLOQ was 10 ng/mL where signal-to-noise ratio was 5 times (Figure.5.89).

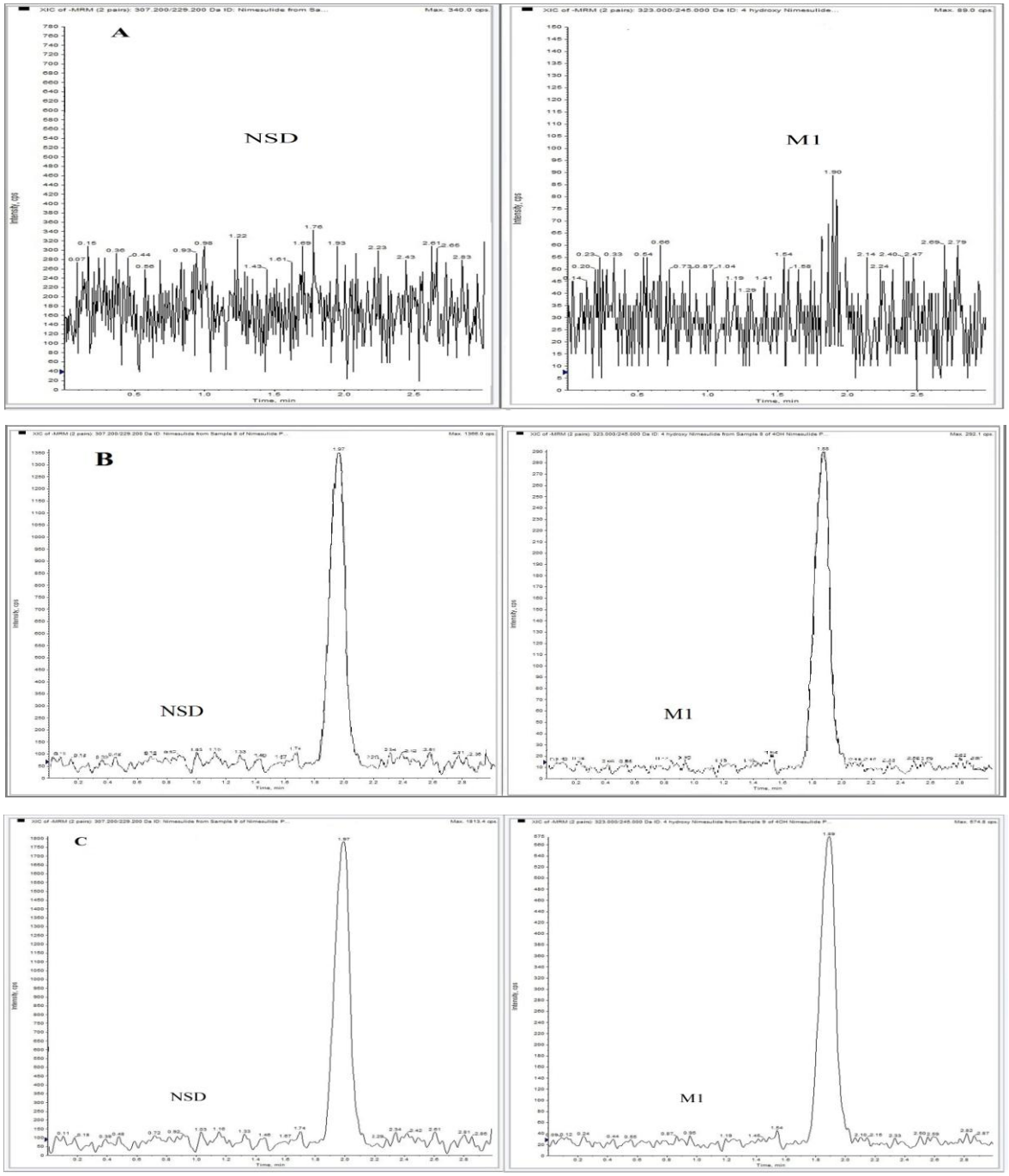


Figure.5.89 Chromatograms for determination of LOD and LLOQ of NSD and M1 : A) chromatogram of blank plasma, B) chromatogram of LOD and C) chromatogram of LLOQ

5.10 Pre-validation study of bioanalytical method for NSD and M1

5.10.1 System suitability test (SST) for NSD and M1 LC-MS/MS method

The system suitability test (SST) was evaluated by replicate (n=6) injection of the same reference standard (RS) solution containing NSD, M1 and IS (CXB) at the concentration of 500 ng/mL. During sample run or at the beginning of bioanalysis, this mixed solution (i.e.,

RS 500 ng/mL) was injected. Hence, it was proved from the Table.5.111 that the low RSD% (CV%) of NSD, M1 and CXB (IS) peak areas and retention times (RTs) were within or below 2% as per the guidelines. Therefore, it was established that the system was reproducible for the present analytical run and working or functioning properly.

Table.5.111 System suitability test (n=6) of NSD, M1 and CXB (IS) during sample run

No. of injection	NSD (conc. 500 ng/mL)		M1 (conc. 500 ng/mL)		CXB (IS, conc. 500ng/mL)	
	Peak area	RT (min.)	Peak area	RT (min.)	Peak area	RT (min.)
1	4.36E+05	1.95	7.28E+05	1.89	4.17E+04	2.34
2	4.35E+05	1.96	7.27E+05	1.90	4.16E+04	2.33
3	4.27E+05	1.98	7.29E+05	1.90	4.19E+04	2.34
4	4.33E+05	1.97	7.27E+05	1.89	4.19E+04	2.34
5	4.30E+05	1.97	7.37E+05	1.90	4.18E+04	2.34
6	4.31E+05	1.96	7.26E+05	1.89	4.16E+04	2.33
Mean	**4.32E+05**	**1.9666**	**7.29E+05**	**1.8961**	**4.17E+04**	**2.3364**
SD	**3176.6948**	**0.0098**	**4083.3797**	**0.0032**	**129.3734**	**0.0050**
RSD% (CV%)	**0.7352**	**0.4996**	**0.5602**	**0.1668**	**0.3099**	**0.2141**

RSD% (or CV%), Relative Standard Deviation [(SD/Mean) x 100]

5.10.2 Reproducibility test for NSD and M1 LC-MS/MS method

The reproducibility of the present bioanalytical method was determined on the basis of the coefficient of variance (CV%) i.e., the relative standard deviation (RSD) of the area ratio of both NSD/CXB (IS) and M1/CXB (IS) for replicate (n=3) assay with conc. 100 ng/mL and conc. 1000 ng/mL.

Table.5.112 Reproducibility test of the plasma spiked with 100 ng/mL of NSD, M1 and CXB (IS)

No.	Peak area of NSD (conc. 100 ng/mL)	Peak area of M1 (conc. 100 ng/mL)	Peak area of CXB (IS) (conc. 100 ng/mL)	Area ratio (NSD)	Area ratio (M1)
1	1.28E+05	2.54E+04	2.21E+04	5.81E+00	1.15E+00
2	1.29E+05	2.51E+04	2.22E+04	5.82E+00	1.13E+00
3	1.28E+05	2.52E+04	2.21E+04	5.81E+00	1.14E+00
Mean	**1.29E+05**	**2.52E+04**	**2.21E+04**	**5.81E+00**	**1.14E+00**
SD	**450.0370**	**172.1753**	**51.5978**	**0.0068**	**0.0102**
CV% (RSD%)	**0.3499**	**0.6820**	**0.2332**	**0.1173**	**0.8967**

Table.5.113 Reproducibility test of the plasma spiked with 1000 ng/mL of NSD, M1 and CXB (IS)

No.	Peak area of NSD (conc. 1000 ng/mL)	Peak area of M1 (conc. 1000 ng/mL)	Peak area of CXB (IS) (conc. 1000 ng/mL)	Area ratio (NSD)	Area ratio (M1)
1	9.36E+05	1.99E+05	1.51E+05	6.21E+00	1.32E+00
2	9.45E+05	1.94E+05	1.52E+05	6.23E+00	1.28E+00
3	9.38E+05	1.96E+05	1.52E+05	6.15E+00	1.29E+00
Mean	**9.40E+05**	**1.97E+05**	**1.52E+05**	**6.20E+00**	**1.30E+00**
SD	**4858.7070**	**2224.6872**	**864.3495**	**0.0412**	**0.0203**
CV% (RSD%)	**0.5171**	**1.1319**	**0.5701**	**0.6655**	**1.5629**

Results of reproducibility test are described in Table.5.112 and Table.5.113 for the concentration 100 ng/mL and 1000 ng/mL, respectively. The low CV% (RSD%) value indicates the reproducibility of the method to be satisfactory and therefore CV% (RSD%) of both concentrations for NSD and IS; M1 and IS were found to be within the acceptable limit.

5.11 Validation of method for bioanalytical study of nimesulide (NSD) and 4-hydroxynimesulide (M1) by liquid chromatography tandem-mass spectrometry (LC-MS/MS)

The method of NSD and M1 was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation.

5.11.1 Specificity and selectivity

According to the representative chromatograms of the double blank plasma sample (see Figure.5.90), blank plasma sample spiked with IS (see Figure.5.91) and blank plasma spiked with the analytes (NSD and M1) at 10 ng/mL and IS (CXB), i.e., at LLOQ (see Figure.5.92), the total chromatographic run time was 3.0 minutes and the retention time of NSD, M1 and CXB (IS) were about 1.97, 1.89 and 2.32 min., respectively. Now, on repeated injection of the samples, the retention time never shifted beyond 10 to 15 seconds. In Figure.5.92, the chromatogram shown that there was no significant interference from the endogenous plasma matrix was found at the retention times of analytes and IS. Similar things were observed in the chromatogram of blank plasma spiked with IS (single blank, Figure.5.91) and in the chromatogram of blank plasma (double blank, Figure.5.90).

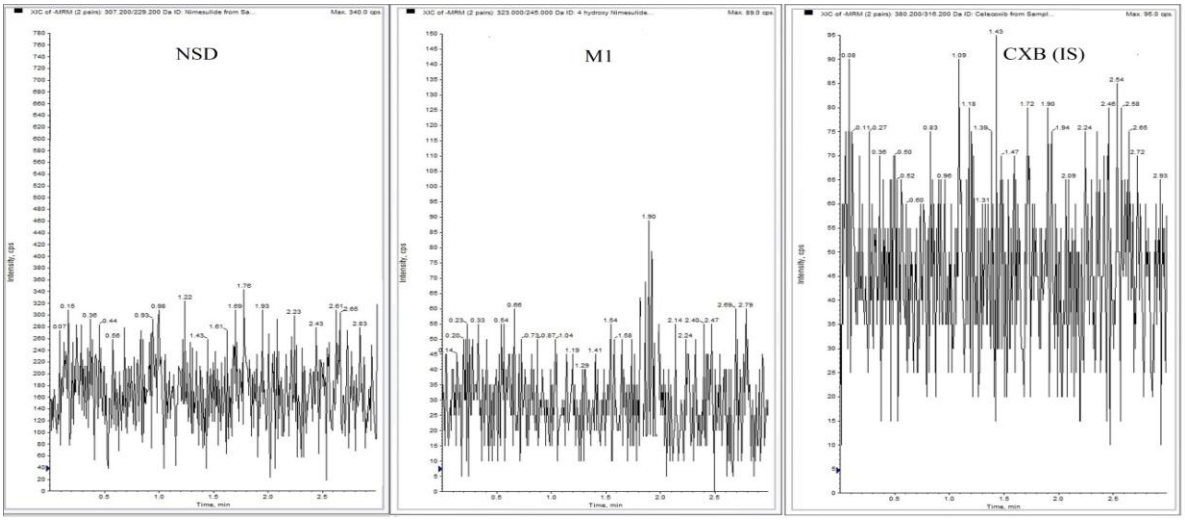


Figure.5.90 Chromatogram of blank human plasma or double blank plasma [free of NSD, M1 and CXB (IS)]

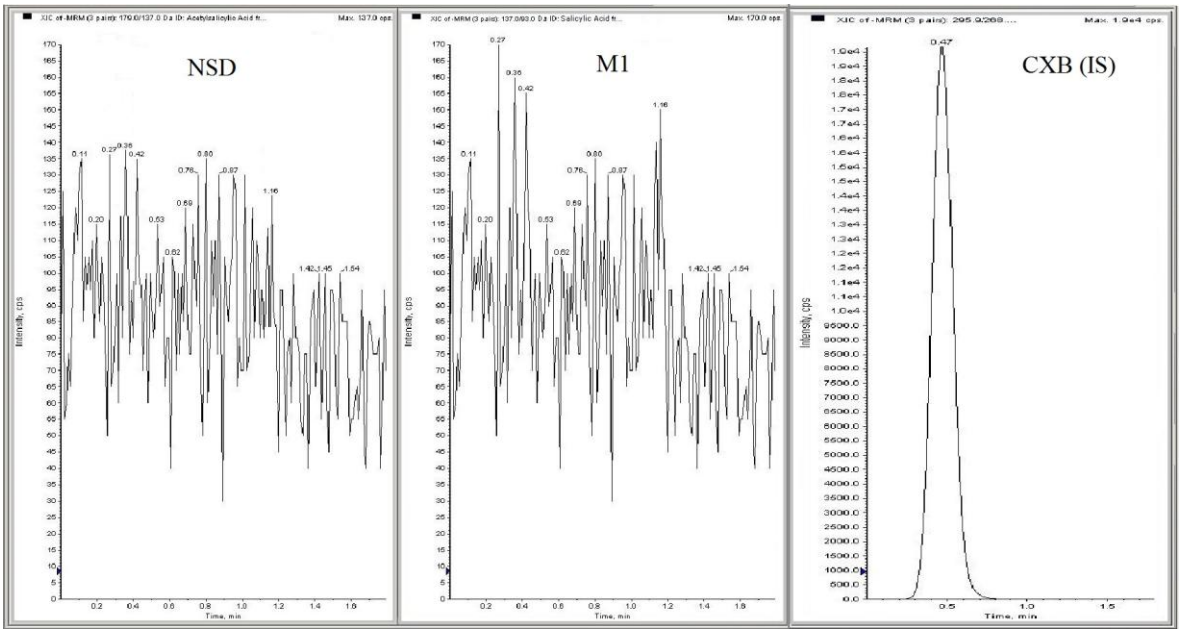


Figure.5.91 Chromatogram of single blank plasma spiked with IS (CXB)

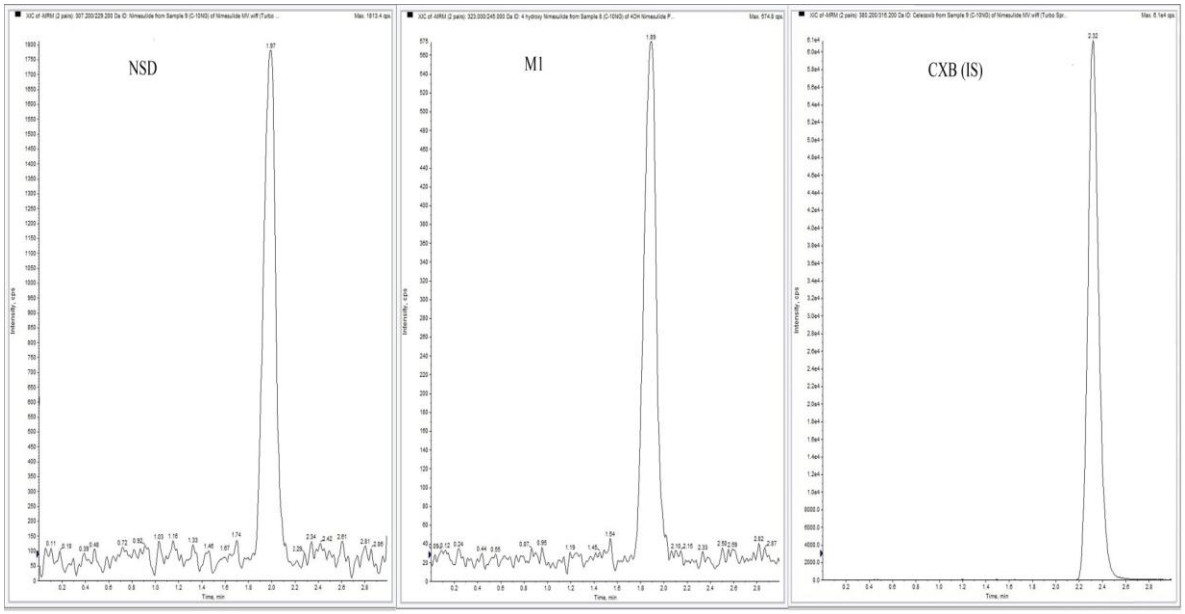


Figure.5.92 Chromatogram of blank human plasma spiked with analytes (NSD and M1) at LLOQ 10 ng/mL and IS (CXB)

5.11.2 Sensitivity

As per the result, the method of NSD and M1 was found very sensitive. Hence, the limit of detection (LOD) and the lower limit of quantification (LLOQ) were found at 3 ng/mL and 10 ng/mL, respectively (see Figure.5.93). The signal to noise (S/N) ratio was maintained 3:1 for

LOD and 5:1 for LLOQ. Now, all LLOQ samples were found within the acceptance criteria as per the guidelines.



Figure.5.93 Chromatograms showing A) LOD and B) LLOQ of NSD and M1

5.11.3 Linearity and calibration (standard) curve of NSD and M1 in human plasma

The validation was carried out to assess the calibration, precision and accuracy of both NSD and M1 in human blank plasma. The standard or calibration curves were constructed by plotting the peak area ratio of the analytes (NSD and M1) to IS (CXB) versus the nominal calibration concentrations (CC) of the analytes. The standard curves have consistently good reproducibility over the standard concentrations across the calibration range and the representative calibration curves (LIN I) for NSD and M1 are demonstrated in Figure.5.94 and Figure.5.95. The linearity of the calibration curve was assessed by an unweighted ($1/x^2$) least square or linear regression analysis. Thereafter the regression equation of the calibration curve was used to determine the concentrations of both NSD and M1 in human plasma. All these procedures were achieved by the Analyst 1.5 software in quantitate mode during analysis.

The back calculated values of the concentrations were determined statistically. These back calculated concentrations from the representative calibration standards by LC-MS/MS determination for NSD and M1 and corresponding regression equations are described in Table.5.114 and Table.5.116. The assay result was found better linear response over the linearity range of 10 ng/mL to 6000 ng/mL for both NSD and M1 in plasma. The regression equations were $y = 0.0014x + 0.0056$ ($r = 0.9992$) for NSD and $y = 0.00110x + 0.00653$ ($r = 0.9993$) for M1 (see Table.5.115 and Table.5.117), where 'y' is the peak area ratio of analyte to IS and 'x' is the nominal plasma concentration of the analyte. According to the Table.5.114 and Table.5.116, the CV% and accuracy (%) values ranging from 0.80% to 8.62% and 95.17% to 114.13% for NSD and from 0.17% to 2.45% and 87.20% to 106.83% for M1, respectively.

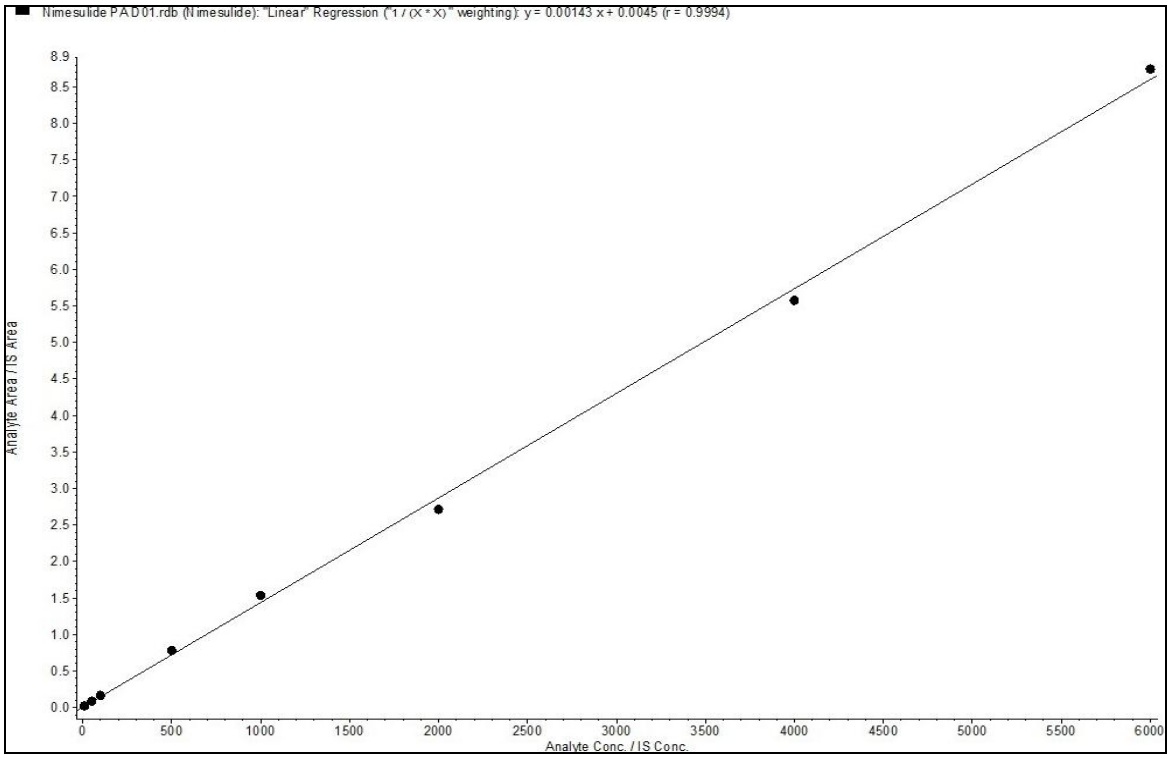


Figure.5.94 Plasma calibration curve of NSD

Table.5.114 Linear regression data for the determination of NSD in human plasma

Linearity	Plasma calibration concentration (ng/mL) of NSD							
	10 (CC1)	**50 (CC2)**	**100 (CC3)**	**500 (CC4)**	**1000 (CC5)**	**2000 (CC6)**	**4000 (CC7)**	**6000 (CC8)**
LIN 1	10.50	56.30	111.00	541.00	1070.00	1890.00	3890.00	6100.00
LIN 2	9.10	57.70	108.00	550.00	1000.00	1900.00	4010.00	6020.00
LIN 3	9.05	57.20	115.00	554.00	1080.00	1920.00	3750.00	6180.00
Mean	**9.55**	**57.07**	**111.33**	**548.33**	**1050.00**	**1903.33**	**3883.33**	**6100.00**
S.D.	**0.82**	**0.71**	**3.51**	**6.66**	**43.59**	**15.28**	**130.13**	**80.00**
CV%	**8.62**	**1.24**	**3.15**	**1.21**	**4.15**	**0.80**	**3.35**	**1.31**
Accuracy (%)	**95.50**	**114.13**	**111.33**	**109.67**	**105.00**	**95.17**	**97.08**	**101.67**

Table.5.115 Pre study linearity of detector response of NSD

Linearity	Statistics		
	Slope (M)	**Intercept-C**	**R square**
LIN 1	0.0014	0.0045	0.9994
LIN 2	0.0015	0.0059	0.9998
LIN 3	0.0014	0.0062	0.9984
Mean	**0.0014**	**0.0056**	**0.9992**
S.D.	**0.0001**	**0.0009**	**0.0007**
CV%	**4.0233**	**16.7031**	**0.0722**

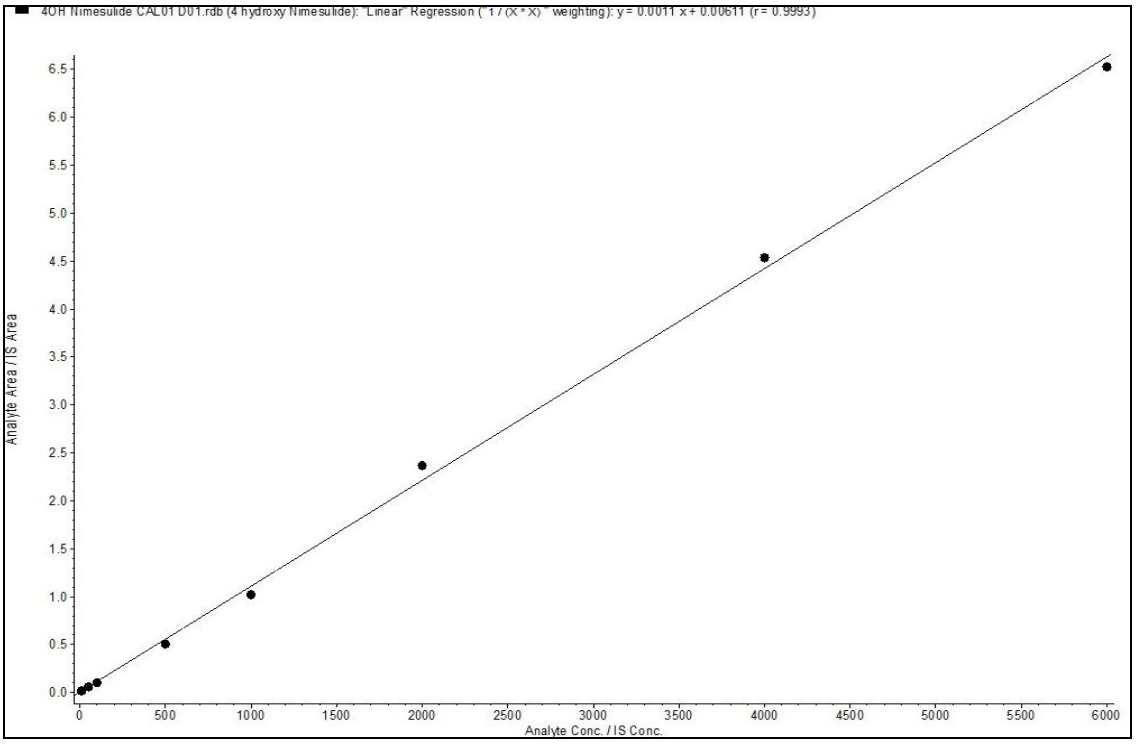


Figure.5.95 Plasma calibration curve of M1

Table.5.116 Linear regression data for the determination of M1 in human plasma

Linearity	Plasma calibration concentration (ng/mL) of M1							
	10 (CC1)	50 (CC2)	100 (CC3)	500 (CC4)	1000 (CC5)	2000 (CC6)	4000 (CC7)	6000 (CC8)
LIN 1	9.26	48.80	87.90	453.00	917.00	2140.00	4100.00	5900.00
LIN 2	8.99	49.60	88.10	453.00	916.00	2140.00	4110.00	5910.00
LIN 3	9.44	49.80	85.60	460.00	914.00	2130.00	4120.00	5930.00
Mean	**9.23**	**49.40**	**87.20**	**455.33**	**915.67**	**2136.67**	**4110.00**	**5913.33**
S.D.	**0.23**	**0.53**	**1.39**	**4.04**	**1.53**	**5.77**	**10.00**	**15.28**
CV%	**2.45**	**1.07**	**1.59**	**0.89**	**0.17**	**0.27**	**0.24**	**0.26**
Accuracy (%)	**92.30**	**98.80**	**87.20**	**91.07**	**91.57**	**106.83**	**102.75**	**98.56**

Table.5.117 Pre study linearity of detector response of M1

Linearity	Statistics		
	Slope (M)	Intercept-C	R square
LIN 1	0.00110	0.00611	0.9993
LIN 2	0.00107	0.00653	0.9993
LIN 3	0.00113	0.00696	0.9993
Mean	**0.00110**	**0.00653**	**0.9993**
S.D.	**0.00003**	**0.00043**	**0.0000**
CV%	**2.72727**	**6.50525**	**0.0000**

5.11.4 Precision and accuracy of NSD and M1

The intra-day and inter-day assay precision, i.e., CV% or RSD% values for different concentrations ranged from 1.87% to 5.97% and 4.65% to 5.97% for NSD and 2.54% to 5.61% and 2.75% to 4.87% for M1, respectively. Whereas, the percentage of accuracy (accuracy %) for the same concentrations were in the range of 89.30% to 109.50% (intra-day) and 91.00% to 106.01% (inter-day) for NSD and 95.86% to 113.36% (intra-day) and 95.87%

to 111.79% (inter-day) for M1, respectively. Both the precision and accuracy data were in the acceptable range according to the USFDA and EMA guidelines. The assay method shown high degree of precision and accuracy. The precision and accuracy data for both intra-day and inter-day assays of LLOQ and three QC samples (LQC, MQC and HQC) are given in Table.5.118 and Table.5.119.

Table.5.118 Intra-day and inter-day precision and accuracy data of NSD in human plasma (n = 6)

Intra-day (Within run)					
Quality control (QC) sample	**Spiked/Nominal Concentration (ng/mL)**	**Mean concentration (ng/mL)**	**SD**	**CV% (RSD%)**	**Accuracy (%)**
LLOQ	10	9.7800	0.5848	5.9796	97.8000
LQC	30	32.8500	1.3982	4.2564	109.5000
MQC	2500	2371.6667	44.4597	1.8746	94.8667
HQC	5000	4465.0000	120.7891	2.7052	89.3000
Inter-day (Between run)					
LLOQ	10	9.5708	0.5714	5.9703	95.7083
LQC	30	31.8042	1.5009	4.7191	106.0139
MQC	2500	2330.4167	108.4066	4.6518	93.2167
HQC	5000	4550.4167	212.8988	4.6787	91.0083

SD = Standard deviation; CV (%) coefficient of variation or RSD (%) relative standard deviation = [(SD / mean) x 100]; Accuracy (%) = [(measured concentration / spiked or nominal concentration) x 100]; LLOQ = Lower limit of quantification; LQC = Low quality control; MQC = Middle quality control; HQC = High quality control; n = number of samples.

Table.5.119 Intra-day and inter-day precision and accuracy data of M1 in human plasma (n = 6)

Intra-day (Within run)					
Quality control (QC) sample	**Spiked/Nominal Concentration (ng/mL)**	**Mean concentration (ng/mL)**	**SD**	**CV% (RSD%)**	**Accuracy (%)**
LLOQ	10	9.5860	0.5379	5.6112	95.8600
LQC	30	33.3333	1.3049	3.9146	111.1111
MQC	2500	2770.0000	136.0882	4.9129	110.8000
HQC	5000	5668.3333	144.4184	2.5478	113.3667
Inter-day (Between run)					
LLOQ	10	9.5879	0.4673	4.8737	95.8792
LQC	30	33.3542	0.9731	2.9175	111.1806
MQC	2500	2749.5833	101.1447	3.6785	109.9833
HQC	5000	5589.5833	154.0886	2.7567	111.7917

SD = Standard deviation; CV (%) coefficient of variation or RSD (%) relative standard deviation = [(SD / mean) x 100]; Accuracy (%) = [(measured concentration / spiked or nominal concentration) x 100]; LLOQ = Lower limit of quantification; LQC = Low quality control; MQC = Medium quality control; HQC = High quality control; n = number of samples.

5.11.5 Extraction recovery of NSD and M1

Extraction recoveries were calculated in the percentage (%) by measuring the peak areas of the analytes at low, medium and high quality control samples. The peak areas of the extracted plasma at low, medium and high quality control samples were compared to the peak areas of the unextracted standards containing the same concentrations of the NSD and M1. Recovery results shown that the maximum recovery was found at low (30 ng/mL), medium (2500 ng/mL) and high (5000 ng/mL) quality control (QC) samples ranged from 101.49% to

106.53% for NSD and 99.13% to 102.64% for M1, respectively and also recovery of the internal standard (IS, CXB) was found 97.81% (see Table.5.120). Now, the recovery of both the ananlytes was indicated to be satisfactory and also enough high and data found with better sensitivity as it was precise, reproducible and consistent.

Table.5.120 Absolute recovery of NSD and M1 in human plasma

Analyte	Spiked/Nominal Concentration (ng/mL)	Mean Concentration (ng/mL)	Recovery (%) (Mean ± S.D.)
NSD	30	32.5667	101.4969 ± 7.1118
	2500	2353.3333	106.5360 ± 1.8145
	5000	4456.6667	101.8007 ± 1.5661
M1	30	33.8167	101.8708 ± 3.9955
	2500	2810.0000	102.6476 ± 3.6854
	5000	5548.3333	99.1336 ± 1.6253
CXB (IS)	5000	5020.0000	97.8123 ± 2.4326

5.11.6 Matrix effect of NSD and M1

No significant matrix effect was found for NSD, M1 and CXB (IS) by comparing the area under the curve (AUC) ratios of the extracted QCs (LQC, MQC and HQC) with the AUC of unextracted QCs (LQC, MQC and HQC) obtained from the injecting raw or aqueous solution prepared at same concentration levels (n = 6) as described in Table.5.121, Table.5.122, Table.5.123 and Table.5.124.

Table.5.121 Evaluation of matrix effect for NSD

Sample name	Analyte peak area		% Matrix effect	Matrix factor
	Spiking in aqueous sample	Spiking in extracted blank matrix (plasma)		
LQC-1	16300	15800	96.93	0.9693
LQC-2	16700	17300	103.59	1.0359
LQC-3	17200	16900	98.26	0.9826
LQC-4	16900	17100	101.18	1.0118
LQC-5	17300	18900	109.25	1.0925
LQC-6	16800	17200	102.38	1.0238
MQC-1	1080000	1060000	98.15	0.9815
MQC-2	1080000	1090000	100.93	1.0093
MQC-3	1060000	1080000	101.89	1.0189
MQC-4	1070000	1040000	97.20	0.9720
MQC-5	1060000	1090000	102.83	1.0283
MQC-6	1090000	1050000	96.33	0.9633
HQC-1	1810000	1960000	108.29	1.0829
HQC-2	1800000	1780000	98.89	0.9889
HQC-3	1840000	1920000	104.35	1.0435
HQC-4	1830000	1880000	102.73	1.0273
HQC-5	1800000	1740000	96.67	0.9667
HQC-6	1799000	1890000	105.06	1.0506

Table.5.122 Evaluation of matrix effect for M1

Sample name	Analyte peak area		% Matrix effect	Matrix factor
	Spiking in aqueous sample	Spiking in extracted blank matrix (plasma)		
LQC-1	5860	5920	101.02	1.0102
LQC-2	5810	5760	99.14	0.9914
LQC-3	6080	6210	102.14	1.0214
LQC-4	6230	6030	96.79	0.9679
LQC-5	6120	6370	104.08	1.0408
LQC-6	5920	5840	98.65	0.9865
MQC-1	411000	414000	100.73	1.0073
MQC-2	414000	406000	98.07	0.9807
MQC-3	436000	427000	97.94	0.9794
MQC-4	436000	446000	102.29	1.0229
MQC-5	438000	437000	99.77	0.9977
MQC-6	429000	406000	94.64	0.9464
HQC-1	883000	876000	99.21	0.9921
HQC-2	876000	891000	101.71	1.0171
HQC-3	905000	942000	104.09	1.0409
HQC-4	912000	886000	97.1491	0.9715
HQC-5	893000	883000	98.88	0.9888
HQC-6	906000	956000	105.52	1.0552

Table.5.123 Evaluation of matrix effect for CXB (IS)

Sample name	IS peak area		% Matrix effect	Matrix factor
	Spiking in aqueous sample	Spiking in extracted blank matrix (plasma)		
LQC-1	132000	143000	108.33	1.0833
LQC-2	128000	134000	104.69	1.0469
LQC-3	141000	135000	95.74	0.9574
LQC-4	143000	146000	102.10	1.0210
LQC-5	138000	134000	97.10	0.9710
LQC-6	140000	138000	98.57	0.9857
MQC-1	139000	147000	105.76	1.0576
MQC-2	137000	129000	94.16	0.9416
MQC-3	136000	143000	105.15	1.0515
MQC-4	137000	140000	102.19	1.0219
MQC-5	140000	135000	96.43	0.9643
MQC-6	141000	139000	98.58	0.9858
HQC-1	142000	151000	106.34	1.0634
HQC-2	142000	143000	100.70	1.0070
HQC-3	139000	134000	96.40	0.9640
HQC-4	138000	137000	99.28	0.9928
HQC-5	142000	140000	98.59	0.9859
HQC-6	137000	136000	99.27	0.9927

Table.5.124 Matrix effect of NSD and M1 calculated from six different source of human plasma

Analytes	Matrix Effect (ME %) [a]						Mean	SD	CV%
	SET 1*	SET 2	SET 3	SET 4	SET 5	SET 6			
NSD									
LQC (30 ng/mL)	96.9325	103.5928	98.2558	101.1834	109.2486	102.3810	101.9323	4.3718	4.2889
MQC (2500 ng/mL)	98.1481	100.9259	101.8868	97.1963	102.8302	96.3303	99.5529	2.6827	2.6948
HQC (5000 ng/mL)	108.2873	98.8889	104.3478	102.7322	96.6667	105.0584	102.6635	4.2524	4.1421
M1									
LQC (30 ng/mL)	101.0239	99.1394	102.1382	96.7897	104.0850	98.6486	100.3041	2.6315	2.6235
MQC (2500 ng/mL)	100.7299	98.0676	97.9358	102.2936	99.7717	94.6387	98.9062	2.6610	2.6905
HQC (5000 ng/mL)	99.2072	101.7123	104.0884	97.1491	98.8802	105.5188	101.0927	3.2542	3.2191

[a] ME% expressed as the ratio of the mean peak area of an analyte spiked postextraction to the mean peak area of the same analyte standards multiplied by 100. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression.

*SET 1-6 represent the ME% from six different source of human plasma.

5.11.7 Stability study of NSD and M1

5.11.7.1 Stability of NSD, M1 and CXB (IS) stock solutions

The stock solutions of both the analytes (NSD and M1) and IS (CXB) were kept in three different storage conditions and peak area was determined of each condition. It has described in the Figure.5.96, Figure.5.97 and Figure.5.98, where NSD, M1 and CXB (IS) shows significant decrease in peak area with time, under the condition of room temperature and normal deep freezer. The stock solution was stable at −20 ºC for 30 days , i.e., 720 hours and stable at normal deep freezer for 7 days.

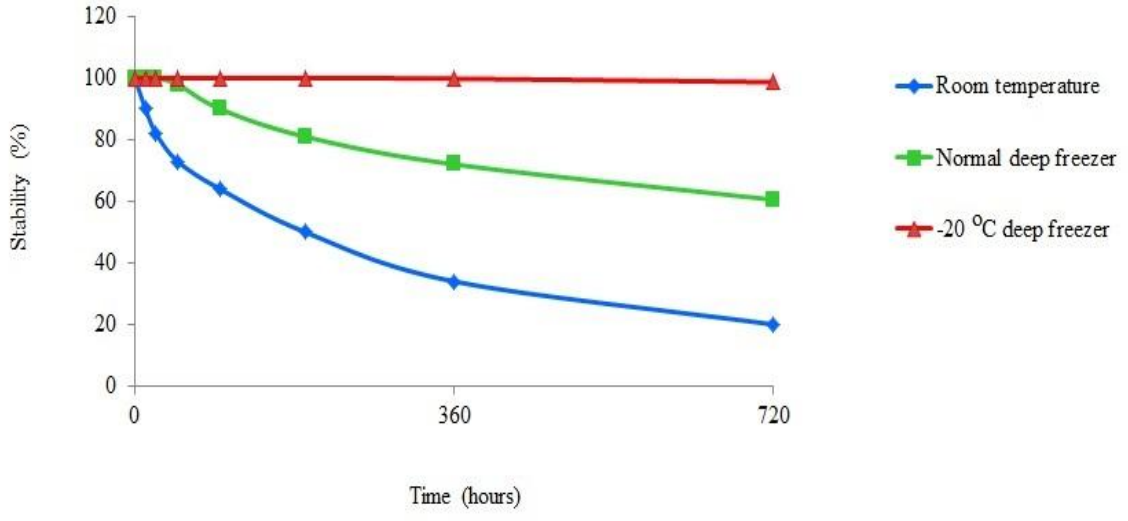


Figure.5.96 Stability of stock solution of NSD in three different conditions

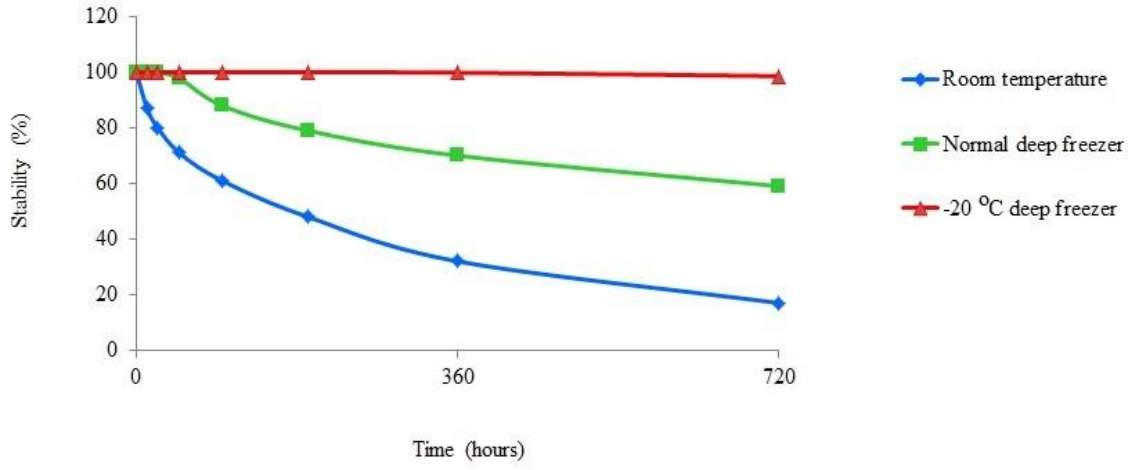


Figure.5.97 Stability of stock solution of M1 in three different conditions

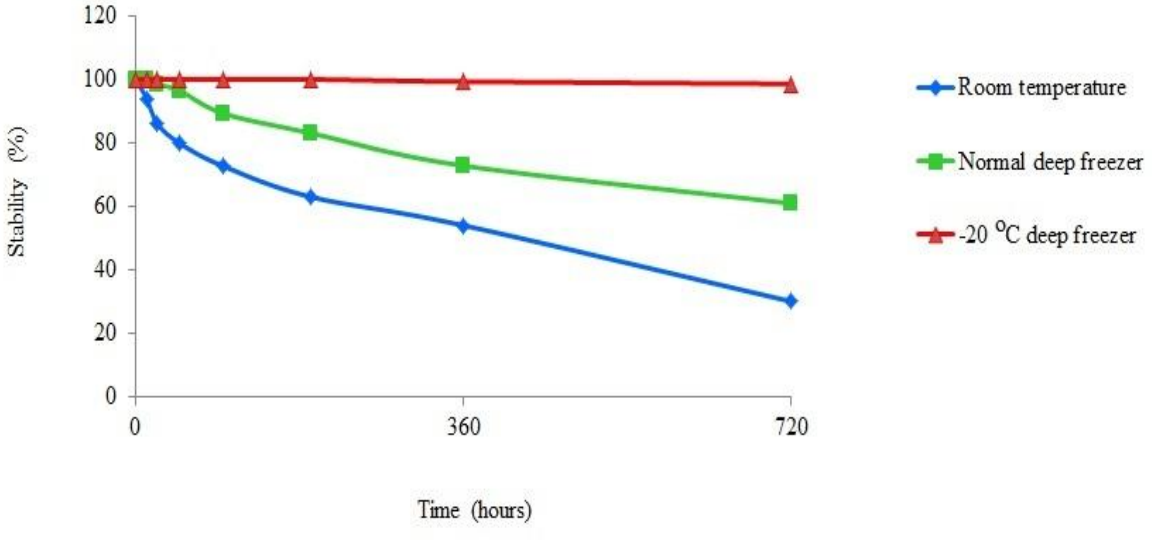


Figure.5.98 Stability of stock solution of CXB (IS) in three different conditions

5.11.7.2 Freeze and thaw stability study of NSD and M1

This stability study or test included six replicates (n = 6) of three levels of QC samples (LQC, MQC and HQC) along with a standard or calibration curve (n = 1), the linear regression correlation coefficient (r or r^2) were determined after three freeze thaw cycles comparing against freshly thaw samples of the same concentration. The freeze thaw stability of NSD and M1 ranged between 91.92% to 97.85% and 95.01% to 100.54%, respectively (see Table.5.125 and Table.5.126). Now, results prove that calculated concentrations for the analytes at LQC, MQC and HQC samples varied within the assay acceptance limits (±15%) of the nominal concentrations as per the guidelines (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.125 Freeze thaw stability study of NSD

	Freshly thaw (Day - 0)			After three freeze thaw cycles		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	35.5000	2320.0000	4460.0000	33.9000	2160.0000	4280.0000
2	31.2000	2310.0000	4370.0000	29.7000	2130.0000	4350.0000
3	31.8000	2280.0000	4380.0000	30.5000	2140.0000	4290.0000
4	32.9000	2360.0000	4460.0000	31.0000	2170.0000	4320.0000
5	32.1000	2380.0000	4390.0000	31.5000	2130.0000	4340.0000
6	30.8000	2470.0000	4560.0000	30.1000	2250.0000	4470.0000
Mean	32.3833	2353.3333	4436.6667	31.1167	2163.3333	4341.6667
SD	1.6916	67.4290	72.2957	1.5052	45.4606	68.5322
CV%	5.2238	2.8653	1.6295	4.8373	2.1014	1.5785
% Freeze thaw stability				**96.0885**	**91.9263**	**97.8588**

Table.5.126 Freeze thaw stability study of M1

	Freshly thaw (Day - 0)			After three freeze thaw cycles		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	32.6000	2660.0000	5690.0000	33.0000	2630.0000	5260.0000
2	33.3000	2760.0000	5700.0000	34.0000	2550.0000	5280.0000
3	34.3000	2730.0000	5540.0000	33.5000	2590.0000	5400.0000
4	33.4000	2710.0000	5540.0000	34.3000	2590.0000	5370.0000
5	34.2000	2770.0000	5480.0000	34.2000	2560.0000	5260.0000
6	32.5000	2620.0000	5390.0000	32.4000	2520.0000	5310.0000
Mean	33.3833	2708.3333	5556.6667	33.5667	2573.3333	5313.3333
SD	0.7627	58.4523	120.4436	0.7501	38.2971	59.2171
CV%	2.2846	2.1582	2.1676	2.2347	1.4882	1.1145
% Freeze thaw stability				**100.5492**	**95.0154**	**95.6209**

5.11.7.3 Bench top stability study of NSD and M1

The bench top stability was done for 8 hours in room temperature, around at 24 °C and assessed at three concentrations of low, medium and high QC levels (LQC, MQC and HQC) in six replicates (n = 6) along with a standard or calibration curve (n = 1). According to Table.5.127 and Table.5.128, this stability ranged from 96.70% to 100.90% for NSD and 97.78% to 101.04% for M1, respectively and all results were found within the acceptable limit.

Table.5.127 Bench top stability study of NSD

	Day - 0			**Bench top stability for 8 hours**		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	35.5000	2320.0000	4460.0000	31.4000	2290.0000	4570.0000
2	31.2000	2310.0000	4370.0000	32.1000	2470.0000	4490.0000
3	31.8000	2280.0000	4380.0000	30.7000	2260.0000	4340.0000
4	32.9000	2360.0000	4460.0000	30.2000	2180.0000	4280.0000
5	32.1000	2380.0000	4390.0000	32.6000	2320.0000	4630.0000
6	30.8000	2470.0000	4560.0000	30.9000	2380.0000	4550.0000
Mean	32.3833	2353.3333	4436.6667	31.3167	2316.6667	4476.6667
SD	1.6916	67.4290	72.2957	0.9020	100.1332	137.9372
CV%	5.2238	2.8653	1.6295	2.8804	4.3223	3.0812
% Bench top stability				**96.7061**	**98.4419**	**100.9016**

Table.5.128 Bench top stability study of M1

	Day - 0			Bench top stability for 8 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	32.6000	2660.0000	5690.0000	34.8000	2740.0000	5570.0000
2	33.3000	2760.0000	5700.0000	32.5000	2590.0000	5610.0000
3	34.3000	2730.0000	5540.0000	35.1000	2670.0000	5540.0000
4	33.4000	2710.0000	5540.0000	32.7000	2680.0000	5720.0000
5	34.2000	2770.0000	5480.0000	33.9000	2580.0000	5630.0000
6	32.5000	2620.0000	5390.0000	33.4000	2630.0000	5420.0000
Mean	33.3833	2708.3333	5556.6667	33.7333	2648.3333	5581.6667
SD	0.7627	58.4523	120.4436	1.0708	60.4704	100.2829
CV%	2.2846	2.1582	2.1676	3.1744	2.2833	1.7966
% Bench top stability				**101.0484**	**97.7846**	**100.4499**

5.11.7.4 Post-preparative or autosampler stability study of NSD and M1

The post-preparative or autosampler stability study was carried out at three concentrations of low, medium and high QC levels (LQC, MQC and HQC) in six replicates (n = 6) along with the calibration standards (n = 1). The samples were placed in the autosampler cabinet after preparation (extraction) at 10 to 15 °C for 24 hours. Then the samples were run and analyzed and the obtained concentrations were compared with the actual values. The percentage (%) of post-preparative or autosampler stability was varied from 95.41% to 99.06% for NSD and 92.56% to 107.40% for M1, respectively (Table.5.129 and Table.5.130). Results express that expected concentrations for the analytes at LQC, MQC and HQC samples deviated within the acceptance criteria (±15%) of the nominal concentrations as per the guidelines (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.129 Post-preparative or autosampler stability study of NSD

	Day - 0			Autosampler stability for 24 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	35.5000	2320.0000	4460.0000	33.8000	2490.0000	4650.0000
2	31.2000	2310.0000	4370.0000	31.5000	2270.0000	4430.0000
3	31.8000	2280.0000	4380.0000	30.7000	2390.0000	4270.0000
4	32.9000	2360.0000	4460.0000	29.6000	2530.0000	4410.0000
5	32.1000	2380.0000	4390.0000	30.7000	2480.0000	4320.0000
6	30.8000	2470.0000	4560.0000	31.4000	2260.0000	4290.0000
Mean	32.3833	2353.3333	4436.6667	30.9000	2403.333	4395.0000
SD	1.6916	67.4290	72.2957	1.40772	116.5619	140.5347
CV%	5.2238	2.8653	1.6295	4.5557	4.8500	3.1976
% Autosampler stability				**95.4195**	**96.1333**	**99.0609**

Table.5.130 Post-preparative or autosampler stability study of M1

	Day - 0			Autosampler stability for 24 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	32.6000	2660.0000	5690.0000	33.7000	2580.0000	5380.0000
2	33.3000	2760.0000	5700.0000	32.5000	2670.0000	5530.0000
3	34.3000	2730.0000	5540.0000	32.6000	2680.0000	5410.0000
4	33.4000	2710.0000	5540.0000	35.1000	2690.0000	5680.0000
5	34.2000	2770.0000	5480.0000	31.9000	2740.0000	5290.0000
6	32.5000	2620.0000	5390.0000	34.7000	2750.0000	5640.0000
Mean	33.3833	2708.3333	5556.6667	30.9000	2685.000	5488.3333
SD	0.7627	58.4523	120.4436	1.29370	60.9098	154.0671
CV%	2.2846	2.1582	2.1676	4.1867	2.2685	2.8072
% Autosampler stability				**92.5612**	**107.4000**	**98.7702**

5.11.7.5 Dry state stability study of NSD and M1

Table.5.131 Dry state stability study of NSD

	Day - 0			Dry state stability for 24 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	35.5000	2320.0000	4460.0000	35.2000	2290.0000	4380.0000
2	31.2000	2310.0000	4370.0000	32.4000	2250.0000	4290.0000
3	31.8000	2280.0000	4380.0000	31.6000	2240.0000	4310.0000
4	32.9000	2360.0000	4460.0000	32.7000	2290.0000	4340.0000
5	32.1000	2380.0000	4390.0000	31.8000	2320.0000	4270.0000
6	30.8000	2470.0000	4560.0000	31.4000	2350.0000	4520.0000
Mean	32.3833	2353.3333	4436.6667	32.5167	2290.0000	4351.6667
SD	1.6916	67.4290	72.2957	1.4034	41.4729	91.0860
CV%	5.2238	2.8653	1.6295	4.3161	1.8110	2.0931
% Dry state stability				**100.4117**	**97.3088**	**98.0841**

Table.5.132 Dry state stability study of M1

	Day - 0			Dry state stability for 24 hours		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	32.6000	2660.0000	5690.0000	32.4000	2620.0000	5630.0000
2	33.3000	2760.0000	5700.0000	32.9000	2680.0000	5660.0000
3	34.3000	2730.0000	5540.0000	33.7000	2700.0000	5420.0000
4	33.4000	2710.0000	5540.0000	33.2000	2640.0000	5460.0000
5	34.2000	2770.0000	5480.0000	33.8000	2720.0000	5370.0000
6	32.5000	2620.0000	5390.0000	33.0000	2590.0000	5240.0000
Mean	33.3833	2708.3333	5556.6667	33.1667	2658.3333	5463.3333
SD	0.7627	58.4523	120.4436	0.5241	49.9667	159.3319
CV%	2.2846	2.1582	2.1676	1.5802	1.8796	2.9164
% Dry state stability				**99.3510**	**98.1538**	**98.3203**

5.11.7.6 Short term stability study of NSD and M1

The short term stability study was carried out at three QC levels; LQC, MQC and HQC samples in six replicates (n = 6) along with a standard or calibration curve (n = 1) and were stored in deep freezer at -20 ºC for 7 days. The samples were analysed after 7 days and the obtained concentrations were compared with the actual values of QC samples containing the same concentrations of the NSD and M1. Now, the short term stability of NSD and M1 ranged between 97.37% to 98.50% and 98.41% to 98.58%, respectively (see Table.5.133 and Table.5.134). The results of calculated concentrations for the analytes at LQC, MQC and HQC samples were within the assay acceptable limit, i.e., ±15% of the nominal concentrations as per the guidelines (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.133 Short term stability study of NSD

	Day - 0			**Short term stability for 7 days**		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	35.5000	2320.0000	4460.0000	35.2000	2300.0000	4420.0000
2	31.2000	2310.0000	4370.0000	30.7000	2270.0000	4280.0000
3	31.8000	2280.0000	4380.0000	31.4000	2240.0000	4330.0000
4	32.9000	2360.0000	4460.0000	32.0000	2290.0000	4400.0000
5	32.1000	2380.0000	4390.0000	31.9000	2260.0000	4270.0000
6	30.8000	2470.0000	4560.0000	30.2000	2390.0000	4460.0000
Mean	32.3833	2353.3333	4436.6667	31.9000	2291.6667	4360.0000
SD	1.6916	67.4290	72.2957	1.7595	52.6941	78.2304
CV%	5.2238	2.8653	1.6295	5.5158	2.2994	1.7943
% Short term stability				**98.5075**	**97.3796**	**98.2720**

Table.5.134 Short term stability study of M1

	Day - 0			Short term stability for 7 days		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	32.6000	2660.0000	5690.0000	32.2000	2610.0000	5520.0000
2	33.3000	2760.0000	5700.0000	32.7000	2740.0000	5680.0000
3	34.3000	2730.0000	5540.0000	33.4000	2640.0000	5430.0000
4	33.4000	2710.0000	5540.0000	33.0000	2690.0000	5500.0000
5	34.2000	2770.0000	5480.0000	33.9000	2750.0000	5370.0000
6	32.5000	2620.0000	5390.0000	32.2000	2590.0000	5310.0000
Mean	33.3833	2708.3333	5556.6667	32.9000	2670.0000	5468.3333
SD	0.7627	58.4523	120.4436	0.6753	67.2309	130.1409
CV%	2.2846	2.1582	2.1676	2.0525	2.5180	2.3799
% Short term stability				**98.5522**	**98.5846**	**98.4103**

5.11.7.7 Long term stability study of NSD and M1

The long term stability study was carried out at LQC, MQC and HQC in six replicates (n = 6) along with a standard or calibration curve (n = 1) and were stored in deep freezer at -20 °C for 30 days. After 30 days, the samples were analysed and the obtained concentrations were compared with the actual values of QC samples containing the similar concentrations of both the NSD and M1. The long term stability was varied from 87.80% to 97.33% for NSD and 96.37% to 97.10% for M1, respectively (see Table.5.135 and Table.5.136). The results of measured concentrations for the analytes at LQC, MQC and HQC samples were within the assay acceptance criteria, i.e., ±15% of the nominal concentrations as per the guidelines (United States Food and Drug Administration, 2001 and European Medicines Agency, 2011).

Table.5.135 Long term stability study of NSD

	Day - 0			Long term stability for 30 days		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	35.5000	2320.0000	4460.0000	25.8000	2140.0000	4280.0000
2	31.2000	2310.0000	4370.0000	28.0000	2180.0000	4320.0000
3	31.8000	2280.0000	4380.0000	29.8000	2160.0000	4380.0000
4	32.9000	2360.0000	4460.0000	29.2000	2170.0000	4300.0000
5	32.1000	2380.0000	4390.0000	29.5000	2230.0000	4350.0000
6	30.8000	2470.0000	4560.0000	28.3000	2310.0000	4280.0000
Mean	32.3833	2353.3333	4436.6667	28.4333	2198.3333	4318.3333
SD	1.6916	67.4290	72.2957	1.4652	62.4233	40.2078
CV%	5.2238	2.8653	1.6295	5.1529	2.8396	0.9311
% Long term stability				**87.8024**	**93.4136**	**97.3328**

Table.5.136 Long term stability study of M1

	Day - 0			Long term stability for 30 days		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	32.6000	2660.0000	5690.0000	32.8000	2610.0000	5360.0000
2	33.3000	2760.0000	5700.0000	30.9000	2580.0000	5570.0000
3	34.3000	2730.0000	5540.0000	31.7000	2730.0000	5460.0000
4	33.4000	2710.0000	5540.0000	32.6000	2670.0000	5210.0000
5	34.2000	2770.0000	5480.0000	33.4000	2560.0000	5340.0000
6	32.5000	2620.0000	5390.0000	32.1000	2630.0000	5190.0000
Mean	33.3833	2708.3333	5556.6667	32.2500	2630.0000	5355.0000
SD	0.7627	58.4523	120.4436	0.8826	62.2896	145.4304
CV%	2.2846	2.1582	2.1676	2.7368	2.3684	2.7158
% Long term stability				**96.6051**	**97.1077**	**96.3707**

5.11.7.8 Dilution integrity of NSD and M1

The dilution integrity was achieved by preparing the concentration four times of ULOQ (upper limit of quantification) i.e., 24000 ng/mL (24 µg/mL), which was further diluted five and ten times (5 fold and 10 fold) with human blank plasma (drug free plasma) in six replicates (n = 6) and these diluted samples were run against calibration standards. The dilution integrity data are summarized in Table.5.137 of both NSD and M1, where the precision (%RSD) values were 1.60% and 1.38% for NSD and 1.68% and 2.36% for M1 and also with the accuracy of 99.86% and 99.51% for NSD and 98.88% and 98.81% for M1, respectively. These results support sample dilution up to 10 fold for bioanalysis.

Table.5.137 Dilution integrity of NSD and M1 in human plasma

	Dilution for NSD		**Dilution for M1**	
	5 fold	**10 fold**	**5 fold**	**10 fold**
	Concentration		**Concentration**	
Serial No.	**4800 ng/mL**	**2400 ng/mL**	**4800 ng/mL**	**2400 ng/mL**
1	4700.0000	2380.0000	4820.0000	2350.0000
2	4910.0000	2370.0000	4740.0000	2430.0000
3	4790.0000	2420.0000	4640.0000	2290.0000
4	4730.0000	2390.0000	4850.0000	2350.0000
5	4850.0000	2340.0000	4750.0000	2370.0000
6	4780.0000	2430.0000	4680.0000	2440.0000
Mean	4793.3333	2388.3333	4746.6667	2371.6667
SD	77.1146	33.1160	79.9166	56.0060
Accuracy	99.8611	99.5139	98.8889	98.8194
Precision (%RSD)	1.6088	1.3866	1.6836	2.3615

SD, Standard deviation; RSD (%), Relative standard deviation [(SD / mean) x 100]

5.12 Application to bioequivalence study and evaluation of pharmacokinetic parameters in healthy human subjects : a single oral dose, open label, randomized, two period, two treatment and two-way cross over study

The validated bioanalytical method was successfully applied to determine the concentration of both NSD and M1 in human plasma for a bioequivalence (BE) study in 24+6 healthy Indian male subjects after an oral administration of Nimesulide 100 mg tablets under fasting conditions. The BE study was approved by the Bio-Ethics Committee (Reg. No. ECR/93/Indt/WB/2013) of Bioequivalence Study Centre, Jadavpur University, Kolkata, India.

Thirty (24+6) healthy adult male subjects were participated in this comparative, i.e., bioequivalence study at Clinical Pharmacology Unit (CPU) of Bioequivalence Study Center, Jadavpur University and the whole work was supported by TAAB Biostudy Services, Jadavpur, Kolkata. The mean age of the selected volunteers was 25.23 ± 5.41 years with a range of 19 to 38 years; the mean body weight was 65.60 ± 3.64 kg with a range of 58 to 74 kg; the mean body height was 170.40 ± 5.13 cm with a range of 163 to 180 cm and finally, the BMI was 22.65 ± 1.81 Kg/m2 with range of 19.05 to 24.98 Kg/m2 (see Table.5.138).

5.12.1 Reference and Test preparation for Nimesulide (NSD)

- **Reference Preparation (A) :**

  Tablet NISE (containing Nimesulide 100 mg)

  Mfg. by : DR. REDDY'S LABORATORIES LTD., VILLAGE – MAUJA THANA, BADDI – 173 205 DIST. SOLAN, (HP), INDIA

  Batch No. : E300161

  Mfg. Date : April, 2013 Exp.Date : March, 2016

- **Test Preparation (B) :**

  Tablet containing Nimesulide 100 mg

  Mfg. by : PHARMLAND JV LLC, OFFICE 3, 124 LENINSKAYA STR., 222603 NESVIZH, MINSK REGION, REPUBLIC OF BELARUS

  Batch No. : NMS/12120948

  Mfg. Date : December, 2012 Exp. Date : November, 2017

Table.5.138 Demographic data of 24+6 volunteers

Vol. No.	Sex	Age (yr)	Height (cm)	Weight (kg.)	BMI (Kg/m^2)
1	M	24	170	65	22.49
2	M	32	169	68	23.81
3	M	28	170	59	20.42
4	M	19	165	64	23.51
5	M	21	170	60	20.76
6	M	22	177	66	21.07
7	M	21	178	68	21.46
8	M	26	164	65	24.17
9	M	24	170	70	24.22
10	M	23	170	69	23.88
11	M	21	166	65	23.59
12	M	20	165	68	24.98
13	M	29	177	65	20.75
14	M	23	168	70	24.80
15	M	34	166	68	24.68
16	M	22	172	58	19.61
17	M	20	163	64	24.09
18	M	30	163	65	24.46
19	M	21	174	64	21.14
20	M	21	179	65	20.29
21	M	28	180	64	19.75
22	M	24	175	74	24.16
23	M	35	175	69	22.53
24	M	20	170	65	22.49
25	M	21	164	63	23.42
26	M	20	167	69	24.74
27	M	27	164	64	23.80
28	M	36	171	70	23.94
29	M	27	176	59	19.05
30	M	38	174	65	21.47
Mean		**25.23**	**170.40**	**65.60**	**22.65**
S.D.		**5.41**	**5.13**	**3.64**	**1.81**

Table.5.139 Mode of treatment

Subject No.	PERIOD I	PERIOD II
1	A	B
2	A	B
3	A	B
4	B	A
5	B	A
6	A	B
7	B	A
8	B	A
9	B	A
10	B	A
11	A	B
12	A	B
13	A	B
14	A	B
15	A	B
16	B	A
17	B	A
18	A	B
19	B	A
20	B	A
21	B	A
22	B	A
23	A	B
24	A	B
25	A	B
26	A	B
27	A	B
28	B	A
29	B	A
30	B	A

A – Reference Preparation B – Test Preparation

Table.5.140 Bioequivalence of TABLET NISE containing NIMESULIDE 100 mg
Plasma concentration of Nimesulide (ng/mL) of the Reference Preparation (A) in 24 volunteers

Vol. No.	Time (in hours)														
	0	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**8.0**	**12.0**	**18.0**	**24.0**
1	0	1510	2750	2790	2810	2470	2500	2490	2480	1760	1010	710	360	170	20
2	0	1660	2180	2250	2430	2860	2750	2540	2200	1170	970	410	250	150	60
3	0	810	1050	1260	1620	1890	920	1570	1450	1300	1310	910	450	200	10
4	0	880	370	770	1770	1870	1460	1300	1200	850	700	440	290	150	00
5	0	180	610	1060	1730	2410	2950	3000	3030	2550	1830	1430	850	290	50
6	0	380	1450	2560	3560	3760	3250	2610	2240	1720	1230	1010	720	130	20
7	0	140	610	1100	1340	2410	2960	3030	3130	2410	1530	1060	530	210	130
8	0	50	810	1490	2370	2740	2940	2520	2150	1740	1400	820	440	140	30
9	0	170	1380	1550	1730	2120	2340	2200	2230	1650	1280	890	330	220	30
10	0	150	1250	1450	1640	1940	2520	2020	1840	1240	1090	1260	260	130	20
11	0	280	1660	1720	1920	2330	2150	1910	1630	1450	1290	950	320	140	30
12	0	1250	2470	2320	2200	2040	1880	1660	1500	1320	1100	870	650	350	20
13	0	1060	1230	1640	2500	3110	2710	2590	2380	1410	1620	1110	660	140	00
14	0	430	810	1460	1870	2910	3070	3240	3760	2270	1460	1080	710	460	30
15	0	630	1070	1680	1940	2250	1780	1530	1410	1270	1110	930	510	100	00
16	0	1170	1980	2650	2940	1800	1520	1320	1300	1220	990	620	300	160	20
17	0	720	1900	2460	2930	2510	3690	3700	3720	3090	2640	1370	730	520	30
18	0	1800	2010	2850	3550	3260	3170	2860	2390	1800	1200	920	640	450	30
19	0	870	1210	1770	2140	2440	3060	2860	2570	2440	1240	970	590	150	00
20	0	710	1020	1460	2320	2230	2100	1710	1620	1500	1110	710	580	150	00
21	0	720	1690	2110	2570	3310	3920	3820	3640	2640	1230	890	590	410	30
22	0	650	1020	1700	2830	2680	2470	2070	1730	1410	1030	950	420	240	20
23	0	120	650	1540	2330	4640	4370	4010	3660	3070	2330	1530	1270	670	30
24	0	330	890	1030	1290	1700	2330	2590	2910	1540	1490	1210	630	520	30
Mean	**0**	**694.583**	**1336.250**	**1777.917**	**2263.750**	**2570.000**	**2617.083**	**2464.583**	**2340.417**	**1784.167**	**1341.250**	**960.417**	**545.000**	**260.417**	**26.667**
S.D.	**0**	**508.048**	**626.480**	**589.613**	**620.903**	**685.147**	**800.655**	**769.579**	**816.171**	**621.036**	**428.641**	**277.575**	**230.123**	**159.904**	**26.811**
C.V.%	**0**	**73.144**	**46.883**	**33.163**	**27.428**	**26.659**	**30.593**	**31.226**	**34.873**	**34.808**	**31.958**	**28.901**	**42.224**	**61.403**	**100.542**

A – Reference Preparation; B – Test Preparation

Table.5.141 Bioequivalence of TABLET NISE containing NIMESULIDE 100 mg
Plasma concentration of 4-Hydroxynimesulide (ng/mL) of the Reference Preparation (A) in 24 volunteers

Vol. No.	Time (in hours)														
	0	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**8.0**	**12.0**	**18.0**	**24.0**
1	0	184	207	443	432	760	744	289	302	160	122	96	63	46	ND
2	0	558	597	1120	1270	1660	1070	1060	712	262	221	176	109	48	ND
3	0	613	654	1260	1450	1670	1230	1120	856	277	198	144	110	56	ND
4	0	206	110	457	427	767	725	280	294	154	112	92	71	46	ND
5	0	296	468	1110	1134	1180	1290	847	619	462	398	253	123	72	ND
6	0	181	112	417	409	765	732	276	286	156	118	89	59	44	ND
7	0	51.1	166	182	585	810	1190	1450	833	173	138	104	92	61.5	ND
8	0	182	108	401	402	776	752	275	280	160	123	87	59	43	ND
9	0	550	626	1620	1730	1870	1190	1080	956	276	212	156	103	73	ND
10	0	345	509	610	774	1200	1290	808	610	373	298	205	98	46	ND
11	0	60.8	177	193	607	803	629	1320	946	183	129	87	68	43	ND
12	0	26.4	159	632	477	655	885	778	660	472	265	189	96	48	ND
13	0	458	527	1000	1170	1410	1043	999	680	235	197	157	93	37	ND
14	0	196	222	460	453	783	771	527	397	217	146	115	79	49	ND
15	0	513	584	1097	1400	1521	1203	1059	824	250	174	125	94	45	ND
16	0	218	125	474	448	890	752	418	326	181	136	108	87	57	ND
17	0	308	483	827	955	1103	1317	845	651	459	322	268	164	96	ND
18	0	193	127	434	430	788	759	314	318	183	142	108	75	45	ND
19	0	63.1	181	199	606	833	1217	1465	865	200	162	123	108	72.5	ND
20	0	194	123	418	423	799	779	313	312	187	147	106	55	39	ND
21	0	450	556	1186	1299	1351	1063	860	697	249	151	96	67	42	ND
22	0	357	524	627	795	1223	1317	846	642	400	322	224	114	57	ND
23	0	72.8	192	210	628	826	656	1358	978	210	153	106	77	50	ND
24	0	38.4	174	649	498	678	912	816	692	499	289	205	110	55	ND
Mean	**0**	**263.108**	**321.292**	**667.750**	**783.417**	**1046.708**	**979.833**	**808.458**	**614.000**	**265.750**	**194.792**	**142.458**	**90.583**	**52.958**	**ND**
S.D.	**0**	**180.983**	**205.554**	**393.984**	**408.565**	**361.286**	**242.795**	**394.866**	**240.677**	**113.528**	**79.660**	**55.153**	**25.272**	**13.691**	**ND**
C.V.%	**0**	**68.786**	**63.977**	**59.002**	**52.152**	**34.516**	**24.779**	**48.842**	**39.198**	**42.720**	**40.895**	**38.715**	**27.899**	**25.853**	**ND**

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.142 Bioequivalence of TABLET containing NIMESULIDE 100 mg
Plasma concentration of Nimesulide (ng/mL) of the Test Preparation (B) in 24 volunteers

Vol. No.	Time (in hours)														
	0	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**8.0**	**12.0**	**18.0**	**24.0**
1	0	1040	2260	2000	1730	1830	1550	1380	1290	1000	750	500	340	140	00
2	0	850	1020	1430	1770	1900	960	1730	1550	1200	1410	900	450	190	10
3	0	220	600	1250	1660	2700	2860	3030	3550	2510	2180	1610	930	370	70
4	0	420	1050	1470	1730	2040	1850	1710	1530	1350	1130	820	620	180	30
5	0	960	1770	2440	2730	1770	2010	2000	2120	1990	1780	1710	770	370	70
6	0	510	1690	2250	2720	2300	3480	3490	3510	2880	2430	1160	520	310	70
7	0	1590	1800	2640	3340	3050	2960	2650	2180	1590	990	710	430	240	30
8	0	660	2000	2560	2930	3230	3850	3650	3360	2230	1030	760	380	260	80
9	0	890	1250	1730	2110	2020	1830	1630	1520	1480	1290	1000	590	130	30
10	0	510	1480	1900	2360	3100	3710	3610	3430	2430	1020	680	380	200	20
11	0	580	1240	1430	1810	2100	1580	1460	1210	980	860	530	310	210	00
12	0	350	1030	1560	1790	2060	1930	1730	1520	1350	1050	660	370	250	10
13	0	160	540	970	1530	2370	3500	2690	2240	1740	1060	900	500	160	20
14	0	1660	1900	2380	2960	2620	2470	2060	2010	1910	1160	860	510	320	10
15	0	1810	2330	2400	2580	3010	2900	2690	2350	1320	1120	560	400	300	20
16	0	960	1200	1410	1770	2040	1670	1470	1420	1050	960	710	600	130	00
17	0	330	520	920	1920	2020	1610	1450	1350	1000	850	590	440	300	20
18	0	330	760	1210	1880	2560	3100	3150	3180	2700	1980	1580	1000	440	20
19	0	530	1460	1710	3710	2910	2310	2040	1790	1600	1400	1120	710	410	10
20	0	290	760	1250	1490	2560	3110	3180	3280	3120	2850	1720	1070	500	30
21	0	200	960	1640	2520	2890	3090	2940	2650	2520	2290	1820	1530	150	00
22	0	320	1530	1700	1880	2270	2490	2350	2040	1800	1430	1040	480	240	00
23	0	300	1400	1600	1790	2610	2270	2170	1990	1390	1240	710	410	280	20
24	0	250	580	860	1230	1580	2280	2880	3520	2000	1680	1050	300	160	00
Mean	**0**	**655.000**	**1297.083**	**1696.250**	**2164.167**	**2397.500**	**2473.750**	**2380.833**	**2274.583**	**1797.500**	**1414.167**	**987.500**	**585.000**	**260.000**	**23.750**
S.D.	**0**	**477.557**	**539.698**	**526.034**	**634.507**	**472.406**	**779.860**	**741.479**	**822.916**	**635.187**	**561.968**	**410.527**	**292.976**	**103.209**	**24.639**
C.V.%	**0**	**72.909**	**41.609**	**31.012**	**29.319**	**19.704**	**31.525**	**31.144**	**36.179**	**35.337**	**39.738**	**41.572**	**50.081**	**39.696**	**103.742**

A – Reference Preparation; B – Test Preparation

Table.5.143 Bioequivalence of TABLET containing NIMESULIDE 100 mg
Plasma concentration of 4-Hydroxynimesulide (ng/mL) of the Test Preparation (B) in 24 volunteers

Vol. No.	Time (in hours)														
	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.0	6.0	8.0	12.0	18.0	24.0
1	0	576	618	1151	1400	1660	1190	1100	881	279	198	109	69	43	ND
2	0	21.5	161	630	1040	700	989	707	712	230	179	109	84	43	ND
3	0	187	106	432	434	783	773	269	294	153	105	81	56	39	ND
4	0	553	584	1520	1290	1770	1160	1050	816	264	201	157	101	67	ND
5	0	197	103	415	423	803	767	266	293	151	108	81	59	43	ND
6	0	61.6	115	470	653	520	937	560	321	244	203	164	109	71	ND
7	0	530	581	1640	1330	1710	1150	1050	887	266	199	146	97	52	ND
8	0	64.8	130	496	727	610	948	599	368	266	212	182	120	71	ND
9	0	195	116	430	430	1038	873	594	451	267	141	99	57	44	ND
10	0	24.1	148	548	457	625	829	446	657	565	335	258	141	83	ND
11	0	177	234	498	659	789	764	484	392	266	153	95	56	39	ND
12	0	69.4	118	536	662	589	928	582	351	278	187	109	77	52	ND
13	0	564	603	1104	1379	1637	1463	1162	849	452	274	164	97	56	ND
14	0	33.5	176	647	1061	1423	1016	645	562	257	203	104	78	38	ND
15	0	453	514	1085	1155	1417	1133	930	757	237	157	124	85	46	ND
16	0	199	121	449	561	1106	800	307	326	180	129	100	72	46	ND
17	0	209	318	532	744	1631	1094	503	425	204	132	100	75	54	ND
18	0	73.6	130	487	674	543	964	598	353	271	227	183	125	82	ND
19	0	430	511	1079	1195	1357	1123	930	828	239	175	127	81	44	ND
20	0	76.8	145	513	748	633	975	637	400	293	236	201	136	82	ND
21	0	207	131	447	651	961	1284	954	735	521	332	198	108	65	ND
22	0	36.1	163	565	478	648	1056	784	689	592	359	277	157	94	ND
23	0	189	125	415	432	1653	1491	1022	724	532	236	156	84	42	ND
24	0	136	201	553	683	756	1055	720	483	351	211	128	93	59	ND
Mean	**ND**	**219.308**	**256.333**	**693.417**	**802.750**	**1056.750**	**1031.750**	**704.125**	**564.750**	**306.583**	**203.833**	**143.833**	**92.375**	**56.458**	**ND**
S.D.	**ND**	**189.215**	**190.977**	**360.498**	**334.545**	**448.163**	**198.359**	**267.878**	**212.947**	**127.772**	**67.756**	**52.497**	**27.938**	**16.563**	**ND**
C.V.%	**ND**	**86.278**	**74.503**	**51.989**	**41.675**	**42.410**	**19.226**	**38.044**	**37.707**	**41.676**	**33.241**	**36.499**	**30.244**	**29.337**	**ND**

*ND – Not Detected; A – Reference Preparation; B – Test Preparation

Table.5.144 Pharmacokinetic parameters

C_{max} (ng/mL), t_{max} (hr.), AUC_{0-t} (ng. hr./mL), $AUC_{0-\infty}$, $t_{1/2}$, K_{el}

in 24 volunteers with the Test and Reference Preparation of Nimesulide (NSD)

Volunteer no.	C_{max}		t_{max}		AUC_{0-t}		$AUC_{0-\infty}$		$t_{1/2}$		K_{el}	
	A	B	A	B	A	B	A	B	A	B	A	B
1	2810.00	2260.00	2.0	1.0	1879.00	1259.00	1890.00	1345.00	3.30	4.18	0.21	0.17
2	2860.00	1900.00	2.5	2.5	1613.00	1540.00	1647.00	1544.00	3.80	3.27	0.18	0.21
3	1890.00	3550.00	2.5	4.0	1508.00	2644.00	1513.00	2679.00	3.36	3.76	0.21	0.18
4	1870.00	2040.00	2.5	2.5	1023.00	1606.00	1115.00	1623.00	4.33	0.83	0.16	0.18
5	3030.00	2730.00	4.0	2.0	2397.00	2446.00	2424.00	2493.00	3.61	4.68	0.19	0.15
6	3760.00	3510.00	2.5	4.0	2148.00	2554.00	2156.00	2588.00	3.04	3.65	0.23	0.19
7	3130.00	3340.00	4.0	2.0	2031.00	1950.00	2113.00	1963.00	4.36	3.43	0.16	0.20
8	2940.00	3850.00	3.0	3.0	1747.00	2171.00	1760.00	2216.00	3.26	3.88	0.21	0.18
9	2340.00	2110.00	3.0	2.0	1674.00	1708.00	1686.00	1721.00	3.43	3.62	0.20	0.19
10	2520.00	3710.00	3.0	3.0	1562.00	2010.00	1573.00	2020.00	3.29	3.05	0.21	0.23
11	2330.00	2100.00	2.5	2.5	1603.00	1204.00	1618.00	1344.00	3.52	4.69	0.20	0.15
12	2470.00	2060.00	1.0	2.5	1906.00	1465.00	1917.00	1470.00	3.94	3.25	0.18	0.21
13	3110.00	3500.00	2.5	3.0	2005.00	1708.00	2080.00	1716.00	3.67	3.12	0.19	0.22
14	3760.00	2960.00	4.0	2.0	2382.00	2024.00	2397.00	2028.00	3.46	3.16	0.20	0.22
15	2250.00	3010.00	2.5	2.5	1509.00	1912.00	1565.00	1922.00	3.84	3.36	0.18	0.21
16	2940.00	2040.00	2.0	2.5	1472.00	1431.00	1483.00	1517.00	3.53	4.42	0.20	0.16
17	3720.00	2020.00	4.0	2.5	2979.00	1349.00	2993.00	1360.00	3.24	3.90	0.21	0.18
18	3550.00	3180.00	2.0	4.0	2387.00	2699.00	2403.00	2708.00	3.69	3.11	0.19	0.22
19	3060.00	3710.00	3.0	2.0	1970.00	2180.00	2044.00	2184.00	3.47	3.26	0.20	0.21
20	2320.00	3280.00	2.0	4.0	1566.00	2974.00	1659.00	2988.00	4.22	3.23	0.16	0.21
21	3920.00	3090.00	3.0	3.0	2448.00	2864.00	2462.00	2951.00	3.37	3.89	0.21	0.18
22	2830.00	2490.00	2.0	3.0	1746.00	1799.00	1756.00	1946.00	3.45	4.29	0.20	0.16
23	4640.00	2610.00	2.5	2.5	3318.00	1669.00	3333.00	1679.00	3.48	3.47	0.20	0.20
24	2910.00	3520.00	4.0	4.0	2106.00	1713.00	2122.00	1785.00	3.71	3.15	0.19	0.22
N	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**
Mean	**2956.667**	**2857.083**	**2.750**	**2.750**	**1957.458**	**1953.292**	**1987.875**	**1991.250**	**3.599**	**3.527**	**0.195**	**0.193**
S.D.	**676.633**	**662.397**	**0.794**	**0.794**	**510.902**	**515.445**	**502.764**	**509.170**	**0.343**	**0.764**	**0.018**	**0.024**
C.V.%	**22.885**	**23.184**	**28.873**	**28.873**	**26.100**	**26.389**	**25.2915**	**25.570**	**9.525**	**21.659**	**9.090**	**12.369**

A – Reference Preparation B – Test Preparation

Table.5.145 Pharmacokinetic parameters

C_{max} (ng/mL), t_{max} (hr.), AUC_{0-t} (ng. hr./mL), $AUC_{0-\infty}$, $t_{1/2}$, K_{el}

in 24 volunteers with the Test and Reference Preparation of 4-Hydroxynimesulide (M1)

Volunteer no.	C_{max}		T_{max}		AUC_{0-t}		$AUC_{0-\alpha}$		$t_{1/2}$		K_{el}	
	A	B	A	B	A	B	A	B	A	B	A	B
1	760.00	1660.00	2.5	2.5	2978.00	6014.25	3248.60	6193.98	4.08	2.90	0.17	0.24
2	1660.00	1040.00	2.5	2.0	6156.18	4161.78	6380.38	4368.22	3.24	3.33	0.21	0.21
3	1670.00	783.00	2.5	2.5	6532.68	2780.20	6795.66	2995.32	3.25	3.82	0.21	0.18
4	767.00	1770.00	2.5	2.5	2935.68	6519.20	3213.76	6849.07	4.19	3.41	0.17	0.20
5	1290.00	803.00	3.0	2.5	6491.93	2815.95	6892.45	3061.67	3.85	3.96	0.18	0.17
6	765.00	937.00	2.5	3.0	2819.68	3910.75	3075.01	4406.96	4.02	4.84	0.17	0.14
7	1450.00	1710.00	3.5	2.5	4362.98	6460.45	4721.22	6698.74	4.04	3.18	0.17	0.22
8	776.00	948.00	2.5	3.0	2816.68	4219.60	3063.07	4705.64	3.97	4.74	0.17	0.15
9	1870.00	1038.00	2.5	2.5	7065.18	3500.95	7424.78	3713.96	3.41	3.35	0.20	0.21
10	1290.00	829.00	3.0	3.0	5426.68	5075.87	5651.21	5641.00	3.38	4.72	0.20	0.15
11	1320.00	789.00	3.5	2.5	3840.08	3391.00	4053.99	3585.56	3.45	3.46	0.20	0.20
12	885.00	928.00	3.0	3.0	4505.88	3587.98	4749.38	3887.59	3.52	3.99	0.20	0.17
13	1410.00	1637.00	2.5	2.5	5502.18	6768.78	5666.31	7018.33	3.07	3.09	0.23	0.22
14	783.00	1423.00	2.5	2.5	3473.93	4413.78	3748.51	4600.46	3.88	3.40	0.18	0.20
15	1521.00	1417.00	2.5	2.5	5932.68	5456.78	6133.70	5667.40	3.10	3.17	0.22	0.22
16	890.00	1106.00	2.5	2.5	3393.18	3325.53	3744.55	3582.42	4.27	3.87	0.16	0.18
17	1317.00	1631.00	3.0	2.5	6549.43	4235.28	7164.54	4510.25	4.44	3.53	0.16	0.20
18	788.00	964.00	2.5	3.0	3126.18	4277.08	3388.85	4874.19	4.05	5.05	0.17	0.14
19	1465.00	1357.00	3.5	2.5	4717.98	5485.03	5160.15	5682.77	4.23	3.11	0.16	0.22
20	799.00	975.00	2.5	3.0	2993.18	4585.93	3201.31	5170.84	3.70	4.94	0.19	0.14
21	1351.00	1284.00	2.5	3.0	5255.93	5411.78	5441.70	5744.91	3.07	3.55	0.23	0.20
22	1317.00	1056.00	3.0	3.0	5793.18	5692.33	6088.88	6292.58	3.60	4.43	0.19	0.16
23	1358.00	1653.00	3.5	2.5	4147.58	5232.53	4403.48	5407.12	3.55	2.88	0.20	0.24
24	912.00	1055.00	3.0	3.0	4829.38	4284.78	5119.04	4614.98	3.65	3.88	0.19	0.18
N	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**
Mean	**1183.916**	**1199.708**	**2.792**	**2.667**	**4651.935**	**4650.316**	**4938.772**	**4969.748**	**3.709**	**3.775**	**0.189**	**0.189**
SD	**348.512**	**333.658**	**0.388**	**0.282**	**1395.943**	**1156.823**	**1427.068**	**1172.206**	**0.411**	**0.677**	**0.022**	**0.031**
CV%	**29.437**	**27.812**	**13.890**	**10.587**	**30.008**	**24.876**	**28.895**	**23.587**	**11.087**	**17.927**	**11.407**	**16.565**

A – Reference Preparation B – Test Preparation

Table.5.146 Pharmacokinetic parameters in 24 volunteers with the Test and Reference Preparation of Nimesulide 100 mg tablet

Pharmacokinetic parameters		Nimesulide (NSD)		4-Hydroxynimesulide (M1)	
		Reference Preparation (A)	Test Preparation (B)	Reference Preparation (A)	Test Preparation (B)
C_{max} (ng./mL.)	Mean	2956.667	2857.083	1183.916	1199.708
	± S.D.	676.633	662.397	348.512	333.658
t_{max} (hr.)	Mean	2.750	2.750	2.792	2.667
	± S.D.	0.794	0.794	0.388	0.282
AUC_{0-t} (ng. hr./mL.)	Mean	1957.458	1953.292	4651.935	4650.316
	± S.D.	510.902	515.445	1395.943	1156.823
$AUC_{0-\infty}$ (ng. hr./mL.)	Mean	1987.875	1991.250	4938.772	4969.748
	± S.D.	502.764	509.170	1427.068	1172.206
k_{el} ($hr.^{-1}$)	Mean	0.195	0.193	0.189	0.189
	± S.D.	0.018	0.024	0.022	0.031
$t_{1/2}$ (hr.)	Mean	3.599	3.527	3.709	3.775
	± S.D.	0.343	0.764	0.411	0.677
Relative Bioavailability (%)		**100%**	**99.78%**	**100%**	**99.97%**

Table.5.147 90% confidence interval (CI) with the Test and Reference Preparation

Confidence Interval (CI)		Nimesulide (NSD)	4-Hydroxynimesulide (M1)
C_{max}	Untransformed Data	0.81507 – 1.12065	0.82773 - 1.19467
	Ln transformed Data	0.82372 – 1.11154	0.97249 - 1.03314
AUC_{0-t}	Untransformed Data	0.81764 – 1.17808	0.80864 - 1.19077
	Ln transformed Data	0.94002 – 1.05744	0.97464 - 1.02869
$AUC_{0-\infty}$	Untransformed Data	0.82967 – 1.17356	0.81314 - 1.19780
	Ln transformed Data	0.94421 – 1.05601	0.97599 - 1.02859

Table.5.148 ANOVA Summary for C_{max} Values untransformed data

		Nimesulide (NSD)				4-Hydroxynimesulide (M1)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	23	11.45600	0.49809	1.20451	N.S.	2651841.8125	115297.47	0.94069	N.S.
Treatment	1	0.119916	0.11992	0.28999	N.S.	2992.520833	2992.521	0.02442	N.S.
Period	1	0.080786	0.08079	0.19536	N.S.	5830.020833	5830.021	0.04757	N.S.
Error	22	9.097423	0.41352	-	-	2696460.958	122566.41	-	-
Total	47	20.75413	-	-	-	5357125.3125	-	-	-

Table.5.149 ANOVA Summary for in Ln C_{max} Values Ln transformed data

		Nimesulide (NSD)				4-Hydroxynimesulide (M1)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	23	1.45622	0.06331	1.29664	N.S.	1.917000	0.08335	0.94108	N.S.
Treatment	1	0.01619	0.01619	0.33157	N.S.	0.00500	0.00500	0.05646	N.S.
Period	1	0.00466	0.00466	0.09552	N.S.	0.00592	0.00592	0.06685	N.S.
Error	22	1.07424	0.04883	-	-	1.948456	0.08857	-	-
Total	47	2.55131	-	-	-	3.87638	-	-	-

Table.5.150 ANOVA Summary for AUC_{0-t} Values untransformed data

		Nimesulide (NSD)				4-Hydroxynimesulide (M1)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	23	654.226	28.4446	1.18920	N.S.	31052205.34	1350095.88	0.66703	N.S.
Treatment	1	0.02213	0.02213	0.00093	N.S.	31.612664104	31.61266410	0.00002	N.S.
Period	1	30.8233	30.8233	1.28865	N.S.	17228.828542	17228.8285	0.00851	N.S.
Error	22	526.219	23.9190	-	-	44529219.294	2024055.42	-	-
Total	47	1211.29	-	-	-	75598685.080	-	-	-

D.F. – Degree of Freedom

S.S. – Sum of Squares

M.S.S. – Mean Sum of Squares

F. – M.S.S. / Error M.S.S.

P - Probability

N.S. – Non significant

S – Significant

Table.5.151 ANOVA Summary for Ln AUC_{0-t} Values Ln transformed data

		Nimesulide (NSD)				4-Hydroxynimesulide (M1)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	23	1.70569	0.07416	1.30660	N.S.	1.544265	0.06714	0.67674	N.S.
Treatment	1	0.00017	0.00017	0.00306	N.S.	0.00247	0.00247	0.02490	N.S.
Period	1	0.09617	0.09617	1.69429	N.S.	0.00020	0.00020	0.00202	N.S.
Error	22	1.24869	0.05676	-	-	2.182697	0.09921	-	-
Total	47	3.05072	-	-	-	3.72963	-	-	-

Table.5.152 ANOVA Summary for AUC_{0-inf} Values untransformed data

		Nimesulide (NSD)				4-Hydroxynimesulide (M1)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	23	633.2174	27.53119	1.17576	N.S.	30571046.83	1329175.95	0.61108	N.S.
Treatment	1	0.0135611	0.0135611	0.00058	N.S.	11513.1661239	11513.166124	0.00529	N.S.
Period	1	29.20286	29.20286	1.24715	N.S.	19640.441286	19640.4413	0.00903	N.S.
Error	22	515.14404	23.41564	-	-	47852894.571	2175131.57	-	-
Total	47	1177.5779	-	-	-	78455095.008	-	-	-

Table.5.153 ANOVA Summary for Ln AUC_{0-inf} VALUES Ln transformed data

		Nimesulide (NSD)				4-Hydroxynimesulide (M1)			
Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	23	1.55646	0.06767	1.28391	N.S.	1.334756	0.05803	0.61351	N.S.
Treatment	1	0.00000	0.00000	0.00003	N.S.	0.00470	0.00470	0.04966	N.S.
Period	1	0.08310	0.08310	1.57653	N.S.	0.00022	0.00022	0.00234	N.S.
Error	22	1.15958	0.05271	-	-	2.080998	0.09459	-	-
Total	47	2.79913	-	-	-	3.42067	-	-	-

D.F. – Degree of Freedom

S.S. – Sum of Squares

M.S.S. – Mean Sum of Squares

F. – M.S.S./Error M.S.S.

P - Probability

N.S. – Non significant

S – Significant

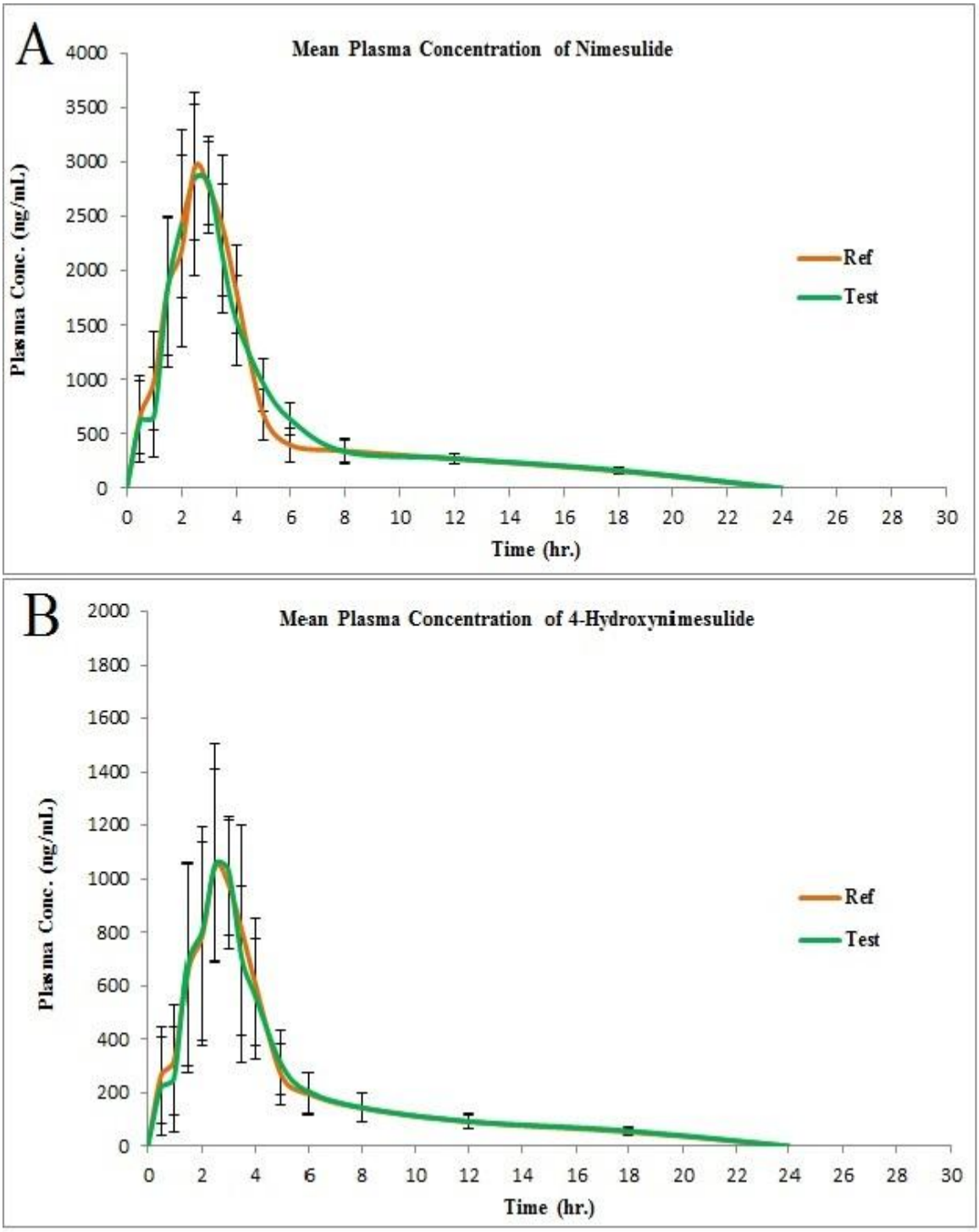


Figure.5.99 Graphical representation of mean plasma concentration versus time of (A) NSD and (B) M1 following 100 mg single oral dose of test and reference preparation to 24 healthy human volunteers

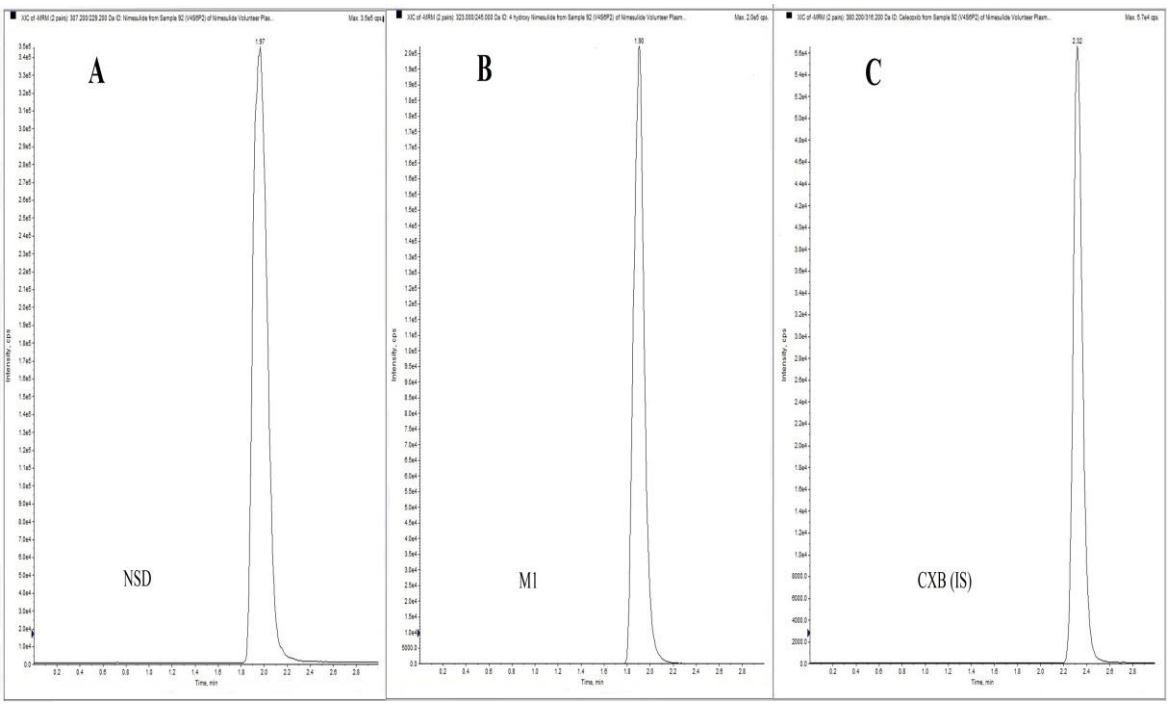


Figure.5.100 Representative MRM chromatograms of volunteer plasma samples : (A) *in vivo* plasma sample showing NSD peak obtained following single oral dose of 100 mg tablets to healthy humans, (B) *in vivo* plasma sample showing M1 peak obtained following single oral dose of 100 mg tablets to healthy humans and (C) CXB (IS) spiked in human volunteer plasma at 5 µg/mL

5.12.2 Pharmacokinetics and statistical analysis

The volunteer plasma samples were analysed by the developed and validated LC-ESI-MS/MS method which has already mentioned in the section 5.9.10 and 5.9.11, respectively and also the plasma concentrations and mean plasma concentrations were determined and reported in Table.5.140 to Table.5.143. The mean plasma concentration-time profile graphs of both NSD and M1 were demonstrated in Figure.5.99 for both Test and Reference preparation and also representative chromatograms of volunteer plasma samples of both NSD and M1 with IS (CXB) were shown in Figure.5.100. The reference tablet as a single oral dose in the fasting state produced the maximum plasma concentration of 2956.667 ± 676.633 ng/mL (C_{max}) at the time of 2.750 ± 0.794 hr. (t_{max}) for NSD and 1183.916 ± 348.512 ng/mL (C_{max}) at the time of 2.792 ± 0.388 hr. (t_{max}) for M1 whereas the test product in the fasting state produced the maximum plasma concentration of 2857.083 ± 662.397 ng/mL (C_{max}) at

the time of 2.750 ± 0.794 hr. (t_{max}) for NSD and 1199.708 ± 333.658 ng/mL (C_{max}) at the time of 2.667 ± 0.282 hr. (t_{max}) for M1, respectively (see Table.5.146).

After administration of the reference tablet produced the area under plasma concentration time curve (AUC_{0-t}) 1957.458 ± 510.902 ng. hr./mL for NSD and 1953.292 ± 515.445 ng. hr./mL for M1, whereas in case of the test tablet produced the area under plasma concentration curve (AUC_{0-t}) 4651.935 ± 1395.943 ng. hr./mL for NSD and 4650.316 ± 1156.823 ng. hr./mL for M1, respectively (Table.5.146).

When administered as a single oral dose, in the fasting state, the reference product of Nimesulide (NSD) produced the area under plasma concentration time curve upto infinity ($AUC_{0-\infty}$) 1987.875 ± 502.764 ng. hr./mL (NSD) and 4938.772 ± 1427.068 ng. hr./mL (M1), and for the test product ($AUC_{0-\infty}$) 1991.250 ± 509.170 ng. hr./mL (NSD) and 4969.748 ± 1172.206 ng. hr./mL (M1) (Table.5.146).

Administration of Nimesulide (NSD) produced the plasma elimination half-life, i.e., $t_{1/2}$ 3.599 ± 0.343 hr. (NSD) and 3.709 ± 0.411 hr. (M1) for the reference and 3.527 ± 0.764 (NSD) and 3.775 ± 0.677 (M1) for the test, respectively (Table.5.146). While administration of the reference and test preparation produced the plasma elimination rate constant, i.e., k_{el} 0.195 ± 0.018 $hr.^{-1}$ and 0.193 ± 0.024 $hr.^{-1}$ for NSD and 0.189 ± 0.022 $hr.^{-1}$ and 0.189 ± 0.031 $hr.^{-1}$ for M1, respectively (Table.5.146).

As an outcome of comparison of the AUC_{0-t} for Reference and Test after single oral dose administration, the relative bioavailability (%) of the test product of Nimesulide (NSD) tablet was 99.78% for NSD and 99.97% for M1 (Table.5.146). The 90% confidence interval (CI) for Untransformed data of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values of the test product were 0.81507 – 1.12065; 0.81764 – 1.17808 and 0.82967 – 1.17356 for NSD and 0.82773 - 1.19467; 0.80864 - 1.19077 and 0.81314 - 1.19780 for M1, respectively of that the reference product. Whereas Ln transformed data of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values of the test product were 0.82372 – 1.11154; 0.94002 – 1.05744 and 0.94421 – 1.05601 for NSD and 0.97249 - 1.03314; 0.97464 - 1.02869 and 0.97599 - 1.02859 for M1, respectively of that the reference product (see Table.5.147).

5.12.3 Statistical inference

The 90% CI, i.e., confidence interval for C_{max}, Ln C_{max}, AUC_{0-t}, Ln AUC_{0-t}, $AUC_{0-\infty}$ and Ln $AUC_{0-\infty}$ values of the Test preparation of Nimesulide (NSD) tablet were within the accepted

limit of that of the Reference preparation, i.e., CI = 0.8 to 1.2. ANOVA (Subjects, Treatment, Period) was applied to the C_{max}, Ln C_{max}, AUC_{0-t}, Ln AUC_{0-t}, $AUC_{0-\infty}$ and Ln $AUC_{0-\infty}$ values. Finally the results were found statistically nonsignificant, i.e., there was no statistically significant difference for the analysis values of the C_{max}, Ln C_{max}, AUC_{0-t}, Ln AUC_{0-t}, $AUC_{0-\infty}$ and Ln $AUC_{0-\infty}$ (see Table.5.148 to Table.5.153).

5.12.4 Discussion

The present ESI-LC-MS/MS bioanalytical method has been developed and validated for the simultaneous determination of both Nimesulide (NSD) and its active metabolite 4-hydroxynimesulide (M1) in human plasma which is rapid, specific, highly sensitive, robust, simple and high-throughput tandem mass spectrometric method. Now, the proposed bioanalytical method has significant advantages over the other techniques or previously reported methods, like Barrientos-Astigarraga *et al*., 2001, Carini *et al*., 1998 and Kucukguzel *et al*., 2005. The limit of detection (LOD) is 3 ng/mL and the lower limit of quantification (LLOQ) is 10 ng/mL for both NSD and M1 which is more sensitive than some published HPLC and LC-MS/MS methods (Sora *et al*., 2007; Kim *et al*., 2012; Bianchi *et al*., 2006; Carini *et al*., 1998; Giachetti and Tenconi. 1998; Kumar *et al*., 2006). Extraction recovery of both NSD and M1 is in between 99% to 106% and that is much better than others methods, such as Sora *et al*., 2007; Barrientos-Astigarraga *et al*., 2001; Giachetti and Tenconi. 1998; Kucukguzel *et al*., 2005. The plasma extraction procedure is very simple one step liquid-liquid extraction (LLE) technique using just only 100 µL of plasma and it is very economic, i.e., cost-effective, also provides higher levels of reproducibility, sensitivity and more precise assessment. Whereas in the other published methods (like Sora *et al*., 2007; Kim *et al*., 2012; Barrientos-Astigarraga *et al*., 2001; Giachetti and Tenconi. 1998; Kumar *et al*., 2006; Kucukguzel *et al*., 2005), plasma extraction procedures were complicated, using many solvents, lengthy process, i.e., more time consuming, not so cost-effective or economic and also using more quantity of plasma than the present method. Now, the total chromatographic run time of the present method is 3.0 min. that is comparatively shorter than the other methods such as Sora *et al*., 2007; Kim *et al*., 2012; Reddy. 2009; Barrientos-Astigarraga *et al*., 2001; Carini *et al*., 1998; Giachetti and Tenconi. 1998; Kumar *et al*., 2006; Kucukguzel *et al*., 2005. So, because of the suitable sample preparation and the shorter run time provides the method potentiality for high sample throughput, i.e., a large number of plasma samples can be analysed per day. The present bioanalytical method has been

successfully validated as per the USFDA and EMA guidelines and all the validation data were within the acceptance criteria.

The developed and fully validated method was successfully employed for simultaneous quantification of both NSD and M1 in human plasma after single oral dose administration of Nimesulide 100 mg tablets in 24 healthy Indian male volunteers under fasting state. Now in some published methods, only NSD was quantified in human plasma for the pharmacokinetic as well as bioequivalence study but not its active metabolite M1 (Sora *et al*., 2007; Kim *et al*., 2012; Reddy. 2009; Barrientos-Astigarraga *et al*., 2001; Kumar *et al*., 2006). Accordingly review from the literature, the main and biologically more active metabolite of drug NSD is 4-hydroxynimesulide (M1) than others such as M2, M3, M4 and M5. M1 has maintained anti-inflammatory and analgesic properties. After 14 days of treatment, plasma concentrations of M1 is significantly higher than those measured on day 1. It can be detected in plasma samples in the same time interval as required for plasma concentration profiles for the parent drug or prodrug (i.e., NSD) in human subjects. Because its biological activity is similar to the parent drug, its concentration was also measured in plasma samples of healthy human subjects during bioavailability or bioequivalence studies (BA/BE) (Sora *et al*., 2007 and Macpherson *et al*., 2013). According to Bianchi *et al*., (2006), M1 exhibits anti-inflammatory and analgesic properties to a lesser extent than its parent drug. Therefore, it is very important to quantify both NSD and M1 in human plasma by LC-MS/MS and to perform the pharmacokinetic as well as bioequivalence study for the drug development process. So finally it is confirmed that the proposed method has significant advantages over earlier published methods for simultaneous quantification of both the analytes NSD and M1 in human plasma without any interference or matrix effect, shorter run time (3.0 min.), wider linearity range (10 ng/mL to 6000 ng/mL) with a proper sensitivity (LLOQ 10 ng/mL) and simple one-step reproducible LLE extraction with higher recovery. Consequently, the pharmacokinetic parameters are become more essential to provide some guidance to clinical application and investigation and also this approach in the near future will enhance the ability of pharmacokinetics or quantitative study of the active metabolites in plasma.

Chapter – 6:

Summary and Conclusion



6.1 Summary

Bioanalytical method has been developed, validated and also successfully applied for the simultaneous quantification of three drugs such as Aspirin or Acetylsalicylic Acid (ASA), Enalapril (EPL) and Nimesulide (NSD) with their active metabolites like Salicylic Acid (SA), Enalaprilat (EPLT) and 4-Hydroxynimesulide (M1) in human plasma.

Summary of bioanalytical study of aspirin or acetylsalicylic acid (ASA) and salicylic acid (SA) in healthy human subjects by LC-ESI-MS/MS

<table>
<tr><th colspan="14">Aspirin (Acetylsalicylic Acid) 150 mg</th></tr>
<tr><th>Drug, Metabolite and IS</th><th colspan="6">MS Parameters</th><th colspan="2">Chromatographic Conditions</th><th>Plasma Analysis Method</th><th colspan="2">Pharmacokinetic Parameters
Reference Preparation (A) and Test Preparation (B)</th><th colspan="4"></th></tr>
<tr><td rowspan="16">ASA, SA and HCTZ</td><td colspan="6"></td><td colspan="2"></td><td></td><td colspan="2"></td><td colspan="2">ASA</td><td colspan="2">SA</td></tr>
<tr><td colspan="6"></td><td colspan="2"></td><td></td><td colspan="2"></td><td>(A)</td><td>(B)</td><td>(A)</td><td>(B)</td></tr>
<tr><td colspan="6">Compound-Dependent Parameters</td><td colspan="2">Mobile Phase</td><td>Liquid-Liquid Extraction (LLE)</td><td>C_{max} (ng./mL.)</td><td>Mean</td><td>1200.785</td><td>1260.155</td><td>9972.108</td><td>9782.580</td></tr>
<tr><td colspan="6"></td><td colspan="2"></td><td></td><td></td><td>± S.D.</td><td>239.627</td><td>419.985</td><td>938.046</td><td>859.788</td></tr>
<tr><td colspan="6">Acetylsalicylic Acid (ASA)</td><td colspan="2">Acetonitrile : 10 mM ammonium formate in water :: 90:10 (v/v) of total pH 5.9</td><td>Plasma Volume : 300 µL</td><td>t_{max} (hr.)</td><td>Mean</td><td>0.398</td><td>0.421</td><td>1.250</td><td>1.317</td></tr>
<tr><td colspan="6"></td><td colspan="2"></td><td></td><td></td><td>± S.D.</td><td>0.085</td><td>0.086</td><td>0.653</td><td>0.725</td></tr>
<tr><td>Q1 Mass (Da)</td><td>Q3 Mass (Da)</td><td>Dwell (msec)</td><td colspan="3">Parameters (Volt/eVolt)</td><td colspan="2">Stationary Phase or Column</td><td>Extracting Solvent : Ethyl Acetate and Methyl Tert-Butyl Ether (50:50, v/v)</td><td>AUC_{0-t} (ng. hr./mL.)</td><td>Mean</td><td>1913.138</td><td>1833.968</td><td>48941.087</td><td>48127.915</td></tr>
<tr><td></td><td></td><td></td><td colspan="3"></td><td colspan="2"></td><td></td><td></td><td>± S.D.</td><td>383.810</td><td>379.589</td><td>8747.326</td><td>9057.221</td></tr>
<tr><td rowspan="8">179</td><td rowspan="8">137</td><td rowspan="8">200</td><td>DP</td><td>-10</td><td colspan="2">Phenomenex Kinetex C18 column, 50 mm x 3 mm (length x inner diameter), 5 µm of particle size</td><td>Reconstitute Volume : 200 µL</td><td>$AUC_{0-\infty}$ (ng. hr./mL.)</td><td>Mean</td><td>1959.722</td><td>1871.502</td><td>57402.729</td><td>56021.315</td></tr>
<tr><td></td><td></td><td colspan="2"></td><td rowspan="7"></td><td></td><td>± S.D.</td><td>390.308</td><td>380.564</td><td>10738.070</td><td>10784.167</td></tr>
<tr><td>FP</td><td>-400</td><td colspan="2">Column Oven Temperature : 60 °C</td><td>k_{el} (hr.-1)</td><td>Mean</td><td>0.387</td><td>0.416</td><td>0.187</td><td>0.194</td></tr>
<tr><td></td><td></td><td colspan="2"></td><td></td><td>± S.D.</td><td>0.025</td><td>0.022</td><td>0.024</td><td>0.027</td></tr>
<tr><td>EP</td><td>-10</td><td colspan="2">Autosampler Temperature : 15 °C</td><td>$t_{1/2}$ (hr.)</td><td>Mean</td><td>1.798</td><td>1.671</td><td>3.764</td><td>3.648</td></tr>
<tr><td></td><td></td><td colspan="2"></td><td></td><td>± S.D.</td><td>0.111</td><td>0.088</td><td>0.522</td><td>0.538</td></tr>
<tr><td>CEP</td><td>-13</td><td colspan="2">Flow Rate : 0.5 mL/min.</td><td>Relative Bioavailability (%)</td><td></td><td>100%</td><td>95.86%</td><td>100%</td><td>98.34%</td></tr>
</table>

Aspirin (Acetylsalicylic Acid) 150 mg

<table>
<tr><th>Drug, Metabolite and IS</th><th colspan="6">MS Parameters</th><th>Chromatographic Conditions</th><th>Plasma Analysis Method</th><th colspan="5">Pharmacokinetic Parameters
Reference Preparation (A) and Test Preparation (B)</th></tr>
<tr><td rowspan="20"></td><td colspan="6" rowspan="2"></td><td rowspan="2"></td><td rowspan="20"></td><td rowspan="3"></td><td rowspan="3"></td><td colspan="2">ASA</td><td colspan="2">SA</td></tr>
<tr><td>(A)</td><td>(B)</td><td>(A)</td><td>(B)</td></tr>
<tr><td colspan="4"></td><td>CE</td><td>-10</td><td>Sample Injection Volume : 20 µL</td><td colspan="6" rowspan="2"></td></tr>
<tr><td colspan="4"></td><td>CXP</td><td>-6</td><td>Total Run Time: 1.8 min.</td></tr>
<tr><td colspan="4">Salicylic Acid (SA)</td><td colspan="2"></td><td>Anatyte Retention Time (RT) :</td></tr>
<tr><td>Q1 Mass (Da)</td><td>Q3 Mass (Da)</td><td>Dwell (msec)</td><td colspan="2">Parameters (Volt/eVolt)</td><td>ASA : 0.45 min.</td><td colspan="6"></td></tr>
<tr><td rowspan="6">137</td><td rowspan="6">93</td><td rowspan="6">200</td><td>DP</td><td>-19</td><td>SA : 0.43 min.</td><td colspan="6"></td></tr>
<tr><td>FP</td><td>-400</td><td>HCTZ (IS) : 0.46 min.</td><td colspan="6" rowspan="19"></td></tr>
<tr><td>EP</td><td>-10</td><td rowspan="18"></td></tr>
<tr><td>CEP</td><td>-11.95</td></tr>
<tr><td>CE</td><td>-27</td></tr>
<tr><td>CXP</td><td>-10</td></tr>
<tr><td colspan="6"></td></tr>
<tr><td colspan="6">Hydrochlorothiazide (HCTZ, IS)</td></tr>
<tr><td>Q1 Mass (Da)</td><td>Q3 Mass (Da)</td><td>Dwell (msec)</td><td colspan="3">Parameters (Volt/eVolt)</td></tr>
<tr><td rowspan="7">295.90</td><td rowspan="7">268.90</td><td rowspan="7">200</td><td>DP</td><td>-20</td></tr>
<tr><td>FP</td><td>-400</td></tr>
<tr><td>EP</td><td>-10</td></tr>
<tr><td>CEP</td><td>-15.92</td></tr>
<tr><td>CE</td><td>-25</td></tr>
<tr><td>CXP</td><td>-15</td></tr>
<tr><td></td><td></td></tr>
<tr><td colspan="6">Source and Gas Parameters</td></tr>
<tr><td rowspan="7"></td><td>CUR</td><td>20 psi</td><td colspan="3" rowspan="7"></td></tr>
<tr><td>CAD</td><td>4 psi</td></tr>
<tr><td>ISV</td><td>-4500 V</td></tr>
<tr><td>TEM</td><td>400 °C</td></tr>
<tr><td>GS1</td><td>50 psi</td></tr>
<tr><td>GS2</td><td>50 psi</td></tr>
</table>

Summary of bioanalytical study of Enalapril (EPL) and Enalaprilat (EPLT) in healthy human subjects by LC-ESI-MS/MS

<table>
<tr><th colspan="13">Enalapril Maleate 20 mg</th></tr>
<tr><th>Drug, Metabolite and IS</th><th colspan="6">MS Parameters</th><th>Chromatographic Conditions</th><th>Plasma Analysis Method</th><th colspan="3">Pharmacokinetic Parameters
Reference Preparation (A) and Test Preparation (B)</th><th colspan="4"></th></tr>
<tr><td rowspan="20">EPL, EPLT and TBM</td><td colspan="6" rowspan="2"></td><td rowspan="2"></td><td rowspan="2"></td><td colspan="2" rowspan="2"></td><td colspan="2">EPL</td><td colspan="2">EPLT</td></tr>
<tr><td>(A)</td><td>(B)</td><td>(A)</td><td>(B)</td></tr>
<tr><td colspan="6">Compound-Dependent Parameters</td><td>Mobile Phase</td><td>Protein Precipitation Technique (PPT)</td><td>C_{max} (ng./mL.)</td><td>Mean</td><td>254.313</td><td>249.788</td><td>121.321</td><td>117.948</td></tr>
<tr><td colspan="6"></td><td></td><td></td><td></td><td>± S.D.</td><td>16.182</td><td>15.499</td><td>10.766</td><td>11.554</td></tr>
<tr><td colspan="6">Enalapril (EPL)</td><td>0.1% formic acid in water, pH 2.45 at pump A and 0.1% formic acid in methanol at pump B</td><td>Plasma Volume : 300 µL</td><td>t_{max} (hr.)</td><td>Mean</td><td>0.681</td><td>0.806</td><td>2.250</td><td>2.700</td></tr>
<tr><td colspan="6"></td><td></td><td></td><td></td><td>± S.D.</td><td>0.270</td><td>0.253</td><td>0.487</td><td>0.535</td></tr>
<tr><td>Q1 Mass (Da)</td><td>Q3 Mass (Da)</td><td>Dwell (msec)</td><td colspan="2">Parameters (Volt/eVolt)</td><td></td><td>Stationary Phase or Column</td><td>Precipitant : Ice Cold Acetonitrile</td><td>AUC_{0-t} (ng. hr./mL.)</td><td>Mean</td><td>317.479</td><td>315.294</td><td>1043.107</td><td>1052.904</td></tr>
<tr><td colspan="6"></td><td></td><td></td><td></td><td>± S.D.</td><td>16.478</td><td>22.334</td><td>50.296</td><td>60.532</td></tr>
<tr><td rowspan="11">377.2</td><td rowspan="11">234.2</td><td rowspan="11">200</td><td>DP</td><td>30</td><td rowspan="2">Phenomenex Kinetex C18 column, 50 mm x 3 mm (length x inner diameter), 5 µm of particle size</td><td rowspan="11">Supernatant Organic Layer Volume : 300 µL</td><td>$AUC_{0-\infty}$ (ng. hr./mL.)</td><td>Mean</td><td>319.834</td><td>317.747</td><td>1086.819</td><td>1099.888</td></tr>
<tr><td></td><td></td><td></td><td>± S.D.</td><td>16.415</td><td>22.250</td><td>53.305</td><td>56.189</td></tr>
<tr><td>FP</td><td>400</td><td>Column Oven Temperature : N.A.</td><td>k_{el} (hr.-1)</td><td>Mean</td><td>0.497</td><td>0.486</td><td>0.075</td><td>0.073</td></tr>
<tr><td></td><td></td><td></td><td>± S.D.</td><td>0.021</td><td>0.020</td><td>0.004</td><td>0.005</td></tr>
<tr><td>EP</td><td>11</td><td>Autosampler Temperature : 15 °C</td><td>$t_{1/2}$ (hr.)</td><td>Mean</td><td>1.396</td><td>1.429</td><td>9.249</td><td>9.551</td></tr>
<tr><td></td><td></td><td></td><td>± S.D.</td><td>0.059</td><td>0.058</td><td>0.532</td><td>0.619</td></tr>
<tr><td>CEP</td><td>29.73</td><td>Flow Rate : 0.5 mL/min.</td><td>Relative Bioavailability (%)</td><td></td><td>100%</td><td>99.31%</td><td>100%</td><td>100.94%</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>CE</td><td>30</td><td>Sample Injection Volume : 10 µL</td><td colspan="6" rowspan="3"></td></tr>
<tr><td>CXP</td><td>4</td><td>Total Run Time: 3.0 min.</td></tr>
<tr><td colspan="3">Enalaprilat (EPLT)</td><td colspan="2"></td><td>Anatyte Retention Time (RT) :</td></tr>
<tr><td>Q1 Mass (Da)</td><td>Q3 Mass (Da)</td><td>Dwell (msec)</td><td colspan="2">Parameters (Volt/eVolt)</td><td>EPL : 2.13 min.</td><td colspan="6" rowspan="2"></td></tr>
<tr><td rowspan="6">349.1</td><td rowspan="6">206.1</td><td rowspan="6">200</td><td>DP</td><td>30</td><td>EPLT : 2.10 min.</td></tr>
<tr><td>FP</td><td>400</td><td>TBM (IS) : 2.23 min.</td><td rowspan="6"></td><td rowspan="6"></td><td colspan="6" rowspan="6"></td></tr>
<tr><td>EP</td><td>11</td><td rowspan="5"></td></tr>
<tr><td>CEP</td><td>29.08</td></tr>
<tr><td>CE</td><td>30</td></tr>
<tr><td>CXP</td><td>4</td></tr>
<tr><td colspan="6"></td></tr>
</table>

Enalapril Maleate 20 mg

Drug, Metabolite and IS	MS Parameters					Chromatographic Conditions	Plasma Analysis Method	Pharmacokinetic Parameters Reference Preparation (A) and Test Preparation (B)				
										EPL		EPLT
										(A)	(B)	(A)	(B)
	Tolbutamide (TBM, IS)											
	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Parameters (Volt/eVolt)								
	271.1	155.0	200	DP	30							
				FP	400							
				EP	11							
				CEP	27.29							
				CE	30							
				CXP	4							
	Source and Gas Parameters											
		CUR	30 psi									
		CAD	6 psi									
		ISV	5000 V									
		TEM	400 °C									
		GS1	55 psi									
		GS2	45 psi									

Summary of bioanalytical study of Nimesulide (NSD) and 4-hydroxynimesulide (M1) in healthy human subjects by LC-ESI-MS/MS

<table>
<tr><th colspan="13">Nimesulide 100 mg</th></tr>
<tr><th>Drug, Metabolite and IS</th><th colspan="5">MS Parameters</th><th>Chromatographic Conditions</th><th>Plasma Analysis Method</th><th colspan="2">Pharmacokinetic Parameters
Reference Preparation (A) and Test Preparation (B)</th><th colspan="4"></th></tr>
<tr><td rowspan="30">NSD, M1 and CXB</td><td colspan="5"></td><td></td><td></td><td colspan="2"></td><td colspan="2">NSD</td><td colspan="2">M1</td></tr>
<tr><td colspan="5">Compound-Dependent Parameters</td><td>Mobile Phase</td><td>Liquid-Liquid Extraction (LLE)</td><td>C_{max} (ng./mL.)</td><td>Mean</td><td>2956.667</td><td>2857.083</td><td>1183.916</td><td>1199.708</td></tr>
<tr><td colspan="5"></td><td></td><td></td><td></td><td>± S.D.</td><td>676.633</td><td>662.397</td><td>348.512</td><td>333.658</td></tr>
<tr><td colspan="5">Nimesulide (NSD)</td><td>Acetronitrile : water containing 5 mM ammonium formate (pH 3.2) :: 9:1 (v/v)</td><td>Plasma Volume : 100 µL</td><td>t_{max} (hr.)</td><td>Mean</td><td>2.750</td><td>2.750</td><td>2.792</td><td>2.667</td></tr>
<tr><td colspan="5"></td><td></td><td></td><td></td><td>± S.D.</td><td>0.794</td><td>0.794</td><td>0.388</td><td>0.282</td></tr>
<tr><td>Q1 Mass (Da)</td><td>Q3 Mass (Da)</td><td>Dwell (msec)</td><td colspan="2">Parameters (Volt/eVolt)</td><td>Stationary Phase or Column</td><td>Extracting Solvent : Dichloromethane</td><td>AUC_{0-t} (ng. hr./mL.)</td><td>Mean</td><td>1957.458</td><td>1953.292</td><td>4651.935</td><td>4650.316</td></tr>
<tr><td></td><td></td><td></td><td colspan="2"></td><td></td><td></td><td></td><td>± S.D.</td><td>510.902</td><td>515.445</td><td>1395.943</td><td>1156.823</td></tr>
<tr><td rowspan="11">307.2</td><td rowspan="11">229.2</td><td rowspan="11">200</td><td>DP</td><td>-20</td><td>Agilent ZORBAX Eclipse Plus C18 column, 75 mm x 4.6 mm (length x inner diameter), particle size 3.5 µm</td><td rowspan="11">Reconstitute Volume : 400 µL</td><td>$AUC_{0-\infty}$ (ng. hr./mL.)</td><td>Mean</td><td>1987.875</td><td>1991.250</td><td>4938.772</td><td>4969.748</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td>± S.D.</td><td>502.764</td><td>509.170</td><td>1427.068</td><td>1172.206</td></tr>
<tr><td>FP</td><td>-400</td><td>Column Oven Temperature : N.A.</td><td>k_{el} (hr.-1)</td><td>Mean</td><td>0.195</td><td>0.193</td><td>0.189</td><td>0.189</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td>± S.D.</td><td>0.018</td><td>0.024</td><td>0.022</td><td>0.031</td></tr>
<tr><td>EP</td><td>-7</td><td>Autosampler Temperature : 15 °C</td><td>$t_{1/2}$ (hr.)</td><td>Mean</td><td>3.599</td><td>3.527</td><td>3.709</td><td>3.775</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td>± S.D.</td><td>0.343</td><td>0.764</td><td>0.411</td><td>0.677</td></tr>
<tr><td>CEP</td><td>-24.61</td><td>Flow Rate : 0.4 mL/min.</td><td>Relative Bioavailability (%)</td><td></td><td>100%</td><td>99.78%</td><td>100%</td><td>99.97%</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td rowspan="3"></td><td rowspan="3"></td><td rowspan="3"></td><td colspan="4" rowspan="3"></td></tr>
<tr><td>CE</td><td>-25</td><td>Sample Injection Volume : 10 µL</td></tr>
<tr><td>CXP</td><td>-4</td><td>Total Run Time: 3.0 min.</td></tr>
<tr><td colspan="5">4-hydroxynimesulide (M1)</td><td>Anatyte Retention Time (RT) :</td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2"></td><td colspan="4" rowspan="2"></td></tr>
<tr><td>Q1 Mass (Da)</td><td>Q3 Mass (Da)</td><td>Dwell (msec)</td><td colspan="2">Parameters (Volt/eVolt)</td><td>NSD : 1.97 min.</td></tr>
<tr><td rowspan="5">323</td><td rowspan="5">245</td><td rowspan="5">200</td><td>DP</td><td>-25</td><td>M1 : 1.89 min.</td><td rowspan="5"></td><td rowspan="5"></td><td rowspan="5"></td><td colspan="4" rowspan="5"></td></tr>
<tr><td>FP</td><td>-400</td><td>CXB (IS) : 2.32 min.</td></tr>
<tr><td>EP</td><td>-7</td><td rowspan="3"></td></tr>
<tr><td>CEP</td><td>-24.61</td></tr>
<tr><td>CE</td><td>-25</td></tr>
</table>

Nimesulide 100 mg

Drug, Metabolite and IS	MS Parameters					Chromatographic Conditions	Plasma Analysis Method	Pharmacokinetic Parameters Reference Preparation (A) and Test Preparation (B)				
										NSD		M1
										(A)	(B)	(A)	(B)
				CXP	-4							
	Celecoxib (CXB, IS)											
	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Parameters (Volt/eVolt)								
	380.2	316.2	200	DP	-45							
				FP	-400							
				EP	-10							
				CEP	-26.73							
				CE	-32							
				CXP	-20							
	Source and Gas Parameters											
		CUR	30 psi									
		CAD	6 psi									
		ISV	-4500 V									
		TEM	400 °C									
		GS1	55 psi									
		GS2	50 psi									

6.2 Conclusion

Over the last two to three decades, LC-MS/MS has become essential for the day-to-day purposes in different laboratories within different areas or various disciplines of science. So now, it is confirmed that "LC-MS/MS" will persist to be a very key and useful analytical tool for a long period to come. Pharmacokinetic and bioequivalence study are more important aspects for the drug development process. Sometimes active metabolite of the drug plays a key role in pharmacological action, i.e., to some extent the pharmacological activity of the metabolite is more compared to its prodrug or parent drug in the body. So because of this, simultaneous quantification of both drug and its active metabolite in human plasma is needed for the drug development process. Validated methods are required to analyse these drugs and metabolites in human plasma to evaluate BA/BE study. Now after literature review, there is limited data or sometimes no data for the simultaneous quantification of these drugs and metabolites till date. Previous methods for the analysis of Acetylsalicylic Acid (Aspirin), Enalapril and Nimesulide with their active metabolites - Salicylic Acid, Enalaprilat and 4-Hydroxynimesulide have limitations and disadvantages in sensitivity, selectivity, extraction recovery, extraction procedure, method throughput, chromatographic run time and application of the methods in human subjects. Now through the literature review, I came to know about how to develop a different LC-MS/MS methods which helps me to develop my own method development. Therefore, the major concern and primary focus of the present thesis has to develop simple, sensitive, reproducible, selective, robust, rapid, high-throughput and specified newly bioanalytical methods to analyze these drugs and metabolites in plasma and successfully applied these methods to quantify large number of plasma samples in bioequivalence study to established their pharmacokinetic profile in healthy human subjects to correlate the therapeutic efficacy. So, present validated ESI-LC-MS/MS methods have been improved and developed very precisely and delicately for above mentioned areas.

In this thesis, LC-MS/MS instrument has been used for betterment of the bioanalytical methods. Because, HPLC coupled with UV, PDA or fluorescence detector does not give to high and better sensitivity, specificity and as well as selectivity. Likewise, LC-MS/MS has been selected over HPLC or GC for higher and better sensitivity, i.e., low detection limits, specificity and selectivity that gives shorter run time which help in high throughput analysis of samples with minimal sample treatment, i.e., using very low amount of plasma. Also, LC-MS/MS method is more precise and accurate than a HPLC method. Whereas, plasma sample preparation or extraction procedures are very simple and convenient for present studies and

i.e., either liquid-liquid extraction (LLE) or protein precipitation technique (PPT). But the solid phase extraction (SPE) technique was avoided because it is complicated and not so economic or cost-effective. During method development, various parameters were used and selected after appropriate confirmation and different trial and errors. In present thesis, LC-ESI-MS/MS integrated methods were developed by the proper optimization of methodologies. Now, the optimization criteria, the application of proper optimization strategies or procedures and also the data interpretation were principal attributes of the methodologies. The main purpose of the present methodologies are to describe and explore various parameters which associated with LC-MS/MS using mainly electrospray ionization (ESI) source experimental set-up. In results and discussions section, all selected optimized specific parameters are reported appropriately. In the experimental set-up, a stepwise optimization strategy is also considered that confidently assist to optimize the performance. Furthermore, the thesis contains analyses of some model drugs, such as Acetylsalicylic Acid (Aspirin), Enalapril and Nimesulide which form their active metabolites, like Salicylic Acid, Enalaprilat and 4-Hydroxynimesulide after single oral administration in healthy human subjects by the number of specific applications of LC-ESI-MS/MS. Finally, obtain their dose dependent comparative bioavailability based on LC-ESI-MS/MS developed and validated methods that already have been described in Table.6.1, Table.6.2 and Table.6.3, respectively.

In this thesis, the proposed bioanalytical methods are very simple, specific, sensitive, selective, reproducible, robust and rapid. Also, these methods have been developed in our bioanalytical laboratory employing simple liquid–liquid extraction (LLE) and protein precipitation technique (PPT) which make the method high throughput for bioanalysis. These bioanalytical methods were successfully validated and all the validation data were within the range of acceptance criteria of the USFDA and EMA guidelines. Furthermore, these methods have been applied successfully to the pharmacokinetic as well as bioequivalence studies of respective or specific drugs with their active metabolites in plasma of human subjects. The obtained values of the main pharmacokinetic parameters, like C_{max}, t_{max}, AUC_{0-t}, $AUC_{0-\infty}$, K_{el} and $t_{1/2}$ were comparable between the reference and test preparations. So therefore, the proposed specified newly LC-ESI-MS/MS methods occurs to be an unique approach of its kind reported so far in the literature and also the bioanalytical methods will be more convenient for performing the bioanalysis of respective drugs with their active metabolites in other laboratories worldwide.

6.3 Future research scope

We believe that, these bioanalytical methods will be used to assess compartmental pharmacokinetic study under the framework of long-term therapeutic effect in the next 5 to 10 years and can also be used as a therapeutic drug monitoring method in a hospital or clinic to examine the plasma concentration of respective drugs in patients. Now, this will also help to establish the therapeutic efficacy of the drugs. The pharmacokinetic or quantitative study of active metabolites in plasma is an important aspect of drug development research because it often shows that active metabolites are more pharmacologically active substances than the prodrug or parent drug after entering the systemic circulation. Present time in India, *in vivo* pharmacokinetic study of active metabolite is limited. Therefore in the near future, this kind of approach will enrich the potentiality of pharmacokinetic or quantitative study of the drugs and its active metabolites in plasma.

Now, further investigation will be needed for more comprehensive study of upcoming drugs and their active metabolites. Although, the similar studies could be further extended with fed condition in large number of volunteers and in presence of viable concomitant drug application to determine preclinical study, drug-drug interaction, toxicokinetic study, toxicokinetic parameters and effects on pharmacokinetic (PK) and pharmacodynamic (PD) parameters. The concept of average bioequivalence (ABE) has also applied in the present thesis. Moreover without establishing the pharmacodynamic effects, the studies were performed in healthy human subjects. But, therapeutic equivalency of the drug can be doubtful or uncertain if it is administered to the actual patients. Concomitant drug application consistently perform in therapeutic efficacy of dosage forms during therapeutic inter-changeability from reference preparation to test preparation, for example pharmacological factors such as health conditions of patients. For the purpose of developing the PK-PD correlation, this thesis can be further extended to explore the pharmacodynamic aspects of Acetylsalicylic Acid (Aspirin), Enalapril and Nimesulide to actual patient population.

Chapter – 7: Bibliography



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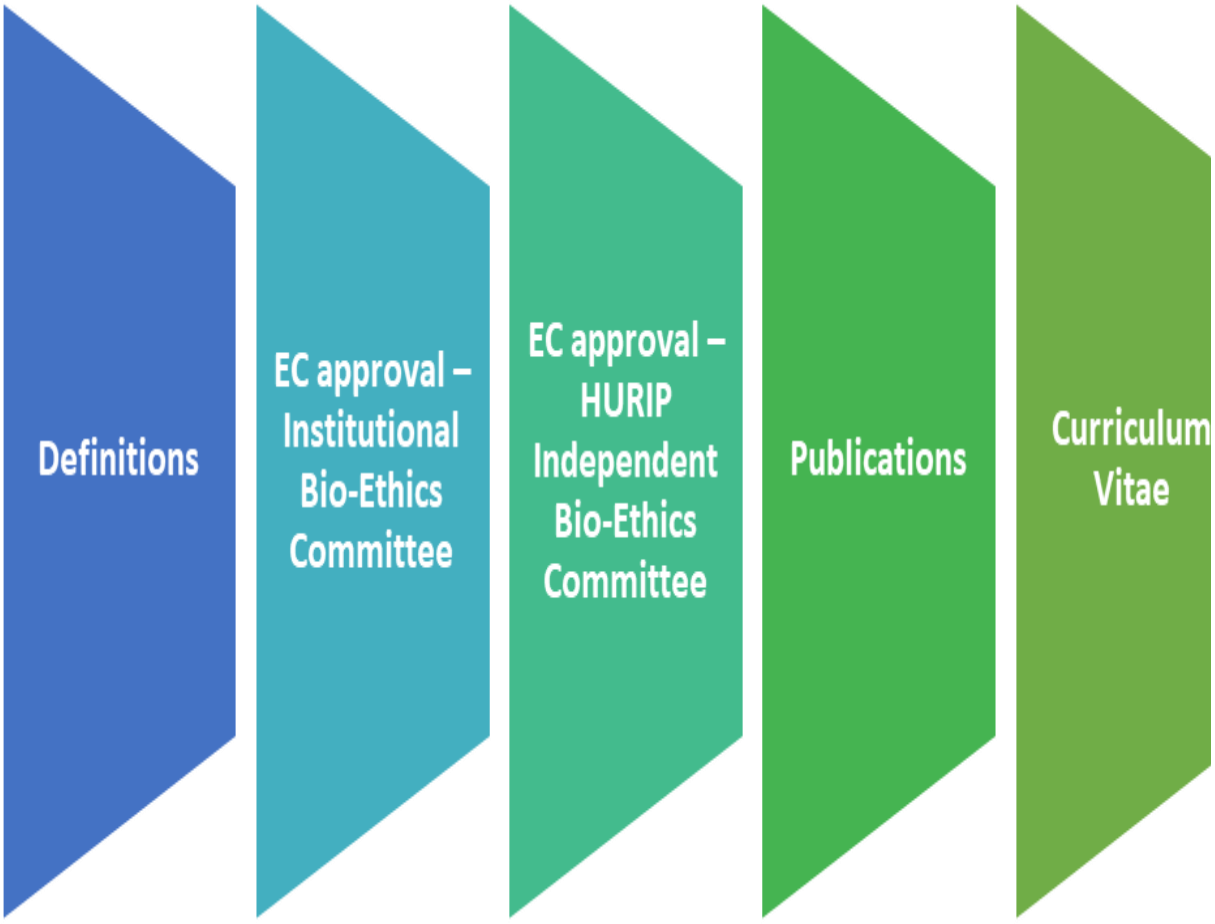
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Annexure


Definitions
EC approval – Institutional Bio-Ethics Committee
EC approval – HURIP Independent Bio-Ethics Committee
Publications
Curriculum Vitae

DEFINITIONS

- **Accuracy :** The accuracy of an analytical procedure expresses the closeness of determined value to the true value, i.e., value to the value which is accepted either as a conventional true value or an accepted reference value. Generally, recovery of added analyte over an appropriate range of concentrations is taken as an indication of accuracy. (The concentration range chosen should bracket the concentration of interest). Accuracy is defined as (determined value/true value) x100%.

- **Precision :** The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision is defined as the ratio of standard deviation/mean (%).

- **Analyte :** A specific chemical moiety being measured, which can be intact drug, biomolecule or its derivative, metabolite, and/or degradation product in a biologic matrix.

- **Analytical run (or batch) :** A complete set of analytical and study samples with appropriate number of calibration standards and QC samples for their validation. Several runs (or batches) may be completed in one day, or one run (or batch) may take several days to complete.

- **Analytical Procedure :** The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analysis.

- **Biological matrix :** A discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples are blood, serum, plasma, urine, feces, saliva, sputum, and various discrete tissues.

- **Blank :** A sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method.

- **Calibration range :** The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure meets the requirements for precision, accuracy and response function.

- **Calibration standard :** A biological matrix to which a known amount of analyte has been added or spiked. Calibration standards are used to construct calibration curves from which the concentrations of analytes in QCs and in unknown study samples are determined.

- **Carry over :** Carry-over is the appearance of an analyte signal in blank sample after the analysis of samples with a high analyte concentration.
- **Validation/Full validation :** Establishment of all validation parameters to apply to sample analysis for the bioanalytical method for each analyte.
- **Cross validation :** Comparison of validation parameters of two bioanalytical methods.
- **Partial validation :** Series of analytical experiments where only relevant parts of the validation are repeated after modifications are made to the validated bioanalytical method.
- **Incurred samples :** Study samples from dosed subjects or animals.
- **Incurred sample reanalysis :** The analysis of a portion of the incurred samples to determine whether the original analytical results are reproducible.
- **Internal standard :** Test compound(s) (e.g. a structurally similar analogue, or stable isotope labelled compound) added to calibration standards, QC samples and study samples at a known and constant concentration to facilitate quantification of the target analyte(s) and also to correct for experimental variability during sample preparation and analysis.
- **Lower limit of quantification (LLOQ) :** The lower limit of quantification of an individual analytical procedure is the lowest amount of an analyte in a sample which can be quantitatively determined with pre-defined suitable precision and accuracy.
- **Matrix effect :** The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.
- **Method :** A comprehensive description of all procedures used in sample analysis.
- **Nominal concentration :** Theoretical or expected concentration.
- **Response function :** Response function is a function which adequately describes relationship between instrument response (e.g. peak area or height ratio) and the concentration (amount) of analyte in the sample. Response function is defined within a given range.
- **Processed :** The final extract (prior to instrumental analysis) of a sample that has been subjected to various manipulations (e.g., extraction, dilution, concentration).
- **Recovery :** The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.

- **Reproducibility :** The precision between two laboratories. It also represents precision of the method under the same operating conditions over a short period of time.

- **Sample :** A generic term encompassing controls, blanks, unknowns, and processed samples, as described below :

a) **Blank :** A sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method.

b) **Quality control (QC) sample :** A spiked sample used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples analysed in an individual batch.

c) **Unknown :** A biological sample that is the subject of the analysis.

- **Selectivity :** Selectivity is the ability of the bioanalytical method to measure and differentiate the analyte(s) of interest and internal standard (IS) in the presence of components which may be expected to be present in the sample. These could include metabolites, impurities, degradants, or matrix components.

- **Specificity :** Specificity is the ability to measure the analyte unequivocally in the presence of other compounds, either exogenous or endogenous, in the matrix.

- **Stability :** The chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

- **Standard curve :** The relationship between the experimental response value and the analytical concentration (also called a calibration curve).

- **System suitability :** Determination of instrument performance (e.g., sensitivity and chromatographic retention) by analysis of a reference standard prior to running the analytical batch.

- **Upper limit of quantification (ULOQ) :** The upper limit of quantification of an individual analytical procedure is the highest amount of analyte in a sample which can be quantitatively determined with pre-defined precision and accuracy.

- **Standard Operating Procedure (SOP) :** Document which describes the regularly recurring operations relevant to the quality of the investigation and enabling to carry out the operations correctly and always in the same manner.

File No. ECR/86/Jadavpur/Indt/WB/2013

From:

O/o The Drugs Controller General (India)
Directorate General of Health Services

New Delhi – 110 002
Dated: 02 AUG 2013

To,

The Chairman,
Bioethics Committee, Jadavpur University,
Bioequivalence Study Centre,
Department of Pharmaceutical Technology,
Jadavpur University, Raja S.C. Mullick Road,
Jadavpur, Kolkata-700032, West Bangal,
India.

SUB: - Ethics Committee Registration No. **ECR/ 93 /Indt/WB/2013** issued under Rule 122DD of the Drugs & Cosmetics Rules1945.

Dear Sir,

Please refer to your application no. Nil dated 06.03.2013 submitted to this office for the Registration of Ethics Committee.

Based on the documents submitted by you, this office hereby Registers the **BIOETHICS COMMITTEE, JADAVPUR UNIVERSITY** situated at **BIOEQUIVALENCE STUDY CENTRE, DEPARTMENT OF PHARMACEUTICAL TECHNOLOGY, JADAVPUR UNIVERSITY, RAJA S.C. MULLICK ROAD, JADAVPUR, KOLKATA-700032, WEST BANGAL, INDIA** with Registration number **ECR/ 93 /Indt/WB/2013** as per the provisions of Rule 122DD of the Drugs and Cosmetics Rules, 1945 subject to the following conditions:

1. **The Ethics Committee shall review and approve only the study protocols and related documents of Bioavailability/Bioequivalence studies of the approved drug molecules and also carry ongoing review of such studies.**
2. The Ethics Committee shall review and accord its approval to Bioavailability/Bioequivalence studies and also carry ongoing review of such studies at appropriate intervals, as specified in Schedule Y and the Good Clinical Practice Guidelines for Clinical Trials in India and other applicable regulatory requirements for safeguarding the rights, safety and well being of the trial subjects.
3. In the case of any serious adverse event occurring during Bioavailability/Bioequivalence studies, the Ethics Committee shall analyze and forward its opinion as per procedures specified under APPENDIX XII of Schedule Y.

4. The Ethics Committee shall allow inspectors or officials authorized by the Central Drugs Standard Control Organization to enter its premises to inspect any record, data or any document related to Bioavailability/Bioequivalence studies and provide adequate replies to any query raised by such inspectors or officials, as the case may be, in relation to the conduct of Bioavailability/Bioequivalence studies.

5. The licensing authority shall be informed in writing in case of any change in the membership or the constitution of the ethics committee takes place.

6. All the records of the ethics committee shall be safely maintained after the completion or termination of the study for not less than five years from the date of completion or termination of the trial (Both in hard and soft copies).

7. If the Ethics Committee fails to comply with any of the conditions of registration, the Licensing Authority may, after giving an opportunity to show cause why such an order should not be passed, by an order in writing stating the reasons therefore, suspend or cancel the registration of the Ethics Committee for such period as considered necessary.

8. This registration shall be in force for a period of three years from the date of issue, unless it is sooner suspended or cancelled.

9. Ethics Committee shall consist of not less than seven members and is subject to a maximum of 15. One among its members, who is from outside the institute, shall be appointed as chairman, one member as a Member Secretary and rest of the members shall be from Medical, Scientific, Non Medical and Non-scientific fields including lay public.

10. The committee shall include at least one member whose primary area of interest or specialization is Non-scientific and at least one member who is independent of the institution besides; there should be appropriate gender representation on the Ethics Committee.

11. The Ethics committee can have as its members, individuals from other Institutions or Communities, if required.

12. Members should be conversant with the provisions under Schedule Y, Good Clinical Practice Guidelines for clinical trials in India and other regulatory requirements to safeguard the rights, safety and well-being of the trial subjects.

For review of each protocol the quorum of Ethics Committee shall be at least five members with the following representations:

i. Basic medical scientist (preferably one pharmacologist)
ii. Clinician
iii. Legal expert
iv. Social scientist or representative of non-governmental voluntary agency or philosopher or ethicist or theologian or a similar person.
v. Lay person from community

13. The members representing medical scientist and clinicians should have Post graduate qualification and adequate experience in their respective fields and aware of their role and responsibilities as committee members.

14. As far as possible, based on the requirement of research area such as HIV, Genetic disorder, etc., specific patient group may also be represented in the Ethics Committee.

15. There should be no conflict of interest. The members shall voluntarily withdraw from the Ethics Committee meeting while making a decision on an application which evokes a conflict of interest which may be indicated in writing to the Chairman prior to the review and be recorded so in the minutes. All members shall sign a declaration on conflict of interest.

16. Subject experts or other experts may be invited to the meetings for their advice. But no such expert shall have voting rights.

17. This certificate is issued to you on the basis of declaration/ submission by you that yours is an independent and registration is sought for Independent Ethics Committee

18. Ethics Committee should review such number of protocols of Bioavailability/Bioequivalence studies of approved drug molecules which should be commensurate to the infrastructure and facilities available with them.

19. Status report of the functioning of the Ethics Committee should be submitted to the CDSCO headquarters and concerned zonal office on quarterly basis.

20. The details of funding support and amount of honorarium, if any, payable to the ethics committee members should be defined in the Standard Operating Procedure (SOP) of the committee and records to this extent shall be maintained.

21. Ethics committee should have dedicated office with required infrastructure and supporting staff.

However, it is informed that this Ethics Committee can carry out periodic review of ongoing clinical trials already approved by them prior to 30.01.2013

Yours Faithfully,

8/8/2013

(A. Visala)
Deputy Drugs Controller (I) & Licensing Authority.

A. Visala
Deputy Drugs Controller (I)
Dte. General of Health Services
Central Drugs Standard Control Organisation
FDA Bhawan, Kotla Road, New Delhi-110002

File No. ECR/746/Hurip/Indt/WB/2013

From:

o/o The Drugs Controller General (India)
Directorate General of Health Services

New Delhi – 110 002
Dated: 16 AUG 2013

To,

The Chairman,
Hurip Bioethics Committee,
Flat No.1C, 69, Ibrahimpur Road, Jadavpur,
Kolkata – 700 032, West Bengal,
India.

SUB: - Ethics Committee Registration No. **ECR/ 103 /Indt/WB/2013** issued under Rule 122DD of the Drugs & Cosmetics Rules1945.

Dear Sir,

Please refer to your application no. Nil, dated 07th March 2013 submitted to this office for the Registration of Ethics Committee.

Based on the documents submitted by you, this office hereby Registers the **HURIP BIOETHICS COMMITTEE** situated at **FLAT NO.1C, 69, IBRAHIMPUR ROAD, JADAVPUR, KOLKATA – 700 032, WEST BENGAL, INDIA** with Registration number **ECR/ 103 /Indt/WB/2013** as per the provisions of Rule 122DD of the Drugs and Cosmetics Rules, 1945 subject to the following conditions:

1. **The Ethics Committee shall review and approve only the study protocols and related documents of Bioavailability/Bioequivalence studies of the approved drug molecules and also carry ongoing review of such studies.**
2. The Ethics Committee shall review and accord its approval to Bioavailability Bioequivalence studies and also carry ongoing review of such studies at appropriate intervals, as specified in Schedule Y and the Good Clinical Practice Guidelines for Clinical Trials in India and other applicable regulatory requirements for safeguarding the rights, safety and well being of the trial subjects.
3. In the case of any serious adverse event occurring during Bioavailability Bioequivalence studies, the Ethics Committee shall analyze and forward its opinion as per procedures specified under APPENDIX XII of Schedule Y.
4. The Ethics Committee shall allow inspectors or officials authorized by the Central Drugs Standard Control Organization to enter its premises to inspect any record, data or any document related to Bioavailability/Bioequivalence studies and provide adequate replies to

any query raised by such inspectors or officials, as the case may be, in relation to the conduct of Bioavailability/Bioequivalence studies.

5. The licensing authority shall be informed in writing in case of any change in the membership or the constitution of the ethics committee takes place.

6. All the records of the ethics committee shall be safely maintained after the completion or termination of the study for not less than five years from the date of completion or termination of the trial (Both in hard and soft copies).

7. If the Ethics Committee fails to comply with any of the conditions of registration, the Licensing Authority may, after giving an opportunity to show cause why such an order should not be passed, by an order in writing stating the reasons therefore, suspend or cancel the registration of the Ethics Committee for such period as considered necessary.

8. This registration shall be in force for a period of three years from the date of issue, unless it is sooner suspended or cancelled.

9. Ethics Committee shall consist of not less than seven members and is subject to a maximum of 15. One among its members, who is from outside the institute, shall be appointed as chairman, one member as a Member Secretary and rest of the members shall be from Medical, Scientific, Non Medical and Non-scientific fields including lay public.

10. The committee shall include at least one member whose primary area of interest or specialization is Non-scientific and at least one member who is independent of the institution besides; there should be appropriate gender representation on the Ethics Committee.

11. The Ethics committee can have as its members, individuals from other institutions or Communities, if required.

12 Members should be conversant with the provisions under Schedule Y, Good Clinical Practice Guidelines for clinical trials in India and other regulatory requirements to safeguard the rights, safety and well-being of the trial subjects.

For review of each protocol the quorum of Ethics Committee shall be at least five members with the following representations:

i. Basic medical scientist (preferably one pharmacologist)
ii. Clinician
iii. Legal expert
iv. Social scientist or representative of non-governmental voluntary agency or philosopher or ethicist or theologian or a similar person.
v. Lay person from community

13. The members representing medical scientist and clinicians should have Post graduate qualification and adequate experience in their respective fields and aware of their role and responsibilities as committee members.

14. As far as possible, based on the requirement of research area such as HIV, Genetic disorder, etc., specific patient group may also be represented in the Ethics Committee.

15. There should be no conflict of interest. The members shall voluntarily withdraw from the Ethics Committee meeting while making a decision on an application which involves a conflict of interest which may be indicated in writing to the Chairman prior to the review and be recorded so in the minutes. All members shall sign a declaration on conflict of interest.

16. Subject experts or other experts may be invited to the meetings for their advice, But no such expert shall have voting rights.

17. This certificate is issued to you on the basis of declaration/ submission by you that yours is an independent and registration is sought for Independent Ethics Committee

18. Ethics Committee should review such number of protocols of Bioavailability/Bioequivalence studies of approved drug molecules which should be commensurate to the workload and facilities available with them.

19. Status report of the functioning of the Ethics Committee should be submitted to the CDSCO headquarters and concerned zonal office on quarterly basis.

20. The details of funding support and amount of honorarium, if any, payable to the ethics committee members should be defined in the Standard Operating Procedures (SOPs) of the committee and records to this extent shall be maintained.

21. Ethics committee should have dedicated office with required infrastructure and supporting staff.

However, it is informed that this Ethics Committee can carry out periodic review of ongoing clinical trials already approved by them prior to 30.01.2013

Yours Faithfully,

(Dr. V. Vida)
Deputy Drugs Controller (I) & Licensing Authority

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LC-MS/MS determination of 4-hydroxynimesulide, an active metabolite of nimesulide and application to bioequivalence study in Indian subjects

Dhiman Halder[1], Shubhasis Dan[1,2], Pradipta Sarkar[1], Dibya Das[2], Umesh Chandra Halder[3] and Tapan Kumar Pal[1]

Abstract

A simple and highly sensitive bioanalytical method was developed and validated for simultaneous quantification of nimesulide (NSD) and its active metabolite 4-hydroxy-nimesulide (M1) in human plasma by liquid chromatography-tandem mass spectrometer (LC-MS/MS) and applied in a bioequivalence study performed on Indian subjects. The bioanalytical method was carried out by LC-MS/MS with celecoxib (CXB) as an internal standard (IS) using liquid–liquid extraction technique. The chromatographic separation was performed on a reversed-phase Agilent eclipse plus C18 (75 mm × 4.6 mm, particle size 3.5 μm) column with a mobile phase of acetronitrile and water containing 5 mM ammonium formate (9:1, v/v). Method validation and clinical sample were analysed as per USFDA and EMA guidelines and results met the acceptance criteria. The lower limit of quantitation of NSD and M1 was found 10 ng/mL with a large linearity range from 10 to 6000 ng/mL for both NSD and M1 using only 100 μL of plasma and reported no matrix effect. The multiple reaction monitoring transitions of m/z 307.20 → 229.20, m/z 323.00 → 245.00 and m/z 380.20 → 316.20 were used to measure NSD, M1 and CXB (IS), respectively. The assay method was successfully applied for the simultaneous quantification of both NSD and M1 in plasma samples after oral administration of nimesulide 100 mg tablet in healthy human subjects.

Keywords

Nimesulide and 4-hydroxy-nimesulide, LC-MS/MS, method validation, pharmacokinetics, bioequivalence study

Received 20 August 2018; accepted 9 December 2018

Introduction

Nimesulide (4-nitro-2-phenoxymethanesulfonanilide) is a nonsteroidal anti-inflammatory drug (NSAID) with a non-acidic property (pKa = 6.5).[1,2] It is a selective inhibitor of cyclo-oxygenase-2 (COX-2) and effectively provides relief from a wide variety of pain (like analgesic and antipyretic) and inflammatory conditions (like anti-inflammatory).[3–6] Apart from this potent anti-inflammatory effect, it has been stated that long-term administration of NSD caused hepatotoxicity[7,8] and gastrointestinal toxicity[9] in human being. NSD has various mechanisms of action in its pharmacological activities. It is a concern that the major mechanisms are the selective inhibition of COX-2 with 5- to 16-fold selectivity for COX-2. COX-1 regulates gastric cytoprotection and vascular haemostasis in many tissue expressions.[10,11] Unwanted gastrointestinal and renal adverse effects are occurred due to the synthesis of cytoprotective compounds which are reduced by the inhibition of COX-1 like prostacyclin. Selective inhibition of COX-2 reduces the production of pro-inflammatory prostaglandins, and its beneficial effects in inflammation and pain relief, with modest gastrointestinal (GI) toxicity.[12–14] Ex vivo determination in human whole blood after oral administration of 100 mg nimesulide (NSD) causes complete suppression of COX-2 activity and partial reduction in COX-1 activity. In vitro, in the bronchial tree during prostaglandin synthesis it does not cause any affects and in case of constitutive COX-1 which exerts a bronchoprotective role in the gastric mucosa, where COX-1 preserves mucosal

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Research Article

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LC–MS/MS assay for quantitation of enalapril and enalaprilat in plasma for bioequivalence study in Indian subjects

Dhiman Halder[1], Shubhasis Dan[1], Murari Mohun Pal[3], Easha Biswas[1], Nilendra Chatterjee[3], Pradipta Sarkar[1], Umesh Chandra Halder[2] & Tapan Kumar Pal[*,1]

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Background: Enalapril (EPL) is an angiotensin-converting enzyme inhibitor for the treatment of hypertension and chronic heart failure. Enalaprilat (EPLT) is an active metabolite that contributes to the overall activity of EPL. **Aim**: To quantitate EPL along with its metabolite EPLT using LC–MS/MS, a bioanalytical method was developed and validated with tolbutamide in human plasma using a protein precipitation technique. **Results:** The sensitive and selective method has an LLOQ of 1 ng/ml with a linearity range of 1–500 ng/ml for both EPL and EPLT using 300 µl of plasma without any matrix effect. **Conclusion:** Linearity, specificity, accuracy, precision and stability, as well as its application to the analysis of plasma samples after oral administration of 20 mg of EPL maleate in healthy volunteers demonstrate applicability to bioavailability/bioequivalence studies.

Lay abstract: The present study describes a sensitive, selective, simple, accurate and reproducible LC–MS/MS method for the simultaneous determination of both enalapril (EPL) and enalaprilat (EPLT) in human plasma. The results obtained indicate the high sensitivity of the described method for analysis of EPL and EPLT, which render this method particularly useful for pharmacokinetic or bioequivalence studies. The proposed method has been applied for the analysis of EPL and EPLT in the plasma of healthy volunteers in a single-dose pharmacokinetic study.

First draft submitted: 27 September 2016; Accepted for publication: 21 November 2016; Published online: 2 February 2017

Keywords: bioequivalence study enalapril • enalaprilat • human plasma • LC–MS/MS • protein precipitation

Hypertension and cardiovascular disease are a major cause of morbidity in India and abroad. Antihypertensives are a class of drugs that are used to treat hypertension (high blood pressure) [1,2] and cardiovascular disease [3,4]. Enalapril maleate [(S)-1-(N-(1-(ethoxycarbonyl)-3-phenylpropyl)-L-alanyl)-L-proline, (Z)-2-butenedioate salt] is a salt of both enalapril (EPL) and maleic acid [1,5–7]. EPL is a potent antihypertensive drug, a vasodialator and also an angiotensin-converting enzyme (ACE) inhibitor [5,8–13] which is widely used for the treatment of hypertension and congestive heart failure [1,4,14–17]. In the kidneys, rennin is synthesized and after release into the circulation it acts on a plasma precursor for the production angiotensin I. ACE is a peptidyl dipeptidase which catalyzes the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor substance that stimulates the release of aldosterone from the adrenal cortex and also increases endogenous bradykinin concentrations [16]. Thus, there is an increase in blood pressure and cardiac failure. ACE inhibitors such as EPL inhibit the conversion of angiotensin I to angiotensin II decreasing blood pressure and aldosterone secretion, slightly

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A Rapid LC-ESI-MS/MS Method for the Quantitation of Salicylic Acid, an Active Metabolite of Acetylsalicylic Acid: Application to *in vivo* Pharmacokinetic and Bioequivalence Study in Indian Healthy Male Volunteers

Dhiman Halder[1], Shubhasis Dan[1], Easha Biswas[1], Pradipta Sarkar[1], Umesh C. Halder[2] and Tapan K. Pal[1,*]

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Dhiman Halder

Abstract: A very simple and rapid bio-analytical method with high-throughput, appropriate sensitivity, selectivity, accuracy and precision has been developed and validated for the quantification of acetylsalicylic acid (ASA) and its main metabolite salicylic acid (SA) in human plasma using hydrochlorothiazide (HCTZ) as internal standard (IS) by high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) with electronspray ionization (ESI). A simple technique of liquid-liquid extraction was developed for plasma samples preparation of the analytes and the chromatographic separation was done under isocratic conditions by Phenomenex Kinetex C18 column (50 mm × 3 mm, 5 μm) with a run time of 1.8 min. The composition of mobile phase was acetonitrile and 10 mM ammonium formate in water (90:10, v/v). The precursor ion was deprotonated to product ion transitions for ASA, SA and HCTZ (IS) were measured on an operating in the multiple reaction monitoring (MRM) with negative ion mode by triple quadrupole mass spectrometer. The assay exhibited a calibration range of 10-15000 ng/mL for both ASA and SA. The bio-analytical validated method was successfully applied for bioavailability or bioequivalence studies to analyze human plasma samples.

Keywords: Acetylsalicylic acid, liquid chromatography, salicylic acid, tandem mass spectrometry.

1. INTRODUCTION

Aspirin (2-acetoxy benzoic acid) is also known as acetylsalicylic acid (ASA), it's a non-steroidal analgesics and has antipyretic, anti-inflammatory and anti-thrombic actions [1-4]. It is most frequently used in headache, transient musculoskeletal pain, dysmenorrheal and gastric irritation [4]. Salicylic acid (SA) is an active metabolite of ASA and its formed in the gastrointestinal tract and the liver in human by rapid hydrolysis of ASA [5]. Other metabolites of ASA are gentisic acid (GA) and salicyluric acid (SUA) which are formed again by hydroxylation and by conjugation, respectively [6]. Platelet aggregation and vasoconstriction are occurred by thromboxane A2 which is a potent activator of these two. This thromboxane A2 is blocked by platelet cyclooxygenase (COX)-1 which is irreversibly inhibited by ASA [7]. Also, ASA is 166 fold more effective in inhibiting COX-1 than COX-2 enzyme [8]. Due to inhibition of cyclooxygenase, production of prostaglandins decreases with the release of pain [4].

Primarily, salicylic acid (SA) is responsible for the pharmacological activity of ASA which is hydrolyzed in the human body rapidly to produce SA [2]. If ASA in human body metabolizes slowly then it's become more effective than in those people who metabolize this drug very fast [3]. In case of both ASA and its metabolite SA, the rate of elimination is an important factor to determine their efficacy in the body. Either by conjugation or by oxidation process, SA is metabolized with glycine or glucuronic acid. In the urine, the most abundant metabolite has found due to conjugation of glycine with SA and in otherhand salicylic acid phenolic glucuronide (SAPG) and salicylic acid acyl glucuronide (SAAG) are formed by the conjugation of glucuronide with SA [9].

T_{max} of intravenous ASA is 0.02 hours and oral ASA is 0.5 hours, respectively. That means intravenous has a faster action than oral. The mean C_{max} per intravenous dose is 11 times higher than oral ASA 500 mg and the mean $AUC_{0-\infty}$ per dose is twice as high. In case of SA, there is slight increase in $AUC_{0-\infty}$ per dose with inhibition of 6-keto-$PGF_{1\acute{\alpha}}$ production followed by oral or intravenous ASA is only ten-fold or less. During PGI_2 synthesis, the effects are compared after administration of 250-500 mg doses either orally or intravenously. So, the only reason either intravenous or oral dosing except at 5 and 20 minutes after 500 mg dose administration there are few significant differences in 6-keto-PGF2ά concentrations [7].

The aim and objective of the present study is to develop and validate a liquid chromatography tandem mass spectrometric method (LC-MS/MS) for the simultaneous quantification of both ASA and its main metabolite SA in human plasma using HCTZ as the internal standard which is very

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Poster 24

Bioanalytical method development & validation of metabolites by LCMC/MS under the framework of BA/BE study

Dhiman Halder[2], Sangjukta Pradhan[1], Easha Biswas[1], Tania Chakraborty[1], Dr. Nilendra Chatterjee[1], Dr. Balaram Ghosh[1], Prof. Dr. T.K.Pal[2]

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Abstract: The first liquid chromatography-tandem mass spectrometry (LC/MS/MS) method for determination of acetylsalicylic acid (aspirin, ASA) and one of its major metabolites, salicylic acid (SA), in human plasma using hydrochlorthiazide as an internal standard has been developed and validated. For ASA analysis, plasma sample was extracted using a mixture of ethyl acetate and methyl terbutyl ether (50:50) in a single step liquid-liquid extraction procedure. The compounds were separated on a reversed-phase column with an isocratic mobile phase consisting of acetonitrile and water containing 10 milli-molar ammonium formate (9:1 v/v). The ion transitions recorded in multiple reaction monitoring mode were *m/z* 179 →137, 137 →93 and 295.9 →268.9 for ASA, SA and IS respectively. The co-efficient of variation of the assay precision was less than 9.3% and the accuracy exceeded 86.5%. The lower limits of quantification for ASA and SA was 10 ng/ml,for both. The developed assay method was successfully applied for the evaluation of pharmacokinetics of ASA and SA after single oral administration of 100 mg of aspirin to 10 Indian healthy male volunteers.

CURRICULUM VITAE

DHIMAN HALDER



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Objective

To seek a challenging career in research and development where I can utilize my education and skill to its full potential while effectively serving and contributing to the success of the organization.

Area of Research Interests

- Liquid chromatography mass spectrometry (LC-MS/MS) and high-performance liquid chromatography (HPLC) Techniques.
- Analysis of samples (specially for drugs and metabolites) in biological matrix.
- Pharmacokinetic and Bioavailability studies.
- Novel analytical techniques.

Research and Working Experiences

- Ph.D. Research Scholar of Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700 032, India from 5th July, 2013 to till date.
- Non-NET Fellow of University Grants Commission (UGC), Govt. of India from 5th July, 2013 to 4th July, 2017.
- Project Fellow of Department of Science and Technology (DST), Govt. of India from 20th April, 2012 to 1st March, 2013.
- Bioanalyst of Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700 032, India from 1st August, 2008 to 23rd April, 2012.
- Visiting Teacher of Khantura Pritilata Shiksha Niketan (Boy's), Khantura, 24 Pgs. (N), West Bengal, India from 5th September, 2005 to 12th July, 2008.

Academic Background

- Doctor of Philosophy (Ph.D.) (in Science, 2013 to till date) from Department of Life Science and Biotechnology, Jadavpur University, Kolkata, West Bengal, India.

- Master of Science (M.Sc.) in Zoology (2003-2005) from Ballygunge Science College, Department of Zoology, University of Calcutta, Kolkata, West Bengal, India.
- Bachelor of Science (B.Sc.) in Zoology (Hons., 2000-2003) from Asutosh College, University of Calcutta, Kolkata, West Bengal, India.

Thesis/Project Titles

- ***Doctor of Philosophy (Ph.D.) (Science) :*** *Bioanalytical Method Development and Validation of Drugs and Metabolites by LC-MS/MS with a Special Emphasis on Bioequivalence Study.*
- ***Master of Science (M.Sc.) :*** *Erythropoiesis and Erythropoietin.*

Scholastic Achievements (Awards and Honours)

- "Young Achiever Award 2020" has given for the publication of "LC-MS/MS Determination of 4-Hydroxynimesulide, an active metabolite of Nimesulide and Application to Bioequivalence Study in Indian Subjects" from Institute of Scholars (InSc), Bangalore, India.
- Research Fellow under UGC-NON NET scheme, Govt. of India from 2013 to 2017.
- Project Fellow, Department of Science and Technology (DST), Govt. of India from 2012 to 2013.

Research Competency and Technical Skills

Bioanalytical activities

- Bioanalytical method development of drugs, metabolites and Pharmaceuticals by LC-MS/MS (API-2000, API-4000 and API-4000 QTRAP) and HPLC (UV and PDA) methods.
- Validation of bioanalytical methods as per regulatory guidelines (USFDA, EMA, ICH and CDSCO).
- Preparation of protocol for BE study and coordination for study initiation and completion.
- Analysis of pharmacokinetic study samples.
- Preparation of bioanalytical validation study report.
- Performing stability studies of the dosage formulations as per protocol.
- Day to day monitoring and maintenance of Good Laboratory Practice (GLP) in laboratory.
- Bioavailability and Bioequivalence studies.
- Statistical analysis and interpretation.

Instrument handling capabilities and Software knowledge

- Liquid chromatography mass spectrometry (LC-MS/MS) – API-2000, API-4000 and API-4000 QTRAP with Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) sources and Analyst Software version 1.6.3, 1.6.2, 1.5, 1.4.2 and 1.4.1.

- High performance liquid chromatography (HPLC) – Shimadzu, Knauer, Waters and Jasco systems with LC Solution, ClarityChrom, Clarity Lite and ChromNAV softwares.
- Single and Double beam UV – Visible spectrophotometer – Shimadzu, Jasco and Chemito Technologies Ltd with SpectraManager software.
- Automated Dissolution Tester USP XXIII – Electrolab and other minor equipments.
- Dreamweaver, Photoshop, Flash (Web Designing Softwares).
- WinNonlin version 5.2 pharmacokinetic evaluation software (Statistical Software).

Participations and Contributions

- Acting as **Management Representative** of the Quality Management Team of Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata for **ISO 9001: 2008** accreditation which received certification in August 2013.
- **TUV-NORD** certified **Inernal Auditor** for internally audit in a QMS.
- Acting as **Lab In-charge as well as LCMS/MS Incharge of Bioequivalence Study Centre,** Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032.
- Acting as **course co-ordinator of Bioequivalence Study Centre,** Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032 for conducting various short term course on HPLC and LC-MS/MS.

Membership

- Lifetime Professional Member of **Institute of Scholars (InSc),** Bangalore, India.

  Membership Id : InSc20201027

Industrial / CRO Exposures

- TAAB Biostudy Services, 69, Ibrahimpur Road, Jadavpur, Kolkata – 700 032, 2017 onwards.
- Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata – 700 032, from 2008 to 2017.

Organizing Committee Experiences

- Acted as **LCMS/MS demonstrator** as well as one of the **key member** of the **working committee** of International Conference on ***"Present Scenario of Bioavailability and Bioequivalence Studies and its Regulatory Affairs"*** held on February 12, 2020 at Dr. Triguna Sen Auditorium, Jadavpur University, Kolkata, organized by Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University and TAAB Biostudy Services, Kolkata, India.
- Participated as **organizing committee member** in the 11th Annual National Conference on ***"Clinical Toxicology and Analytical Pharmacology"*** by Indian Society for Rational Pharmacotherapeutics,

ISRPTCon2019 Pre-Conference Workshop held on November 27, 2019 at Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

- Participated as **organizing committee member** in the DST Sponsored International Seminar cum Workshop on ***"Recent Advances in Clinical Research with a Special Emphasis on BA/BE Study"*** held from September 11 to September 15, 2014 at Dr. Triguna Sen Auditorium and Vivekananda Hall, Jadavpur University, Kolkata, organized by Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

- Participated as **organizing committee member** in the National Workshop on ***"Registration of Bioethics Committee for Clinical Research in India"*** held on April 18, 2013 at Dr. K. P. Basu Auditorium, Jadavpur University, Kolkata, organized by Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

- Participated as **organizing committee member** in the DST sponsored International Seminar on ***"Clinical Research and Regulatory Affairs : Present Scenario"*** held on July 16 and July 17, 2012, organized by Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

- Acted as a **core member** of short term training course on on ***"Bioequivalence study of drug by HPLC and LC-MS/MS"*** held during April 2010 and February 2012 at Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

- Acted as a **key member** of the DST sponsored 5 day National Workshop on ***"Application of Chromatography and Mass Spectrometry In Clinical Research"*** held during September 2010, organized by Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

Workshops / Symposia / Conferences Attended / Professional Trainings

- Participated in three days online international conference, ***"Colloquium 2020"*** on the theme ***"Innovations in Pharmacy and Green Chemistry in Post Covid-19 Era"*** organized by Dept. of Pharm. Tech., Dept. of Biotech. and Dept. of Chemistry, JIS University, Kolkata, India, from 11th June to 13th June, 2020.

- Attended and presented poster in the International Conference on ***"Present Scenario of Bioavailability and Bioequivalence Studies and its Regulatory Affairs"*** at Dr. Triguna Sen Auditorium, Jadavpur University, Kolkata, organized by Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University and TAAB Biostudy Services, Kolkata, India held on 12th February, 2020.

- Participated in the 11th Annual National Conference on ***"Clinical Toxicology and Analytical Pharmacology"*** by Indian Society for Rational Pharmacotherapeutics, ISRPTCon2019 Pre-Conference Workshop at Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India held on 27th November , 2019.

- Participated in ***"Good Clinical Practice (GCP) Training for Investigators"*** by Prof (Dr.) Suparna Chatterjee and Dr. Supriyo Choudhury at Bioequivalence Study Centre, Department of Pharmaceutical

Technology, Jadavpur University, Kolkata, India on 2019, 2018, 2017, 2016, 2015, 2014, 2013, 2012 and 2011.

- Attended the seminar on ***"Evolving Trend in Pharmaceutical Regulation and Opportunities in Pharma Industries"*** at Dr. K. P. Basu Auditorium, Jadavpur University, Kolkata, India held on 20th May, 2017.

- Participated and presented poster in the DST Sponsored International Seminar cum Workshop on ***"Recent Advances in Clinical Research with a Special Emphasis on BA/BE Study"*** at Dr. Triguna Sen Auditorium and Vivekananda Hall, Jadavpur University, Kolkata, organized by Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India held from 11th September to 15th September, 2014.

- Attended the seminar on ***"Good Clinical Practice and Indian Regulation (Schedule-Y) Workshop"*** organized by Novo Nordisk and Apollo Glenegales Hospitals and Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India held on 25th May, 2013.

- Attended in the National Workshop on ***"Registration of Bioethics Committee for Clinical Research in India"*** at Dr. K. P. Basu Auditorium, Jadavpur University, Kolkata, organized by Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India held on 18th April, 2013.

- Participated and presented poster in the DST sponsored International Seminar on ***"Clinical Research and Regulatory Affairs : Present Scenario"*** organized by Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India held on 16th July and 17th July, 2012.

- Participated in the DST sponsored 5 day National Workshop on ***"Application of Chromatography and Mass Spectrometry In Clinical Research"*** organized by Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India held during September 2010.

- Participated in the seminar on ***"Biological Research-Now and Beyond"*** organized by Department of Zoology, College of Science and Technology, University of Calcutta, Kolkata, India held on 30th March, 2005.

- Participated in the seminar on ***"Pollution in Natural Waters and Methods of Conservation of Aquatic System through Water Surveillance"*** under the National Environment Awareness Campaign (NEAC), a project of the Ministry of Environment and Forests, Govt. of India, organized by The Zoological Society, Kolkata, India and Department of Zoology, College of Science and Technology, University of Calcutta, Kolkata, India held on 31st March, 2004.

- Participated in the National Symposium on ***"Zoology – A Neorealistic Approach"*** organized by Department of Zoology, College of Science and Technology, University of Calcutta, Kolkata, India held on 26th March and 27th March, 2004.

Publications

1. Dibya Das, **Dhiman Halder,** Anirbandeep Bose, Chiranjit Saha, Himangshu Sekhar Maji and Tapan Kumar Pal. "Bioequivalence study of azelnidipine 16 Mg tablet to evaluate pharmacokinetic profile of single dose in healthy, adult, human volunteers under fasting condition". ***International Journal of Applied Pharmaceutics.*** 2021; 13(4), 154-159.

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2. Dibya Das, **Dhiman Halder,** Himangshu Sekhar Maji, Pintu Kumar De and Tapan Kumar Pal. "Special emphasis on bioanalytical method development and validation of an anti-hypertensive drug azelnidipine by LC-ESI-MS/MS in healthy human volunteer's blood plasma". ***Research Journal of Pharmacy and Technology.*** 2021; 14(7), 3572-3578.
[DOI: 10.52711/0974-360X.2021.00618]

3. Dibya Das, **Dhiman Halder,** Himangshu Sekhar Maji, Pintu Kumar De, Sudipta Saha, Navjot Singh and Tapan Kumar Pal. "Introduction of an innovative approach for bioanalytical method development and validation of febuxostat by using LC-ESI-MS/MS in human plasma". ***Research Journal of Pharmacy and Technology.*** 2021; 14(8), 4060-4066.
[DOI: 10.52711/0974-360X.2021.00703]

4. Dibya Das, **Dhiman Halder,** Anirbandeep Bose, Chiranjit Saha, Himangshu Sekhar Maji and Tapan Kumar Pal. "Pharmacokinetic determination of methylprednisolone in Indian healthy volunteers by LC-MS/MS". ***Journal of Medical Pharmaceutical and Allied Sciences.*** 2021; 10-I 3 (1094), 2732-2736.
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Abstract Publications

1. Dibya Das, **Dhiman Halder,** Himangshu Sekhar Maji and Tapan Kumar Pal, "Method Development and validation of Methylprednisolone by LC-ESI-MS/MS in human plasma and its Application in BA/BE Studies", 8th World Conference on "Pharmaceutical Science and Drug Manufactering" (BioLEAGUES Worldwide, 17-18th April, 2021). [E-Oral Presentation]

2. Dibya Das, **Dhiman Halder,** Himangshu Sekhar Maji and Tapan Kumar Pal, "Human Plasma Analysis of Aceclofenac by LC-ESI-MS/MS following Method Development and Validation", 1st International e-Conference on "Changing Waves in Healthcare Research : Focus on Post-Covid Era" [ADAMAS University (School of Pharmaceutical Technology), 5-6th April, 2021]. [E-Oral Presentation]

3. Dibya Das, **Dhiman Halder,** Himangshu Sekhar Maji and Tapan Kumar Pal, "A Novel Investigative Approach of Quantitation of Antiretroviral Drug in Human Plasma by Rapid, Sensitive and Simple Validated LC-MS/MS Method", 55th Annual Conference of the Indian Hospital Pharmacists' Association (IHPA) and Hospital Pharmacy Foundation [Bengal School of Technology (A College of Pharmacy), 16th January, 2021]. [E-Oral Presentation]

4. **Dhiman Halder,** Easha Biswas, Anurupa Sasmal, Tapan Kumar Pal and Umesh Chandra Halder, "Bioanalytical Method Development and Validation of Newer Drugs in Human Plasma by LC-MS/MS under the Framework of BA/BE Study", International Conference on "Present Scenario of Bioavailability and Bioequivalence Studies and its Regulatory Affairs" (Jadavpur University, 12th February, 2020). [Poster Presentation]

5. Easha Biswas, **Dhiman Halder,** Anurupa Sasmal, Tapan Kumar Pal, Sanmoy Karmakar and Sanat Karmakar, "Bioalalytical Method Development of Newer Drug Eprosartan Mesylate with Rosuvastatin Calcium By LC-ESI-MS/MS", International Conference on "Present Scenario of Bioavailability and Bioequivalence Studies and its Regulatory Affairs" (Jadavpur University, 12th February, 2020). [Poster Presentation]

6. Dibya Das, **Dhiman Halder,** Himangshu Sekhar Maji, Pintu Kumar De and Tapan Kumar Pal, "Human Plasma Analysis of Rabeprazole by LC-ESI-MS/MS Following Method Development and Validation", COLLOQUIUM-2020 International e-Conference on "Innovation in Pharmacy and Green Chemistry during Post Covid-19 Era" (JIS University, 11-13th June, 2020). [Poster Presentation]

7. Dibya Das, **Dhiman Halder,** Himangshu Sekhar Maji and Tapan Kumar Pal, "Azelnidipine an Anti-Hypertensive Drug : Bioanalytical Method Development and Validation by LC-MS/MS in Human Plasma", Proceedings of the International Webinar on "Chemistry at the Bio – Chemical Interface" (St. Paul's Cathedral Mission College, 29-30th August, 2020). [E-Oral Presentation]

8. Dibya Das, **Dhiman Halder,** Himangshu Sekhar Maji, Tapan Kumar Pal and Pintu Kumar De, "Bioanalytical Method Development and Validation of Raltegravir by LC-ESI-MS/MS in Human Plasma", 9th International Science Conference World Science Congress (Jadavpur University, 28th February to 1st March, 2020). [Poster Presentation]

9. Dibya Das, **Dhiman Halder,** Himangshu Sekhar Maji, Tapan Kumar Pal and Pintu Kumar De, "Bioanalytical Method Development and Validation of Azelnidipine by LC-ESI-MS/MS in Human Plasma", SPER International Conference (Kathmandu University, 7-8th June, 2019). [Poster Presentation]

10. **Dhiman Halder,** Sangjukta Pradhan, Easha Biswas, Tania Chakraborty, Nilendra Chatterjee, Balaram Ghosh and Tapan Kumar Pal, "Bioanalytical Method Development and Validation of Metabolites by LC-MS/MS under the Framework of BA/BE Study", DST Sponsored International Seminar cum Workshop on "Recent Advances in Clinical Research with a Special Emphasis on BA/BE Study" (Jadavpur University, 11-15th September, 2014). [Poster Presentation]

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12. Bapi Gorain, Hira Choudhury, **Dhiman Halder,** Debotri Ghosh, Sangita Agarwal, Shubhasis Dan, Balaram Ghosh and Tapan Kumar Pal, " Bioequivalence Study of Fixed Dose Combination Tablet containing Olmesartan, Amlodipine and Hydrochlorothiazide in Healthy Subjects", DST Sponsored International Seminar on "Clinical Research and Regulatory Affairs : Present Scenario" (Jadavpur University, 16-17th July, 2012). [Poster Presentation]

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