Rationale of Using Hydrochlorothiazide in Combination with Losartan as Antihypertensive Drugs can be Evidenced by Evaluating Pharmacokinetic Parameters of Parent Drug and Metabolite Using LC-MS/MS (API-2000)

Thesis Submitted For Degree of Master of Pharmacy of Jadavpur University

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Dedicated to My Lovely Mother

JADAVPUR UNIVERSITY

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Title of the thesis:

"Rationale of Using Hydrochlorothiazide in Combination with Losartan as Antihypertensive Drugs can be Evidenced by Evaluating Pharmacokinetic Parameters of Parent Drug and Metabolite Using LC-MS/MS (API-2000)"

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CERTIFICATE OF APPROVAL

This is to certify that the thesis entitled **"Rationale of Using Hydrochlorothiazide in Combination with Losartan as Antihypertensive Drugs can be Evidenced by Evaluating Pharmacokinetic Parameters of Parent Drug and Metabolite Using LC-MS/MS (API-2000)"** submitted by **Pallab Mandal,** Class Roll No.-001711402003, Registration.Number.116517 of 2011-2012**,** Exam Roll No-M4PHA19005, under the supervision of **Prof.(Dr.) Jasmina Khanam** at the Department of Pharmaceutical Technology, Jadavpur University, for the award of Master of Pharmacy Degree of Jadavpur University is absolutely based upon his own work and that neither his thesis nor any part of the thesis has been submitted for any Degree/ Diploma or any other academic award anywhere before.

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work pursed by me. My thesis work entitled "**Rationale of Using Hydrochlorothiazide in Combination with Losartan as Antihypertensive Drugs can be Evidenced by Evaluating Pharmacokinetic Parameters of Parent Drug and Metabolite Using LC-MS/MS (API-2000)"**

All the information in this document have obtained and presented in accordance with academic rules and ethical conduct.

I also declare that as required by thesis rules and conduct, I have fully cited and referenced all the materials and results that is absolutely based upon my own work and that neither my thesis nor any part of the thesis has been submitted for any Degree/ Diploma or any other academic award anywhere before.

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PREFACE

The Present Thesis Work entitled "Rationale of Using Hydrochlorothiazide in Combination with Losartan as Antihypertensive Drugs can be Evidenced by Evaluating Pharmacokinetic Parameters of Parent Drug and Metabolite Using LC-MS/MS (API-2000)" deals with the formulation of fixed dose combination containing Losartan and Hydrochlorothiazide and single dose containing only Losartan for the treatment of hypertension. The research work can be divided into eight main parts: Preparation of Protocol of this work, Human Ethics Committee permission, written Informed consent form the volunteer, carry out pre-study screening of healthy volunteers to estimate the biochemical or pathological parameters and physical examination by doctor, blood sampling and plasma separation after dosing by single dose losartan drug and then fixed dose combination containing losartan and hydrochlorothiazide by randomization, then analytical method development and validation by LC-MS/MS for analysis of hydrochlorothiazide, losartan, losartan carboxylic acid, analysis of plasma samples obtained from the volunteers for analysis of hydrochlorothiazide, losartan, losartan carboxylic acid, evaluation of pharmacokinetic parameters Cmax, Tmsx, AUC, Kel, T1/2 of hydrochlorothiazide, losartan, losartan carboxylic acid, established the effect of hydrochlothiazide in releasing metabolite of losartan, rationale of the fixed dose combination for evaluating the therapeutic efficacy in preview of pharmacological action.

The introduction part of the thesis **(Chapter-1)** describes about pharmacokinetic and pharmacodynamic, symptoms, causes, pathophysiology of cardiac diseases and specially hypertension, drugs and its metabolite, fixed dose vs single dose of drug, about pharmacokinetic parameters, bioequivalence study, ethical principle, informed consent, randomanization of the volunteers, about rennin angiotensin aldosterone system and its function and effect of this system on the disease hypertension, regulatory guidelines, general bioanalytical method development, general bioanalytical method validation.

The second chapter represents a careful selection and presentation of the published material related to my present work. This **chapter 2** describes about single dose of the drug and fixed

dose of the cardiovascular drugs and its factors on which it dependent and the advantages of the fixed dose combination of the cardiovascular drugs, pharmacokinetics of the drugs, chemical structure with chemical properties, pharmacology, mechanism of action, pharmacodynamics, drug interaction, adverse drug reaction and side effect of the drugs losartan and its metabolite losartan carboxylic acid and hydrochlorothiazide, about liquid chromatography tandem quadruple mass spectrometry, instrument condition, bioanalytical method developmental parameters.

The main aims and objectives of this work are highlighted in **Chapter 3**

Chapter 4 opens with the list of materials used in this study. The material & methods part is described in the following points: materials, ethical clearance, sample collection time, sample extraction procedure, bioanlytical method development, bioanalytical method validation, volunteers report, pharmacokinetic parameter analysis, biochemical and pathological parameters.

The result and discussion of the work are elaborated in **Chapter 5.**

The summary of the overall work done And the conclusion drawn from the entire work are detailed in **Chapter 6.**

Chapter 7 gives a comprehensive list of references cited in the text.

During the whole project work principles of human care followed and instructions given by **Hurip Independent Bioethics Committee** (**Registered by DCG (I), vide ECR/746/Hurip/Indt/WB/2016**) and Study protocol (**Protocol Number: Research/JU/01/2018, Version no. 1.0, Date: 30/03/2018),** which was approved by the members of the Hurip Independent BioEthics Committee, were followed throughout the experiment.

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ABBREVIATIONS:

1. API: Atomic Pressure Ionization
2. LC-MS/MS: Liquid Chromatography Quadruple Tandem Mass Spectrometry
3. ADME : Absorption, Distribution, Metabolism, Excretion
4. CYP: Cytochrome P
5. NDA : New Drug Application
6. ANDA: Abbreviated New Drug Application
7. CHD : Chronic Heart Disease
8. RAS : Renin-Angiotensin system
9. ACE : Angiotensin Converting Enzyme
10. ACEIs : Angiotensin converting enzyme inhibitors
11. ARBs : Angiotensin receptor blocker
12. DRIs : Direct rennin inhibitors
13. AUC : Area Under the Curve
14. FDC : Fixed dose combination
15. COPD : Chronic Obstruction Pulmonary Disease
16. ICF : Informed Consent Form
17. IRB : Institutional Review Board
18. EC : Ethics Committee
19. CPU: Clinical Pharmacology Unit
20. Cmax : Maxium Concentration
21. CI: Confidence Interval
22. Tmax : Time to maximum concentration.
23. T1/2 : Elimination half-life.
24. MEC : Minimum Effective Concentration
25. MSC : Maximum Safety Concentration
26. ESI : Electrospray Ionization
27. APCI : Atmospheric pressure chemical ionization
28. APPI : Atmospheric pressure photoionization
29. ACD: Advanced Chemistry Development
30. GPCR: G-protein coupled receptor
31. RO5: Rules of 5
32. HCTZ: Hydrochlorothiazide
33. LLOQ: Lower Limit of Quantification
34. LQC: Low Quality Control
35. MQC: Medium Quality Control
36. HQC: High Quality Control
37. CDSCO: Central Drugs Standard Control Organization

CHAPTER:-1.

INTRODUCTION

1. INTRODUCTION

Cardiac disease is common. Minor congenital abnormalities affect one in one hundred live births and more serious abnormalities approximately one in five hundred. Acquired heart disease becomes increasingly common with age and in western societies heart disease is the commonest cause of death from the fourth decade onwards (A. Anglada *et al.* 2010).

Over the last decade mortality from heart disease has begun to decline in the United States of America, in Australia, and very recently this decline has also been noted in the United Kingdom. It is not yet clear whether this reflects changing life styles, improved diagnosis and management, or other factors as yet unrecognized (A. Foroumadi *et al*. 2007).

Heart disease has two peculiarities when compared with disease with other organs. First, it is very commonly latent, that is a disease process of, for example, the coronary arteries can proceed to an advanced stage before the patient notices any symptoms. Second, the number of symptoms attributable to heart disease is limited and it is common for much different pathology to present through a final common symptomatic pathway (A. Mendoza *et al*. 2015).

The growing prevalence of obesity, type 2 diabetes mellitus and metabolic syndrome, which are important risk factors for atherosclerosis, now threatens to reverse the progress that has been made in the age-adjusted reduction in the mortality rate of coronary heart disease. For many years cardiovascular disease was considered to be more common in men than in women. In fact, the percentage of all deaths secondary to cardiovascular diseases is higher among women (43%) than among men (37%) (A. Pereira, et al. 2015).

Successful drug therapy depends not only upon the choice of an appropriate drug but also upon the choice of suitable formulation. The delivery system needs to be reliable and its formulation needs to be technically feasible. This means the pharmaceutical quality of the delivery systems needs to be assured, drug release from the system needs to be reproducible and the negative influence of the body on drug release should be minimized. The task of the research and development scientists involved in the formulation of a new product is to focus on that point (Das R.et al, 2014).

Over the last 25 years, **Pharmacokinetics** has emerged as an integral part of drug development, especially when identifying a drug's biological properties. By pharmacokinetics, one means the

application of kinetics to a Pharmakon, the Greek word used to specify drugs and poisons. The term thereby implies the time course and fate of drugs in the body. This general definition broadly embraces absorption, distribution, metabolism (biotransformation) and *excretion* (ADME). Drug metabolism is the chemical alteration of a drug by the body. Some drugs are chemically altered by the body (metabolized). The substances that result from metabolism (metabolites) may be inactive, or they may be similar to or different from the original drug in therapeutic activity or toxicity (Karen Whalen, *et.al,* 6th edition).

This chemical alteration of drug in the body is also called biotransformation. It is needed to render nonpolar (lipid soluble) compound polar (lipid insoluble) so that they are not reabsorbed in the renal tubules and are excreted. Some drugs, called prodrugs, are administered in an inactive form, which is metabolized into an active form(A. Rosiak et al, 2006). The resulting active metabolites produce the desired therapeutic effects. Metabolites may be metabolized further instead of being excreted from the body. The subsequent metabolites are then excreted. Excretion involves elimination of the drug from the body, for example, in the urine or bile. In general biotransformation reactions generate more polar inactive metabolites that are readily excreted from the body. Many of the enzyme systems that transform drugs to inactive metabolite also generate biologically active metabolite of endogenous compounds. Lipophilic substances are eliminated efficiently by the kidney. Consequently, most lipophilic drugs are metabolized to more polar products, which are then excreted in urine. Drug metabolism occurs predominantly in the liver, especially by the cytochrome P450 (CYP) system (Goodman and Gilman, 12th edition).

The linking of **Pharmacodynamics** (response) and **pharmacokinetics** offers a composite understanding both about how the drug affects the body and how the body affects the drug.The most comprehensive insight about a drug's inherent pharmacokinetic properties is gained by studying an intravenous dose (A. Schulze, *et. al*. 2006). This route of administration has the greatest quantitative potential, as it permits a mass balance approach to be applied to distribution, clearance and the body processes associated with excretion and metabolic elimination (e.g. renal, hepatic). The administration of a drug by other routes, notably oral, introduces an uncertainty that reflects the unknown fraction that is actually absorbed. Consequently, such doses alone

cannot accurately identify the distribution and clearance processes (H.P.Rang, 8th edition).The most important property of any non-intravenous dosage form, intended to treat a systemic condition, is the ability to deliver the active ingredient to the bloodstream in an amount sufficient to cause the desired response. This property of a dosage form has historically been identified as physiologic availability, biologic availability or bioavailability. Bioavailability captures two essential features, namely how fast the drug enters the systemic circulation (rate of absorption) and *how much* of the nominal strength enters the body (extent of absorption) (A. Shafiee. *et.al*, 1997). Given that the therapeutic effect is a function of the drug concentration in a patient's blood, these two properties of non-intravenous dosage forms are, in principle, important in identifying the response to a drug dose. Onset of response is linked to the *rate* of drug absorption whereas the time-dependent extent of response is linked to the *extent* of drug absorption. While the bioavailability of each type of non-intravenous product (e.g. oral, inhalation, topical (e.g. patch), rectal, etc.) could be discussed, this chapter will of necessity focus only on orally administered products (A.M. Sales Solanoa, et.al, 2013). They certainly represent the major pharmaceutical class in drug development and patient treatment. The majority of drugs are formulated in such a dosage form which delivers the drug to the systemic circulation at a rate which is controlled by the physiological process in the body and not controlled by the dosage forms themselves (K.D.Tripathi, 7th edition). However, for many diseases the selected drug, on administration, should be released from the dosage form in required amount at a constant rate to the target organ over the desired period of time. The continuous administration of drug administration by conventional drug delivery systems seems impractical due to patient incompliance caused by accidental miss of dose and frequent drug administration. Therefore the rational drug therapy is that by which an acceptable therapeutic condition is immediately attained at the site of action and is then maintained constant for the desired period of the treatment (Goodman and Gilman, 12th edition).

Bioavailability following oral doses may vary because of either patient-related or dosage-form-related factors. Patient factors can include the nature and timing of meals, age, disease, genetic traits and gastrointestinal physiology. The dosage form factors include

- Chemical form of the drug (e.g. salt vs. acid),

- Physical properties (e.g. crystal structure, particle size), and
- Array of formulation (e.g. non-active ingredients) and manufacturing (e.g. tablet hardness) variables (Aghera *et. al*, 2012).

Not surprisingly, bioavailability is of clinical, academic, and regulatory interest. The latter includes agencies that approve the sale of products in their nation(s), as well as reimbursement agencies. Applications from manufacturers seeking regulatory approval for a new drug (e.g. New Drug Application (NDA)) must furnish exhaustive information about a drug's pharmacokinetics. Typically, such evidence entails studies wherein the drug has been orally administered. While such trials may broadly be viewed as bioavailability studies, many are ostensibly designed to assess the drug's safety and efficacy via strategies of dose escalation and chronic administration (Agnel M, *et.al*. 1996). These studies will not be entertained in this chapter. The more pertinent interest in bioavailability relates to questions about absolute extent of absorption (absolute bioavailability), the importance of product formulation changes that are made during a new drug's development process, the comparability of different oral dosage forms (e.g. modified-release versus conventional products), and whether the products can be administered with meals (B. Gargouri, *et.al.* 2014). These facets will receive attention in this chapter. Manufacturers seeking regulatory approval of competitive (generic) products (e.g. Abbreviated New Drug Application [ANDA]), must provide detailed bioavailability evidence showing head-to-head comparative performance of their product against the innovator's product. Such trials are fundamentally designed to establish clinical equivalence particularly as it relates to interchangeability or substitutability (21-CFR-300.50).

1.1 Clinical Features of Heart Disease:

The various symptoms of heart disease are

1.1.1 Cardiac Death

Cessation of the heart’s activities is a traditional death. There are three forms of cardiac death. These are Asystole; is a lack of electrical activation of the ventricle. Ventricular fibrillation; is due to Inco-ordinate activation of ventricular muscle with consequent lack of ventricular contraction. Electrochemical dissociation occurs when the ventricle is activated but is activated but is unable to contract or to expel blood (Antiplatelet Trialist Collaboration, 1994).

- **Causes of Cardiac Death:**

➢ Asystole:

i. Heart block
ii. Myocardial infarction
iii. Hypoxia

➢ Ventricular Fibrilation

i. Myocardial infarction
ii. Myocardial ischaemia
iii. Myocarditis
iv. Cardiomyopathy
v. Electrolyte disturbances: low or high potassium ion, low calcium ion or magnesium ion
vi. Electric shock
vii. WPW
viii. Long QT syndromes

➢ Electromechanical dissociation

i. Cardiac rupture
ii. Cardiac tamponade
iii. Low calcium ion
iv. Cardiac depressant drugs

1.1.2 Breathlessness (Dyspnoea)

It is a common symptom of cardiac disease. This is breathlessness which comes on during exertion and subsides on resting. It is commonly due either to heart failure or to lung disease (B. Prasaja, *et.al*. 2009).

1.1.3 Pulmonary oedema

This is persistent breathlessness resulting from fluid accumulation in the lung as a manifestation of acute left heart failure. The patient

1.1.4 Orthopnea

This is breathlessness brought on by lying flat. It is usually due to failure of the left side of the heart, and is attributed to redistribution of fluid from the lower extremities to the lungs (B. Schmidt,et.al. 2003)

1.1.5 Paroxysmal nocturnal dyspnoea

This is variant of orthopnea in which the patient awakes from sleep extremely breathless, has a persistant cough, and may produce white frothy sputum. It is usually relieved at least initially by sitting upright. It is a manifestation of acute left heart failure (B.R.Travis *et.al*, 2003).

1.1.6 Cheyne Stokes breathing

This is periodic breathing in which both the rate and depth of breathing increase to a maximum over a period of a few minutes, then decrease until breathing virtually ceases, when the cycle is repeated. It is a feature of severe heart failure (Bonfiglio. R. *et.al*. 1999).

1.1.7 Chest pain

1.1.7.1 Angina

This is a choking or constricting chest pain which comes on with exertion, is relieved by rest and is due to myocardial ischaemia. It is commonly felt retrosternally and may radiate to the left or more rarely the right arm to the thorat, jaws and teeth, or through to the back. The pain may be squeezing, crushing, burning or aching, but seldom stabbing. The pain may be brought on or exacerbated by emotion, and is frequently made worse by large meals or cold wind (Boyd. R. K. *et.al*. 2008).

1.1.7.2 Myocardial Infarction

The pain is similar in nature and distribution to angina but is more severe, persist at rest. There are usually features of sympathetic nervous system activation, and vomiting is common. There may be anxiety and a feeling of impending death (Brunton. L.L. *et.al*, 2007).

1.1.7.3 Dissecting aortic aneurysm

The pain is severe sharp and tearing often felt or penetrating through to the back. It may be accompanied by vomiting. The pulse may be accompanied by vomiting (Buhl A.E *et.al*. 1990).

1.1.7.4 Pericarditic pain

This is retrosternally, to the left of the sternum, or in the left or right shoulder. It characteristically varies in intensity with the phase of respiration (C.Carlsson, *et.al*, 2006)

1.1.7.5 Musculo-skeletal pain

This is very variable in site and intensity but it may or may not vary with posture or movement, it may be brought on by exertion but often does not cease instantly on rest and it is very commonly accompanied by local tenderness over a rib or costal cartilage (C.Espinoza *et.al*. 2014)

1.1.7.6 Oesophageal spasm

The pain can mimic that of angina very closely, is sometimes precipitated by exercise and may be relieved by nitrates (C.Kramer, et.al, 2002).

1.1.8 Oedema

1.1.8.1 Peripheral oedema

This is a feature of chronic heart failure and is due to excessive salt and water retention. It usually affects the ankles, leg, thighs and lower abdomen in that order. In a patient who is lying down it is most apparent over the sacrum. The oedema of heart failure is usually accompanied by at least some other symptoms of heart failure and by a raised jugular venous pressure. Unless it is long standing and the skin is very tense the oedema pits easily on pressure (C.A.Martinez *et.al*.2006).

1.1.8.2 Oedema of chronic venous insufficiency

It usually affects the ankles and lower legs only. The oedema pits readily and redistributes after a night's sleep. It is a relatively late and unreliable feature of deep venous thrombosis can cause severe venous congestion and oedema (C.A.Martinez-Huitle,*et.al*. 2009)

1.1.8.3 Oedema of nephrotic syndrome

It tends to be more severe and more widely distributed than the oedema of heart failure and it affects the face and arms but normal cardiac output and normal or reduced circulating blood volume. The presence of proteinuria confirms the diagnosis. Hyperproteinaemic oedema may also occur in liver disease and in protein losing enteropathy (G.Chen. *et.al.* 2009).

1.1.9 Palpitation

It is an abnormal subjective awareness of the heart beat. Patient can usually distinguish between sporadic and continuous palpitation and between an irregular and a regular pulse.

1.1.10 Syncope

It is loss of consciousness resulting from an inadequate blood supply to the brain. This may be due to sudden vasodilatation, to a sudden fall in cardiac output or to both simultaneously. Postural syncope when due to vasodilator or anti hypertensive drugs is an example of the former, and diminished cardiac output from complete heart block or a very rapid tachycardia of the latter (Chalmers J. *et.al*. 1993)

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1.1.11 Vasovagal fainting

This involves both a reflex cardiac slowing mediated by the vagus and a sudden withdrawal of peripheral sympathetic tone. It is a complex centrally mediated reflex which tends to be initiated when pain or a powerful emotional stimulus is inflicted against a background of intense sympathetic stimulation (Chang.M.S *et.al*. 2007).

1.1.12 Other symptoms

Tiredness is a common complaint with severe heart failure and ischemic heart disease.

1.2 Hypertension:

Hypertension and coronary heart disease (CHD) are of great importance. Hypertension affects above 20% of the total population of the USA with its major impact on those over age 50. CHD is the cause of death in 30% of males and 22% of females in England and Wales (Connor J. *et.al.* 2004).

Hypertension is defined as either a systolic blood pressure of greater than 140mmHg or a sustained diastolic blood pressure of greater than 90mmHg. Hypertension results from increased peripheral vascular arteriolar smooth muscle tone, which leads to increased arteriolar resistance and reduced capacitance of the venous system. Hypertension is also an important risk factor in the development of chronic kidney disease and heart failure, although chronic hypertension can lead to heart disease and stroke (C.Kerzia *et.al*. 2014)

1.2.1 Etiology of Hypertension:

Hypertension may occur secondary to other disease processes. Family history develops hypertension likelihood that an individual develop hypertension. Persons with diabetes, obesity or disability status are all more likely to have hypertension than those without. In addition, environmental factors, such as stressful life style, high dietary intake of sodium, and smoking, may further predispose an individual to hypertension (D.A.Sica. *et. al*. 2005).

1.2.2 Mechanism of controlling blood pressure

Arterial blood pressure is regulated within a narrow range to provide adequate perfusion of the tissues without causing damage to the vascular system, particularly the arterial intima (endothelium). Arterial blood pressure is directly proportional to cardiac output and peripheral vascular resistance (D.J.Triggle,*et.al*. 1995)

Cardiac output and peripheral resistance, in turn are controlled mainly by two overlapping control mechanisms: the baro-reflexes and the renin-angiotensin-aldosterone system.

1.2.2.1 Baroreceptors:

Baroreflexes act by changing the activity of the sympathetic nervous system. Therefore, they are responsible for the rapid, moment to moment regulation of blood pressure. A fall in blood pressure causes pressure-sensitive neurons (baroreceptor in aortic arch and carotid sinuses) to send fewer impulses to cardiovascular centers in the spinal cord. This prompts a reflex response of increased sympathetic and decreased parasympathetic output to the heart and vasculature, resulting in vasoconstriction and increased cardiac output. These changes result in a compensatory rise in blood pressure (D.Bortoli *et.al*. 2013)

1.2.2.2 Renin Angiotensin Aldosterone System :

The Renin-Angiotensin system (RAS) participate significantly in the pathophysiology in the hypertension, congestive heart failure, myocardial infarction and diabetic nephropathy.

In 1898, Tiegerstedt and Bergman discovered rennin, and in 1934 Goldblatt demonstrated that constriction of the renal arteries produced persistant hypertension, in 1940, Braun – Menendez and Page and Helmer in the US reported that rennin was an enzyme that acted on a plasma protein substrate to catalyze the the formation of the actual pressure material, a peptide, that was named hypertension and angiotonin by the latter, then after 20years this substances named was angiotensin and to call the plasma substrate angiotensinogen. In the mid 1950s two forms of angiotensin were recognized, a decapeptide angiotensin I [AngI] and an octapeptide angiotensin II[Ang II] formed by proteolytic cleavage of AngI by an enzyme termed as angiotensin-converting enzyme (ACE).

Kidney are important for aldostrone regulation and that angiotensin potently stimulates the production of aldosterone in humans. Renin secretion increased with depletion of sodium, ion. Thus, the Ras came to be recognized as a mechanism to stimulate aldosterone synthesis and secretion and an important homeostatic mechanism in the regulation of blood pressure and electrolyte composition (Dean J.R. Jones *et.al.* 2002).

In the early 1970s, polypeptide were discovered that either inhibited that either inhibited the formation of Ang II or blocked Ang II receptors. These inhibitors revealed important physiological and pathophysiological roles for the RAS and developed a new and broadly

efficacious class of anti-hypertensive drugs: the orally active ACE inhibitors. ACE inhibitors have roles on the RAS in the pathophysiology of hypertension, heart failure, vascular disease and renal failure. Selective and competitive antagonist of AngII receptors were developed that yielded losartan, the first orally active, highly selevctive and potent nonpeptide AngII receptor antagonist (E.Brillas.et.al. 2005)

1.2.2.2.1. **Components of the Renin-Angiotensin System :**

Angiotensin II , the most active angiotensin peptide, is derived from angiotensiogen in two proteolytic steps.

i. Renin an enzyme released from the kidneys, claves the decapeptide AngI from the amino terminus of angiotensionogen(rennin substrate) (E.Hermann.et.al. 1998)

ii. ACE removes the carboxy-terminal dipeptide of AngI to produce the octapeptide AngII. AngII acts by binding to two heptahelical GPCRs AT_1 and AT_2

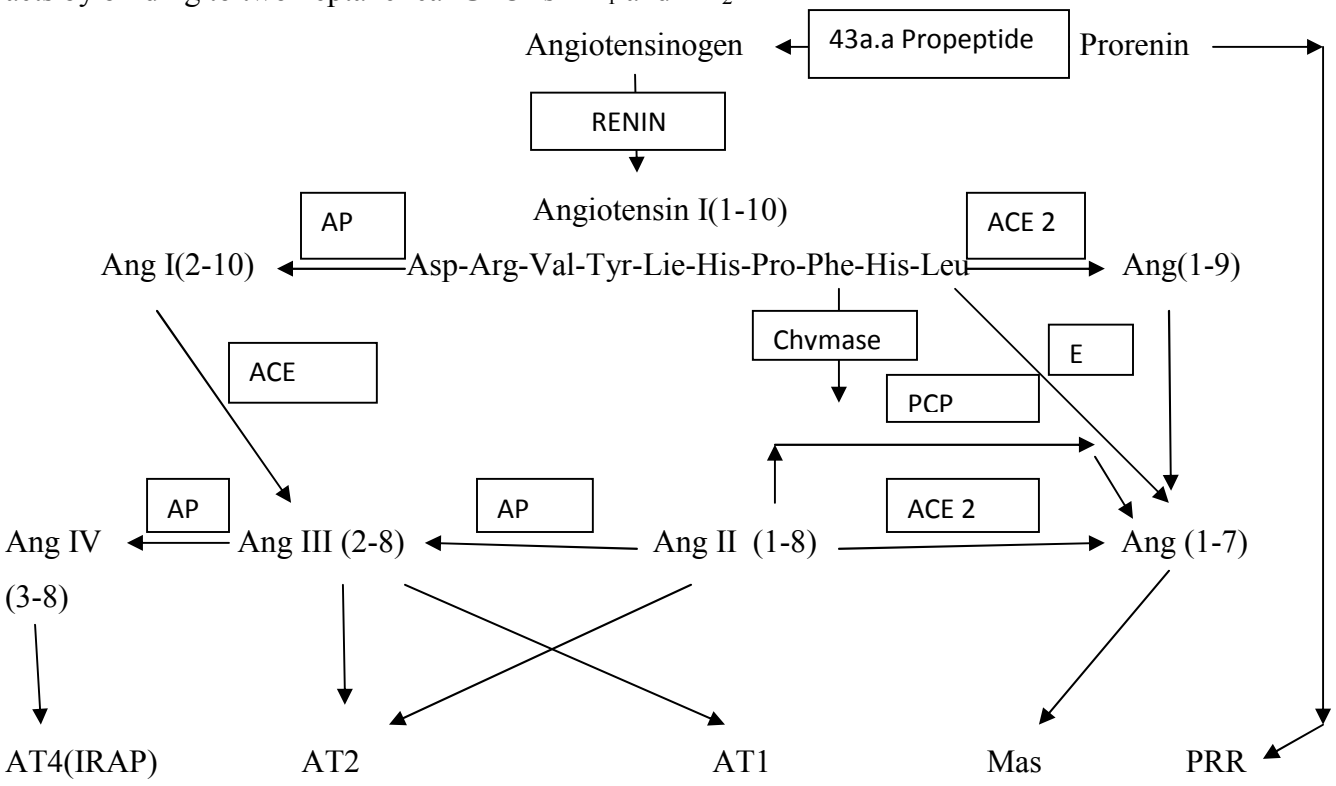


Figure: 1 Component of the RAS. The heavy arrows show the classical pathway, and the light arrows indicate alternative pathways. ACE, angiotensin converting enzyme; Ang, angiotensin; AP, aminopeptidase; E, endopeptidases; IRAP, insulin-regulated amino peptidases; PCP, prolylcarboxylpeptidase; PRR, (pro)renin receptor. Receptors involved: AT1 , AT2, M as, AT4, and PRR.

Recently it was observed that RAS has also a local RAS in tissue which is the alternative pathway of AngII synthesis and ACE dependent and formation of other angiotensin peptides AngIII, AngIV and to AngVII and additional angiotensin binding receptor AT_1, AT_2, AT_4 that precipitate in cell growth differentiation, hypertrophy, inflammation, fibrosis and apoptosis (E.Guinea.*et.al*.2009)

A. Renin:

Renin is the major determinant of the rate of AngII production. It is synthesis , stored and secreted by exocytosis into the renal arterial circulation by the granular juxtaglomerular cells located in the walls of affarent arterioles that enter the glomeruli.

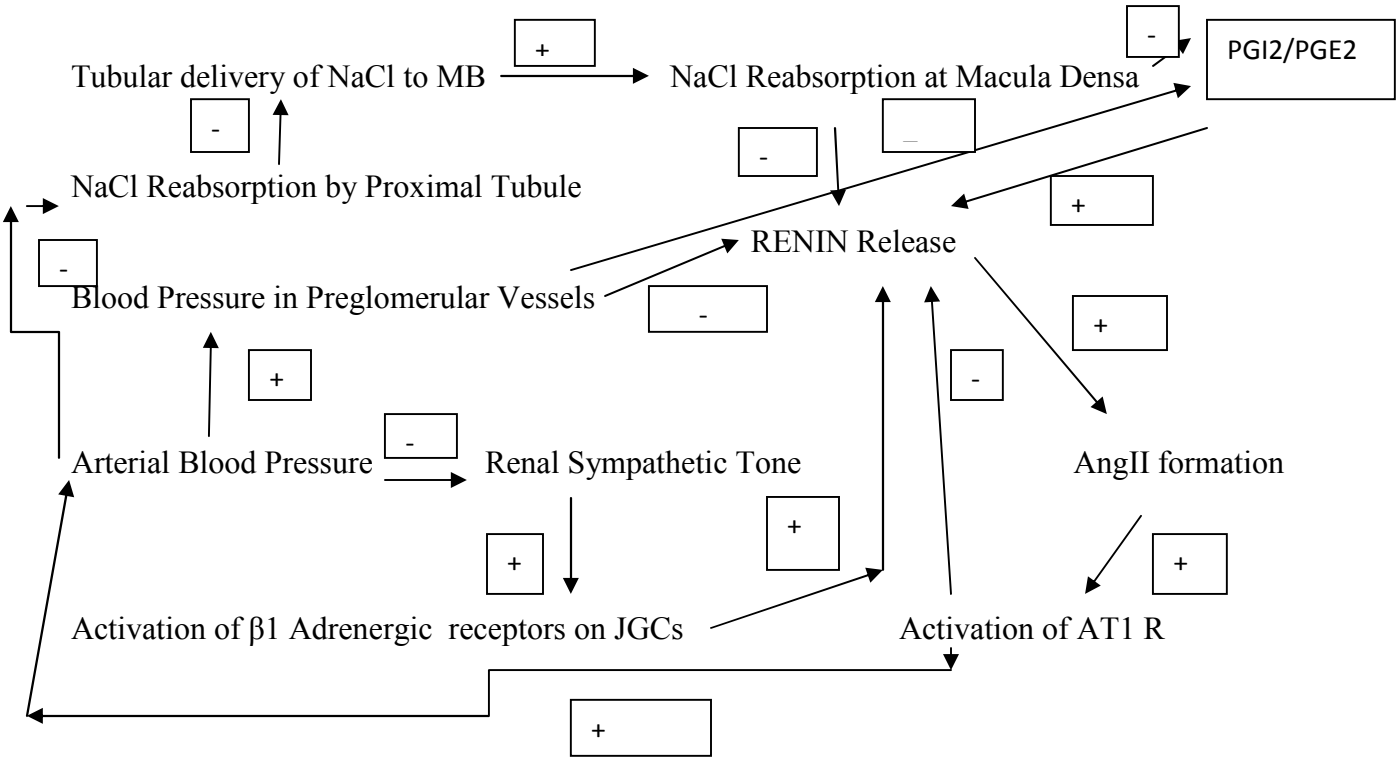


Figure: 2 Schematic portrayal of the three major physiological pathways regulating renin release. *See* text for details.

Renin is aspertyl protease enzyme that claves the bond between residues 10 and 11 at the amino terminus of angiotensinogen to generate AngI (E.Hermann. *et.al*.1998)

The active form of rennin is a glycoprotein that contains 340 amino acid. It is synthesized as a preproenzyme of 406 amino acid residues that is processed to prorenin. This prorenin is

proteolytically activated by proconvertase 1 or cathepsin B enzymes that removes 43 amino acids (propeptide) from its amino terminus to uncover the active site of renin. The active site of rennin is located in a cleft between two homologous lobes of the enzyme aspartyl proteases. Non proteolytic activation of prorenin, central to the activation of local tissue RAS, occurs when prorenin binds to the prorenin or rennin receptors, resulting in the conformational changes that unfold the propeptide and expose the catalytic site of the of the enzyme. Both rennin and prorenin are stored in juxtaglomerular cells and when released circulate in the blood. ARB interrupt both the long and short loop negative feedback mechanism and increase the rennin release (Ereshefsky L.*et.al.* 1999)

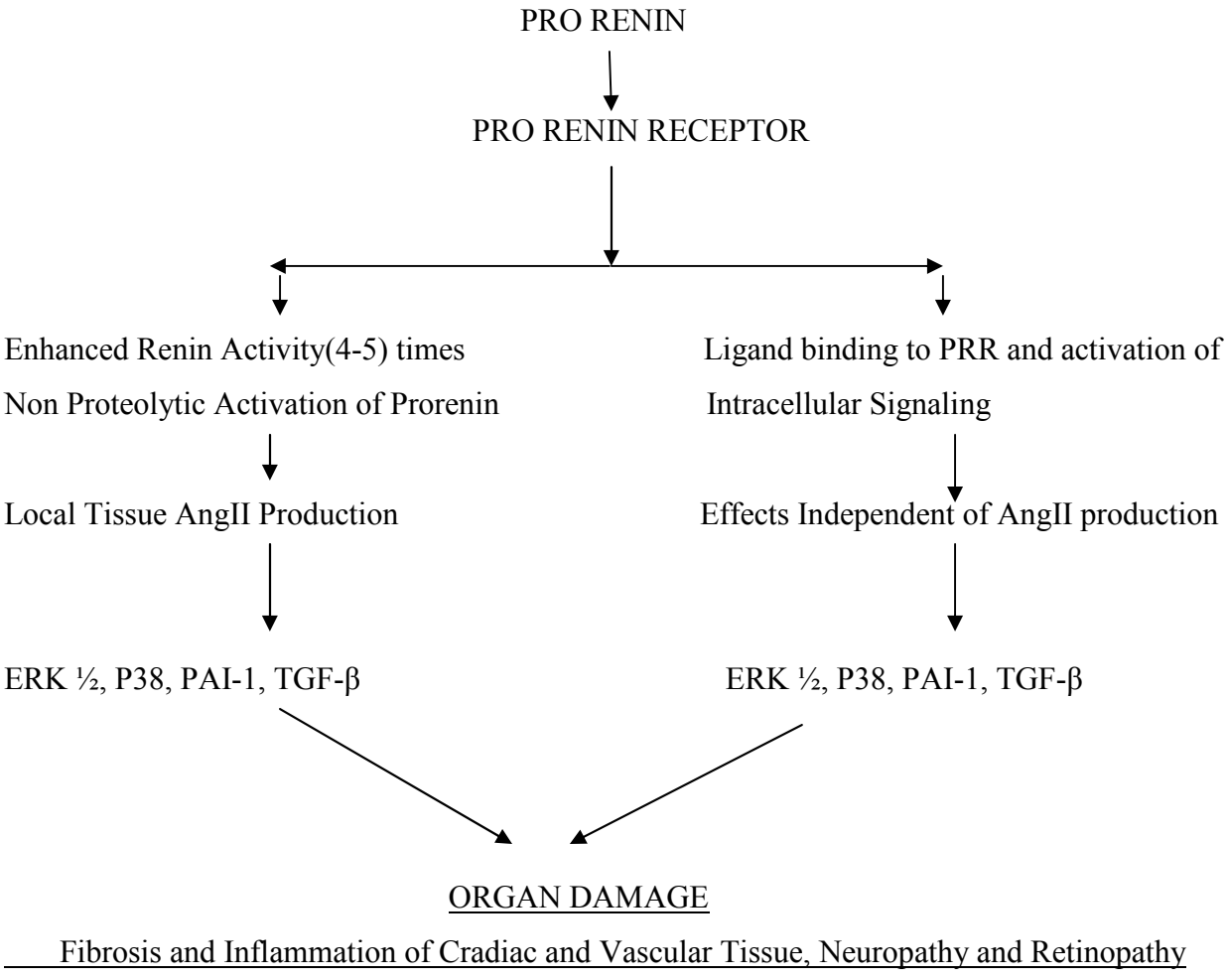


Figure: 3 Ang II-dependent and Ang II-independent actions of prorenin.

B. Angiotensinogen:

Angiotensinogen is an abundant globular glycoprotein which molecular weight 55000 to 60000 and it is the substrate of rennin. This angiotensinogen is the receptor of rennin. Angiotensin I cleaves from the amino terminus of the angiotesinogen and this angiotensinogen contains 452 aminoacid and is synthesized as preangiotensinogen which has 24-33 amino acid signal peptide. Angiotensinogen is synthesized and secreted from the liver, certain region of central nervous system and the kidney. This synthesis is stimulated by inflammation, insulin , estrogen, glucocoticoid, thyroid hormone and angiotensin II (Farolfi M. *et,al*. 1999)

C. **Angiotensin –Converting Enzyme:**

It is an ectoenzyme and glycoprotein with an apparent molecular weight 170000, it contains 1277 amino acid residue and has two homologous domain, each with a catalytic site and a zinc ion binding region. ACE has a large amino-terminal extra cellular domain, a short intracellular domain, and a 17- amino acid hydrophobic region that anchors the ectoenzyme to the cell membrane. It is rather nonspecific and cleaves the dipeptide units from substrate with diverse amino acid sequences and it proffered substrate have only one free carboxyl group in the carboxyl 1-amino acid, the enzyme does not degrade the Angiotensin II (Ferreira AC, *et.al*. 2013) This enzyme is identical to kininase II , the enzyme that inactivates bradykinin and other vasidilated peptides.. Although slow conversion of AngI to AngII occurs in plasma and very rapid metabolism occurs in tissue due to present of ACE in the luminal surface of endothelial cells throughout the vascular system. The ACE gene contains an insertion or deletion polymorphism in intron 16 that explains 47% of the phenotypic variance in serum ACE level.This deletion allele, associated with higher level of serum ACE increased the metabolism of bradykinin, increased risk of hypertension, cardiac hypertrophy atherosclerosis and diabetic nephropathy but may be prptective against Alzheimer's disease (Garrison SR. *et.al*.2017)

D. Angiotensin –Converting Enzyme 2:

This is carboxypeptidase and contains 805 amino acids in length and contain single catalytic domain that is 42% identical to two catalytic domain of ACE.It has opposing effect of ACE and cannot bind with inhibitors of ACE and has no effect on bradykinin (F.L. Migliorini *et.al*.2011)

E. Angiotensin Peptides:

When given intravenously , AngI is rapidly converted to AngII. AngI is less potent than AngII on smooth muscle, AngIII also called Ang(2-8) can be formed either by aminopeptidase on AngII or by the action of Ang(2-10). AngII and AngIII has similar effect and stimulate aldosterone secretion with equal potency (Gautam CS.*et,al*. 2006)

AngI can be converted to Ang(1-7) by endopeptidase and AngII can be converted to Ang(1-7) by polycarboxypeptidase. ACE2 converts AngI to Ang(1-9) and AngII to Ang(1-7). ACE metabolizes Ang(1-9) to Ang(1-7) (Gerbino PP. *et.al*. 2007).

F. Angiotensinases:

This includes amino peptidases, endopeptidases, carboxypeptidases, and other peptidases that degrade and inactivate angiotensin peptides (Giuseppe Mncia.*et.al*. 2013)

G. Local(Tissue) Renin-Angiotensin System

Local tissue RAS is an AngII –producing system that is being recognized for its role in hypertrophy, hypertension, inflammation, remodeling and apoptosis. Activation of tissue RAS and local Angiotensin II production require the binding of rennin or prorenin to the specific rennin receptor (PRR), located on cell surface (Gowda KV *et.al*. 2006)

H. Extrinsic Local RAS:

ACE is present on the luminal face of vascular endothelial cell throughout the circulation, and circulating rennin of renal origin can be taken up by the arterial wall and by other tissues (Grossman E. *et.al*. 2012)

I. Intrinsic Local RAS:

Many tissues including brain, pituitary, blood vessels, heart, kidney and adrenal gland,express mRNAs for rennin, angiotensinogen, and ACE.

J. The (Pro)Renin Receptor:

The prorenin receptor is the fuctional receptor, located on the cell surface that binds prorenin and rennin with affinity and specificity (H.P.Rang. 8th edition)

K. Alternative Pathway for Angiotensin Biosynthesis:

Angiotensinogen may be converted to AngI or directly to AngII by cathepsin G and tonin. Other enzyme that convert AngI to AngII include cathepsin G, chymostatin-

sensitive AngII generating enzyme and heart chymase. Chymase contributes to the local tissue conversion of AngI to AngII , particularly to the heart and kidneys.

L. Angiotensin Receptors:

AngII and AngIII couple to Specific GPCRs designated AT1 and AT2. AT1 receptor has 10,000 fold higher affinity for losartan and related biphenyl tetrazole derivatives than the AT2 receptor. Most of the biologic effect of AngII are mediated by the AT1 receptor because the AT1 receptor gene contains a polymorphism associated with hypertension, hypertrophic cardiomyopathy and coronery artery vasoconstrioction (HANSSON L. *et.al*. 1998)

1.2.2.2.2. Function of RAAS:

AngII increases total peripheral resistance via direct and indirect effect on blood vessels. The main effects of AngII on the cardiovascular System include:

I. Rapid pressor response
II. Slow pressor response
III. Vascular and cardiac hypertrophy and remodeling

1.2.2.2.3. Inhibitors of the RAAS :

Three types of inhibitors are utilized therapeutically:

I. Angiotensin converting enzyme inhibitors(ACEIs)
II. Angiotensin receptor blocker (ARBs)
III. Direct rennin inhibitors(DRIs)

1.3. Drugs and Metabolite

Metabolism of drugs may lead to the formation of inactive metabolite as for example clopidogrel transformed into clopidogrel carboxylic acid, active metabolite from an active drug as for example diosmin to diosmetin and losartan to losartan carboxylic acid , also activation of inactive drug as for example olmesartanminoxedil to olmesartan. These transformation occurs by two reactions(K.D.Tripathi,7th edition)

i. Nonsynthetic/Phase-I / Functionalization reaction
ii. Synthetic / Phase-II / Conjugation reaction

Nonsynthetic reaction done by oxidation, reduction, hydrolysis, cyclization, decyclization and synthetic reaction done by glucuronide conjugation, acetylation, methylation, sulfate conjugation, glycine conjugation, glutaythione conjugation, ribonucleoside synthesis. These reactions occur by the help of microsomal and nonmicrosomal enzyme and sometimes by Hofmann elimination (Goodman and Gilman, 12th edition).

Phase-I reactions introduce or expose a functional polar groups (e.g. OH, COOH, NH_2, SH) on the parent compound and loss of pharmacological activity or enhance the activity and produce a more water soluble compounds. As for example prodrugs are pharmacologically inactive compounds designed to maximize the amount of active compounds that reaches in the site of action.All ACE inhibitors like olmesartan[olmesatanminoxedil to olmesartan], enalapril[enalapril to enalaprilat by esterase activity], employed in the management of high blood pressure (Lubega and Prichard, 1991).

Phase-II conjugation reactions lead to the formation of a covalent linkage between a functional group on the parent compound or phase-I metabolite and endogenously derived glucuronic acid, sulfate, glutathione, amino acid, and acetate. So highly polar conjugates generally are inactive and excreted rapidly in the urine and faces (Lutz JD,*et.al,* 2012).

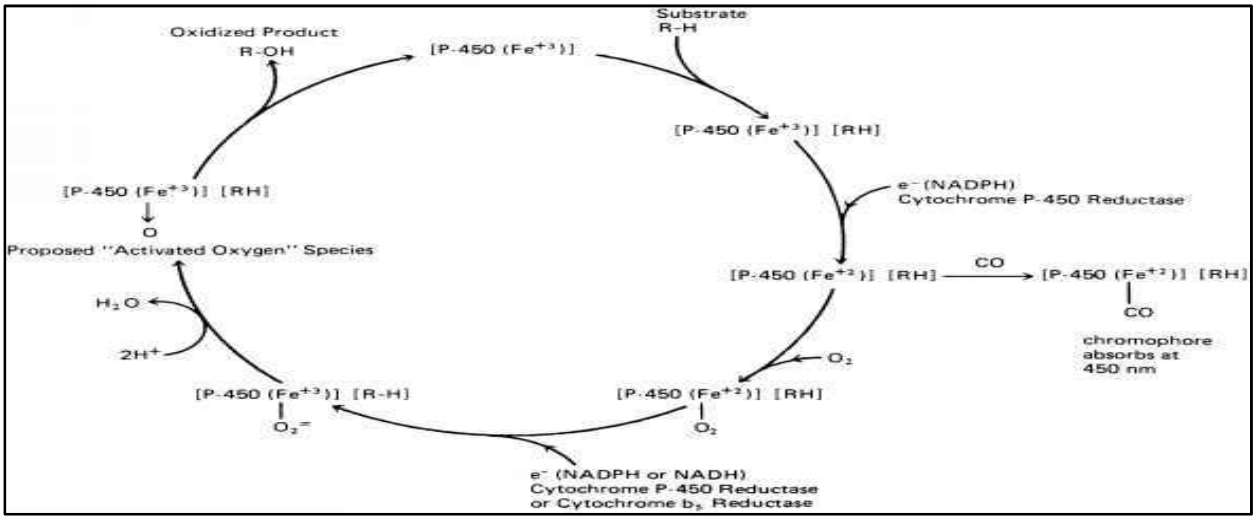


Figure-4: Proposed catalytic reaction cycle involving cytochrome P450 in the oxidation of xenobiotic

The purpose of phase-ii reactions is to attach small, polar and ionizable endogenous compounds such as glucuronic acid, sulfate, glycine and other amino acids to the functional handles of phase-I metabolites or parent compounds that already have suitable existing functional group to form water soluble conjugated products. Conjugated metabolites are readily excreted in the urine and are generally devoid of pharmacological activity and toxicity in humans.The enzyme systems involved in phase I reactions are located primarily in the endoplasmic reticulum, whereas the phase II conjugation enzyme systems are mainlycytosolic. Often, drugs bio transformed through a phase I reaction in the endoplasmic reticulum are conjugated at this same site or in the cytosolic fraction of thesame cell in a sequential fashion. These bio transforming reactions are carried out by CYPs (cytochrome P450 isoforms) and by a variety of transferases.

Importance of drug metabolite is that metabolites have undergone enough chemical change from the parent drug that their capabilities to bind to the target macromolecule are greatly diminished or abolished altogether (Fujioka Y,et.al, 2010).

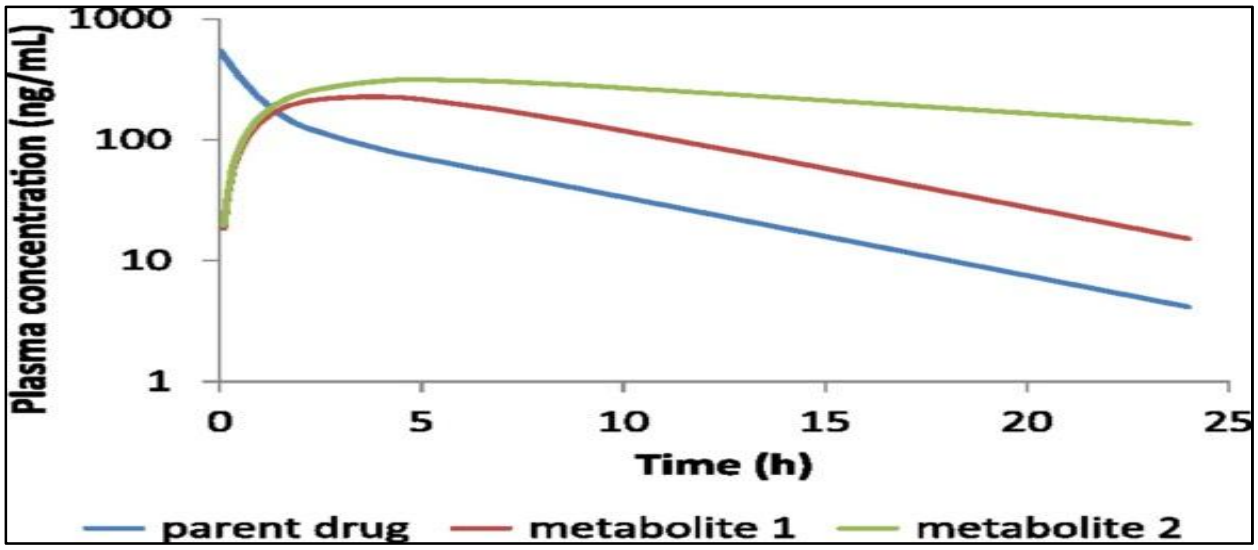


Figure-5: Example illustrating formation rate–limiting and elimination rate–limiting kinetics for metabolites. In this example, plasma concentrations of the parent drug and two metabolites are measured following intravenous administration of parent drug.

Metabolite 1 demonstrates formation rate–limiting kinetics since its $t_{1/2}$ is the same as the parent drug. Metabolite 2 demonstrates elimination rate–limiting kinetics because its $t_{1/2}$ is longer than that of the parent drug (Linnolia M,*et.al,* 1982).

However, in some cases a metabolite can retain enough intrinsic activity (*i.e.* binding to the target in the absence of any of the other factors that occur in a more complex system such as a whole organism, tissue, or in vitro cellular assay) at the target receptor such that it can contribute to the in vivo pharmacological effect(s) to a meaningful extent (Buhl, *et al.,* 1990; Messenger and Rundegren, 2004).

In case (A), the drug, represented as possessing a hydrophobic region, a hydrogen bonding region, and a solvent-exposed region, is shown binding to the receptor with points of interaction between atoms on each. (HA and HD refer to hydrogen bond acceptor and donors, respectively.) In case (B), the metabolic modification, represented by the triangle, does not affect these interactions, so the metabolite would be active. In case (C) the metabolic modification occurs on a position not involved in binding, so the metabolite would be active. In (D), the metabolic modification is on a position that disrupts interaction, so the metabolite would be inactive. Such disruptions can include introduction of a polar or ionic substituent, or a substituent that provides steric bulk(Mc Taggart F,*et.al,*2001).

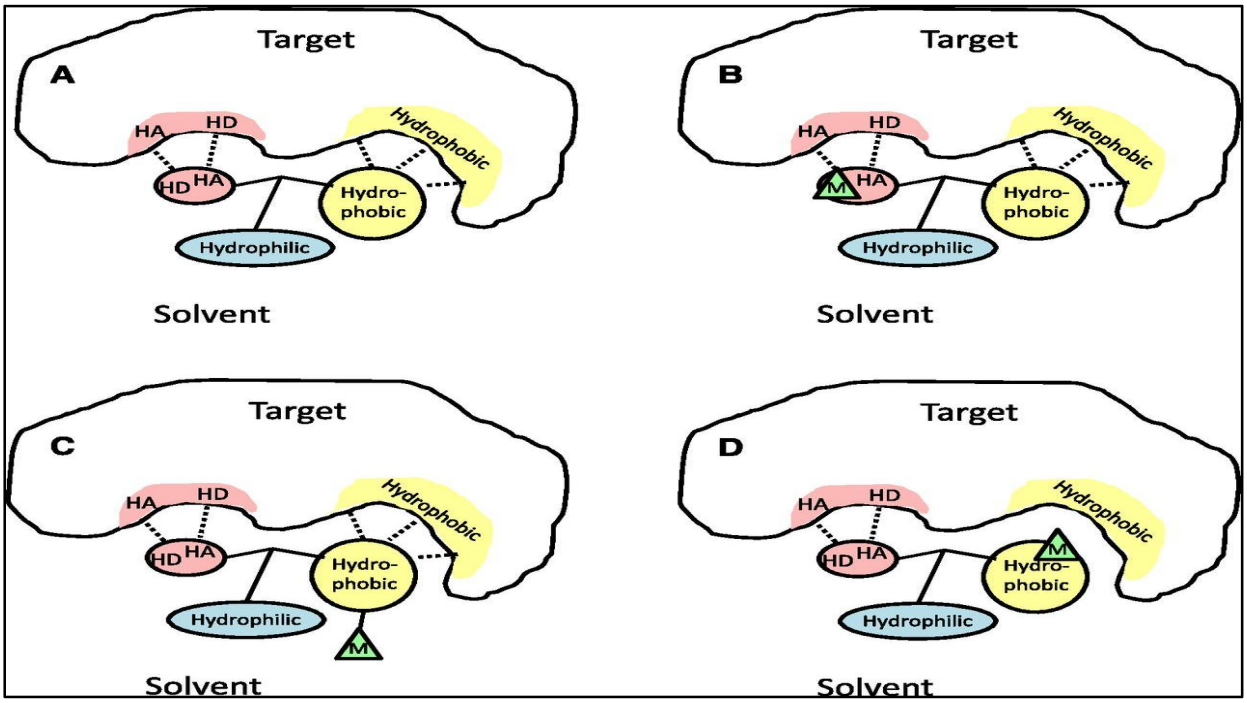


Figure-6: Schematic illustrating drug and metabolite binding to a target receptor

In the case of prodrugs, the parent drug is not pharmacologically active itself, but it is converted into a drug, which essentially is an "active metabolite" when administered. The metabolite possesses all the pharmacological activity, the parent none. Prodrugs are poor absorption, short half-life but in active metabolite improve these characters and also change its protein binding so change its elimination half-life as for example protein binding of Losartan and its metabolite LCA 0.013 and 0.002 respectively (Pluss*et al.,* 1972).

From a pharmacokinetic standpoint, metabolites (active and inactive) are frequently referred to as to having formation rate–limiting kinetics or elimination rate–limiting kinetics (Houston, 1981). A metabolite cannot have an elimination rate that exceeds the elimination rate of the parent drug from which it is formed. This is referred to as formation rate–limiting kinetics (Fig. 2). If an experiment is done wherein the metabolite is administered directly, then its $t_{1/2}$ may be shorter than when it is generated after administration of the parent drug. Many metabolites exhibit this type of behavior by virtue of having higher clearances and/or lower volumes of distribution than the parent drug. In elimination rate–limiting kinetics, the $t_{1/2}$ of the metabolite is longer than that of the parent drug (Fig. 2), and this value will be the same irrespective of whether the metabolite arises after administration of the parent drug or whether it is administered directly. If a metabolite exhibits elimination rate–limiting kinetics, then with repeated administration of parent drug the metabolite has the potential to accumulate to a greater extent than the parent drug at steady state.When considering an active metabolite, an important metric in understanding the potential contribution of the metabolite to effect relative to the parent drug is the area-under-thecurve (AUC) ratio. This can be defined as:

$$\mathbf{AUC_{metabolite} / AUC_{parent} = (f_{CL;m}) X \ (F_m) \ X \ (CL_{parent} / CL_{metabolite})}$$

The terms are defined as follows: $f_{CL;m}$ is the fraction of the clearance of the parent drug that yields the metabolite, F_m is the portion of the total metabolite generated within an organ that is released into the systemic circulation before it is either further metabolized or released into bile (in the case of the liver) or urine (in the case of the kidney), CL_{parent} is the total clearance of the parent drug, and $CL_{metabolite}$ is the total clearance of the metabolite (Lutz *et al.,* 2010; Yeung*et al.,* 2011; Lutz and Isoherranen, 2012; Smith and Dalvie, 2012).

Metabolites are frequently less protein bound in plasma than their respective parent drugs, presumably due to their decreased hydrophobicity. Thus, the potential decreases in membrane permeability and free tissue–to–free plasma concentration ratio of metabolites relative to their parent drugs can be offset by increases in free fraction (Whitehouse *et al.,* 1994).

So, from this point of view it is observed that-

- Active metabolite wherein the metabolite contributes the majority of the activity, even though the parent drug has intrinsic target potency.
- Active metabolites that contribute to target activity at a level comparable to parent drug.
- Active metabolite that possess target affinity but would be estimated to contribute little to in vivo effect, relative to the parent, and
- Metabolites that have activity at alternate pharmacological targets closely related to intended target that the parent drug interact with, and putatively contribute to clinical effect via this alternate target.

Examples:

A. Drugs with active metabolite that dominate the activity:

Drug	Metabolite
Albendazole	S-oxide metabolite
Allopurinol	Oxypurinol
Artesunate	Dihydroartimisinin
Astemizole.	o-desmethylastemizole
Codeine	Morphine
Dolasetron	Hydrodolasetron
Ebastine	Carebastine
Loratadine	Desloratadine
Losartan	Losartan carboxylic acid
Risperidone	Paliperidone

B. Drugs with Active Metabolites That Contribute Comparably to the Parent:

Drug	Metabolite
Acebutolol	Diacetolol
Acetohexamide	Hydroxyhexamide
Amiodarone	Desethylamiodarone
Carbamazepine	Carbamazpine-10,11- epoxide
Clarithromycin	14-hydroxyclarithromycin
Fluoxetine	Norfluoxetine
Itraconazole	Hydroxyitraconazole
Metoprolol	α-hydroxymetoprolol
Quinidine	3-hydroxyquinidine
Spironolactone	Canrenone

C. Drugs with Metabolites That Possess Target Potency But Contribute Little to *In Vivo* Effect:

Drug	Metabolite
Alprazolam	1′hydroxyalprazolam
Carisoprodol	Meprobamate
Chloroquine	N-desethylchloroquine
Diazepam	Desmethyldiazepam
Granisetron	7-hydroxygranisetron
Halofantrine	Desbutylhalofantrine
Lumefantrine	Desbutyllumefantrine
Lidocaine	Monoethylglycinexylidide and glycine xylidide
Primidone	Phenobarbital
Rosuvastatin	N-desmethylrosuvastatin

D. Drugs with Metabolites possessing activity at related targets

Drug	Metabolite
Amitryptyline	Nortriptyline
Clozapine	Norclozapine

Clomipramine	8-hydroxyclomipramine
Doxepin	N-desmethyldoxepin
Imipramine	Desipramine
Loxapine	Amoxapine
Nefazodone	Hydroxynefazodone

E. Drugs That Generate Active Metabolites but Assessment of *In Vivo* Contribution Is Ambiguous

Drug	**Metabolite**
Atorvastatin	2 and 4- hydroxyatorvastatin
Bromhexine	Ambroxol
Bupropion	Hydroxybupropion
Etretinate	Acitretin
Ivabradine	N-desmethyivabradine
Mycophenolic acid	Glucuronide derivative

So, there are many examples of drugs used in clinical practice that were originally observed as metabolites of other drugs which possess advantages as drugs themselves i.e. greater potency or efficacy, superior dispositional properties, improved safety profile etc (Shipkova*et al.,*2002; Ragueneau*et al.,*1998; Pilkington and Brogden, 1992;Malerba and Ragnoli, 2008; Taylor *et al.,* 1995; Owens *et al.,*1997;Ereshefsky, 1999;Mundo*et al.,*1974; Thomas and Jones, 1977; Linnoila*et al.,* 1982; Nunez and Perel, 1995; Agnel*et al.,* 1996; Tatsumi*et al.,* 1997 ; McTaggart *et al.,* 2001)

1.4. Fixed dose combination vs. Single dose

Fixed dose combination (FDC) drugs, are formulations of two or more active ingredients in a single tablet (Orloff DG, 2005). According to the US FDA, two or more drugs may be combined into a single

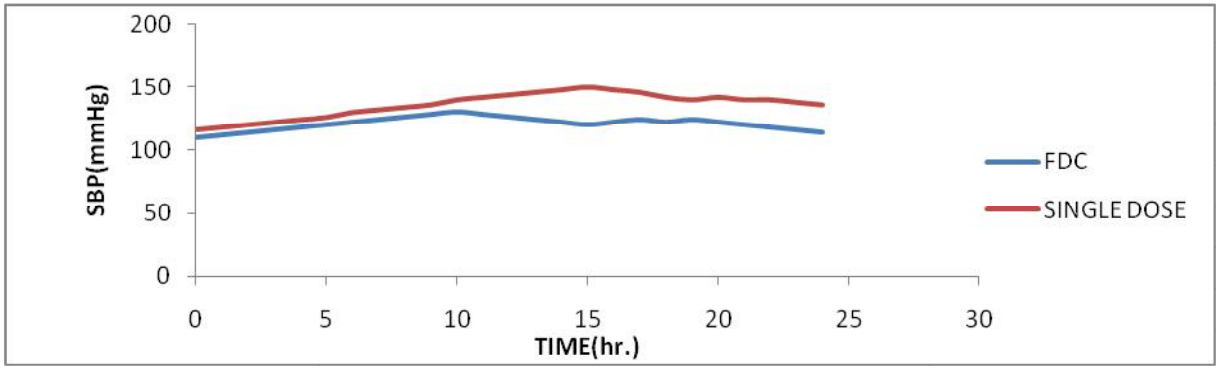


Figure-7: Change in SBP within 24hr. using FDC and Single Dose combination of a Antihypertensive drug

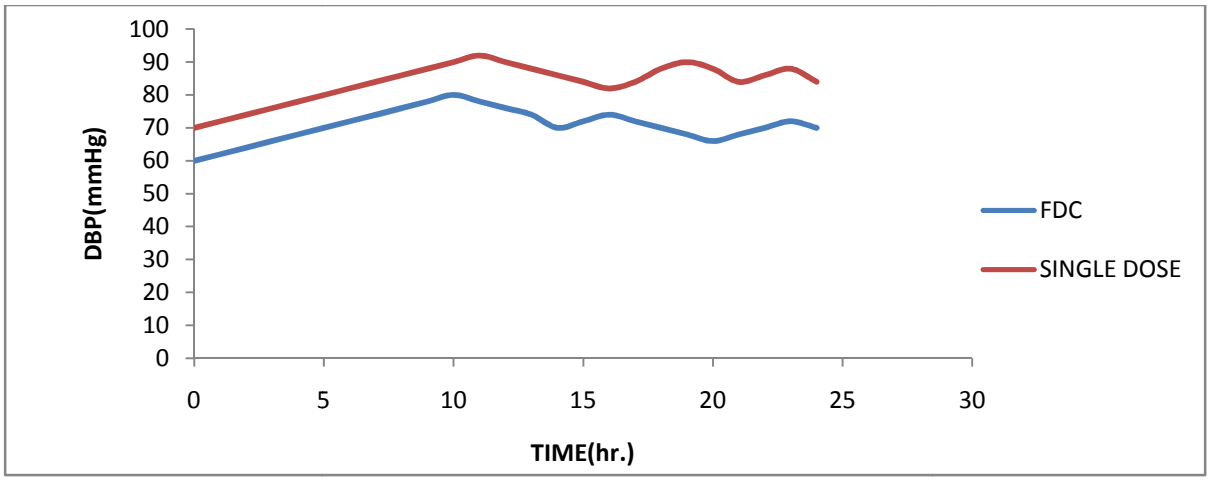


Figure-8: Change in DBP within 24hr. using FDC and Single Dose combination of a Antihypertensive drug

dose when each component makes a contribution to the claimed effect and dosage of each component (*i.e.* amount, frequency, and distribution) produces a safe and effective treatment for a significant patient population requiring such ocurrent therapy (21-CFR-300.50; FDA, 1975)

Hypertension is the most significant risk factor for preventable death worldwide so blood pressure control is very importance (Krause *et.al.* 2011). The use of fixed dose combination can provide more effective blood pressure control than single dose combination by increasing compliance and adherence (Gerbino*et. al.,* 2007;Schroeder K, Fahey T*et.al,* 2004). FDCs established good BP control in both the moderate and intensive treatment groups is well tolerated, has low side-effect profile (Operil*et.al.* 2011).

Fixed-dose combinations (FDCs) of drugs for TB treatmenthave been advocated internationally to prevent the emergence of drug resistance attributable to inappropriate drugintake (Bull Int Union Tuberc Lung Dis. 1988; 63(2):60–64; Ferreira AC.*et.al*. 2013).Use of FDCs can reduce the risk of an incorrect dosage, simplify drug procurement, and aid in ensuringadherence without changing the drug dosage.

Fixed dose combinations have become an alternative to monotherapies in the treatment of diseases as hypertension, diabetes, Helicobacter pylori, AIDS-HIV infections and tuberculosis, asthma, COPD by offering several advantages including (Drug Update,2008 ; FDC,2013)

- Patient compliance
- Simple dosage schedule
- Greater efficacy compared to monotherapy
- Reduced risk of adverse events
- Synergistic effect
- Inhibition of microbial resistance
- Cheaper shipment and packaging activities
- FDCs improve glycemic control, showing better efficacy.
- Medical expenditures due to hospitalization can be reduced.
- It prevents polypharmacy.

Several disadvantages also present but it's lower than advantages

- Reduced dosage flexibility
- Drug interactions
- Misidentifying the causative ingredient when the patient experiences side effect
- The combination may affect the bioavailability of agents
- Dose titration will be difficult.

Though some critical issues occur during evaluation FDC's

- Safety/efficacy
- BA\BE
- Stability

Fixed Dose Combination (FDC) is highly popular in the Indian pharmaceutical market and has been particularly flourishing in the last few years.

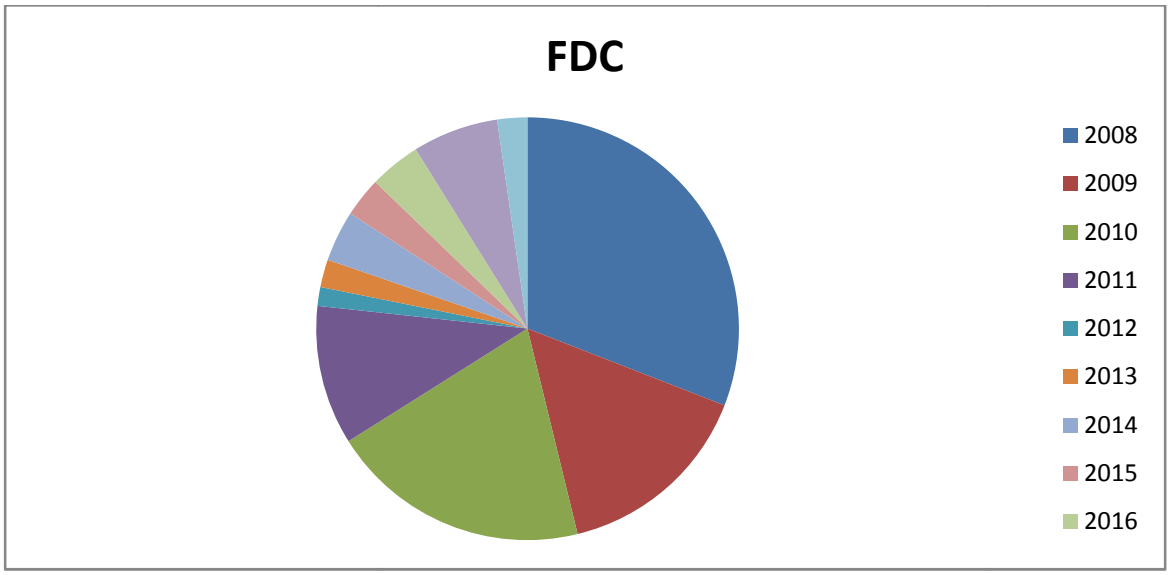


Figure-9: Pie chart of FDC approved by CDSCO in last ten year

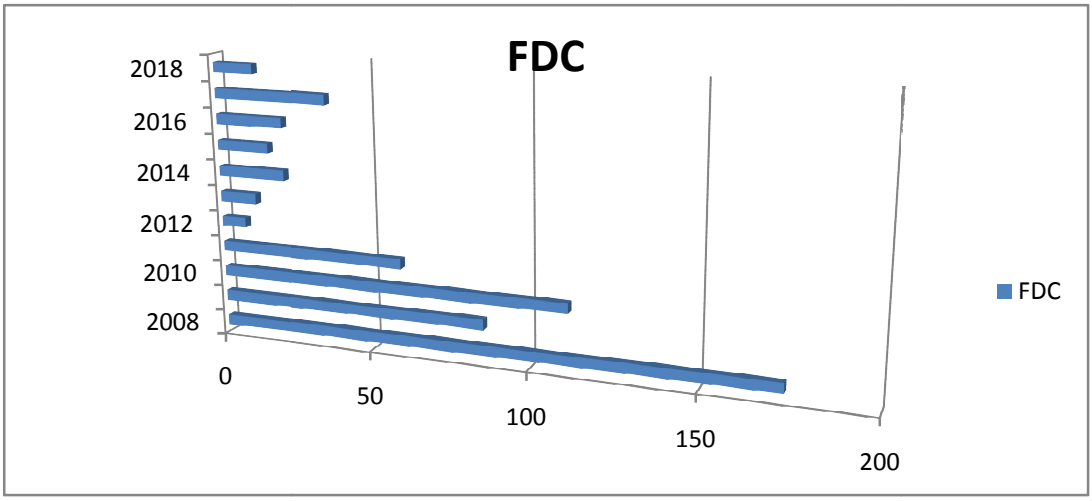


Figure. 10: Bar chart of FDC approved by CDSCO in last ten year

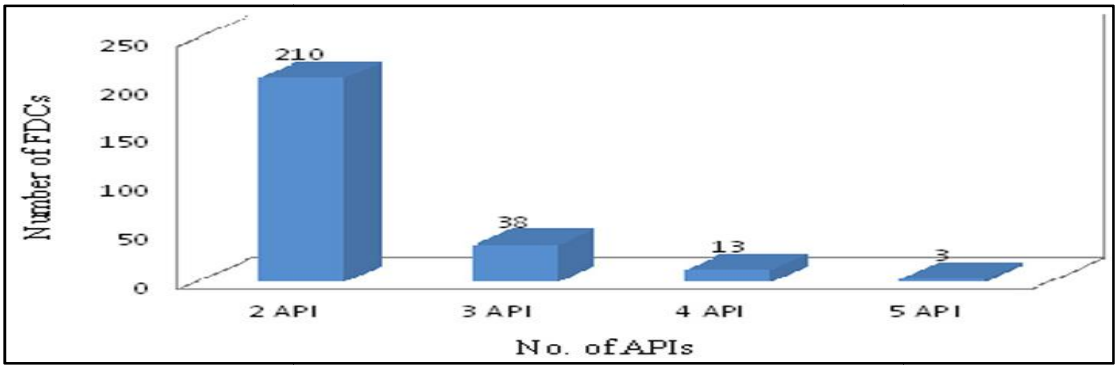


Figure-11: Number of Active Pharmacological Ingredients (API) in FDCs.

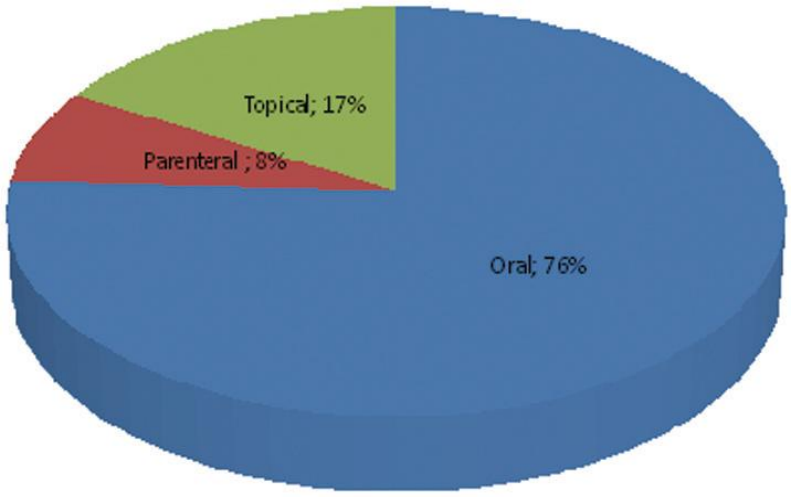


Figure-12: Distribution of FDCs according to dasage form

From these valuable information it is confirm that FDC is more valuable combination than single dose combination of any particular drug for the prophylactic and maintenance treatment of any diseases.

1.5. Pharmacokinetic studies in humans

1.5.1 Bioequivalence 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, or serum and/or urine to indicate the release of the drug substance from the drug product into the systemic circulation (Mandal U, *et.al,*2004).

This approach resets on the understanding that measuring the active moiety or ingredient at the site of action is not generally possible and, furthermore, that some relationship exists between the efficacy/safety and concentration of the active moiety and / or its important metabolite or metabolites 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 (Malerba U,*et.al*. 2004) A common design for a bioequivalence study involves administration of the test and reference products on two occasions to volunteer subjects,

with each administration separated by a washout period. The washoutperiod is chosen to ensure that drug given in one treatment is entirely eliminated prior to administration of the next treatment.Just prior to administration, and for a suitable period afterwards,blood and/or urine samples are collected and assayed for the concentration of the drug substance and/or one or more metabolites. The rise and fall of these concentrations over time in each subject in the study provide an estimate of how the drug substance is released from the test and reference products and absorbed into the body. To allow comparisons between the two products, these blood (to include plasma or serum) and/or urine concentration time curves are used to calculate certain bioequivalence metrics of interest. These metrics are calculated for each subject in the study and the resulting values are compared statistically (Mandal U, *et.al,* 2005).

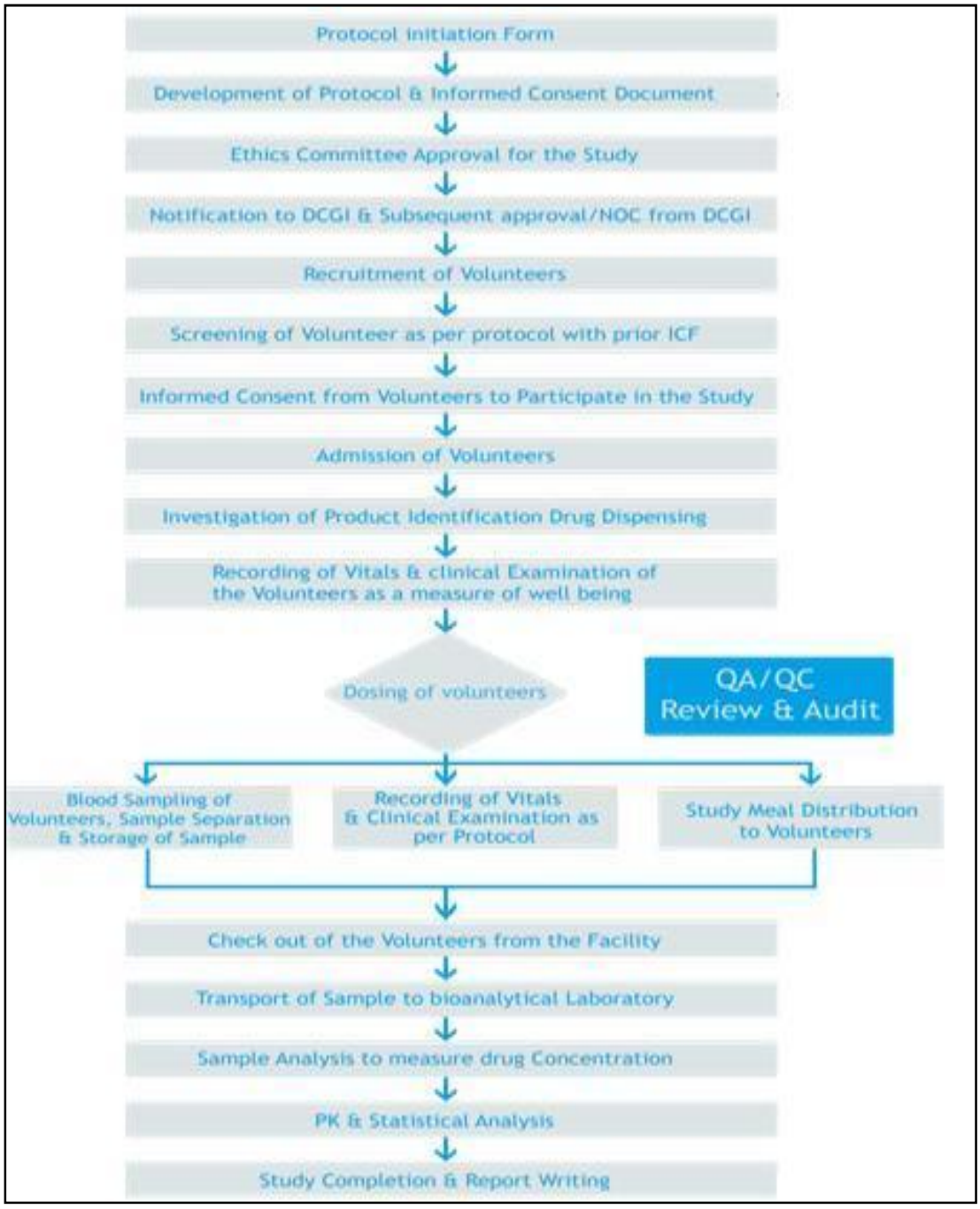


Figure-13: Flow chart of BA/BE Study

1.5.2 Subjects

For a sound bioequivalence study the sponsor should enroll a number of subjects sufficient to ensure adequate statistical results, which is based on the power function of the parametric statistical test procedure applied. The number of subjects "should be not less than 12"

(sometimes more than 24 are needed as in case of highly variable drugs) and should be determined using appropriate methods taking into account the error variance associated with the primary parameters to be studied (as estimated for a pilot experiment, from previous studies or from published data), the significance level desired ($\alpha = 0.05$), and the deviation from the reference product compatible with bioequivalence (± 20%) and compatible with safety and efficacy. In most of the cases 18-24 normal healthy subjects (sometimes more than 24) preferably nonsmoking, between 18-50 years in age and within 10% of ideal body weight for height and body build (Metropolitan Life Insurance Company Statistical Bulletin, 1983) are enrolled in a crossover bioequivalence study. Fora parallel design study a greater number of subjects may be required to achieve sufficient study power. Sponsors should enter a sufficient number of subjects in the study to allow for dropouts. Because replacement of subjects could complicate the statistical model and analysis, dropouts generally should not be replaced. The major objective of using a selective demographic profile is to minimize the magnitude of inter-subject variability(Hauscheke D, *et.al,* 1990).

In some cases, for bioequivalence studies on specific classes of drugs, for example cytotoxic drugs, drugs solely recommended for a very specific population or gender, etc., and for studies using pharmacodynamic or clinical endpoints, a targeted patient population may be enrolled in the study. If females are included in the study, the effects of gender differences and menstrual cycle (if applicable) are examined statistically.A physical examination, medical history (administered within 30 days prior to theinitiation of the study), routine blood chemistry and the hematology tests and urinalysis should be performed to ensure normal hepatic, hematological and renal functions of the volunteers selected for the study. Subjects should be free of any history of serious gastrointestinal, renal, hepatic, cardiovascular or hematological disorders and should have no history of adverse reactions to the drug (or its class) under study (Farolfi M,*et.al,* 1999).Exclusion and inclusion criteria should be stated in the study protocol. The subjects are not permitted to take any prescription or over-the-counter drug products within two weeks of the start of the study. Ingestion of alcohol or caffeine or related xanthines containing food or beverages is not allowed within 48 hours. Each subject is enrolled after signing an Informed Consent Form (ICF). Both the study protocol and ICF are approved by an appropriate

Institutional Review Board (IRB) prior to the start of the study. A prior provision is made for the replacement of dropout subjects by enrolling additional subjects(S Dan, *et.al.*2014)

1.5.3 Ethical Principles

All research involving human subjects should be conducted in accordance with the ethical principles contained in the current version of the Declaration of Helsinki. It is essential to have a review committee confirm the protocol complies with ethical standards for research on human subjects. The voluntary informed written constant of the healthy volunteers to participate in the study must be obtained. Information given to each volunteer should include details of the study, risks associated with participation and information regarding the right to withdraw at any time from participation without jeopardy.

As per revised Schedule Y of Drugs & Cosmetics Act, 1940, amended in 2005 and the recent changes (2013) in the act, the ethics committee approving drug trials should have in the quorum at least one representative from the following groups:

- One basic medical scientist (preferably one pharmacologist).
- One clinician
- One legal expert or retired judge
- One social scientist/ representative of non-governmental organization/ philosopher/ ethicist/ theologian or a similar person
- One lay person from the community

The Ethics Committee (EC) can have as its members, individuals from other institutions or communities with adequate representation of age and gender to safeguard the interests and welfare of all sections of the community/society.It is mandatory to have Ethics Committee approval before initiation of clinical study.

1.5.4 Informed consent

Before recruitment and enrollment into the study, each prospective candidate will be given a full explanation of the study. Once this essential information is provided to the subject and once the physician in charge has the conviction that he understands the implications of participating in the study, the subject will be asked to sign the informed consent form.

1.5.5 Randomization and dosing of the volunteers

The volunteers were randomized on the previous day of study initiation. During the study session I and II, each volunteer will receive either the test or reference preparation as a single dose, only on the study day, at a fixed time with 240-ml. of water after overnight fasting of at least 10 hrs. The test product should be from a production lot or from a lot produced under production conditions. Each drug product should be clearly identified by its lot number, manufacture, and expiration dates.

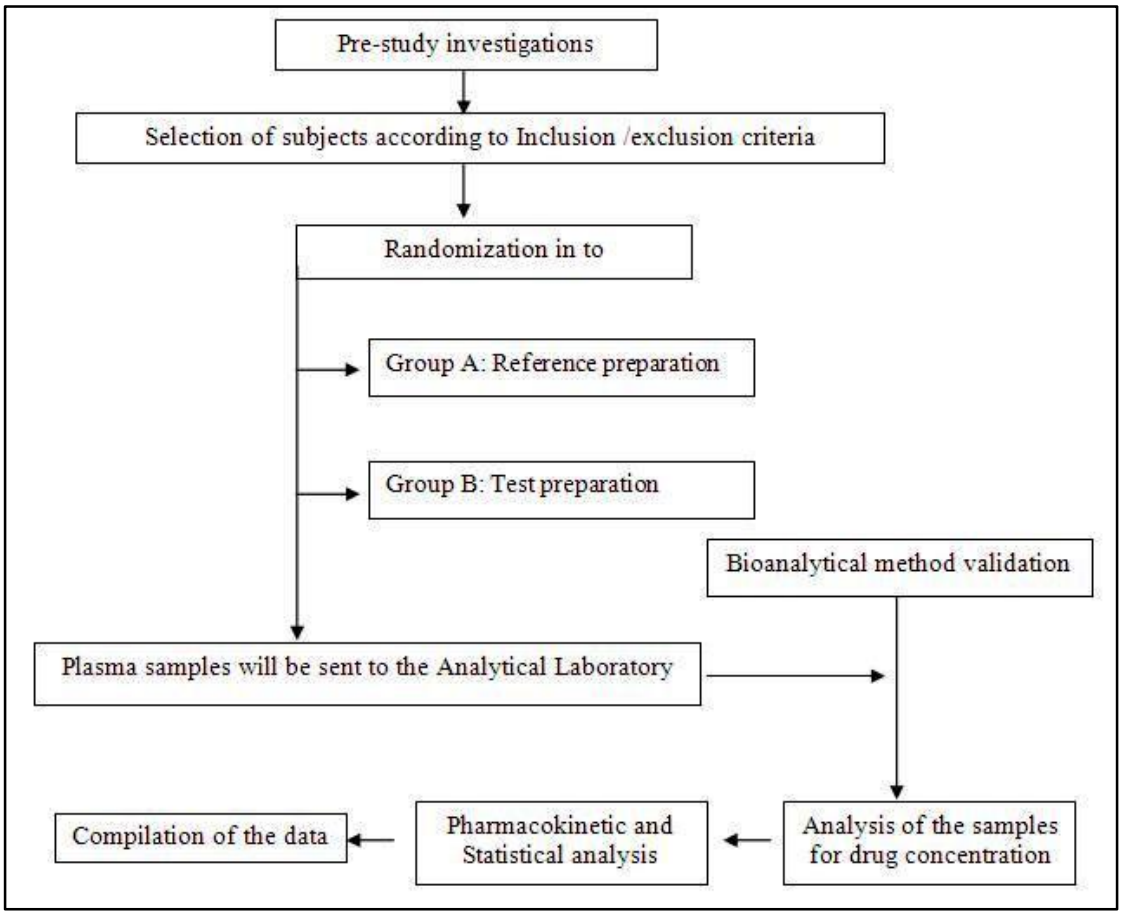


Figure-14: Comparative pharmacokinetic study flow chart

An approved product serves as the reference drug. A generic drug product (Test Drug) is compared to the designated innovator product (Reference Drug). A reference product is a pharmaceutical product with which the new product is intended to be interchangeable in clinical practice. The reference product would normally be the innovator product for which efficacy,

safety and quality have been established. Bioequivalence studies on generic products are usually conducted on the highest approved strength, unless there are safety concerns preventing the use of this strength. The administered dose does not ordinarily exceed the dose recommended in the labeling.

Typically, in a single dose study, the test and reference drug products whose potencies do not vary more than ± 5%, are administered to the subjects according to their randomization schedule and pre-assigned sequence. The dose is administered with sufficient fluid after at least 10 hours of fasting which is continued for at least 4 hours post-dose. Appropriate restrictions on fluid intake and physical activities are made, and all vital signs and adverse events are monitored post-dose.

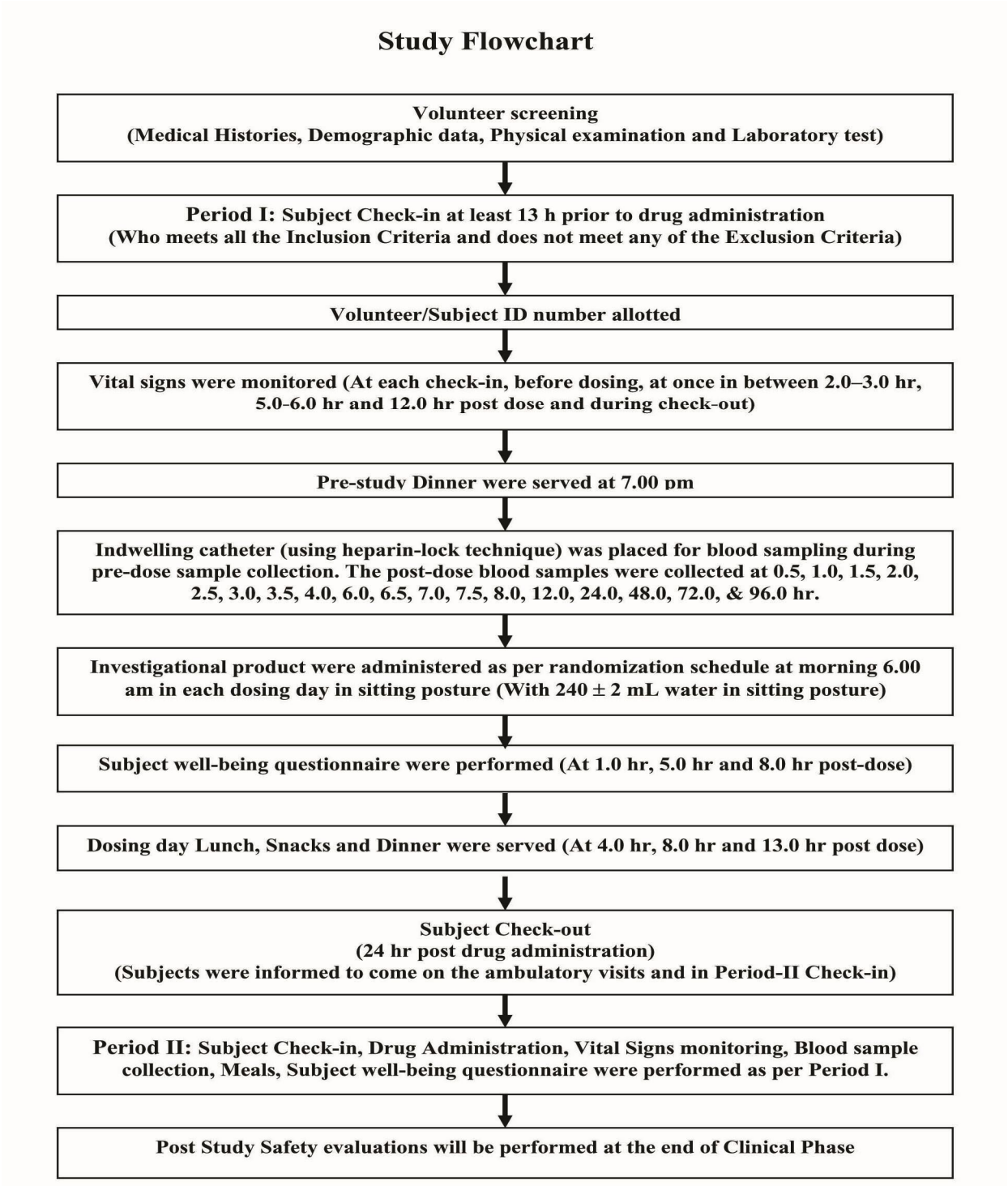


Figure-15: Flow Chart: General Treatment at the Clinical Pharmacology Unit (CPU)

1.5.6. Statistical analysis

Usual descriptive analysis including the mean and standard deviation (SD) were used for variables such as the height, weight and age. These statistical parameters including coefficient of variance also used to describe plasma concentrations at each individual time point as well as the pharmacokinetic parameters. Following statistical test was applied on untransformed (Tmax, Cmax, $AUC_{(o-t)}$, $AUC_{(o-\infty)}$) and log-transformed pharmacokinetic data. ANOVA of Tmax, Cmax, $AUC_{(o-t)}$, $AUC_{(o-\infty)}$was subjected to a 3-way ANOVA accounting for subjects, period and treatment.90% confidence interval (CI) consistent with two-one sided t-test with the significance level of 5% for untransformed and log transformed parameters. Products will be considered to be bioequivalent, if the 90% confidence interval (CI) of difference in the average values of logarithmic AUC and Cmax between test and reference preparations is within the acceptable range of Log (0.8) to Log (1.25).

1.5.7 Drug Accountability Record

The quantity of drugs supplied by the sponsor must be recorded and kept in secure place under the responsibility of the principle investigator. The left over drugs are properly stored for a period not less than the expiration date and not more than 5 years.

1.6. Pharmacokinetic Parameters (Bioavailability Metrics)

Examination of the plasma analyte concentration versus time profile provides an overview of the comparative absorption and elimination of the test and reference drug products. In addition, several observed and estimated parameters are also evaluated to assess bioequivalence.

In a single dose bioequivalence study, the following pertinent pharmacokinetic parameters are examined:

- $AUC_{0\rightarrow t}$ = Area under the curve (from time 0 to time of Last Quantifiable Concentration).
- $AUC_{0\rightarrow \infty}$ = Area under the curve (from time 0 to infinity).
- Cmax = Maximum concentration.
- Tmax = Time to maximum concentration.
- $T_{1/2}$ = Elimination half-life.

A sufficient number of blood samples should be taken to cover at least 80% of the area under the curve as extrapolated to infinity in each individual.

1.7.Bio-analytical method development

Bio-analytical methods for bioequivalence studies should be accurate, precise, selective, sensitive and reproducible. The FDA guidance entitled "Guidance for Industry: Bio-analytical Method Validation" (Published in May 2001), should be adapted in validating bio-analytical, methods. Collection of Biological Matrix and Sampling Schedule Several samples of appropriate biological matrix (blood, plasma/serum, urine) are collected at various time intervals post dose. The sampling schedule depends on the pharmacokinetic characteristics of the drug tested. In most cases, plasma or serum is the matrix of choice. However, if the parent drug is not metabolized and is largely excreted unchanged and can be suitably assayed in the urine, urinary drug levels as well as plasma levels may be used to assess bioequivalence (EMEA, 2002).

Sufficient numbers of samples are collected during the absorption phase to adequately define the ascending portion of the plasma drug level versus time curve. Intensive sampling is carried out around the time of the expected peak concentration. Sufficient numbers of samples should also be collected in the log-linear elimination phase of the drug so that the terminal elimination rate constant and half-life of the drug can be accurately determined. A sampling period extending to at least four to five terminal elimination half-lives of the drug or the four to five longest half-lives of the pertinent analyte. The samples are appropriately processed and stored carefully under conditions that preserve the integrity of the analyte(s).

1.8.Bio-analytical method validation

All analytical test methods used to determine the active compound and/or its biotransformation product in the biological fluid must be well characterized, fully validated and documented. The objective of the validation is to demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum or urine, is reliable and reproducible for the intended use. Applicable principles of GLP should be followed in the conduct of chemical analysis. Bio-analytical methods should meet the requirements of specificity, sensitivity, accuracy, precision and reproducibility. Knowledge of the stability of the API and/or its biotransformation product in the sample material is a prerequisite for obtaining reliable results.Validation comprises pre-study and within-study phases. During the pre-study

phase stability of the stock solution and spiked samples in the biological matrix, specificity, sensitivity, accuracy, precision and reproducibility should be provided. Within-study validation proves the stability of samples collected during a clinical trial under storage conditions and confirms the accuracy and precision of the determinations. Validation must cover the intended use of the assay (USFDA)

The calibration range must be appropriate to the study samples. A calibration curve should be prepared in the same biological matrix as will be used for the samples in the intended study by spiking the matrix with known concentrations of the analyte. A calibration curve should consist of a blank sample, a zero sample, and 6–8 non-zero samples covering the expected range. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study.Spiked quality control samples at a minimum of three different concentrations in duplicate should be used for accepting or rejecting the analytical run (USFDA)

All the samples from one subject (all periods) should be analyzed in the same analytical run, if possible. Validation procedures, methodology and acceptance criteria should be specified in the analytical protocol, and/or the SOP. All experiments used to support claims or draw conclusions about the validity of the method should be described in a report (method validation report). Any modification of the method during the analysis of study samples will require adequate revalidation. The results of study sample determination should be given in the analytical report together with calibration and quality control sample results, repeat analyses (if any), and a representative number of sample chromatograms (Li W.*et.al*.2010)

1.9.Regulatory guidelines

Regarding AUCs, the 90% confidence interval should generally be within the acceptance range 80% to 125% (when log transformed data are used). For drugs with a particularly narrow therapeutic range, the AUC acceptance range may need to be smaller, and this should be justified clinically. Cmax does not characterize the rate of absorption particularly well in many cases and there is no consensus at present time on any other concentration-based parameter, which might be more suitable. The acceptance range for Cmax may be wider than for the AUC. The

recommended range is between 70% to 143% (when log-transformed data are used). The range used should be justified taking into account safety and efficacy. In general, the choice of the appropriate bioequivalence range should be made on clinical grounds, thus, for a drug with a narrow therapeutic range, tighter limits may have to be considered, e.g., 90% to 111% for AUC and 80% to 125% for Cmax (when log-transformed data are used). Statistical evaluation of Tmax (Tmax differences) only makes sense if there is a clinically relevant claim for rapid release or action or signs for a relation to adverse effects. The parametric 90% confidence interval (untransformed data are used) and the nonparametric 90% confidence interval for this measure of relative bioavailability should lie within a clinically relevant range (ICH)

For multiple dose studies (steady-state) the pharmacokinetic parameters: $AUC_{0\rightarrow Tss}$, C_{maxss}, C_{minss}, C_{avgss}, % swing and % fluctuation should be analyzed statistically after logarithmic transformation and the 90% confidence interval should be within the acceptance range 80% to 125%.

1.10. Instrumentation:

1.10.1. Liquid Chromatography Quadruple Tandem Mass Spectrometry(LCMS-MS)

1.10.1.1. Various features using LC-MS/MS for quantitation:

The preferred tool until the turn of the millennium for separating a mixture and providing definitive identification of its components was the gas chromatography/mass spectrometer. This technique was limited by three main factors (Novakova L. *et.al*. 2009):

i. Sample volatility
ii. The fact that aqueous samples require extraction.
iii. Thermal degradation of samples in the GC oven.

For LC/MS to be a major player in the analytical laboratory there are main factors limiting performance that must be overcome.

I. Analyzer signal swamping by the elution solvent.
II. Solvent composition changing in gradient elution.
III. Buffers use for PH control.
IV. Ionization of neutral peak components.

The benefited features of the technique using LCMS/MS for quantitation are (Pavia 5^{th} edition)

a. **Selectivity:** Combining the two separation mechanisms by LC and MS/MS allows the analysis of complex mixtures. The resulting selectivity allows a particular analyte or analytes to be isolated from the mixture and gives confidence that the correct component is being measured. Since analytes are separated by their mass-to-charge ratio (m/z) the technique allows for the use of isotopically labeled internal standards, which may not separate by LC but can be separated by their mass deference. The use of stable isotopically labeled internal standards can help control variability in a quantitative assay.
b. **Speed:** Since the MS will distinguish compounds based on mass, the chromatographic method does not have the separate every single component in the sample, so co-elution of non isobaric analytes is possible. This allows fast analysis times and reduced sample preparation, which helps with method development and high throughput method analysis.
c. **Sensitivity:** Mass spectrometry is an inherently sensitive technique. Good selectivity also leads to reduced noise, allowing very low levels($fgmL^{-1}$) to be detected.

But some disadvantages also present like expense, complexity, limited dynamic range, excessive selectivity.

1.10.1.2.Types of Mass Spectrometer:

Mass Spectrometer are several types like (Patrovic M. *et.al*. 2005)

i. **Single Quadrupole**: Good scan function sensitivity and good selectivity or sensitivity via SIM scanning, high duty cycle with SIM, good dynamic range (3-4 orders), fast positive and negative ionization. But some disadvantages also there like limited mass range(generally upto 3000m/z), SIM functionality can be prone to matrix interferences thus limit detection limits, low resolution(0.7Da)

ii. **Triple Quadrupole**: Good scan function sensitivity, good SIM function, excellent selectivity with MRM, even with matrix, excellent duty cycle with MRM. Ability to run multiple analytes simultaneously with MRM, high dynamic range(4-5 orders), fast positive and negative ionization, other scan function available like neutral loss, product and precursor ion. Disadvantages are low resolution generally (0.7Da) and limited mass range(generally upto 3000m/z).

iii. **Ion Trap (low resolution):** Very high full scan sensitivity, full scan MSMS which is ideal for structural identification and it can perform targeted quantitation with 3

orders of dynamic range using SIM scan functions, some linear ion traps can perform simultaneous full scan and MRM experiments, Disadvantages are, it can suffer from matrix interferences, duty cycle is generally slower when compared to a triple quadrupole, specially when doing simultaneous full scan and MRM acquisitions, low resolution generally 0.7Da but can run at higher resolution.

iv. **Ion Trap(high resolution):** High full scan sensitivity in MS, MSMS mode and good dynamic range, high resolution can provide good selectivity using exact mass measurement. Disadvantages are resolution can be affected by scan speed that is faster the acquisition speed, the lower the resolution; limited dynamic range can be affected by matrix and limited mass range up to 4000m/z typically.

v. **TOF (high resolution):** Good scan functionality and sensitivity, high resolution provides high selectivity through exact mass measurement, good dynamic range, ability to get quantitation on multiple analytes in a single acquisition, mass range in excess of 20000m/z. Disadvantages are no MS/MS functionality or other scan functions, generally lower sensitivity when compared to a triple quadrupole running MRM and sensitivity can be effected by scan speed.

vi. **q TOF (high resolution):** Good full scan sensitivity, good MSMS scan function, high resolution providing high degree of selectivity via exact mass measurement and good dynamic range with newer ADC based detection systems, ability to get quantitation on multiple analytes during a single run, mass range in excess of 20000m/z and resolution not affected by increased scan speed. Disadvantages are generally, lower sensitivity when compared to a triple quadrupole running MRM and sensitivity can be affected by scan speed.

1.10.1.3.Tandem Mass Spectrometer:

Current drug discovery efforts have been focused on identification of drug metabolism and pharmacokinetic issues (Rao RN. Et.al.2012). Here the liquid chromatography/mass spectrometry (LC/MS) is becoming the preferred tool of liquid chromatographers or analysts. It is a powerful analytical technique that combines the resolving power of liquid chromatography with the detection specificity of mass spectrometry. The development of electrospray ionization (ESI) providing a simple and robust interface, this can be applied to a wide range of biological

molecules and the use of tandem MS and stable isotope internal standards allows highly sensitive and accurate assays to be developed although some method optimization is required to minimize ion suppression effects.

The liquid chromatography (LC) separates the sample components and then introduces them to the mass spectrometer (MS). The MS creates and detects charged ions. The LC/MS data may be used to provide information about the molecular weight, structure, identity and quantity of specific sample components and provides additional useful information (e.g. metabolite profiles) (Pitt, 2009). Mass spectral data add specificity that increases confidence in the results of both qualitative and quantitative analyses. Mass spectrometers work by ionizing molecules and then sorting and identifying the ions according to their mass-to-charge (m/z) ratios. Major applications of the LC-MS/MS in the field of Pharmaceuticals are to analyze and quantify drugs and metabolites in biological samples (plasma). LC-MS/MS--- its works by 5 steps

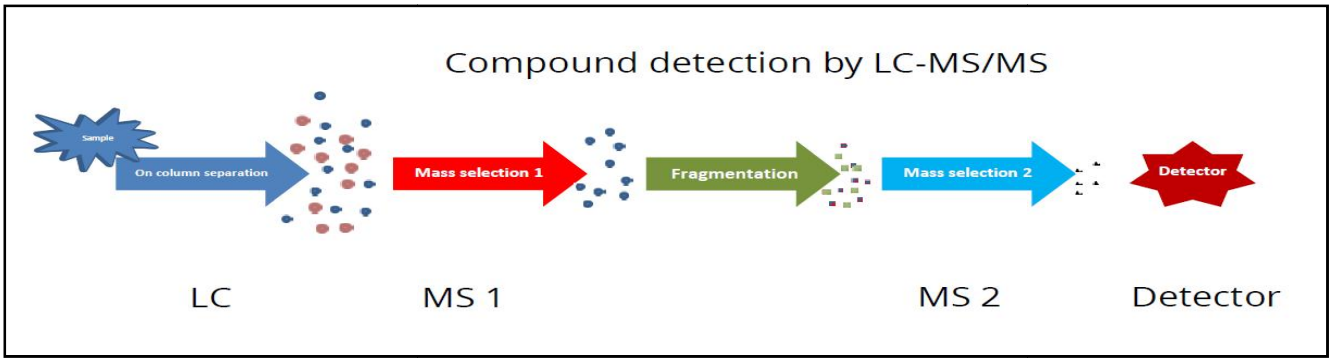


Figure-16: Detection process of compound by LC-MS/MS

These are

- Ion production—Atmospheric Pressure ionization (API)
- Transition to vacuum
- m/z-selection in a quadrupole, linear ion trap, and time of flight mass analyzer
- MS/MS fragmentation in a collision cell
- Ion detection using an electron multiplier.
- Signal and Data processing.

Tandem mass spectrometry (MS/MS) is the combination of two MS experiments. The aim for this experiment is to get structural information by fragmenting the ions isolated during the first experiment and to achieve better sensitivity and selectivity for quantitative analysis by selecting

representative ion transitions. Common atmospheric pressure ionization techniques are (Agilent Technologies, 2001):

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

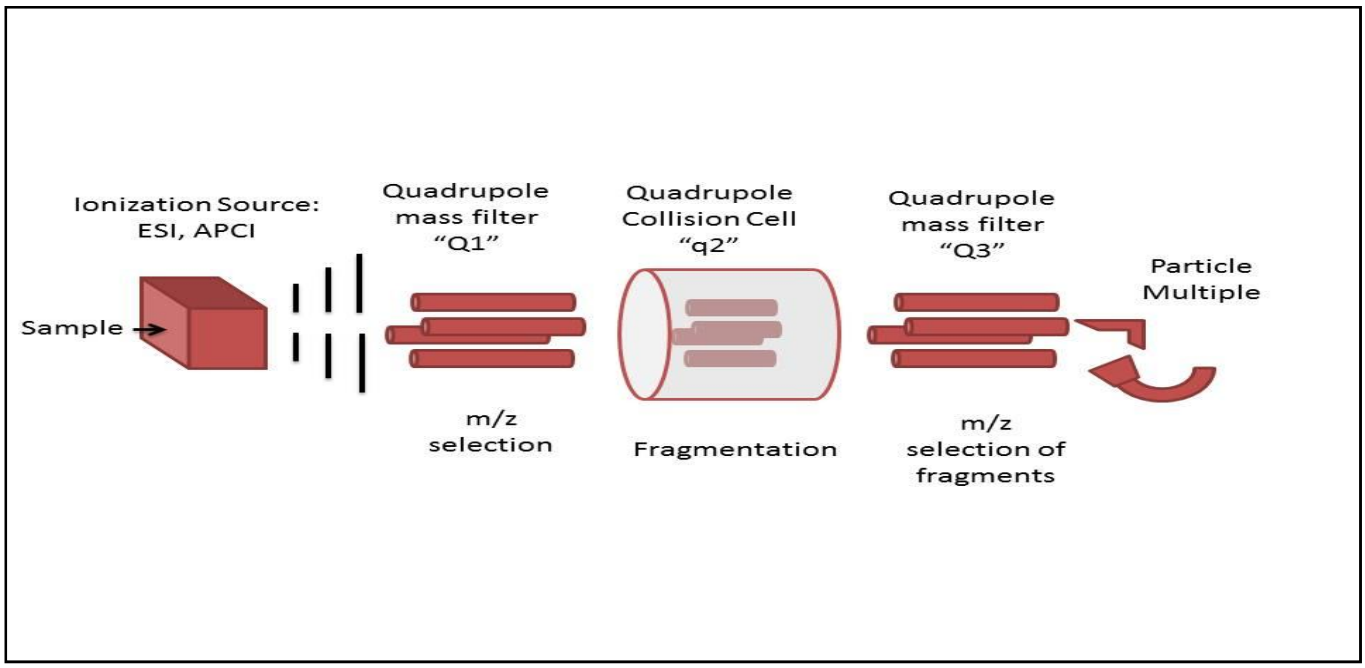


Figure-17: Schematic of Triple Quadruple Tandem Mass Spectrometer

Adequate sample preparation is a key aspect of quantitative bioanalysis and it is usually the most time consuming part of analyses. Interfering matrix compounds, such as proteins, lipids, salts, other endogenous and background compounds, should be removed in sample pretreatment, not only to avoid column clogging and instrument soiling, but also to improve the sensitivity, selectivity and reliability of analyses. Commonly and widely applied sample preparation techniques includes-

- Protein precipitation (PP)
- Liquid-liquid extraction (LLE) and
- Solid-phase extraction (SPE)

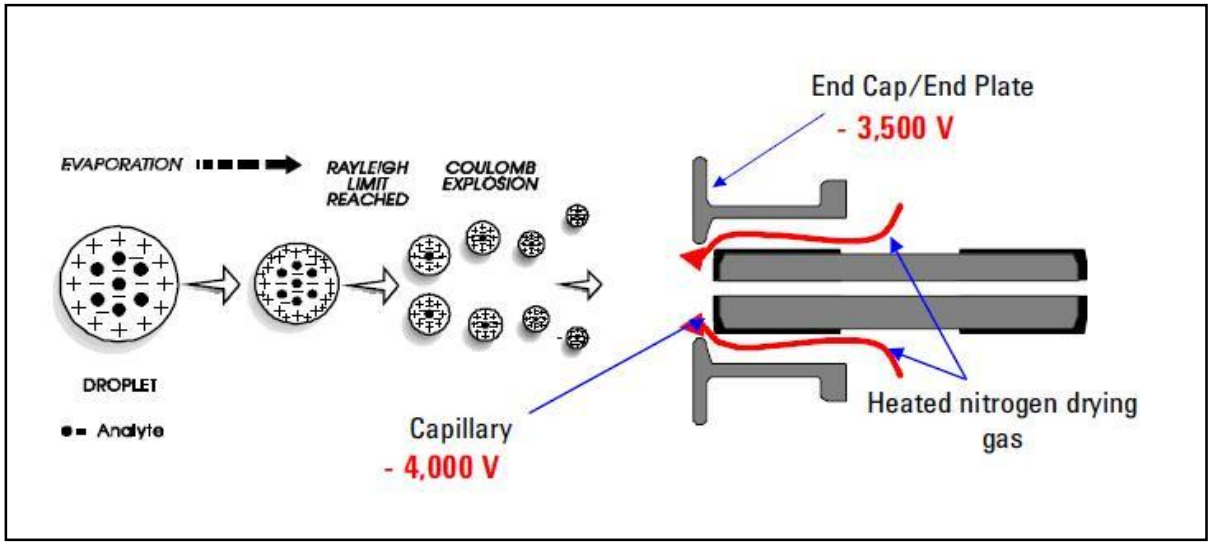


Figure-18: Working Principle of LC-MS/MS

The literature survey revealed that there are several methods described for the determination of Losartan Carboxylic acid and Losartan with Hydrochlorothiazide either alone or in combination with drugs, in liquid chromatography (Aghera NJ,*et. al,* 2012) and LC/MS-MS (Nie, J.; Zhang, *et. al,* 2005).

Simultaneous estimation method described in the present study will help the researchers as the drug used in this method is available on the market with a fixed dose combination. The present work describes a simple, rapid and sensitive method that employs plasma extraction technique for sample preparation and gradient method for liquid chromatography with electrospray ionization–tandem mass spectrometry for simultaneous quantitation of Losartan Carboxylic acid, Losartan and Hydrochlorothiazide in human plasma. The application of this assay method to a clinical pharmacokinetic study in healthy male volunteers following oral administration is described. To the best of our knowledge, no LC-MS/MS method was reported yet for the simultaneous determination of the studied drugs in biological fluids.

1.10.1.4. Key Chromatographic Parameters impacting on quantification using LC-MS/MS:

The key parameter affecting quantification in resolution which is expressed in terms of three parameters that are directly related to experimental conditions.

i. Retention or capacity factor

ii. Selectivity

iii. Column efficiency or column plate number.

1.10.1.5.LC-MS/MS Column:

Column is a packed tube filled with a stationary-phase particles used to achieve liquid chromatography separations. Different bonded phase can be attached to the stationary phase to achieve partition, ion exchange, affinity or size separations.

For initial condition column selection is the main criteria. The column should be

- HPLC system: C18 3µm or<3µm SPS, 3mm i.d * 50 or 100mm
- UHPLC system: C18<2µm, 2.1 or 3mm i.d. *50mm
- Fitted and appropriate guard column and pre column filter.

1.10.1.6.Mobile Phase for LC-MS/MS:

Aqueous component: Ammonium formate (<10Mm) with or without 0.1% formic acid (positive ion mode) or 0.1% ammonia for negative ion mode. Organic component methanol. Flow rate 0.3-1.0 mL/min depending on column dimensions at 40-60^{0}c

1.10.1.7.Application of LC-MS/MS:

There are currently three principal application areas in LC/MS, but the technique has much wider potential application and is in fact already to a variety of fields.

- The first main area is compound discovery and identifies confirmation in pharmaceutical manufacturing or drug discovery.
- The second application area called proteomics is protein structure determination by LC/MS. A growing subset of these studies is in the field of DNA/RNA structure studies, an appropriate term is nucleomics.
- The third area of application is in metabolite and trace contaminates studies
- Another applications are
    i. Arson residue investigation
    ii. Industrial water and pesticide analysis
    iii. Toxicology and drugs of abuse.
    iv. Clinical therapeutic drug screening
    v. Pesticide manufacturing.

1.11. Sample preparation

This is probably the most technically demanding stage in development. The aim is to remove as much interference as possible while maximizing recovery of the analytes. Where low concentration Liquid-liquid extraction (LLE) procedure or plasma precipitation technique (PPT) extraction procedure or SPE (Solid Phase Extraction) procedure should be used for the extraction of the drug from the plasma. Collected blood samples were centrifuged immediately, plasma was separated and stored frozen at -20^0C with appropriate labeling of volunteer code no., study date and collection time. Abnormal signs / symptoms were monitored, during the study period and for one week after the study period and if noticed, their details were entered in the case report sheets and tabulated at the end of the study (Rice N.M. *et.al.* 1993)

This is technically demanding stage of method development. The aim is to remove as much interference as possible while maximizing recovery of the analytes. Where low concentrations are being measured it may also be desirable to introduce a concentration step. Typical extraction technique include solvent extraction, solid phase extraction, protein precipitation for biological matrices and if the matrices is simple enough, dilution. As part of this developmental phase it will be necessary to measure the effect of matrix on the ionization process (matrix effect) and analyte recovery. If sufficient selectivity cannot be achieved during the extraction it may be necessary to adjust the LC conditions to separate out any interference (Taylor P. *et.al*. 2005)

- **General Consideration of sample preparation:**

  The entire environment and management of the sample must be considered during the sample preparation procedure for a successful outcome. These considerations include:

  - The suitability of the sample containers-some analytes are known to absorb onto plastic and certain solvent mixtures can dissolve molecular components from the container into sample thereby increasing its complexity.
  - The thermal stability and photo stability of the analyte-whether the preparation needs to be carried out at a reduced temperature or in amber glass vials (Tech tip Thermoscientific)

    After selection of the container methods of preparation and extractrion can be used in one of the two ways:

1. To remove and concentrate the target analyte.

2. To selectively remove other components of the sample that may interfere with the analysis due to their high abundance and inability to the distinguished from the target analyte.

1.11.1. Liquid Liquid Extraction:

This is relatively simple and quick wet chemistry technique based on the solubility of an analyte between two immiscible solvents, where the target analyte passes from its solvent of origin into a polarity compatible solvent in which it is more soluble. In the case of a highly non polar analyte, contained in an aqueous biological matrix such as urine the analyte will partition into the added immiscible non polar solvent. The partition ratio is the relative measure of separation at equilibrium conditions often referred to as distribution constant or partition coefficient or log P.

The extraction solvent can be strategically choosen according to its immiscibility with the sample solution and compatibility (polarity or hydrophobicity) with the target analyte. The partition ratio may be used as a gauge of good solubility for an efficient and high recovery extraction. Ionic analytes (dissociated) typically will remain in the aqueous layer unless neutralized. Extraction is typically not 100% efficient unless K is large, some analyte will still remain in the liquid phase then repeated extraction of the sample liquid re often required (amc lcms guide)

- If the organic solvent has lower density than water e.g. diethyl ether, hexane, ethylacetate-the organic solvent phase is the top layer, above the water.
- If the organic solvent is greater density than water like chloroform , dichloromethane-the organic solvent phase is the bottom layer, below the water.
- To concentrate the analyte or transfer the analyte to a solvent more suited to the subsequent analysis (Zendelovska D.*et.al*. 2004)
    - Organic phase: evaporate with nitrogen gas and reconstitute in a smaller volume of suitable organic solvent for analysis
    - Aqueous phase: Lyophilize and reconstitute in a smaller volume of aqueous solvent.

1.11.2. Protein Precipitation:

This is one of the straightforward sample preparation methods used for generating partially clean extract for LCMS quantitation from samples with a relative high abundance of protein. Protocols typically involve (Yeung P. *et.al*. 2000)

- Addition of an organic solvent(Acetonitrile, methanol, acetone), ammonium sulfate, trichloroacetic acid to a biological sample such as blood, serum or plasma.
- Following precipitation which may require mixing or vortexing, the sample mixture may be centrifuged to draw the protein precipitate to the bottom of the sample vial, leaving other components in the liquid layer.
- Removal of the protein free liquid which may be analysed directly, or evaporated to dryness and reconstituted in more suitable solvent prior to analysis by LC-MS

  Addition of precipitate-inducing additives which function using defferent mechanisms , including dehydration using ammonium sulphate or an organic solvent acetone and denaturing of the proteins through an adeverse change in PH (TCA).

1.11.3. Solid Phase Extraction:

This preparation technique, which is commonly used in both envioronmental and clinical applications uses a solid stationary phase sorbent normally contained in a cartridge, to clean up and concentrate sample components prior to analysis. For trace analysis, SPE is generally used to selectively isolate and concentrate a known target analyte within the sample. Typical SPE protocols for trace analysis involve these steps:

i. Wetting and conditioning
ii. Sample loading/retention
iii. Wash / rinse
iv. Elution

The choice of SPE procedure and sorbent characteristics will depend upon a range of issues such as (Morvan 5th edition)

- The sample volume
- The sample solvent characteristics and content of the sample matrix
- The target analyte characteristics
- The amount of target analyte.

1.11.4. Method Development and Optimization:

The most important and widely used LC separation technique for quantitative LC-MS/MS is reversed phase separation this utilizes differences in hydrophobicity to achieve patitionning between an apolar stationary phase and a polar mobile phase. Typically mobile phases use an aqueous blend of water with a miscible polar organic solvent, such as acetonitrile or methanol. This competes effectively with analyte molecules for sites on the apolar stationary phase, displacing analyte causing them to move faster through the column. With control of solvent composition, P^H temperature and flow rate, RP can provide good peak shape and enable separation of many analytes from each other, as well as from isobaric interferences or coextractive responsible for ion suppression.

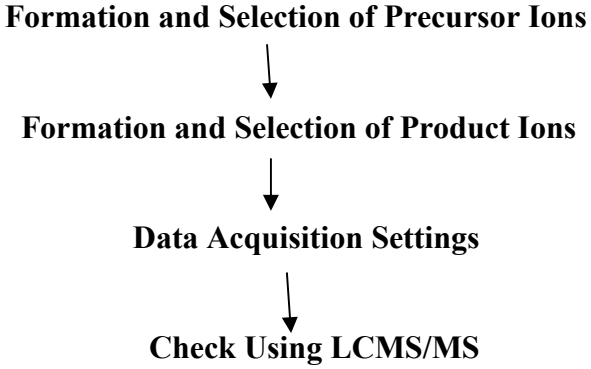


Figure: 19 Overview of the optimization strategy.

CHAPTER: 2.

LITERATURE REVIEW

2. LITERATURE REVIEW:

2.1 Single Dose of Drug:

Cardiac disease is the main typical disease nowadays, because it effect in all ages of life. Cardiac disease is a life threatening disease. Death rates from cardiovascular disease are stabilizing higher in developing countries. In many developing countries, the primary problem to be addressed with fixed drug combinations does not concern incomplete treatment. Single dose therapy offers a number of potential advantages, it can combine different classes of drugs to increase efficacy while mitigating the risk of treatment-related adverse event, reduce dugs burden, lower medical cost and improve patient adherence. In hypertension single dose of the drugs include standard to lower doses of each drug than would be necessary to achieve goals with monotherapy. In ischemic heart disease the concept of prevention of fixed dose combination established (Karen Whalen. 6th edition)

It is well established that adequate blood pressure control is essential to reduce cardiovascular risk. Hypertension is the main cardiovascular risk factor due to its high incidence and a linear relationship exist between blood pressure and cardiovascular events. Non adherence to antihypertensive treatment is common and is much higher in patients with resistant hypertension than in the general hypertensive population. In addition non adherence is difficult to monitor because most objective measures do not confirm ingestion of the medication. Single dose medication in hypertension has been associated with increased patient adherence. Current guidelines suggest initiating antihypertensive therapy using single drug or a pharmacological association on the basis of blood pressure values and presence of concomitant risk factors.

Potential advantages of using single dose therapy as first line treatment include: a faster condition of blood pressure and greater possibility of achieving target blood pressure, opposition to the counter regulatory pathways activated by monotherapies, improving tolerability and decreasing the adverse effect arising from up treating single agent (Kasper 19th edition)

2.2. Fixed Dose Combination of Cardiovascular Drugs:

Fixed dose combination drugs can be defined as two or more drugs in a single formulation, each drug having independent modes of action, the combination of which are synergistic or complimentary in their effect. Free combinations can be defined as two or more drugs in separate formulations, usually taken at the same time. Combination therapy is essential for the treatment of hypertension or otherwise cardiovascular conditions. The main advantages of fixed dose combination drugs increased patient adherence (J. Urzua.*et.al* 2012) The USFDA formalized the combination rule for prescription drug products, and states two or more drugs combined in a single dosage form when each component makes a contribution to the claimed effect and the dosage of each component such as amount, frequency, composition, duration, is such that the combination is safe and effective for a significant patient population requiring such concurrent therapy is defined in the labeling for the drug (Hunink M.*et.al*. 1997)

2.3.Advantages of Fixed Dose Combination than Single Dose Combination:

The presumed advantages of fixed dose combinations are as following:

i. Simpler dosage schedule improves compliance and therefore improves treatment outcomes.
ii. Reduction of inadvertent medication errors.
iii. Prevents and/or slow attainment of resistance by eliminating monotherapy (one drug is never in circulation by itself).
iv. Synergistic combinations allow each drug to selective interface with successive steps.
v. Reduced drug shortages by simplifying drug handling and therefore lowering the risk the risk of being out of stock.
vi. Only one expiry date simplifies dosing (single products have different expiry dates).
vii. The procurement, management and handling of drug is simplified.
viii. Lower production, packing and shipping cost.
ix. Side effects may be reduced by using one drug of the combination for the purpose.

But have some disadvantages like expensive, quality problem, side effect, flexible dosing, and incompatible pharmacokinetics (Oparil S. *et.al*. 2011)

The key advantages of FDC are that all the individual active pharmaceutical ingredients would be available at low costs; high quality, safe and efficacious product would be assured by compliance of research and development with the process. Manufacturing and quality assurance is technically more demanding the FDC and bioequivalence, pharmacodynamic, pharmacokinetics; stability would have to be assessed. It improves the patient adherence and reduces the complexity of dosing regimen and reduction of side effect with reduces dosage. Clinical trials are more adequate and reduced expenditure in packaging of medication by pharmaceutical companies, storage, handling, distribution cost will be for single agent (Orloff DG. *et.al*. 2005)

2.4. Factors Affecting Fixed Dose Combination

Fixed dose combination of drug depends on the amount of the drug, frequency dosage form of the drug, composition of the drug, and duration or drug interval dosing period of the drug. But in the case of cardiovascular drugs fixed dose combination depends on the cardiovascular risk factors like hypercholeterolomia, dislipidemia, high baseline blood pressure, high basal metabolic index, low density lipoprotein level, smoking, alcohol consumption status etc (Nunez R.*et.al*. 2005)

2.5. Pharmacokinetics of Drug:

"What the body does to the drug"-this refers to movements of the drug in and alteration of the drug by the body; includes absorption, distribution, binding/localization/storage, biotransformation, and excretion of the drug, a small fraction metabolized in the liver to inactive products and is excreted unchanged by glomerular filtration in kidney, has a total body clearance mL/min. approx. and a plasma half life of approx. 40.hrs (Owens MJ. *et.al.* 1997)

The duration of drug therapy ranges from a single dose of a drug taken for relieving an acute condition to drugs taken lifelong for chronic condition such as hypertension.

The frequency of administration of a drug in a particular dose is called as dosage regimen. Rational and optimal therapy with a drug depends upon: choice of a suitable drug and a balance between the therapeutic and the toxic effects. Both the therapeutic and the toxic effects depend upon the concentration of drug at the site of action which is difficult to measure. However it corresponds to a specific concentration of drug in plasma which can be measured with accuracy. The drug fails to elicit a therapeutic response when the concentration is below the effective level and precipitates adverse reaction when above the toxic level. The plasma drug concentration between these two limits is called as the therapeutic concentration range or therapeutic window (the ratio of maximum safe concentration to minimum effective concentration of the drug is called as therapeutic index).

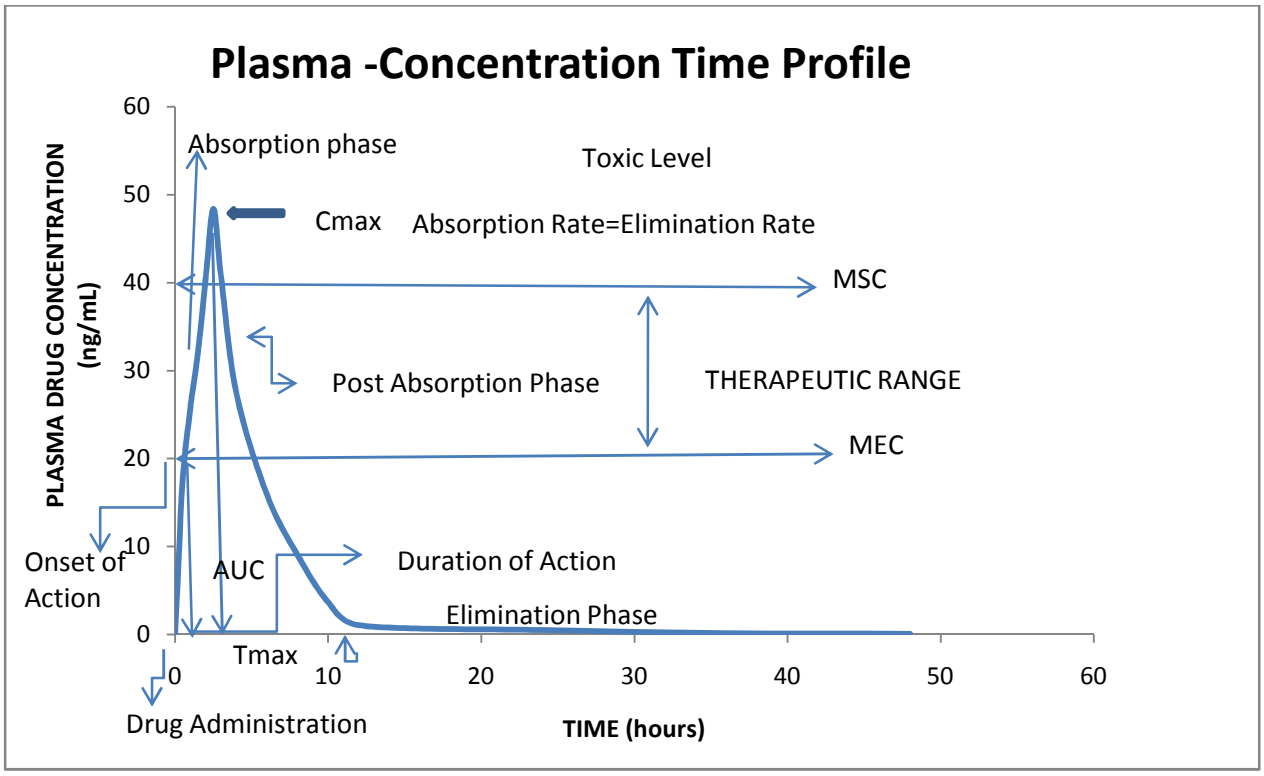


Figure: 20 Plasma Drug Concentration- time profiles

The point of maximum concentration of drug in plasma is called as the peak and the concentration of drug at peak is known as peak plasma concentration. It is also called as peak height concentration or maximum drug concentration, expressed as C_{max} in µg /mL. The peak represents the point of time when absorption rate equals the elimination rate of the drug. The portion of the curve to the left of peak represents absorption phase, when the rate of absorption is greater than the rate of elimination. The section of curve to the right of peak generally represents elimination phase, when the rate of elimination exceeds rate of absorption (Pal.T.K.*et.al*. 2011). The time for drug to reach peak concentration in plasma after extravsacular administration is called as the time of peak concentration, expressed as T_{max} in hours, useful in estimating the rate of absorption. Area under curve represents the total integrated area under the plasma level-time profile and expresses the total amount of drug that comes into the systemic circulation after its administration. It is expressed as µg /mL. in hours. AUC is important for drugs that are administered repetitively for the treatment of chronic conditions (Pilkington T. *et.al*. 1992).

Minimum Effective Concentration is defined as the minimum concentration of drug in plasma required to produce the therapeutic effect. It reflects the minimum concentration of

drug at the receptor site to elicit the desired pharmacologic response. The concentration of drug below MEC is said to be in the sub therapeutic level. Maximum safe concentration is the concentration of drug in plasma above which adverse or unwanted effects are precipitated. Concentration of drug above MSC is said to be in the toxic level. The beginning of pharmacologic response is called as onset of action. It occurs when the plasma drug concentration just exceeds the required MEC. Onset of time required for the drug to start producing pharmacologic response (Plus RG. *et.al*. 1972) It is the time for the plasma concentration to reach MEC after administration of drug. The time period for which the plasma concentration of drug remains above the MEC level is called as duration of drug action. Intensity of action is the maximum pharmacologic response produced by the peak plasma concentration of drug. It is also called as peak response. The drug concentration between MEC and MSC represents the therapeutic range (Quan A. *et.al*. 2006)

Some Pharmacokinetic Formulae:

- Elimination Rate Constant: $K_e=CL/Vd$
- Half Life : $t_{1/2}=0.693/K_e$
- Minimum Concentration in case of multiple dose of drug: $C_{min.}=C_0.e^{-K_e\iota}/(1- e^{-K_e\iota})$
- Average plasma steady state concentration: $\overline{Cp}_{ss}=D/CL.\iota$
- Plasma concentration of single dose of drug after oral administration: $C=F.D.K_a(e^{-K_e\iota}- e^{-K_a\iota})/Vd(K_a-K_e)$
- Time of maximum concentration after single oral administration: $t_{max}=\ln(K_a/K_e)/(K_a-K_e)$
- Clearance: $Cl=Dose.F/AUC$ or $Cl=K_e.\ Vd$ or $Cl=Dose/AUC$
- Average Plasma concentration of drug in steady state: $\overline{C}=F.D/CL.\iota$
- Calculated Peak: $C_{max}=C^{*}_{max}/\ e^{-K_e\iota}$
- Male ideal body weight: IBW=50kg+2.3kg for each inch over 5ft in height
- Female ideal body weight: IBW=45kg+2.3kg for each inch over 5ft in height
- Obese: ABW=IBW+0.4*(TBW-IBW)
- Absolute Bioavailability: $F*(t_{1/2})_{oral}/(t_{1/2})_{IV}$

2.6.General Information of Losartan

AngiotensinII receptor blocker like losartan (LOW-sar-tan) is alternative to the ACE inhibitors. This drug block the AT1 receptor, decresing the activitation of AT1 receptors by Angiotensin II and the produde arterioaler and venous dilatation like ACE inhibitors and block aldosterone secretion, thus lowering the blood pressure and decreasing the salt and water retention

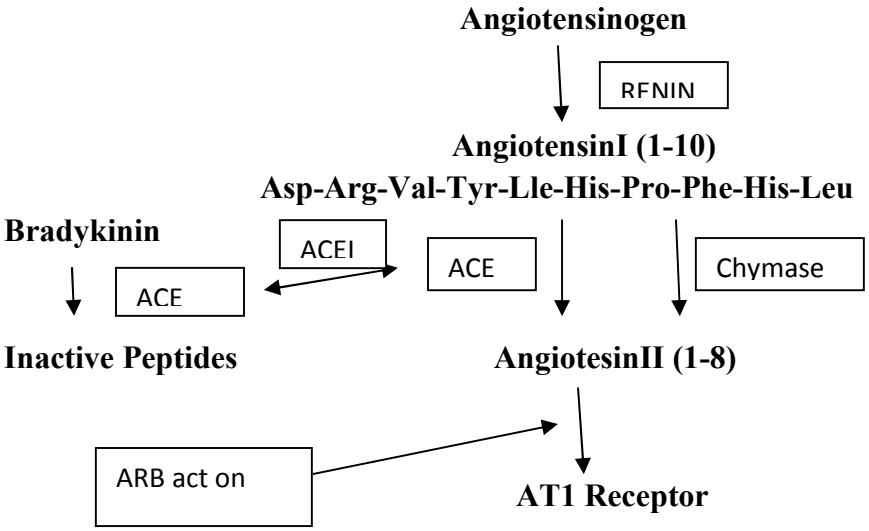


Figure: **21** Inhibitor of RAS

They do no increase the bradykinin level, they may be used as first line agents for the treatment of hypertension, especially in patients with a compelling indication of diabetes, heart failure or chronic kidney disease .This drug should not be combined with an ACE inhibitors because of adverse effect and teratogenic effect during hypertension treatment and due to pregnant woman.

Losartan is a white crystalline powder and is freely soluble in water, methanol and soluble in chloroform. Its melting point 180^0C – 184^0C and belongs to Class III drug (High Solubility & low Permeability). This drug Stored at -20^oC stable for at least two years and Indicated for the treatment of hypertension (Tripathy 7^{th} edition)

2.6.1. Chemistry of Losartan

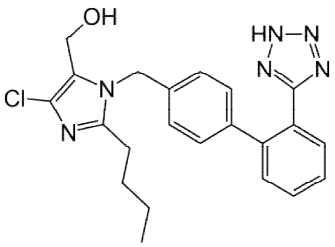


Figure 22 LOSARTAN

Losartan which chemical formula $C_{22}H_{23}ClN_6O$ and CAS number 114798-26-4.

Chemical name of Losartan is 2-butyl-5-chloro-3-[[4-[2-(2H-tetrazol-5-yl) phenyl] phenyl] methyl] imidazol-4-yl] methanol and molecular weight 422.92 and monoisotropic molecular weight 422.1622. Losartan is generally marketed as the basic potassium salt of the aromatized negatively charged tetrazole. Losartan contain Imidazole and Tetrazole heterocyclic ring. Tetrazole are a class of synthetic organic heterocyclic compound consisting of a five membered ring of four nitrogen atom and one carbon atom. Tetrazole Pka value 4.90 that is stronger acidic in character. Tertrazole can act as a bioisosteres for carboxylate group because they have similar pka and are deprotonated at physiological P^H Angiotensin II receptor

blocker. Losartan contain tetrazole. Imidazole is a heterocyclic compound containing two nitrogen. It is a moderately strong base which pka value 7.0 and a week acid which pka value 14.9, so imidazole is not ionized significantly between two pka value. Losartan AlogP value 4.27, rotable bond count 8 and polar surface area 92.51, hydrogen bond acceptor count 6 and hydrogen bond donor count 2 . According to Lipinski rule hydrogen bond acceptor count 7 and hydrogen bond donor count 2 , so losartan does not violate RO5 rules . From the rotable bond count and polar surface area it was concluded that losartan maintain Veber's rule, so predictable this compound is orally active and good oral bioavailablity. ACD log P value shows 3.46 that is positive so losartan compound is more lipophilic and ACD logD at P^H 7.4 is 1.48 also shows losartan highly lipophilic in the body and highly permeable through lipid membrane. Losartan contain tetrazole and imidazole heterocyclic ring as a result it contain two pka value 4.15 and 4.39 and total molecule is acidic (Gisvold medicinal chemistry 12th edition)

2.6.2. Pharmacology of Losartan

2.6.2.1.Mechanism of Action

It is a nonpeptide angiotensin II receptor antagonist with high affinity and selectivity for the AT_1 receptor. It blocks the vasoconstrictor and aldosterone-secreting effects of angiotensin II by inhibiting the binding of angiotensin II to the AT_1 receptor.

2.6.2.2.Pharmacokinetics

In healthy subjects, maximum plasma concentrations (Cmax) and AUC of drug increased in a dose proportional manner following single and multiple doses of 25 mg to 50 mg. There is no accumulation when therapeutic doses are administered every 24 hours. It has an apparent mean terminal elimination half-life (t½) of approximately 6 to 9 hours. The pharmacokinetic parameters for patients with hypertension estimated by population pharmacokinetic analyses were similar to those estimated in healthy subjects (Poulter NR. *et.al*. 2015).

- **Absorption**: Following oral administration, It is well absorbed (based on absorption of radiolabeled) and undergoes substantial first-pass metabolism; the systemic bioavailability of drug is approximately 33%. About 14% of an orally administered dose of it is converted to the active metabolite (K,D.Tripathy,7thedition).
- **Distribution**: The volume of distribution of it is about 34 liters.

- **Metabolism**: It undergoes substantial first-pass metabolism by cytochrome P450 enzymes. In addition to the active carboxylic acid metabolite, several inactive metabolites are formed. Following oral and intravenous administration of 14C -labeled drug, circulating plasma radioactivity is primarily attributed to parent drug and its active metabolite. In vitro studies indicate that cytochrome P450 2C9 and 3A4 are involved in the biotransformation of parent drug to its metabolites. Minimal conversion of parent drug to the active metabolite (less than 1% of the dose compared to 14% of the dose in normal subjects) was seen in about one percent of individuals studied(Rodgers A. *et.al*. 2003)
- **Elimination**: When it is administered orally, about 4% of the dose is excreted unchanged in the urine and about 6% is excreted in urine as active metabolite. Biliary excretion contributes to the elimination of parent drug and its metabolites. Following oral 14C-labeled Losartan, about 35% of radioactivity is recovered in the urine and about 60% in the faeces. Following an intravenous dose of 14C-labeled of parent drug, about 45% of radioactivity is recovered in the urine and 50% in the faeces. The apparent mean terminal elimination half-life (t½) of parent drug was approximately 6 to 9 hours. (Wald NJ et.al. 2003)

2.6.2.3.Pharmacodynamics: It is a non-peptide molecule, is chemically described as 2-butyl-4-chloro-1- [p-(o-1H-tetrazol-5-ylphenyl) benzyl] imidazole-5-methanol, angiotensin II receptor (type AT1) antagonist (Goodman and Gilmann,12thedition). Drug block the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor found in many tissues, (e.g., vascular smooth muscle, adrenal gland) (Vaidya A.*et.al*. 2010)

2.6.2.4.Therapeutic Indications

Losartan used in the treatment of hypertension as AngiotensinII receptor blocker.

2.6.2.5.Dosage and Administration

For treatment of hypertension, It is recommended at 25 mg or 50 mg once daily.

2.6.2.6.Drug Interactions

It is mainly metabolized by P450 chiefly in the liver. A P450 inducer, phenobarbital, has no significant effects on the pharmacokinetics of it. Cimetidine, known to inhibit P450 activity,

has no remarkable effects on the metabolism of it. Side effects of it have been very few in the clinical trials of this drug on several thousands of patients so far. The rate was as low as that of placebo. It may cause hyperkalemia when used with potassium-sparing diuretics, such as spironolactone or triamterene. Angioedema and acute hepatitis had been reported in 3 patients among thousands of millions of hypertensive under its treatment. The etiology was unclear, simple coincidence may not be ruled out. It should not be administered to pregnant women and breast-feeding mothers, because it may disturb the fetal growth or may be harmful to the newborn. It should be cautiously used in patients with renal failure or liver dysfunction (WHO)

2.6.2.7.Tolerability/ Adverse Effect/ Side effect:

Side effect include-

- diarrhoea
- stomach pain
- muscle cramps
- leg or back pain
- dizziness
- headache
- sleep problems (insomnia)
- tiredness, and
- cold or flu symptoms such as stuffy nose, sneezing, sore throat, fever, and cough

2.6.2.8.Conclusion

Losartan is an Angiotensin II receptor blocker used as an antihypertensive drug and also uses for diabetic kidney diseases, heart failure and left ventricular enlargement and it is orally active and predictable highly oral bioavailability. Losartan have various common side effect like stuffy nose, cough and high blood potassium, angioedema , kidney problem but it used as an essential medicine for hypertension and it always used in combination with hydrochlorothiazide. Losartan metabolized by the liver enzyme and form active metabolite losartan carboxylic acid.

2.7.Drug Metabolite Losartan Carboxylic acid :

Losartan may be used as a first line agent to treat uncomplicated hypertension, isolated systolic hypertension, and left ventricular hypertrophy. It may be used as a first line agent to delay progression of diabetic neuropathy. Losartan may be also used as a second line agent in the treatment of congestive heart failure, systolic dysfunction, myocardial infarction, and coronery artery disease in those intolerance of ACE inhibitors. Losartan is the first of a class of antihypertensive agent called angiotensin II receptor blockers. Losartan and its longer acting active metabolite Losartan Carboxylic acid (E-3174) are specific and selective type-I angiotensin II receptor anatagonist(AT1) which block the blood pressure increasing effect angiotensin II via the Renin Angiotensin Aldosterone Sysytem (RAAS) . RAAS is a homeostatic mechanism for regulating hemodynamics, water and electrolyte balance. During sympathetic stimulation or when renal blood pressure or blood flow is reduced, renin is released from granular cells of the juxtaglomerular apparatus in the kidneys. Renin cleaves circulating angiotensinogen to angiotensin I, which is cleaved by angiotensin converting enzyme (ACE) to angiotensin II. Angiotensin II increases blood pressure by increasing total peripheral resistance, increasing sodium and water reabsorption in the kidneys via aldosterone secretion, and altering cardiovascular structure. Angiotensin II binds to two receptors: AT1 and type-2 angiotensin II receptor (AT2). AT1 is a G-protein coupled receptor (GPCR) that mediates the vasoconstrictive and aldosterone-secreting effects of angiotensin II. Studies performed in recent years suggest that AT2 antagonizes AT1-mediated effects and directly affects long-term blood pressure control by inducing vasorelaxation and increasing urinary sodium excretion. Angiotensin receptor blockers (ARBs) are non-peptide competitive inhibitors of AT1. ARBs block the ability of angiotensin II to stimulate pressor and cell proliferative effects. Unlike ACE inhibitors, ARBs do not affect bradykinin-induced vasodilation. The overall effect of ARBs is a decrease in blood pressure. Losartan competitively inhibits the binding of angiotensin II to AT1 in many tissues including vascular smooth muscle and the adrenal glands. Losartan is metabolized to its active metabolite, E-3174, which is 10 to 40 times more potent than losartan and acts as a non-competitive AT1 antagonist. Inhibition of angiotensin II binding to AT1 inhibits its AT1-mediated vasoconstrictive and aldosterone-secreting effects and results in decreased vascular resistance

and blood pressure. Losartan is 1,000 times more selective for AT1 than AT2. Inhibition of aldosterone secretion may increase sodium and water excretion while decreasing potassium excretion. Losartan is effective for reducing blood pressure and may be used to treat essential hypertension, left ventricular hypertrophy and diabetic nephropathy. Losartan is well absorbed and undergoes substantial first-pass metabolism; the systemic bioavailability of losartan is approximately 33%. Mean peak concentrations of losartan and its active metabolite are reached in 1 hour and in 3-4 hours, respectively. While maximum plasma concentrations of losartan and its active metabolite are approximately equal, the AUC of the metabolite is about 4 times as great as that of losartan. When given with a meal, absorption is slows down and Cmax decreases. Hepatic. Losartan is metabolized to a 5-carboxylic acid derivative (E-3174) via an aldehyde intermediate (E-3179) primarily by cytochrome P450 (CYP) 2C9 and CYP3A4. E-3174 is an active metabolite with 10- to 40-fold higher potency than its parent compound, losartan. Approxiamtely 14% of losartan is converted to E-3174; however, the AUC of E-3174 was found to be 4- to 8-fold higher than losartan and E-3174 is considered the main contributor to the pharmacologic effects of this medication. The terminal $t_{1/2}$ of losartan is 2 hours. The active metabolite has a half-life of 6-9 hours. Total plasma clearance = 600 mL/min losartan. Total plasma clearance = 50 mL/min [active metabolite]. Renal clearance = 75 mL/min losartan. Renal clearance = 25 mL/min [active metabolite]

2.7.1. Chemistry of Drug Metabolite Losartan Carboxylic acid:

Losartan carboxylic acid is an active metabolite of losartan which CAS number is124750-92-1 and molecular weight 436.9 and which exact or monoisotropic mass is 436.141 . The IUPAC name of losartan carboxylic acid is 2-butyl-5-chloro-3-[[4-[2-(2H-tetrazol-5-yl)phenyl] phenyl]methyl]imidazole-4-carboxylic acid and chemical formula is $C_{22}H_{21}ClN_6O_2$. Losartan carboxylic acid is a biphenyltetrazole that is losartan with the hydroxymethyl group at position 5 on the imidazole ring replaced with a carboxylic acid. It has a role as a metabolite. It is a biphenyl tetrazole, a member of imidazole and an organochlorine compound. It derives from a losartan.

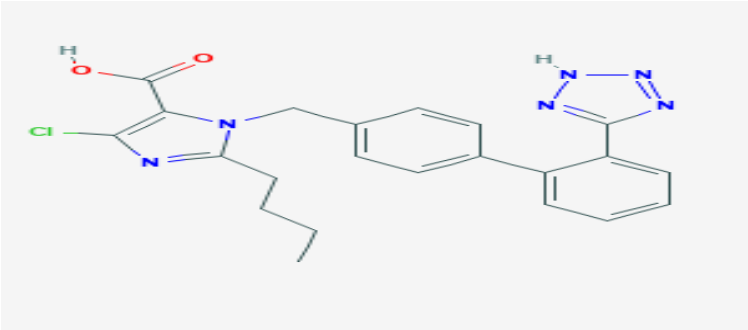


Figure: **23**. Losartan Carboxylic Acid

Losartan carboxylic acid contain Imidazole and Tetrazole heterocyclic ring. Tetrazole are a class of synthetic organic heterocyclic compound consisting of a five membered ring of four nitrogen atom and one carbon atom. Tetrazole Pka value 4.90 that is stronger acidic in character. Tertrazole can act as a bioisosteres for carboxylate group because they have similar pka and are deprotonated at physiological P^H Angiotensin II receptor blocker. Imidazole is a heterocyclic compound containing two nitrogen. It is a moderately strong base which pka value 7.0 and a week acid which pka value 14.9, so imidazole is not ionized significantly between two pka value. Losartan carboxylic acid also contain biphenyl group which is a bezenoid aromatic compound that consist of two benzene ring connected ny a single covalent bond. This part absorbed from the mucous membrane and metabolized in the liver to water soluble hydroxyl derivative. Biphenyl is neutral structure and ACD log D at ph 7.4 value is 4.09 that is highly permeable through the lipid membrane that is lipophilic. Hydrogen bond donar count , hydrogen bond acceptor count of losartan carboxylic acid are 2,6 respectively. According to Lipinski rule hydrogen bond acceptor count 6 and hydrogen bond donor count 2 , so losartan carboxylic acid does not violate RO5 rules . Losartan carboxylic acid contain 8 rotable bond and its polar surface area 110 $Å^2$, so according to veber's rule it is predictable that losartan carboxylic acid also orally active and high orally good bioavailability. Log p value of losartan carboxylic acid 4.5 and two typ[es of pka value 7.4 and 4.12 which one is neutral deu to biphenyl ring and one is strongest acidic due to tetrazole and imidazole and carboxylic acid residue. Losartan carboxylic acid is the active metabolite and 5-carboxylic acid derivative of losatan.

2.7.2. Process of Formation of Metabolite from Losartan:

Losartan is readily absorbed from the gastrointestinal tract after oral doses, but undergoes first pass metabolism resulting in a systemic bioavailability of about 33%. It is metabolite to an active carboxylic acid metabolite E3174(EXP3174), which predictable has a greater pharmacological

activity than losartan; some active metabolite are also formed. Metabolism is mainly by CYP isoenzymes CYP2C9 and CYP3A4. Peak plasma concentrations of losartan and its active metabolite occur about 1 and 3-4 hour respectively after an oral dose. Both losartan and its active metabolite are more than 98% bound to plasma protein. Losartan is excreted in the urine , and in feces via bile , as unchanged drug and metabolites. About 4% of an oral dose is excreted unchanged in the urine and about 6% is excreted in urine as the active metabolite. The terminal elimination half life of losartan and its active metabolite are about 1.5-2.5 and 3-9 hour respectively.

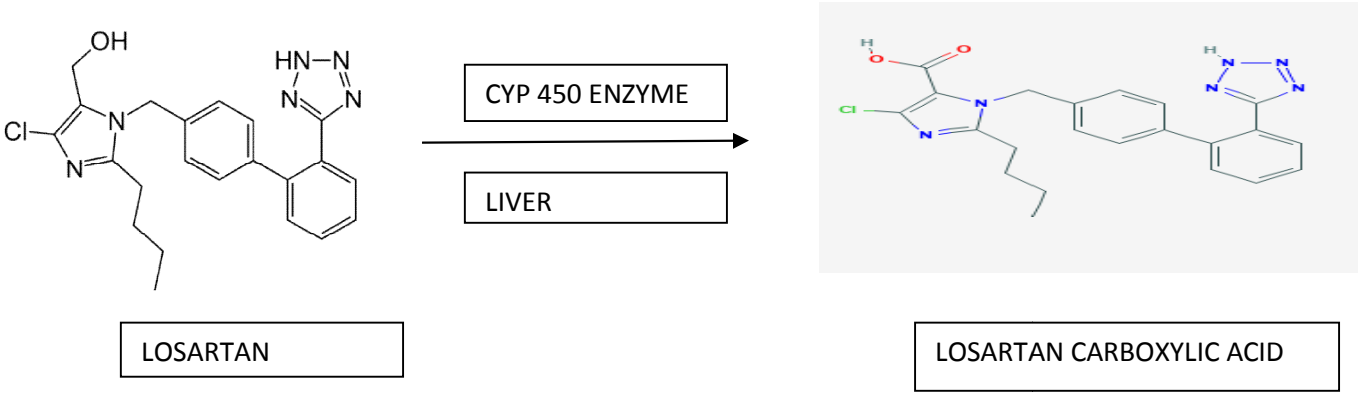


Figure: 24 Conversion of Losartan to active metabolite Losartan Carboxylic Acid in Liver by Hepatic Enzyme

After oral administration, losartan is mainly converted to an active metabolite losartan carboxylic acid. This active metabolite is the consequences of the oxidation of 5-hydroxymethyl group to the corresponding 5-caboxaldehyde and finally 5-carboxylic acid group. The isoenzyme of cytochrome P450 in liver are responsible for metabolism and conversion of losartan to its major active metabolite. On the other hand, 5-carboxaldehyde metabolite is responsible for for anti-inflammatory and antiaggregatory activities of losartan.

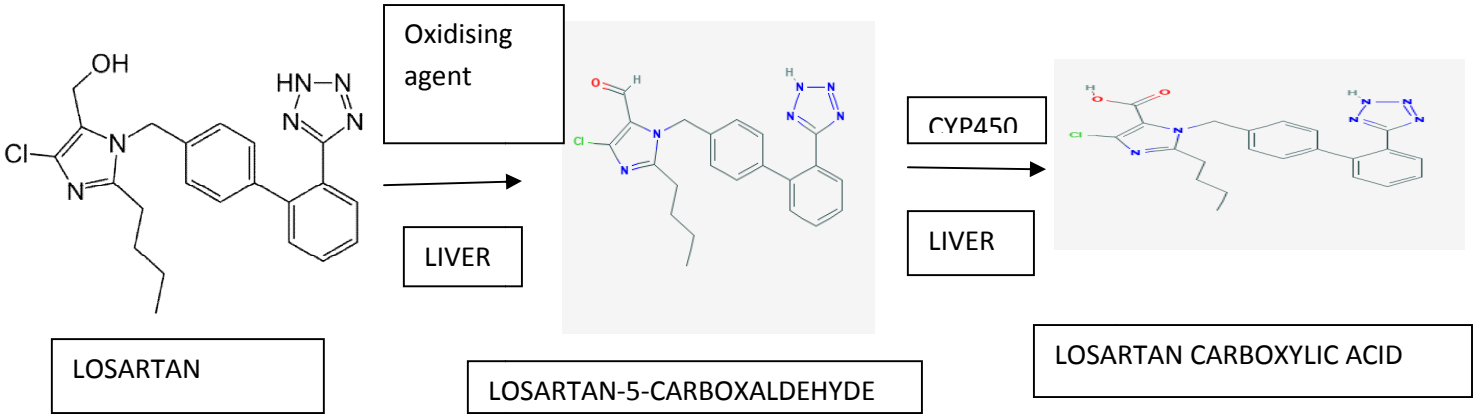


Figure: 25 Conversion Losartan to LCA through an intermediate compound Losartan -5-carboxaldehyde

Losartan to losartan carboxylic acid formation is the two steps chemical reaction in which losartan converted to the losartan 5-carboxaldehyde by the action of some oxidixing agent and Oxidation of the 5-hydroxymethyl group on the imidazole ring results in the active metabolite of losartan and this aldehyde form converted to the active metabolite or target metabolite losartan carboxylic acid by CYP 450 enzyme.

2.7.3. Pharmacology of Losartan Carboxylic Acid

2.7.3.1.Mechanism of Action

It is a nonpeptide angiotensin II receptor antagonist with high affinity and selectivity for the AT $_1$ receptor. It blocks the vasoconstrictor and aldosterone-secreting effects of angiotensin II by inhibiting the binding of angiotensin II to the AT $_1$ receptor. More potent than parent drug.

2.7.3.2.Pharmacokinetics

In healthy subjects, maximum plasma concentrations (Cmax) and AUC of drug increased in a dose proportional manner following single and multiple doses of parent drug. There is no accumulation when therapeutic doses are administered every 24 hours. It has an apparent mean terminal elimination half-life (t½) of approximately 1.5 to 2 hours. The pharmacokinetic parameters for patients with hypertension estimated by population pharmacokinetic analyses were similar to those estimated in healthy subjects.

- **Absorption**: Following oral administration, It is well absorbed (based on absorption of radiolabeled) and undergoes substantial first-pass metabolism; the systemic bioavailability of drug is approximately 25%. About 35 % of an orally administered dose of it is converted to the active metabolite (K,D.Tripathy,7thedition).

- **Distribution**: The volume of distribution of it is about 10 liters.
- **Metabolism**: It is a metabolite product
- **Elimination**: It is eliminated through kidney and renal clearance 26ml/min and elimination half-life 6.3hrs.

2.7.3.3.Pharmacodynamics:

It is a non-peptide molecule, is chemically described as 2-butyl-5-chloro-3-[[4-[2-(2H-tetrazol-5-yl)phenyl]phenyl]methyl]imidazole-4-carboxylic acid, angiotensin II receptor (type AT1) antagonist (Goodman and Gilmann,12thedition). Drug block the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor found in many tissues, (e.g., vascular smooth muscle, adrenal gland).

2.7.3.4.Therapeutic Indications

For treatment of hypertension, antiarrhythmia

2.7.3.5.Drug Interactions

Drug interaction like parent drug is almost absent.

2.7.3.6.Tolerability/ Adverse Effect

No significant side effect was observed

2.7.3.7.Conclusion

Losartan carboxylic acid is the active metabolite of losartan which is more potent than losartan according literature review it was observed that its elimination rate higher than losatan and its peak plasma concentration more than losartan and it is 10-40 times more potent than losartan. Losartan converted to losartan carboxylic acid through an intermediate compound losartan -5-carboxaldehyde which is also an active intermediate metabolite by which losartan act as an anti-inflammatory and antiaggregatory activities.

2.8.Drug Review of Hydrochlorothiazide:

A thiazide diuretic often considered the prototypical member of this class. It reduces the reabsorption of electrolytes from the renal tubules. This results in increased excretion of water

and electrolytes, including sodium, potassium, chloride, and magnesium. It is used in the treatment of several disorders including edema, hypertension, diabetes insipidus, and hypoparathyroidism.Hydrochlorothiazide is a low ceiling thiadiazide diuretic. Hydrochlorothiazide is a white or almost white crystalline odorless powder. It is slightly or very slightly soluble in water; sparingly soluble in alcohol; soluble in acetone; freely soluble in dimethylformamide; n-butylamine; and solutions of alkali hydroxides; insoluble in ether, chloroform, and dilute mineral acids. Indications: Hydrochlorothiazide is a diuretic which reduces the reabsorption of electrolytes from the renal tubules. Used to treat hypertensive disease and to manage the edema due to mild-to-moderate congestive heart failure. Edema due to chronic hepatic or renal disease may also respond favorably. It may also be used in patients with diabetes insipidus, due to a paradoxical effect. May be used in the treatment of hypercalciuria in patients who have recurrent urinary calculi composed of calcium salts. The use of hydrochlorothiazide has been indicated for the edema of the premenstrual tension, if there is evidence of fluid retention. This agent's metabolite appears to preferentially bind to and accumulate in red blood cells. This agent is primarily excreted by the kidneys. In human exposure main risks and target organs: Hydrochlorothiazide is generally a very safe diuretic, as the distance between therapeutically effective and frankly toxic doses is large. Clinical toxicity is relatively infrequent and may result from overdosage, adverse reactions or unexpected hypersensitivity. Main risks: Electrolytes imbalances that may lead to cardiac arrhythmias and orthostatic hypotension. Metabolic disturbances, such as hyperglycemia and hyperuricemia. Aggravation of hepatic and/or renal insufficiency, hypersensitivity reactions, blood dyscrasias, acute noncardiogenic pulmonary edema. Gastrointestinal irritability and Central Nervous System manifestations. Target organs: Kidneys, heart, central nervous system. Summary of clinical effects: Hematological: Thrombocytopenia, granulocytopenia, leucopenia, a plastic anemia, and hemolytic anemia. Hypersensitivity: Purpura, intravascular immunohemolysis, pneumonitis, skin rashes, urticaria, eczema, lichen planus-like reactions; photosensitivity, similar to subacute cutaneous lupus erythematosis; vasculitis, Stevens Johnson Syndrome. Cardiovascular: Cardiac arrhythmias, increasing the effect of digitalis on cardiac muscle, orthostatic hypotension. Gastrointestinal: Anorexia, gastric irritation, nausea, vomiting, cramping, diarrhoea, constipation, jaundice due to intrahepatic cholestasis, pancreatitis, sialadenitis, dry mouth,

hepatic insufficiency, intestinal ulceration. Central Nervous System: Dizziness, vertigo, paraesthesias, headache, xanthopsia. Respiratory tract: Acute noncardiogenic pulmonary edema. Renal tract: Renal insufficiency. Others: Attacks of gout, hyperuricaemia, hyperglycaemia, glycosuria, thirst, weakness, muscle pain, lethargy, drowsiness, restlessness, increasing in plasma concentrations of cholesterol and triglycerides, impotence. Edema after abrupt suspension. Contraindications: Anuria and hypersensitivity to sulfonamide-derived drugs. Precautions: Hydrochlorothiazide should be used with caution in: patients with impaired hepatic function since it may increase the risk of hepatic encephalopathy, patients with renal impairment since it can further reduce renal function, and precipitate azotemia. Cumulative effects of the drug may develop in patients with impaired renal function, patients with gout since it can precipitate attacks of the disease, the patients should be carefully observed for signs of fluids and electrolyte imbalance. Hydrochlorothiazide may enhance the toxicity of digitalis glycosides by depleting serum-potassium concentrations. The possibility of exacerbation or precipitation of systemic lupus erythematosis has been reported. Thiazides cross the placental barrier and appear in umbilical cord blood. Thiazides appear in breast milk. Routes of entry: Oral: Oral route is the common route of administration. Accidental or deliberate ingestion of large doses may occur. Absorption by route of exposure: Hydrochlorothiazide is variably but fairly rapidly absorbed from the gastrointestinal tract. Bio-availability of hydrochlorothiazide after oral administration is approximately 60 to 80 percent. Peak plasma level occurs after 1 to 2 hours. Distribution by route of exposure: Hydrochlorothiazide is widely distributed in body tissue. Protein binding in the plasma is estimated at 58%. Biological half-life by route of exposure: A plasma half-life of about 9.5 hours has been estimated. The red blood cells half-life is 2.7 to 7 hours. Metabolism: Hydrochlorothiazide is not modified by organic biochemical processes. Elimination by route of exposure: Elimination of hydrochlorothiazide is mainly due to renal clearance. It is excreted unchanged in the urine. Hydrochlorothiazide crosses the placental barrier and appears in breast milk. Total systemic clearance of drug from the plasma is 4.9 mL/min/kg, decreasing in patients with uremia or congestive heart failure. Mode of action Toxicodynamics: Most of the toxicodynamic manifestations are due to electrolyte imbalances including hypochloremic alkalosis, hyponatremia, hypokalemia and hypomagnesaemia. The mechanism of hypercalcemia and hypophosphatemia are unknown. Clinical studies indicate that depletion of potassium has a

role in glucose intolerance, probably by inhibition of insulin secretion. For reasons that are unexplained thiazides increase the concentrations of cholesterol and triglycerides in plasma. Other toxic effects produced by hydrochlorothiazide are due to hypersensitivity reactions. Pharmacodynamics: Hydrochlorothiazide acts directly on the kidney, increasing the excretion of sodium chloride an potassium and consequently water, mainly in the distal tubule. Carcinogenicity: There is inadequate evidence for the carcinogenicity of hydrochlorothiazide in experimental animals. Teratogenicity: In general the exposure to diuretics was not associated with teratogenicity. A slight association with respiratory malformation was suggested. Other risks include fetal or neonatal jaundice, thrombocytopenia, and possible other adverse reactions which have occurred in the adult. Interactions: Hydrochlorothiazide may increase the toxicity of digitalis glycosides by depleting serum-potassium concentrations. Due to the potassium depletion it may enhance the neuromuscular blocking action of competitive muscle relaxants such as tubocurarine or gallamine triethiodide. It may increase the effect of anti-hypertensive agents such as guanethidine sulfate, methyldopa, or a ganglionic blocking agent. The postural hypotension due to thiazide diuretic therapy may be increased by concomitant ingestion of alcohol, barbiturates, or opiates. The potassium-depleting effect of thiazide diuretic may be enhanced by corticosteroids, corticotrophin, carbenoxolone, and amphotericin. Hydrochlorothiazide has been reported to reduce the response to pressor amines, such as noradrenaline, but the clinical significance of this effect is uncertain. Concomitant administration of thiazide diuretic and lithium salts is not recommended since the association may lead to toxic blood concentration of lithium. The pharmacological effects of oral hypoglycemic agents may be reduced. The urinary excretion of chloramphenicol in healthy subjects was decreased by thiazide diuretics.Cholestyramine may produce a decrease of 30 to 35% in the absorption of hydrochlorothiazide. Hydrochlorothiazide may reduce the tubular secretion of amantadine. The non-steroidal anti-inflammatory drugs may antagonise the diuretic actions of thiazides. The hyperglycemic, hypotensive and hyperuricemic effects of diazoxide can be potentiated by thiazides.

Probenecid enhances excretion of calcium, magnesium and citrate during thiazide therapy, butdoes not affect excretion of sodium, potassium, ammoniumchloride, bicarbonate and phosphate and titratable acid.

Thiazides increase urinary pH and may decrease urinary excretion of amphetamines and quinidine. Main adverse effects: The thiazide diuretic may cause a number of metabolic disturbances. Hydrochlorothiazide may induce hyperglycemia and may aggravate pre-existing diabetes mellitis. Thiazide diuretics increase the concentrations of cholesterol and triglycerides in plasma by unknown mechanisms. Borderline renal and/or hepatic insufficiency may be aggravated by hydrochlorothiazide. In patients with hypertensive disease and decreased renal reserve, the manifestations of renal insufficiency may be aggravated after intensive or prolonged therapy. Increased concentrations of ammonia in the blood have been reported. Cases of cholestatic jaundice, dermatitis, necrotising vasculitis have been reported. Pancreatitis has been reported. Intestinal ulceration has occurred following the administration of tablets containing thiazides with an enteric-coated core of potassium chloride. Thiazide diuretics can produce acute renal failure by producing saline depletion and hypovolaemia and also by a hypersensitivity reaction. They can cause the formation of non-opaque urate calculi. After two weeks of abrupt suspension of hydrochlorothiazide, 8 patients developed an intense edema. In animal and plant studies showed Carcinogenicity: There is inadequate evidence for the carcinogenicity of hydrochlorothiazide in humans. Teratogenicity: In rats, no teratogenic, embryotoxic or foetotoxic effect was observed. Mutagenicity: Hydrochlorothiazide induced gene mutations in mouse lymphoma cells and sister chromatid exchange in Chinese hamster cells. It did not induce chromosomal aberrations in Chinese hamster cells in vitro or sex-linked recessive lethal mutations in Drosophila. Hydrochlorothiazide induced mitotic recombination and non-disjunction in Aspergillus. It was not mutagenic to Salmonella typhimurium or Escherichia coli.

2.8.1. Chemistry of Hydrochlorothiazide:

Hydrochlorothiazide is a diuretic medication used to treat high blood pressure and swelling due to fluid buildup. Hydrochlorothiazide molecular weight 297.75 and which monoisotropic molecular weight is 296.9645 and chemical formula $C_7H_8ClN_3O_4S_2$ which IUPAC name is 6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide.

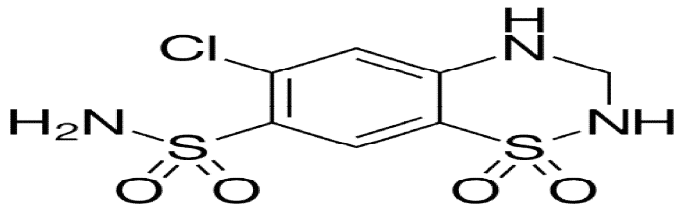


Figure: 26 Hydrochlorothiazide

Hydrochlorothiazide CAS number is 58-93-5, H-bond donor count, acceptor counts are 3,5 respectively. On the other hand hydrochlorothiazide has P^{Ka} values of 7.9 and 9.2 for secondary amine and sulfonamide group, respectively. The value of AlogP of hydrochlorothiazide is -0.35 and rotable bond count 1 and polar surface area 118.36$Å^{2,}$ so according to veber's rule it is predictable that hydrochlorothiazide also orally active and high orally good bioavailability. According to Lipinski rule hydrogen bond acceptor count 7 and hydrogen bond donor count 4 so does not violate RO5 rule. The value of ACD logP -0.02 so compound has higher affinity for the aqueous phase that is hydrochlorothiazide is more hydrophilic and at P^{H} 7.4 ACD logD value -0.04 so it is concluded that this compound is more susceptible to higher aqueous solubility and of lower lipophilicity in the body. As a result we would expect membrane permeability to be poor. Due to the presence of secondary amine and sulfonamide group ACD pka value is 8.95 nearly 9.2 so compound is strongly basic and weakly acidic. But the total molecule is neutral in character because pka value of total compound is 7.9. Hydrochlorothiazide is a low ceiling thiadiazide diuretic and white or almost white crystalline odorless powder. It is slightly and very slightly soluble in water, sparingly soluble in alcohol, soluble in acetone, freely soluble in dimethylformamide; n butylamine and solution of alkali hydroxide; insoluble in ether, chloroform and dilute mineral acid. At neutral P^{H} hydrochlorothiazide can be degraded by electrochemical oxidation in water due to the attack of hydroxyl radical, as a result aromatic intermediates, aliphatic carboxylic acid and inorganic anions were detected as main products (J.Urzua.et.al.2013)

$C_7H_8ClN_3O_4S_2 + 24\ H_2O \rightarrow 7\ CO_2 + 52\ H^+ + 2\ SO4^{2-} + NH_4^+ + 2\ NO_3^- + Cl^- + 46\ e–$

HCTZ

Figure: 27 Electrochemical Oxidation Reaction of Hydrochlorothiazide

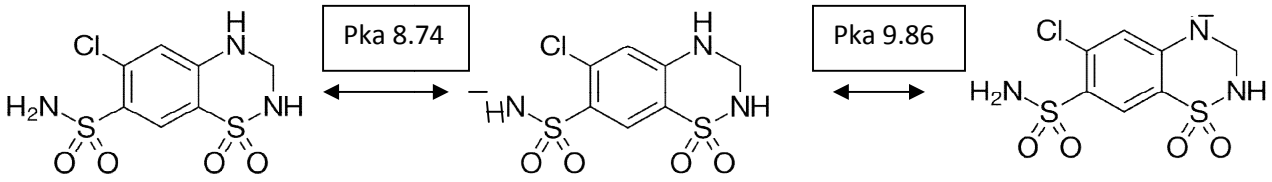


Figure: 28. Degradation Reaction of Hydrochlorothiazide by Electrochemical Oxidation at neutral P^H

During electrochemical oxidation reaction of HCTZ, the influence of initial P^H significantly influence the generation of hydroxyl redical and then presence of an acidic sulfonamide moiety ($-SO_2NH_2$) in the chemical structure of hydrochlorothiazide (HCTZ) allows hydrochlorothiazide to be present in its neutral and deprotonated form in biological fluid.

2.8.2. Pharmacology of Hydrochlorothiazide

2.8.2.1. Mechanism of Action

It belongs to thiazide class of diuretics. It reduces blood volume by acting on the kidneys to reduce sodium(Na^+) reabsorption in the distal convoluted tubule. The major site of action in the nephron appears on an electroneutral NaCl co-transporter by competing for the chloride site on the transporter. By impairing Na^+ transport in the distal convoluted tubule, it induces a natriuresis and concomitant water loss. It increases the reabsorption of calcium in this segment in a manner unrelated to sodium transport. Additionally, by other mechanisms, it is believed to lower peripheral vascular resistance. Hydrochlorothiazide, a thiazide diuretic, inhibits water reabsorption in the nephron by inhibiting the sodium-chloride symporter (SLC12A3) in the distal convoluted tubule, which is responsible for 5% of total sodium reabsorption. Normally, the sodium-chloride symporter transports sodium and chloride from the lumen into the epithelial cell lining the distal convoluted tubule. The energy for this is provided by a sodium gradient established by sodium-potassium ATPases on the basolateral membrane. Once sodium has entered the cell, it is transported out into the basolateral interstitium via the sodium-potassium ATPase, causing an increase in the osmolarity of the interstitium, thereby establishing an osmotic gradient for water reabsorption. By blocking the sodium-chloride symporter, hydrochlorothiazide effectively reduces the osmotic gradient and water reabsorption throughout the nephron (Goodman 12^{th} edition)

2.8.2.2.Pharmacokinetics

In healthy subjects, maximum plasma concentrations (Cmax) and AUC of drug increased in a dose proportional manner following single and multiple doses of drug. There is no accumulation when therapeutic doses are administered every 24 hours. It has an apparent mean terminal elimination half-life (t½) of approximately 5.6 to 14.8 hours. The pharmacokinetic parameters for patients with hypertension estimated by population pharmacokinetic analyses were similar to those estimated in healthy subjects (Rang & Dale. 5th edition)

- **Absorption**:Following oral administration, it is variably but fairly rapidly absorbed from the gastrointestinal tract. Bio-availability ofit after oral administration is approximately 60 to 80 per cent. Peak plasma level occurs after 1 to 2 hours(K,D.Tripathy,7thedition).
- **Distribution**:It is widely distributed in body tissue and its volume of distribution following oral administration corresponds to 0.83 L/Kg.Protein binding in the plasma is estimated at 58%.
- **Metabolism**: It is not modified by organic biochemical processes.
- **Elimination**: Elimination of it is mainly due to renal clearance that occurs in about 320 mg/ml .It is excreted unchanged in the urine. Hydrochlorothiazidecrosses the placental barrier and appears in breast milk. Total systemic clearance of drug from the plasma is4.9 mL/min/kg, decreasing in patients with uremia orcongestive heart failure.

2.8.2.3 Pharmacodynamics:

It acts directly on the kidney, increasing the excretion of sodium chloride and potassium and consequently water in the distal tubule of the kidney. Thiazides such as hydrochlorothiazide promote water loss from the body (diuretics). They inhibit Na^+/Cl^- reabsorption from the distal convoluted tubules in the kidneys. Thiazides also cause loss of potassium and an increase in serum uric acid. Thiazides are often used to treat hypertension, but their hypotensive effects are not necessarily due to their diuretic activity. Thiazides have been shown to prevent hypertension-related morbidity and mortality although the mechanism is not fully understood. Thiazides cause vasodilation by activating calcium-activated potassium channels (large conductance) in vascular smooth muscles and inhibiting various carbonic anhydrases in vascular tissue.

2.8.2.3. Therapeutic Indications:

For treatment of hypertension, congestive heart failure, edema, diabetes insipidus at a dose of 12.5mg to 25mg. A patient with hypercalciuria was treated with hydrochlorothiazide, 100 mg/day. On the 3rd day the urinary calcium dropped from a pretreatment value of 416 to 55 mg/24 hr. Hydrochlorothiazide has been evaluated for use in lessening sodium and water retention in conditions of preeclampsia during pregnancy. Although prophylactic use of hydrochlorothiazide apparently does not prevent preeclampsia, some benefit may be derived when hydrochlorothiazide is administered during the second trimester to pregnant women with underlying hypertension. It indicated in the management of hypertension, and as adjunctive therapy in edema associated with congestive heart failure, hepatic cirrhosis, and corticosteroid and estrogen therapy. It also useful in edema due to various forms of renal dysfunction such as nephrotic syndrome.

2.8.2.4.Drug Interactions

It may increase the toxicity of digitalis glycosides by depleting serum-potassium concentrations. Due to the potassium depletion it may enhance the neuromuscular blocking action of competitive muscle relaxants such as tubocurarine or gallaminetriethiodide.It may increase the effect of anti-hypertensive agents such as guanethidinesulfate, methyldopa, or a ganglionic blocking agent. The postural hypotension due to thiazide diuretic therapy may be increased by concomitant ingestion of alcohol, barbiturates, or opiates.

The potassium-depleting effect of thiazide diuretic may be enhanced by corticosteroids, corticotrophin, carbenoxolone, and amphotericin.It has been reported to reduce the response to pressor amines, such as noradrenaline, but the clinical significance of this effect is uncertain.

Concomitant administration of thiazide diuretic and lithium salts is not recommended since the association may lead to toxic blood concentration of lithium.The pharmacological effects of oral hypoglycaemic agents may be reduced.

The urinary excretion of chloramphenicol in healthy subjects was decreased by thiazide diuretics .Cholestyramine may produce a decrease of 30 to 35% in the absorption of hydrochlorothiazide. It may reduce the tubular secretion of amantadine. The non-steroidal anti-inflammatory drugs may antagonise the diuretic actions of thiazides.The hyperglycaemic, hypotensive and hyperuricaemic effects of diazoxidecan be potentiated by thiazides.Probenecid

enhances excretion of calcium, magnesium and citrate during thiazide therapy, but does not affect excretion of sodium, potassium, ammonium chloride,bicarbonate and phosphate and titratable acid.Thiazides increase urinary pH and may decrease urinary excretion of amphetamines and quinidine.

2.8.2.5. Tolerability/Adverse Effect/ Side effect:

Side effect include

- Cramps and muscle weakness
- Dizziness, headache, or thirst.
- Stomach pain, nausea, vomiting, diarrhea, or loss of appetite.
- Dizziness.
- Blurred vision.

Use of hydrochlorothiazide in glaucoma does merit caution. ... In presence of glaucomatously elevated intraocular pressure, it appears to be disadvantageous to optic nerve to reduce systemic blood pressure excessively by any agent. Thiazide diuretics also decrease glucose tolerance, and latent diabetes mellitus may be unmasked during therapy. Sexual impotence is the most common troublesome side effect of thiazide-class diuretics... Gout may be a consequence of uricemia induced by these diuretics. The occurrence of either of these adverse effects is reason for considering alternative approaches to therapy. Distal tubule diuretics, including hydrochlorothiazide, increased urinary output of zinc through a poorly understood mechanism which could involve both direct and hormone-mediated processes. Significant Zn depletion may occur during long-term administration of distal tubule diuretics principally in conditions associated with diminished total body zinc levels such as hepatic cirrhosis, diabetes mellitus, gastrointestinal disorders, and several renal diseases. May cause hypokalemia, and serum potassium levels should be determined periodically. Reduction in potassium levels may increase the action and toxicity of digoxin and related glycosides. Concurrent use with lithium is best avoided because of an increased risk of lithium toxicity. May cause hyperglycemia and hyperuricemia, and therapy in patients having diabetes or gout should be closely monitored. Some commercially available formulations of hydrochlorothiazide contain sulfites that may cause allergic-type reactions, including anaphylaxis and life-threatening or less severe asthmatic episodes, in

certain susceptible individuals. Hydrochlorothiazide has been reported to cause noncardiogenic pulmonary edema. Thiazide diuretics cross the placenta and appear in cord blood. ... thiazide diuretics can cause fetal harm when given to pregnant women. Fetal or neonatal jaundice has been reported. Thiazide diuretics do not prevent development of toxemia of pregnancy, and there is no satisfactory evidence that they are useful in the treatment of toxemia. Thiazide diuretic are indicated only in the treatment of edema due to pathological causes or as a short course of treatment in patients with severe hypervolemia. Possible hazards include fetal thrombocytopenia or other adverse reactions seen in adults. Thiazide diuretic are distributed into breast milk. The American Academy of pediatrics recommends that nursing mothers avoid thiazide diuretics during the first month of lactation because of reports of suppression of lactation. Although not strictly antiresorptive, thiazides reduce urinary calcium excretion and constrain bone loss in patients with hypercalciuria. Hydrochlorothiazide, 25 mg once or twice daily, may achieve substantial reductions in calciuria. Side effects ... associated with the use of hydrochlorothiazide include hypokalemia with resultant muscle cramps, cardiac arrhythmia, hyperglycemia, and hyperlipidemia. A variety of hypersensitivity reactions have also been reported. Electrolyte imbalances, in particular hypokalemia and hypomagnesemia, may be involved in increased incidences of sudden death in patients with preexisting electrocardiographic abnormalities. Results of the large Multiple Risk Factor Intervention Trial, a 10 yr, multicenter study of factors involved in heart disease, indicated that high dose hydrochlorothiazide therapy (100 mg/day) was associated with greater incidences of sudden death in patients with both high blood pressure and electrocardiographic abnormalities. The involvement of hypokalemia and hypomagnesemia in this observation remains a point of controversy. Thiazide diuretics also induce a transient increase in serum cholesterol and triglyceride levels, raising the possibility that long term treatment may contribute to atherosclerosis. Hypertensive individuals receiving 50 mg hydrochlorothiazide per day for 4 wk had increased concentrations of total plasma cholesterol, of high density, low density, and very low density lipoproteins, and of triglycerides. Immunologic reactions to hydrochlorothiazide therapy were reported, including cases of severe allergic pneumonitis, a photoallergic dermatitis resembling subacute cutaneous lupus erythematosus and several types of hematologic dyscrasias. Neutropenia was

reported in several patients with a pattern of onset which suggested a toxic depression of the bone marrow. On the other hand, thromboyctopenia also was reported with hydrochlorothiazide therapy and with other thiazides and appears to be immunologically mediated. In one person, a specific IgM antibody was identified as an antiplatelet factor associated with hydrochlorothiazide-induced thrombocytopenia. Photosensitive eruptions with clinical and histologic features of subacute cutaneous lupus erythematosus and antibodies to SS-A(Ro) antigen occurred in five patients taking hydrochlorothiazide. After drug therapy was discontinued, the eruptions cleared. In one patient anti-SS-A antibodies disappeared after discontinuation of thiazide, and in another rechallenge with hydrochlorothiazide produced an acute dermatitis with a photodistribution. These eruptions may represent a new type of photosensitive drug reaction in which the photoactive drug may be synergistic with anti-SS-A antibody in producing cutaneous lesions of photosensitive subacute cutaneous lupus erythematosus.

2.8.3. Conclusion

Hydrochlorothiazide is a thiazide diuretic and contains many side effects but it used in the treatment of hypertension as a diuretic and reduced the blood volume. Thiazides affect the renal tubular mechanisms of electrolyte reabsorption, directly increasing excretion of sodium and chloride in approximately equivalent amounts. Indirectly, the diuretic action of hydrochlorothiazide reduces plasma volume, with consequent increases in plasma renin activity, increases in aldosterone secretion, increases in urinary potassium loss, and decreases in serum potassium. The renin-aldosterone link is mediated by angiotensin II, so coadministration of an angiotensin II receptor antagonist tends to reverse the potassium loss associated with these diuretics. Thiazide diuretics are commonly used at lower doses because of concerns about side effect.

2.9.Hypertension:

Hypertension also known as high blood pressure is a long term medical condition in which the blood pressure in the arteries is persistently elevated(Lackland et.al 2005). Long term high blood pressure is major risk factor for coronery artery diseases, stroke , heart failure, atrial fibrillation, peripheral vascular diseases, vision loss, chronic kidney disease, dementia(Lau, DH;

Nattel,et.al,2017).High blood pressure is classified as either primary (essential) high blood pressure or secondary high blood pressure(Poulter et.al,2015) About 90–95% of cases are primary, defined as high blood pressure due to nonspecific lifestyle and genetic factors. Lifestyle factors that increase the risk include excess salt in the diet, excess body weight, smoking, and alcohol use. The remaining 5–10% of cases are categorized as secondary high blood pressure, defined as high blood pressure due to an identifiable cause, such as chronic kidney disease, narrowing of the kidney arteries, an endocrine disorder, or the use of birth control pills.

Blood pressure is expressed by two measurements, the systolic and diastolic pressures, which are the maximum and minimum pressures, respectively. For most adults, normal blood pressure at rest is within the range of 100–130 millimeters mercury(mmHg) systolic and 60–80 mmHg diastolic(Giuseppe et. al ,2013) For most adults, high blood pressure is present if the resting blood pressure is persistently at or above 130/90 or 140/90 mmHg. Different numbers apply to children.[13] Ambulatory blood pressure monitoring over a 24-hour period appears more accurate than office-based blood pressure measurement.

Lifestyle changes and medications can lower blood pressure and decrease the risk of health complications. Lifestyle changes include weight loss, decreased salt intake, physical exercise, and a healthy diet. If lifestyle changes are not sufficient then blood pressure medications are used. Up to three medications can control blood pressure in 90% of people. The treatment of moderately high arterial blood pressure (defined as >160/100 mmHg) with medications is associated with an improved life expectancy(Musini*et.al,* 2009) The effect of treatment of blood pressure between 130/80 mmHg and 160/100 mmHg is less clear, with some reviews finding benefit and others finding unclear benefit(*Garrison et.al.,2017)* High blood pressure affects between 16 and 37% of the population globally. In 2010 hypertension was believed to have been a factor in 18% of all deaths (9.4 million globally).

2.9.1. Causes of hypertension

Two types of hypertension caused by (Grossman *et. al,* 2012; Lawlar*et.al,*2005; Vaidya*et.al,* 2010)

- **Primary hypertension:**
    - Metabolic syndrome

- Genetical cause
- High salt intake
- Depression
- Vitamin D deficiency
- Insulin resistance
- Maternal smoking
- Lack of breast feeding
- High blood urea

❖ **Secondary hypertension**

- Kidney disease
- Cushing syndrome
- Hyper and hypo thyroidism
- Pheochromocytoma
- Hyperparathyroidism
- Acromegaly
- Conn's syndrome
- Renal arterial stenosis
- Pregnancy
- Sleep apnea
- obesity

2.9.2. Treatment of hypertension:

Blood pressure is the product of cardiac output and total peripheral resistance. Cardiac output is dependent on total blood volume, heart rate and the pumping action of the heart whereas peripheral resistance is determined by the diameter of arterioles. Sympathetic system stimulates heart directly(β_1), causes vasoconstriction (α) and also stimulates the RAAS. All these factors result in increased in blood pressure. Four main group of drugs used for controlling hypertension are

i. **Diuretics (Decrease blood volume and sodium retention)**: Hydrochlorothiazide, Chlorothalidone, Indapamide, Furosemide, Torsemide, Spironolactone, Triamterene, Bedroflumethiazide, Bumetanide, Indacrinone, Epleronone, amiloride

ii. **Sympathoplegics (Decrease the activity of sympathetic system)**

   a. **Drugs inhibiting central sympathetic outflow:** Clonidine, α-methyl dopa, Moxonidine, Rilmenidine, Atenolol, Metoprolol, Propanolol

   b. **Ganglionic blockers:** Trimethapan, Mecamylamine

   c. **Adrenergic receptor antagonist : α blocker**: Phenoxybenzamine, Phentolamine, Tolazoline, and **β blockers** are Celiprolol, Nevibolol, Metoprolol, Esmolol, Atenolol, Acebutolol, Betaxolol, Bisoprolol, Pindolol, Oxyprenolol

      Combined α and β blockers: Labetalol, Carvedilol

   d. **Adrenergic neuron blockers :** Reserpine, Bretylium, Guanethidine

iii. **Vasodilators (causes vasodilation):**

   a. **Potassium channel openers :** Hydralazine, Minoxidil, Diazoxide

   b. **Nitric oxide releasers :** Sodium nitroprusside, Hydralazine

   c. **Dopamine antagonist :** Fenoldopam

   d. **Calcium channel blockers :** Phenylalkylamines: Verapamil, Nor verapamil Benzodiazepine:Diltiazem; Dihydropyridines: Nifedipine, Nicardipine, Nimodipine, Nisoldipine, Nitrendipine, Isradipine, Lacidipine, Felodipine, Amlodipine

iv. **Drugs decreasing the action of RAAS:**

   a. **Renin inhibitors:** Aliskiren, Remikiren, Enalkiren

   b. **Angiotensin converting enzyme inhibitors (ACE inhibitors):** Captopril, Enalapril, Lisinopril, Ramipril, Perindopril, Trandolapril, Fosinopril, Moexipril,

v. **Angiotensin receptor blockers (ARBs):**Losartan, Valsartan, Irbesartan, Candesartan, Telmisartan, Eprosartan.

2.10. Bioanalytical method development

Validated LC-MS/MS method will be employeed for determination of drugs and

metabolites concerntration in plasma samples. During the analysis, standard and quality control samples will be distributed throughout each batch of study samples analyzed. The analyst will not have access to the randomization scheme.

Plasma samples of the subjects who completed the study will be analyzed. Subjects who are withdrawn from the study due to an adverse event related to study drug will also be assayed. Their concentration data will be provided in separate table and will not be included in pharmacokinetic and statistical analysis.

2.11. Bioanalytical Method Validation

Development of a LC-MS/MS method was essential to determine the concentration of Losartan and Losartan Carboxylic acid and Hydrochlorothiazide in human plasma after oral administration of the drugs for a fixed dose pharmacokinetic study.

Chromatographic analysis was performed on a Shimadzu HPLC system equipped with LC-20AD Binary pump, SIL- 20A Autosampler, CTO-10ASvp Oven and CBM-20A Lite System Control Compartment. The developed method was validated according to industrial guidelines for the bioanalytical method validation (Dams, *et al.,* 2003; Guidance for Industry Bio analytical Method Validation, Food and Drug Administration, US, 2013).

LC-MS/MS Mass Spectrometer (Triple Quadrupole)		**Shimadzu LC Method Parameters**	
Component ID	API 2000	Pump A Model	LC-20AD
Manufacturer	AB Sciex Instruments	Pump B Model	LC-20AD
Model	029345/Q	Pumping Mode	Binary Flow
Serial Number	B20510910	Total Flow	0.6000 mL/min.
Column	PhenomenexKinetex (5µ C18 100A 50*3mm)		
Scan Type	MRM (MRM)	**Autosampler**	
Scheduled MRM	No	Model	SIL-20AC
Polarity	Negative	Rinsing Volume	200 uL
Scan Mode	N/A	Needle Stroke	52 mm
Ion Source	Turbo Spray	Rinsing Speed	35 uL/sec.
Resolution Q1	Unit	Sampling Speed	15.0 uL/sec.
Resolution Q3	Unit	Purge Time	25.0 min
Intensity Thres	0.00 cps	Rinse Dip Time	0 sec.
Settling Time	0.0000 msec	Rinse Mode	Before and after aspiration
MR Pause	5.0070 msec	Cooler Enabled	Yes
MCA	No	Cooler Temperature	15 ºC

Step Size	0.00 Da	Control Vial Needle Stroke	52 mm
Shimadzu LC system		**System Controller**	
Equlibration Time	0.00 min	Model	CBM-20A Lite
Injection Volume	10.00 ul	Power	On
		Event 1	Off
Column Oven		Event 2	Off
Model	CTO-10ASvp	Event 3	Off
Temp. Control	Disabled	Event 4	Off

List of Instruments

Name of the Instrument	Make/Model
HPLC Pump	Shimadzu LC20AD
HPLC Autosampler	Shimadzu SIL20AC
Triple QuadrupoleMassSpectrometerAPI 2000	AB Sciex Instruments
Deep Freezer (-20ºC)	Celfrost
Centrifuge	REMI group
Evaporator	Home made
pH meter	Sartorius
Top loading balance	Sartorious

Table: 1 LCMS/MS Parameter

2.11.1. Linearity and LLOQ

Calibration curve considered as the relationship between instrument response and known concentrations of the analyte. The relationship between response and concentration should be continuous and reproducible. A calibration curve should be generated for each analyte in the sample. (Guidance for Industry Bioanalytical Method Validation, Food and Drug Administration, US, 2013). The lowest concentration on the calibration curve with a detector response greater than five timers of the blank plasma was considered as LLOQ (Nandi, *et al.,* 2015).

2.11.2. Accuracy and precision

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the actual value (concentration) of the analyte and 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 homogeneous volume of

biological matrix.Intra-day and inter-day precision and accuracy was determined by analyzing six replicates at four different QC levels (LLOQ, LQC, MQC and HQC).

2.11.3. 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 analyte in solvent. 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 pertains to the extraction efficiency of an analytical method within the limits of variability (Guidance for Industry Bioanalytical Method Validation, Food and Drug Administration, US, 2013). Recovery was determined by comparing responses of the analytes extracted from replicate QC samples with the response of analytes from post-extracted plasma standard sample at equivalent concentrations.

2.11.4. Stability

Stability testing should evaluate the stability of the analytes during sample collection and handling, after long-term and short-term storage, and after freeze and thaw cycles and the analytical process. (Guidance for Industry Bioanalytical Method Validation, Food and Drug Administration, US, 2013). . Samples were considered to be stable if assay values were within the acceptable limits of accuracy (*i.e.* ± 15% SD) and precision (*i.e.* ±15% RSD) (Dams, *et al.,* 2003; Guidance for Industry Bioanalytical Method Validation, Food and Drug Administration, US, 2013).

CHAPTER: 3

AIMS & OBJECTIVES

❖ AIMS AND OBJECTIVES:

The main aim and object is to evaluate the pharmacokinetic parameters, of FDC containing Losartan (Containing 50mg each) and Hydrochlorothiazide 12.5mg where meytabolite Losartan Carboxylic Acid (LCA) is formed in plasma due to the presence of Hydrochlorothiazide. This LCA an active metabolite plays a vital role compared to losartan as a single dose towards therapeutic efficacy for hypertensive patients, in 6 healthy human volunteer. The study will be carried out by utilizing a typical two period, randomized, two-way complete crossover design. However the main objectives of this study are as follow.

- ❖ To obtain Ethical Approval for carrying out the proposed study on human volunteers.
- ❖ To get written informed consent from the volunteers
- ❖ To be carry out pre-study screening of healthy volunteers.
    - To be estimate the biochemical/pathological parameters.
    - Physical examination by doctor
- ❖ Blood sampling and plasma separation
- ❖ Analytical method development and validation by LC-MS/MS for analysis of
    - Hydrochlorothiazide
    - Losartan
    - Losartan Carboxylic Acid
- ❖ Analysis of plasma samples obtained from the volunteers for analysis of
    - Hydrochlorothiazide
    - Losartan
    - Losartan Carboxylic Acid
- ❖ To evaluate pharmacokinetic parameters—**C_{max},T_{max}, AUC, K_{el}, $t_{1/2}$**of
    - Hydrochlorothiazide
    - Losartan
    - Losartan Carboxylic Acid
- ❖ To establish the effect of Hydrochlorothiazide in releasing metabolite of losartan.
- ❖ Rationale of the fixed dose combination for evaluating the therapeutic efficacy in purview of pharmacological action.

CHAPTER: 4

MATERIAL & METHODS

4. Materials and Methods

4.1 Chemical and reagents:

Acetonitrile , methanol, and isopropyl alcohol were purchased from Merck (MERCK India Ltd., Mumbai). All solvents used in the analysis were of HPLC grade. Other chemicals and reagents of analytical grade were used throughout the study. Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Elix, Milli-Q A10 Academic, Bedford, MA, USA) until a resistively of 18.2MΩ was achieved.The blank human plasma with EDTA-K3 anticoagulant was collected from Clinical Pharmacological Unit (CPU) of TAAB Biostudy Services, Kolkata and was stored at −20°C until analysis.

4.2 Ethical clearance, volunteer consenting and study design details

The study protocol and related documents *eg;* informed consent form, case record form, subject information sheet was submitted to the HURIP Independent Bio-ethics committee, Kolkata, India [Central Drugs Standard Control Organization (CDSCO) registration: ECR/746/Hurip/Indt/WB/2016]. The ethical clearance was obtained prior to initiation of the study .In presence of the clinical investigator and other study team members, volunteers were informed about the aspects in regards to the clinical study comprising written information approved by ethics committee. It was ensured that the participation of the volunteers in the present study was completely voluntarily. After giving the written informed consent, eight volunteers were screened and six volunteers were included in the study. Volunteers' rights and health protection during the study were ensured by ethics committee and study team. The volunteers were randomized and blinded. As the study was a comparative pharmacokinetic study, the volunteers were exposed in test drug (Single dose and FDC) after the completion of two treatments. A washout period of 7days was maintained between two study periods or two treatment periods.

4.3 Drug information and dosing

A two period, randomized, two-way complete crossover design was done using the random number generator after their clinical and vital parameters examination.

Sample product: Marketed sample containing Losartan Potassium 50 mg + Hydrochlorothiaizde 12.5 mg. as a fixed dose drug and single dose drug containing Losartan Potassium 50 mg.

The volunteers were received fixed dose and single dose product based on the randomization code in each clinical period as specified in the **Table-2.** Drugs were taken with 240ml of drinking water on an empty stomach with at least 8-10hrs fasting condition in single dose without chewing.

Treatments

- **A1:** Losartan
- **A2:** FDC (Losartan + Hydrochlorothiazide)

Phase-1			Phase-2		
Volunteers No.	**Volunteers Name**	**Treatment**	**Volunteers No.**	**Volunteers Name**	**Treatment**
1.	V1	A1	1.	V1	A2
2.	V2	A1	2.	V2	A2
3.	V3	A2	3.	V3	A1
4.	V4	A2	4.	V4	A1
5.	V5	A1	5.	V5	A2
6.	V6	A2	6.	V6	A1

Table-2 Randomization of Dosing in between 6 volunteers

Volunteers No.	Sex	Age	Height (cm.)	Weight (kg.)
1.	M	22	166	55
2.	M	24	155	56
3.	M	30	165	62
4.	M	32	162	68
5.	M	28	166	63
6.	M	33	171	70
Mean		28.16	164.1	62.33
S.D.		4.30	4.455	5.36

Table-3 Demographic Data of 6 volunteers

4.4 Sampling schedule and blood collection

Total 16 blood samples were taken from the volunteers according to the following schedule--0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 24, 36, and 48hrs. The sampling was done by cubital vein puncture with installing of cubital catheter by the study team member (phlebotomist). 5ml of blood was taken at each time point.

4.5. Bioanalytical method development by gradation LC-MS/MS

Losartan which chemical formula $C_{22}H_{23}ClN_6O$ and CAS number 114798-26-4. Chemical name of Losartan is 2-butyl-5-chloro-3-[[4-[2-(2H-tetrazol-5-yl) phenyl] phenyl] methyl] imidazol-4-yl] methanol and molecular weight 422.92 and monoisotropic molecular weight 422.1622. Losartan is generally marketed as the basic potassium salt of the aromatized negatively charged tetrazole. Losartan contain Imidazole and Tetrazole heterocyclic ring. Tetrazole are a class of synthetic organic heterocyclic compound consisting of a five membered ring of four nitrogen atom and one carbon atom. Tetrazole Pka value 4.90 that is stronger acidic in character. Losartan contain tetrazole. Imidazole is a heterocyclic compound containing two nitrogen. It is a moderately strong base which pka value 7.0 and a week acid which pka value 14.9, so imidazole is not ionized significantly between two pka value. Losartan AlogP value 4.27, rotable bond count 8 and polar surface area 92.51, hydrogen bond acceptor count 6 and hydrogen bond donor count 2 . According to Lipinski rule hydrogen bond acceptor count 7 and hydrogen bond donor count 2 , so losartan does not violate RO5 rules . From the rotable bond count and polar surface area it was concluded that losartan maintain Veber's rule, so predictable this compound is orally active and good oral bioavailablity. ACD log P value shows 3.46 that is positive so losartan compound is more lipophilic and ACD logD at P^H 7.4 is 1.48 also shows losartan highly lipophilic in the body and highly permeable through lipid membrane. Losartan contain tetrazole and imidazole heterocyclic ring as a result it contain two pka value 4.15 and 4.39 and total molecule is acidic. Due to significant difference in P^{Ka} value for tetrazole ring, imidazole ring , it was imperative to set optimum condition for plasma extraction, chromatographic and mass detection for their simultaneous determination.

For quantitation used positive polarity to achieve adequate response for their simultaneous analysis. Moreover positive ionization mode is selective and highly sensitive for compounds

with low electron affinity. Thus positive ionization mode was selected to fragment the losartan to obtain intense and consistent product ions.

Losartan carboxylic acid is an active metabolite of losartan which CAS number is124750-92-1 and molecular weight 436.9 and which exact or monoisotropic mass is 436.141. The IUPAC name of losartan carboxylic acid is 2-butyl-5-chloro-3-[[4-[2-(2H-tetrazol-5-yl) phenyl] phenyl]methyl]imidazole-4-carboxylic acid and chemical formula is $C_{22}H_{21}ClN_6O_2$. Losartan carboxylic acid is a biphenyltetrazole that is losartan with the hydroxymethyl group at position 5 on the imidazole ring replaced with a carboxylic acid. It has a role as a metabolite. It is a biphenyl tetrazole, a member of imidazole and an organochlorine compound. It derives from a losartan. Losartan carboxylic acid contain Imidazole and Tetrazole heterocyclic ring. Losartan carboxylic acid is very weak acid compounds with 5.4 and 4.2, 6.9 P^{Ka}values for acidic nitrogen proton in the tetrazole ring and due to carboxy group and imidazole ring respectively.H-bond donor count, acceptor count and rotable bond counts are 2, 6 and 8 respectively. Losartan carboxylic acid also contain biphenyl group which is a bezenoid aromatic compound that consist of two benzene ring connected ny a single covalent bond. This part absorbed from the mucous membrane and metabolized in the liver to water soluble hydroxyl derivative. Biphenyl is neutral structure and ACD log D at ph 7.4 value is 4.09 that is highly permeable through the lipid membrane that is lipophilic. Hydrogen bond donar count , hydrogen bond acceptor count of losartan carboxylic acid are 2,6 respectively. According to Lipinski rule hydrogen bond acceptor count 6 and hydrogen bond donor count 2 , so losartan carboxylic acid does not violate RO5 rules . Losartan carboxylic acid contain 8 rotable bond and its polar surface area 110 $Å^2$, so according to veber's rule it is predictable that losartan carboxylic acid also orally active and high orally good bioavailability. Log p value of losartan carboxylic acid 4.5 and two typ[es of pka value 7.4 and 4.12 which one is neutral deu to biphenyl ring and one is strongest acidic due to tetrazole and imidazole and carboxylic acid residue. Losartan carboxylic acid is the active metabolite and 5-carboxylic acid derivative of losatan. Due to significant difference in P^{Ka} value for tetrazole ring, imidazole ring , it was imperative to set optimum condition for plasma extraction, chromatographic and mass detection for their simultaneous determination.For quantitation used positive polarity to achieve adequate response for their simultaneous analysis. Moreover positive ionization mode is selective and highly

sensitive for compounds with low electron affinity. Thus positive ionization mode was selected to fragment the losartan to obtain intense and consistent product ions.

Candesartan use as an internal standard for quantitation of Losartan and losartan carboxylic acid. Candesartan has similar in structure like losartan. CAS number of candesartan is 139481-59-7. The molecular formula of candesartan is $C_{24}H_{20}N_6O_3$ and chemical name 2-ethoxy-3-[[4-[2-(2*H*-tetrazol-5-yl)phenyl]phenyl]methyl]benzimidazole-4-carboxylic acid. The molecular weight of candesartan 440.463 and monoisotropic mass 440.16. Like losartan candesartan also contain tetrazole and imidazole heterocyclic group but candesartan contain imidazole group with benzene so called bezimidazole which pka value 5.3. Tetrazole are a class of synthetic organic heterocyclic compound consisting of a five membered ring of four nitrogen atom and one carbon atom. Tetrazole Pka value 4.90 that is stronger acidic in character. Candesartan ACD pka value 2.06 and 5.6 and total molecular species acidic in character. Candesartan AlogP value 4.03, rotable bond count 7 and polar surface area 118.81, hydrogen bond acceptor count 7 and hydrogen bond donor count 2 . According to Lipinski rule hydrogen bond acceptor count 7 and hydrogen bond donor count 2 , so candesartan does not violate RO5 rules . From the rotable bond count and polar surface area it was concluded that candesartan maintain Veber's rule, so predictable this compound is orally active and good oral bioavailablity. ACD log P value shows 4.65 that is positive so candesartan compound is more lipophilic and ACD logD at P^H 7.4 is 0.54 also shows candesartan highly lipophilic in the body and highly permeable through lipid membrane. Due to significant difference in P^{Ka} value for tetrazole ring, imidazole ring , it was imperative to set optimum condition for plasma extraction, chromatographic and mass detection for their simultaneous determination.For quantitation used positive polarity to achieve adequate response for their simultaneous analysis. Moreover positive ionization mode is selective and highly sensitive for compounds with low electron affinity. Thus positive ionization mode was selected to fragment the losartan to obtain intense and consistent product ions.

Hydrochlorothiazide is a diuretic medication used to treat high blood pressure and swelling due to fluid buildup. Hydrochlorothiazide molecular weight 297.75 and which monoisotropic molecular weight is 296.9645 and chemical formula $C_7H_8ClN_3O_4S_2$ which IUPAC name is 6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide. Hydrochlorothiazide CAS number is 58-93-5, H-bond donor count, acceptor counts are 3,5 respectively. On the other

hand hydrochlorothiazide has P^{Ka} values of 7.9 and 9.2 for secondary amine and sulfonamide group, respectively. The value of AlogP of hydrochlorothiazide is -0.35 and rotable bond count 1 and polar surface area 118.36$Å^2$, so according to veber's rule it is predictable that hydrochlorothiazide also orally active and high orally good bioavailability. According to Lipinski rule hydrogen bond acceptor count 7 and hydrogen bond donor count 4 so does not violate RO5 rule. The value of ACD logP -0.02 so compound has higher affinity for the aqueous phase that is hydrochlorothiazide is more hydrophilic and at P^H 7.4 ACD logD value -0.04 so it is concluded that this compound is more susceptible to higher aqueous solubility and of lower lipophilicity in the body. As a result we would expect membrane permeability to be poor. Due to the presence of secondary amine and sulfonamide group ACD pka value is 8.95 nearly 9.2 so compound is strongly basic and weakly acidic. But the total molecule is neutral in character because pka value of total compound is 7.9.

Letrozole used as an internal standard for quantification of hydrochlorothiazide. CAS number of letrozole is 112809-51-5 and molecular formula $C_{17}H_{11}N_5$ and chemical name 4-[(4-cyanophenyl)-(1,2,4-triazol-1-yl)methyl]benzonitrile.Molecular weight of letrozole is 285.31 and monoisotropic mass is 285.101. According to Lipinski rules hydrogen bond acceptor count is 5 but hydrogen bond donor count 0 so does not violate RO5 rule. Due to the presence of triazole its pka value 9.4 and 1.2 and pka of benzonitrile -10.1 so letrozole contain basic pka value 1.52. The value of AlogP of letrozole is 2.66 and rotable bond count 3 and polar surface area 78.29$Å^{2,}$ so according to veber's rule it is predictable that hydrochlorothiazide also orally active and high orally good bioavailability. The value of ACD logP 0.43 so compound has higher affinity for the lipid phase that is letrozole is more lipophilic and at P^H 7.4 ACD logD value 0.43 so it is concluded that this compound is more susceptible to higher lipid solubility and of lower aqueous soluble. So letrozole molecular species neutral in character.

Due to significant difference in P^{Ka} value for triazole ring, bezonitrile, carboxyl group, secondary amine and sulfonamide group it was imperative to set optimum condition for plasma extraction, chromatographic and mass detection for their simultaneous determination.Negativeionization mode is highly selective and sensitive for compounds with low electron affinity. Thus negative ionization mode was selected in the present study to fragment the analytes and IS to obtain intense and consistent product ions.

The protonated precursor ions**[M+H]**$^{+}$at m/z 423.0(highest peak),453.3(2nd peak),475.0(3rd peak),491.2(4th peak) were observed in Q1 MS scanning for Losartan and characteristic product ions or fragment ions found in Q3 MS scanning were at m/z 207.2,191.8,171.2,405.5,234.8.

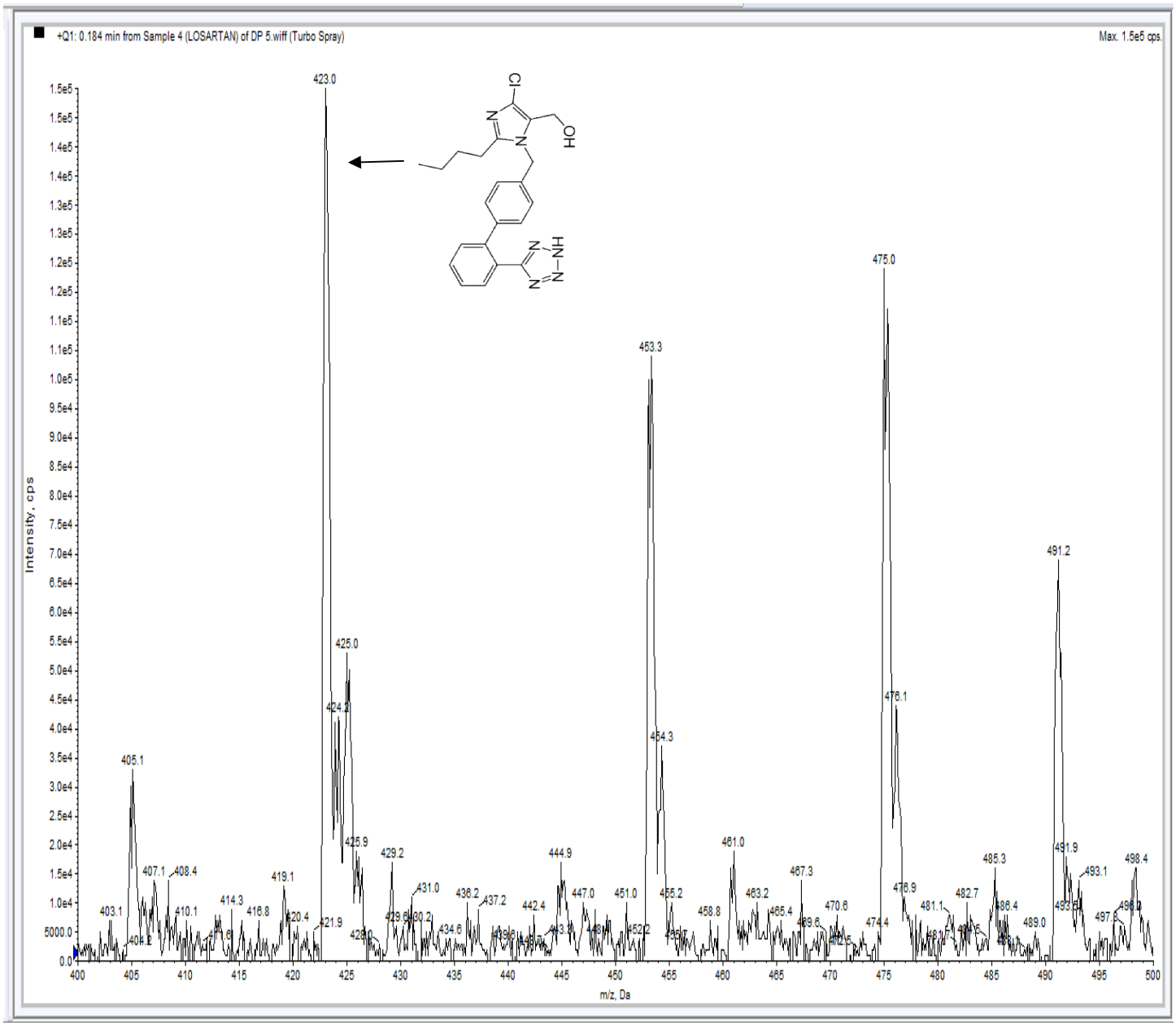


Figure: 29 Parent Ion (Q1) Scan of Losartan

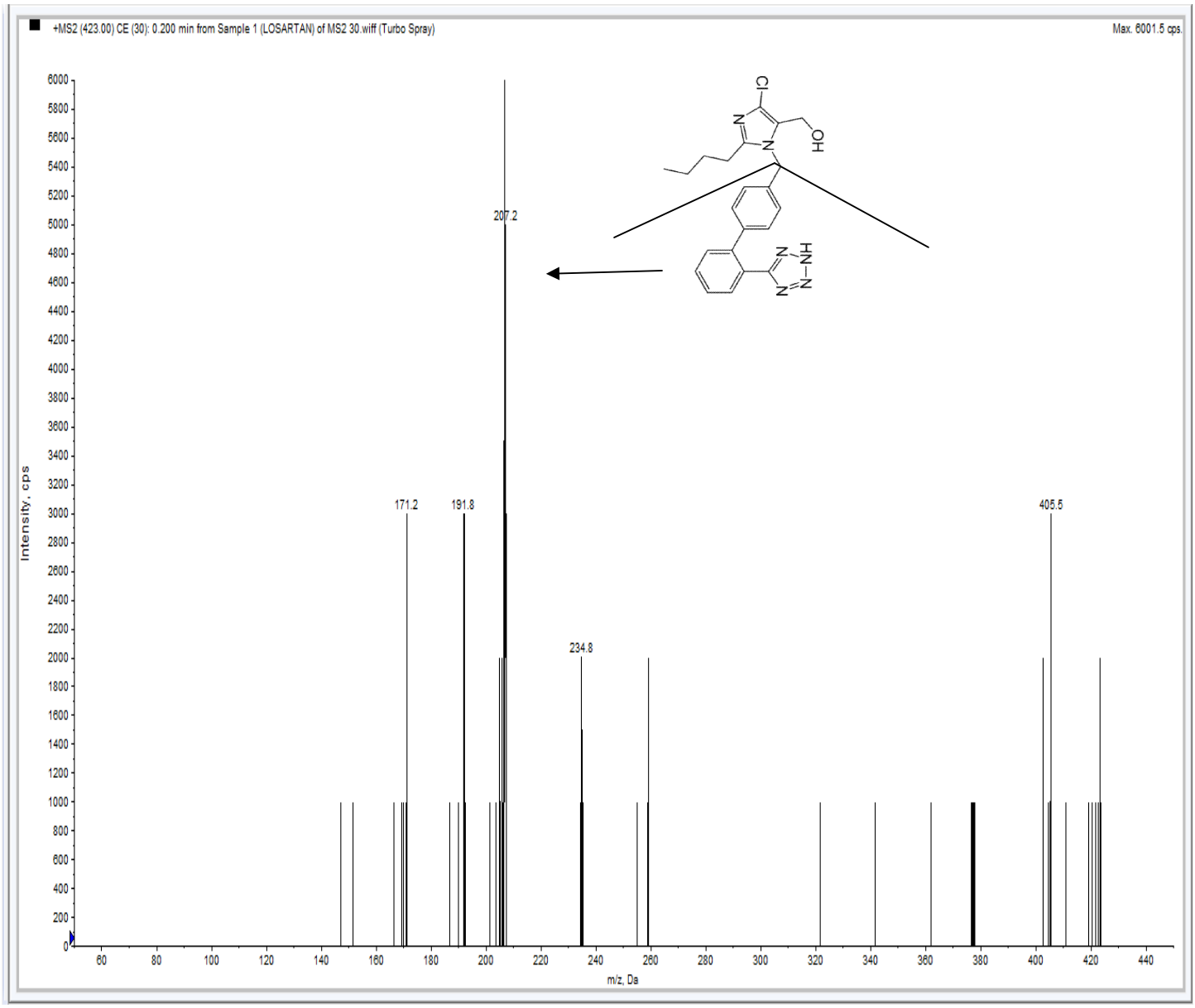


Figure: 30 Product Ion (MS2 or Q3) Scan of Losartan

However the most stable and consistent fragment ion selected was m/z 207.2 for 2-(2H-tetrazol-5-yl) di-phenyl.

The protonated precursor ions$[M+H]^+$at m/z 437.1(highest peak),391.2(2^{nd} peak), were observed in Q1 MS scanning for Losartan Carboxylic acid and characteristic product ions or fragment ions found in Q3 MS scanning were at m/z 235.1,206.8,435.6 . However the most stable and consistent fragment ion selected was m/z 235.1 for 2-butyl-4-chloro-1H-imidazole-5-carboxylic Acid.

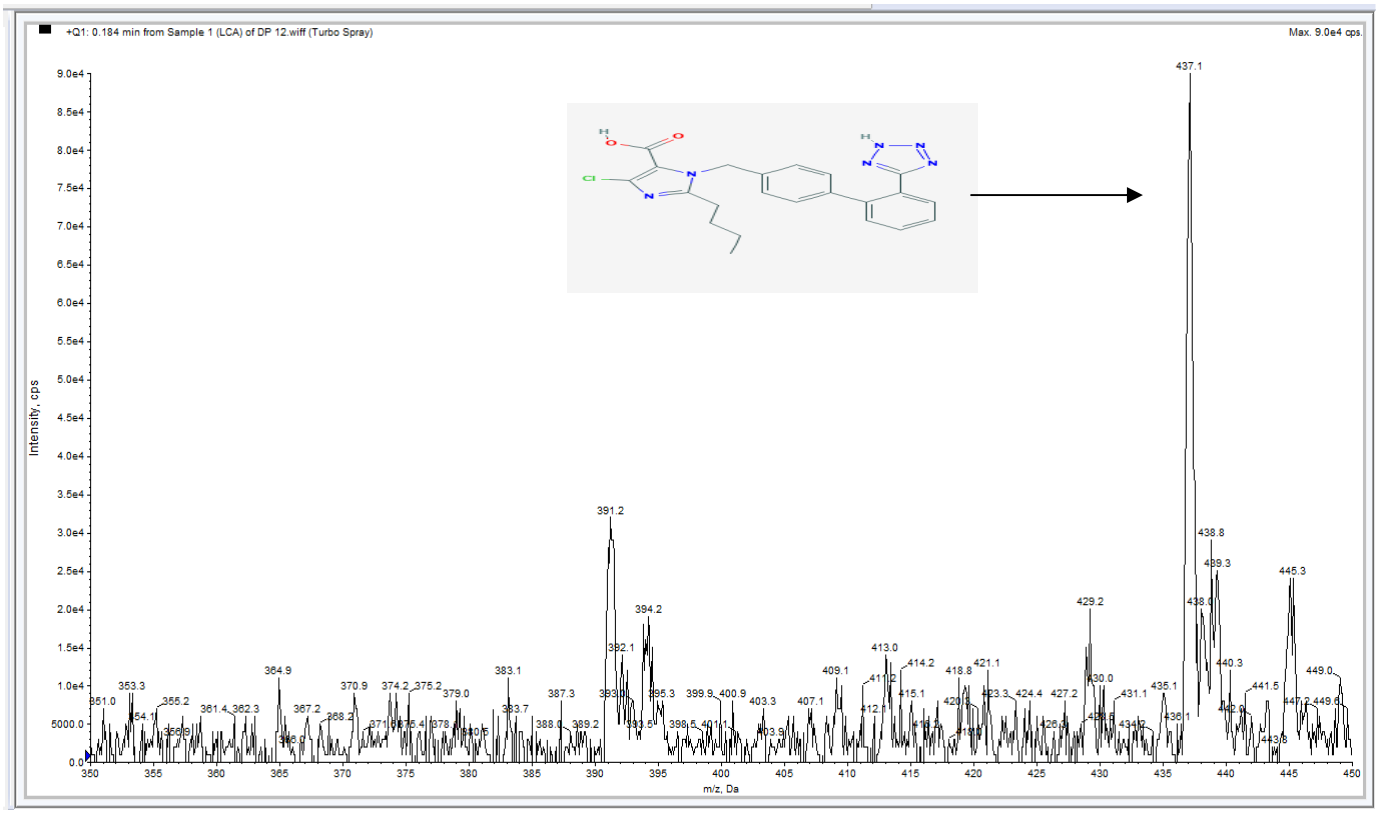


Figure: 31 Parent Ion (Q1) Scan of Losartan Carboxylic Acid

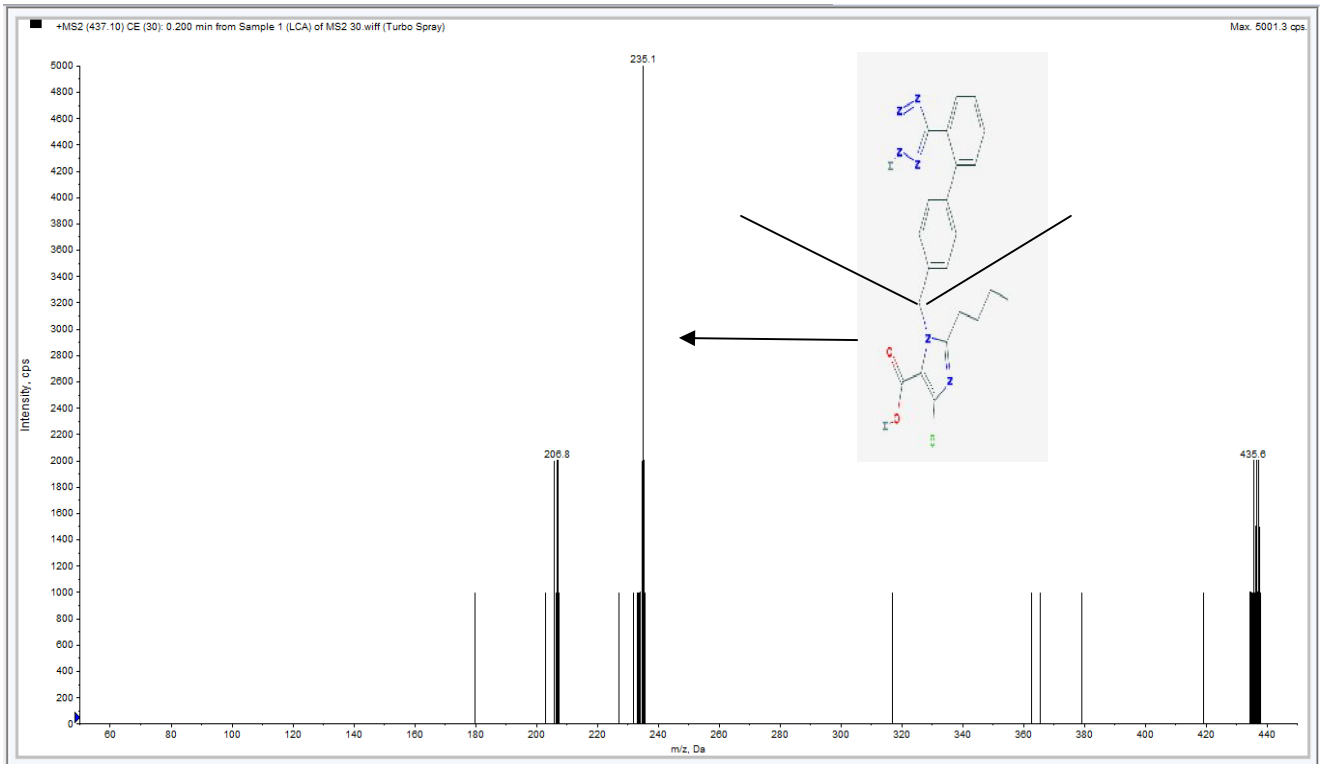


Figure: 32 Product Ion (MS2 or Q3) Scan of Losartan Carboxylic Acid

Candesartan use as an internal standard for quantitation of Losartan and losartan carboxylic acid. The protonated precursor ions $[M+H]^+$at m/z 441.1 (highest peak), 423(2^{nd} peak) was observed in Q1 MS scanning for candesartan and characteristic product ions or fragment ions found in Q3 MS scanning was m/z 263.2 .

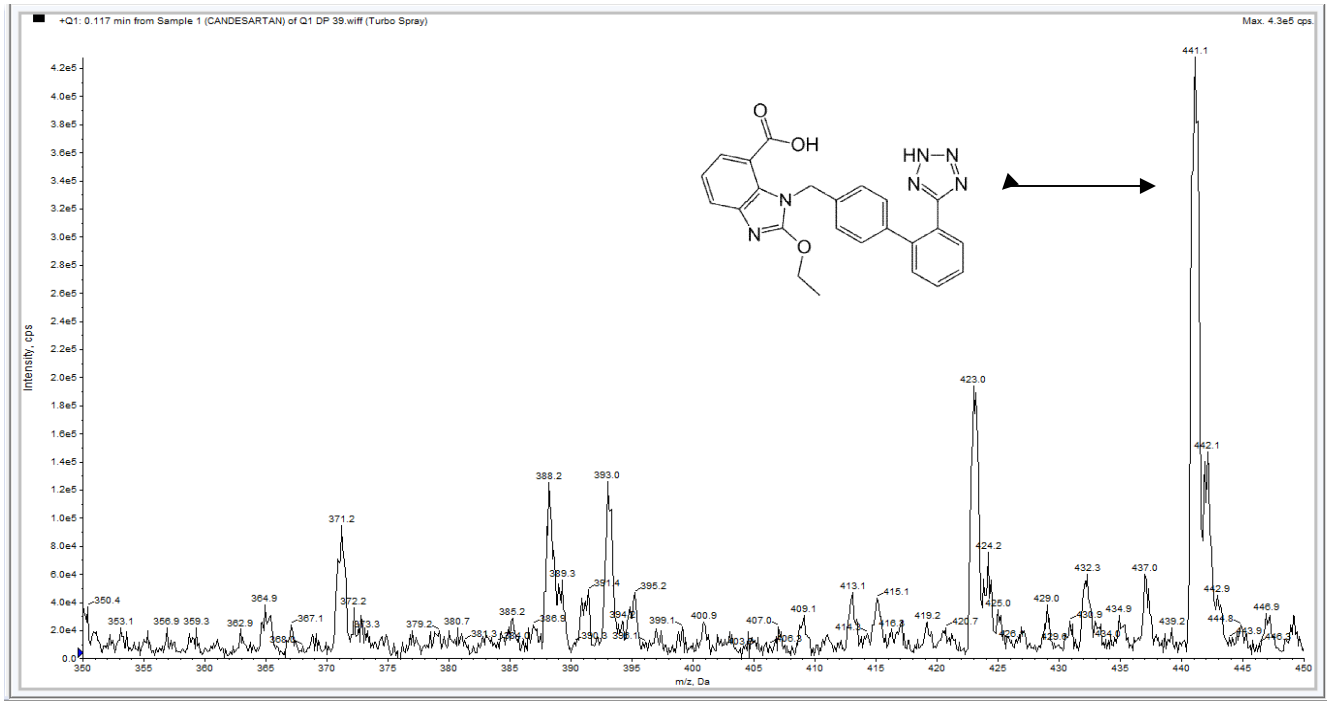


Figure: 33 Parent Ion (Q1) Scan of Candesartan (IS)

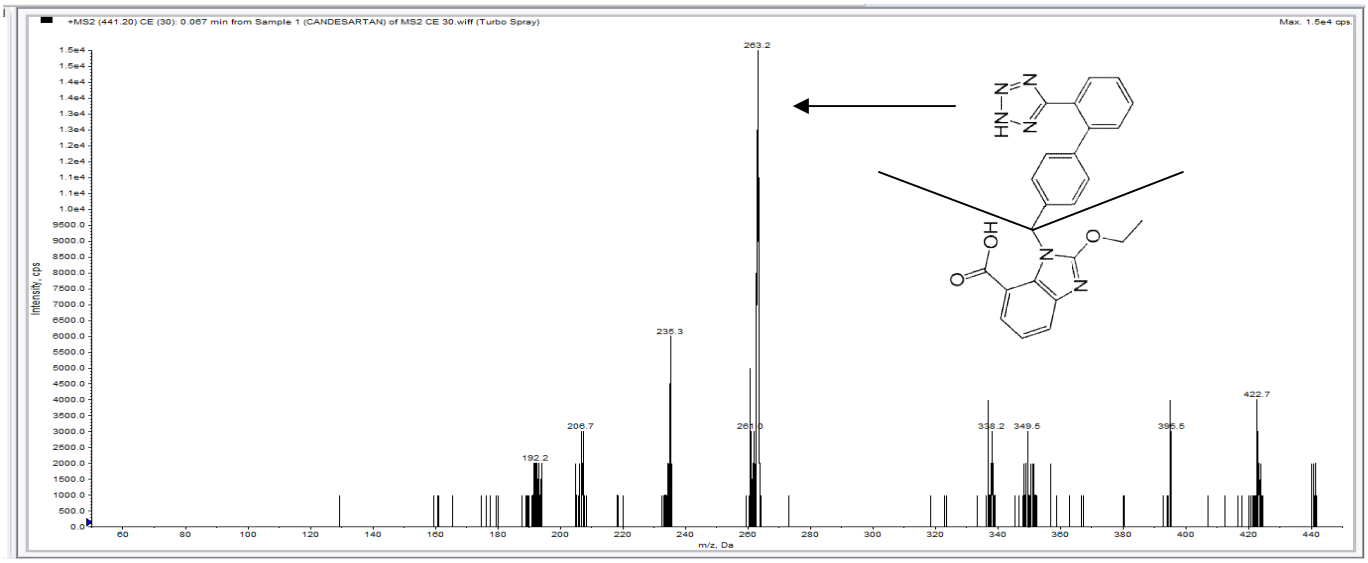


Figure: 34 Product Ion (MS2 or Q3) Scan of Candesartan (IS)

Whereas the deprotonated precursor ions **[M-H]**$^{-}$at m/z 295.8(highest peak), 331.9(2nd peak), 255.1(3rd peak), 297.7(4th peak), were observed in Q1 MS scanning for Hydrochlorothiazide and characteristic product ions or fragment ions found in Q3 MS scanning were at m/z 268.9, 231.8, However the most stable and consistent fragment ion selected was m/z 268.9 for elimination of HCN **[M-H-HCN]**$^{-}$from precursor ion.

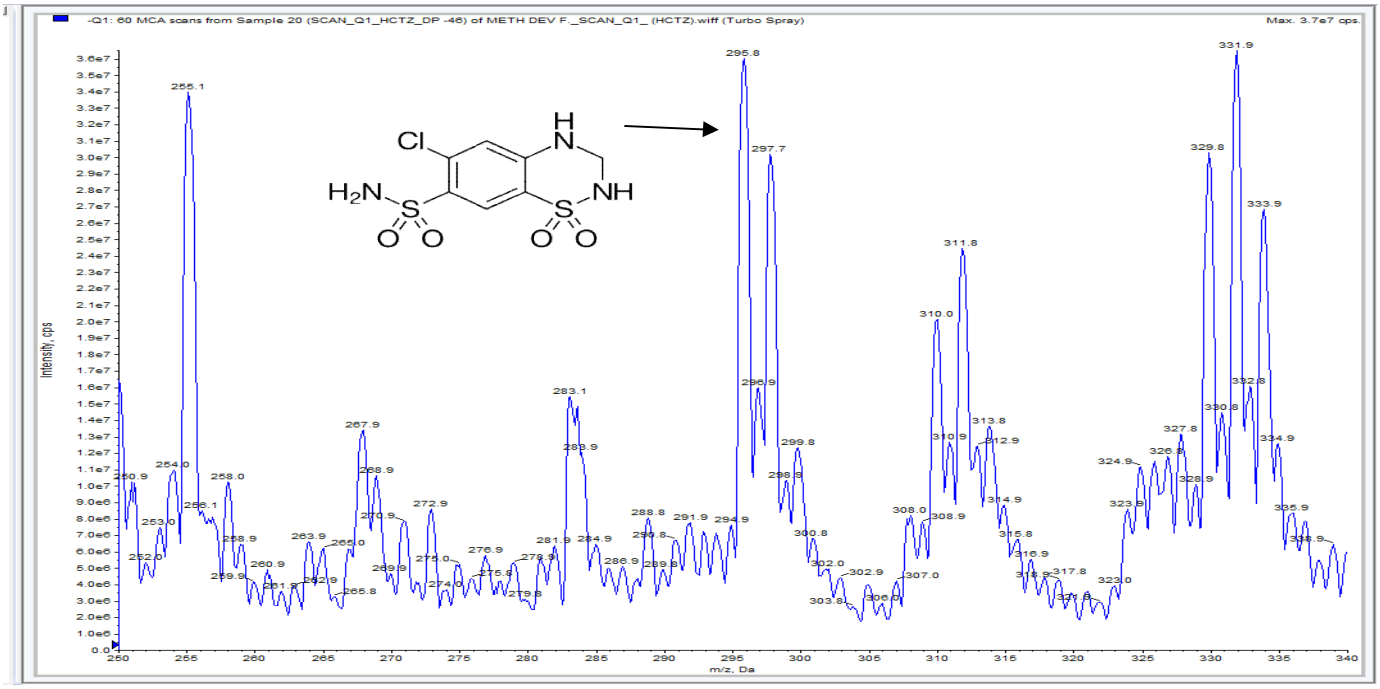


Figure: 35 Parent Ion (Q1) Scan of Hydrochlorothiazide

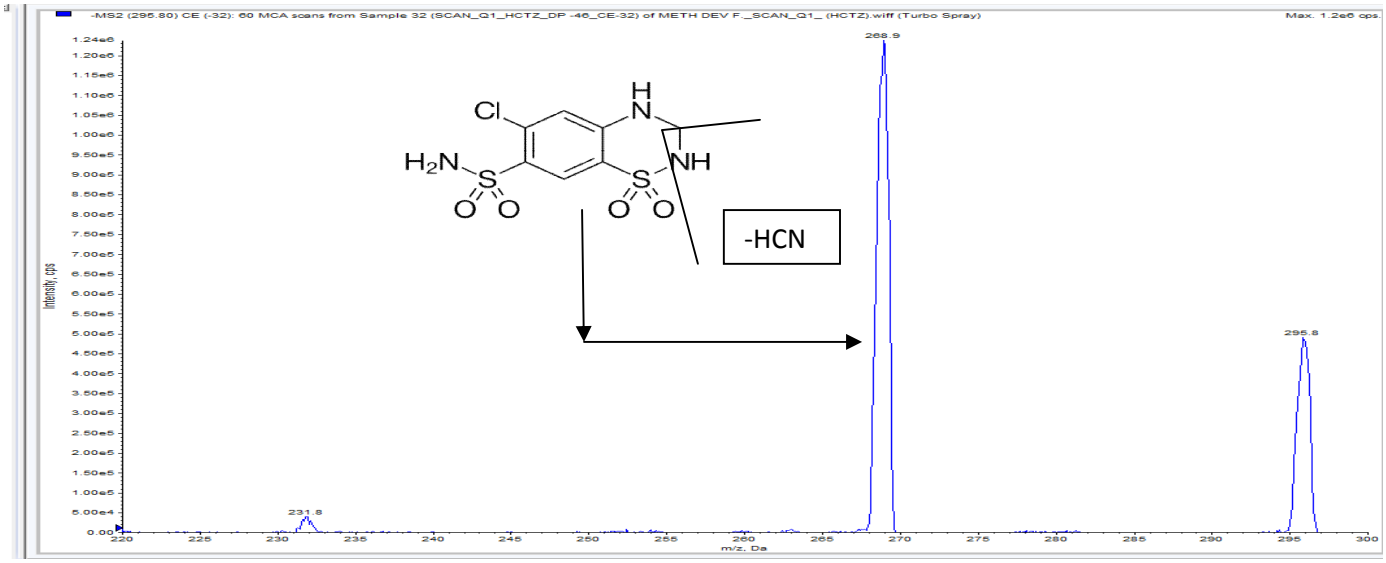


Figure: 36 Product Ion (MS2 or Q3) Scan of Hydrochlorothiazide

Letrozole (chemical formula $C_{17}H_{11}N_5$ and molecular weight 285.303) used as an internal standard (IS) for quantitation of Hydrochlorothiazide. The deprotonated precursor ions $[M-H]^-$ at m/z 283.9 (highest peak)was observed in Q1 MS scanning for letrozole and characteristic product ions or fragment ions found in Q3 MS scanning was m/z 241.9 $[M-H- CH_3CN]^-$ for release CH_3CN (Methyl Cyanide)

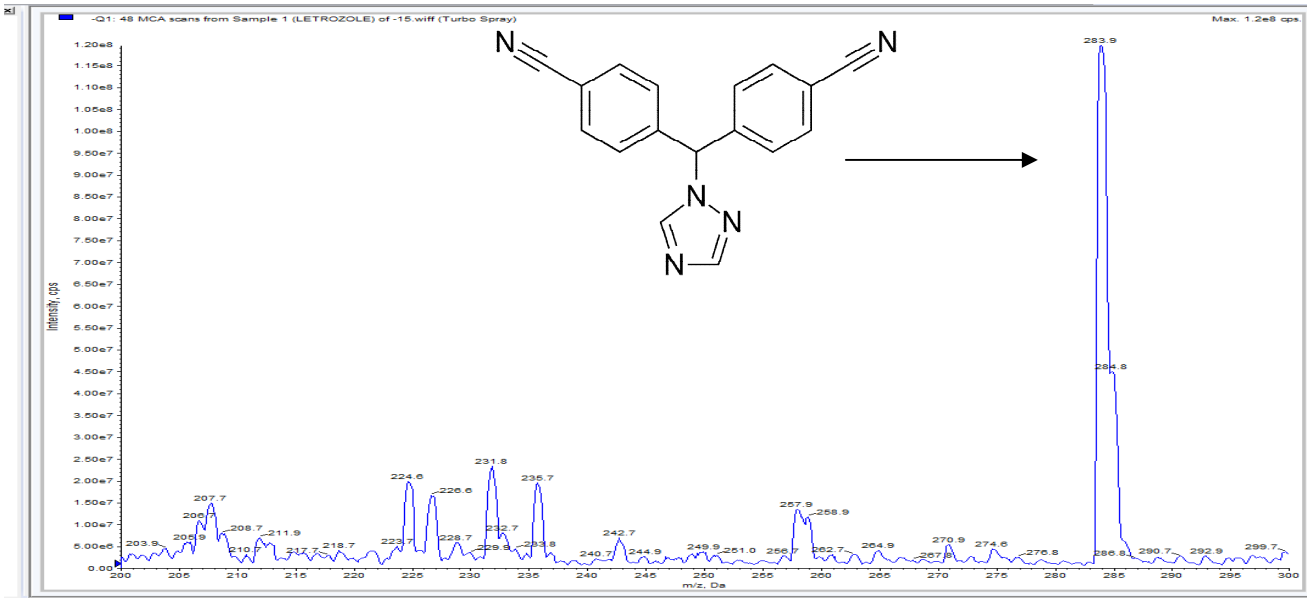


Figure: 37 Parent Ion (Q1) Scan of Letrozole (IS)

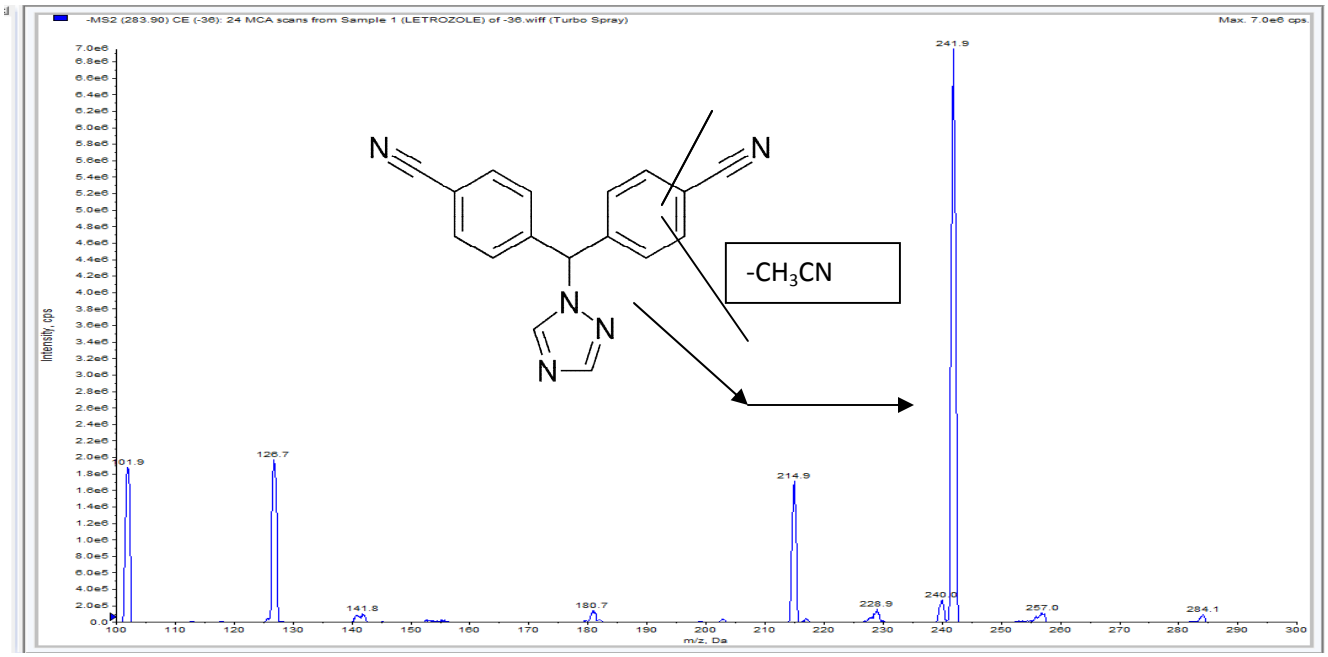


Figure: 38 Product Ion (MS2 or Q3) Scan of Letrozole (IS)

The representative MRM chromatograms were showed in

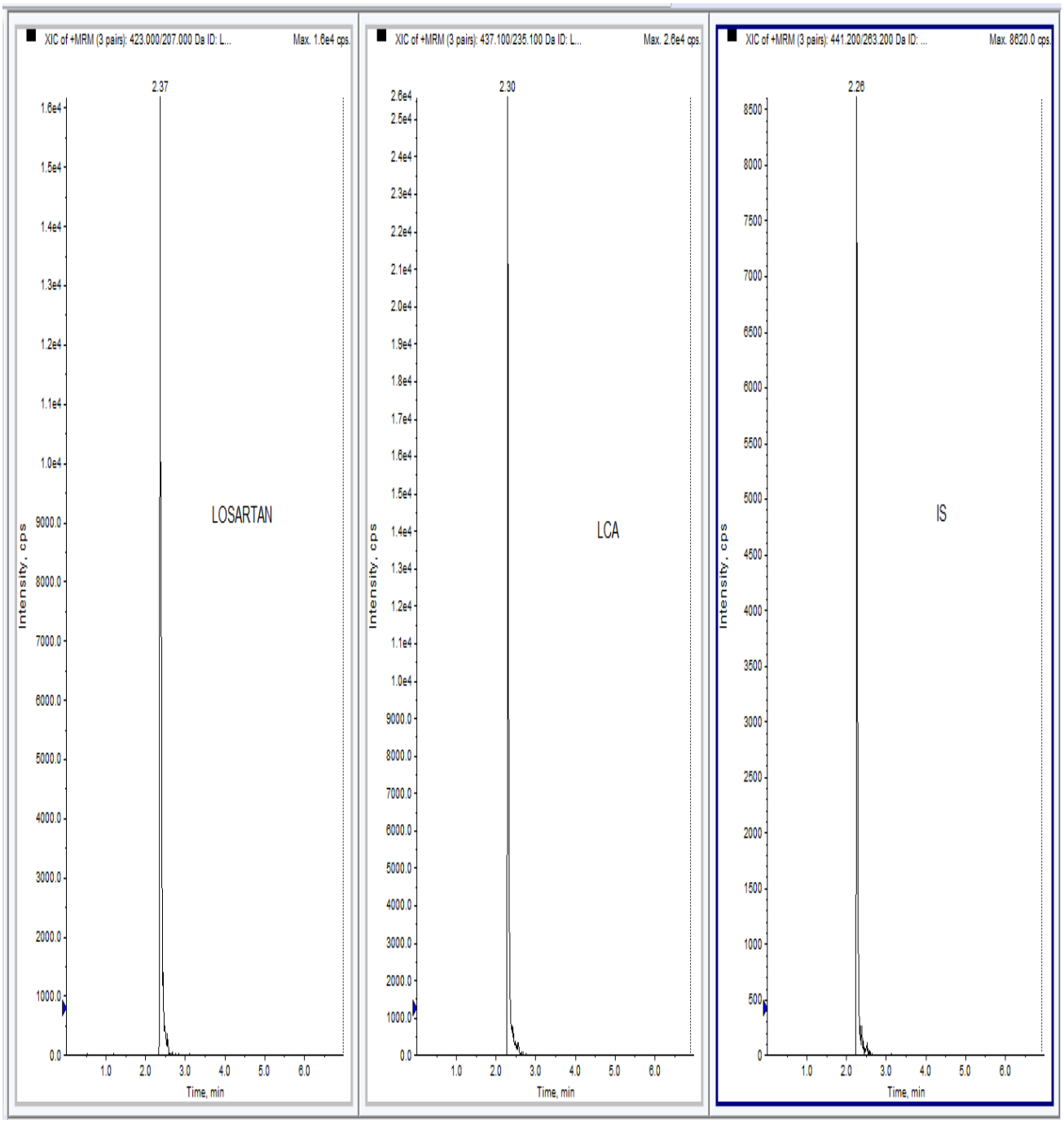


Figure: 39 MRM (Multiple Reaction Monitoring) Chromatogram of Losartan and Losartan Carboxylic Acid with Internal Standard Candesartan

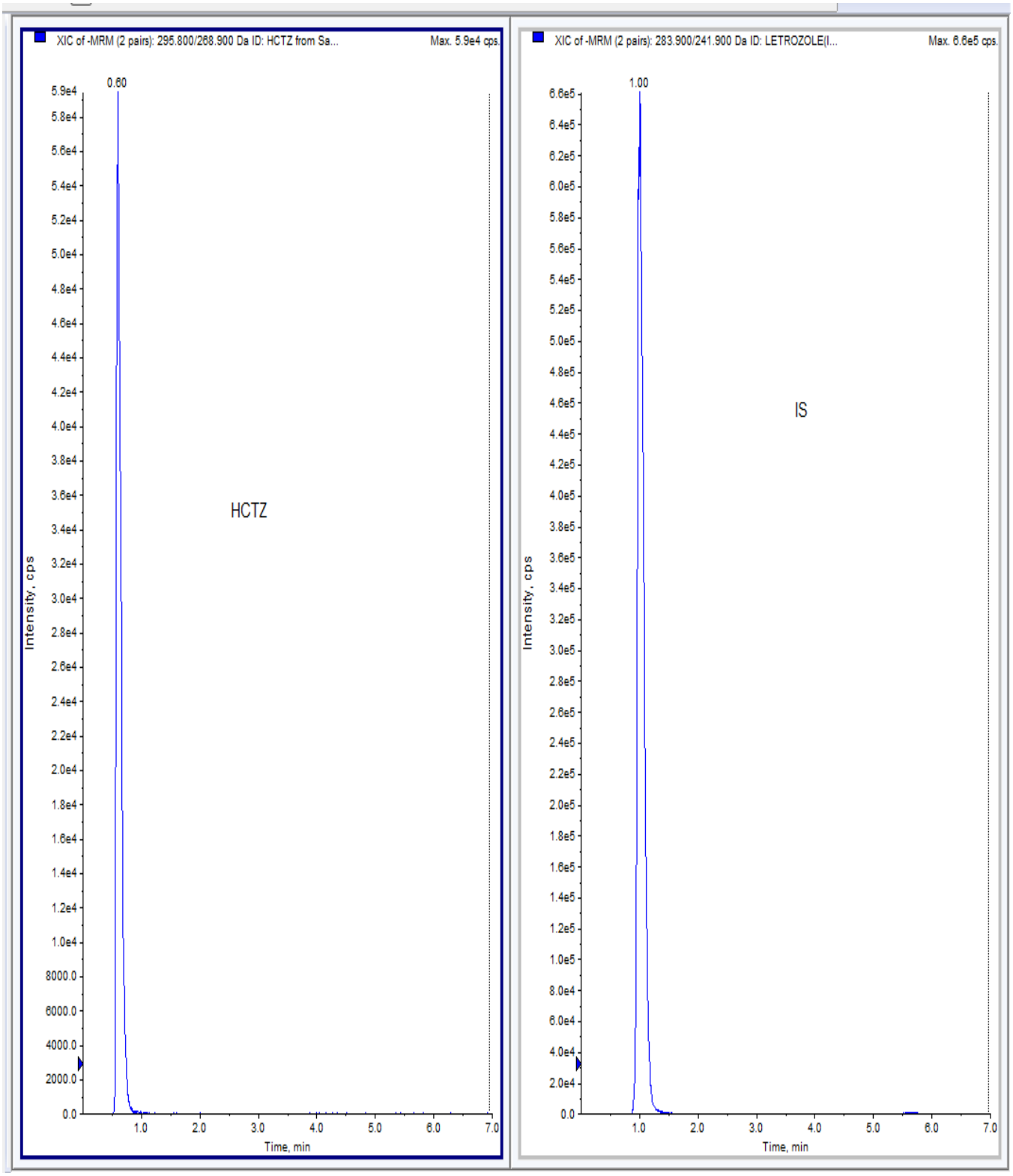


Figure: 40 MRM (Multiple Reaction Monitoring) Chromatogram of Hydrochlorothiazide with Internal Standard Letrozole.

Chromatographic analysis with gradation technique was performed on a Shimadzu HPLC system equipped with LC-20AD binary pump, SIL-20A autosampler, CTO-10ASvp Oven and CBM-20A lite system control compartment. Mass spectrometric detection was performed on an API 2000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, ON, Canada) equipped with an Turbo electrospray ionization (ESI) interface. The selection of mobile phase was crucial for synchronized determination of the drugs having imidazole and sulfonamide group. Thus, the pH of the mobile phase, buffer concentration and choice and proportion of diluents were very important for chromatographic resolution with adequate response to achieve the desired sensitivity. The optimal mass parameters for both the analytes and IS were elaborated in the table.

Parameter(s)	Value
Ionization mode	MRM (+ve)
Source temperature (ºC)	400
Dwell time per transition (msec)	100
Curtain gas (psi)	30
CAD gas (psi)	8
Ion spray voltage (V)	5500.00
Ion source gas 1 (psi)	55
Ion source gas 2 (psi)	45
Focussing potential (V)	400
Declustering potential (V)	5 and 12 (Losartan and LCA) and 39 (IS)
Entrance potential (V)	11
Collision energy (V)	30 (Losartan and LCA and IS)
Collision cell exit potential (V)	15 (analytes and IS)
Transition pair of Losartan and LCA (analyte)	423.0/207.0, 437.1/235.1
Transition pair of candesartan (IS)	441.2/263.2

Table-4: Mass spectrometric conditions of Losartan & Losartan Carboxylic Acid

Parameter(s)	Value
Ionization mode	MRM (-ve)
Source temperature (ºC)	400
Dwell time per transition (msec)	100
Curtain gas (psi)	30
CAD gas (psi)	8
Ion spray voltage (V)	-4500.00
Ion source gas 1 (psi)	55
Ion source gas 2 (psi)	45
Focussing potential (V)	-400
Declustering potential (V)	-46 (Hydrochlorothiazide) and -15 (IS)
Entrance potential (V)	-11
Collision energy (V)	-32 and -36 (analyte and IS)
Collision cell exit potential (V)	-15 (analyte and IS)
Transition pair of Hydrochlorothiazide (analyte)	295.8/268.9
Transition pair of letrozole (IS)	283.9/241.9

Table-5: Mass spectrometric conditions of Hydrochlorothiazide

The chromatographic elution of the analytes on a Phenomenex Kinetex 5µ C18 100A 50*3mm column was initiated as a rapid, sensitive and rugged analytical method covering the dynamic linear range. The selection of mobile phase was crucial for synchronized determination of the drug having di pKa values. Thus, the pH of the mobile phase, buffer concentration, and choice and proportion of diluents were varying which was important for chromatographic resolution with adequate response to achieve the desired sensitivity. Initially, acetonitrile/methanol with 5 mM ammonium acetate buffer (pH 6.5) gave response for losartan and losartan carboxylic acid. However, the response was not reproducible. The signal was severely compromised at lower limit of quantitation (LLOQ) levels even after altering the concentration of buffer from 5 mM to 10 mM. Further, the chromatography was better with a higher response using an methanol-buffer as compared to a acetonitrile-buffer combination. Moreover, highering the methanol content in

the mobile phase resulted in an increase in the retention of losartan and losartan carboxylic acid, candesartan and thereby the analysis time. Subsequent efforts were directed to optimize the pH of the mobile phase and the concentration of the buffer solution as they had significant impact on analyte retention, peak shape, and resolution. At P^H above 5.0 the resolution of losartan and losartan carboxylic acid was not affected, and increase the resolution (narrow peak) with increase in pH. Thus, to achieve greater reproducibility and better chromatography, high pH buffers were tried. Better reproducibility and peak shape were observed in 0.1% Ammonia in methanol having, but the signal to noise ratio was not adequate at LLOQ level. Finally, a superior signal to noise ratio (≥ 22) and baseline resolution was obtained for the analyte by 10 Mm ammonium acetate buffer with 0.1% (v/v) Ammonia solution together with Milli Q water having apparent pH 8.90 at a flow rate of 0.5000 mL/min. There were no additional peaks due to endogenous plasma components as observed in one report when column was used even under MRM mode. The chromatographic elution time for losartan and losartan carboxylic acid & IS (candesartan) was 2.40, 2.31 & 2.27 min, respectively, in a run time of 7.00 min. This analysis was done by gradation method in which 0.01min to 1.00 min organic solvent 10% and then 1.00 min to 4.00min organic solvent 90% and then from 4.00min aqueous solvent 90% run upto 7.00 min for washing purpose.

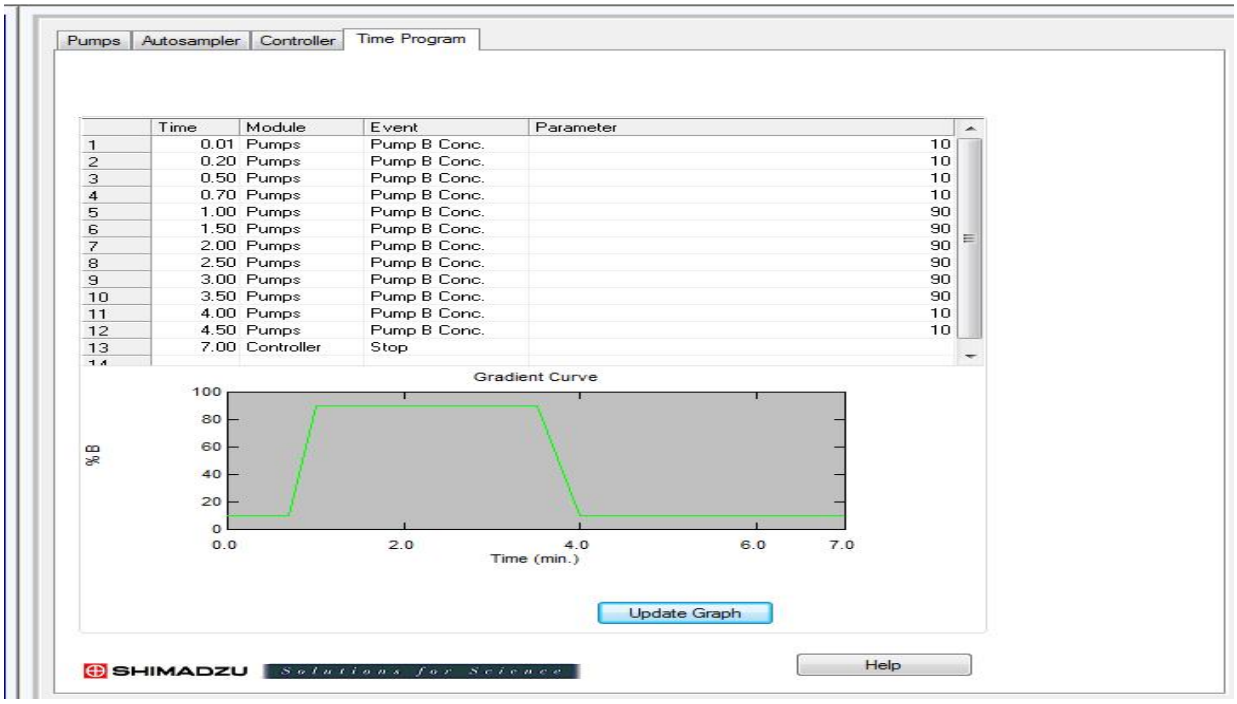


Figure: 41 Gradient Curve of Losartan & Losartan Carboxylic Acid.

For Hydrochlorothiaziode the chromatographic elution of the analytes on a Phenomenex Kinetex 5µ C18 100A 50*3mm column was initiated as a rapid, sensitive and rugged analytical method covering the dynamic linear range. The selection of mobile phase was crucial for synchronized determination of the drug having di pKa values. Thus, the pH of the mobile phase, buffer concentration, and choice and proportion of diluents were varying which was important for chromatographic resolution with adequate response to achieve the desired sensitivity. Initially, acetonitrile/methanol with 5 mM ammonium acetate buffer (pH 6.5) gave response for hydrochlorothiazide. However, the response was not reproducible. The signal was severely compromised at lower limit of quantitation (LLOQ) levels even after altering the concentration of buffer from 5 mM to 10 mM. Further, the chromatography was better with a higher response using an acetonitrile-buffer as compared to a methanol-buffer combination. Moreover, lowering the methanol content in the mobile phase resulted in an increase in the retention of hydrochlorothiazide and letrozole and thereby the analysis time. Subsequent efforts were directed to optimize the pH of the mobile phase and the concentration of the buffer solution as they had significant impact on analyte retention, peak shape, and resolution. At pH above 5.0 the resolution of hydrochlorothiazide and letrozole were affected, and decrease the resolution with increase in pH. Thus, to achieve greater reproducibility and better chromatography, low pH buffers were tried. Better reproducibility and peak shape were observed in 1.0% formic acid in acetonitrile having, but the signal to noise ratio was not adequate at LLOQ level. Finally, a superior signal to noise ratio (≥ 22) and baseline resolution was obtained for the analyte by replacing 10 Mm ammonium acetate buffer with 1.0% (v/v) formic acid together with Milli Q Water having apparent pH 2.5 at a flow rate of 0.5000 mL/min. There were no additional peaks due to endogenous plasma components as observed in one report when column was used even under MRM mode. The chromatographic elution time for Hydrochlorothiazide & IS (Letrozole) was 0.60 & 0.97 min, respectively, in a run time of 7.00 min. This analysis was done by gradation method in which 0.01min to 2.70 min organic solvent 40% and then 2.70 min to 4.70min organic solvent 60% and then from 4.70min aqueous solvent 40% run upto 7.00 min for washing purpose.

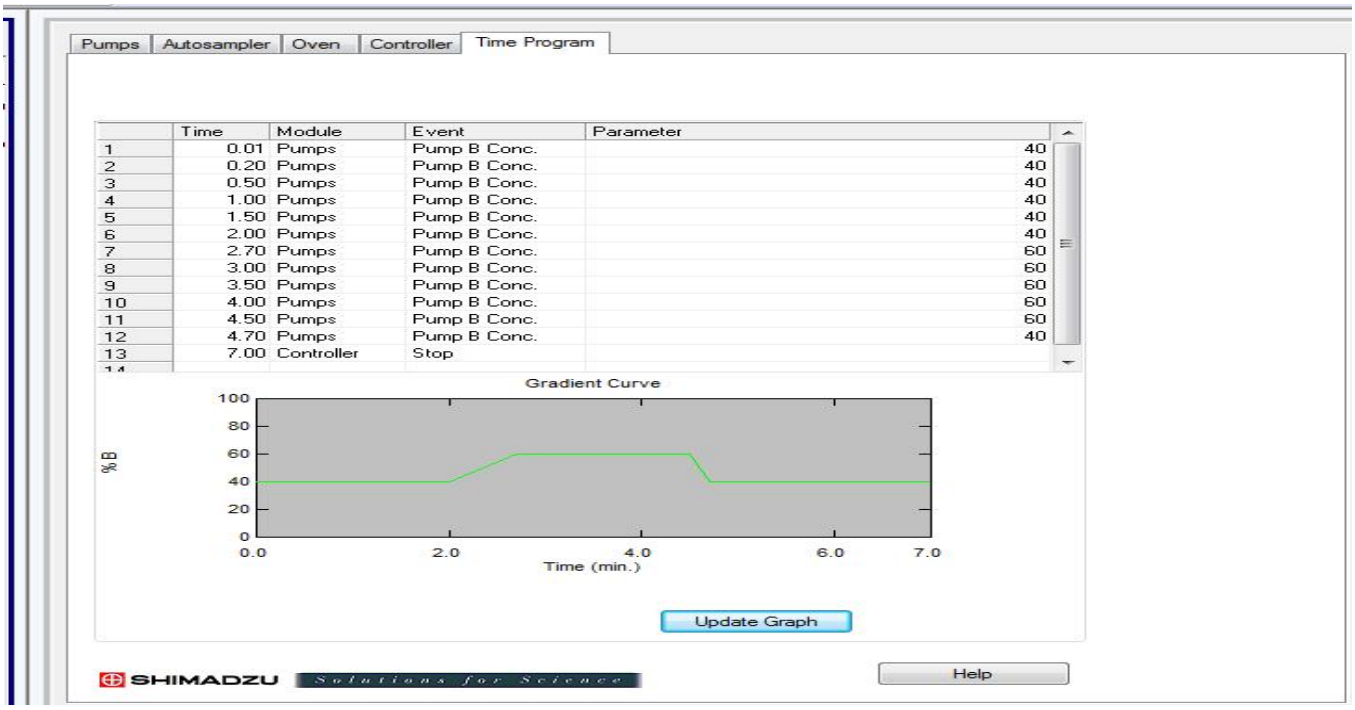


Figure: 42 Gradient Curve of Hydrochlorothiazide

4.6 Plasma extraction and sample preparation

Plasma extraction was performed by **Protein precipitation technique**

4.6.1 Extraction Procedure of Losartan and Losartan Carboxylic acid:

Plasma extraction was performed by **Protein precipitation technique,** 100 µl of plasma was taken and precipitated with 400 µl of MeCN containing 1000ng/ml **Candesartan** (IS) and vortexed for 10 min, followed by Centrifugation for 10 mins at 12,000 rpm at4^{0}C.300µl supernatant was taken and transferred to autosampler vials for injection.

4.6.2. Extraction Procedure of Hydrochlorothiazide:

Plasma extraction was performed by **Protein precipitation technique,** 100 µl of plasma was taken and precipitated with 400 µl of MeCN containing 1000 ng/ml Letrozole (IS) and vortexed for 10 mins, followed by Centrifugation for 10 min. at 10,000 rpm at -20^{0}C. 300µl. Supernatant was taken and transferred into auto sampler vials for injection.

4.6.3. Stock solution and calibration standards preparation

Stock solutions of Losartan, LCA, HCTZ and IS (Candesartan and Letrozole respectively) were prepared by dissolving accurately weighed samples in the DMSO to obtain concentrations of 1mg/ml. The stock solutions of Losartan and LCA were then gradually diluted with Methanol: water: 50: 50 (v/v) to obtain calibration samples.

❖ **PLASMA CALIBRATION STANDARDS (ng/ml):**

Losartan Carboxylic Acid: 50.00, 100.0, 200.0, 400.00, 800.00, 1600.00, 3200.00.

Losartan: 9.37, 18.75, 37.50, 75.00, 150.00, 300.00, 600.00.

The stock solutions of Hydrochlorothiazide was then gradually diluted with Acetonitrile: water 50: 50 (v/v) to obtain calibration samples of

❖ **PLASMA CALIBRATION STANDARDS (ng/ml):**

HCTZ: 10, 20, 40, 80, 160, 320, 640.

4.7 Method validation

The method validation was conducted in accordance with the guidelines US-FDAfor selectivity, sensitivity, linearity, precision, accuracy, recovery and stability.

4.7.1 *Specificity, selectivity and linearity*

The specificity and selectivity of the assay was illustrated by the chromatograms of mobile phase run and extract of blank plasma recorded for samples near the C_{max} for 2.00 to 3.00 hr for Losartan Carboxylic Acid and 1.00 to 2.50 hr for Losartan and 2.5 to 3.0 hr. for Hydrochlorothiazide. The linearity of the calibration curve was determined by a un weighted least square regression analysis. Representative calibration curves of Losartan, Losartan Carboxylic Acid and Hydrochlorothiazide from human plasma were depicted in the linearity graph.

4.7.2 *Precision and accuracy*

Between–run precision and accuracy were determined from the low, medium and high QC samples (LQC, MQC and HQC). A total of 5 replicates of each QC concentration were assayed on day 1 and a total of 5 replicates each QC concentration were assayed on day 2 and 3. The QC

samples concentrations were determined from three different calibration curves that were assayed with QC samples.

Within-run precision and accuracy were determined from a total of 5 replicates of each QC concentration. The low, medium and high QC samples (LQC, MQC and HQC) were assayed on day 2. The same was carried out separately for Losartan, Losartan Carboxylic Acid and Hydrochlorothiazide. The QC samples concentrations were determined from calibration curves LIN 3 for Losartan LIN3 for LCA and LIN2 for HCTZ. Precision was expressed as percent variation (%CV), while accuracy was measured as the percent nominal.

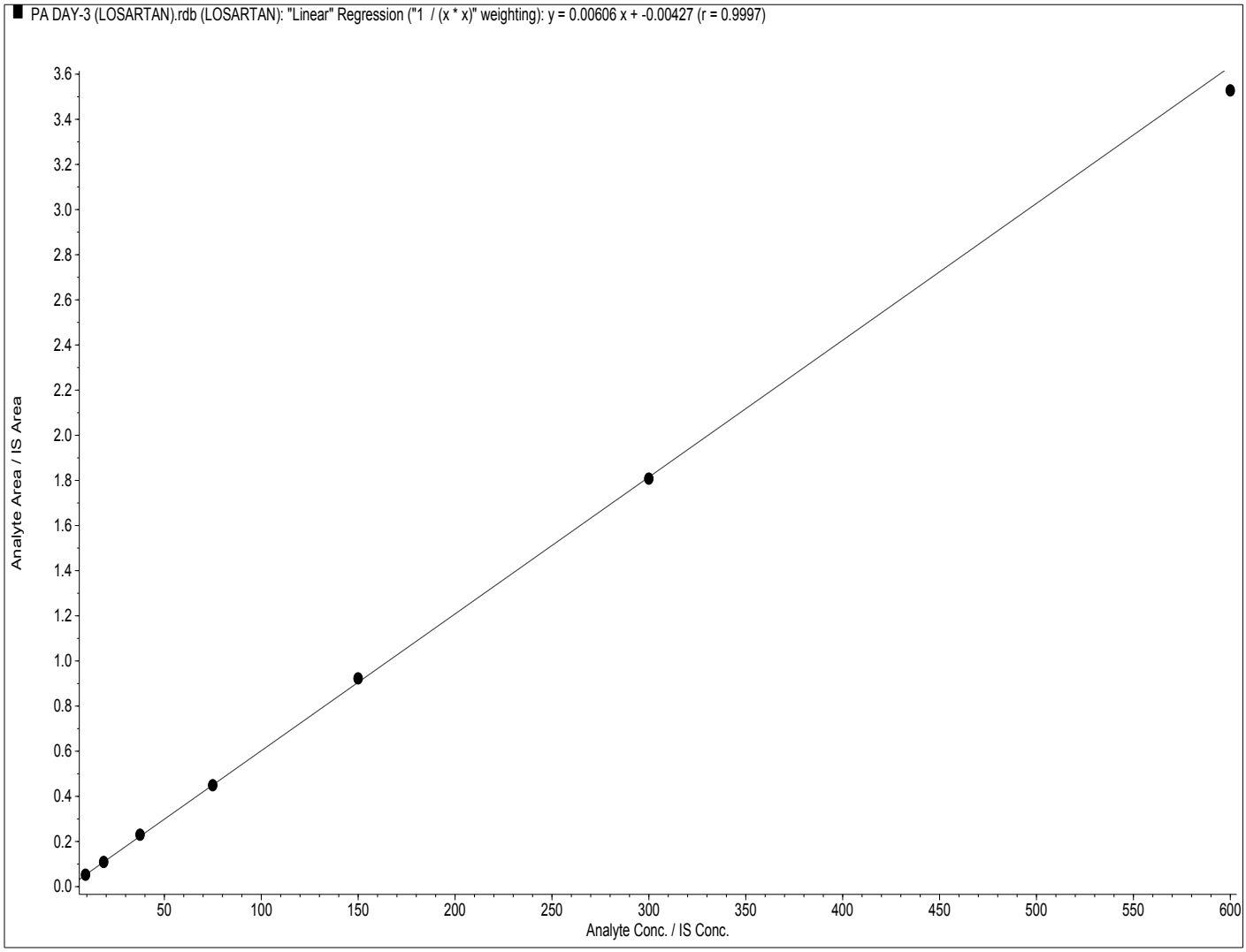


Figure: 43 Calibration Curve of Losartan

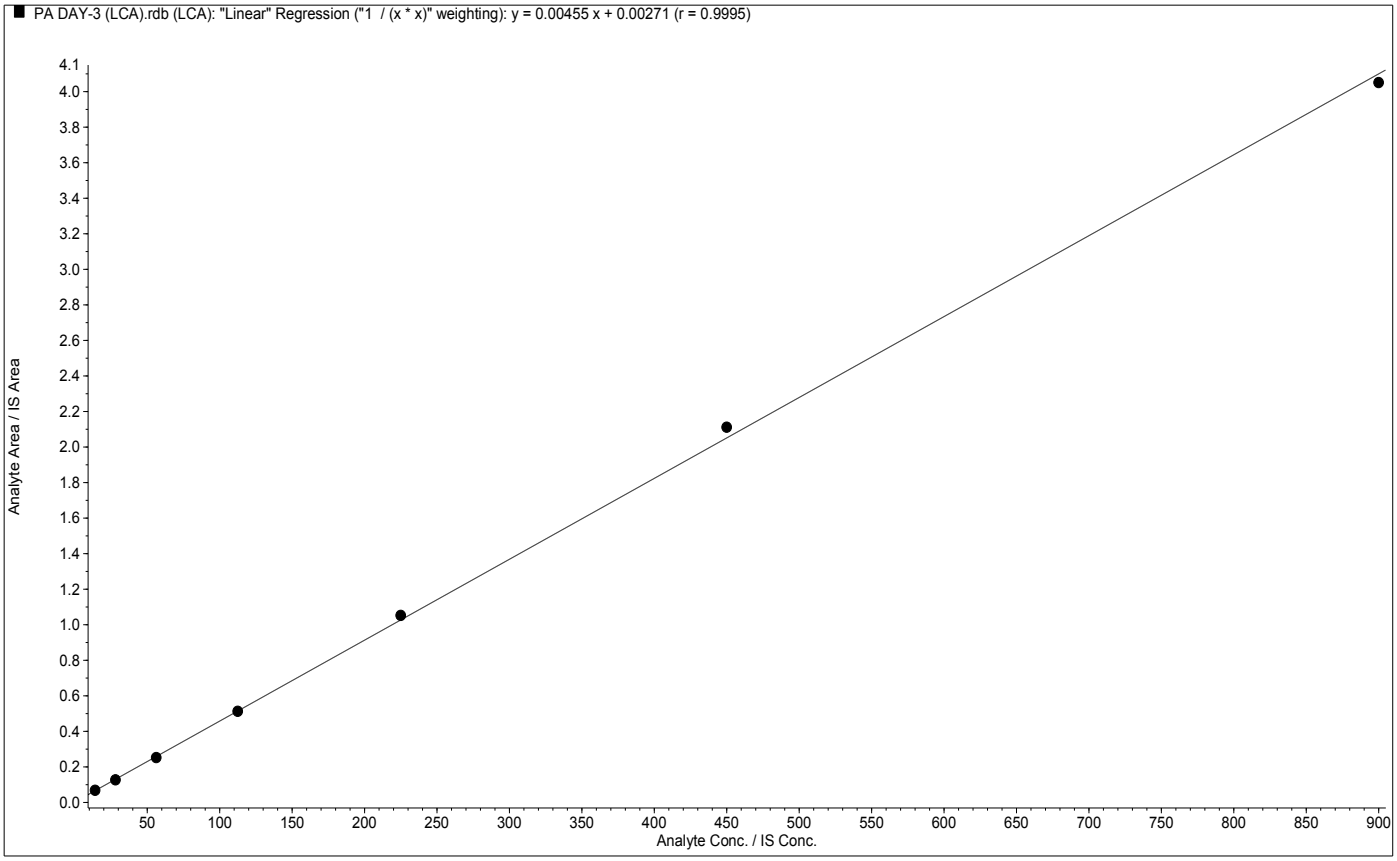


Figure: 44 Calibration Curve of Losartan Carboxylic Acid

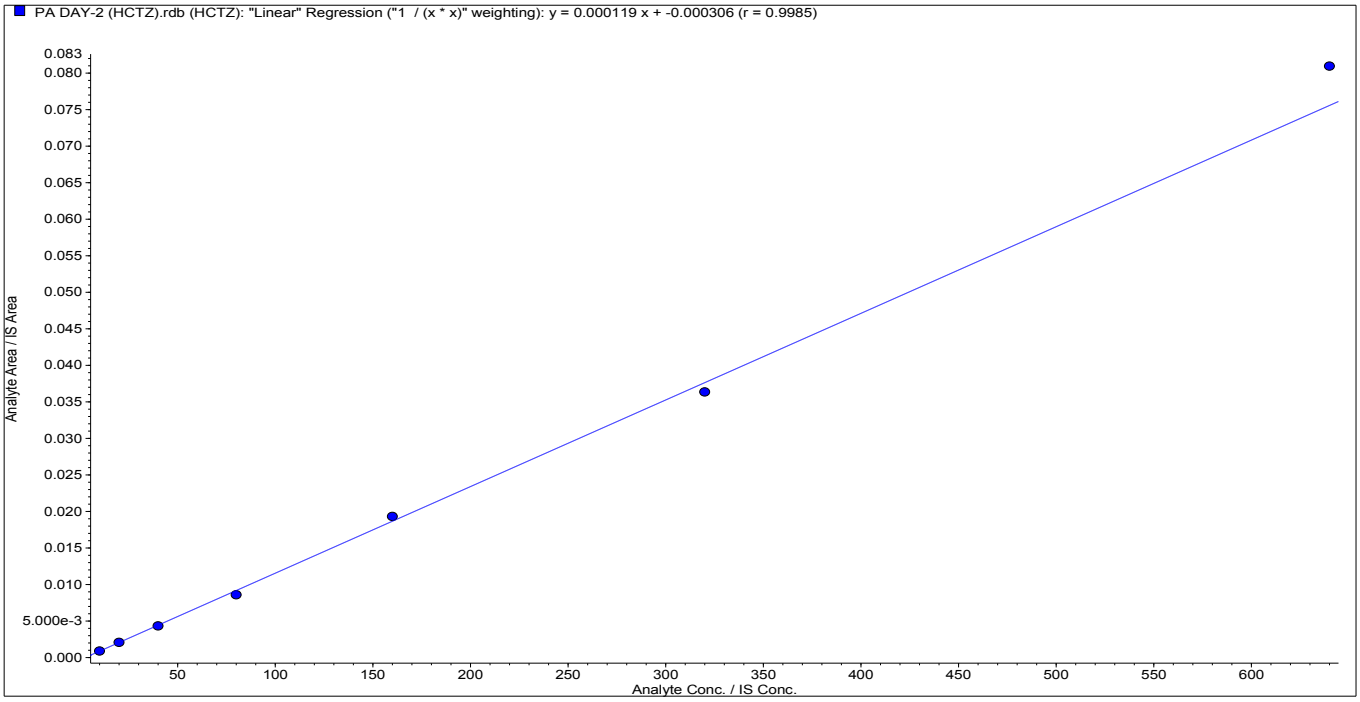


Figure: 45 Calibration Curve of Hydrochlorothiazide

Linearity	Calibration Concentration (ng/ml)								Calibration Curve statistics		
	9.37	18.75	37.5	75	150	300	600		Slope(M)	Intersept [C]	R square
Lin 1	9.39	17.5	41.09	80.21	155.29	275.52	567.62		5.72E-03	2.80E-03	0.9964
Lin 2	9.29	18.91	38.34	74.82	150.19	291.16	605.05		4.88E-03	8.58E-04	0.9998
Lin 3	9.33	18.64	38.57	74.73	152.8	298.76	582.47		6.06E-03	4.27E-03	0.9997
mean	9.337	18.350	39.333	76.587	152.760	288.480	585.047		**0.005553333**		**0.998633333**
±s.d.	0.050	0.748	1.526	3.138	2.550	11.850	18.848		**0.000607399**		**0.00193477**
% c.v.	0.539	4.078	3.879	4.098	1.669	4.108	3.222		**10.9376**		**0.193741739**
% nominal	99.64	97.87	104.89	102.12	101.84	96.16	97.51				

Table: 6 LCMS/MS Data Table of Pre-study linearity Value for Losartan

Linearity	**Calibration Concentration (ng/ml)**								Calibration Curve statistics		
	14.06	**28.12**	**56.25**	**112.5**	**225**	**450**	**900**		**Slope(M)**	**Intersept [C]**	**R square value**
Lin 1	13.38	30.26	57.35	111.38	236.94	436.02	871.67		5.05E-03	1.32E-02	0.9957
Lin 2	13.38	30.26	57.35	119.47	225.16	423.1	854.57		4.42E-03	3.02E-03	0.9977
Lin 3	14.35	27.25	54.75	111.99	230.52	463.14	889.15		4.55E-03	2.71E-03	0.9995
mean	13.703	29.257	56.483	114.280	230.873	440.753	871.797		**0.004673333**		**0.997633333**
±s.d.	0.560	1.738	1.501	4.505	5.898	20.435	17.290		**0.000332616**		**±0.00304**
% c.v.	4.087	5.940	2.658	3.942	2.555	4.636	1.983		**7.1173**		**0.305**
% nominal	97.463	104.042	100.415	101.582	102.610	97.945	96.866				

Table: 7 LCMS/MS Data Table of Prestudy linearity Value for Losartan Carboxylic Acid

Linearity	Calibration Concentration (ng/ml)								Calibration Curve statistics		
	10	**20**	**40**	**80**	**160**	**320**	**640**		Slope(M)	Intersept [C]	R square
Lin 1	10.08	20.16	40.46	71.15	150.67	328.4	713.64		0.00009	0.00001	0.9963
Lin 2	10.09	20.1	39.1	75.02	165.46	309.18	685.33		0.00012	0.00031	0.9985
Lin 3	10.68	17.18	41.8	72.28	165.63	324.83	687.91		0.00014	0.00002	0.9942
mean	**10.283**	**19.147**	**40.453**	**72.817**	**160.587**	**320.803**	**695.627**		**0.00013**		**0.9964**
±s.d.	**±0.344**	**±1.703**	**±1.350**	**±1.990**	**±8.589**	**±10.223**	**±15.653**		**±0.00002**		**±0.00304**
% c.v.	**3.341**	**8.897**	**3.337**	**2.733**	**5.348**	**3.187**	**2.250**		**12.955**		**0.305**
% nominal	**102.83**	**95.73**	**101.13**	**91.02**	**100.37**	**100.25**	**108.69**				

Table: 8 LCMS/MS Data Table of Prestudy linearity Value for Hydrochlorothiazide

4.7.3 Stability

In the present study the freeze thaw, short term (ST), long term (LT) and auto sampler (AS) stability had been performed as per the regulatory guidelines (US-FDA).As per guidelines the freeze thaw stability percentage should be within 80–115%. As per guidelines the both the ST and LT stability percentage should be within 90–110% and the AS stability percentage should be within 85–115%.

4.7.4 *Matrix effect and Recovery*

In the present study the matrix effect for the internal standard (Letrozole and Candesartan) and analytes (Losartan, Losartan Carboxylic Acid and Hydrochlorothiazide) were also carried out. The matrix effect percentage should be within 85–115% as per US-FDA guidelines.

The percentage recovery was determined by measuring the peak areas of the analyte and IS from the prepared plasma low, medium and high quality control samples. The peak areas of the plasma low, medium and high quality control samples were compared to the absolute peak area of the unextracted standards containing the same concentrations of the analytes and IS.

CHAPTER: 5

RESULTS & DISCUSSION

5. Results and discussion

5.1 Method validation

5.1.1 Specificity, selectivity and linearity

Following plasma calibration standards (ng/ml) were prepared- 50.00, 100.0, 200.0, 400.00, 800.00, 1600.00, 3200.00 for LCA 9.37, 18.75, 37.50, 75.00, 150.00, 300.00, 600.00.

for Losartan and 10, 20, 40, 80, 160, 320, 640. HCTZ respectively. The proposed assay was found linear. The representative calibration curves were showed as linearity graphs **[Figure: 43, 44, 45]**, Back calculated concentrations of the calibrant samples of the linearity's for Losartan. Losartan Carboxylic Acid and Hydrochlorothiazide were also represented in **Table-6, 7, 8**. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were found 4.50 ng/ml, 9.37 ng/ml respectively for Losartan, 1.5ng/ml and 50ng/ml respectively for LCA and for HCTZ it were 1.00ng/ml (LLOD) and 10ng/ml (LLOQ).

5.1.2 Precision and accuracy

Between – run precision values (%CV) ranged from 3.880% to 7.660% for Losartan, 4.592% to 6.583% for LCA and 5.123% to 7.839% for HCTZ. Between – run accuracy values (% nominal) were 103.145% for Losartan, 103.31% for LCA and 99.59% for HCTZ for low (LLOQ), 98.547% for Losartan , 103.14% for LCA and 99.25% for HCTZ for low QC (LQC), 94.174% for Losartan ,100.68% for LCA and 95.98% for HCTZ for medium QC (MQC) and 94.679% for Losartan , 100.99% for LCA and 102.22% for HCTZ for high QC (HQC) samples.

Within-run precision values (%CV) ranged from 3.441% to 6.103% for Losartan, 1.450% to 2.265% for LCA and 3.134% to 7.150% for HCTZ. Within-run accuracy values (% nominal) were 107.663% for Losartan, 99.29% for LCA and 107.12% for HCTZ for low (LLOQ), 98.073% for Losartan, 97.40% for LCA and 99.82% for HCTZ for low QC (LQC), 96.575% for Losartan, 95.67% for LCA and 95.28% for HCTZ for medium QC (MQC) and 97.167% for Losartan, 96.11% for LCA and 95.94% for HCTZ for high QC (HQC) samples. The between run and within run precision results were represented in Table-9, 10, 11.

	Between Run			Within run		
	Mean ± SD	C.V.%	Absolute bias (%)	Mean ± SD	C.V.%	Absolute bias (%)
LLOQ (9.37ng/ml)	9.665± 0.740	7.660	103.145	10.088±0.347	3.441	107.663
LQC(28.12ng/ml)	27.711± 1.486	5.363	98.547	27.578±1.683	6.103	98.073
MQC (225ng/ml)	211.892±8.222	3.880	94.174	217.294±8.719	4.013	96.575
HQC (450 ng/ml)	426.054±18.161	4.262	94.679	437.252±21.344	4.881	97.167

Table: 9 Between run and within run accuracy and precision of Losartan

	Between Run			Within run		
	Mean ± SD	C.V.%	Absolute bias (%)	Mean ± SD	C.V.%	Absolute bias (%)
LLOQ (14.06 ng/ml)	13.819± 1.143	8.271	98.288	14.636±1.330	9.086	104.097
LQC(42.18 ng/ml)	41.034± 1.189	2.898	97.283	41.360±1.597	3.861	98.056
MQC (337.5 ng/ml)	321.164± 16.819	5.237	95.160	338.146±11.525	3.408	100.191
HQC (675 ng/ml)	644.973± 20.581	3.191	95.552	662.918±17.250	2.602	98.210

Table: 10 Between run and within run accuracy and precision of Losartan Carboxylic Acid

	Between run			Within run		
	Mean ± SD	C.V.%	Absolute bias (%)	Mean ± SD	C.V.%	Absolute bias (%)
LLOQ(10ng/ml)	9.959±0.722	7.254	99.59	10.712±0.336	3.134	107.12
LQC(30ng/ml)	29.775±2.334	7.839	99.25	29.946±1.490	4.977	99.82
MQC (240ng/ml)	230.351±11.800	5.123	95.98	228.674±16.349	7.150	95.28
HQC(480ng/ml)	490.669±30.557	6.228	102.22	460.492±28.875	6.270	95.94

Table: 11 Between run and within run accuracy and precision of Hydrochlorothiazide

5.1.3 Stability

The stability study data were elaborated in Table-12, 13, 14,

Bench top: QC samples were kept for 24 hrs. at room temperature and then processed and analyzed. Percentage stability was found within 103.14% to 109.17% for Losartan, 92.98% to 97.58% for LCA and 91.95% to 107.03% for HCTZ.

Auto sampler stability: The auto sampler stability of Losartan ranged between 105.33% to 108.72%, LCA ranged between 94.92% to 97.80% and 90.31% to 104.33% for HCTZ.

Freeze thaw stability: The stability of low, medium and high quality control samples were determined after three cycles comparing against freshly thawed samples of the same concentration. The stability found for Losartan ranged between 105.20% to 107.72%, LCA ranged between 93.41% to 98.09% and 88.67% to 102.43% for HCTZ.

Short term stability: The percentage stability was found within 104.02% to 109.06% for Losartan, 92.18% to 98.19% for LCA and 93.17% to 103.00% for HCTZ.

Long term stability: The percentage stability range was found within 97.79% to 108.08% for Losartan, 91.16% to 92.86% for LCA and 95.00% to 102.46% for HCTZ.

		Inj No.	**LQC (28.12ng/ml)**	**MQC (225ng/ml)**	**HQC (450ng/ml)**
	Freshly thawed sample (PA Batch)	1	27.83	210.36	399.12
		2	26.32	203.66	447.84
		3	26.32	209.41	395.80
		4	29.61	220.54	397.01
		5	27.77	199.40	468.74
		Mean	**27.57**	**208.67**	**421.70**
Freeze thaw stability	After Three Freeze Thaw Cycle	1	24.44	225.66	460.64
		2	29.07	225.65	431.52
		3	29.57	224.08	433.35
		4	31.14	218	442.77
		5	31.86	230.48	449.96
		Mean	**29.22**	**224.77**	**443.65**
	% Stability		**105.97**	**107.72**	**105.20**
Short Term Stability	After 24 hours of freezing	1	30.34	226.86	439.04
		2	30.77	243.8	436.54
		3	29.92	221.49	418.38
		4	26.4	212.94	464.42
		5	31.38	232.8	434.8
		Mean	**29.76**	**227.58**	**438.64**
	% Stability		**107.95**	**109.06**	**104.02**
Long Term Stability	Long Term stability 7days of Freezing	1	24.82	223.33	431.66
		2	26.04	224.28	409.45
		3	30.28	235.39	411.42
		4	28.78	215.79	402.24
		5	29.57	228.92	407.23
		Mean	**27.90**	**225.54**	**412.40**
	% Stability		**101.19**	**108.08**	**97.79**

Bench Top Stability	Bench Top Stability after 24 hours	1	31.29	211.80	452.86
		2	29.16	227.79	429.09
		3	29.76	216.90	424.12
		4	31.16	229.52	439.53
		5	29.12	215.77	429.08
		Mean	**30.10**	**220.36**	**434.94**
	% Stability		**109.17**	**105.60**	**103.14**
Autosampler stability	Auto-sampler after 24 hours	1	29.94	228.69	437.58
		2	29.27	223.53	461.7
		3	30.16	229.65	412.45
		4	29.2	219.32	446.94
		5	28.03	233.11	462.19
		Mean	**29.32**	**226.86**	**444.17**
	% Stability		**106.35**	**108.72**	**105.33**

Table: 12 Freeze Thaw Stability, Short term stability (ST), Long term stability (LT), Bench Top Stability, and Auto sampler stability (AS) study data of Losartan

		Inj No.	**LQC (42.18 ng/ml)**	**MQC(337.5 ng/ml)**	**HQC (675ng/ml)**
	Freshly thawed sample (PA Batch)	1	41.40	311.44	605.99
		2	42.74	292.27	584.80
		3	41.06	323.08	613.83
		4	41.69	317.18	571.88
		5	40.88	296.41	679.59
		Mean	**41.55**	**308.08**	**611.22**
Freeze thaw stability	After Three Freeze Thaw Cycle	1	41.8	328.36	671.19
		2	39.64	308.07	637.26
		3	42.25	323.19	696.57
		4	45.73	327.38	662.23
		5	44.25	331.39	689.91
		Mean	**42.73**	**323.68**	**671.43**
	% Stability		**102.84**	**105.06**	**109.85**
Short Term Stability	After 24 hours of freezing	1	43.96	328.36	671.19
		2	44.96	308.07	637.26
		3	42.95	323.19	696.57
		4	44.86	327.38	662.23
		5	39.16	331.39	689.91
		Mean	**43.18**	**323.68**	**671.43**
	% Stability		**103.91**	**105.06**	**109.85**
Long Term Stability	Long Term stability after 7 days of Freezing	1	38.68	334.17	649.01
		2	38.26	354.13	631.66
		3	39.04	338.6	612.87
		4	40	304.26	624.36
		5	38.76	324.03	655.22
		Mean	**38.95**	**331.04**	**634.62**
	% Stability		**93.73**	**107.45**	**103.83**

Bench Top Stability	Bench Top Stability after 24 hours	1	41.86	309.49	653.15
		2	40.54	320.04	670.50
		3	41.85	347.99	618.18
		4	40.35	307.99	635.64
		5	41.64	327.71	639.33
		Mean	**41.25**	**322.64**	**643.36**
	% Stability		**99.26**	**104.73**	**105.26**
Autosampler stability	Auto-sampler after 24 hours	1	39.77	308.62	616.73
		2	39.9	339.73	652.86
		3	38.33	322.91	670.2
		4	38.44	309.22	617
		5	40.30	336.01	630.27
		Mean	**39.35**	**323.30**	**637.41**
	% Stability		**94.69**	**104.94**	**104.29**

Table: 13 Freeze Thaw Stability, Short term stability (ST), Long term stability (LT), Bench Top Stability, and Auto sampler stability (AS) study data of Losartan Carboxylic acid.

		Inj No.	LQC 30ng/ml	MQC 240ng/ml	HQC 480ng/ml
	Freshly thawed sample (PA Batch)	1	32.15	242.54	489.06
		2	26.44	240.18	489.62
		3	31.49	219.88	519.99
		4	33.03	214.91	516.10
		5	30.07	234.36	469.47
		Mean	**30.64**	**230.37**	**496.85**
Freeze thaw stability	After Three Freeze Thaw Cycle	1	26.11	232.42	470.29
		2	28.84	247.83	466.71
		3	27.44	237.91	487.74
		4	27.13	232.95	472.32
		5	26.30	228.73	473.21
		Mean	**27.16**	**235.97**	**474.05**
	% Stability		**88.67**	**102.43**	**95.41**
Short Term Stability	After 24 hours of freezing	1	27.18	237.55	491.09
		2	30.32	236.45	487.25
		3	29.07	242.38	495.72
		4	29.14	241.18	486.88
		5	27.01	228.81	532.55
		Mean	**28.54**	**237.27**	**498.70**
	% Stability		**93.17**	**103.00**	**100.37**
Long Term Stability	Long Term stability after 7 days of Freezing	1	28.58	260.75	464.28
		2	31.95	228.51	463.58
		3	29.76	230.28	476.98
		4	30.36	232.25	481.76
		5	28.00	228.47	473.45
		Mean	**29.73**	**236.05**	**472.01**
	% Stability		**97.04**	**102.46**	**95.00**

Bench Top Stability	Bench Top Stability after 24 hours	1	27.44	261.65	513.78
		2	31.14	240.89	501.09
		3	27.29	242.78	515.91
		4	28.05	248.21	504.19
		5	26.93	239.36	496.90
		Mean	**28.17**	**246.58**	**506.37**
	% Stability		**91.95**	**107.03**	**101.92**
Autosampler stability	Auto-sampler after 24 hours	1	26.88	259.11	483.09
		2	29.66	234.36	482.57
		3	27.9	236.63	477.9
		4	27.31	238.68	474.33
		5	26.58	232.94	532.08
		Mean	**27.67**	**240.34**	**489.99**
	% Stability		**90.31**	**104.33**	**98.62**

Table: 14 Freeze Thaw Stability, Short term stability (ST), Long term stability (LT), Bench Top Stability, and Auto sampler stability (AS) study data of Hydrochlorothiazide.

5.1.4 *Matrix effect and recovery*

The matrix effect of internal standard (letrozole) ranged between 87.32% - 89.71%, for Hydrochlorothiazide, it ranged between 88.47% - 89.86% and the matrix effect of internal standard (candesartan) ranged between 93.77% - 98.28%, same was found between 95.97 % - 96.78 % in case of Losartan and 95.41 % - 98.39 % in case of Losartan Carboxylic Acid (Table-6). The values were within the limit and hence accepted. (Table-15, 16, 17)

		Internal Standard (Area)			
Sample	Statistical Evaluation	Extracted Blank Plasma	Aqueous	Matrix Effect %	Matrix Factor
LQC (28.12ng/ml)	Mean ± SD	**19363.91±3129.96**	**20604.20±2829.71**	**93.77±2.54**	**0.94±0.03**
	C.V.%	**16.16**	**13.73**	**2.71**	**2.79**
MQC (**225**ng/ml)	Mean ± SD	**20752.79±1272.92**	**21308.87±1143.54**	**97.38±2.27**	**0.97±0.02**
	C.V.%	**6.13**	**5.37**	**2.33**	**2.13**
HQC (**450**ng/ml)	Mean ± SD	**22465.93±6293.66**	**22876.32±6494.99**	**98.28±1.94**	**0.98±0.02**
	C.V.%	**28.01**	**28.39**	**1.97**	**1.77**
		Analyte Area (LOSARTAN)			
LQC (28.12ng/ml)	Mean ± SD	**2867.71±215.58**	**2973.72±145.94**	**96.32±2.91**	**0.97±0.03**
	C.V.%	**7.52**	**4.91**	**3.02**	**2.71**
MQC (**225**ng/ml)	Mean ± SD	**24305.49±744.16**	**25325.22±472.52**	**95.97±1.97**	**0.96±0.02**
	C.V.%	**3.06**	**1.87**	**2.05**	**2.12**
HQC (**450**ng/ml)	Mean ± SD	**44341.95±1812.78**	**45818.19±941.11**	**96.78±3.30**	**0.97±0.03**
	C.V.%	**4.09**	**2.05**	**3.41**	**3.16**

Table: 15 Matrix effect of Candesartan (IS) and Losartan (Analyte)

		Internal Standard (Area)			
Sample	Statistical Evaluation	Extracted Blank Plasma	Aqueous	Matrix Effect %	Matrix Factor
LQC (42.18 ng/ml)	Mean ± SD	19363.91±3129.96	20604.20±2829.71	93.77±2.54	0.94±0.03
	C.V.%	16.16	13.73	2.71	2.79
MQC (**337.5** ng/ml)	Mean ± SD	20752.79±1272.92	21308.87±1143.54	97.38±2.27	0.97±0.02
	C.V.%	6.13	5.37	2.33	2.13
HQC (**675**ng/ml)	Mean ± SD	22465.93±6293.66	22876.32±6494.99	98.28±1.94	0.98±0.02
	C.V.%	28.01	28.39	1.97	1.77
		Analyte Area (LCA)			
LQC (42.18 ng/ml)	Mean ± SD	3396.09±380.47	3543.94±403.11	95.86±2.04	0.96±0.02
	C.V.%	11.20	11.37	2.13	2.08
MQC (**337.5** ng/ml)	Mean ± SD	23935.45±598.85	25601.66±507.21	93.49±1.16	0.94±0.01
	C.V.%	2.50	1.98	1.24	1.41
HQC (**675**ng/ml)	Mean ± SD	49325.61±4206.55	51379.53±4606.50	96.08±3.22	0.96±0.03
	C.V.%	8.53	8.97	3.36	3.45

Table: 16 Matrix effect of Candesartan (IS) and Losartan Carboxylic Acid (Analyte)

		Internal Standard (Area)			
Sample	Statistical Evaluation	Extracted Blank Plasma	Aqueous	Matrix Effect %	Matrix Factor
LQC(30 ng/ml)	Mean ± SD	**5082808.80±55056.72**	**5676762.05±273700.01**	**89.71±4.68**	**0.92±0.06**
	C.V.%	**1.08**	**4.82**	**5.21**	**6.34**
MQC(240 ng/ml)	Mean ± SD	**5216288.21±139457.99**	**5863613.25±184067.34**	**89.02±3.20**	**0.89±0.03**
	C.V.%	**2.67**	**3.14**	**3.60**	**3.60**
HQC(480 ng/ml)	Mean ± SD	**5138935.00±155776.93**	**5885501.23±196496.84**	**87.32±0.66**	**0.87±0.01**
	C.V.%	**3.03**	**3.34**	**0.76**	**0.76**
		Analyte Area (HCTZ)			
LQC(30 ng/ml)	Mean ± SD	**16620.13±826.79**	**18556.21±1254.08**	**89.73±4.57**	**0.92±0.06**
	C.V.%	**4.97**	**6.76**	**5.09**	**6.65**
MQC(240 ng/ml)	Mean ± SD	**139679.04±8826.88**	**155514.84±10962.27**	**89.86±1.73**	**0.90±0.02**
	C.V.%	**6.32**	**7.05**	**1.93**	**1.93**
HQC(480 ng/ml)	Mean ± SD	**281266.93±9624.33**	**318011.52±12822.39**	**88.47±1.23**	**0.88±0.01**
	C.V.%	**3.42**	**4.03**	**1.39**	**1.39**

Table: 17 Matrix effect of Letrozole (IS) and Hydrochlorothiazide (Analyte)

The percentage recoveries were determined by measuring the peak areas of the drug from the prepared low, medium and high quality control plasma samples. The peak areas of the low, medium and high quality control plasma samples were compared to the absolute peak area of the unextracted standards containing the same concentrations of the Losartan, Losartan Carboxylic Acid and Hydrochlorothiazide. Recovery after extraction was found 91.21% - 98.49% for Losartan, 92.04% - 97.52% for LCA and IS (candesartan) was 94.31% - 97.86% and 93.79% - 96.96% for HCTZ and IS (Letrozole) was 92.41% - 95.93 %(Table-18, 19, 20).

Recovery data.

Dilluent Sample (Area)			Plasma sample (Area)		
LQC (28.12ng/ml)	**MQC (225**ng/ml)	**HQC (450**ng/ml)	**LQC (**28.12ng/ml)	**MQC (225**ng/ml)	**HQC (450**ng/ml)
2975.75	23090.27	38945.50	2948.25	22149.21	39463.37
2924.66	25792.23	42224.31	2990.75	25223.03	39630.28
3082.44	24581.52	43971.23	2783.12	24091.51	40083.88
3175.76	24308.50	43434.01	2641.06	24856.40	37908.49
2819.35	24468.45	44274.75	2804.16	24078.84	37054.85
2995.59	24448.19	42569.96	2833.47	24079.80	38828.17
% Recovery			94.59	98.49	91.21

Table: 18 Recovery Data of Losartan (Analyte)

Recovery data.

Dilluent Sample			Plasma sample		
LQC (42.18 ng/ml)	**MQC (337.5 ng/ml)**	**HQC (675ng/ml)**	**LQC (42.18 ng/ml)**	**MQC (337.5 ng/ml)**	**HQC (675ng/ml)**
3620.09	25581.18	46097.34	3381.81	25235.97	44613.87
3340.85	25978.23	48901.98	3024.81	24705.12	48227.24
3085.17	28762.01	44618.57	2944.98	28028.01	43635.74
3420.50	27799.81	47164.97	3331.52	26451.46	43901.66
3353.32	27211.64	52778.96	3199.78	26742.50	41330.95
3363.99	27066.57	47912.36	3176.58	26232.61	44341.89
% Recovery			94.43	96.92	92.55

Table: 19 Recovery Data of Losartan Carboxylic Acid (Analyte)

Recovery data.

Dilluent Sample			Plasma sample		
LQC (30 ng/ml)	**MQC (240 ng/ml)**	**HQC (480 ng/ml)**	**LQC (30 ng/ml)**	**MQC (240 ng/ml)**	**HQC (480 ng/ml)**
19815.55	179865.91	325107.06	18524.22	152256.51	318889.78
21038.76	161688.21	310128.80	20066.44	156524.84	307522.50
20148.57	163034.80	343271.86	18576.77	157885.91	331446.45
19896.12	154261.72	316454.49	18582.04	149678.67	304438.05
18206.54	153545.65	326853.98	17639.58	145585.57	310279.95
19821.11	**162479.26**	**324363.24**	**18677.81**	**152386.30**	**314515.35**
% Recovery			**94.23**	**93.79**	**96.96**

Table: 20 Recovery Data of Hydrochlorothiazide (Analyte)

Dilluent Sample			Plasma sample		
LQC(30 ng/ml)	**MQC(240 ng/ml)**	**HQC(480 ng/ml)**	**LQC(30 ng/ml)**	**MQC(240 ng/ml)**	**HQC(480 ng/ml)**
21003.57	18278.07	17166.85	18864.94	18002.20	17430.01
18830.43	18808.63	16494.92	15608.43	18229.57	16226.14
18735.91	19360.19	16885.94	19575.88	19407.70	16260.58
18320.21	18713.25	16169.66	18944.24	18041.30	15877.65
19395.70	19918.97	18830.40	17811.99	19359.15	15466.32
19257.16	**19015.82**	**17109.55**	**18161.10**	**18607.98**	**16252.14**
% Recovery			**94.31**	**97.86**	**94.99**

Table: 21 Recovery Data of Losartan and Losartan Carboxylic Acid (IS)

Dilluent Sample			Plasma sample		
LQC(30 ng/ml)	**MQC(240 ng/ml)**	**HQC(480 ng/ml)**	**LQC(30 ng/ml)**	**MQC(240 ng/ml)**	**HQC(480 ng/ml)**
6053903.95	6226837.96	5943190.48	4694625.56	5910015.23	5872303.94
5897934.75	6079704.46	5776791.99	5596011.53	5843130.25	5488083.54
5909869.08	6080151.16	6199941.67	5712462.73	5837082.34	5982875.22
6000447.73	5837819.58	5849106.74	5658161.68	5642561.05	5585740.73
5607183.02	5773140.60	5960156.86	5572747.24	5500159.68	5590687.38
5893867.71	**5999530.75**	**5945837.55**	**5446801.75**	**5746589.71**	**5703938.16**
% Recovery			**92.41**	**95.78**	**95.93**

Table: 22 Recovery Data of Hydrochlorothiazide (IS)

5.2 Comparative pharmacokinetic study in human volunteers

The comparative pharmacokinetic study was carried out in 6 healthy human Indian volunteers under fasting condition with single dose and fixed dose administration. The volunteers were exposed in both the preparations *i.e.* single dose containing only losartan Potassium and fixed dose containing Losartan Potassium with Hydrochlorothiazide.

After oral administration of Losartan Potassium 50 mg tablet as single dose produced the maximum plasma concentration of 217.11 ± 50.10ng/ml (C_{max}) at the time 1.75± 0.75hr. (t_{max}) for Losartan and the average maximum plasma concentration of 6 volunteers is 207.93 ng/ml and Tmax 1.50hrs. and AUC_{0-t} 788.56 ng/ml at 48hr. and AUC $_{0-\alpha}$ 791.34 ng/ml at infinitive time, plasma half life of single dose losartan 5.21hr and elimination constant K_{el} value 0.13hr. After

administration of Losartan tablet 50mg only 14% losartan converted into its active metabolite Losartan Carboxylic acid in the liver by hepatic enzyme and acts on the Renin Angiotensin Aldesterone System. The maximum plasma concentration produced in the body is 462.45±62.22 ng/ml (C_{max}) at the time(t_{max}) 2.75±0.25 hr. and the average maximum plasma concentration of 6 volunteers is 458.74 ng/ml and t_{max} 2.67hrs. and AUC_{0-t} 2457.57 ng/ml at 48hr. and AUC $_{0-\alpha}$ 2468.56 ng/ml at infinitive time, plasma half life ($T_{½}$) of losartan carboxylic acid 5.71hr and elimination constant K_{el} value 0.12hr.

Whereas after administration of fixed dose combination drug in which losartan combined with hydrochlorothiazide (containing Losartan 50mg and Hydrochlorothiazide 12.5mg), it was found 49.63± 8.87 ng/ml (C_{max}) at the time 2.75± 0.25hr. (t_{max}) for HCTZ and the average maximum plasma concentration of 6 volunteers is 49.87 ng/ml and t_{max} 2.75hrs. and AUC_{0-t} 227.02 ng/ml at 48hr. and AUC $_{0-\alpha}$ 227.67 ng/ml at infinitive time, plasma half life of hydrochlorothiazide 4.91hr and elimination constant K_{el} value 0.14hr.The preparation of FDC tablet formulation containing Losartan 50mg and Hydrochlorothiazide 12.5mg drugs, produced the C_{max} of 530.635± 66.425ng/ml and 399.06± 73.29ng/ml at the time 2.50± 0.50hr. and 1.25.± 0.25 (t_{max}) for Losartan Carboxylic Acid and Losartan respectively. The average maximum plasma concentration of 6 volunteers is 543.56 ng/ml and t_{max} 2.42hrs. and AUC_{0-t} 1662.73 ng/ml at 48hr. and AUC $_{0-\alpha}$ 1666.18 ng/ml at infinitive time, plasma half life 5.35hr and elimination constant K_{el} value 0.13hr for Losartan Carboxylic Acid and the average maximum plasma concentration of 6 volunteers is 383.59 ng/ml and t_{max} 1.25hrs. and AUC_{0-t} 1467.90 ng/ml at 48hr. and AUC $_{0-\alpha}$ 1473.02 ng/ml at infinitive time, plasma half life 5.53hr and elimination constant K_{el} value 0.13hr for Losartan. (Table-8). The other pharmacokinetic parameters were also elaborated in the Table. Representative chromatograms of the volunteer plasma samples analysis was represented in Figure-35, 36, 37. The mean plasma concentration vs time profile for both the formulations was represented in Figure-38, 39, 40

Sample	Time(hr.)	Concentration (ng/ml)					
		V1	V2	V3	V4	V5	V6
S1	0	0	0	0	0	0	0
S2	0.25	46.11	91.34	29.28	111.45	31.62	29.24
S3	0.5	129.15	132.17	78.65	184.11	84.2	78.95
S4	1	194.32	178.68	94.32	267.21	110.17	88.59
S5	1.5	183.21	264.59	123.59	238.91	171.75	167.01
S6	2	141.86	223.42	166.68	210.68	135.17	142.64
S7	2.5	100.33	189.75	182.71	158.07	97.19	104.91
S8	3	82.91	151.05	102.14	113.56	69.94	85.49
S9	4	51.14	91.44	54.77	88.71	55.43	40.51
S10	6	28.2	46.63	34.64	56.1	29.36	22.71
S11	8	10.26	29.52	22.75	34.07	13.54	14.3
S12	10	5.11	17.57	11.76	20.25	9.44	10.81
S13	12	1.62	8.63	5.02	12.66	5.27	5.81
S14	24	0.71	4.02	2.83	5.85	1.78	1.56
S15	36	0.57	1.88	0.83	1.19	0.89	0.73
S16	48	0.2	0.5	0.32	0.56	0.45	0.14
	C_{max} (ng/ml)	**194.32**	**264.59**	**182.71**	**267.21**	**171.75**	**167.01**
	T_{max} (hr.)	**1**	**1.5**	**2.5**	**1**	**1.5**	**1.5**
	Auc_{0-t} (ng hr/ml)	**623.75**	**1072.72**	**698.5**	**1149.87**	**608.53**	**578**
	Auc_{0-a} (ng hr/ml)	**625.1**	**1076.61**	**701.05**	**1154.15**	**612.14**	**578.98**
	K_{el} ($hr.^{-1}$)	**0.148**	**0.129**	**0.126**	**0.131**	**0.125**	**0.143**
	$T_{½}$ (hr.)	**4.68**	**5.39**	**5.51**	**5.29**	**5.56**	**4.85**

Table: 23 Volunteers Plasma Concentration of Losartan (Single Dose)

	C_{max} (ng/ml)	T_{max} (hr)	Auc_{0-t}(ng. hr/ml)	Auc 0-t(ng. hr/ml)	K_{el}($hr.^{-1}$)	$T_{½}$ (hr)
Vol 1	194.32	1	623.75	625.1	0.148	4.68
Vol 2	264.59	1.5	1072.72	1076.61	0.129	5.39
Vol 3	182.71	2.5	698.5	701.05	0.126	5.51
Vol 4	267.21	1	1149.87	1154.15	0.131	5.29
Vol 5	171.75	1.5	608.53	612.14	0.125	5.56
Vol 6	167.01	1.5	578	578.98	0.143	4.85
Mean PK values	**207.93**	**1.50**	**788.56**	**791.34**	**0.13**	**5.21**

Table: 24 Volunteers Mean Plasma Concentration of Losartan (Single Dose)

Sample	Time(hr.)	Concentration (ng/ml)					
		V1	V2	V3	V4	V5	V6
S1	0	0	0	0	0	0	0
S2	0.25	103.7	164.91	156.69	172.6	98.81	160.26
S3	0.5	200.01	257.18	307.13	272.36	211.92	301.02
S4	1	249.48	374.51	385.68	335.39	272.03	380.4
S5	1.5	325.77	174.66	298.38	472.35	362.81	308.59
S6	2	305.73	158.29	205.34	301.68	358.42	218.47
S7	2.5	183.43	140.71	147.19	189.73	195.29	147.05
S8	3	138.56	103.03	100.91	149.48	153.5	104.05
S9	4	78.28	90.21	80.83	108.03	110.79	80.04
S10	6	35.57	62.64	64.46	78.52	81.59	61.11
S11	8	15.63	40.87	42.82	56.61	61.65	40.8
S12	10	10.13	30.11	27.72	39.47	43.55	30.9
S13	12	5.58	20.63	19.04	25.86	31.83	23.55
S14	24	1.12	10.35	4.85	12.62	10.58	9.66
S15	36	0.73	5.64	1.06	5.15	4.44	2.65
S16	48	0.15	0.85	0.56	0.99	0.48	0.64
	C_{max} (ng/ml)	**325.77**	**374.51**	**385.68**	**472.35**	**362.81**	**380.4**
	T_{max} (hr.)	**1.5**	**1**	**1**	**1.5**	**1.5**	**1**
	Auc $_{0-t}$ (ng hr/ml)	**1039.29**	**1382.97**	**1347.13**	**1818.42**	**1759.27**	**1460.33**
	Auc $_{0-a}$ (ng hr/ml)	**1040.24**	**1390.84**	**1351.27**	**1827.13**	**1763.07**	**1465.56**
	K_{el} ($hr.^{-1}$)	**0.158**	**0.108**	**0.135**	**0.114**	**0.126**	**0.122**
	$T_{½}$ (hr.)	**4.39**	**6.42**	**5.12**	**6.1**	**5.49**	**5.66**

Table: 25 Volunteers Plasma Concentration of Losartan (Fixed Dose)

	C_{max} (ng/ml)	**T_{max} (hr)**	**Auc $_{0-t}$(ng. hr/ml)**	**Auc 0-t(ng. hr/ml)**	**K_{el}($hr.^{-1}$)**	**$T_{½}$ (hr)**
Vol 1	325.77	1.5	1039.29	1040.24	0.158	4.39
Vol 2	374.51	1	1382.97	1390.84	0.108	6.42
Vol 3	385.68	1	1347.13	1351.27	0.135	5.12
Vol 4	472.35	1.5	1818.42	1827.13	0.114	6.1
Vol 5	362.81	1.5	1759.27	1763.07	0.126	5.49
Vol 6	380.4	1	1460.33	1465.56	0.122	5.66
Mean PK values	**383.59**	**1.25**	**1467.90**	**1473.02**	**0.13**	**5.53**

Table: 26 Volunteers Mean Plasma Concentration of Losartan (Fixed Dose)

Sample	Time(hr.)	Concentration (ng/ml)					
		V1	V2	V3	V4	V5	V6
S1	0	0	0	0	0	0	0
S2	0.25	44.88	93.55	43.95	43.07	99.04	89.01
S3	0.5	130.24	111.67	92.31	91.8	138.82	135.43
S4	1	217.13	149.42	127.18	169.9	180.41	180.02
S5	1.5	279.44	219.64	157.18	260.85	271.62	269.97
S6	2	369.48	322.95	232.3	352.79	346.35	394.07
S7	2.5	416.72	383.13	400.23	379.62	514.22	524.67
S8	3	365.01	458.22	250.21	438.4	451.93	476.83
S9	4	262.9	297.46	183.07	318.74	363.5	367.84
S10	6	167.31	201.16	98.1	208.39	203.36	207.46
S11	8	100.3	120.31	44.78	129.54	121.16	105.74
S12	10	45.97	58.19	26.18	63.69	58.88	48.94
S13	12	20.19	22.31	17.03	24.26	29.69	27.67
S14	24	10.61	8	8.79	14.83	15.75	18.12
S15	36	5.53	3.54	5.16	5.26	4.91	3.27
S16	48	1.2	0.8	1.54	2.1	1.39	0.78
	C_{max} (ng/ml)	**416.72**	**458.22**	**400.23**	**438.4**	**514.22**	**524.67**
	T_{max} (hr.)	**2.5**	**3**	**2.5**	**3**	**2.5**	**2.5**
	Auc_{0-t} (ng hr/ml)	**2338.92**	**2451.46**	**1600.28**	**2660.42**	**2848.21**	**2846.12**
	Auc_{0-a} (ng hr/ml)	**2348.89**	**2457.5**	**1614.65**	**2679.07**	**2859.37**	**2851.85**
	K_{el} ($hr.^{-1}$)	**0.12**	**0.132**	**0.107**	**0.113**	**0.125**	**0.136**
	$T_{½}$ (hr.)	**5.76**	**5.23**	**6.46**	**6.15**	**5.57**	**5.1**

Table: 27 Volunteers Plasma Concentration of LCA (Single Dose)

	C_{max} (ng/ml)	T_{max} (hr)	Auc_{0-t}(ng. hr/ml)	Auc 0-t(ng. hr/ml)	K_{el}($hr.^{-1}$)	$T_{½}$ (hr)
Vol 1	416.72	2.5	2338.92	2348.89	0.12	5.76
Vol 2	458.22	3	2451.46	2457.5	0.132	5.23
Vol 3	400.23	2.5	1600.28	1614.65	0.107	6.46
Vol 4	438.4	3	2660.42	2679.07	0.113	6.15
Vol 5	514.22	2.5	2848.21	2859.37	0.125	5.57
Vol 6	524.67	2.5	2846.12	2851.85	0.136	5.1
Mean PK values	**458.74**	**2.67**	**2457.57**	**2468.56**	**0.12**	**5.71**

Table: 28 Volunteers Mean Plasma Concentration of LCA (Single Dose)

Sample	Time(hr.)	Concentration (ng/ml)					
		V1	V2	V3	V4	V5	V6
	0	0	0	0	0	0	0
	0.25	22.63	34.52	24.77	23.3	36.95	25.2
	0.5	54.85	55.25	81.3	63.05	60.61	84.25
	1	102.92	104.08	175.01	113.84	114.66	172.87
	1.5	205.12	206.63	301.92	246.2	224.52	296.01
	2	329.63	597.06	425.93	464.21	365.22	373.54
	2.5	532.25	538.83	515.82	363.06	472.97	585.22
	3	316.06	445.48	395.41	304.71	566.79	392.79
	4	160.36	353.48	186.01	116.96	349.05	171.84
	6	22.56	56.77	27.37	24.06	62.85	36.84
	8	11.63	32.99	26.86	13.94	36.17	25.96
	10	9.21	22.74	12.22	9.69	25.21	12.42
	12	7.16	12.19	10.29	7.58	11.88	10.65
	24	5.43	5.96	6.79	5.55	6.53	7.01
	36	2.7	1.53	4.2	4.07	1.98	4.54
	48	0.7	0.53	0.42	0.17	0.38	0.44
	C_{max} (ng/ml)	532.25	597.06	515.82	464.21	566.79	585.22
	T_{max} (hr.)	2.5	2	2.5	2	3	2.5
	Auc_{0-t} (ng hr/ml)	1327.45	2020.37	1660.92	1292	2005.34	1670.3
	Auc_{0-a} (ng hr/ml)	1333.56	2023.99	1664.31	1293.24	2008.11	1673.85
	K_{el} ($hr.^{-1}$)	0.115	0.147	0.124	0.137	0.137	0.124
	$T_{½}$ (hr.)	6.05	4.73	5.6	5.05	5.06	5.6

Table: 29 Volunteers Plasma Concentration of LCA (Fixed Dose)

	C_{max} (ng/ml)	T_{max} (hr)	Auc_{0-t}(ng. hr/ml)	Auc 0-t(ng. hr/ml)	K_{el}($hr.^{-1}$)	$T_{½}$ (hr)
Vol 1	532.25	2.5	1327.45	1333.56	0.115	6.05
Vol 2	597.06	2	2020.37	2023.99	0.147	4.73
Vol 3	515.82	2.5	1660.92	1664.31	0.124	5.6
Vol 4	464.21	2	1292	1293.24	0.137	5.05
Vol 5	566.79	3	2005.34	2008.11	0.137	5.06
Vol 6	585.22	2.5	1670.3	1673.85	0.124	5.6
Mean PK values	**543.56**	**2.42**	**1662.73**	**1666.18**	**0.13**	**5.35**

Table: 30 Volunteers Mean Plasma Concentration of LCA (Fixed Dose)

S1	**0**	0	0	0	0	0	0
S2	**0.25**	6.35	10.33	10.83	5.95	10.21	10.77
S3	**0.5**	10.1	19.68	23.17	10.45	19.3	23.83
S4	**1**	20.23	29.37	27.64	20.4	27.99	29.2
S5	**1.5**	28.04	33.51	34.38	27.23	34.31	35.48
S6	**2**	34.19	44.36	42.15	32.96	45.29	44.41
S7	**2.5**	38.57	50.51	53.3	38.04	55.13	54.78
S8	**3**	41.37	37.13	33.17	40.76	58.5	33.73
S9	**4**	24.3	22.65	26.86	30.44	32.94	27.51
S10	**6**	10.27	12.5	17.32	17.49	20.88	16.97
S11	**8**	5.61	7.64	9.52	10.74	10.22	10.3
S12	**10**	1.48	4.02	2.27	4.54	5.02	4.66
S13	**12**	0.57	1.38	0.82	1.08	1.01	1.45
S14	**24**	0.25	0.51	0.25	0.72	0.71	0.54
S15	**36**	0.08	0.14	0.08	0.19	0.29	0.16
S16	**48**	0.04	0.07	0.27	0.05	0.06	0.04
	C_{max} (ng/ml)	**41.37**	**50.51**	**53.3**	**40.76**	**58.5**	**54.78**
	T_{max} (hr.)	**3**	**2.5**	**2.5**	**3**	**3**	**2.5**
	Auc $_{0-t}$ (ng hr/ml)	**176.28**	**216.86**	**224.84**	**225.27**	**276.11**	**242.78**
	Auc $_{0-a}$ (ng hr/ml)	**176.55**	**217.35**	**226.91**	**225.62**	**276.54**	**243.04**
	K_{el} ($hr.^{-1}$)	**0.145**	**0.142**	**0.13**	**0.142**	**0.139**	**0.152**
	$T_{½}$ (hr.)	**4.78**	**4.89**	**5.33**	**4.89**	**5**	**4.56**

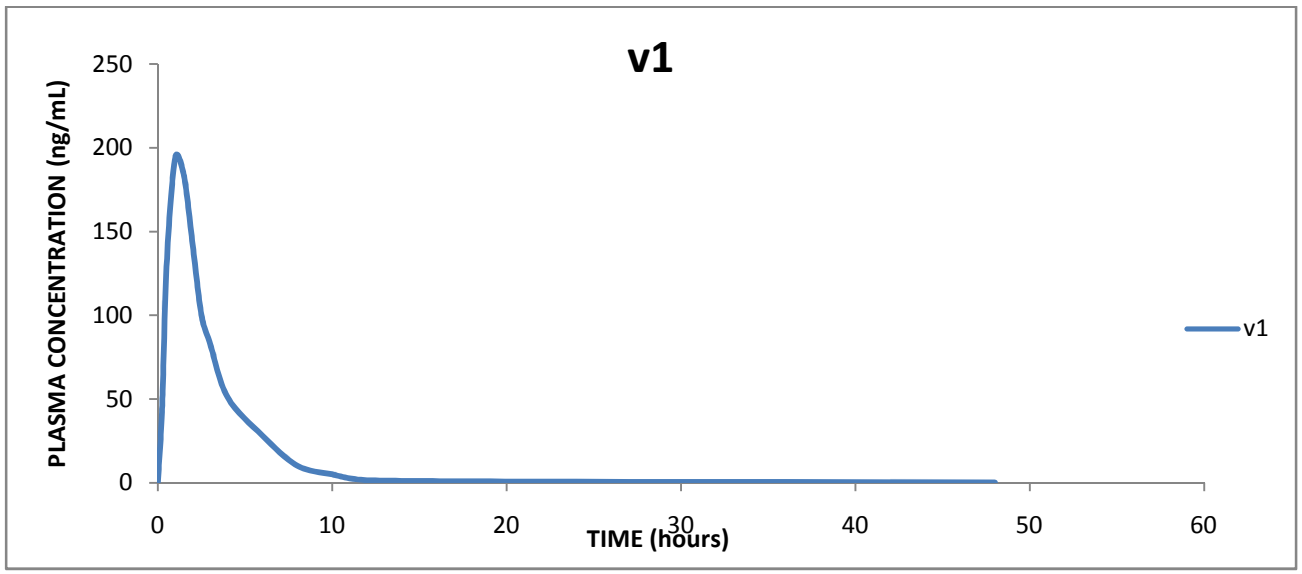
Table: 31 Volunteers Plasma Concentration of Hydrochlorothiazide (Fixed Dose)

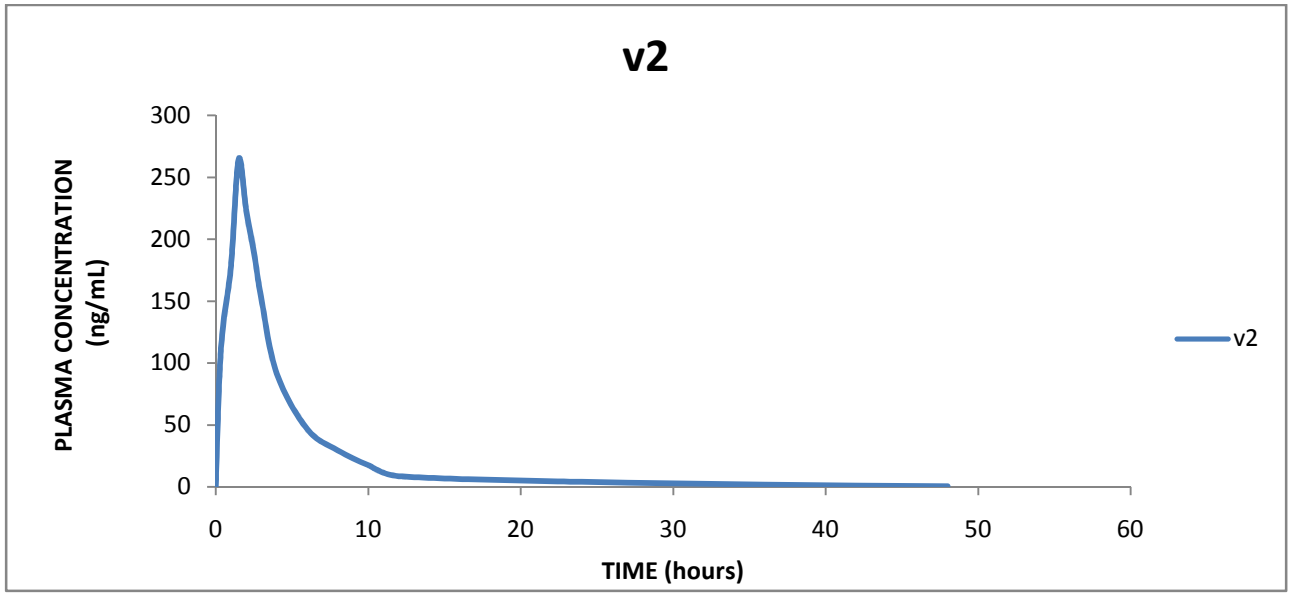
	C_{max} (ng/ml)	**T_{max} (hr)**	**Auc $_{0-t}$(ng. hr/ml)**	**Auc 0-t(ng. hr/ml)**	**K_{el}($hr.^{-1}$)**	**$T_{½}$ (hr)**
Vol 1	41.37	3	176.28	176.55	0.145	4.78
Vol 2	50.51	2.5	216.86	217.35	0.142	4.89
Vol 3	53.3	2.5	224.84	226.91	0.13	5.33
Vol 4	40.76	3	225.27	225.62	0.142	4.89
Vol 5	58.5	3	276.11	276.54	0.139	5
Vol 6	54.78	2.5	242.78	243.04	0.152	4.56
Mean PK values	**49.87**	**2.75**	**227.02**	**227.67**	**0.14**	**4.91**

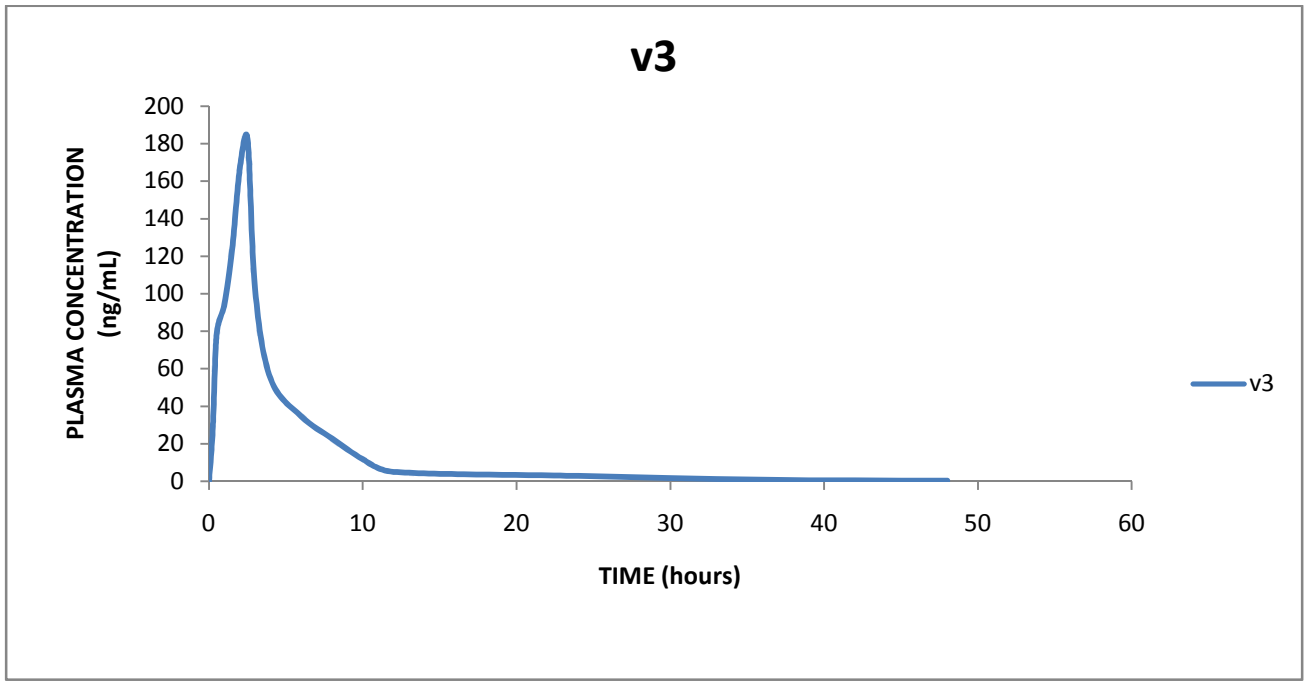
Table: 32 Volunteers Mean Plasma Concentration of Hydrochlorothiazide (Fixed Dose)

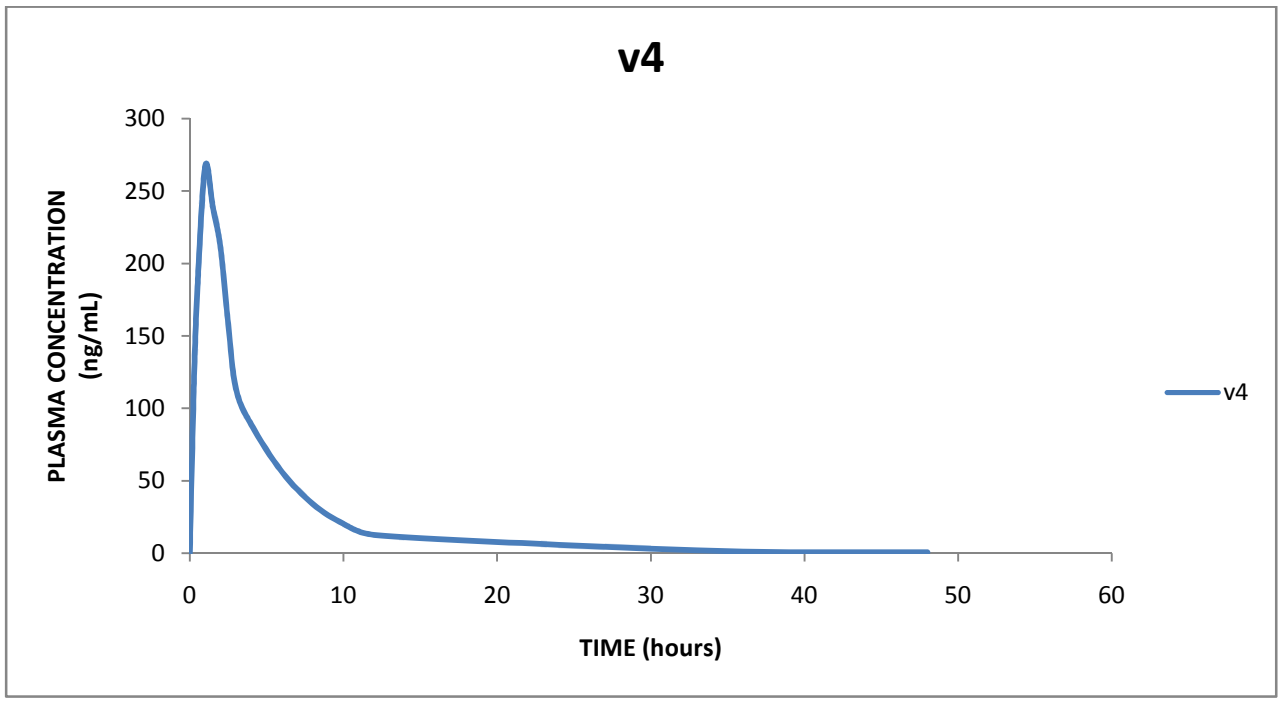
Drug / Mean PK	Losartan single dose	Losartan fixed dose	LCA single dose	LCA fixed dose	HCTZ fixed dose
C_{max} (ng/ml)	207.93	383.59	458.74	543.56	49.87
T_{max} (hr)	1.50	1.25	2.67	2.42	2.75
Auc $_{0-t}$ (ng. hr/ml)	788.56	1467.90	2457.57	1662.73	227.02
Auc 0-t(ng. hr/ml)	791.34	1473.02	2468.56	1666.18	227.67
K_{el} ($hr.^{-1}$)	0.13	0.13	0.12	0.13	0.14
$T_{½}$ (hr)	5.21	5.53	5.71	5.35	4.91

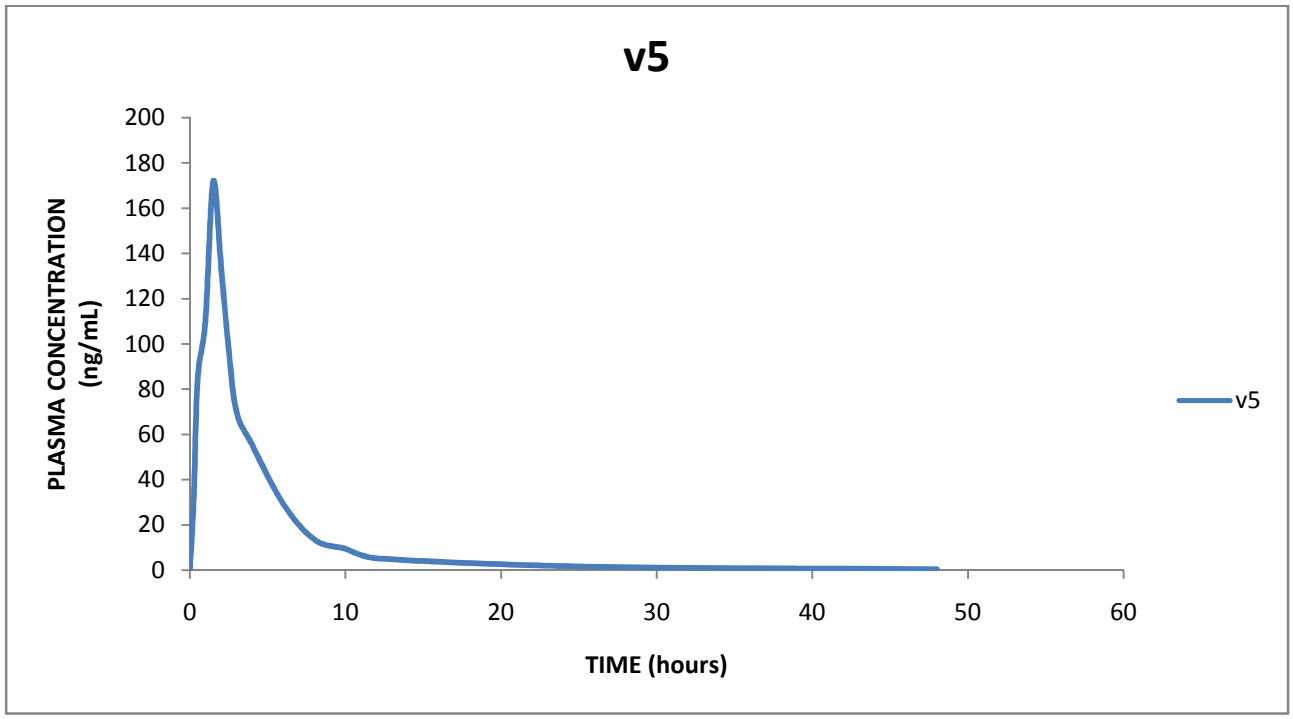
Table: 33 Comparative studies of Pharmacokinetic parameters

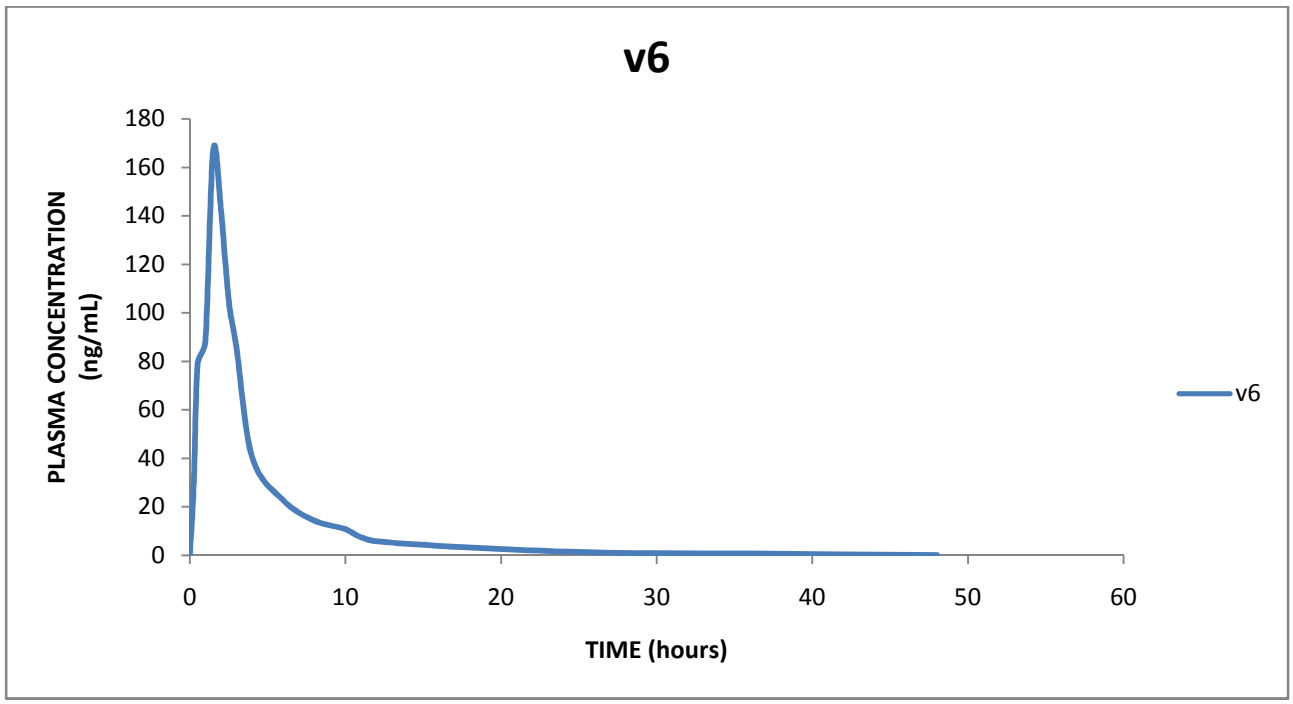





v3
PLASMA CONCENTRATION (ng/mL)
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
TIME (hours)
v3


v4
PLASMA CONCENTRATION (ng/mL)
300
250
200
150
100
50
0
0
10
20
30
40
50
60
TIME (hours)
v4


v5
PLASMA CONCENTRATION (ng/mL)
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
TIME (hours)
v5


v6
PLASMA CONCENTRATION (ng/mL)
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
TIME (hours)
v6

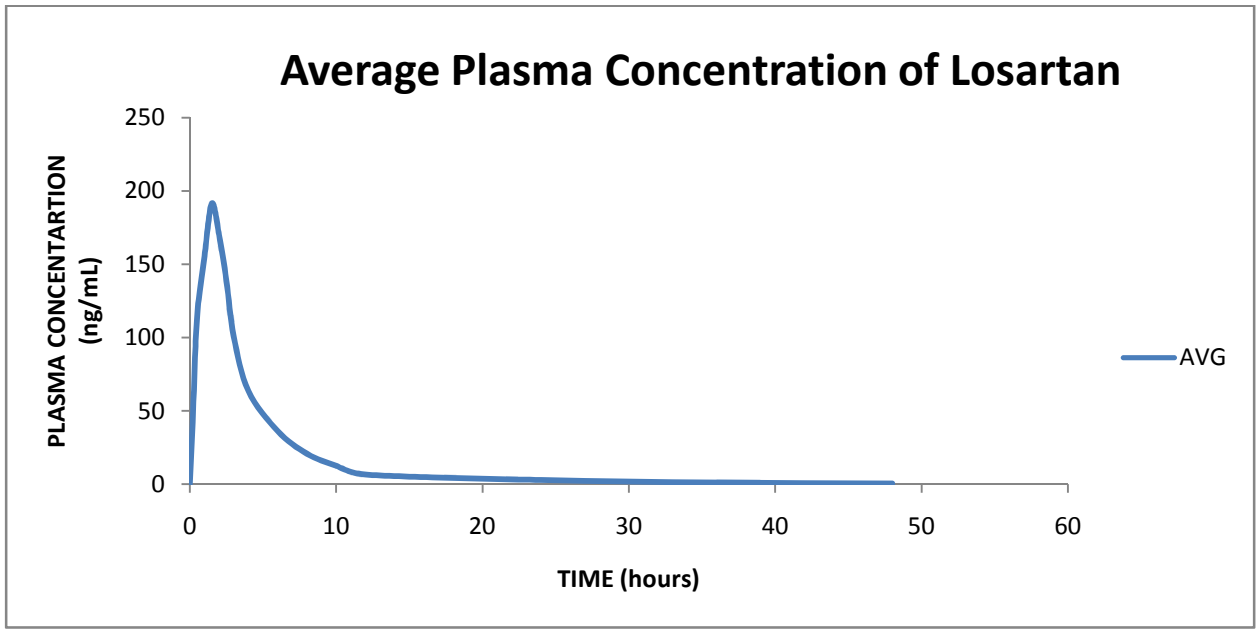
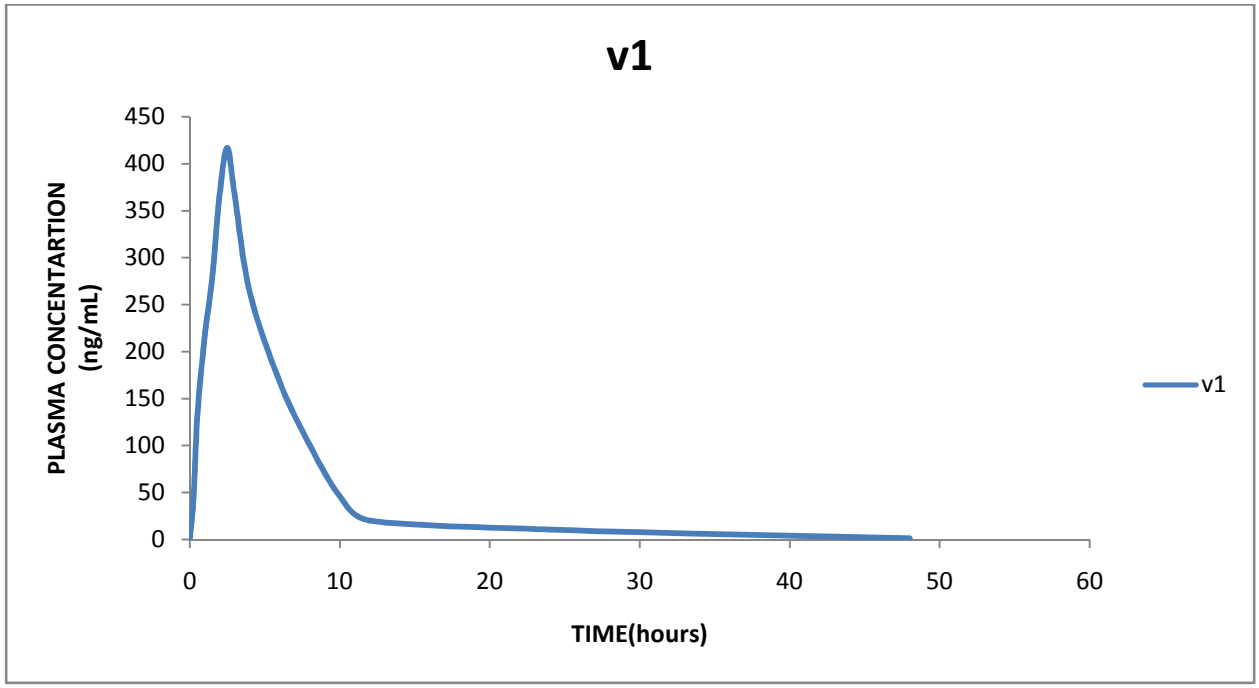
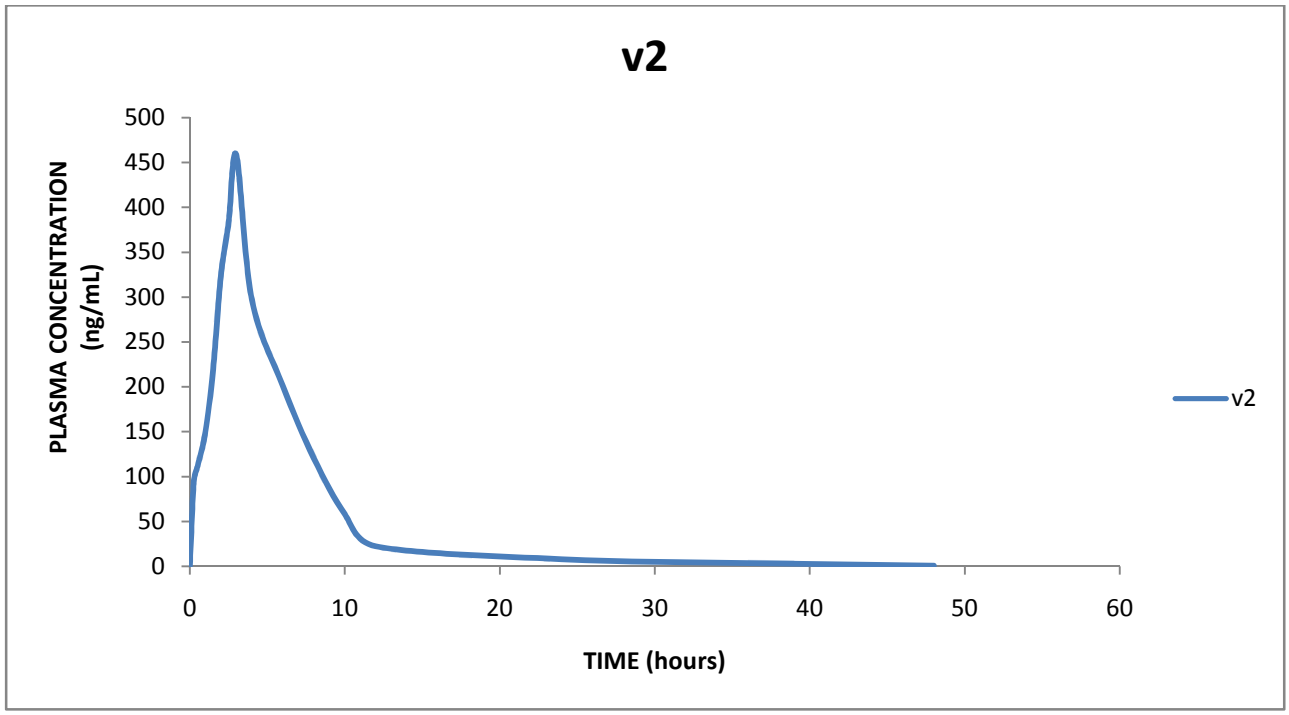
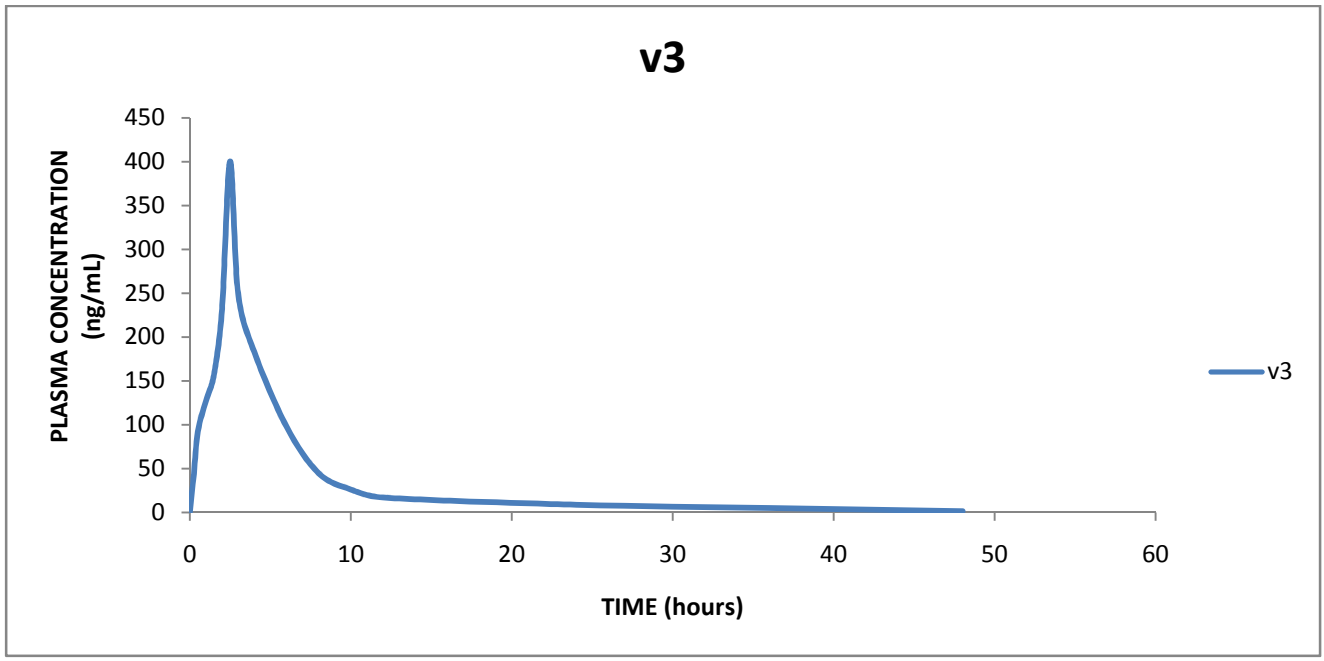
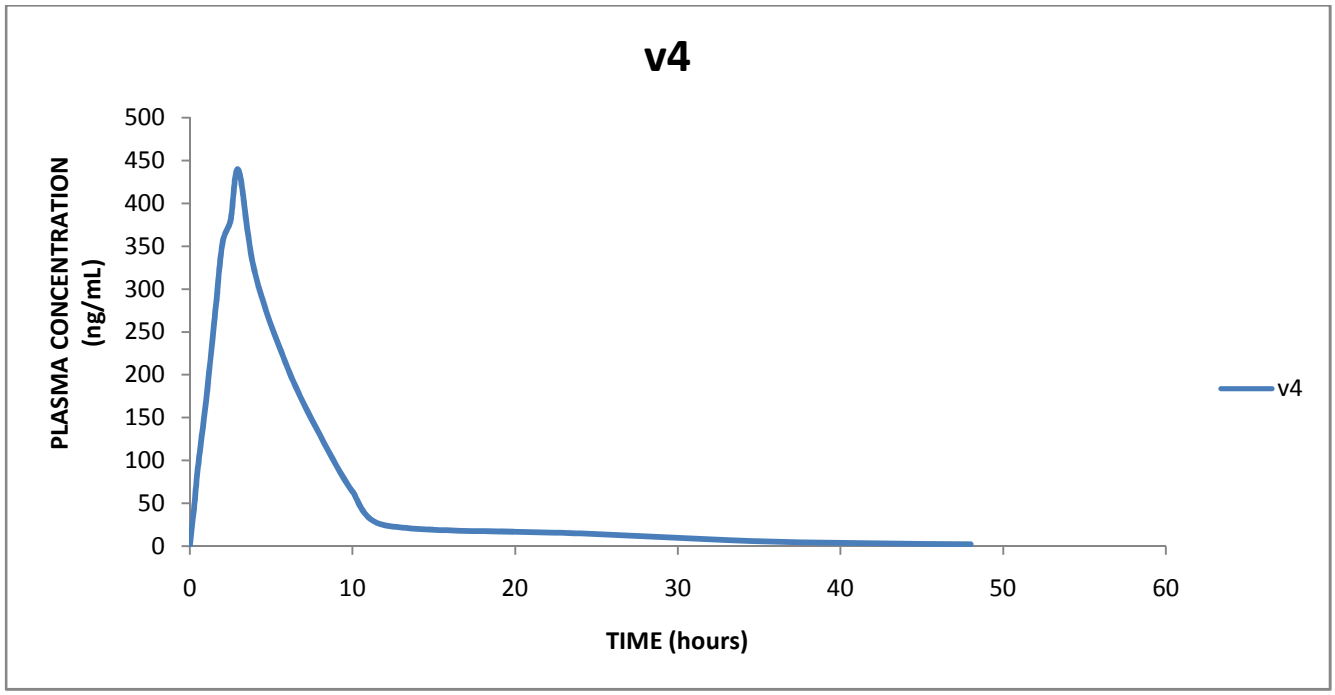


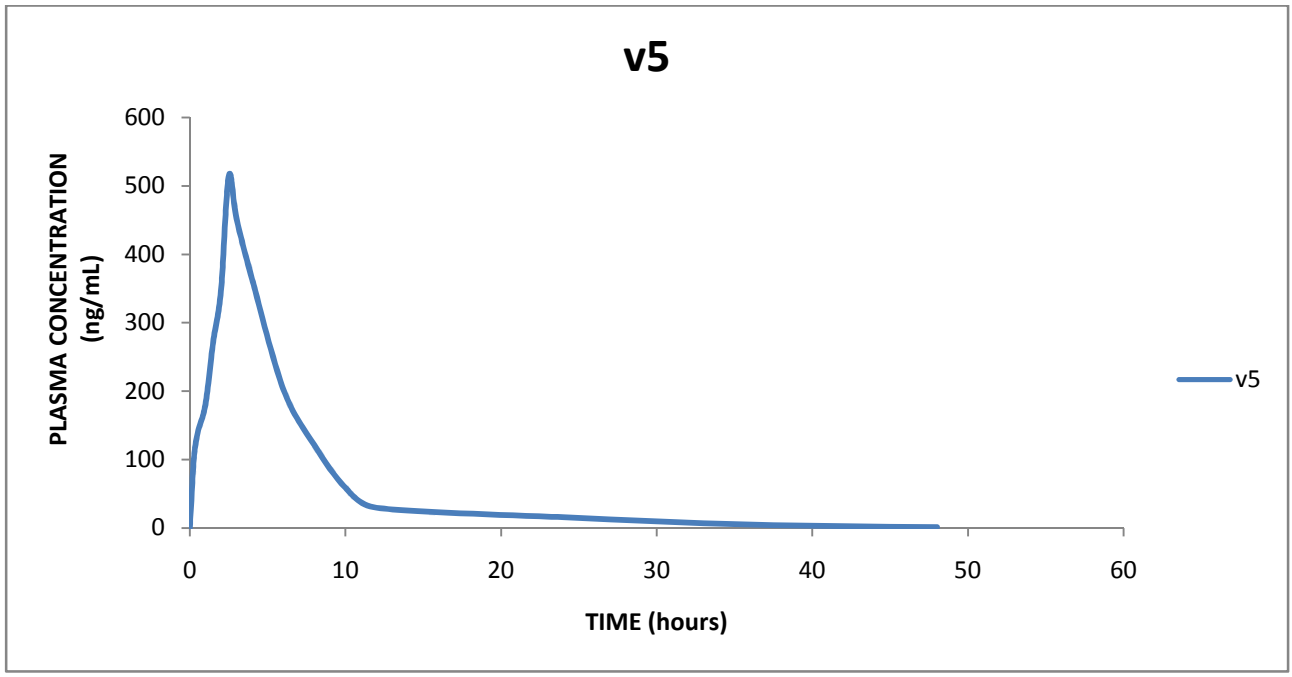
Figure-46 Individual Volunteer Plasma Concentration Curve and Average Plasma Concentration Curve of Losartan as a single Dose

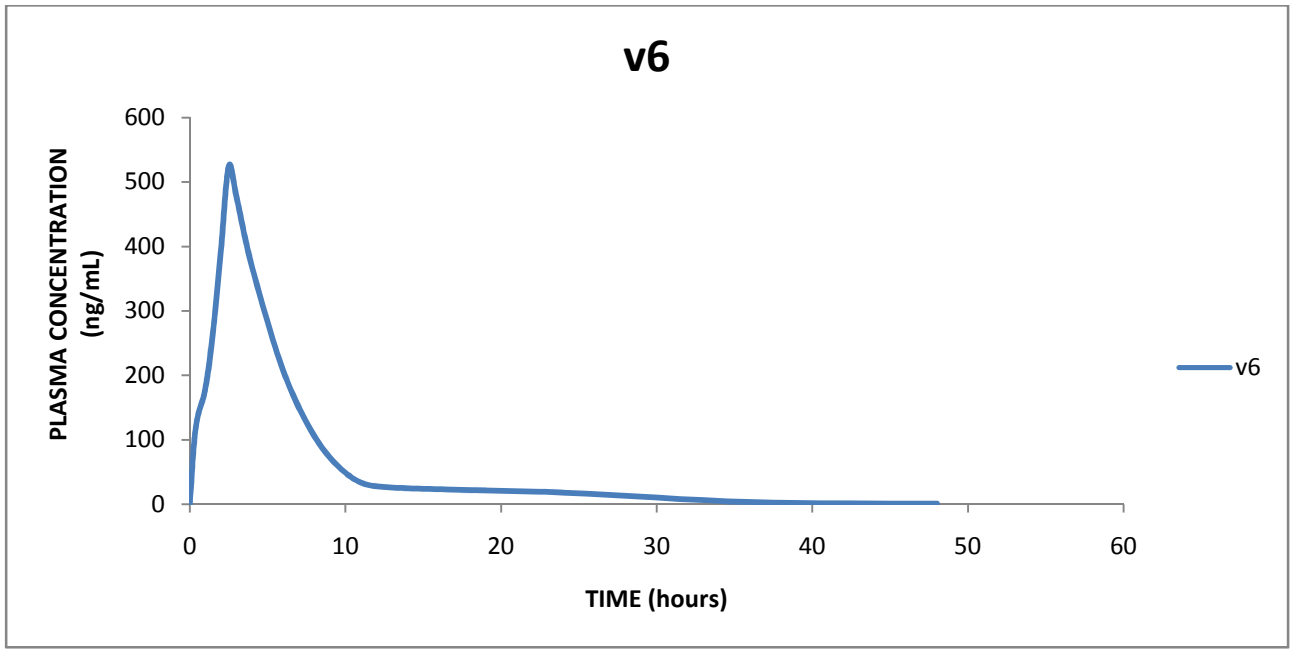



v2
PLASMA CONCENTRATION (ng/mL)
500
450
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
TIME (hours)
v2


v3
PLASMA CONCENTRATION (ng/mL)
450
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
TIME (hours)
v3


v4
PLASMA CONCENTRATION (ng/mL)
500
450
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
TIME (hours)
v4


v5
PLASMA CONCENTRATION (ng/mL)
600
500
400
300
200
100
0
0
10
20
30
40
50
60
TIME (hours)
v5



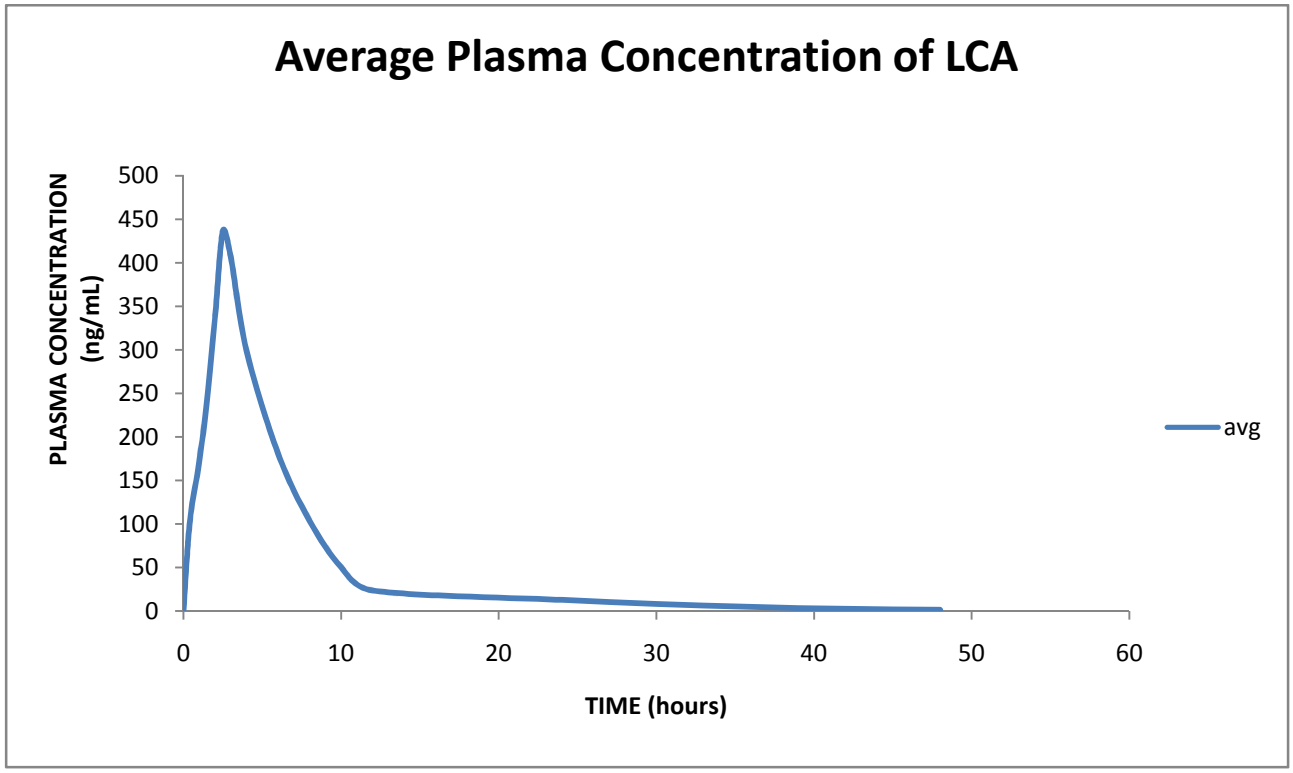
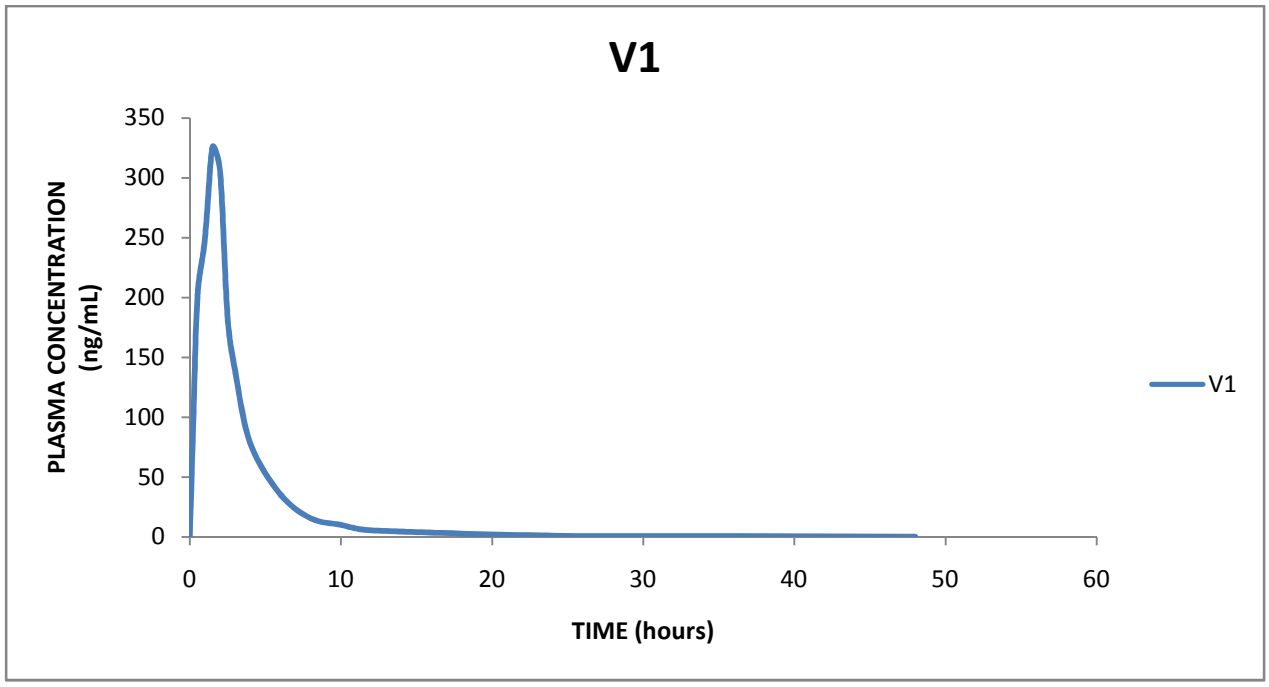
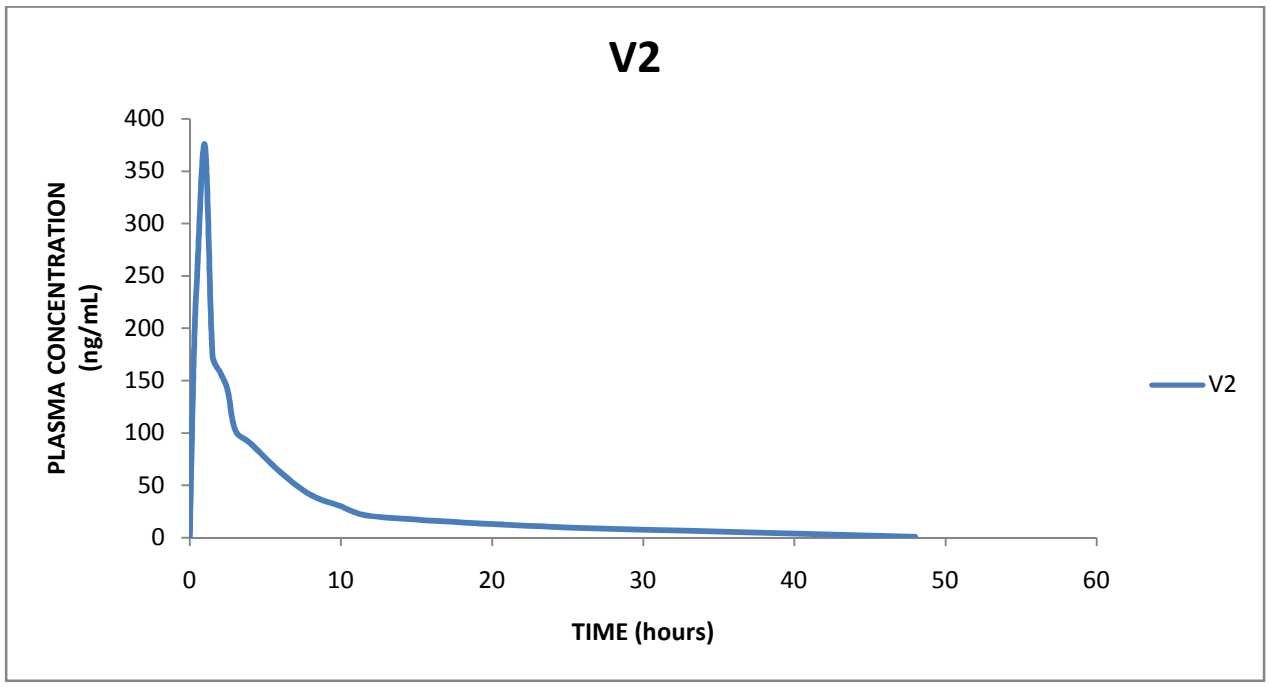
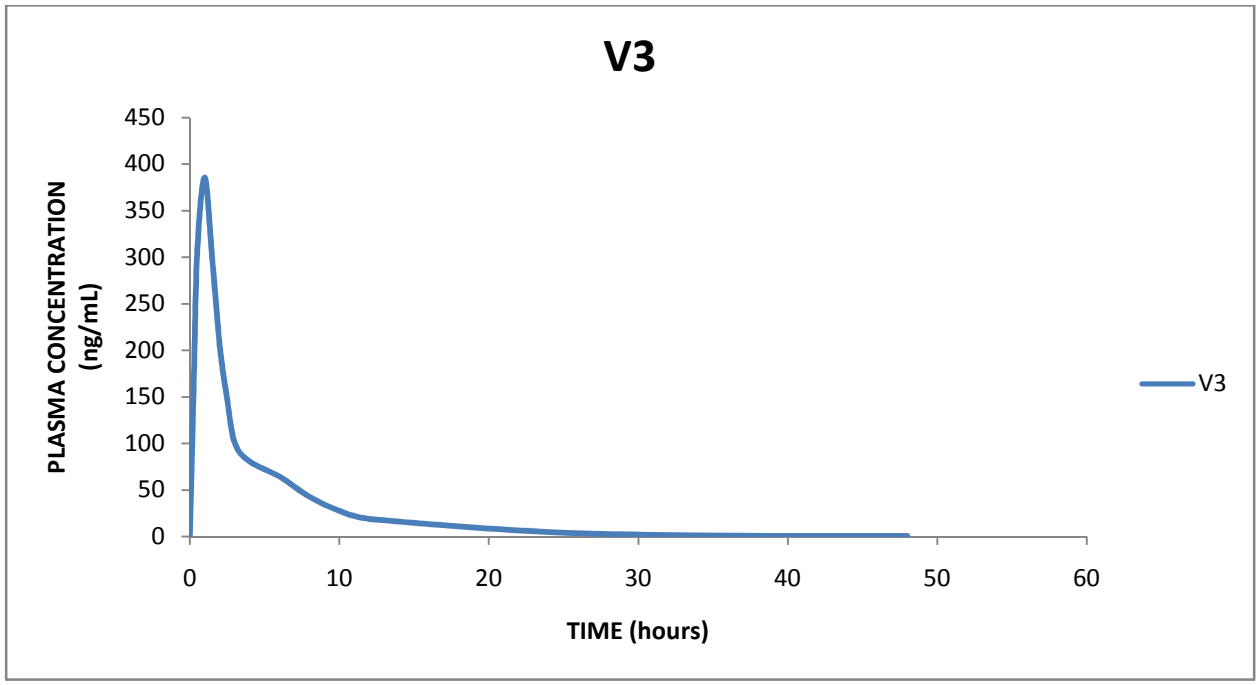
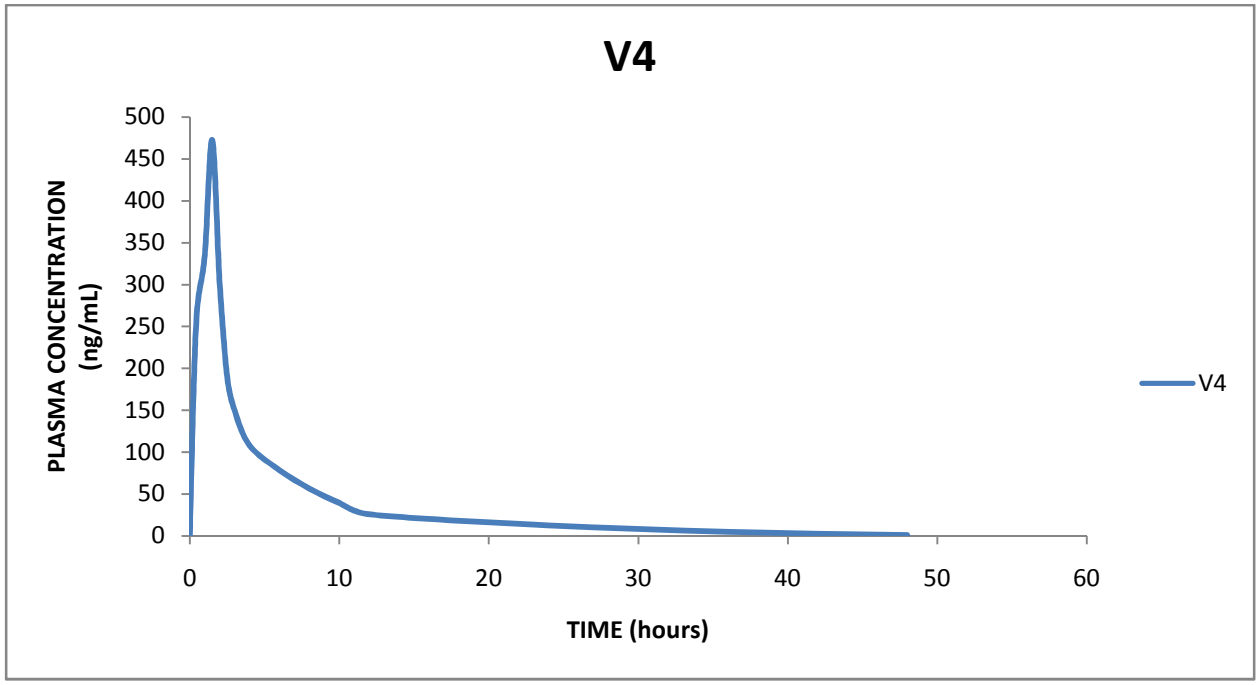


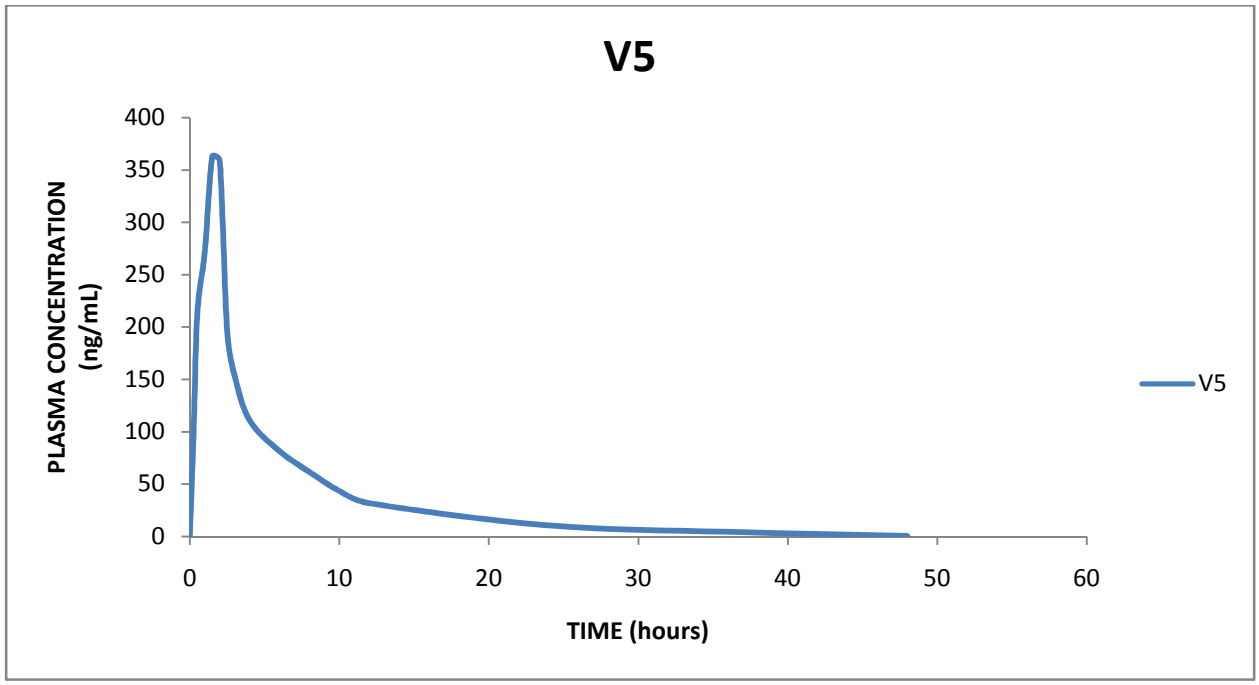
Figure-47 Individual Volunteer Plasma Concentration Curve and Average Plasma Concentration Curve of Losartan Carboxylic Acid as a single Dose

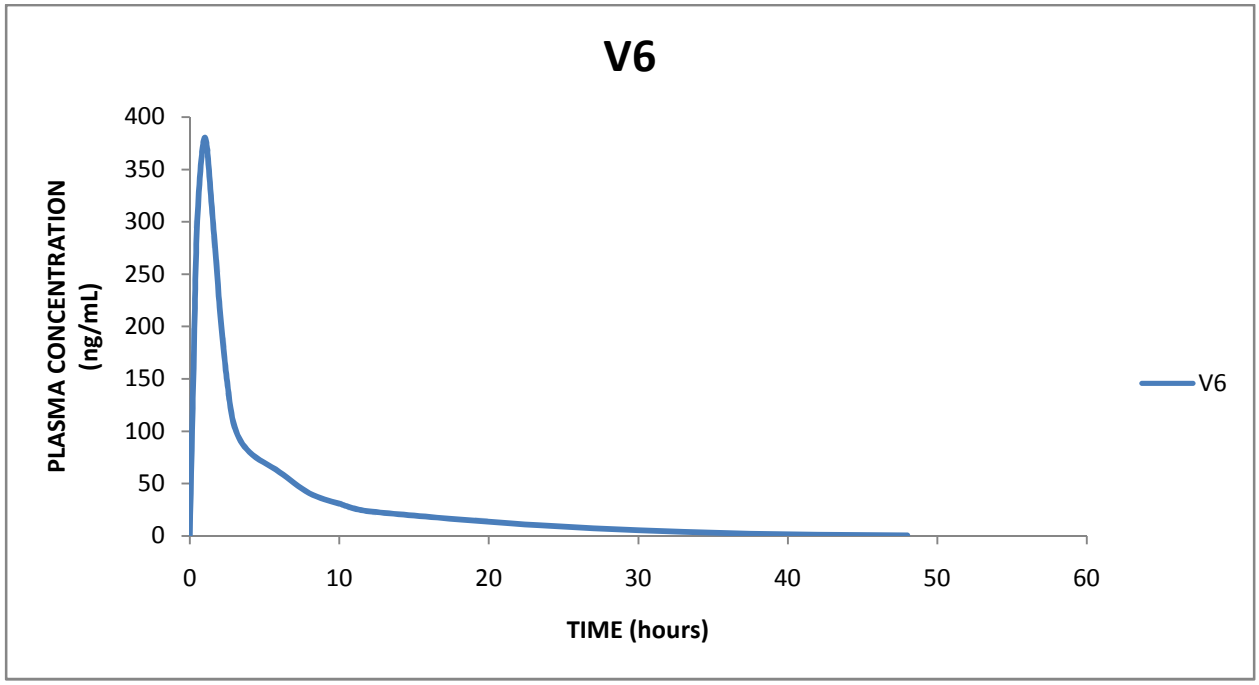

V1
PLASMA CONCENTRATION (ng/mL)
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
TIME (hours)
V1


V2
PLASMA CONCENTRATION (ng/mL)
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
TIME (hours)
V2


V3
PLASMA CONCENTRATION (ng/mL)
450
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
TIME (hours)
V3


V4
PLASMA CONCENTRATION (ng/mL)
500
450
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
TIME (hours)
V4


V5
PLASMA CONCENTRATION (ng/mL)
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
TIME (hours)
V5


V6
PLASMA CONCENTRATION (ng/mL)
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
TIME (hours)
V6

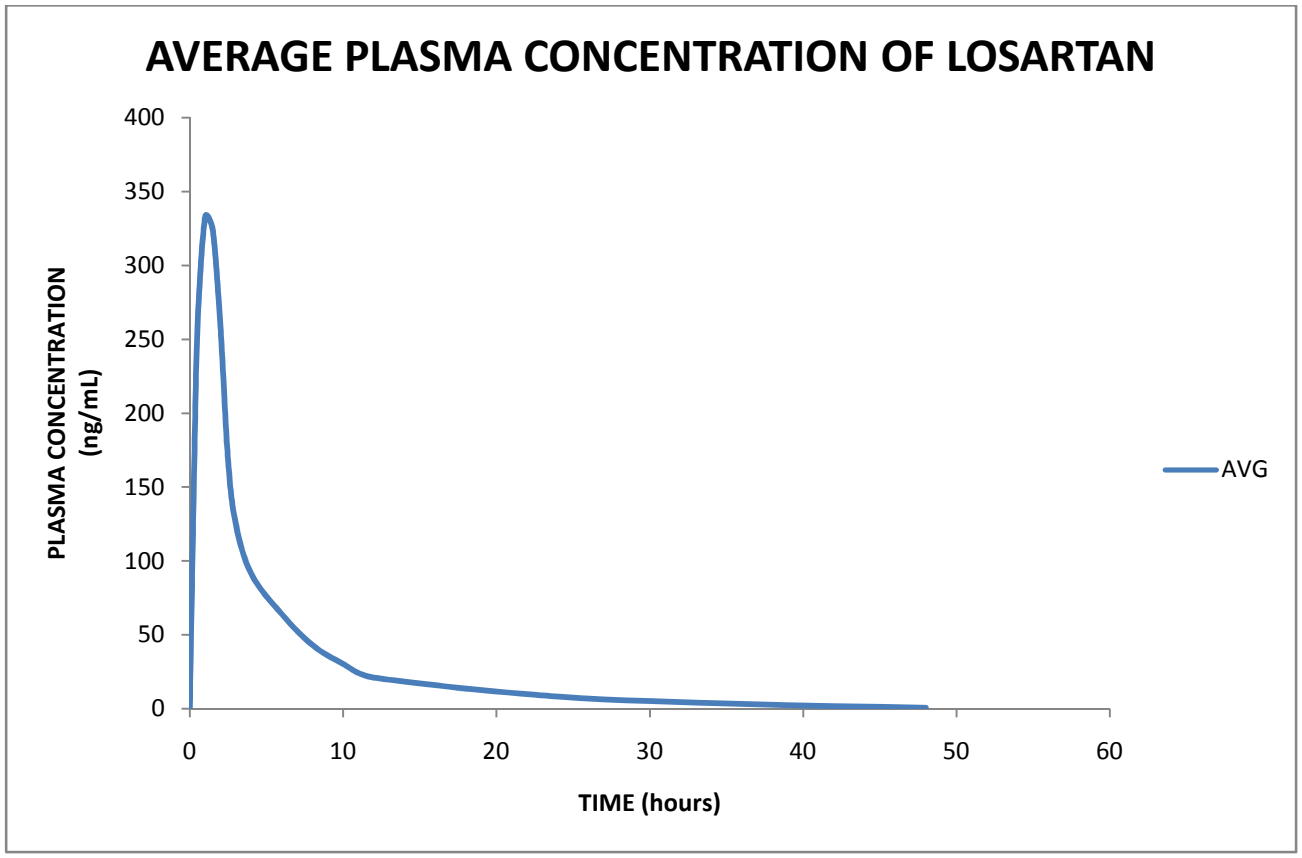
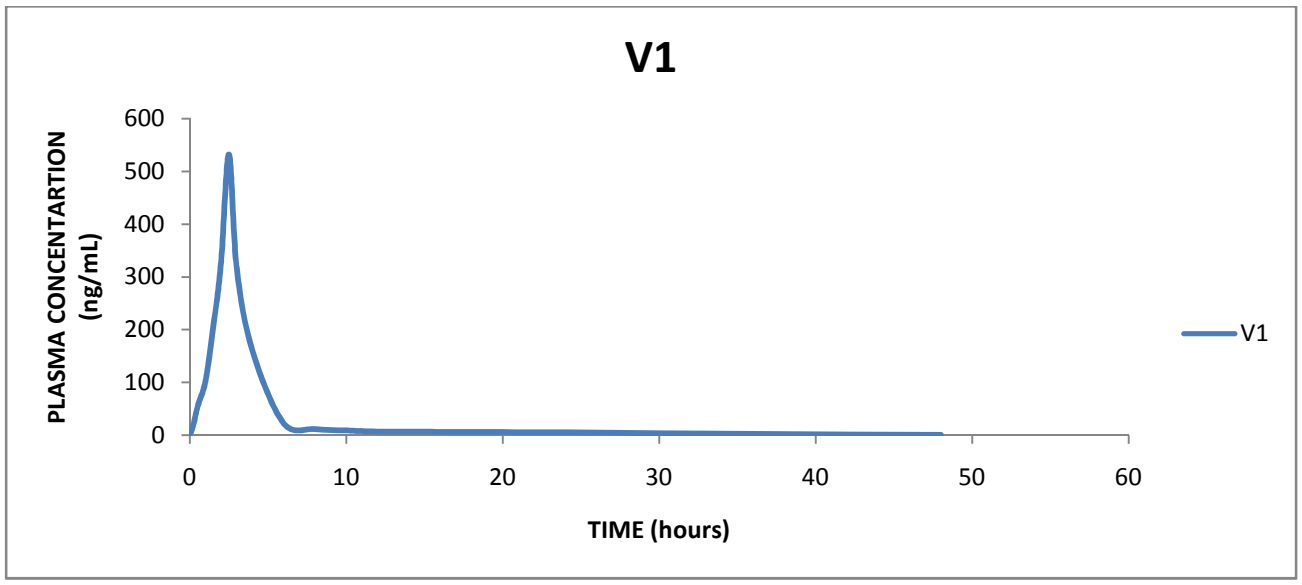
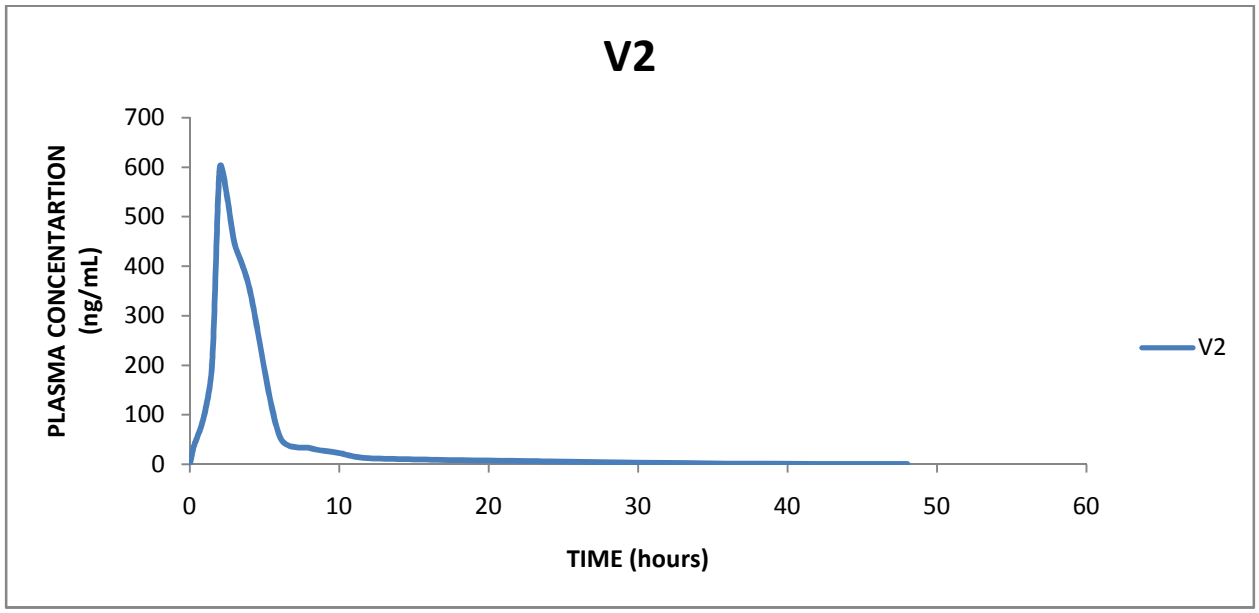
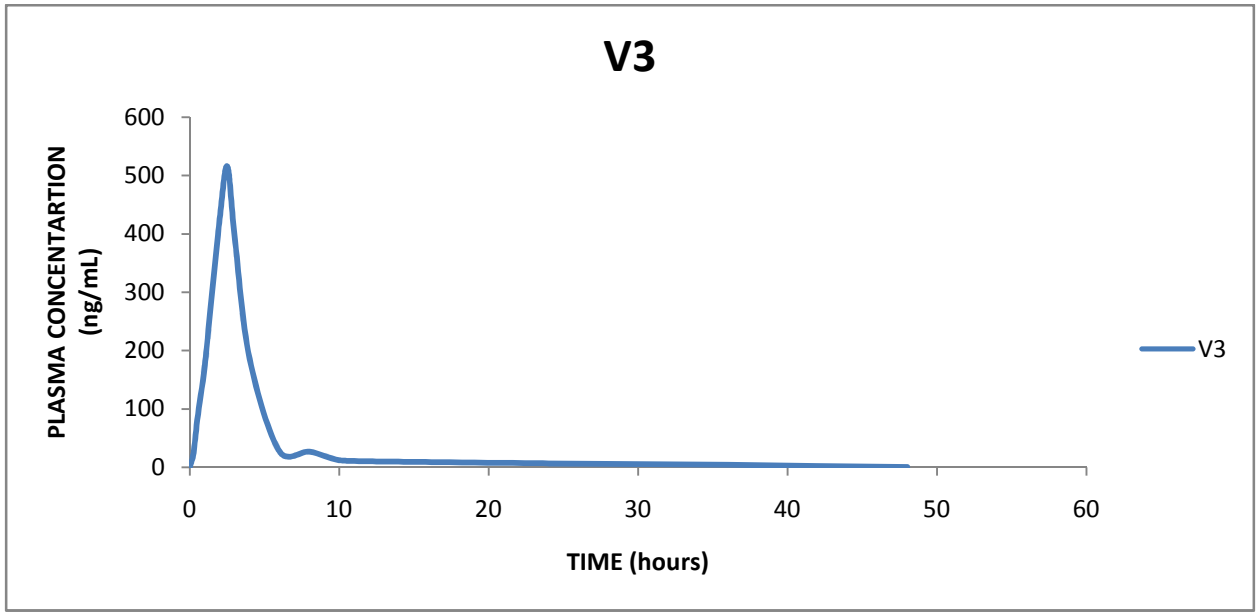
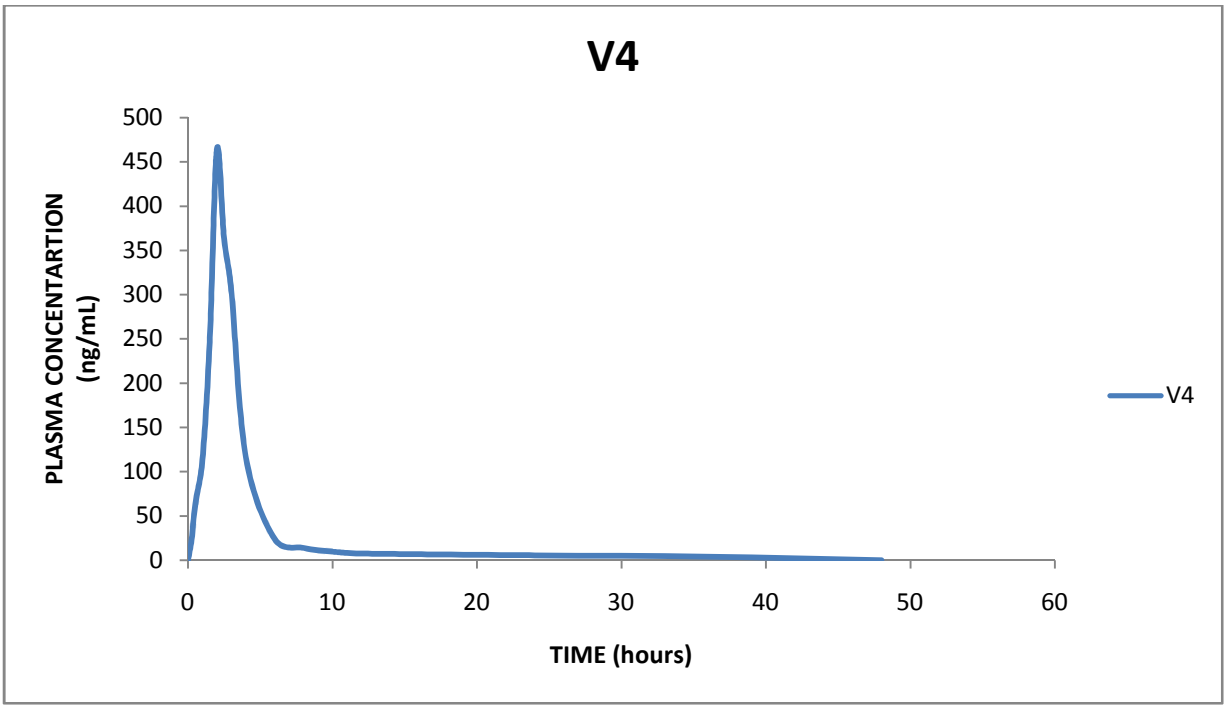


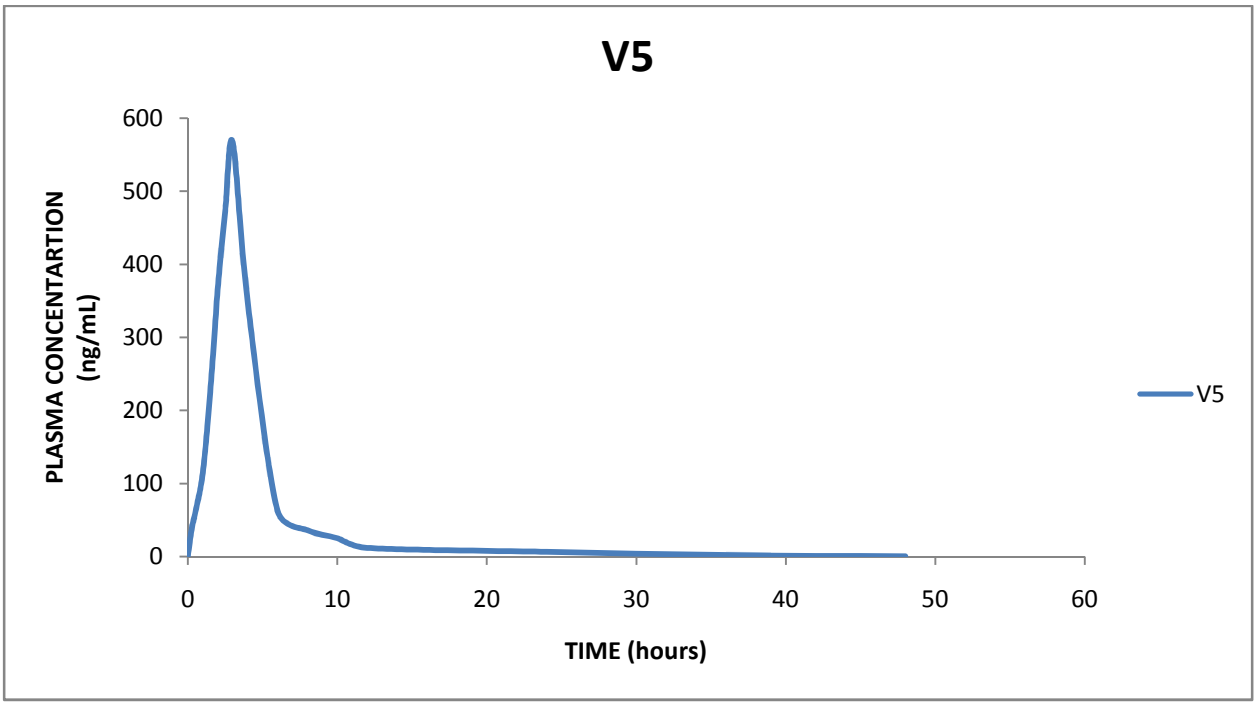
Figure-48 Individual Volunteer Plasma Concentration Curve and Average Plasma Concentration Curve of Losartan as a Fixed Dose

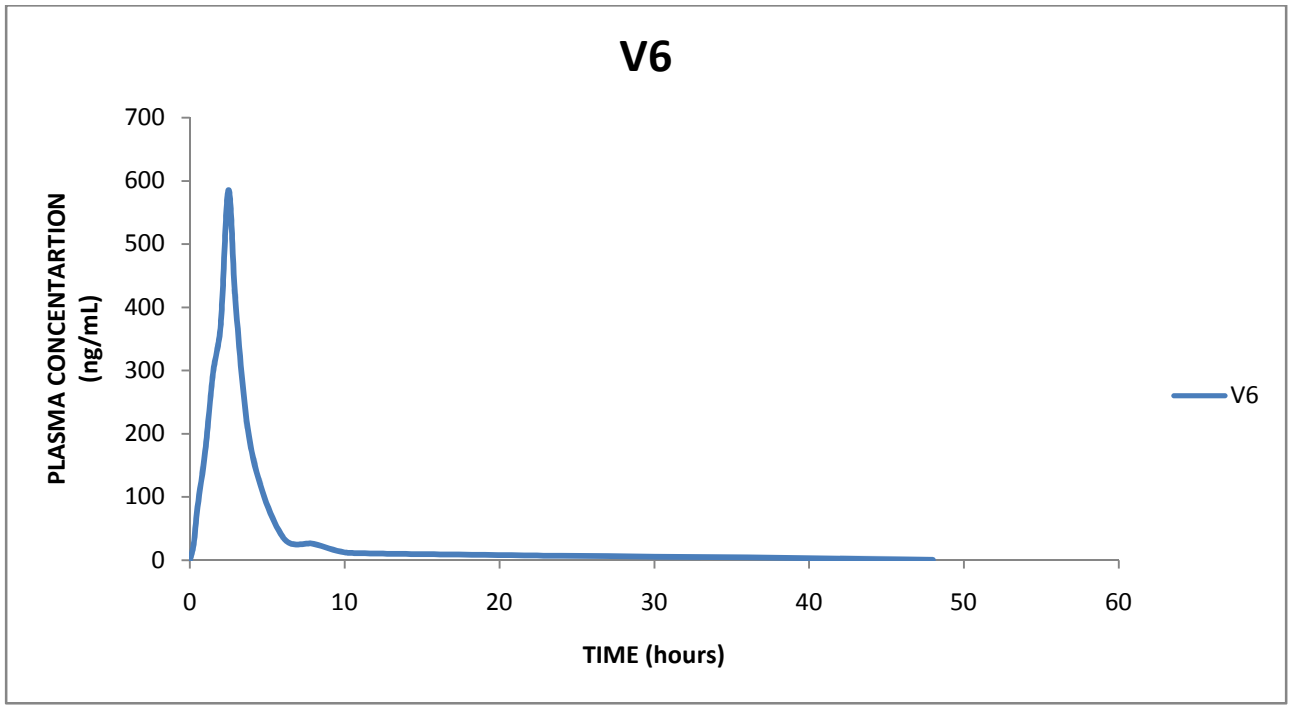



V2
PLASMA CONCENTARTION (ng/mL)
700
600
500
400
300
200
100
0
0
10
20
30
40
50
60
TIME (hours)
V2


V3
PLASMA CONCENTARTION (ng/mL)
600
500
400
300
200
100
0
0
10
20
30
40
50
60
TIME (hours)
V3


V4
500
450
400
350
300
250
200
150
100
50
0
PLASMA CONCENTARTION (ng/mL)
0
10
20
30
40
50
60
TIME (hours)
V4


V5
600
500
400
300
200
100
0
PLASMA CONCENTARTION (ng/mL)
0
10
20
30
40
50
60
TIME (hours)
V5



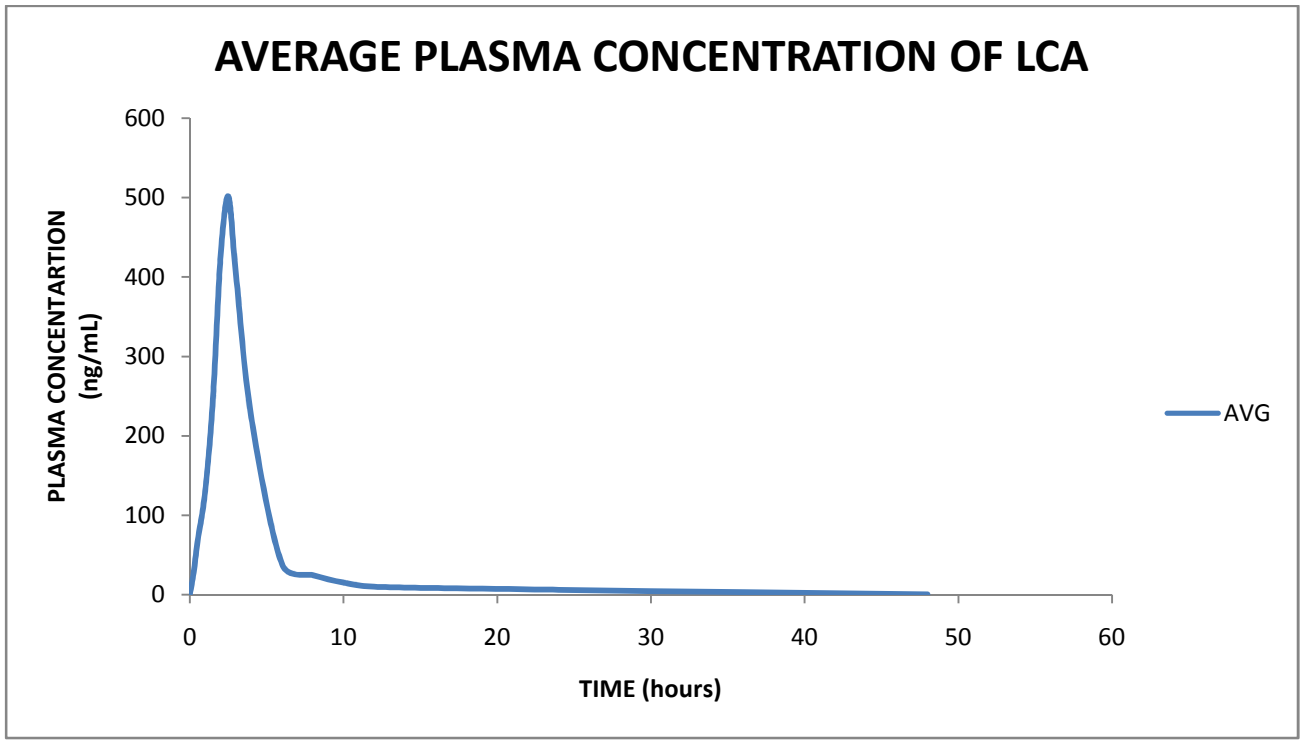
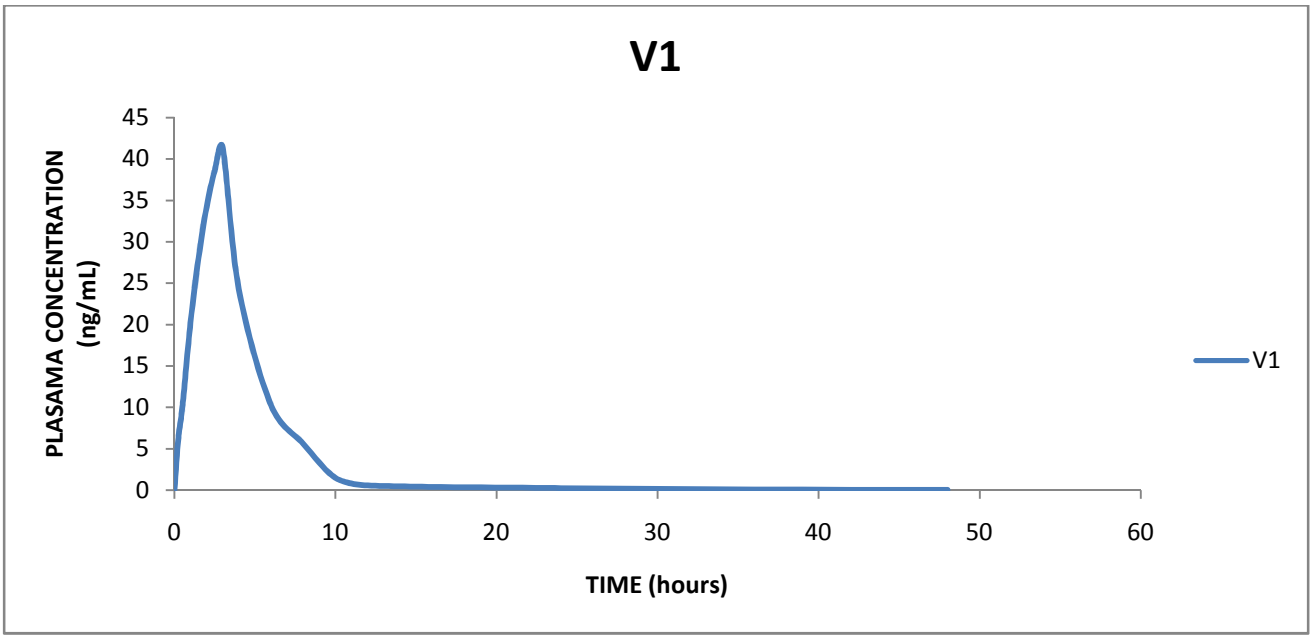
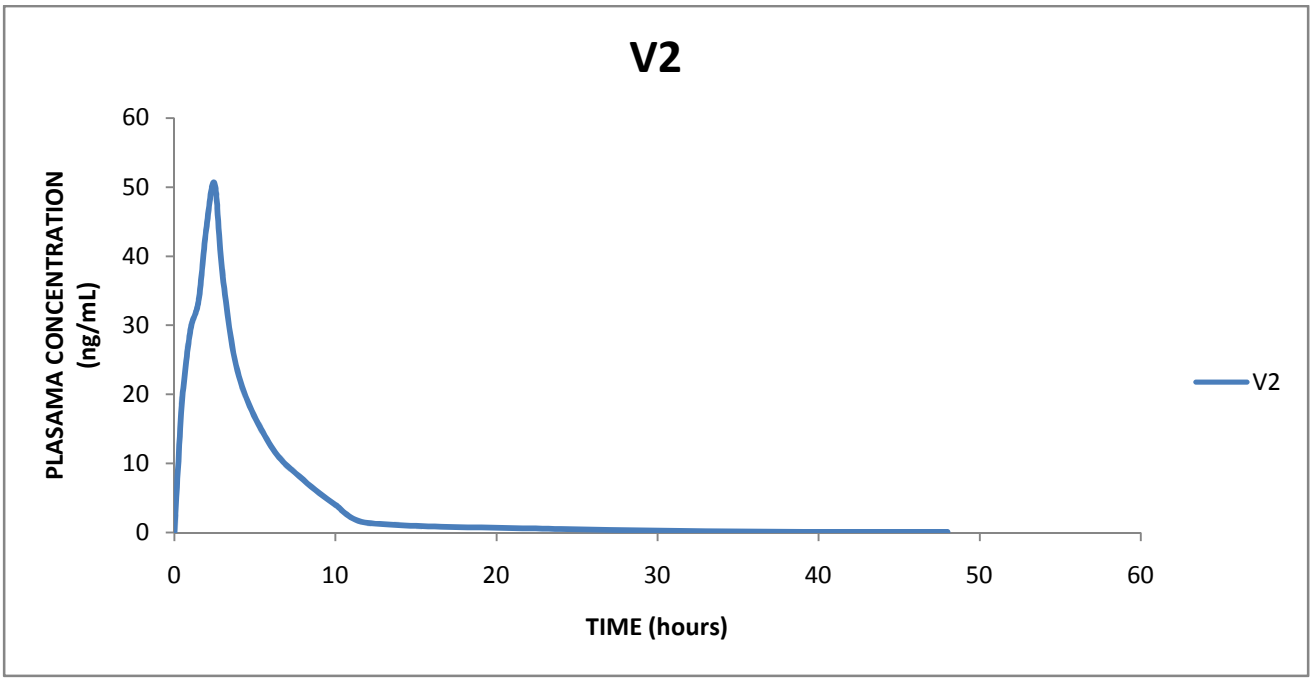
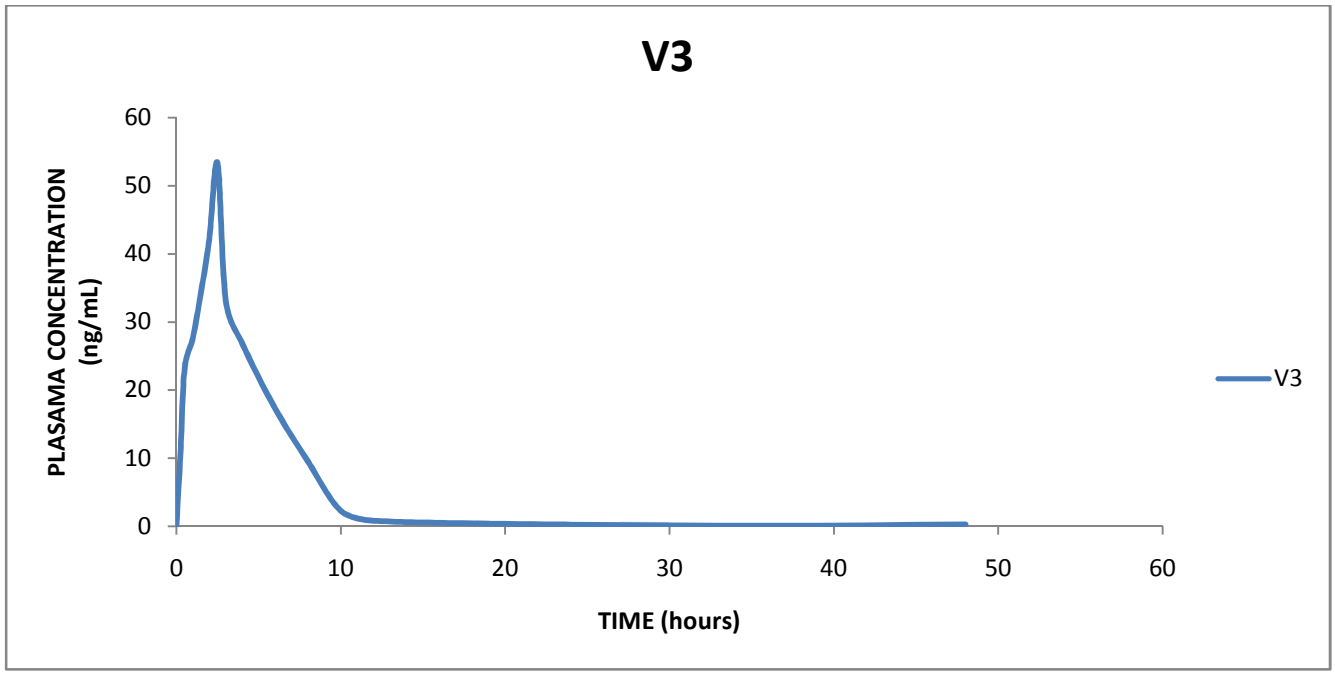
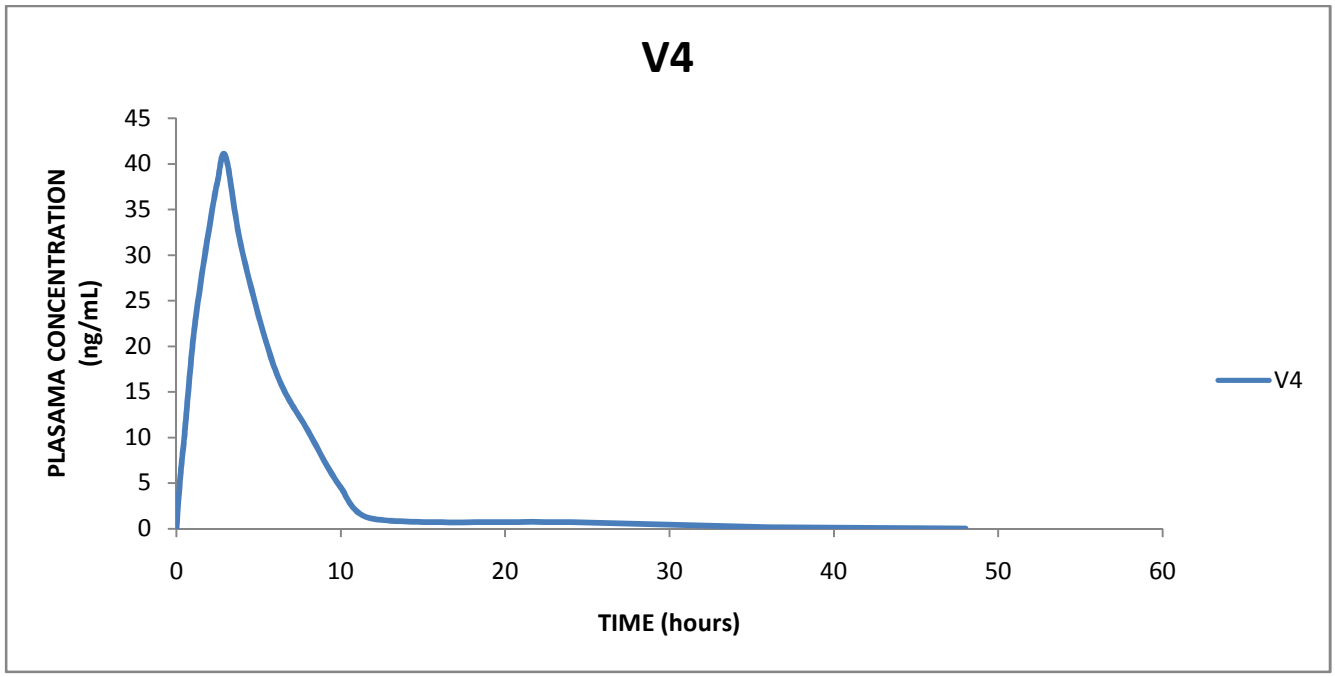


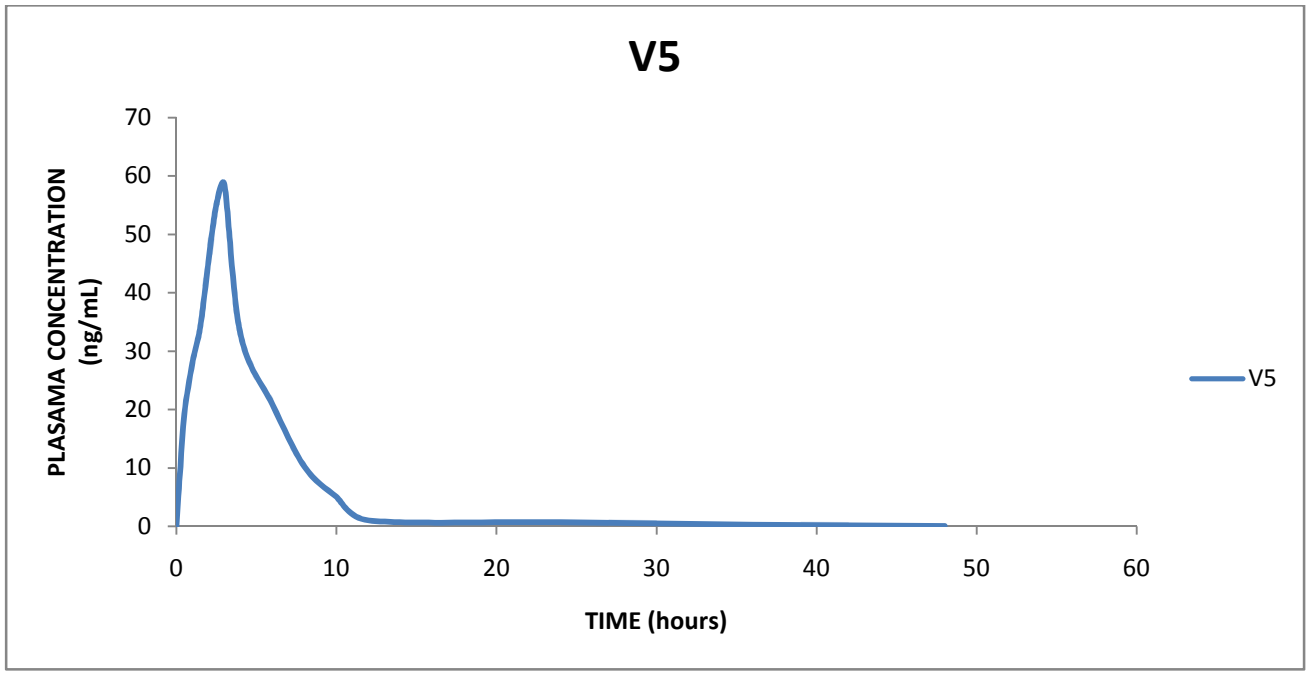
Figure-49 Individual Volunteer Plasma Concentration Curve and Average Plasma Concentration Curve of Losartan Carboxylic Acid as a Fixed Dose

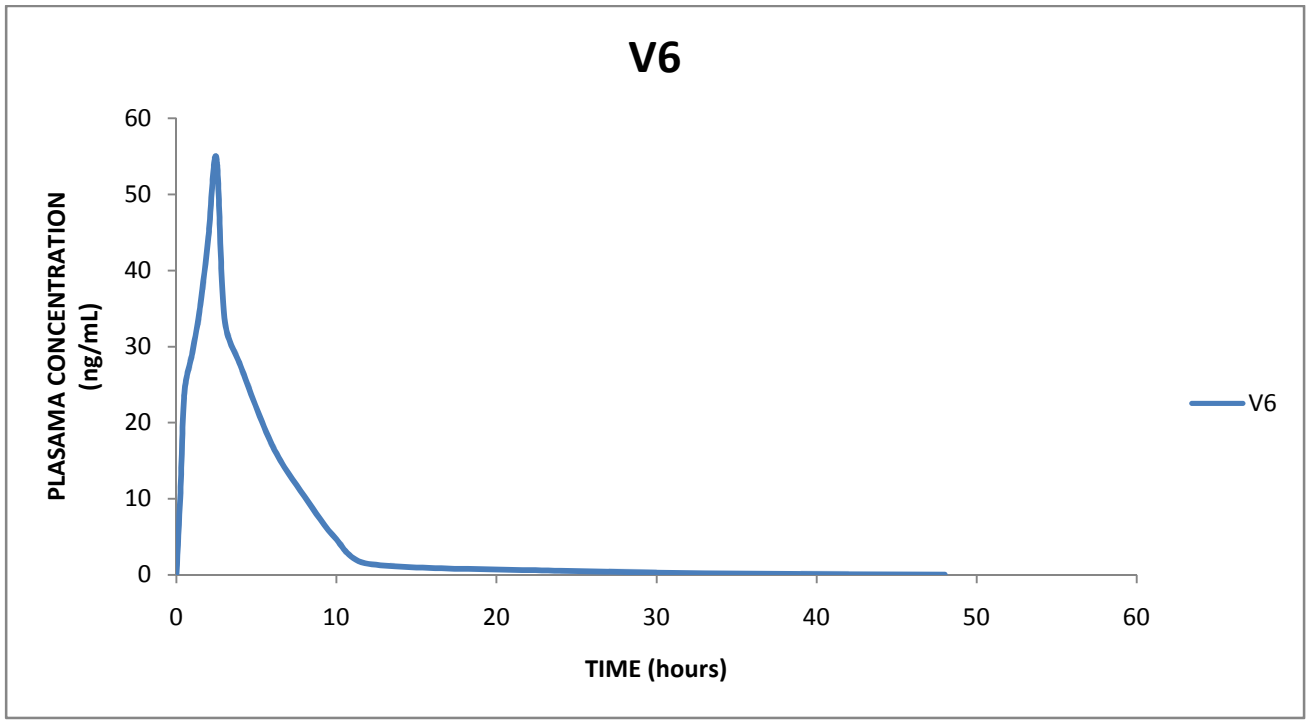

V1
PLASAMA CONCENTRATION (ng/mL)
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
TIME (hours)
V1


V2
PLASAMA CONCENTRATION (ng/mL)
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (hours)
V2


V3
PLASAMA CONCENTRATION (ng/mL)
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (hours)
V3


V4
PLASAMA CONCENTRATION (ng/mL)
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
TIME (hours)
V4


V5
PLASAMA CONCENTRATION (ng/mL)
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (hours)
V5


V6
PLASAMA CONCENTRATION (ng/mL)
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (hours)
V6

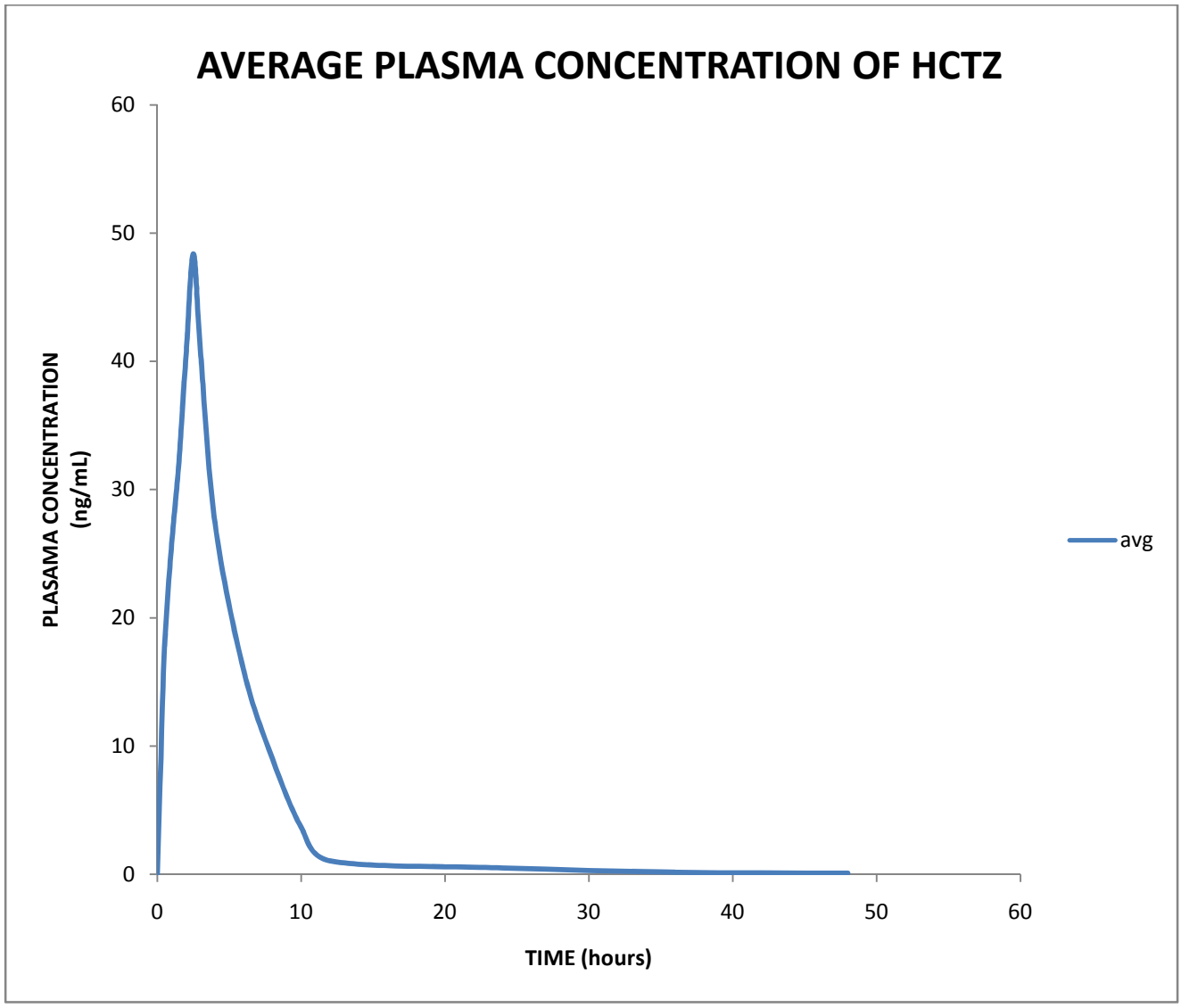


Figure-50 Individual Volunteer Plasma Concentration Curve and Average Plasma Concentration Curve of Hydrochlorothiazide as a Fixed Dose

1.3.Comparative Blood Pressure study in human volunteers:

Blood pressure is recorded with two numbers. The systolic pressure (higher number) is the force at which heart pumps blood around the body. The diastolic pressure (lower number) is the resistance to the blood flow in the blood vessels. Ideal blood pressure is considered to be between 90/60mm/Hg and 120/80mm/Hg.

Before dosing of drugs each volunteer blood pressure was recorded, called 0hour dosing and then after dosing each volunteer blood pressure was recorded after 5hour interval that is 5, 10, 15, 20, 25, 30 hrs. Before dosing each volunteer systolic blood pressure was normal 135±5mm/Hg but after dosing single drug losartan it was observed that systolic blood pressure of each volunteer varied like 131±7mm/Hg. The average maximum systolic blood pressure of 6 volunteers is 134.67mm/Hg and lowest blood pressure is 129.67mm/Hg. Like diastolic pressure before dosing of single drug containing losartan 50mg each volunteer blood pressure was recorded and normal blood pressure was observed 75±5mm/Hg but after dosing of single drug diastolic blood pressure of each volunteer was varied like 82±8mm/Hg and the average maximum blood diastolic blood pressure of 6 volunteers is 86mm/Hg and lowest blood pressure 78.33mm/Hg.

Like before dosing and after dosing of fixed dose drug containing Losartan 50mg with Hydrochlorothiazide 12.5mg same condition and time schedule were maintained and observed that systolic blood pressure of each volunteer was normal before dosing 136±6mm/Hg but after dosing fixed dose combination drug it was observed that systolic blood pressure of each volunteer was varied like 128±8mm/Hg. The average maximum systolic blood pressure of 6 volunteers is 135.33mm/Hg and lowest systolic blood pressure 121.33mm/Hg. Like diastolic pressure before dosing of fixed dose combined drug containing losartan 50mg with hydrochlorothiazide 12.5mg each volunteer blood pressure was recorded and normal blood pressure was observed 80±6mm/Hg but after dosing of fixed dose combined drug diastolic blood pressure of each volunteer was varied like 76±6mm/Hg and the average maximum blood diastolic blood pressure of 6 volunteers is 81.33mm/Hg and lowest diastolic blood pressure 73.33mm/Hg. The total blood pressure report of 6 volunteers were also elaborated in Table -9 (a,b) and Table-10 (a,b) and the mean and each volunteer systolic blood pressure vs time profile

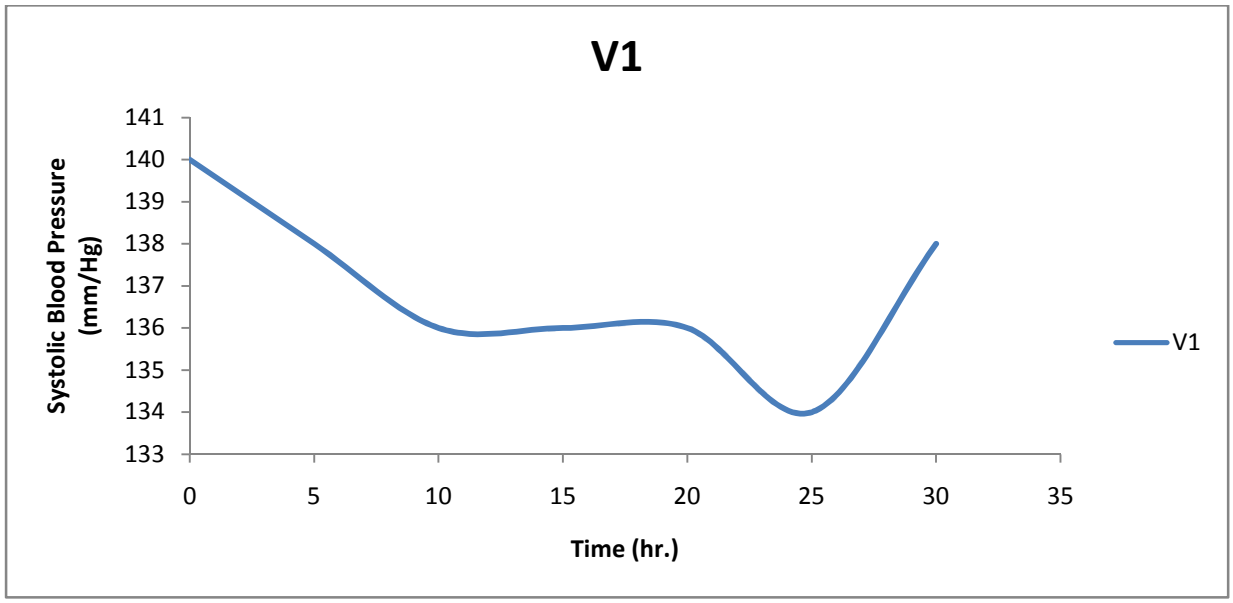
and diastolic blood pressure vs time profile for both the formulations was represented in Figure-51(a,b,), 52(a,b), 53(a,b), 54(a,b).

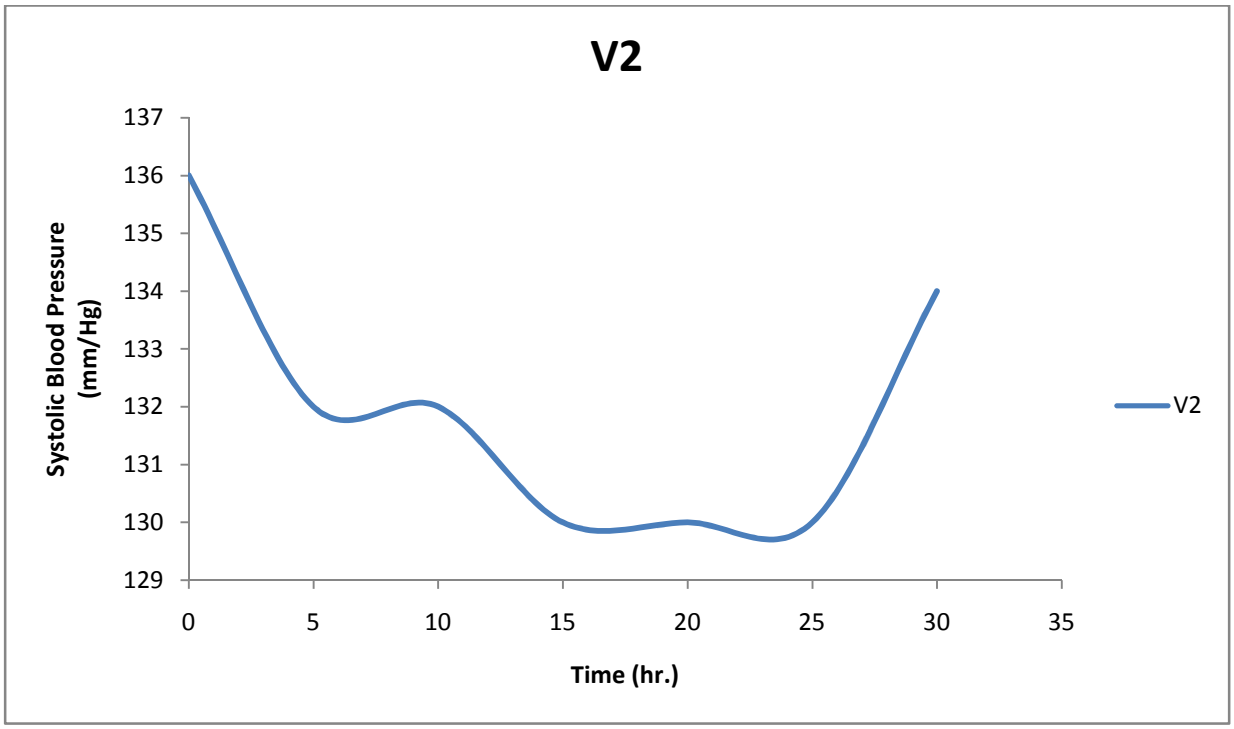
	Blood Pressure(mm/Hg)						
Time(hr.)	Vol 1 BP	Vol 2 BP	Vol 3 BP	Vol 4 BP	Vol 5 BP	Vol 6 BP	Vol Avg BP
0	140/80	136/70	130/82	140/78	132/84	130/76	134.67/78.33
5	138/80	132/76	130/80	136/80	130/86	128/80	132.33/80.33
10	136/78	132/74	128/80	134/76	128/84	124/80	130.33/78.67
15	136/76	130/80	126/82	132/76	128/82	126/76	129.67/78.67
20	136/80	130/82	126/82	134/82	126/84	128/80	130.00/81.67
25	134/82	130/82	126/84	132/84	130/86	130/82	130.33/83.33
30	138/84	134/86	130/86	132/84	134/90	132/86	133.33/86.00

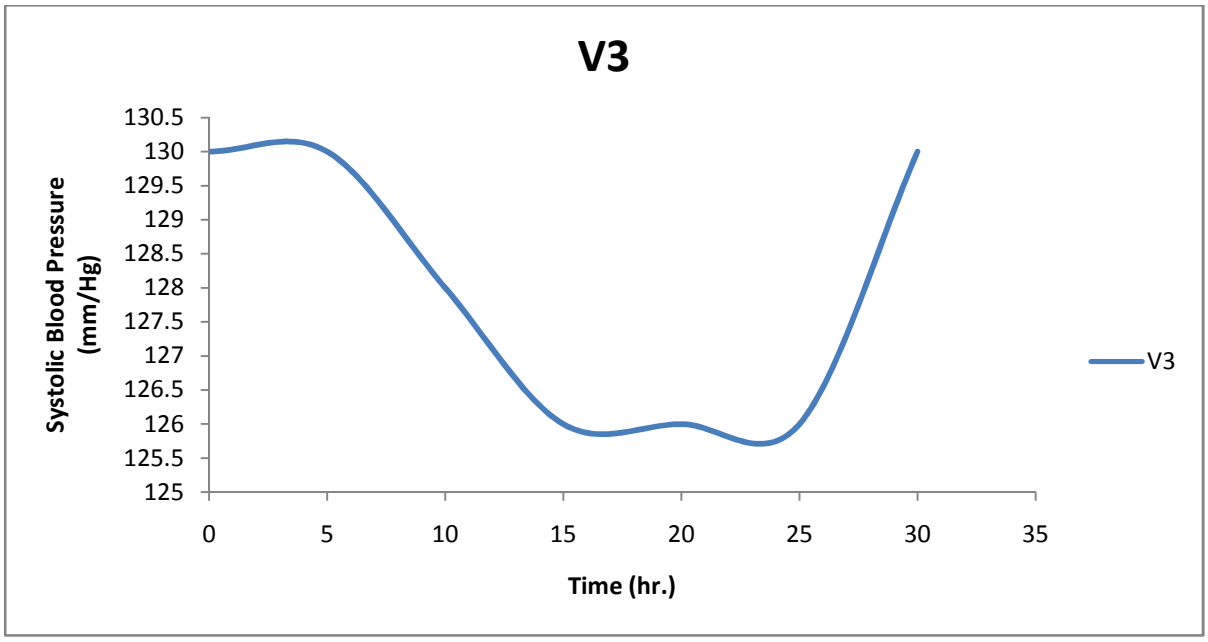
Table 34: Measurement of Blood Pressure after Single Dose Losartan administration.

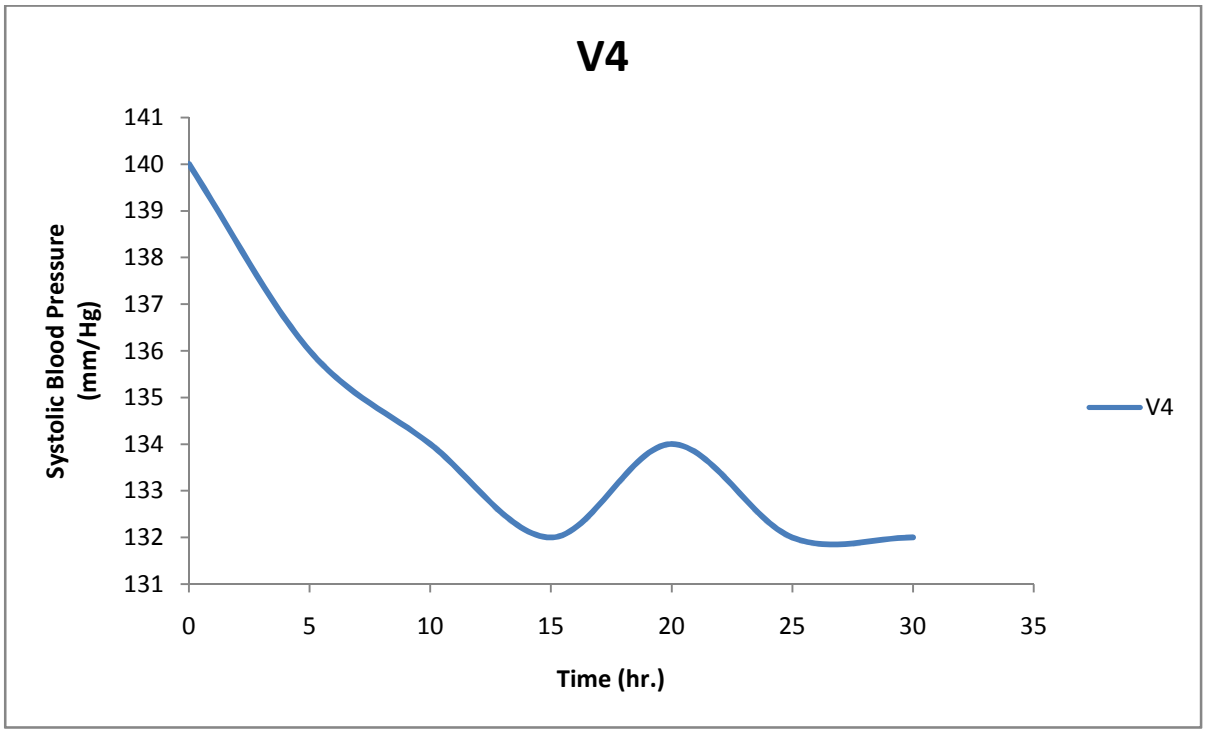
Time(hr.)	Vol 1 BP	Vol 2 BP	Vol 3 BP	Vol 4 BP	Vol 5 BP	Vol 6 BP	Vol Avg BP
0	138/82	142/74	134/84	136/82	130/86	132/80	135.33/81.33
5	136/78	130/72	128/78	132/78	128/82	126/74	130.00/77.00
10	134/76	130/72	128/78	130/76	126/82	126/72	129.00/76.00
15	130/74	126/74	124/76	130/74	124/82	124/72	126.33/75.33
20	13070	124/70	120/76	128/74	124/82	122/74	124.67/74.33
25	128/70	124/72	120/74	126/72	120/80	120/74	123.00/73.67
30	126/70	120/72	120/74	124/72	120/80	118/72	121.33/73.33

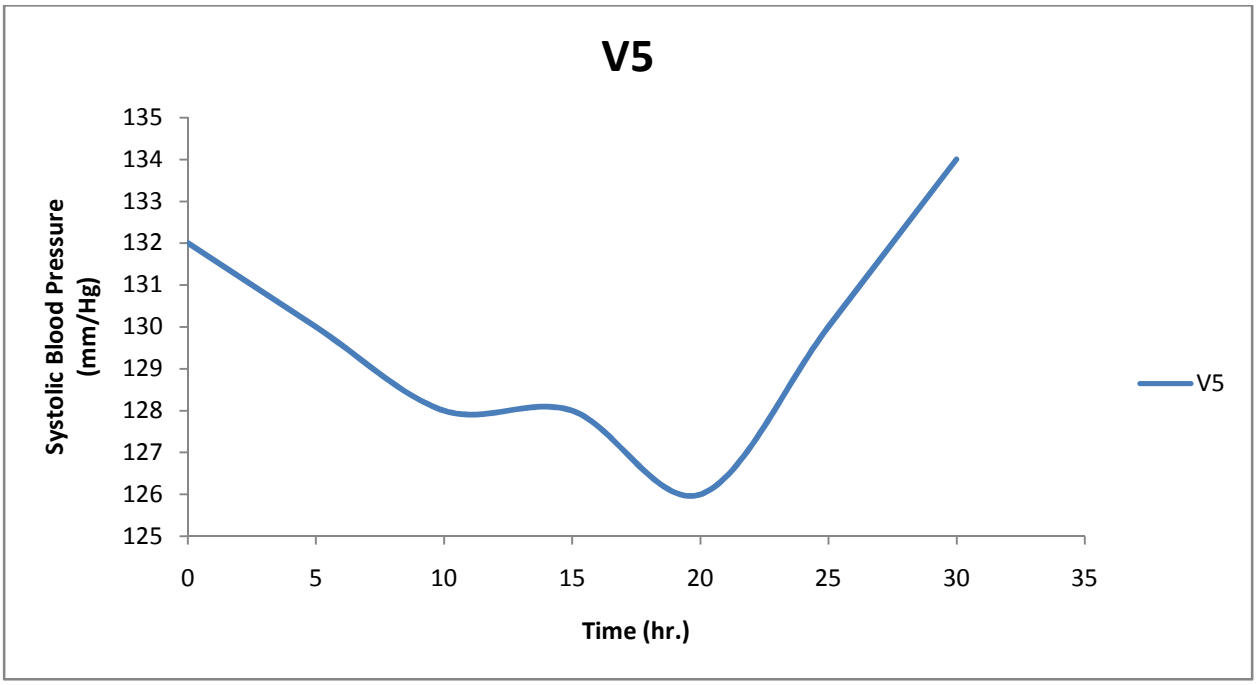
Table 35: Measurement of Blood Pressure after Fixed Dose Losartan with Hydrochlorothiazide administration.

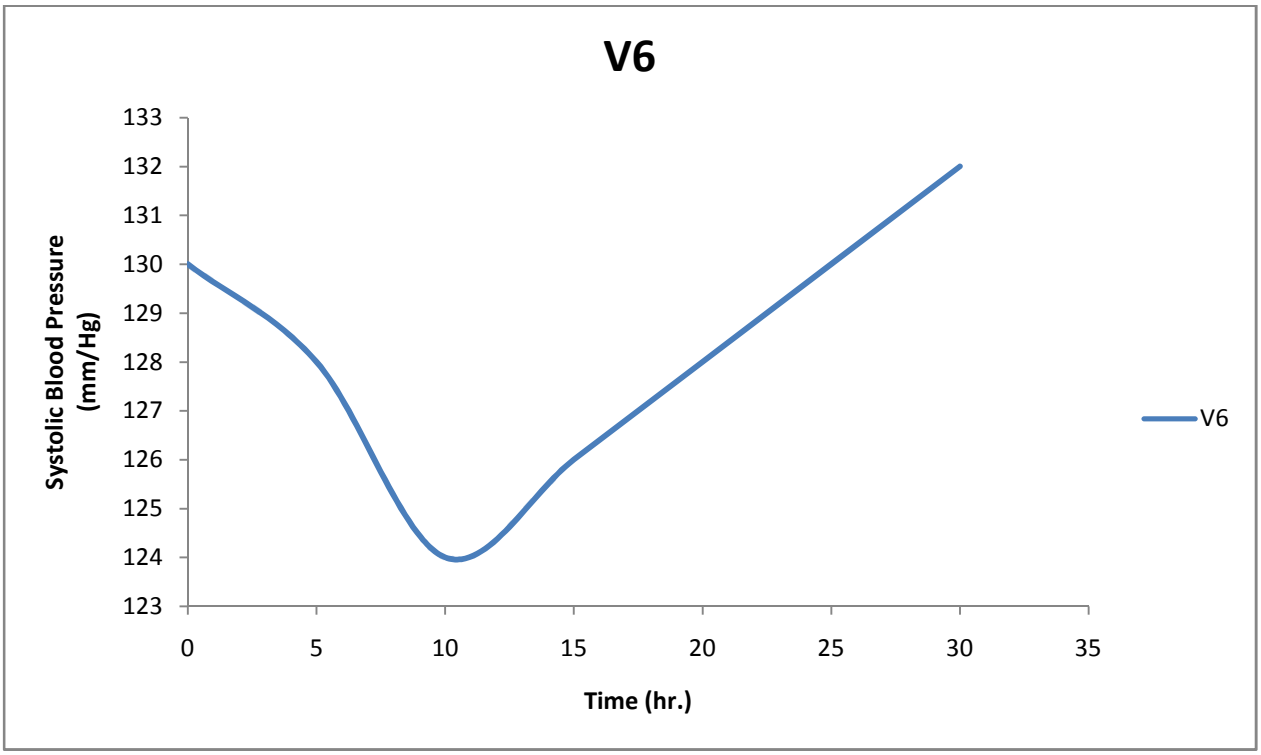

V1
Systolic Blood Pressure (mm/Hg)
141
140
139
138
137
136
135
134
133
0
5
10
15
20
25
30
35
Time (hr.)
V1


V2
Systolic Blood Pressure (mm/Hg)
137
136
135
134
133
132
131
130
129
0
5
10
15
20
25
30
35
Time (hr.)
V2


V3
Systolic Blood Pressure (mm/Hg)
130.5
130
129.5
129
128.5
128
127.5
127
126.5
126
125.5
125
0
5
10
15
20
25
30
35
Time (hr.)
V3


V4
Systolic Blood Pressure (mm/Hg)
141
140
139
138
137
136
135
134
133
132
131
0
5
10
15
20
25
30
35
Time (hr.)
V4


V5
Systolic Blood Pressure (mm/Hg)
135
134
133
132
131
130
129
128
127
126
125
0
5
10
15
20
25
30
35
Time (hr.)
V5


V6
Systolic Blood Pressure (mm/Hg)
133
132
131
130
129
128
127
126
125
124
123
0
5
10
15
20
25
30
35
Time (hr.)
V6

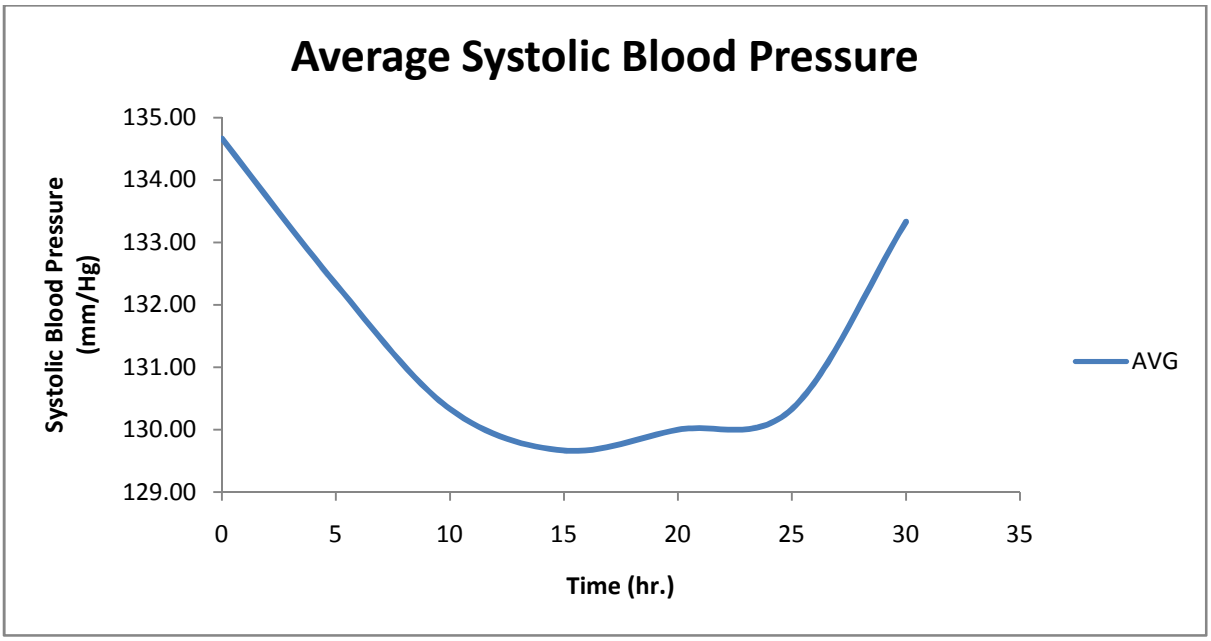
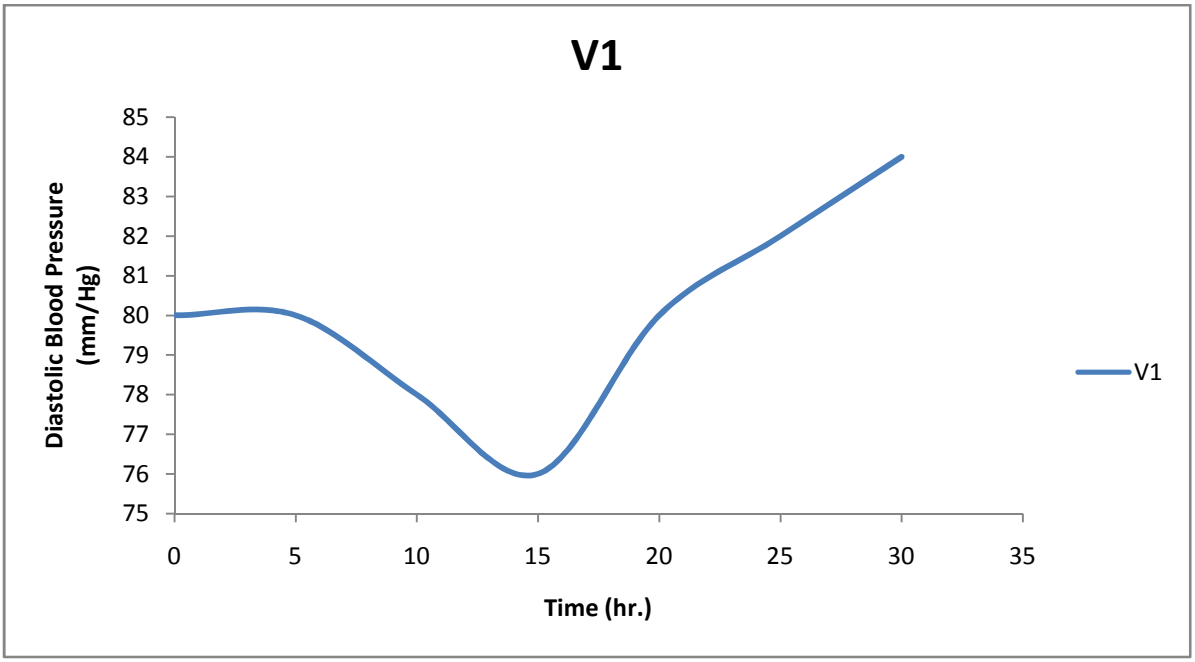
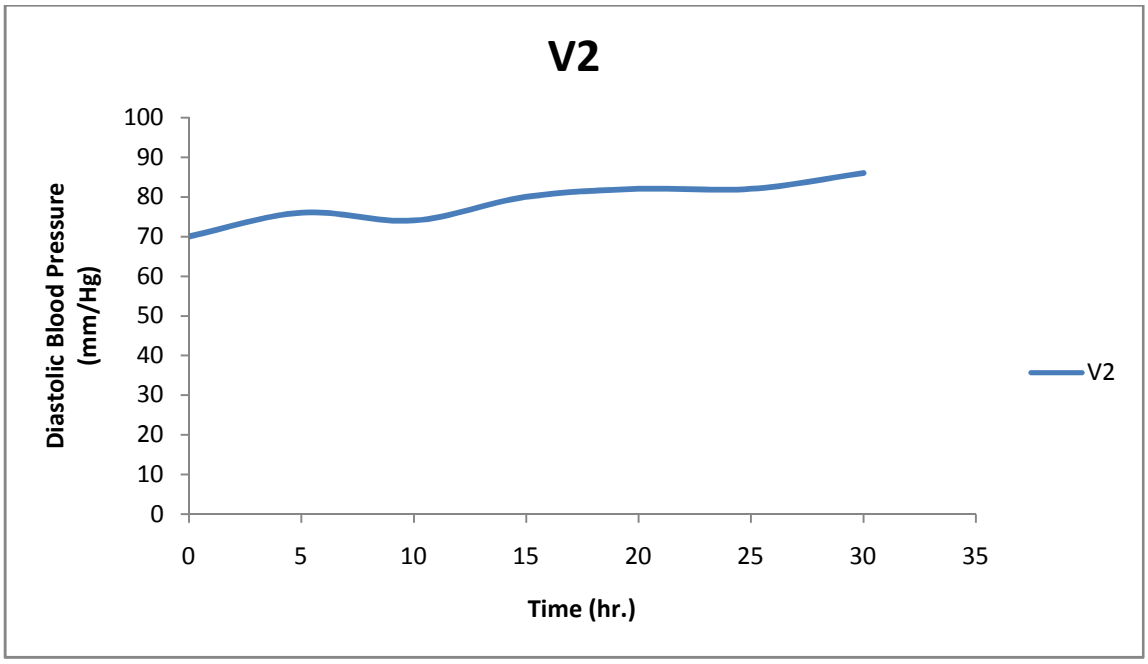
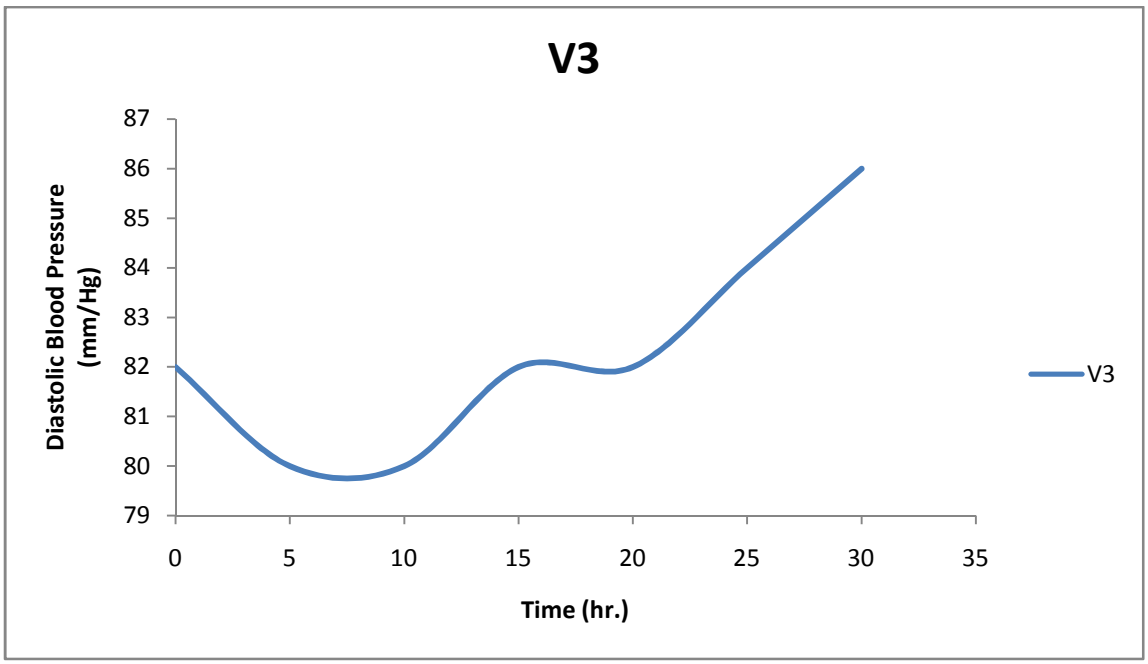
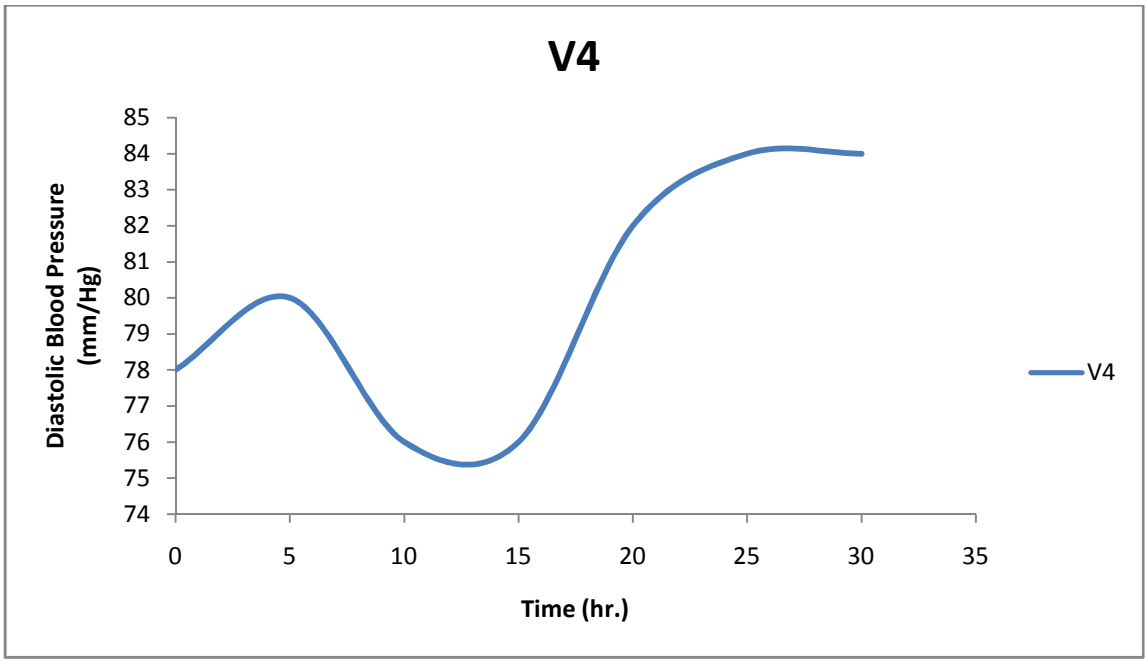


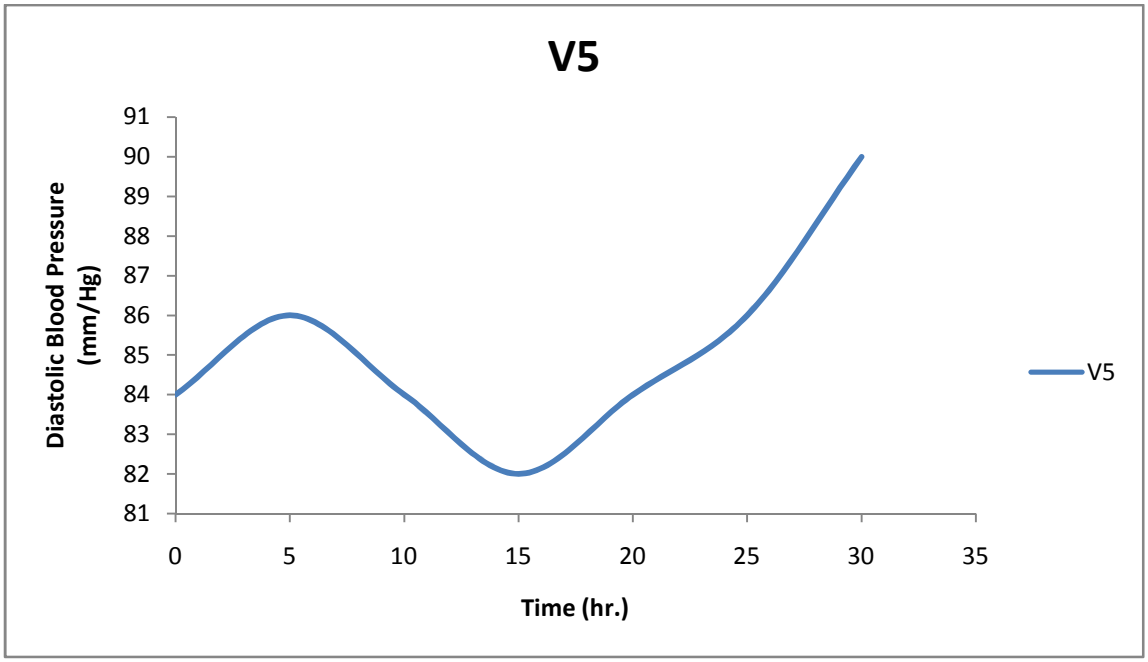
Figure-51 Individual Volunteer Systolic Blood Pressure Curve and Average Systolic Blood Pressure Curve During Single Dose Containing Losartan Treatment

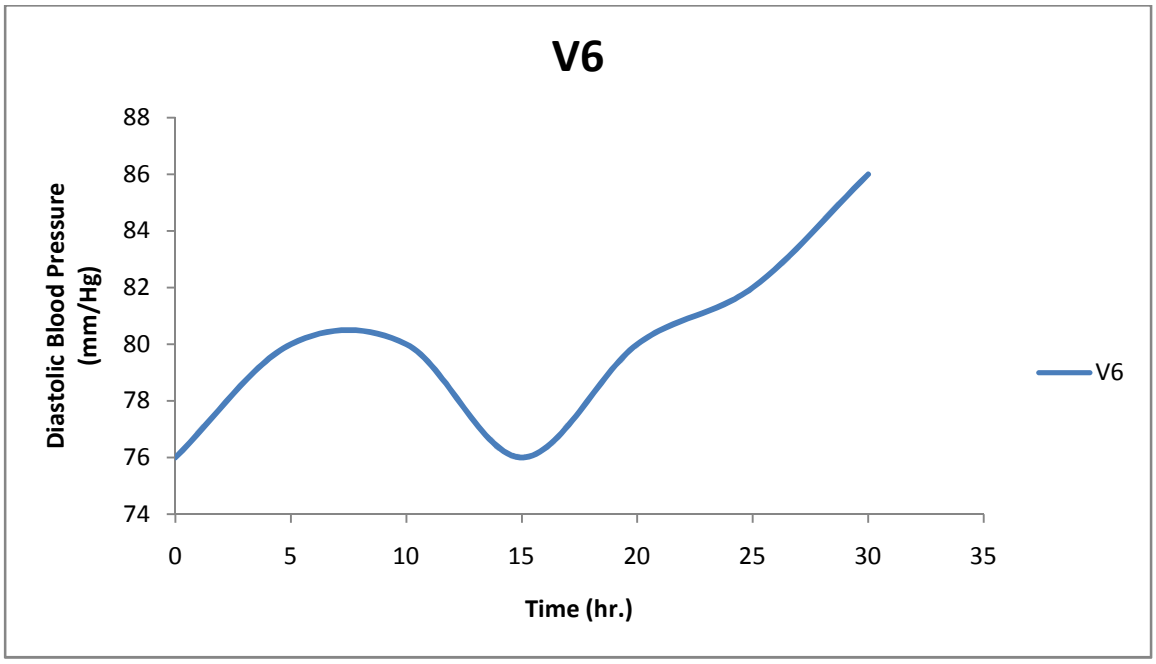



V2
Diastolic Blood Pressure (mm/Hg)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Time (hr.)
V2


V3
Diastolic Blood Pressure (mm/Hg)
87
86
85
84
83
82
81
80
79
0
5
10
15
20
25
30
35
Time (hr.)
V3


V4
Diastolic Blood Pressure (mm/Hg)
85
84
83
82
81
80
79
78
77
76
75
74
0
5
10
15
20
25
30
35
Time (hr.)
V4


V5
Diastolic Blood Pressure (mm/Hg)
91
90
89
88
87
86
85
84
83
82
81
0
5
10
15
20
25
30
35
Time (hr.)
V5



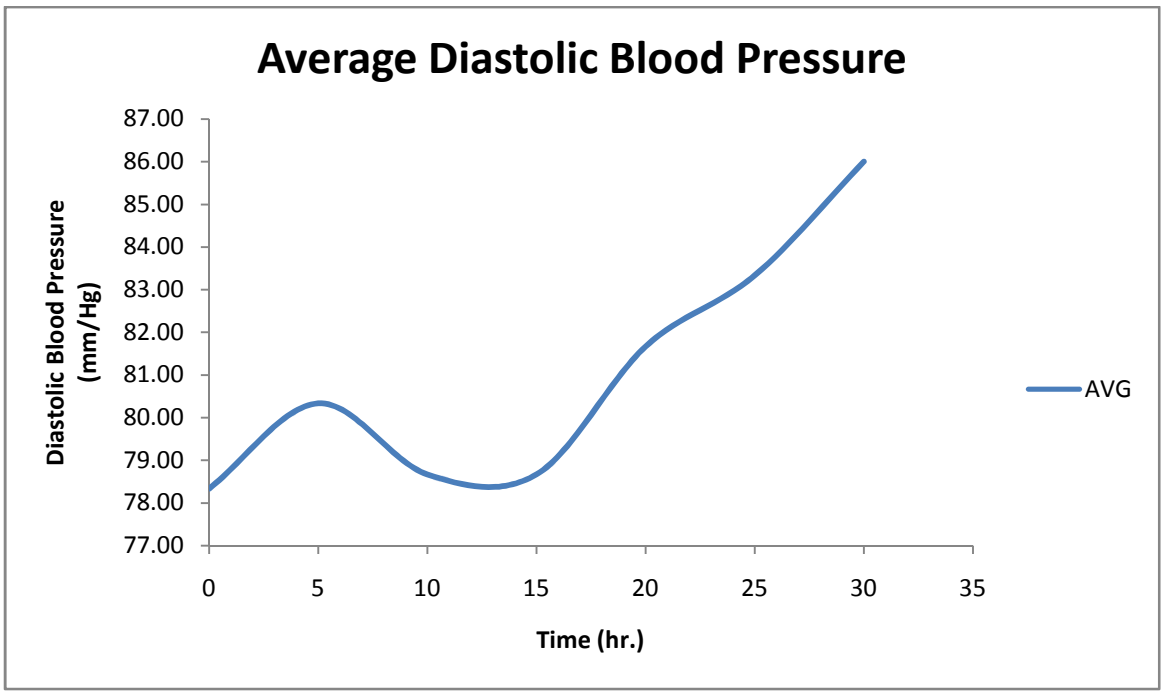
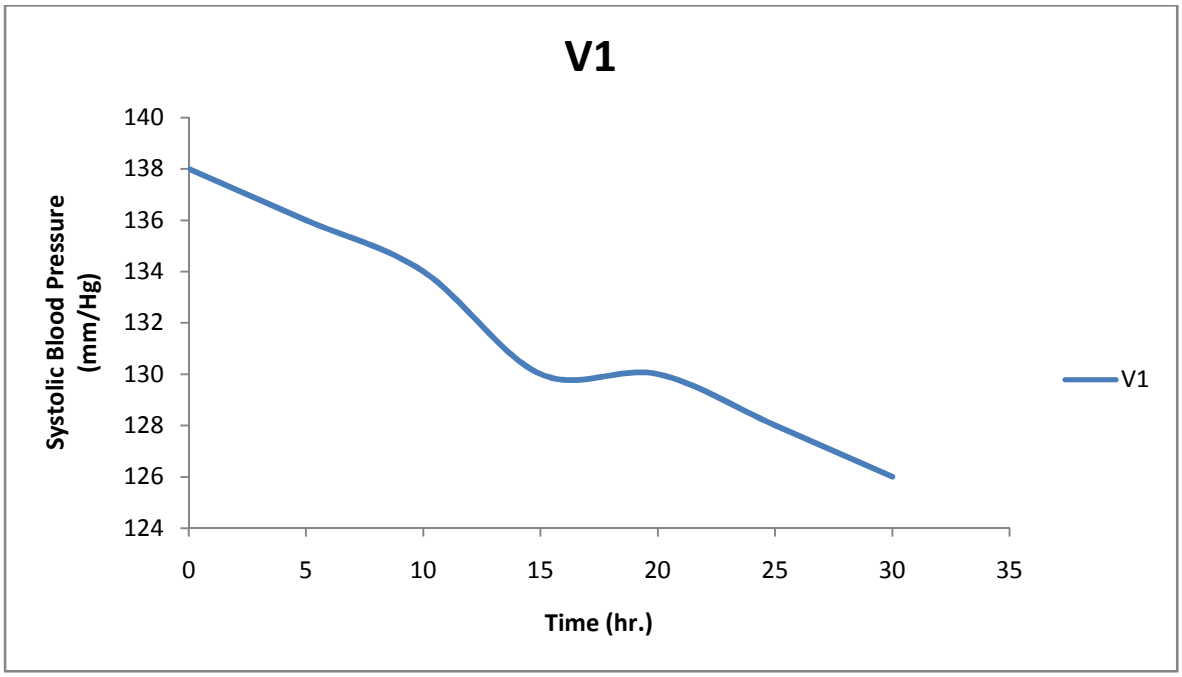
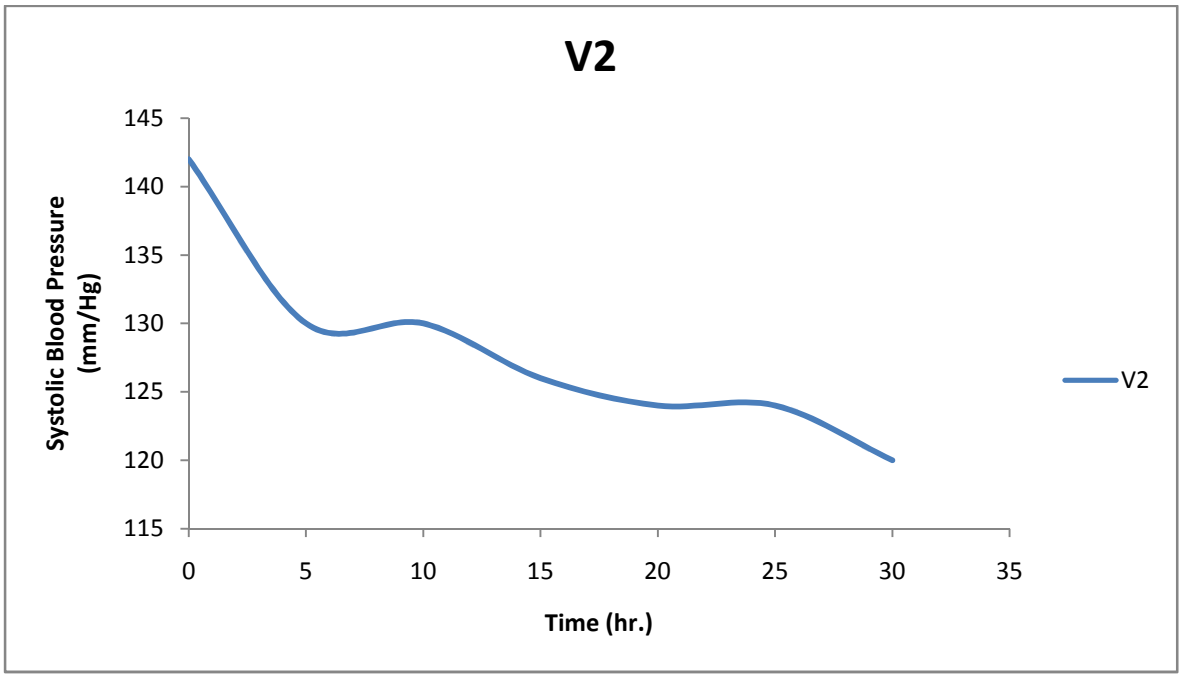
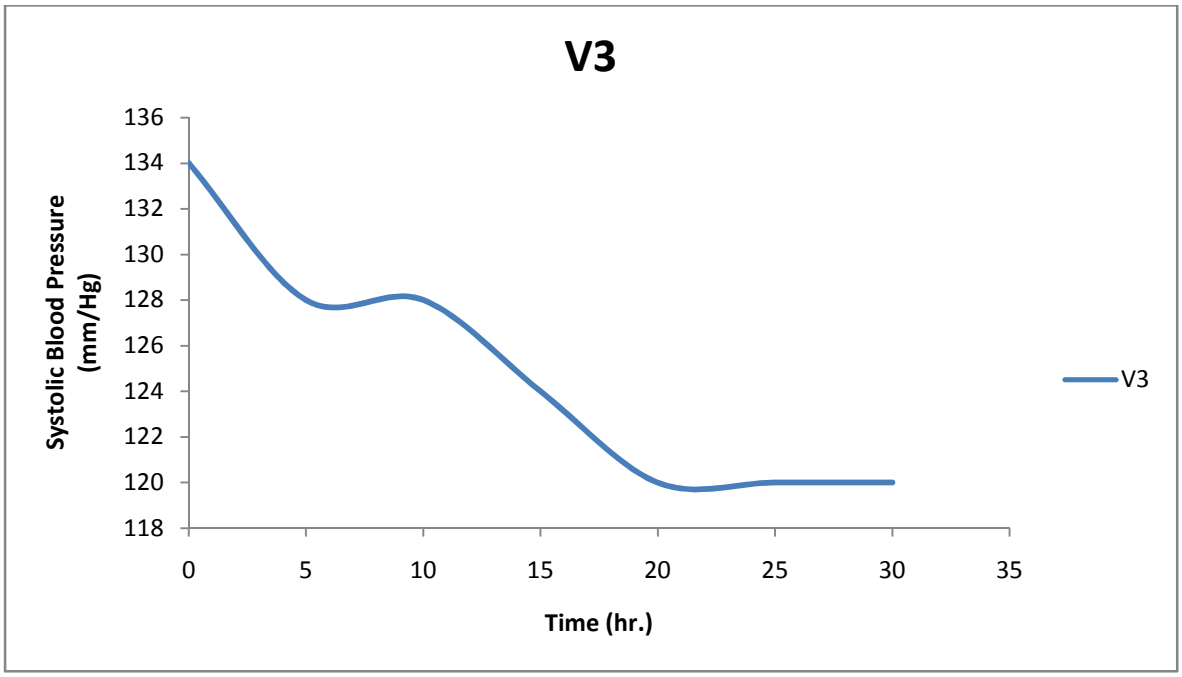
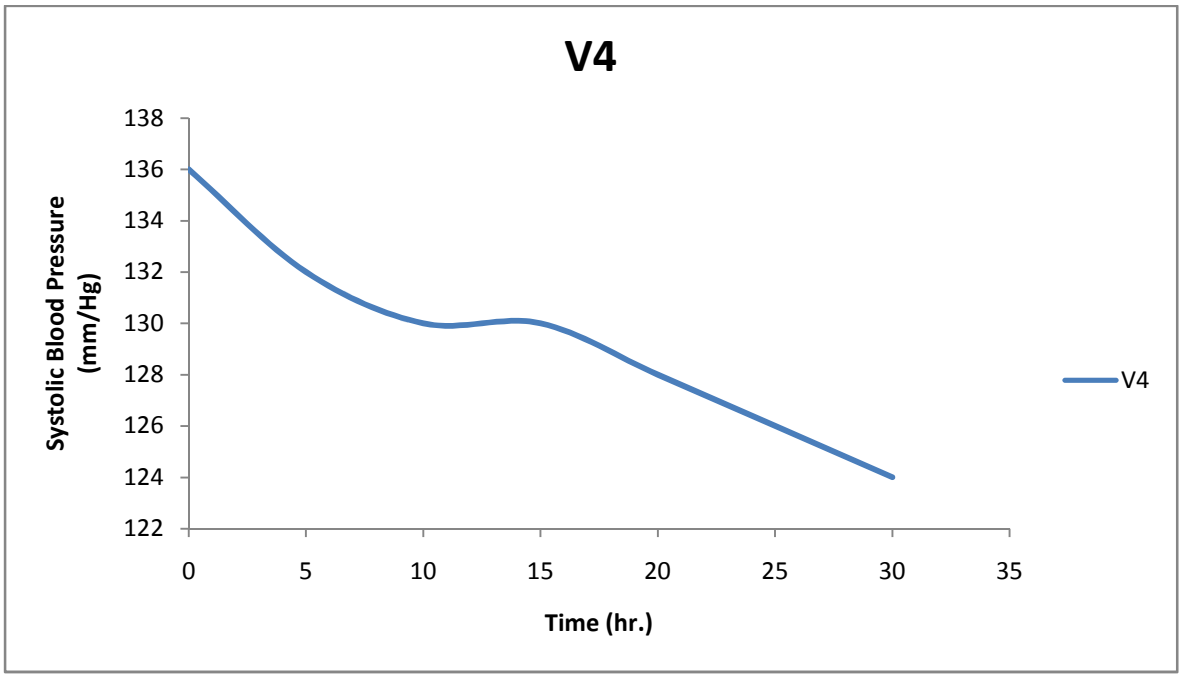


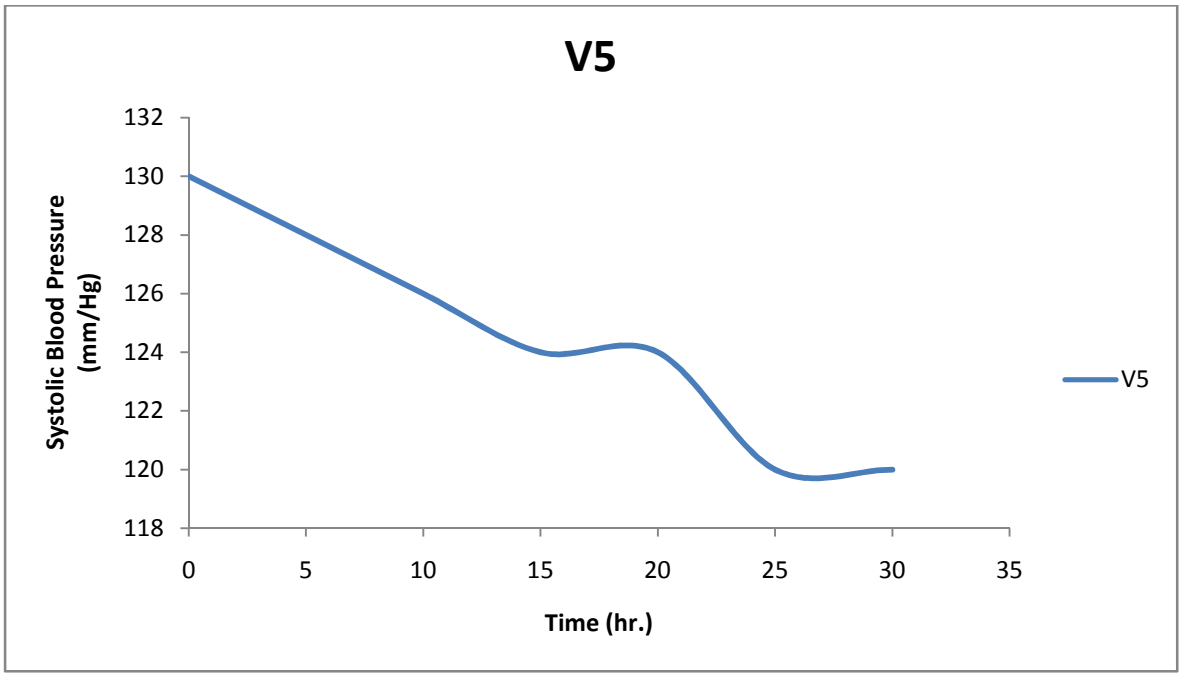
Figure-52 Individual Volunteer Diastolic Blood Pressure Curve and Average Diastolic Blood Pressure Curve During Single Dose Containing Losartan Treatment

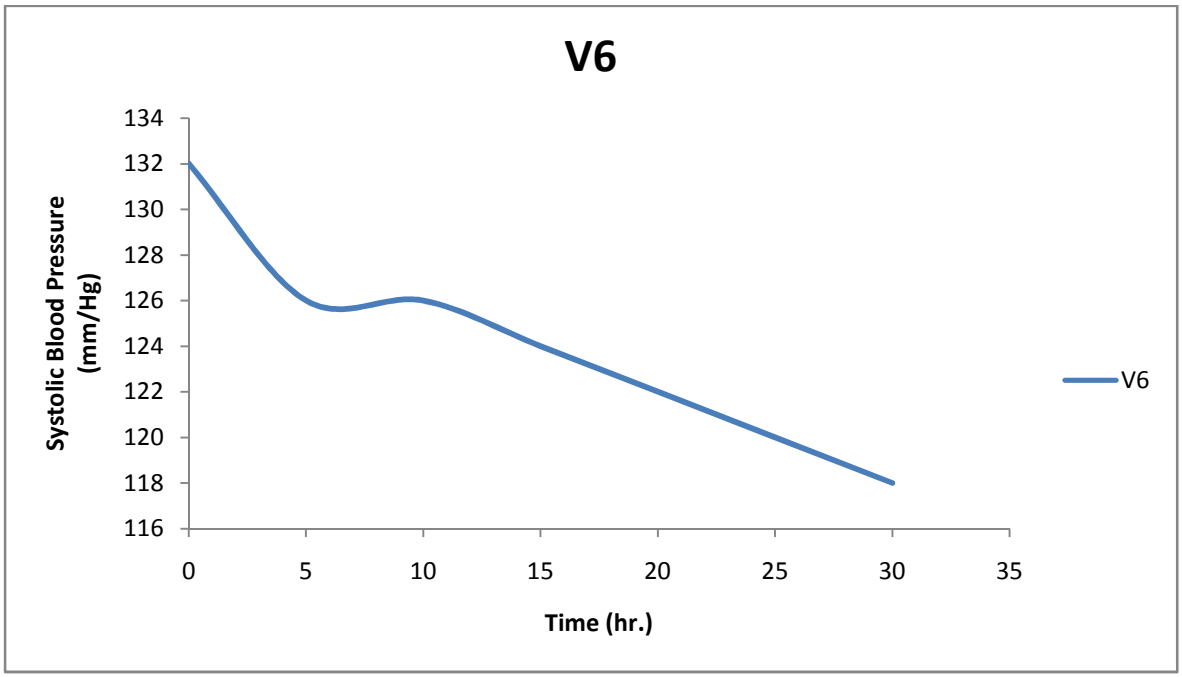

V1
Systolic Blood Pressure (mm/Hg)
140
138
136
134
132
130
128
126
124
0
5
10
15
20
25
30
35
Time (hr.)
V1


V2
Systolic Blood Pressure (mm/Hg)
145
140
135
130
125
120
115
0
5
10
15
20
25
30
35
Time (hr.)
V2


V3
Systolic Blood Pressure (mm/Hg)
136
134
132
130
128
126
124
122
120
118
0
5
10
15
20
25
30
35
Time (hr.)
V3


V4
Systolic Blood Pressure (mm/Hg)
138
136
134
132
130
128
126
124
122
0
5
10
15
20
25
30
35
Time (hr.)
V4


V5
Systolic Blood Pressure (mm/Hg)
132
130
128
126
124
122
120
118
0
5
10
15
20
25
30
35
Time (hr.)
V5


V6
Systolic Blood Pressure (mm/Hg)
134
132
130
128
126
124
122
120
118
116
0
5
10
15
20
25
30
35
Time (hr.)
V6

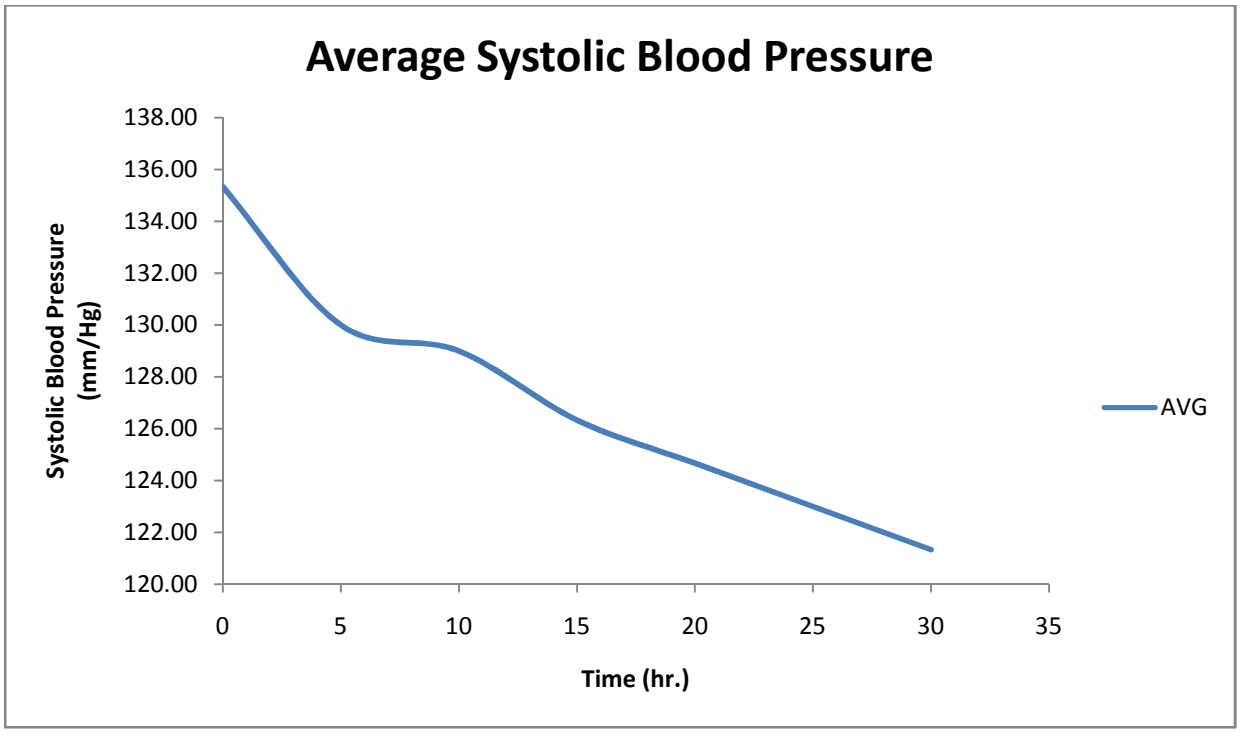
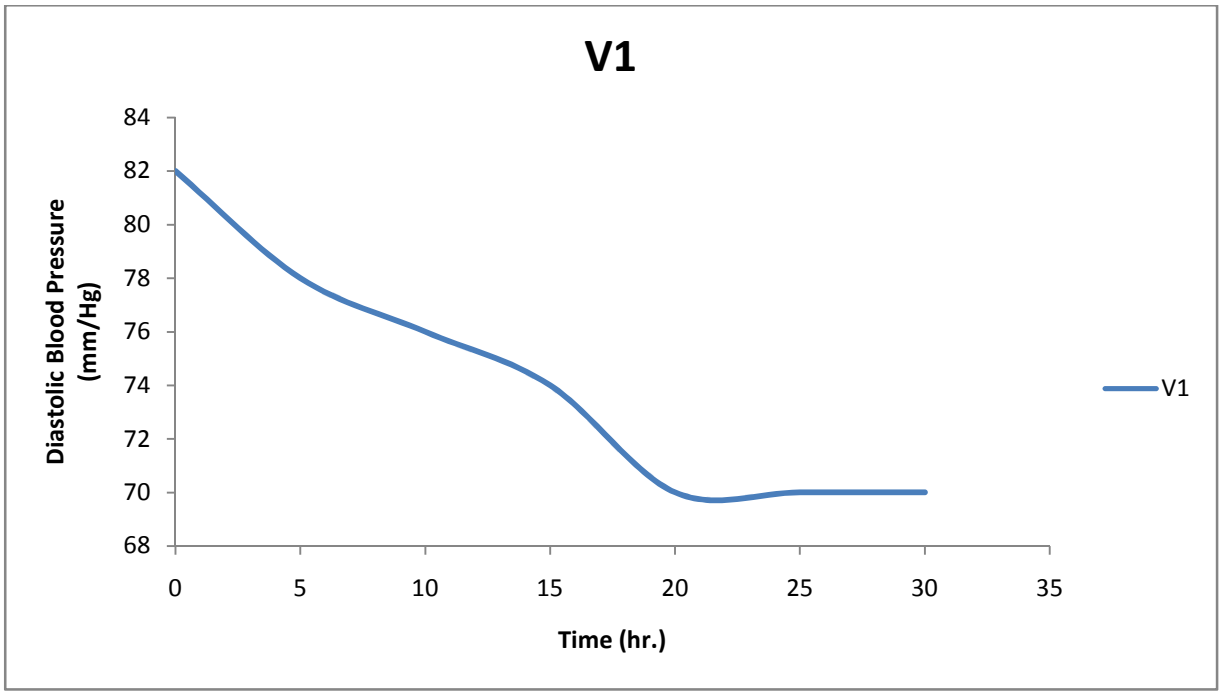
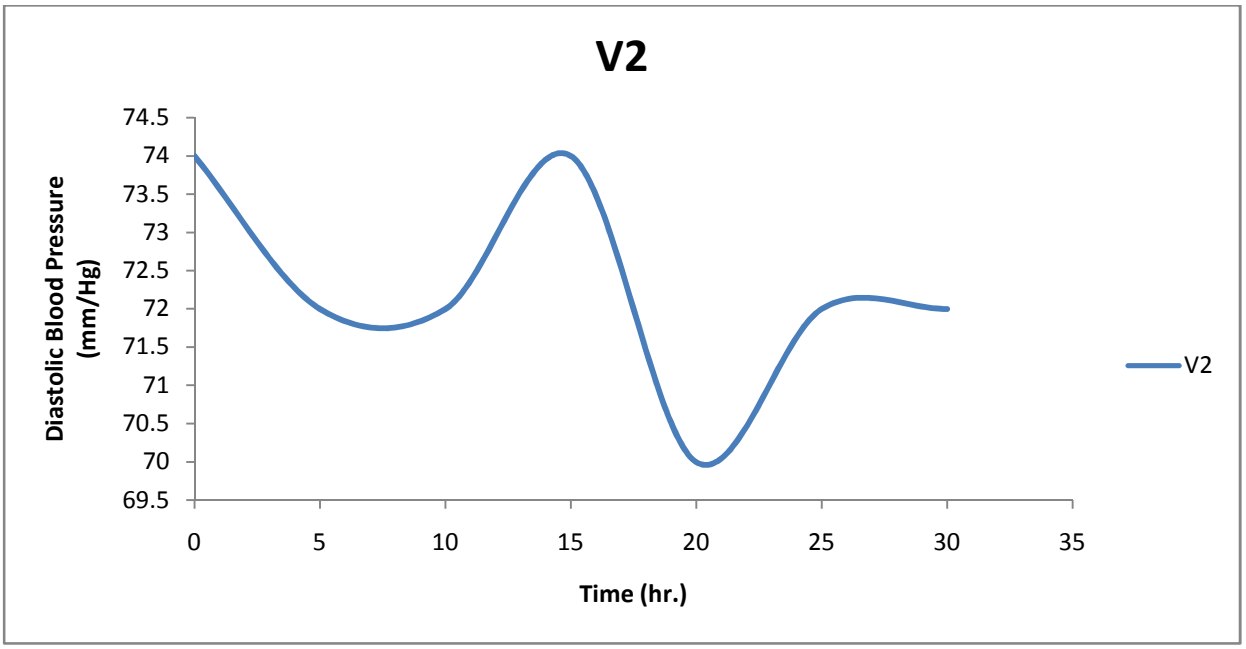
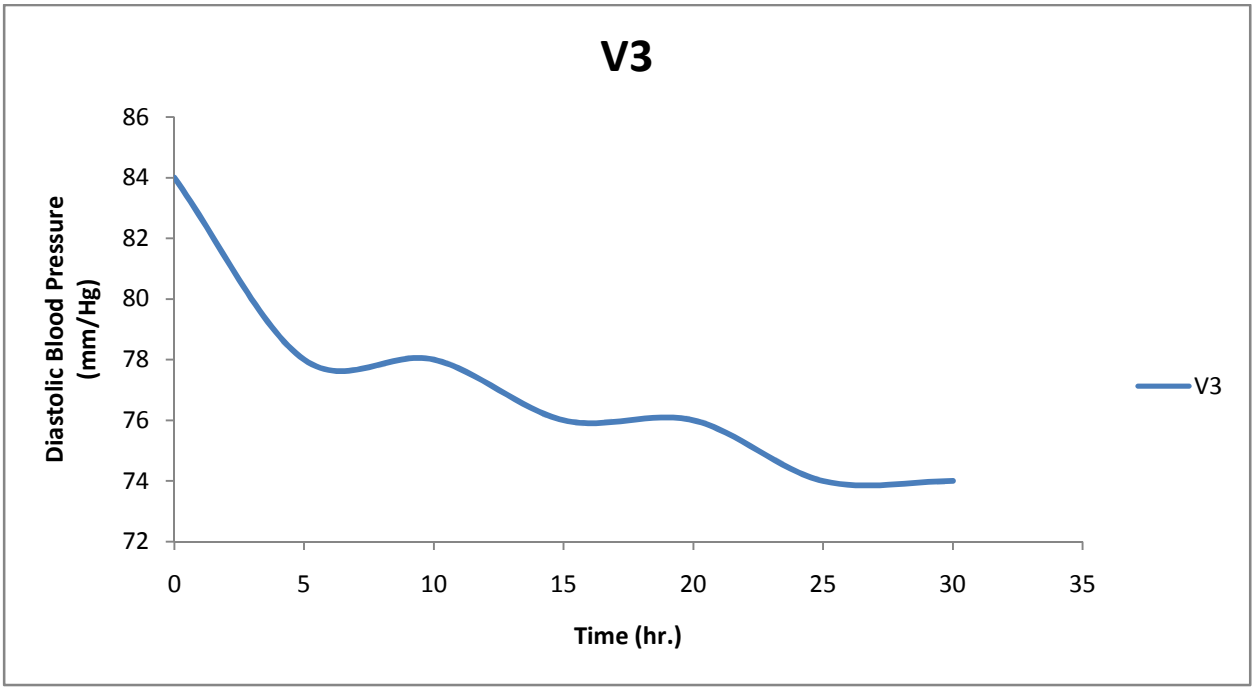
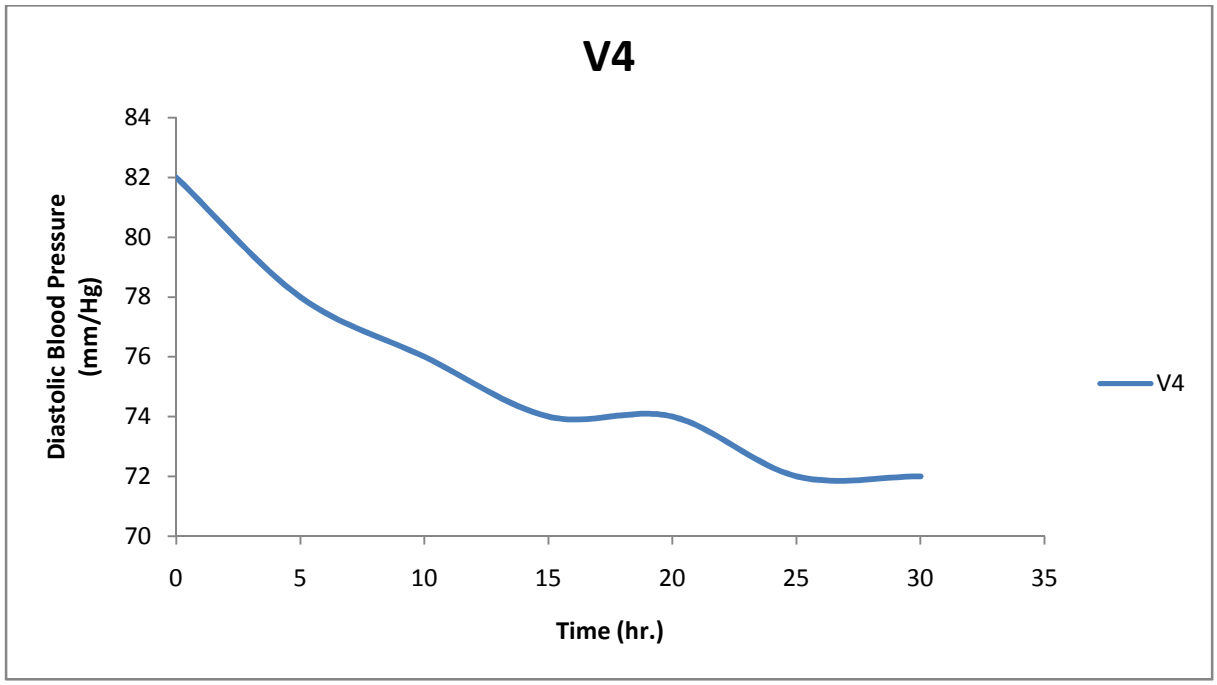


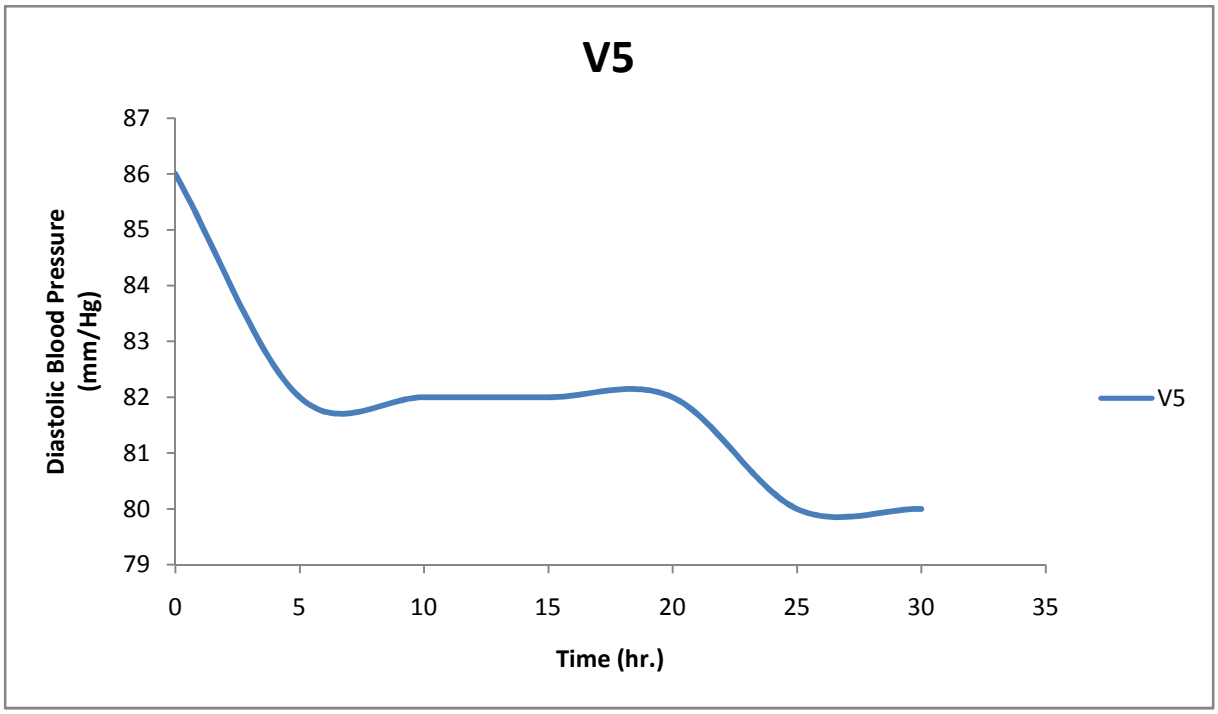
Figure-53 Individual Volunteer Systolic Blood Pressure Curve and Average Systolic Blood Pressure Curve During Fixed Dose Containing Losartan with Hydrochlorothiazide Treatment

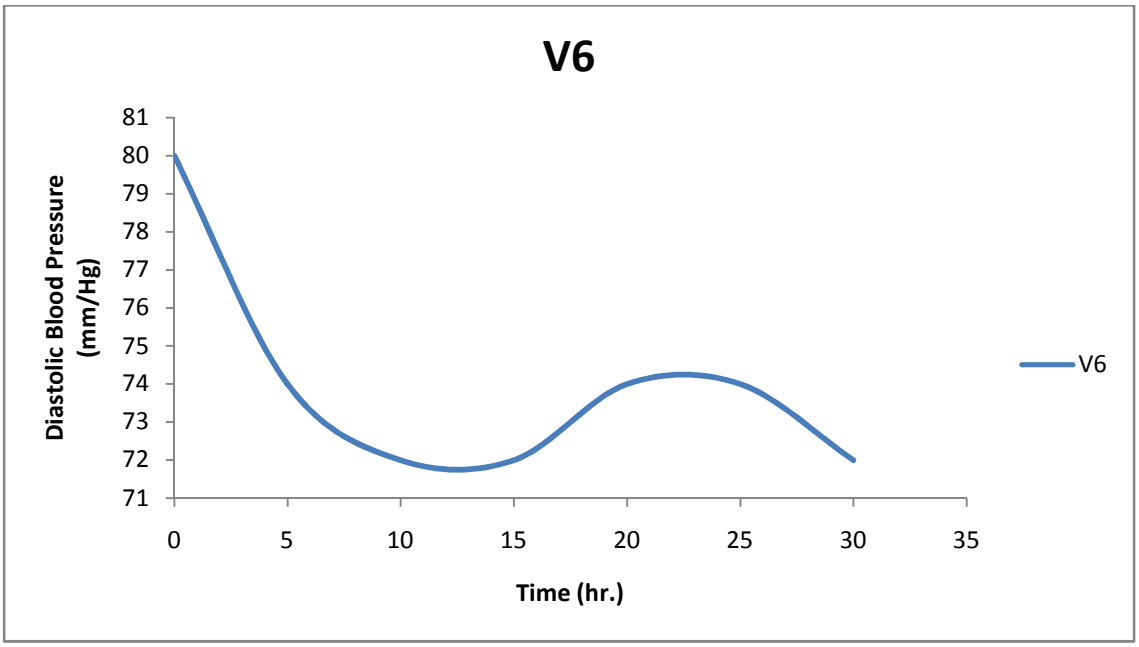



V2
Diastolic Blood Pressure (mm/Hg)
74.5
74
73.5
73
72.5
72
71.5
71
70.5
70
69.5
0
5
10
15
20
25
30
35
Time (hr.)
V2


V3
Diastolic Blood Pressure (mm/Hg)
86
84
82
80
78
76
74
72
0
5
10
15
20
25
30
35
Time (hr.)
V3


V4
Diastolic Blood Pressure (mm/Hg)
84
82
80
78
76
74
72
70
0
5
10
15
20
25
30
35
Time (hr.)
V4


V5
Diastolic Blood Pressure (mm/Hg)
87
86
85
84
83
82
81
80
79
0
5
10
15
20
25
30
35
Time (hr.)
V5



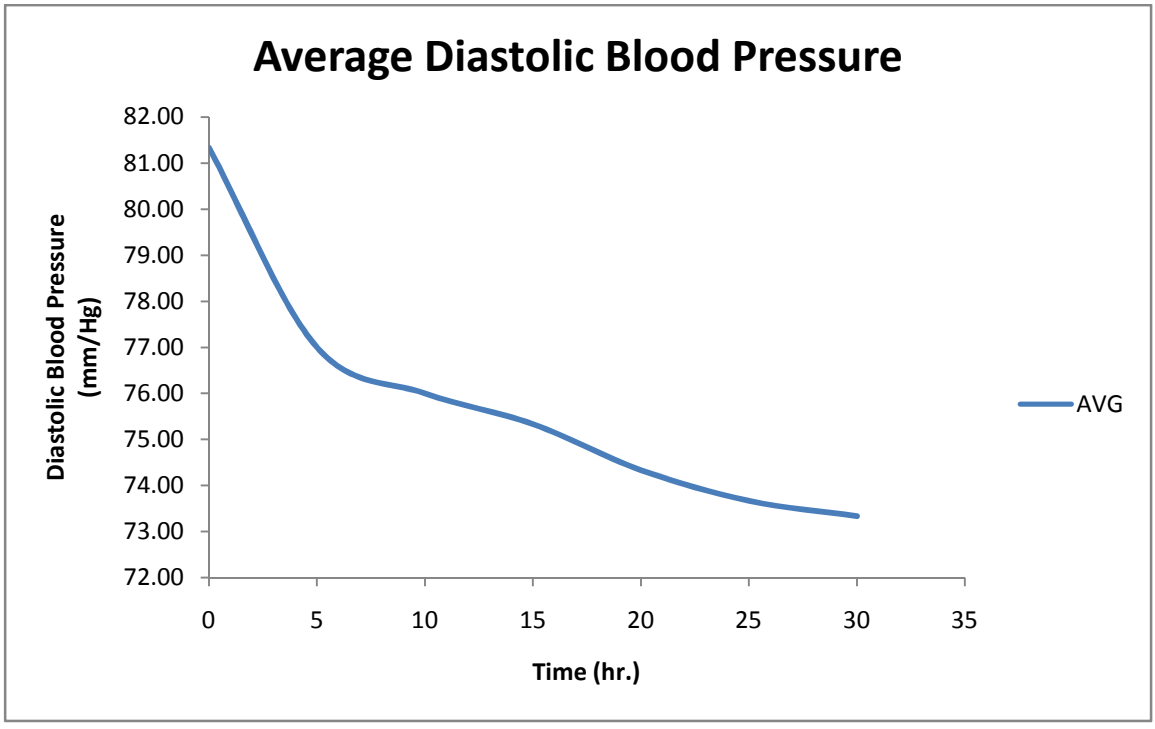


Figure-54 Individual Volunteer Diastolic Blood Pressure Curve and Average Diastolic Blood Pressure Curve During Fixed Dose Containing Losartan with Hydrochlorothiazide Treatment

CHAPTER: 6

OVERALL SUMMARY & CONCLUSION

6.Overall Summary and conclusion :

Hypertension depends on peripheral resistance and blood volume of the body. It also depends on cardiac output which rate depends on heart muscle. Among the various medication of hypertension one of the medication is the Angiotensin receptor blocker in which Losartan is one of the important drug. Losartan is a nonpeptide angiotensin II receptor antagonist acts on Renin Angiotensin Aldesterone system. Losartan block the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor found in many tissues, so decrease the peripheral resistance by blocking the vasoconstrictor action and decrease the total blood volume in the body. Other hand Hydrochlorothiazide is a low celing thiazide diuretic. It reduces the reabsorption of electrolytes from the renal tubules. This results in increased excretion of water and electrolytes, including sodium, potassium, chloride, and magnesium. So hydrochlorothiazide decreases the total blood volume of the body.

After dosing of single drug containing Losartan Potassium 50mg oral tablet and fixed dose combination drug containing Losartan Potassium 50mg with Hydrochlorothiazide 12.5mg as a single dose with randomization in 6 human healthy volunteers and was collect blood plasma after dosing at several times interval and calculate the concentration of the Losartan and Hydrochlorothiazide in this human healthy volunteers plasma which is binding with the plasma protein of the blood. After literature survey and during analysis it was observed that 14% losartan drug molecule converted into its active metabolite Losartan Carboxylic Acid by hepatic enzyme CYP450 in the liver. According to literature survey it was observed that losartan carboxylic acid is 40 times more potent than actual drug molecule losartan. These drugs concentration analysis by Liquid Chromatography Quadruple Tandem Mass Spectrometry (API-2000).

The literature survey reveals that there were already plenty of published articles describing the methods for determination of the Losartan, Losartan Carboxylic acid and Hydrochlorothiazide separately in human plasma. Method development and validation for the simultaneous determination of both the drugs in human plasma is still not available. The present study deals with development of a LC-MS/MS method using gradation technique. The developed method for simultaneous determination and quantification of Losartan, Losartan Carboxylic acid and Hydrochlorothiazide in human plasma was also validated as per the US-FDA guidelines. The

validation parameters found within the specified regulatory limit, hence acceptable. The present method also has a short run time (7.0 min) and easy extraction process. Therefore, the developed method was found to be simple, specific, highly selective, sensitive and reproducible. This was applied in the analysis of the volunteer plasma samples obtained from the comparative pharmacokinetic study.

The clinical phase of the comparative pharmacokinetic study was carried out in accordance with the supervisions of the Ethics Committee and all other pertinent requirements of the ICH [Step 6] 'Guidance on Good Clinical Practice'. Total six healthy human volunteers of 32.61 ± 6.31 years (average age) and 20.86 ± 1.48 kg/m^2 (average BMI) were exposed to the drugs in a randomize manner (table-1). None of the volunteers complained of any adverse reaction during the entire clinical study period.

On the comparative pharmacokinetic study it was observed that when single drug containing Losartan Potassium 50mg administered produced the maximum plasma concentration of 217.11 ± 50.10ng/ml (C_{max}) at the time 1.75± 0.75hr. (t_{max}) for Losartan and the average maximum plasma concentration of 6 volunteers is 207.93 ng/ml and Tmax 1.50hrs. and AUC_{0-t} 788.56 ng/ml at 48hr. and AUC $_{0-\alpha}$ 791.34 ng/ml at infinitive time, plasma half life of single dose losartan 5.21hr and elimination constant K_{el} value 0.13hr and at that same time the maximum plasma concentration of Losartan Carboxylic acid produced in the body is 462.45±62.22 ng/ml (C_{max}) at the time(t_{max}) 2.75±0.25 hr. and the average maximum plasma concentration of 6 volunteers is 458.74 ng/ml and t_{max} 2.67hrs. and AUC_{0-t} 2457.57 ng/ml at 48hr. and AUC $_{0-\alpha}$ 2468.56 ng/ml at infinitive time, plasma half life ($T_{½}$) of losartan carboxylic acid 5.71hr and elimination constant K_{el} value 0.12hr. During this time blood pressure reading observed which showed that during the treatment of single drug systolic blood pressure of each volunteer varied like 131±7mm/Hg. The average maximum systolic blood pressure of 6 volunteers is 134.67mm/Hg and lowest blood pressure is 129.67mm/Hg and diastolic blood pressure of each volunteer was varied like 82±8mm/Hg and the average maximum blood diastolic blood pressure of 6 volunteers is 86mm/Hg and lowest blood pressure 78.33mm/Hg.

But when treated with fixed dose combined drug containing Losartan Potassium 50mg with Hydrochlorothiazide 12.5mg then it was found 49.63± 8.87 ng/ml (C_{max}) at the time 2.75± 0.25hr. (t_{max}) for HCTZ and the average maximum plasma concentration of 6 volunteers is

49.87 ng/ml and t_{max} 2.75hrs. and AUC_{0-t} 227.02 ng/ml at 48hr. and AUC $_{0-\alpha}$ 227.67 ng/ml at infinitive time, plasma half life of hydrochlorothiazide 4.91hr and elimination constant K_{el} value 0.14hr. and Losartan Carboxylic Acid and Losartan produced C_{max} of 530.635± 66.425ng/ml and 399.06± 73.29ng/ml at the time 2.50± 0.50hr. and 1.25.± 0.25 (t_{max}) and The average maximum plasma concentration of 6 volunteers is 543.56 ng/ml and t_{max} 2.42hrs. and AUC_{0-t} 1662.73 ng/ml at 48hr. and AUC $_{0-\alpha}$ 1666.18 ng/ml at infinitive time, plasma half life 5.35hr and elimination constant K_{el} value 0.13hr for Losartan Carboxylic Acid and the average maximum plasma concentration of 6 volunteers is 383.59 ng/ml and t_{max} 1.25hrs. and AUC_{0-t} 1467.90 ng/ml at 48hr. and AUC $_{0-\alpha}$ 1473.02 ng/ml at infinitive time, plasma half life 5.53hr and elimination constant K_{el} value 0.13hr for Losartan. During this time blood pressure reading observed which showed that during the treatment of fixed dose combined drug systolic blood pressure of each volunteer was varied like 128±8mm/Hg. The average maximum systolic blood pressure of 6 volunteers is 135.33mm/Hg and lowest systolic blood pressure 121.33mm/Hg and diastolic blood pressure of each volunteer was varied like 76±6mm/Hg and the average maximum blood diastolic blood pressure of 6 volunteers is 81.33mm/Hg and lowest diastolic blood pressure 73.33mm/Hg.

From this report it is concluded that after fixed dose combined drug(Losartan with Hydrochlorothiazide) treatment the maximum plasma concentration of losartan and losartan carboxylic acid were too much higher than the maximum plasma concentration of losartan and losartan carboxylic acid after single drug containing losartan only. From the literature survey it was revealed that losartan carboxylic acid is 40 times more potent than the losartan itself. So during fixed dose treatment systolic blood pressure was too much lower than the systolic blood pressure during the treatment of single drug and diastolic blood pressure also too much lower during treatment of fixed dose drug than the treatment of single dose drug. Although diastolic blood pressure depend on the cardiac itself but systolic blood pressure depends on the peripheral resistance and also on the blood volume of the body. Losartan cannot affect on the cardiac muscle but losartan carboxylic acid 10-40times more potent on AT1 receptor and also on the AT2 receptor and has minor effect on the cardiac muscle by blocking the aldesterone effect. Losartan Carboxylic acid area under curve greater than the losartan and volume of distribution more than the losartan and eliminartion constant rate approximately similar. Again fixed dose

combination contains hydrochlorothiazide which act as a diuretic and reduced the blood volume by increasing electrolyte excretion and water excretion by increasing dieresis effect. But maximum plasma concentration of losartan carboxylic acid increase during treatment of fixed dose combination because hydrochlorothiazide act on the metabolism of losartan to losartan carboxylic acid.

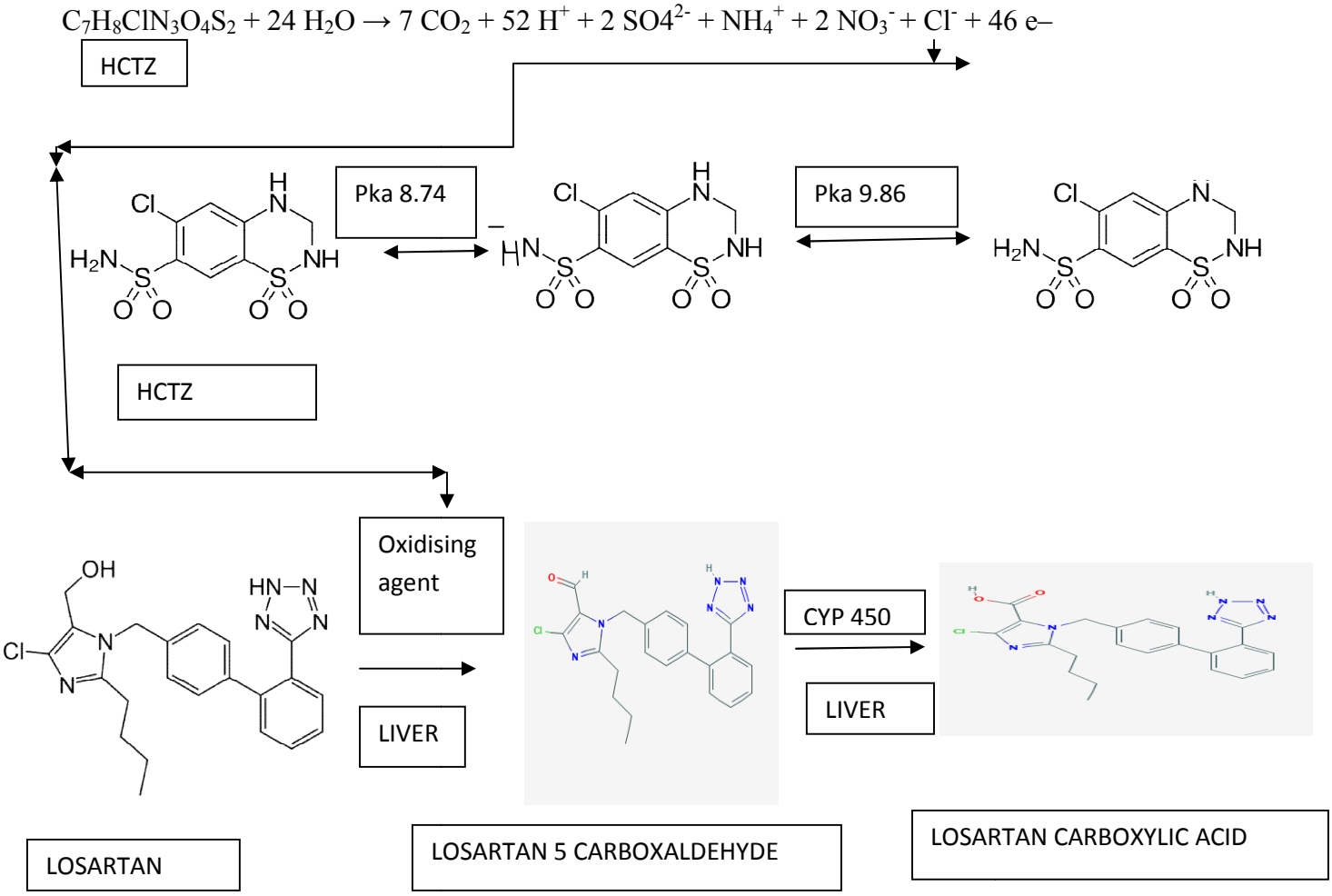


Figure: 55 Action of Hydrochlorothiazide on conversion of Losartan to Losartan Carboxylic Acid

At neutral P^H hydrochlorothiazide can be degraded by electrochemical oxidation in water due to the attack of hydroxyl radical, as a result aromatic intermediates, aliphatic carboxylic acid and inorganic anions were detected as main products. During electrochemical oxidation reaction of HCTZ, the influence of initial P^H significantly influence the generation of hydroxyl redical and then presence of an acidic sulfonamide moiety ($-SO_2NH_2$) in the chemical structure of

hydrochlorothiazide (HCTZ) allows hydrochlorothiazide to be present in its neutral and deprotonated form in biological fluid. In the electrochemical oxidation reaction chloride ion was formed at neutral P^H which act as an oxidizing agent and converted into losartan 5-carboxaldehyde which act as an anti-inflammatory and antiaggregatory agent then it converted into losartan carboxylic acid by CYP 450 enzyme in the liver. Losartan also act as an anti-inflammatory and antiaggregatory agent due to the formation of losartan 5-carboxaldehyde. Losartan carboxylic acid is the main AT1 receptor blocker.

It was concluded that fixed dose combination (Losartan with Hydrochlorothiazide) drug is more susceptible than single dose of Losartan as therapeutic sight in terms of therapeutic efficacy because hydrochlorothiazide act as an diuretic and decrease the blood volume so synergistic effect occurs with losartan and reduce the hypertension more than the single dose of Losartan alone. Again hydrochlorothiazide acts as an oxidizing agent with its degradation product and converts more efficiently from losartan to losartan carboxylic acid to increase the action of AT1 blocker more than the single dose of Losartan. Losartan with Hydrochlorothiazide is therapeutically active more than single dose containing Losartan only.

Finally it is concluded that fixed dose combination drug better than single drug and in this case hydrochlorothiazide is more susceptible combined drug with losartan for the treatment of hypertension.

CHAPTER-7

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ANNEXURE

Appendix I	Protocol of this study
Appendix II	Hurip Independent BioEthics committee

PROTOCOL TITLE

A pilot, open label, single dose, oral bioavailability study of marketed single tablet containing losartan potassium 50 mg and FDC tablet containing losartan potassium 50 mg and hydrochlorothiazide 12.5 mg in 6 healthy, adult, human, male subjects under fasting conditions to establish the Rationale of Hydrochlorothiazide in Combination with Losartan.

Protocol Number: Research/JU/01/2018

Version no. 1.0

Date: 30/03/2018

Clinical Investigator

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Declaration by the Study Team

We, the undersigned have read and understood this protocol and hereby agree to conduct the study in accordance with this protocol and to comply with all requirements regarding the obligations of investigators and all other pertinent requirements of the International Conference on Harmonization (ICH) [Step 4] 'Guideline for Good Clinical Practice (GCP) E6 [R1].', Good Laboratory Practice (GLP), Amended version of Schedule Y [2005], Central Drugs Standards Control Organization CDSCO India, Indian Council on Medical Research (ICMR) Guidelines for Biomedical Research on Human Participants, Declaration of Helsinki and other regulatory requirements.

We agree to comply with all relevant Standard Operating Procedures (SOPs) required for the conduct of this study. We further agree to ensure that all associates assisting in the conduct of this study will be informed regarding their obligations.

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- Appendix I Informed Consent Form [English]
- Appendix II Informed Consent Form [Bengali]
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- Appendix VII Laboratory parameters with reference range
- Appendix VIII Table of Study Diet

PROTOCOL SYNOPSIS

Study Title	A pilot, open label, single dose, oral bioavailability study of marketed single tablet containing losartan potassium 50 mg and FDC tablet containing losartan potassium 50 mg and hydrochlorothiazide 12.5 mg in 6 healthy, adult, human, male subjects under fasting conditions to establish the Rationale of Hydrochlorothiazide in Combination with Losartan.
Objective:	To establish the Rationale of Hydrochlorothiazide in Combination with Losartan.
Trial Design:	A pilot, open label, single dose, oral bioavailability study under fasting conditions.
Subjects:	6 healthy, adult, human, male subjects.
Screening Procedures:	Demographic data, medical and medication histories, physical examination, height, weight, vital signs, hematology, biochemistry and serology will be done at screening.
Housing:	Housing from at least 11 hr prior to drug administration until after the 24 hr blood sampling.
Treatments	• FDC tablet containing losartan potassium 50 mg and hydrochlorothiazide 12.5 mg • Tablet containing losartan potassium 50 mg
Drug Administration:	Single tablet of marketed product will be orally administered to each subject in each period with 240± 2 mL of water in sitting posture after an overnight fast of at least 10 hr. The subjects will receive a standardized meal 4.0 hr post-dose. Water will be permitted *ad libitum* except for 1.0 hr before and until 1.0 hr post dose.
Blood sampling:	In each of the study period, 16 blood samples will be collected in 5 mL K_2EDTA vacutainers via an indwelling catheter placed in one of the forearm vein. Blood samples will also be collected by direct venipuncture during ambulatory blood sampling visit as well as wherever necessary for any practical reason. The post-dose blood samples will be collected at 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 24.0, 36.0 and 48.0 hrs. All the blood samples for a particular sample time point, will be centrifuged under refrigeration at 3500 rpm and 4°C for 10 min. The resulting plasma will be separated and stored in suitably labeled polypropylene tubes at –20 ± 5°C for pending assay. The total volume of blood drawn including the volume necessary for the laboratory tests [screening & safety sample], PK analysis and volume of blood discarded before each blood draw will be about 182 mL per subject for the entire study.
Subject Monitoring:	Clinical examination and vital signs measurements [blood pressure, pulse rate, respiration rate and oral temperature] will be recorded at each check-in, check-out. Vital signs will also be recorded before dosing of investigational products. Clinical examination and measurement of vital signs may also be carried out at any time during the conduct of the study if the Investigator/Doctor feels necessary. In case of abnormality in vital signs during pre-dose vitals recording, medical opinion will be taken whether to dose the subject or not. During recording of vital signs, each subject will be asked about his well being.
Washout:	At least 7 days gap between two consecutive dosing days will be maintained.
Pharmacokinetic Parameters:	C_{max}, T_{max}, AUC_{0-t}, $AUC_{0-\infty}$, K_{el}, $t_{1/2}$
Analytical Methods:	Losartan, Losartan Carboxylic Acid (LCA) & Hydrochlorothiazide in plasma will be quantified using validated LC-MS/MS method.

ABBREVIATIONS

ADR	Adverse Drug Reaction
AE	Adverse Event
AUC_{0-t}	The area under the plasma concentration versus time curve from time 0 to t
$AUC_{0-\infty}$	The area under the plasma concentration versus time curve from time 0 to infinity
BMI	Body Mass Index
CDSCO	Central Drugs Standards Control Organization
C_{max}	Maximum measured plasma concentration
CFR	Code of Federal Regulation
CRF	Case Record Form
DCGI	Drugs Controller General of India
EDTA	Ethylene diamine tetra acetic acid
GCP	Good Clinical Practice
Hb	Hemoglobin
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
h/hr	Hour
ICF	Informed Consent Form
ICH	International Conference on Harmonization
ICMR	Indian Council of Medical Research
K_{el}	Elimination rate constant
LC-MS/MS	Liquid Chromatography-Tandem Mass spectrometry
LFT	Liver Function Test
LH	Luteinizing Hormone
LOQ	Limit of Quantification
mL	Milliliter
mmHg	Millimeter of Mercury
PK	Pharmacokinetic
QA	Quality Assurance
RPM/ rpm	Revolutions per minute
SAE	Serious Adverse Event
SD	Standard Deviation
SEM	Standard Error of the Mean
SOP	Standard Operating Procedure
$t_{1/2}$	Elimination half life
T_{max}	Time to maximum concentration
USFDA	United States Food and Drug Administration

1.0 BACKGROUND AND PHARMACOKINETICS

DESCRIPTION

Losartan is an angiotensin II receptor (type AT_1) antagonist. Losartan potassium, a non-peptide molecule, is chemically described as 2-butyl-4-chloro-1-[p-(o-1H-tetrazol-5-ylphenyl) benzyl] imidazole-5-methanol monopotassium salt. Its molecular formula is $C_{22}H_{22}ClKN_6O$.It is a white to off-white powder with a molecular weight of 461.01. It is freely soluble in water and slightly soluble in acetonitrile. Oxidation of the 5-hydroxymethyl group on the imidazole ring results in the active metabolite of Losartan.

Hydrochlorothiazide is a diuretic and antihypertensive. It is the 3,4-dihydro derivative of chlorothiazide. It is chemically designated as 6-chloro-3,4-dihydro-2 H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide. It is in a class of medications called Thiazide Diuretics.It is a white, or practically white, crystalline powder which is slightly soluble in water, freely soluble in sodium hydroxide solution, in n-butylamine, and in dimethylformamide; sparingly soluble in methanol; insoluble in ether, in chloroform, and in dilute mineral acids. The molecular weight is 297.74. The mechanism of action depends on renal prostaglandin production.

CLINICAL PHARMACOLOGY

Pharmacodynamics

The combination therapy of Losartan and Hydrochlorothiazide have been shown to have an additive effect on blood pressure reduction, reducing blood pressure to a greater degree than either component alone. This effect is thought to be a result of the complimentary actions of both components. Further, as a result of its diuretic effect, hydrochlorothiazide increases plasma renin activity, increases aldosterone secretion, decreases serum potassium, and increases the levels of angiotensin II.

Administration of losartan blocks all the physiologically relevant actions of angiotensin II and through inhibition of aldosterone could tend to attenuate the potassium loss associated with the diuretic.

Losartan has been shown to have a mild and transient uricosuric effect. Hydrochlorothiazide has been shown to cause modest increases in uric acid; the combination of losartan and hydrochlorothiazide tends to attenuate the diuretic-induced hyperuricemia.

The antihypertensive effect of fixed dose combination of Losartan and Hydrochlorothiazide is sustained for a 24-hour period. In clinical studies of at least one year's duration, the antihypertensive effect was maintained with continued therapy. Despite the significant decrease in blood pressure, administration of combination of losartan and hydrochlorothiazide had no clinically significant effect on heart rate. In clinical trials, after 12 weeks of therapy with losartan 50 mg/hydrochlorothiazide 12.5 mg, trough sitting diastolic blood pressure was reduced by an average of up to 13.2 mmHg. Combination therapy of losartan and hydrochlorothiazide is effective in reducing blood pressure in males and females, blacks and non-blacks and in younger (<65 years) and older (≥65 years) patients and is effective in all degrees of hypertension.

Losartan

Losartan is a synthetically produced oral angiotensin-II receptor (type AT1) antagonist. Angiotensin II, a potent vasoconstrictor, is the primary active hormone of the renin-angiotensin system and an important determinant of the pathophysiology of hypertension. Angiotensin II binds to the AT1 receptor found in many tissues (e.g. vascular smooth muscle, adrenal gland, kidneys and the heart) and elicits several important biological actions, including vasoconstriction and the release of aldosterone. Angiotensin II also stimulates smooth-muscle cell proliferation. Losartan selectively blocks the AT_1 receptor. *In vitro* and *in vivo* losartan and its pharmacologically active metabolite, Losartan carboxylic acid blocks all physiologically relevant actions of angiotensin II, regardless of the source or route of its synthesis.

Losartan does not have an agonist effect nor does it block other hormone receptors or ion channels important in cardiovascular regulation. Furthermore, losartan does not inhibit ACE (kininase II), the enzyme that degrades bradykinin. Consequently, there is thus no increase in bradykinin-mediated undesirable effects.

During the administration of losartan the removal of the angiotensin II negative feedback on rennin secretion leads to increased plasma-renin activity (PRA). Increase in the PRA leads to an increase in angiotensin II in plasma. Despite these increases, antihypertensive activity and suppression of the plasma aldosterone concentration are maintained, indicating effective angiotensin II receptor blockade. After the discontinuation of losartan, PRA and angiotensin II values fell within 3 days to the baseline values. Both losartan and its principal active metabolite have a far greater affinity for the AT_1 receptor than for the AT_2 receptor. The active metabolite is 10- to 40-times more active than losartan on a weight for weight basis.

Losartan has no effect on autonomic reflexes and no sustained effect on plasma norepinephrine. In patients with left ventricular failure, 25 mg and 50 mg doses of losartan produced positive hemodynamic and neurohormonal effects characterized by an increase in cardiac index and decreases in pulmonary capillary wedge pressure, systemic vascular resistance, mean systemic arterial pressure and heart rate and a reduction in circulating levels of aldosterone and norepinephrine, respectively. The occurrence of hypotension was dose related in these heart failure patients.

Hydrochlorothiazide

Hydrochlorothiazide is a thiazide diuretic. The mechanism of the antihypertensive effect of thiazide diuretics is not fully known. Thiazides affect the renal tubular mechanisms of electrolyte reabsorption, directly increasing excretion of sodium and chloride in approximately equivalent amounts. The diuretic action of hydrochlorothiazide reduces plasma volume, increases plasma renin activity and increases aldosterone secretion, with consequent increases in urinary potassium and bicarbonate loss, and decreases in serum potassium. The renin-aldosterone link is mediated by angiotensin II and therefore co-administration of an angiotensin II receptor antagonist tends to reverse the potassium loss associated with thiazide diuretics.

After oral use, diuresis begins within 2 hours, peaks in about 4 hours and lasts about 6 to 12 hours the antihypertensive effect persists for up to 24 hours.

Pharmacokinetics

Losartan

Absorption: Following oral administration, losartan is well absorbed and undergoes first-pass metabolism, forming an active carboxylic acid metabolite and other inactive metabolites. The systemic bioavailability of losartan tablets is approximately 33%. Mean peak concentrations of losartan and its active metabolite are reached in 1 hour and in 3-4 hours, respectively. There was no clinically significant effect on the plasma concentration profile of losartan when the drug was administered with a standardized meal.

Distribution: Both losartan and its active metabolite are ≥99% bound to plasma proteins, primarily albumin. The volume of distribution of losartan is 34 lit. Studies in rats indicate that losartan crosses the bloodbrain barrier poorly, if at all.

Biotransformation: About 14% of an intravenously- or orally-administered dose of losartan is converted to its active metabolite. Following oral and intravenous administration of ^{14}C-labeled losartan potassium, circulating plasma radioactivity primarily is attributed to losartan and its active metabolite. Minimal conversion of losartan to its active metabolite was seen in about one percent of individuals studied.

In addition to the active metabolite, inactive metabolites are formed, including two major metabolites formed by hydroxylation of the butyl side chain and a minor metabolite, an N-2 tetrazole glucuronide.

Elimination: Plasma clearance of losartan and its active metabolite is about 600 mL/min and 50 mL/min, respectively. Renal clearance of losartan and its active metabolite is about 74 mL/min and 26 mL/min, respectively. When losartan is administered orally, about 4% of the dose is excreted unchanged in the urine, and about 6% of the dose is excreted in the urine as active metabolite. The pharmacokinetics of losartan and its active metabolite are linear with oral losartan potassium doses up to 200 mg. Following oral administration, plasma

concentrations of losartan and its active metabolite decline polyexponentially with a terminal half-life of about 2 hours and 6-9 hours, respectively. During oncedaily dosing with 100 mg, neither losartan nor its active metabolite accumulates significantly in plasma.

Both biliary and urinary excretion contribute to the elimination of losartan and its metabolites. Following an oral dose of ^{14}C-labeled losartan in man, about 35% of radioactivity is recovered in the urine and 58% in the feces.

Hydrochlorothiazide

Absorption: Hydrochlorothiazide is well absorbed (65% to 75%) following oral administration with onset of action occurring within one hour, and the duration of action is 6 to 12 hours.Absorption of hydrochlorothiazide is reduced in patients with congestive heart failure. After single oral dose, Hydrochlorothiazide achieves peak concentrations in 1 to 2 hours and has a half-life of 6.5 to 9 hours.The bioavailability is 71%.When it is administered with food, its bioavailability is reduced by 10%, the maximum plasma concentration is reduced by 20%, and the time to maximum concentration increases from 1.6 to 2.9 hours. The absorption efficiency of hydrochlorothiazide appears to be independent of dose

Distribution: It is distributed throughout the extracellular space, with essentially no tissue accumulation except in the kidney. Hydrochlorothiazide is approximately 40% protein bound and accumulates in erythrocytes by an unknown mechanism. The ratio between red blood corpuscles and plasma is 3.5:1. The volume of distribution of Hydrochlorothiazide is approximately 3 – 4 L/kg.

Metabolism: Hydrochlorothiazide is not metabolized but is eliminated rapidly by the kidney.

Excretion: When plasma levels have been followed for at least 24 hours, the plasma half-life has been observed to vary between 5.6 and 14.8 hours with long-term dosing. At least 61% of the oral dose is eliminated unchanged within 24 hours. Hydrochlorothiazide crosses the placental but not the blood-brain barrier and is excreted in breast milk. In patients with renal disease, plasma concentrations of hydrochlorothiazide are increased and the elimination half-life is prolonged.

USE IN SPECIFIC POPULATIONS

Pregnancy: The Losartan and Hydrochlorothiazide combined therapy is not recommended during the first trimester of pregnancy. The use of Losartan and Hydrochlorothiazide combined therapy is contra-indicated during the 2nd and 3rd trimester of pregnancy. Epidemiological evidence regarding the risk of teratogenicity following exposure to ACE inhibitors during the first trimester of pregnancy has not been conclusive; however a small increase in risk cannot be excluded. Whilst there is no controlled epidemiological data on the risk with Angiotensin II Receptor Inhibitors (AIIRAs), similar risks may exist for this class of drugs. Unless continued ARB therapy is considered essential, patients planning pregnancy should be changed to alternative antihypertensive treatments which have an established safety profile for use in pregnancy. When pregnancy is diagnosed, treatment with Losartan and Hydrochlorothiazide combined therapy should be stopped immediately and, if appropriate, alternative therapy should be started.

Losartan and Hydrochlorothiazide combined therapy exposure during the second and third trimesters is known to induce human fetotoxicity (decreased renal function, oligohydramnios, skull ossification retardation) and neonatal toxicity (renal failure, hypotension, hyperkalaemia). Should exposure to Losartan and Hydrochlorothiazide combined therapy have occurred from the second trimester of pregnancy, ultrasound check of renal function and skull is recommended. Infants whose mothers have taken Losartan and Hydrochlorothiazide combined therapy should be closely observed for hypotension.

Hydrochlorothiazide may reduce both plasma volume and uteroplacental blood flow. Thiazides pass the placental barrier and are found in cord blood. They may cause fetal electrolyte disturbances and possibly other reactions that have been observed in adults. Cases of thrombocytopenia in neonates and fetal or neonatal jaundice were reported after treating the mothers with thiazides.

Lactation: It is not known whether losartan is excreted in human milk. However, losartan is excreted in the milk of lactating rats. Thiazides pass into human milk and may inhibit lactation. Because of the potential for adverse effects on the nursing infant, Losartan and Hydrochlorothiazide combined therapy is contraindicated during breast-feeding.

Geriatric Use: Dosage adjustment is not usually necessary for the elderly.

Pediatric and adolescent Use: There is no experience in children and adolescents. Therefore, losartan/hydrochlorothiazide should not be administered to children and adolescents.

Hepatic Impairment: Losartan and Hydrochlorothiazide combined therapy is contraindicated in patients with severe hepatic impairment

Use in patients with renal impairment and hemodialysis patients: No initial dosage adjustment is necessary in patients with moderate renal impairment (i.e. creatinine clearance 30-50 ml/min). Losartan and hydrochlorothiazide tablets are not recommended for haemodialysis patients. Losartan/Hydrochlorothiazide tablets must not be used in patients with severe renal impairment (i.e. creatinine clearance <30 ml/min)

Use in patients with intravascular volume depletion: Volume and /or sodium depletion should be corrected prior to administration of losartan/hydrochlorothiazide tablets.

INTERACTIONS

Losartan

Rifampicin and fluconazole have been reported to reduce levels of active metabolite. The clinical consequences of these interactions have not been evaluated.

As with other drugs that block angiotensin II or its effects, concomitant use of potassium-sparing diuretics (e.g., spironolactone, triamterene, amiloride), potassium supplements, or salt substitutes containing potassium may lead to increases in serum potassium. Co-medication is not advisable.

As with other medicines which affect the excretion of sodium, lithium excretion may be reduced. Therefore, serum lithium levels should be monitored carefully if lithium salts are to be co-administered with angiotensin II receptor antagonists.

When angiotensin II antagonists are administered simultaneously with NSAIDs (i.e. selective COX-2 inhibitors, acetylsalicylic acid at anti-inflammatory doses) and non-selective NSAIDs, attenuation of the antihypertensive effect may occur. Concomitant use of angiotensin II antagonists or diuretics and NSAIDs may lead to an increased risk of worsening of renal function, including possible acute renal failure, and an increase in serum potassium, especially in patients with poor pre-existing renal function. The combination should be administered with caution, especially in the elderly. Patients should be adequately hydrated and consideration should be given to monitoring renal function after initiation of concomitant therapy, and periodically thereafter.

Hydrochlorothiazide

Alcohol, barbiturates, narcotics or antidepressants: Potentiation of orthostatic hypotension may occur.

Antidiabetic drugs (oral agents and insulin): The treatment with a thiazide may influence the glucose tolerance. Dosage adjustment of the antidiabetic drug may be required. Metformin should be used with caution because of the risk of lactic acidosis induced by possible functional renal failure linked to hydrochlorothiazide.

Other antihypertensive drugs: It may increase the effect of anti-hypertensive agents such as guanethidine sulfate, methyldopa, or a ganglionic blocking agent. Hydrochlorothiazide has been reported to reduce the response to pressor amines, such as noradrenaline, but the clinical significance of this effect is uncertain.

Cholestyramine and colestipol resins: Absorption of hydrochlorothiazide is impaired in the presence of anionic exchange resins.

Skeletal muscle relaxants, nondepolarizing (e.g., tubocurarine): Possible increased responsiveness to the muscle relaxant.

Lithium: Diuretic agents reduce the renal clearance of lithium and add a high risk of lithium toxicity; concomitant use is not recommended.

Anticholinergic agents (e.g. atropine, biperiden): Increase of the bioavailability to thiazide-type diuretics by decreasing gastrointestinal motility and stomach emptying rate.

Salicylates: In case of high dosages of salicylates hydrochlorothiazide may enhance the toxic effect of the salicylates on the central nervous system.

Methyldopa: There have been isolated reports of haemolytic anaemia occurring with concomitant use of hydrochlorothiazide and methyldopa.

Digitalis glycosides: Thiazide-induced hypokalaemia or hypomagnesaemia may favour the onset of digitalis-induced cardiac arrhythmias.

Calcium salts: Thiazide diuretics may increase serum calcium levels due to decreased excretion. If calcium supplements must be prescribed, serum calcium levels should be monitored and calcium dosage should be adjusted accordingly.

Carbamazepine: Risk of symptomatic hyponatremia. Clinical and biological monitoring is required.

Iodine Contrast Media: In case of diuretic-induced dehydration, there is an increased risk of acute renal failure; especially with high doses of the iodine product. Patients should be rehydrated before the administration.

Amphotericin B (parenteral), corticosteroids, ACTH or stimulant laxatives: Hydrochlorothiazide may intensify electrolyte imbalance, particularly hypokalaemia

CONTRAINDICATIONS

- Hypersensitivity to losartan, sulphonamide-derived substances (hydrochlorothiazide) or any excipients
- Therapy resistant hypokalaemia or hypercalcaemia
- Severe hepatic impairment; Cholestasis and biliary obstructive disorders.
- Refractory hyponatraemia
- Symtomatic hyperuricaemia/gout
- 2nd and 3rd trimester of pregnancy
- Lactation
- Severe renal impairment (i.e. creatinine clearance <30 ml/min)
- Anuria

INDICATIONS

Hypertension: Fixed dose combination of Losartan and Hydrochlorothiazide is indicated for the treatment of hypertension. This fixed dose combination is not indicated for initial therapy of hypertension, except when the hypertension is severe enough that the value of achieving prompt blood pressure control exceeds the risk of initiating combination therapy in these patients.

Hypertensive Patients with Left Ventricular Hypertrophy: Fixed dose combination of Losartan and Hydrochlorothiazide is indicated to reduce the risk of stroke in patients with hypertension and left ventricular hypertrophy, but there is evidence that this benefit does not apply to Black patients.

WARNING & PRECAUTIONS

Losartan

Angioedema: Patients with a history of angiooedema (swelling of the face, lips, throat, and/or tongue) should be closely monitored.

Hypotension and Intravascular volume depletion: Symptomatic hypotension, especially after the first dose, may occur in patients who are volume and/ or sodium-depleted by vigorous diuretic therapy, dietary salt restriction, diarrhoea or vomiting. Such conditions should be corrected before the administration of fixed dose combination of Losartan and Hydrochlorothiazide tablets.

Electrolyte imbalances: Electrolyte imbalances are common in patients with renal impairment, with or without diabetes, and should be addressed. Therefore, the plasma concentrations of potassium and creatinine clearance values should be closely monitored; especially patients with heart failure and a creatinine clearance between 30-50 ml/ min should be closely monitored.

Liver function impairment: Based on pharmacokinetic data which demonstrate significantly increased plasma concentrations of losartan in cirrhotic patients, Fixed dose combination of Losartan and Hydrochlorothiazide should be used with caution in patients with a history of mild to moderate hepatic impairment. There is no therapeutic experience with losartan in patients with severe hepatic impairment. Therefore fixed dose combination of Losartan and Hydrochlorothiazide is contraindicated in patients with severe hepatic impairment.

Renal function impairment: As a consequence of inhibiting the renin-angiotensin-aldosterone system, changes in renal function, including renal failure, have been reported (in particular, in patients whose renal function is dependent on the renin-angiotensin-aldosterone system, such as those with severe cardiac insufficiency or pre-existing renal dysfunction).

As with other drugs that affect the renin-angiotensin-aldosterone system, increases in blood urea and serum creatinine have also been reported in patients with bilateral renal artery stenosis or stenosis of the artery to a solitary kidney; these changes in renal function may be reversible upon discontinuation of therapy. Losartan should be used with caution in patients with bilateral renal artery stenosis or stenosis of the artery to a solitary kidney.

Renal transplantation: There is no experience in patients with recent kidney transplantation.

Primary hyperaldosteronism: Patients with primary aldosteronism generally will not respond to antihypertensive drugs acting through inhibition of the renin-angiotensin system. Therefore, the use of fixed dose combination of Losartan and Hydrochlorothiazide tablets is not recommended.

Coronary heart disease and cerebrovascular disease: As with any antihypertensive agents, excessive blood pressure decrease in patients with ischemic cardiovascular and cerebrovascular disease could result in a myocardial infarction or stroke.

Heart failure: In patients with heart failure, with or without renal impairment, there is - as with other drugs acting on the renin-angiotensin system - a risk of severe arterial hypotension, and (often acute) renal impairment.

Aortic and mitral valve stenosis, obstructive hypertrophic cardiomyophathy: As with other vasodilators, special caution is indicated in patients suffering from aortic or mitral stenosis, or obstructive hypertrophic cardiomyopathy.

Ethnic differences: As observed for angiotensin converting enzyme inhibitors, losartan and the other angiotensin antagonists are apparently less effective in lowering blood pressure in black people than in nonblacks, possibly because of higher prevalence of low-renin states in the black hypertensive population.

Pregnancy: Fixed dose combination of Losartan and Hydrochlorothiazide should not be initiated during pregnancy. Unless continued Losartan/ Hydrochlorothiazide therapy is considered essential, patients planning pregnancy should be changed to alternative anti-hypertensive treatments which have an established safety profile for use in pregnancy. When pregnancy is diagnosed, treatment with fixed dose combination of Losartan and Hydrochlorothiazide should be stopped immediately, and, if appropriate, alternative therapy should be started.

In January 2014, the FDA issued a black box warning that losartan can cause fetal toxicity, and should be discontinued as soon as pregnancy is detected. Using losartan while pregnant could result in fetal injury or death.

Hydrochlorothiazide

Hypotension and electrolyte/fluid imbalance: As with all antihypertensive therapy, symptomatic hypotension may occur in some patients. Patients should be observed for clinical signs of fluid or electrolyte imbalance, e.g., volume depletion, hyponatremia, hypochloremic alkalosis, hypomagnesemia or hypokalemia which may occur during intercurrent diarrhea or vomiting. Periodic determination of serum electrolytes should be performed at appropriate intervals in such patients. Dilutional hyponatraemia may occur in oedematous patients in hot weather.

Metabolic and endocrine effects: Thiazide therapy may impair glucose tolerance.Dosage adjustment of antidiabetic agents, including insulin, may be required.Latent diabetes mellitus may become manifest during thiazide therapy.

Thiazides may decrease urinary calcium excretion and may cause intermittent and slight elevation of serum calcium. Marked hypercalcemia may be evidence of hidden hyperparathyroidism.

Hepatic impairment: Thiazides should be used with caution in patients with impaired hepatic function or progressive liver disease, as it may cause intrahepatic cholestasis, and since minor alterations of fluid and electrolyte balance may precipitate hepatic coma.

Fixed dose combination of Losartan and Hydrochlorothiazide is contraindicated for patients with severe hepatic impairment.

Other: In patients receiving thiazides, hypersensitivity reactions may occur with or without a history of allergy or bronchial asthma. Exacerbation or activation of systemic lupus erythematosus has been reported with the use of thiazides.

Excipient: Patients with rare hereditary problems of galactose intolerance, the Lapp lactase deficiency or glucose-galactose malabsorption should not take this medicine.

ADVERSE REACTIONS

In clinical trials with losartan potassium salt and hydrochlorothiazide, no adverse events peculiar to this combination of substances were observed. The adverse events were restricted to those which were formerly observed with losartan potassium salt and/or hydrochlorothiazide. In controlled clinical trials for essential hypertension, dizziness was the only adverse experience reported as substance-related that occurred with an incidence greater than placebo in 1% or more of patients treated with losartan and hydrochlorothiazide.

Next to these effects, there are further adverse reactions reported after the introduction of the product to the market as follows:

- Hepato-biliary disorders: Hepatitis
- Investigations: Hyperkalaemia, elevation of ALT

Additional adverse events that have been seen with one of the individual components and may be potential adverse events with losartan potassium/ hydrochlorothiazide are the following:

Losartan

- Blood And Lymphatic System Disorders: Anaemia,Henoch-Schönlein purpura, ecchymosis, haemolysis
- Immune System Disorders: Anaphylactic reactions, angioedema, urticaria
- Metabolism And Nutrition Disorders: Anorexia, gout
- Psychiatric Disorders: Insomnia, anxiety disorder, panic disorder, confusion, depression, abnormal dreams, sleep disorder, somnolence, memory impairment.
- Nervous system disorders: Headache, dizziness, nervousness, paraesthesia, peripheral neuropathy, tremor, migraine, syncope
- Eye disorders: Blurred vision, burning/stinging in the eye, conjunctivitis, decrease in visual acuity
- Ear and labyrinth disorders: Vertigo, tinnitus
- Cardiac disorders: Hypotension, orthostatic hypotension, sternalgia, angina pectoris, grade II-AV block,cerebrovascular event, myocardial infarction, palpitation, arrhythmias (atrial fibrillations, sinus bradycardia, tachycardia, ventricular tachycardia, ventricular fibrillation)
- Vascular disorders: Vasculitis
- Respiratory, thoracic and mediastinal disorders: Cough, upper respiratory infection, nasal congestion, sinusitis, sinus disorder, pharyngeal discomfort, pharyngitis, laryngitis, dyspnoea, bronchitis, epistaxis, rhinitis, respiratory congestion
- Gastrointestinal disorders: Abdominal pain, nausea, diarrhoea, dyspepsia, constipation, dental pain, dry mouth, flatulence, gastritis, vomiting
- Hepato-biliary disorders: Liver function abnormalities
- Skin and subcutaneous tissue disorders:Alopecia, dermatitis, dry skin, erythema, flushing, photosensitivity, pruritus, rash, urticaria, sweating
- Musculoskeletal and connective tissue disorders: Muscle cramp, back pain, leg pain, myalgia, Arm pain, joint swelling, knee pain, musculoskeletal pain, shoulder pain, stiffness, arthralgia, arthritis, coxalgia, fibromyalgia, muscle weakness
- Renal and urinary disorders: Nocturia, urinary frequency, urinary tract infection
- Reproductive system and breast disorders: Decreased libido, impotence
- General disorders and administration site conditions: Asthenia, fatigue, chest pain, facial oedema, fever
- Investigations: Hyperkalaemia, mild reduction of haematocrit and haemoglobin,mild increase in urea and creatinine serum levels,Increase in hepatic enzymes and bilirubin.

Hydrochlorothiazide

- Blood and lymphatic system disorders: Agranulocytosis, aplastic anaemia, haemolytic anaemia, leukopenia, purpura, thrombocytopenia
- Immune system disorders: Anaphylactic reaction
- Metabolism and nutrition disorders: Anorexia, hyperglycaemia, hyperuricaemia, hypokalaemia, hyponatraemia
- Psychiatric disorders: Insomnia
- Nervous system disorders: Cephalalgia
- Eye disorders: Transient blurred vision, xanthopsia
- Vascular disorders: Necrotizing angiitis (vasculitis, cutaneous vasculitis)
- Respiratory, thoracic and mediastinal disorders: Respiratory distress including pneumonitis and pulmonary oedema
- Hepato-biliary disorders: Icterus (intrahepatic cholestatis), pancreatitis
- Skin and subcutaneous tissue disorders: Photosensitivity, urticaria, toxic epidermal necrolysis
- Musculoskeletal and connective tissue disorders: Muscle cramps
- Renal and urinary disorders: Glycosuria, interstitial nephritis, renal dysfunction, renal failure
- General disorders and administration site conditions: Fever, dizziness

OVERDOSE

No specific information is available on the treatment of overdosage with fixed dose combination of Losartan and Hydrochlorothiazide. Treatment is symptomatic and supportive. Therapy with fixed dose combination of Losartan and Hydrochlorothiazide should be discontinued and the patient observed closely.Suggested measures include induction of emesis if ingestion is recent, and correction of dehydration, electrolyte imbalance, hepatic coma and hypotension by established procedures.

Losartan: Limited data are available in regard to over dosage in humans. The most likely manifestation of over dosage would be hypotension and tachycardia; bradycardia could occur from parasympathetic (vagal) stimulation. If symptomatic hypotension should occur, supportive treatment should be instituted. Neither losartan nor the active metabolite can be removed by hemodialysis.

Hydrochlorothiazide: The most common signs and symptoms observed are those caused by electrolyte depletion (hypokalemia, hypochloremia, hyponatremia) and dehydration resulting from excessive diuresis. If digitalis has also been administered, hypokalemia may accentuate cardiac arrhythmias.

DOSAGE AND ADMINISTRATION

Hypertension: Dosing must be individualized. The usual starting dose of losartan is 50 mg once daily, with 25 mg recommended for patients with intravascular volume depletion and patients with a history of hepatic impairment. Losartan can be administered once or twice daily at total daily doses of 25 to 100 mg. If the antihypertensive effect measured at trough using once a day dosing is inadequate, a twice-a-day regimen at the same total daily dose or an increase in dose may give a more satisfactory response.

Severe Hypertension: The starting dose for initial treatment of severe hypertension is one tablet of Losartan/Hydrochlorothiazide 50/12.5 once daily. For patients who do not respond adequately to Losartan/Hydrochlorothiazide 50/12.5 after 2 to 4 weeks of therapy, the dosage may be increased to one tablet of Losartan/Hydrochlorothiazide 100/25 once daily. The maximum dose is one tablet of Losartan/Hydrochlorothiazide 100/25 once daily. Losartan/Hydrochlorothiazide is not recommended as initial therapy in patients with hepatic impairment because the appropriate 25 mg starting dose of losartan cannot be given. It is also not recommended for use as initial therapy in patients with intravascular volume depletion (e.g., patients treated with diuretics, Hypotension-Volume-Depleted Patients).

Hypertensive Patients with Left Ventricular Hypertrophy: Treatment should be initiated with Losartan 50 mg once daily. Hydrochlorothiazide 12.5 mg should be added or Losartan/Hydrochlorothiazide 50/12.5 substituted if the blood pressure reduction is inadequate. If additional blood pressure reduction is needed, Losartan 100 mg and hydrochlorothiazide 12.5 mg or Losartan/Hydrochlorothiazide 100/12.5 may be substituted, followed by Losartan 100 mg and hydrochlorothiazide 25 mg or Losartan/Hydrochlorothiazide 100/25. For further blood pressure reduction other antihypertensive should be added. Losartan/Hydrochlorothiazide may be administered with other antihypertensive agents. Losartan/Hydrochlorothiazide may be administered with or without food.

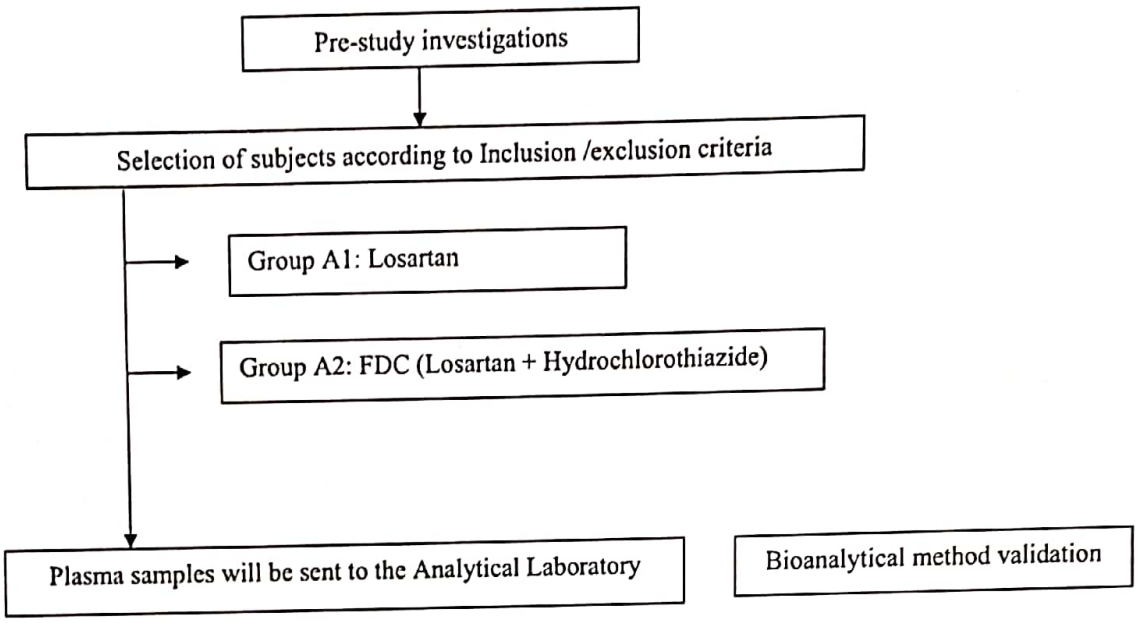
STORAGE: Store in a cool 20-25°C (68-77°F) and dry place.

2.0 OBJECTIVE

The objective of this study is to establish the Rationale of Hydrochlorothiazide in Combination with Losartan by evaluating pharmacokinetic parameters.

3.0 STUDY DESIGN

FLOW CHART OF THE STUDY:



3.1 Treatments

- **A1:** Losartan
- **A2:** FDC (Losartan + Hydrochlorothiazide)

3.2 Number of Subjects

6 healthy, adult, human, male subjects will be enrolled in the study to meet the study objective.

3.3 Blood Samples

In each of the study period, 16 blood samples will be collected in 5 mL K_2EDTA vacutainers via an indwelling catheter placed in one of the forearm vein. Heparin-lock technique will be used to prevent clotting of blood in the indwelling catheter. Before each blood sample is drawn through catheter, 0.5 mL of blood will be discarded so as to purge the heparinised blood sample in the catheter. Blood samples will also be collected by direct venipuncture during ambulatory blood sampling visit as well as wherever necessary for any practical reason. The post-dose blood samples will be collected at 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 24.0, 36.0 and 48.0 hrs. The total volume of blood drawn including the volume necessary for the laboratory tests [screening & safety sample], PK analysis and the volume of blood discarded before each blood draw will be about 182 mL per subject for the entire study.

Amount of blood drawn for analysis [16 samples × 5 mL each× 2 period]	160 mL
Volume of blood discarded before sampling [12 samples× 0.5 mL each × 2 period]	12 mL
Amount of blood drawn at the time of screening [including repeat samples, if required]	10 mL
Total volume of blood drawn per subject for the entire study	**182 mL**

3.4 Study Meals

Supervised fast for at least 10.0 hr before dosing will be maintained in each period. On dosing days lunch, snacks and dinner will be served at 4.0, 8.0 and 14.0 hr post dose respectively. Meal plans will be identical for both the periods. Water will be permitted *ad libitum* except for 1.0 hr before drug administration & until 1.0 hr post-dose.

3.5 Housing

Housing from at least 11 hr prior to drug administration until after the 24 hr blood sampling.

3.6 Washout Period

At least 7 days gap between two dosing days will be maintained.

3.7 Vital Signs

Clinical examination and vital signs measurement [blood pressure, pulse rate, respiration rate and oral temperature] will be carried out and recorded at each check-in and at check-out. Vital signs [blood pressure and pulse rate] will also be recorded before dosing of investigational products and post dose. Clinical examination and measurement of vital signs may also be carried out at any time during the conduct of the study if the Investigator/Doctor feels necessary. In case of abnormality in pre-dose vital signs, medical opinion will be taken whether to dose the subject or not. During recording of vital sign each subject will be asked about his well-being.

3.8 Analytical Methods

Validated LC-MS/MS method will be used for determination of Losartan, Losartan Carboxylic Acid & Hydrochlorothiaizde concentration in plasma at Bioanalytical Laboratory of Bioequivalence Study Centre, Jadavpur University, Kolkata – 700 032.

4.0 SUBJECT SELECTION AND RESTRICTION

4.1 Subjects

The study will include 6 healthy, adult, human, male subjects, within age of 18-45 years, normal BMI [18.5 to 24.99 kg/m^2] and with a minimum weight of 50 kg.

4.2 Subject Screening

Medical histories and demographic data including initial, sex, age, race, weight [kg], height [cm], BMI [kg/m^2], occupation, alcohol consumption and smoking habits will be recorded. Each subject will undergo a complete physical examination and laboratory tests of haematopoietic, hepatic and renal functions as listed below. Only medically healthy adult human subjects with clinically acceptable/clinically not significant laboratory profiles will be enrolled into the study. For selection into the study, the subjects will have to undergo physical and laboratory tests within 21 days prior to enrollment into the study and should fulfill all the clauses of inclusion and none of the exclusion criteria.

Laboratory tests to be performed during screening visit :

1. Haematology

- Haemoglobin
- WBC Count
- Neutrophils
- Lymphocytes
- Monocytes
- Eosinophils
- Basophils

2. Serum Chemistry

- Cholesterol
- Random Blood Sugar
- Blood urea & Creatinine
- Sodium, potassium
- Uric acid
- LFT
  - Alkaline phosphatase
  - SGPT
  - Total proteins
  - Albumin, globulin and A/G ratio
  - Total and Direct bilirubin

4.3 Inclusion Criteria

- Healthy adult human male subjects within the age range of 18 to 45 years.
- Weight not less than 50 kg.
- Normal BMI [18.5 to 24.99 kg/m^2].
- Willingness to provide written Informed Consent to participate in the study.
- Normal blood pressure and heart rate as measured after resting supine for three minutes.
- 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.
- No history of drug abuse (benzodiazepines and barbiturates) for the last one month and other illicit drugs for the last 6 months.
- To be a non-smoker.
- 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 as evidence by written informed consent.

4.4 Exclusion Criteria

- History or presence of significant cardiovascular, pulmonary, hepatic, renal, hematological, gastro-intestinal, endocrine, immunologic, dermatologic, neurological, psychiatric disease.
- History or presence of significant:
  - Alcohol dependence, alcohol abuse or drug abuse during past one year.
  - Asthma, urticaria or other allergic type reactions after taking aspirin or any other drug.
  - Ulceration or history of gastric and / or duodenal ulcer.
  - Jaundice in the past 6 months.
- Bleeding disorder.
- Maintenance therapy with any drug
- Poor mental development or impaired cerebral function.
- Allergy to the Test drug or any drug chemically similar to the drug or to the excipients of the products under investigation.
- 1 unit or 350 mL blood loss within 56 days prior to the start of study.
- Subjects who have 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 h prior to drug administration and for at least 4 h after drug administration in each period.
- Refuse to abstain from fluid for at least 1 h prior to drug administration and until 1 h after drug administration.
- Found positive in breath alcohol test on the day of check-in.
- 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 Losartan Potassium & Hydrochlorothiazide and other related class of drugs.

4.5 Criteria for Subject Withdrawal

Subjects will be free to withdraw at any time without stating any reason. The Investigator may withdraw a subject from the study if:

a) The subject suffers from significant intercurrent illness or undergoes surgery during the course of the study.
b) The subject experiences adverse event, when withdrawal would be in the best interest of the subjects.
c) If the subject requires concomitant medications which may interfere with the pharmacokinetics of study drug.

d) If the Investigator thinks it is necessary to further protect the health of the subject or the integrity of the study.
e) If subject vomits at or before the time equivalent to 2 median T_{max} after administration of investigational product.
f) The subject fails to comply with the requirements of the protocol.

Any subject discontinuing the trial medication prematurely because of reasons 1 or 5 will be replaced. The final report will include reasons for withdrawals.

5.0 CLINICAL PROCEDURES

5.1 Investigational Product Administration

As per the randomization schedule, either single tablet or FDC tablet will be orally administered to each subject in each period with 240± 2 mL of water in sitting posture after an overnight fast of at least 10 hr. Subjects will be instructed not to chew or crush the tablet but to swallow it. Compliance for the dosing will be assessed by checking the oral cavity immediately after dosing. Subjects will remain in upright position [sitting or ambulatory] for 2.0 hr post-dose in each period except when clinically indicated to change the posture. The subjects will receive a standardized meal only after 4.0 hr post-dose. Water will be permitted *ad libitum* except for 1.0 hr before and until1.0 h post-dose.

5.2 Blood Sample Handling

Blood samples will be collected in 5 mL K_2EDTA vacutainers at specified time. Any blood draws after the window period of 2 min will be noted as a sampling time point deviation except predose blood sample. Blood samples will be drawn via an indwelling catheter using heparin-lock technique or by direct venipuncture. Before each in-house blood draw, 0.5 mL of blood will be discarded so as to purge heparinised blood in the catheter. Immediately after collection of blood, the sample will be kept in ice bath. All the blood samples for a particular sample time point, will be centrifuged under refrigeration [at 3500 rpm and 4°C for 10 min]. The time interval between sample collection and the start of centrifugation should not exceed more than 45 min. The resulting plasma will be separated and stored in suitably labeled polypropylene tubes at –20 ± 2°C for pending assay.

5.3 Activity Levels

Subjects will remain upright [sitting or ambulatory] for the first 2.0 hr post-dose in both the study periods. However, should medical events occur at any time, subject may be placed in an appropriate position. Subjects will be monitored throughout confinement for adverse events. No strenuous exercises will be permitted.

5.4 Adverse Event Monitoring and Reporting

The Principal Investigator will monitor safety data throughout the course of the study. A qualified medical officer experienced in conducting bioequivalence study will be available during housing in the clinical center. Subjects will be monitored throughout the study period for occurrence of adverse events. A nearby nursing home with PEERLESS HOSPITEX HOSPITAL AND RESEARCH CENTER LTD. 360, PANCHASAGAR, KOLKATA-700 094 capable of handling emergency situations will be informed about the study. In case of adverse event, if required, the physicians attached to the hospital will treat adverse events as appropriate, either at the study center or with PEERLESS HOSPITEX HOSPITAL AND RESEARCH CENTER LTD. 360, PANCHASAGAR, KOLKATA-700 094. Subjects experiencing adverse events will be followed up until resolution of adverse event. Subjects who at least receive one dose of the study medication will be included in the safety analysis.

5.5 Adverse Events

The following definitions will be used in assessment and recording of an adverse event:

Adverse event [AE]: An AE is any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and that does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory

finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product

Adverse drug reaction [ADR]: All noxious and unintended responses to a medical product related to any dose should be considered an adverse drug reaction.

The expectedness of an ADR to the study medication should be graded as follows:

Expected drug reaction: An adverse reaction, the nature or severity of which is consistent with applicable product labeling (e.g. the investigator brochure for an approved experimental drug; the data sheet (or package insert) for a marketed product).

Unexpected adverse drug reaction: An adverse reaction, the nature or severity of which is not consistent with applicable product labeling (e.g. the investigator brochure for an approved experimental drug; the data sheet (or package insert) for a marketed product).

Serious adverse event: A serious adverse event or reaction is an untoward medical occurrence that at any dose:

- Results in death;
- Is life-threatening;
- Requires in-patient hospitalization or prolongation of existing hospitalization;
- Results in persistent or significant disability/incapacity;
- Leads to any congenital anomaly;
- Necessitates medical or surgical intervention to preclude permanent impairment of a body function or permanent damage to body structure.

The term "life-threatening" in the definition of "serious" refers to an event in which the patient was at risk of death due to the adverse event at severity it occurred; it does not refer to an event which might have caused death if it occurred in more severe form. Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm; blood dispraises or convulsions that do not result in hospitalization; or development of drug dependency or drug abuse. All serious adverse events will be reported within 7 working days to the IEC/IRB (Ethics Review Committee) approving the study. Occurrence of any Serious Adverse Event [SAE] needs to be notified by the Investigator to the sponsor immediately [within 24 hr of becoming aware of the occurrence of the event].
As soon as new information about the SAE becomes known the Investigator has to forward it without delay to the sponsor.

Non-serious adverse event: All AEs which are not considered to be serious whether expected or not should be described as 'non-serious' for the purposes of reporting. All adverse events, unless specified, refer to non-serious adverse events.

The causal relationship of an AE to the study medication/device should be graded as follows:

None: The AE is definitely not associated with the study medication/device administered.

Unlikely/Remote: The temporal association is such that the study medication/device is not likely to have had an association with the observed AE.

Possible: This causal relationship is assigned when the AE: (a) follows a reasonable temporal sequence from medication/device administration but: (b) could have been produced by the study subject's clinical state or other modes of therapy administered to the study subject.

Probable: This causal relationship is assigned when the AE: (a) follows a reasonable temporal sequence from medication/device administration; (b) abates upon discontinuation of the treatment; (c) cannot be reasonably explained by known characteristics of the study subject's clinical state.

Highly probable: This causal relationship is assigned when the AE: (a) follows a reasonable temporal sequence from medication/device administration; (b) abates upon discontinuation of the treatment; and (c) is confirmed by reappearance of the AE on repeat exposure (rechallenge).

In assessable: (a) more data for proper assessment needed; cannot be judged because information is insufficient or contradictory at the time of assessment; (b) additional details under examination; (c) Details cannot be supplemented or verified.

Severity (intensity) of AE should be assessed according to following definitions:

Mild: The AE is transient, requires no treatment, and does not interfere with the study subject's daily activities.

Moderate: The AE introduces a low level of inconvenience or concern to the study subject and may interfere with daily activities, but is usually ameliorated by simple therapeutic measures.

Severe: The AE interrupts the study subject's usual daily activity and requires systematic therapy or other treatment.

Subjects will be monitored throughout confinement for adverse events. A doctor will be on the premises during the drug administration in each period and until the end of the housing period. Clinical examination and vital signs [blood pressure, pulse rate and oral temperature] will be carried out and recorded at each check-in and at check-out. Vital signs [blood pressure and pulse rate] will also be recorded before dosing of investigational products and post-dose. Clinical examination and measurement of vital signs may also be carried out at any time during the conduct of the study if the Investigator/Doctor feels it necessary. In case of abnormality in pre-dose vital signs, medical opinion will be taken whether to dose the subject or not. During recording of vital signs each subject will be asked about his well-being. At the end of clinical stay an exit interview will be conducted during check-out. Any subject who develops any adverse event or clinically significant abnormal laboratory test values will be evaluated, and will be treated and /or followed up until the symptoms or values return to normal or acceptable levels, as judged by the Investigator /Doctor.

6.0 DATA ANALYSIS

6.1 Pharmacokinetic Analysis

Pharmacokinetic parameters for Losartan, Losartan Carboxylic Acid & Hydrochlorothiaizde will be calculated using 'SAS Version 9.1.3'/'WinNonlin™ Enterprise V:5.3,' as follows:

AUC_{0-t}	The area under the plasma concentration versus time curve, from time 0 to the last measurable concentration, as calculated by the linear trapezoidal method.
$AUC_{0-\infty}$	The area under the plasma concentration versus time curve from time 0 to infinity. $AUC_{0-\infty}$ is calculated as the sum of the AUC_{0-t} and the ratio of the last measurable plasma concentration to the elimination rate constant.
C_{max}	Maximum measured plasma concentration over the time span specified
T_{max}	Time of the maximum measured plasma concentration. If the maximum value occurs at more than one time point, T_{max} is defined as the first time point with this value.
$t_{½}$	The elimination or terminal half-life will be calculated as $0.693/K_{el}$, where K_{el} = elimination rate constant estimated from the slope of terminal linear portion of the plasma concentration time curve
K_{el}	Elimination rate constant estimated from the slope of terminal linear portion of the plasma concentration-time curve

Pharmacokinetic analysis will be performed for all the subjects who complete both the periods of the study. If necessary, an unequal number of subjects per sequence will be used for pharmacokinetic analysis. No value of K_{el}, $AUC_{0-\infty}$ or $t_{1/2}$ will be reported for cases that do not exhibit a terminal log-linear phase in the concentration versus time profile.

7.0 ETHICAL CONSIDERATIONS

7.1 Basic Principles

This research will be carried out in accordance with the clinical research guidelines established by the basic principles and the principles enunciated in The Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects [59th WMA General Assembly, Seoul, October 2008] and as per ICMR and Indian GCP guidelines.

7.2 Independent Ethics Committee

This protocol, Informed Consent Form along with protocol appendices will be reviewed by HURIP Independent Bioethics Committee, Jadavpur, Kolkata – 700032. The study subjects will be dosed only after the IEC has approved the protocol, Informed Consent Form along with protocol appendices or a modification thereof, except for medical screening of volunteers to record their health status after their consent for screening. HURIP consultants operate in compliance with ICH-GCP, ICMR guidelines, Guidelines for Clinical Trials – CDCSO (India) and Schedule Y of Drugs & Cosmetics Act & Rules .

7.3 Informed Consent

Prior to screening activity, the screening consent will be obtained from each of the volunteers. Prior to enrollment into the study, the Investigator/designated person will inform the volunteer about purpose of the study, the procedures to be carried out, the potential hazards and rights of the volunteer in a language that the subject comprehends. Ample time and opportunity will be provided to volunteer for deciding to or not to participate in the study. The subjects will be required to read and sign the consent form summarizing the discussion prior to enrollment. A copy of the Informed Consent Form will be given to the subject, which describes the study procedures and potential hazards in non-technical terms in conformity with regulatory requirements. By signing the consent form, the subject attests that the information in the consent form and any other written information was accurately explained to and apparently understood by the subject and that the Informed Consent was given freely by the subject.

7.4 Confidentiality

All data generated from the study will be regarded as confidential. The monitor(s), the auditor(s), the IRB/IEC, and the regulatory authority(ies) will be granted direct access to the subject's original medical records for verification of study procedures and/or data, without violating the confidentiality of the subject, to the extent permitted by the applicable laws and regulations and that, by signing a written Informed Consent Form, the subject or the subject's legally acceptable representative will be authorizing such access.The records identifying the subject will be kept confidential and, to the extent permitted by the applicable laws and/or regulations, will not be made publicly available. If the results of the trial are published, the subject's identity will remain confidential. No publication or dissemination of the data will be permitted without prior written agreement with the Sponsor.

8.0 ANALYTICAL PROCEDURES

Validated LC-MS/MS method will be employeed for determination of Losartan, Losartan Carboxylic Acid & Hydrochlorothiaizde concerntration in plasma samples. During the analysis, standard and quality control samples will be distributed throughout each batch of study samples analyzed. The analyst will not have access to the randomization scheme. All concentration values below the limit of quantification will be set to zero for all pharmacokinetic and statistical evaluation. Any missing samples will be reported as 'M' and for any unreportable concentration values will be reported as 'NR' and will not be included for pharmacokinetic or statistical analysis.

Plasma samples of the subjects who completed the study will be analyzed. Subjects who are withdrawn from the

study due to an adverse event related to study drug will also be assayed. Their concentration data will be provided in separate table and will not be included in pharmacokinetic and statistical analysis.

ANALYSIS OF PLASMA SAMPLES:

Standard solutions

Separate solutions containing 1 mg/ml of analytes and I.S. should be prepared using mobile phase respectively. These solutions will be further diluted suitably with the mobile phase to obtain a stock solution of 1 µg/ml. The stock solutions prepared for the drugs will be diluted further to obtain eight working solutions for calibration standards. All solutions must be stored at 2 – 8°C.

Calibration curves

An eight point standard calibration solutions of analyte(s) should be prepared by spiking appropriate amounts of analyte(s) and IS in human plasma to yield final concentrations. Three quality control (QC) samples will be prepared at three concentration levels of analyte. Calibration curves will be plotted with peak area ratio of drug and IS on Y – axis and concentration on X- axis.

Sample preparation & extraction

Liquid-liquid extraction procedure should be used for the extraction of the drug from the plasma. Calibration standards, quality control samples will be treated with 5 ml of organic solvent. 100 µl of internal standard be added with each 1ml plasma sample and vortex mixed for 10 min followed by centrifugation for another 10 min. The organic layer containing the analyte(s) will be separated, transferred to a separate test tube and evaporated to dryness under a stream of N2 at 40 °C. The residue obtained on drying will be reconstituted with the 250 µl of mobile phase/diluent. The reconstituted sample will be manually with the micro liter syringe into the injector with fixed volume loop and will be injected into the liquid chromatography system.

9.0 CHANGES IN PROTOCOL

The Investigator will not implement any changes to the approved protocol without agreement from the Sponsor and prior review and documented approval from the IEC of an amendment, except where necessary to eliminate an immediate hazard(s) to trial subjects, or when the change(s) involves only logistical or administrative aspects of the trial.

The following definitions and details will be used while assessment and recording of Protocol Deviations/Violation:

Protocol Deviation: Any alteration/modification to the IEC-approved protocol. The protocol includes the detailed protocol, protocol summary, consent form, recruitment materials, questionnaires, and any other information relating to the research study. Protocol deviation can also be known as planned protocol deviation because it protocol deviation will be approved by IEC prior to its implementation.

Major Deviation: A major protocol deviation is a deviation that has an impact on subject safety, may substantially alter risks to subjects, may have an effect on the integrity of the study data, or may affect the subject's willingness to participate in the study.

Minor Deviation: A minor protocol deviation is one that does not impact subject safety, compromise the integrity of the study data, or affect the subject's willingness to participate in the study.

The Protocol deviations will be recorded in "Protocol Amendment Form" and same will be reported in final study report.

Protocol Violation: Any protocol deviation that is not approved by the IEC prior to its initiation or implementation. Protocol Violation is also known as unplanned protocol deviation.

Major Violation: A major protocol violation is a deviation that has an impact on subject safety, may substantially alter risks to subjects, may have an effect on the integrity of the study data, or may affect the subject's willingness to participate in the study.

Minor Violation: A minor protocol violation is one that does not impact subject safety, compromise the integrity of the study data, or affect the subject's willingness to participate in the study.

The Protocol violations will be recorded in "Protocol Violation Form" and same will be reported in final study report.

10.0 REFERENCES

1. P. Yang, L. Li, J. Sun and Z. G. He, "Bioequivalent Evaluation of two Losartan/Hydrochlorothiazide Com-pound Tablets in Healthy Chinese Male Volunteers," Journal of Chinese Pharmaceutical Sciences, Vol. 15, No. 3, 2006, pp. 162-167.
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3. M. Lusina, T. Cindri, J. Tomai, et al., "Stability Study of Losartan/Hydrochlorothiazide Tablets," International Jour- nal of Pharmaceutics, Vol. 291, 2005, pp. 127-137. doi:10.1016/j.ijpharm.2004.07.050
4. Sha Liu1, Fan-Long Bu1, Chun-Min Wei1, Gui-Yan Yuan1, Ben-Jie Wang1, Rui-Chen Guo, "Pharmacokinetics of Hydrochlorothiazide, Losartan and E3174 after Oral Doses of Losartan and Losartan/Hydrochlorothiazide in Healthy Chinese Male Volunteers", Pharmacology & Pharmacy, 2012, 3, 7-14
5. Kumar S,Monif T, Khuroo A, Reyar S, Jain R, Singla AK, Kurachi K "Pharmacokinetic comparison and bioequivalence evaluation of losartan/ hydrochlorothiazide tablet between Asian Indian and Japanese volunteers", International Journal of Clinical Pharmacology and Therapeutics [2014, 52(1):39-54]
6. http://www.druglib.com/druginfo/hyzaar/
7. http://www.medicines.org.uk/emc/medicine/25426/SPC/Losartan+Potassium+++Hydrochlorothiazide+50+mg+12.5+mg+Filmcoated+Tablets
8. http://www.rxlist.com/hyzaar-drug/medicationguide.htm

11.0 LIST OF APPENDICES

Appendix I	Informed Consent Form [English]
Appendix II	Informed Consent Form [Bengali]
Appendix III	Subject Information Sheet (English)
Appendix IV	Subject Information Sheet (Bengali)
Appendix V	Case Report Form
Appendix VI	Serious adverse event form
Appendix VII	Laboratory parameters with reference range
Appendix VIII	Table of Study Diet

HURIP INDEPENDENT BIOETHICS COMMITTEE

Ground Floor, 27 Central Road, Kolkata-700032
Ph No. (033) 6450 5885, e-mail : hurip.iec@gmail.com

Date: 29/06/2018

To
Mr. Pallab Mandal
C/O, Prof. Jasmina Khanam
Dept. of Pharm. Tech.,
Jadavpur University
Kolkata-32

Subject: Ethics committee approval of research protocol.

Dear Mr. Mandal,

The HURIP Independent Bioethics Committee reviewed and discussed your application to conduct the research work in relation to your M. Pharm thesis of the following--

Study Title: "Rationale of Hydrochlorothiazide in Combination with Losartan by Evaluating Pharmacokinetic Parameters of Parent Drug and Metabolite Using LC-MS/MS"

Submitted By: - Pallab Mandal, B. Pharm

Supervisor: - Prof (Dr.) Jasmina Khanam,
Professor, Department of Pharmaceutical Technology,
Jadavpur University

Submitted study protocol, sample informed consent form and related documents have been approved by the members of the Independent Ethics Committee in the meeting held on ~~meeting held on~~ 29/06/2018 at 4:00 PM in office of HURIP (Ground Floor, 27 Central Road, Kolkata-700032).

HURIP Independent Bioethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of study, any changes in the protocol or informed consent form and asked to be provided a copy of the final report.

Yours sincerely,

INDEPENDENT BIOETHICS COMMITTEE HURIP KOL-32

DR. P JAISANKAR
Secretary, HURIP

PROF (DR.) A K GHOSH
Chairman, HURIP

Re-Registered by DCG (I), vide ECR / 746 / Hurip / Indt / WB / 2013 / Re-Registration-2016