Development and Evaluation of Microemulsion Based Lipoidal Delivery Systems for Improving Biopharmaceutical Characteristics of Cardiovascular Therapeutics

Thesis submitted by

Kumar Anand

Doctor of Philosophy (Pharmacy)

Department of Pharmaceutical Technology

Faculty Council of Engineering & Technology

Jadavpur University

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Jadavpur University

Kolkata -700032, India

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1. Title of the Thesis:

Development and Evaluation of Microemulsion Based Lipoidal Delivery Systems for Improving Biopharmaceutical Characteristics of Cardiovascular Therapeutics

2. Name, Designation and Institute of the Supervisor(s):

Prof. Sanmoy Karmakar

Head of the Department

Professor of Pharmacology and Toxicology,

Dept. of Pharmaceutical Technology,

Jadavpur University , Kolkata 700032.

Prof. Subhabrata Ray

Principal,

Dr.B.C.Roy College of Pharmacy and Allied Health Sciences,

Dr.Meghnad Saha Sarani, Bidhan Nagar,

Durgapur-713206.

3. List of publications

i. **Nano-emulgel: Emerging as a Smarter Topical Lipidic Emulsion-based Nanocarrier for Skin Healthcare Applications**

Anand K, Ray S, Rahman M, Shaharyar A, Bhowmik R, Bera R, Karmakar S. Nano-emulgel: emerging as a smarter topical lipidic emulsion-based nanocarrier for skin healthcare applications. Recent patents on anti-infective drug discovery. 2019 May 1;14(1):16-35.

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Bhowmik R, Shaharyar MA, **Anand K**, Mazumdar H, Mandal A, Mandal P, Chakraborty S, Panday P, Karmakar S. Mechanism of action of drugs used in hypertension. InHow Synthetic Drugs Work 2023 Jan 1 (pp. 349-367). Academic Press.

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Kumar Anand[1]*, Sanmoy Karmakar[1], Subhabrata Ray[2]

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Topic: Key Concerns and Considerations in Pharmaceutical sciences and Technology: South-East Asian Perspective on 4-5 February 2019.

"Statement of Originality"

I Kumar Anand registered on **27th April 2015 (Registration no: 1021513001 and Index No: 231/15/Ph)** do hereby declare that this thesis entitled "**Development and Evaluation of Microemulsion Based Lipoidal Delivery Systems for Improving Biopharmaceutical Characteristics of Cardiovascular Therapeutics**" contains literature survey and original research work done by the undersigned candidate as part of Doctoral studies.

All information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I have fully cited and referred all materials and results that are not original to this work.

I also declare that I have checked this thesis as per the "Policy on Anti Plagiarism, Jadavpur University, 2019", and the level of similarity as checked by iThenticate software is **7%.**

Signature of Candidate: Kumar Anand

Date : 15.11.2022

Certified by Supervisor(s):
(Signature with date, seal)

17/11/22

Prof. Sanmoy Karmakar
Head of the Department
Professor of Pharmacology and Toxicology,
Dept. of Pharmaceutical Technology,
Jadavpur University , Kolkata 700032.

Sanmoy Karmakar, M.Pharm., Ph.D.
Professor & Head
Dept. of Pharmaceutical Technology
Jadavpur University, Kol-32, INDIA

19/11/2022

Prof. Subhabrata Ray
Principal,
Dr.B.C.Roy College of Pharmacy and
Allied Health Sciences,
Dr.Meghnad Saha Sarani, Bidhan Nagar,
Durgapur-713206.

Principal
Dr. B. C. Roy College of Pharmacy & A.H.S.
Bidhannagar, Durgapur-713206, Burdwan

CERTIFICATE FROM THE SUPERVISOR/S

This is to certify that the thesis entitled "**Development and Evaluation of Microemulsion Based Lipoidal Delivery Systems for Improving Biopharmaceutical Characteristics of Cardiovascular Therapeutics**" submitted by Shri Kumar Anand who got his name registered on 27th April 2015 (Registration no: 1021513001 and Index No: 231/15/Ph) for the award of Ph. D. (Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the supervision of Prof. Sanmoy Karmakar and Prof. Subhabrata Ray 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.

Sanmoy Karmakar 17/11/22

Prof. Sanmoy Karmakar
Head of the Department
Professor of Pharmacology and Toxicology,
Dept. of Pharmaceutical Technology,
Jadavpur University , Kolkata 700032.

Sanmoy Karmakar, M.Pharm., Ph.D.
Professor & Head
Dept. of Pharmaceutical Technology
Jadavpur University, Kol-32, INDIA

Subhabrata Ray 19/11/2022

Prof. Subhabrata Ray
Principal,
Dr.B.C.Roy College of Pharmacy and
Allied Health Sciences,
Dr.Meghnad Saha Sarani, Bidhan Nagar,
Durgapur-713206.

Principal
Dr. B. C. Roy College of Pharmacy & A.H.S.
Bidhannagar, Durgapur-713206, Burdwan

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Date: 15.11.2022 ..... Kumar Anand

KUMAR ANAND

Dedicated

To

My Parents

&

My Beloved wife

PREFACE

Hypertension among all cardiovascular diseases remains the leading cause of death globally. Accounting for millions of deaths per year it is one of the most important risk factors for cardiovascular diseases, including cerebral infarction, ischemic heart diseases and heart failure. Despite several initiatives, the prevalence of raised blood pressure and adverse impact on cardiovascular morbidity and mortality are increasing worldwide. During a couple of decades, a number of antihypertensive drugs have been developed, and the choice of hypertension treatment has been expanded. Among many recently approved antihypertensive drugs, calcium channel blockers, which inhibit L /N type or both voltage-gated calcium channels, are potent vasodilators, and have been used as a first- or second-line drug. Regardless of having desired therapeutic properties many of these recently approved CCBs are unable to achieve their clinical significance due to low solubility or low permeability or both and barred from the development pipeline. Cilnidipine is one of the recently approved fourth generation calcium channel blocker showing both L and N type calcium channel blocking properties. Cilnidipine belongs to BCS class II drug and exhibits low bioavailability. Enhancement in bioavailability can meet the requirement of desired therapeutic effects. Lipid based drug delivery systems are hope for such drugs. Self micro emulsifying drug delivery system (SMEDDS) is one of the nano lipoidal drug delivery systems which can enhance the all relevant pharmacokinetic parameters for achieving desired therapeutic properties of Cilnidipine. Enhance bioavailability can also helps to reduce dose related adverse effects. In the present study with an aim to enhance the bioavailability Cilnidipine is developed as a SMEDDS. With an aim to attain a pharmaco-economically optimized process here low energy method is used for development of Cilnidipine SMEDDS. With help of Pseudoternary phase diagrams and statistically optimization process the final formulation is developed and all relevant *in vitro* and *in vivo* parameters are analyzed with robust analytical methods. As a result of this present work the optimized SMEDDS was found to enhance the bioavailability of Cilnidipine 2.4 time in comparison to the marketed Cilnidipine tablet.

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ABBREVIATIONS

ANS:	Autonomic nervous system
AHA:	American Heart Association
ANOVA:	Analysis of Variance
API:	Active Pharmaceutical Ingredients
ARB:	Angiotensin receptor blocker
ACE:	Angiotensinogen converting enzyme
ANP:	Atrial natriuretic peptide
APCI:	Atmospheric pressure chemical ionization
BCS:	Bio pharmaceutical classification system
BP:	Blood pressure
BHT:	Butyl Hydroxy Toluene
CCB:	Calcium channel blockers
CPP:	Critical Packing Parameter
CKD:	Chronic kidney disease
CVD:	Cardiovascular diseases
CNS:	Central nervous system
CPP:	Critical packing parameter
CPCSEA:	Committee for the purpose of control and supervision of experiments on animal
DSC:	Differential Scanning calorimetry
DLS:	Dynamic Light Scattering
DHA:	Docosahexaenoic Acid
DBP:	Diastolic blood pressure
DHPs:	Di hydro pyridines
DSS:	Doppler shift spectroscopy
EIP:	Emulsion Inversion Point
EMA:	European medicinal agency
ESH:	European society of hypertension
ESC:	European society of cardiology

EDRF:	Endothelial derived relaxing factor
EDTA:	Ethylene di amine tetra acetic acid
FDA:	Food and Drug Administration
GIT:	Gastro Intestinal Tract
GRAS:	Generally Recommended As Safe
HLB:	Hydrophilic lipophilic balance
HPMC:	Hydroxy Propyl Methylcellulose
HIV:	Human Immune Virus
HTN:	Hypertension
HPMC:	Hydroxypropyl methyl cellulose
IRB:	Irbesartan
IHD:	Ischemic heart disease
IUPAC:	International Union of Pure and applied chemistry
IAEC:	Institutional Animal Ethical Committee
LBDD:	Lipid-based drug delivery
LCT:	Long Chain Triglycerides
LSCM:	Laser Scanning Confocal Microscopy
LCMS:	Liquid chromatography mass spectrometry
LCT:	Long chain triglycerides
L-SEDDS:	Liquid self emulsifying drug delivery system
L-NAME:	N-nitro L –arginine methyl ester
MCT:	Medium Chain Triglycerides
MONCPT:	Campothecin derivative 10-methoxy-9-nitrocampothecin
MSNA:	Muscle sympathetic nerve activity
MCT:	Medium chain triglycerides
NIBP:	Non invasive blood pressure
NLC:	Nanostructured lipid carriers
NO:	Nitrous oxide
O/W:	Oil in Water
OPF:	Optimized formulation

PPI:	Polymeric Precipitation Inhibitor
PEG:	Polyethylene Glycols
PVP:	Polyvinylpyrrolidone
PIC:	Phase Inversion Composition
PIT:	Phase Inversion Temperature
POE:	Polyoxyethylenes
PDI:	Polydispersity Index
PDI:	Permissible daily Intake
PCS:	Photon Correlation Spectroscopy
PEG:	Polyethylene Glycol
PHCO:	PEG-40 hydrogenated castor oil
PCS:	Photon correlation spectroscopy
RAAS:	Renin angiotensin aldosterone system
SLN:	Solid Lipid Nanoparticle
SEDDS:	Self Emulsifying Drug Delivery Systems
SEF:	Self Emulsifying Formulation
SMEF:	Self Micro Emulsifying Formulations
SNEF:	Self Nano Emulsifying Formulations
SNEDDS:	Self Nano Emulsifying Drug Delivery System
SMEDDS:	Self Micro Emulsifying Drug Delivery
S-SEDDS:	Solid Self Emulsifying Drug Delivery Systems
SDEDDS:	Self Double Emulsifying Drug Delivery System
SCT:	Short Chain Triglycerides
SI:	Spontaneous Emulsification
SEM:	Scanning Electron Microscopy
SBP:	Systolic blood pressure
SCT:	Short chain triglycerides
SET:	Self emulsification time
SANS:	Small angle neutron scattering
SDS:	Sodium dodecyl sulfate

T2DM:	Type 2 diabetes mellitus
TEM:	Transmission Electron Microscopy
UARC:	Urinary albumin to creatinine ratio.
VSMC:	Vascular smooth muscle cells
WHO:	World health organization
W/O:	Water in Oil
WPC:	Whey Protein Concentrate
WPI:	Whey Protein Isolate
WPH:	Whey Protein Hydrolysate

CHAPTER I

INTRODUCTION

In today's era the world of pharmaceutical industry is full of newly synthesized drugs proven to be highly therapeutic effective and pharmacologically active. Almost every year many active pharmaceutical ingredients get approval and enter the development pipeline. Many of these highly promising drug agents are dropped from the development pipeline because of their low aqueous solubility as well as poor membrane permeability. According to a classification system named as biopharmaceutical classification system there are four categories of drugs namely BCS class I, II, III and IV. Here the drugs are classified on the basis of their solubility and permeability. These are highly soluble-highly permeable, low soluble-high permeable, high soluble-low permeable and low soluble-low permeable for class I, II, III and IV respectively [1]. Figure1.

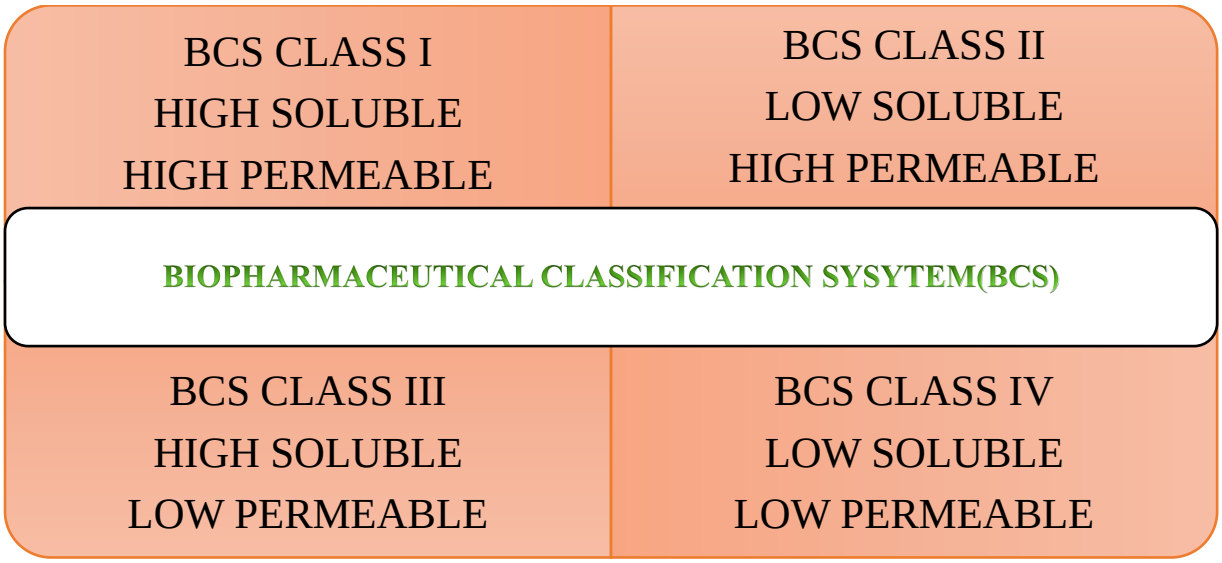

Figure 1: BCS classification system

As these BCS class II and IV drugs are solubility and permeability limited they need a novel drug delivery system to achieve their desired pharmacokinetic profile and therefore desired therapeutic action. In the present work Cilnidipine is selected as the model cardiovascular drug with an objective to develop as a novel drug delivery system to achieve the desired pharmacokinetic profile and even better than the available dosage form. Cilnidipine is a 1, 4 di- hydro pyridine derivative, a calcium channel blocker antihypertensive. Unlike other CCBs cilnidipine shows blocking activity to both L and N receptors. Being extensively studied in preclinical and clinical development phases it has been found that Cilnidipine has sufficient cardio protective, reno-protective and neuro protective properties [2]. In comparison to various other calcium channel blockers Cilnidipine is found to be better than the others.

However regardless of having desired antihypertensive properties clinical use of the drug is limited. This is because Cilnidipine belongs to BCS class II and so exhibits very poor solubility in water. This poor solubility in water leads to dissolution limited and hence results in low bioavailability of the drug. A lipoidal drug delivery system can be a hope for such a drug [3-7]. In last decades lipids have attracted a lot of interest as carriers for the delivery of poorly soluble drugs mainly belongs to BCS class II and IV. The development of lipid-based formulations as a method of drug delivery has been aided by the availability of innovative lipid excipients with favorable regulatory and safety profiles as well as their capacity to increase oral bioavailability. Lipid-based drug delivery (LBDD) systems are found enable increase the solubility and bioavailability of BCS class II or IV drugs [8]. Particle size, emulsifying capacity, rate of dispersion, and the precipitation of the drug upon dispersion are most important variables that can affect absorption of the drug from a lipid-based formulation [9, 10]. Based on the type of excipients and formulation variables, a variety of lipid-based systems can be developed, ranging from basic oil solutions to complicated blends of oils, co-solvents, surfactants, and co-surfactants [11]. The most common surfactants used in the development of self-emulsifying drug delivery systems are those that are water-soluble. Materials with HLB value 12 or more when dissolve in pure water above their critical micellar concentration, can produce micellar solutions at low concentrations and results in formation of microemulsions [12, 13].

Fortunately Cilnidipine is a lipophilic drug moiety and a suitable candidate to develop in a lipid based novel drug delivery system. In recent approaches to drug delivery systems there are many lipoidal dosage forms and can be seen as SLNs, NLCs, smart lipids, microparticles, nanoparticles, liposomes, and nano-emulsions. Nano-emulsions and micro-emulsions are emulsions whose size of emulsion ranges from 5 to 500 and 5 to 50 nm respectively [14-17]. This is a thermodynamic unstable biphasic dosage form consisting of an oil phase and an aqueous phase and stabilized with the help of a third material known as emulsifier. It can be primarily of two types O/W or W/O. Nano-emulsions and micro-emulsions are clear and isotropic in nature. Further this nano-emulsion and micro-emulsion formulation capable of self-emulsification after reaching GIT can be categorized in various subtypes depending on the purpose of fabrication such as SEDDS, SMEDDS and SNEDDS. Here SEDDS, SMEDDS and SNEDDS can be differentiated on the basis of size of the emulsion and component used in the fabrication. Unlike the nano-emulsion these self-emulsifying formulations are different as they are devoid of water in the formulation i.e., these

formulations are actually fabricated as pre-concentrate. In the present work Cilnidipine is developed as Liquid self-micro-emulsifying drug delivery system. SMEDDS has the ability to form oil-in-water (O/W) micro-emulsion in a spontaneous manner under mild agitation in gastrointestinal tract (GIT) fluids after oral administration. Enhancement in the solubility and permeability of the BCS class II drugs, spontaneous formation, thermodynamic stability, improved bioavailability and feasibility of the preparation are among the primary advantages of SMEDDS [18, 19]. In addition, a SMEDDS prolongs the gastric residence time of the drug and hence facilitates absorption of the same. As far as the Formulation and development of SMEDDS is concerned, comparatively it is easy to emulsify as the developmental process involves low energy consumption and the process is devoid of any critical steps. SMEDDS can be an advantageous approach for Cilnidipine, a drug with high lipophilicity (log >4.4). Here presence of triglyceride in composition of self-micro-emulsifying pre-concentrate is expected to enhance the bioavailability by improving lymphatic transport bypassing the portal circulation, which provides a greater interfacial area for absorption and improvement of the physical and chemical stability of drugs [20,21] .In this present work a SMEDDS pre-concentrate of Cilnidipine is developed and formulated using triacetin, Tween 20 and transcutol® HP as oil surfactant and co- surfactant respectively. Most significantly considering the chronic use of the developed antihypertensive formulation all the used components of the SMEDDS are selected considering PDI (permissible daily intake). Triacetin is a GRAS listed excipient and used as a food additive in many preparations [22]. Pseudo-ternary phase diagram was used for the quantification of used components.

LCMS/MS is a well-developed and advanced method for quantification and analysis of drug samples. Here the analysis of pharmacokinetic data is done with the help of LCMS/MS API 2000. After pharmacokinetic profiling of the drug to understand the pharmacodynamic effect NIBP method is used. Prediction of the pharmacodynamic effects of the drug is done by exploiting the pharmacokinetic profiles using the NIBP method.

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CHAPTER II

AIMS AND OBJECTIVES

In the recent era of drug design, many chemical entities are designed and invented as therapeutically active pharmaceutical ingredients. But in spite of showing desirable pharmacological activity most of these newly approved drugs entities are hydrophobic in nature and exhibit low bioavailability hence found unfit for their expected pharmacokinetic behaviors. In order to achieve the desired therapeutic value many of such drugs are used in higher doses and can precipitate various organ related toxicities. As far as cardiovascular therapeutics are concerned, in every year many chemical entities are approved for various cardiovascular disorders and found to belong either of BCS class II or IV. Hypertension is a cardiovascular disorder and a leading cause of morbidity and mortality every year all over the world. As a therapeutic agent calcium channel blockers are approved as one of the best agents as antihypertensive. Cilnidipine is one of the recently approved 1, 4 dihydropyridine derivative antihypertensive agents. It is a unique antihypertensive agent that exhibits both L and N type calcium channel receptor blocking property. It shows inhibition of cellular influx of calcium, thus causing vasodilation. It has potent selectivity for vascular smooth muscle. Regardless of all these therapeutic properties this Cilnidipine is highly water insoluble and belongs to BCS class II. Understanding the need of time this drug is thought to formulate and develop as a novel drug delivery system. To achieve the desired pharmacological and pharmacokinetic profiles of the Cilnidipine as an antihypertensive, the present study is done with below given aims and objectives.

1. Preparation, characterization and optimization of a thermodynamically stable lipoidal nano sized dispersion of Cilnidipine.
2. Improvement and enhancement of the pharmacokinetic profile of the drug such as bioavailability.
3. With the enhancement of bioavailability of the drug reduction in the dose of the selected drug to counter the dose related toxicity.
4. All relevant physicochemical characterization of the developed formulation.
5. Pharmacokinetic profiling of the developed formulation using appropriate animal models.
6. Clinical correlation with pharmaceutical outcomes with Pharmacodynamic study of the developed formulation using NIBP method.

CHAPTER III

LITERATURE SEARCH

Aim of this chapter is to focus on the brief description of the components used in the research work. As the objective of the present research work is formulation and developing a novel lipoidal drug delivery system of CCB antihypertensive, it will be important to understand the role of oil and surfactants used in the formulation. Further a brief idea of hypertension will be an important concern. Therefore this chapter is divided and described in three parts as given below.

1. Hypertension
2. SMEDDS as a novel drug delivery System.
3. Analysis

3.1. Hypertension:

The present research work is all about the development of a novel drug delivery system for cardiovascular therapeutics. Further here the selected therapeutic out of cardiovascular system is antihypertensive. In recent decades hypertension has emerged as one of the most crucial phenomena leading to millions of deaths only in a few years. However WHO has recognized hypertension which is also known as high blood pressure as a preventable disease. For the preventive measures a hypertensive patient needs chronic or lifelong treatment. Parallel to the treatment the hypertensive patient needs proper diagnostics and management of lifestyle. In different time frames relating various clinical outcomes of continuous research many guidelines were proposed for classification, measurement and maintenance of high blood pressure (BP). The story of hypertension can be narrated with the beginning of development of relevant techniques for blood pressure measurement. Reverend Stephen Hales will be credited as the first person who measured the arterial pressure and direct intra arterial pressure in a horse in 1733 and most interestingly the sphygmomanometer was discovered after a century for measurement of human blood pressure non invasively[1]. As far as the clinical significance of high blood pressure is concerned, the insurance industry of the United States provided early and consistent evidence for the same. In 1911, the medical director of the Northwestern Mutual Life Insurance Company wrote, “The sphygmomanometer is indispensable in life insurance examinations, and the time is not far distant when all progressive life insurance companies will require its use in all examinations of applicants for life insurance. In recent decades there are many regarding techniques of blood pressure measurement. However the clinical significance and risk is unchanged. Out of the many definition of high blood pressure the latest one is “In accordance with most major

guidelines it is recommended that hypertension be diagnosed when a person's systolic blood pressure (SBP) in the office or clinic is ≥140 mm Hg and/or their diastolic blood pressure (DBP) is ≥90 mm Hg following repeated examination" [2]. According to the American Heart Association (AHA), the latest definition of hypertension is "Hypertension is diagnosed when blood pressure is consistently ≥130 (systolic) and/or ≥80 mm Hg (Diastolic)" [3].

There are several guidelines proposed to define hypertension as well as to set the diagnostic criteria and treatments.

According to ESH/ESC guideline (European society of hypertension) 2018, hypertension is considered when BP value ≥140/90 mmHg upon repeated measures at the clinic. This guideline also defines various categories of risk with elevation of blood pressure (4). The same classification is used in younger, middle aged, and older people, whereas BP centiles are used in children and teenagers, in whom data from interventional trials are not available. Details on BP classification in boys and girls (ESH 2018).

3.1.1 Definition of Hypertension and related guidelines according to AHA:

According to the American Heart Association the Hypertension can be termed when a patient will be diagnosed and blood pressure is consistently ≥130 and/or ≥80 mm Hg systolic and diastolic pressure respectively. The majority of individuals with hypertension ranging from 130 to 139/80 to 89 mm Hg (stage 1 hypertension) do not, however, qualify for rapid pharmacological treatment. Some of the recommendations in the guideline are groundbreaking. When blood pressure is 130-139/80-89 mm Hg (Stage 1 hypertension) and high-risk patient characteristics/comorbidities are absent, such as age 65 and older, diabetes, chronic kidney disease, and known cardiovascular disease, absolute cardiovascular risk is used for the first time to determine high-risk status; high-risk individuals begin drug therapy when blood pressure is 130/80 mm Hg. Secondary stroke prevention in drug-naive persons is uncommon among high-risk individuals, as drug therapy is started when blood pressure is less than 140/90 mm Hg. When blood pressure is less than 140/90 mm Hg, non-high-risk people will begin taking medication. In most cases, the target blood pressure is less than 130/80 mm Hg, regardless of the blood pressure threshold for starting medication therapy. However, target BP is <130 systolic amongst those 65 and older as the committee made no recommendation for a diastolic blood pressure (DBP) target [3]. Treatment should be initiated with two drugs having complementary mechanisms of action when blood pressure is >20/10 mm Hg above goal. In the general non-black population, a thiazide diuretic, CCBs,

ACE inhibitor, or ARB should be used as an initial antihypertensive treatment, but in the general black population, a thiazide diuretic or CCBs should be used[5,6].

3.1.2 Epidemiology of hypertension:

In cardiovascular disorders elevated blood pressure or hypertension is one of the leading causes of mortality worldwide [7]. Especially in LMICs (low and middle income countries) hypertension has emerged as a potential health risk. However, global use of various antihypertensive drugs has reduced the global mean blood pressure [8]. The reason for the elevation of blood pressure can be attributed due to several reasons such as high sodium intake, low potassium intake, obesity, alcohol consumption, physical inactivity and unhealthy diet [9]. According to a recent data obtained from summarization of 844 studies performed in more than 150 countries with about 86.9 lacs participants indicates that in 2015 that the mean aged global standardized systolic blood pressure in 2015 for men was 127.0 whereas it was 122.3for women. For global mean diastolic pressure it was 78.7 for men and 76.7 mm of hg for women. In countries of south Asia, sub Saharan Africa as well as eastern and central Europe both mean systolic and diastolic pressure was found higher for men and women whereas the same was found low for high income western and Asia pacific regions. Main reasons attributed towards the difference in status of hypertension are the economical condition of countries which actually decides the social management of health care [10]. To some extent geological and environmental conditions also is a reason for the above condition [11]. According to the same study it has been also found that for the past forty years the mean BP has moderately decreased or remained persistent globally.

Being a serious concern, this elevated blood pressure, whether it is systolic or diastolic, is directly related with cardiovascular diseases and CKD and leading to uncontrolled mortality [12]. In a study done by Forouzanfar MH et al. it is shown that in 2015 millions of deaths were caused due to systolic blood pressure elevation. About 10.7million deaths were caused by elevation of systolic blood pressure ≥ 110-115 mm of Hg whereas elevation ≥ 140mm of Hg caused a total death of more than 7.8 million[13]. IHD (ischemic heart disease) is also a potential reason for death due to CVD and is associated with elevated systolic blood pressure. A continuous monitoring of epidemiology of HTN has shown a strong relation of elevated blood pressure with cardiovascular diseases. In the process of epidemiological study very interestingly it has been found that adults who are middle aged or older (≥35 years), systolic BP is a more important determinant of CVD risk than diastolic BP [14].

As elevation in blood pressure is triggered by many reasons there are medicines of different chemical moieties according to the need. With the help of continuous clinical trials many agents have been invented and developed for lowering the blood pressure. These agents include CCBs, ARBs, diuretics and ACE inhibitors. All these antihypertensive drugs were used to lower the morbidity rate [15].

3.1.3 Pathophysiology & prognosis of hypertension

Reason of HTN is very uncertain and attributed to multidimensional physiological and lifestyle related grounds. According to these reasons hypertension can be of two type's primary hypertension or essential and secondary hypertension or non essential. It has been observed that just 2-5% of patients are associated with hypertension due to some defined reasons such as adrenal and renal diseases [16]. Apart from these two reasons, pathophysiology of hypertension is unclear and then grouped in essential and non essential hypertension. In a detailed study it has been observed that there are several mechanisms on which the elevation and lowering of blood pressure depends. Most important and primary physiological reasons of blood pressure control is cardiac output and peripheral vascular resistance [17]. Having normal cardiac output many patients have been found with increased vascular peripheral resistance which leads to elevated blood pressure. The peripheral resistance is actually controlled by small arterioles where walls are made-up of smooth muscle cells.

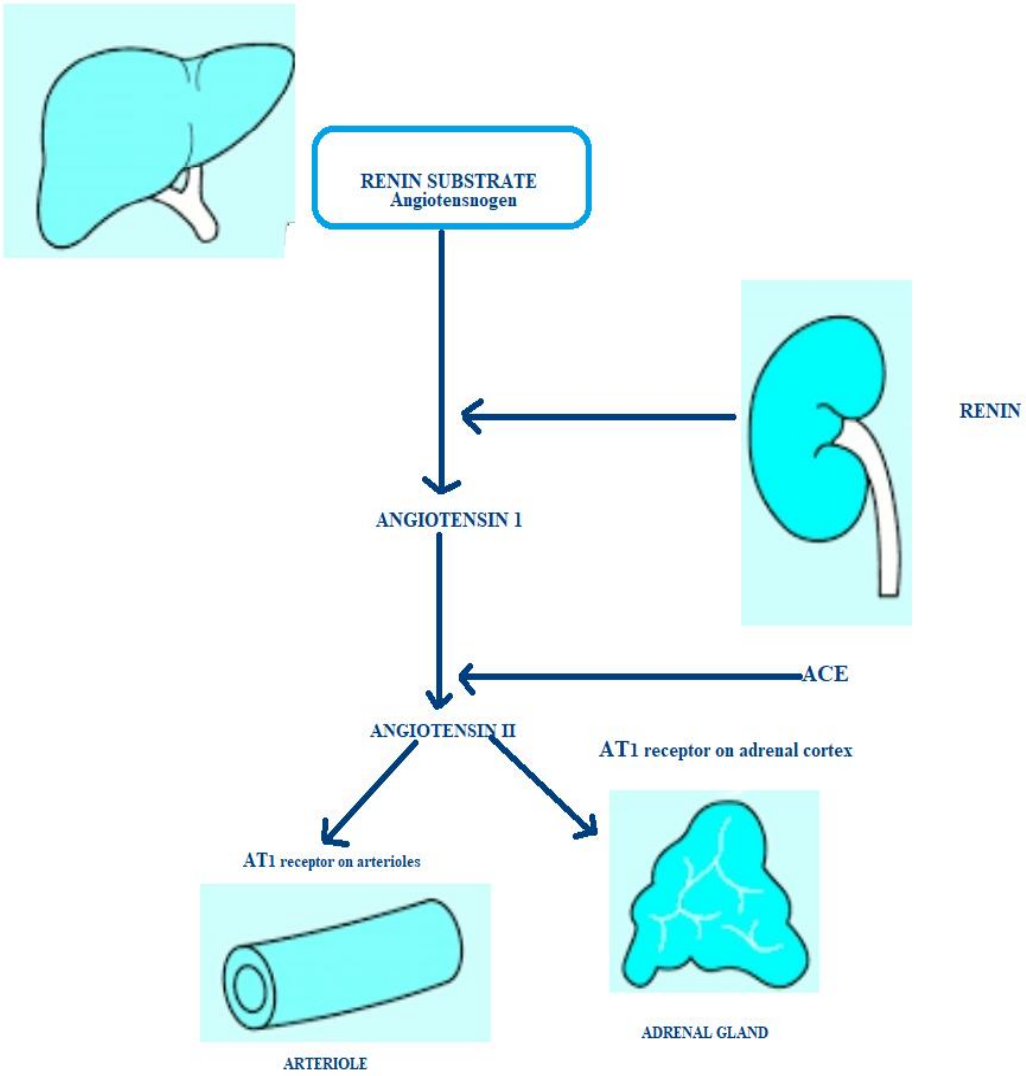

Figure 1: **Pathophysiology & prognosis of hypertension**

Smooth muscle cell contraction is assumed to be linked to an increase in intracellular calcium concentration, which could explain why medicines that block calcium channels have a vasodilatory effect. Long-term smooth muscle constriction is hypothesized to cause structural changes in the arteriolar artery walls, potentially mediated by angiotensin, which results in an irreversible increase in peripheral resistance. In a very interesting fact it has been noted that in early symptoms of hypertension the peripheral resistance does not increase but the cardiac output increases hence results in the elevation of blood pressure[18, 19]. Main physiological reasons for the occurrence of elevation of blood pressure or in other words development of primary hypertension includes RAAS, autonomic nervous system, Bradykinin, Endothelin, EDRF (endothelial derived relaxing factor), ANP (atrial natriuretic peptide)), ouabain, cardiac output, peripheral resistance and in some cases genetic factors[20-23]. These factors can be briefly described as follows.

1. RAAS: Arterial hypertension is a common condition in cardiovascular diseases and an important predictor of many diseases. Renin angiotensin aldosterone system (RAAS) is one of the critical physiological controlling systems of blood pressure. RAAS functions through regulating the blood volume and systemic vascular resistance. In the process of blood pressure regulation the RAAS system plays a role for chronic alteration whereas the baroreceptor alters the pressure for a short while. Elevation in blood pressure for a chronic period is a matter of damage to the vascular system of the heart and kidneys. Further various stresses like mechanical and oxidative stresses along with some inflammation due to fibrosis also results in chronic elevation in blood pressure. As the name of the system suggests itself the system is composed of three components namely rennin, angiotensin II and aldosterone. So here the RAAS manage the system through a hormonal mechanism. It controls the blood pressure by balancing the hemodynamic stability controlling the fluid volume and sodium potassium balance. RAAS locally acts on tissue level and transports the required components. [24, 25]

RAAS primarily controls hypertension by releasing a hormone present in the kidney known as Renin. Renin is the hormone secreted from juxtaglomerular cells and regulated mainly by two pathways locally within the kidney and through the central nervous system (CNS). In CNS it is controlled by norepinephrine release from renal noradrenergic nerves [26].

Renin is an enzyme released into the bloodstream by specialized cells that surround the arterioles at the kidney's glomeruli's entry (the renal capillary networks that are the filtration units of the kidney). The juxtaglomerular apparatus's renin-secreting cells are sensitive to variations in blood flow and blood pressure. Reduced blood supply to the kidneys is the major stimulus for increased renin secretion, which can be induced by sodium and water loss (as a result of diarrhoea, continuous vomiting, or profuse perspiration) or by a constriction of a renal artery. Renin catalyzes the conversion of angiotensinogen, a plasma protein, into angiotensin I, a decapeptide (a ten-amino-acid peptide). An enzyme in the blood called angiotensin-converting enzyme (ACE) transforms angiotensin I into an octapeptide called angiotensin II, which is made up of eight amino acids. Angiotensin II promotes the release of aldosterone, which stimulates salt and water reabsorption by the kidneys, as well as the constriction of small arteries (arterioles), resulting in an increase in blood pressure, via receptors in the adrenal glands. Angiotensin II constricts blood vessels further by inhibiting the reuptake of the hormone norepinephrine into nerve terminals [26]. Mechanism is shown in **figure 1.**

2. ANS: In the mechanism of controlling the hypertension autonomic nervous system also plays an important role [27]. With the help of a sympathetic arm this system regulates the arterial pressure for a short period of time via baroreceptors. Apart from the short period of effect, the long period effect on BP through ANS on young and adult people is an important matter of concern. In order to understand these activities the sympathetic nervous system is taken into concern. As a sympathetic ANS three important phenomena's muscle sympathetic nerve activity (MSNA), regional norepinephrine and plasma norepinephrine are taken into account [28,29].

3. Endothelial dysfunction: In primary hypertension a defect in L arginine NO pathway results in endothelial dysfunction. Nitrous oxide, a colorless sweet smelly gas which is primarily used as an anesthetic agent significantly decreases the mean arterial pressure in dose-dependent matter. Here the main mechanism behind controlling hypertension through endothelial dysfunction is the production of oxygen free radicals which results in Nitrous oxide breakdown and hence availability of NO [30].

4. Cardiac output and peripheral resistance [31]

5. Genetic factors [32]

3.1.4 Risk assessment:

Considering the epidemiology it is now well understood that elevation in blood pressure can precipitates various cardiovascular diseases. Here comes the significance of assessment of related risk due to levels of blood pressures and other factors related with that.

3.1.5 Antihypertensive therapy:

Pharmacotherapy: Continuous randomized clinical trials on large scale have led several antihypertensive such as Angiotensin receptor blocker (ARBs), calcium channel blockers (CCBs), ACE inhibitors, beta blockers, diuretics, alpha blockers and alpha 2 receptor agonist. As the present work is related to calcium channel blocker, a brief drescription of CCBs can be seen as given below.

3.1.5.1 Calcium Channel Blockers (CCBs):

Calcium channel blockers or antagonists are molecules universally used as indication for many cardiovascular therapeutics. Calcium channel blockers are of two type's dihydropyridine derivative and non dihydropyridine derivative [33]. A number of cardiovascular diseases such as hypertension, angina pectoris, Supraventricular dysrhythmias,

hypertrophic cardiomyopathy, coronary spasm and pulmonary hypertension can be treated by CCBs.[34] Along with all these indication the calcium channel blockers are also prescribed for Raynaud phenomenon, subarachnoid hemorrhage, and migraine headaches[35]. In comparison to the Non dihydropyridinic group CCBs such as verapamil, a phenylalkylamine, and diltiazem, a benzothiazepine the dihydropyridine group CCBs whose name ends with "pine" is preferred more in monotherapy as well as cardiovascular therapeutics with multiple drugs [36,37]. Further research revealed that they inhibited calcium entry and interacted with binding sites on voltage-dependent calcium channels to diminish artery contraction. Calcium channel blockers were made in the context of this. CCBs lower arterial pressure in short-term studies by lowering total peripheral resistance. CCBs increase myocardial oxygenation by unloading the heart and enhancing coronary blood flow. In long-term treatment, the decrease in blood pressure is more pronounced in hypertensive than in normotensive patients. Among various categories the CCBs inspite of having similar interaction with L-type voltage-dependent transmembrane calcium channels they possess different pharmacodynamic, pharmacokinetic properties, selectivity as well as difference in duration of therapeutic action [38-40]. Due to extensive first pass metabolism most of the CCBs posses low oral bioavailability [41]. Calcium channel blockers can be subdivided into different classes as:

i) Dihydropyridine derivative: Amlodipine, nicardipine, lercanidipine, felodipine, azelnidipine, nifedipine etc
ii) Non dihydropyridine derivative:

Phenyl alkyl amine: Fendiline, Gallopamil, Verapamil

Benzothiazepine: Diltiazem

3.1.5.1a General pharmacology of calcium channel blockers:

Calcium channel blockers are a collection of chemical entities that block calcium-selective channels in the plasma membranes of excitable cells. Calcium channel blockers decrease cell function by allowing the calcium ion (Ca2+) to gain access to the cell interior, where calcium serves as an activator messenger. Calcium channel blockers reduce excitatory activities that rely on calcium entry across the plasma membrane by lowering depolarizing currents induced by the entry of positively charged Ca2+ into the negatively charged core of resting cells [42]. The majority of the effects of calcium channel blockers on the cardiovascular system are explained by these concepts. Calcium channel blockers reduce the heart rate and prolong atrioventricular conduction by inhibiting contractile activity in the heart and vascular smooth muscle. Calcium channels carry the initial depolarizing currents in the sinoatrial and

atrioventricular nodes.[43] Calcium channel blockers' negative inotropic and vasodilatory properties, both of which can lower systemic blood pressure, provide a theoretical basis for their use in the treatment of hypertension. Some calcium channel blockers have tissue selectivity, which may improve their therapeutic efficacy in some hypertension patients. Out of the three CCBs Diltiazem and verapamil are nearly equal in their ability to suppress calcium channel function in the heart and vascular smooth muscle, although nifedipine is more effective in smooth muscle [44]. In the treatment of hypertension, this tissue selectivity can be beneficial. The emerging recognition of the potential value of calcium channel blockers in the treatment of hypertension is based on these pharmacologic concepts. General pharmacology of CCB's is shown in **figure 2**.

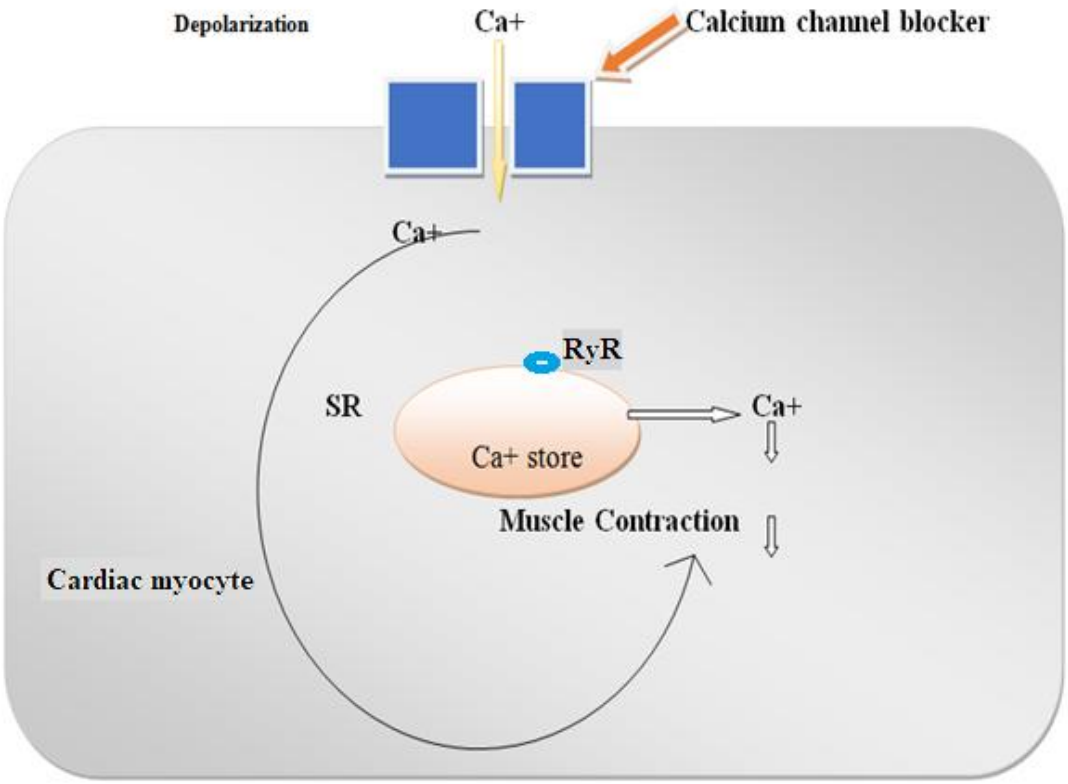

Figure 2: Pharmacology of calcium channel blockers where SR: Sarcoplasmic reticulam and RyR: Ryanodine receptors

3.1.5.1b Pharmacokinetics of calcium channel blockers:

Calcium antagonists are a biochemically diverse collection of medications that all have the property of preventing calcium from entering cells through voltage-operated channels in cardiac and smooth muscle. They can help with angina pectoris and hypertension treatment.

Nifedipine, verapamil, and diltiazem are currently accessible medications. Low and variable bioavailability, high first-pass metabolism, short elimination half-life, and active metabolites are all pharmacokinetic features shared by all three medicines. The pharmacokinetics of calcium antagonists is important since the intensity and duration of the pharmacological impact in individual patients is related to the medication level in plasma. Amlodipine, a novel dihydropyridine calcium antagonist in advanced clinical development, is one of the CCBs. Its pharmacokinetic profile is very different. It has a longer half-life of 35-50 hours and is water soluble and photostable. Amlodipine is a slow-acting medication with a high absolute bioavailability and significant hepatic metabolism. A lengthy half-life is linked to long pharmacodynamic action duration. Amlodipine may offer practical advantages over conventional calcium antagonists in the long-term therapy of cardiovascular disease due to its new pharmacokinetics. Emerging technology and drug development processes has led invention of another calcium channel blocker Cilnidipine a fourth generation and both L and N type receptor blocker in nature [45-51].

According to evolution of calcium channel blockers they are again categorized in four generation:

First generation: Nifedipine

Second generation: Benidipine, Efonidipine, isradipine, nicardipine, felodipine

Third generation: Amlodipine

Fourth generation: Cilnidipine

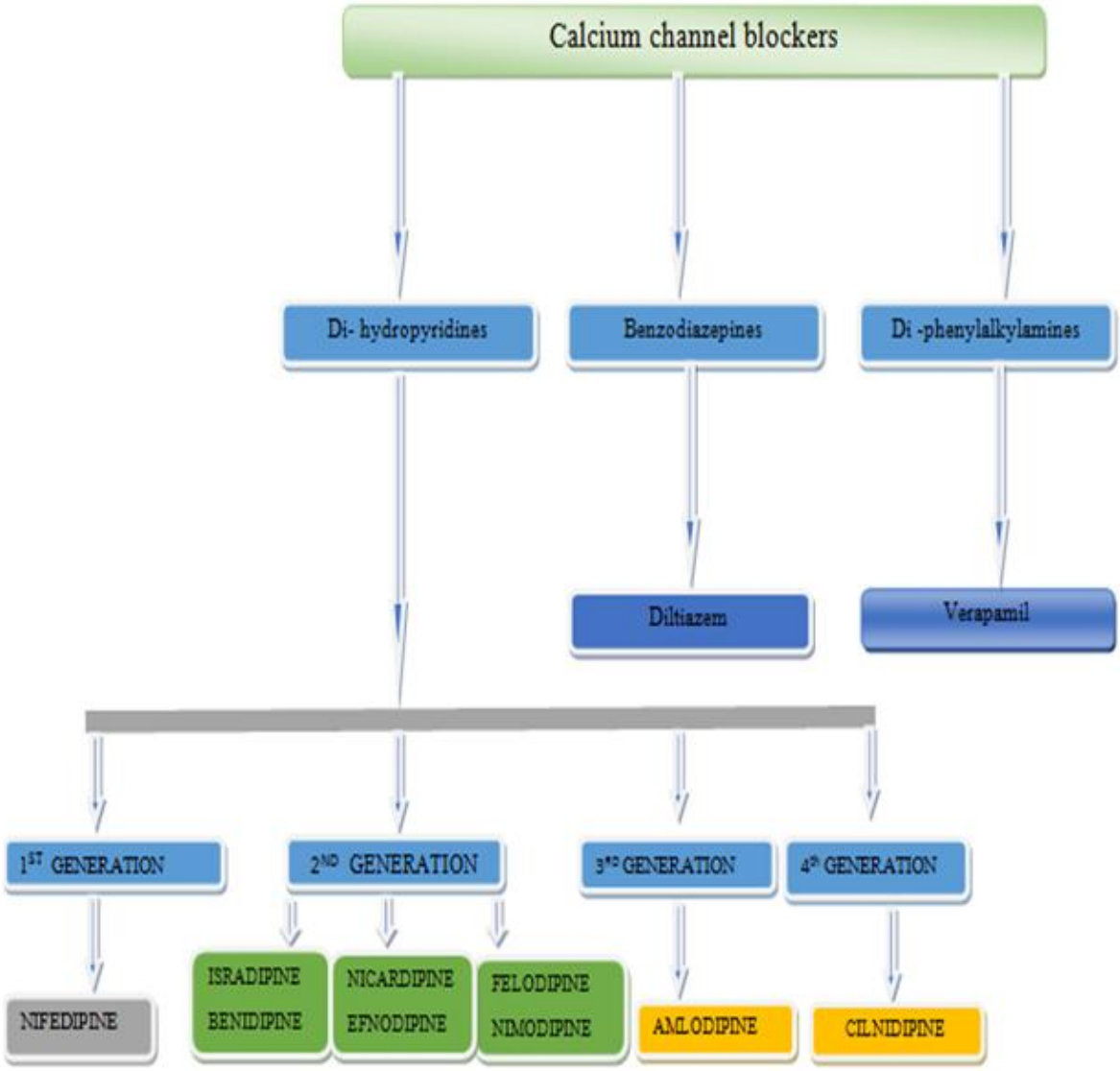

Figure 3: Various calcium channel blockers

Being the first generation calcium channel blockers Nifedipine is the only L type calcium channel blocker. Effect of Nifedipine on N type calcium channel blockers is almost nil. On other hand being first generation CCB Nifedipine is not the drug of choice for emergency hypertensive patients [52, 53]. It shows a rapid onset of action resulting in an unpredictable effect on blood pressure. Nifedipine is found to cause reflex tachycardia as well as it also activates the renin angiotensin system. Second generation CCBs such as Benidipine, efonidipine, manidipine are found to induce the vasodilation slowly in compare to first generation CCBs [54]. Further the second generation CCBs shows better pharmacokinetic profile compare to the first one that encompasses longer duration of action. Third generation CCBs are exemplified by amlodipine and azelnidipine. Amlodipine is one of the most

prescribed CCBs for hypertension. It shows a unique pharmacokinetic profile. Half life of amlodipine is much longer (36hrs.) than any second generation calcium channel blockers results in slower induction of vasodilation [55]. Very interestingly it has been observed that an increased dose of amlodipine can cause increased heart rate as well as enhanced level of plasma nor-epinephrine concentration via sympathetic reflex. Amlodipine is also found to cause pedal oedema [56]. Cilnidipine is the fourth generation dihydropyridine derivative CCB. Cilnidipine is unique and it exhibits both L and N type calcium channel receptor blocking properties [57]. It is also well observed that Cilnidipine acts as antisympathetic from cell to human level. With its ability to block N-type Ca2+ channels, the Cilnidipine directly suppresses the release of sympathetic neurotransmitters, offering an efficient method for treating cardiovascular disorders [58]. Cilnidipine is found to suppress the RAAS at antihypertensive doses whereas many other calcium channel blockers activate such systems after repeated or acute doses. Cilnidipine is also understood to be a better choice for combination therapy with ARBs (Angiotensin receptor blockers) as it has been studied that where some ARBs are found to activate the RAS the Cilnidipine suppress the same [59]. Hence Cilnidipine can provide synergistic effects with ARB and can be used as a combination therapy. Human adrenocortical cells' N-type calcium channels are found to be a source of intracellular calcium transport in this study. Cilnidipine and a particular N-type Ca2+ channel blocker, ω-conotoxin, efficiently limit angiotensin II's effects on aldosterone synthesis. [60]

3.1.6 Cilnidipine

3.1.6.1 Pharmacology of Cilnidipine:

Cilnidipine (FRC-8653) is a fourth generation 1,4 dihydropyridine derivative Calcium channel blocker developed in Japan [61]. Despite being a calcium channel blocker pharmacology of cilnidipine is different from other CCBs.[62-63] Cilnidipine is found to block the influx of Ca^{2+} ions into the vascular Smooth muscles as well as neuronal muscles both. In smooth muscles it takes place at level of L type calcium channel whereas in neuronal muscle it occurs at level of N type calcium channel. Cilnidipine reduces blood pressure by blocking L-type channels and inhibiting reflex sympathetic activation by blocking N-type channels, resulting in lower blood pressure, less reflex tachycardia, and lower HR variability [64-66]. The action of cilnidipine is distinct in that it reduces sympathetic activity and catecholamine release. [67]

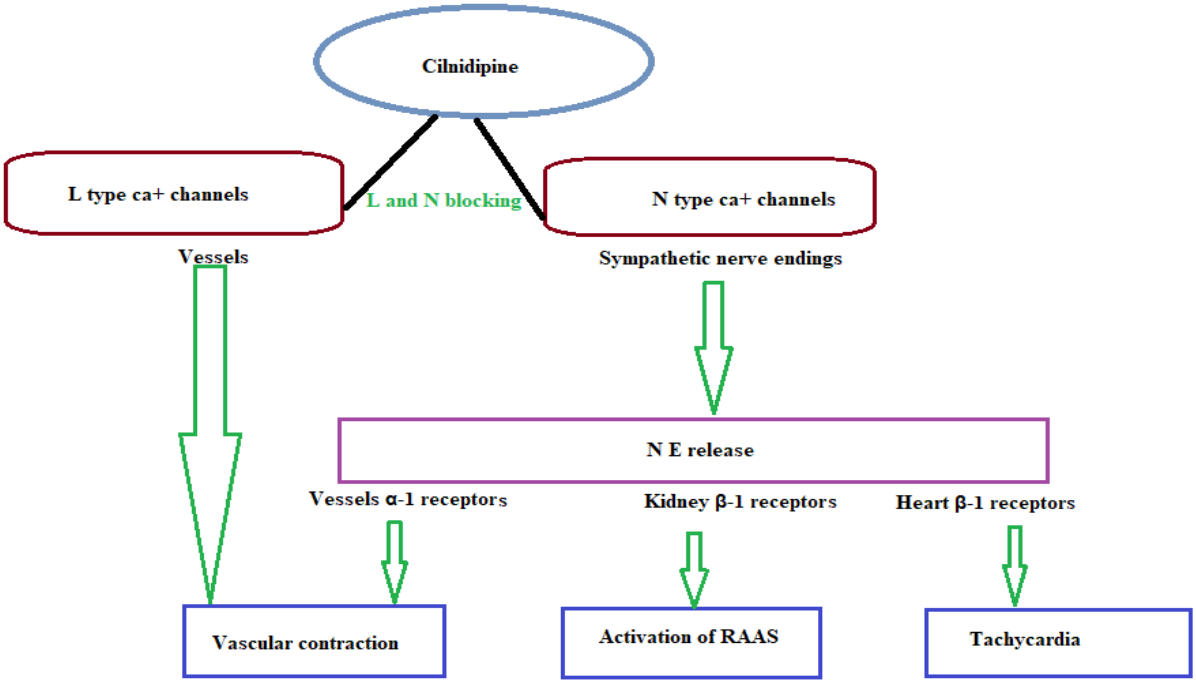

Figure 4: MOA of Cilnidipine

Further Calcium entry causes an increase in arterial tone, peripheral vascular resistance, and arterial hypertension in vascular smooth muscle cells (VSMC) [68].

Calcium entrance through N-type voltage-gated channels in sympathetic nerve terminals enhances the presynaptic release of noradrenaline and sympathetic tone in the arteries, kidney, and heart.

The cilnidipine-induced blockage of L-type Ca2+ channels primarily affects vascular smooth muscle, resulting in dilatation of peripheral resistance vessels and coronary arteries. The inhibition of N-type Ca2+ channels primarily affects sympathetic neuron peripheral nerve terminals, dilating blood vessels by reducing plasma catecholamine levels. In individuals with hypertension, cilnidipine reduced blood pressure more than in healthy volunteers [69]. Although tests with typical L-type selective DHPs showed increases in heart rate, changes in heart rate with cilnidipine were insignificant, even in patients with rapid blood pressure decrease. Thus, it appears that this inhibitory activity on N-type Ca2+ channels dampens hypotension-induced baroreflex sympathetic activation, and that cilnidipine has an even higher blood pressure-lowering impact. In addition, cilnidipine inhibits the rise in blood pressure that occurs in response to acute cold stress, which is generally not suppressed by L-

type Ca2+ channel antagonists. Direct negative inotropic effects in hypertension individuals treated with cilnidipine, on the other hand, were not observed.

Antihypertensive Ca2+ channel antagonists have recently been developed with two objectives in mind:

1) To limit the risk of unwanted side effects, tissue selectivity among L-type Ca2+ channels is used, and
2) To increase compliance and minimize sympathetic activation caused by hypotensive baroreflexes, a slow onset and lengthy duration of action is used.

However, Because of the extensive tissue distribution of L-type Ca2+ channels, comprehensive regulation of this sympathetic stimulation appears to be quite challenging. As a result of our experience with cilnidipine, we now feel that regulating N-type Ca2+ channels can also help with sympathetic issues. We now believe that modulating N-type Ca2+ channels can also help with sympathetic disorders as a consequence of our experience with cilnidipine.

As a cardiovascular drug Cilnidipine acts as an antihypertensive as well as vascular relaxing agent. In order to suppress the renin angiotensin- aldosterone system Cilnidipine decreases the plasma level of angiotensin II and aldosterone. In the similar process it also inhibits the aldosterone production related to adrenocortical cells and results in hypotension. In other pharmacological functions related to antisympathetic action leading to hypotension Cilnidipine is found to decrease the catecholamine release and tissue norepinephrine level. Cilnidipine also inhibits NADPH oxidase-derived superoxide production.

Cilnidipine as an antihypertensive agent was established much better in comparison to other CCBs in case of hypertensive patients having chronic kidney diseases. This fourth generation calcium channel blocker significantly decreased urinary albumin excretion without affecting serum creatinine concentration. It has also been found that the renal protective effect of cilnidipine was greater than pure L-type Ca2 channel blockers. [70-72]

As far as the various responses of Cilnidipine towards vital organs are concerned there are many functions of Cilnidipine towards kidney, heart and brain. In the kidney it enhances the renal blood flow, dilates the afferent and efferent arterioles, decreases in renal angiotensin II content and decreases albuminuria and urinary protein. It also inhibits the renal nerve stimulation induced antinatriuresis [73]. In the heart cilnidipine increases the coronary blood

flow [74]. It also suppresses the vasopressin-induced ST depression and reduces the myocardial infarct size as well as incidence of ventricular premature beats after ischemia-reperfusion. Similarly in the brain it improves the cerebral blood flow and helps in reduction of cerebral infarction size. [75]

3.1.6.2 Pharmacokinetic profile of Cilnidipine:

Cilnidipine has a good pharmacokinetic profile, with quick and full oral absorption; peak plasma concentrations are reached at 1.8 to 2.2 hours following oral administration [69]. Cilnidipine is metabolized in the liver and converted to inactive metabolites by the cytochrome P450 3A4 iso-enzyme, further the same is eliminated in urine [76]. Dihydropyridines have a strong hepatic first-pass action, with bioavailability ranging from 6% to 30% [77].

Cilnidipine has a shorter half-life than other typical CCBs, with a T1/2 of roughly 2.5 to 3 hours [78]. Additionally, Cilnidipine appears to have a linear pharmacokinetic profile as oral dosage is increased.

3.1.6.3 Therapeutic Safety and Tolerability of Cilnidipie:

Cilnidipine is a ten-fold more effective coronary vasodilator with stronger vascular selectivity than nicardipine, and its unique action completely relaxes potassium-precontracted human internal thoracic arteries.

Cilnidipine is an antihypertensive medication that reduces plasma renin activity and Ang-II levels while also inhibiting the RAAS.

Cilnidipine dilates both afferent and efferent arteries, lowering inter-glomerular pressure, urinary albumin excretion, and plasma noradrenaline levels in renal damage models.

3.1.6.4 The role of Cilnidipine in Amlodipine-induced pedal edema:

Pedal edema is a typical side effect associated with amlodipine therapy. Initiation of Cilnidipine treatment in exchange of amlodipine totally eliminates amlodipine-induced edema without causing hypertension or tachycardia to worsen. Cilnidipine reduces cardiac sympathetic nerve activity more effectively than amlodipine. [78] Blocking of N type calcium channels disrupts SNS activity and reduces catecholamine release from neuronal terminals, thus cilnidipine causes arteriole and venule dilatation. Cilnidipine's unique method

of action causes dilatation of both precapillary and postcapillary resistance vessels. Cilnidipine also reduces capillary hypertension and fluid hyperfiltration to inter-spatial parameters [79, 80].

The clinical significance of cilnidipine as compared to other antihypertensive drugs has been depicted in **Table 1**.

Table 1: Summary of some clinical studies of Cilnidipine with other anti-hypertensive drug:

Number of Patients and disease	Study design/period	Drugs used (mg/day)	Primary outcomes	Outcomes	Reference
28, Chronic kidney disease	PA, 12 months	CIL: 10 AML: 5-10	Compare their effects on renal function in hypertensive patients with proteinuria	CIL produced a greater suppression of proteinuria and a greater reduction in GFR	81
43, Suffering with proteinuria on CCBs other than CIL	PA, 6 months	CIL: 2.5-15 CCBs	Compare the antiproteinuric effect	CIL decreased proteinuria in patients with essential hypertension	82
110 Hypertension	OL, R 16 weeks	CIL: 10-20 AML: 2.5-5	Assess the effect of both drugs on 24-h ambulatory BP monitoring	Both drugs significantly reduced ambulatory BP, but CIL did not increase HR	83

38, T2DM, albuminuria on AML & ACEI/ARB	3 months	CIL: 10-20	Renoprotective effect of CIL	CIL resulted in a significant reduction in albuminuria	84
2,920, uncontrolled with an ARB	R, PA 12 weeks	CIL: 5 AML: 5	Safety and efficacy of adding CIL to an ARB	CIL reduced SBP/DBP and HR, particularly in patients with a baseline	85
339, Hypertension and Chronic kidney disease treated with RAASIs	OL, R, PA 12 months	CIL: 5-10 AML: 2.5-10	Changes in the urinary protein/Cr ratio	A similar decrease in BP. Proteinuria is reducing effect better with CIL. The similar antiproteinuric effect in patients with macro-proteinuria	86
50 Untreated hypertension	R, PA 24 weeks	CIL: 10 AML: 5	Compare their effects on UAE, vascular endothelial function, and arterial stiffness	CIL was more effective than AML to decrease proteinuria and arterial stiffness	87
40 uncontrolled with diabetes mellitus receiving CIL	R, PA 3 months	CIL: 5-10 BND: 8	Effects of switching from CIL to BND on BP and renal function	Switching to BND significantly reduced BP and urinary protein excretion	88

35 proteinuric patients treated with RAASIs	O, R, PA 48 weeks	CIL:5-10 AML:5	Compare their antiproteinuric effect when coupled with RAS inhibitors	A similar reduction in SBP/DBP. Proteinuria decreased only in the CIL group	89
60 Hypertension controlled with ARB and CKD	OL, R, C, PA 12 months	CIL: 5-10; AML: 5-10 NIF: 40; BND: 4	Effects on renal function after switching from a CCB to CIL	CIL and T-type CCBs similarly reduced BP. Proteinuria can be further reduced by CIL	90
90, T2DM, and urinary artery embolization	OL, non-R, CO, 12 months	CIL Other CCBs	Evaluating the influence of switching from CCBs to CIL or vice versa	CIL, but not other CCBs, decreased proteinuria	91
2,319 Hypertension	OL 12 weeks	CIL: 5-20	Effects of CIL on morning hypertension	CIL significantly reduced BP and HR	92
45, Chronic kidney disease treated with RAASIs and one CCB	R, OL, PG, PA, 24 weeks	CIL: up to 20 Various CCBs	Compare CIL with other CCBs on BP and HR and cardio-renal function	CIL improved ambulatory BP and decreased HR and LV mass index	93
87, T2DM With normal/microalbuminuria treated with VAL	PA, OL, R, C 12 months	CIL (5-20)+VAL: 40-80	Percent change in the urinary ACR	CIL+VAL reduced more effectively than VAL, despite both treatments produce a similar reduction in BP	94

AML: Amlodipine, ARB: Angiotensin II receptor blocker, OB: open blinded, BID: twice daily, BND: benidipine, BNZ: benazepril, BP: blood pressure, CKD: chronic kidney disease, CCB: calcium channel blocker, CIL: Cilnidipine, CO: crossover, CV: cardiovascular, CVD: cardiovascular disease, DB: double-blind, HR: heart rate. HTN: hypertensive patients. O: observational, OL: open-label, OD: once daily, PA: Parallel- assignment, PC: placebo-controlled, PG: parallel-group, R: Randomized, RAASI: Renin-angiotensin-aldosterone system inhibitors, SB: single-blind, T2DM: type 2 diabetes mellitus, UAE: urinary albumin/Cr ratio. UARC: urinary albumin to creatinine ratio. VAL: Valsartan.

3.2 Novel drug delivery system:

With the help of drug design or drug discovery processes in recent decades, numerous chemical entities have been discovered [95, 96]. Regardless of having achievable and desirable therapeutic properties many of these drug entities are found unsuitable for use as a therapeutic agent or drug moiety due to poor water solubility or permeability. A large portion of the newly discovered drugs are of high molecular weight and lipophilic and hydrophobic in nature [97-100]. Out of recently developed APIs, 70% of the drugs are being reported to be water insoluble and hence found to be low bioavailable.[101-103] This results in poor bioavailability of the drug and hence less suitable for desired therapeutic effect. Not only the low bioavailability but the dissolution limited factors also creates problems like inter and intra subject variability of the drug and leads to difference in the dose. Many drugs are there which are affected with the presence of food. Here food is found to alter the bioavailability. For example, presence of food can enhance the bioavailability of Danazol and Halofantrine and risk of toxicity can precipitate [104]. Now it is obvious that the drugs belonging to either BCS class II or IV are unfit to deliver as conventional dosage forms. Novel drug delivery system is hoped for drug entities classified under BCS class II and IV. In novel drug delivery system bioavailability of the API (active pharmaceutical ingredient) is enhanced by enhancing the solubility of the same with the help of various processes such as micronisation, complexation with cyclodextrin, using surfactants, permeation enhancers, making solid dispersions etc.[105] Among the various novel drug delivery systems lipoidal drug delivery systems are one of the emerging systems. Challenges related to BCS class II or IV can be better addressed with the help of lipid based deliveries where a large portion of the drug is lipophilic in nature [106, 107]. Lipid based drug delivery system (LBDDS) enhances the absorption of drugs and hence enhances the bioavailability. Many factors such as particle size, degree of emulsification, rate of dispersion, and precipitation of drug from dispersion

can affect the absorption of drug and hence bioavailability. In the present scenario of the commercial market there are several examples of successful lipid based formulation. Based on route of application LBDDS can be of both types oral and parenteral. Parenteral route comprising lipid material is reported to have some drawbacks like extravasation of drug or blood, catheter infections, and thrombosis. Along with that pain and patient non compliance is also reported. Oral lipid based drug delivery system is the popular and well accepted drug delivery system. There are several types of oral LBDDS according to composition and physicochemical properties viz. SLNs, nanoemulsion, microemulsion, liposomes, niosomes, SEDDS, LNS etc.

a. Liposome: These are nanosized spherical lipoidal delivery systems capable of entrapment and delivery of both hydrophilic and hydrophobic drug candidates because of its specialized structural characteristics. Liposomal vesicles may compose of natural and /or synthetic surface active agents like phospho or sphingo-lipids. These are natural surfactants and can arrange themselves in a tail to tail manner forming a bilayer flexible membrane containing some water [108]. Liposomes can vary from 0.5 µ to 5µ in size and are a unique delivery system for drugs with highly variable lipophilicity [109].

b. Microemulsion: According to the definition of Danielsson and Lindman "a microemulsion is a system of water, oil and an amphiphile which is a single optically isotropic and thermodynamically stable liquid solution"[110]. These are transparent or translucent formulations and categorized as water in oil (w/o) or oil in water (o/w) systems in the size range of 5 to 50 nm [110]. Microemulsion system is usually much different than emulsion (with a size range of >0.1 µ) and nanoemulsion (thermodynamically unstable). According to IUPAC, in an emulsion liquid droplets and/or liquid crystals are dispersed in a liquid. So As for the characteristics of the microemulsion is concerned microemulsion is excluded from this definition if the word "dispersed" is interpreted as non-equilibrium and opposite to "solubilized", a term that can be applied to microemulsion and micellar systems[111]. Unlike microemulsion the latter ones are not thermodynamically stable.

c. Nanoemulsion: In recent decades the branch of science dealing with fast screening of material and unique ability of combinatorial chemistry has gifted the idea of nanoemulsion formulation. Approaches for nanoemulsion remain more or less similar in comparison to microemulsion. It also requires synthetic and / or natural oil, with surfactants and co-surfactants for fabrication into oil in water (o/w) or water in oil (w/o) emulsion system having

a droplet size of 5 to 500 nm [112]. The membrane stability and flexibility of the system comes as the result of used surfactant and co-surfactant mixture. Nanoemulsion is thermodynamically unstable but having long term kinetic stability [110]. The drug loading capacity of nanoemulsion is quite high because of its increased surface area. Micro and nanoemulsion bears much hope for successful delivery of hydrophobic drug candidates.

d. Solid lipid nanoparticles (SLNs): Solid lipid nanoparticles deals mainly with particle science and can be seen as an alternative approach for nano/micro emulsions and liposomes. In solid lipid nanoparticle drug delivery systems the lipid droplets convert themselves in a crystallized state and orient themselves in highly ordered structures with the drug molecules present with them [111]. The particles presented in nanoemulsion are mainly lipid in nature and hence the delivery system will be as advantageous as nano/micro emulsions. These SLNs can be coated with hydrophilic molecules which can improve the bio-distribution, stability and other important pharmacokinetic parameters like bioavailability of incorporated drug moieties.

e. Nanostructure lipid carriers (NLCs): Nanostructure lipid carriers are the modified form of solid lipid nanoparticles. In SLNs the lipid droplets are made to fully crystallize containing the therapeutic material in the core of it. It is seen that after a level of crystallization or re-crystallization the solubility of drug molecules present in the core of formulation starts to decline.[113] NLCs thus contain a mixed form of lipid i.e. solid and liquid both. As recently approved lipophilic drug molecules are concerned, similar to the nanoemulsion and microemulsion formulations the loading capacity of NLCs is also more because of oils in the formulation [113].

Considering all the above mentioned systems as major lipid based formulations having more or less differences in each other either in the sense of formulation processes, component or in pharmacokinetic properties, here the aim of the present work is to develop the nano size emulsion formulation and study of different characterization of the same.

As far as the nano sized emulsion formulations are concerned these also can be of different types depending on component and scope of formulation. Nanoemulsion, microemulsion and self emulsifying drug delivery are the different forms of nano sized emulsion formulation. Present research work is concerned on nano sized self emulsifying formulation and characterization.

3.2.1 Self emulsifying formulation (SEFs):

Self-emulsifying formulations are isotropic solutions, transparent in nature and can be prepared by gentle agitation of the components. The components of the SEFs involve oils, surfactants, co-surfactants or co-solvents [114]. The nature of self mixing after oral administration with merely little agitation is quite beneficial for the formulation. When the pre-concentrate form of the formulation reaches the gastrointestinal tract the presence of lipid materials in SEFs trigger secretion of the bile salts and then formulation get emulsified in the presence of GIT fluids with a little agitation produced by peristalsis motion. These self emulsifying therapeutic systems may be present either as pre-concentrate or anhydrous powder form of an emulsion and itself can be of different kind like self emulsifying formulations (SEFs), self micro emulsifying formulations (SMEFs) and self nano emulsifying formulations (SNEFs) [114-118] .Regardless of similarity in components the value of HLB (hydrophilic lipophilic balance) changes with scope of changing the globule size. Surfactants generally with an HLB value greater than 12 are used in self micro emulsifying (SMEFs) and self nano emulsifying formulations (SNEFs) [119,120]

3.2.1a. Process of self emulsification:

After oral intake of self emulsifying formulation, the lipids present in formulation induces the secretion of bile salts which results in conversion of oil present in formulation in emulsified form containing drug moieties. According to the thermodynamics, process of self emulsification occurs when changes in entropy occur which favors the dispersion and is also greater than the energy required to increase the surface area of the dispersion. In a typical emulsion, free energy is proportional to the energy required to establish a new surface between oil and water phase [121], and the emulsion is stabilized when the emulsifier forms a monolayer around the emulsion droplets, resulting in lower interfacial tension. In the case of a traditional emulsion, the free energy (G) is a direct function of the energy necessary to form a new surface between the water and oil phases. This self-emulsification process is represented mathematically by equation 1.

$$\Delta G = \sum N_i \; 4\pi r_i 2\sigma \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where ΔG= free energy related to the process, r_i is the radius of the droplet, N_i is the number of droplets, and $\boldsymbol{\sigma}$ is the interfacial energy.

In the case of a self-emulsifying formulation, emulsification occurs spontaneously, requiring very little free energy.

Self-emulsifying formulations are preferable to emulsions in terms of ease of manufacture and stability. This is because emulsions are very sensitive and metastable, but self-emulsifying formulations are physically stable and will be a better delivery choice for any drug moieties that are lipophilic in nature and have a slow dissolution rate. The bioavailability of self-emulsifying formulations is increased, resulting in a more consistent blood time profile. [122]

3.2.2 Types and nature of different self emulsifying formulations:

3.2.2.1. Self emulsifying drug delivery system (SEDDS):

There are a variety of lipoidal dispersions available. Hydrophobic and low permeable drug moieties have a lot of hope in self-emulsifying drug delivery systems because they improve solubility, membrane permeability, dissolution, and hence bioavailability. All of these improvements, when combined, result in a reduction in dose-related adverse effects. Drug, oil/lipid, co-solvent, and surface active agents make up self-emulsifying drug delivery systems, which are isotropic mixtures. [123] Typical SEDDS creates larger droplets, ranging in size from 200 nm to 5m, as well as a turbid emulsion. SNEDDS (self nano emulsifying drug delivery system) and SMEDDS (self micro emulsifying drug delivery system) generate clear and translucent emulsions with sizes of less than 500 and 200 nm, respectively [124,125]. SEDDS is made up of different triglyceride oils and nonionic ethoxylated surfactants. Proportion of the surfactants in this formulation is high and greater than 25%. Selection and consideration of proportion of the surfactants is an important step in dosage form fabrication and here safety should be a serious concern. Non-ionic surfactants have been proven to be safer and less harmful than ionic surfactants; yet, they can cause reversible alterations in intestinal lumen permeability. [126]. Furthermore, long-term usage of surfactants alters the intestinal epithelium by causing changes in the tissue junction, which can lead to undesirable absorption of pathogens and poisons.[127] Different adjustments in SEDDS are introduced, taking surfactant concentration and related deleterious effects into account. Self-emulsifying systems that require a large proportion of surfactants can be solidified, resulting in two varieties of SEDDS: L-SEDDS (liquid SEDDS) and S-SEDDS (solid SEDDS). L-SEDDS are solidified to make S-SEDDS.

Different dosage forms of solid self-emulsifying drug delivery devices are designed and distributed. Self emulsifying capsules, self emulsifying control or sustained release tablets, self emulsifying control or sustained release pellets, sustained release microspheres, nanoparticles, self emulsifying dispersions, self emulsifying suppositories, and self emulsifying implants are all examples of self emulsifying dosage forms[128].By various solidification methods of the liquid self-emulsifying component, self-emulsifying drug delivery systems can be transformed to solid or powdered SEDDS, which is known as solid self-emulsifying drug delivery system (S-SEDDS). S-SEDDS powder can be used to make alternative solid dosage forms such as pellets and tablets, which are referred to as self emulsifying pellets and self emulsifying tablets. Converting liquid self-emulsifying drug delivery systems to solid form enhances the dosage form's stability, as well as other benefits such as patient compliance and manufacturing cost. Aside from powder production, alternative methods for converting SEDDS to solid form include adsorption of SEDDS on various carriers such as colloidal silica, high-grade HPMC, microcrystalline cellulose, and high-melting-point excipients such as Lutrol® and Gelucire.®

Advantages of SEDDS:

- Increases the solubility and permeability of BCS class II, III & IV drugs.
- Eliminates the variability in absorption of poorly aqueous soluble drugs.
- Helpful in taste masking
- Rapid and efficient penetration of the drug moiety in tissue
- Reduced energy requirement for emulsion formation.
- Many fold reduction in the dose of the drug hence reduces the dose related toxicity
- Control in the delivery profile
- Helps to bypass the first pass and hepatic metabolism and delivers the API directly to the systemic circulation.
- Capable of formation of various liquid and solid dosage forms hence improving patient compliance.
- Capable of high drug loading.
- Can enhance the bioavailability of such drugs whose absorption is limited due to food interference.
- Ease of manufacturing and scale up.

3.2.2.2 SMEDDS AND SNEDDS:

Self nanoemulsifying drug delivery systems (SNEDDS) and self microemulsifying drug delivery systems (SMEDDS) are isotropic preparations comprising oil, surfactants, and co-surfactants, similar to self emulsifying drug delivery systems (SEDDS). SNEDDS and SMEDDS differ from conventional SEDDS in terms of droplet size and formulation appearance. Lipoidal formulations in the form of SEDDS have a murky appearance, whereas SMEDDS and SNEDDS are clear. A control strategy for component selection, including type of triglycerides, surfactants, and co-surfactants, as well as a specific and controlled temperature, is required for successful formulation of SNEDDS and SMEDDS. Controlled selection and comprehension of the spontaneous emulsification process leads in the conversion of big emulsion droplets to the nano and micro scales, as well as the production of clear, transparent emulsion droplets. Many investigations have shown that a small number of specialized medicinal adjuvants or their combination can form a self-emulsifying system, corroborating these findings SNEDDS and SMEDDS have been proven to improve the pharmacokinetic profile of medicines with reproducible results when compared to SEDDS formulation [129-134]. In the case of dose-sensitive medicinal compounds, this also helps to limit toxicity and dose-related side effects. Many therapeutically active compounds, including as cardiovascular, anticancer, anti-inflammatory, and antidiabetic medicines, are produced as SNEDDS, and SMEDDS studies demonstrate improvements in pharmacokinetic profiles like solubility and bioavailability [135-137]. Paclitaxel and curcumin are two anticancer medicines that have been successfully fabricated as SNEDDS and demonstrate improved bioavailability and other pharmacokinetic parameters [138,139]. Many antihypertensive, fibrinolytic, and lipid-lowering medicines are manufactured as SNEDDS and SMEDDS in cardiovascular therapies, and have been shown to be superior to their usual dose form. Fenofibrate, a water-insoluble medication, was effectively manufactured as SNEDDS. When compared to pure medication, the oral bioavailability of Fenofibrate was enhanced by 1.7 times in SNEDDS. [140]. Dabigatran is an anticoagulant (blood thinner) that can also be referred to as a direct thrombin inhibitor. For improved bioavailability, a solid self nano-emulsifying system of Dabigatran etexilate was developed. When compared to standard Dabigatran etexilate capsule preparation, experimental results showed that the optimized SNEDDS formulation had 531.80 percent relative bioavailability. [141] SNEDDS were improved using a ternary phase diagram and subsequently solidified into dispersible tablets in this work. Many antihypertensives, such as Valsartan, Olmesartan, and Irbesartan, are

formulated as SNEDDS or SMEDDS, which have demonstrated improved bioavailability and other pharmacokinetic features when compared to their traditional dose form [142-146].

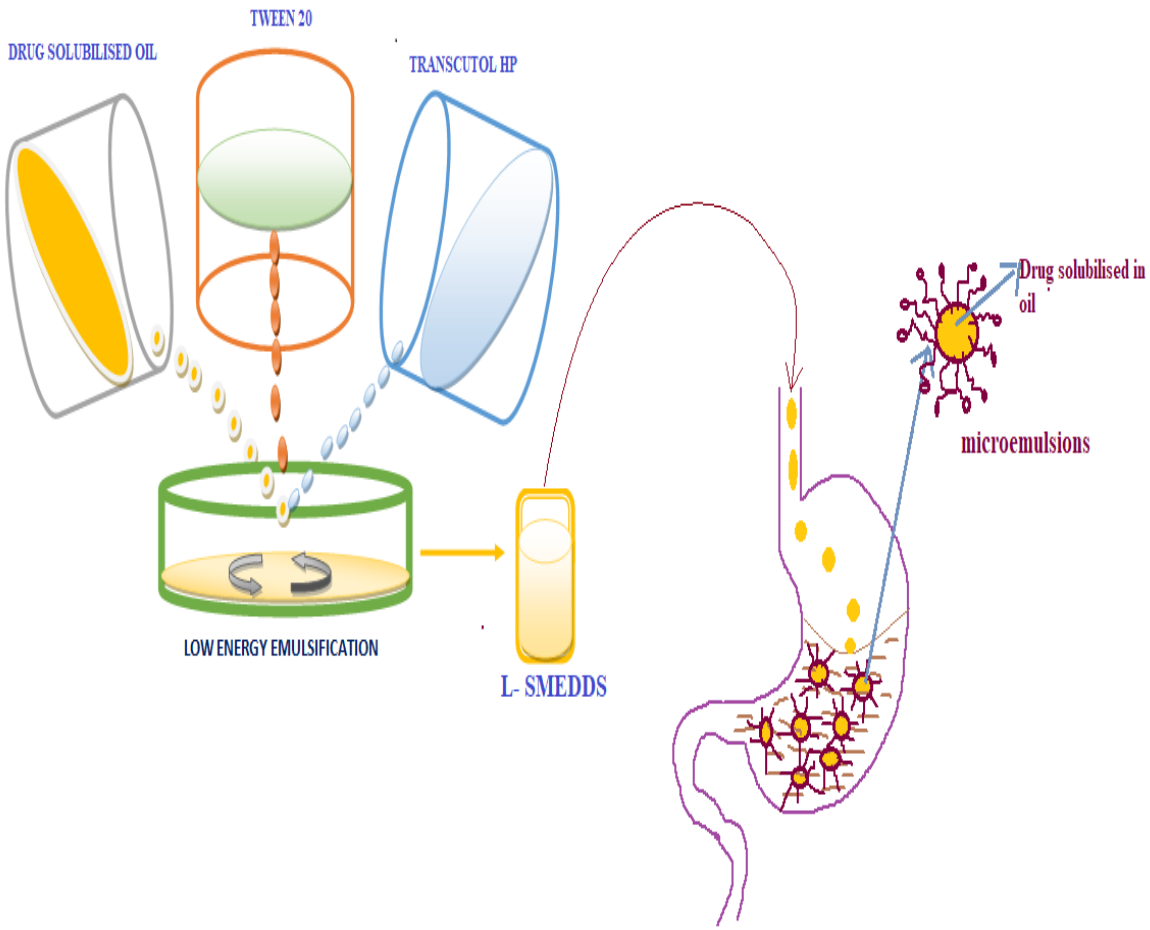

Figure 5: Self emulsifying system

3.2.3 Materials required for self emulsifying preparation

3.2.3.1 OILS AND LIPIDS

For the manufacture of SEDDS whether it is SMEDDS or SNEDDS a variety of oils and lipids can be employed. Long chain triglycerides (LCTs), medium chain triglycerides (MCTs), and short chain triglycerides are some of the lipids used in this process (SCTs). In addition, various fatty acids and glycerol, as well as fatty acids and alcohols, are used [147]. Clinically approved long chain triglycerides include rice bran oil, sesame oil, olive oil, safflower oil, arachis oil, and soybean oil. Sefsol 218, Captex 300, Capmul MCM, Capmul PG, and Miglyol 812 are some of the most often utilized medium chain triglycerides [148]. Short chain fatty acids such as triacetin, tributyrin, tricaproin, and tricaprylin can all be used

to make nanoemulsions. The solubility and stability of the active pharmaceutical ingridients (API) in the individual oils, as well as the stability of the dosage form, determine which oils to use among the various SCTc, MCTs, and LCTs [149]. In terms of solubility and emulsion type, a water in oil (W/O) emulsion is better for hydrophilic medications while an oil in water (O/W) emulsion is better for lipophilic drugs. Most active pharmacological agents, including newer ones, are classified as BCS class II or IV, with medium chain triglycerides having a higher solubility than long chain triglycerides. The oil phase must be sufficiently pure and free of any contaminants, such as decomposed products, pigments, and different lipid peroxides, in order to form a stable emulsion. Aside from that, unsaponifiable elements such as sterols and polymers are unlikely to be present. Peroxide-free lipid is critical since even a small amount of peroxide can start the oxidation process, resulting in an unstable formulation. Some approved antioxidants may be used in some instances to improve stability or prevent oxidation. [150,151].

Viscosity of oils also plays a major role in selection of oil as far as the SET is concerned.

3. 2.3.2 EMULSIFIERS:

Emulsifying agents, commonly known as emulsifiers, are chemical compounds that help to stabilize emulsions and improve their physicochemical qualities. Emulsifiers act by lowering the interfacial tension between water and oil and altering the repulsion force between nanosized droplets by manipulating the surface charge [148, 152-155]. Emulsifiers of various ionic natures, such as anionic, cationic, nonionic, and zwitterionic, can be used for this purpose. Emulsifiers come in a variety of forms, including small molecule surfactants, proteins, phospholipids, and polysaccharides. The type and percentage of emulsifiers used in emulsion formulation are determined by the delivery site, stability potential, and other toxicological concerns. For the preparation of SMEDDS, small molecule surfactants like Tweens, Spans, Labrasole, etc are mainly used [156, 157]. Small molecule surfactants can have a positive or negative ionic charge, as well as non-ionic and net-ionic properties. Net ionic surfactants are zwitterionic in nature, carrying net positive and negative charges as the PH of the solution changes. Non-ionic small molecule surfactants are preferred for SMEDDS preparation because they are less hazardous than others and remain stable when the pH changes [158].

Role of surfactants: In self microemulsifying drug delivery systems for stabilization of nano sized emulsion surfactants such as tweens, span labrasoles are used and they mainly

contribute to maintaining the homogeneity of the system. This happens due to repulsion among the globules. The surfactants attributes towards reducing the interfacial tension on the surface of the globules.

Role of co surfactants: Co surfactants are alcohols and amines in nature and mainly C4 to C10 in structure. These co surfactants usually stabilize the emulsion system by attributing flexibility to the structure. Glycerol, transcutol, IPA, IPM etc are used as co surfactants in formation of SMEDDS.

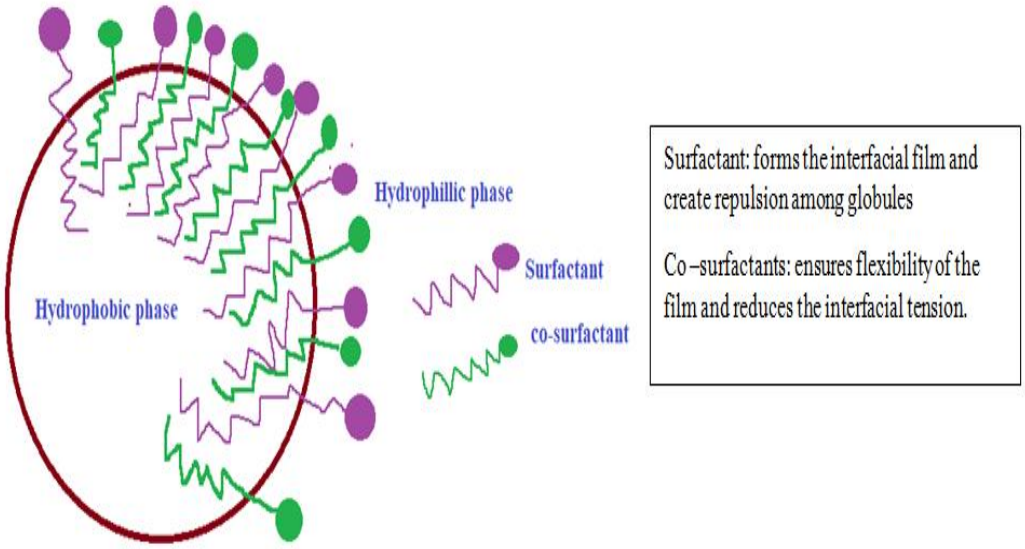

Figure 6: surfactants and co-surfactants in microemulsion

3.2.3.3 Preservatives and antioxidants:

As SMEDDS formulations are made up of oil, and active medicinal components are entrapped inside the oil, any physicochemical deterioration of the oil could represent a risk to the formulation. Inappropriate storage conditions or microbiological growth might also cause this degradation. As a result, the formulation requires chemical protection and/or physicochemical stability augmentation. Some selective preservatives and antioxidants may be required for this reason. Pre-formulation studies are particularly important before selecting and using preservatives because they can ensure that the chosen preservative is compatible with taste and odor, is readily available, and has broad spectrum antibacterial action. Antifungal properties can be found in many preservatives, including propionic acids, benzoic

acids, di-hydro acetic acid, and sorbic acid. Because the included oils are particularly susceptible to oxidation, anti-oxidants such as Butyl Hydroxy Toluene (BHT), Thiourea, Ascorbic acid, and Sodium Metabisulfite are utilized in the emulsion. Chemical stability can sometimes be improved with the application of pH stabilizers [159].

3. 2.4 Methods of preparation of microemulsion:

Nano Lipoidal formulations like microemulsion and nanoemulsions comprise numerous tiny globules of nano (10^{-9}m) range. Here globules are composed of oil entrapping drug, surfactant and cosurfactant. Each of these fabrication materials has their unique role. Hence in the overall process of developing a nano lipoidal formulation mixing of the components is a prime concern. In this process the amount of formulation components, force of mixing and time are critical parameters [160]. Describing the method of fabrication this is very interesting to differentiate between nanoemulsions and microemulsions. Both are termed as a nano lipoidal formulation and found in nanosize range. According to Schulman in 1959, a microemulsion (μE) is a thermodynamically stable mixture of oil, water, and surfactants that is optically isotropic and clear. The prefix "micro" was employed in this context to mean "very small," with no reference to the actual length scale. Because of the μE's optical transparency, their microstructure must be described by submicrometric length scales (usually below 100 nm). Because the interfacial tension (Y) is exceedingly low (in many cases found to be as low as 10^{-4} mJ/m2), such small diameters are associated with a large interfacial area that can be achieved without any energy input. As far as the nanoemulsions are concerned these are thermodynamically unstable and kinetically stable in nature. These nanoemulsions can be greater than 100 nm but less than 500 nm in size. Nanoemulsions and microemulsions can be differentiated in the amount of surfactant used to prepare them. Usually a nanoemulsion needs less concentration of surfactants but high energy for preparation and a microemulsion needs more concentrated surfactants but low energy for preparation. On the basis of fabrication microemulsions are self-assembling nano-scale emulsions, whereas nanoemulsions are nano-scale emulsions generated under high mechanical shear [161].

Manufacturing of nano sized lipoidal drug delivery systems involves both the methods as high energy and low energy. Usually nanoemulsions preparations involve high energy methods whereas microemulsion and its derived drug delivery systems are produced by low energy methods. Various methods of preparation and related reasons are discussed below.

3.2.4.1 High energy methods:

Extreme disruptive forces are used with various mechanical devices in the high-energy approach of nano lipoidal production. High-shear stirrers, high-pressure homogenizers, and ultrasonic generators are examples of these devices [162, 163]. These devices make nano-sized globules very quickly and with a high kinetic energy using such massive amounts of energy. The size of nanoemulsion globules might vary depending on the formulation's components and their properties, the instruments' condition, and, most crucially, the operating conditions such as time and temperature [160]. Most significant advantage of this method is the involvement of a low quantity of surfactants. Here produced nano-sized emulsions are thermodynamically unstable but kinetically stable in nature. Further participation of such a high degree of energy is a major source of concern, both in terms of cost and formulation of long-term stability.

3.2.4.1 a. HIGH PRESSURE HOMOGENIZATION

In this approach, a macro size emulsion or coarse emulsion is first created by mixing oil and water phases with high shear, resulting in large oil droplets, and then this coarse emulsion is employed to flow through a very narrow orifice while being subjected to a high pressure of 500 to 5000 psi. Different forces such as hydraulic shear, severe turbulence, and cavitation work together to form nanosized globules [164]. The desired globule size is achieved in this process, and the size of the globule is determined by the pressure and number of cycles applied. The number of cycles employed and the increase in homogenization pressure leads to smaller emulsion droplets, and the size of the droplets may be determined using the Laplace pressure equation. The equation can be seen as: $\Delta P = 4Y/ d$ where ΔP = Laplace pressure, Y= interfacial tension, d = droplet diameter [164].

3. 2.4.1 b. MICROFLUIDISER

Microfluidiser, a patented technology for nano-sized emulsion preparation, requires a very high pressure of roughly 500 to 20000 psi, unlike the high-pressure homogenizer Microfluidiser. The pre-homogenized coarse emulsion is forced into micro channels placed in the interaction chamber with the help of a high pressure positive displacement pump, where the emulsion is divided into two opposite streams and impinges on each other at a very high pressure and velocity to create high disruptive force [165]. As a result, the entire process

produces emulsion droplets that are exceedingly small and stable. The bulk emulsion is then filtered in the presence of nitrogen to produce uniformly sized nanoemulsion droplets.

3. 2.4.1 c. ULTRASONICATION

Ultrasonication is a process that uses high-frequency ultrasound waves to form imploding cavitation bubbles, which subsequently generate high-intensity shockwaves that shatter the large pre-homogenized emulsion into nanometer-scale size. The use of a sonication probe aids in the production of high-frequency ultrasound waves of around 20 kHz. [166].

3.2.4.2 Low energy methods:

As the name implies, these methods are low-energy and are dependent on factors such as changes in various interfacial phenomena, phase transitions, and, most importantly, the physicochemical properties of various components used in the preparation of nano lipoidal drug delivery system such as oils, surfactants, and co-surfactants. The primary approaches, which can be defined as low energy emulsification processes, are spontaneous emulsification (SE), phase inversion temperature (PIT), phase inversion composition (PIC), and emulsion inversion point (EIP). [167-170].

3.2.4.2 a. Spontaneous emulsification

This method of forming microemulsions is widely accepted and practical at both the laboratory and industrial scales. In this method nano sized emulsions are prepared by mixing two liquid phases together [171]. Both phases are liquids, with an organic and aqueous phase. Microemulsion produced spontaneously using this approach due to the quick diffusion of the organic phase. Organic solvents that have been included can be removed using a suitable procedure such as vacuum evaporation [172-175]. Both the self microemulsifying and self nano-emulsifying drug delivery systems use the same mechanism. Under spontaneous emulsification the pseudo ternary phase diagram is a well accepted system and very feasible to the system. Pseudo ternary phase diagrams are ternary phase diagrams utilized in the development of microemulsions and derived formulations like SMEDDS (176, 177). If there are more than three formulation components, the corners of these equilateral triangles often reflect a binary mixing of two components, such as surfactant/cosurfactant, water/drug, or oil/drug. Oil (drug entrapped) + surfactant + co-surfactant + water can be described as a self-micro-emulsifying drug delivery system. These diagrams are used to identify the micro emulsion’s existence region and to investigate the impact of various surfactant/co-surfactant

weight ratios on the size of a stable microemulsion area [178]. In vivo, water comes from the aqueous phase. Constructing ternary phase diagrams is a time-consuming operation, but it is the most critical and crucial step in microemulsion formulation preparation. Following this, the appropriate formulation is identified by evaluating at the centroid of the highest microemulsion zone. On the computer, one can get various Microsoft Excel templates or many software's for area calculation that may be used to calculate the area of the highest emulsification zone. Different factors like rate and order of addition, stirring rate, and the system's environmental conditions, such as temperature, PH, and ionic strength, may all play a major role in the size and qualities of emulsion droplets. [179]. Example of construction of Pseudoternary phase diagram is shown below **[figure 7].**

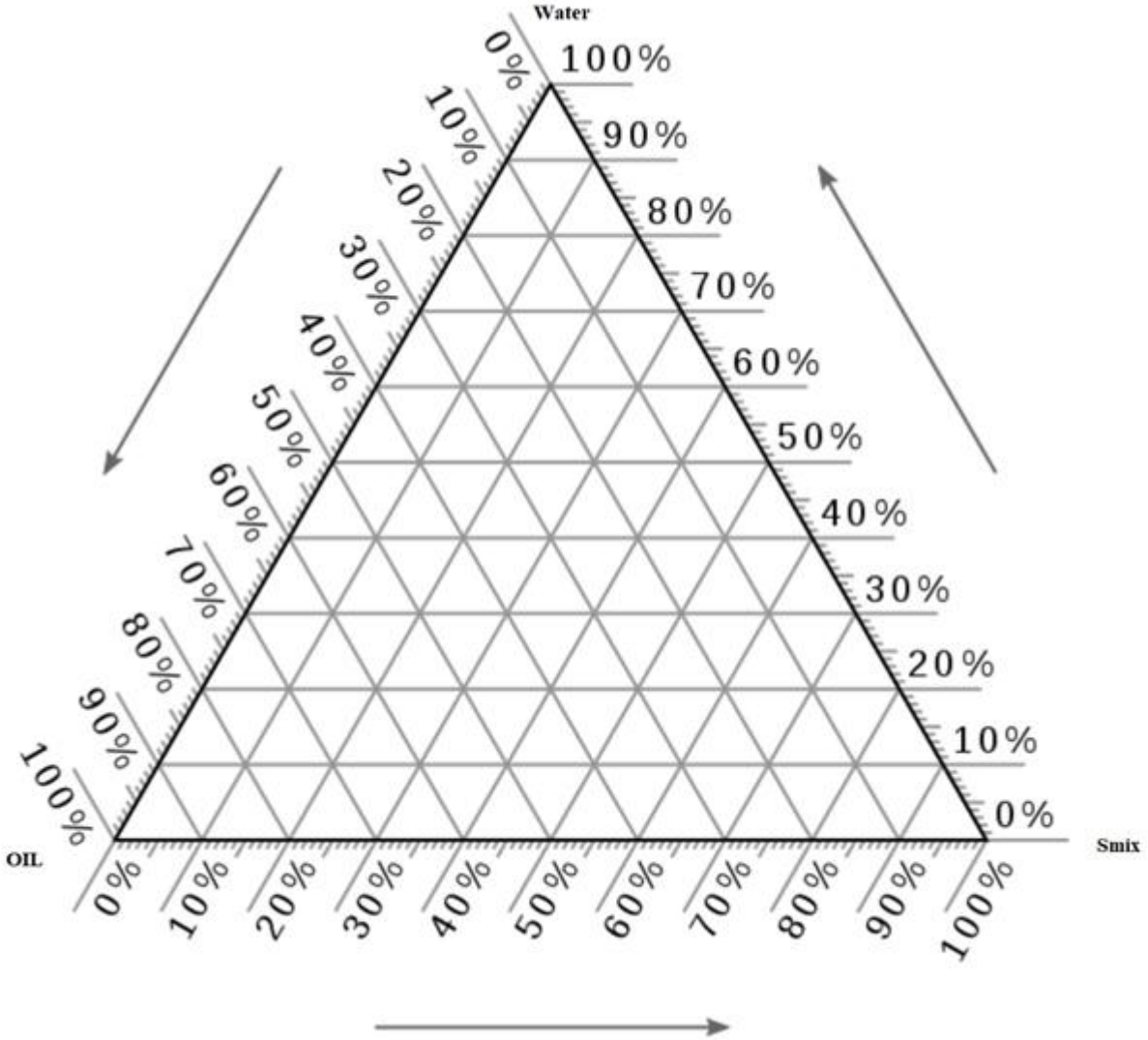

Figure 7: Example of construction of Pseudo ternary phase diagram

3.2.4.2 b. Phase inversion methods.

Phase inversion is mainly of two types: phase inversion temperature (PIT) and phase inversion composition (PIC) [180]. It is feasible to generate phase inversion in each of these approaches by modifying the composition or ambient parameters like temperature, PH, and ionic strength. In PIT, emulsion droplets are formed by altering the molecular geometry, i.e. the curvature of the surface of the involved surfactants (mostly nonionic), as well as modulating the solubility of such surfactants, with temperature being a key factor. [180]. An emulsion can be changed from one form to another by changing the temperature–time profile, for example, from O/W to W/O. All of these changes to an emulsion are caused by physicochemical factors, with temperature being a crucial contributor. This is due to the fact that low temperature causes an increase in hydrophilicity and thus solubility of nonionic surfactants, which makes the surfactant's head more hydrated, whereas high temperatures cause an increase in hydrophobicity and causes the surfactant's head to become less hydrated [148]. Surfactants assemble at optimum curvature, which is primarily determined by the critical packing parameter (CPP), which is one of their most essential physicochemical parameters [181].

3.2.4.2 c. Emulsion inversion points:

Emulsion inversion point is a catastrophic phase inversion method that produces nanosize emulsions by shifting the water and oil phase ratio [148]. An emulsion turns from O/W to W/O and vice versa using this method of progressively adding the dispersed phase into the continuous media. With constant stirring and a suitable emulsifier, an O/W emulsion with a high oil to water ratio is generated, and an increasing amount of water is progressively added. After reaching a high concentration, water molecules begin to pack and coalesce, causing the emulsion to reach the phase inversion point, and then the system switches from W/O to O/W. [182].

3.2.5 Characterization of SMEDDS:

SMEDDS encompasses a wide range of characterisation procedures and is available in liquid or solid dose forms. However, the focus of the characterisation approaches in this review will be liquid SMEDDS. Light scattering, microscopy, electrical properties and zeta potential, optical properties, and properties linked to interfacial and rheology are among the most significant physicochemical and stability investigations in all of these approaches. Aside from

these characterization approaches, the SMEDDS formulation process involves differential scanning calorimetry (DSC) based compatibility analyses of several formulation components. The techniques and instruments used in SMEDDS are as follows:

3.2.5.1 Light scattering methods

Static or dynamic light scattering techniques are one of the most important ways to determine the size of droplets in emulsion formulation. Some of the major and very significant properties such as physicochemical stability factors, solubility, release kinetics etc. of nano sized emulsions depend on particle (droplet) size and size distribution. The droplet size of nano size emulsion in SMEDDS can range from 5 to 50nm. Particle size measurements by static light scattering technique is based on the Mie theory following Maxwell electromagnetic field equations and calculating the scattering intensity by assuming the shape of the particle as spherical in a homogeneous dilute dispersion[183,184]. Dynamic light scattering techniques also known as photon correlation spectroscopy is nowadays mainly used for the determination of particle size and size distribution. DLS techniques are used normally for analysis of particle size ranging from a few nanometers to about 3 micron [185]. DLS calculates the particle size of the droplets in nanoemulsion when they move randomly in solution following Brownian motion [186]. It works on the translational diffusion coefficient for measurements on the basis of analyzing the interaction between a laser beam and dispersion whereas Photon correlation spectroscopy (PCS) and Doppler shift spectroscopy (DSS) are conventional design of DLS and use to measure the particle size of dilute dispersions. Some time particle size can be determined through Stock's Einstein equation in absence of particle particle interaction [183]. Stock's equation can be written as: $r = \frac{kT}{6\pi\eta_1 D}$

where r is radius of droplet, k is Boltzmann constant, T is absolute temperature, and η_1 is the viscosity of continuous phase.

3.2.5.2 Zeta potential and other electrical characteristics

SMEDDS preparations are colloidal dispersion and zeta potential being the electro kinetic potential at the shear plane is a very important factor for the physical stability of the nano sized emulsion droplets [187]. SMEDDS preparation involves a number of surfactants carrying numerous kinds of positive and negative ions attached to the surface of droplets. Carrying an anionic emulsifier the surface of the droplets can be negative whereas with a cationic emulsifier it will be positive. This zeta potential measures the electrical potential

related to the same ions. Nanoemulsion formulations on storage can show changes in stability due to electrostatic repulsion and that can be measured in relation with zeta potential. Using electrophoretic techniques the electrical charges of the emulsion droplets can be measured. In this electrophoretic technique the charges are measured from particle velocity [188].Generally physical stability of nanoemulsion should be measured in value of ± 30 mV during its storage. For the convenient determination of zeta potential of emulsion droplets generally dynamic light scattering (DLS) and photon correlation spectroscopy (PCS) are used. Characteristics of emulsion in terms of charge of droplets are very important and zeta potential comes in the same purpose. But considering only zeta potential study for stability of emulsion is limited. This is because characterization of emulsion charge and potential through zeta potential is limited to emulsion formulated using hydrocolloid and showing low zeta potential determination value. Other than zeta potential determination there are few other advanced technologies for the measurement of charge. These advanced techniques and related instruments can not only be used for different other characterizations. Some of them are photon correlation spectroscopy and Rheo small angle neutron scattering (SANS). Using these techniques one can study the formulation component, related rheological properties and structure as well [189].

3. 2.5.3 Rheological properties

Depending on the component and nature of material used in formulation, an emulsion shows different flow properties. Most importantly they can differ in viscosity and can be of low viscosity or high viscosity depending on different factors. For the SMEDDS formulation viscosity is one of the critical parameters as it can affect the SET as well as ease in dosage form formulation such as capsulation. Change in viscosity can be well observed in food emulsion. Conventional food emulsions are actually concentrated products. Regardless of composition and process of formulation one of the reasons for change in viscosity can be attributed to droplet interaction. Generally emulsions with small droplets can attract and coalesce many times and more than large sized conventional emulsions and result in change to high viscosity. Considering all these factors it is very important to understand the rheological properties of emulsion. Measurement of viscosity can be carried out with different kinds of viscometers [190].

3.2.5.4 Stability study

Like every formulation, stability is an important concern for Self emulsifying formulations. There are different factors which can affect formulation such as droplet attraction, phase separation, phase inversion, gravitational separation, etc. phase separation or creaming can be defined as a stage when the droplet of emulsion either goes upward or downward. On long storage phase inversion can take place where o/w emulsion can change to w/o or vice versa.

Stock's law states an equation regarding the stability of emulsion system:

$$V = \frac{g}{18} D^2 \frac{\rho_d}{\eta}$$

Where: V= rising velocity of the droplet (m/s)

g= gravitational constant (9.81m/s^2)

D= droplet diameter (m)

ρ_d = density difference between heavy phase and light phase (kg/m^3)

η = dynamic viscosity of the fluid

3.3 Analysis

3.3.1 LCMS/MS:

In recent decades technology of chromatography has developed itself on a high. Starting from simple liquid chromatography it was HPLC and now one of the most desired and robust processes is liquid chromatography/mass spectrometry LC/MS. Coupling of MS to chromatographic techniques has made it desirable as it enhances the sensitive and highly specific nature of MS compared to other chromatographic detectors. With the development of electrospray ionization (ESI) technique, liquid chromatography mass spectrometry nowadays is a robust process and routine method for several drug development processes as well as development of many biological molecules. LCMS is a dreadfully selective, accurate and ultrasensitive technique. In this technique tandem use of mass spectroscopy and stable isotope internal standard provide ultra sensitive and precise assay. In the field of pharmacokinetics it is one of the most frequently used techniques. As far as the cost of study is concerned the LCMS method is affordable.

LCMS basic principle and instrumentation:

Liquid chromatography or high pressure liquid chromatography is a surface phenomenon. As far as the principle of HPLC is concerned it is based on the process of separation where the analyte molecule is distributed between a specific mobile phase and stationary phase. It is the chemical structure of the sample which decides the separation while passing through the stationary phase. Principle of mass spectrometer operation is converting the analyte molecule to an ionized state. Here mass to charge ratio (m/z) of produced ions after ionization are the main factor. So in LCMS here first the individual component is separated by liquid chromatography then on the basis of m/z ratio the ionized mass is separated and quantified by mass spectroscopy. In MS the ionization of intact molecules is a critical step and it is possible with atmospheric pressure chemical ionization (APCI) or Electrospray ionization and it also depends whether the analyte is polar or non polar in nature.

The basic components of LCMS can be briefly seen as:

Parts of liquid chromatography: Liquid chromatographies consist of four components.

1. PUMP
2. Auto sampler
3. Column
4. Detector

Now comprising a LC the LCMS components are.

1. LC
2. An interface between LC and MS
3. An ion source that ionizes the analyte.
4. An ion Guide
5. A mass analyzer
6. Detector.

Advantages of LCMS:

1. A robust analytical technique which furnishes highly sensitive and selective method of analyte detection with exact molecular weight of a broad range of samples.
2. Combination of liquid chromatography and mass spectrometry reduces the experimental errors and enhances the accuracy of the experimental results.

3. Ease of the process and affordability results in vast utilization of this method in many pharmaceutical product developments and clinical studies.
4. Very low concentrate analytes are possible to analyse.
5. It is widely applied in regulatory compliance.

Figure 8: LCMS/MS System

3.3.2 Non invasive blood pressure (NIBP):

NIBP stands for non invasive blood pressure. NIBP measurement involves the detection of systolic blood pressure in rodents by observing the generation of pulse. A cuff is put round the tail of the rodent which is inflated in the order to stop the blood flow. As gradually the applied pressure decreases, a time point comes when the pulse reappears on the skin. This pertcular point marks the systolic pressure as blood flow is restored since the externally applied pressure and internal pressure get equal. Usually an average of 3 or more consecutive ascending peaks of pulse is taken to determine the BP.

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CHAPTER IV

MATERIALS AND METHODS

4. 1. Materials:

Seletion of appropriate materials is the first priority for development and formulation of any dosage form. In the present work the development, formulation and analysis of a nanolipoidal formulation as liquid SMEDDS (preconcantrate) involves the entire chemical belongs to HPLC grade and analytical grade. Here Milli Q water is used in study. The name of materials and source of the same is given below.

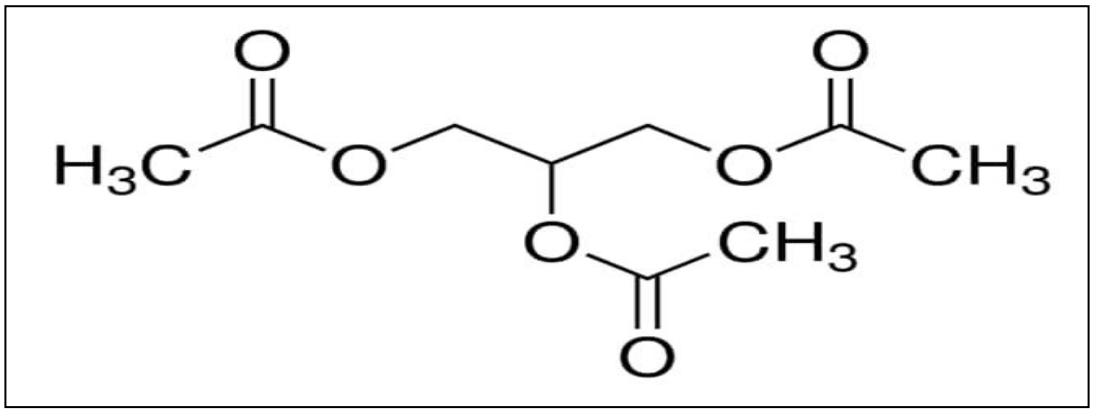
Table 1: List of materials used in formulation development and analysis.

Sl no	Materials	Sources
1	Cilnidipine (selected drug)	Pure Chem Pvt Ltd (Gift)
2	Tween 20 (selected surfactant)	Merck Millipore
3	Tween 80	Merck Millipore
4	Labrasole	Gattefoss
5	Span 80	Merck Millipore
6	IPA	Merck Millipore
7	Glycerol	Merck Millipore
8	Transcutol HP (selected co-surfactants)	Sigma Aldrich
9	Soyabean oil	Lipoid GmbH, Ludwingshafen, Germany
10	Capmul MCM	ABITEC
11	Triacetin (Selected oil)	Sigma
12	Flaxseed oil	Local purchase
13	Almond oil	Local purchase
14	Rice bran oil	Local purchase
15	Seesem oil	Local purchase
16	Olive oil	Local purchase
17	Acetonirile	Merck Millipore
18	Milli Q water	Sartorius Stedium
19	Methanol	Merck Millipore
20	α-Napthol	Gift sample
21	Ketoconazole	Gift sample
22	PEG 400	Loba chem.
23	Sodium Dodecyl Sulphate	Merck Millipore
24	Larcanidipine	Gift sample
25	Tolbutamide	Gift sample
26	Propylene Glycol	Gift sample

4.1a: Selected formulation materials in brief

Triacetin:

Structure

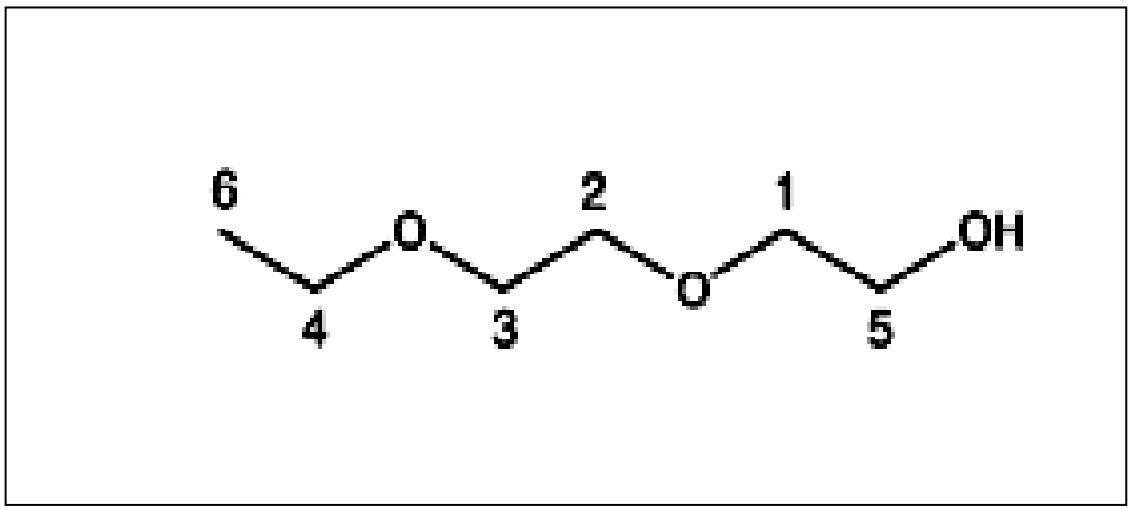


Chemical formula: **$C_9H_{14}O_6$**

Triacetin is basically a triglyceride derived by acetylation of three hydroxyl groups of glycerol. It is also known as glyceryl triacetate. Triacetin is an edible oil in short chain triglyceride form [1,2]. Triacetin is a GRAS listed ingredient. It is preferably used as a plasticizer in chewing gum, food additive carrier and food humectants. Molecular weight of triacetin is 218.20 g/mol and it is a colorless liquid. It reveals a slightly fruity or fatty odor with mild sweet taste. As far as daily permissible intake is concern the joint FAO/WHO expert Committee on food additive (JECFA) considered it unnecessary to assign an acceptable daily intake (ADI) as triacetin is metabolized in the same manner as other dietary triglycerides [3] .In present formulation triacetin is used as oil.

Transcutol® HP:

Structure:

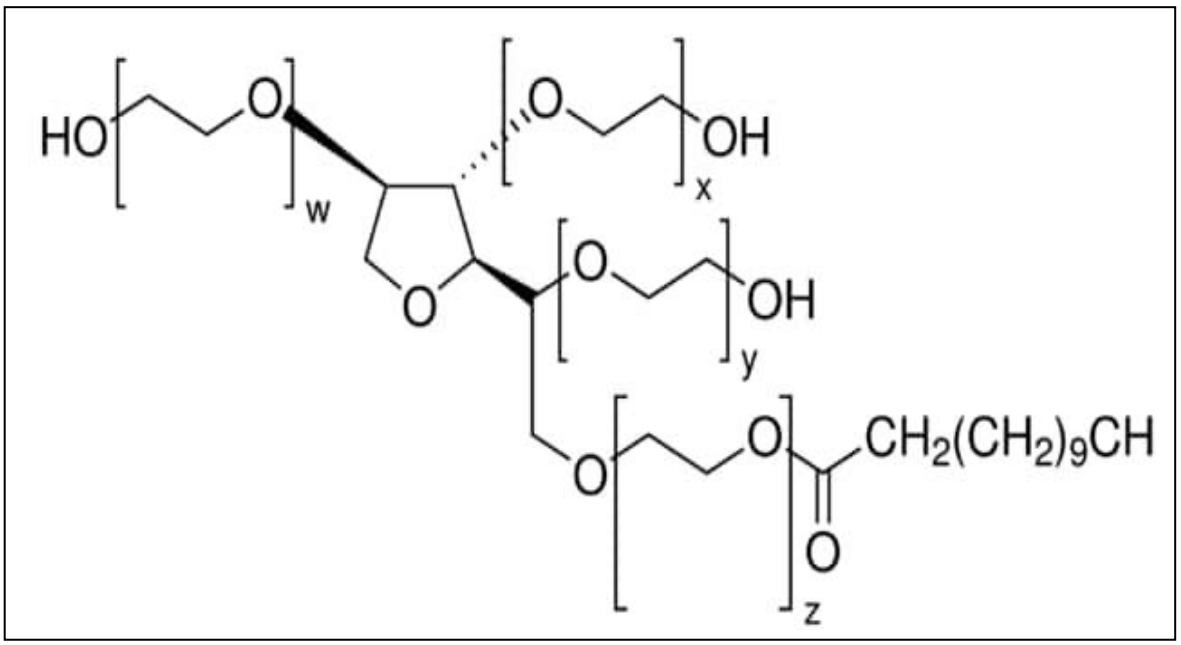


Chemical formula: **$C_6H_{14}O_3$**

It is a colorless, slightly viscous liquid with a mild pleasant odor. It has a molecular weight 134.17g/mol. In the formulation and several production procedures of medications, cosmetics, and food additives, Transcutol® HP is frequently utilised as a vehicle. In the formulation process of various nanolipoidal formulations transcutol is used as a co-solvent or co-surfactant [4,-6]. Permissible daily intake of Transcutol® HP is more than 10mg/Kg/day [7]. In present formulation Transcutol HP is used as co surfactant.

Tween 20:

Structure:



Chemical formula: **$C_{58}H_{114}O_{26}$**

Tween 20 is Polyoxyethylene glycol sorbitan monolaurate. Molecular weight of tween 20 is 1227.54g/mol. It is a commonly excipients and used as surfactant in various nanolipoidal formulations such as emulsion and suspension [7-10]. Other than this it is used as a wetting agent in flavored mouth drops such as Ice drops. Permissible daily intake of tween 20 is reported to be 25mg/kg body weight [11]. In present work tween 20 is used as a surfactant.

4.2. Methods of experimental studies:

4.2. 1. Pre-formulation studies of Cilnidipine:

In order to ensure the purity of procured drug (Cilnidipine) following pre-formulation studies have been performed:

4.2.1a UV visual spectroscopy study of Cilnidipine:

Machine: Spectramax

Maximum wavelength found: 240 nm

Media: Methanol

Method:

Determination of λmax of Cilnidipine:

For determination of maximum wavelength and absorbance for different concentration of Cilnidipine, the Standard stock solution of Cilnidipine was prepared by dissolving 4.8 mg of powder Cilnidipine working standard in 48 mL of methanol to get concentration of 100 µg/ml. Aliquots of stock solutions were further diluted with methanol to get working solutions of 1 µg/ml to 100 µg/ml and the working standards were scanned between 200-400 nm to find λmax of solution using SpectraMax®. The maximum wavelength of Cilnidipine was found to be 240 nm.

Preparation of Cilnidipine calibration curve:

The Standard stock solution of Cilnidipine was prepared by dissolving 4.8 mg of powder Cilnidipine working standard in 48 ml of methanol. Then various concentration 1µg/ml, 2.5 µg/ml,5 µg/ml,7.5 µg/ml,10 µg/ml, 15 µg/ml,20 µg/ml,25 µg/ml,30 µg/ml,50 µg/ml and 100 µg/ml of Cilnidipine in methanol was prepared. Pure methanol was taken as blank. At 240 nm wavelength the absorbance of various concentrations was observed and plotted to obtain the Cilnidipine standard curve.

4.2.1b XRD of Cilnidipine:

For the analysis of crystalline nature as well as polymorphic character of the powder drug, X-ray powder diffraction (PXRD) study was carried out (MODEL- ULTIMA-III ,RIGAKU, MAKE :JAPAN).

4.2.1c FTIR study of Cilnidipine:

In order to find the different functional groups of Cilnidipine FTIR study was done using BRUKER MODEL-ALPHA Germany.

4.2.1d Determination of purity of Cilnidipine with the help of DSC (Differential scanning calorimetry):

The purity of the procured Cilnidipine was determined by DSC i.e differential scanning calorimetry (MODEL NO.:- Pyris Diamond TG/DTA, MAKE:- PerkinElmer (SINGAPORE), and the melting point was done with thiele tube method.

4.2.1e HPLC Method development for in vitro dissolution and membrane permeability study using HPLC:

HPLC technique was used to determine in-vitro dissolution tests and the permeability of goat intestinal membranes for developed formulations. The system consists of **Shimadzu series** with a UV detector. An octadecylsilane column (5µ, 4.6250 mm) with a thermostat set at 25 °C was used to analyze all of the samples. Acetonitrile, Methanol, and water were used as mobile phases in the method development. Flow rates of 1 ml/min, 0.8 ml/min, and 0.5 ml/min were tested. For this purpose, both isocratic and gradient separation methods were investigated. Lercanidipine, α-Naphthol, and Ketoconazole were employed for selection of internal standards.

Standard curve of Cilnidipine using HPLC:

Freshly prepared 100 µg/ml stock solutions of Cilnidipine and internal standard diluted with Acetonitrile was used for preparation of intermediate concentrations. Total 9 different concentrations were made to get the standard curve. These concentrations were 10µg/ml, 5 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml, 0.0625 and 0.03125 µg/ml. Acetonitrile was used as a diluting solvent. Then the samples were run for method development of Cilnidipine.

4.2.2 Formulation development of Cilnidipine Loaded SMEDDS:

4.2.2.1 Selection of suitable oil on the basis of maximum solubility:

The basis of selection of suitable oil for the formulation of Cilnidipine SMEDDS was maximum solubility of the Cilnidipine in various oils. Oil, which is both safe for long-term usage and inexpensive, was also an important concern. Hence solubility of Cilnidipine was determined in various oils (olive oil, almond oil, Castor oil, Triacetin, Sesame oil, flaxseed oil, Rice Bran oil and Capmul MCM). Cilnidipine was put in excess to 2 mL of each type of oil in a 5-mL eppendorf tube and mixed with a Cyclo mixer. To achieve equilibrium, all of the tubes were held at 25 ± 1 °C in an isothermal shaker for 72 hours. After that, the samples were removed from the shaker and centrifuged for 15 minutes at 10,000 rpm. The supernatant fluid was removed and filtered using a 0.22 m pore size membrane filter. For spectroscopic analysis, the supernatant fluid was diluted many times in methanol. Methanol was used as blank media. The concentration of Cilnidipine in several types of oils was determined using UV/VIS spectroscopy at 240 nm (Intech, Model No: 295). [12]

4.2.2.2 Selection of Surfactant and co-surfactant: For development of SMEDDS surfactant and co-surfactant were chosen one by one for the current formulation. [12]

4.2.2.2 a. Selection of surfactants:

Tween 20, Tween 80, Span 80, and Labrasol were among the surfactants used for selection and subsequent formulation of desired SMEDDS in this investigation. 2.5 ml of 15 percent by weight of surfactant solution was prepared in the study for selecting the acceptable surfactant initially in water, and then 4 µl of selected oil (triacetin) was added with continuous violent vortexing. Following the observation of a one-phase clear solution, the oil was added in stages until the solution turned hazy and turbid.

4.2.2.2b. Selection of co-surfactants:

In the process of selecting co-surfactants, the selected surfactant was mixed with different solubilizers (co-surfactants) namely, glycerol, isopropyl alcohol, PEG 400, Transcutol HP, and propylene glycol. A pseudoternary phase diagram was created using a fixed mix ratio of 1:1. The maximum ratios were covered by ten different combinations in varied weight ratios of oil and Smix, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, and 1:3.5, to highlight the borders of phases generated exactly in the phase diagrams.

4.2.2.3 Development of pseudo-ternary phase diagram:

Various oils and Smix ratios were used to create the pseudo-ternary phase diagram. In 10 ml borosil glass tube, several ratios of surfactants and co-surfactants (1:1, 2:1, 3:1, and 4:1) were combined with the selected oil in varied ratios (1:9, 2:8, 1:3.5, 3:7; 4:6,5:5,6:4,7:3, 8:2, 9:1) for each phase diagram. Using the water titration technique, a pseudo-ternary phase diagram was created. Titration was performed with Milli Q water in increasing proportions, with each step resulting in a change in the combination. At 25 °C, water was added in stages using a vortex mixer. Using software, phase diagrams were created, and then the suitable Smix was chosen by observing the best microemulsifying zone. The same region was used to design the formulations. One axis represents the aqueous phase, the second axis represents the oil phase, and the third represents the mix surfactant phase, i.e. Smix, in the resulting pseudo ternary component phase diagram.

4.**2.2.4 Method of selection of formulation composition:**

According to the pseudo ternary phase diagram, the formulation composition is the region of maximal emulsification where maximum emulsion dilution with water is achievable. Utilizing Design-Expert^{TM} (Stat-Ease Inc.) v7.0, a formulation design was created using the D-Optimal design and point exchange technique. All the formulations were tested for all relevant physicochemical characterizations. The optimum formulation was chosen based on the obtained parameters using Design-ExpertTM (Stat-Ease Inc.) v7.0 again and was then examined for relevant in vitro characterizations.

4.2.3: characterization of formulations:

4.2.3.1 Thermodynamic stability study

1. Different thermodynamic stability experiments have been performed on the 16 produced formulations (Cilnidipine SMEDDS and SMEDDS with assigned amount of water in formulation both) to assess their physical stability, and observations were made for instability such as phase separation or turbidity.
2. Heating–cooling cycle: This technique was utilized to carry out the stability studies of all developed formulations. The formulations were stored at 4 °C (refrigerated temperature) and 45 °C for at least 48 hours and observed for the phase separation phenomenon. The process was repeated for six times.

3. Centrifugation test: All the developed formulations were centrifuged at 10000 rpm for 15 min, and observed for the phase separation phenomenon.
4. Freeze–thaw cycle: The formulations were frozen and thawed three times between -20°C and +25°C storage temperatures for at least 48 hours and observed for the phase separation phenomenon.

4.2.3.2 Robustness to dilution in GI lumen:

The dilution resistance of Cilnidipine SMEDDS was investigated by diluting it 125, 250, and 500 times in water, acidic buffer of pH 1.2, and phosphate buffer of pH 6.8. The diluted SMEDDS were then stored for 6, 12, 24, and 72 hours and observed for drug precipitation and/or phase separation. The diluted (500 times) SMEDDS formulations were evaluated using UV spectroscopy at 640 nm against Milli-Q water and other media as a blank. [13, 14]

4.2.3.3 Method of globule size, zeta potential (Surface Charge), polydispersity index (PDI), measurement

The mean globule size of the developed SMEDDS was determined using dynamic light scattering method (DLS-nano ZS, Zetasizer, Nanoseries, Malvern Instruments). The polydispersity index (PDI), which represents the broadness of the globule size distribution and the surface charge (zeta potential) of the formed SMEDDS was obtained using the same instrument. [15,16, 17]

4.2.3.4 Determination of pH

The pH of the optimized SMEDDS was determined using a pH meter (Sartorius PB-11) at 25°C. [18]

4.2.3.5 Refractive index

Abbe refractometer was used to evaluate the refractive index of SMEDDS samples and placebo samples (SMEDDS without medication) at 25°C. The isotropic character of SMEDDS is determined by the refractive index.

4.2.3.6 Viscosity

The viscosity of the developed SMEDDS was measured by Brookfield Viscometer DV II+ Pro (Brookfield Engineering Laboratories, Inc., MA) with spindle CPE 41 at 10 rpm, and 25± 0.5°C. [19]

4.2.3.7 Electrical conductivity

The electrical conductivity of each formulation was investigated to identify the type of emulsion developed i.e., O/W or W/O, as well as the percolation effect. Electrical current was passed through the samples in this experiment. For the O/W emulsion type, a variance in the digital conductivity meter with cell (Systronic 304) is expected.

4.2.3.8 In-vitro dissolution of developed formulations:

To find the best formulation, the developed formulations were subjected to in-vitro drug release studies. The developed self micro emulsifying formulations were immediately introduced into the release medium (phosphate buffer 6.8) held in a 900 ml basket of the USP apparatus II at rpm 100 and maintained at 37±.5ºC. The samples were taken at intervals of 1, 5, 10, 15, 20, and 30 minutes, after which they were centrifuged at 10000 rpm and filtered through a 0.22m membrane filter. The filtrate was analyzed by HPLC for release of Cilnidipine at various time periods. Further the comparative dissolution study of Optimised SMEDDS with marketed tablet was done in the same manner and the analysis was done with the help of LCMS/MS for better confirmation [20].

4.2.3.9 Ex-vivo permeation studies using goat intestinal sac technique:

Intestinal permeation studies were employed to determine the most permeable SMEDDS among all developed formulations. Fresh goat intestine was brought from a neighboring butchery and maintained at - 20 ºC for the investigation. The mucus and undesirable intestinal content were removed from the intestinal segment used in the experiment using Krebs-Ringer solution. Both ends of the intestinal membrane were used to make a sac, with one end open. The luminal section was filled with Cilnidipine powder suspension containing 0.2 percent SDS and developed SMEDDS formulations comparable to 2 mg of Cilnidipine. Both the ends of the intestinal segment were tied off and the sac was immersed in an organ bath maintained at 50 rpm using a magnetic stirrer with continuous aeration. 250 mL Phosphate buffer of pH 6.8 with 0.5 percent SDS was used in the receptor compartment of the organ bath maintained at a temperature of 37 ± 0.5ºC. Samples were taken at regular intervals of 5 minutes, 30 minutes, 60 minutes, 1 hour, 2 hours, 3 hours, and 4 hours and replenished with the same quantity of fresh phosphate buffer to keep the sink condition. Lab Companion was used to conduct this ex-vivo permeation research (Model No. IST-3075R). The samples were filtered

through a 0.22m membrane filter. The filtrate was analyzed by HPLC for permeability of Cilnidipine. [21,22]

4.2.4 LCMS method development and validation for in vivo animal study:

4.2.4.1 Bio-analytical method development by gradation in LC-ESI-MS/MS:

Cilnidipine, a calcium channel blocker, is one of the most prominent dihydropyridines among the numerous dihydropyridines. Cilnidipine (CAS No. 132203-70-4) is a dihydropyridine dicarboxylate with the molecular formula C27H28N2O7. **[Figure: 1].**

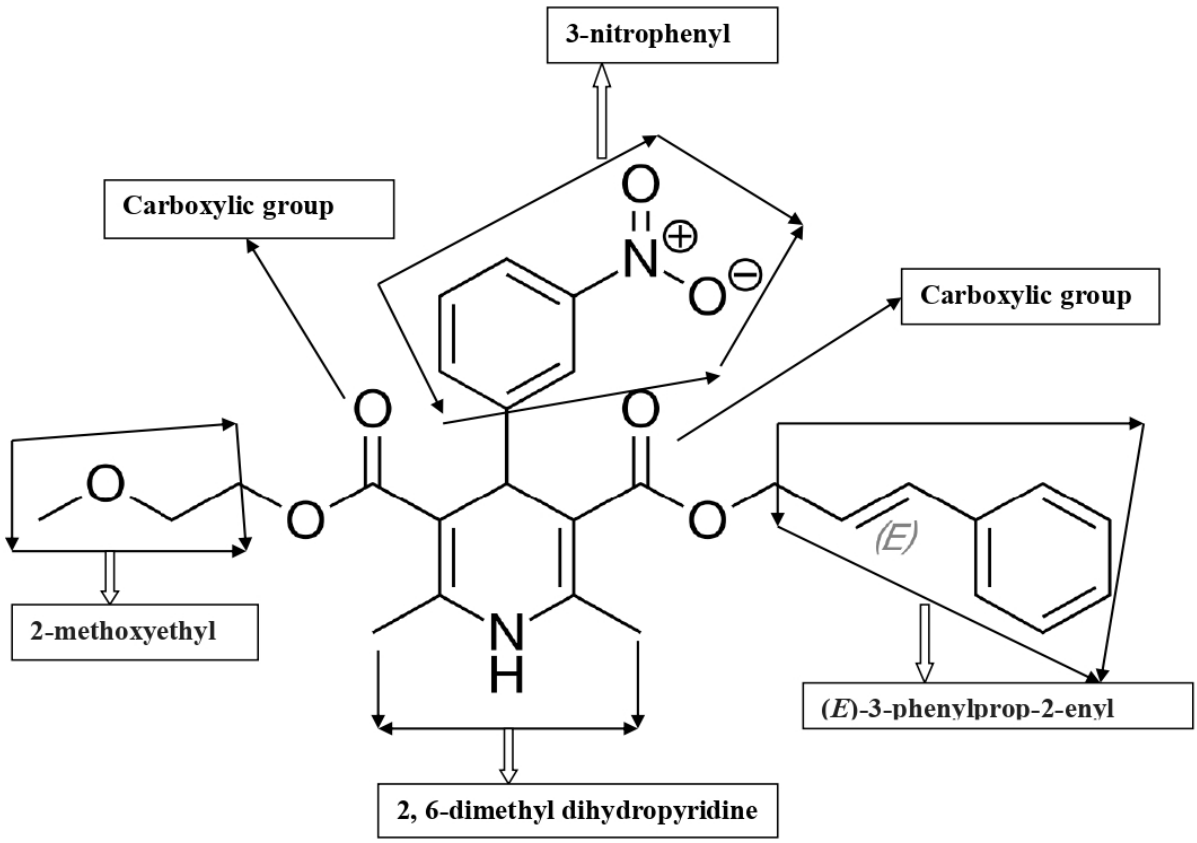


Figure 1: Cilnidipine chemical structure

Cilnidipine has a pKa value of 8.36 due to the presence of 3-nitrophenyl. Because the nitro group is an electron withdrawing group, the CH alpha bond (nearby) is acidic. Cilnidipine, on the other hand, contains 2, 6-dimethyl dihydropyridine, resulting in a pKa value of 5.0 to 6.60, giving it a slightly acidic property. Cilnidipine also contains 2-methoxyethyl, which has a strong basic character, as well as two carboxylic acid groups, which are acidic in nature, and one of the sections contains 3-phenylprop-2-enyl, which has a basic character. Overall,

the compound has a neutral character, but because of the greater acidic character, the proton is tightly bonded inside the molecule, resulting in a pKa value of 19.46, which is strongly basic. Cilnidipine has a molecular weight of 492.53, however its precise mass is 492.18. Negative mode was chosen to achieve adequate chromatogram response because the electron withdrawing nitro group removes electrons from the solvent and neutralizes the positive ionization character of nitrogen present in the nitro group. On the other hand, fragmentation of 3-phenylprop-2-enyl during production in the carboxylic group, negative mode was chosen to achieve adequate chromatogram response.

Cilnidipine was deprotonated into parent ion [M-H] at m/z 491.2 in parent ion scanning (Q1), and product ions were discovered at m/z 237.0, 224.0, 208.0 in product ion scanning (Q3), with the consistent and selected ion at m/z 237.0 [(M-C15H12NO3-H) -] by releasing 2-[(2-phenylacetyl)amino]benzoate or 1-hydroxy-2-(4-methoxyphenyl)indole-3-olate. Parent and product ions scanning in negative mode was presented in **[Figure: 2].**

Phenomenex Kinetex 5 C18 100A 50*3 mm column was used to elute Cilnidipine in triple quadrupole liquid chromatography mass spectrometry (API-2000, Analyst software version 1.5, Applied Biosystem/ MDS SCIEX, Toronto, ON, Canada) using ESI interface. The chemical properties of the drug and the ionization method, which are required for adequate high sensitivity, influences the selection of mobile phase, pH of the mobile phase, and buffer concentration.

For analysis of Cilnidipine, 0.1 percent formic acid in Milli-Q water with 10 mM Ammonium acetate at a flow rate of 0.5 ml/min as an aqueous mobile phase and 0.1 percent formic acid in (Methanol) MEOH as an organic mobile phase were used that results in a superior S/N ratio (22), as well as resolution of peak baseline. The retention time of Cilnidipine and internal standard were 3.11 minutes and 2.92 minutes respectively. The gradient curve of method development has been shown in figure. **[Figure: 3]**

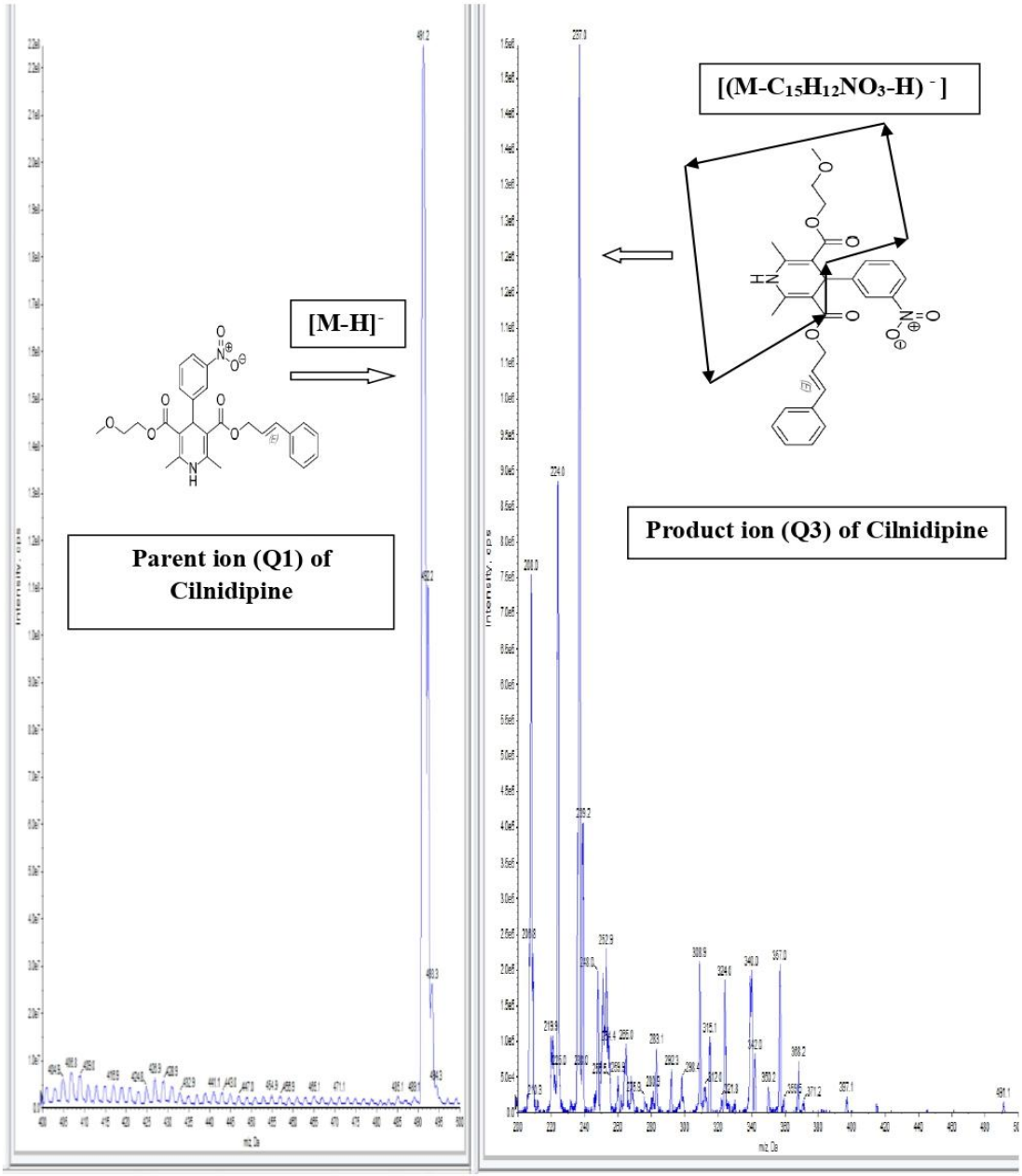


Figure 2: Parent ion (Q1) and product ion(Q3) scan of Cilnidipine.

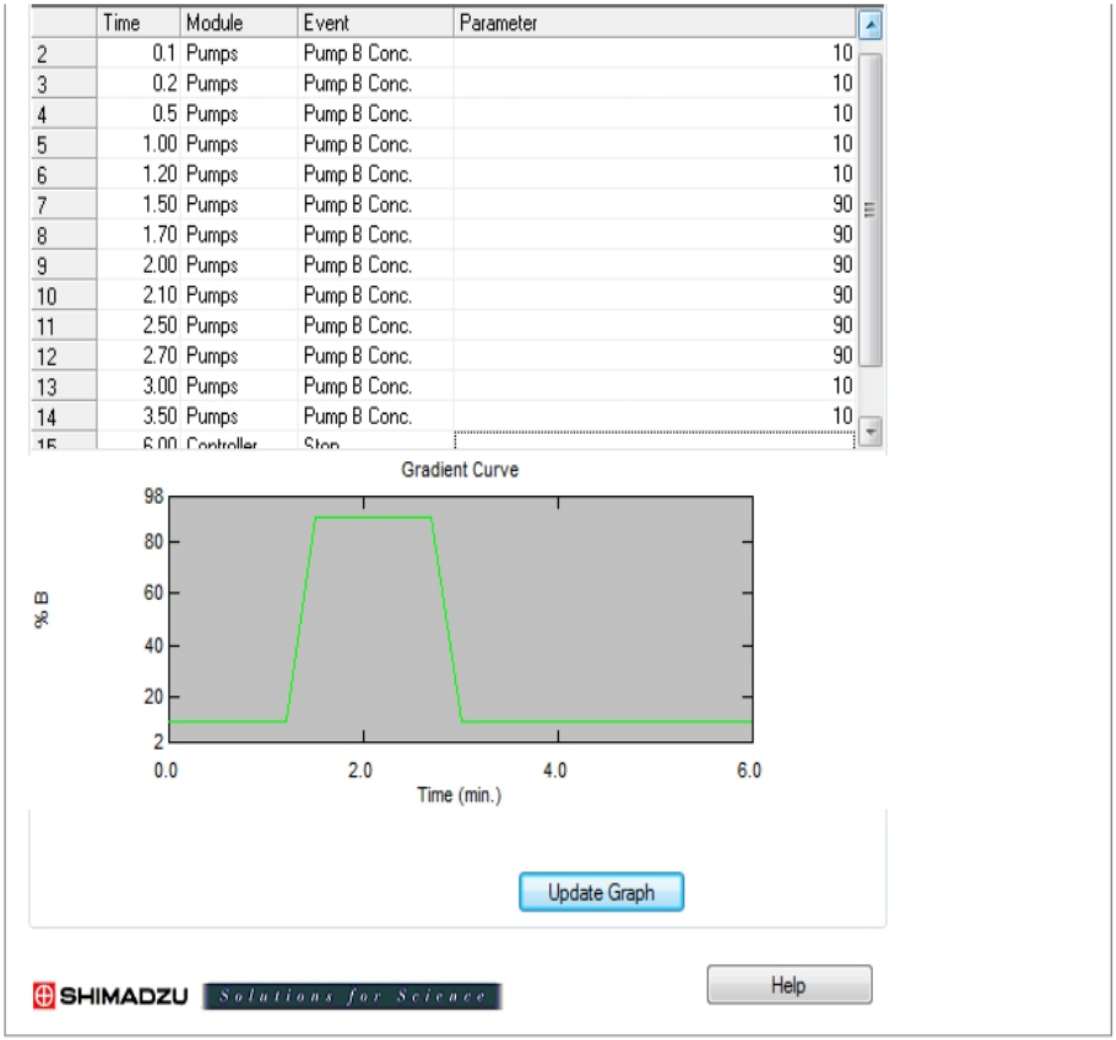


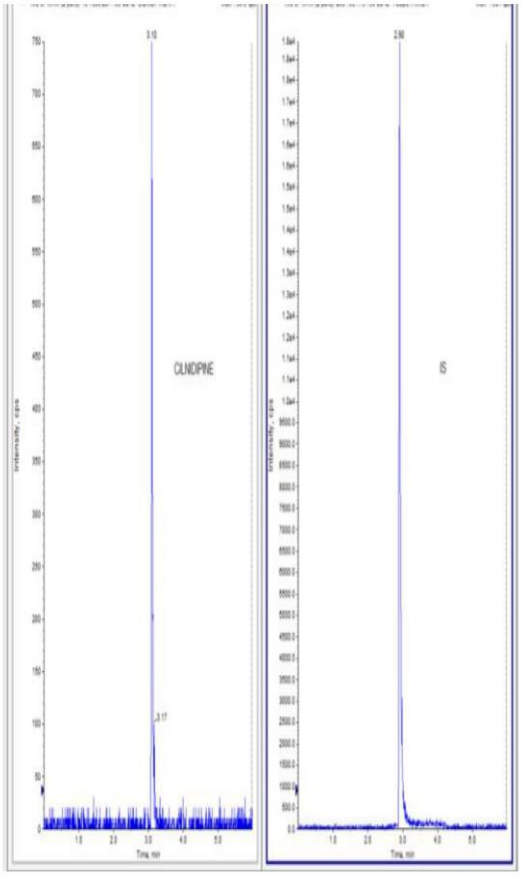
Figure 3: Gradient curve of method development of Cilnidipine.

This study was conducted using a gradient technique, where 10% organic solvent was run for 0.01 min to 1.50 min, 90% organic solvent was run from 1.50 min to 3.00 min, and 90% aqueous solvent was run for washing during rest of the total run time (6.00min). The mass optimized conditions are indicated in [**Table-2]**.

Table 2: Optimized mass condition

Sl no	**Parameter(s)**	**Value**
1	Ionization mode	MRM (Negative)
2	Source temperature (ºC)	400
3	Dwell time per transition (msec)	100
4	Curtain gas (psi)	30
5	CAD gas (psi)	8
6	Ion spray voltage (V)	-4500.00
7	Ion source gas 1 (psi)	55
8	Ion source gas 2 (psi)	45
9	Focussing potential (V)	-400
10	Declustering potential (V)	-20 (Cilnidipine) and -30 (IS)
11	Entrance potential (V)	-11
12	Collision energy (V)	-40 (Cilnidipine) and -25 (IS)
13	Collision cell exit potential (V)	-4 (Cilnidipine and IS)
14	Transition pair of Cilnidipine (analyte)	491.2/237.0
15	Transition pair of Tolbutamide (IS)	269.1/170.1

The representative chromatograms were shown in **[Figure-4a-4d].**



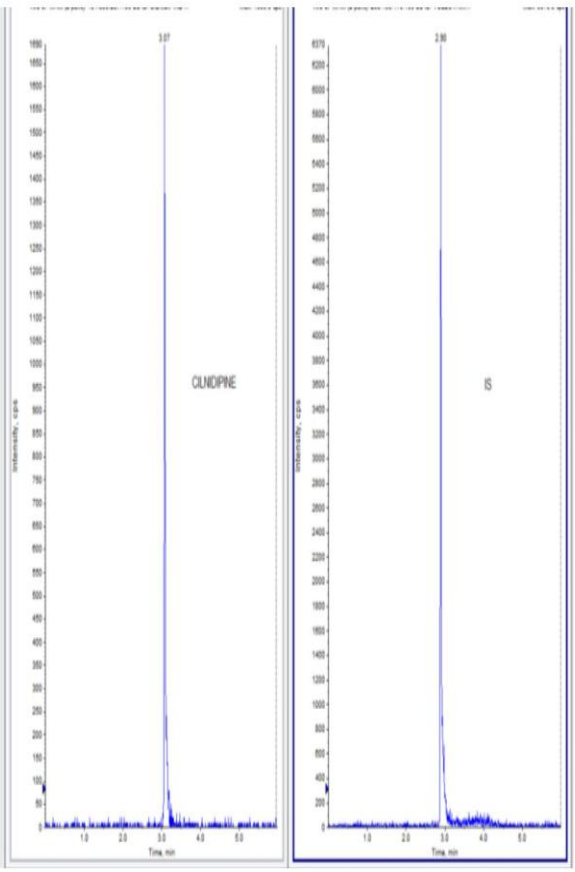
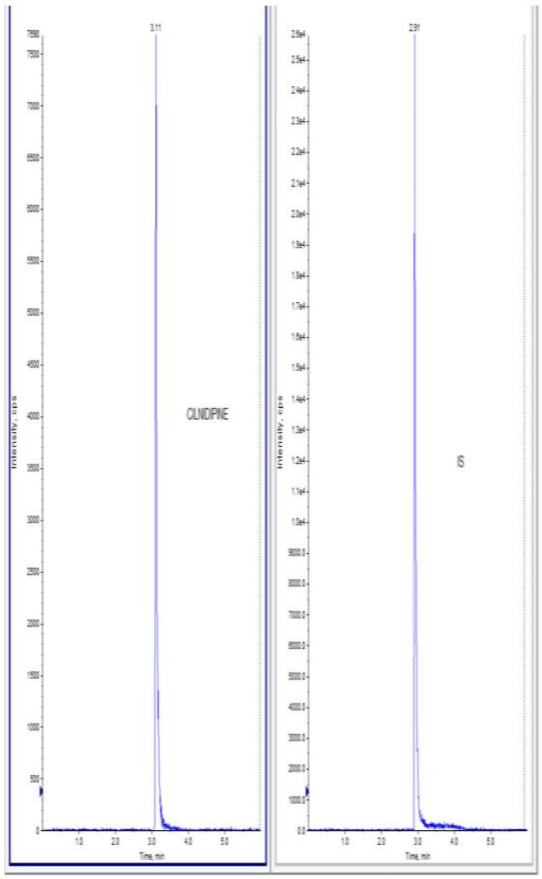


Figure: 4a

Figure: 4b

Figure 4a, 4b: Representative chromatograms of Cilnidipine and IS



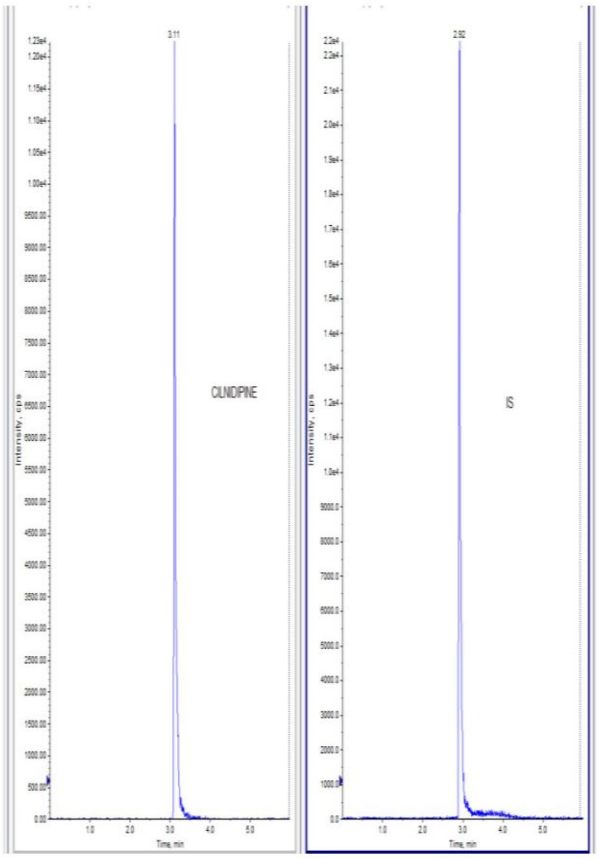


Figure: 4c

Figure: 4d

Figure 4c, 4d: Representative chromatograms of Cilnidipine and IS

4.2.4.2 Plasma extraction and sample preparation method:

4.2.4.2.1 Plasma extraction procedure:

Plasma extraction was performed by Protein precipitation technique. For this purpose 100 µl of plasma was mixed with 400 µl of acetonitrile (MeCN) containing 1000 ng/ml Tolbutamide (IS) and vortexed for 10 minutes followed by Centrifugation at 10,000 rpm for 10 minutes at -20 °C. After centrifugation 300µl of supernatant (plasma) sample was taken and transferred into autosampler vials for injection.

4.2.4.2.2 Stock solution and calibration of standards preparation:

4.2.4.2.2 a Preparation of Cilnidipine stock solution (W/V):

Accurately weighing 1.0 mg of Cilnidipine was dissolved in 1.0 ml of dimethyl sulfoxide. The final concentration of Cilnidipine was achieved at 1 mg/ml. This stock solution was used for preparation of intermediate concentration of Cilnidipine. It was stored in a refrigerator at 2-8°C.

4.2.4.2.2b Preparation of Tolbutamide (ISTD) stock solution (W/V):

Accurately weighing 1.0 mg of Tolbutamide was dissolved in 1.0 ml dimethyl sulfoxide. So the final concentration of Tolbutamide was achieved at 1 mg/ml. This stock solution was used for preparation of intermediate concentration of Tolbutamide. It was stored in a refrigerator at 2-8°C.

4.2.4.2.2 c Preparation of calibration concentrations in plasma:

Previously prepared and stored stock solutions (1 mg/ml) of Cilnidipine and internal standard (Tolbutamide) were diluted with methanol: water:: 50: 50 (v/v) and was used for preparation of intermediate concentrations. From each of this intermediate concentrations 500 µl of Cilnidipine was transferred into 500 µl blank plasma to prepare calibration standard concentration 1.87, 3.75, 7.50, 15.00, 30.00, 60.00 ng/ml in which LLOQ 1.87 ng/ml, LQC 5.62 ng/ml, MQC 22.50 ng/ml, HQC 45.00 ng/ml.

4.2.4.3 Validation of LC-MS/MS bioanalytical method:

Bioanalytical method for Cilnidipine was validated in accordance with US-FDA and EMA regulatory criteria [23, 24]. The linearity of a plasma sample was measured using a freshly produced sample on three separate days. Y= mX+c, where m is the slope, was used to calculate the regression value. The freeze-thaw stability, bench-top stability, autosampler stability, short-term, and long-term stability of LQC, MQC, and HQC samples were all measured to determine plasma sample stability. The matrix effect determined the minimum suppression of cilnidipine ionization from plasma and the maximum extraction of cilnidipine from plasma was determined by sample recovery. Figure 1 depicts the plasma calibration curve. **[Figure: 5]**

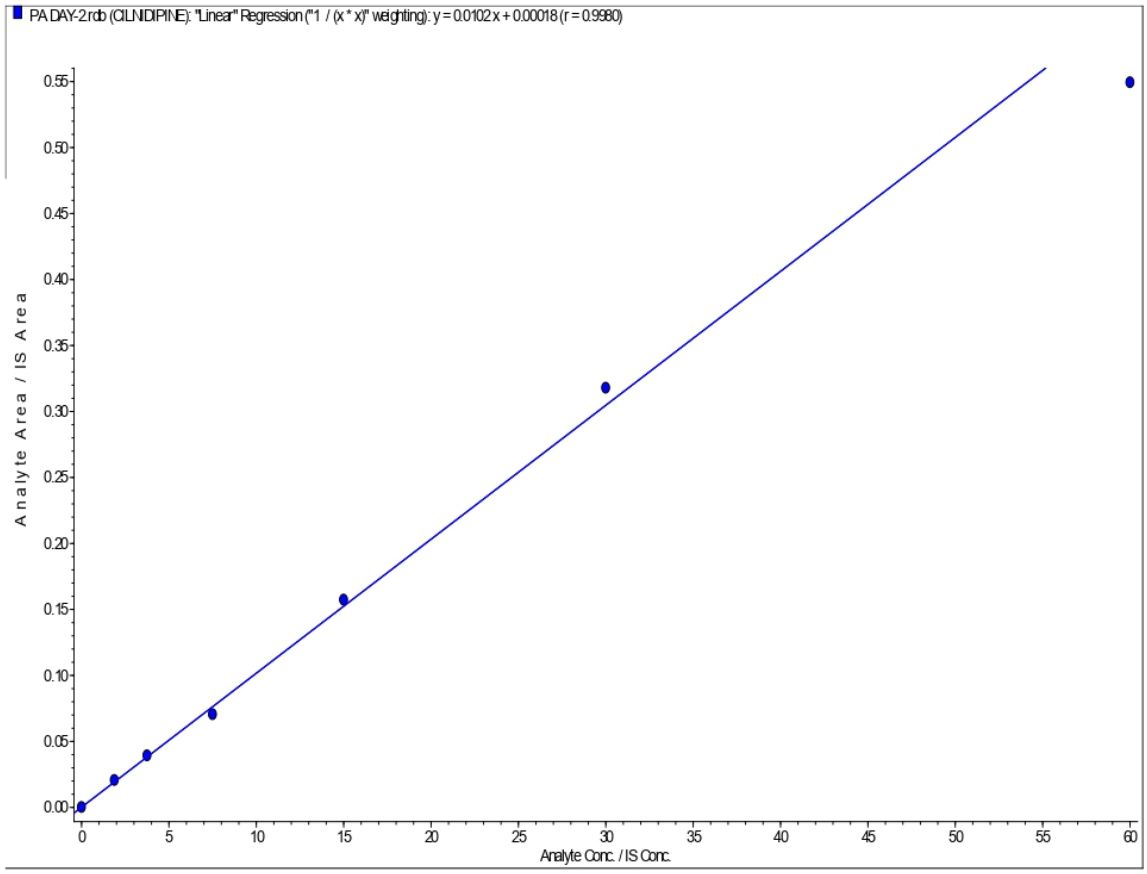


Figure 5: Plasma calibration curve of Cilnidipine.

4.2.5 Pharmacokinetic and pharmacodynamic studies using Wister rat:

4.2.5.1 Ethical Clearance and animal handling:

This research study was approved by the Institutional Animal Ethical Committee (IAEC), TAAB BIOSTUDY SERVICES, 69, Ibrahimpur road, Jadavpur , Kolkata-700032, West Bengal, India bearing Registration No: 1938/PO/Rc/S/17/CPCSEA dated 06.10.2018. This protocol was followed according to the guidelines of the CPCSEA of India for animal handling. All the procured animals were adapted and kept at standard laboratory condition.

4.2.5.2 Pharmacokinetic behavior in animal:

All the animals i.e., wistar rats were fasted for 12 hours before the experiment with sufficient water. Animals were divided into two groups with four animals in each group. Parallel study design was followed for optimized SMEDDS and marketed tablets (equivalent weight of powder tablet with 0.2% SDS). Administered dose of optimized SMEDDS and marketed

tablet was 1.2 mg/kg body weight of wistar rat. 300 µL of blood was collected into 500 µl centrifuge tubes containing EDTA solution at 0.0 (pre-dose) and 0.25, 0.5, 0.75, 1.0, 2.0, 4.0, 6.0, 8.0 10, and 12.0 h post-dose. All the samples were centrifuged at 4°C and 4000 rpm for 10 minutes.The samples (plasma) were collected in eppendorf tubes and stored at −20 °C prior to the analysis with LC-MS/MS (API2000).

4.2.5.3 Pharmacodynamic studies:

4.2.5.3a Development of hypertension in Wister rat:

Male Wister rats weighing 150-160 grams were chosen for this study. Hypertension in Wistar rats was developed using L-NAME (N-nitro L –arginine methyl ester) at a dose of 185 Mol/kg twice daily for the next seven days. [25,26].

4.2.5.3b Measurement of blood pressure:

The BIOPAC-MP36 system was used to measure blood pressure (BIOPAC Inc., USA). The body temperature of Wister rats was maintained at 37°C using a system-attached animal heating device. The MP36 software was employed for data processing and measurement of animal tail NIBP (noninvasive blood pressure). Animals' blood pressure was measured until a steady pressure was achieved. [26, 27]

4.2.5.3c Effect of optimized SMEDDS and marketed tablet on hypertensive rats:

In order to investigate the effect of optimized SMEDDS and marketed tablets, the animals are divided into three groups. Each group contains six wistar rats. Group 1 was assigned as a control group and treated with a control vehicle (water). Group 2 and group 3 animals were treated with the optimized SMEDDS and marketed tablet (equivalent weight of powder tablet with 0.2% SDS) at a dose of 0.5 mg/kg body weight and 1.2 mg/kg body weight respectively. The treatment runs up to 14 days and L-NAME was administered concomitantly as already discussed. The selection of dose was done with reference to the pharmacokinetic findings.

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CHAPTER V

RESULTS & DISCUSSIONS

5. Results

5.1 Pre-formulation studies of Cilnidipine:

5.1.1 Maximum wavelength of Cilnidipine Using UV-visual spectroscopy and preparation of calibration curve: Maximum wave length of Cilnidipine was found 240nm.

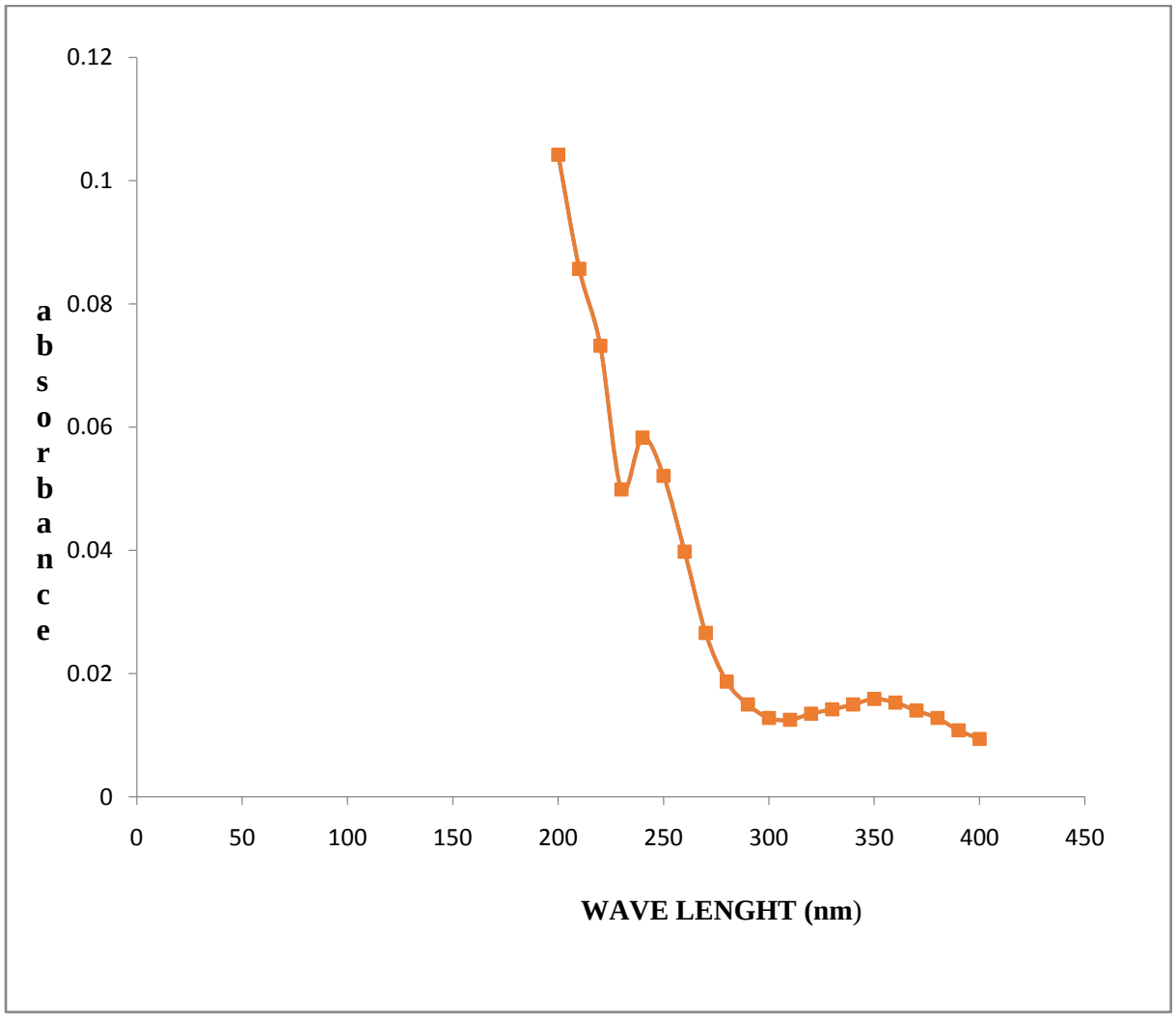


Figure 1: Cilnidipine maximum wave length

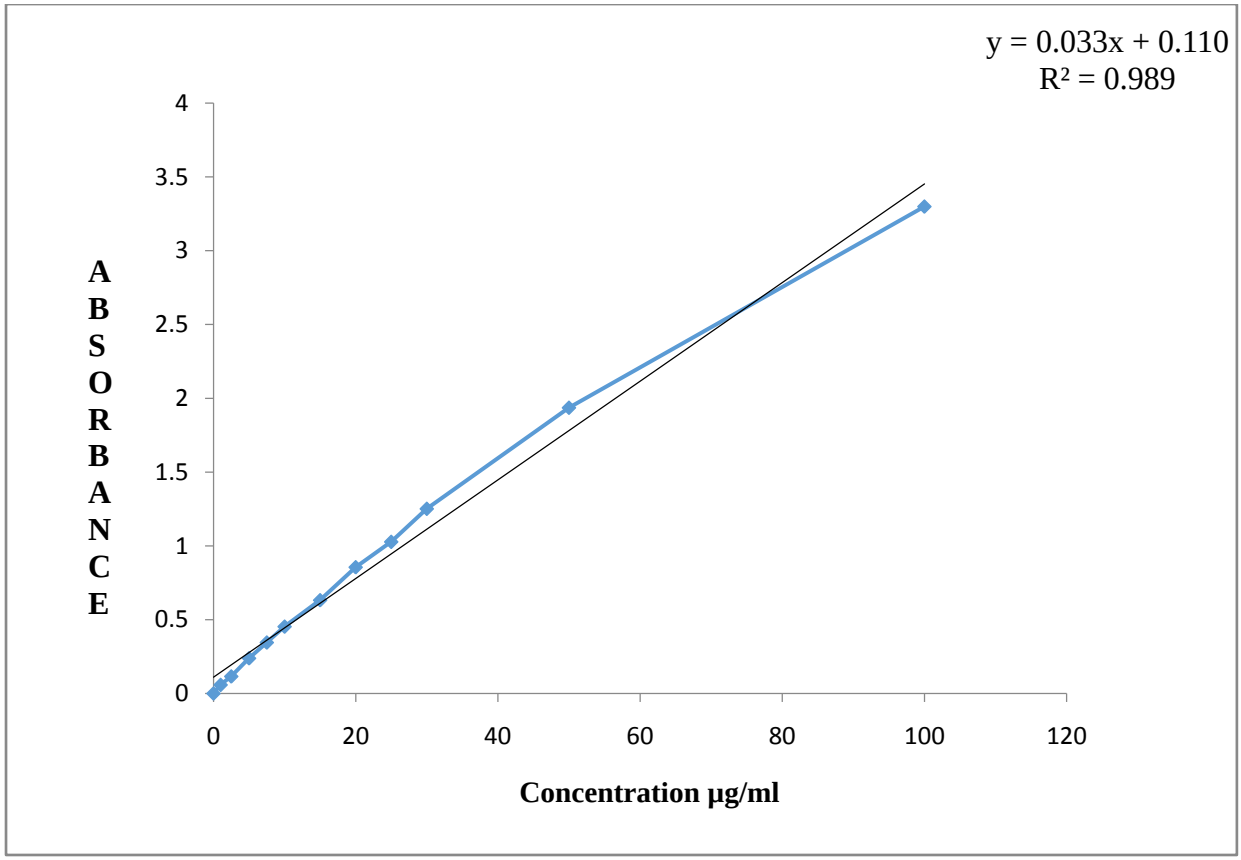


Figure 2: Calibration curve of Cilnidipine

5.1.2 XRD study of Cilnidipine:

X-ray powder diffraction (PXRD) study was carried out (MODEL- ULTIMA-III ,RIGAKU, MAKE :JAPAN) for polymorphic and crystalline character of the powder Cilnidipine. The PXRD spectrogram of the model drug exhibited characteristic sharp peaks at numerous 2θ and indicated its crystalline nature. PXRD methods illustrate the amorphous or crystalline nature and define the extent of hydrophilic or hydrophobic character of the drug. Polymorphic characters have their own effect on the dissolution and solubility of APIs characteristic sharp intensified peaks of Cilnidipine revealing its hydrophobic nature. **Figure 3**

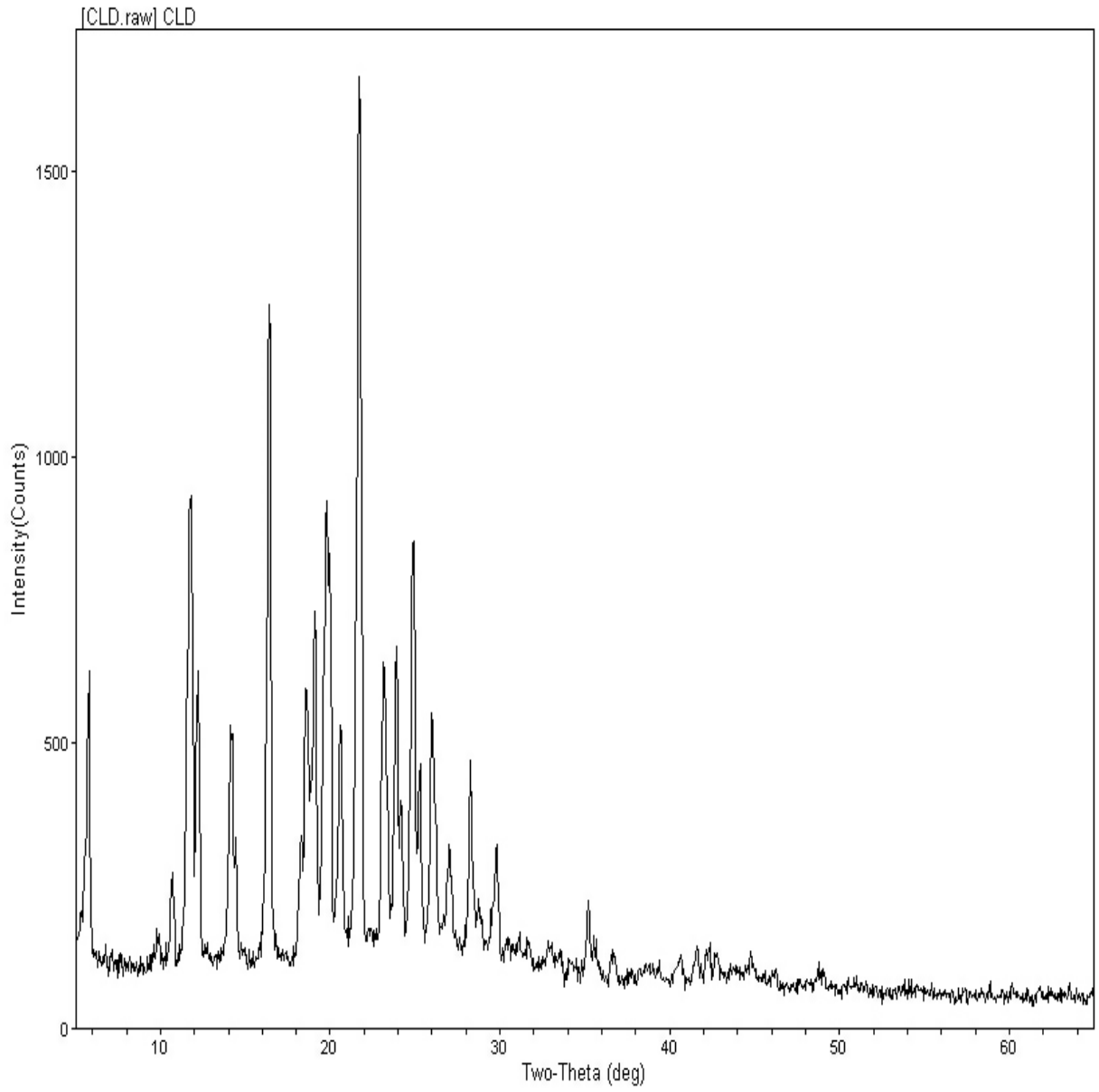


Figure 3: XRD of Cilnidipine

5.1.3 FTIR study of Cilnidipine:

Infrared spectroscopy (FTIR) has been carried out using BRUKER MODEL-ALPHA and it shows most distinct peaks of drug lay in the N- H stretch (3285 cm−1) and the C=C stretch (1649 cm−1). Moreover, the characteristic absorption band at 1347 cm−1 and 1251 cm−1 contribute to the existence of N-O and N=O [1]. **Figure 4 Table1**

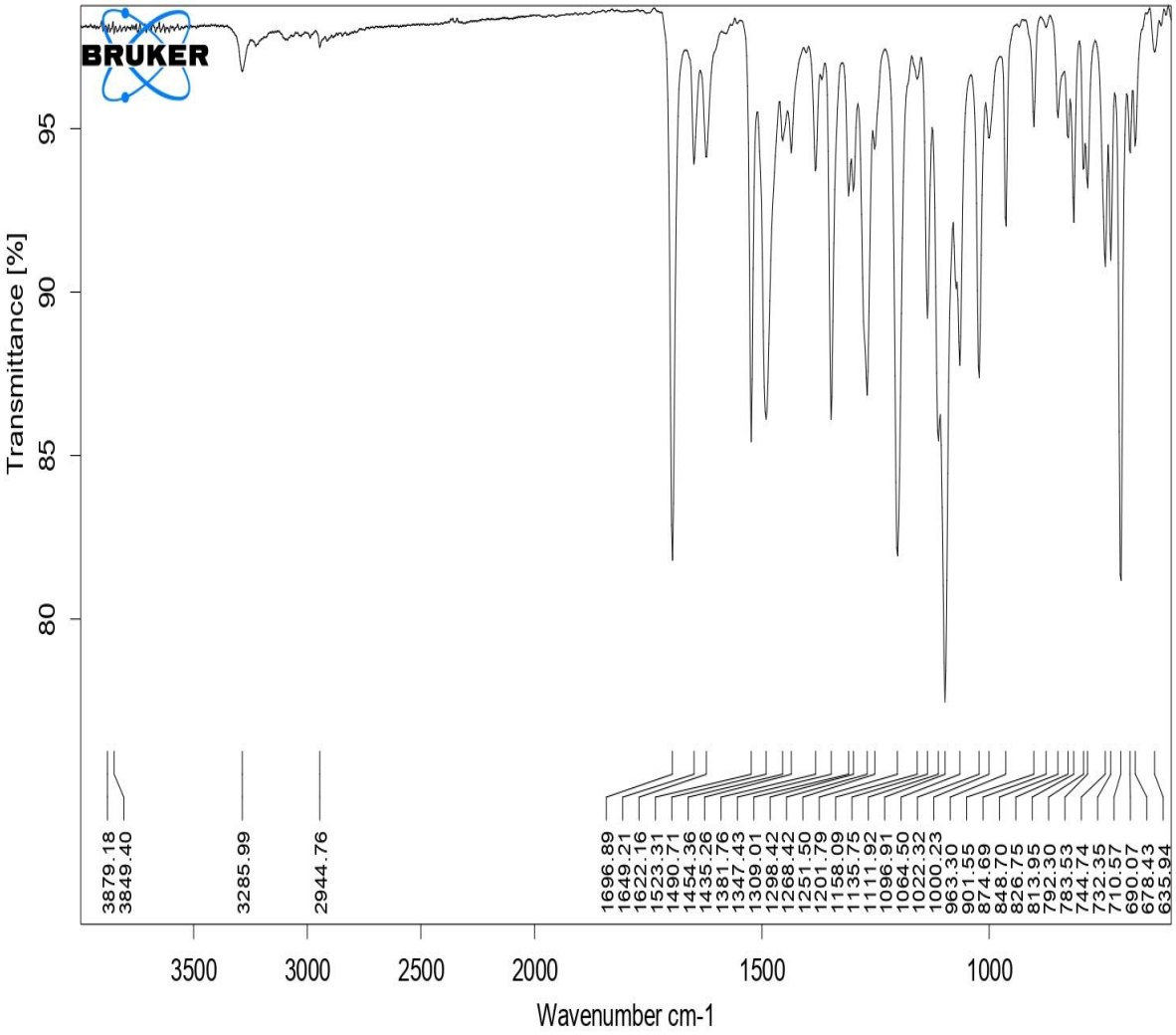


Figure 4: FTIR of Cilnidipine

Table 1: Interpretation of FTIR spectrum of Cilnidipine

Functional group	**FTIR frequency (cm^{-1}) theoretical peak**	**FTIR frequency (cm-1) Practical peak**
Aromatic 2º amine stretch N-H stretch	3320-3270	3285cm^{-1}
C= C stretch	1680-1625	1649cm^{-1}
Nitro(-N-O) stretch	1357-1318	1347cm^{-1}
Nitroso (N=O)	1290-1190	1251 cm^{-1}

5.1.4 Determination of purity of Cilnidipine: DSC (Differential scanning calorimetry):

The purity of the procured CILNIDIPINE was examined by DSC i.e differential scanning calorimetry (MODEL NO.:- Pyris Diamond TG/DTA, MAKE: PerkinElmer (SINGAPORE), and by the melting point determination with Thiele's tube method. The melting point of Cilnidipine was found to be 109.73 ºC and 113.33±2.51 ºc following DSC and Thiele's tube respectively. According to the DSC thermo gram, the unique melting peak of the drug is 109.73 ºC, indicating the melting point of the drug. The differential scanning calorimetry significantly indicates the purity of the powder drug and hence Cilnidipine is found pure to proceed for the formulation development. [2,3] **Figure 5**

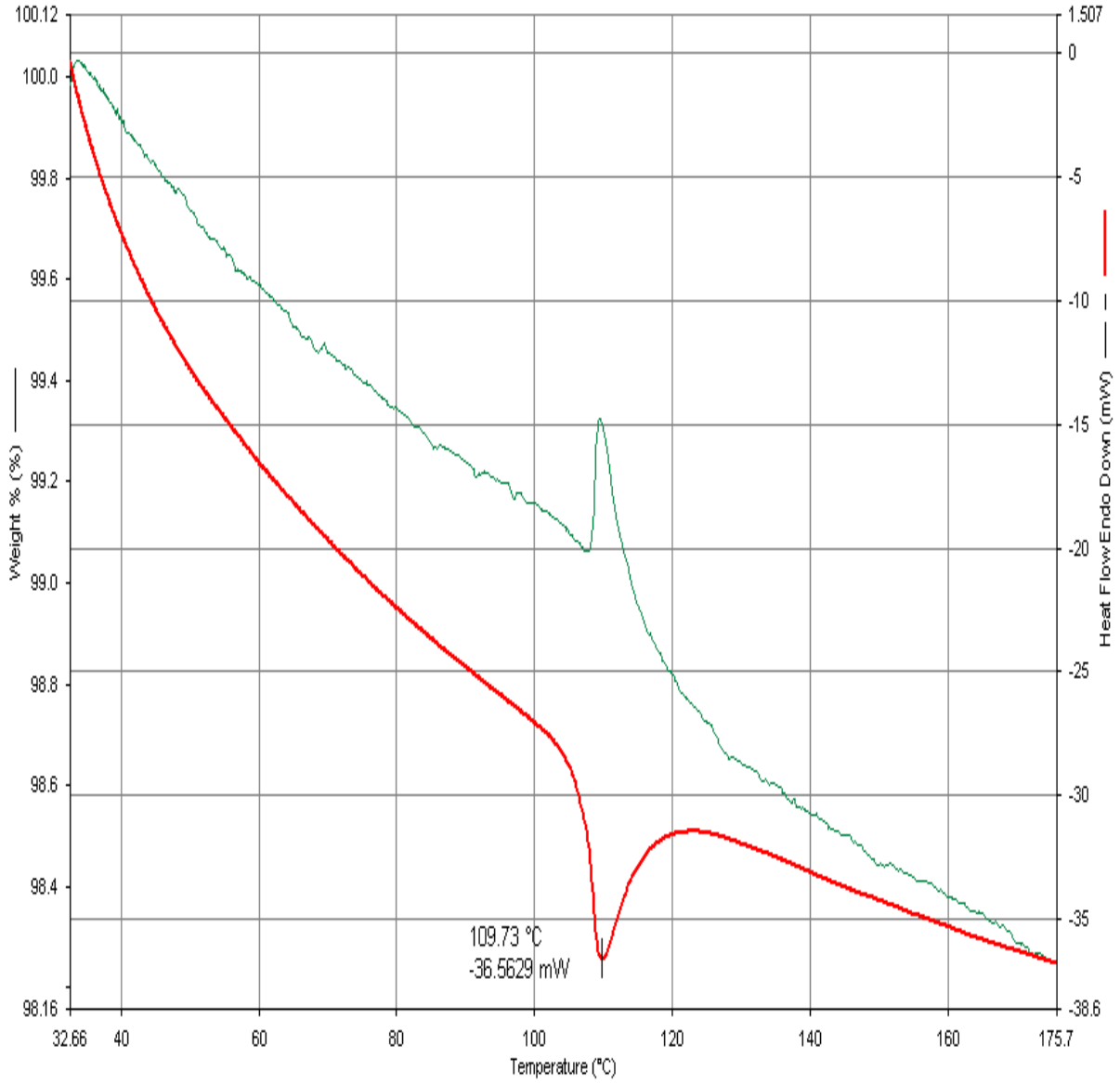
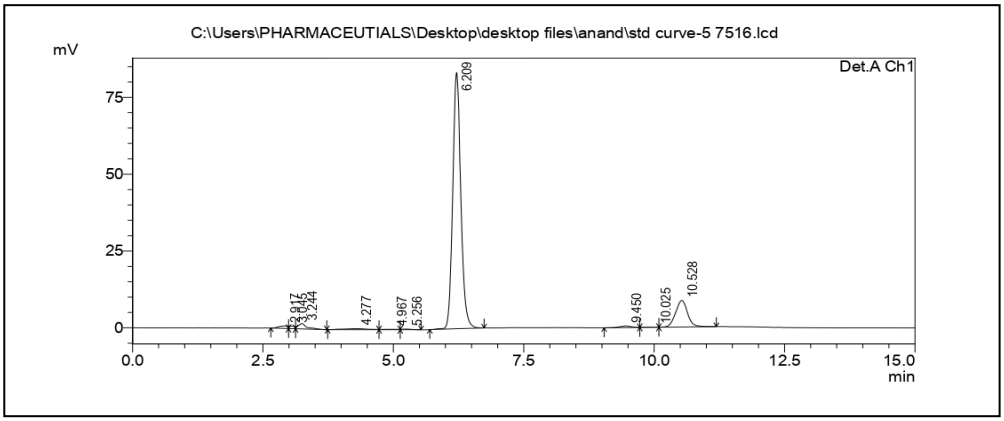
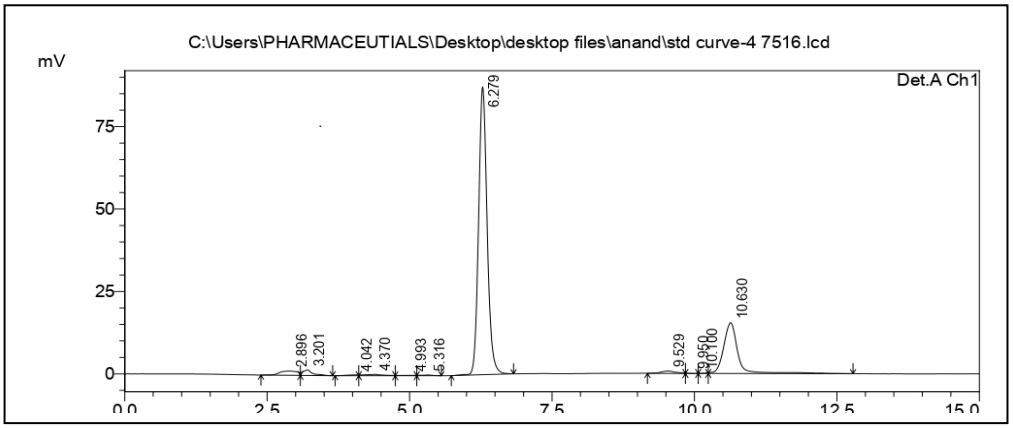


Figure 5: DCS thermogram of Cilnidipine

5.1.5 HPLC method development:

Method development: Acetonitrile and Ammonium acetate with water were selected as mobile phase (70:30 v/v) and run at a flow rate of 0.8 ml/min. Different concentrations of Cilnidipine were recorded at 254 and 237 nm wavelengths. Ketoconazole was selected as an internal standard.





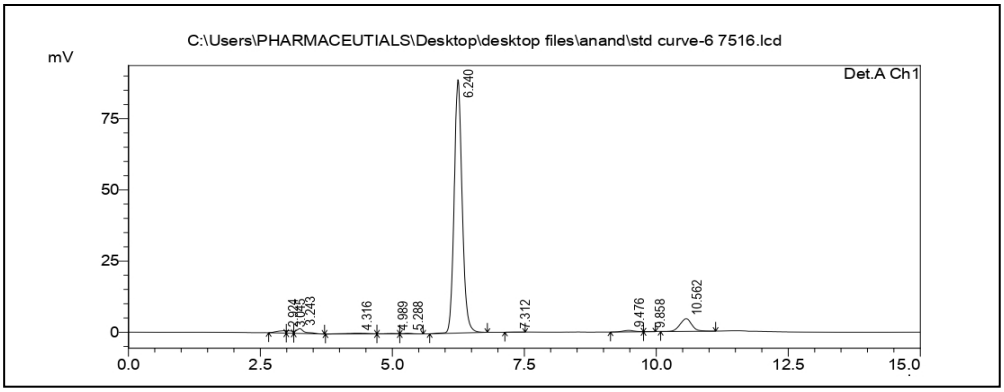


Figure 6 a 6 b 6 c: HPLC chromatogram of Cilnidipine (showing retention time of 10.528, 10.630, 10.562 min.) and Ketaconazole (IS showing retention time 6.209, 6.279 and 6.240min.)

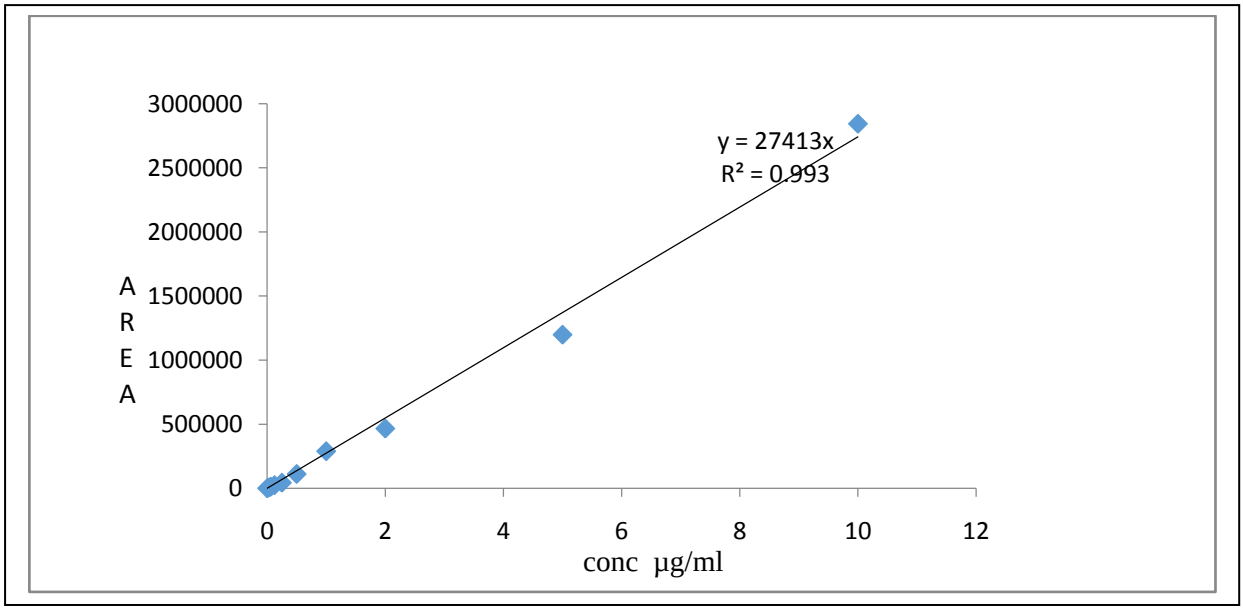


Figure 7: Calibration curve of Cilnidipine by HPLC

5. 2 Development and optimization of SMEDDS:

5.2.1 Selection of suitable oil:

The initial step was finalization of suitable oil for SMEDDS based on the optimum drug solubility and therapeutic appropriateness. The concentration of both polar (high water solubility and low log P) and non-polar (low water solubility and high log P) components increased with increasing oil fold, which is an important fact regarding the nature of oil used for the preparation of microemulsion. Further oil folding affects the rate and extent of solubilization, as well as the stability of triacetin droplets [4]. The study showed highest solubility of Cilnidipine in triacetin (**59.53±3.541mg/ml**) and comparatively low solubility in other edible oils. The solubility of the drug was almost two times in triacetin and found very less in different other edible oils as compared to Capmul MCM (**31.71±1.575mg/ml**). Triacetin was considered as the selected oil because of its high loading capacity, ease of emulsification and thermodynamic stability. Triacetin is a GRAS-listed short-chain triglyceride that can be used as a food additive with no restrictions, hence it is considered to be safe. [5]. Triacetin is frequently used in beverages, meals, cosmetics, and household products as a flavoring component. It has been used for more than 75 years as a cosmetic biocide (most commonly as a fungicide), plasticizer, and solvent in cosmetics. The antihypertensive medicines are used for longer periods. It is very important to consider the safety of the ingredients used for the formulation development. Triacetin was chosen as oil as it is safe for long-term use in antihypertensive based on information. The optimal

formulation of Cilnidipine was developed with components under the permitted daily intake on the basis of statistical design and formulation stability. Results of solubility of Cilnidipine in various oils are depicted in **fig: 8**

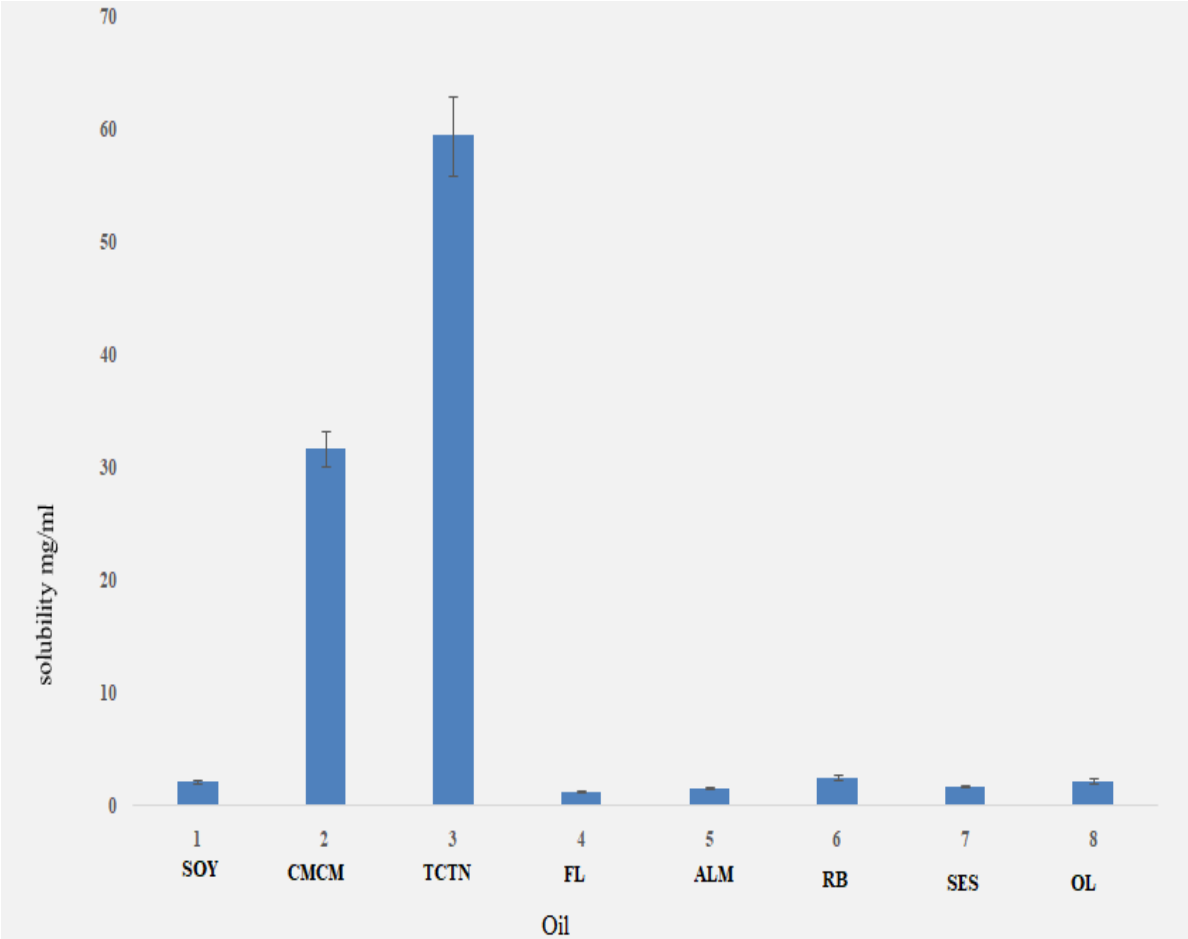


Figure 8: Solubility of Cilnidipine (presented in mean ±SD n=3) in various drugs, where soy: Soybean oil, CMCM: Capmul MCM, TCTN: Triacetin, FL: Flaxseed oil, ALM: almond oil, RB: Rice Bran oil, SES: Sesame oil, OL: olive OIL.

5.2.2 Selection of surfactant:

Selection of surfactant was crucial in order to achieve the desired size of globules of the microemulsion and for the preparation of an isotropic mixture. Surfactant was primarily utilized for effectively lowering the surface tension between oil and water of an o/w microemulsion. In order to expand the surface area, the surface energy is required to be reduced and the water or oil droplets should be present in a thermodynamically stable equilibrium. The efficiency and frequency with which a surfactant micro emulsifies specified oil depends on different factors like drug solubilizing capacity, safety (depending on the route of administration), the type of emulsion to be formulated and the surfactant's cloud point.

Non-ionic surfactants are less harmful and more stable than ionic surfactants, according to many experimental studies. [6]. Non-ionic or zwitter-ionic surfactants were shown to be more appropriate for distribution through oral route in pH-related in-vivo stability and toxicity studies. [7]. Surfactants with HLB values ranging from 4-6 are preferably used for W/O(water in oil), whereas those with HLB values ranging from 8-18 are favored for O/W (oil in water) microemulsion.[8-10] Surface active agents with a small polar head group and an appropriate length alkyl chain play an important role in the development of a microemulsion system because they fill in the gaps that surfactants alone cannot, resulting in a reduction in interfacial tension and the formation of a strong structure that can eventually contribute as a novel drug carrier. Following the procedure of selection of surfactants tween 20 was observed to induce maximum solubilization for the selected oil triacetin. [11]. Tween 20 has an HLB value of 16.7, which is greater than 10, indicating that it is possible to produce an O/W emulsion.

5.2.3 Selection of co-surfactant:

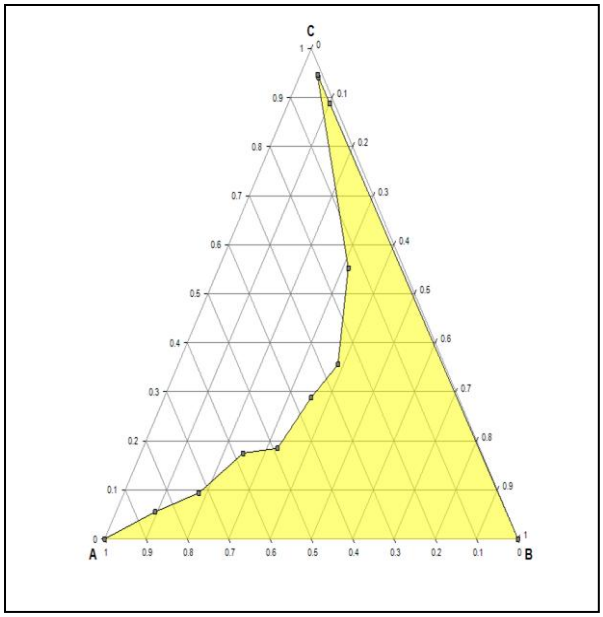
To increase the fluidity of the interface as well as to destroy liquid crystalline or gel structure, a co-surfactant is used. By altering the surfactant partitioning characteristic, it will aid in the adjustment of the HLB value and spontaneous curvature of the interface [11,12,13]. An appropriate co-surfactant can lower interfacial tension, enhance the flexibility allowing for better penetration of the hydrophobic zone of the oil phase and greater fluidity at the microemulsion system's interface. [14]. Hence to achieve the targeted form of isotropic mixture a suitable co-surfactant was investigated. Using the water titration method different co-surfactants and Tween 20 in an equal ratio (Smix ratio = 1:1) with different weight of triacetin were used to produce pseudo ternary phase diagrams. On the basis of the clear nature of the phase diagrams and homogeneity of the same, Transcutol HP was chosen as a suitable co-surfactant for the formulation development with triacetin and Tween 20.

5.2.4 Development of optimized SMEDDS

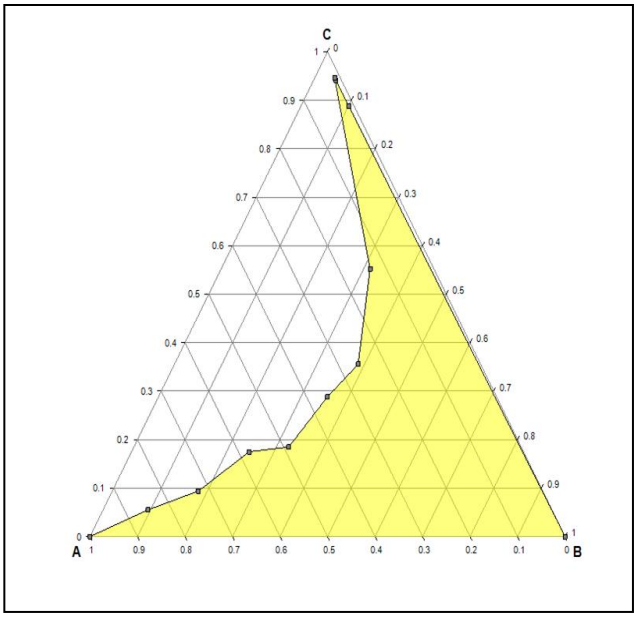
For formulation development, the finalization of the ratio of the selected oil (Triacetin), surfactant (Tween 20), and co-surfactants (Transcutol HP) was done by pseudo-ternary diagrams. Using the water titration method, four separate pseudo-ternary diagrams were prepared with changing ratios of Smix to oil in order to fix the ratio. A pseudoternary phase diagram is a tool that optimizes the components of a microemulsion, namely water, oil, and surfactant(s), in order to determine the concentration range of these components for the

stabilization of the microemulsion [15-18]. On the three sides of the triangle, the three components of a system were plotted. These phase diagrams can be utilized for identification of the type of emulsion, the globule size, miscibility, phase behavior, and the thermodynamic stability of the emulsion. Phase diagrams can be utilized for the prediction of optimum percentage of an aqueous phase, an oil phase, and surfactant concentration. It can also anticipate the working concentration range and mechanism of various surfactants, such as micellar concentration, reverse micellar concentration, lamellar concentration, and bicontinuous concentration [19]. In order to understand the stability of mixtures formed after water titration to the mixture of oil and Smix, all the tubes were kept for observation up to 48 hrs. Now, the most significant and critical reasons are the maximum emulsifying area obtained in different phase diagrams, permitted daily intake, and extent of homogenous behavior of mixtures after 48 hours.

Four different Pseudoternary phase diagrams were developed using triacetin, tween 20 and transcutol HP. The ratio of oil and smix used were 1:9 to 9:1 (1:9, 2:8, 1:3.5, 3:7; 4:6,5:5,6:4,7:3, 8:2, 9:1) for specific ratios of Smix 1:1, 2:1, 3:1 and 4:1 (surfactant : co-surfactant). In a mixture, the proportion of surfactants was increasing to achieve the best emulsification of oil. The result of the phase diagrams showed little difference in area of emulsification when comparing specific ratios of Smix 2:1, 3:1 and 1:1. However, the plotted graph (diagram) showed much better results in area of emulsification for the specific ratios of Smix 1:1. From the observations specific ratio of Smix 1:1 was selected considering the permissible daily intake of surfactants and co surfactants as well as 24 to 48 hours observation of diluted formulation (in water, pH 1.2 and pH 6.8 buffer). 1: 1 out of all four ratios contains fewer amounts of surfactants and further proportion of the same in daily dose with 5 mg and 10 mg Cilnidipine is under PDI. As the pharmacokinetic results of the study shows that bioavailability enhances 2.4 times it means daily dose of the drug reduces to the same proportion for the same therapeutic effect (proved and explained in NIBP study). Hence for the best fit diagram, a 1:1 Smix ratio with triacetin (selected oil) was chosen. All the phase diagrams are depicted in **fig: 9a (all phase diagram) 9b (selected diagram)**



1:1



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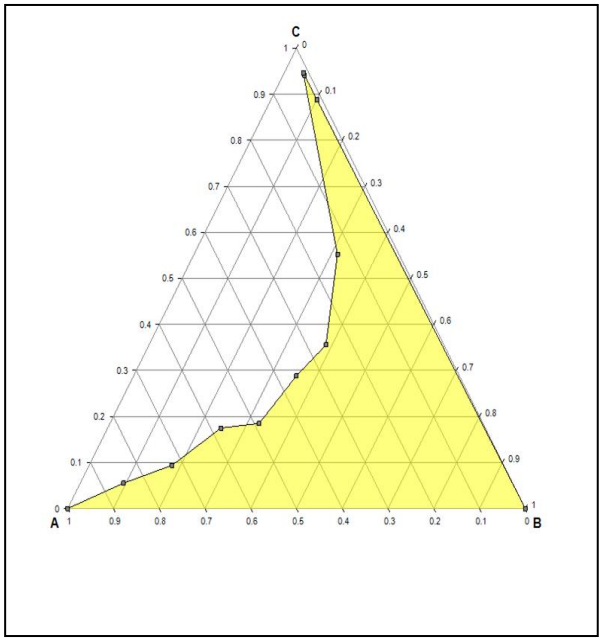

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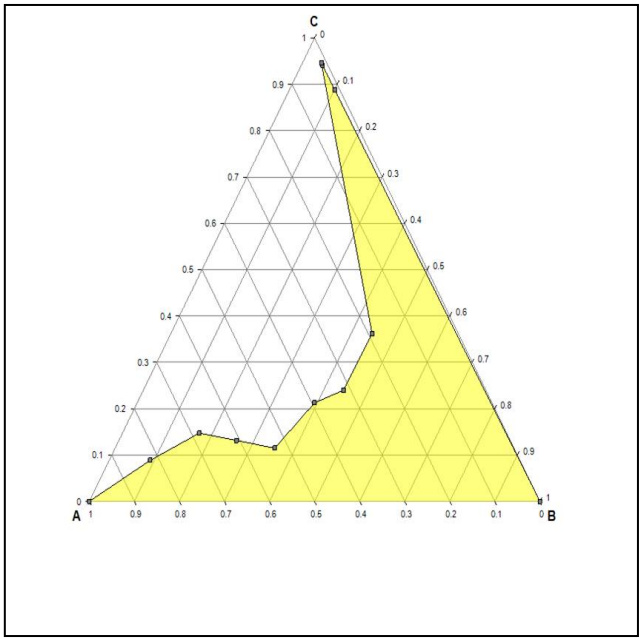


4:1

Where A: oil B: Smix and C: water

Figure 9a: Pseudoternary phase diagrams.

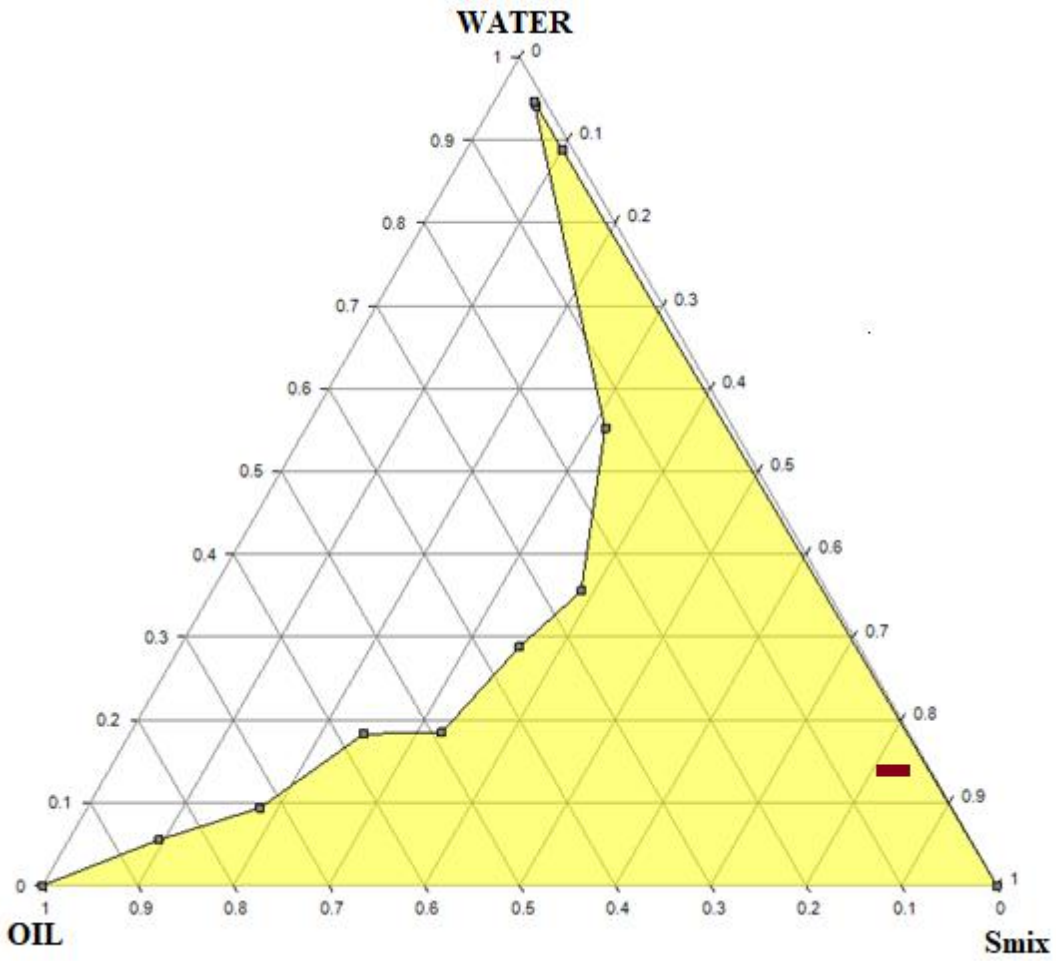


Figure 9b: Selected Pseudoternary phase diagram.

The proportion of oil, surfactant, and co surfactant, along with drug in a pre-concentrate is very important for the physicochemical properties of the dosage form concerned with gastric dilution, dispersion and drug absorption. In order to achieve the optimized pre-concentrate (SMEDDS), 16 distinct formulations were developed from the selected phase diagram which actually provides the proportion of formulation components shown in **Table 2** (using maximum area of emulsification as shown in fig 10b).Further all the developed formulations were investigated for characterization. All the formlations were developed using a software Design-ExpertTM (Stat-Ease Inc.) v7.0.

All 16 formulations were found to be stable and suitable for further characterizations when subjected to various known stresses and thermodynamic stability studies and further same are focused towards finding optimum pharmaco-economical formulation (optimized SMEDDS).

Further the formulations were characterized for globular size, zeta potential, PDI, viscosity, percentage transmittance, self emulsification time, in-vitro permeability, in-vitro dissolution, and conductivity. The optimized SMEDDS was identified by statistical data analysis based on the characterization. To corroborate the clinical relevance, optimized SMEDDS was assessed for in-vivo pharmacokinetic and pharmacodynamic studies. Table view of the 16 formulation with evaluation results is depicted in **Table 3**

Table 2: Formulation variable of the SMEDDS derived from selected phase diagram.

Name	Lower limit	Upper limit
Oil	5	10
S_{mix}	80	85
Water	10	15

Table 3: statistically designed formulations and results of various characterizations.

Formulations	oil	Smix	Water used for preapring emulsion	Size(nm)	PDI	ZP(mv)	Transmittance	Conductivity µs	Viscosity (cps)	SET Sec
F1	10	80	10	8.825	0.134	-1.62	97.949	168	33	24
F2	7.50844	82.4916	10	8.763	0.183	-0.58	97.27	106	28	26
F3	5	85	10	8.81	0.121	-0.865	97.78	166	26.5	24
F4	7.48991	80	12.5101	8.38	0.108	-0.58	95.27	158	27	23
F5	6.25328	83.7467	10	8.463	0.153	-0.342	98.8553	190	25	25
F6	8.34826	80.8125	10.8393	8.45	0.169	-0.432	98.4	136	32	24
F7	5	80	15	8.596	0.125	-0.432	93.1108	100.8	26	24
F8	5.0723	82.4547	12.437	8.636	0.164	-1.28	97.72	176	25	22
F9	7.48991	80	12.5101	23.75	0.134	-1.8	97.72	177.6	24	27
F10	6.7285	81.6439	11.6276	8.144	0.152	1.81	97.72	168.4	28	22
F11	5	83.7279	11.2721	8.693	0.112	-1.38	96.38	184	26	23
F12	5.0723	82.4547	12.437	9.281	0.226	-0.353	97.35	173.8	26	24
F13	5.87889	80.7496	13.3715	8.88	0.179	-0.78	98.34	167	24	25
F14	5.0723	82.4547	12.437	8.3	0.187	-1.87	97.37	176	24	26
F15	7.48991	80	12.5101	8.523	0.141	-0.703	98.3	149	29	25
F16	7.50844	82.4916	10	8.237	0.197	0.167	97.72	164	31	26

5.2.5 Formulation of optimized SMEDDS: On the basis of statistical data analysis of all the developed formulations using Design-ExpertTM (Stat-Ease Inc.) v7.0, physicochemical characterizations and permeability profile, the optimized SMEDDS was prepared with the formula shown in **Table: 4 fig:10** for further study.

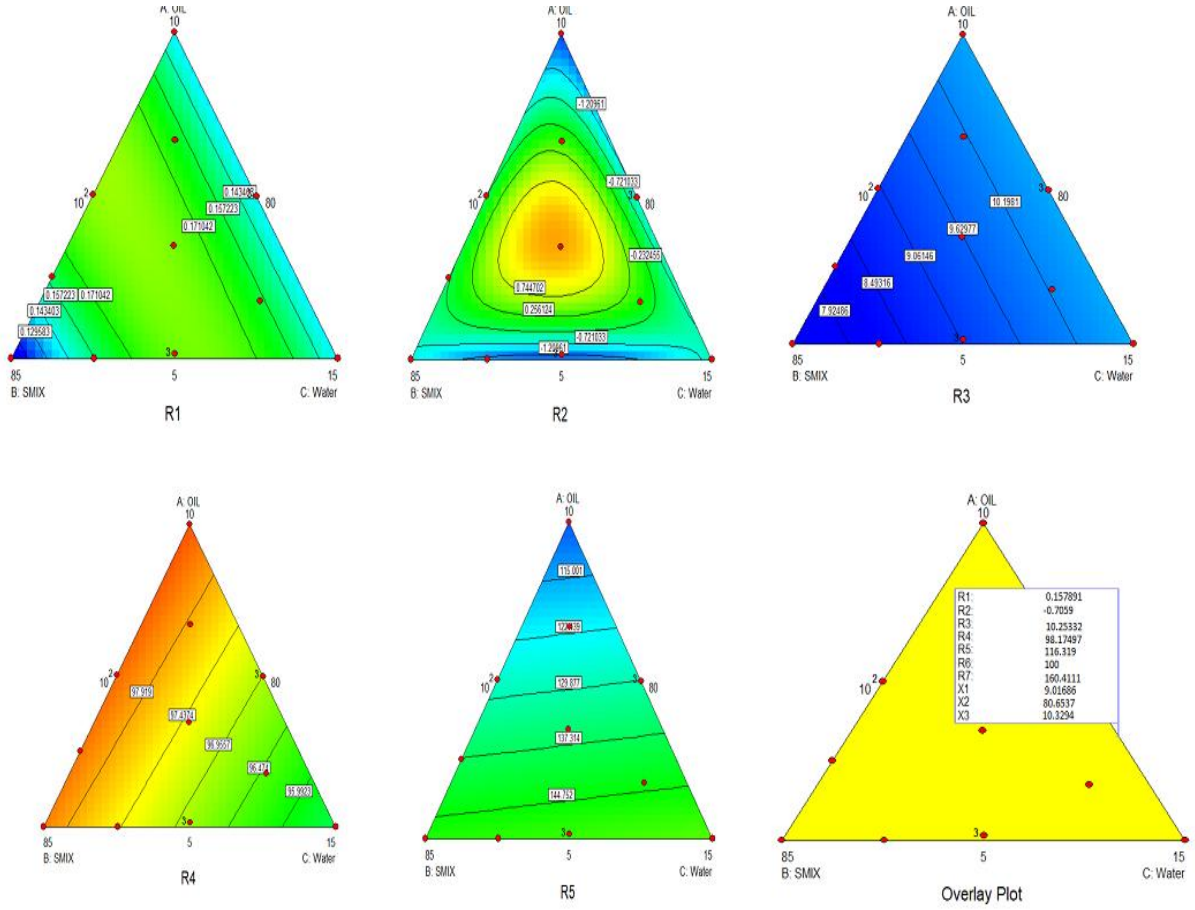


Figure10: Contour plots. Where (R1) polydispersity index, (R2) zeta potential, (R3) globule size, (R4) transmittance, (R5) conductivity and overlay plot is shown.

Table 4: Optimized formulation of SMEDDS

Ingredients	Weight in milligram	Weight in % of total formulation
Cilnidipine	10 mg	0.84
Triacetin (selected oil):	130 mg	10.92
Tween 20(selected surfactant)	500 mg	42
Transculto HP(selected co surfactant)	550 mg	46.2
Total	1190mg	

5.3 *In vitro* Characterization of optimized SMEDDS

5.3.1 Robustness of optimized SMEDDS in GI solution:

It is undesirable that an optimized pre-concentrate gets precipitated or phase separated when introduced into the gastrointestinal fluid during in-vivo study. Various factors such as sharp pH change in dilution of formulation with body fluids or dilution of solubilizing excipients in formulations etc. may play a vital role behind drug precipitation or phase separation [20]. Due to this undesirable precipitation, the ultimate concentration of the drug falls in the GI fluid which results in inability of the drug delivery system for immediate therapeutic action. A normal human stomach contains around 250 to 300 ml GI fluid [21, 22]. The results showed no phase separation after dilutions of optimized SMEDDS by 125, 250, and 500 times with different solvent (pH 1.2 buffer, pH 6.8 buffer, and water) for 24 hours duration. As a result, the optimized SMEDDS is robust to any tested solvent. This result ensures the homogeneous dispersion of API (active pharmaceutical ingredients) in the whole fluid as well as in presence of used excipients.

5.3.2 Globule size, PDI and Zeta potential:

Globule size, PDI, and Zeta potential of SMEDDS are critical parameters for drug release. Globule of SMEDDS is expected to be below 50 nm in size (23). As the drug delivery system is self microemulsifying in nature and produces microemulsion after introduction to the GI fluid, the expected globule size of emulsion should be below 50 nm. It was observed that the globule size was remarkably unchanged after 150 times dilution of SMEDDS. The globule size of optimized formulation was found to be **9.045** nm.

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -2.32	Peak 1:	-2.32	100.0	4.18
Zeta Deviation (mV): 4.18	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.123	Peak 3:	0.00	0.0	0.00

Result quality : Good

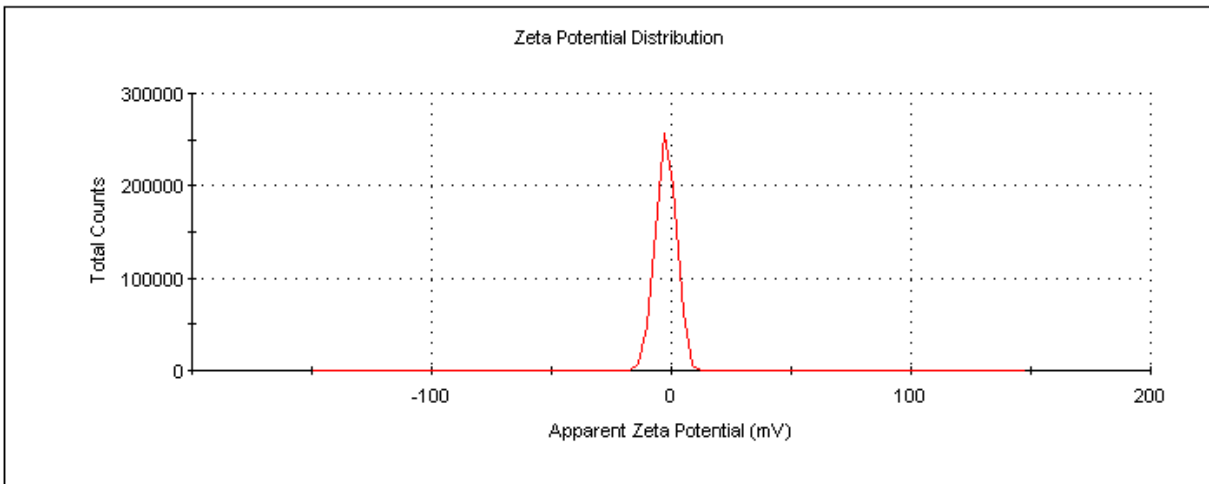


Figure 11 a Zeta potential of optimized SMEDDS

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 9.045	Peak 1:	9.832	100.0	2.860
PdI: 0.203	Peak 2:	0.000	0.0	0.000
Intercept: 0.964	Peak 3:	0.000	0.0	0.000

Result quality : Good

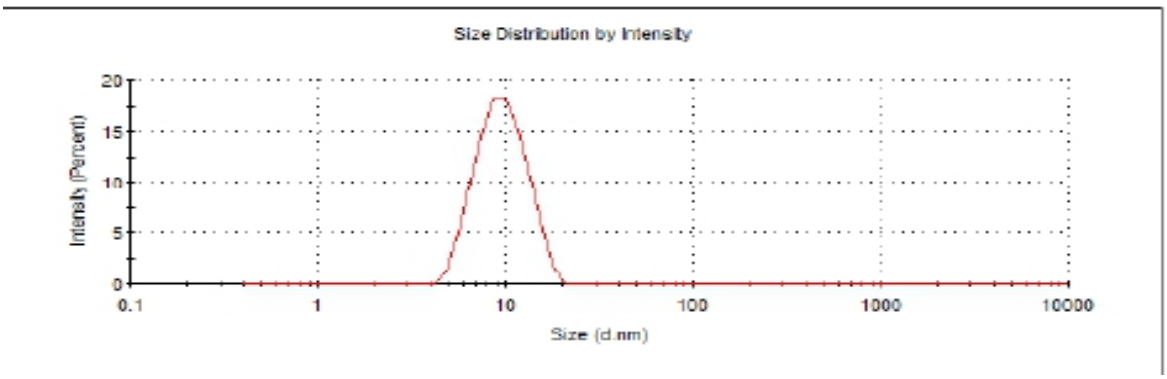


Figure 11b: Globule size and PDI of optimized SMEDDS

The zeta potential indicates the electrical charge at the surface of the hydrodynamic shear surrounding the colloidal particles as a significant characterization of nano size formulations [24,25]. Zeta potential depends on the composition of the formulation and decides the environment of the developed dosage form (here nano sized globules). Zeta potential of a developed formulation defines the extent of repulsion and chances of aggregation of particles. Hence zeta potential is a critical parameter for the developed formulation. The formulation

was developed using triacetin, Tween 20 and transcutol HP. Tween 20 is a non ionic surfactant and contributes negligible charge to the formulation. Net charge of triacetin is also very low or about to be zero. Rest of the materials such as transcutol HP, Cilnidipine and water after dilution may be attributed to the charge in the formulation. A negative zeta potential is an indication of better stability of nano sized formulations, facilitation of drug permeability and hence effectiveness of the formulation [25, 26, 27]. Zeta potential of the optimized formulation was measured using Zetasizer (DLS-nano ZS, Nanoseries, Malvern Instruments). The zeta potential of the developed formulation was found to be **-2.32mv**, indicating the repulsion of the nanosized globules and a stable formulation with a net negative charged dispersion system. This total negative charge helps the emulsion globules to remain dispersed and stable due to globular repulsion.

The polydispersity index (PDI) is a size-based measure of a sample's heterogeneity. Polydispersity can arise as a consequence of a sample's size distribution or aggregation during isolation or analysis. PDI can also detect nanoparticle aggregation, as well as the consistency and efficacy of particle surface changes across a particle sample. Dynamic light scattering (DLS) instruments or electron micrographs can also be used to calculate the PDI. A PDI value of 1 indicates that the sample has a wide size range of particles and also may contain bigger particles or aggregates that are slowly sedimenting. As PDI is a significant indicator of size distribution a PDI value 1 indicates that he sample has a broad size distribution and may contain large particles or globules leads to non uniformity of the mixture. To achieve a uniform and homogeneous preparation a PDI value less than one is desirable [28]. The low PDI indicates the quality of size distribution i.e. uniformity of dispersed systems such as microemulsion [28,29]. From the result, it was observed the PDI of the optimized formulation was found to be **0.203** indicating that optimized composition can be used to produce stable microemulsions of Cilnidipine loaded globules with a relatively narrow size distribution.

5.3.3 Conductivity:

The electrical conductivity test for nanoemulsions and microemulsions was carried out to identify the type of the emulsion i.e., o/w or w/o. The optimized formulation showed a conductivity of **157** µs. The optimized SMEDDS has a higher conductivity than the dispersion medium, indicating the presence of the percolation effect, in which the charge is

supposed to move from one surface to another, resulting in a dramatic increase in conductivity. [30].

5.3.4 Refractive index:

Refractive index of a material is a dimensionless number indicating how fast light can travel through it as compared to vacuum. It is already stated above that SMEDDS of Cilnidipine is an isotropic mixture and clear in nature. And it is also observed that on desired dilution, SMEDDS was found to be clear. The refractive indices of 100 times diluted optimized SMEDDS were found to be **1.378, 1.396 and 1.382** or **1.385333±0.009452** at 25 ºC. These values are nearly identical, consistent, and comparable with water (R.I of water 1.333). The constant refractive index indicates the thermodynamic stability, homogeneity and isotropic nature of the developed formulation. [31]

5.3.5 Viscosity:

Viscosity is one of the critical parameters of a dosage form specially where the pre-concentrate is designed and fabricated to fill in the capsule. The low viscosity of the pre-concentrate will help in the filling of the formulation and aids in the patient compliance. The viscosity of the optimized SMEDDS was found to be **31 cps** which is advantageous for capsule filling.

5.3.6 Self emulsification time (SET):

Self emulsification time (SET) is one of the indicators of quality of SMEDDS. The faster the emulsification, the faster the drug entrapment and absorption. The faster the emulsification, the faster the release and absorption of the drug and hence the onset of therapeutic effect. Nature of components of the formulation can affect the self emulsification of the pre-concentrate. From the result, it was observed that the optimized formulation self-emulsified within **26 seconds** which is less than 30 seconds that indicates the developed Cilnidipine SMEDDS pre-concentrate forms a homogeneous dispersion which is a necessary condition for in-vitro and in-vivo dissolution. [32,33].

5.3.7 P^H: The pH of the optimized formulation was found to be **5.9 ± 0.5**, indicating slight acidic character which is an important property for patient compliance. The formulation's mild acidic nature also makes it less likely to cause stomach irritation.

5.3.8 Percentage transmittance:

The percentage light transmittance of the optimized formulation was found to be **98.95**, which is nearer to 100. This signifies exceptionally clear formulation indicating it is an entirely soluble system. Complete drug solubility in the system is essential for the improvement of the bioavailability, which was studied subsequently in *in-vitro* and in *in-vivo* tests. [34].

5.3.9 *In-vitro* dissolution of statistically designed formulations:

In-vitro drug dissolution studies reveal that 100 percent of drugs were released from the developed formulations within 15 minutes. In 15 minutes, all of the statistically designed formulations show 100 percent release. Comparative dissolution of optimized SMEDDS with marketed tablet was done and analysed with LCMS/MS. Here the optimized SMEDDS also showed 100 percent release within 15 minutes.

5.3.10: *Ex-vivo* permeation studies using goat intestinal sac technique:

The drug permeability across the gut lining was performed for Cilnidipine powder drug suspension with 0.2 percent SDS and developed formulations. It was found that Cilnidipine powder drug suspension with 0.2 percent SDS did not pass through the intestinal membrane in 4 hours, but all developed formulations exhibit permeability through the membrane. It highlights the importance of formulation composition and aids in the development of the best formulation. The permeation test was conducted for four hours only, with the integrity of the intestinal membrane as a top priority. The penetration of 184 µg Cilnidipine across the goat intestinal barrier in the first hour from the developed formulation demonstrated an optimized formulation. **Figure 12** shows the results of the permeability of all developed formulations along with optimized SMEDDS.

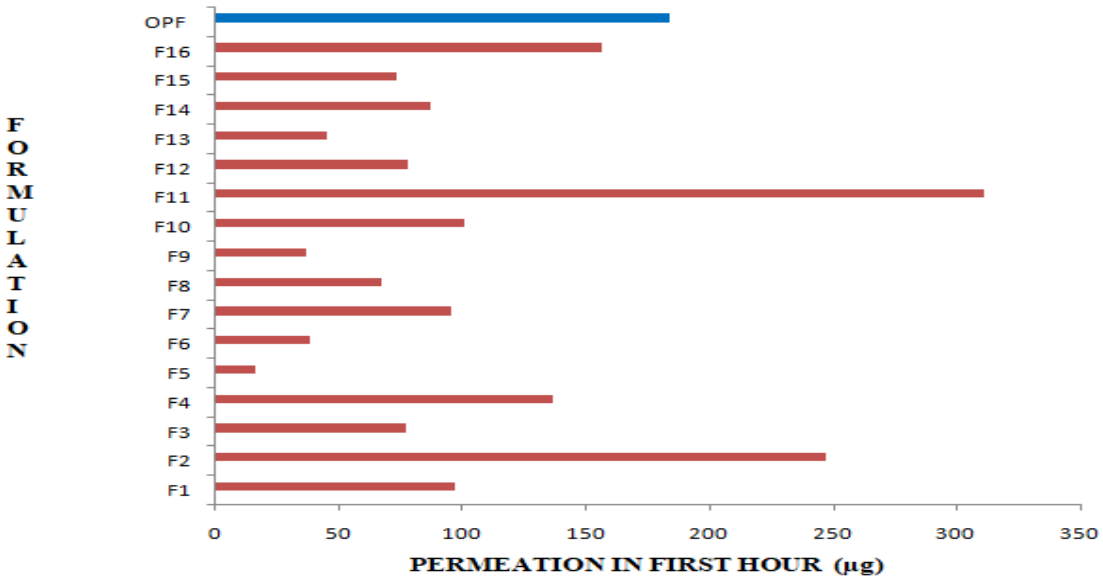


Figure 12: *Ex-vivo* Permeation of different developed formulations. (in µg) from goat intestinal membrane. Here F indicates the number of formulations. OPF is optimized formulation.

5.4. Result of method validation of bioanalytical method in LC-MS/MS:

The calibration concentrations of Cilnidipine were 1.875-60.00 ng/ml for which accuracy was 94.38% to 103.90% and LOD value was 0.82ng/ml. The accuracy of all stable samples were 94.44-100.90%, 94.35-95.76%, 98.06-105.65%, 90.81-101.65% and 95.33-99.94% for freeze thaw, short term, bench top, autosampler and long term stability respectively. The recovery was 87.08-91.51% and the matrix factor was found to be 0.94-0.97. **[Table: 5(a,b), 6, 7(a,b), 8, 9,)].** All the results are acceptable according to US-FDA and EMA guideline. **Annexure 1**

Table-5a: Pre-study linearity of detector response (n=3)

Linearity	Concentration (ng/ml)					
	1.88	**3.75**	**7.5**	**15**	**30**	**60**
LIN 1	1.81	3.96	7.8	14.24	32.29	54.73
LIN 2	2.02	3.85	6.93	15.48	31.30	54.10
LIN 3	2.03	3.87	7.09	15.85	26.13	61.05
Average	**1.953**	**3.893**	**7.273**	**15.190**	**29.907**	**56.627**
S.D	**0.124**	**0.059**	**0.463**	**0.843**	**3.308**	**3.308**
% C.V.	**3.191**	**1.505**	**6.367**	**5.551**	**11.061**	**5.842**
Nominal % NOMINAL	**103.90**	**103.82**	**96.98**	**101.27**	**99.69**	**94.38**

Table-5b: Pre-study linearity of detector response statistics (n=3)

LINEARITY	STATISTICS		
LINEARITY CODE	**SLOPE (m)**	**INTERCEPT (c)**	**R square**
LIN 1	0.01040	0.01010	0.9965
LIN 2	0.01020	0.00018	0.9980
LIN 3	0.00618	0.00105	0.9975
MEAN	**0.00819**		**0.9978**
S.D.	**0.00284**		**0.00035**
C.V.%	**34.708**		**0.035**

Table-6: Precision and accuracy (n = 5)

	Between run			Within run		
	Mean ± SD	**C.V.%**	**Absolute bias (%)**	**Mean ± SD**	**C.V.%**	**Absolute bias (%)**
LLOQ (1.88ng/ml)	1.937±0.135	6.984	103.01	1.896±0.146	7.721	100.85
LQC (5.63ng/ml)	5.941±0.249	4.199	105.52	5.874±0.205	3.496	104.33
MQC (22.5 ng/ml)	22.997±1.690	7.348	102.21	23.002±2.153	9.358	102.23
HQC (45.00 ng/ml)	43.387±3.613	8.328	96.41	43.086±3.832	8.893	95.75

Table-7a: Recovery of Cilnidipine

INJ No.	Diluent Sample			In Plasma		
	LQC 5.63ng/ml	**MQC 22.5ng/ml**	**HQC 45.00ng/ml**	**LQC 5.63ng/ml**	**MQC 22.5ng/ml**	**HQC 45.00ng/ml**
1	4750316.16	4598167.18	4212772.60	4505199.32	3881642.41	3847457.84
2	5308605.01	4685812.57	5112450.30	4872707.09	3990744.40	5495812.96
3	4695590.15	4461829.04	4276308.27	4238577.61	4094830.18	3571132.41
4	4410610.42	4627922.87	4569647.40	4007340.29	3971332.73	4173644.98
5	4591823.20	4559404.61	4250767.26	4009008.29	4031978.22	3429915.38
Mean	**4751388.99**	**4586627.25**	**4484389.17**	**4326566.52**	**3994105.59**	**4103592.71**
% Recovery				**91.06**	**87.08**	**91.51**

Table-7b: Recovery of IS

INJ No.	Diluent Sample			In Plasma		
	LQC 5.63ng/ml	**MQC 22.5ng/ml**	**HQC 45.00ng/ml**	**LQC 5.63ng/ml**	**MQC 22.5ng/ml**	**HQC 45.00ng/ml**
1	9576481.81	9965772.62	7975233.40	8498481.38	8880068.39	8859991.94
2	9619123.92	9593272.96	7815211.23	8939775.07	8867580.65	8247471.52
3	9676111.26	9466372.22	8517740.08	8673734.93	8937093.49	8115143.48
4	9371911.94	9360864.06	8058814.21	8495924.93	8846871.55	8096364.77
5	9759629.56	9392947.71	8325793.74	8784523.90	9000156.92	7068261.56
Mean	**9600651.70**	**9555845.91**	**8138558.53**	**8678488.04**	**8906354.20**	**8077446.65**
% Recovery				**90.39**	**93.20**	**99.25**

Table-8: Matrix effect (area) (N = 5)

	Matrix effect IS		Matrix effectCilnidipine	
	% of ME	**Matrix factor**	**% of ME**	**Matrix factor**
LQC (5.63 ng/ml)	91.77±2.21	0.94±0.04	96.59±1.48	0.97±0.02
MQC (22.50 ng/ml)	90.74±4.05	0.91±0.04	93.36±3.12	0.94±0.03
HQC (45.00ng/ml)	91.39±3.57	0.91±0.04	97.15±1.50	0.97±0.02

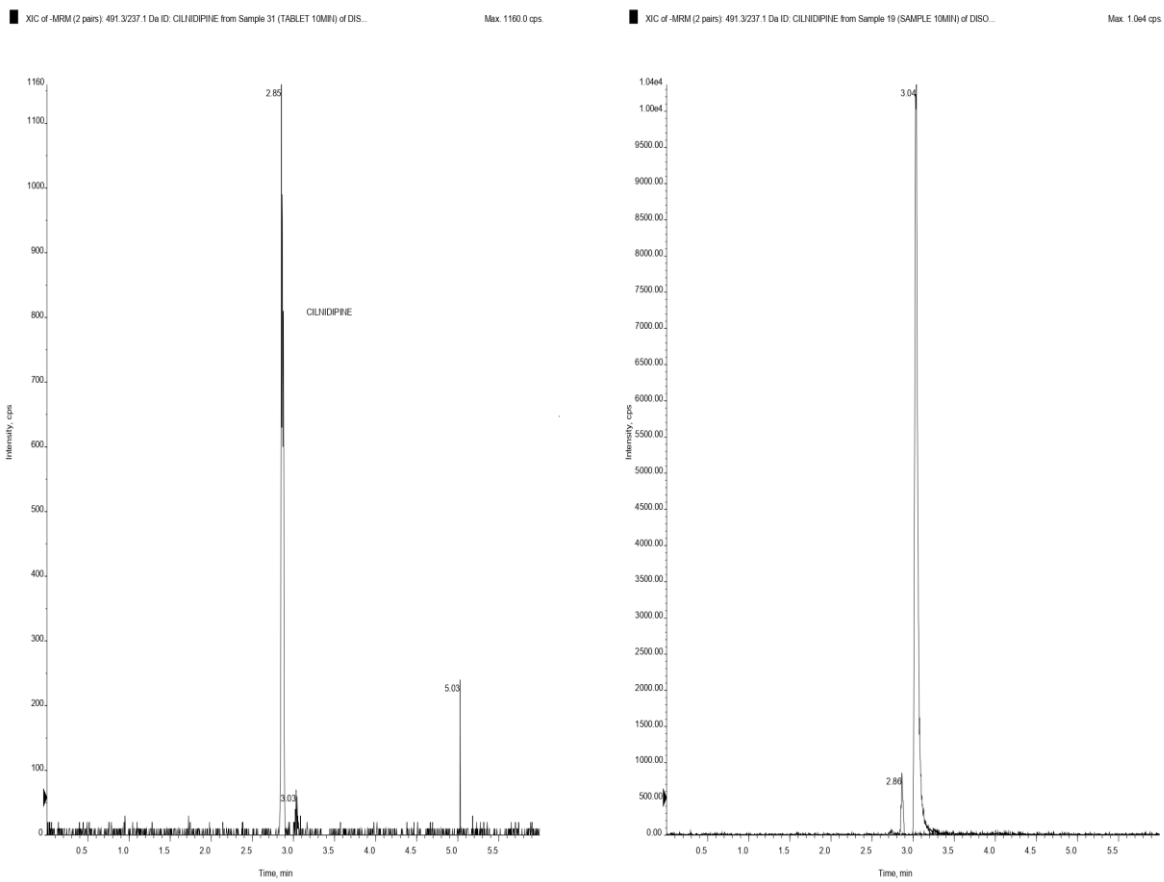
Table 9: Stability Study (Freeze thaw, Short term, Auto sampler, Bench top stability, Long term stability).

		Inj No.	**LQC (5.63 ng/ml)**	**MQC (22.5 ng/ml)**	**HQC (45.00 ng/ml)**
	Freshly Thawed	1	5.64	24.04	42.92
		2	6.35	21.62	46.01
		3	5.48	23.07	50.23
		4	6.10	21.20	41.52
		5	6.34	23.54	46.67
		Mean	**5.98**	**22.69**	**45.47**
		1	5.76	20.52	43.27
		2	6.09	24.54	43.8

Freeze thaw stability	After 3 cycle	3	6.42	23.17	44.27
		4	5.88	21.54	40.87
		5	6.03	21.19	42.51
		Mean	**6.04**	**22.19**	**42.94**
	% Stability		**100.90**	**97.79**	**94.44**
Short term stability	After 24 hours.	1	5.52	20.17	45.02
		2	5.26	20.44	45.49
		3	5.37	19.76	39.64
		4	6.01	23.11	44.61
		5	6.06	25.18	40.65
		Mean	**5.64**	**21.73**	**43.08**
	% Stability		**94.35**	**95.76**	**94.75**
Autosampler stability	After 24 hours in auto sampler (15ºc)	1	5.5	21.26	46.98
		2	5.24	23.58	45.55
		3	5.21	22.88	44.14
		4	5.61	23.17	46.56
		5	5.60	24.45	47.81
		Mean	**5.43**	**23.07**	**46.21**
	% Stability		**90.81**	**101.65**	**101.62**
Bench top stability	After 24 hours in laboratory room temperature	1	5.76	24.79	46.43
		2	6.19	24.18	48.88
		3	6.42	24.08	42.96
		4	5.24	24.09	44.87
		5	5.72	22.74	49.19
		Mean	**5.87**	**23.98**	**46.47**
	% Stability		**98.06**	**105.65**	**102.19**
Long term stability		1	5.98	21.05	48.73
		2	5.96	24.6	45.07
		3	5.41	20.18	46.01
		4	6.24	20.23	44.37
		5	5.58	22.11	43.04
		Mean	**5.83**	**21.63**	**45.44**
	% Stability		**97.53**	**95.33**	**99.94**

5.5 In-vitro dissolution of optimized SMEDDS and marketed tablet analysed with LC-MS/MS:

The release of Cilnidipine from optimized SMEDDS and marketed tablets using LC-MS/MS (API2000) demonstrated that 100% of Cilnidipine from SMEDDS was released within 10 minutes whereas marketed tablets showed <30% drug release. These results support the improvement of bioavailability hypothesis with SMEDDS as compared to the marketed tablets. Chromatographic result of the experiments is shown in **Figure 13 a 13 b.**



13a 13b

Figure 13a, b: In-vitro dissolution of marketed tablet and optimized SMEDDS respectively

5.6 Comparative result of pharmacokinetic study:

After oral administration of both optimized SMEDDS and marketed tablet, non-compartmental analysis was applied in the current investigation to generate a pharmacokinetic profile. Findings of the pharmacokinetic study in rat plasma are shown in [**Table 10]**. Following oral administration of the optimised formulation, C_{max} (peak plasma concentration) was reached at 21.02±3.17 ng/ml at 0.866 ± 0.11hours (T_{max}), compared to 10.16±0.89 ng/ml at 0.93±0.11 hours (Tmax) in the case of the commercially available tablet. This outcome indicates an enhancement in the solubility of a BCS class II drug Cilnidipine, formulated as SMEDDS when compared to the marketed tablet. The elimination half-life ($T_{1/2}$) of L-SMEDDS was found 2.27±0.25hr, whereas the elimination half-life (T1/2) of the

commercially available tablet was 1.88±0.030 hr. This significant enhancement in the half life can be due to increase in the volume of distribution of the Cilnidipine SMEDDS as compared to marketed tablet. The optimised L-SMEDDS of cilnidipine and the commercially available tablet had area under curves (Auc $_{0\text{-}t}$) of 49.15±22.62 ng.hr/ml. and 20.14±3.24 ng hr/ml, respectively. Interestingly, the area under the curve ($AUC_{0\text{-}\alpha}$) values for L-SMEDDS and commercially available tablets, respectively, were 51.20 ±22.63ng.hr/ml and 20.62±3.37ng.hr/ml. These findings are indicating towards the significant increase in the exposure of the drug from the SMEDDS dosage form as compared to commercial tablet. Further the optimised L-SMEDDS and commercial tablet's elimination constants (K_{el}) were reported to be 0.307±0.03 hr.-1 and 0.36±0.005 hr.-1, respectively. This pharmacokinetically translates into an increase in protein binding with prolonged drug effect accompanied by an enhancement in AUC which is evident from our result. This indicates towards better therapeutic performance and dose reduction of the developed SMEDDS as compared to tablet. Finally when compared to the commercial tablet, the relative oral bioavailability of L-SMEDDS in the plasma was observed to be increased by 2.4 fold based on the AUC values after oral administration. Comparative studies of pharmacokinetic profiles of optimized formulation and marketed tablet are shown in **figure14.**

Table10: Pharmacokinetic parameters of marketed Cilnidipine and SMEDDS (Data expressed as Mean±SD) (n=4).

Pharmacokinetic parameters	Marketed Cilnidipine	SMEDDS of Cilnidipine
C_{max} (ng/ml.)	**10.16±0.89**	**21.02 ±3.17**
t_{max} (hr.)	**0.93±0.11**	**0.866 ± 0.11**
AUC 0-t (ng. hr./ml.)	**20.14±3.24**	**49.15±22.62**
AUC 0-∞ (ng. hr./ml.)	**20.62±3.37**	**51.20 ±22.63**
k_{el} ($hr.^{-1}$)	**0.36±0.005**	**0.307±0.03**
$t_{1/2}$ (hr.)	**1.88±0.030**	**2.27±0.25**
Relative Bioavailability (%)	**100 %**	**244.04%**

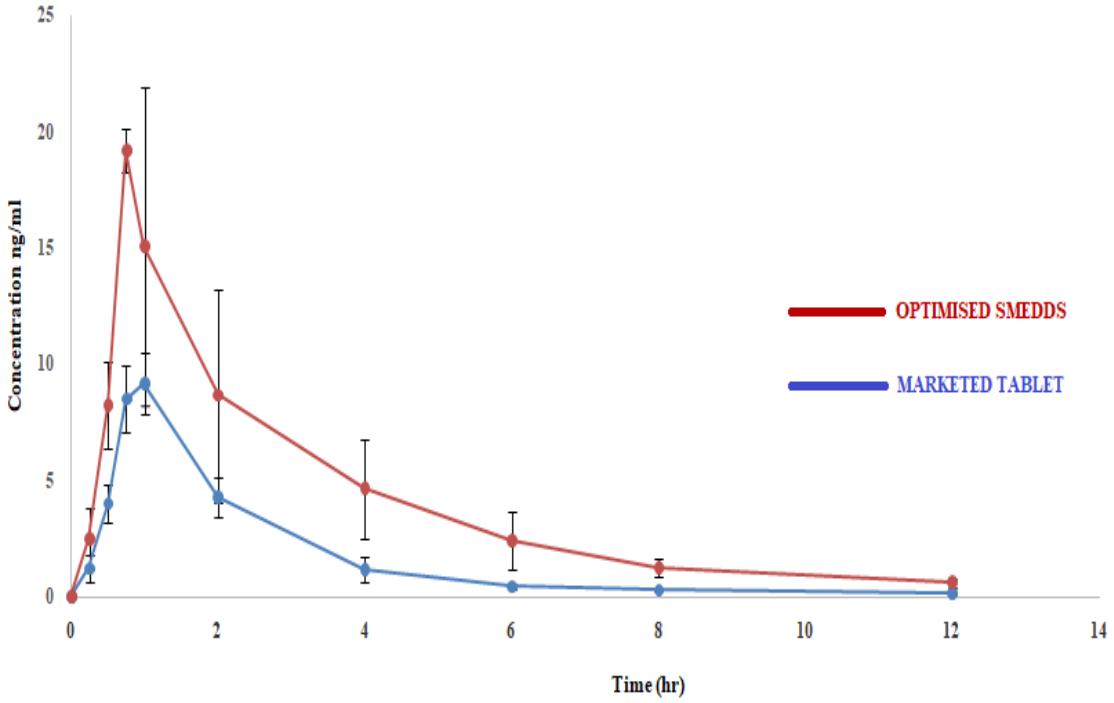


Figure 14: Mean concentration vs Time comparison of marketed tablet and optimized SMEDDS formulation. Data were expressed as Mean±SD (n=4).

5.7 Pharmacodynamic study:

In response to various physical and chemical stimuli, several vasoactive paracrine factors like nitric oxide released by endothelium that modulates the tone of the smooth muscles, L-NAME (N^G-Nitro-L-arginine-methyl ester) in experimental conditions reduces endothelial release of NO and artificially induces arterial hypertension in laboratory animals [35, 36] were investigated.

Following 7 days of administration of L-NAME, significant ($p<0.001$) increase in the SBP was noted in the control group and was significantly ($p<0.05$) reduced following administration of 1.2 mg/kg body wt of Cilnidipine in the marketed tablet (equivalent weight of powder, tablet with 0.2% SDS). Interestingly, SMEDDS loaded with 0.5 mg/kg body wt of Cilnidipine dose significantly ($p<0.01$) reduced the SBP in L-NAME induced hypertensive wistar rats. Greater reduction of systolic blood pressure was obtained by SMEDDS than the marketed tablet which explicitly corresponds to the 2.4 fold enhanced relative oral bioavailability of the former than the latter. The remarkable outcome of the development of SMEDDS is its reduced dose as compared to the conventional marketed tablet. For statistical analysis, one way ANOVA (analysis of Variance) followed by Tukey's test was done. The

NIBP observation of the SBP reduction in case of optimized SMEDDS is depicted in **fig: 15, 16 Table 11**. Therefore, this observation probably confirms the hypothesis of increased bioavailability of an otherwise less bioavailable drug (Cilnidipine) achievable with the use of SMEDDS.

Table 11: Pharmacodynamic data of blood pressure (Data expressed as Mean±SD) (n=6).

Formulation	Before treatment (mm of Hg)	Hypertension induction (mm of Hg)	After treatment (mm of Hg)	Elevation (mm of Hg)	Reduction (mm of Hg)	% Elevation	% Reduction
Tablet (Marketed)	124.996±9.72	146.582±16.72	131.446±16.79	21.586±16.32	15.136±5.32	17.852±14.51	12.073±3.57
SMEDDS	125.81±14.29	149.868±17.53	128.92±13.181	24.058±11.41	20.948±10.09	19.463±10.009	16.126±5.9

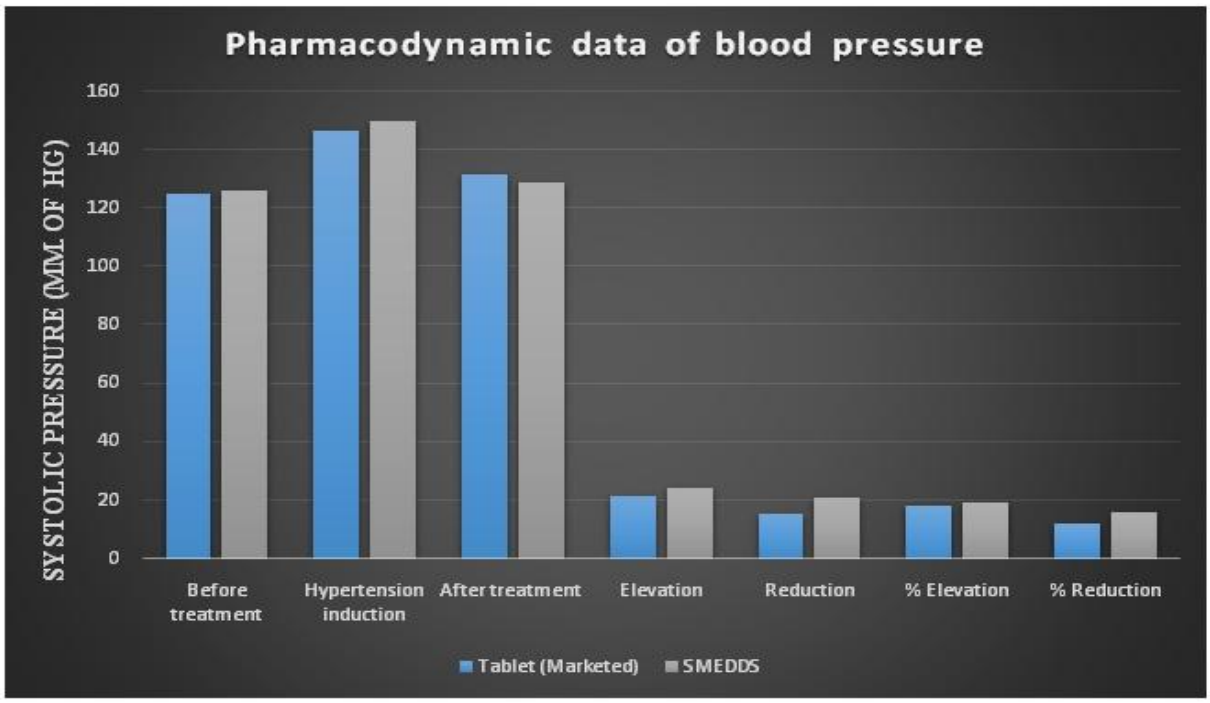
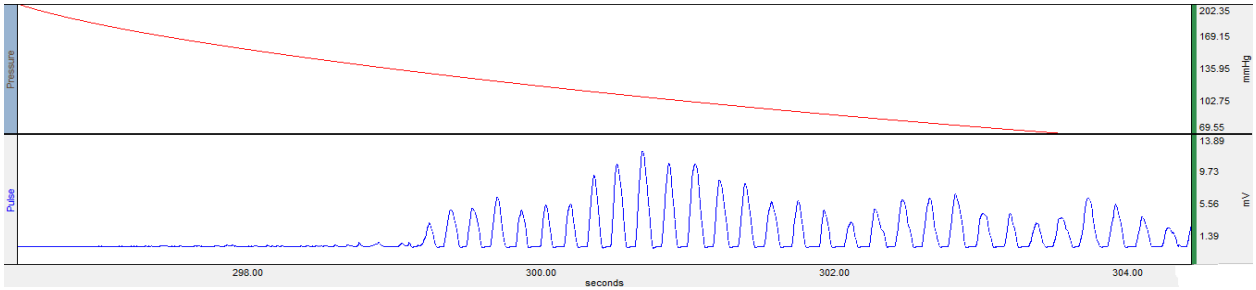
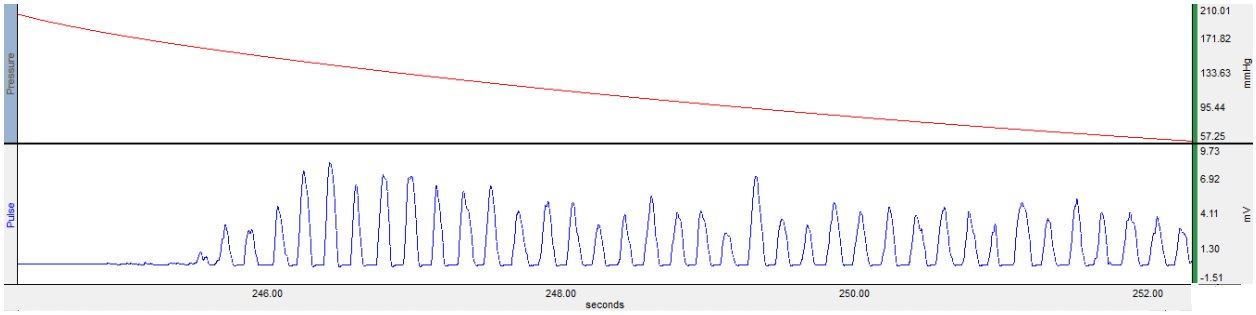


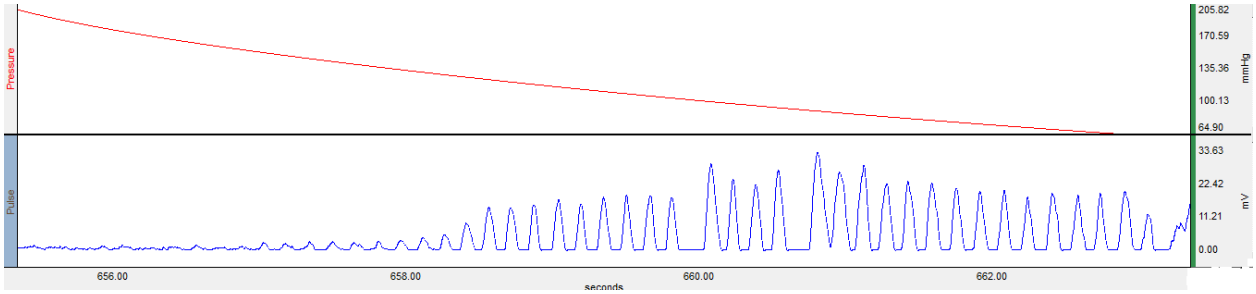
Figure 15: Pharmacodynamic data of blood pressure (Data expressed as Mean±SD) (n=6).



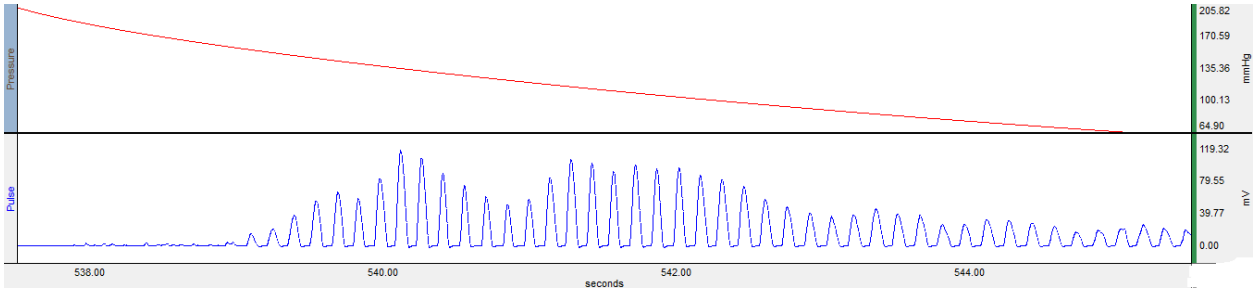
A: SBP At day 0 i.e. before treatment



B: SBP after inducing hypertension with L-NAME



C: SBP after 14th Day, treatment with SMEDDS



D: SBP after 14th Day, treatment with the Marketed Tablet

Figure 16: Systolic blood pressure at 0 and 14th days with different treatments A, B, C and D

5.8 Conclusion:

In this research work, L-SMEDDS of Cilnidipine, a BCS class II and very poorly water soluble drug has been successfully developed. The developed formulations of Cilnidipine were pre-concentrate consisting of Cilnidipine, Triacetin, Tween 20 and Transcutol HP as drug, oil, surfactant and co- surfactant respectively. The study was supported by experimental design mediated statistical optimization and computer assisted prediction of optimized formulation. The surfactant and co-surfactants were used in a ratio of 1:1 and the amount of the selected surfactants was kept at a low concentration in accordance with the permissible daily intake limits [37-39]. The total weight of the optimized pre-concentrate was around 1190 mg consisting of 10 mg of anti-hypertensive CCB Cilnidipine. Triacetin is GRAS listed and fulfills the criteria for the chronic use of the anti-hypertensive dosage forms. Pharmacokinetic studies of the SMEDDS showed an increase in the relative bioavailability by about 2.4 fold as compared to the marketed Cilnidipine tablet with the help of robust chromatographic technique LC-ESI MS/MS. The validation of pharmacokinetic parameters corroborate with the pharmacodynamic findings which involved NIBP measurements in L-NAME challenged animals. This further indicates that the formulation of L-SMEDDS may offer the advantage of reduction in dose and adverse effects associated with the dose. The result supports chronic use of L-SMEDDS for antihypertensive treatment. Finally, the developed SMEDDS of Cilnidipine is determined to be a more viable alternative to currently available marketed antihypertensive medications that can fulfill clinical relevance.

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Kumar Anand 15.11.2022

Annexure 1

Accuracy: 85--115%

Precision (CV%) = +/- 15

Regression<1,00

Short term, long term, bench top stability: 90-110%

Matrix factor: 0.8-1.00

Freeze-thaw, Autosampler, Matrix effect: 85-1115%

Recovery: 80-120%

PURE CHEM
PRIVATE LIMITED

Dt. 02/12/2015

CERTIFICATE OF ANALYSIS

Product Name	**Cilnidipine**	A. R. No.	QCFP/069/15-16
Batch No.	CLD 151208P2	COA No.	FP/COA/069/15-16
Mfg Date	DEC 2015	Exp. Date	NOV 2018

Test	**Requirements**	**Results**
Physical Appearance	Yellowish or yellow powder	Yellowish powder
Solubility	Sparingly Soluble in ethanol, Practically insoluble in water.	Conformed
Melting Range	$105^0 - 110^0$ C	$106^0 - 108^0$ C
Residue after ignition	Not more than 0.2% w/w	0.05% w/w
Loss On Drying	Not more than 0.5% w/w	0.25% w/w
Heavy metals	Not more than 10ppm	Less than 10ppm
Related substance Individual Impurity Total impurities	 Not more than 0.5% w/w Not more than 1.0% w/w	 0.19% w/w 0.34% w/w
Assay (on dried base)	Not less than 98.5% w/w Not more than 101.5% w/w	99.21% w/w
Particle Size	D(90) less than 10 micron	Complies
Conclusion	The material **complies** with respect to the above specification.	

Prepared By		Checked By	Harit	Approved By	
Date	02.12.15	Date	02/12/2015	Date	02/12/2015

PURE CHEM
PRIVATE LIMITED

QC/F024

Dt.14/02/2018

CERTIFICATE OF ANALYSIS

Product Name	**Cilnidipine**	A. R. No.	QCFP/17-18/126
Batch No.	CLD 180213P4	COA No.	COA/FP/17-18/127
Batch Size	150 KG	Analysed Qty.	05 gm
Mfg Date	FEB 2018	Exp. Date	JAN 2021

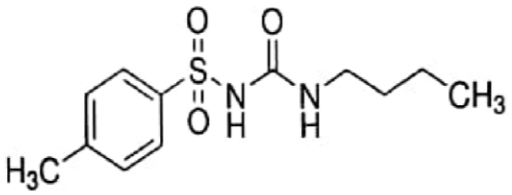
Test	Requirements	Results
Physical Appearance	Yellowish or yellow powder	Yellowish powder
Solubility	Sparingly Soluble in ethanol, Practically insoluble in water.	Conformed
Melting Range	$105^0 - 110^0$ C	$108^0 - 110^0$ C
Residue after ignition	Not more than 0.2% w/w	0.05% w/w
Loss on Drying	Not more than 0.5% w/w	0.09% w/w
Heavy metals	Not more than 10ppm	Less than 10ppm
Related substance Individual Impurity Total impurities	 Not more than 0.5% w/w Not more than 1.0% w/w	 0.10% w/w 0.20% w/w
Assay (on dried base)	Not less than 98.5% w/w Not more than 101.5% w/w	99.94% w/w
Particle Size	D(90) less than 20 micron	Complies
Conclusion	The material **complies** with respect to the above specification.	

Prepared By		Checked By		Approved By	
Date	14.02.'18	Date	14/02/18	Date	14/02/2018

SHUBHAM
BIOPHARMA

CERTIFICATE OF ANALYSIS

Product Name : **Tolbutamide**
Structure :



Molecular Formula : $C_{12}H_{18}N_2O_3S$
Molecular Weight : 270.35

Batch No. : SBP-TBTM-045	Retested Date : 12 November, 2020
Quantity : 500 mg	Expiry Date : 12 November, 2022

SR NO.	TEST	SPECIFICATION	RESULT
1	Description	Solid	Complies
2	Solubility	Chloroform	Complies
3	NMR	Conforms to Structure	Conforms
4	Mass	Conforms to Structure	Conforms
5	Assay (by HPLC)	NLT 95.0 %	97.16 %

For Long term Storage condition: Store at 2-8 ° C, in a well closed container.
***OBSERVATIONS**: The material complies with the above specification*

	Signature	Date
CHECKED BY:	Geeth	12 November, 2020
APPROVED BY:	Mavade	12 November, 2020

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website : www.shubhambiopharma.com | Email : shubhambiopharma@gmail.com

ORIGINAL ARTICLE e-ISSN: 2349-0659 p-ISSN; 2350-0964

Formulation Development, Optimization, and Characterization of Cilnidipine-Loaded Self-microemulsifying Drug Delivery System

Kumar Anand[1], Samit Karmakar[2]*, Pallab Mandal[1], Md. Adil Shaharyar[1], Rudranil Bhowmik[1], Avishek Mondal[1], Subhabrata Ray[3], Sanmoy Karmakar[1]*

Abstract

Cilnidipine, a 2, 4-dihydropyridine antihypertensive, is poorly bioavailable and belongs to Biopharmaceutical Classification System Class II. The present study was carried out to develop and evaluate a cilnidipine-loaded self-microemulsifying drug delivery system (SMEDDS) using food grade oil for enhanced pharmacokinetic parameters. The SMEDDS was prepared by low-energy method. A pseudo-ternary phase diagram was developed using triacetin, Tween 20, and Transcutol HP as oil, surfactants, and cosurfactants, respectively. The statistically optimized formulation was obtained and was evaluated for relevant *in vitro* characterizations. Globule size, zeta potential, and polydispersity index (PDI) of the optimized formulation were found to be 9.045 nm, −2.32 mv and 0.203, respectively, indicating stable and uniformly distributed microemulsion nature of the formulation. Developed SMEDDS of viscosity 31 cps was found to be clear in 500 times dilution in water and phosphate buffer pH 1.2. Selection of the optimized SMEDDS was followed by various formulation characteristics, including goat intestinal membrane permeability. The *in vitro* dissolution study of optimized SMEDDS exhibited much better result as compared to the marketed tablet of cilnidipine.

Keywords: Biopharmaceutical classification system Class II, Cilnidipine, Design expert, Goat intestinal membrane permeability, Pseudo-ternary phase diagram, Self-microemulsifying drug delivery system

Asian Pac. J. Health Sci., (2021); DOI: 10.21276/apjhs.2021.9.1.05

Introduction

Drugs falling under Biopharmaceutical Classification System (BCS) Class II and IV are known to have very low absorption, which is difficult to overcome using conventional dosage forms. Accordingly, lipoidal drug delivery systems might become helpful to overcome the above-mentioned constraints and may achieve desired clinical benefits at a lower dose with the added advantage of reduced toxicities. Cilnidipine is one of the recently approved drugs entity for better antihypertensive management in comparison to other calcium channel blockers.[1] In the present study, self-microemulsifying drug delivery system (SMEDDS) of cilnidipine, a newer congener with problems characteristic of the BCS Class II group, was developed, investigated, and evaluated. SMEDDS, a novel drug delivery system, is an isotropic mixture of oil, surfactant, cosurfactant, and drug. SMEDDS has the ability to form oil-in-water (O/W) microemulsion in a spontaneous manner under mild agitation in gastrointestinal tract fluids after oral administration. Enhancement in the solubility and permeability of the BCS Class II drugs, spontaneous formation, thermodynamic stability, improved bioavailability, and feasibility of the preparation are among the primary advantages of SMEDDS.[2] Here, the presence of triglyceride in composition of self-microemulsifying pre-concentrate is expected to enhance the bioavailability by improving lymphatic transport bypassing the portal circulation, which provides a greater interfacial area for absorption and improvement of the physical and chemical stability of drugs.[3,4]

Materials and Methods

Chemical Reagent

Triacetin (glycerol triacetate) and diethylene glycol monoethyl ether (Transcutol HP) were purchased from Sigma-Aldrich. Tween 20, Tween 80, and Span 80 were purchased from Merck Millipore.

[1]Department of Pharmaceutical Technology, Jadavpur University, Kolkata, West Bengal, India, [2]Medical College, Kolkata, West Bengal, India, [3]Dr. B. C. Roy College of Pharmacy and Allied Health Sciences, Dr. Meghnad Saha Sarani, Durgapur, West Bengal, India

Corresponding Author: Sanmoy Karmakar, Department of Pharmaceutical Technology, Director, Bio-Equivalence Study Centre, Jadavpur University, Kolkata, West Bengal, India. E-mail: sanmoykarmakar@gmail.com

Samit Karmakar, Medical College, Kolkata, West Bengal, India. E-mail: samitkarmakar2015@gmail.com

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Conflicts of interest: None.

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Labrasol was a gift sample from Gattefosse, India. Analytical grade sodium dodecyl sulfate (SDS) was purchased from E-Merck, India. Glycerol, isopropyl alcohol, and PEG 400 were purchased from Merck, India. Cilnidipine was a gift from PURE CHEM PVT. LTD., Gujarat, India, lercanidipine, α-napthol, and ketoconazole were obtained as a gift sample. All chemicals used were of analytical grade. Milli-Q water was used for the present study.

Methods

Selection of oil

Selection of oil for the formulation of cilnidipine was done on the basis of solubility of the cilnidipine in various oils. Oil, safe for chronic use, and low cost was also a concern. Hence, solubility of cilnidipine was determined in various oils (olive oil, almond oil, castor oil, triacetin, sesame oil, flaxseed oil, rice bran oil, and Capmul MCM).

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BCRCPiCON-19-P-083

STATISTICAL OPTIMIZATION OF A NANOLIPOIDAL PLATFORM FOR ENHANCING BIOPHARMACEUTICAL PARAMETERS

Kumar Anand[1]*, Sanmoy Karmakar[1], Subhabrata Ray[2], Rudranil Bhowmik[1]

[1]*Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, West Bengal, India.*

[2]*Dr. B.C. Roy College of Pharmacy & Allied Health Sciences, Durgapur, West Bengal-713206, India.*

**kr.anand.pharmamrit@gmail.com*

Many recently approved drug moieties are classified as either belonging to Class II / IV of BCS (Biopharmaceutical Classification System). A suitable delivery system is necessary to overcome the bioavailability related problems as also to deliver the drug optimally. A nanotechnological approach having lipoidal carriers bears much hope for improving the pharmacokinetic parameters along with reduction in dose related toxicities. Nano size emulsions, out of various lipoidal formulations, are better delivery systems with a globule size range of 1 to 500nm. Formulation optimization for the purpose of enhancing pharmacokinetic parameters and reducing dose related toxicities are accomplished following statistical techniques. In this work, a Dihydropyridine Calcium channel blocker with poor bioavailability was chosen as model drug. To formulate the nanolipoidal systems, various oils and surfactantswere used. Economy of the product was a major goal of the study. The components proportions were chosen based on pseudo ternary phase diagrams and prepared using low energy method.As for the statistical optimization of the formulations, various parameters such as globule size, zeta potential, viscosity, PDI, in vitro release, membrane permeation study, conductivity, and percentage transmittance were considered. A mixture experimental design strategy was adopted. The presentation will detail these results.Developed formulation was stable and meets qualities of a suitable nanolipoidal formulation. The results of various characterizations implied that the formulated dosage form will enhance the bioavailability and can decrease the dose related toxicities. Such cost-effective technologies are essential for "health for all" in SOUTH-EAST ASIA.

BCRCPiCON-19-P-084

A BRIEF REVIEW ABOUT THE IMPLICATIONS OFDRUG-DRUG INTERACTION

J Bondyopaddhyay, *M Chattopadhyay, P Chakraborty

Bengal School of Technology, Sugandha, Hooghly, West Bengal 712102

**moit179@rediffmail.com*

In the present work the implications of Drug-Drug interactions (DDI)have been considered as it is a crucial parameter in prescribing the drugs to patients. Concurrent use of medicine by patient is the cause of Drug-Drug Interaction. Polypharmacy is very common in India. The practice of polypharmacy is sometimes due to necessity of the treatment and sometimes is the cause of self-medication. In this condition the chances of DDI increases. The DDI is usually broadly classified into two sections – Pharmacokinetic interactions and Pharmacodynamic interactions; this usually depends on the mechanism by which interaction between drugs occur. These interactions show two prominent effects in the body – Synergism and Antagonism. The synergism increases the therapeutic value, whereas the antagonisms are the causes of reduced treatment efficacy, adverse drug reactions and toxicity. There are multiple factors that control the DDIs and many times it has been found to vary among individuals. Identifying the DDIs has become the important topic for the developed countries and developing countries. Certain common examples of the DDIs are: Increased gastric irritation by NSAIDs and alcohol; Risk of bleeding increases with the co-administration of antiplatelet drugs and anticoagulants; Severe drowsiness was observed with antihistaminics and alcohol. Therefore, here the effects of DDIs have been elaborated.

BCRCPiCON-19-P-085

POLYMORPHISM: A KEY CONCERN IN DEVELOPMENT, MANUFACTURING AND STABILITY OF PHARMACEUTICAL SOLID PRODUCTS

Prasun Mukherjee, Parthasarathi Panda*

Dr. B.C. Roy College of Pharmacy & Allied Health Sciences, Durgapur- 713206, W.B. India

**panda.ju06@gmail.com*

Polymorphism is a phenomenon where pure molecules exist in various crystalline forms having divergent arrangements and/or diverse molecular confirmation

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Insights into the Approach, Fabrication, Application, and Lacunae of Nanoemulsions in Drug Delivery Systems

Kumar Anand,[a] Mahfoozur Rahman,[b] Subhabrata Ray,[c] & Sanmoy Karmakar[a,*]

[a]Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, West Bengal, India; [b]Department of Pharmaceutical Sciences, Faculty of Health Sciences, SIHAS, SHUATS, Allahabad, Uttar Pradesh, India, 211007; [c]Dr. B.C. Roy College of Pharmacy & Allied Health Sciences, Durgapur, West Bengal-713206, India

*Address all correspondence to: Sanmoy Karmakar, Professor, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, West Bengal, India; Tel.: +033-24572056; Fax: +0091-33-2414604, E-mail: sanmoykarmakar@gmail.com

ABSTRACT: Many of the recently approved drug molecules that are therapeutically successful are found to be incompatible for the development of a novel delivery system and to take part in various health care management. Regardless of having better therapeutic properties, these molecules are barred from their effective clinical uses. The main reason attributed to it is poor solubility and/or poor permeability of drugs which finally emerges the drug to be low bioavailable. Nanoemulsions are one of the most acceptable nanolipoidal drug delivery system and appears to be a hope for the delivery of many of the Biopharmaceutical Classification System (BCS) class II and IV drugs. A nanoemulsion is a thermodynamically unstable isotropic mixture of oil, surfactant, and co-surfactants and is biphasic in nature. It can be either water in oil or oil in water and droplets are found in the range of 5 to 500 nm. The manufacturing and fabrication of nanoemulsions involve various natural, synthetic and semi synthetic materials using either low or high-energy methods. Application of nanoemulsions as a novel drug delivery system through several routes, especially oral, transdermal, ophthalmic, and intranasal, have been increased for various pharmacological aspects such as cardiovascular, anticancer, antimicrobial, and ophthalmic due to their stability, high solubilization capacity, and ease of preparation. The objective of this review is to focus on the aspects of manufacturing, fabrication, application, and some toxicological concerns related to nanoemulsions.

KEY WORDS: nanoemulsion, Biopharmaceutical Classification System (BCS) class II/IV, bioavailability, manufacturing, fabrication, application

I. INTRODUCTION

In previous decades, drug molecule developments have been quite successful and one can expect the availability of innumerable drug molecules for currently persisting diseases. Unfortunately, when we look for the desirable pharmacological responses it becomes difficult due to lack of proper delivery of the drug moiety to the desired tissue or cell. A successful approach for a novel drug delivery system can hopefully solve the above problems and can improve the related pharmacological responses of the delivered drug moiety. With progress in time the advancement of science and technology has resulted in a number of delivery systems counting from solution to tablets and then novel delivery systems such as nanostructured lipid carriers (NLCs), smart lipids, microparticle, nanoparticles, liposomes, and nanoemulsions. The novel drug delivery systems are

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REVIEW ARTICLE

Nano-emulgel: Emerging as a Smarter Topical Lipidic Emulsion-based Nanocarrier for Skin Healthcare Applications

Kumar Anand[1,*], Subhabrata Ray[2], Mahfoozur Rahman[3], Adil Shaharyar[1], Rudranil Bhowmik[1], Rammohan Bera[1] and Sanmoy Karmakar[1]

[1]*Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, West Bengal, India;* [2]*Dr. B.C. Roy College of Pharmacy & Allied Health Sciences, Durgapur, West 713206 Bengal, India;* [3]*Shalom Institute of Health and Allied sciences, Allahabad 211007, India*

Abstract: ***Background*:** In recent decades, enormous efforts for different drug discovery processes have led to a number of drug molecules available today to overcome different challenges of the health care system. Unfortunately, more than half of these drugs are listed in either BCS (biopharmaceutical classification system) class II/ IV or both are eliminated from the development pipeline due to their limited clinical use. A nanotechnological approach bears much hope and lipoidal fabrication is found to be suitable for the delivery of such drugs. Nanoemulsion based gel *i.e.* nanoemulgel out of different nanolipoidal formulations has been found to be a suitable approach to successful drug delivery through topical routes. In past few years many herbal and synthetic active pharmaceutical ingredients (APIs) has been patented as nano sized emulsified gel for various therapeutic activities.

***Methods*:** Nanoemulgel is basically an emulsion-based topical gel formulation, where nano-sized emulsion globules can be prepared with the help of high energy or low energy methods and further converted into nanoemulgel by adding a suitable gelling agent. Nanoemulgel fabrication enlists various kinds of polymeric materials, surfactants and fatty substances of natural, synthetic and semi-synthetic nature with a globule size range from 5 to 500 nm.

***Results*:** Nanoemulgel can be applicable to various acute and chronic diseases through topical routes.

***Conclusion*:** Nanoemulgel preparations of many recently approved drugs are being used successfully in different areas of health care and have re-defined the significance of topical route of delivery as compared to other routes. However, along with various improvements in the current state of the delivery system, the safety factor needs to be taken into account by toxicological studies of the materials used in such formulations.

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Keywords: Health care, topical route, BCS class II/IV, bioavailability, nanotechnology, nanoemulgel.

1. INTRODUCTION

In the history of ancient medicine, the skin has been extensively used as the most prior organ for the application of various medicaments and subsequently, achieving the desired therapeutic activity. Similarly, in the modern medical practice over the past decades, Transdermal Drug Delivery System (TDDS) has made an important contribution to health care by providing an attractive alternative to oral drug delivery. Accepting many challenges in the delivery of recently approved drugs, the Transdermal drug delivery has also been advanced and emerged as the first generation, second generation and third generation TDDS. These generations were classified on the basis of size and physicochemical properties of the active pharmaceuti-

*Address correspondence to this author at the Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, West Bengal, India; E-mail: sanmoykarmakar@gmail.com

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How Synthetic Drugs Work

Insights Into Molecular Pharmacology of Classic and New Pharmaceuticals

2023, Pages 349-367

Chapter 15 - Mechanism of action of drugs used in hypertension

Rudranil Bhowmik [1], Md. Adil Shaharyar [1], Kumar Anand [1], Hindol Mazumdar [1], Avishek Mandal [1], Pallab Mandal [1], Suparno Chakraborty [2], Parag Panday [3], Sanmoy Karmakar [1]

https://doi.org/10.1016/B978-0-323-99855-0.00015-4

Abstract

Hypertension is a cardiovascular disease that is related to different physiological parameters. Several pathways are involved in regulation of blood pressure, such as a sympathetic reflex pathway, renin–angiotensin–aldosterone system, cardiac contractility and vascular physiology. Induction of stress, change in food consumption behavior, sedentary lifestyle are precursors of hypertension nowadays. If untreated for a prolong period, it can damage vascular curvature, cardiac wall, renal perfusion, and several other physiological problems. In this context, we are trying to understand pathophysiological condition of hypertension and agents that can reduce it clinically through different pathways.