Studies on Liposomal Delivery of Dopamine to Brain via Nasal Route

THESIS

Submitted in partial fulfilment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

by

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Under the Supervision of

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BITS Pilani
Pilani | Dubai | Goa | Hyderabad

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

2015

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on Liposomal Delivery of Dopamine to Brain via Nasal Route"** and submitted by **Vibhu** ID No **2009PHXF406P** for award of Ph.D. Degree of the Institute embodies original work done by her under my supervision.

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Acknowledgements

"It is your attitude not your aptitude that determines your altitude. Defeat never comes to any man until he admits it...Zig Ziggler"

It is a moment of gratification and pride to look back with a deep sense of contentment and gratitude at the long travelled path, to be able to capture some of the finest moments, to be able to thank the infinite number of people, some who were with me from the beginning and some who joined me later, whose love and blessing have made this day possible for me.

From the inner core of my heart, I express profound indebtedness and gratefulness towards my research supervisor, Dr. Ranendra N. Saha, Shri B.K. Birla and Shrimati Sarala Birla Chair Professor and Director, BITS-Pilani, Dubai campus. He has an insurmountable "never say die" attitude. A true embodiment of "To strive to seek and not to yield"...He single handed transformed me from a student to a researcher. During these years I have known him as sympathetic and principle-centered person. His enthusiasm and integral view on research and his mission for providing 'only high-quality work and not less', has made a deep impression on me. I owe him lots of gratitude for having me shown this way of research. He taught me to look for solutions to problems rather than focus on the problem. It goes without saying that mere acknowledging with words is inadequate to express my gratefulness to him and he is always an inspiration to me in research and personal life.

I am thankful to Prof. B. N. Jain, Vice-Chancellor, Dr. G. Raghurama, Director and Prof M.M.S. Anand, Registrar, BITS, Pilani, for providing me the opportunity to pursue my doctoral research work in the institute.

I express sincere gratitude to Prof. R. Mahesh, Dean Faculty Affairs, Prof. S.K. Verma, Dean, Academic Research Division and Dr. Hemanth R Jadhav, Associate Dean, ARD, BITS, Pilani for extending support during my research work.

I am highly indebted to Dr S. Murugesan, Head Department of Pharmarcy, BITS, Pilani, Dr. Shrikant Charde, Head Department of Pharmacy BITS, Pilani, Hyderabad

Campus for their timely suggestions and support during various stages of my research.

I take this opportunity to specially thank to Dr. Atish Paul, Convener, Departmental Research Committee and Dr. Anil Jindal, Doctoral Advisory Committee member for their valuable comments and intellectual guidance while compiling this thesis.

I gratefully acknowledge all the faculty members of Department of Pharmacy. My special thanks go to Dr Sunil K Dubey, Mr. Gautham Singhvi, Ms. Archana, Mr. Jaipal, Dr. Rajeev Taliyan and Dr. Anil Gaikward. I thank Animal House In-charge, Dr. Sushil for his co-operation in my research. I must thank all the non-teaching staff members of Department of Pharmacy and Biology, Lakshman, Vishal, Puran, Sajjanji, Naveen, Mukesh, Mahenderji, Virender, Kaushailya, Mahipalji, Tarachand and Ramsutar who have always been helpful during my research work. I also thank staff members of Education Development Division, Academic Research Division and Account Department for their help when it is required.

I am thankful to all my colleagues who have helped me immensely, Mr Prashant Raut, Mr Satish Reddi, Ms Garmia, Mr Muthu and Mr Ashok Penta. Special mention to Mr. Yashwant Kurhe, for his day and night support especially during animal studies. I thank my lab senior Dr. VasanthaKumar Sekar for his indirect support. I thank all research scholars and M. Pharmacy students of BITS, Pilani who passed out during my stay in Pilani for their support and enc+ouragement.

There are some elements which are indispensable for the very existence of our lives. They come in myriad of forms. My eternal gratitude to Mr Emil Joseph a friend in moments of tears and laughter, the silent and suave spectator of all my errands… Your image emanates phenomenal serenity to keep me ticking…

I acknowledge service of Sophisticated Analytical and Instrument facilities at Panjab University, Chandigarh, for Electron Microscopic and ^{1}H nuclear magnetic resonance analysis. I am very grateful to Dr. B.N. Datta, Medico centre, Chandigrah for preparing histopath slides and Dr. Rakesh Vasishtha, Head department of histopathology, PGI, Chandigarh for analysis of histopath slides. I am very grateful to Dr. R.K. Gupta, Department of Physics, BITS-Pilani, for providing access to Atomic Force Microscopy.

I deeply acknowledge, Torrent Research Centre (TRC: India) for providing Fellowship, with special mention to Ms Jaya Abraham. I would like to thank the Department of Science and Technology (DST) for providing the full travel grant and registration under Young Scientist International Travel Support Scheme (ITS) to present part of my Ph.D. work in 2013 AAPS Annual Meeting and Exposition, San Antonia, Texas, USA and XX World Congress for providing funding to attend XX World Congress on Parkinson's Disease held in Geneva, Switzerland in 2013. My special thanks to Vav Life Sciences, India and Lipoid, Germany for the generous gift samples of Lipids.

The people who make your life worth are only few… fewer are those who are the sculptors of your entire persona. I express my deepest regards to Prof. M.A. Potdar, my masters' supervisor for always being there…

"There are people you know for your life and then there are people you want to know for rest of your lives". I take the opportunity to express my unending gratitude towards "Friends forever". They helped me to claw out of the inextricable depths of loneliness and melancholy. Pallavi, Rituparna (gf), Aishwarya and Preeti who were here for the relation that is God gifted and may be the result of good deeds that I have done.

I take this opportunity to say my very special thanks to Mrs. Dipa Saha for her continuous support and encouragement throughout my Ph.D. tenure. I thank Mr. Soumik and Mr. Shaunak for their support and encouragement

The 'perfect mate' does not exist and I have found mine. Words fail me to express my sincere love and appreciation to my husband Dr. Puneet Rinwa to stand by me through thick and thin, even when I may not have deserved it. I owe him for being unselfishly let his intelligence, passions and ambitions collide with mine. Without him I would not have survived a day. I only pray he always be there like a pole star in the peregrination called life. I cannot finish without saying how grateful I am to my mother in law Ms Pushpa Rinwa and father in law Mr. Rajinder Rinwa whose affection and support was like mellifluous confluence of blessings of almighty.

Some angels make our life worth, who enlighten the very essence of our existence. My most precious possession Ishan my dearest younger brother who enlightened the very

fabric of my life with his innocence and was a bundle of joy which helped me wade through sea of difficult moments.

It is said that 'accomplishments must be credited to those who had put up the foundations of the particular chore'. Here, I thanks to my parents for lifting me up till this phase of life. Let me pay my deepest homage and regards to the unending affection of Papa who is the fount of my existence and who has ensconced me from all hardships and catastrophe to make me what I am. It is a Jewish Saying that "God could not be everywhere and therefore he made mothers". I want to extend my deepest regards to Mummy for guiding me through inextricable errands.

Beside this there are several other people who have knowingly or unknowingly helped me in the successful completion of this project. I thank all the people for every ounce of efforts they contributed.

Above all and most essentially my humble solicitations to The Supreme Power- The GOD for giving me chance as a human to live and survive and being my constant companion, strongest source of inspiration, and my ultimate Guardian.

Finally I owe a great debt of gratitude to the innocent rats who sacrificed their lives to unearth the facts that are necessary foundation of this research.

Needless to say, errors and omissions are mine

Date: ***Vibhu***

Abstract

Drug delivery to brain remains the major challenge for treatment of central nervous system (CNS) disorders due to formidable mechanisms protecting the brain from unwanted substances from main circulation system. The emerging loom is to bypass blood brain barrier (BBB) by using a novel, practical, simple and non invasive approach such as intranasal delivery. This route is already investigated to found to bypass BBB and target CNS in treatment of various neurological disorders. The strategy of applying drugs encapsulated into nanocarriers (polymeric and lipid based) to the olfactory epithelium could potentially improve the efficacy and direct CNS delivery of drugs. The aim of this work is to deliver Dopamine directly to brain via nasal application using liposomal drug carriers to improve selective biodistribution to brain and its therapeutic efficacy to suggest future prospective that may benefit in progressive treatment for neurodegenerative disorders. To achieve this broad objective, following studies were carried out in this research work.

Appropriate analytical and bioanalytical methods based on spectrophotometric and liquid chromatography were developed and validated for estimating Dopamine HCl. The developed methods were simple, selective, sensitive, accurate and precise in the determination of Dopamine HCl. The analytical method was applied to determine Dopamine in liposomes and in the in-vitro dissolution sample and the bioanalytical method was used to estimate Dopamine in plasma, brain, nasal fluid, lungs, trachea and oesophagus samples in pharmacokinetic and biodistribution studies. Prior to the formulation development, preformulation studies were performed to establish necessary physicochemical data of the drug. Studies were performed to concentrate on product specific questions related to drug-excipient compatibility, stability etc. The drug-excipient compatibility studies indicated no physical or chemical changes at ambient and accelerated storage conditions. Thus, drug-excipient compatibility studies would support the rationale for selection of various excipients and justify the product life span.

Design of suitable dosage form is very essential for delivering a drug to achieve effective therapy. An attempt was made to deliver dopamine to the brain using liposomes as carriers. Formulation development was carried out by identifying and optimizing the critical formulation and process parameters. Reverse phase evaporation technique coupled with freeze and thaw cycles were found to be best for the preparation of Dopamine HCl liposomes (LPs) using Leciva S70 and Lipova E120 lipids. The prepared liposomes were characterized for the size, size distribution,

polydispersity index, zeta potential, encapsulation efficiency, loading efficiency and in-vitro dissolution using appropriate methods. In addition the surface morphology of the prepared LPs was examined using transmission electron microscopy and atomic force microscopy. The characterization result confirms that the prepared LPs were stable, spherical in shape with low and uniform size. The various formulation parameters such as lipids cholesterol ratio, lipid and drug amount along with other processing parameters were found to affect critical properties of liposomes including particle size, size distribution, entrapment efficiency, loading efficiency and in vitro drug release profile. The liposomes prepared using biodegradable lipids were found to be suitable for the drug delivery application of Dopamine HCl with high drug entrapment and better loading efficiency. The prepared LPs showed control release of drug from 37 h to beyond 48 h depending on lipid nature and formulation, which could be explained by first order kinetics. Moreover, the thermal studies have confirmed uniform distribution of the drug, at molecular level, within the lipid vesicles without showing any chemical and physical interactions between the drug and lipids or other excipients. The stability study result showed that the prepared liposomes were stable by selected method with acceptable reproducibility.

In vivo pharmacokinetic and biodistribution behavior of the optimized formulations were performed in rats to assess the suitability of the formulations for direct delivery to brain and extent of release. The histopathological study was done to see any toxic effect from prepared formulations to respective tissues. The histopathological studies in rat nasal cavity and brain suggest that the prepared LPs did not show change in the architecture of respective organ cells. The biodistribution studies confirmed that Dopamine LPs are rapidly distributed in high extent to brain via nasal route when compared with i.v. administration. Drug targeting efficacy (DTE %) and nose to brain direct transport (DTP %) were calculated using tissue/organ distribution data following intranasal and intravenous administration. Leciva S70 and Lipova E120 LPs showed higher DTE % and DTP % suggesting that LPs has better brain targeting efficiency via nose. Thus the delivery of Dopamine HCl using liposomes via nose would be advantageous for CNS targeting.

Collectively, these results indicate that the prepared LPs have a great potential as a nasal delivery system for brain targeting in treatment of Parkinson's disease.

List of Tables

List of Figures

List of Abbreviations and Symbols

AA	Ascorbic acid
AD	Alzheimer's disease
ABC	Area between the drug dissolution curve and its asymptote
ADHD	Attention deficit hyperactivity disorder
AFM	Atomic force microscopy
cAMP	3',5'-cyclic adenosine monophosphate
ANOVA	Analysis of variance
AT	Accelerated temperature (40 ± 2 °C/ 75 ± 5% RH)
AUC	Area under the concentration versus time curve
AUC_{∞}	Area under the concentration versus time curve for time zero to infinity
AUDC	Area under the dissolution curve
AUMC	Area under first moment curve of concentration versus time profile
BBB	Blood brain barrier
CDDS	Conventional drug delivery systems
CI	Confidence intervals
C_{max}	Maximum concentration of drug in serum or tissue
Cl	Clearance
CNS	Central nervous system
CMC	Critical micelle concentration
CPCSEA	Committee for the purpose of control and supervision of experiments on animals
DA	Dopamine
DC	Drug content
DCM	Dichloro methane
°C	Degree centigrade
DEE	Diethylether
DDS	Drug delivery system
DSC	Differential scanning calorimetric
DE	Dissolution efficiency
DMPC	Dimyristoylphosphatidyl choline
DMPG	Dimyristoyl-phosphatidylglycol
DOE	Design of experimentation
DOPA	3,4-dihydroxy-phenyl-alanine
DOPAC	3-4-dihydroxy-phenylacetic acid
DOPE	Dioleoyl-phosphatidylethanolamine
DPPC	Dipalmitoylphosphatidyl choline
DPPS	Dipalmitoyl-phosphatidylserine
DSPE	Distearoyl-phosphatidylethanolamine
ECD	Electrochemical detector
EDTA	Ethylene di amine tetra acetic acid
EE	Entrapment/Encapsulation efficiency
EM	Electron microscopy
FDA	Federal Drug Administration
FPC	French pressure cell technique
FRR	Flow rate ratio

FT	Refrigerated temperature (5 ± 2°C)
FTIR	Fourier transform infrared spectrum
GH	Galanthamine hydrobromide
GIT	Gastrointestinal tract
g	Grams
h	hour
HCl	Hydrochloric acid
HEFF	Height equivalent to one effective plate
HETP	Height equivalent to theoretical plates
HPLC	High performance liquid chromatography
HPE	High pressure extrusion technique
HQC	High quality control
HVA	Homovanillic acid
IAEC	Institutional animal ethics committee
ICH	International conference on harmonization
IC_{50}	Concentration producing 50% of the maximum inhibitory response
i.n	Intranasal
IPA	Isopropylacohcol
i.v	Intravenous
IUPAC	International union of pure and applied chemistry
k'	Capacity factor
K	Release rate constant
K_{deg}	Degradation rate constant
L	Liter
LC	Liquid chromatography
LCMS	Liquid chromatography coupled with mass spectrophotometer
L-DOPA	3,4-levo-dihydroxy-phenyl-alanine
LE	Loading efficiency
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantitation
LPs	Liposomes
LPDDS	Liposomal drug delivery system
LQC	Lower quality control
LUVs	Large unilamellar vesicles
mg	Milligram
min	Minutes
mL	Millilitre
mm	Millimetre
M	Molar
MAO	Monoamine oxidase
MAT	Mean absorption time
MDT	Mean dissolution time
MEC	Minimum effective concentration
MET	Methanol
MFH	Microhydrodynamic focusing
MLVs	Multilamellar vesicles

MQC	Medium quality control
MRTD	Mean residence time of the drug molecules in the dosage form
MRT	Mean residence time
MSC	Model selection criterion
MST	Mean survival time
MTL	Maximum tolerable level
MTE	Minimum therapeutic effective level
MXST	Maximal survival time
n	Diffusional exponent indicative of release mechanism in krosmeyer-peppas model
N	Number of theoretical plates
NC	Negative control
NDDS	Novel drug delivery systems
Neff	Effective plate number
NLCs	Nanostructured lipid carriers
NMR	^{1}H nuclear magnetic resonance
NPDDS	Nanoparticulate drug delivery systems
NPs	Nanoparticles
OVAL	Ovalbumin
p-value	Significance level in statistical tests (probability of a type I error)
PBS	Phosphate buffer
PC	Phosphatidyl choline
PD	Parkinson's disease
PDA	Photodiode array detector
PDI	Polydispersity index
pH	Negative log to the base 10 of hydrogen ion concentration
PK	Pharmacokinetics
PK/PD	Pharmacokinetics and pharmacodynamics correlation
PNS	Peripheral nervous system
PS	Particle size
p-value	Significance level in statistical tests (probability os a type I error)
QC	Quality control
R	Correlation coefficient
R^2	Regression coefficient
RD	Relative dispersion of dissolution time
RES	Reserpine
REV	Reverse phase evaporation
R_f	Retention factor
RH	Relative humidity
RLS	Restless legs syndrome
rpm	Revolutions per minute
Rt	Retention time
RT	Room temperature (25 ± 2°C/ 60± 5% RH)
RTD	Log % remaining to degrade
s	Seconds
S	Slope of the least square regression line
SAXS	Small angle X-ray scattering
SD	Standard deviation

SDC	Sodium deoxycholate
SEM	Scanning electron microscopy
SPE	Solid phase extraction
SUVs	Small unilamellar vesicles
T	Tailing factor
$t_{1/2}$	Half life
$t_{90\%}$	Time taken for drug concentration to come down to 90 % of original concentration
T_m	Transition temperature
$T_{25\%}$	Time taken to release 25 % of drug from LPs
$T_{50\%}$	Time taken to release 50 % of drug from LPs
$T_{90\%}$	Time taken to release 90 % of drug from LPs
TDW	Triple distilled water
TEM	Transmission electron microscopy
TFH	Thin film hydration
Tmax	Time to reach maximum concentration of drug
TST	Thermal stress treatment
UFLC	Ultra Fast Liquid Chromatography
USP	United States Pharmacopoeia
ULVs	Unilamellar vesicles
UV	Ultraviolet
Vd	Apparent volume of distribution
VDT	Variance of dissolution time
VMAT2	Vesicular monoamine transporter
Vss	Apparent volume of distribution at steady-state
λ_{max}	Wavelength of maximum for UV- absorbance
XRD	X-ray powder diffraction patterns
ZP	Zeta potential
% RSD	Percent relative standard deviation / coefficient of variation
3D	Three dimensions
σ	Standard deviation of y-intercept of regression equation

Table of Content

Chapter 1. Introduction

1.1 Introduction

In the phase of mammoth research in central nervous system (CNS), brain disorders are still the foremost cause of disability. It is reported that patients with CNS disorders go for more hospitalization and expanded care when compared with all other diseases collectively (1). The treatment of brain disorders is one of the greatest challenges in drug delivery due to presence of blood brain barrier (BBB) which prevents or controls penetration of substances into the CNS and becomes primary obstacle for delivery of drugs to the brain. This barrier is designed to regulate brain homeostasis and to permit selective transport of molecules that are vital for brain function (1). Drugs effective against CNS disorders must pass these barriers to reach brain. There is an ongoing quest from last one decade of circumventing the BBB. CNS diseases like Parkinson's, Alzheimer's, Huntington's, schizophrenia, migraine, brain tumor and meningitis needs transport of drug to the brain for treatment (1). There are principally two potential approaches to deliver drugs to brain, namely the invasive or the non-invasive approaches (2). Patient compliance and risk-benefit ratio put forward the use of non-invasive methods of drug delivery over invasive methods. Conventional non invasive methods lead to more systemic availability of drugs than targeted organ which often cause severe side effects in other tissues. In addition, delayed onset of action, stability issues in GIT, extensive biotransformation, rapid elimination and widespread distribution into non targeted organs and tissues require the administration of high dose and often owing to nonspecific toxicity. Thus another challenge is delivering sufficient amount of drug to target organs, which is otherwise poorly accessible to drugs e.g. poor brain penetration (2). Advances in pharmaceutical technology have led to development of sophisticated drug delivery systems/techniques that allow drugs to be delivered through the skin, ocular, transmucosal membranes (nose, buccal or bronchial). Intranasal administration has drawn extensive interest seeing as it provides a non invasive method for bypassing the BBB and delivering directly therapeutic drugs to CNS (3). Targeting the brain via the nasal route offers potential for drug delivery as the olfactory region is in direct contact with the CNS as well as outer environment. Therefore, targeting brain via the nasal route is a prospective for drug delivery. Formulation scientists are endlessly working on novel drug delivery system (NDDS), as conventional dosage forms have various shortcomings. New generation drugs, though lesser in numbers, have entered the market, but most of them found to have drawbacks, such as poor water solubility, low gastro-intestinal permeability, high first pass metabolism, poor stability, non-selective distribution, poor permeability through BBB and many

more (4, 5). A promising strategy for drug delivery to overcome one or more of these shortcomings of conventional dosage forms is using colloidal carriers either polymer or lipid based. Liposomes are interesting candidates for brain targeting, due to rapid uptake by the brain, bioacceptability, biodegradability, and less toxicity compared to the polymeric nanoparticles. The approach of delivering drugs that are encapsulated into liposomes to the olfactory region could potentially improve the direct CNS delivery of drugs. Indeed, feasibility in scale-up and absence of burst effect make this delivery system a promising carrier for drug delivery. Additionally, liposomal delivery showed the potential in terms of higher delivery efficiency especially to brain and lower side effects (6, 7).

1.2 Challenges in treating CNS diseases

The existence of BBB turns out to be the only reason for the clinical breakdown of even a highly potent neurotherapeutic agent. The principal anatomical and functional site of the BBB is the brain endothelium, formed by complex cellular system. Physiologically, it includes endothelial cells, astroglia, extracellular basal lamina, adjoining pericytes and perivascular macrophages. These components mutually with adjoining neurons, form a composite and efficient "neurovascular unit" (8). Compared to other tissues brain has high resistance, epithelial-like tight junctions and minimal endothelial pinocytosis (9, 10). These tight junctions prevent cell migration or cell movement across endothelial cells. There are about 100 billion capillaries in human brain, providing a collective length of brain capillary endothelium of 650 km and a total surface area of about 20 m^2 (11). The creation of constantly even basement membrane surrounds the brain capillaries (12). Pericytes are enclosed in this basal lamina forming an intermittent layer which probably play role in phagocytosis and defence if the BBB is breached. The brain capillaries are covered by astrocytic end feet, to maintain the integrity of BBB. The brain capillary network have spacing of approximately 40 microns which is so dense that each brain cell essentially has its own vessel for nutrient supply (13). The BBB which consists of the endothelium of the brain vessels, the basal membrane and neuroglial cells, limits transfer of materials into the brain (14). Due to its inflexible permeability, there is restricted entry of promising drugs to target brain and is supposed to be the key obstacle in delivering CNS drugs.

Diffusion of molecules into the brain is generally divided into paracellular and transcellular pathways. Water-soluble drugs of lesser molecular weight may diffuse only through the tight junctions but not to any great extent. Even small lipid soluble substances like alcohol and steroid

hormones penetrates transcellularly by dissolving themselves in lipid plasma membrane. Actually various polar drugs are unable to reach CNS due to virtually absent of paracellular pathways because of unique properties of tight junctions in the BBB. The existence of only some endocytic vesicles in the CNS capillaries additionally removes a transcellular route for free diffusion of drugs into the interstitium (15, 16). But, a huge number of highly lipophilic drugs and other substances including essential materials like amino acids and glucose have to go with transport proteins (carriers), specific receptor-mediated or vesicular mechanisms (adsorptive transcytosis) to pass BBB (17). If the molecules fulfilled certain criteria they can diffuse transcellular transport by passive diffusion. These criteria includes low molecular weight (<500 Da); unionized drugs; log P close to 2; cumulative number of hydrogen bonds in drug molecule should not be more than 10 (18-20). Unluckily very few drugs fit these criteria. Transport across BBB for other therapeutic drugs rely on either the integrity of the BBB or the drug or drug carrier properties and their interaction with or affinity for receptors expressed at the BBB, as well as other biological or immunological processes occurring at the BBB. Therefore difficulty of delivering drugs across the BBB resulted in lower therapeutic efficacy for treating CNS disorders (21, 22). The answer to the problem of BBB "bottleneck" is the development of novel drug delivery (23).

1.3 Drug delivery to brain

Drug delivery to the brain remains the major challenge for treatment of CNS disorders due to formidable mechanisms protecting the brain from the outside world. Unfortunately, the same mechanisms that prevent intrusive environmental chemical accessing the brain also prevent the access of therapeutic chemicals. Hence various approaches have been developed to deliver drugs across the BBB. This impediment created by BBB can be overcome by 3 broad strategies namely delivery across the BBB, direct delivery to the brain (invasive approach) and bypassing the BBB (non-invasive approach). Delivery across the BBB includes physiological approach (prodrugs, pseudonutrients, ligand binding proteins, chimeric peptides) and pharmaceutical approach (carriers or transporters: liposomes, nanoparticles, nano-conjugates). These methods are complex and require drugs to possess certain specific characteristics and hence do not work effectively for all therapeutic agents. Moreover drugs are administered by classical i.v. or i.p. routes, which are subjecting to distribute different parts and to being metabolized in the liver, resulting in a modification in the amount of circulating drug available to the brain. Another part of the dosage may be excreted by the kidney before entry into the CNS, rendering the precise amount of the

drug that finally enters the brain difficult to be estimated. Direct delivery to the brain by invasive approach (BBB disruption, intracerebral implants, intraventricular infusion) allows the administration of a precise amount of drug to the brain but are not practical for use in humans due to risk, inconvenience and cost factor (24). The third and the emerging approach is to bypass BBB by using a novel, practical, simple and non invasive approach i.e. intranasal delivery. Intranasal administration offers an alternative route of administration for rapid drug delivery to brain (25-27). This route is already investigated to bypass BBB and target CNS in treatment of various neurological disorders (28). The intranasal delivery of drugs to the brain has previously been proven in human clinical trials. Intranasal insulin delivery has been reported to get better memory, attention, and functioning in patients with Alzheimer's disease with no change in the blood levels of insulin or glucose. These results put forward that intranasal insulin administration have therapeutic benefits without the risk of peripheral hypoglycemia (29).

The pathway involved in nasal route is olfactory or trigeminal nerve which starts in the brain and terminated in the nasal cavity. This is the only region of brain which is outwardly exposed and hence offers direct method of non invasive access to the brain. For studying aspects of nasal delivery to brain, it is essential to have understanding of the nasal anatomy and physiology. It is discussed here the olfactory region and trigeminal nerve system which jointly play role in transport of drugs from nose to the brain. The olfactory epithelium is situated at the roof of nasal cavity. Like all epithelium nasal epithelium too has a pseudostratified, columnar organization and is composed of 3 cell types: a) olfactory (receptor) cells, b) supporting cells and c) basal cells. The receptor cells in olfactory region are lengthened bipolar neurons that have cell bodies situated at different depths within the epithelium, with one end in the nasal olfactory epithelium and other end extending through the holes in the cribiform plate of the ethmoid bone, terminating in the olfactory bulb (30, 31). The supporting cells are roofed with microvilli and expand from the mucosal surface of the neuroepithelium to the basal membrane. The basal cells are situated at the basal surface of the neuroepithelial layer and continually differentiate for forming new receptor cells (32). The nasal submucosal interstitial space and the olfactory perinueronal space has free communication, which appears to be continuous with a subarachnoid extension that surrounds the olfactory nerve as it penetrates the cribiform plate (33-35). The diameter of olfactory axons in humans estimated by electron microscopy is in range of 100-700 nm. Therefore theoretically transcellular transport of upto 700 nm is possible in humans via this route (36).

Compounds in the range of nanosize be capable of entering the olfactory neurons by receptor mediated uptake, bulk endocytosis or pinocytosis (37-39). After entering the olfactory neuron, the unmyelinated axon projecting through the cribriform plate and synapsing in the olfactory bulb permits direct transport of compounds from nose to the brain (40). Both indirect and direct projection through the olfactory bulb innervates seizurogenic regions in the temporal lobe, such as the amygdale, piriform and entorhinal cortices, and hippocampal formation (37, 41). Non-neuronal epithelial pathway, additionally, due to presence of supporting cells or Bowman's glands allow entry of compounds into the brain by way of pinocytosis, diffusion or paracellular transport through cell junctions (41, 42). Transport of drugs through non neuronal pathway appeared in brain within few minutes of nasal delivery (35). This transport is really interesting for polar drugs which are not able to cross BBB. For example, it was investigated that [^{3}H]-dopamine reached the olfactory lobe after nasal administration and 4 h after administration it attained a concentration 27 times higher than that of i.v. administration (43, 44). From literature it is clear that a specific and/or rapid targeting of drugs to the CNS would be feasible in many therapeutic situations, such as for the management of Parkinson's disease, Alzheimer's disease or pain. Parkinson's disease (PD) is the second most likely progressive neurodegenerative disorder with pervasiveness reaching 1-2 % in people more than the age of 50, irrespective of gender (45). There are reports of promising transfer of adequate amount to brain via olfactory nerve by use of morphine, zolmitriptan, sumatriptane, carbamazepin, nimodipine and clonazepam.

The incorporation of drugs into nanocarriers came out to be promising approach. These colloidal formulations not only protect drugs from degradation but also facilitate their transport through nasal cavity to brain. It is demonstrated clearly that the efficacy of intranasal administered nanostructured lipid carriers of valproic acid in rats was much higher (46). The results of this study showed that brain:plasma concentration ratio increased to about 20 times after intranasal administration of nanostructured carriers of valproic acid compared to i.p administration (46). To take advantage of these researches, attempt has to be at present taken for development of nasal delivery systems which are competent of raising the amount of drug that will reach the brain after nasal delivery.

1.4 Nanocarriers based intranasal brain targeting in neurodegenerative diseases

The strategy of applying drugs encapsulated into nanocarriers (polymeric and lipid based) to the olfactory epithelium could potentially improve direct CNS delivery and thus the efficacy of drugs. In therapeutics, nanocarriers may show significant change in both pharmacokinetic and pharmacodynamic levels. At the nanoscale level, often the materials demonstrate significantly different quantum mechanical properties leading to a fundamental change in their physical, chemical and biological properties as compared to their bulk forms. They can carry much more drug payload and are capable of controlling drug release. These nanocarriers can carry a range of drugs and their surface properties can be modified (47). These properties make nanocarriers an attractive and alternative for transporting drug across the BBB. In spite of a large variety of nanocarriers developed so far, it is noteworthy that only amphiphilic molecule-formed liposomes and polymeric nanoparticles have been extensively exploited for drug delivery to brain (48). In a recent study, the University of Regensburg in collaboration with Essex Pharma (Schering-Plough) Germany has completed a phase II clinical trial of pegylated liposomal doxorubicin and prolonged temozolomide in combination through i.v route with radiotherapy in newly diagnosed glioblastoma (49). Intranasal liposomes showed longer half-life in the brain when investigated in Alzheimer's disease. Rivstagmine liposomes were used for delivery into the brain through nasal route and results demonstrated significant higher level of drug in brain than intranasally or orally administered free drug. Sustained release of liposomes reduced the administration frequency and hence increases patient compliance (50). Liposomal drug delivery, due to its immense advantages, has gained considerable attention and has been used in variety of applications in research, particularly as carriers of therapeutic and diagnostic agents for brain targeting. The relationship among the lipid solubility of a drug and its CNS diffusion considered to represent a direct correlation. Thus designing a carrier system with lipid vesicles for CNS penetration retains a significant activity.

1.5 Liposomes

Liposomes have been considered from last many years as delivery systems for drugs (51). The advantages like good therapeutic efficacy, increased biological half life, enhanced tissue penetration, reduced side effects due to low dose, targeting to tumor tissues (doxorubicin), preventing drugs from enzymatic degradation make these carrier systems more useful in

delivering various drugs using different routes (52-54). Structurally liposomes are concrete bi-layered vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bi-layer mainly composed of natural or synthetic phospholipids. The main structural components of liposomes are Phospholipids and Cholesterol (55). The most common phospholipid used is phosphatidylcholine (PC). Phosphatidylcholine is an amphipathic molecule which exist a hydrophilic polar head group phosphocholine, a glycerol bridge and a pair of hydrophobic acyl hydrocarbons chains. Phospholipid molecules assemble themselves in planar bilayer sheets i.e. they orient themselves so that the fatty acid chains face each other, and the polar head face the aqueous phase. These bilayer sheets start separation in aqueous media and later on folds on themselves to form closed sealed vesicles called liposomes (55) (Fig.1.1).

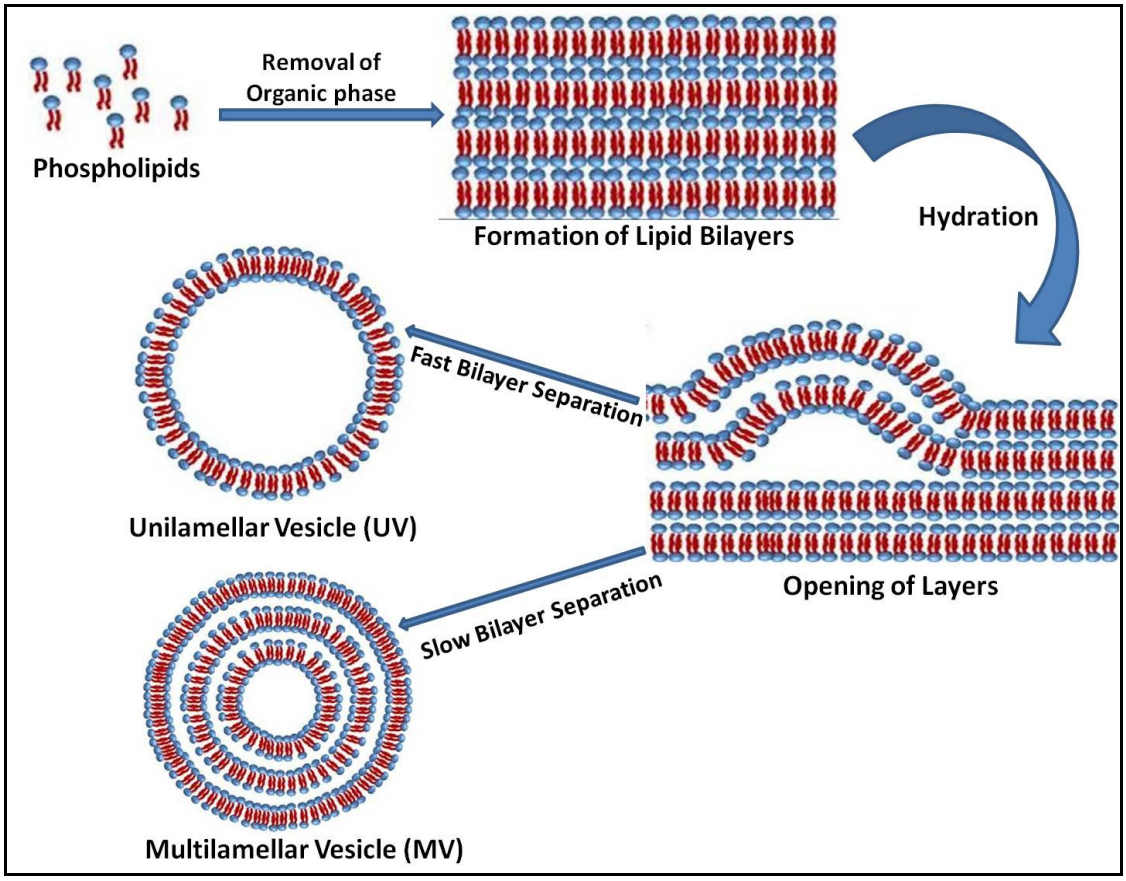

Fig 1.1 Formation of vesicles: Schematic display of phospholipids for vesicle formation

Cholesterol is used to improve the fluidity and stability of the lipid bilayer membrane. It improves the stability of liposomes by reducing the permeability of hydrophilic drugs when comes in contact with biological fluids. Instability of liposomes in plasma due to their interaction

with albumin, blood proteins, macroglobulin and m-transferrin was biggest challenge which has been overcome by addition of cholesterol. Cholesterol molecules adjust themselves between the phospholipid molecules by its hydroxyl group in front of the aqueous phase whereas the tricyclic ring stuck between the first few carbons of the fatty acyl chains, existing in the hydrocarbon nucleus of bilayer (56, 57). The unique ability of liposomes is that these carrier systems are able to encapsulate drugs both in a lipid and aqueous phase making it suitable for both lipophilic and hydrophilic drugs. Hydrophilic drugs generally entrapped inside the aqueous core of vesicles whereas lipophilic drugs get incorporated completely in lipid bilayers of liposomes (58).

More amount of non polar drug gets encapsulated in multiple lamellar (MLVs) liposomes and polar drugs in unilamellar (ULVs.) because of larger aqueous space in ULVs. Incorporation of charged lipids e.g., phosphatidylserine or osmotic swelling helps in increasing aqueous volume in vesicles (59, 60). The extent of drug entrapment markedly varies in MLVs due to leakage of hydrophilic drugs. As discussed above cholesterol improves the fluidity, thus influencing the extent of retention of drugs by liposomes by means of stabilizing these carrier systems from enzymatic degradation (59). Small drug molecules diffuse easily through lipid bilayers than large peptides and proteins which are comparatively better retained. On the other hand lipophilic drugs can have high entrapment efficiency (as 100 %, eg. camptothecin) (61). Also in aqueous biological environment retention of liposomes carrying lipophilic drugs is more because of high lipid-water partition coefficient.

1.6 Lipids used in liposomes preparation

Liposomes are generally prepared from pure lipids or using combination of lipids. The commonly used biocompatible & biodegradable lipids are phospholipids and sphingolipids for preparing liposomes. These lipids are both of natural and synthetic origin. Among all lipids phosphatidylcholines (PC) are used widely because of highest stability and capability to act when there is change in pH or salt concentration in biological environment.

Naturally derived phosphatidylcholines are soya phosphatidylcholine from soya lecithine and egg phosphatidylcholine from egg, whereas synthetic are dipalmitoyl-DL-α-phosphatidylcholine (DPPC), dimyristoyl-phosphatidylcholine (DMPC) and Dioleoyl-phosphatidylcholine. Other phospholipids are Phosphatidylethanolamine; Dioleoyl-phosphatidylethanolamine (DOPE) and Distearoyl-phosphatidylethanolamine (DSPE), Phosphatidylglycerol; Dimyristoyl-phosphatidylglycol (DMPG) and Dipalmitoyl-phosphatidylserine (DPPS), sphingomyeline,

phosphatidylinositol and ovolecithine. The basic lipid used for preparation of liposomes is a zwitterionic or non-ionic. The incorporation of charged lipids positively or negatively such as stearylamine or phosphatidyl glycerol, phosphatidyl serine, diacetylphosphate respectively modified the net surface charge of liposomes (55). The presence of positive or negative charge leads to larger overall volume for aqueous entrapment and also reduces probability of aggregation after preparation of liposomes (60). Cationic liposomes due to stearylamine have disadvantages of cytotoxic and cytolytic activities showed by in vivo toxicity studies in rabbits. This consequence was recognized to haemolysis of the erythrocytes and was straight related to the quantity of stearylamine used in the liposome composition. (62). The other additives used in liposomes preparation to modify its structure and properties are cholesterol and sphingomyelin, basically added to stabilize the liposomes. Stability here refers to retain entrapped drugs into liposomes vesicles. To reduce leakage and membrane fluidity a supplementary layer of lipid is formed using cholesterol (63). Further, lamellarity is also one of the important factors in deciding rate of leakage of drug molecule from vesicles. Multilamellar vesicles are less prone to leakage as compared to unilamellar vesicles (64). While using unsaturated phospholipids in liposomes preparation oxidation has to be avoided. α-tocophenol can be used as antioxidant in these cases, or it can be avoided by de-aeration, nitrogen or argon exposure.

1.7 Method of liposomes preparation

As abovementioned, liposomes are instinctively created as phospholipids goes under hydration. Additional some steps are essential to transform the size allocation and lamellarity of vesicles. Liposome preparation generally involves 3 major steps: a) vesicle formation, b) vesicle size reduction, c) purification. Several preparation methods have been established based on vesicles shape and size, encapsulation efficiency, the drug's physicochemical characteristics, and route of drug administration. Depending on method of preparation liposomes are classified as: Multilamellar vesicles (MLVs) ranging from 500-10,000 nm and consists of multiple lipid layers, maximum upto 14 layers each separated by an aqueous phase (onion skin arrangement) (65). Unilamellar liposomes further divided as small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs). The average diameter of SUVs is from 25-100 nm and structurally they contain only one lipid layer (66, 67). And LUVs has a diameter from 200-800 nm with single lipid layer. As ULVs hold a large central aqueous core, they are fit for entrapment of hydrophilic drugs. The liposomes of very large size range are giant liposomes falls in range of 10,000-10,00,000 nm.

These liposomes may be unilamellar or multilamellar. Based on mechanism of delivering drugs and composition, the liposomes are classified as conventional liposomes, polymorphic liposomes (pH-sensitive, thermo-sensitive, and cationic liposomes), decorated liposomes (surface-modified liposomes and immunoliposomes) and long-circulating liposomes. In hypothesis, drugs encapsulated in liposomal systems released through 3 possible mechanisms: a) passive diffusion, b) vesicle erosion and c) vesicle retention in the circulation. Near the beginning of 1970s so many drugs of different categories have been incorporated into the lipid or aqueous phase of liposomes of a range of sizes based on different method of preparation.

1.7.1 Multilamellar liposomes

i) Thin film hydration technique (TFH)

a) This method is used widely for preparing MLVs since was first described by Bangham et al., 1965 (68). In this process; the phospholipids are dissolved in an organic solvent (generally a chloroform/methanol mixture) and using rotary evaporation under reduced pressure deposited as a thin film on the wall of a round bottom flask (Fig.1.2). MLVs form automatically when an excess volume of aqueous buffer containing the hydrophilic drug is added to the dried lipid film. This hydration step is done at a temperature above Tc (gel-liquid crystalline transition temperature) of the lipid or above the Tc of the highest melting component in the lipid mixture. The drug to be entrapped in vesicles are added either to organic phase in which lipids are dissolved if drug is lipophilic or in the aqueous phase if drug is hydrophilic. The entrapment of drug into vesicles depends on the time allowed for hydration of the dried film and conditions of agitation. For illustration, it is reported that more of the aqueous phase can be sequestered when the lipid is hydrated for 20 h with gentle shaking, compared with a hydration period of 2 h, with vigorous shaking of the flask, even though size distribution of the MLVs was unaffected (69). This way hydration at slower rate is directly related with larger entrapment of drug. Unloaded drug from liposomes can be separated by various purification processes like centrifugation, chromatography, ultrafilteration by gel filtration or dialysis. These vesicles are simple to prepare using this method and varieties of drugs are reported to be entrapped in liposomes using this method. Low internal volume, low encapsulation efficiency and heterogeneous size distribution are drawbacks of this method (68).

b) Encapsulation efficiency in MLVs can be increased if hydration of lipids is done in presence of immiscible solvents like diethylether and petroleum ether. With this emulsion is formed using sonication or vigorous shaking. The formation of MLVs is immediately after addition of aqueous

phase after organic phase removal. The drawback of this method is direct exposure of the drugs to organic solvents (70, 71).

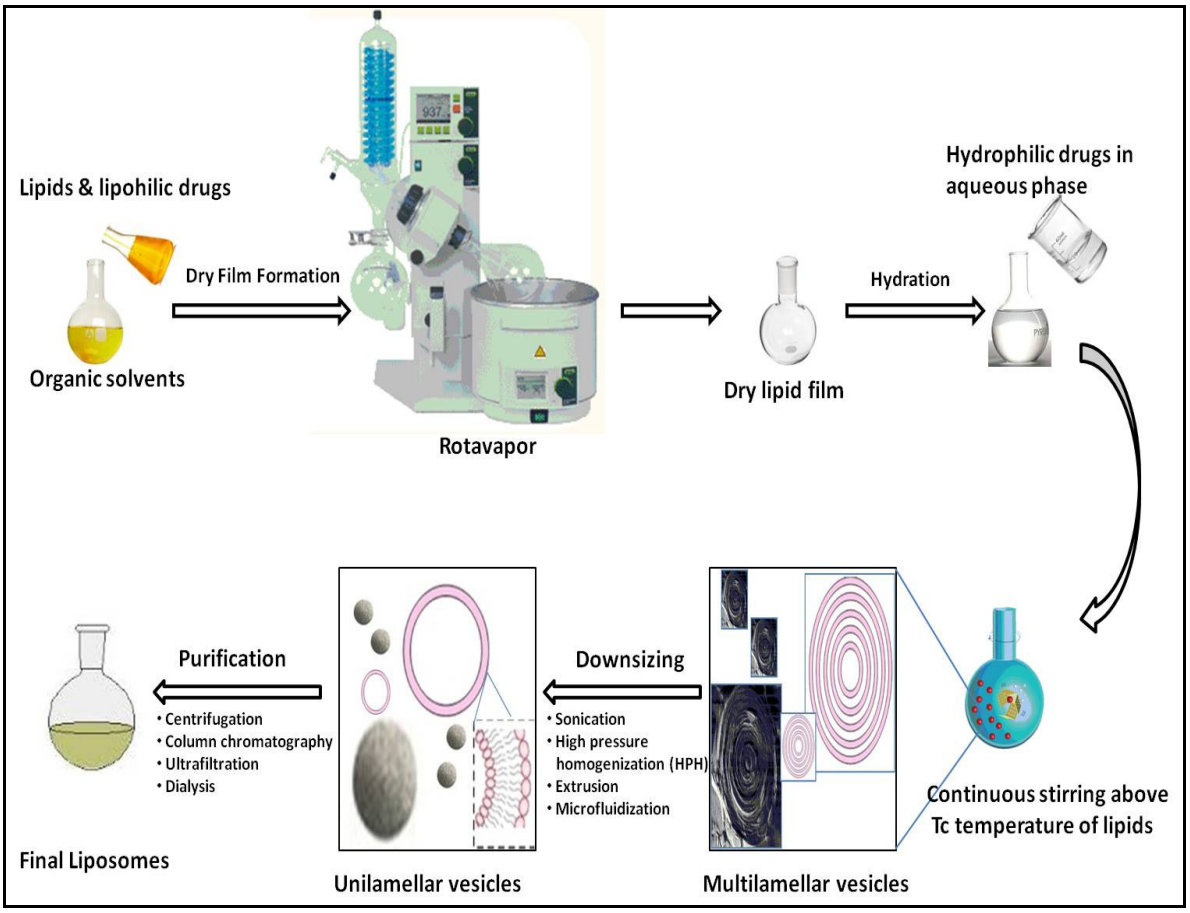

Fig.1.2 Schematic representation for liposome formation by thin film hydration technique

ii) Solvent spherule technique

Kim et al. in 1985 proposed this method for preparing MLVs of homogeneous size distribution. This technique involved dispersion of small spherules of lipids dissolved in volatile hydrophobic solvent in aqueous phase. At controlled evaporation of organic solvent in a water bath there is formation of MLVs (72).

1.7.2 Small unilamellar liposomes (SUVs)

i) Sonication method

This method involves sonication of MLVs prepared using above methods either by using probe sonication technique or bath sonication under inter atmosphere using argon or nitrogen. The theory of sonication involves the exercise of pulsed, high frequency sound waves (sonic energy) to stir up a suspension of the MLVs. This sonication produces SUVs with homogeneous

dispersion and much smaller diameter with a potential for greater tissue penetration. Comparatively, probe sonication results in smaller vesicles with fast breakdown of MLVs that can be achieved by a bath sonication. However, the reduction in size of liposomes decreases the amount of aqueous phase inside vesicle, thereby decreasing the entrapment of hydrophilic drugs. As compared to bath sonicator a probe tip sonicator delivers high sonic energy but has the shortcoming of overheating the lipid suspension which causes degradation. Also probe tip tends to liberate titanium particles into suspension, which has to be removed by centrifugation later. However, degradation of lipids and titanium particles shedding from probe tip could cause serious biohazards which are not encountered with bath sonication. Therefore comparatively, bath sonication is more commonly used. As bath sonication is a closed system so due to temperature control it minimizes thermo-degradation of the lipids and entrapped drugs. Using bath sonicator, sonication is done for 5-10 minutes above the transitional temperature of the lipid. The disadvantage of this technique is oxidation of unsaturated bonds in the fatty acid chains of phospholipids and hydrolysis to lysophospholipids and free fatty acids. As well as denaturation of thermolabile substances to be entrapped is another drawback of sonication technique (73).

ii) High pressure extrusion technique (HPE)

This method is another technique for converting MLVs to SUVs. In this method, suspension of MLVs (prepared by convectional method) are passed over and over again from one side to another side of filter polycarbonate membranes with small pore diameter of 0.8-1.0 μm under high pressure of 250 psi (74). Liposomes of desirable diameters can be produced by selecting filters of appropriate pore sizes (74-76). The mechanism behind HPE appears to be similar to an onion peeling. MLVs suspension is forced via the small pores, which results in peeling off consecutive layers in anticipation of single layer remains. Besides reducing the liposome size, the extrusion method produces liposomes of homogeneous size distributions (74, 77). Extrusion at low pressures $\leq$1 Mpa is possible when lipid concentration is low but the most commonly used pressures are about 5 Mpa (77, 78). A variety of lipids can be used for forming stable vesicles using this method (79). This method is agreeable to small scale production only. Schneider and co-workers in order to overcome this disadvantage designed continuous extrusion equipment, which operates at high pressures of up to 10.5 Mpa with the benefit of an elevated output (80).

iii) French pressure cell technique (FPC)

This method involves extrusion of MLVs through small orifice at 20,000 psi at 4 ^{o}C. This method is suitable for unstable drugs and simple, rapid and reproducible (81). The disadvantages of this

method are difficulty in achieving temperature and smaller working volumes (50 mL maximum) and larger SUVs as compared to sonication method.

1.7.3 Large unilamellar liposomes (LUVs)

These vesicles have highest internal volume resulting is highest encapsulation efficiency. Now days these liposomes are used widely for entrapment of various drug.

i) Solvent injection methods

a) Ether infusion method: Solution of lipids is dissolved in methanol/ether or diethyl ether and injected slowly to an aqueous phase of drug to be encapsulated at 55-65 ^{o}C at reduced pressure. The subsequent removal of organic solvent under vacuum leads to formation of liposomes. The limitations of this method are heterogeneous population of vesicles from 70-190 nm and direct exposure of drugs with organic solvents used, as well the high temperature (82, 83).

b) Ethanol injection method: In this method lipids is dissolved in ethanol and injected to vast excess of aqueous buffer. LUVs formation is immediate but its limitation is heterogeneous population (30-110 nm). Liposomes formed using this method is very dilute and removal of ethanol is very difficult as there is formation of azeotrope with water. This resulted in inactivation of biologically active macromolecules (84).

ii) Detergent removal and solubilization method

This method is for preparing LUVs and involves use of detergents as surfactant for solubilization of lipids (85). The detergents at their critical micelle concentration (CMC), solubilize the lipids. The surfactants (detergents) used are nonionic (e.g., n-octyl-bete-D-glucopyranose (octyl gluside)), anionic (e.g., dodecyl sulphate) and cationic (e.g., hexadecyltrimethyl ammoniumbromide) (59). At the removal of surfactant micelles become gradually more affluent in phospholipid and lastly unite to form LUVs. The surfactant is subsequently detached by column chromatography or dialysis (86-88). Other purification methods used are: gel chromatography using Sephadex G-25 column, binding to Amberlite XAD-2 beads and Bio-Beads SM-2 for detergent octyl glucoside and Triton X-100 respectively (89-91). The main limitation of this method is traces of detergent presence within vesicles. LIPOPREP (version of dialysis system) is a commercial available device for separation of detergents (Diachema AG, Switzerland). With the detergent removal method, homogeneous population LUVs of range 0.08-0.2 μm is produced.

iii) Reverse phase evaporation technique (REV)

This technique consists of two steps. First is rapid injection of aqueous buffer containing drug into an organic solvent containing dissolved lipids with immediate bath sonication of the mixture leading to the creation of a "water-in-oil" emulsion. Second step is removal of organic solvent in a rotary evaporator under vacuum which causes phospholipid covered water droplets to come jointly resulting into a semi solid gel matrix. Further removal of organic phase under reduced pressure with vigorous agitation converts solid gel matrix into smooth paste consistency. This induces a phase reversal from water-in-oil to oil-in-water dispersion (i.e., an aqueous suspension of the vesicles). During the agitation, a few water droplets collapse to form the external phase whereas the left over portion forms the entrapped aqueous volume. LUVs formed in this process are in range of 0.1-1 μm in size. High encapsulation efficiency (60-65 %) especially for hydrophilic drugs is the most important advantage of this method. This can be achieved if low ionic strength medium is used for liposomes preparation. This method has been used to encapsulate small, large and macromolecules such as RNA and various enzymes without loss of activity. The main limitation of this method is the exposure of the material to be encapsulated to organic solvents, brief sonication and mechanical agitation, which may lead to denaturation of some proteins or breakage of DNA strands. Reports of such limitations are however rare in the literatures. Also heterogeneous size distribution is achieved using this method. (92).

iv) Miscellaneous Methods

Other techniques reported to prepare LUVs are:

a) Calcium induced fusion method: Using this method LUVs can only be obtained using acidic phospholipids. This procedure is based on calcium addition to SUVs which induces fusion and results in formation of MLVs in spiral configuration. SUVs are converted to LUVs by addition of EDTA (93). These prepared liposomes are large unilamellar and falls in heterogeneous size range.

b) Microfluidization method: This method is also known as **Microemulsification/ Homogenization** first proposed by Mayhew et al. 1984 for large scale manufacturing. The reduction in liposomes size is achieved by recycling. Size range of 150-160 nm has been achieved with good aqueous volume approximately after repeating 25 cycles. Microfluidization involves fluid flow in channels having high velocities up to 10,000 psi and cross-sectional diameter in range of 5-500 μm (94).

In the previous decade pretty good modifications has been done in microfluidics technique for making large scale liposomes production continent and simple. One among these includes microhydrodynamic focusing (MHF) in which aqueous buffer flows from opposite side in closed rectangular channel whereas phospholipids dissolved in isopropylacohcol (IPA) flow in between the aqueous phase along the axis of the channel (Fig. 1.3). Diffusion of aqueous and organic phase counterally, results in regions of low alcohol fraction where phospholipids are enforced to self-assemble into bilayers which ultimately close into vesicles (liposomes). As the aqueous buffer to phospholipid solution flow rate ratio (FRR) was increased from 5:1 to 50:1, the mean radius of liposomes decreases asymptotically from 140 to 40 nm after which the liposome size remained largely unchanged (95).

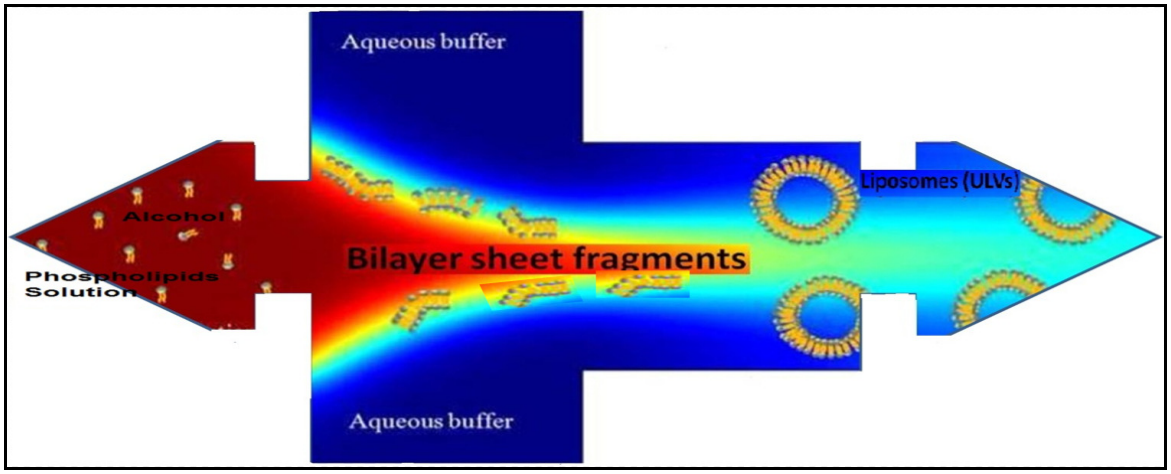

Fig.1.3 Representation of liposomes formation using Microhydrodynamic focusing method introduced by Jahn et al., 2004 (96).

c) Freeze and thaw method: Using this method SUVs are prepared by rapidly freezing followed by slow thawing. During the process of freezing and thawing SUV fusion takes place. The brief sonication has been done which disperses aggregated vesicles to LUVs (97-99).

1.7.3 Giant Liposomes

Earlier production of giant liposomes includes dialysis of phosphatidylcholine dissolved in methanol in presence of methylglucoside (detergent) with aqueous phase of 1M NaCl solution (100).

i) Microfluidic droplets for formation of giant vesicles.

Principally when two immiscible phases like water and oil are forced to flow in microchannel, small droplets of one phase of uniform size is generated under definite conditions (101, 102).

Sugiura and co-workers formulated a water-in-hexane emulsion using this method in which sorbitan monooleate and stearylamine act as emulsifiers (103, 104). The frozen water droplets were used to prevent coalescence and remove from the dispersed hexane phase by precipitation (104). The frozen ice particles were again added to phospholipid dissolved in hexane, which replaced the sorbitan monooleate at the droplet surface (water–hexane interface). Finally hexane was evaporated and water added to the system to obtain giant phospholipid vesicles. These have mean diameters in the range 4–20 μm which were similar to that of the water droplets (104). Recently, a novel microfluidic device was developed in which all the steps of generating water in oil-emulsion, subsequent removal of the oil phase and transfer of giant vesicles into an aqueous phase takes place simultaneously on the same device (105). A feasible drawback of double emulsion technique is presence of residual organic solvent in the final product and stability of liposomes (106).

ii) Pulsed jet flow microfluidics for giant vesicles.

This is another method for generating giant vesicles. It involves a pulsed jet flow of an aqueous solution from a micro-capillary at a phospholipid bilayer (107). The bilayer is generated by bringing two macro-sized aqueous drops together in a phospholipid containing oil phase (Fig. 1.4). The most important advantage of this method is the high encapsulation efficiency but on the other hand, automation is a challenge as the microcapillary positioning near the bilayer has to be controlled manually.

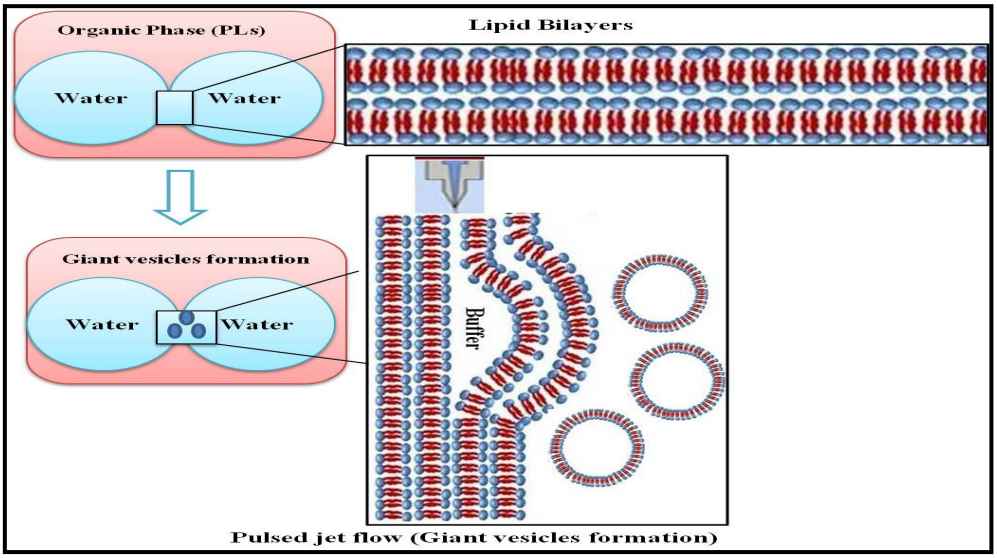

Fig.1.4 Giant vesicles formation using pulsed jet flow technique: Funakoshi et al., 2007 (106)

1.7.4 The pH gradient remote drug loading

The above discussed methods of liposomes preparation include mechanism of passive loading for drugs entrapment. This pH gradient method is used for loading drug into already formed liposomes called active loading. For implementing this method, drug should be fairly soluble and capable to get converted to uncharged species. As uncharged species can diffuse across the lipid membrane whereas charged ones are not competent of it. Drugs which fulfills these requirement are either amphipathic weak acids or bases e.g. daunorubicin, dopamine, doxorubicin, epirubicin, lodocaine, serotonin, vinblastine (108).

Also for remote loading there is prerequisite of driving force which may be generated by the trans-membrane gradient, which will "pump" drug from the external medium into the liposome and uphold there. Nichols and Deamer[a] were 1st to reveal this approach by loading amphipathic amines into liposomes via a pH gradient (109) (Fig 1.5). Barenholz's laboratory upgraded this technique in which pH and ion gradients were created by salts of either weak bases (e.g., ammonium) or weak acids (e.g., acetate). These ions can be present as charged and uncharged species (depending on pH) and can traverse the liposome membrane only in their uncharged form. Hence, the trans-membrane gradient of such ions acts as the driving force for remote loading as they can be exchanged with amphipathic drugs. Let's take an example from *Haran et al* of loading Dopamine using ammonium sulfate gradient (Fig. 1.5). The driving force of the gradient is related with a considerable difference in coefficients of permeability through the lipid bilayer (P) of different compounds and ions: $P_{(NH4)2SO4} < P_{SO4}^{2-}\ P_{NH}^{4+} << P_{H}^{+} <<< P_{NH}^{3+}$. An extremely high permeability coefficient for the ammonia molecule (P_{NH3}) results into a quick dispersal of neutral NH_3 molecules that are formed through the deprotonation of ammonium cations inside liposomes (Fig. 1.5, equation 2). Such rapid ammonia diffusion from liposomes along with the concentration gradient (the NH3 concentration outside liposomes is negligibly small in the initial moment) shifts the equilibrium of reaction 2 to the right; i.e., pH values inside liposomes are decreased. In their turn, Dopamine in neutral form is capable of penetrating through the bilayer. Though, Dopamine acquires positive charge in the acidic medium of the inner liposome and completely loses its penetration ability, since the permeability coefficients for their charged forms are substantially less than those for neutral ones (Fig. 1.5, equations 3–6). Therefore, the influx rate for dopamine would be higher than the efflux rate until concentration of its neutral forms inside and outside liposomes became equal (110).

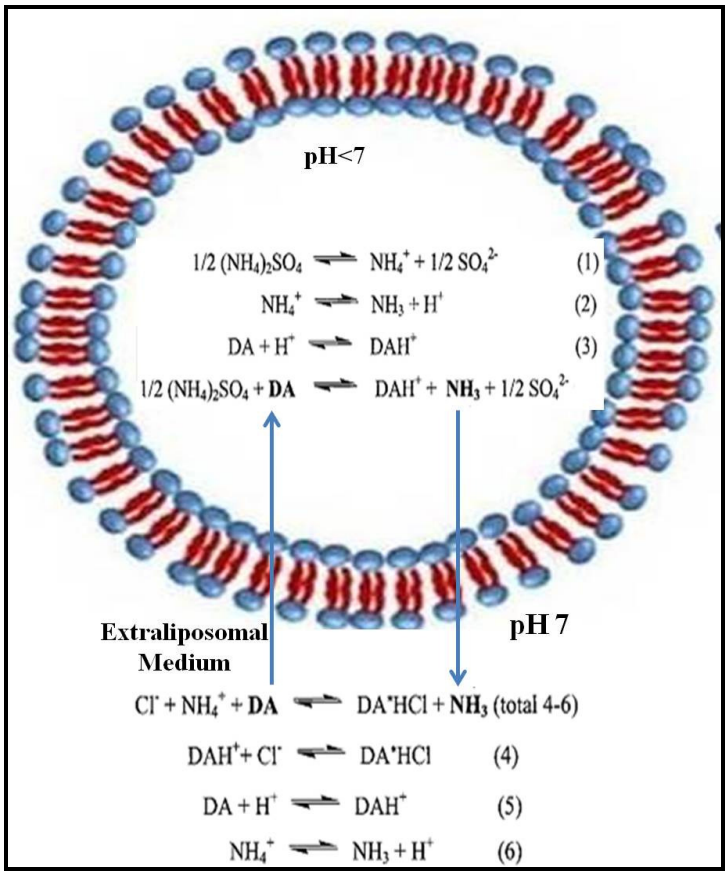

Fig.1.5 pH gradient remote loading of Dopamine

1.8 Critical steps during and after liposomes preparation

1.8.1 Protection of phospholipids from oxidation

Mostly used phospholipids are unsaturated and undergo oxidative degradation or lipid peroxidation. This oxidation takes place readily during and after liposomes preparation if lipids are not protected. Oxidation of lipids can be minimized by using antioxidants such as α-tocopherol, or by using inert atmosphere with nitrogen or argon, or by removal of heavy metals by addition of chelating agents (EDTA).

1.8.2 Size reduction

Size reduction technique is important to consider for decreasing size of liposomes to nanoscale. As high heat is generated during size reduction liposomes may undergoes disruption. Therefore, bath ultra sonication at lower temperature is preferred for liposomes size reduction as compared to ultra probe sonication.

1.8.2 Removal of trace organic solvent

Generally liposomes are composed of lipids, cholesterol and antioxiadnat. The organic solvents used in liposomes preparation for dissolution of various phospholipids are choloform, methanol, diethylether, methylene chloride and their combination. Although these solvents are evaporated from preparation but residual left over of solvents in final preparation may cause biohazards to users. So it should be kept below toxic level limits. While selecting any of these solvent its limits, solubility and purity should be kept in mind.

1.8.3 Lyophilization of liposomes

Lyophilization/freeze-drying is done to increase shelf-life of pharmaceuticals. It has been reported that liposomes containing drug molecules has been lyophilized and reconstituted with significant drug retention measured as % encapsulated drug, and without significant change in vesicles size. Cryoprotection sugars such as sucrose, mannitol, lactose and trehalose was needed during lyophilization to protect during freezing stage of lyophilization. On rehydration water molecules quickly replace sugar and liposomes reseal themselves before leakage occurs (111).

1.8.4 Removal of free-drug

In aspect of using liposomes for controlled release in dosage form, it is required to remove un-entrapped drug from trapped. Different approaches have been setup in laboratory to remove free drug from liposomes. This includes ion exchange chromatography, ultrafiltration, size-exlusion chromatography, dialysis and ultracentrifugation (112-114).

1.9 Liposomes characterization and its important

Even though the Federal Drug Administration (FDA) has not released any specific guidelines for Liposomal Drug Delivery System (LPDDS), the following characterizations are usual for liposomal formulations; liposome lamellarity, particle size and its distribution, surface morphology (surface charge/zeta potential) and surface properties (poly-dispersity index, PDI), drug entrapment efficiency (EE)/ loading efficiency (LE) and in-vitro drug release evaluation. Techniques used to determine these properties are liposome lamellarity determination using 31P NMR, size, distribution, mean size and PDI of the LPs are identified using laser light scattering or photon correlation spectroscopy. Particles morphology is examined by microscopy techniques, such as, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM). Photon correlation spectroscopy, X-ray photon correlation spectroscopy, Fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy

and electrophoretic mobility can be used to examine surface properties of LPs, such as, zeta potential (ZP) and surface hydrophobicity (115). The drug release from LPs is mainly governed by diffusion and biodegradation processes. The methods normally used to study the in-vitro release of LPs include the use of side-by-side diffusion cells with an artificial or biological membrane, the dialysis bag diffusion technique, the reverse dialysis sac technique, ultra-centrifugation, ultra-filtration and /or centrifugal ultra –filtration (116). Other commonly monitored parameter includes phase transitions through differential scanning calorimetry (117-119), and quantification of residual solvents through gas chromatography (120).

1.9.1 Lamellarity determination

As mentioned above liposomes (LPs) lamellarity varies widely based on different method of preparation and plays important role in encapsulation of drugs. If aqueous core is greater than lipid membranes (ULVs aqueous part >> MLVs aqueous part) chances of more hydrophilic drugs to get entrapped in ULVs than MLVs. Lamellarity is also one of the important factors in deciding rate of leakage of drug molecule from vesicles. Multilamellar vesicles are less prone to leakage as compared to unilamellar vesicles (64). Liposome lamellarity can be determined using 31P NMR. Briefly, in this method, on Mn2+ addition there occurs a quenching in 31P NMR signal from phospholipids on the exterior face of the liposomes. As Mn2+ interact with phospholipids due to negatively charged phosphate groups and results in broadening and reduction of the quantifiable signal (121, 122). The degree of lamellarity is estimated from the signal ratio before and after Mn2+ addition. This method has found to be quite sensitive because of Mn2+ and buffer concentration as well as types of liposomes under analysis (122, 123). Therefore other techniques used for lamellarity determination are electron microscopy (124, 125) small angle X-ray scattering (SAXS) (126-128) and methods based on the change in the visible or fluorescence signal of marker lipids upon addition of reagents (123).

1.9.2 Particle Size

Several variations on electron microscopy (EM) such as transmission EM using negative staining (127, 129), freeze-fracture TEM (130, 131) and cryo EM (132-134) gave best resolution in determination of size and shape of particles. Particle size plays a major role in determining the in-vivo fates of LPs. Discussion of particle size here is relevant to liposomes and intranasal delivery. The smallest particles, less than 10microns in size, can be carried by air currents more superiorly in the nasal cavity and impact on superior turbinate and possibly reach the olfactory region and nerve. The average diameter of olfactory axons reported in humans is from 100-700 nm (36).

Ideally particles in this range can be delivered to brain via nose. Owing to large size of liposomes, small ULVs 40-80 nm does not undergo significant transport and retention in brain (135-137). Although MLVs on the order of 0.3-2 μm in size retained by brain for longer duration due to embolization within the brain microvasculature (135).

1.9.3 Surface properties

Surface properties of LPDDS are critical in determining their drug delivery potential as these properties govern the overall in-vivo performance of the drug delivery system. These properties also modulate the in-vitro performance such as stability, drug entrapment efficiency and drug release kinetics. Polar head part of any lipid and pH is responsible for bearing a negative, positive or neutral charge on liposomes surface. Neutral liposomes have lower propensity to get cleared by reticuloendothelial system (RES) and highest propensity of aggregation (138). On the other hand negatively charged liposomes were observed to be endocytosed to a greater extent and at faster rate than neutral liposomes and lesser tendency of aggregation. Negative charge on surface is easily recognized by RES due to presence of receptors on it but can be overcome by including some glycolipids which inhibits uptake by RES and hence increase circulation time (139). While positively charged liposomes have a tendency to get interacted with serum proteins, high uptake by RES and clearance by lung, liver and spleen. Even high dose of cationic liposomes has shown tendency of tissue inflammation (140). Hence the specific surface area, surface charge and surface hydrophobicity are very important as these govern the physiochemical and electrostatic interactions with biological membranes and the overall biodistribution of drug loaded LPs.

1.9.4 Entrapment efficiency (EE) and loading efficiency (LE)

Ideally liposomes should have high EE as well as DC to reduce the volume or mass of delivery system required to be administered. Numerous different terms have been used to represent the drug efficiency of the preparation method. These include EE, LPs recovery, LE, process efficiency, loading capacity, association efficiency and drug incorporation efficiency. The entrapment of drug into the LPs can be determined directly or indirectly. In the indirect method the prepared LPs are recovered or separated by ultracentrifugation (100,000 g, 25 min) and the supernatant is analyzed for the free drug content. In the direct method the incorporated drug in LPs is determined by dissolving the LPs in a suitable solvent, followed by analysis of drug content (115).

1.10 Intranasal liposomes: Applications of direct drug delivery to brain

There are thousands of reports on numerous applications of Liposomal Drug Delivery System in CNS disorders. Nasal route via olfactory pathway and liposomes targeted drug delivery has separately explored in detail for brain targeting with exceptional findings. However, here application of intranasal liposomal delivery for brain targeting would be focused. Although these findings are very few in number but has shown a great potential to be explored. The liposomes are biodegradable and are nontoxic, nonimmunogenic, noncarcinogenic and nonthrombogenic so proposed remarkably for brain delivery (141). In addition a negative biological response that generally occurs when a foreign material is introduced in the brain has been elicited using liposomes.

Recently a study was reported by Yang et al on brain distribution and pharmacodynamics of rivastigmine for treating Alzheimer's disease by liposomes following intranasal administration. They formulated rivastigmine containing liposomes and cell penetrating peptide modified liposomes to improve rivastigmine distribution in brain followed by intranasal administration. Intranasal delivery of rivastigmine solution and liposomes demonstrated the capacity to improve rivastigmine delivery and adequate retention in CNS regions especially in hippocampus and cortex, which were the region's most affected by Alzheimer's disease, than that of i.v. administration. The data suggest that rivastigmine containing liposomes improve the brain delivery and enhance pharmacodynamics with respect to BBB penetration and nasal olfactory pathway into brain after intranasal administration, and also decrease the hepatic first pass metabolism and gastrointestinal adverse effects (142). K. Arumugam et al also studied rivastigmine liposomes for delivery into brain through nasal route. When AUCs of oral and nasal administration of free drug and after intranasal administration of liposomal formulation were compared, the intranasal liposome showed 5-fold higher AUC (36.13±1.87 μg min/mL) as compared to free drug (6.58±0.26 μg min/mL) given orally and almost 3-fold higher when compared with free drug administered intranasally (12.99±0.87 μg min/mL). Intranasal liposomes also showed longer half-life in brain than nasally or orally administered free drug (50). Intranasal administration of this drug in liposomal form significantly increased the exposure and resulted in its higher concentration in brain. Also sustained release effect of rivastigmine from liposomes may be useful for reducing administration frequency thereby increasing patient convenience.

In another study by Eskandari et al Valproic acid is delivered intranasal in nanostructured lipid carriers (NLCs) for treatment of seizers. In vivo data presented by them showed significant protective effects in rats after treatment by NLCs of valproic acid via intranasal administration. Results of valproic acid determination in brain and plasma showed that brain:plasma concentration ratio was much higher after intranasal administration of NLCs of valproic acid than i.p. administration suggesting benefits of nasal delivery (143).

HA Salama et al studied brain targeting of olanazapine via nasal route. In this study, olanzapine is encapsulated in new phospholipid based colloidal nanocubic vesicles for its brain targeting via the nasal route. The mean diameter of the vesicles was in the range of 363–645 nm. These vesicles showed efficient transport of drug to brain via nasal administration with good bioavailability when compared to other route of administration. The developed nanocubic vesicles enhanced the brain delivery of olanzapine via the noninvasive intranasal route with 37.9 % absolute bioavailability and 100 % brain targeting efficiency (144). Brain delivery of olanzapine by intranasal administration of transfersomal vesicles was also reported. This study investigated the presence of a probable direct correlation between vesicle elasticity and the amount of drug reaching the brain intranasally. Therefore, transfersomes were developed using phosphatidylcholine (PC) as the lipid matrix and sodium deoxycholate (SDC), Span® 60, Cremophor® EL, Brij® 58, and Brij® 72 as surfactants. The prepared transfersomes were mainly spherical in shape, with diameter ranging from 310 to 885 nm. Values for absolute drug bioavailability in rat plasma for transfersomes containing SDC and those containing Span 60 were 24.75 and 51.35 %, whereas AUC (0-360 min) values in rat brain were 22,334.6 and 36,486.3 ng/mL/min, respectively. The present study revealed that the deformability index is a parameter having a direct relation with the amount of the drug delivered to the brain by the nasal route (145).

Proteins necessary for brain function are also explored using intranasal liposomal technique by MM Migliore et al. Their study is to evaluate the effectiveness of cationic liposomes for intranasal administration of proteins to the brain. Cationic liposomes were loaded with a model protein, ovalbumin (OVAL), and were administered intranasally to rats. In qualitative studies, by 6 and 24 h after administration, Alexa 488-OVAL deposits were widely distributed throughout brain, with apparent cellular uptake in midbrain by 6 h after administration. In quantitative studies, liposomes were loaded with 111In-OVAL, and distribution to brain and peripheral tissues was monitored by gamma counting at 1, 4, 6, and 24 h after administration. The highest brain

concentrations were achieved at the shortest time point, 1 h, for both liposomal and aqueous OVAL. However, the liposomes yielded higher 111In-OVAL concentrations in brain than 111In-OVAL in aqueous. Moreover, a 2 mg/mL form of liposomal OVAL yielded a higher percentage of dose in brain, and a lower percentage in stomach and intestines, than twice the volume of a 1 mg/mL preparation. This research suggested that cationic liposomes may provide a novel, noninvasive strategy for delivery of neuroactive proteins to the brain for treatment of central nervous system disorders (146).

Galanthamine hydrobromide (GH) has been approved for symptomatic treatment of Alzheimer's disease (AD) and vascular dementia. Hence, the effects of intranasal administration of GH loaded flexible liposomes have been investigated for the first time on the efficiency of acetylcholinesterase inhibition, as well as the pharmacokinetic behavior of GH in rat brain. The inhibition of acetylcholinesterase was investigated using rat brain homogenates as an enzyme resource and microdialysis was used to determine the pharmacokinetic behavior of GH in rats brain. The rat pheochromocytoma PC-12 cell line was used in this study to evaluate the cytotoxicity of GH loaded flexible liposomes. The results revealed that: (i) the efficiency of acetylcholinesterase inhibition of GH was greatly enhanced by intranasal administration compared with oral administration, especially GH loaded in flexible liposomes; (ii) the C(max) and AUC (0→10) for intranasal administration of GH loaded flexible liposomes were 3.52 and 3.36 times higher than those of orally administered GH, moreover, the T(max) was greatly shortened from 1.5h for oral administration to 0.75 h for intranasal administration of GH loaded flexible liposomes; and (iii) PC- 12 cells viability tests showed that the flexible liposome carrier is not toxic to the cultured cells and the cytotoxicity of GH to cells was clearly decreased by loading in flexible liposomes. These results indicate that intranasal administration of GH loaded flexible liposomes may possibly transport GH into brain tissues, suggesting some promise for this approach in successful brain-drug targeting in AD treatment (147).

1.11 Objective of work

For more than 30 years, considerable efforts have been made to enhance delivery of therapeutic molecules across the vascular barriers of CNS. The current challenge is to develop drug delivery strategies that will allow the drug molecules to enter brain in a safe and effective manner. Treatment of PD is still very challenging, as to begin with it was treated by exogenous dopamine supply to restore dopaminergic transmission. However, DA which is known to be hallmark in

PD treatment was not used for therapy as it could not cross BBB. In addition, because of high first-pass metabolism after oral administration, the drug is usually only given by i.v. infusion at present to treat acute cardiovascular diseases. The levodopa, which is immediate precursor of dopamine, is administered orally to treat Parkinson. As levodopa can pass BBB easily and is converted to dopamine rapidly in brain. But almost 95 % of the oral levodopa is decarboxylated to dopamine peripherally and thus required to be administered with a peripheral dopa decarboxylase inhibitor (148, 149). Moreover the conventional delivery systems fail to deliver drugs effectively into brain due to distribution of more amount of drug into general circulation and different organs/tissues. This inefficient utilization of drug will require higher dose leading to high plasma drug concentrations that cause side effects, such as nausea, sleepiness and dysknesis resulting into only small proportions entering the brain (150). The intense search for alternative routes of drug delivery has been driven by the need to overcome the physiological barriers of the brain and to achieve high drug concentrations within the brain. Nasal delivery with novel systems appears to be a useful alternative for delivering dopamine directly to brain. An important consideration in delivering drug to brain via nasal route is to administer formulation to posterior region of nose (olfactory neuroepithelium) than to be simply delivering into respiratory epithelium. Human nasal applicators commercially exist that target the olfactory epithelium (151, 152). These new devices can advance the development of intranasal nanocarrier's drug delivery.

The aim of this work was to deliver Dopamine via nasal route to brain using liposomes as drug carriers and to suggest future prospective that may benefit progress in neurodegenerative disorders. Brain targeting by nasal route is not a new approach, but its combination with nanocarriers has developed renewed interest in this concept of delivery. In the present research, Dopamine loaded liposomes were prepared using biodegradable and biocompatible lipids Leciva S70, Lipova E120, 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), (1,2-Dimyristoyl-sn-glycero-3-phosphocholine) DMPC, Lipoid S 100 and Cholesterol. Prior to preparation and characterization, some preformulation studies of dopamine were performed to develop an efficient formulation. Dopamine loaded liposomes were prepared using thin film formation and reverse phase evaporation method coupled with freeze and thawing technique, the ideal methods to load hydrophilic drug in liposomes. The prepared liposomes were characterized for their particle size, size distribution, PDI and surface charge/zeta potential by photon correlation spectroscopy using zetasizer. The surface morphology of the prepared liposomes was examined using microscopy techniques, simple microscopy, TEM and AFM. The encapsulation efficiency,

loading efficiency and in-vitro dissolution studies were performed for the prepared LPs using standard procedures. Formulations of drug loaded liposomes are mainly influenced by method of preparation, amount of lipids/cholesterol, hydration media and amount of dopamine. The influences of all these parameters on characterization like size, polydispersity index, encapsulation efficiency, loading efficiency, zeta potential, % recovery of liposomes and in-vitro release were studied and analyzed in order to optimize the parameters for delivering dopamine effectively. The optimized dopamine loaded liposomes were subjected for stability study. The therapeutic efficacy and selective biodistribution of dopamine loaded liposomes were studied in rats by delivering through nasal route.

The proposed research work required simple and sensitive analytical and bioanalytical methods to estimate dopamine in different samples like, bulk powder, formulations, in-vitro dissolution samples, stability samples and biosamples. So, high performances liquid chromatography (HPLC) and UV (ultraviolet) - spectrophotometry methods were developed and validated for dopamine estimation.

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Chapter 2. Drug Profile

2.1 Introduction

Dopamine (DA) is one of the most important catecholamine neurotransmitters that is synthesized by decarboxylation of 3,4-dihydroxy-phenyl-alanine (DOPA). DOPA is derived from the amino acid tyrosine in the hypothalamus and is present in the central and the peripheral nervous systems (CNS and PNS) (1, 2). DA, a typical neurotransmitter, is known to activate five types of dopamine receptors -D_1, D_2, D_3, D_4, and D_5 with their variants (3). Most of CNS disorders are associated with dysfunctions of the DA system. Parkinson's disease (PD), a degenerative condition causing tremor and motor impairment, is most significantly related to the loss of DA-secreting neurons in the midbrain area, called the substantia nigra. PD first described by James Parkinson in 1817, is the second most common neurodegenerative disorder, affecting 1 in every 500 people, totaling approximately to about 6.3 million people worldwide (4). There is indication that schizophrenia also involves altered levels of dopamine activity, and the antipsychotic drugs that are commonly used to treat it have a key effect of attenuating DA activity. Attention deficit hyperactivity disorder (ADHD) and restless legs syndrome (RLS) are also believed to be associated with DA depletion (5, 6). Exterior to brain, in peripheral system, also DA functions quite a few as a local chemical messenger. Like it inhibits norepinephrine release in blood vessels acting as vasodilator, it also increases sodium excretion in kidney and in pancreas reduces insulin production (7-9).

Dopamine HCl is available as intravenous injection/infusion. Dopamine cannot be administered by the oral route because of its fast metabolism in the gastrointestinal tract. Following injection, DA is well distributed in body, but it does not cross blood brain barrier. Even though a trademark of Parkinson's disease, it cannot reach the brain from the bloodstream and thus cannot be used for this therapy. Its peripheral effects make it functional in the management of heart failure or shock, particularly in newborn babies, due to myocardial infarction, trauma, open-heart surgery, renal failure and chronic cardiac decompensation as in congestive failure (10-12). There is a need for administering DA through different route which bypasses blood brain barrier (e.g. nasal route via olfactory pathway). Thus, Dopamine is an ideal representative drug for any formulation scientist to work with for better CNS delivery and improved therapeutic efficacy.

2.2 Physicochemical properties

Dopamine HCl has higher solubility in water (≈ 600 mg/mL) (Table 2.1). The drug comes under BCS class III high solubility and poor permeability (log P, 0.9) (13).

Table 2.1 Physico-chemical properties of Dopamine HCl

S.NO	Parameters	Description
1	Generic name	Dopamine
2	IUPAC name	4-(2-aminoethyl)benzene-1,2-diol hydrochloride
3	CAS registry number	62-31-7
4	Empirical formula	$C_8H_{11}NO_2.HCl$
5	Molecular weight	189.65
6	Melting point	241°C
7	Appearances	White- off white crystalline powder
8	Log P	0.9 (experimental)
9	pKa	8.93 (experimental)
10	Water Solubility	Dopamine is highly hydrophilic drug which is freely soluble in water (600 mg/mL) (13).

2.3 Chemistry

Dopamine hydrochloride is chemically 3,4-dihydroxyphenylethylamine hydrochloride (Fig. 2.1) with a molecular weight of 189.65 g/mol (molecular weight of free base is 153.15). It is well known that DA, due to presence of catechol group, is sensitive to air and light and is readily oxidisable to a quinoid form (1, 14).

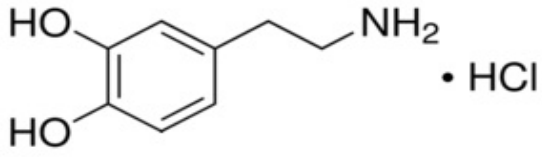

Fig. 2.1 Chemical structure of Dopamine hydrochloride

DA similar to the majority of amines is a natural base. At neutral/acidic pH levels DA is usually protonated. This protonated form of DA is comparatively stable. It is highly soluble in water and goes under oxidation if exposed to oxygen or other oxidants. On the other hand at basic pH levels, DA undergoes deprotonation. In this free base form it is least stable, highly reactive/easily oxidized and also less soluble (1). Therefore, DA is supplied for chemical and pharmaceutical use in the form of Dopamine HCl.

2.4 Mechanism of action

Dopamine is a neurotransmitter in the central nervous system and also a predecessor in noradrenergic nerves to norepinephrine. DA produces positive chronotropic and inotropic effects on the myocardium, resulting in increased heart rate and cardiac contractility. This is accomplished either directly by exerting an agonist action on β-adrenoceptors or indirectly by causing norepinephrine release from storage sites in sympathetic nerve endings (15).

In the brain, DA acts as an agonist to the five DA receptors. All are G protein coupled receptors and are grouped into two families: D_1-like receptors (D_1 and D_5) are excitatory which act by increasing cAMP (3',5'-cyclic adenosine monophosphate) formation, whereas D_2-like receptors (D_2, D_3 and D_4) are inhibitory which act by inhibiting adenylyl cyclase (3). The PD occurs due to DA deficiency in brain (basal ganglia region). The neural cells, which produce DA, start deteriorating in PD. When these neurons start to disappear, the rate of DA production decreases. It was made known that signals from the cortex are led through the basal ganglia, to the thalamus, which finally influences motor control centers in the brain. (16, 17). From the cortex there is a direct and an indirect signal pathway, which are maintained by circuits that utilize different neurotransmitters (GABA, glutamate, enkaphalin and substance P). Also the balance between these 2 pathways is maintained by dopamine release from the substantia nigra to the striatum. DA release inhibits the indirect pathway by stimulating D2 receptors, and excites the direct pathway by stimulating D1 receptor. The substantia nigra (black substance) had lost its color in Parkinson patients (Fig.2.1) due to drastically reduction in DA level. Because of DA depletion, both direct and indirect pathway gets misbalanced from the striatum, which causes the thalamus to be over-stimulated. As a result the frontal cortex is less activated which would account for most of the Parkinson's symptoms (18-20).

Dopamine deficiency and changes in homovanillic acid (HVA), the major metabolite of DA, due to progressive loss of dopaminergic neurons in different regions of human brain, have been also reported in PD (21). The cause of degeneration of nigrostriatal DA neurons in idiopathic PD is still not completely known. Recently a study reported that intra-neuronal DA is principally confined to synaptic vesicles, where it is cosseted from metabolic breakdown. In the cytoplasm, conversely, free DA may give rise to creation of cytotoxic free radicals. Usually, the concentration of cytoplasmic DA is held in reserve to a minimum by continuous pumping activity of the vesicular monoamine transporter (VMAT2). Defects in handling of cytosolic DA by VMAT2

increases level of DA-generated oxygen radicals ultimately resulting in degeneration of dopaminergic neurons (22).

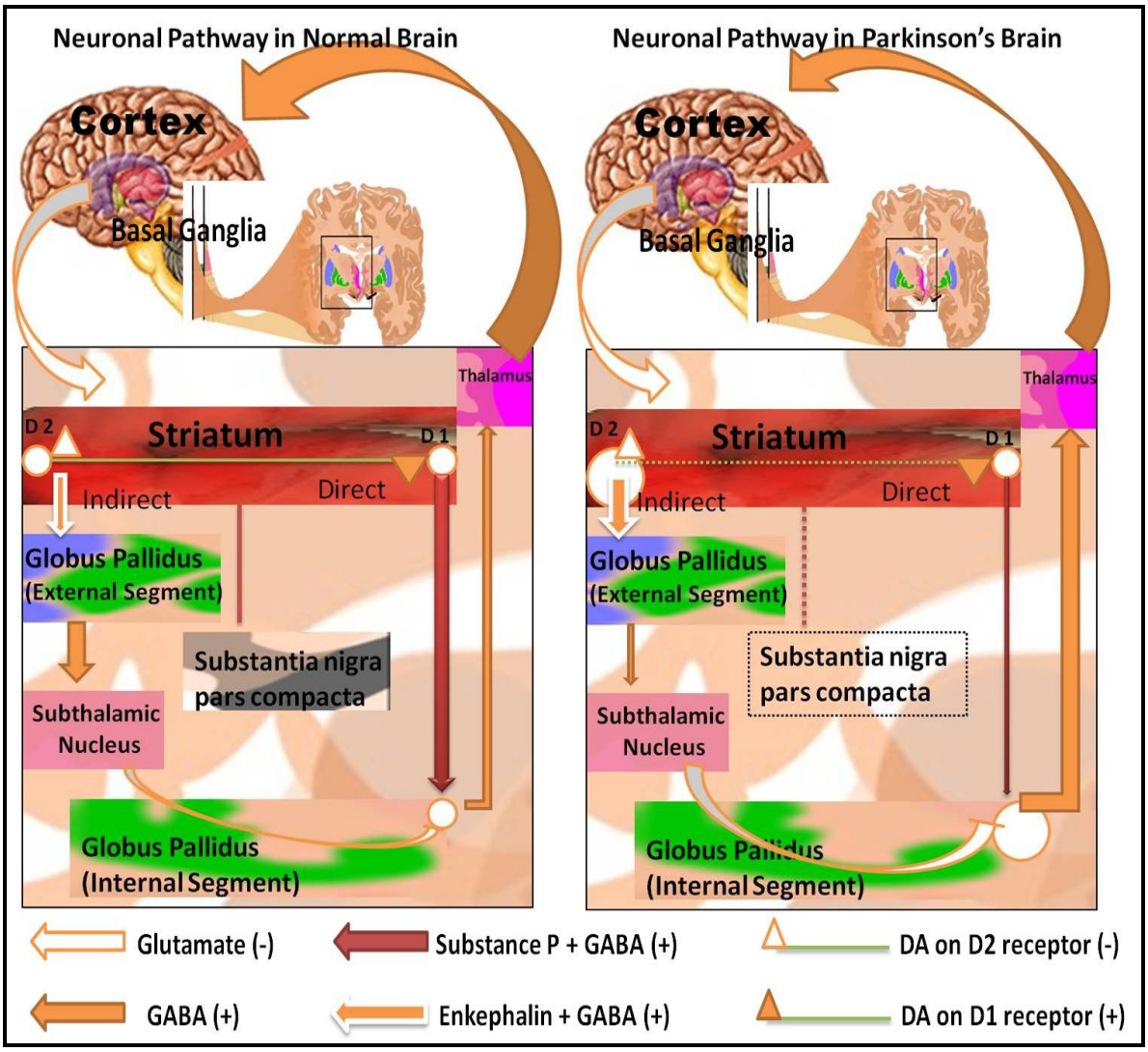

Fig. 2.2 Neuronal pathway in normal and Parkinson's brain.
***(The arrow thickness represents strength of the signal)

2.5 Pharmacokinetics and metabolism

Dopamine is used in treatment of renal failure/hypotensive conditions. Dopamine pharmacokinetics was studied after i.v. infusion. When 5 μg/kg.min was infused for 30 min, steady state levels were reached in 5 min. After termination of infusion DA elimination had a biphasic course with $t_{1/2}$ α = 1 min and $t_{1/2}$ β = 9 min, indicating very short half-life (23).

Pharmacokinetics of DA studied in infants and children

Plasma DA concentration in these subjects was measured at steady state. The rate of infusion was normalized at 5 μg/kg.min, the plasma concentration at this rate varied from 1.4 to 572.5 ng/mL. The half life of distribution and elimination determined were 1.8±1.1 and 26±14 min respectively. The apparent volume of distribution was 2952±2332 mL/kg and the rate of clearance was 454±900 mL/kg.min. The steady state DA concentrations varied significantly (24).

Pharmacokinetics of DA studied in healthy male subjects

In healthy subjects, constant i.v. infusion of DA was given at changeable doses (200, 400 and 800 μg/min) upto 90 min. It was seen that DA achieved a steady state level within 15-30 min after commencement of the infusion. The steady state levels averaged 36.5 μg/L at 200 μg/min, 73.8 μg/L at 400 μg/min and 207 μg/L at 800 μg/min. As the infusion stopped plasma DA concentrations declined very rapidly within the first 15 min, with an apparent half-life of elimination about 7 min. The corresponding total clearances were 5.81 min^{-1}, 5.51 min^{-1} and 3.91 min^{-1} at the doses of 200, 400 and 800 μg/min, respectively suggesting non-linear kinetics. Dopamine kinetics could not be described by a simple compartmental model (25).

Metabolism and elimination

Aforementioned after injection, DA is well distributed in body, but not able to cross BBB. Dopamine is absorbed rapidly from the small intestine also and it does not bind to plasma proteins. Biotransformation of DA takes place rapidly in liver, kidney and plasma to yield 3-4-dihydroxy-phenylacetic acid (DOPAC), 3-methoxy-4-hydroxy-phenylacetic acid and homovanillic acid (HVA) as major metabolites. Finally it has been reported that about 80 % of the drug is excreted in the urine within 24 hours, primarily as HVA and its sulfate and glucuronide conjugates and as DOPAC. A very small portion of DA is excreted unchanged in urine (21).

2.6 Dosage and treatment

Dopamine in cardiac indication

DA currently used as i.v. infusion for cardiac indication. In neonates and children continuous infusion is given at a rate of 1-20 μg/kg/min (maximum: 50 μg/kg/min) to achieve desired response. And in adults infusion at 1-5 μg/kg/min upto 20 μg/kg/min was given (13).

Dopamine in hemodynamic imbalance

Also the hemodynamic effect of DA is dose dependent. Low dose (1-3 μg/kg/min) increases renal flow and urine output. Intermediate dose (3-10 μg/kg/min) increases renal blood flow,

heart rate, cardiac contractility and cardiac output. High dose (> 10 μg/kg/min) α-adrenergic effects begin to predominate, vasoconstriction and increased blood pressure (26).

Dopamine in Parkinson's disease

Due to high polarity of DA, it is unable to cross BBB; thus for treatment of Parkinson's by DA its prodrug Levodopa is most widely used. Levodopa can pass through BBB relatively easily than DA. This treatment is not able to refurbish the dopamine cells that have been vanished, but it causes the remaining cells to produce more DA, in this manner compensating for the loss to at least a little degree. In advanced stages this treatment begins to be unsuccessful because the cell damage is so rigorous that the left over ones cannot produce enough DA in spite of Levodopa levels. At this stage of PD the DA regulation through metabolic mechanism become erratic resulting into DA dysregulation syndrome. In this syndrome patient fluctuate between state of paralysis and hyperactivity (27-29).

2.7 Drug-drug interactions

The tricyclic antidepressants amitriptyline, amoxapine, clomipramine, desipramine and doxepin, increases the sympathomimetic effect of DA significantly. Entacapone also increases the DA sympathomimetic effect and toxicity (30, 31). There is increase in arterial pressure if DA is given with reserpine, rasagiline, phenelzine, methyldopa, midodrine and isocarboxazid. Dopamine increased toxicity of lurasidone due to enhanced hyotensive effects. Administration of phenytoin to patients receiving DA leads to severe risk of hypotension and bradycardia. Cyclopropane/halogenated hydrocarbon anesthetics may increase cardiac autonomic irritability and sensitize the myocardium to the action of i.v. administered DA. This interaction is associated with pressor activity and to the β-adrenergic stimulating property of DA, and may result in ventricular arrhythmias. As DA is metabolized by monoamine oxidase (MAO), inhibition of this extends and potentiates the DA effect. Therefore those patients who have been treated with MAO inhibitors earlier (within 2-3 weeks) to DA administration should be given lesser doses of DA HCl (around 1/10 of the lesser dose). Simultaneous administration of low-dose DA and diuretic agents possibly produce an additive or potentiating effect on urine flow (13).

2.8 Delivery systems and problems

Dopamine is currently marketed as DA injections; Dopamine HCL 40 mg/mL, 80 mg/mL and 160 mg/mL (5 mL, single-dose vials) injection (American Laboratories, Inc. Shirley, NY, Boots

Phamaceuticals, UK and Sanofi-Aventis, India) under trade names Intropin, Dopastat or Revimine (13). Treating PD with DA is a clinical challenge, as DA is not able to enter brain due to BBB. Moreover, Levodopa/Carbidopa (Dopamine precursor) which is used as gold standard in PD has its aforementioned disadvantages. These issues create additional pressure for treatment of Parkinson's disease along with other CNS disorders. In drug discovery, the development cost of a new drug is very high (about $250-300 million) and time consuming (about 12-15 years) to reach the market, on the other hand an existing drug molecule can get a second life with novel drug delivery systems (NDDS) that can be developed in half the time and with only 20 % of cost of a new drug discovery. This made the formulation scientist to work for alternative NDDS and hence there is abundance of literature in the last few years in the development of novel formulations, liposomes, nanoparticles, micelles, microemulsion, cyclodextrin complex, prodrug exploring through different routes. One of the routes is nasal route which attained great success in administering these NDDS directly to brain bypassing BBB via olfactory pathway. The outcome of the growing demand increases interest and investment in the development of alternate formulation for treatment of PD. Hence results in biocompatible NDDS through noninvasive methods which provide patient's fulfillment.

2.9 Conclusion

Dopamine is the trademark for treatment of Parkinson's disease, but is ineffective due to its high polar nature and restriction by BBB. But DA is one of the most important drugs required in treatment of PD. Thus, there is a need for making novel delivery systems to deliver Dopamine efficiently into brain.

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Chapter 3. Analytical and Bio-analytical Methods

3.1 Introduction

In pursuing research and development of any drug delivery system, estimation of drug is an essential practice and for this, the formulation scientists need a suitable, precise and highly sensitive analytical method. As analysis is an important and integral component in the formulation process, it significantly influences the product development process. A simple, rapid, accurate, precise and stability indicating method will make the product development process faster with greater success. Therefore development of analytical method of any drug molecule is very much important for its characterization before starting designing any drug delivery system (1). Estimation of drug in variety of samples like bulk powders, drug loaded delivery systems, in the dissolution media and stability samples is everyday work in the development of novel delivery systems (NDDS) (1, 2). Additionally, estimation of drug in plasma and other biological samples is another important aspect of product development (3). Hence, a need for more simple and sensitive analytical method and procedure was felt to determine Dopamine (DA) in different matrix samples as there were no suitable methods available. Chromatographic analysis technique is very basic and sensitive method, explored extensively in the research and development of any drug delivery systems (4). In the present work, apart from UV-spectrophotometric method, liquid chromatographic (LC) analytical and bioanalytical method was developed and validated for the estimation of DA in bulk and other samples. The developed methods were successfully applied for the estimation of DA in liposomes, dissolution samples, stability samples and in different biomatrix.

3.2 Analytical methods for estimation of Dopamine HCl

Dopamine is a catecholamine and plays important role in brain functions. Among growing understanding of the participation of DA in regulation of brain functions and association of CNS disorders with dysregulated availability of DA, its determination has become important and been given remarkable attention by neuroscientists and analyst in bioanalytical research while its invention in 1950s (5). As well as changes in DA concentrations in biological samples gives indication of probable body abnormalities. Seeing that DA has shown tremendous therapeutic benefit in treatment of brain disorders; there is an increase in demand for formulation of NDDS for DA. Therefore, there have been regular attempts over the years to develop simplified, sensitive, and reliable techniques for extraction and quantification of DA present in different brain regions.

There are number of methods reported to estimate the endogenous concentrations of DA along with other neurotransmitters and its metabolites in discrete brain-regions. Methods which are reported in literature have been mainly developed for the determination of DA in biological samples but not for bulk, formulations or related samples. Methods reported are spectrophotometry (6, 7), spectrofluorimetry (8-12), capillary electrophoresis (13), liquid chromatography (14, 15), chemiluminescence (16) and electrochemical methods (17-19). The spectrophotometry and spectrofluorimetry methods lack specificity and sensitivity as compared to liquid chromatography. Capillary electrophoresis method need only a small amount of samples and their sensitivity is very low. The reported liquid chromatography methods have some drawbacks, including the requirement for sample pretreatment, poor chromatographic techniques, questionable uncharacterized peaks, long run time, complex mobile phase, long analysis time and high cost. These drawbacks have prevented these methods from being applied in routine analysis.

Also many chromatographic techniques are available for the measurement of multiple classes of neurotransmitters, but analysis of specific neurotransmitter DA generally involves separate tissue preparation protocols and separate analytical processing procedures (20-26). Already developed bioanalytical methods to determine DA along with other neurotransmitters in brain were based on, fluorescence (27), gas chromatography, mass-spectrometry (28) and radio-enzymatic-methods (29, 30). The earlier methods involving fluorometric detection of catecholamines needed larger volumes of body fluids or tissue weights, lacked specificity, and had limited sensitivity (31, 32). Although, the gas chromatography and mass spectrometry combined with mass fragmentography used earlier, were highly specific and sensitive, they were time consuming and required expensive instrumentation for derivatization and detection of these bioamines (33, 34). DA possesses high electrochemical activity and has been widely studied by electroanalytical techniques to significantly benefit biosciences (35). An approach of using HPLC-Electrochemical detector (ECD) for analysis of catecholamines first reported by Refshauge et al. (36) and later applied by Maruyama and Kusaka (37) and Hefti (38), is considered to be the most suitable for small body fluid volumes and has also been used for measurement of catecholamines in small brain tissue weight homogenates using methods which involved alumina adsorption (31, 36) or extraction with organic solvents, butanol/heptanes (39). Reversed-phase high-performance liquid chromatography with electrochemical detection is now an accepted technique for measuring plasma catecholamines (40-42). But it has also experienced great problem with its selectivity. The

main interference in a neural biological environment for electrochemical detection of DA is from ascorbic acid (AA), which is another electroactive species that coexists with DA in the CNS. It is very difficult to selectively detect DA in the presence of AA by electrochemical methods because the two species have nearly the same redox potential ranges and comparable sensitivities on the known plain electrodes (43). For DA normal levels for plasma and tissue catecholamines reported in the literature vary and appear to depend on the analytical method employed, (44) as it is highly conjugated to sulfates (t< 2min) (45). This has made cross-correlation and validation of data difficult. However, the maximum recovery of catecholamines by these methods was low and remained in the range of 50–60 % (36, 39, 46). These techniques require either deproteinization of plasma, complex column separation steps, or multiple solvent extractions, which often result in low sample recovery and are time-consuming procedures. In an effort to standardize these measurements we have developed a sensitive, selective and rapid method for determination of DA in biological samples.

Extensive literature survey did not reveal any simple, sensitive and validated analytical liquid chromatography method for the determination of DA in bulk and pharmaceutical preparations and their evaluation.

As necessitated, it was decided to develop ultraviolet spectrophotometric method for estimation of DA in the proposed stability samples and compatibility of DA with different formulation excipients. Liquid chromatographic method was developed for estimation of drug content and encapsulation efficiency in liposomes, in vitro release samples and drug content in stability samples. As well as present investigation has been made in quantification of DA in micro-volume biosample (plasma and brain tissue) using solid phase extraction technique (SEP-PAK Alumina B cartridges). The impact of the study can probably be attributed to the simplicity of the chromatographic system (mobile phase) with very quick sample preparation and the selectivity and flexibility of specific neurotransmitter DA measurement with higher recovery in micro volume plasma. Most important, however, is probably the fact that the validated method has been first time successfully applied for pharmacokinetic investigation of intravenous as well as nasal delivery to brain of DA in Wistar rats. The developed methods were validated as per the International Conference on Harmonization (ICH) as well as USP guidelines (47, 48). Suitable statistical tests were performed to validate the developed method (49, 50).

3.3 UV-Spectrophotometric method

3.3.1 Experimental

a) Materials and methods

Dopamine hydrochloride (assay 99.95 %) was obtained from Sigma-Aldrich, India. All other chemicals used were of analytical grade procured from S.D. Fine Chemicals Ltd, Mumbai, India. A commercially available i.v. infusion – Dopamine Hydrochloride U.S.P. (Sterile Specialities India Pvt. Ltd. India) labeled 200 mg/5mL Dopamine HCl was procured from the local Indian market.

b) Instruments

A double-beam Jasco (Japan) UV–Vis–NIR spectrophotometer, model V570 and for intermediate precision study a double-beam Jasco (Japan) UV–Vis spectrophotometer, model V630 was used. Both instruments have an automatic wavelength correction and a wavelength accuracy of 0.1 nm with 10 mm matched quartz cells. The instrument was connected to a computer loaded with Spectramanager software for computational purpose.

3.3.2 Method development and validation

a) Preparation of stock, calibration and quality control (QC) standards

Stock solution of 100 μg/mL of Dopamine HCl was prepared in 100 mM phosphate buffer (pH 6.8) by dissolving 10 mg of drug in 100 mL of media. The λ_{max} of DA was determined by scanning a suitable dilution of the stock using spectrophotometer. For preparation of different concentrations, aliquots of stock solutions were transferred into a series of 10 mL standard flasks and volumes were made with respective media. To get desired calibration curve, standards six different concentrations were prepared in the range of 15–90 μg/mL in the phosphate buffer. Dopamine was estimated at 280 nm in phosphate buffer. Least square regression analysis was carried out for the obtained data and calibration equation was developed. On three consecutive days of validation**,** three separate series of six calibration curve standards were prepared fresh and their absorbance was recorded in fixed wavelength mode at 280 nm keeping diluent as blank. QC samples were prepared at three concentrations, lower (LQC 20 μg/mL) medium (MQC 50 μg/mL) and higher (HQC 80 μg/mL) from the master stock solution like the calibrator sample.

b) Specificity and selectivity

Specificity and selectivity of the method was assessed by scanning a solution with drug concentration of 25 μg/mL from pure drug stock and commercial sample stock in selected media (N=5). In other selectivity study, DA solution was prepared in the selected media along with and without common excipients (mannitol, lactose, sucrose, cholesterol, propyl parabene, lipids, bile

salts, sodium metabisulfide and benzalkonium chloride) separately. All the solutions were scanned from 450 to 200 nm at a speed 400 nm/min and checked for change in the absorbance at respective wavelengths. The accuracy in terms of % recovery confirms the selectivity of the method. Paired t-test was performed at 95 % level of significances to the spiked sample and to the pure drug sample.

c) Precision

Precision of the developed method was tested with intra-day precision (repeatability) precision and intermediate/inter-day precision (reproducibility). Intra-day precision experiment was performed by analyzing six separate QC samples within a day on three different occasions, which gives the precision within the same concentration (N=6) in a day and between the same set of concentrations (N=18) in a day. Inter-day precision was performed with the QC samples between days for three days (N=18). In addition, UV-spectrophotometric method was validated for inter-instrument repeatability where six set of QC samples were analyzed using a different instrument with same specification. The precision of the developed method was represented by actual mean concentration and % RSD.

d) Accuracy

As a part of determining accuracy of the proposed method, different levels of drug concentrations (LQC, MQC and HQC) were prepared from independent stock solution and analyzed (N=6). Accuracy was assessed as the percentage relative error and mean percentage recovery. To give additional support to accuracy of the developed assay method, standard addition method was used. In this study, different concentrations of pure drug (10, 20 and 30 µg/mL in the phosphate buffer) were added to a known pre-analyzed formulation sample and the total concentration was determined using the proposed methods (N = 3). The accuracy results were represented by calculating mean absolute recovery and % bias, where % bias = [(Predicted conc – Nominal conc)/Nominal conc] x 100. The percent recovery of the added pure drug was calculated as, % Recovery = [(Cv – Cu)/Ca] × 100, where Cv is the total drug concentration measured after standard addition; Cu, drug concentration in the formulation; Ca, drug concentration added to formulation.

e) Sensitivity

The sensitivity of the developed UV-Spectrophotometric method was represented by limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were determined using the standard formula 3.3 σs^{-1} and 10.00 σs^{-1} respectively, where 's'- is the slope of the calibration

curve and 'σ'- is the standard deviation (SD) of y-intercept of regression equation or SD of intercept.

f) Robustness and stability study

Robustness of the developed UV-Spectrophotometric method was determined by making a small deliberate change to the optimized internal parameter. In this study pH of media was changed by ± 0.1 units. Three different concentrations (LQC, MQC and HQC) were prepared in media with changed pH. The robustness result was represented by mean % recovery and % RSD. The bench top stability of Dopamine HCl in the diluent medium was monitored using the validated UV-Spectrophotometric method. The QC samples were prepared fresh and their respective spectrum was recorded using the developed method. All the QC samples were recorded at a predetermined time point of 2 h, 6 h and 12 h. The overlay of the 12^{th} h spectrum, over the fresh QC sample spectrum demonstrates the stability of DA in the selected diluent. The stability of drug was represented by % recovery.

3.3.3 Application of developed method for analysis of commercial injection

The developed method was applied to determine the drug content of DA in the commercial available injections (200 mg/5 mL). The samples were processed in triplicate as per the sample processing procedure. Aliquot of dopamine injection was taken and suitably diluted with the media separately to get a 40 µg/mL concentration and the sample was analyzed by the developed method.

3.3.4 Results and Discussion for: UV Spectrophotometric Method

a) Selection of media

Different media (phosphate buffer pH 6.8, 7.0, 7.4 (DA undergoes oxidation above pH 7.0)) were investigated to develop a suitable UV-spectrophotometric method for the analysis of DA HCl in formulations. For selection of media the criteria employed were stability of the drug in media, ease of sample preparation, solubility of the drug, cost of solvents and applicability of method to various purposes (in vitro nasal dissolution studies). Finally 100 mM phosphate buffer pH 6.8 was selected on the basis of sensitivity, applicability and stability.

b) Linearity

Plot of absorbance vs concentration at λ_{max} 280 nm is presented in Fig. 3.1. The obtained regression equation was, absorbance = 0.0138 x concentration of DA (µg /mL) + 0.0195. The goodness of fit of regression equation in the selected concentration range, 15-90 µg/mL for DA was linear with significant regression coefficient $r^2 = 0.9999$. The absorption recorded for the

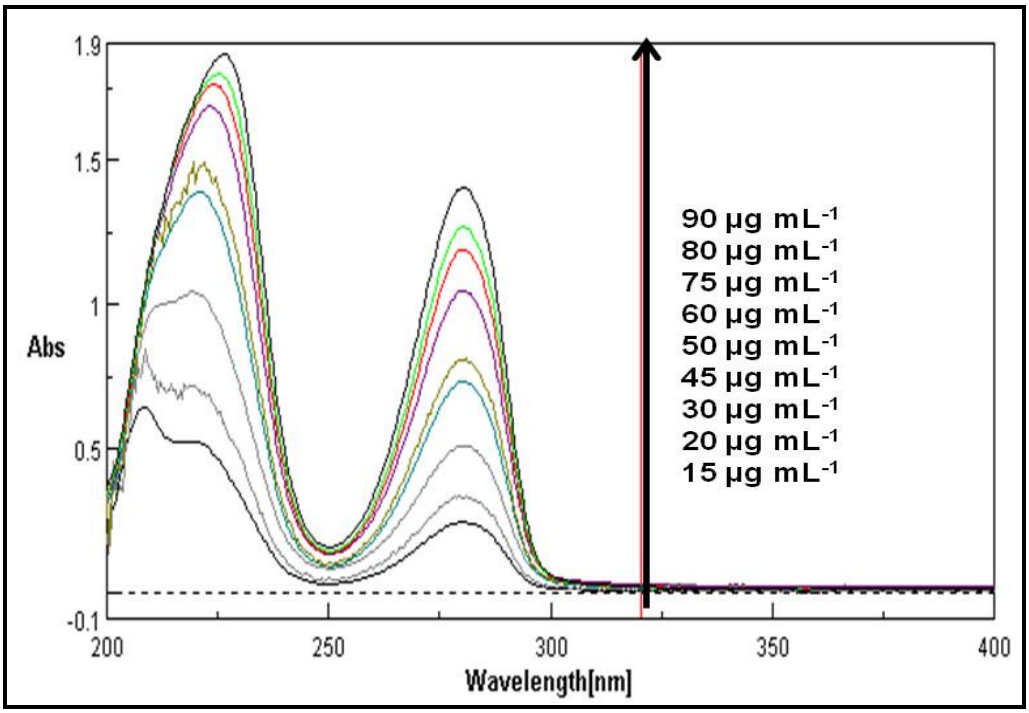

Fig. 3.1 UV-visible absorption spectra of calibration points and QC standards of Dopamine HCl

calibration curve concentrations 15-90 µg/mL (N=9) shows very low % RSD. The back calculated concentration of the obtained absorbance using regression equation illustrated low % bias (Table 3.1). The statistical analysis, confidence interval, standard deviation and standard error further confirmed the excellent linearity in the concentration range of 15-90 µg/mL of DA (Table 3.2).

Table 3.1 Calibration data of the developed UV-spectrophotometric method

Concentration (µg/mL)	Average abs ±SD (280 nm)[a]	% RSD[b]	Predicted conc[c] (µg/mL)	Mean % recovery	% Bias
15	0.223 ± 0.003	1.24	14.71	98.12	-1.88
30	0.483 ± 0.006	1.32	30.06	100.21	0.21
45	0.643 ± 0.004	0.65	45.16	100.35	0.35
60	0.854 ± 0.012	1.43	60.45	100.75	0.75
75	1.059 ± 0.010	1.01	75.34	100.46	0.46
90	1.256 ± 0.007	0.57	89.60	99.55	-0.45

[a] Average of nine separate determinations with standard deviation, [b] Percent relative standard deviation, [c] Predicted concentration is calculated from average absorbance.

Table 3.2 Summary of statistical data of optical characteristics and validation parameters of the developed spectroscopic method

Parameters	Observations
Calibration range (λ_{max})	15-90 μg/mL (280 nm)
Linearity (Regression coefficient)	$r^2 = 0.9999$
Regression equation	Absorbance (AU) = 0.0138×conc (μg/mL) + 0.0195
Confidence interval of slope [a] (S.E.)	0.01372 to 0.01391 (4.60×10^{-3})
Confidence interval of intercept (S.E.)	0.01408 to 0.02497 (6.50×10^{-3})
Standard deviation of intercept ($Ś_c$)	2.89×10^{-3}
Standard deviation of slope	7.61×10^{-5}
t-value for intercept [b] (tab = 2.78)	2.40 (P-value 0.5)
Calculated *F*-value [c] (critical *F*-value)	6.00×10^{-4} (4.45)
Standard error of estimate	8.73×10^{-3}
Limit of detection	0.69 μg/ mL
Limit of quantification	2.09 μg/ mL
Absolute recovery	99.95 – 101.52 %
Precision (% RSD)	Repeatability - 1.85 % (intra-day)
	Intermediate Precision - 1.45 % (inter-day)
System suitability (Optical characteristics)	Molar Absorptivity - 2.11×10^3 L M^{-1} cm^{-1}
	Sandell's sensitivity - 7.24×10^{-2} μg cm^{-2}
Specificity and Selectivity- t_{Cal} (t_{Crit}) [d]	0.161 (2.78)
Robustness (mean % recovery ± S.D.)	100.45±1.22

[a] Standard error of mean, [b] calculated at 0.05 level of significance, using the test of the intercept ($t_{df} = | C - \alpha | / Ś_c$), [c] Calculated based on one-way ANOVA test at P = 0.05 level of significance, [d] Calculated at P = 0.05 level of significance (d.f. 2) based on paired t-test.

The ANOVA results of the developed UV method showed that the calculated F-value, 6×10^{-4}, was found to be much lower than the critical F-value 4.45 at 95% level of significant which supports the best-fit linear equation. The test of the intercept was performed using the t-statistic at 95 % confidence interval and the t_{df} value of 2.40 showed that the intercept was not significantly (0.05 level) different from zero**,** thus**,** once again supports the best fit linear equation.

c) Selectivity

The developed method was found to be selective and it differentiates effectively DA from the other excipients substances in the commercial formulation samples. The method produced response only to DA at 280 nm where it is monitored and quantified in the formulation samples studied. The experimental result of selectivity showed that spectrum of DA spiked to commercial sample overlaid with the freshly prepared calibrator sample spectrum of the same concentrations respectively. The % recoveries of the analyzed samples were 99.88 ± 1.02 and 100.23 ± 0.45 respectively. These indicated that the excipients in the formulations do not interfere in the estimation of DA. The paired t-test result of the developed method showed that the calculated t-value 0.161 was less than the critical t–value 2.78, indicating that there is no significant (P=0.05) differences between mean absorbance of DA spiked injection and pure drug calibrator samples.

d) Precision

The repeatability study results (% RSD) of the developed UV method ranged from 0.77 to 1.85 respectively. The intra-day precision (% RSD) of the QC samples on three different occasions within a day was between 0.77 to 1.85. In case of inter-day precision the % RSD were in the range of 1.20 to 1.45 (Table 3.3). This study result showed that there was no significant variation in absorbance, which demonstrates that the entire calibration range, 15-90 μg/mL has very good repeatability and reproducibility. The repeatability of the QC samples set on each day of validation was less than 1.85 and the LOQ was always less than 1.56 μg/mL with least % RSD of 0.96 respectively. The concentration range of QC samples during inter-day precision ranged 19.60 to 20.20, 50.5 to 51.22 and 79.8 to 80.81 μg /mL respectively with % RSD ranged from 0.43 to 1.11. The inter-instrument repeatability (% RSD) of the method was in the range of 0.16 to 0.96.

e) Accuracy

Results of the accuracy study showed % RSD less than 1.12 with mean % recovery range from 99.95 to 101.52 and the % relative error range from -0.048 to 1.525 respectively (Table 3.4). The mean % recovery of standard addition method was in the range of 100.21 to 101.17 with % RSD less than 1.36 (Table 3.5). This indicated that the DA recovery was high at each time of analysis and the developed extraction procedure was efficient in extracting DA and the developed method was accurate in determining DA. This was supported by the low % bias range obtained. The important inference from this accuracy study was that there was no significant interference of the excipients at wavelength 280 nm used for estimation of drug.

Table 3.3 Results of intermediate precision study for the developed spectroscopic method

QC Levels µg/mL	Repeatability (Intra-day) (N=9)						Intermediate Precision (Inter-day) (N=27)		Inter-instrument repeatability % R.S.D (N = 3)
	Day (1)		Day (2)		Day (3)				
	Mean	% RSD	Mean	% RSD	Mean	% RSD	Mean	% RSD	% RSD
LQC (20)	0.291	1.56	0.293	0.96	0.293	1.39	0.292	1.33	0.66
MQC (50)	0.708	1.86	0.719	0.96	0.719	0.84	0.716	1.46	0.16
HQC (80)	1.115	1.64	1.124	1.01	1.12	0.77	1.12	1.21	0.96

Table 3.4 Accuracy and precision data for the developed spectroscopic method (each value is result of nine separate determinations)

QC Level (µg/mL)	Predicted conc. (µg/mL) [a]			Mean (%) recovery	Accuracy (%) [b]
	Range	Mean (± S.D)	% RSD		
LQC (20)	19.6-20.0	19.9±0.23	1.12	99.95	-0.048
MQC (50)	50.5-51.2	50.7±0.27	0.53	101.52	1.525
HQC (80)	79.8-80.8	80.2±0.35	0.43	100.33	0.338

[a] Predicted concentration of dopamine HCl. was calculated by linear regression equation, [b]Accuracy is given in % relative error (= 100 × [(predicted concentration – nominal concentration)/nominal concentration)].

f) Sensitivity

The LOD and LOQ of the developed method were found to be 0.69 and 2.09 µg/mL respectively. The developed calibration curve of the method has showed high magnitude of slope (0.0138 ± 0.00007), less SD of intercept (0.0195±0.000007). The developed UV method was found to be very sensitive**,** even a small change in concentration can be detected.

Table 3.5 Results of standard addition method in commercial injection using spectrophotometric method (each value is result of three separate determinations)

Concentrations of drug in formulations (µg/mL)	Concentration of pure drug added (µg/mL)	Total concentration of drug found (µg/mL)	Mean absolute recovery (%)	% RSD
40	10	50.11±0.63	100.21	1.25
40	20	60.54±0.38	100.90	0.63
40	30	70.82±0.95	101.17	1.36

g) Robustness and stability

A deliberate change in pH of the media in estimation of DA at 280 nm in UV-method showed that there was no significant change in absorbance at the selected variation of concentrations of solvent. The mean % recovery ranged from 98.78 to 100.45 with % RSD less than 1.23. The stability of DA in the selected diluent was monitored at 4°C by the method. The superimposed spectrum of the 12th h samples over the fresh sample spectrum (Fig. 3.2) of the selected QC samples indicated that DA is stable over the evaluated time in the selected media.

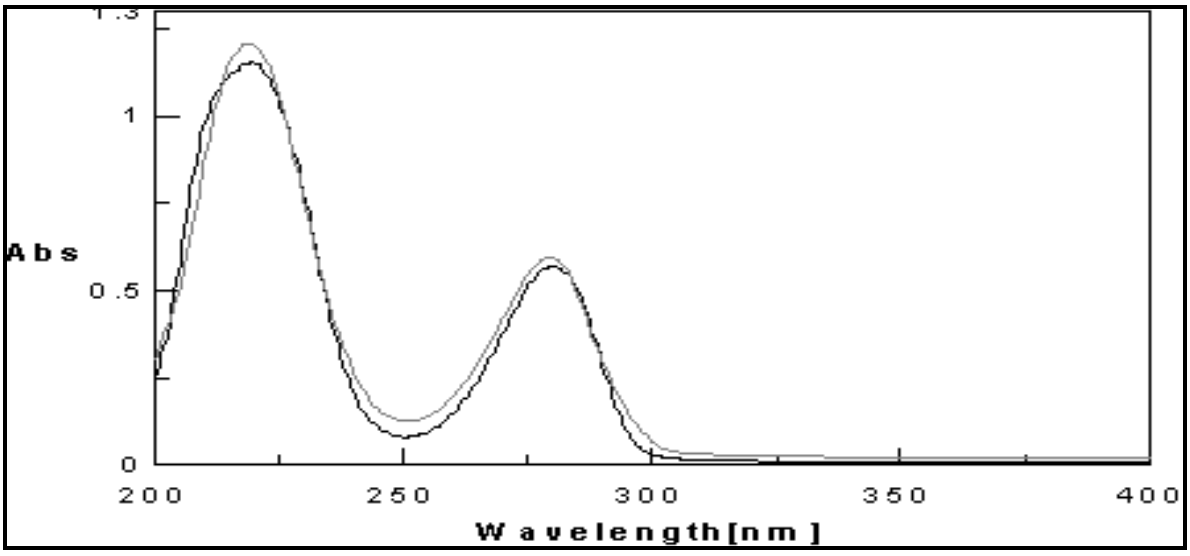

Fig. 3.2 Overlaid UV-absorption spectra of 12th h stability sample with freshly prepared sample for same concentration

3.3.5 Application of developed method-analysis of commercial injection

The results of the estimation of DA in commercial available injectable by the proposed method ranged from 101.39 to 104.83 of the claimed amount with maximum SD of 0.69. The recovery of

DA from the commercial was close to 100 %, which indicated the accuracy of the method in determining DA. It is also estimated that interference of excipient matrix is insignificant in estimation of DA by proposed method. The estimated drug content with low values of SD further established precision of the proposed method.

3.4 LC analytical method

3.4.1 Experimental

a) Materials and methods

Dopamine hydrochloride (assay 99.95 %) was obtained from Sigma-Aldrich, India. HPLC grade methanol was purchased from Spectrochem, India. Fresh triple distilled water (TDW) was prepared in quartz distillation assembly and filtered through a 0.22 μm membrane using Millipore ultra-filtration system (Millipore, France) before use. Potassium dihydrogen orthophosphate, orthophosphoric acid, citrate, ammonium acetate, perchloric acid, sodium metabisulphite, acetic acid, oxalic acid, L-cysteine, ascorbic acid and chloroform were purchased from Merck, Mumbai, India. All other chemicals and reagents were of analytical grade. For preparation of liposomes soya phosphatidyl choline (PC), Leciva S70, Lipova E120 and cholesterol were purchased from Avanti Polar Lipids, Inc. Alabaster, Alabama, Vav Life Sciences, Mumbai and Sisco Research Lab, Mumbai, India, respectively. Ammonium sulphate and sucrose were purchased from Khimmed (Russia) and Sephadex G-50 from Sigma-Aldrich. A commercially available i.v. infusion – Dopamine Hydrochloride U.S.P. (Sterile Specialities India Pvt. Ltd. India) labeled 200 mg/5 mL dopamine HCl was procured from the local Indian market. Liposomes loaded with DA and without it (blank) were prepared in-house using soya PC and cholesterol by thin film evaporation technique.

b) Instrument

An UFLC (Ultra Fast Liquid Chromatography) system (Shimadzu Prominence Japan) consisting of two pumps (LC-20AD), integrated system controller auto-sampler (SIL-20AC) and Prominence diode array detector (SPD-M20A) was used. The chromatographic separation of DA was carried out on BDS Hypersil C18 (250 mm x 4.6 mm, 5 μm, 80 Å) double end-capped RP-HPLC column (Thermo Scientific, Mumbai, India). Data acquisition and analysis were performed using 21 CFR part 11 compliant workstation LC solutions 1 (Shimadzu, Japan).

3.4.2 Method development and validation

a) Preparation of stock, calibration and quality control (QC) and formulation standards

A primary stock solution of DA was prepared in 0.1 M perchloric acid containing 0.1 % (w/v) sodium metabisulphite having concentration of 100 μg/mL. Working standards (1, 2, 4, 10, 20, 40, 60 and 80 μg/mL) were prepared from primary stock solution using phosphate buffer pH 4.0. Three separate series of eight calibration standards containing 25, 50, 100, 250, 500, 1000, 1500 and 2000 ng/mL of DA were prepared freshly by spiking 25 μL of respective working standard in diluent on three different days of validation. On each day of validation QC samples were prepared at 60, 800, 1600 ng/mL from independent stock and working standard same as calibration standards. Formulation standards were prepared by adding known amounts of drug to water for injection and to blank liposomes preparation at five levels—50, 75, 100, 125 and 150 % of the labeled claim. Similarly, placebo standards were prepared without adding any drug. Prepared standards were processed independently as described in their respective sample preparation section and analyzed. The quantitation was carried out at 227 nm with an injection volume of 50 μL for all analysis. Analysis was performed at ambient temperature (25°C) after baseline stabilization for at least 60 min.

b) Formulation sample preparation

For injections, a quantity of the product equivalent to 200 mg of DA was transferred to a 100 mL calibrated flask and volume was made up with 0.1M perchloric acid containing 0.1 % (w/v) sodium metabisulphite. Samples were vortexed for 5 min. Finally, 0.1 mL of sample was transferred to a 100 mL calibrated flask and diluted to volume with diluent.

For estimation of DA in liposome preparations, a quantity of the product equivalent to 40 mg of DA was transferred to a 100 mL calibrated flask and liposomes were digested with 10 mL of methanol by ultra-sonication (15 min, 25°C). The volume was made with 0.1M perchloric acid containing 0.1 % (w/v) sodium metabisulphite and the samples were centrifuged (10,000 rpm, 15 min, 20°C). Finally, 0.2 mL of the supernatant was transferred to a 100 mL calibrated flask and diluted to volume with diluent and analyzed.

c) Linearity and range

To set up linearity of the proposed method, nine separate series of calibration standards prepared at eight concentration levels ranging from 25-2,000 ng/mL and were analyzed. Average peak area at each concentration level was subjected to linear regression analysis with the least square method. Calibration equation obtained from regression analysis was used to calculate the corresponding predicted concentrations. One-way analysis of variance (ANOVA) was performed

on each replicate response obtained at eight concentration levels. Analytical range of the method was established by analysis of residuals and test of the intercept using t-statistic.

d) Specificity and Selectivity

The selectivity of the developed HPLC method was determined by placebo and spiked-placebo analysis technique. In this method, on three consecutive days chromatograms of the placebo and formulations standards were prepared and analyzed. Each standard (commercial injection and in house prepared soya liposomes) were prepared in triplicate. The samples were processed for analysis by the procedure given in the sample preparation. Obtained chromatograms were compared with fresh calibration standards. Paired t-test at 5 % level of significance was performed to compare the means of peak areas.

e) Precision

Precision was determined through repeatability (intra-day) and intermediate (inter-day) precision. Study was conducted using quality control (QC) standards prepared at lower (LQC =60 ng/mL), medium (MQC =800 ng/mL) and higher (HQC =1,600 ng/mL) concentration levels. Precision of the method was expressed as percent relative standard deviation (% RSD) of assay results. For repeatability (intra-day), six series of three QC standards were prepared freshly and analyzed. Similarly, standards were prepared and analyzed on three consecutive days for intermediate precision (inter-day) (N=27). The intra-day and inter-day assay % RSD obtained at three QC levels were used to assess repeatability and intermediate precision, respectively.

f) Accuracy

For determination of accuracy, recovery study was carried out using two different techniques viz. placebo spiking method and standard addition method for injections and liposomes, separately. In the placebo spiking method, a known amount of standard solution was added to placebo blank at five concentration levels 50, 75, 100, 125 and 150 % of the labeled claim. In the standard addition technique, a known amount of pure drug was added in sample solution at 50 and 100 % concentration level of the labeled claim of previously analyzed injection and liposome preparations. Each concentration level was processed in six replicates on three different days and the results are expressed as mean absolute recovery, % RSD and % Bias.

g) Sensitivity

The sensitivity of the developed LC method was represented by LOD and LOQ. The LOD and LOQ of the developed method were obtained by slope calculation and by signal-to-noise ratio. The LOD and LOQ were determined using the standard formula 3.3 σs^{-1} and 10.00 σs^{-1}

respectively, where 's' is the slope of the calibration curve and 'σ-' is the SD of y-intercept of regression equation or SD of intercept. In signal-to-noise ratio method, the LOQ determination was performed on five different days (n=5) by spiking an aliquot from working standard to diluent to get the desired concentration of the lowest calibrator. The results with precision, RSD less than 20 %, accuracy of 80-120 % and signal-to-noise ratio of 10:1 confirm the LOQ of the developed method.

h) Robustness

A robustness study was conducted during the final phase of method development by using design of experimentation (DOE) technique (Stat-Ease DOE). Robustness of the developed method was checked by deliberately making small changes to the optimized method parameters. The experimentation design technique was used to identify critical chromatographic factors and their effect on method performance (49, 50). Critical parameters are identified which may influence operating procedure such as percent organic component, buffer strength and pH. The critical factors were investigated in a range that covers the variation due to intra- and inter-laboratory conditions. A three-factor face centered design consisting of eighteen experiments was carried out. Three selected factors were: percentage of methanol (MET: X_1, 3–5 %, v/v), buffer strength in aqueous component (phosphate buffer: X_2, 5–15 mM) and pH of aqueous phase (pH: X_3, 3.5–4.5), adjusted with orthophosphoric acid. For each experiment, triplicate injections of DA (800 ng/mL) were made after baseline stabilization. Chromatographic parameters such as retention time, peak area and peak height along with system suitability parameters (k, T_f, N and HETP) were recorded as experimental responses. For statistical analysis, one-way ANOVA and lack of fit analysis were carried out for model fitting and validation.

i) System suitability and drug stability

The repeatability or routine analysis of the developed method within the validated limits was checked by system suitability parameters. The parameters checked are capacity factor (k), tailing factor (T), number of theoretical plates, height equivalent to theoretical plates (HETP), effective plate number (Neff), height equivalent to one effective plate (HEFF) and reduced plate height (h). All the above parameters were calculated using the standard formula (1). Further, the stability of dopamine was assessed at varying conditions that are expected under routine use of the method.

3.4.3 Application of developed method

As part of the validation procedure, the developed method was tested for drug content analysis of real world samples such as marketed injection and in-house prepared DA loaded liposomal preparations. For commercial injection, quantity equivalent to 200 mg of DA was processed as described in sample processing section. Finally, 50 μL of resulting solution was injected in triplicate and analyzed by the method. For in-house prepared liposome preparations, a quantity of liposome preparation equivalent to 40 mg of DA was processed as described in sample processing section. Finally, 50 μL of resulting solution was injected in triplicate and analyzed by the method.

3.4.4 Results and Discussion for: LC Analytical Method

a) Selection of media

In preliminary studies, peak properties and response function were optimized by changing organic to aqueous solvents ratio, buffer type, buffer strength and pH. In order to develop a selective and sensitive method, primary concern during development was to achieve high height to area ratio and peak symmetry. Different buffers of different pH, such as phosphate buffer (pH 3–7, 10 mM), citrate buffer (pH 3–5, 10 mM), ammonium acetate buffer (pH 3–5, 10 mM) and acetate buffer (pH 3–5, 10 mM) were studied in combinations with methanol (3, 4, 5 and 10 %, v/v). The effects of various organic modifiers such as acetonitrile, methanol and their combinations, on peak properties (peak height and symmetry) and response function were investigated. Considering chromatographic parameters, use of potassium dihydrogen phosphate buffer (pH 4.0; 10 mM) in combination with methanol (96:4, v/v) produced a symmetric peak (T_f ≈1.11) with sensitive and reproducible results. Various antioxidants such as perchloric acid, acetic acid, oxalic acid, L-cysteine, ascorbic acid and sodium metabisulphite, were used to enhance drug stability. Decrease in pH led to peak asymmetry (T_f ≈1.85), whereas pH above 6.0 showed poor peak properties and loss in selectivity with DA oxidation at higher pH. As the catechol group in DA is readily oxidized, to prevent oxidation of DA, stock has been prepared with optimized antioxidant (0.1 M perchloric acid containing 0.1 % w/v sodium metabisulphite). A double end-capped C18 column was used for better peak symmetry and enhanced stability at selected pH. Moreover, wavelength was optimized at 227 nm for better sensitivity and selectivity. Thus, the optimized mobile phase consisting of aqueous phase (10 mM potassium dihydrogen phosphate buffer, pH adjusted to 4.0 using 0.1 M orthophosphoric acid) and methanol (96:4, v/v) was found to provide quick retention time (Rt =6.0±0.25 min) with better peak properties, selectivity and reproducibility. Mobile phase was delivered in isocratic elution mode at a flow rate of 1

mL/min. The peak profile of DA is presnted in Figure 3.3A. Moreover this method utilizes green chemistry approach in mobile phase optimization with less than 5 % organic phase and only 10 min to run each sample. Thus our method is environmentally friendly. In literature there are reports using very complicated and time consuming extraction procedure to estimate DA only in the in-vivo samples (14, 15). The developed method did not require any complicated extraction procedure to estimate in vitro DA samples and applied successfully in estimating drug content in injections and liposome samples. In the present method, there were no interferences of excipients (lipids and stabilizers) in the estimation of DA as clearly shown by the standard addition and placebo spiking method and their respective chromatograms (Fig. 3.3 B, C).

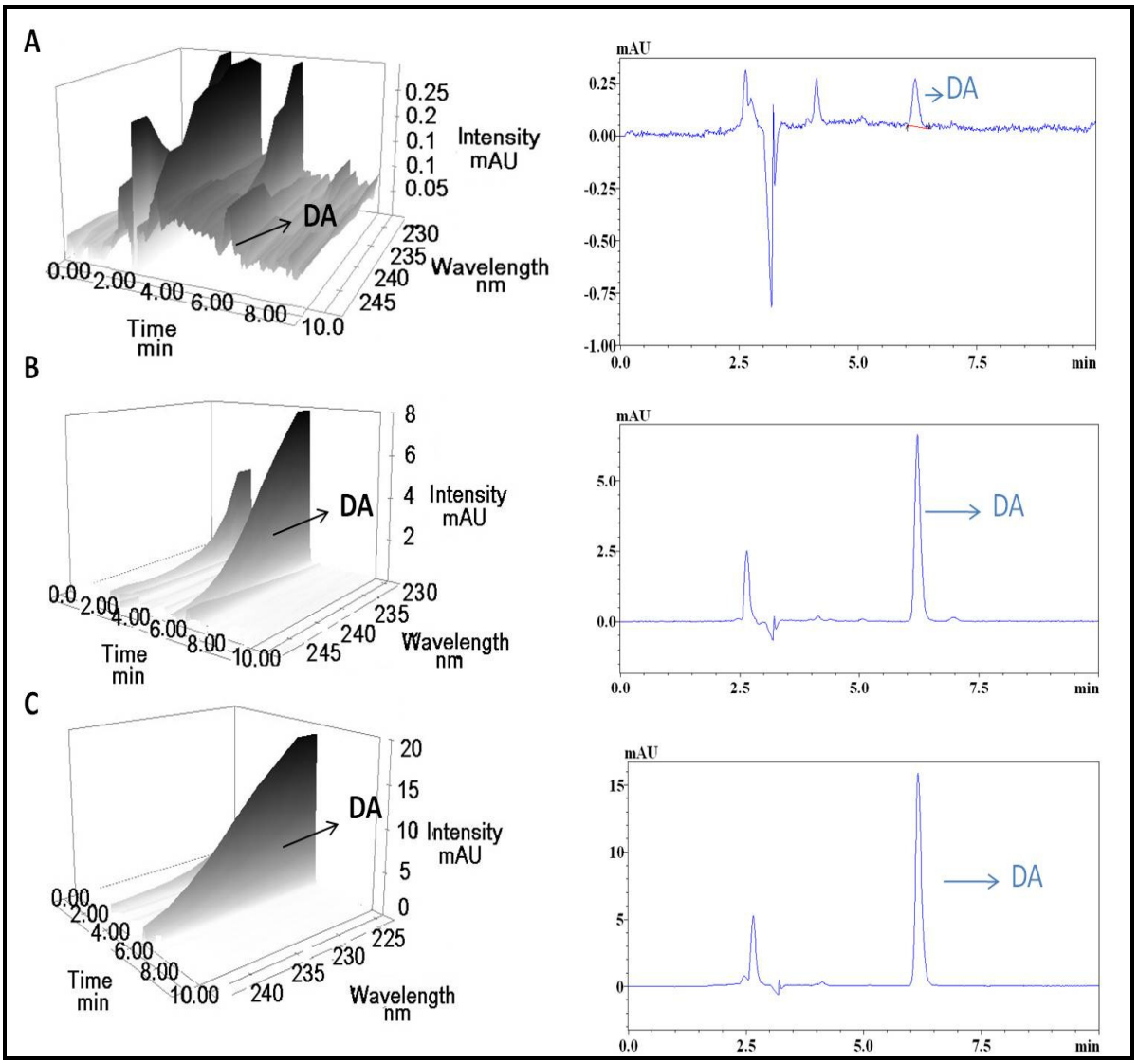

Fig. 3.3 Representative 3D (time, wavelength and intensity) chromatograms of DA (left) and normal view [time and intensity (right)] at 227nm: A) standard at quantitation limit; B) formulation standard in house prepared liposomes; C) test sample commercial injections

b) Linearity and range

The best-fit linear equation obtained was, average peak area (mV s) =72.091 x concentration (ng/mL) + 141.77 for the concentration range 25-2,000 ng/mL. Calibration curve obtained by the least square regression analysis between average peak area and the concentration showed linear relationship with a regression coefficient of 0.9997. At all concentration levels, standard deviation of peak area was significantly low and % RSD was below 2.08 %. This low % RSD supports the selected linear model with univariant regression and it also shows the goodness of fit (Table 3.6).

Table 3.6 Calibration curve data of DA by LC method

Concentration (ng/mL)	Average absorbs ± SD (227 nm [a])	% RSD	Predicted conc [b] (ng/mL)	% Bias
25	2006.90 ± 39.13	1.95	25.87	0.034
50	3920.44 ± 75.16	1.92	52.41	0.048
100	7515.00 ± 110.93	1.47	102.27	0.023
250	18959.00 ± 394.60	2.08	261.02	0.044
500	35992.40 ± 523.55	1.45	497.29	-0.005
1000	70912.00 ± 797.96	1.12	981.67	-0.018
1500	107173.67 ± 843.36	0.78	1484.68	-0.010
2000	1457493.83 ± 1126.36	0.77	2019.77	0.009

[a] Average of nine separate determinations with standard deviation, [b] Predicted concentration is calculated from average peak area, using regression equation

The statistical results of one way ANOVA illustrate that the calculated F-value (1.783×10^{-4}) was found to be lower than the critical F-value of 2.18 at 95 % level of significant which supports the best-fit linear equation. The test of the intercept was performed using the t-statistic at 95 % confidence interval the t_{df} value of 0.28 indicated that the intercept was not significantly (0.05 level) different from zero**,** thus**,** once again supports the obtained best-fit linear regression equations (Table 3.7)

c) Specificity and Selectivity

Low calculated t-values than that of the critical t-value, revealed that statistically there was no significant difference between mean peak areas of standards prepared from pure drug sample and marketed formulation sample (Table 3.7).

Table 3.7 Statistical data summary for chromatographic method

Parameters	Value
Calibration range	25 - 2000 ng/mL
Linearity (Regression coefficient)	$r^2 = 0.9997$
Regression equation	Peak Area (mV s)=72.091×conc (ng/mL)+ 141.77
Confidence interval of slope [a] (S.E)	71.74 - 72.44
Confidence interval of intercept [a] (S.E)	-200.30 - 483.83
Standard deviation of intercept ($Ś_c$)	5.13×10^2
t-value for intercept [a,b] (tab = 2.45)	0.28
F-value [c]	1.783×10^{-4}
Standard error of estimate	1.042×10^3(12.48 ng/mL)
Specificity and selectivity - t_{Cal} (t_{Crit})[d]	1.73 (2.45)
Limit of detection	7 ng/ mL
Limit of quantification	21 ng/ mL
Absolute recovery (% RSD)	99.13-100.95% (≤1.90%)
Precision (% RSD)	Repeatability: 2.01 % (intra-day)
	Intermediate Precision: 1.79 % (inter-day)
System suitability	System precision: 0.77% (n=10)
	Tailing factor: 1.11 ± 0.01
	Number of plates: 10485 ± 50
	HETP= 14.43 ± 0.15 μm
	Retention factor: 1.24 ± 0.09
Selectivity (resolution)[e)]	6.08
Robustness	% MET ± 1.0
	pH ± 0.50
	Buffer strength ± 5.0

a) Calculated at 0.05 level of significance, b) Calculated using the test of the intercept ($t_{diff} = |a-\alpha|/Sa$), c) Calculated using Fisher test with one way ANOVA (P-value<0.05), d) t_{Cal} is calculated value and t_{Crit} is theoretical value (at 6 d.f.) based on paired t-test at P=0.05 level of significance, e) Acceptable resolution is >2

In case of placebo samples, no interference was observed within the vicinity of drug peak, which demonstrate the selectivity of the method for DA in presence of formulation excipients. At LOQ level, drug spiked placebo samples showed no significant change in response and peak properties

(Figure 3.3 A). Finally almost 100 % recovery of DA at different samples at the same concentration and drug spiked placebo injections and liposome formulation samples confirmed that the method was selective and specific in determining DA in presence of expected excipients (Table 3.7)

e) Precision

In the repeatability study, variation in measured response of freshly prepared six standards at three quality control levels was found to be insignificant, which demonstrated that the developed method was repeatable with % RSD values below 2.01%. Similarly, % RSD values for inter-day variation were significantly low (≤1.79%) for intermediate precision. Low RSDs indicated the repeatability and intermediate precision of the method (Table 3.8).

Table 3.8 Results of repeatability and intermediate precision study for chromatographic method

QC Levels (ng/mL)	Repeatability (Intra-day)						Intermediate Precision (inter-day)	
	Day (1)		Day (2)		Day (3)			
	Mean conc[a] (ng/mL)	% RSD	Mean conc[a] (ng/mL)	% RSD	Mean conc[a] (ng/mL)	% RSD	Mean conc[a] (ng/mL)	% RSD
LQC (60)	61.01	1.76	60.81	2.01	60.50	1.62	60.77	1.79
MQC (800)	804.87	0.94	795.19	0.60	799.19	1.57	0.716	1.37
HQC (1600)	1601.9	1.52	1594.9	0.88	1594.9	0.94	1597.2	1.15

a) Each determination is average of six replicates

d) Accuracy

The method showed consistent and high absolute recoveries at all five concentration levels in the placebo spiking method with a mean absolute recovery ranging from 99.13 to 100.95%. Placebo spiking method indicated that the obtained absolute recoveries were normally distributed around the mean with uniform RSD across five concentration levels signifying homoscedastic nature of the data. Thus, it can be summarized that there was no significant interference of excipients and the proposed method was found to be accurate with low % Bias (<1.0). Further, consistent and high absolute recoveries obtained from standard addition method were in agreement with

placebo spiking technique. Recovery study indicated that the method was suitable for determination of DA from injections and liposome preparations (Table 3.9). As per literature review this is the first work which describes the accuracy of the developed method in terms of standard addition and placebo spiking for DA estimation.

Table 3.9 Recovery studies by placebo spiking and standard addition technique for chromatographic method

Product	Technique	Amount of drug added (% of label claim)	Mean absolute recovery[a] (%)	% RSD	% Bias
Injections	Placebo spiking[b]	50	100.27	1.90	0.27
		75	100.43	0.25	0.43
		100	99.81	1.29	0.19
		125	100.33	0.41	0.33
		150	100.95	0.39	0.95
	Standard addition[d]	0	100.59	1.85	0.59
		50	99.86	0.70	0.14
		100	100.58	0.64	0.58
Liposomes	Placebo spiking[c]	50	99.35	1.44	0.65
		75	100.21	0.51	0.21
		100	100.19	1.80	0.19
		125	100.44	1.77	0.44
		150	99.17	1.50	0.83
	Standard addition[d]	0	100.50	1.60	0.50
		50	99.18	0.67	0.82
		100	99.13	0.51	0.87

a) Each level was processed independently and injected in six replicates, b) Placebo injection equivalent to unit dose, c) Placebo liposomes preparation equivalent to unit dose, d) Commercial injection preparation containing 40 mg/mL of equivalent DA, e) In-house prepared nanoparticulate preparation containing 40 mg of equivalent dopamine

f) Sensitivity

The LOD, found to be 7 ng/mL of the developed method satisfies the signal to noise ratio 2:1. LOQ was found to be 21 ng/mL and calibration point starts from 25 ng/mL as it satisfies the signal to noise ratio condition 10:1. The method has shown high magnitude of slope and low standard error (Table 3.7) indicating sensitivity of proposed method. Upon repeated injection at quantitation limit, retention time, peak area and T_f were not affected and mean absolute recovery

was consistently high and acceptable with low % RSD and % Bias. Thus, the method was found to be highly sensitive.

g) Robustness

Response surface plots indicate that the obtained response remains unaffected by small changes in critical method parameters such as percent organic component, buffer strength and pH. The statistical analysis revealed that there was good agreement between experimental and predicted values. The developed quadratic model indicates good correlation between obtained responses and studied factors. The model coefficients estimated by least square regression analysis were successfully used to determine qualitative and quantitative relationship between the critical method parameters and chromatographic response function using the following equation.

$$\hat{Y} = \beta 0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_{3+} \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where, $\hat{Y}$: predicted response; X: coded variables: β: model coefficients.

Figures 3.4 A-G show the 3-D surface plots of predicted responses (on Z-axis) for DA as a function of two significant factors (on X- and Y-axis) while the third factor (third factor) was considered constant at optimum level. Figures 3.4 A-C shows peak area of DA against % MET versus pH, % MET versus buffer strength and pH versus buffer strength, where there was no significant change in the mean peak area over the studied range of all three factors indicating robustness of response for the studied factors. However, % MET showed significant effect on retention time of DA, as shown in Figures 3.4 D-E indicating sensitivity of retention time for % MET amongst the studied factors. The peak height has showed sensitivity towards % MET with significant drop in retention time and retention factor, as shown in Figures 3.4 F-G. Selected factors did not show significant effect on peak symmetry (T_f) within the vicinity of optimized parameters. Further, none of the factors studied showed significant effect on system efficiency. Although few factors have shown effect on retention time and peak symmetry (T_f), but peak area, the principle chromatographic response function was almost unaffected by any of the studied factors suggesting that the proposed method was robust.

h) System suitability and DA stability

Primary system suitability parameters such as retention factor (k ≥2.5), resolution (Rs ≥2.0), number of theoretical plates (N ≥3,900) were above acceptable limits and height equivalent to theoretical plates (HETP 14.43 μm) was well below limit, indicating that optimized method was suitable in terms of system performance (Table 3.7). Moreover, method demonstrated good peak symmetry for DA (T_f ≈1.11) with consistently low variability in peak areas and retention times

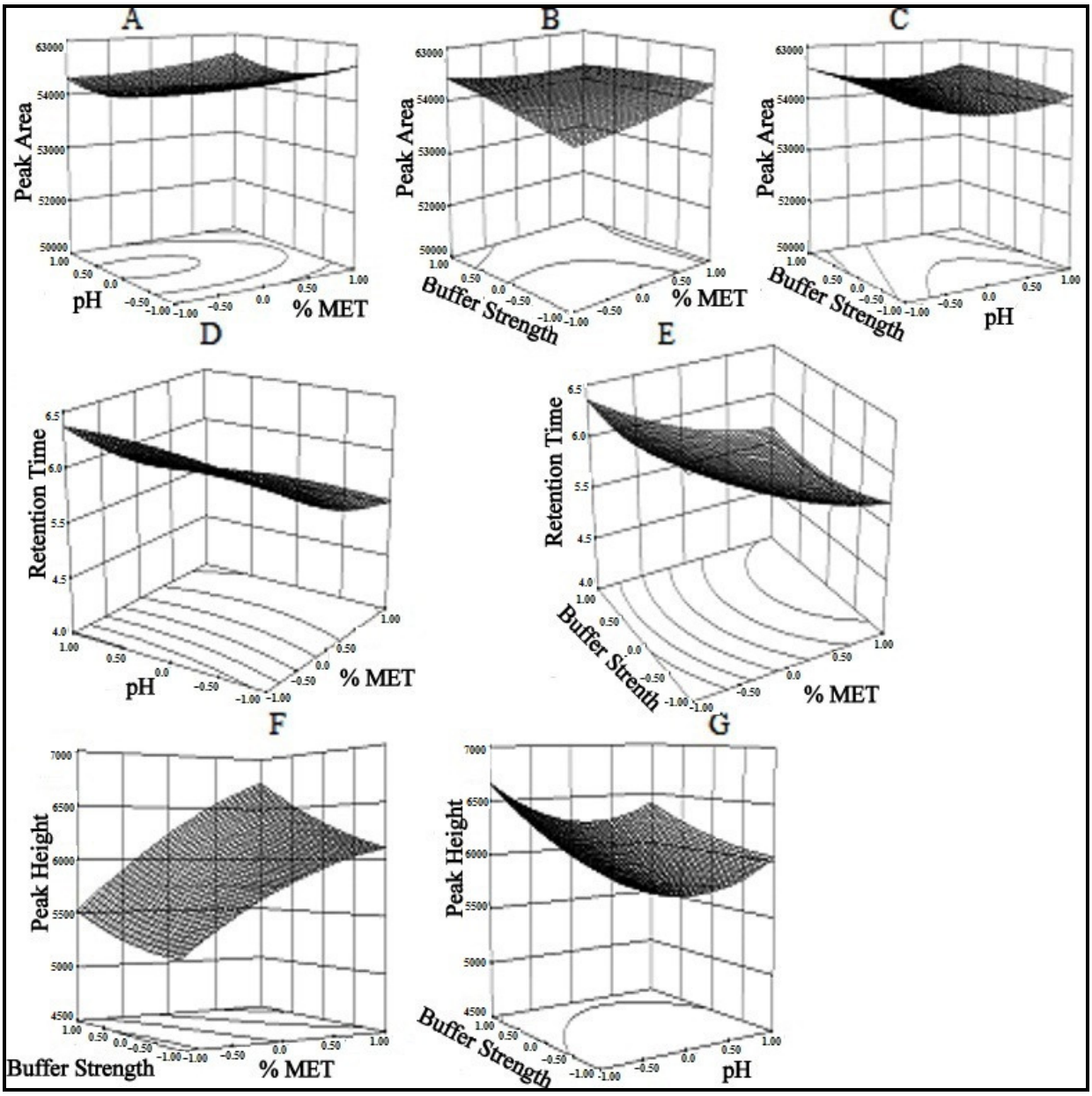

Fig. 3.4 Three-dimensional surface plots of predicted responses: A-C: peak area, (mVS): A) as a function of % methanol (v/v) and pH; B) as a function of % methanol (v/v) and buffer strength (mM); C) as a function of pH and buffer strength (mM); D-E: retention time (min): D) as a function of % methanol (v/v) and pH; E) as a function of % methanol (v/v) and buffer strength (mM); and F-G: peak height (mV): F) as a function of % methanol (v/v) and buffer strength (mM); G) as a function of pH and buffer strength (mM)

after repeated injections. System suitability study confirmed that the method was specific, precise and stable for estimation of DA. The stock stability of dopamine was studied at two different temperatures. At 25°C, the DA without antioxidant was not stable even for 2 h (recovery 75.74-85.44 %). However, with antioxidant, DA was found to be stable for 48 h at 25°C (recovery

99.86-100.46 %). Similarly, at 4°C in the auto-injector, DA without antioxidant was degraded within 4 h (recovery 73.24-89.09 %), however was stable, with antioxidant in selected stock for more than one month (recovery 99.83-101.08 %). Chromatograms of samples stored at room temperature for 48 hr and at 2-8°C for 30 days showed similar response and absence of additional peaks when compared to chromatograms of freshly prepared samples. The results indicated that the drug was stable in stock at room temperature with an RSD ≤ 1.60 % and at 2-8°C with an RSD ≤ 1.21 % for at least 48 hr and one month, respectively. Further peak purity index (1.0000) was greater than peak purity threshold (0.99003) which indicates that there is no merging of impurity or degraded peaks and peak is pure (Figure 3.5).

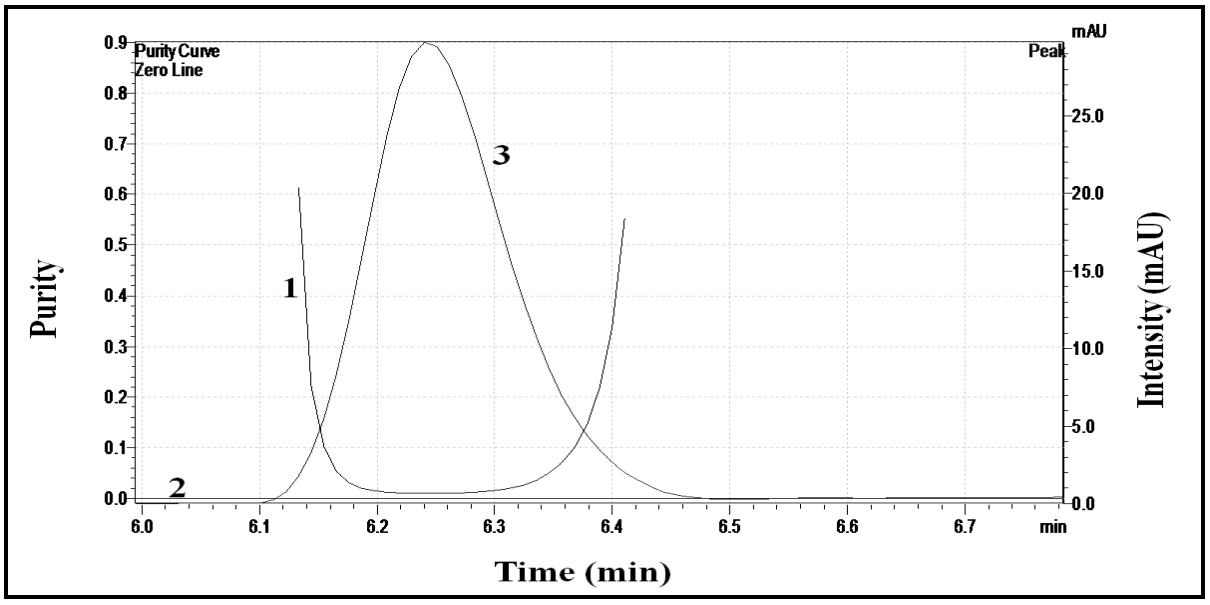

Fig. 3.5 Peak purity of DA between time; min (X axis), purity (left Y axis) and intensity; mAU (right Y axis) after 30 days (one month stability sample): 1) purity curve; 2) zero line; 3) DA peak

3.4.5 Application of developed method-analysis of commercial injection and in house prepared liposomes.

A typical chromatogram of DA extracted from in house prepared liposome preparations and commercial injection are presented in Figs 3.3B and 3.3C, respectively. The mean recoveries for each formulation were found to be in good agreement with the labeled claim of the individual product. The method was found to be accurate with a mean absolute recovery of 100.33 % for injection and 99.86 % for liposomes preparations, with % RSD not exceeding 0.40 and 1.51 % for injection and liposome preparations, respectively. Moreover, formulation excipients did not show interference in the determination of DA, as % Bias was below 0.95 and 0.87 for injection

and liposome preparations, respectively. Thus, the method was found to be suitable for determination of drug content from both formulations and can be applied for in vitro drug release and stability sample analysis for DA.

3.5 Bioanalytical LC method

3.5.1 Experimental

a) Materials and methods

Dopamine hydrochloride (assay 99.95 %) and Reserpine (assay 100 %) was purchased from Sigma-Aldrich, India. HPLC grade methanol was purchased from Spectrochem (Mumbai, India). Fresh triple distilled water (TDW) was prepared in quartz distillation assembly and filtered through a 0.22 μm membrane using Millipore ultra-filtration system (Millipore, France) before use. Potassium dihydrogen orthophosphate, orthophosphoric acid, perchloric acid, formic acid and sodium metabisulphite, were purchased from Merck (Mumbai, India). Tris (hydroxymethyl) Aminomethane (Tris buffer) were obtained from SRL Lab (New Delhi, India). Analytical grade EDTA-2Na was obtained from Himedia (Mumbai, India). Alumina B cartridges (100 mg bed size) were purchased from Orochem (Mumbai, India).

b) Instrument

Same as mentioned in analytical LC method.

3.5.2 Method Development

a) Preparation of stock and standard solution

A master stock solution of 0.2 mg/mL was prepared in 0.1 M perchloric acid containing 0.1 % (w/v) sodium metabisulphite and stored at 4°C. Further serial dilution was done from this master stock to get working standard solutions in the range of 100–0.4 μg/mL; all the dilutions were made in diluent (10 mM potassium dihydrogen phosphate buffer pH 4.0). Eight analytical standards containing 25, 50, 100, 250, 500, 1000, 2000 and 2500 ng/mL was prepared freshly from each working stock, in triplicate on three different days of validation. Eight plasma standards containing 25, 50, 100, 250, 500, 1000, 2000 and 2500 ng/mL were prepared freshly by spiking 10 μL of each working stock in 190 μL of blank rat plasma. Similarly, brain, nasal cavity, lungs, trachea and esophagus samples were prepared at 25-2000 ng/mL in brain fluid and 50-2000 ng/mL were prepared in fluids of nasal cavity, lungs, trachea and esophagus homogenates. Each standard were prepared fresh daily on three different days of validation. All the stock

solutions were prepared freshly on the day of validation and care was taken to store DA stock in glass at 4°C.

b) Sample collection and preparation

Rat blood samples, about 1.5 mL from each animal, were withdrawn from 15 healthy Wistar rats by retro-orbital bleeding under mild anesthesia (diethyl ether) with a fine capillary. After blood collection, all animals were recovered from anesthesia. The blood was collected into 2 mL polypropylene microtubes (Tarsone, India) containing EDTA-2Na (0.1 mL of 10 % solution for 2 mL of plasma) for preparation of plasma, and care was taken to mix the blood with EDTA-2 Na. After centrifugation at 12000 rpm for 15 min at 4°C, the plasma was pooled into one tube and stored at –20°C and thawed before analysis.

c) Sample processing and Extraction procedure

An alumina-based solid-phase sample preparation approach (51-53) was used. The Alumina B cartridges of 100 mg bed size with 1 mL capacity were conditioned with methanol (1 mL) and equilibrated with de-ionised water (1 mL). To aliquot of plasma sample (200 μL) or tissue homogenate (200 μL), 10 μL each of 250 mmol/L solutions of EDTA and sodium metabisulfite were added and finally 1 mL of 0.2 M Tris buffer (pH 9.0) was added and vortexed for 2 min in multiholder vortex mixer (SPINIX Multilab, Mumbai, India). The prepared samples were then loaded onto cartridges and the cartridges were washed with one cycle each of 20 % methanol (1 mL) and water (1 mL) and finally eluted with 400 μL of 2.5 % formic acid. The total sample processing time before injection was not more than 10 min. Finally the quantification was carried out at 227 nm with an injection volume of 75 μL.

d) System suitability parameters

The column efficiency for the separation of DA was evaluated using the following formula:

$N = 16\ (t/W)^2$ or $N = 5.54\ (t/W_{h/2})^2$

where, N is the number of theoretical plates, t is the retention time of DA, and W the peak width of DA at the baseline or $W_{h/2}$ the width of DA peak at half height.

The capacity factor (k') for DA were calculated as follows:

$k' = (t\text{-}t_o)/t_o$

where t and t_0 are the retention time of DA and the non-retained sample (solvent front), respectively.

The tailing factor (T) for DA were calculated as follows

$T = W_{0.05}/2f$

where $W_{0.05}$ is the width of peak (DA) at 5 % height and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5 % of the peak height from the baseline.

3.5.3 Method validation

a) Selectivity and peak purity

The selectivity of the method was studied by investigating the interference from various endogenous matrix components (mainly proteins) and exogenous substances that may come in contact with the sample (intentional or accident) during the process. Blank blood, brain, nasal fluid, lungs, trachea and esophagus samples from six different rats were collected for this study, and the blood samples were processed for plasma and all samples were stored at –20°C until analysis. Six individual samples of drug, blank sample (drug free plasma) and lower limit of quantification (LLOQ) (25 ng/mL) samples were processed individually and analyzed by the proposed method. The obtained chromatograms of blank samples were compared against analytical, calibration standards, and real time pharmacokinetic samples to investigate possible interference in the determination. The guidelines for LLOQ require that the peak area of compounds co-eluting with the DA should not exceeds 20 % of the DA peak area at LLOQ. The selectivity is further confirmed by peak purity determination by using the ratiograms and purity curve. The ratiograms is constructed by plotting the ratio of absorbance/response of DA over a retention time (7.5±0.50). The rectangular ratiograms showed that the peak is pure.

b) Linearity and quality control samples

Calibration standards in drug free rat plasma were prepared at concentrations of 25, 50, 100, 250, 500, 1000, 2000 and 2500 ng/mL of DA from respective working stocks. Calibrator samples were prepared by spiking 190 µL of blank rat plasma with 10 µL of the respective DA working stock solutions in 2.0 mL polypropylene microcentrifuge tubes. As per guidelines this satisfies the limit of 5% addition of organic solution to plasma. The calibration samples consist of a blank sample (matrix sample processed without DA) and eight non-zero samples including LLOQ. Quality control (QC) samples were prepared at concentrations of 25, 75, 1500 and 2250 ng/mL from respective working stock solutions similar to the calibration sample. On each day of validation, calibration standards and QC samples were prepared fresh and analyzed.

Similarly three separate series of six standards each of brain (25-2000 ng/mL), lungs (50-2000 ng/mL), nasal cavity (50-2000 ng/mL) and trachea esophagus (50-2000 ng/mL) were used for

linearity and range. Average area at each level was plotted against concentration and the curves were subjected to linear regression analysis by least square method.

c) Determination of LLOQ and limit of detection

The LLOQ determination was performed on five different days (n = 5), by spiking an aliquot of blank rat plasma (190 μL) with DA (10 μL) at a concentration of the lowest calibrator with a precision less than 20 %, accuracy of 80–120 %, and signal-to-background noise ratio greater than 6:1. The limit of detection (LOD) was defined as the lowest concentration of DA that the method can detect with a signal-to-noise ratio greater than or equal to 3 (i.e., the response: peak height/area or height/area ratio of peaks in the case of DA concentration should be equal to or greater than 3 times of the base line noise of the instrument).

d) Recovery

The determination of the processing method efficiency was done by calculating the recovery of DA in spiked plasma samples. The recovery was calculated by comparing the DA peak area of the spiked plasma samples (extracted sample) with their respective aqueous samples. DA recovery study was carried out in all calibration points. All the prepared calibration standards were subjected to sample processing and analyzed by the proposed method. Recovery of DA from brain, nasal cavity, trachea, esophagus and lungs samples were also calculated as follows:

Recovery (%) = area of extracted standard /area of aqueous standard x 100.

e) Intra-day and inter-day precision and accuracy

The precision and accuracy were determined by taking four concentrations (QC samples) in the range of calibration curve (all measurement were five determinations per concentration). The intra-day precision and accuracy were determined by analyzing the spiked QC plasma samples prepared within a day on three different occasions. The inter-day precision and accuracy were determined by analyzing the spiked QC samples prepared on three different days. On each day of validation separate calibration curves were constructed to determine the calculated concentration or actual concentration of the prepared samples. After the concentrations were calculated by using the regression equation, the % relative standard deviation (RSD) was calculated using the mean value and the standard deviation (SD); the % Bias was calculated from the calculated concentration and known concentration (concentration prepared); the % recovery was calculated by using the standard formula at each concentration of the QC samples. The limit for precision is reached when the % RSD value did not exceed 15 %, except for LLOQ, where it should not exceed 20 %. A low percent relative error shows the accuracy of the proposed method.

f) Stability

During the pharmacokinetic study, the collected blood samples were processed to separate the plasma and then stored in respective storage condition (–20°C). Hence, it was necessary to determine the stability of DA in the biological samples at these respective storage conditions. The stability studies are conducted as follows: freeze-and-thaw stability, short-term stability, long-term stability, post-preparative stability, and stock solution stability. On each day of the stability study, separate calibration standards were processed and analyzed with the stability samples. All stability studies were conducted as per USFDA guidelines in LQC and HQC standards.

i) Freeze-and-thaw stability

The LQC and HQC standards were prepared in plasma and stored at –20°C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 18 hr under same conditions. The freeze-and-thaw cycles were repeated four more times, after which the samples were analyzed with the proposed method on the fifth cycle. Hence, DA stability in plasma was determined for five freeze-and-thaw cycles. The stability of DA was determined by calculating % Bias and % recovery. Each concentration was measured in triplicate.

ii) Short and long-term stability

The short term stability was conducted up to 24 h at room temperature (25°C ± 0.5), based on the expectation that DA in plasma will be maintained at this temperature for a maximum of 24 h. The selected QC standards were thawed at room temperature, and then processed and analyzed at 1, 6, 12 and 24 h. The long-term stability time points were selected by considering the time between the date of first sample collection and the date of last sample analysis. The time points chosen for long-term stability were 7, 15, and 30 days. On the respective time points the samples were thawed unassisted at room temperature, processed, and analyzed by using the proposed method. The concentration of all stability samples were compared to the mean of back calculated values of the fresh QC standards at the appropriate concentration. As in the freeze-and-thaw stability study, QC standards were analyzed in triplicates.

iii) Post-preparative stability

This is to determine the stability of DA in the reconstitution solution during the time the sample rests in the auto-sampler (4°C ± 0.2). The time points were selected based on the anticipated run time for the batch size in validation samples. The time points selected for this study were 1, 3, and 5 days. The QC samples were prepared by spiking respective aliquots from working stock to

the plasma, then all the samples were processed and loaded into the auto-sampler, and the analysis was done as per the time points.

iv) Stock solution stability

The stability of DA in the stock solution is evaluated at room temperature and at –20°C for one month. Each determination was performed in duplicate.

3.5.4 Application of developed method- analysis of in vivo samples

The developed method was used to estimate DA in plasma and tissue samples (brain, trachea, esophagus, nasal cavity and lungs) for pharmacokinetic and biodistribution studies. (Discussed in Chapter 6- In vivo bioavailability and biodistribution studies)

3.5.5 Results and Discussion- Bioanalytical LC method

a) Chromatographic separation

Objective was to develop bioanalytical method to determine specific DA in biosamples (plasma and tissue). During the chromatographic separation, in order to get good resolved peaks for DA, different aqueous phases were tried while keeping organic phase (methanol) constant. Optimized mobile phase consisted of an aqueous phase 10 mM potassium dihydrogen phosphate buffer and methanol (pH 4.0, 97:3, v/v), where the aqueous phase pH was adjusted using 0.1 M orthophosphoric acid. This was selected with respect to peak symmetry (T = 1.02) and simplicity. The method utilizes green chemistry approach in solvent selection with use of only 3 % organic phase as compared to reported methods with more than 15 % organic phase (54-56) and this method takes less than 10 min to run each sample (56, 57).

Thus our method is environmental friendly. Mobile phase was delivered in isocratic elution mode at a flow rate of 1 mL/min. The mobile phase used in this method is very simple in preparation when compared to those previously reported (54, 56-58) and the extraction procedure is also comparatively simple to Maycock and Frayn (52) procedure, which has multiple extraction steps. The chromatographic separation was achieved on BDS Hypersil C18 (250 mm x 4.6 mm, 5 µm, 80 A°) double end-capped RP-HPLC column (Thermo Scientific, Mumbai, India). The optimized mobile phase provided moderate and quick retention time, with better peak properties, resolution and selectivity for DA. The retention times for DA were 7.5 ± 0.50. The quantitation was carried out at 227 nm with an injection volume of 75 µL. Analysis was performed at ambient temperature (25°C) after baseline stabilization for at least 60 min. Dopamine lacks strong UV absorption; detection at the more permissive wavelength of 280 nm requires the use of large volumes (0.5-1 mL) of sample. There are many endogenous substances from biomatrix with

similar amines as that of Dopamine, which also have strong absorption at 280 nm and they get strongly retain in C18 column. Because of the above two reason DA was determined at 227 nm where endogenous substance absorption is very much low (Fig. 3.6 A) and hence estimated with high accuracy and precision.

This study reports an improved bioanalytical methodology for quantifying dopamine in micro volume rat plasma using a combination of simple solid-phase extraction (SPE) and liquid chromatography. Although the sample preparation approach used here was reported previously using in-house alumina columns for the isolation and trace enrichment of this neurotransmitter along with other catecholamines from biological samples (31, 52-53), we have redesigned and optimized the choice of extraction conditions so that the procedure can be made simple, reproducible, readily compatible with UFLC-PDA and which gives high recovery using very less volume of plasma for samples.

In this method we have used Alumina B cartridges of 100 mg bed size with 1 mL capacity to estimate DA (average % recovery 90.9 ± 3.2) from micro volume rat plasma. The extraction procedure described is very economical than the reported method using solid phase extraction (SPE). In the reported SPE (52) for the estimation of DA in biomatrix, used more than 1 mL of sample but in the present method, only 190 μL of sample was used for extraction and estimation of DA from plasma and tissues. In this method we have found very high recovery (average recovery 90.9 %± 3.2) when eluted with 2.5 % formic acid as compared to reported methods with low recovery (average recovery 70 %) in which the commonly used elution solvent was perchloric acid. In the reported method there are many complicated and sophisticated steps as well as the supernatant is concentrated by evaporation which requires longer time. As, in this method final elution was in aqueous phase so no evaporation step was required. The total sample processing time before injection in present method is not more than 10 min. There was no interference from the matrix during total analysis time of 10 min and even there were no high interfering peaks up to 30 min.

The sample clean up procedure using the alumina B cartridge was chosen because it provided clean chromatograms, rapid sample preparation and easily handling when compared with the liquid-liquid extraction methods and the protein precipitation (59, 60). The plasma samples can be directly loaded onto the cartridges without de-proteinization. The relatively clean chromatograms obtained by this method of sample preparation may be accounted for by two factors: firstly adsorption of undesired substances to the alumina matrix of the cartridge, and

secondly, elution of contaminating species in washing step. The effectiveness of this clean-up contributed to prolongation of column life, and more than 1500 injections of sample extraction have been injected with no change in column performance. By using this simple SPE method DA was extracted efficiently from plasma, tissue samples, brain, nose, trachea and esophagus with good % recovery. All of the reported methods make the process more complicated and difficult for routine analysis. As proposed method is simple and rapid, this method can be used for routine analysis of DA in rat biosamples. The procedure has been characterized by assessing the precision, accuracy, recovery and selectivity of the method in accordance with standards generally accepted and has found utility in studies of the central nervous system where elevated levels dopamine are anticipated after appropriate doses.

b) Selectivity and peak purity

Fig.3.6 A-F illustrate representative chromatogram of a blank rat plasma, control plasma spiked with 25, 50, 1000 and 2500 ng/mL of DA and plasma and plasma sample of i.v. bolus pharmacokinetic study. Chromatogram of blank sample revealed that there was no interfering peak present in the eluting window of DA. Further, the real time i.v. pharmacokinetic chromatogram confirm that there was no interference from metabolites or degradation products or other exogenous xenobiotics in the near vicinity of DA. There was no co-eluting peak, > 20 % of the DA at LLOQ at their respective retention time. These results confirm the selectivity of the developed method for extracting DA from micro-volume rat plasma. The chromatograms recorded at the elution time at different wavelengths (225-230 and 280 nm) shows the peaks homogeneity of DA (Fig. 3.7A-B) with good overlay of peak shape and retention match. There is no interfering, co-eluting, or co-migrants endogenous or exogenous or metabolite substance at the selected wavelength, this shows the efficiency of the method and maximum sensitivity of determining DA at 227 nm. This is the reason, in addition to extraction efficiency, for the lower limit of quantification (25 ng/mL).

In the obtained rectangular ratiograms, the ratio of response (area/absorbance) at two selected wavelengths (225 and 230) are less than one (Fig. 3.7 C-E) and it's constant across the elution time. The rectangular ratiograms shows that the DA is quantified with high selectivity at 227 nm. The peak purity index and single point threshold (Fig. 3.7 F) value is always close to one (1.0000 and 0. 0.989232). The positive minimum peak purity index value (10756) also shows the purity of peak (DA) and it's selective. The obtained purity curve data shows selectivity of the method in determining the DA. Likewise, there were no interferences from the tissue samples eluting at the

retention time of DA, which confers the selectivity of the method in estimating DA in tissue samples. There is no interfering/co-eluting/ co-migrants endogenous or exogenous or metabolite substance at the selected wavelength indicating the efficiency of the SPE method and selectivity of determining DA at 227 nm.

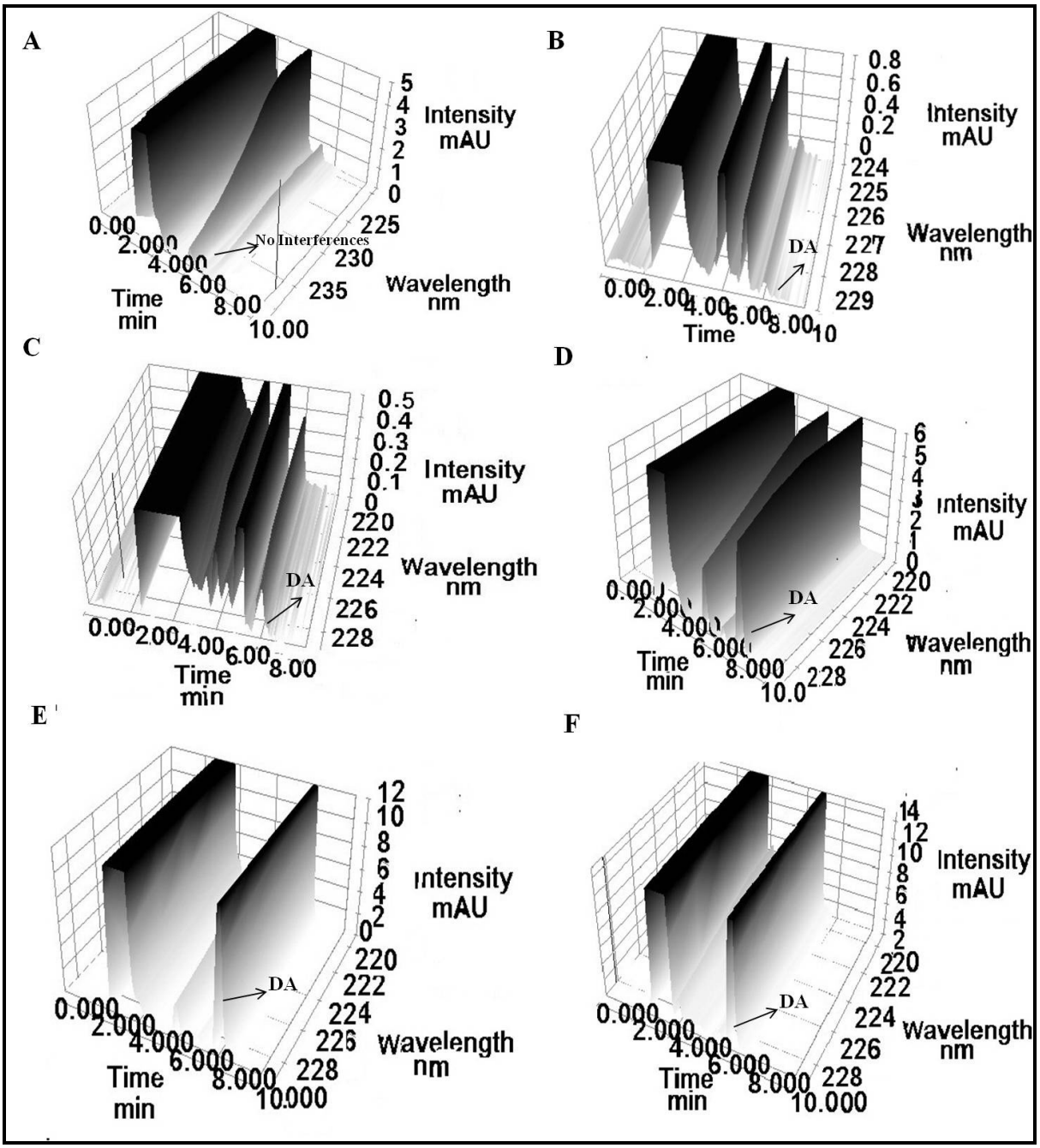

Fig. 3.6 Representative 3D (Time, Wavelength and Intensity) chromatograms of DA: (A) Blank plasma, (B) Control plasma spiked with 25 ng/mL (C) Control plasma spiked with 50 ng/mL (D) Control plasma spiked with 1000 ng/mL (E) Control plasma spiked with 2500 ng/mL (F) Plasma sample at 4 min after i.v. bolus of DA

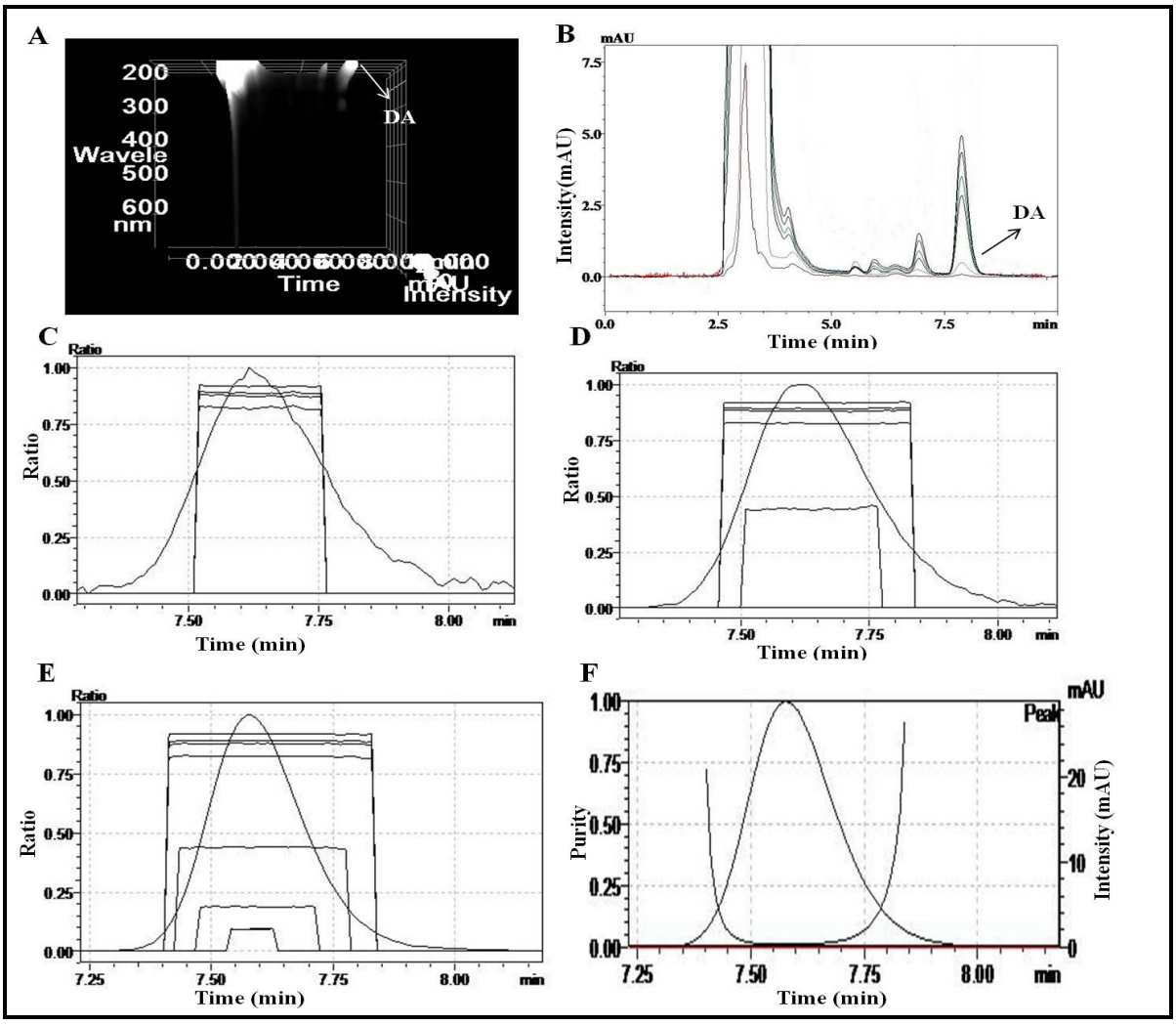

Fig. 3.7 Representative 3D chromatogram for selectivity and sensitivity: (A) Top 3D view chromatogram of 1000 ng/mL, (B) Chromatogram of 1000 ng/mL recorded at the same time at different wavelength, (C) Ratiograms of 25 ng/mL, (D) Ratiograms of 1000 ng/mL, (E) Ratiograms of 2500 ng/mL, (F) Peak purity

b) Linearity and quality control samples

Calibration curves were prepared on each day of analysis for known concentration of DA in rat plasma samples. Calibration curves were constructed by plotting peak area vs concentrations. The typical best-fit linear regression equation for the calibration curve in the range of 25 -2500 ng/mL, average peak area (μV s) = 83.569 x concentration of Dopamine (ng/mL) – 60.786, r^2 = 1.000. Mean response obtained for individual concentrations are indicated in Table 3.10. Goodness of fit of regression equation for DA in rat plasma was linear with high mean regression coefficient of 1.000 ± 0.0023, the standard deviation and % RSD of slope were found to be 3.54 and 4.24 respectively (Table 3.10). ANOVA test (one-way) was performed for peak area ratio

obtained at individual concentration levels and calculated F-value was low than critical F- value at 95 % level of significant, which supports the best-fit linear equation.

Table 3.10 Slope and Intercept of Calibration Curve of DA in Micro-Volume Rat Plasma

Calibration curve[a]	Slope	Intercept	Correlation Coefficient
1	84.065	-1386.80	0.9983
2	78.262	1078.10	0.9980
3	87.220	518.32	0.9994
4	80.521	-1376.00	0.9981
5	81.677	-184.95	0.9997
6	84.089	-590.00	0.9987
7	82.667	-128.74	0.9991
8	83.352	1374.20	0.9921
9	90.272	149.68	0.9994
Mean	83.569	-60.68	0.9990
SD.	3.540	-	0.0023
% RSD	4.240	-	0.2330

[a]Each calibration curve is obtained using eight points

Similarly good linear relationship existed between the average peak area and concentration prepared in different rat tissues as shown in Table 3.11. The standard deviation of peak area was significantly low across the selected range in all samples with % RSD less than 10.16.

Table 3.11 Best-fit linear regression equations for calibration curves in different rat tissues

Sample	Range (ng/mL)	Regression equation	r^2
Brain	25-2000	Average peak area (μV s) = 71.95 x concentration (ng/mL) – 1281	0.9990
Nasal fluid	50-2000	Average peak area (μV s) = 89.06 x concentration (ng/mL) – 1455	0.9994
Lungs	50-2000	Average peak area (μV s) =108.08 x concentration (ng/mL) – 580.8	0.9991
Trachea & esophagus	50-2000	Average peak area (μV s) = 97.69 x concentration (ng/mL) – 93.73	0.9995

c) Determination of LLOQ and LOD

The LLOQ on the calibration curve (25 ng/mL) was accepted as the limit of quantification as its response was more than six times to blank response or background noise. The peak obtained for LLOQ was identifiable, discrete and reproducible with precision (% RSD) of 10.87 % and

accuracy (% recovery) of 90.90, the repeatability is very high as compared to method reported by Lee, Rossi et al, (57, 61). The method was found to be sensitive with a high signal-to-noise ratio and acceptable precision and accuracy. By using micro-volume (200 μL) of rat plasma the LOD was found to be 10 ng/mL with signal to noise ratio greater than three, and is lower than the reported method by Lee, Oh et al (57) 50 ng/mL. The LOD of present method is almost equal to the Li, Rossi et al (61) who used sophisticated techniques. The clinical pharmacokinetic reviles that the bioanalytical method with less than 50 ng/mL as quantification limit is required for the estimation of Dopamine in the bio matrix (62). This suggests that the method is suitable for various pharmacokinetic investigations of Dopamine in rodents, which demands high sensitivity and repeatability. The developed method was able to detect Dopamine in i.v. pharmacokinetic study with 10 mg/kg dose in rats and 1 mg/kg in brain via nasal delivery.

d) Recovery

The recovery of DA after SPE was studied at all calibration standards in triplicates. The results are summarized in Table 3.12. The efficiency of extraction was found to be in the range of 86.7 ± 2.1 to 95.3 ± 4.2 %, with average recovery of 90.9 ± 3.2 which are good in comparison to other reported methods. Poor recovery was indicted as a significant problem encountered with the aluminum oxide extraction. Overall recovery was reported previously as 42 ± 2 % for dopamine (63). Using a larger volume of elution solvent (0.5 ml) increased the overall recovery to 80–90 %, but the trade off was undesirable dilution. Higher recoveries were achieved using other forms of SPE for sample clean up. The recoveries were 64 % by a Toyopak IC-SP S cartridge (64) and 90 % for other catecholes by Bond-Elut C18 (65). The present sample clean up method indicated that the processing method is simple and efficient in extracting the DA in micro-volume of rat plasma using solid-phase extraction. The results show that the extraction efficiency of the method is consistent, precise and reproducible (% RSD ≤ 7.10). The efficiency of extracting DA form the brain samples of the studied QC samples were, 86.15 to 95.49 with low standard deviation ranging from 0.19 to 2.24.

e) Intra-day and inter day precision and accuracy

Precision and accuracy data of DA in rat plasma are shown in Table 3.13. The intra-day precision of Dopamine in micro-volume of rat plasma showed % RSD less than 6.04. The percent relative standard deviation (% RSD) in inter-day precision of all QC sample were less than 5.56. The precision results (% RSD) are very low as compared to highly sophisticated method reported by

Table 3.12 Recovery of Dopamine from micro volume rat plasma

Concentration (ng/mL)	Mean absolute recovery (%, ± SD)[a]	% RSD
25	93.3 ± 5.7	5.73
50	92.5 ± 7.1	7.09
100	95.3 ± 4.2	4.24
250	91.3 ± 0.6	0.62
500	90.1 ± 3.5	3.56
1000	89.5 ± 3.7	3.59
2000	86.7 ± 2.1	2.06
2500	88.5 ± 1.2	1.20
Average recovery (± SD)	90.9 ± 3.2	

[a] Average of six determination (n=3 on two days)

Li, Rossi et al (57) which showed intra-run precision 15 % and the inter-run precision and accuracy 11 %.This showed that the present method is highly reproducible; hence it can be used for routine analysis of DA in rat plasma. The intra-day accuracy study showed % bias ranged from 1.8 to 8.9. The inter-day accuracy (% bias) at all QC levels ranged from 0.02 to 9.0. The % recovery for intra-day and inter-day precision were in the range of 91.03 to 98.16 and 90.94 to 97.94.

f) System suitability parameters

The number of theoretical plates (N) of the column for separation of DA was 4460. The capacity factor (k´) for Dopamine was 3.5. The tailing factor for DA (1.05 ± 0.06) was around unity, which showed that the peaks are perfectly symmetrical. The system suitability parameters show that the method is reproducible with good resolution.

g) Stability

The results of freeze-and-thaw stability at all QC levels demonstrated that DA was stable in rat plasma up to five freeze-and-thaw cycles (Table 3.14). Results are expressed in terms of % RSD and % recovery, which ranges from 1.28 to 7.20 and 93.51 to 100.01, respectively. Hubbard, Wells et al (66) showed that DA is stable (% accuracy and % RSD) up to three freeze-and-thaw cycles, and the present study results showed that DA is stable up to five freeze-and-thaw cycles. This confirms that DA is stable for five freeze-and-thaw cycles, making it suitable for subzero storage condition. The short- and long-term stability results showed that DA was stable up to 24 hr in bench top conditions and for 30 days at –20°C (Table 3.13).

Table 3.13 Intra-day and inter-day precision and accuracy of DA in micro-volume rat plasma

QC Levels		*Predicted Concentration (ng/mL)		% RSD		% Recovery	
	Range	Intra-Day (mean)	Intra-Day (mean)	Intra-Day (mean)	Intra-Day (mean)	Intra-Day (mean)	Intra-Day (mean)
LLOQ (25 ng/mL)	24.38-24.50	24.54	24.48	0.05	0.10	98.16	97.94
LQC (75 ng/mL)	67.90-68.63	68.27	68.20	6.04	5.41	91.03	90.94
MQC (1500 ng/mL)	1402.71-1477.50	1440.12	1432.81	2.38	1.94	96.01	95.52
HQC (2250 ng/mL)	2153.42-2175.02	2158.94	2164.31	5.94	5.56	95.95	96.19

* Predicted concentration of DA was calculated from linear regression equation. (Each Value is Determination of Twelve Values)

The % RSD and % recovery for short-term stability ranged from 4.41 to 12.18 and 92.81 to 101.68, respectively. At all QC standards in long term stability the % RSD and % recovery ranged from –0.34 to 2.38 and 95.21 to 100.10, respectively. The post preparative study results demonstrated that DA can be stored in an auto-sampler (4°C ± 0.2) for up to 5 days. The % RSD and % recovery of post preparative study ranged from 1.86 to 6.35 and 92.65 to 100.23 respectively. It has been shown that the DA can be reanalyzed even after five days at 4°C ± 0.2. This allows the analyst to re-analyze the samples if required in situations like machine failure, which is very common for the researchers who work with HPLC. This post-preparative stability results gives confidence to reanalysis for DA rat plasma sample up to five days. The % RSD calculated for all stability samples were well within the acceptable range of ± 20 % at LQC and ± 15 % at HQC concentration levels. These confirm that the DA was stable under various processing and storage conditions stated in the method. The stock solution stability data shows that DA is stable at room temperature (mean % recovery 99.99±0.57) and at –20°C (mean % recovery 99.95 ± 0.59) in 0.1 M perchloric acid containing 0.1 % (w/v) sodium metabisulphite for one month. After a complete survey of the literature, this is the first complete report with all stability study in micro volume rat plasma for DA.

Table 3.14 Stability study of DA in rat plasma

Parameters	Concentration (ng/ml)	% RSD	% Accuracy
Freeze Thaw stability (for 5 cycles)	75 (LQC)	4.49-7.20	97.42-100.01
	2250 (HQC)	1.23-3.48	93.51-9.99
Bench Top stability (for 24hrs)	75 (LQC)	4.41-12.18	92.81-101.68
	2250 (HQC)	3.67- 6.60	94.41-99.25
Long term stability (for 30 days)	75 (LQC)	1.21-2.38	95.50-100.10
	2250 (HQC)	0.34-0.98	95.21-98.99
Post processing refrigeration stability (for 5 days)	75 (LQC)	1.86-6.35	92.65-99.91
	2250 (HQC)	3.72-5.99	93.99-100.23

(Each value is determination of triplicate)

3.5.6 Application of developed method- analysis of in vivo samples

The validated method was successfully applied to estimate Dopamine in rat plasma and tissue samples as discussed in Chapter 6.

3.6 Conclusion

A simple, sensitive, accurate and precise UV-spectrophometric method was developed and validated for the determination of DA in bulk and formulations. The selectivity of the developed method was well established at the limit of quantification. The LOD and LLOQ of the method, 0.69 μg/mL and 2.09 μg/mL respectively. A rapid, sensitive, accurate, precise and specific-stability indicating LC method was developed and validated for the estimation of DA in Liposome formulations. The accuracy of the developed method was tested by placebo spiking and standard addition method. The LOD and LLOQ of the method, 7 ng/mL and 21 ng/mL respectively. Further this study confidently stands for a new, simple, rapid, and sensitive HPLC-diode array UV method for the estimation of DA in micro volume (200 μL) rat plasma samples, brain, nasal cavity, lungs, trachea and esophagus samples by simple solid phase extraction technique. The LOD and LLOQ of the developed method was 10 and 25 ng/mL. The rectangular ratiograms and purity curve demonstrates selectivity of the method. The validated method has been shown to be suitable for pharmacokinetic study of DA in wistar rats. All the stability study results show that DA is stable in plasma with less % RSD and high % recovery.

Thus, as the pace of drug development quickens, we cannot ignore the potential for our methods application or the time savings it can present thus encouraging clinical and exploratory studies.

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Chapter 4. Preformulation

4.1 Introduction

Preformulation studies are an assessment of physical and chemical properties of the active pharmaceutical ingredients and are important to design an effective, safe, stable and marketable product. The aim of preformulation study is to consider critical physicochemical parameters of the drug, which addresses the identity, purity, strength of the active pharmaceutical ingredients and produce a meticulous understanding of stability of it under various conditions. Preformulation data confirm the presence or absence of potential barriers to the development of optimally bioavailable and stable formulation for respective drug (1, 2). An adequate understanding of this prior information minimizes the efforts in the later stages and finally helps formulation scientists for design and development of any successful formulation. Non-availability of this information can affect drug performance and can lead to stability problems (3, 4). Dopamine is well-known drug since long time and good amount of physico-chemical information are available in literature. In the present work, as aim is to formulate liposomal drug delivery system for Dopamine using chosen preformed lipids, some preformulation studies are required.

Still now US-FDA has not framed any specific guideline for nanotechnology based products, these investigations were carried out as per standard product development guidelines. Based on the need, some important preformulation experiments were planned and performed. Study covered the bulk characterization, the drug-excipient compatibility and stability of Dopamine as per the need of formulation (4, 5).

4.2 Experimental

4.2.1 Materials and Methods

Dopamine hydrochloride (assay 99.95 %) was obtained from Sigma-Aldrich, India. Lyso-Phosphatidyl choline (Lipova E120) and Soya-Phosphatidyl choline (Leciva S70) from VAV Life Sciences Mumbai, India and Dipalmitoylphosphatidyl choline (DPPC), Dimyristoylphosphatidyl choline (DMPC) and Soya PC (Lipoid S100) from Lipoid GmbH, Germany was received as gift sample. Cholesterol was purchased from Sisco Research Lab, Mumbai, India. Other chemicals are obtained from market and are pharmaceutical or AR grade.

4.2.2 Instruments and equipments

A digital pH meter (pH Tutor, Eutech Instruments, Singapore) equipped with glass electrode and automatic thermal compensation probe; digital analytical balance (AG135, Mettler Toledo, Switzerland) with sensitivity ± 0.01mg; humidity and temperature control cabinet (MSW-125, Macro Scientific Works, India); refrigerator (Frost-free 200L, Godrej, India); ultrasonicator (1201, Systronics Instruments, India); vortex mixer (Spinix, India) were used for study. All other analytical instruments were of standard grade and used after calibration.

4.2.3 Methods

Frequently, wide ranges of analytical methods are required to perform the preformulation studies. For present study, analysis of drug was carried out using either the reversed phase liquid chromatographic method or the UV-spectroscopic method as described in Chapter 3. A Fourier transform infrared spectrophotometer model IRPrestige-21 (Shimadzu, Japan) equipped with a diffuse reflectance attachment was used to record infrared absorption spectrum of all samples. The individual samples were suitably mixed with moisture-free spectral grade potassium bromide. The infrared absorption spectra were recorded in a range of 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} using a high-energy ceramic source, CsI beam splitter and DLATGS detector. The diffuse reflectance FTIR spectra were acquired using a Labsolutions® workstation (CreonLab Control, Japan) and the data was transformed using Kubelka-Munk conversion before interpretations.

Thermal analysis was carried out using a previously calibrated differential scanning calorimeter-DSC-60 (Shimadzu, Japan) with TA-60WS thermal analyzer. For each measurement, around 4 mg of individual sample was loaded into aluminum pan and covered by crimping the lid. Considering the melting of individual component, each sample was suitably scanned between 25 to 300 ^{o}C at 5 ^{o}C min^{-1} heating rate. Inert environment was ensured by purging nitrogen gas at 30 mL/min flow rate. The thermograms were acquired using a TA-60WS workstation (Shimadzu, Japan) and the melting temperatures (Tm) were recorded.

4.3 Preformulation studies

4.3.1 Bulk characterization

The UV-spectrum of Dopamine was recorded by preparing, 100 μg/mL fresh stock in 100 mM phosphate buffer (pH 6.8) with sodium metabisulfide as described earlier. To characterize the powdered drug its thermal properties were studied using Differential Scanning Calorimetric (DSC) and the integrity of the functional groups was confirmed using infra red spectrum analysis.

4.3.2 Compatibility study

a) FTIR spectroscopy

This study was carried out to check the compatibility of Dopamine with the selected lipids. The physical mixture of drug (Dopamine) and excipients (Leciva S70, Lipova 120, DMPC, DPPC, S100, Cholesterol, Mannitol and sodium metabisulfide) were prepared in 1:1 ratio and kept at room temperature (25 ± 3 ºC). FTIR was taken after 6 months of observation to monitor any incompatibility.

b) DSC study

DSC study was carried out for pure DA, individual selected lipids and combination of DA with lipids (mixed in 1:1 ratio). Around 4mg of sample (pure DA, individual lipid and combination of DA with lipids) was taken and sealed in standard pan with lid. The temperature range used was 25 to 300ºC at 5ºC min^{-1}.

c) Accelerated stability test

i) Physical stability

The accelerated physical stability test of the drug in mixture with excipients was performed at two different conditions, controlled room temperature 25 ± 2ºC/60 ± 5 % RH and 40 ± 2°C/75 ± 5 % Relative Humidity (RH). Duplicates of drug and excipients mixture were taken in the amber colored bottle and these mixtures were kept in the selected storage condition. The mixtures were observed for any physical change up to four weeks (5, 6).

ii) Chemical stability

The accelerated chemical stability test of the drug in mixture with excipients was performed at two different conditions, controlled room temperature 25 ± 2ºC/60 ± 5 % RH and at 40 ± 2°C/75 ± 5 % RH. Triplicate of drug (1 mg) and excipients mixture were taken in the amber colored bottle and these mixtures were kept in the selected storage condition. The mixtures were estimated for Dopamine using HPLC method reported in Chapter 3 for any chemical change (degradation) up to four weeks (5, 6).

4.3.3 Stability study

The stability study of DA was performed under two heads, solution state stability and solid state stability.

a) Solution state stability

The solution state stability of DA was studied in different buffered pH solutions of pH 1.2, 3.0 4.8, 6.8, 7.4 and 11 with and without antioxidant (sodium metabisulfide). A stock solution of 1

mg/mL DA was prepared in 0.1% perchloric acid with 0.1 % sodium metabisulfide and from that 1 μg/mL of DA solutions were prepared in different pH solutions for the stability analysis. The prepared samples were stored at 25 ± 3°C and at different time points (1, 2, 3, 4, 5 and 6 days) samples were taken out for drug content analysis using developed HPLC method. The results were analyzed to determine order of degradation and rate constants. The shelf life was expressed as $t_{90\%,}$ which indicates the time taken for drug concentration to come down to 90 % of original concentration.

b) Solid state stability

In this study, 10 mg of DA was weighed and transferred to three clean glass containers. These containers were stored in three separated conditions, ambient temperature, 25 ± 3°C, 60 ± 5 % RH, accelerated conditions, 40 ± 2°C, 75 ± 5% RH and refrigerated condition, 5 ± 3°C for the predetermined time intervals. All the samples were analyzed for drug content at pre determined time points, 1, 2, 3, 4, 5 and 6 months. The results were analyzed to determine order of degradation, rate constants and $t_{90\%}$.

4.4 Results and Discussion

4.4.1 Bulk characterization

The UV-spectrum of DA showed λ_{max} at 280 nm. The FTIR absorption spectra of pure drug illustrated characteristic bands, which were in agreement with the reported data in USP (7) (Fig 4.1).

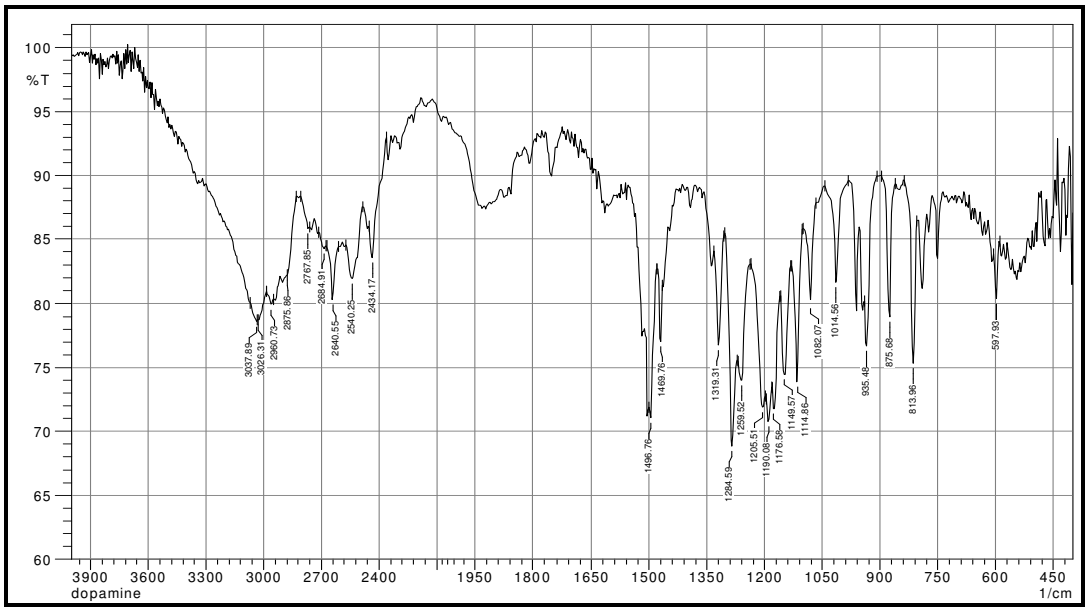

Fig. 4.1 IR spectrum of Dopamine HCl in potassium bromide.

In brief, a strong and broad singlet was attributed to O-H stretching at 3037 cm^{-1}, a strong signal at 2960 cm^{-1} and at 2875 cm^{-1} was assigned to C-H stretching. Two distinct bands due to C=C (aromatic) stretching was observed at 1496 cm^{-1} and at 1469 cm^{-1}. The N-H bending was around 1600 cm^{-1}, C-N stretching at 1284 cm^{-1} and C-O stretching at 1149 cm^{-1} and 1114 cm^{-1}. The differential scanning calorimeter (DSC) showed characteristic melting endothermic peak at 252.17°C, onset 248.93°C, endset 255.00°C and heat -109.24 J/g clearly indicated the crystallinity of DA (Fig.4.2). All these studies showed that the sample obtained was crystalline white powder DA.

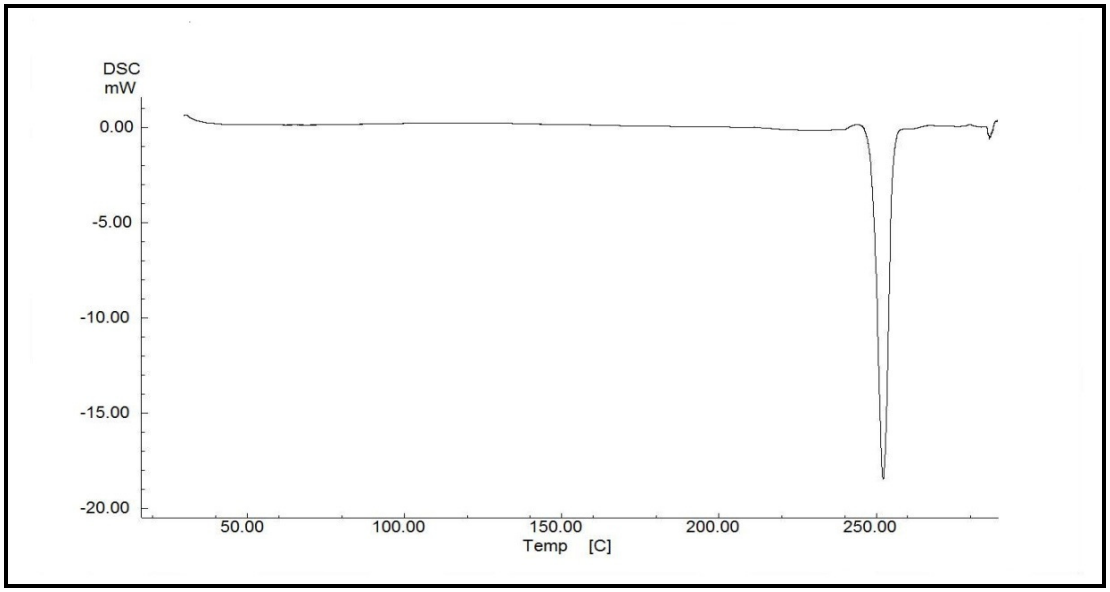

Fig. 4.2 DSC Thermogram of pure Dopamine HCl

4.4.2 Compatibility studies

The FTIR study of physical mixture of drug and excipients showed characteristic IR bands that can be attributed to DA as presented above. In all the drug-lipids, and other excipient mixtures studied, these drug bands were retained representing absence of chemical interaction between the drug and the excipients selected for formulation. FTIR study further established that there is no chemical interaction between drug and the excipients studied. Similar results were obtained when the study was repeated on the samples stored at controlled room temperature for 6 months. DSC thermogram showed above (Fig.4.2) a distinct melting endotherm of DA at 252.17°C. Endothermic peaks recorded in the thermograms are directed downwards. Figure 4.3 to 4.8 represent thermograms of lipids and physical mixture of dopamine with different lipids (1:1) selected for the study. Melting endotherm of drug was well preserved in most of the cases. However a slight change in drug peak shape with little broadening and shifting to higher or lower

temperature was observed in some physical mixtures, which could be attributed to the earlier melting and decomposition of lipids that lowers the purity of each component of the mixture. All the peaks were retained in the physical mixture of drug and lipids indicating lack of interaction between selected lipids and Dopamine.

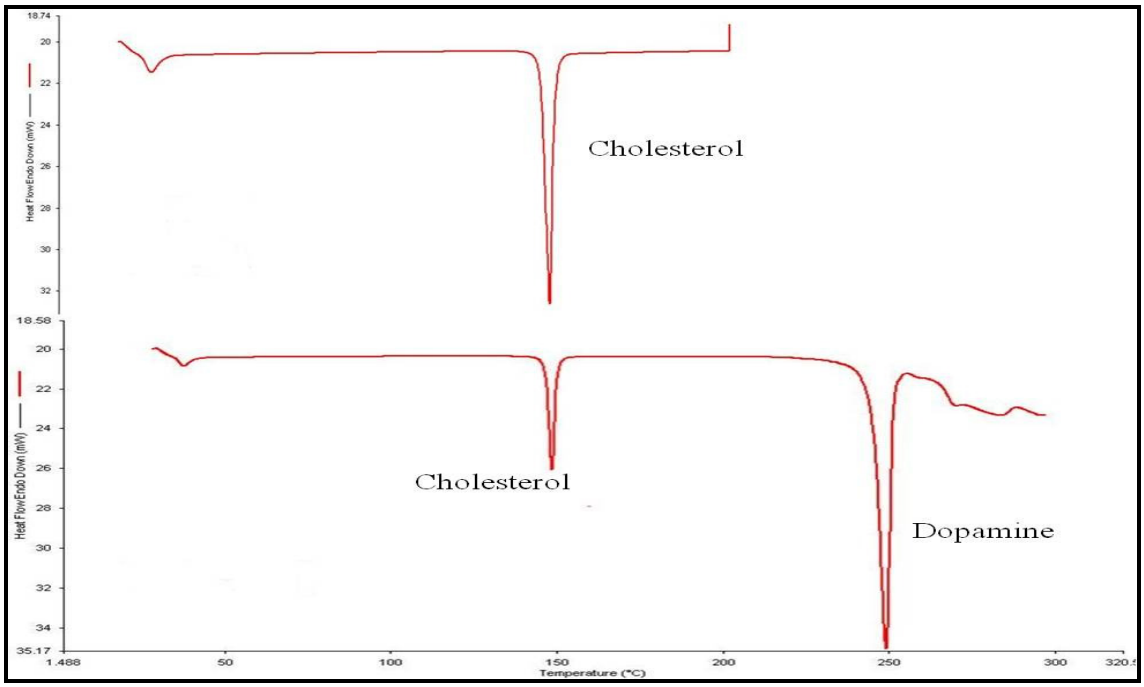

Fig. 4.3 DSC Thermogram of Cholesterol and Cholesterol:Dopamine physical mixture (1:1)

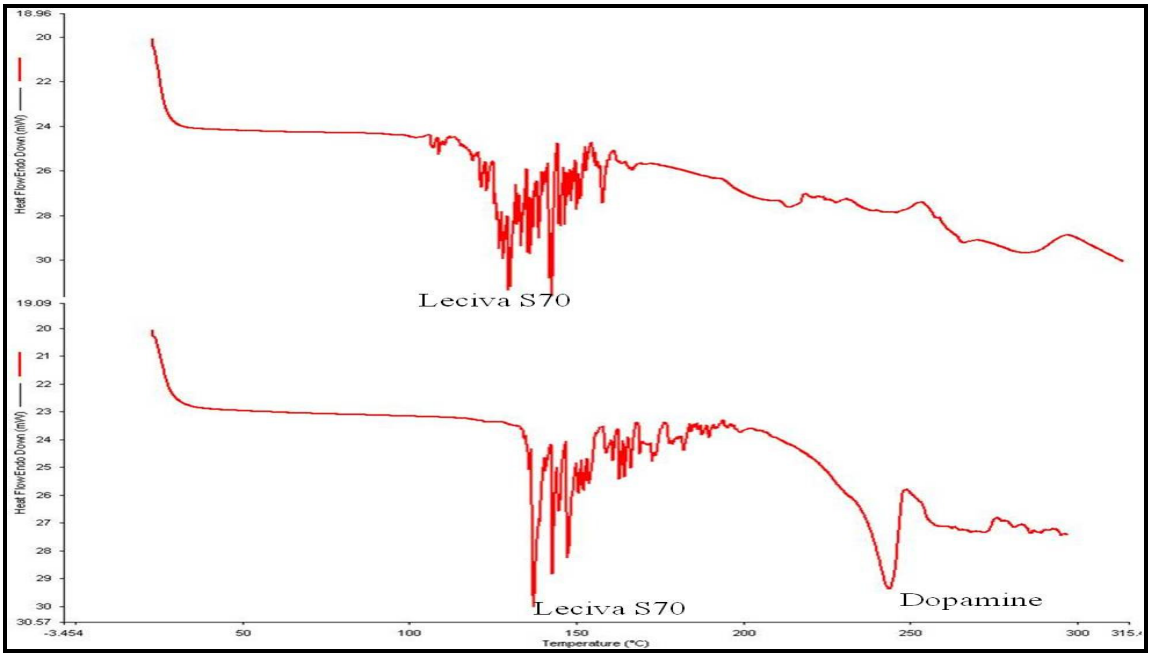

Fig. 4.4 DSC Thermogram of Leciva S70 and Leciva S70:Dopamine physical mixture (1:1)

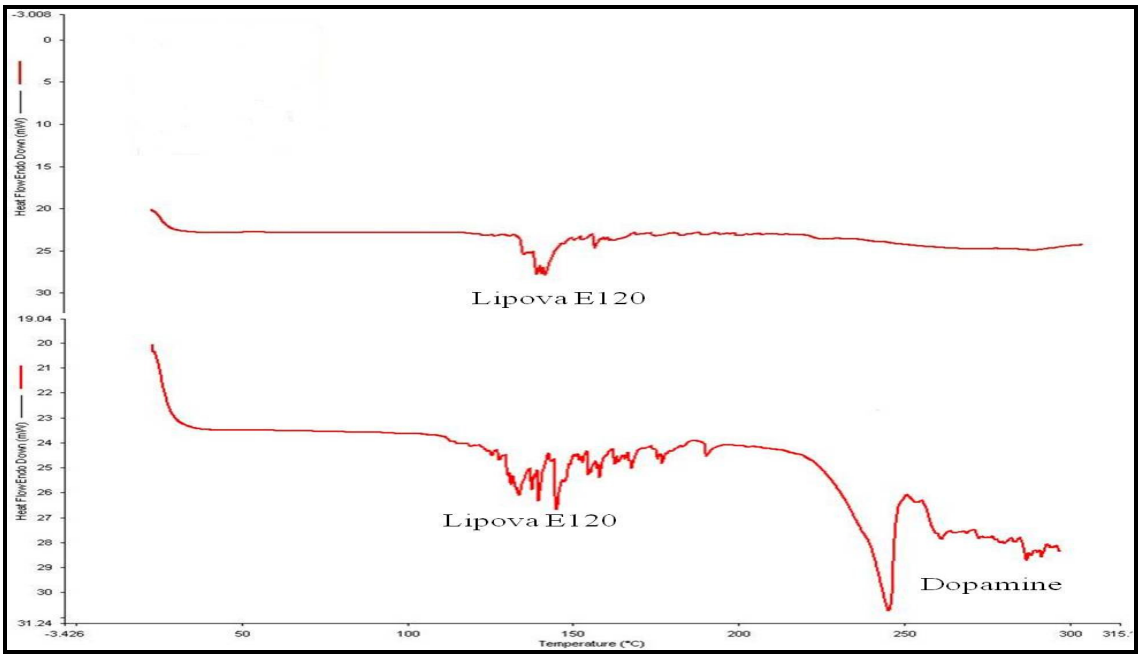

Fig. 4.5 DSC Thermogram of Lipova E120 and Lipova E120:Dopamine physical mixture (1:1)

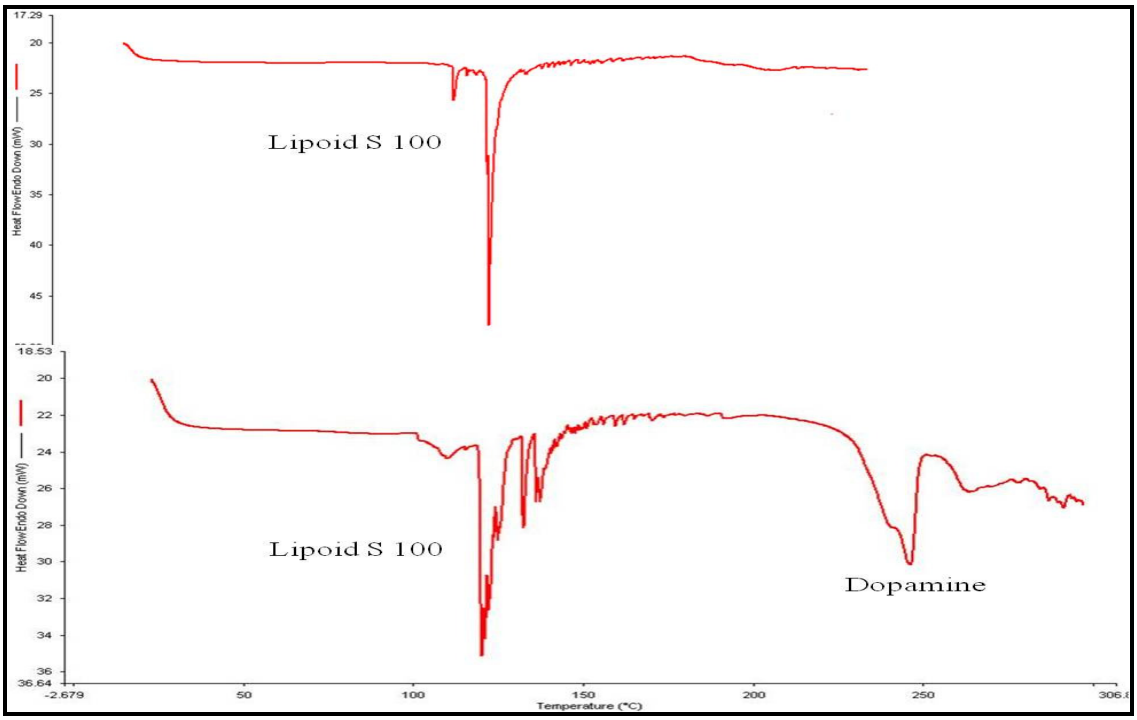

Fig. 4.6 DSC Thermogram of Lipoid Soya PC and Lipoid Soya PC:Dopamine physical mixture (1:1)

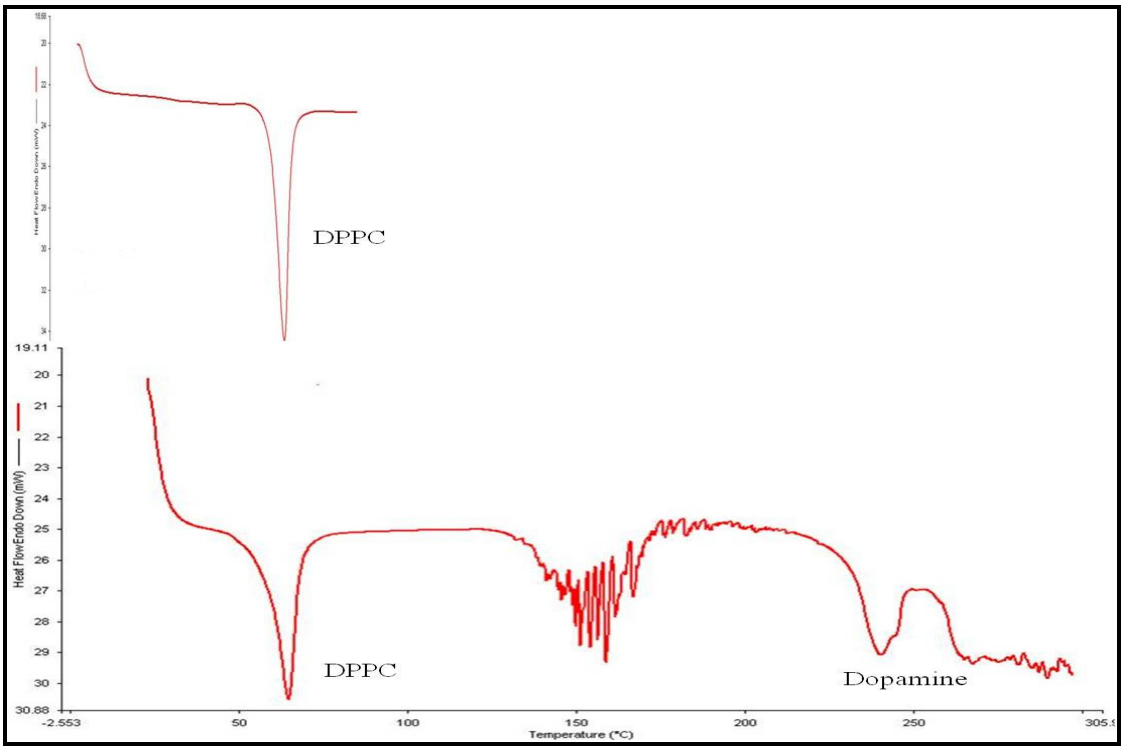

Fig. 4.7 DSC Thermogram of DPPC and DPPC:Dopamine physical mixture (1:1)

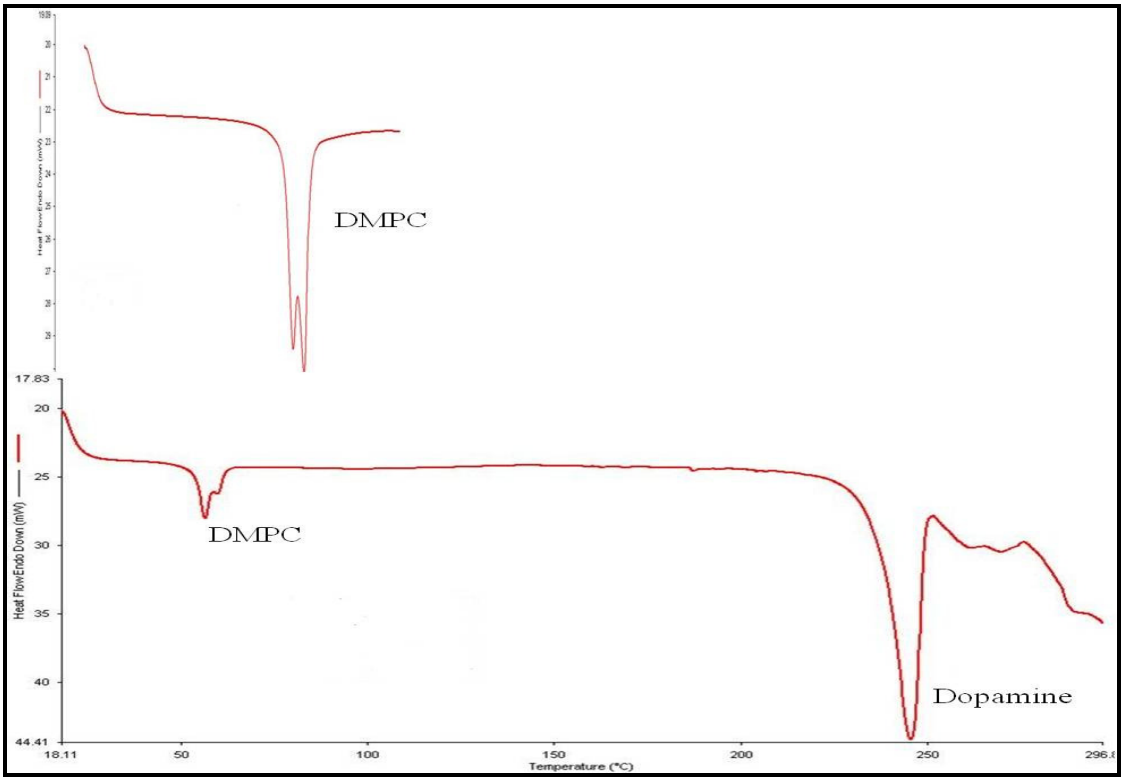

Fig. 4.8 DSC Thermogram of DMPC and DMPC:Dopamine physical mixture (1:1)

The accelerated physical stability test at controlled room temperature of the entire sample showed no characteristic change in the initial color till 4th week (Table 4.1). However, all samples became sticky in nature after 1th week. Though DA has high melting point (252°C), but due to low melting point of the lipids, DA in presence of these lipids became sticky. The drug with lipids and stabilizer stored at 40 ± 2°C/75 ± 5% RH became sticky on first day onwards and at the end of 1st week almost all the tested samples were in sticky nature but no major color change was observed (Table 4.2). In 4th week, all the tested samples were in melt form and it has become solid mass. However, no degradation of products or color change was observed in any sample. The accelerated chemical stability study result showed good % recovery ranging from 100.05 to 100.16. This suggested that there was no chemical interaction of DA with the lipids mixture studied.

4.4.3 Stability study

a) Solution state stability

Dopamine was found to be sensitive towards various pH conditions 1.2, 3.0, 4.8, 6.8, 7.4 and 11. The log % remaining to degrade (RTD) was plotted as a function of time and slope was used to determine the first order degradation rate constants (K_d) at the respective pH conditions. The obtained degradation data of DA in different pH solutions with 1 % sodium metabisulfide as antioxidant and without antioxidant could be well described by the first order degradation kinetics indicating degradation dependent on drug concentration. The regression coefficient (R^2) value was in the range of 0.8647 to 0.9744 without antioxidant and 0.9276 to 0.9833 with antioxidant (1% sodium metabisulfide). Significant difference was observed in the degradation of Dopamine in different pH solution 1.2 to 11 (Fig. 4.9, 4.10). However, in case of pH solutions with antioxidant it was observed that the DA is more stable in acidic pH.

The degradation rate constant (K_{deg}) for all the pH solutions were, 1.1 x 10^{-2}, 52.80 x 10^{-2}, 65.21 x 10^{-2}, 79.29 x 10^{-2}, 86.25 x 10^{-2} and 98.95 x 10^{-2} $days^{-1}$ respectively without antioxidant and 0.32 x 10^{-2}, 0.41 x 10^{-2}, 0.23 x 10^{-2}, 0.76 x 10^{-2}, 3.61 x 10^{-2} and 5.34 x 10^{-2} with antoxidant. The highest K_{deg} was observed in pH 11.00 and the lowest was observed in pH 1.2. The shelf life was expressed as $T_{90\%}$ which indicates the period of storage of the drug without significant loss of the potency. The $t_{90\%}$ (days) of DA in different pH solution were, 9.11, 0.198, 0.161, 0.132, 0.106 and 0.121 days without antioxidant and 32.56, 25.33, 45.59, 13.81, 2.90 and 1.96 days with 1% sodium metabisulfide respectively. This shows that DA is more stable in pH 1.2 without antioxidant and upto pH 6.8 with antioxidant and it is least stable in pH 7.4 and 11.00.

Table 4.1 Dopamine and excipients compatibility study at Controlled Room Temperature (25 ± 2^{O}C/60 ± 5 %RH)

S.NO	Sample	Initial Observation	1st Week	4th Week
1	Dopamine + Leciva S70	Brown color sample	No characteristic change	No color change, sample was sticky
2	Dopamine + Lipova E120	Yellow color sample	No characteristic change	No color change, sample was sticky
3	Dopamine + DPPC	White color sample	No characteristic change	No characteristic change
4	Dopamine + DMPC	White color sample	No characteristic change	No characteristic change
5	Dopamine + S 100	White color sample	No characteristic change	No color change, sample was sticky
6	Dopamine + Cholesterol	White color sample	No characteristic change	No characteristic change
7	Dopamine + Mannitol	White color sample	No characteristic change	No characteristic change
8	Dopamine + Sodium metabisulfide	White color sample	No characteristic change	No characteristic change
9	Leciva S70 + Lipova E120	Brown color sample	No characteristic change	No color change, sample was sticky
10	Leciva S70 + Cholesterol	Brown color sample	No characteristic change	No color change, sample was sticky
11	Lipova E120 + Cholesterol	Yellow color sample	No characteristic change	No color change, sample was sticky
12	Dopamine + Leciva S70 + Cholestrol + Mannitol + Sodium metabisulfide	Brown color sample	No characteristic change	No color change, sample was sticky
13	Dopamine + Lipova E120 + Cholestrol + Mannitol + Sodium metabisulfide	Yellow color sample	No characteristic change	No color change, sample was sticky
14	Dopamine + Leciva S70 + lipova E120 + Cholestrol + Mannitol + Sodium metabisulfide	Brown color sample	No characteristic change	No color change, sample was sticky
15	Dopamine + DPPC + Cholestrol + Mannitol + Sodium metabisulfide	White color sample	No characteristic change	No characteristic change
16	Dopamine + DMPC + Cholestrol + Mannitol + Sodium metabisulfide	White color sample	No characteristic change	No characteristic change

Table 4.2 Dopamine and excipients compatibility study at accelerated storage condition (40 ± 2 °C/75 ± 5% RH)

S.NO	Sample	Initial Observation	1st Week	4th Week
1	Dopamine + Leciva S70	Brown color sample	No color change, sample was sticky	No color change, sample was in melt form
2	Dopamine + Lipova E120	Yellow color sample	No color change, sample was sticky	No color change, sample was in melt form
3	Dopamine + DPPC	White color sample	No color change, sample was sticky	No color change, sample was in melt form
4	Dopamine + DMPC	White color sample	No color change, sample was sticky	No color change, sample was in melt form
5	Dopamine + S 100	White color sample	No color change, sample was sticky	No color change, sample was in melt form
6	Dopamine + Cholesterol	White color sample	No color change, sample was sticky	No color change, sample was in melt form
7	Dopamine + Mannitol	White color sample	No color change, sample was sticky	No color change, sample was in melt form
8	Dopamine + Sodium metabisulfide	White color sample	No color change, sample was sticky	No color change, sample was in melt form
9	Leciva S70 + Lipova E120	Brown color sample	No color change, sample was sticky	No color change, sample was in melt form
10	Leciva S70 + Cholesterol	Brown color sample	No color change, sample was sticky	No color change, sample was in melt form
11	Lipova E120 + Cholesterol	Yellow color sample	No color change, sample was sticky	No color change, sample was in melt form
12	Dopamine + Leciva S70 + Cholestrol+ Mannitol + Sodium metabisulfide	Brown color sample	No color change, sample was sticky	No color change, sample was in melt form
13	Dopamine + Lipova E120 + Cholestrol + Mannitol + Sodium metabisulfide	Yellow color sample	No color change, sample was sticky	No color change, sample was in melt form
14	Dopamine + Leciva S70 + lipova E120 + Cholestrol + Mannitol + Sodium metabisulfide	Brown color sample	No color change, sample was sticky	No color change, sample was in melt form
15	Dopamine + DPPC + Cholestrol + Mannitol + Sodium metabisulfide	White color sample	No color change, sample was sticky	No color change, sample was in melt form
16	Dopamine + DMPC + Cholestrol + Mannitol + Sodium metabisulfide	White color sample	No color change, sample was sticky	No color change, sample was in melt form

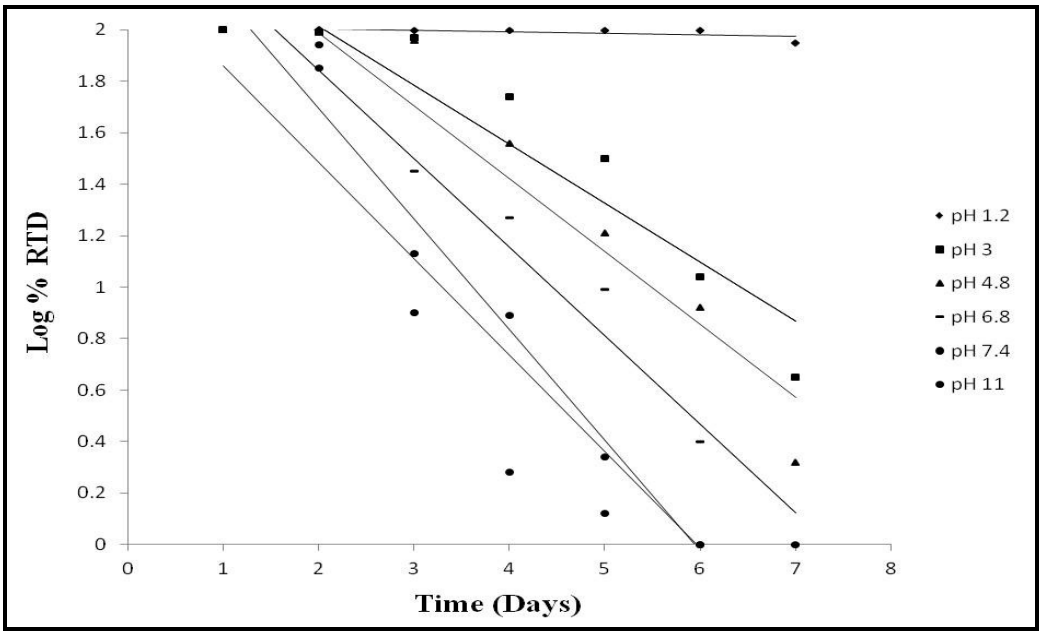

Fig. 4.9 Log % RTD vs. time graph of solution state stability of Dopamine at different pH

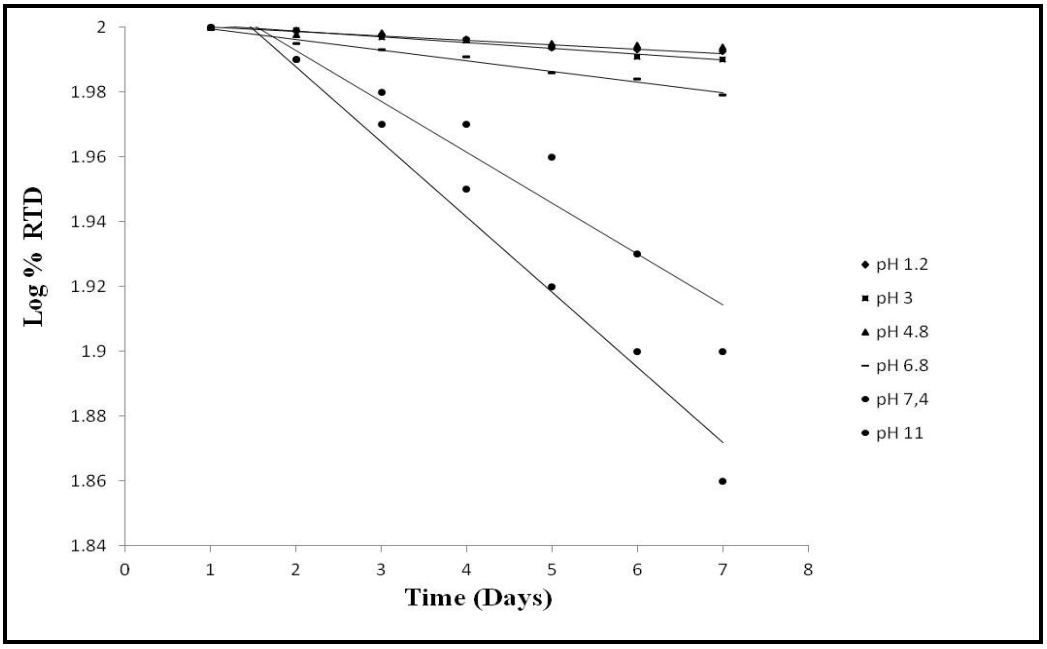

Fig. 4.10 Log % RTD vs. time graph of solution state stability of Dopamine at different pH with 1 % sodium metabisulfide.

The K_{deg} and $t_{90\%}$ values clearly indicated that there was significant effect of pH on the degradation of DA, at highly alkaline condition rapid degradation of DA was observed.

b) Solid state stability

The log % RTD versus time profile of DA in different temperature gave straight line (Fig. 4.11) demonstrating first order degradation with R^2 value close to one, ranging from 0.9292 to 0.9744 respectively. DA was stable up to 6 months at all the stability conditions with DA remaining to degrade was in the range of 99.15 to 100 % respectively. The degradation rate constant (K_{deg}) values obtained were 1.84 x 10^{-3}, 4.145 x 10^{-3} and 4.145 x 10^{-3} $months^{-1}$ respectively. The $t_{90\%}$ of Dopamine in all the three storage conditions, 5 ± 3°C, 25 ± 2°C and 40 ± 2°C were 56.99 ± 4.11, 25.39 ± 8.03 and 25.33 ± 9.32 months respectively. Similarly, photo-stability samples did not show significant degradation (<2 %, 1 month) suggesting that the drug is photo-stable.

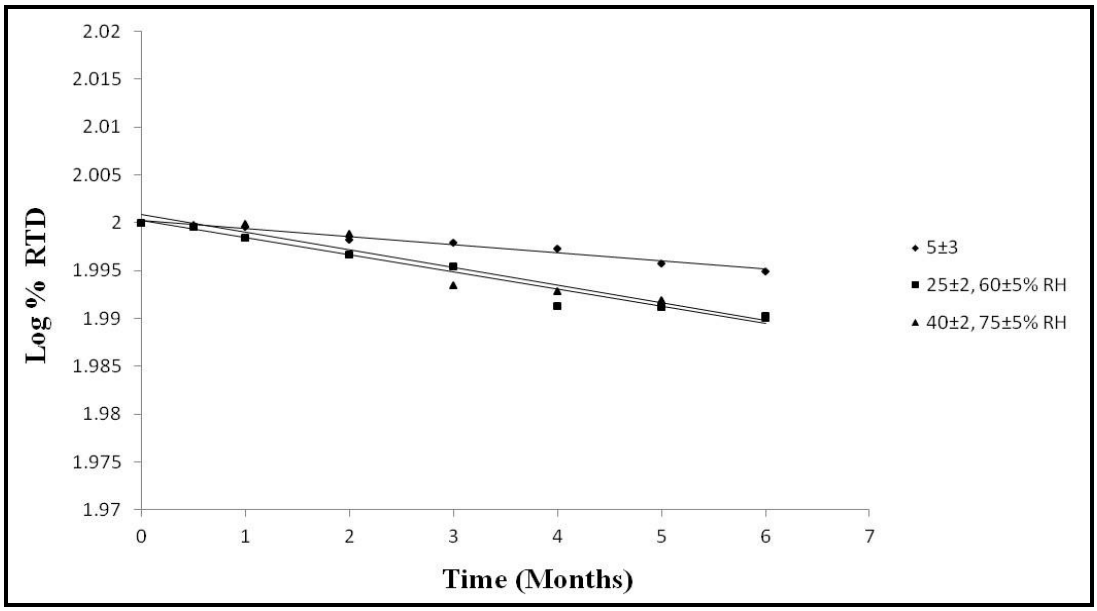

Fig. 4.11 Log % RTD vs. time graph of solid state stability of DA at different temperature

4.5 Conclusion

The UV, FTIR and DSC analysis clearly showed that obtained DA is crystalline nature. The drug-excipient compatibility studies indicated no physical or chemical changes at ambient and accelerated storage conditions. Thus, drug-excipient compatibility studies would support the rationale for selection of various excipients and justify the product life span. The solution state stability studies revealed that the drug follows first order degradation kinetics. Stability studies of solutions indicated that DA is sensitive for alkaline environment as the degradation rate constants were found to be higher at alkaline conditions because of DA oxidation at alkaline pH.However drug was found to be

stable upto pH 6.8 when 1 % sodium metabisulfide was used as antioxidant. Solid state stability confirmed that the drug is stable at refrigerated and ambient temperature.

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Chapter 5.Formulation Design, Development and Characterization of LPDDS

5.1 Introduction

Drug delivery to brain is challenging and needs design of specialized delivery systems and approaches. The management of brain disorders with presently available delivery systems is still difficult and needs betterment because insufficient drug reaching the brain due to presence of highly lipophilic blood brain barrier (BBB). Design of suitable dosage form is very essential for delivering a drug to achieve effective therapy. Continuous efforts are there to have better delivery systems, as conventional dosage forms have various shortcomings in therapy. For direct delivery to brain via nasal route, by-passing the BBB, the olfactory region can be useful to deliver drugs to the cerebrospinal space, for effective treatment of CNS disorders. Though some work was done to overcome resistance of BBB but without much success. It has been proposed that nasal administration may allow a substrate to reach a target in the brain at a higher concentration than would be feasible with other routes of administration (1- 6). Therefore, an attempt was made to deliver dopamine (DA) to the brain using liposomes (LPs) as carriers via nasal route, bypassing BBB, for effective management of CNS disorders. However, the preparation of this delivery system was challenging with influence of several process and formulation parameters. These parameters affect the various characteristics and properties of liposomal formulations and ultimately govern the availability and therapeutic performance of the product.

In this study attempts were made to prepare and characterize DA loaded LPs for better and extended therapy. As an effort to formulate more efficient formulations for nasal delivery, this research work is an endeavour to optimize the amount of lipids and amount of DA required to get the model LPs using different biocompatible lipids. The LPs prepared using Leciva S70, Lipova E120 and Leciva S70 + Lipova E120 lipids were also compared with LPs prepared using well known Soya S100, 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) lipids by various in vitro evaluation. Selected formulations, based on in vitro evaluation results, were further used for in vivo pharmacokinetic and biodistribution studies on animal model.

5.2 Experimental

5.2.1 Materials and Methods

Dopamine hydrochloride (assay 99.95 %) and cholesterol were procured from Sigma-Aldrich chemicals, Bangalore, India. Leciva S70 and Lipova E120 from Vav Life Sciences, Mumbai, India and 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-Dipalmitoyl-sn-glycero-3-

phosphocholine (DPPC) and Lipoid soya S 100 from Lipoid AG, Steinhausen, Switzerland were received as gift sample. All other chemicals and solvents procured were of the highest purity available, either pharmaceutical or analytical grade. Purified water was prepared by filtering the freshly collected Milli-Q® water (Millipore®, France) through 0.22 μm membrane filter (Millipore®, France).

5.2.2 Equipments/Instruments

Buchi rotavapor (Rotavapor Buchi, Switzerland), was used for thin film preparation and organic solvents removal under inert environment at reduced pressure. Bath sonicator (6.5 L Toshibha Laboratory testing instruments, Delhi, India) was used for preparation of emulsion and size reduction. Ultracentrifuge (5340R, Eppendorf AG, Hamburg, Germany) was used for liposomes separation from un-entrapped drug. A lyophilizer (Heto-Dry-Lyo, Heto-Holten, Denmark) was used for freeze drying after freezing sample at -20 ^{o}C in a refrigerator (Frost-free, Vest-Frost, India).

A photon correlation spectroscopy Zetasizer, Nanoseries (Nano-ZS, Malvern Instruments, UK) was used for particle size, polydispersity index (PDI) and zeta-potential (ZP) analysis. Olympus fluorescence phase-contrast inverted microscope (Cambridge Scientific) was used for initial characterization of formulations. A transmission electron microscopy (TEM, Hitachi, H-7500, copper grids/carbon coated) equipped with CCD camera and plate film camera with HC, HR magnification of 6,00,000 X available at Sophisticated Analytical Instrumentation Facility, Panjab University, Chandigarh was employed for acquisition of the images. An atomic force microscopy (AFM, Nanoscope II, USA) using Nova AFM [NTMDT] in semi contact mode at Surface Chemistry Lab, Physics Department, BITS-Pilani was used for morphological (size and shape analysis) characterization.

5.2.3 Preparation of liposomes

The choice of particular method of preparation of LPs is principally determined by the physicochemical properties of the drug. In the present study liposomes were tested for their capacity to encapsulate Dopamine HCl, prepared using different techniques; thin film hydration, reverse phase evaporation and reverse phase evaporation combined with freeze and thaw method. LPs prepared by altered methods with different lipids have shown different properties, like size, loading efficiency (LE), encapsulation efficiency (EE), in vitro release, stability etc.

Thin film hydration (TFH) is a simple technique, in which lipid films were first deposited on a substrate and subsequently hydrated to give LPs. But one of the major disadvantages of this method

is relatively poor encapsulation efficiency (EE) (5-15 %) of hydrophilic drugs, which moreover get reduced on further reduction of LPs size (7- 9).

In Reverse phase evaporation (REV) technique, first water in oil emulsion was formed followed by removal of organic layer, resulting in vesicles formation. REV technique is more advantageous than thin film hydration technique (TFH) especially for encapsulation of hydrophilic drugs (EE≈ 65 %) (10). Freeze and thaw cycles (FTC) are known to further increase the encapsulation efficiency (11). In this method, formation of unilamellar vesicles were achieved by fusion of SUV during freezing and slow thawing cycles (12- 13).

All liposome formulations were characterized for drug entrapment and loading efficiency, average particle size and size distribution, particle morphology, in vitro drug release studies etc.

a) Thin film hydration technique (TFH)

DA loaded LPs were prepared by thin film hydration technique as described previously (Chapter 1, Fig. 1.2). Leciva S70 and cholesterol were weighed in different ratios and dissolved in 10 mL chloroform: methanol (9:1) mixture in 250 mL round bottom flask attached to Buchi rotavapor. The organic solvent was evaporated at 40 ^{o}C under reduced pressure of 345 ± 5 mbar in a stream of nitrogen at 60 rpm. This process was continued until all organic phase gets evaporated and a dry thin film was formed on flask walls. The flask was left in vacuum desiccator overnight to ensure complete removal of residual solvent. Further, dried film was hydrated with 15 mL aqueous phase containing DA and sodium metabisulfide dissolved in phosphate buffer (pH 5.0). The film was hydrated and flask was kept on rotavapor at 100 rpm until the film got dispersed in aqueous phase. The hydration was performed at above the phase transition temperature (T_m) of the lipids and under a stream of nitrogen in reduced pressure to avoid oxidation of drug and lipids. After hydration, this dispersion was allowed to stand for some time for vesicle formation. Finally, the multilamellar liposomes suspension was bath sonicated for 30 min (ice bath) for size reduction and stored at 4^{o}C for 24 h. DA containing LPs were separated from un-entrapped drug by centrifugation at 17,500 rpm at 4 ^{o}C for 30 min and washed with buffer twice and used for further study.

b) Reverse phase evaporation technique (REV)

DA loaded LPs were prepared by REV technique using Leciva S70, Lipova E120, S 100, DPPC, DMPC alone or combination with or without and cholesterol in different ratios. This procedure was based on two important steps. In first step the lipids with cholesterol in different ratios was dissolved in 50 mL diethyl ether in 250 mL round bottom flask. The aqueous phase was prepared by dissolving DA in 15 mL phosphate buffer pH 5.0 with sodium metabisulfide. In next step aqueous

phase was added to organic phase maintaining inert environment using nitrogen (Fig. 5.1). The resulting two-phase system was sonicated using bath sonicator (ice bath) for 30 min until the mixture becomes either clear one-phase dispersion or a homogeneous opalescent dispersion. Basically this resulted into w/o kind of emulsion ensuring that emulsion was not separated at least for 30 min. The mixture was then attached to rotavapor under reduced pressure in stream of nitrogen at 20-25 °C, rotating at 100 rpm to remove organic solvent.

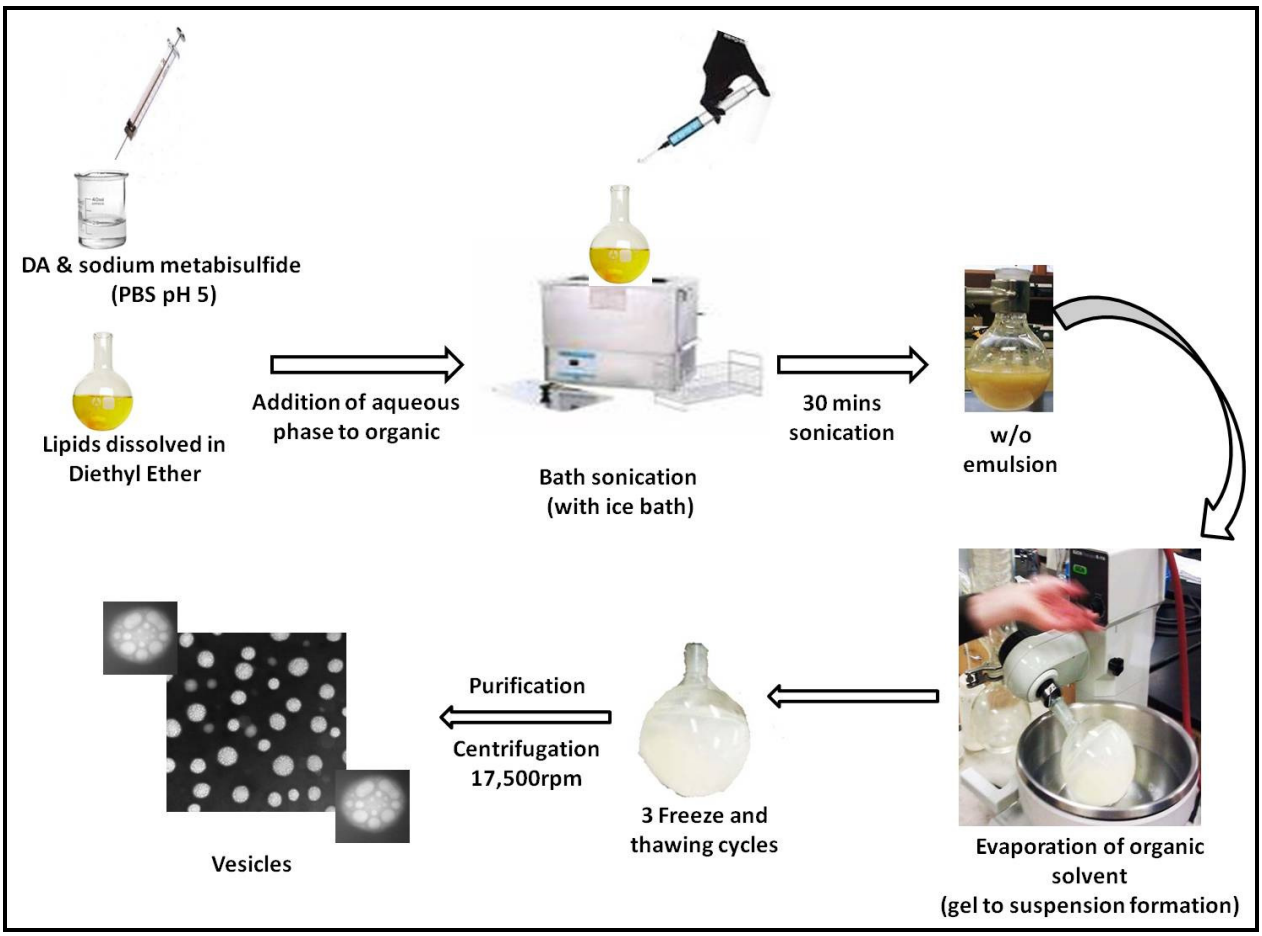

Fig. 5.1 Schematic representation of reverse phase evaporation technique used for preparation of DA loaded LPs

During evaporation of the organic solvent, the system produced froths. As the majority of solvent got removed, the material first formed a viscous gel and subsequently (within 5-10 min) it became an aqueous suspension. Additional buffer was added as the suspension was more viscous at this stage and evaporated for additional time to remove residual solvent. After complete removal of organic solvent, this suspension was kept at 4 °C for 2-3 h until vesicles formed.

For freeze and thaw cycles (FTC), the suspension was stored at -80 °C and thawed at room temperature. Same way, three cycles were repeated. Finally DA containing LPs were separated from un-entrapped drug by centrifugation at 17,500 rpm at 4 °C for 30 min and washed with buffer two times and were used for further study.

c) Freeze drying and storage

The prepared liposomes, separated by centrifugation technique, were immediately freeze dried using mannitol (10 %) as a cryoprotective agent. For this purpose, individual formulation was conveniently transferred in glass container and were frozen to -20 °C for at least 24 h. Freeze drying was carried out with vacuum (1 mbar, -110 °C), until free flowing powder was obtained. Processed samples were stored at refrigerated conditions in sealed glass containers.

5.2.4 Effect of formulation and process variables

Liposomes prepared by different methods containing Dopamine HCl, were tested for various parameters. The effect of various formulation parameters and process variables on characteristics of Leciva S70, Lipova E120 and combination of Leciva S70 and Lipova E120 liposomes were investigated. Liposomes prepared using these lipids were also compared with LPs prepared using lipids; soya S100, DMPC and DPPC in respect of size and shape characteristics, encapsulation efficiency (EE), loading efficiency (LE) and in vitro drug release. Different parameters varied are preparation techniques, composition of hydration media, drug and lipid ratio, cholesterol amount, sonication strength (intensity and duration of sonication), evaporation rate (vacuum conditions) and freeze and thawing cycles by varying one selected variable at a time keeping all other parameters constant. The influences of drug amount and lipid: cholesterol ratios on the EE and LE, PDI**,** ZP were also studied comprehensively. In addition, effect of amount of cholesterol on in-vitro dissolution was studied. Specific formulation composition and physical characters for individual liposome formulations are presented in Table 5.1-5.6.

5.2.5 Characterization of liposomes

All the prepared liposomal formulations were extensively characterized as per following procedures.

a) Determination of Dopamine in liposomes

The drug analysis was performed to determine the entrapment efficiency and loading efficiency of the individual liposomal formulations. Accurately weighed freeze-dried LPs were transferred to a fresh calibrated flask. In order to release entrapped drug, the LPs were digested by dissolving in IPA with ultra-sonication (30 min, 25 °C). Then it was suitably diluted with buffer (pH 4.0) and after filtering through 0.22 μm membrane filter (Millipore®) was used for analysis. The amount of drug

was determined using analytical method described in Chapter 3. Each determination was performed in triplicate and values are represented as average with standard deviations.

The encapsulation efficiency (EE) was calculated from the total amount of the drug entrapped by using following formula

$$EE\ (\%) = \frac{\text{Amount of drug in LPs (mg)}}{\text{Initial amount of drug taken (mg)}} \times 100$$

The loading efficiency (LE) was calculated from the amount of the drug present per unit weight of the final product using following formula

$$LE\ (\%) = \frac{\text{Amount of drug in LPs}}{\text{Amount of LPs}} \times 100$$

b) Particle size and size distribution

The particle size and size distribution of individual formulation were analyzed by photon correlation spectroscopy using a Zetasizer. Freeze-dried liposomal formulations were suitably dispersed in Milli-Q® water. For each measurement, sufficiently diluted liposome dispersions (1 mg/mL) were assessed for average particle size and polydispersity index (PDI).

c) Particle shape and morphology

The morphological characterization and direct visualization of the prepared liposomal formulations were performed using microscopy, transmission electron microscopy (TEM) and atomic force microscopy (AFM). The prepared LPs were dispersed in pure water by sonication and filtered using Millipore® ultrafiltration system for analysis.

For TEM, a drop of liposome formulation containing 0.02 % phosphotungstic acid was placed on a carbon coated grid of TEM and allowed to equilibrate. Excess liquid was removed and the grid was dried at room temperature. The prepared samples were micro-graphed at 80 to 100 kV on a digital TEM station. For AFM, a drop of liposomal formulation was loaded onto an AFM silicone stub by spin coat technique and immediately dried under vacuum. Processed samples were scanned at 20 Hz in semi contact mode using a digital multicode scanning probe microscope station.

d) In vitro drug release studies

The in-vitro drug release from the prepared liposome formulations were performed with dialysis bag (Spectrapor, molecular weight cut off: 12,500 Da, USA) as previously reported by several authors (16, 17). The appropriate amount of each DA loaded freeze dried liposome formulations were

accurately weighed and dispersed in 2 mL phosphate buffer solution (100 mM, pH 5.0). This liposome dispersion was introduced into a dialysis membrane bag and the sealed bag was immediately placed in release media. The release study was carried out in 100 mL of phosphate buffer (pH 5.0) with 1 % (w/v) sodium metabisulfide maintained at 37 ± 2 °C and the medium was covered to prevent evaporation losses. In addition, flux conditions were ensured by continuous stirring of the medium at 100 rpm. Samples, 1 mL aliquots were withdrawn from the beaker at predetermined time points, with spontaneous replacement of equal volume of fresh buffer media.

All samples were suitably diluted in mobile phase and DA concentration was estimated using analytical method mentioned in Chapter 3. The percent cumulative release was calculated and plotted as a function of time. The obtained dissolution data were fitted to various kinetic equations, Zero order, First order, Higuchi, Korsmeyer-Peppas, Hixson-Crowell, Makoid- Banakar and Baker-Lonsdate model to find the order and mechanism of DA release from the LPs (18).

Zero order kinetic equations

$$F = k_0 \times t \quad (1)$$

First order kinetic equations

$$F = 100\left(1 - e^{-kt}\right) \quad (2)$$

Higuchi square-root equation

$$F = k_H t^{0.5} \quad (3)$$

Korsmeyer-Peppas equation

$$F = k_{kp} t^n \quad (4)$$

Hixson-Crowell equation

$$F = 100\left[1 - (1 - k_{hc} t)^3\right] \quad (5)$$

Makoid-Banakar (MB) equation

$$F = k_{MB} t \hat{} n \; Exp(-kt) \quad (6)$$

Baker-Lonsdate (BK) equation

$$BL = 3/2\left[1 - (1 - F/100)^{2/3}\right] - F/100 = k_{BL} t, k_{BL} = \left[3 \times D \times C_s / \left(r_0^{\,2} \times C_0\right)\right] \quad (7)$$

Where, F is the percentage drug released in time t, k_0 (μg h^{-1}) is the zero-order release constant, k (μg h^{-1}) is the first-order release constant, k_H (μg $(h^{-0.5})^{-1}$) is the Higuchi release constant, k_{KP} (μg $(h^{-n})^{-1}$) is the release constant incorporating structural and geometric characteristics of the drug-dosage form; n is the diffusional exponent indicating the drug-release mechanism, in case of non-Fickian release, n

falls between 0.5 and 1.0 ($0.5 < n < 1.0$), while for Fickian diffusion n = 0.5, for zero order release (case II transport) n = 1, and for supercase II transport, n > 1. The values of n as estimated by linear regression of F vs. log (t). k_{HC} (μg $(h^{-3})^{-1}$) is the release constant in Hixson–Crowell model, k_{MB} (μg h^{-1}) is the combined constant in Makoid-Banakar method, k_{BL} (μg h^{-1}), is the combined constant in Baker–Lonsdale model, D is the diffusion coefficient, Cs is the saturation solubility, r_0 is the initial radius for a sphere or cylinder or the half-thickness for a slab, C_0 is the initial drug loading in the matrix. The model providing relatively high regression coefficient (nearing 1) were considered suitable. The time required to release 25 %, 50 % and 90 % of the drug was expressed as $t_{25\%}$, $t_{50\%}$ and $t_{90\%}$ (h) respectively.

The in-vitro dissolution data was used to find, area under the dissolution curve (AUDC), area between the drug dissolution curve and its asymptote (ABC), mean dissolution time (MDT), variance of dissolution time (VDT), relative dispersion of dissolution time (RD) and dissolution efficiency (DE). All the parameters were calculated using a model-independent nonparametric method based on the linear trapezoidal rule, using standard formula (18).

e) Stability studies

The physical state of entrapped drug in the optimized liposome formulations were assessed by performing thermal studies using a differential scanning calorimeter (DSC). All measurements were carried out on a previously calibrated DSC, as described earlier. The glass transition temperature (T_g) was recorded as the midpoint of the curve between pre and post transitions baselines. The peak melting temperature (T_m) and heat of fusion were determined using software and were used to determine the percent crystallinity. Reported data of the heat of fusion for complete crystalline state of the lipids was compared with standard values reported in literature (19- 21). The thermograms of the optimized liposome formulations loaded with and without DA were compared with the pure drug, pure lipids and physical mixture as mentioned in Preformulation Chapter 4.

US-FDA has not framed any specific guidelines for liposomal drug delivery systems (LPDDS), production, characterization, handling and use. There is no protocol and limits available from any international bodies to conduct stability studies of these formulations. In the present work, the claimed stability of optimized liposome formulations, in dispersed and freeze-dried state, was investigated over a period of time by exposing samples in three different conditions, ambient temperature (15 ± 5 °C), refrigerator (5 ± 3 °C) and freeze (-20 ± 5 °C) over a period of 4 months. The LPs were evaluated at 0, 1 and 4 months for their size, PDI, ZP, LE, EE and in-vitro

dissolution. In addition any change in physical appearances was observed and the samples were characterized for their shape and structure using AFM.

5.3 Results and Discussion

5.3.1 Preparation of Liposomes

Composition and physical characters of prepared liposomes are presented in Table 5.1-5.6. REV technique coupled with freeze and thaw method was found to be best suitable for preparation of DA loaded liposomes. REV is the most reproducible and economical method among all for incorporation of hydrophilic drugs with high encapsulation efficiency. Based on the initial experiment, organic to aqueous phase ratio, 2:1 for thin film hydration technique and 10:3 for reverse phase evaporation technique was fixed constant throughout the study when other formulation parameters were changed. In thin film hydration technique (TFH) the encapsulation efficiency and loading efficiency was very low when compared to reverse phase evaporation technique (REV). This might be as drug is not coming in contact with organic phase in which lipids are solubilised, and its high solubility in aqueous phase, it is not getting well entrapped into formed LPs.

On the other hand in case of REV, the initial sonication of organic and aqueous phase formed primary w/o emulsion which shaped into small water droplets stabilized by a phospholipid monolayer (23). On subsequent addition of water these droplets became worm-like micelles (24- 26), due to hydrogen bonds between the phosphate groups in lipids and aqueous phase (27). As the organic phase begins evaporating, the extended micelles start overlapping after attaining a threshold length, forming a thermo-reversible organogel (28). As a consequence of further water addition to organogel, these micelles coalesce into vesicles. Further this end, organogel precipitated and the solution viscosity decreased. The nonpolar chains of phosphatidylcholine (PC) self organised, to prevent interactions with water, while the polar heads face the water molecules. In case of REV along with freeze and thaw method, there was further increase in encapsulation efficiency with lesser particle size and PDI. This is due to well reported fact that the freeze thawing of LPs increases the trapped volume (29- 31). This process reflected a physical disruption of lamellar structure, due to ice crystal formed in freezing step resulting into expansion of inner water phase (32) and dehydration of hydrated head group of lipids. Further, increase in trapped volume resulted in significant decrease in number of lamellarity as well as absence of dense concentric inner lamellar.

Table 5.1 Composition and characterization of DA loaded Leciva S70 LPs prepared by "Thin film hydration technique" *

Batch Code	Drug (mg)	Organic Phase (mL) $CHCl_3$:MET	Tween 80 (%v/v)	Mean Size (nm ±SD)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]LE (% w/w)
LecivaS70/TF/01	50	6.6:3.4	----	209.51 ± 3.28	0.40 ± 0.15	-24.30 ± 0.72	3.68 ± 0.14	2.4 ± 1.11
LecivaS70/TF/02	100	6.6:3.4	----	202.83 ± 7.63	0.42 ± 0.12	-22.11 ± 0.34	2.38 ± 0.54	4.46 ± 0.83
LecivaS70/TF/03	200	6.6:3.4	----	195.32 ± 9.44	0.30 ± 0.14	-22.63 ± 0.61	1.79 ± 1.10	7.43 ± 0.42
LecivaS70/TF/04	250	6.6:3.4	----	197.63 ± 3.24	0.37 ± 0.12	-21.02 ± 0.22	1.78 ± 1.07	16.93 ± 1.06
LecivaS70/TF/05	2.5	9:1	----	258.02 ± 4.31	0.27 ± 0.14	-51.82 ± 0.22	33.35 ± 0.03	0.06 ± 0.51
LecivaS70/TF/06	5	9:1	----	240.32 ± 5.31	0.26 ± 0.12	-48.01 ± 0.19	23.02 ± 0.21	0.25 ± 0.73
LecivaS70/TF/07	10	9:1	----	229.04 ± 6.56	0.20 ± 0.12	-48.52 ± 1.29	16.25 ± 0.20	1.09 ± 0.32
LecivaS70/TF/08	20	9:1	----	220.13 ±9.92	0.26 ± 0.13	-44.44 ± 0.51	10.75 ± 0.10	1.17 ± 1.03
LecivaS70/TTF/01	2.5	9:1	3	405.61 ± 7.32	0.30 ± 0.09	-23.66 ± 0.89	39.94 ± 1.32	0.17 ± 0.86
LecivaS70/TTF/02	5	9:1	3	367.90 ±4.21	0.37 ± 0.09	-16.45 ± 0.40	33.87 ± 1.09	0.67 ± 1.04
LecivaS70/TTF/03	10	9:1	3	307.52 ±3.95	0.32 ± 0.11	-16.43 ± 0.17	24.64 ± 2.98	1.23 ± 0.12
LecivaS70/TTF/04	20	9:1	3	309.7 ± 4.61	0.22 ± 0.13	-14.45 ± 0.70	17.17 ± 1.42	1.57 ± 0.90

In all formulations amount of phospholipid Leciva S70 was 205.72 mg and Cholesterol was 45.64 mg.

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Loading Efficiency

Table 5.2 Composition and characterization of DA loaded Leciva S70 LPs prepared by "Reverse phase evaporation technique" *

Batch Code	Drug (mg)	Leciva S70 (mg)	Cholest-erol (mg)	Hydration Media	Mean Size (nm ±SD)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]LE (% w/w)
LecivaS70/REV/01	---	100	----	PBS(pH5.0)	491.81 ± 9.28	0.62 ± 0.09	-25.80 ± 0.17	----	----
LecivaS70/REV/02	---	50	50	PBS(pH5.0)	232.90 ± 10.3	0.87 ± 0.02	-26.70 ± 0.82	----	----
LecivaS70/REV/03	10	100	----	PBS(pH5.0)	142.92 ± 9.44	0.23 ± 0.04	-20.43 ± 0.19	31.83 ± 2.11	2.12 ± 0.42
LecivaS70/REV/04	10	50	50	PBS(pH5.0)	491.53 ± 7.24	1.0 ± 0.00	-19.62 ± 0.20	24.37 ± 5.17	1.56 ± 0.06
LecivaS70/REV/05	50	500	500	PBS(pH5.0)	271.32 ± 4.33	1.0 ± 0.00	-35.32 ± 0.60	17.38 ± 0.13	0.60 ± 0.11
LecivaS70/REV/06	100	500	500	PBS(pH5.0)	294.52 ± 5.41	1.0 ± 0.01	-48.01 ± 0.99	28.77 ± 1.01	0.99 ± 0.83
LecivaS70/REV/07	150	500	500	PBS(pH5.0)	294.50 ± 6.36	1.0 ± 0.02	-48.52 ± 1.09	16.25 ± 0.20	1.09 ± 0.02
LecivaS70/REV/08	200	500	500	PBS(pH5.0)	220.13 ± 9.92	0.26 ± 0.01	-44.44 ± 1.01	10.75 ± 0.09	1.17 ± 0.13
LecivaS70/REV/09	10	700	300	PBS(pH5.0)	425.4 ± 3.32	0.501 ± 0.09	-40.93 ± 0.14	51.36 ± 4.32	0.36 ± 0.08
LecivaS70/REV/11	10	500	500	PBS(pH5.0)	638.52 ±1.95	1.0 ± 0.00	-46.76 ± 0.87	40.94 ± 3.10	0.28 ± 0.12
LecivaS70/REV/H-04	100	500	500	10% Sucrose	449.6 ± 8.61	1.0 ± 0.01	-33.25 ± 0.10	21.62 ± 1.42	1.32 ± 0.71
LecivaS70/REV/H-07	50	500	500	10% Sucrose	397.9 ± 5.11	1.0 ±0.00	-35.5 ± 0.73	28.7 ±2.10	0.92 ±0.01
LecivaS70/REV/H-08	100	700	300	10% Sucrose	404 ± 9.41	0.720 ± 0.11	-35.5 ±1.00	36.99 ±1.00	2.32 ± 0.05
LecivaS70/REV/H-09	10	700	300	10% Sucrose	657.63 ± 2.91	0.503 ± 0.03	-23 ± 1.05	36.87 ±2.93	0.25 ± 0.16
LecivaS70/REV/H-11	10	500	500	10% Sucrose	683.9 ± 1.10	1.0 ±0.00	-19.8 ± 0.99	21.90 ±0.99	0.14 ± 0.51

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Loading Efficiency

Table 5.3 Composition and characterization of DA loaded Leciva S70 LPs prepared by "Reverse phase evaporation technique with FTC"*

Batch Code	Drug (mg)	Leciva S70 (mg)	Cholest-erol (mg)	Mean Size (nm ±SD)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]LE (% w/w)
LecivaS70/REV/FTC/09	10	700	300	438.52 ± 9.28	0.59 ± 0.09	-46.60 ± 0.17	74.55 ± 0.23	0.54 ± 0.04
LecivaS70/REV/FTC/11	10	500	500	321.90 ± 10.3	0.93 ± 0.02	-48.70 ± 0.82	61.04 ± 0.20	0.40 ± 0.02
LecivaS70/REV/FTC/12	20	700	300	374.92 ± 9.44	0.63 ± 0.04	-42.9 ± 0.19	74.83 ± 2.11	1.04 ± 0.42
LecivaS70/REV/FTC/13	20	500	500	348.53 ± 7.24	1.0 ± 0.00	-45.62 ± 0.20	45.2 ± 5.17	0.63 ± 0.06
LecivaS70/REV/FTC/14	30	700	300	338.32 ± 4.33	0.58 ± 0.8	-45.8 ± 0.60	64.28 ± 0.13	1.34 ± 0.11
LecivaS70/REV/FTC/15	30	500	500	277.52 ± 5.41	0.87 ± 0.1	-42.7 ± 0.99	44.25 ± 1.01	0.92 ± 0.83
LecivaS70/REV/FTC/16	40	700	300	259.50 ± 6.36	0.89 ± 0.2	-44.50 ± 1.09	36.75 ± 0.20	1.07 ± 0.02
LecivaS70/REV/FTC/17	40	500	500	215.50 ± 6.36	0.96 ± 0.2	-45.60 ± 1.09	30.97 ± 0.20	0.858 ± 0.02

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Loading Efficiency

Table 5.4 Composition and characterization of DA loaded Lipova E120 LPs prepared by "Reverse phase evaporation technique with FTC"*

Batch Code	Drug (mg)	Lipova E120 (mg)	Cholest-erol (mg)	Mean Size (nm ±SD)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]LE (% w/w)
LipovaE120/REV/FTC/01	--	100	---	353.52 ± 9.28	0.90 ± 0.09	-23.60 ± 0.17	----	----
LipovaE120/REV/FTC/02	--	50	50	232.60 ±10.30	0.71 ± 0.02	-21.80 ± 0.82	----	----
LipovaE120/REV/FTC/03	10	500	500	590.92 ± 9.44	0.80 ± 0.04	-43.65 ± 0.19	62.07 ± 2.11	0.43 ± 0.42
LipovaE120/REV/FTC/04	10	700	300	378.25 ± 7.24	0.87 ± 0.00	-46.00 ± 0.20	44.75 ± 5.17	0.19 ± 0.06
LipovaE120/REV/FTC/05	20	500	500	496.32 ± 4.33	1.00 ± 0.80	-43.4 ± 0.60	58.58 ± 0.13	0.62 ± 0.11
LipovaE120/REV/FTC/06	20	700	300	434.50 ± 6.36	0.96 ± 0.20	-42.60 ± 1.09	30.97 ± 0.20	0.76 ± 0.02
LipovaE120/REV/FTC/07	30	500	500	385.38 ± 9.54	1.00 ± 0.14	-41.20 ± 3.12	35.55 ± 2.65	0.71 ± 0.03
LipovaE120/REV/FTC/08	30	700	300	327.11 ± 3.65	1.00 ± 0.01	-40.22 ± 2.45	28.58 ± 1.32	0.67 ± 0.01

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Loading Efficiency

Table 5.5 Composition and characterization of DA loaded Leciva S70 + Lipova E120 LPs prepared by "Reverse phase evaporation technique with FTC"*

Batch Code	Drug (mg)	Lipova E120 (mg)	Leciva S70 (mg)	Cholest-erol (mg)	Mean Size (nm ±SD)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]LE (% w/w)
S70+E120/REV/FTC/01	10	250	250	500	221.52 ± 6.79	1.0 ± 0.09	-52.60 ± 0.17	39.6 ± 6.61	0.27 ± 0.93
S70+E120/REV/FTC/02	10	350	350	300	768.60 ± 10.3	0.66 ± 0.02	-39.30 ± 0.82	76.7 ± 1.98	0.54 ± 0.75
S70+E120/REV/FTC/03	20	250	250	500	398.92 ± 9.44	0.72 ± 0.04	-46.35 ± 0.19	51.78 ± 2.11	0.72 ± 0.42
S70+E120/REV/FTC/04	20	350	350	300	878.25 ± 7.24	0.77 ± 0.00	-35.60 ± 0.20	46.25 ± 5.17	0.65 ± 0.06
S70+E120/REV/FTC/05	30	250	250	500	164.32 ± 4.33	0.63 ± 0.8	-35.4 ± 0.60	40.40 ± 0.13	0.85 ± 0.11
S70+E120/REV/FTC/06	30	350	350	300	438.50 ± 6.36	0.96 ± 0.2	-32.60 ± 1.09	30.97 ± 0.20	0.76 ± 0.02
S70+E120/REV/FTC/07	20	210	490	300	392 ± 4.67	0.513 ± 0.01	-43.80 ± 0.61	58.3 ± 3.19	0.81 ± 0.33
S70+E120/REV/FTC/08	20	150	350	500	335 ± 3.76	0.82 ± 0.14	-41.1 ± 0.70	35 ± 4.58	0.49 ± 0.27
S70+E120/REV/FTC/09	20	490	210	300	607.8 ± 4.59	0.674 ± 0.02	-43.5 ± 1.95	26 ± 2.99	0.37 ± 0.03
S70+E120/REV/FTC/10	20	350	150	500	633 ± 2.89	0.95 ± 0.04	-38.9 ± 0.28	18 ± 0.12	0.25 ± 1.68
S70+E120/REV/FTC/11	30	490	210	300	422.8 ± 5.32	1.0 ± 0.08	-39.1 ± 0.81	25.59 ± 1.90	0.54 ± 1.96

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Loading Efficiency

Table 5.6 Composition and characterization of Dopamine loaded DPPC/DMPC/S-100 LPs prepared by "Reverse phase evaporation technique with FTC"*

Batch Code	Drug (mg)	DPPC (mg)	DMPC (mg)	S 100	Cholest-erol (mg)	Mean Size (nm ±SD)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]LE (% w/w)
DPPC/REV/FTC/01	30	700	---	---	300	23.52 ± 15.28	0.88 ± 0.09	-4.63 ± 0.17	46.7 ± 2.93	0.97 ±0.05
DMPC/REV/FTC/02	30	---	700	---	300	151.60 ± 10.30	0.73 ± 0.02	-4.58 ± 0.82	16.74 ± 1.50	0.35 ± 0.03
S-100/REV/FTC/03	30	---	---	700	300	429.00 ± 3.05	0.62 ± 0.16	-14.5 ± 1.34	61.6 ± 0.51	1.29 ± 0.02

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Loading Efficiency

During thawing, exposed hydrophobic cores fused within adjacent lamellae to form new vesicles with decreased lamellarity (33). This indicated that almost all the LPs are oligolamellar vesicles or unilamellar in nature. Subsequent increase in number of freeze and thaw cycles resulted in further increase of trapped volume judged by increase in EE. After 3 freeze and thaw cycles, the EE was observed to be constant due to no further increase in trapped volume inside LPs. The REV+FTC method produced stable, narrow size LPs without using any toxic chlorinated solvents with good encapsulation efficiency and was found to be reproducible.

5.3.2 Physiochemical characterization of liposomes

a) Surface properties of liposomes

The detailed morphological properties of the prepared LPs were investigated and presented using the sophisticated microscopic techniques (Fig. 5.2), transmission electron microscopy (TEM) (Fig. 5.3) and atomic force microscopy (AFM) (Fig. 5.4). The simple microscopic image showed that all the prepared LPs using Leciva S70 lipid were spherical in shape with homogeneous solid matrix structure and no evidences of aggregation (Fig. 5.2).

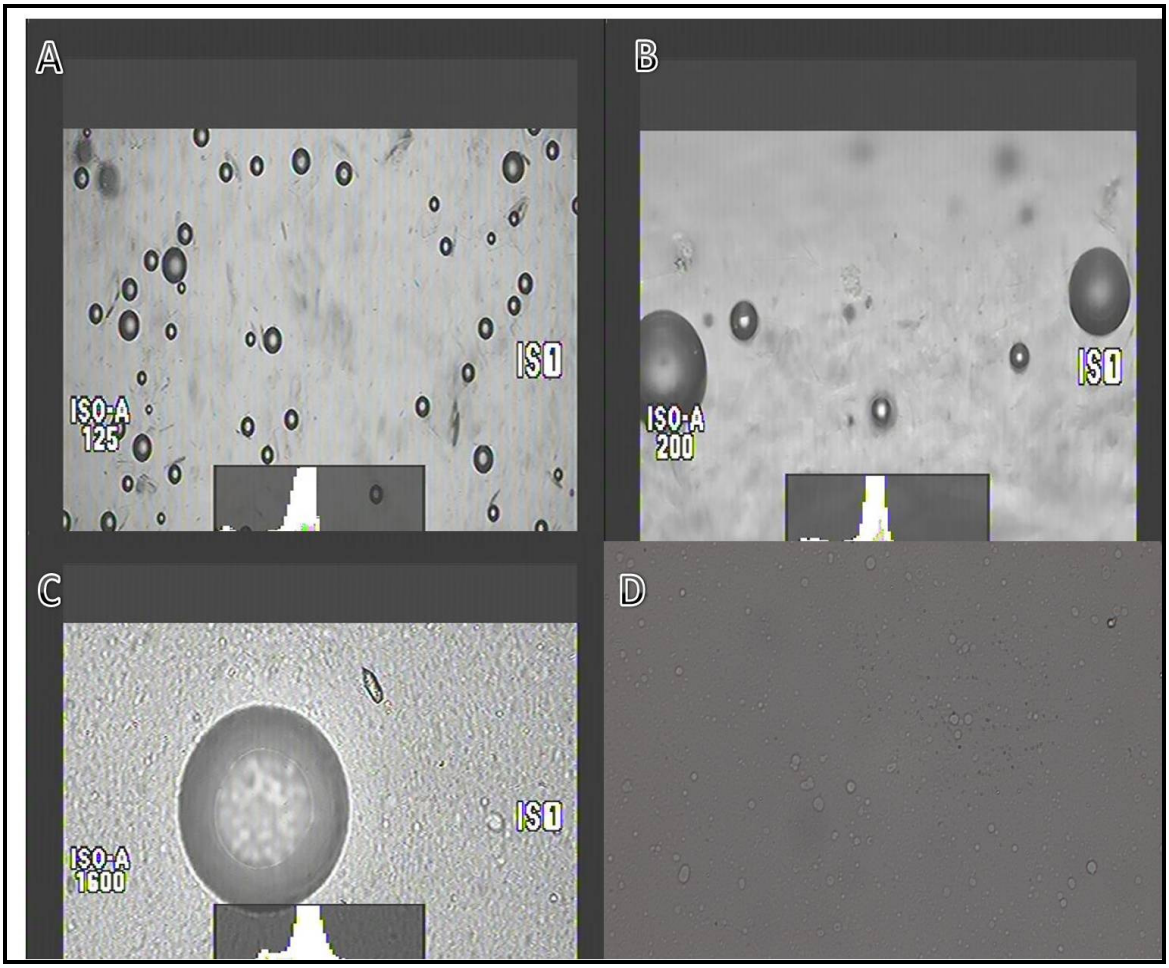

Fig. 5.2 Characterization of Dopamine loaded LPs by microscopy, prepared using Leciva S70 by REV+FTC [A (100x), B (400x) and C (1000x)], and Thin film hydration [D (1000x)] method

This examination was done before sonication of the vesicles. It was observed that the size of vesicles prepared using REV technique (5.2 A-C) was bigger than that of vesicles prepared using TFH method (5.2 D). Vesicles appeared as concentric bilayer with a large internal aqueous space.

Surface morphological studies on shape of prepared systems using TEM indicated that the prepared LPs lamellarity was based on different lipids and techniques used for their preparation. The size and organization of the vesicles from different lipids and techniques are being illustrated using TEM. Images revealed that the particles are uniformly distributed and particle surface is smooth in nature without any amorphous arrangement on particle surface (Fig 5.3).

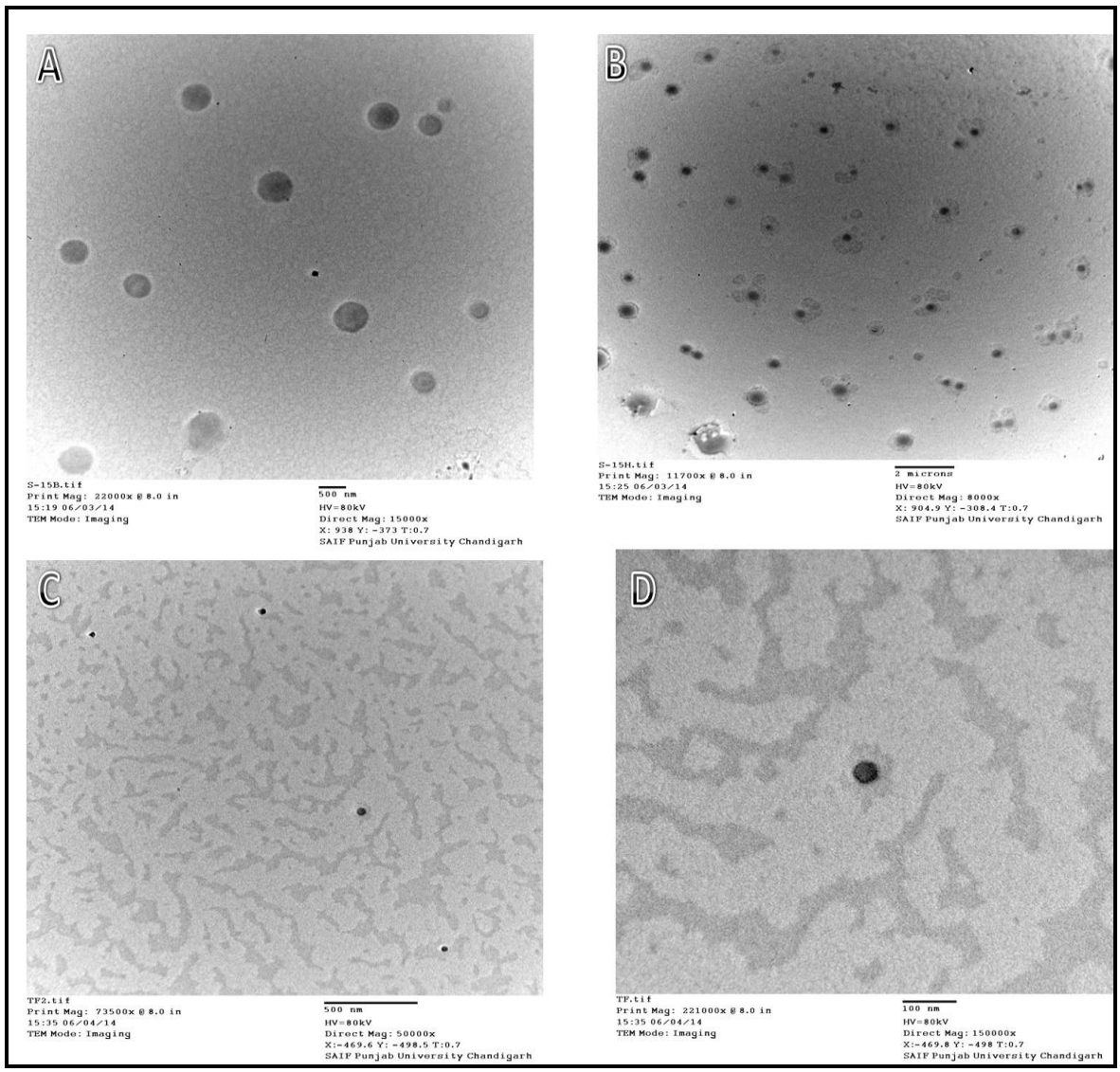

Fig. 5.3 a) Characterization of Dopamine loaded LPs by TEM, prepared using Leciva S70 by REV+FTC (A and B) and Thin film hydration (C and D) method

The effect of phospholipid composition on vesicles structure was examined, and there was slight difference in the morphology of samples. The vesicles prepared using thin film hydration showed

lesser number of vesicles with smaller size as compared to reverse phase evaporation technique (Fig. 5.3 a)-C,D). Leciva S70 liposomes prepared using TFH and REV illustrated singular unilamellar vesicles (Fig. 5.3 a). The average size data and distribution was in agreement with PCS analysis results.

The Leciva S70 and Lipova E120 liposomes prepared using REV in combination with FTC technique were spherical in shape with integrated bilayers and fingerprint-like surface (Fig. 5.3 b)-B,D). These images confirm the spherical vesicles and provide no evidence of aggregation.

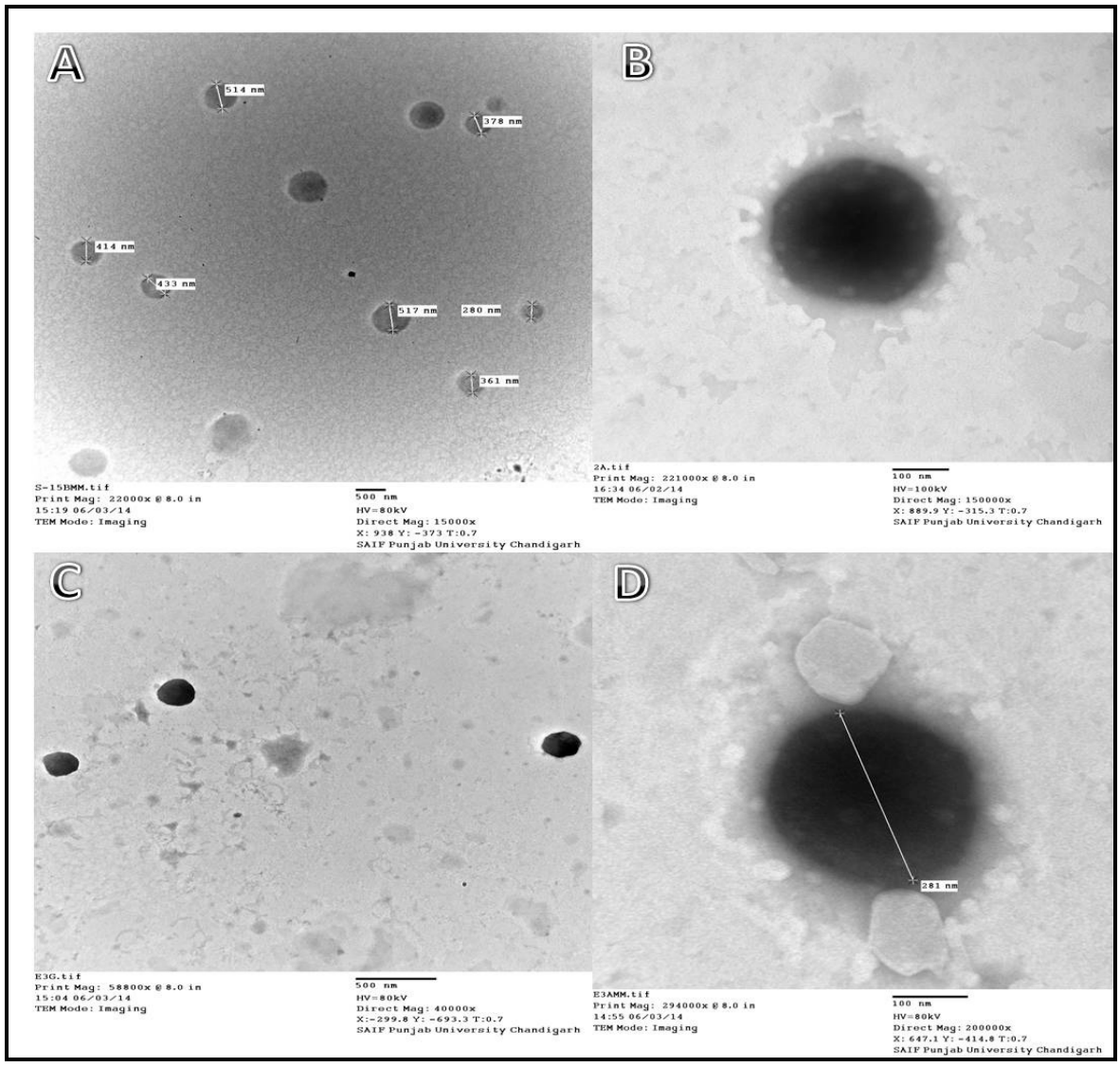

Fig. 5.3 b) Characterization of Dopamine loaded LPs by TEM, prepared using Leciva S70 (A and B) and Lipova E120 (C and D) by REV+FTC method

The liposomes prepared using combinations of both lipids (Leciva S70 and Lipova E120) were found to be smaller and in agreement with PCS analysis results. In case of morphology and structure these vesicles were similar to the vesicles prepared using individual lipid. As shown in

Fig. 5.3 c) the vesicular structure was maintained in the presence of different loading amount of drug and lipid to cholesterol proportion.

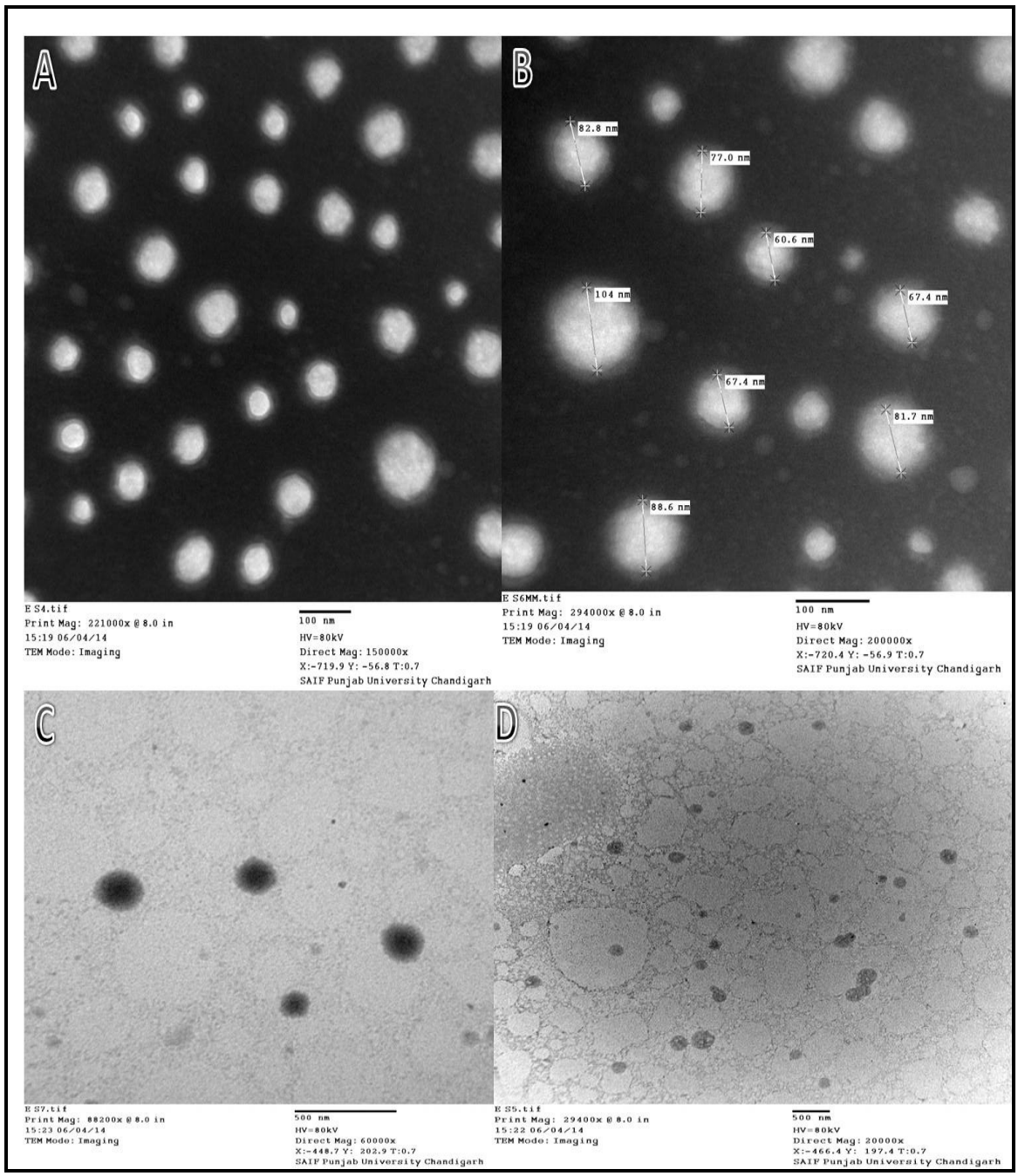

Fig. 5.3 c) Characterization of Dopamine loaded LPs by TEM, prepared using combination of Leciva S70 and Lipova E120 by REV+FTC method

LPs prepared with lipids DPPC, DMPC and S-100 using REV technique collective with FTC showed similar morphology as Leciva S70 and Lipova E120 lipids (Fig. 5.3 d-e). However, the surface of DMPC and DPPC prepared vesicles were found to be smoother than other used lipids.

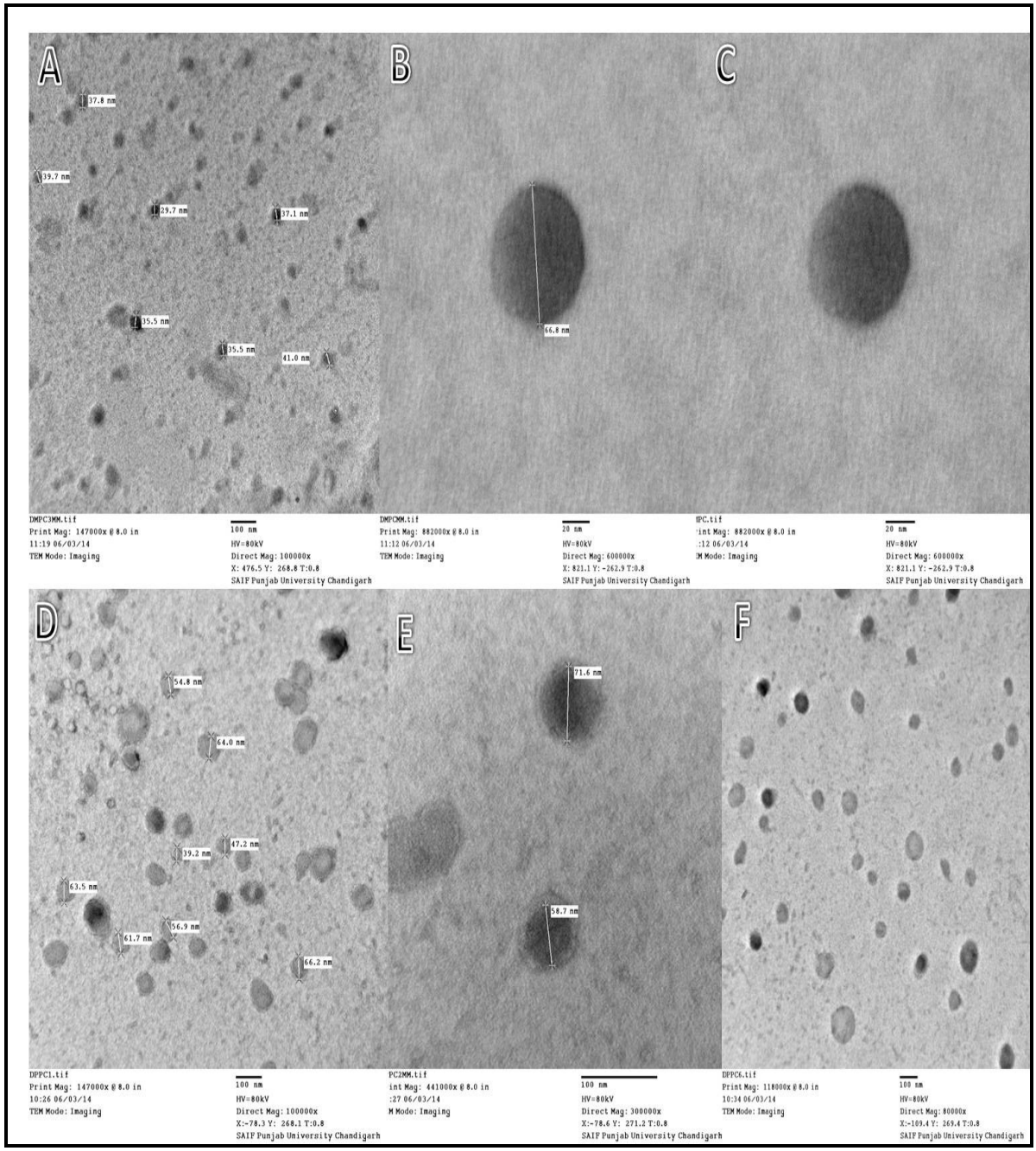

Fig. 5.3 d) Characterization of Dopamine loaded LPs by TEM, prepared using DMPC (A, B & C) and DPPC (D, E & F) by REV+FTC method

Size of DMPC and DPPC liposomes were found to be much smaller than Leciva S70 and Lipova 120. The morphology of S-100 vesicles were found to be exactly similar in size and character as compared to vesicles prepared with Leciva S70 and combination of Leciva S70 and Lipova E120 LPs. These structures demonstrate the oligolamellar vesicle structure (Fig. 5.3 e) with uniform distribution. The average size data and distribution was in agreement with PCS analysis results.

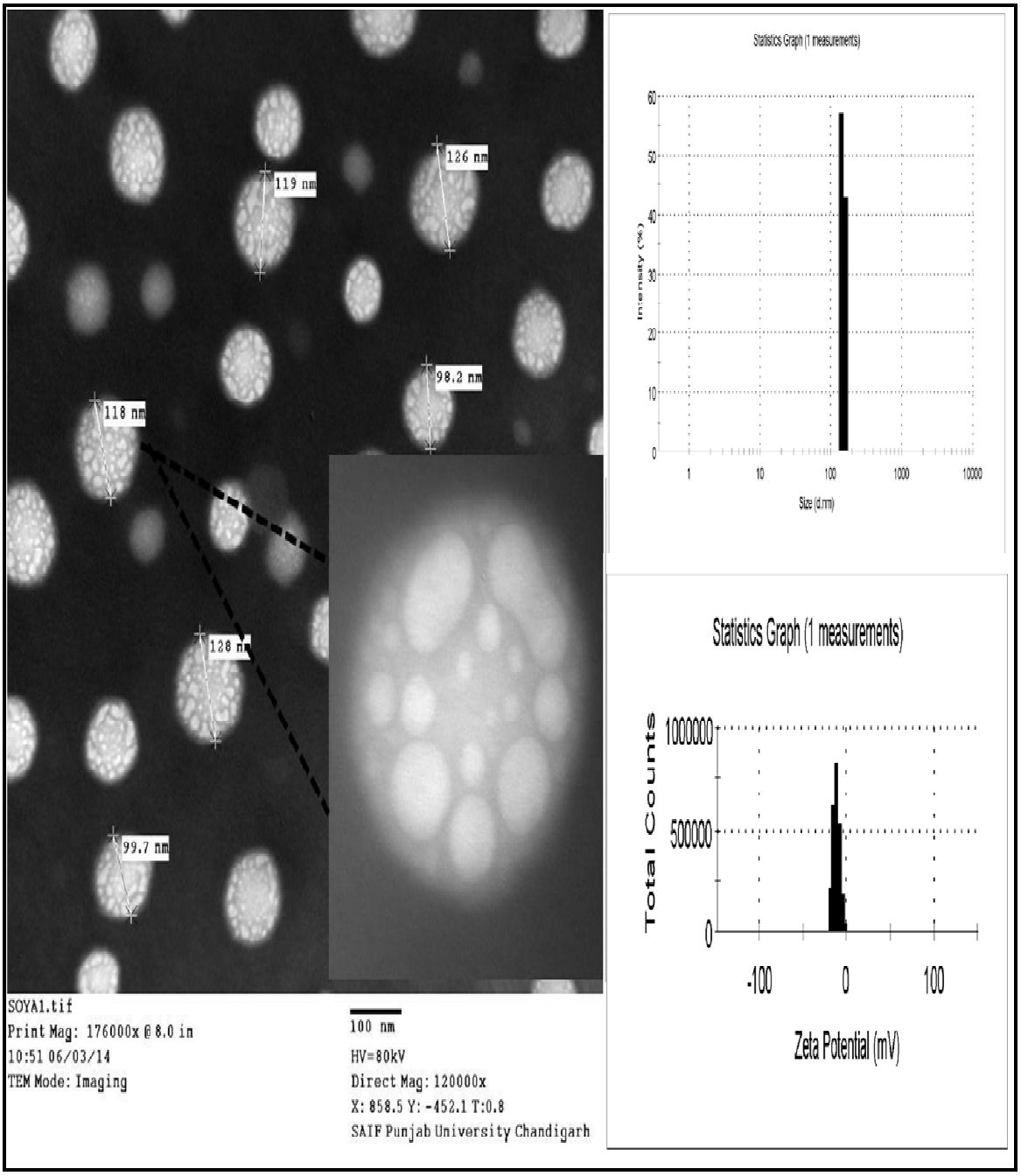

Fig. 5.3 e) Characterization of Dopamine loaded LPs by TEM and Zetasizer prepared using Soya S 100 by REV+FTC method

However, the AFM studies revealed good shape with uniform size distribution (Fig. 5.4) and crystals of DA on the surface in some formulations (Fig 5.4 B). Due to small size of the LPs the close investigation of single particle was done using AFM which gave clear 3D morphological images (Fig. 5.4). The 3D view of multiple particles showed smooth surface. The 2D AFM images clearly demonstrate the spherical shape of LPs.

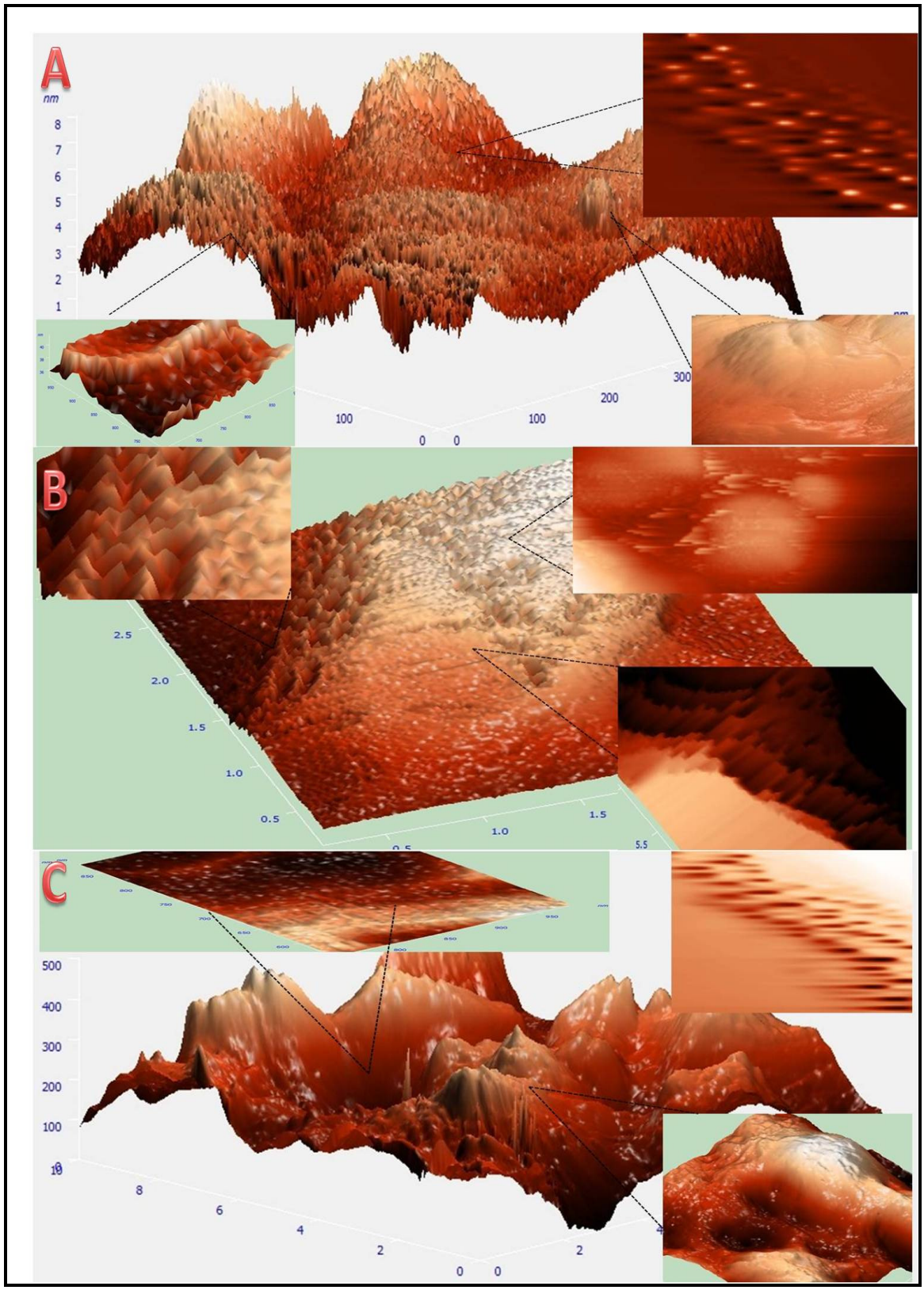

Fig. 5.4 a) Characterization of DA loaded LPs by AFM, cluster, single particle (3D), particles (2D-right corner) prepared by REV+FTC method with A) Leciva S70, B) Lipova E120 and C) Leciva S70+Lipova E120

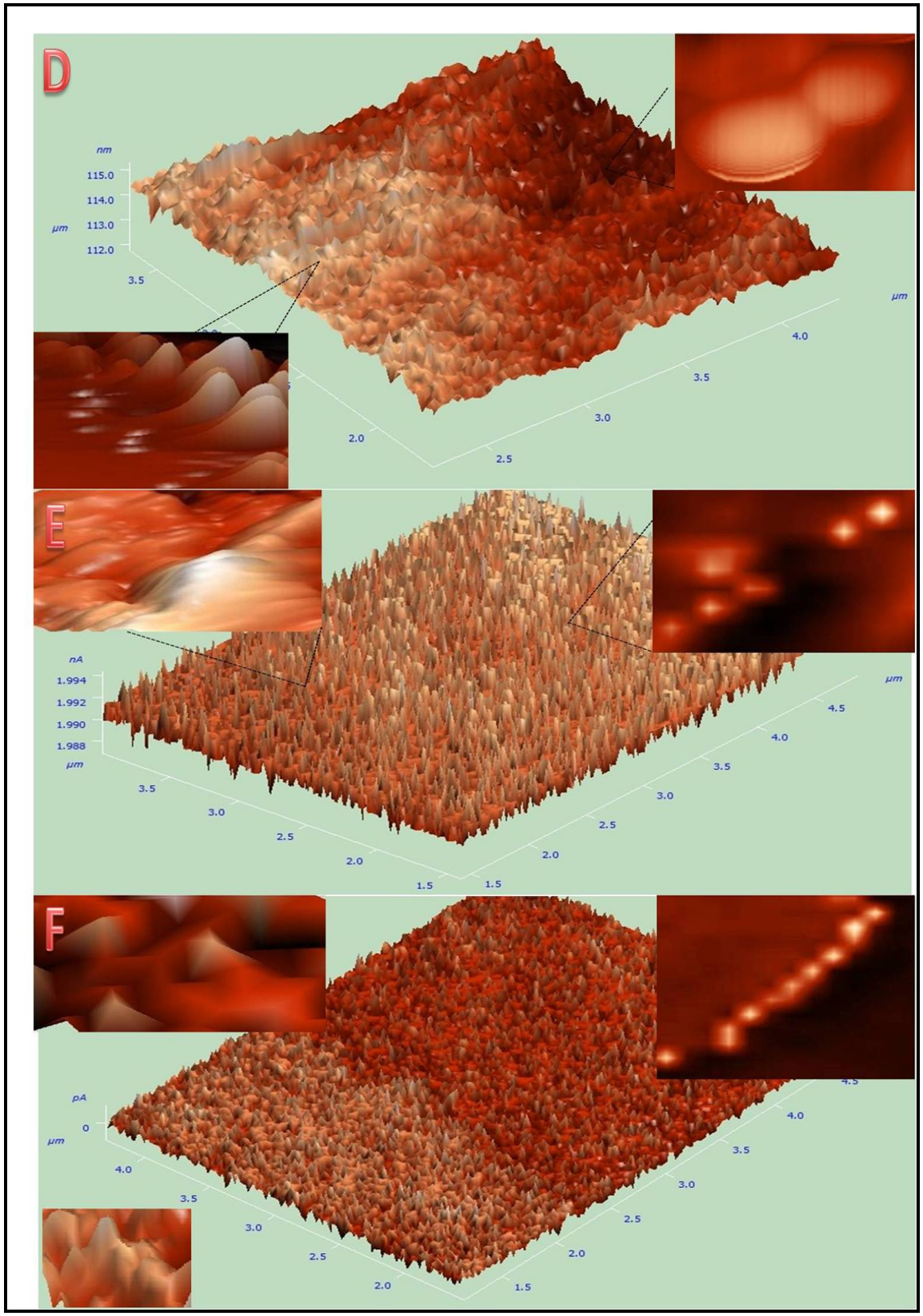

Fig. 5.4 b) Characterization of DA loaded LPs by AFM, cluster, single particle (3D), particles (2D-right corner) prepared by REV+FTC method with D) Lipoid S-100, E) DMPC and F) DPPC

5.3.3 Effect of formulation parameters and processing variables on LPs characterization.

a) LPs prepared by different techniques using Leciva S70 lipid vs. LPs characterization

For preparation of LPs, different techniques were tried using Leciva S70 lipid, however REV coupled with freeze and thaw cycles (FTC) provided maximum entrapment efficiency (≈75 ± 0.23 %) and loading efficiency (Table 5.1-5.5). In thin film hydration technique (TFH) both loading efficiency and encapsulation efficiency (≈16.25 ± 0.20 %) were found to be lowest (Fig. 5.5). Also, average particle size (≈229.04 ± 6.56 nm) and polydispersity index (PDI) were lower (≈0.25 ± 0.12) in TFH as compared to REV which showed a narrow size distribution. However there was small increase in encapsulation efficiency (≈24.64 ± 2.98 %) in TTF technique, in which 3% tween 80 was used as surfactant, but also there was increase in average particle size (≈307.52 ± 3.95 nm) and PDI (≈0.32 ± 0.11). This observation was due to stearic repulsion between tween 80, which was exposed from the outer to inner leaflets of liposomal bilayer membrane, resulted in increased liposome particle curvature (34).

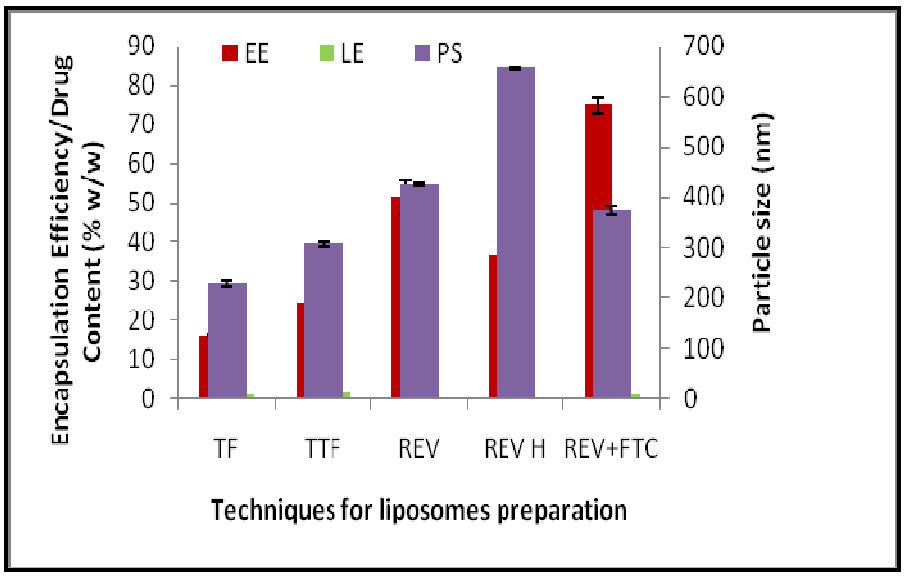

Fig. 5.5 Effect of various techniques of LPs preparation on characteristics of Leciva S70 LPs

Further, REV technique increased the encapsulation efficiency, but in case of REV H where 10 % sucrose was used instead of PBS (pH 5.0) as hydration media there was significant increase in particle size (≈657.63 ± 2.91 nm). However, REV technique coupled with FTC showed maximum entrapment efficiency (≈74.83 %) with average particle size of 374.92 nm and lesser PDI as compared to REV (Table 5.3). There was no significant difference observed in zeta potential of formulations prepared by different technique using Leciva S70.

b) Effect of amount of drug on LPs characteristics

i) Effect of amount of drug on characteristics of Leciva S70 LPs prepared by TFH method

The results indicated that the drug proportion affects encapsulation efficiency and loading efficiency of prepared LPs to a greater extent (Table 5.1). However, variation observed in particles size with respect to drug amount was lesser as compared to loading efficiency and encapsulation efficiency in TFH. The results of Leciva S70 LPs indicate that LE was highest (≈17 %) in 1:1 (drug:lipid) ratio but EE was lowest (≈1.78 %). As the amount of drug decreased EE increased (≈33.35 %) while the LE decreased (≈0.06 %). The average particle size was almost constant (≈200 nm) for drug amount varied from 50-250 mg. However, the average particle size was maximum (≈ 258 nm) with lowest drug amount of 2.5 mg (Fig. 5.6).

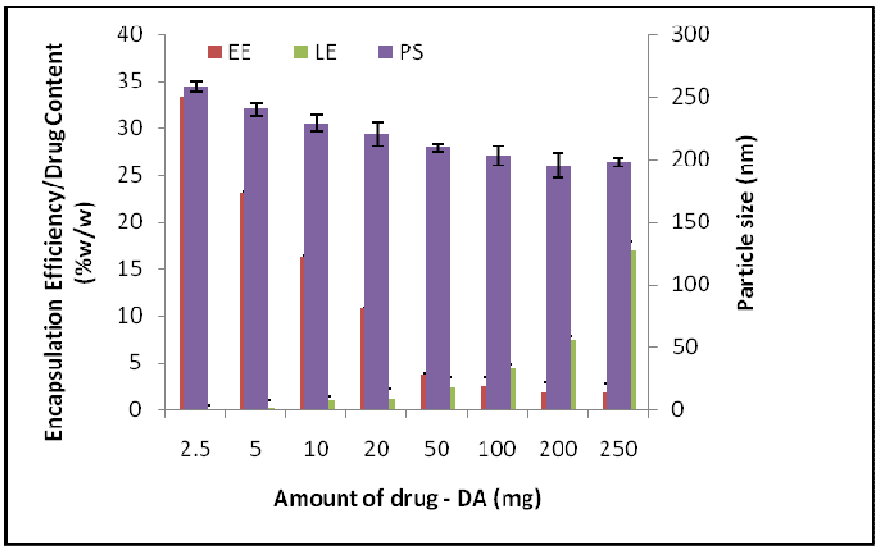

Fig. 5.6 Effect of amount of drug on characteristics of Leciva S70 LPs prepared by TFH method

ii) Effect of amount of drug on characteristics of Leciva S70 LPs prepared by REV method

In REV technique using Leciva S70 lipid, encapsulation efficiency was found to be more as compared to TFH with improved loading efficiency (Table 5.2). But in this case variation in average particle size was significant with respect to change of drug amount. Particle size for different amount of drug mottled from 220 nm to 638 nm. The average particle size was highest (≈ 638 nm) with 10 mg DA and lowest (220 nm) with 200 mg DA (Fig. 5.7). As compared to TFH method particle size increased as the amount of drug decreased in this technique. Therefore, the results are line with the literature that the vesicle size formed using REV technique is greater as compared to TFH method.

The EE (≈ 51.36 %) and LE (≈ 0.36 %) were observed higher when prepared by REV than TFH method. PDI observed in this study was also lower (≈ 0.501) with less amount of drug. However, there was not a significant difference on zeta potential with change of amount of drug.

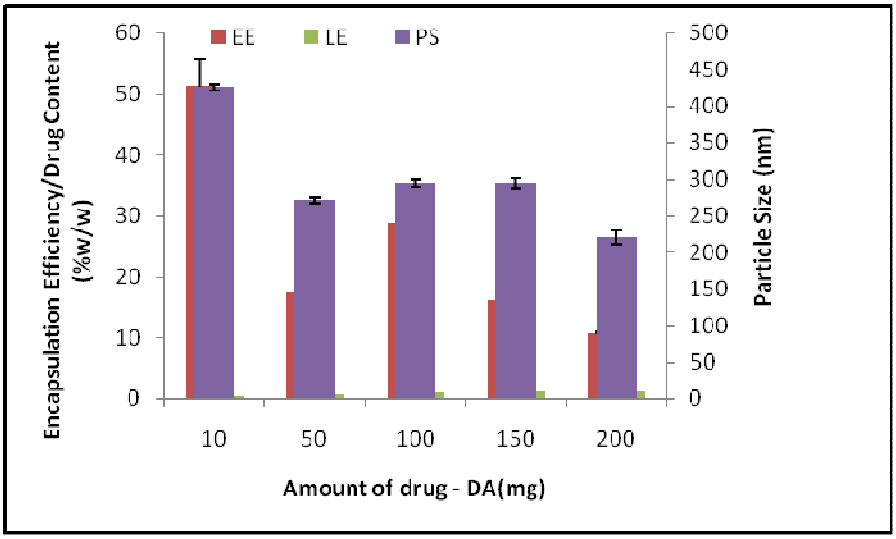

Fig. 5.7 Effect of amount of drug on characteristics of Leciva S70 LPs prepared by REV

iii) Effect of amount of drug on characteristics of Leciva S70 LPs prepared by REV+FTC method

With varied drug amount (10-40 mg), the maximum EE (≈ 75 %) was observed with 10 mg and 20 mg. The LE was lesser with 10 mg drug amount (≈ 0.54 %) and almost doubled with 20 mg drug (≈ 1.04 %) without decrease in EE. As drug amount increased further, EE decreased with 30 mg drug amount showing 65 % EE and 40 mg produced only 36.75 % EE. However, LE increased with maximum of 1.34 % for 30 mg drug amount but later decreased to 1.07 % for 40 mg drug amount. Therefore, it became clear that the drug amount could be increased only upto a certain extent. Highest EE (≈75 %) and LE (≈1.34 %) was found using this technique (Table 5.3), due to increased trapped volume during vesicles freezing and conversion of multilamellar structure to unilamellar or oligolamellar. Also, in terms of vesicle size this combined technique produced lesser size as compared to REV only. The size of vesicles were found in range 215.50 nm to 438.52 nm, which was maximum 638 nm in case of REV (Fig. 5.8). Similar to REV method, particle size increased as the amount of drug decreased using this technique. On the other hand, there was not much effect seen on PDI and zeta potential. Since REV coupled with FTC showed maximum EE and LE, therefore this technique was used in rest of the study.

iv) Effect of amount of drug on characteristics of Lipova E 120 LPs prepared by REV + FTC method

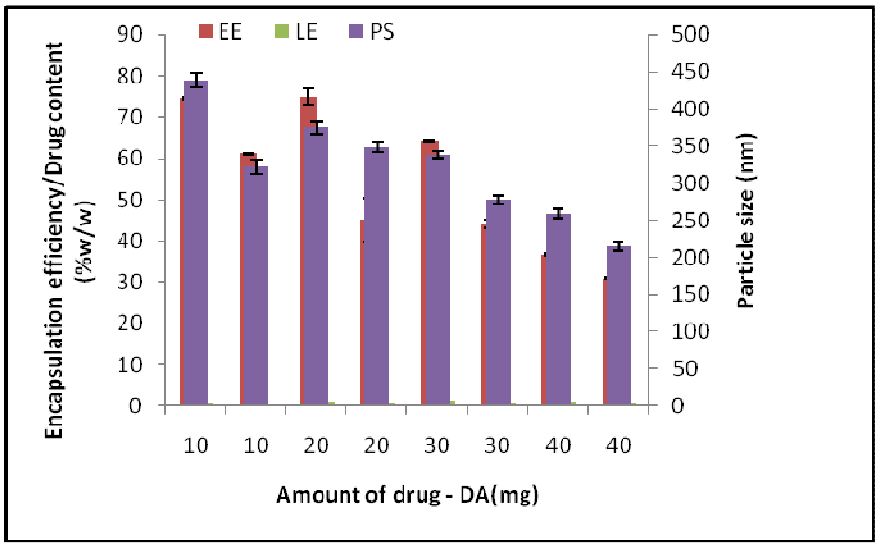

Fig. 5.8 Effect of amount of drug on characteristics of Leciva S70 LPs prepared by REV+FTC method

Lipova E 120 (egg phosphatidylcholine) was used in LPs preparation with varied drug amount by optimized REV+FTC technique. The effect of different types of phospholipids on different parameters was studied in detail. With varying drug amounts (10-40 mg), maximum EE (≈ 62 %) and lowest LE (≈ 0.43 %) with highest average particle size (≈ 590 nm) were observed with 10 mg. Increasing drug amount to 20 mg lead to an enhanced LE (≈ 0.62 %) with EE of 59 % and particle size of ≈ 496 nm (Fig. 5.9).

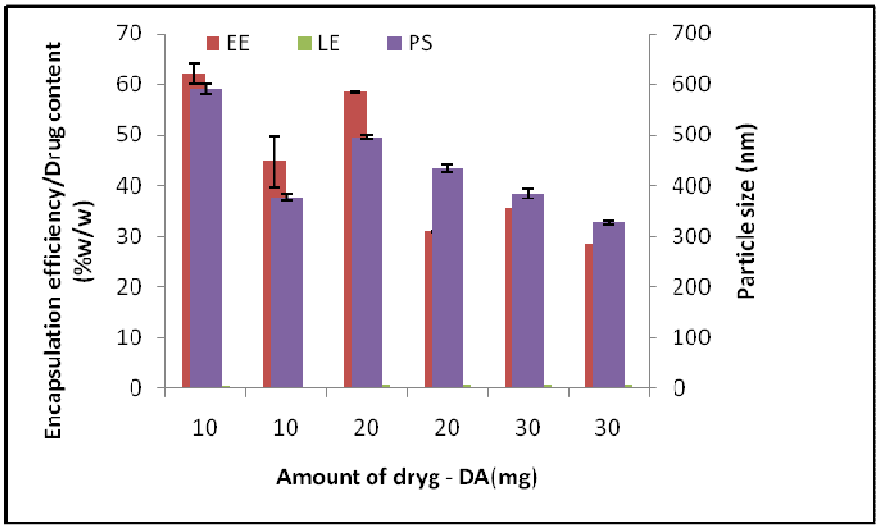

Fig. 5.9 Effect of amount of drug on characteristics of Lipova E120 LPs prepared by REV+FTC method

Further increase in drug amount to 30 mg resulted in decrease in EE (≈ 36 %) and increase in LE (≈ 0.71 %) with particle size reduced to ≈385 nm. With respect to particle size and drug amount, Lipova E120 showed similar trend like Leciva S70 i.e. decrease in

particle size with increase in drug amount. Further, there was no variation observed in zeta potential and PDI, with different amount of drug in the study.

v) Effect of amount of drug on characteristics of LPs prepared using both Leciva S70 and Lipova E 120 lipids by REV+FTC method.

LPs prepared using combination of two lipids i.e. Leciva S70 and Lipova E120, maximum EE (≈ 77 %) was observed with 10 mg drug amount which then decreased (≈ 40 %) with increase in drug amount up to 30 mg. Similar to other lipid, LE observed was lower with lesser amount of drug but increased to 0.85 % with maximum amount of drug (Fig. 5.10). There was significant difference observed in vesicles size (≈ 164 nm to 398 nm) using different amount of lipids (Table 5.5). Zeta Potential and PDI did not show much difference in relation to amount of drug.

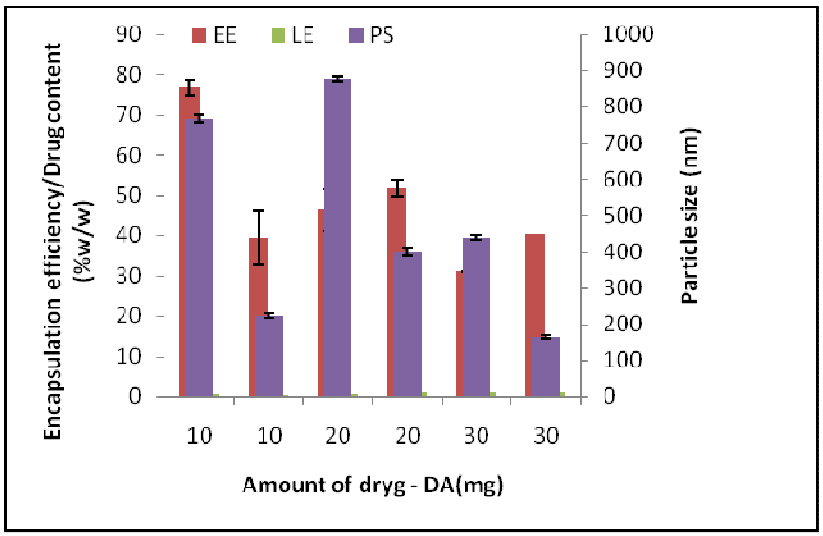

Fig. 5.10 Effect of amount of drug on the characteristics of Leciva S70+Lipova E120 LPs prepared by REV+FTC method

c) Effect of lipids to cholesterol ratios on LPs characteristics

Cholesterol is known to play a significant role in liposome composition since the formation of vesicles and its stability were extremely depended on lipid to cholesterol ratio (23). Cholesterol is an important component of liposomes, which provides rigidity to membrane and improves stability of liposomes in biological fluids (plasma) by reducing the fluidity and permeability of vesicles bilayer (35). Hence, incorporation of different amount of cholesterol significantly affected the various liposome characters and parameters.

i) Effect of Leciva S70 to cholesterol ratios on LPs characteristics

It was demonstrated that cholesterol affects the electrostatic interactions among phosphatidyl-choline Leciva S70 liposome membrane. Also, the appropriate cholesterol content in the liposome improved its stability with good EE and desired vesicles size. In this study, the effect of lipid

Leciva S70 and cholesterol ratio was observed on different parameters. Two optimized ratios of lipid/cholesterol (7:3 and 5:5) were taken for rest of the study (Table 5.3). With varied amount of drug from 10 mg to 40 mg, EE and LE were higher in 7:3 ratio (Leciva S70: Cholesterol) as compared to 5:5 ratios. However, in case of vesicles size the opposite effect was observed since 5:5 ratios showed lower vesicles size as compared to 7:3 ratios (Fig. 5.11). These results showed that higher amount of cholesterol in combination with Leciva S70 resulted in decreased liposome size. Also, if the ratio of cholesterol was further increased, EE and LE decreased drastically. The study with 7:3 lipid/cholesterol showed lesser PDI as compared to 5:5, but no significant effect was observed with zeta potential (Table 5.3).

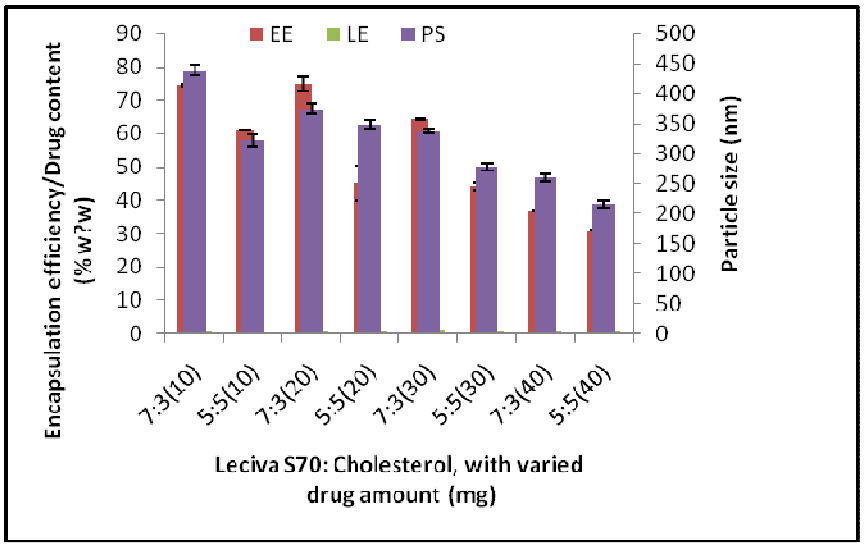

Fig. 5.11 Effect of Leciva S70 to cholesterol ratios on LPs characteristics prepared by REV+FTC method

ii) Effect of Lipova E120 to cholesterol ratios on LPs characteristics

The optimized ratios of Lipova E120/cholesterol (7:3/ 5:5) were taken into account and its effect on all parameters was observed (Table 5.3). Unlike Leciva S70, combination of Lipova E120 with cholesterol (5:5) showed higher EE and LE with varied amount of drug (10 mg to 30 mg) as compared to Lipova E120 with cholesterol in 7:3 ratios. The average particle size also showed significant difference with different ratios of cholesterol (Fig. 5.12). The particle size varied from ≈ 327 nm to 378 nm for 7:3 ratios, but was between 385 nm to 590 nm for 5:5 ratios. On the other hand, there was no significant difference observed on PDI and zeta potential with varied cholesterol amount.

iii) Effect of Leciva S 70+Lipova E120 to cholesterol ratios on LPs characteristics

Higher and effective EE (≈76.7 %) and LE (≈0.54 %) were observed with 10 mg drug amount using 7:3 lipid/cholesterol ratios. On increasing the drug amount to 20 mg and 30 mg, EE and LE

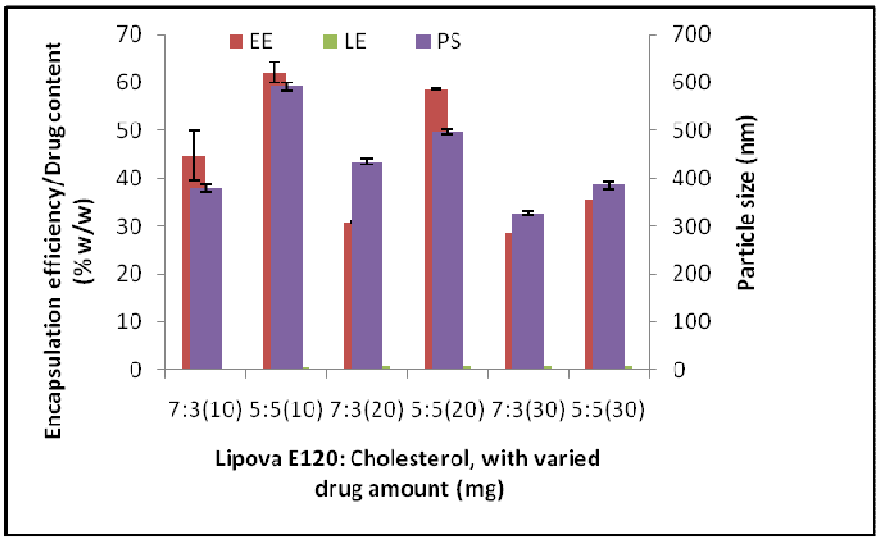

Fig. 5.12 Effect of Lipova E120 to cholesterol ratios on LPs characteristics prepared by REV+FTC method

improved with 5:5 ratios as compared to 7.3 ratios (Table 5.5). Vesicle size was smaller (≈164 nm) with 5:5 lipid/cholesterol as compared to 7:3 ratios (≈ 438 nm) with 30 mg drug amount. Similar trend was observed with 10 mg and 20 mg amount of drug. PDI was also found to be lower with 5:5 ratios as compared to 7:3. However, no difference was observed on zeta potential based on varying cholesterol amount (Fig. 5.13).

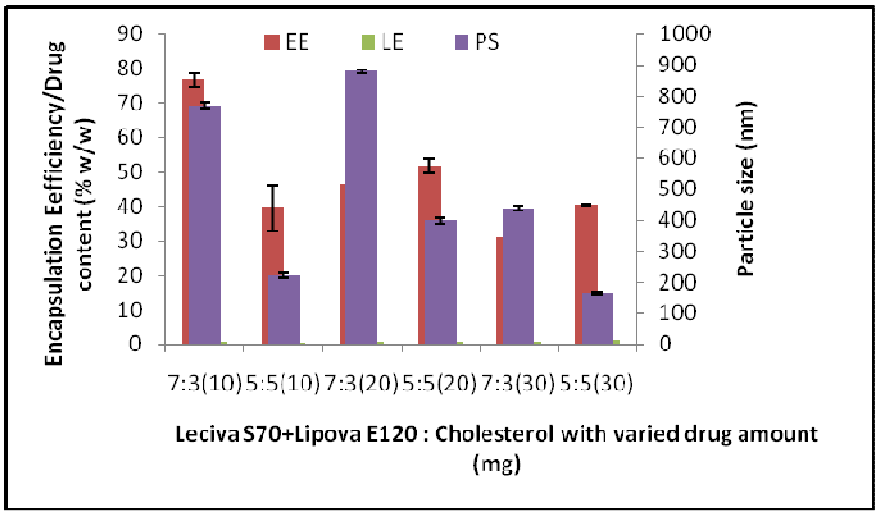

Fig. 5.13 Effect of Leciva S70+Lipova E120 to cholesterol ratios on LPs characteristics prepared by REV+FTC method

e) Effect of different types of lipids on LPs charactistics

Encapsulation efficiency of DA varied to a large degree depending on the lipid composition. The selection of lipids for preparing LPs depends on various factors. Further, experiments were performed for preparing liposomal formulations using different phospholipids. In order to

investigate the role of lipids, phospholipids used are varied in length of acyl chain and degree of saturation of the acyl chains.

The shape and organization of the vesicles prepared from different types of lipids are illustrated above. Further, we have seen their effect on other parameters such as size, EE, LE, PDI and zeta potential. Leciva S70, Lipova E120 and combination of these two were selected lipids, because of natural biocompatible nature and are very economic from commercial point of view. S100, DPPC and DMPC were used as ideal lipids and to compare their effects for different parameters mentioned above. Since, both Leciva S70 and S 100 are soya phosphatidylcholine; therefore, their direct comparison was fruitful. The EE and LE were almost same with Leciva S70 and S100, but vesicles size varied in both cases. With Leciva S70 the size was smaller as compared to S100 (Fig. 5.14). Comparing Leciva S70 with Lipova E120, the EE was found to be lower in Lipova E120 with higher vesicles size. On the other hand, on combining both Leciva S70 and Lipova E 120, the EE increased as compared to Lipova E120 and particle size was lesser (≈ 164 nm) as compared to both Leciva S70 (≈ 338 nm) and Lipova E120 (≈ 385 nm). All these lipids were natural lipids but DPPC and DMPC were taken as synthetic lipids and their effect on similar parameters were also studied.

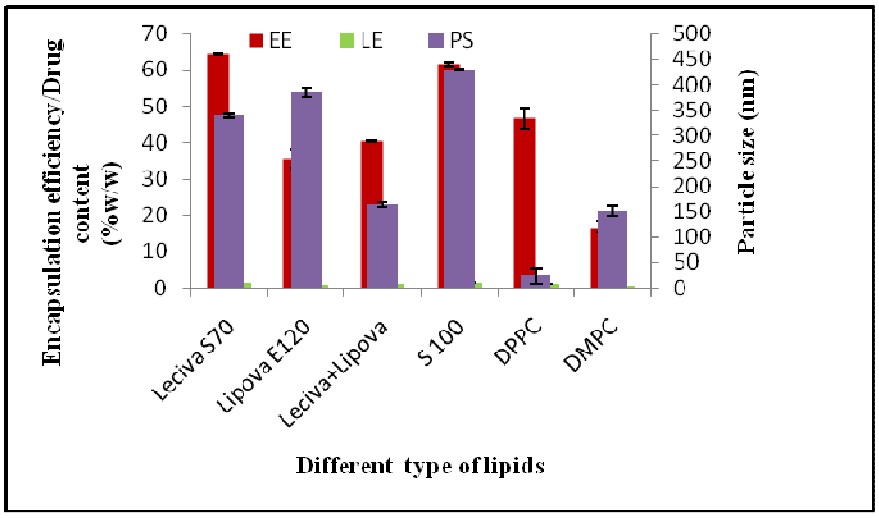

Fig. 5.14 Effect of different type of lipids on the LPs characteristics prepared by REV+FTC method

DPPC showed an effective EE with lower particle size (≈ 23 nm) as compared to DMPC which showed a poor EE and higher particle size (≈ 151 nm). Overall, a higher extent of incorporation in terms of EE and LE was observed in Leciva S70 and S100 followed by DPPC, Lipova E120 and DMPC vesicles. Increase in fatty acid chain length and gel state of vesicles composed of these lipids might be one of the responsible factors (36, 37). However, the particle size in DMPC was

much lower than the natural lipids. PDI and zeta potential obtained from synthetic lipids were also much lower than the natural lipids used (Table 5.6).

f) Effect of hydration media on LPs characteristics

The hydration media had a significant effect on amount of DA encapsulation, zeta potential and particle size. 10 mM was selected as the hydration media as maximum EE was found with minimum particle size. On addition of 3 % tween 80 in hydration media, there was increased EE due to surface active role of tween 80 along with increased particle size (Fig. 5.15). Also, tween 80 increased the vesicle curvature since it was exposed more towards inner leaflet than the outer leaflet of liposomal bilayer (38). A significant change in surface charge (negative charge) was found in vesicles bilayer by addition of tween 80, which was also found to be important in formation of more stabilized liposomes. This occurred due to hydroxyl groups of tween 80 which gets combined with polar region of phosphatidylcholine to produce dipole tropism and resulted in increased liposomal surface negative charge. However, there was no significant effect observed on PDI due to change in hydration media.

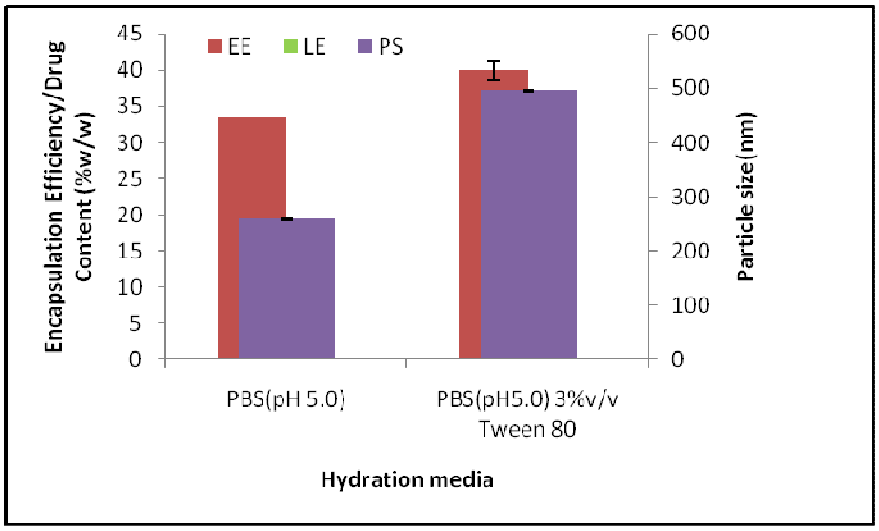

Fig. 5.15 Effect of Hydration media on the characteristics of Leciva S70 LPs prepared by TFH method

The ability of LPs to encapsulate DA varied with the ionic strength of buffer. Since ionic strength of buffer was increased, there was decrease in EE due to decrease in volume of encapsulated aqueous space per mole of phosphate lipid. The maximum EE was achieved in case of DA using REV technique with 10 mM phosphate buffer. Both 5 mM and 15 mM buffer resulted in lower EE of DA. To achieve the maximal encapsulation efficiency of aqueous space it was necessary to use buffer of lower ionic strength (10). Further, addition of different strength of sucrose under similar conditions were tried to increase EE. As 10 % sucrose showed maximum EE

(comparatively lesser than PBS) and significantly larger particle size, so finally PBS was selected as hydration media. Also, there was a significant increase in PDI due to sucrose addition (Table 5.2).

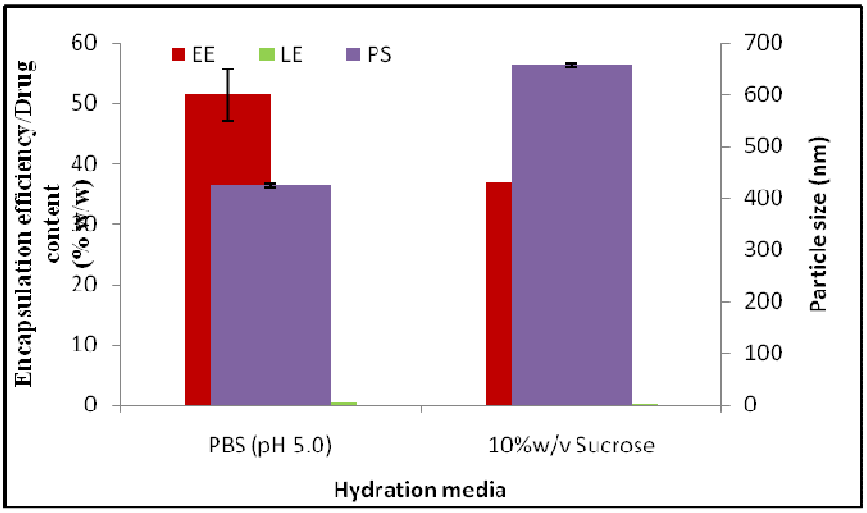

Fig. 5.16 Effect of Hydration media on characteristics of Leciva S70 LPs prepared by REV method

g) Effect of sonication time, organic solvents and freeze/ thaw cycles on LPs characterization

It is known that large number of process parameters might also be responsible for determining the final product in terms of vesicle characters. There was a prominent trend observed for average particle size with change in the intensity and duration of ultrasonication treatment (bath sonication). Another mode of sonication (probe sonication) at different intensity from 10 to 20 W was studied which resulted in smaller particle size as compared to bath sonication but disruption of liposome vesicles were observed. Therefore, ultrasonication treatment using bath sonicator was optimized. It is inferred that the increased emulsification energy associated with either increased duration or intensity of the ultrasonication treatment helped in rapid dispersion of organic phase, forming uniform vesicles with narrow size distribution. Although, sonication was performed in controlled temperature conditions, high sonication treatment might also resulted in rise of temperature which might be a contributing factor to a decrease in EE due to diffusion of drug and increase in solubility. It was also observed that DPPC (≈ 23 nm) and DMPC (≈ 151 nm) produced much smaller vesicles size as compared to Leciva S70, Lipova E120 and S100 under same optimized conditions.

The other variable, responsible for determining vesicles characteristics, was organic solvent. This also includes the type of phospholipid used and its solubility in the organic solvent due to the interfacial tension between the organic solvent and aqueous phase. The specific solvents and relative ratios of solvent/water/lipid studied here represent a system for the highest possible EE.

These optimal conditions were suggested after systemic evaluation of the effect of various solvents and relative ratios of solvent/water/lipid.

Freeze and thaw method is best known for increasing the trapped volume of multilamellar liposomes (MLV) which results in increased EE with appropriate vesicles size. The commotion gradients between water and solute fashioned during freeze and thaw method generate fluxes of water and solute crosses the cell membrane resulting in increased trapped volume. These thermal and osmotic stresses resulted into change in curvature, surface properties and phase behaviour of lipid bilayer. During freezing, the bilayer of liposomes can be destabilized through expansion of inner water phase by ice formation and dehydration of hydrated head group of lipid. During thawing, exposed hydrophobic cores fuse together to form new vesicles with decreased lamellarity.

5.3.4 In-vitro drug release studies

A range of liposomal formulations were prepared to evaluate the effect of various formulation parameters on the in vitro drug release profile. As per hypothesis, drugs encapsulated in liposomal systems are released through 3 possible mechanisms: a) passive diffusion, b) vesicle erosion and c) vesicle retention in the circulation.

a) Influence of lipid-cholesterol ratio on in-vitro drug release and its kinetics

As discussed above, the formation of DA loaded LPs were extremely dependent on lipid: cholesterol ratio and similarly this ratio have direct impact on the release behaviour. In vitro drug release was conducted in order to determine the effect of lipid cholesterol ratio on DA release. In-vitro cumulative percent release profile of LPs prepared with different lipid and cholesterol ratio were represented in Fig. 5.17 to 5.24. It was observed that pure DA and its physical mixture with lipids and cholesterol used in same proportion as formulation got dissoluted completely within 2 h (Fig. 5.17). However, the release pattern from LPs extended from 18 to 48 h depending on the proportion of lipid and cholesterol used with increased mean dissolution time (MDT). Leciva S70 LPs with cholesterol in 7:3 ratio showed controlled release up to 18 h (Fig. 5.18) whereas the same lipid with cholesterol in 5:5 ratio extended the drug release up to 48 h (Fig. 5.19). We could observe a circuitous relationship between the amount of cholesterol and drug release. As the amount of cholesterol increased the release rate decreased. It was concluded that liposomal formulation with 30 % cholesterol content was more beneficial for the efficient encapsulation, but to achieve controlled release behaviour 50 % cholesterol showed better results. Extra cholesterol was unfavourable due to stimulating burst drug release might be because of low encapsulation efficiency. However, formulations with Lipova E120 and cholesterol in 7:3 ratios showed 95 % release till 48 h (Fig. 5.20). As represented in Fig. 5.24, in some combination batches where amount of Lipova E120 was comparatively more than Leciva S70 the release profile was extended to more extent showing about 60 % drug release till 48 h.

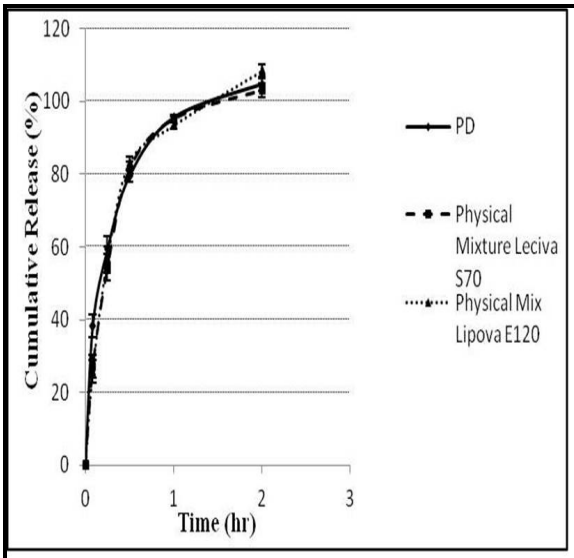

Fig. 5.17 In-vitro drug release from pure drug (PD) and physical mixture of PD with Leciva S70 and Lipova E120 & cholesterol in equal proportion as used in formulation

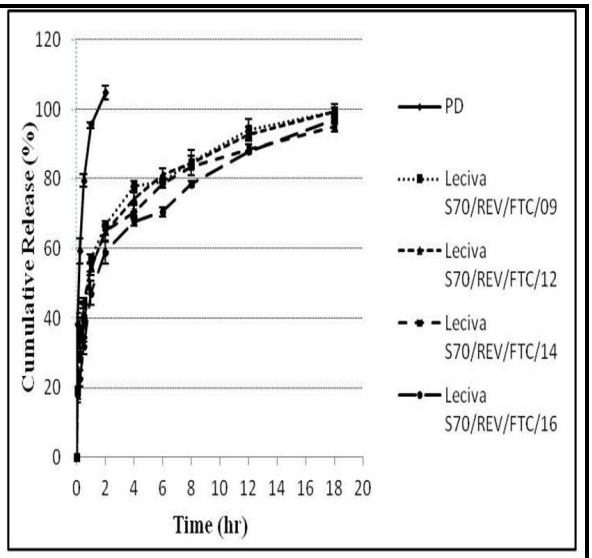

Fig. 5.18 Influence of Leciva S70 and cholesterol in 7:3 proportion with different amount of drug on in-vitro dissolution profile of dopamine loaded LPs

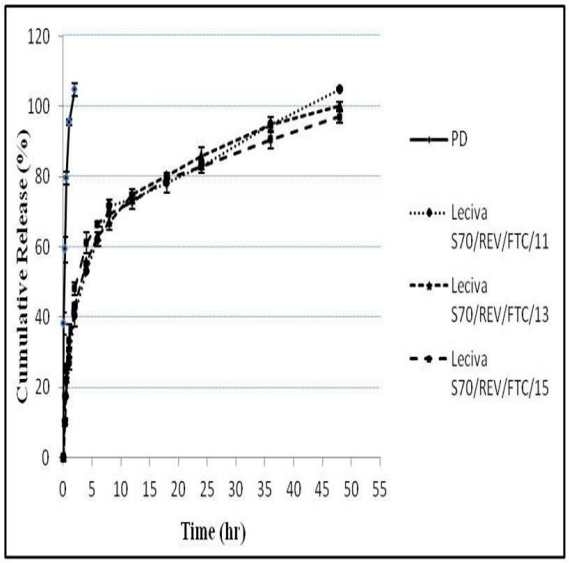

Fig. 5.19 Influence of Leciva S70 and cholesterol in 5:5 proportion with different amount of drug on in-vitro dissolution profile of dopamine loaded LPs

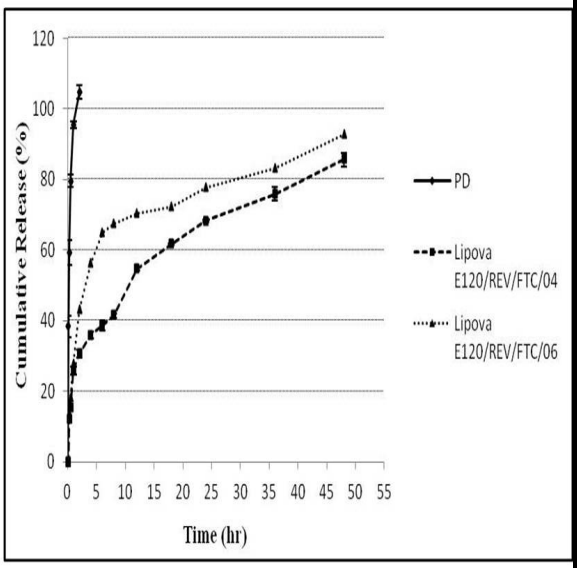

Fig. 5.20 Influence of Lipova E120 and cholesterol in 7:3 proportion with different amount of drug on in-vitro dissolution profile of dopamine loaded LPs

On the other hand, with 5:5 ratios the release observed was more controlled, as up to 80 % of the drug got released till 48 h (Fig. 5.21). Moreover, formulations prepared using Leciva S70 and Lipova E120 in combination, showed better release with both 7:3 and 5:5 lipid cholesterol ratios, extending release from 35 to 48 h respectively (Fig. 5.22-5.23). The drug release from LPs is a complex process, dependent on many factors like nature of drug, lipid degradation rate, water permeability and drug-lipid matrix interaction (7). As the amount of cholesterol was increased in formulation, the time to release 50 % of drug ($T_{50\%}$) was found to increase considerably (Table

5.7). The release profile and value of $T_{50\%}$ indicate that 50 % cholesterol produced more extended release than 30 % due to more rigidity provided by cholesterol in lipid membrane. For better understanding of release process, the dissolution data were fitted to different models (39, 40).

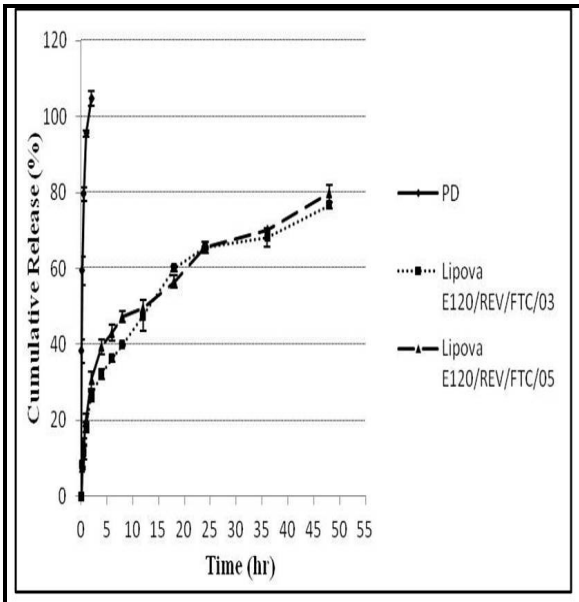

Fig. 5.21 Influence of Lipova E120 and cholesterol in 5:5 proportion with different amount of drug on in-vitro dissolution profile of dopamine loaded LPs

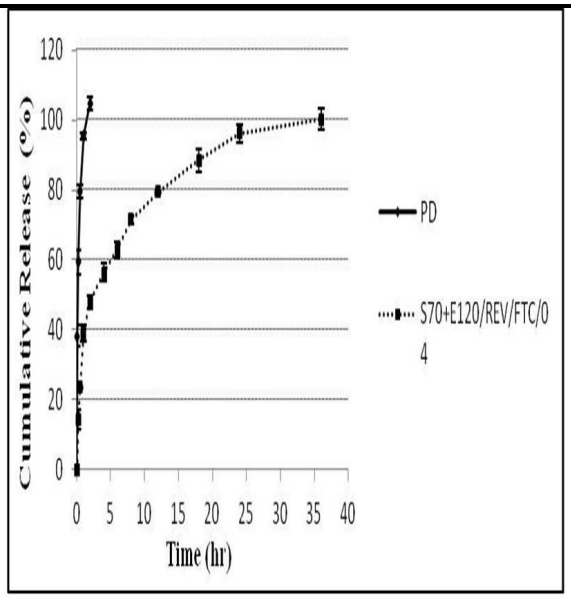

Fig. 5.22 Influence of Leciva S70+Lipova E120 and cholesterol in 7:3 proportion on in-vitro dissolution profile of dopamine loaded LPs

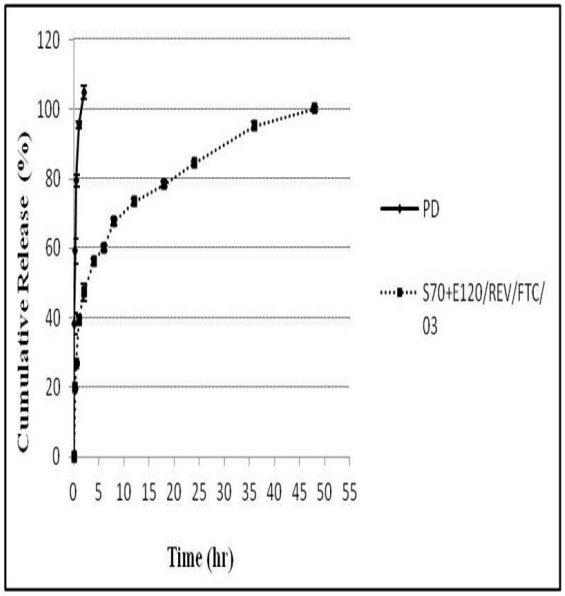

Fig. 5.23 Influence of Leciva S70+Lipova E120 and cholesterol in 5:5 proportion on in-vitro dissolution profile of dopamine loaded LPs

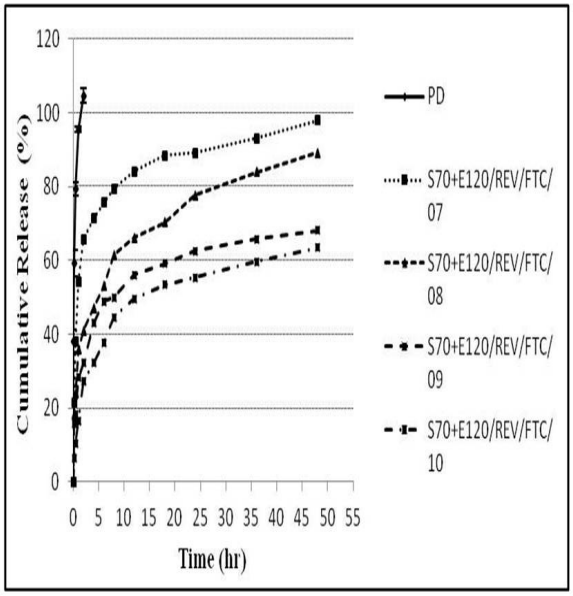

Fig. 5.24 Influence of Leciva S70+Lipova E120 and cholesterol in different proportion on in-vitro dissolution profile of dopamine loaded LPs

Data fitting results indicated that the drug release data can be well described by modified first order kinetics described with Tlag and Fmax. In addition, the Korsmeyer-Peppas model was found to be suitable for explaining drug release mechanism in all the formulations (Table 5.7). In case of Korsmeyer-Peppas model, most of dissolution data follows Fickian diffusion (n=0.185 to 0.431) and some data followed non- Fickian diffusion (0.538) process, known as anomalous transport. The fitting of best model to the dissolution data was selected through three important parameters, R^2, Akaike Information Criterion (AIC) and Model Selection Criterion (MSC). In general, R^2 value close to one, model with low AIC value and MSC value more than 2 to 3 were considered for deciding best model for the analyzed in-vitro dissolution data of the prepared LPs (39). The R^2, AIC and MSC value best fit model of LPs is represented in Table 5.7.

b) Influence of different types of lipids and techniques studied on in-vitro drug release and its kinetics

The influence of different lipids Leciva S70, Lipova E120, their combination, DPPC, DMPC and S-100 using different preparation techniques in drug release is represented in Fig. 5.29 to 5.30 respectively. Among different lipids used, the presence of Lipova E120 extended drug release to a higher extent (Table 5.7). As Lipova E120 is more hydrophobic due to long fatty acyl chains and the gel state of liposomes further extended the release.

LPs prepared using thin film hydration technique showed faster DA release especially when tween 80 was used in hydration media as compared to REV. This could be due to enhanced surfactant property of tween 80 and other due to lower EE with TFH. Further, DPPC showed extended drug release due to better encapsulation as well as more hydrophobic character of this lipid. Finally, when release profile was compared along with EE and structural analysis, both Leciva S70 and S-100 soya PC were found to be similar confirming the steadiness of Leciva S70. Moreover, these LPs consisted of non-concentric smaller vesicles inside a larger particle; therefore the controlled drug release could be due to the longer diffusion pathway or erosion of the small vesicles at the outer region of the larger particles. Fitting of dissolution data of Lipova E120 and its combination with Leciva S70, DPPC and DMPC to different models were represented in Table 5.7.

c) Influence of freeze and thaw cycles on in-vitro drug release and its kinetics

The release profile of Leciva S70 before freeze-thawing cycles was compared with that after freeze-thawing. Fig 5.31-5.32 showed that the Leciva S70 LPs after freeze-thawing to some extent had a higher initial burst release in comparison with that of before freeze-thawing. This burst release might be due to the increased ratio of surface area to volume with the vesicle size reduction upon freeze-thawing. As mentioned above before freeze-thawing the controlled drug release could be due to the longer diffusion pathway.

Table 5.7 Best fitting of in-vitro release data using mathematical modelling

S.NO	Batch	First Order with Tlag and Fmax Model K_{f0} (R^2)	Korsmeyer-Peppas R^2	AIC	MSC	n^z	$T_{50\%}$ (h)	$T_{75\%}$ (h)
1	Leciva S70/TF/05	0.456 (0.9717)	0.9557	67.22	2.28	0.431	2.14	5.47
2	Leciva S70/TTF/01	1.076 (0.9611)	0.9828	49.49	3.17	0.281	0.63	2.68
3	Leciva S70/REV/09	0.676 (0.9106)	0.9851	51.95	3.39	0.282	1.26	5.31
4	Leciva S70/REV/H-09	0.861 (0.9432)	0.9805	59.08	3.18	0.304	0.78	2.98
5	Leciva S70/REV/11	0.105 (0.9886)	0.9492	90.51	2.48	0.538	9.20	19.64
6	Leciva S70/REV/FTC/09	0.849 (0.9427)	0.9800	62.60	3.13	0.243	0.88	4.67
7	Leciva S70/REV/FTC/11	0.191 (0.9566)	0.9652	92.51	2.67	0.325	4.44	15.47
8	Leciva S70/REV/FTC/12	0.808 (0.9348)	0.9866	57.93	3.52	0.242	0.89	4.78
9	Leciva S70/REV/FTC/13	0.209 (0.9627)	0.9693	73.62	2.85	0.314	3.92	14.24
10	Leciva S70/REV/FTC/14	0.692 (0.9455)	0.9752	71.71	2.98	0.239	1.10	6.03
11	Leciva S70/REV/FTC/15	0.313 (0.9512)	0.9509	95.36	2.20	0.275	3.49	15.28
12	Leciva S70/REV/FTC/16	0.328 (0.9394)	0.9861	65.37	3.62	0.279	1.66	7.12
13	Lipova E120/REV/FTC/03	0.098 (0.9690)	0.9891	60.92	3.99	0.369	13.79	41.41
14	Lipova E120/REV/FTC/04	0.086 (0.9471)	0.9925	57.33	4.34	0.350	10.39	33.11
15	Lipova E120/REV/FTC/05	0.134 (0.9447)	0.9847	65.44	3.62	0.332	11.55	39.19
16	Lipova E120/REV/FTC/06	0.310 (0.9661)	0.9499	85.39	2.37	0.269	4.25	19.20
17	Leciva S70+Lipova E120 / REV/FTC/03	0.200 (0.9236)	0.9910	63.62	4.03	0.260	3.05	14.48
18	Leciva S70+Lipova E120 / REV/FTC/04	0.209 (0.9509)	0.9815	68.58	3.33	0.303	2.92	11.14
19	Leciva S70+Lipova E120/ REV/FTC/07	0.926 (0.9561)	0.9430	87.37	2.03	0.185	0.934	8.32
20	Leciva S70+Lipova E120/ REV/FTC/08	0.182 (0.9283)	0.9908	61.055	4.01	0.262	4.7	22.25
21	Leciva S70+Lipova E120/ REV/FTC/09	0.275 (0.9523)	0.9740	68.71	2.95	0.243	10.01	53.24
22	Leciva S70+Lipova E120/ REV/FTC/10	0.185 (0.9766)	0.9634	73.06	2.74	0.313	17.53	64.09
23	DPPC/REV/FTC/01	0.363 (0.9122)	0.9615	81.84	2.60	0.263	3.73	17.51
24	DMPC/REV/FTC/01	0.367 (0.9717)	0.9912	44.08	3.92	0.388	1.72	4.90
25	S-100/REV/FTC/03	0.375 (0.9530)	0.9825	61.19	3.25	0.274	1.61	7.09

All lipids variable formulations, follows first order kinetics and Korsmeyer-Peppas model. [z]Diffusion exponent indicating the drug release mechanism.

Table 5.8 Summary of dissolution parameters for characterizing in vitro drug release curve

S.NO	Batch	Parameters to characterize release curve of dopamine loaded Lps						
		AUC	ABC	MRT	MDT	VDT	RD	DE
1	Leciva S70/TF/05	896.01	258.59	3.09	2.68	7.26	1.01	0.74
2	Leciva S70/TTF/01	638.22	164.52	2.15	1.63	4.76	1.77	0.80
3	Leciva S70/REV/09	876.22	299.80	3.80	3.06	13.75	1.47	0.73
4	Leciva S70/REV/H-09	1061.40	239.57	0.37	2.20	7.73	1.58	0.88
5	Leciva S70/REV/11	2449.34	933.99	10.45	4.90	80.82	0.82	0.68
6	Leciva S70/REV/FTC/09	1505.18	283.32	4.48	1.32	17.46	2.14	0.83
7	Leciva S70/REV/FTC/11	3886.38	1145.13	11.24	10.92	191.41	1.60	0.80
8	Leciva S70/REV/FTC/12	1492.86	297.61	4.57	2.99	18.71	2.09	0.83
9	Leciva S70/REV/FTC/13	3903.36	898.78	12.0	8.98	142.42	1.76	0.81
10	Leciva S70/REV/FTC/14	2018.77	344.58	6.21	3.50	28.21	2,30	0.84
11	Leciva S70/REV/FTC/15	3827.46	829.16	14.62	8.54	155.92	2.14	0.80
12	Leciva S70/REV/FTC/16	1980.00	445.40	5.42	4.41	33.48	1.72	0.82
13	Lipova E120/REV/FTC/03	2780.19	895.14	19. 05	11.69	65.88	1.25	0.58
14	Lipova E120/REV/FTC/04	3031.54	1077.90	17.63	12.60	92.14	1.21	0.63
15	Lipova E120/REV/FTC/05	2871.02	954.49	18.99	11.97	84.99	1.37	0.59
16	Lipova E120/REV/FTC/06	3580.31	874.74	17. 00	9.42	70.96	2.10	0.74
17	Leciva S70+Lipova E120 / REV/FTC/03	3889.92	917.25	12.30	9.16	98.53	1.79	0.81
18	Leciva S70+Lipova E120 / REV/FTC/04	2960.44	644.34	7.79	6.43	63.42	1.53	0.82
19	Leciva S70+Lipova E120/ REV/FTC/07	4153.25	548.74	14.21	5.60	112.32	3.57	0.86
20	Leciva S70+Lipova E120/ REV/FTC/08	3482.73	794. 06	16.88	8.91	144. 09	1.81	0.72
21	Leciva S70+Lipova E120/ REV/FTC/09	2815.31	460.23	20.91	6.74	103.23	2.27	0.58
22	Leciva S70+Lipova E120/ REV/FTC/10	2501.88	549.35	21. 06	8.64	133.79	1.79	0.52
23	DPPC/REV/FTC/01	3659.33	1148.133	15.15	11.46	225.93	1.72	0.76
24	DMPC/REV/FTC/01	921.57	284.37	2.63	2.83	7.95	0.99	0.77
25	S-100/REV/FTC/03	1985.85	346.17	5.65	3.56	19.98	1.57	0.82

AUC (%.h)/mL)-Area under the dissolution curve, ABC (h %) -Area between the drug dissolution curve and its asymptote, MRT (h) -Mean residence time of the drug molecules in the dosage form, MDT (h) -Mean dissolution time, VDT (hr) -Variance of dissolution time, RD (h) -Relative dispersion of dissolution time, DE-Dissolution efficiency

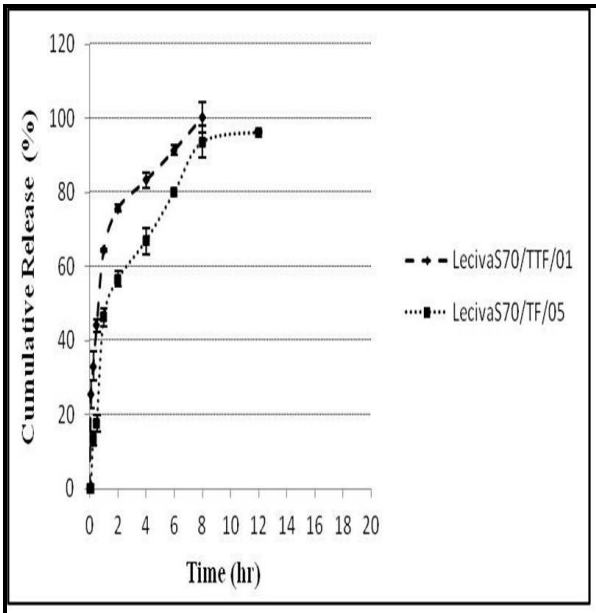

Fig. 5.25 Influence of surfactant using thin film hydration technique on in-vitro dissolution profile of dopamine loaded Leciva S70 and cholesterol LPs

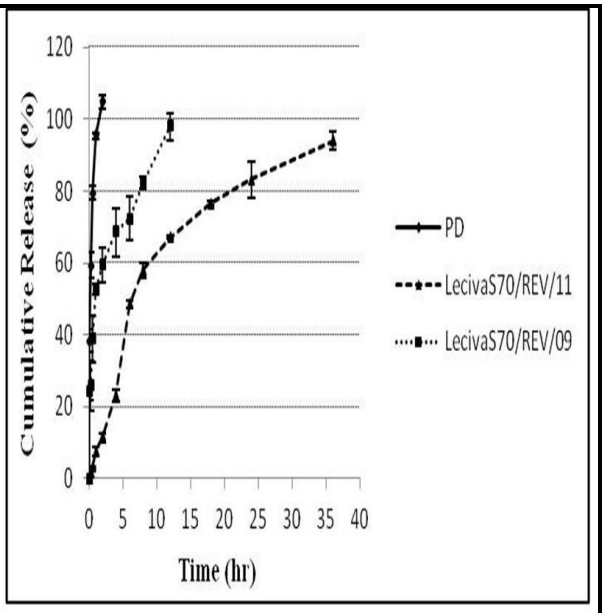

Fig. 5.26 Influence of Leciva S70 and cholesterol in different proportion with reverse phase evaporation technique on in-vitro dissolution profile of dopamine loaded LPs

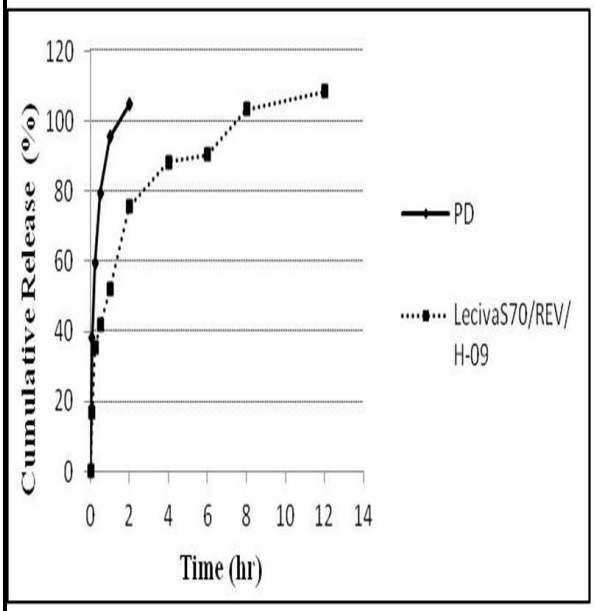

Fig. 5.27 Influence of hydration media using reverse phase evaporation technique on in-vitro dissolution profile of dopamine loaded Leciva S70 and cholesterol LPs

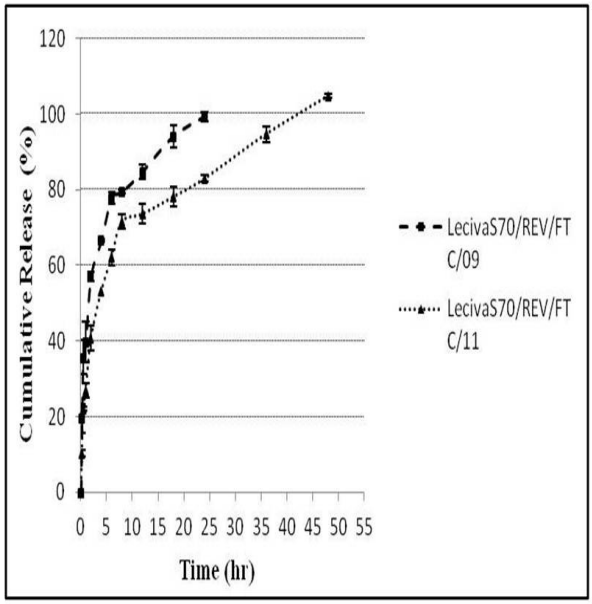

Fig. 5.28 Influence of Leciva S70 and cholesterol in different proportion with reverse phase evaporation and freeze and thaw method technique on in-vitro dissolution profile of dopamine loaded LPs

But after freeze thawing when the vesicle size got smaller, the diffusion pathway became shorter and the erosion became much easier, resulting in relatively quick release of the drug. According to

the release profiles, the integrity of the vesicles and the non-concentric structure might still be retained to some extent and thus provide a controlled drug release.

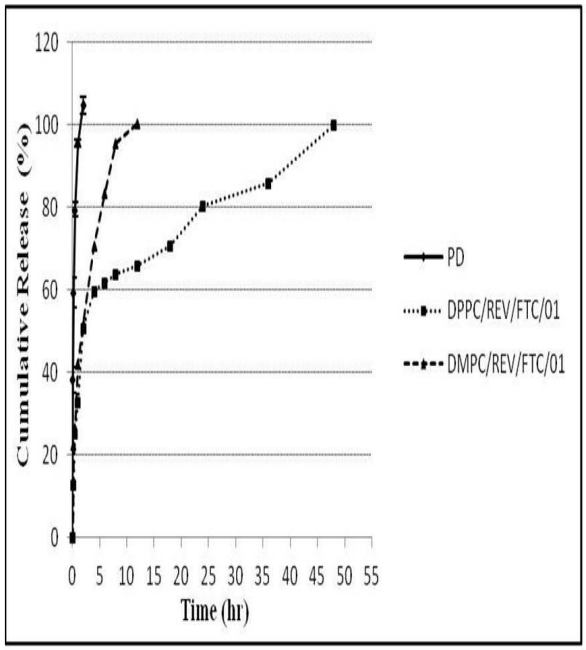

Fig. 5.29 Influence of DPPC and DMPC on in-vitro dissolution profile of dopamine loaded LPs

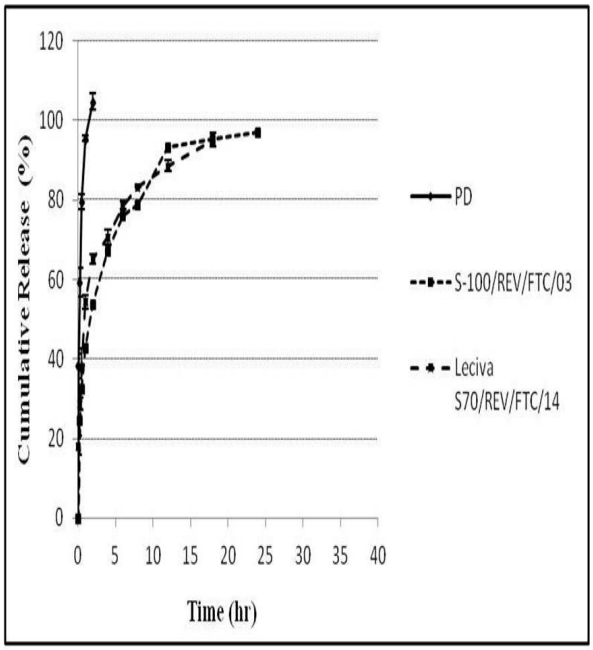

Fig. 5.30 Influence S-100 in comparison to Leciva S70 on in-vitro dissolution profile of dopamine loaded LPs

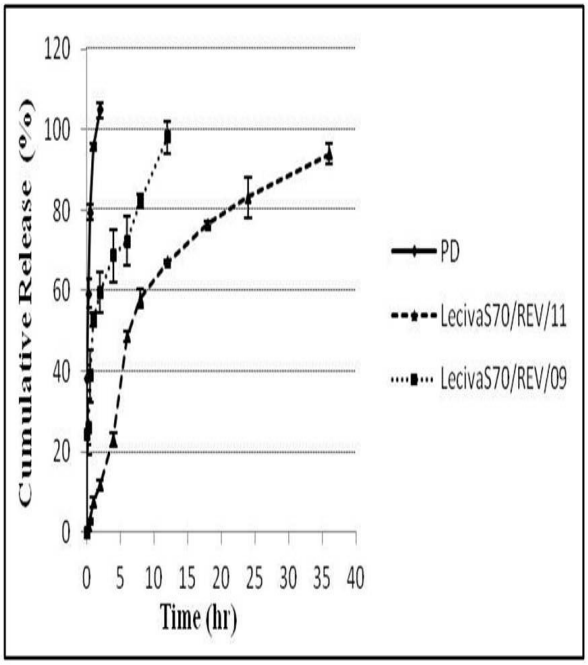

Fig. 5.31 Influence of Leciva S70 and cholesterol in different proportion without freeze and thaw cycles on in-vitro dissolution profile of dopamine loaded LPs

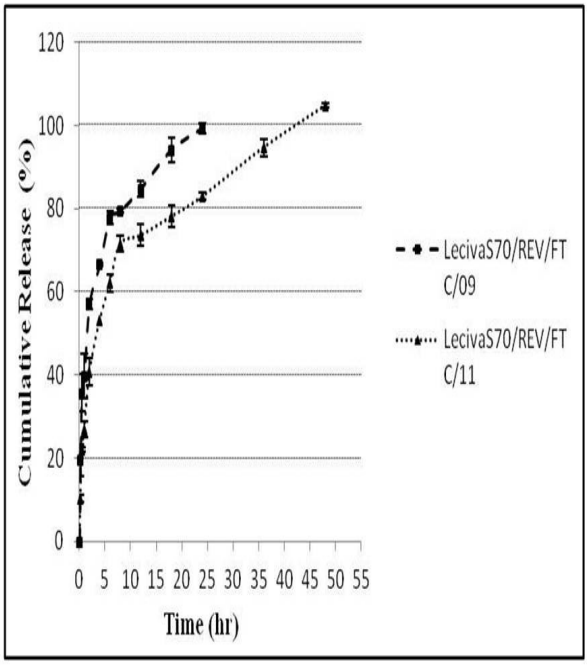

Fig. 5.32 Influence of Leciva S70 and cholesterol in different proportion with freeze and thaw cycles on in-vitro dissolution profile of dopamine loaded LPs

5.3.4 Stability studies

DSC thermograms of Leciva S70, Lipova E120, S-100, DPPP and DMPC showed a melting endotherm corresponding to the pure lipids (Chapter 4). DSC thermograms of the pure drug demonstrated a sharp endothermic peak at 252.17 °C. DSC thermograms of drug loaded liposomal formulations of Leciva S70, Lipova E120, S-100, DPPC and DMPC have shown to preserve the pure drug endotherm at 252.17 °C. Previously, several researchers have observed similar findings, which were attributed to loss of crystallinity resulting from molecular level dispersion of the drug within the lipid bilayers. The DSC thermograms of physical mixtures (Chapter 4) further supported this fact as the melting endotherm and enthalpy values were found to be unaffected. Additionally, the drug present in the physical mixture has shown sharp melting endotherm which was diminished in the formulation suggesting loss in crystallinity. Thermal analysis results indicated that the physical state of drug remains unchanged with the treatments given during manufacturing process and there is less possibility of physical interactions between the drug and lipids or other excipients.

A higher extent of stability was observed in LPs prepared. Freeze dried formulations stored at refrigerated and freeze temperature were found to be stable for 4 months with acceptable change in various product characteristics such as size, size distribution, drug entrapment, loading efficiency and in vitro release. All the LPs stored at 15 ± 5 °C for more than 4 months showed some increase in size, due to aggregation of particles (Table 5.9). Increase in fatty acid chain length as well as gel state of liposomes prepared using selective lipids were the responsible factors. In the presence of rigid acyl chain, freedom of movement of lipophilic chains decreased and this might lead to lower drug-membrane interaction and higher stability of the formulations. All freeze dried formulations showed better redispersibility even after 8 months of storage at -20 °C.

The AFM analysis showed (Fig. 5.33) no change of surface character of stored LPs. The in-vitro dissolution study of all stored formulations confirmed no change in release profile. The stability study result confirmed that the prepared LPs were stable in all the selected temperatures tested.

5.4 Conclusions

Leciva S70, Lipova E120 and combinations of Leciva S70 and Lipova E120 liposomes were prepared successfully using reverse phase evaporation technique coupled with freeze and thaw method. The various formulation variables such as lipids, cholesterol and drug amount along with other processing variables were found to affect critical properties of liposomes including particle size, size distribution, entrapment efficiency, loading efficiency and in vitro drug release profile. Further the studied correlation helped in designing formulations of intended characteristics such as

Table 5.9 Stability study results of optimized Dopamine loaded LPs formulations in three different conditions

Stability Conditions	Evaluation Parameters	LecivaS70 LPs		Lipova E120 LPs		Leciva S70+Lipova E120 LPs	
		Observation (months)					
		0	4	0	4	0	4
-20 ± 3ºC	Physical appearances	White	No change	White	No change	White	No change
	Size (nm)	277.03 ± 2.34	270.13 ± 1. 04	425.52 ± 0.10	416.21 ± 0.11	386.13 ± 2.10	375.23 ± 2.79
	PDI[a]	0.53 ± 0.13	0.51 ± 1.49	0.80 ± 0.04	0.72 ± 1.87	0.47 ± 0.40	0.51 ± 0.82
	ZP[b]	-50.30 ± 0.49	-50.52 ± 0.42	-40.23 ± 1.53	-40.19 ± 0.69	-35.64 ± 0.19	-37.11 ± 0.94
	LE[c]	0.924 ± 0.19	0.950 ± 0.29	0.62 ± 0.21	0.62 ± 0.20	0.725 ± 0.02	0.750 ± 0.22
5 ± 2° C	Physical appearances	White	No change	White	No change	White	No change
	Size (nm)	277.03 ± 2.34	275.13 ± 0.21	425.52 ± 0.10	466.23 ± 1.11	386.13 ± 2.10	354.21 ± 0.23
	PDI[a]	0.53 ± 0.13	0.61 ± 0.34	0.80 ± 0.04	0.82 ± 0.05	0.47 ± 0.40	0.42 ± 0.48
	ZP[b]	-50.30 ± 0.49	-50.12 ± 0.14	-40.23 ± 1.53	-40.56 ± 1.23	-35.64 ± 0.19	-35.11 ± 0.90
	LE[c]	0.924 ± 0.19	1. 04 ± 0.21	0.62 ± 0.21	0.62 ± 0.12	0.725 ± 0.02	0.801 ± 0.41
15 ± 5° C	Physical appearances	White	No change	White	No change	White	No change
	Size (nm)	277.03 ± 2.34	390.13 ± 1. 04	425.52 ± 0.10	516.21 ± 0.11	386.13 ± 2.10	451.23 ± 2.79
	PDI[a]	0.53 ± 0.13	0.91 ± 1.49	0.80 ± 0.04	1.52 ± 1.87	0.47 ± 0.40	0.51 ± 0.82
	ZP[b]	-50.30 ± 0.49	-45.52 ± 0.42	-40.23 ± 1.53	-41.89 ± 0.69	-35.64 ± 0.19	-39.11 ± 0.94
	LE[c]	0.924 ± 0.19	0.750 ± 0.29	0.62 ± 0.21	0.49 ± 0.20	0.725 ± 0.02	0.57 ± 0.22

[a] Polydispersity index, [b] Zeta potential, [c] Loading Efficiency

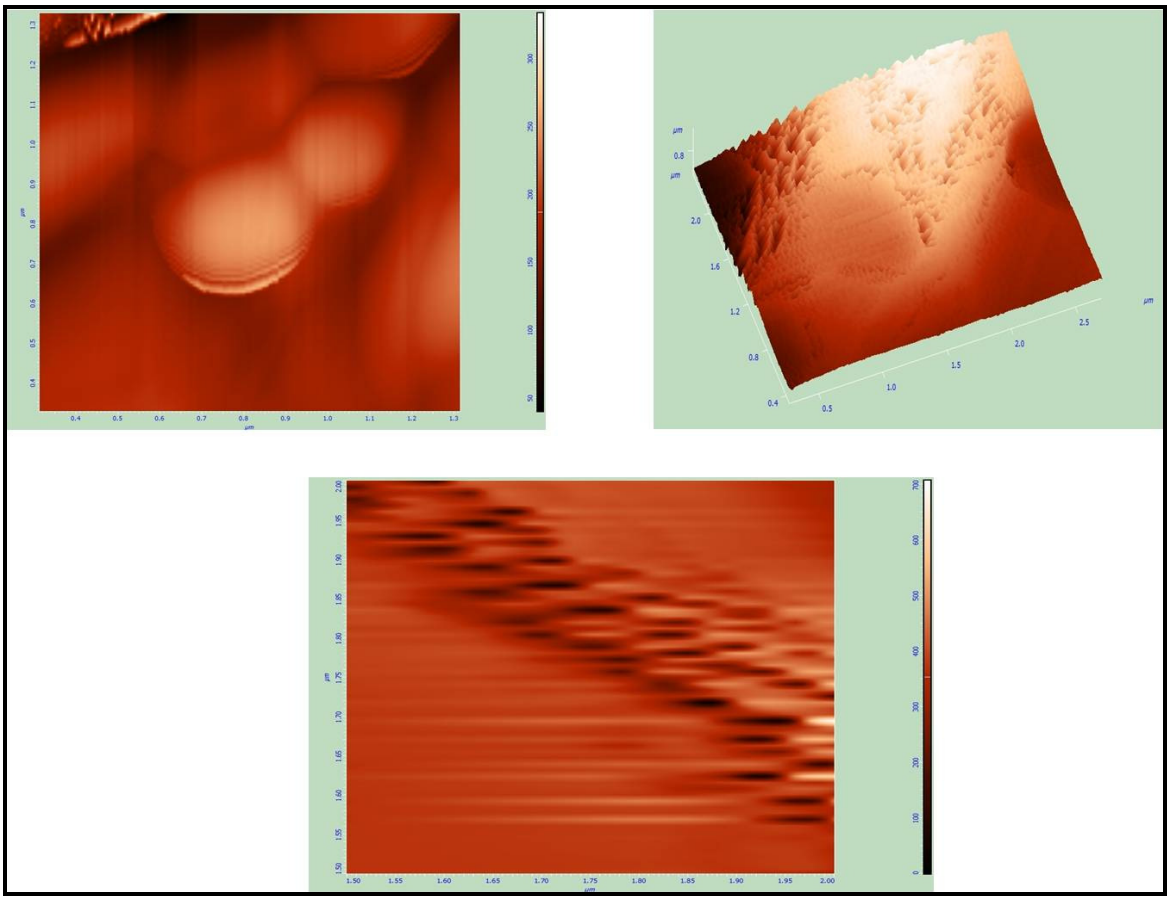

Fig. 5.33 Stability study results, Leciva S70 AFM after 4 month storage (A) Lipova E120 LPs AFM after 4 month storage (B) Leciva S70+Lipova E120 AFM after 4 month storage (C)

optimized particle size, high drug entrapment efficiency, and narrow size distribution by optimization of critical parameters. The prepared LPs were characterized for their shape and structure using TEM and AFM. The classy microscopic examination, TEM and AFM analysis revealed the spherical and smooth surface character of the LPs as well as their homogeneous bilayer vesicles without any amorphous arrangements. The developed formulations have shown a better entrapment within the bilayer vesicles with excellent particle morphology. Leciva S70 and Lipova E120 formulations prepared in combination using reverse phase evaporation technique coupled with freeze and thaw method were found to have an optimum particle size with narrow size distribution. The drug release profiles achieved were found to be more influenced by the proportion of lipid and cholesterol, types of lipid, preparation technique and amount of drug. The in-vitro drug release studies revealed that the developed formulations extended the drug release over 37-48 h depending on formulations, which could be useful for controlled drug delivery and explained the release profile by different drug release mechanisms. The in-vitro dissolution curve of DA loaded LPs was best fitted to First order release kinetics and Korsmeyer-Peppas model. Thus, the liposomes prepared using biodegradable lipids were found to be suitable for the drug

delivery application of hydrophilic drug with high drug entrapment and better loading efficiency. Moreover, the thermal studies have confirmed uniform distribution of the drug, at molecular level, within the lipid vesicles without showing any chemical and physical interactions between the drug and lipids or other excipients. Consequently, the prepared liposomes by selected method were found to be stable with acceptable reproducibility. However, increasing drug loading efficiency remains a significant challenge for hydrophilic drugs. The obtained results also justifed the rationale for conducting preclinical investigations including in vivo pharmacokinetic and biodistribution studies to assess the suitability of the controlled targeted nasal delivery of formulations. It may aid in enhancing the therapeutic efficacy of drug along with improved safety and increased patient compliance.

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Chapter 6. In-vivo Pharmacokinetic and Biodistribution Study

6.1 Introduction

For evaluation of a designed formulation and decide its therapeutic effectiveness, pharmacokinetic and biodistribution study are essential in human volunteers or patients. However, preclinical studies to be carried out in suitable animal model before studying in human volunteers or patients. Pharmacokinetic and biodistribution studies of designed formulations were planned with objective to provide direct intranasal (i.n.) delivery for achieving effective therapeutic concentration with intended duration to produce the desirable pharmacological effects at the site of action with minimal or undesirable side effects. The selection of an appropriate site of drug administration is one of the most important factors for optimizing its therapeutic effects. In the present study liposomes (LPs) delivery to brain through nasal route by- passing blood brain barrier (BBB) were investigated in rats. As discussed in Chapter 1, conventional drug delivery systems, which release the drug in general circulation, normally cannot cross BBB effectively; failing to deliver drugs sufficiently or poorly into the brain, therefore not always preferred for treating CNS diseases. Further excess drug goes to other parts of the body from circulation, which causes side effects or toxic effects and wastage. Liposomal technology combined with appropriate administration site provides more opportunities for targeting Dopamine (DA) delivery for Parkinson's disease (PD) therapy. It was reported that Dopamine delivered via nasal route achieved better permeability in brain via olfactory pathway (1). The connection between the lipid solubility of a drug and its CNS access is evidence to represent a direct correlation between brain penetration and the ability of a drug to partition into the lipid of the cell membrane (2). Thus liposomal carrier system with optimal lipid solubility for BBB penetration that also retains a significant central pharmacological activity of the drug would be the desired solution. The passage from blood circulation to the brain via a lipid mediated carrier system at the BBB is one of the dominant factors influencing the brain concentration of a drug and thus researchers reported enhanced permeability to brain by administering i.v. however, enhancement is not that much. Therefore, the drug Dopamine was encapsulated in LPs to facilitate its delivery from nasal route directly to brain. It was shown that intranasal (i.n.) administration of DA had the capacity to improve distribution and pharmacological effect of Dopamine in CNS regions compared to i.v. administration. Intranasal administration being a non invasive method can be an advantageous strategy for delivering Dopamine into brain. Considering the fact that the in vivo fate of drug in novel drug delivery system (DDS) with specific administration site is significantly different compared to conventional DDS, detailed in vivo investigation to determine the biological fate of prepared liposomal DDS is essential (3). Though FDA has not approved specialized guidelines for nanotechnology based formulations, the pharmacokinetic requirements for these delivery systems covered under federal register are extended to these formulations (21 CFR Part 314, 1998) (4). As of now, these regulations indicate

that pharmacokinetic data obtained from the brain and plasma concentration time profile of a drug under investigation is sufficient for its regulatory approval as nano drug delivery systems. Although the purpose of a pharmacokinetic study is to verify the safety and efficacy of the candidate drug, the application of pharmacokinetic principles in the design and development of the nano DDS provide rational for designing effective and better DDS (5, 6). Thus, the effectiveness of a developed DDS as a new drug product cannot be demonstrated without complete pharmacokinetic and biodistribution studies.

6.2 Materials and Methods

Pure Dopamine HCl and selected liposomal formulations, prepared and mentioned in Chapter 5 were used for these studies. Reserpine (RES) (assay 100 %) was purchased from Sigma-Aldrich, India. Surgical instruments scissors, forceps, glass hypodermic syringes, etc were procured from the Niraj Industries Pvt. Ltd. Mumbai, India. All the animal experiments were done with proper approval from Institute Animal Ethical Committee.

6.3 Residual solvent evaluation

During development of delivery systems, several organic solvents were used for various purposes, which is unavoidable and if are not completely removed from the final products, it may lead to serious adverse effect to the patients. Hence, it is important to ensure that there is no residual solvent left in the finished products. As per USP and ICH (Q3C) guidelines, the residual solvents in pharmaceutical products should be within the limits (7, 8). The residual solvents are classified as, Class 1, solvents to be avoided, known or suspected as human carcinogens and environmental hazards; Class 2 solvents are non-genotoxic animal carcinogens and suspected of other significant but reversible toxicities; Class 3, solvents with low toxic potential and health-based exposure limit is needed (8). In the present study, during LPs preparation diethylether (DEE) (Class-3) solvent was used, which was identified using ^{1}H nuclear magnetic resonance and infrared spectrum (mentioned in Chapter 4).

^{1}H NMR

The presence of residual solvent (DEE) in the blank and DA loaded LPs were analyzed by Bruker NMR spectrometer operating at 400 mHz (available at Sophisticated Analytical Instrumentation Facility, Panjab University, Chandigarh). The LPs were dissolved in $CDCl_3$ and the spectrum was recorded for the respective samples and interpreted for presences of solvent peaks.

6.4 Histopathological studies

6.4.1 Protocol approval and animal grouping

The in-vivo acute toxicity study for formulations was done in Wistar rats (300 ± 25 g) using saline control, DA solution and DA loaded LPs. The study protocol was approved by the Institute Animal Ethical Committee, BITS, Pilani, India (protocol no: IAEC/RES/18/14). Rats were acclimatised for at least one week before the experiment was initiated. Throughout the experiment, animals were housed in polypropylene cages filled with sterile paddy husk and maintained at 22 ± 2°C and 50-60 % RH, under a 12:12 h light-dark cycle. The animals were separated according to body weight and they were marked with picric acid in head, tail and body. Animals were divided into three groups, saline control, DA solution and DA loaded LPs with each group having three animals per cage. At predetermined time points tissue samples (brain, nose, lungs, trachea and oesophagus) were taken from respective animals.

6.4.2 Dosing of animals and sample collection

The acclimatised rats were fasted overnight before sample administration and had access to water ad libitum. Dosing (1 mg/kg) was delivered through i.n. via Portex fine bore polythene tubing of internal diameter 0.40 mm and outer diameter 0.80 mm standardizing dose volume suitable for nasal administration (50 μL). After 12 h, animals were sacrificed and the respective tissue sections such as nasal cavity, brain, lungs, trachea and esophagus were isolated and fixed in 10 % formalin saline solution after washing with cold water.

6.4.3 Histopathological evaluation

The tissue samples were dehydrated by treating with increasing concentration of absolute alcohol and xylene and then embedded in paraffin block. The tissue sections were sliced using a microtome and processed for hydration and for microscopic study. Finally the tissue sections were stained using hematoxylin and eosin then mounted on the microscopic slide and fixed with cover slips using DPX mountant solution. The tissue section slides were observed under light microscope (4X/10X/40X) for any histopathological changes (Histopathological plates were prepared at Medicos Center, Chandigarh and evaluated at Department of histopathology, PGI, Chandigarh).

6.5 In-vivo pharmacokinetic and biodistribution study

The pharmacokinetic and biodistribution characters of DA and DA loaded LPs were performed in Wistar rats (300±25 g) pre-treated with Reserpine (RES). As DA is continuously released and available in brain, and likely to interfere with estimation of extent of delivery of DA from formulations, animals were pre-treated with RES for 5 consecutive days to induce PD by complete DA depletion.

6.5.1 Protocol approval and animal grouping

The study protocol was approved by the Institute Animal Ethical Committee, BITS, Pilani, India (Protocol no: IEAC/RES/13/13/REV/15/9, IEAC/RES/13/13/REV-2/17/15 & IAEC/RES/18/14). Rats were acclimatised for at least one week before the experiment. Throughout the experiment, animals were housed in polypropylene cages filled with sterile paddy husk and maintained at 22 ± 2°C and 50-60 % RH, under a 12:12 h light-dark cycle. The animals were separated according to body weight and they were marked with picric acid in head, tail and body. For DA depletion study, two groups (n=5) of animal (Wistar rats) were taken. First group, of control animals, were administered 5 % glucose solution i.p. Second group of rats were treated with RES (5 mg/kg, i.p).

To determine the extent of delivery of DA from solution and DA loaded LPs directly to brain from i.n. and i.v. administration subsequent groups of animals were used. For i.n studies animals were randomly divided into four groups, DA solution, DA loaded Leciva S70, Lipova E120 and combination of Leciva S70 and Lipova E120 LPs. Each group having ten cages, each cage containing three animals and for each time point samples were taken from three animals. For i.v studies animals were divided into two groups, DA solution and DA loaded Leciva S70 and Lipova E120 liposomes in combination and similarly for each time point samples were taken from three animals.

6.5.2 Preparation of Reserpine solutions, Dopamine solutions and Dopamine loaded LPs dispersion

RES was dissolved in 1 % glacial acetic acid at a concentration of 2.5 mg/mL and then diluted with water for injection to obtain a final concentration of 0.25 mg/mL and not more than 0.1 % glacial acetic acid. DA solution and DA loaded LPs dispersion for i.n. administration were prepared by dissolving 6 mg of pure DA and LPs equivalent to 6 mg drug with 1 % sodium metabisulfide in 1 mL of PBS buffer (pH 4.0). Concentration (6 mg/mL) was made such that about 50 µl contained the required dose based on 1 mg/kg body weight. Similarly for i.v administration 10 mg of pure DA and LPs were prepared with 1 % sodium metabisulfide in 10 mL of sterile saline (0.9 % of sodium chloride) respectively. The DA solution and the selected LPs formulations were administered as aqueous dispersion at dose equivalent to 1 mg/kg. through i.n. as well as i.v route. The volume of drug solution to be administered through i.n. was arround 50 µl and for i.v. administration below 1 mL.

6.5.3 Dosing of animals and sample collection

This RES solution was injected i.p. at a concentration of 5 mg/kg for 5 consecutive days to generate acute DA depletion before formulations administration. The formulations (dose 1 mg/kg of DA) were administered through i.n and i.v. route. For each time point 300 µL of blood samples

were collected through retro-orbital under mild diethyl ether anaesthesia. The samples were immediately transferred into EDTA-Na_2 containing pre-labelled 1.5 mL polypropylene micro tubes, instantly after collection and the micro tubes were gently inverted several times to ensure complete mixing with the anticoagulant. At the same time animals were sacrificed for biodistribution studies and all the organs were collected separately. The sample collection time points were 0.5, 1, 3, 6, 9, 12, 18, 24, 36 and 48 h after administration. Samples were also collected at zero time (pre-administration). In case of biodistribution study, brain, nasal cavity, lungs, trachea and oesophagus samples were used from rats at same time points. After collecting the tissue samples they were cleaned using phosphate buffer to remove the residual blood and blotted dried with Whatman filter paper. All the samples were stored at -80°C until analysis and thawed every time before analysis.

6.5.4 Estimation of Dopamine in plasma and tissue samples

The blood samples were centrifuged at 4000 rpm for 15 min at 4°C to obtain plasma samples. The plasma samples were processed for extraction of DA as mentioned in Chapter 3 for analysis. All the tissue samples were weighed, chopped and transferred to 50 mL tarson tube for homogenizing. The obtained tissue suspension was processed for DA estimation as mentioned in Chapter 3. DA in the plasma and tissue samples was estimated by HPLC method as mentioned in Chapter 3. The plasma concentration of DA vs. time and DA amount in per g of tissue vs, time were plotted for pure drug and all the LPs formulations.

6.5.5 Pharmacokinetic data analysis

All pharmacokinetic parameters were calculated by non-compartment analysis using WinNonlin software (Version 2.1, Pharsight Corporation and USA) and it is listed in Table 6.1, 6.2, 6.3 and 6.4. Results of in vivo studies were statistically evaluated using unpaired t-test at 5 % level of significance.

6.6 Result and Discussion

6.6.1 Residual solvent evaluation

The ^1H NMR spectra of blank Leciva S70 and Lipova E120 LPs and DA loaded Leciva S70 and Lipova E120 LPs in $CDCL_3$ were obtained and there was no proton peak found corresponding to residual solvent diethyl ether, used in the preparation of LPs. All the characteristic peaks of DA, lipids and excipients were observed. This analysis showed that the prepared LPs were free from the residual solvents or below permissible limit and it can be administered safely to rat by i.n. or i.v. route.

6.6.2 Histopathological studies

The in vivo safety of LPs formulations through nasal administration was evaluated using histopathology assay. In this study, intact nasal septal mucosa and brain were used to determine the effects. As observed from histopathological images (Fig. 6.1 & 6.2) there were no microscopic changes in brain regions of treated animals when compared with control animals.

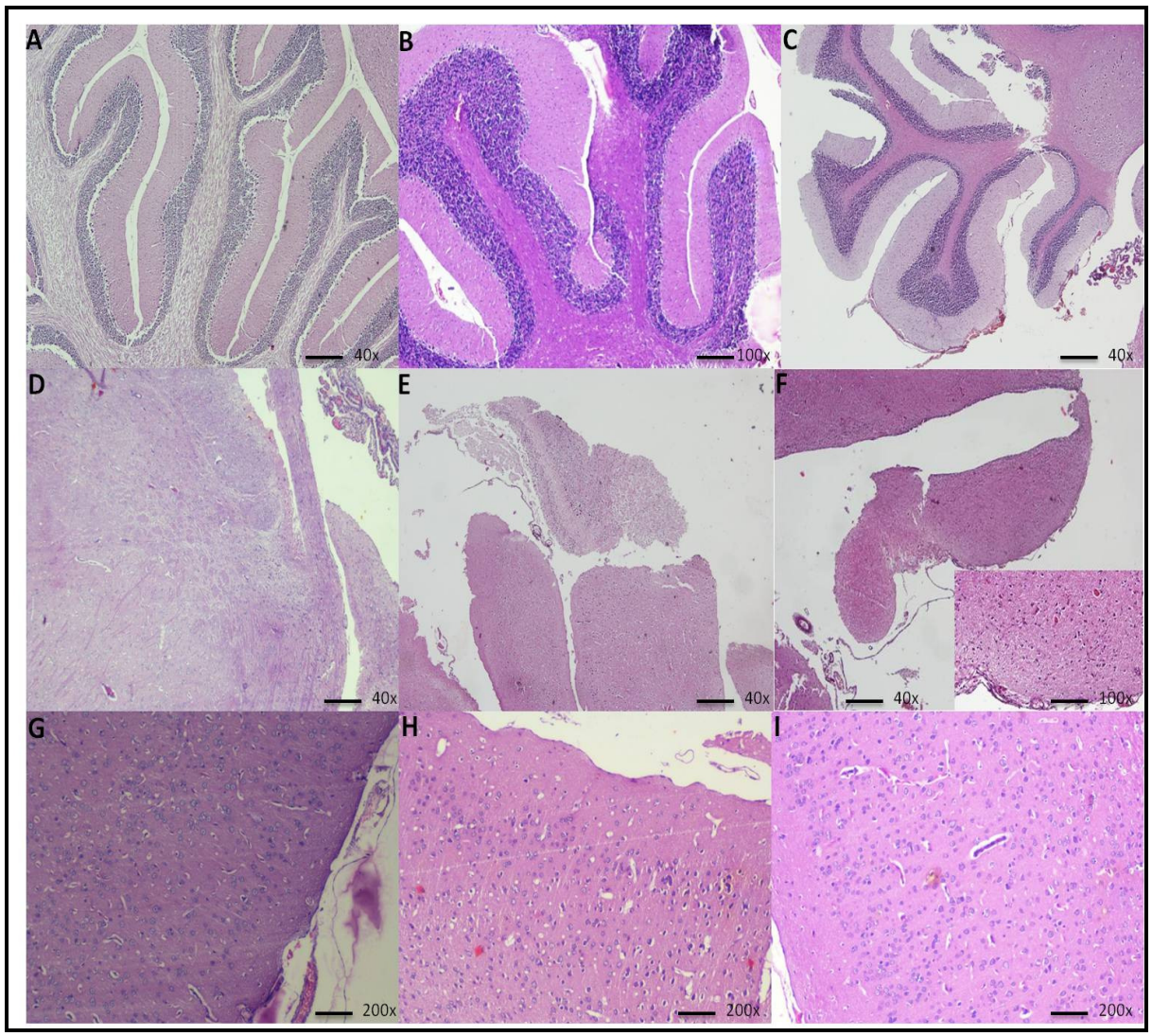

Fig. 6.1 Histopathology staining sections of different brain and olfactory regions, Control animal; cerebellum A), base of brain with olfactory tract D), base of frontal lobe cortex G), Dopamine solution; cerebellum B), olfactory tract in the center E), cortex H), LPs formulation; cerebellum C), olfactory bulb and base of frontal lobe F), cortex at base of brain I)

The nasal glands, blood vessels and nerve tracts of nasal cilia were clearly arranged in order and density, showing no significant difference to those treated with control. This study was conducted in different regions of brain to reflect damage of nerve cells and to evaluate the neuroprotective effect of LPs formulation. As shown in figure above (Fig. 6.1) neither cell necrosis nor removal of

cilia from the nasal mucosa was observed after treating with LPs dispersion. These observations indicate that the LPs formulations had no significant harmful effect on the microscopic structure of nasal mucosa, which is in accordance with the results reported in literature (9).

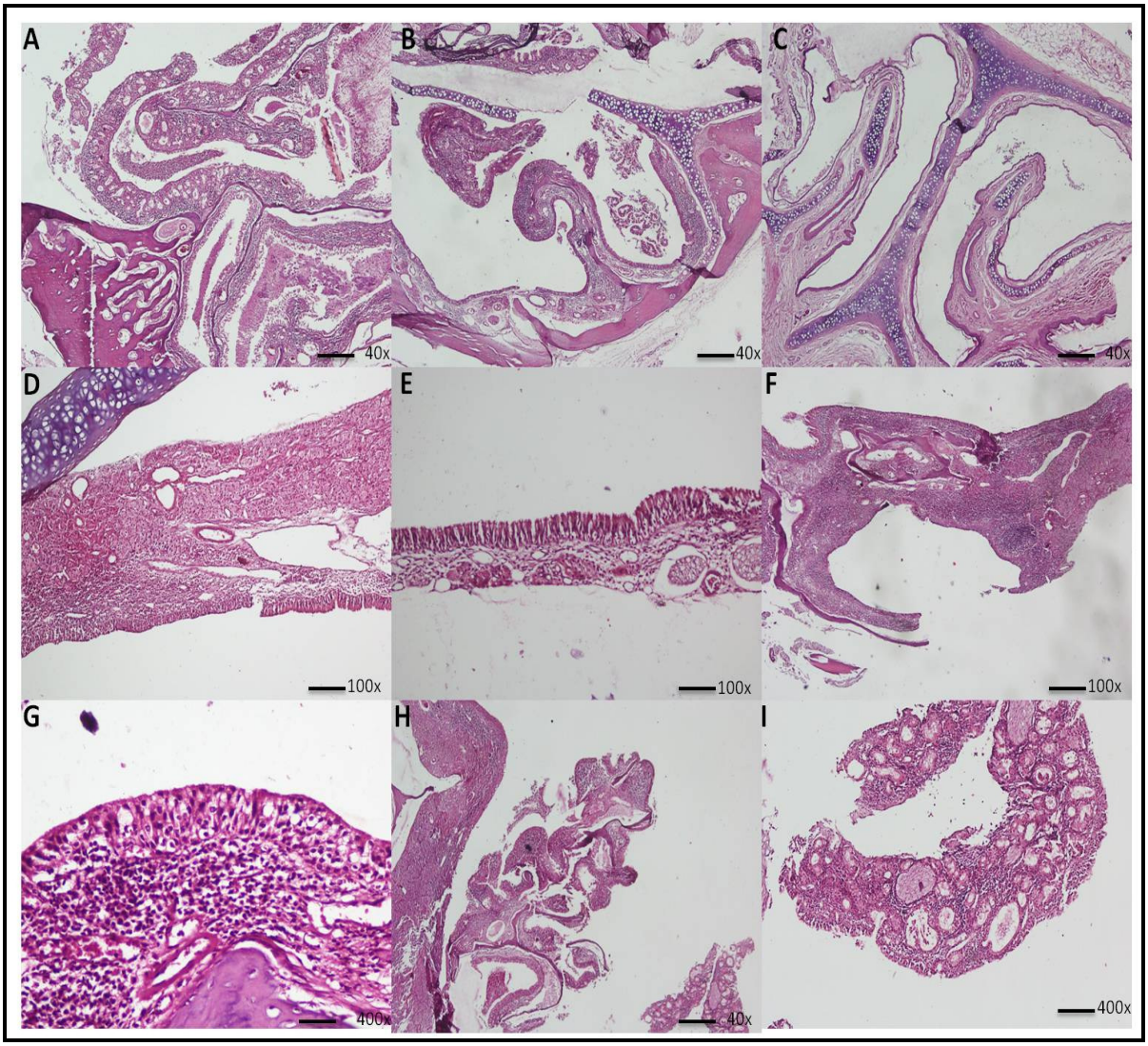

Fig. 6.2 Histopathology staining sections of different nasal cavity regions, Control; turbinate mucosa (A), normal mucosa with cartilage on one surface (D), turbinate mucosa part (G), Dopamine solution; turbinate mucosa part (B), normal epithelium (E), polypoid mucosa (H), LPs formulation; both nasal passages (opening on right) (C), nasophrayngeal area showing severe inflammation an enlarged lymphoid follicle (tonsil or adenoid) (F) mucosa, glands (I)

The nasal epithelial layer was intact, but there was small alteration in the basal membrane and superficial part of the submucosa when compared in all groups including control which could be due to administration of dose through polypropylene tubes. As there was no neuronal damage observed in brain regions of all groups, indicating DA loaded LPs to be safe for intranasal delivery. If the organic volatile impurities are not removed completely it may result in generation of

inflammatory and tissue response at the respective organs (8, 9). Thus the morphology of the respective tissue cells can give clear picture about inflammatory condition after i.n. administration of LPs. The histopathology studies also confirm that the prepared LPs or residual solvents did not, induce any histological damage, when administered i.n. to rats at 1 mg/kg dose. Hence, the solvents were absent or well within the limits and these formulations can be safely administered by i.n. route.

6.6.3 In vivo pharmacokinetics and biodistribution studies

To start with pharmacokinetics and biodistribution studies DA depleted rats from Reserpine study were used. In this study rats were treated with RES through i.p administration for 5 consecutive days and brain from all animals were estimated for Dopamine level. It was found from the study that Dopamine was depleted to non detectable level after Reserpine treatment using HPLC method (Chapter 3). Single dose i.n. pharmacokinetics studies and for comparison i.v. study was carried out for pure drug and selected liposomal preparations. The drug concentration levels in brain (site of action), plasma, lungs, trachea and oesophagus were determined for knowing biodistribution profile, pharmacokinetic parameters and expected therapeutic efficacy. The principle objective was to investigate the specific brain bioavailability and understand the difference between in vivo behaviour of pure drug and drug loaded LPs after i.n. and i.v. administration separately.

a) Brain biodistribution studies

i) Intranasal administration

The drug concentration in rat brain following single intranasal dose of the pure drug, Leciva S70 LPs, Lipova E120 LPs and combination of Leciva S70+Lipova E120 LPs were determined and plotted as a function of time (Fig. 6.3). The various pharmacokinetic parameters were calculated using a data modelling software and is listed in Table 6.1. Brain biodistribution studies after intranasal administration of the pure drug indicated a less penetration of free drug to the brain when compared with liposome formulations. The data suggest dopamine containing LPs improved delivery into brain after intranasal administration via nasal olfactory pathway. The maximum drug concentration (C_{max}) observed in brain was 0.331 μg/g at 1 h (T_{max}) of the post dosing of DA solution. Intranasal administration of Leciva S70, Lipova E120 and Leciva S70+Lipova E120 LPs indicated a significant increase in permeation and retention of the drug in brain. Against pure drug, Leciva S70 and Lipova E120 LPs in combination indicated maximum drug concentration (C_{max}) 2.74 μg g^{-1} at 9 h (T_{max}) in brain. However maximum permeation and retention of the drug was observed in Lipova E120 LPs with $AUC_{0\text{-}inf}$ to 156.98 μg h g^{-1}. Moreover, approximately 42 fold increases in MRT was observed for Lipova E120 LPs against the pure drug indicating increased half life in brain.

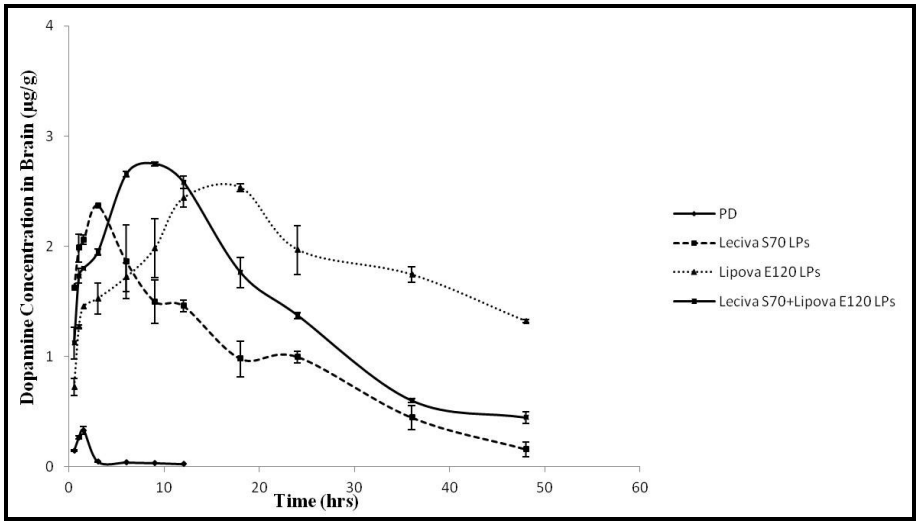

Fig. 6.3 Dopamine concentration in brain verses time profile following intranasal administration of pure drug, Leciva S70 LPs, Lipova E120 LPS and combination of Leciva S70 and Lipova E120 LPs in rats

Table 6.1 Summary of the pharmacokinetic parameters in brain after intranasal administration of Dopamine and Dopamine loaded LPs

Parameters	Pure Drug Dopamine HCl	Leciva S70 LPs	Lipova E120 LPs	Leciva S70+Lipova E120 LPs
$AUC_{0\text{-}inf}$ (µg h g^{-1})	0.416 ± 0.03	49.02 ± 2.81	156.98 ± 2.172	77.16 ± 1.07
C_{max} (µg g^{-1})	0.331 ± 0.04	2.37 ± 0.33	2.53 ± 0.03	2.74 ± 0.18
Cl (g h^{-1} kg^{-1})	2.14 ± 0.18	0.020 ± 0.01	0.0064 ± 0.00	0.0129 ± 0.00
MRT (h)	1.33 ± 0.05	17.46 ± 0.65	55.68 ± 1.89	22.82 ± 1.22

*Each value represents the mean ± standard deviation of 3 independent determinations

Increased AUC and MRT indicated more permeation of the drug to brain via nose in LPS DDS with extended release and retention of drug in brain for longer time. The difference in C_{max} and $AUC_{0\text{-}inf}$ values following intranasal administration of solution and LPs formulations was found to be statistically significant at 5 % level of significance. Low concentration C_{max} in DA solution indicated poor delivery from solution though given via i.n. Delivery of LPs found to be much higher.

ii) Intravenous administration

The drug concentration in the rat brain following single intra-venous dose of the pure drug and Leciva S70+Lipova E120 LPs were also determined and plotted as a function of time (Fig. 6.4). The various pharmacokinetic parameters calculated using a data modelling software is listed in Table 6.2. Brain biodistribution studies of the pure drug after i.v. administration confirmed a poor

distribution of free DA to brain with C_{max} of only 0.024 μg g^{-1}. The drug concentration levels in brain were negligible with $AUC_{0\text{-}inf}$ to 0.034 μg h g^{-1} confirming that Dopamine cannot cross BBB as a free drug. Intravenous administration of LPs revealed that the LPs produced relatively slower absorption rate but increased extent of absorption with the maximum drug concentration (C_{max}) of 1.07 μg g^{-1}.

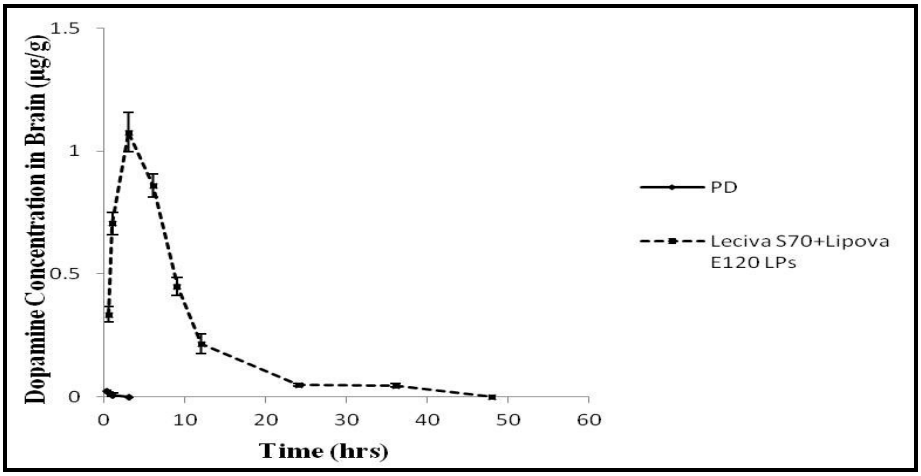

Fig. 6.4 Dopamine concentration in brain verses time profile following intravenous administration of pure drug and combination of Leciva S70 and Lipova E120 LPs in rats

The non compartmental analysis indicated significant increase in the $AUC_{0\text{-}inf}$ to 10.62 μg h g^{-1} for liposomes suggesting enhanced permeation through BBB and distribution to the brain. LPs have shown increased residence time of the drug in the brain indicating significant increase in elimination half-life in the brain. Moreover, MRT of DA from the drug loaded LPs increased considerably in comparison with the pure drug (1.08 to 9.74 h). The DA amount for Leciva S70 and Lipova E 120 LPs was statistically significant in brain ($P<0.05$) tissues in comparison with DA solution. Thus it was concluded that DA loaded LPs increased the extent of drug permeation to the brain against the pure drug.

Table 6.2 Summary of the pharmacokinetic parameters in brain after intravenous administration of Dopamine and Dopamine loaded LPs

Parameters	Pure Drug Dopamine HCl	Leciva S70 +Lipova E120 LPs
$AUC_{0\text{-}inf}$ (μg h g^{-1})	0.034 ± 0.00	10.62 ± 0.91
C_{max} (μg g^{-1})	0.024 ± 0.00	1.07 ± 0.08
Cl (g h^{-1} kg^{-1})	30.08 ± 1.86	0.09 ± 0.07
MRT (h)	1.08 ± 0.11	9.74 ± 0.15

*Each value represents the mean ± standard deviation of 3 independent determinations

The study demonstrated that intranasal delivery of DA solution and liposomes showed the capacity to improve DA delivery and adequate retention in brain than that of i.v. administration. When AUCs of i.v. and i.n. administration of free drug and after i.n. administration of LPs formulation were compared, the intranasal delivery of liposome formulation Leciva S70 + Lipova E120, showed 2269 fold higher AUC (77.16 μg h g^{-1}) as compared to free drug (0.034 μg h g^{-1}) given i.v. and almost 185 fold higher when compared with free drug administration intranasally (0.416 μg h g^{-1}). Intranasal administration of DA in liposomal form significantly increased the exposure and resulted in its higher concentration in brain with increased residence time and significant increase in the elimination half-life. The DA amount for LPs was statistically significant in brain ($P<0.001$) tissues via intranasal administration in comparison with intravenous administration. Also sustained release effect of DA from liposomes may be useful for reducing administration frequency thereby increasing patient compliance.

b) Drug targeting efficiency percentage (%DTE) and nose to brain direct transport percentage (% DTP)

It is already revealed in the literature that the drug uptake into the brain from the nasal mucosa mainly occurs via two different pathways. One is the systemic pathway by which some of the drug is absorbed into the systemic circulation and subsequently reaches the brain by crossing BBB. The other is the olfactory pathway by which the drug partly travels from the nasal cavity to CSF and/or brain tissue (10-12). The drug targeting efficiency (% DTE) and nose to brain direct transport percentage (% DTP) represents the percentage of drug directly transported to the brain via the olfactory pathway and was calculated using two equations mentioned below (13-15). % DTE represents relative exposure of the brain to the drug following intranasal administration vs. i.v. administration. It was calculated using formula as follows:-

$$DTE\ (\%) = \frac{(AUC_{brain}/AUC_{blood})_{i.n.}}{(AUC_{brain}/AUC_{blood})_{i.v.}} \times 100$$

% DTP represents percentage of the dose that is estimated to reach the brain via direct routes (i.e., via trigeminal or olfactory nerves), vs. the overall delivery of the studied drug/compound to the brain (i.e. via all the direct routes and via the BBB). It was calculated according to following formula:-

$$DTP\ (\%) = \left[\frac{B_{i.n.} - B_x}{B_{i.n.}}\right] \times 100$$

Where $B_x = (B_{i.v.}/P_{i.v.}) \times P_{i.n.}$, B_x is the brain AUC fraction contributed by systemic circulation through the BBB following intranasal administration, $B_{i.v.}$ is the $AUC_{0\text{-}inf}$ (brain) following

intravenous administration, $P_{i.v}$. is the $AUC_{0\text{-}inf}$ (blood) following intravenous administration, $B_{i.n}$. is the $AUC_{0\text{-}inf}$ (brain) following intranasal administration, $P_{i.n.}$ is the $AUC_{0\text{-}inf}$ (blood) following intranasal administration, AUC is the area under the curve.

DTE % and DTP % were calculated using tissue/organ distribution data following intranasal and intravenous administration. Leciva S70 and Lipova E120 LPs showed the highest DTE % (112151.16) and DTP % (99.9) as compared to DA solution with DTE % (198.78) and DTP % (51.92). Two fold higher DTP % of LPs as compared to pure drug showed the benefits of LPs formulation through nasal route. The higher DTE % and DTP % suggest that LPs has better brain targeting efficiency via nose.

c) Plasma pharmacokinetic studies

i) Intranasal administration

The drug concentration in plasma versus time curve following intranasal administration of pure drug, Leciva S70, Lipova E120 and Leciva S70+Lipova E120 LPs are shown in Fig. 6.5. The various pharmacokinetic parameters calculated using a data modelling software is listed in Table 6.3. Pharmacokinetic profile of i.n. administration of free DA showed a maximum $AUC_{0\text{-}inf}$ of 3.54 μg h L^{-1} in plasma as compared to all LPs formulations. This indicated that the free DA from solution was entering in systemic circulation more via nasal route, however in LPs formulations significantly lower amount of DA was found in plasma suggesting more nose to brain targeting.

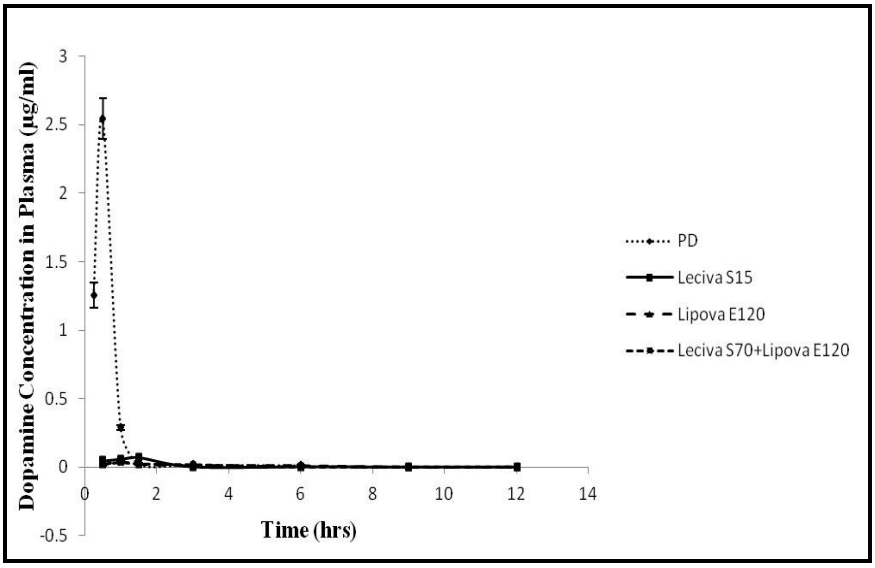

Fig. 6.5 Dopamine concentration in plasma verses time profile following intranasal administration of pure drug, Leciva S70 LPs, Lipova E120 LPS and combination of Leciva S70 and Lipova E120 LPs in rats

Table 6.3 Summary of the pharmacokinetic parameters in plasma after intranasal administration of Dopamine and Dopamine loaded LPs

Parameters	Pure Drug Dopamine HCl	Leciva S70 LPs	Lipova E120 LPs	Leciva S70 +Lipova E120 LPs
$AUC_{0\text{-}inf}$ (μg h L^{-1})	3.54 ± 0.11	0.27 ± 0.12	0.19 ± 0.10	0.16 ± 0.01
C_{max} (μg L^{-1})	2.54 ± 0.15	0.07 ± 0.04	0.04 ± 0.05	0.03 ± 0.01
Cl (L h^{-1} kg^{-1})	0.39 ± 0.01	3.60 ± 0.16	5.17 ± 0.53	6.15 ± 0.53
MRT (h)	1.30 ± 0.03	2.34 ± 0.02	4.20 ± 0.10	3.52 ± 0.19

*Each value represents the mean ± standard deviation of 3 independent determinations

d) Other tissues distribution studies via intranasal administration

i) Nose, lungs, trachea and oesophagus distribution

The drug amount in rat nasal cavity, lungs, trachea and oesophagus vs. time profile and pharmacokinetic parameters of DA and DA loaded LPs after i.n administration are presented in Fig. 6.6.

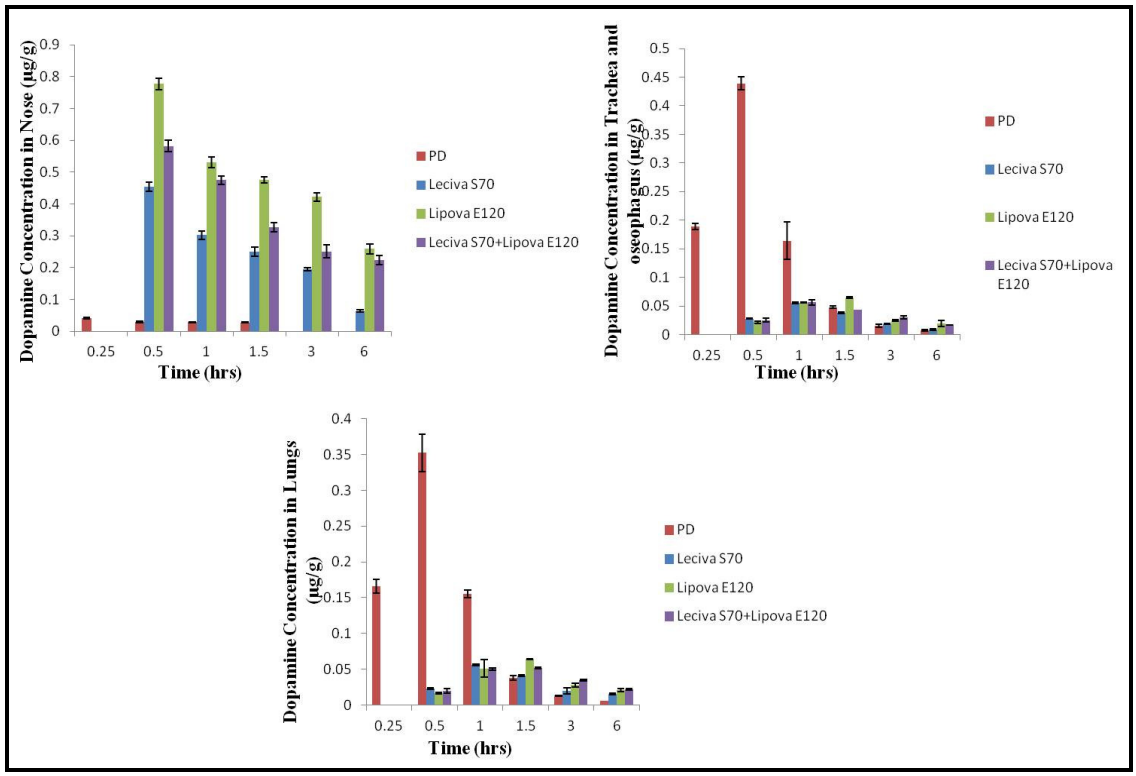

Fig. 6.6 Amount of Dopamine in nose, trachea and oesophagus and lungs following intranasal administration of pure drug, Leciva S70 LPs, Lipova E120 LPS and combination of Leciva S70 and Lipova E120 LPs in rats

*Each value represents the mean ± standard deviation of 3 independent determinations

When compared to DA solution, the DA loaded LPs clearances were less in all tissues The biodistribution profile from nasal administration study clearly showed that DA loaded LPs stayed for significantly longer duration in nasal cavity as compared to drug solution. The amount of DA reached lungs and trachea oseophagus decreased drastically after 0.5 h in case of LPs when compared to pure drug. Overall the biodistribution study results showed that DA loaded LPs distribute selectively to brain than the other tissue systems studied. This study showed that the prepared LPs can be used to treat PD, because of its selective delivery via nose.

6.7 Conclusion

The residual solvent analysis by ^{1}H NMR clearly showed that there was no residual solvent, DEE in the blank and DA loaded LPs. This study indicated that the LPs formulations were free from the residual solvents and it is safe to administer by i.n. to animals. The histopathological studies of nasal tissue and brain, on administration of formulations, also suggested that the prepared LPs did not show any toxic effect on the rat tissues and hence there was no change in the architecture of respective organ cells. The biodistribution studies confirmed that DA LPs were rapidly distributed extensively to brain from the nasal delivery of developed liposomes in comparison to i.v. administration. The brain concentration levels were found to be very high in comparison to plasma concentration levels compared to i.v. administration. Also distribution study of LPs in other tissues (lungs, trachea and oesophagus), where there was more likely chances of entering drug via i.n. route revealed very poor concentration level of DA from LPs in these tissues. Moreover % DTP value of 99.9 % for LPs indicates very effective drug delivery to brain via nasal administration. Thus pharmacokinetic and biodistribution studies confirmed that effective delivery of DA can be achieved by nasal delivery of liposomal system by i.n. route and with much higher availability to brain.

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Chapter 7. Conclusion

7.1 Conclusion

Though nasal route is used from old times for various localized purpose but use of nasal route for systemic action as well as direct delivery to brain has gained importance in last few years. It is more preferred for direct delivery to brain as the drug goes to brain directly bypassing BBB, thus enhancing availability and no or less distribution to circulation or other tissues. Dopamine being a highly hydrophilic drug cannot pass BBB directly. Therefore in the present research work, studies were carried out to design liposomal delivery system to enhance the therapeutic efficacy of DA by enhanced delivery through nasal route into the brain for treating PD disease.

As reported methods are not found for analytical determination of DA and bioanalytical methods available are not suitable for this project due to sophisticated techniques, low sensitivity, less specificity and complex processes. It has been decided to develop methods for routine and specified analysis of DA. UV-spectrophotometric, liquid chromatographic and bioanalytical methods were developed as well as validated. Developed methods have been found to provide advantages over the reported methods. These developed analytical methods were successfully implemented for estimation of Dopamine in various preformulation samples and formulation development studies. The developed bioanalytical method were used for estimating Dopamine selectively with high accuracy in biological samples such as rat plasma, brain, nasal cavity, lungs, trachea and esophagus. This validated bioanalytical method were successfully employed for in-vivo pharmacokinetic and biodistribution studies of the pure DA solution and LPs formulations in rats via i.n. and i.v administration.

In preformulation studies, integrity of Dopamine was confirmed using UV-absorption, DSC analysis and FTIR spectrum analysis successfully. The stability study results showed that in liquid state, stability of Dopamine was highly affected by pH change. There was rapid degradation of Dopamine in pH above 3 with high degradation rate constant because of dopamine oxidation at higher pH. However, drug was found to be stable for appropriate time upto pH 6.8 using 1 % sodium metabisulfide as an antioxidant. There was no chemical and physical interaction between drug and excipients and hence no incompatibility observed between Dopamine, cholesterol and lipids (Leviva S70, Lipova E120, Soya 100, DPPC and DMPC). Solid state stability confirmed that the drug is stable at refrigerated and ambient temperature.

Leciva S70, Lipova E120 and combinations of Leciva S70 and Lipova E120 liposomes were prepared successfully using reverse phase evaporation technique coupled with freeze and thawing method. The microscopic examination revealed the spherical and smooth surface character of the LPs as well as their homogeneous bilayer vesicles without any amorphous arrangements. The developed formulations have shown better entrapment within the bilayer vesicles with excellent particle morphology. The drug release characteristics revealed that the developed formulations

extended the drug release over 37-48 h for most of the formulations, which could be useful for controlled drug delivery purpose. The thermal studies have confirmed uniform distribution of the drug, at molecular level, within the lipid vesicles without showing any chemical and physical interactions between the drug and lipids or other excipients. Consequently, the prepared liposomes by selected method were found to be stable with acceptable reproducibility and for making quality liposomal delivery system.

The histopathological studies in rat nasal cavity and brain suggest that the prepared LPs did not show any toxic effect on the rat nasal tissues and hence there was no change in the architecture of respective organ cells. In vivo pharmacokinetic and biodistribution studies were performed to assess the suitability of formulations for direct delivery to brain by nasal application with controlled release. The biodistribution studies confirmed that Dopamine LPs is rapidly distributed in higher extent to brain from the developed liposomes in comparison to i.v. administration. The brain concentration levels were comparatively much higher than plasma concentration levels through i.n. route. Moreover, two fold higher DTP % of LPs as compared to pure drug showed the benefits of LPs formulation. % DTP value of 99.9 % for LPs indicates very effective drug delivery to brain via nasal administration. Thus pharmacokinetic and biodistribution studies confirmed that the biodistribution of the dopamine directly into brain is modulated efficiently with the aid of LPs delivery systems with nasal route of administration.

7.2 Future scope of work

As achieving high drug loading in liposomes is a great challenge particularly for highly water soluble drugs, further work needs to be done to increase both encapsulation efficiency and loading efficiency. With enhanced encapsulation efficiency and loading efficiency total amount of lipids and total mass for administration can be decreased. Further, optimization of process can be done to produce high quality liposomes.

Though drug distribution to brain and pK studies are carried out but pharmacodynamic study may be carried out on treating PD model in rats. Further, study can be done for not only PD but for other CNS disorders or even targeting drugs for treatment of brain cancer. Designed products are also required to be studied in patients with Parkinson's disease using suitable nasal devices.

Appendix

International Publication

V. Nagpal, E. Joseph, J. Abraham and R.N. Saha. (2015). Three dimentional viewed validated diode array UV-LC method for estimating dopamine in liposomes. Journal of Analytical Chemistry, 70 (3), 1-9 (Accepted).

V. Nagpal, E. Joseph, J. Abraham and R.N. Saha. (2015). Ultra Micro Quantification of Dopamine using Solid Phase Extraction: A Simple and Rapid HPLC method for in-vivo pharmacokinetic and biodistribution studies of the pure drug and LPs formulations in rats via i.n. and i.v administration. Plos one. (Communicated)

Book Chapter

R.N. Saha, G. Balwani, **Vibhu**. (2015). Tramsmucosal drug delivery systems and nanaoparticulate systems. NanoBiomedicine, vol 6. (Accepted)

Review Article

V. Nagpal, R.N. Saha. (2013). Intranasal delivery-opportunities for systemic and brain targeting. Pharmawave, 6(13), 1-11.

International Conferences

V. Nagpal, E. Joseph, J. Abraham, G. Balwani, S. Reddi, R.N. Saha. (2013). Development of controlled release liposome formulation for nasal delivery of candidate hydrophilic CNS active drug. Annual Meeting and Exposition, American Association of Pharmaceutical Scientists, San Antonio, Texas, USA.

V. Nagpal, J. Abraham, E. Joseph, G. Balwani, R. N. Saha. (2013). Ultra micro quantification of Dopamine in rat Plasma using solid phase extraction: A simple and rapid 3D view method for higher recovery, selectivity and sensitivity with UFLC diode array method. Annual Meeting and Exposition, American Association of Pharmaceutical Scientists San Antonio, Texas.

V. Nagpal, R.N.Saha. (2013). Studies on delivery of Dopamine in rat brain via nasal administration through olfactory pathway bypassing blood brain barrier. XX World Congress on Parkinson's Disease and Related Disorders, Geneva, Switzerland.

Biography of Chair Prof. Ranendra N. Saha

Dr. Ranendra N. Saha is Senior Professor of Pharmacy and presently Director of BITS Pilani Dubai Campus, UAE. In 2011 he has been awarded Shri B. K. Birla and Shrimati Sarala Birla Chair Professorship at BITS Pilani. He completed his Bachelor of Pharmacy and Master of Pharmacy (Pharmaceutics) degrees from Jadavpur University, Kolkata and obtained his Doctor of Philosophy degree from BITS, Pilani. Prof. Saha has more than 33 years of teaching, research and administrative experience and guided several doctoral, postgraduates and undergraduate students. He has vast experience in the field of Pharmacy especially in Pharmaceutics, novel drug delivery systems and pharmacokinetics. He received "Pharmacy Professional of the Year 2013" award from Indian Association of Pharmaceutical Sciences and Technologists. He has published book, several book chapters, research articles in renowned international and national journals and presented papers in conferences India and abroad. Prof. Saha received **'Pharmacy Teacher of India of the Year- 2005'** award given by Association of Pharmaceutical Teachers of India (APTI). Dr. Saha has successfully completed several government and industry sponsored projects. He has obtained patent on novel delivery systems entitled, "Stable Controlled Release Pharmaceutical Composition Comprising Aceclofenac", which is commercialized, and brought to Indian market by Ipca Lab Ltd, Mumbai. He has visited abroad on several occasions on invitation from universities and associations to deliver lectures. He has organized several national and international conferences. He organized one International Symposium at Damascus, Syria on invitation for ASST (Arab Societies of Science and Technology, Syria).

He is also a Senate and Research Board Member of BITS, Pilani. He is on advisory board and selection committee member of a number of Universities in India and abroad. He is also member of many scientific associations and societies like Association of Pharmaceutical Teachers of India (APTI), Indian Pharmaceutical Association (IPA), Indian Society of Technical Education (ISTE), Controlled Release Society Inc., USA, American Association of Pharmaceutical Scientists (AAPS) USA, American Chemical Society USA, Indian Chapter Controlled Release Society.

Biography of Vibhu

Ms. Vibhu has completed her bachelor degree B. Pharmacy from Lord Shiva College of Pharmacy, Kurukshetra University, Haryana. She has completed her post graduation, M.Pharmacy from, Poona College of Pharmacy, Bharti Vidyapeeth University, Pune. After completing her post graduation she joined as a research scholar at BITS, Pilani, under Chair Prof. R.N. Saha for her Ph D. She has published research and review articles in renowned journals and presented papers in national and international conferences in India and abroad. During the period of Ph.D. work, she was awarded by Analysis and pharmaceutical section (APQ), AAPS and got full funding from

DST, India under "Young Scientist International Travel Support" scheme to present her research findings in AAPS, Annual Meeting and Exposition, in San Antonio, Texas, USA in the year 2013.

To present part of her Ph.D. work in XX world congress on Parkinson's disease and related disorders, Geneva, Switzerland, 2013, she is also awarded travel grant with full funding by this congress.