# 9. SUMMARY AND CONCLUSIONS

The work presented in this thesis describes the formulation development and characterization of lipid-based drug delivery system for poorly bioavailable drugs.

## 9.1 Introduction

Amidst possible mechanisms to increase oral bioavailability, improved solubilization in GIT is notable means of absorption enhancement. Nanomedicine – Lipid-based drug delivery system (LBDDS) is one of the approach for the same. The primary constituent in LBDDS is the lipid part, which may be either used as single material or blend of several types of lipids. LBDDS range from liquid system, solid systems and vesicular to particulate drug delivery systems. The formulation approaches explored herein were SMEDDS and Niosomes for drugs Iloperidone (ILO) and Vardenafil HCl trihydrate (VDN). The selection of drug candidate was on the basis of their BCS classification (solubility) and absolute bioavailability. Iloperidone and Vardenafil HCl trihydrate are poorly soluble drugs having less bioavailability having oral bioavailability of only 36 and 15% respectively due to first pass metabolism. Hence, LBDDS approach was used to enhance their oral bioavailability.

This thesis precisely focuses on QbD enabled formulation development of SMEDDS and Niosomes of ILO and VDN, their characterization and its ability to bypass the first pass metabolism effect and enhance their relative bioavailability.

## 9.2 Aims and Objectives

The aim of the present research work was development of LBDDS for the selected drugs to increase bioavailability by increasing solubility and bypassing their first pass metabolism by lymphatic targeting which will help to increase uptake of the nano-formulations.

Quality by Design (QbD) enabled formulation development generates a design space. Hence, we focused on development of control space in the design space to make it robust. Another approach for optimization – Artificial Neural Network (ANN) was also considered. The results between Design of Experiment (DoE) and ANN were compared.

*In vitro* cell line studies were performed to study toxicity profile and uptake of formulation. The formulation when subjected for *in vivo* evaluation, the pathway of LBDDS absorption was deduced.

### 9.3 SMEDDS

The development of SMEDDS began with the selection of oil phase depending on the solubility study of drug. Based on the emulsification and %transmittance study, surfactant and co-surfactants were selected. Pseudo-ternary diagram was constructed to identify area of micro-emulsification. For iloperidone (ILO), oleic acid: Capmul mcm c8 (2:1), Cremophor EL and Transcutol HP were chosen as oil, surfactant and co-surfactant respectively.

Preliminary working range was generated for oil, surfactant and co-surfactant. Later, the formulation was optimized using DoE (I-optimal mixture design) and ANN approach. Globule size was taken as a response. On that basis, optimized formula was generated which was further used for different characterizations and in vivo studies. The SMEDDS demonstrated nano size range upon dilution. To find the actual globule size of oil core, Small Angle Neutron Scattering (SANS) was performed along with Dynamic Light Scattering (DLS). Further, the formulation was characterized for surface charge, optical clarity, cryo-TEM, thermodynamic stability study, rheology study, cloud point measurement, dispersibility, drug content, in vitro and ex vivo drug release study and stability.

The optimization by ANN indicated its better predictability of size than I-optimal mixture design. R value for ANN was higher than quadratic model of I-optimal design. The relative contribution of each ingredient on response, globule size, indicated that oil showed the highest effect on the output.

Average globule size was found to be 118.2 nm by DLS whereas actual oil core size was found to be 10.6 nm. This indicated that there was large difference between intensity weighed hydrodynamic diameter, as obtained by DLS, and actual core diameter obtained by SANS. Transmittance of 97% indicated optical clarity of diluted SMEDDS. Morphology study as carried out by cryo-TEM indicated globule size in nano range. Formulation was found to be thermostable when subjected to centrifugation, heating – cooling and freeze thaw cycle. Viscosity of ILO SMEDDS was found to be 109.9 mPa.s which was the desired one, as it fits in the ideal range for capsule filling. Cloud point of 80°C indicated successful formation of a stable formulation. There was no observed drug precipitation upon dilution.

For vardenafil (VDN), Capmul MCM C8, Cremophor EL (C-EL)/Tween 20 (T-20) and PEG 200 were chosen as oil, surfactant and co-surfactant respectively. Amongst two formulations prepared with two surfactants C-EL and T-20, the vardenafil SMEDDS prepared with T-20 gave superior performance over C-EL surfactant.

VDN SMEDDS was optimized by I-optimal mixture design. Control space was generated inside the design space using tolerance interval limits for each response i.e. globule size and %transmittance. DLS based hydrodynamic diameter was found to be around 49.98 nm and 214.50 nm respectively for C-EL and T-20 based SMEDDS. It was found that even tough, DLS based diameter of C-EL based SMEDDS was smaller, actual core size was 10.2 nm which was significantly different from 7.2 nm size of T-20 based VDN SMEDDS by SANS. %transmittance of both the SMEDDS was more than 95%. Cryo-TEM indicated nano size of both the SMEDDS.

*In vitro* and *ex vivo* drug release study of ILO SMEDDS and VDN SMEDDS indicated superior performance of SMEDDS formulation over its counterpart drug suspension.

Comparing two SMEDDS system of VDN made using different surfactants (Cremophor El and Tween 20), it was found that the capacity to enhance the permeation of the drug of these formulations was purely based on the nature of the surfactant and specifically on the chemical structure of the surfactant used. While comparing the formulations, T-20 enhanced permeation of the drug more than C-EL. Surfactants with medium chain were able to interact well with the intestinal cells layer and increase the transport of the drug as in the case of T-20. On the other hand, C-EL showed poor permeation ability for the drug because of its bulky nature which retarded the actual interaction with the cell surface. Hence, judicious selection of the SMEDDS excipients are needed for better *in vitro* and *ex vivo* performance of the formulation.

**9.4 Niosomes**

Niosomes were prepared using thin film hydration method. Using fish-bone diagram followed by Failure Mode Effective Analysis (FMEA) approach, factors were screened and critical factors possessing a potential failure cause for niosomes development were optimized by DoE approach. Here, process variables were optimized by OVAT approach. Process variables taken

into consideration were solvent evaporation time, rotation speed for thin film formation, rotation speed for hydration, hydration time and sonication cycle. Along with these process variables hydration volume (formulation variable) was also optimized using OVAT approach. Formulation factors such as Span 60, cholesterol and drug amount were optimized using Combined D-optimal mixture design. Two responses were studied, particle size and %EE. Here, effect of individual factor and multi-collinearity effect was also studied. The formulation was optimized by numerical approach (desirability) and graphical optimization (generation of control space).

Effect of various levels of Span 60: Cholesterol (surfactant: cholesterol) was studied from 7:3 to 9:1 ratio. The desired responses selected were entrapment efficiency (R1) and size (R2). Smaller size was highly desirable response, as by decreasing the size, permeation increases. At 7:3 level, %EE was low, which indicated requirement of more surfactant to encapsulate the drug. %EE increased when ratio was increased to 8:2. However, still increasing the ratio to 9:1 indicated decrease in %EE. This indicated that by increasing surfactant from 7:3 to 8:2, there might be encapsulation of drug inside the hydrophobic pockets formed by acyl chain of Span 60. Whereas, still increase in surfactant concentration showed negative effect on %EE. This might be due to leakiness of the bilayer, as less quantity of cholesterol is not adequate enough to provide stiffness.

On size also, similar trend was found as %EE. At 7:3 ratio, highest size was observed. After that at 8:2 ratio, there was decrease in size. But at 9:1 ratio, there was increase in size. At higher amount of cholesterol (7:3 ratio), cholesterol molecules get incorporated into SPAN 60's bilayers. The small hydrophilic head group (3ß-hydroxyl) of cholesterol is located in the vicinity of C=O of ester of Span 60, and the hydrophobic steroid ring orients itself parallel to the alkyl group of fatty acid (stearic acid) of Span 60. Thus, the movement of the acyl chains of the Span 60's bilayer gets restricted leading to failure of tight packing arrangement of other Span 60 molecules. This explains the direct increase of niosomes size observed at 7:3 Span 60: Cholesterol ratio. At 8:2 ratio, mean diameter of the niosomes decreased due to increased amount of Span 60 which resulted in tight packing of niosomes. But after this, at 9:1 ratio, as

Span 60 increased, it tends to align itself on outer surface of niosomes, ultimately leading to increase mean diameter of niosomes.

Similarly, effect of drug amount was also studied on size and %EE. Here, the amount of drug varies from 1 to 2. Hence, the ratio of drug: lipid is 1:10 to 2:10 (i.e. 1:5). In this study, >80% EE was found up to 1.5 parts of drug. Highest entrapment was found at 1.25 parts of drug when Span 60: cholesterol is 8:2, which might be due to increased availability of lipid for solubilization and entrapment of drug molecules inside the bilayer formed by surfactant and cholesterol. However, after that, still increasing drug amount did not show any increase in %EE. This might be due to less amount of lipid available for encapsulation. The effect of drug on size was proportionate to amount of drug after 1.25 parts of drug. From 1 to 1.25 parts of drug, there was decrease in size, whereas after that increasing drug amount led to increase in size. This might be correlated to decreased availability of lipid at higher concentration of drug.

For VDN niosomes development, we used optimized process and formulation parameters as of ILO niosomes and optimized VDN Niosomes to get maximum possible entrapment with minimum size by QbD approach.

The developed formulations were lyophilized using mannitol. The formulated niosomes were characterized for bilayer thickness, morphology by Cryo-TEM, surface charge, drug content, DSC, FTIR and stability studies. *In vitro* and *ex vivo* drug release study of ILO niosomes and VDN niosomes indicated superior performance of niosomes formulation over its counterpart drug suspension.

### 9.5 *In vitro* cell line studies

The cellular safety aspects of SMEDDS and Niosomes were studied using Caco2 cell line. Cell viability was nearly 95-100 % at 25 µg/mL concentration after 4 h of incubation for both ILO and VDN formulations. Intracellular uptake study by confocal imaging was carried out to find qualitative characterization. Similarly, FACS was performed for quantification. Coumarin 6 was added as the fluorophore for illumination, whereas for nucleus staining, DAPI dye was added. The drugs, ILO and VDN belong to BCS class II. This indicates the hydrophobic nature of drug. Hence, coumarin 6 was chosen as a hydrophobic model dye to mimic their nature.

From the confocal imaging, it can be concluded that lipid-based drug delivery systems represent a good carrier for intracellular drug delivery. Due to added advantage of nano size, it provides higher surface area which facilitates the contact with the cell membrane. However, as the confocal study is a qualitative study, any generalization cannot be given regarding superiority of SMEDDS over niosomes. Hence, quantitative cell uptake study of the coumarin 6 fluorescent probe by FACS in Caco-2 cells was studied by dot plot. The side scattering was measured for a fixed no of cells (10,000 events) and the graph was plotted. Results indicated rightward shift of dot plot towards P3 population which was corelated with increased cellular uptake of formulations. The rightward shift after treatment of lipid-based formulations depicts that the lipid formulations were easily uptaken by the cells through endocytosis due to their nano size. FACS uptake studies showed that the fluorescence intensity inside the cells got increased by using SMEDDS as compared to Niosomes. Thus, higher endocytosis was seen with SMEDDS formulations compared to its counterpart Niosomes formulation.

*In vitro* permeability study was performed using a monolayer of the Caco-2 cells cultivated on transwell which imitates intestinal barrier. The results indicated medium to high permeability of formulations compared to poorly soluble drugs ILO and VDN. This might be because of nano size of SMEDDS and Niosomes formulation, combined with amphiphilic nature of non-ionic surfactants present in the formulation. In addition to surfactants action, lipidic component of formulations help solubilization of lipophilic drugs in formulation which in presence of surfactants form mixed micelles that facilitate diffusion through the aqueous diffusion layer, thus, improving absorption.

The results of this study indicated that the permeability of ILO and VDN was clearly enhanced by nano-sized lipid-based formulations. However, other pharmacokinetic parameters such as clearance, volume of distribution and elimination half-life could not be predicted by this experimental approach. Therefore, in order to obtain the pharmacokinetic data of the developed formulations, *in vivo* studies were performed.

## 9.6 Animal Studies

### 9.6.1 Pharmacokinetics study

Lipid based formulations have been reported to enhance bioavailability through intestinal lipid transport system after oral administration. Hence, LBDDS were prepared with the hypothesis that; by their virtues of lipidic nature and nanometric particle size range, they would be able to increase bioavailable fraction and thereby reduce the dose required to exhibit the therapeutic activity.

For pharmacokinetic study of ILO and its formulations, less absorption of the pure drug can be attributable to slow and incomplete dissolution of this BCS class II drug in the GIT. Whereas for ILO SMEDDS and Niosomes formulations, high $C_{max}$ was observed. AUC for ILO SMEDDS was around 3 times higher than drug suspension. This might be due to dual advantage of increased concentration in plasma and decreased clearance from plasma. This increase in bioavailability eventually resulted in an escalation in the intensity of therapeutic effect of ILO.

Extent of absorption of drug was significantly increased as evident from $F_{rel}$ for ILO SMEDDS. Compared to drug suspension, ILO SMEDDS enhanced bioavailability by 2.63 folds. To evaluate the mechanism of absorption, when SMEDDS was orally administered to cycloheximide treated rats, there was significant decrease in AUC value. $F_{rel}$ value decreased to 0.808 from the 2.63 folds. Hence, it can be concluded that the prepared SMEDDS increased the bioavailability of ILO via lymphatic pathway.

Comparing the bioavailability of Niosomes with pure drug suspension, niosomes were able to increase bioavailability by 1.46 folds. This might be attributed to colloidal particulate nature of niosomes.

The results of VDN and its formulations pharmacokinetics study indicated that solubilization of drug in oil could overcome the absorption barrier. As the oils used herein increased lipoprotein synthesis and consequent lymphatic absorption, high bioavailability from both the SMEDDS may be attributed to lymphatic transport through transcellular pathway. SMEDDS formulations imparted a higher AUC0→t and enhanced the oral bioavailability of VDN (~1.4 fold for VDN C-EL SMEDDS and ~2.8 folds for VDN T-20 SMEDDS). This may be due to

increased solubility and prevention of precipitation of drug in git environment provided by encapsulation of drug in oil droplets. The enhanced permeation across intestine and direct uptake by enterocytes resulted into bypass of first pass metabolism which led to increased absorption and bioavailability for VDN SMEDDS as compared to pure VDN suspension.

Unambiguously, the capacity to enhance the permeation of the drug of these formulations is purely based on the nature of the surfactant and specifically on the chemical structure of the surfactant used. While comparing the formulations, the one containing the polysorbates T-20 surfactant enhanced permeation of the drug more than C-EL. It can be owing to the ability of the emulsifying dispersion to interact with the intestinal surface. Surfactants with medium chain were able to interact well with the intestinal cells layer and increase the transport of the drug as in the case of T-20. On the other hand, C-EL showed poor permeation ability for the drug because of its bulky nature which retarded the actual interaction with the cell surface. Thus, from the above results it can be concluded that judicial selection of the SMEDDS excipients are need for better *in vivo* performance of the formulation.

Compared to drug suspension, niosomal formulation of VDN increased the relative bioavailability by 2.34 folds.

### 9.6.2 Pharmacodynamics study for ILO

The main objective of the pharmacodynamic study in mice was to evaluate effect of ILO and its formulations; SMEDDS and niosomes, in MK 801 induced Schizophrenia which is a well-established model for negative symptoms. Rotarod test, Forced Swimming test and estimation of brain nitrite levels were performed. Compared to disease control model, drug and formulation treated groups significantly prevented the progression of disease. Fall of time from the rod in rota rod was significantly increased in treated groups. The immobility time was also decreased in treatment group. Quantitative estimation of brain nitrite level in mice indicated superior performance of formulation treatment over drug suspension treated group. Comparing the effectiveness of SMEDDS and niosomes formulation in decreasing reactive nitrogen species (RNS), niosomes were found to be more effective than SMEDDS. Increased reactive nitrogen species (RNS) and altered antioxidant molecules in the brain of schizophrenic subjects

supports the role of nitrosative stress in the pathology of this complex disease. As brain tissue has greater susceptibility factors such as low antioxidant defense system, high oxygen utilization and high levels of polyunsaturated fatty acid which may oxidize easily, nitrosative stress becomes a major pathogenic cause of schizophrenia. Inhibition of nitrosative and oxidative stress mechanisms is one of the novel therapeutic approaches for the reduction of neuronal damage in schizophrenia. The prepared formulation of ILO exhibited anti-RNS generation effect, which helped to slow down the progression of the disease. Comparison between drug and its formulations, the formulations showed superiority in decreasing the RNS level in the brain. This might be due to increased bioavailability of drug via lipid-based drug delivery approach.

### 9.6.3 Pharmacodynamics study for VDN

As seen from the pharmacokinetic studies, T-20 VDN SMEDDS showed better effectiveness compared to C-EL VDN SMEDDS. Considering this fact, only T-20 VDN SMEDDS formulation was further taken into consideration for pharmacodynamic study.

The male rats were rendered diabetic by i.p. injection of streptozotocin. The rats having blood glucose levels >16.6mmol/l were considered as diabetic models and were used for further study. At the end of 8 weeks of treatment period, serum testosterone level and serum nitric oxide levels were measured. No of sperms and % live sperm and % defective sperms were also calculated. Histopathology study of sexual organs was also performed. Hematoxylin – Eosin staining and Masson Trichome Staining of the testis and penis indicated intact architecture of testis and penis in normal control group. In disease control group, there was distorted architecture as indicated by disruption of epithelial tissues and loss of Sertoli cells in testis. Whereas testis sections of the treatment groups indicated somewhat distorted architecture but it comprised of closely packed seminiferous tubules. The spermatogonia and spermatocytes appeared adequate. The seminiferous tubules were separated by fibrovascular septae containing fibroblasts, collagen fibers and vascular spaces. In sections of penis, spongy corpus cavernosum was replaced by fat cells in disease control group, whereas in treated groups, presence of intact corpus spongiosum indicated effectiveness of treatment.

## 9.7 Conclusions

In the present investigation, we have reported formulation development and characterization of lipid-based drug delivery systems of poorly soluble drugs, iloperidone and vardenafil. Herein we have developed SMEDDS and Niosomes by QbD enabled systematic approach. This was the first approach of optimization of SMEDDS formulation by ANN. The formulations indicated superior *in vitro, ex vivo* and *in vivo* performance compared to drug suspension. The lymphatic targeted therapeutic approach will help to improve bioavailability by overcoming p-gp efflux, solubility issues and first pass metabolism effect. The *in vivo* pharmacokinetics and pharmacodynamic study further illustrated efficacy of the developed systems supporting the findings of *in vitro* cell line study and *ex vivo* studies. However, the present research need to be extended in terms of clinical trials and alternative cost-effective raw materials, scale up studies and exhaustive stability studies.