6.1. SUMMARY AND CONCLUSIONS

The development of suitable drug delivery systems remains a major challenge in the drug product development and industrialization process. Many of the orally delivered pharmacologically active compounds often face delivery issues like low solubility, poor permeability, rapid metabolism, positive food effect, low and erratic bioavailability which leads into suboptimal therapeutic response and poor patient compliance. To satisfy these troublesome biopharmaceutical properties of the drugs various concepts and approaches have been employed in drug delivery. The nanotechnology-based drug delivery tools hold a tremendous potential in therapeutic efficacy of such type of compounds. Polymeric nanoparticles and lipid based drug deliveries are one of the important and viable approaches among the arsenal of nano-enabled drug delivery systems.

Lercanidipine, a dihydropyridine calcium antagonist recommended in the treatment of hypertension. Lercanidipine exhibits absolute bioavailability of only 10% due to its extensive and saturable first-pass metabolism. It also shows poor solubility and hence exhibits small concentration gradient across the intestinal mucosa which can result in variable absorption culminating into poor therapeutic response. Thus, in order to overcome these shortcomings of lercanidipine and considering the advantages associated with nano-based drug deliveries, in the present study, two different nanoformulations of lercanidipine were developed. In one part of the study, an effort was made to develop lercanidipine into nanoproliposomal formulation with two important purposes. Firstly, the lipidic proliposomal carriers facilitate to improve oral bioavailability of lercanidipine. Secondly, based on theory that smaller particle size could improve the solubility, lercanidipine proliposomes were concurrently formulated in nanoform that can help to overcome difficulties associated with poor aqueous solubility and consequently its absorption. In another part, potential of polymeric nanoparticles was investigated in improving the poor biopharmaceutical properties of lercanidipine. The results of each chapter are summarized below:

ANALYTICAL AND BIOANALYTICAL METHOD DEVELOPMENT

A rapid, sensitive, specific, accurate and precise RP-HPLC method has been developed and validated for determination of lercanidipine in novel formulations. The developed

method was validated in accordance with ICH Q2(R1) guidelines. The analytical method was successfully applied in analysis of encapsulation efficiency, *in vitro* drug release samples and *in situ* perfusion samples of lercanidipine. The bioanalytical method for determination of lercanidipine in rabbit plasma using RP-HPLC was also developed and was found to be simple, highly sensitive, rapid, specific, accurate and reproducible. The method was validated in accordance with USFDA guidelines. The simple and less cumbersome protein-precipitation method adopted was efficient in extraction of lercanidipine from the plasma matrix of the rabbit. The developed method was successfully applied for preclinical pharmacokinetic studies for estimation of lercanidipine in rabbits.

FORMULATION OF LERCANIDIPINE LOADED NANOPROLIPOSOMES

A conscientious effort was made to develop lercanidipine into nanoproliposomal formulation with two important purposes. Firstly, the lipidic proliposomal carriers facilitate to improve oral bioavailability of lercanidipine. Secondly, based on theory that smaller particle size could improve the solubility, lercanidipine proliposomes were concurrently formulated in nanoform that can help to overcome difficulties associated with poor aqueous solubility and consequently its absorption. Lercanidipine nanoproliposomes were produced by modified thin-lipid film formation method and particle downsizing was achieved by high pressure homogenization technique. The study can be summarized as following:

- Initially, detailed drug-excipient compatibility studies were conducted to understand any possible interaction between lercanidipine and excipients. The study revealed absence of any interactions between drug and excipients. The compatibility was further confirmed by FTIR and DSC analysis.
- The solubility of lercanidipine in water and different pH conditions was investigated. The drug exhibited a solubility of 0.168 mg/mL, 0.6184 mg/mL and 0.040 mg/mL in pH 1.2, 0.01N HCl and pH 4.6 respectively. Solubility above pH 4.6 was found to be almost negligible.
- In order to arrive at the best possible optimized formulation different types of lipids *viz.* SPC, HSPC and EPC were investigated in the preparation of lercanidipine nanoproliposomes. Also, to increase bilayer membrane rigidity cholesterol was

incorporated in the formulation. The batches with SPC yielded a particle size in the range of 93.3 to 741.3 nm, zeta potential of above -40 mV and encapsulation efficiency in the range of 49.91 to 89.95%. The particle size obtained in case of HSPC batches were between 319.4 to 468.9 nm, zeta potential between +3.80 to +25.4 mV and encapsulation efficiency in the range of 50.52 to 78.26%, whereas EPC batch produced particle size in the range of 71.1 to 232.5 nm, zeta potential value of almost above + 29 mV and encapsulation efficiency in the range of 63.62 to 75.51%. In most of the formulations, the increase in particle size was observed with increasing lipid concentrations.

- Another important purpose of this work was to present lercanidipine nanoproliposomal formulation in freeze dried free-flowing solid powder form, for the same, different sugars were tested. Based on physicochemical properties of the formulation, the stabilizing effect of these sugars was ranked in the following order: Trehalose > Fructose > Dextrose > Sucrose > Mannitol and based on this, trehalose was finalized as the cryoprotectant agent in the formulation.
- The influence of homogenization pressure on the downsizing of proliposomes was investigated. Based on the desired physicochemical properties and to avoid heat generation at high pressures, 15000 psi with 10 homogenization cycles was optimized as a homogenization condition in the formulation process.
- Amongst the batches tested for *in vitro* drug release studies, B. No's. PL-06, PN-09 and PL-19 illustrated biphasic release characterized by an initial rapid release followed by a sustained release pattern. These batches released about 25% of lercanidipine within 1 h and followed by sustained release of more than 80% up to 14 h. The initial rapid release was ascribed to the presence of unentrapped drug in the formulation. The slow release in the later phase may be due to the lipophilic nature of nanoproliposomes as well as swelling of the lipid matrix which reduced the degree of diffusion of the dissolution medium thus results in slow release of the drug from the formulation. Based on the results of particle size, PDI, zeta potential, % encapsulation efficiency and *in vitro* drug release studies, B. No's. PL-06, PL-10 and PL-19 from SPC, HSPC and EPC respectively were chosen as optimized formulations for their further characterization.

- AFM and TEM analysis was carried out to evaluate surface characteristics and size range of the developed formulations. The images displayed smooth, spherical to oval shaped vesicles with homogeneous size distribution. The particle size observed with AFM and TEM for all the batches was comparable with that of the size obtained with particle size analyzer.
- To substantiate encapsulation of lercanidipine in nanoproliposomes, FTIR analysis was carried out and the spectral data showed the probability of presence of intact drug molecule in the lipid bilayer.
- DSC studies were carried out to assess transition behaviour of the lercanidipine encapsulated into nanoproliposomes. The results demonstrated the possibility of conversion of lercanidipine into amorphous form. Also, the thermogram suggests even distribution of the drug at the molecular level inside the bilayer structure of the formulations.
- X-ray pattern of pure lercanidipine and lercanidipine loaded nanoproliposomes prepared with SPC (B. No. PL-06) HSPC (B. No. PL-10) and EPC (B. No. PL-19) were evaluated to comprehend the physical state of the drug in the formulation. In case of B. No. PL-06 and B. No. PL-19, predominant increase of d-space in the XRD diffractograms showed that majority of the drug was embeded in the lipid bilayer. B. No. PL-10 shows less intense and broaden peaks in their XRD diagram indicating that the drug was embedded in these lipid vesicles. Overall, XRD pattern of all the three formulations confirms that the drug was encapsulated in the lipidic matrix uniformly in amorphous state.
- The *in situ* absorption study was performed to investigate absorption behaviour of the novel lercanidipine nanoproliposomal formulations. Amongst the lercanidipine loaded nanoproliposomes, B. No. PL-06 and PL-19 exhibited almost similar pattern i.e. steady increase in absorption rate values with respect to time. On the contrary, the absorption of free lercanidipine was found to be plateau within 1 h which may be due to the saturation of intestinal membrane and/or may be poor aqueous solubility of lercanidipine.
- Based on the suitable physicochemical parameters and encouraging results obtained from the dissolution rate and *in situ* absorption studies, B. No. PL-06 (particle size-174.7 nm and entrapment efficiency-85.35%) was selected for further *in vivo*

experiments. From these results, it was hypothesized that lercanidipine nanoproliposomes could enhance the oral bioavailability of lercanidipine, and hence pharmacokinetic studies were conducted in rabbits. The oral administration of lercanidipine showed a mean T_{max} of 1 h with a C_{max} of 543.53 ± 101.43 ng/mL suggesting its rapid absorption. The plasma exposure, $AUC_{(0-18)}$ was found to be 1733.19 ± 167.07 ng.h/mL with absolute oral bioavailability of 13.37%. As opposed to pure drug oral administration of lercanidipine loaded nanoproliposomes showed significant increase in mean T_{max} of 2.67 ± 1.15 h, indicating delayed absorption and decrease in C_{max} may be due to slow diffusion and dissolution of the drug embedded in the lipid matrix. The $AUC_{(0-18)}$ of nanoproliposomes estimated to be 4760.97 ± 512.76 ng.h/mL which was 2.75 times higher than pure drug. The absolute oral bioavailability of nanoproliposomes was found to be 36.73%. Formulation also displayed almost 2-fold increase in mean residence time and 2.15-fold decrease in the volume of distribution and 2.87-fold decrease in clearance values and overall increase in biological half-life to 6.95 ± 0.67 h as compared to 5.26 ± 0.80 h of pure drug attributing to the extended/sustained release property of the developed formulation. Along with extended release property of the developed novel formulation, there was also 2.75-fold increase in the absolute bioavailability as compared to free lercanidipine.

- In order to investigate the possible antihypertensive effect of the developed novel lercanidipine loaded nanoproliposomes, *in vivo* antihypertensive efficacy studies were conducted in DOCA-Salt induced hypertensive rats. Administration of lercanidipine showed significant decline in blood pressure immediately from 140.25 ± 2.62 to 126.83 ± 1.26 mm Hg within 1 h. Later it did not show any significant change in blood pressure. Acute administration of lercanidipine loaded nanoproliposomes, on the other hand, began to attenuate blood pressure immediately from 1 h and effect lasted until the study period of 24 h. These observations seemingly indicate the efficacy of formulated lercanidipine nanoproliposomes in treating hypertension as well as its role in maintaining the therapeutic levels until extended periods of time.
- Accelerated stability studies were conducted to generate quality of the final dosage form during its shelf life. Lercanidipine nanoproliposomes (B. No. PL-06) were pooled out after 3 months did not show any significant change in the physicochemical

parameters compared to initial batch results. The product also retained its appearance and free flowing properties and did not show any shrivelling tendency on storage.

In conclusion, the present investigation illustrates successful preparation of lercanidipine nanoproliposomes by modified thin-film hydration technique using a cryoprotectant solution as film hydration medium. The novel nanoproliposomes developed in the present study demonstrated acceptable size, morphology and excellent encapsulation efficiency. Appreciative extended drug release pattern was obtained when the formulation was tested *in vitro.* The *in situ* single-pass perfusion studies across the rat intestine displayed increased absorption of new formulation following oral administration. The *in vivo* pharmacokinetic studies showed significant improvement in the pharmacokinetic profile of lercanidipine when delivered through nanoproliposomal form. The lercanidipine loaded nanoproliposomes demonstrated 2.75-fold increase in oral bioavailability compared to free lercanidipine. The increase in absorption and bioavailability are attributed to the incorporation of lercanidipine in lipidic vesicles as well as to their nano-size range which in combination helps the drug to get internalized through M-cells of intestinal payer's patches thus bypassing the first-pass metabolism. The efficacy of formulated lercanidipine nanoproliposomes in treating hypertension as well as its role in maintaining the therapeutic levels until extended period of time was observed in DOCA-Salt induced hypertensive rats. By virtue of these properties, lercanidipine nanoproliposomes obtained was an elegant lyophilizate, easy to manipulate powder, which has the potential for designing into suitable oral dosage forms.

FORMULATION OF LERCANIDIPINE LOADED NANOPARTICLES

Polymeric nanoparticles were formulated to investigate their potential in improving poor biopharmaceutical properties of lercanidipine. Lercanidipine loaded nanoparticles were prepared by modified emulsion-diffusion-solvent evaporation technique. The formulation was assessed for different characterization parameters and their summary is provided as following:

- Initially, the drug-excipient compatibility studies were carried out to understand any possible interaction between lercanidipine and other formulation components *viz.* polymer, stabilizer and cryoprotectant used in the development of lercanidipine

nanoparticles. The study revealed absence of any interactions between drug and excipients. The compatibility was also further confirmed by FTIR and DSC analysis.

- The nanoparticles were devised using different type of polymers such as, PLGA 50:50, Eudragit RLPO and Gantrez AN-119BF. PLGA based nanoparticles showed a particle size between 194.4 to 729.6 nm, zeta potential, -13.6 to -27.3 mV and encapsulation efficiency, 16.79 to 82.62%. Eudragit nanoparticles exhibited a particle size in the range of 122.8 to 247.8, encapsulation of 14.48 - 57. 90% and zeta potential was almost above +48 mV. In case of Gantrez based nanopartilces, particle size obtained was above 300 nm whereas, encapsulation ranged between 18.48 to 78.34%. The results suggest the particle size obtained employing single emulsion method can be directly correlated with the viscosity of the dispersion that is formed during emulsification process. The increase in polymer concentration increases the inner organic phase viscosity which results into a higher mass transfer resistance that reduces diffusion of the polymer into the external aqueous phase, which in turn might have increased the particle size of the higher polymer concentration batches.
- Stabilizers are used in the formulation of nanoparticles as to provide stability for the dispersion in aqueous conditions. Some batches were executed to verify the influence of various types of surfactants on the physicochemical properties of lercanidipine nanoparticles. Based on the optimum physicochemical properties and superior nature of the obtained freeze dried product, PVA was selected as a surfactant of choice in the formulation process.
- High pressure homogenization technique was employed to obtain lercanidipine nanoparticles in the present study. To optimize the homogenization conditions, homogenization was carried out at four different speed levels by keeping other formulation parameters constant. From the results, 10,000 rpm was determined as optimal homogenization speed condition for all the batches pertaining to lercanidipine nanoparticles.
- The impact of probe sonication on physicochemical properties of lercanidipine loaded nanoparticles was also assessed as a part of optimization process. The batch processed with 60% amplitude displayed ideal physicochemical parameters and hence this condition was found to be energetically efficient to reduce particle size in the present formulation batches.

- Different cryoprotectants were evaluated in purview of achieving successful and long-term stability of freeze dried lercanidipine loaded nanoparticles. Out of all screened cryoprotectants, mannitol produced completely dry and free flowing powder with a particle size of 200.2 nm and hence it was selected as a cryoprotectant in the present study.
- The drug release behaviour of lercanidipine and lercanidipine loaded nanoparticles was evaluated to gain the predictive estimation of the drug performance *in vivo.* B. No's. PN-05, PN-13 and PN-19 illustrated initial rapid release which may be due to non-encapsulated or weakly bound drug to the large surface area of the nanoparticles. These formulations also gave an incremental drug release values up to 24 h without reaching plateau conditions as observed in case of pure drug. Here, the fact is that, nano-meter particle size must have played a pronounced role in improving the solubility of lercanidipine and hence the drug release was almost complete when it was incorporated in nanoparticles. Apart from this, extended release of the drug from nanoparticles may be explained by several mechanisms such as, drug diffusion through polymer matrix, nanoparticle-polymer matrix erosion and/or degradation and/or combination of these processes. Thus, based on suitable physicochemical and *in vitro* drug release properties, B. No's. PN-05, PN-13 and PN-19, a batch from each polymer was selected for further studies.
- AFM and TEM studies were carried out to access information related to particle size, morphology and surface properties of nanoparticles. The morphology appeared to be substantially spherical, non-aggregated with a smooth nature but somewhat uneven surface characters.
- DSC studies for lercanidipine and selected batches of freeze dried lercanidipine loaded nanoparticles were carried out. From the results it was evident that the drug is completely encapsulated inside the polymer matrix of nanoparticles in a molecular form of dispersion and therefore it was apparent that no peak appeared at the melting point of lercanidipine.
- X-ray pattern of the lercanidipine and lercanidipine loaded nanoparticles prepared with PLGA (B. No. PN-05) Eudragit (B. No. PN-13) and Gantrez (B. No. PN-19) were studied to evaluate the physical nature of the drug in the formulation. The XRD

diffractograms clearly indicated that the formulated nanoparticles are in amorphous state.

- *In situ* absorption technique across the rat intestine was employed to investigate the fate of nanoparticles through oral route. There was a statistically significant increase in absorption rate constant values of all the nanoparticle formulations as compared to free lercanidipine. The batch prepared with PLGA polymer showed highest absorption, which might be due to their particle size (200.2 nm), bioadhesive nature and their uptake via M-cells of payer's patches present in the small intestinal segment. Based on favourable physicochemical properties and encouraging preliminary results obtained from dissolution rate and *in situ* absorption studies, B. No. PN-05 (PLGA based lercanidipine nanoparticles) was selected for further *in vivo* experiments.
- *In vivo* pharmacokinetics in rats showed that orally administered nanoparticles showed 2.24-fold increase in mean residence time, 2-fold decrease in plasma clearance along with slight increase in half-life of the formulation (7.73 ± 1.94 h) as compared to pure drug (5.26 ± 0.80 h). These results indicate the extended release behaviour of the formulation. The absolute oral bioavailability of lercanidipine loaded nanoparticles was found to be 28.45%. These results are also indicative of long-systemic circulation of drug embedded in nanoparticles. Besides possessing the extended release property, the novel polymeric lercanidipine nanoparticles also showed 2.12-fold enhancement in absolute bioavailability as compared to free lercanidipine.
- In order to investigate the possible antihypertensive effect of the developed novel lercanidipine loaded nanoproliposomes, *in vivo* antihypertensive efficacy studies were conducted in DOCA-Salt induced hypertensive rats. The acute administration of lercanidipine loaded nanoparticles exhibited significant decline in blood pressure immediately from 1 h and effect lasted until the study period of 24 h. The results can be attributed to the efficacy of novel formulation in treating hypertension as well as its role in maintaining the therapeutic levels until extended periods of time.
- Accelerated stability studies were conducted for newly developed formulation to generate its quality during shelf-life. The freeze dried batch of product did not show any change in the visual appearance and has also retained its free flowing properties.

The physicochemical parameters of the stability batch were almost in proximity with the initial state of nanoparticles.

In conclusion, the study demonstrates the ample role of polymeric nanoparticles in oral delivery of lercanidipine, to the best of our knowledge, is the first of its kind. Among the different polymers used, PLGA based lercanidipine nanoparticles produced encouraging results. The novel freeze dried formulation presented desirable particle size and excellent encapsulation efficiency. The *in situ* single-pass perfusion study in wistar rats displayed an increased absorption rate across the rat intestinal membrane compared to pure lercanidipine. The oral bioavailability of lercanidipine is enhanced by 2.12-folds compared to free lercanidipine. Besides increased bioavailability it also illustrated appreciable extended drug release pattern releasing the encapsulated drug up to 24 h. The pronounced increase in absorption and bioavailability of lercanidipine may be due to its loading in polymeric nanoparticles which facilitated the drug to get internalized through M-cells of intestinal payer's patches present in the small intestinal segment thus bypassing the first-pass metabolism. Moreover, the lercanidipine nanoparticles expected to be suitable for prolonged control of antihypertensive activity in DOCA-Salt induced hypertensive rats. Overall, the extended drug release profile, increase in intestinal absorption, enhanced bioavailability and prolonged control of antihypertensive activity of the developed lercanidipine nanoparticles may concomitantly improve the therapeutic outcome and can help in patient compliance.

6.2. FUTURE DIRECTIONS

The present research work describes the potential application of nanoproliposomes and polymeric nanoparticles in improving oral delivery of lercanidipine. However, a key parameter which could not be pursued in this work was the detailed intestinal uptake study of these nano-carriers which would have supported the optimal efficacy of the developed systems. Another issue of importance was total amount of formulation bulk required to achieve the desired dose was very high to be realistic. Besides, to further strengthen the formulation work carried out in the present study, application of optimization software's would have represented a better approach in arriving at the optimized formulations.

Future research to investigate the above mentioned parameters would provide valuable information and further strengthens the suitability of developed formulations for their successful use in clinical conditions. It is also of equal importance to investigate the suitability of the developed formulations for designing into a oral dosage forms. Finally, when these studies provide fruitful results, *in vivo* pharmacokinetic studies in healthy human volunteers can be undertaken to demonstrate the clinical effectiveness of the newly developed nanoformulations. Similarly, the approach of encapsulating the drugs with poor biopharmaceutical properties into nanoproliposomes and polymeric nanoparticles can be further applied as platform technology for other suitable active moieties possessing similar kind of delivery issues.