9.1 Summary

HIV, the Human Immuno Deficiency virus is a lentivirus that causes HIV infection and Aquired Immuno Deficiency Syndrome (AIDS). They form one of the world's most serious health and development challenges. AIDS causes progressive failure of the immune system that result in life threatening opportunistic infections. According to statistics in 2013, 35 million people were living with HIV and 39 million people had died due to HIV infection. The average survival time for patient without treatment of HIV is estimated to be 9-11 years. Treatment of HIV includes regimen the therapy called HAART- Highly Active antiretroviral Therapy. HAART aims to minimize the amount of HIV in the body at a low level. It cannot eradicate HIV totally from the body and hence drugs used in this treatment need to be taken throughout the whole life of the patient. Due to resistance developed by HIV against a specific drugs used in treatment, HAART mainly consist of combination of drugs from its different category. One of such category is protease inhibitors (PIs) widely used in first line therapy of HAART. PIs prevent viral replication process by selectively binding to viral proteases and blocks proteolytic cleavage of protein precursors that are necessary for production of infectious virus particles.

Darunavir is a potent protease inhibitor. It is administered as oral immediate release tablet at adult dose of 600 mg with 100 mg Ritonavir twice a day. It has low oral bioavailability of 37 %. It has low aqueous solubility. It undergoes high enzymatic degradation by CYP enzymes in intestine and liver. To avoid this metabolism, it is always administered with Ritonavir which boosts its bioavailability from 37% to 82 %. In spite of co-administration of Ritonavir, the dose of Darunavir is high enough which causes various dose dependent side effects. Long term use of Ritonavir causes its resistance and further cross resistance for other PIs. Moreover, it can cause several serious side effects like perioral and peripheral paraesthesiae, liver problems, pancreatitis, heart rhythm problems, severe allergic reactions etc. Bioavailability is highly food dependent. So in this work, an attempt was made to increase the bioavailability of Darunavir by formulation of lipid based drug delivery systems. Apart from solubility and dissolution enhancement properties of lipids, it confers additional advantages like

decreasing the effect of cytochrome P-450 enzyme and increasing intestinal permeability by reducing the efflux transport activity at intestinal wall. In addition, lipid based drug delivery systems can reduce the effect of food on bioavailability by facilitating solubility of drug. Lipids also enhance the secretion of chylomicrons and thus facilitate the higher absorption as well as lymphatic transport of lipophilic drugs. Hence solid lipid nanoparticles (SLNs) and nanoemulsion were formulated for Darunavir. Nanoemulsion is oil in water emulsion with mean droplet diameters ranging from 50-1000 nm. It has already been used for bioavailability enhancement of many drugs. Solid lipid nanoparticles are lipidic particles of size 50-500 nm. They possess a solid core matrix that can solubilize the lipophilic drug. SLNs have the advantage over nanoemulsion in that SLNs are particulate matters which can also be taken by transcellular transport through the intestinal membrane. The intestinal cells contain specialized cell called M-cells which takes up particulate matters and there by direct the drug to lymphatic system. Thus, first pass metabolism is avoided. However this route has many limitations. One major limitation is the size dependent uptake by M-cells. So Darunavir loaded SLNs was prepared with different mean particle sizes 100 nm, 200 nm and 500 nm and the pharmacokinetics of all three sized SLNs were compared for selection of most suitable size for enhancement in bioavailability of Darunavir. Another protease inhibitor selected was Atazanavir sulfate (ATZ). It differs from other PIs in that it is available as once a day formulation at adult dose of 400 mg per day with an oral bioavailability of 60-68 %. Its bioavailability is also food dependent and is increased in presence of food. The common adverse effect associated with use of ATZ is rise in bilirubin level.

In spite of progresses made in HAART, HIV is able to reside in several anatomical and intracellular sites where antiretroviral drugs have restricted access. Such cellular sites mainly include $CD4^+$T lymphocytes, macrophages and follicular dendritic cells. Anatomical reservoirs includes lymphoid organs (spleen, lymph node and gut associated lymphoid tissue), central nervous system etc. 99% of all viral replication occurs in infected CD4+T cell of blood and lymphoid tissue. As 98% of circulating lymphocytes reside in lymphatic system, the later becomes an important site for antiretroviral drugs to act. Hence in this work, a peptide (that is known to bind specifically to $CD4^+$ receptor containing cells) was used and grafted on the surface of

solid lipid nanoparticles of Darunavir and ATZ. For this, Darunavir loaded SLNs that showed enhanced bioavailability was selected while ATZ loaded SLNs were formulated and optimized.

For determination of Darunavir content in SLNs and in nanoemulsion, UV-spectrophotometric method was developed in methanol: dichloromethane (DCM) at ratio of 7:3 and λ_{max} 267 nm. Beer's law was obeyed between 3 & 21 µg/ml and regression equation for standard plot was y = 0.038x-0.009. UV Method was also developed in simulated gastric fluid (SGF): methanol (1:0.5) and simulated intestinal fluid (SIF): methanol (1:0.5). Beer's law was obeyed in range of 3-21 and 5-30 µg/ml respectively at λ_{max} 267 nm. Regression equations for standard plot for SGF: methanol and SIF: methanol were obtained to be y = 0.0317 x + 0.0073 and y= 0.0324x – 0.0024 respectively. HPLC method for Darunavir was developed with mobile phase acetonitrile: water (1:1) (with pH adjusted to 3 using formic acid) at λ_{max} 267 nm for determination during cellline studies. Beer's range was followed in 2-10 µg/ml and retention time of 7.1 min was obtained with LOD and LOQ values 0.45 and 0.67 µg/ml respectively. For quantification of Darunvair during *in-vivo* studies, LCMS method was developed in plasma and spleen at concentrations ranging from 25-1000 ng/ml. Acetonitrile: water (60:40) as mobile phase and run time of 15 min was kept in both the methods. In method developed in plasma, energy was optimized at m/z 548.1→ m/z 156.20 Da and R^2 value of 0.9982 was obtained. In method developed in spleen, energy was optimized at m/z 548.1→ m/z 241.40 Da and R^2 value of 0.9992 was obtained.

In order to quantify ATZ content in the formulation, UV spectroscopic method was developed in methanol: DCM (7:3) at λ_{max} 249 nm. Regression equation of y = 0.0167x + 0.01 was obtained in Beer's range of 10-60 µg/ml with R^2 value 0.9993. For estimation of drug in the *in-vitro* release media, UV calibration plots of ATZ were developed in SGF: methanol (1:0.5) and SIF: methanol (1:0.5) in concentration range of 10-50 µg/ml at the λ_{max} 249 nm. The regression equations of methods developed in SGF: methanol and SIF: methanol obtained were y = 0.0208x + 0.0122 and y = 0.022x – 0.008 respectively with correlation coefficients of 0.9992 in both methods. For determination of ATZ during cellline studies, HPLC method was developed. The method was found to

have linearity in concentration range of 50-250 ng/ml at λ_{max} of 249 nm and mobile phase consisting of 45% water, 20% methanol and 35% acetonitrile with pH adjusted to 3.55 using acetic acid. Retention time of drug was obtained as 8.1 min and regression equation as y = 2.311x + 22.305 (R^2 = 0.9985). For quantification of drug during pharmacokinetic studies, HPLC method was developed in plasma with mobile phase, λ_{max} and retention time same as in former method. LOD and LOQ were obtained as 1.5 and 4.55 ng/ml. For determining drug concentrations in various organs during biodistribution study, HPLC method was developed in spleen. The Beer's range was obeyed in 50-250 ng/ml concentration and retention time of 8.1 min. The regression equation obtained was y = 2.9612x – 7.1351 (R^2 = 0.9991).

Placebo (without drug) SLNs were developed in order to optimize various process and formulation parameters. SLNs were prepared by emulsification-solvent evaporation technique where by solid lipid was dissolved in an organic solvent and emulsified in an aqueous surfactant solution. Gradual evaporation of solvent led to formation of solid particles which upon reduction in size yields solid lipid nanoparticles. Initially, glyceryl behenate was taken as solid lipid at concentration of 0.6% w/v of aqueous phase. Homogenizer (Ultraturrax) was used for emulsification. Particle size and PDI were measured for each determination and criteria were kept as desired particle size of 100 nm and minimum PDI value. The various parameters optimized were 12 000 rpm Ultraturrax speed, 15 min homogenization time, 1: 0.1 ratio of organic: aqueous phase, 0.7 sec x 70% amplitude x 3 min sonication cycle (Probe sonicator) and dichloromethane as organic solvent. Various solid lipids were taken and SLNs were prepared using 3 different surfactants- pluronic F68, polyvinyl alcohol and sodium oleate. Sodium oleate gave nanoparticles with less particle size (in range of 130-230 nm in various solid lipids) in comparison to other two surfactants and was selected as final surfactant.

Using these optimized parameter values, Darunavir loaded SLNs were formulated with various solid lipids and were evaluated for drug entrapment efficiency and particle size. Drug: lipid ratio and total solid content (drug+ lipid) were also varied. Results suggested hydrogenated castor oil (HCO) as a solid lipid giving highest entrapment of up to 90 % and desirable particle size of 99 nm. So, HCO was selected as final solid lipid for

preparation of SLNs loaded with Darunavir. Further optimization of drug: HCO ratio and sodium oleate concentration was done using High Pressure Homogenization (HPH) technique since industrial production of SLNs is more feasible using this technique. Drug: HCO ratio was varied from 1:4 to 1:6 and sodium oleate concentration from 3-4 % v/v. For each batch, particle size, PDI and drug entrapment efficiencies were estimated. The final selected batch **(Dar-SLN1)** contained 3.5 % v/v sodium oleate and drug: lipid ratio of 1:6 giving particle size of 93.28 nm and 89.09 % drug entrapment obtained using 3 cycles of HPH (1^{st} at 10 000 kpa while 2^{nd} and 3^{rd} cycle at 15 000 kpa). Upon first 2 cycles of HPH, the SLNs obtained were of size 189.45 nm and this batch was also selected as SLNs ~ 200 nm **(Dar-SLN2).** Further, using 1 cycle at 10000 kpa gave SLNs with particle size 527.62 nm and it was also selected as nanoparticle with size ~500 nm **(Dar-SLN3)** for further evaluation. Dar-SLN1, Dar-SLN2 and Dar-SLN3 were lyophilized using Vertis lyophilizer. Two cryoprotectants- sucrose and trehalose were taken with varying concentrations and their effect was observed on the physical appearance and particle size of lyophilized sample. Residual water content was less than 2 % in each sample. Trehalose (50 mg/ml of formulation) gave good results in comparison to sucrose and so was selected as final cryoprotectant.

In-vivo pharmacokinetic study of Dar-SLN1, Dar-SLN2 and Dar-SLN3 was performed and Dar-SLN2 was selected as optimized batch based on highest bioavailability obtained. So, in order to increase binding of nanoparticles to HIV host cells, the selected peptide was grafted onto the surface of Dar-SLN2 by covalent binding forming thioether linkage. DSPE-Mal-mPEG 2000 was used for attachment of nanoparticles to the terminal cysteine amino acid of the peptide. The obtained formulation was named as **Pept-Dar-SLN**.

In order to formulate nanoemulsion loaded with Darunavir, solubility of drug was checked in various oils. Soyabean oil gave highest solubility and so it was selected for further formulation. Tween-80 was selected as emulsifier since it is reported to inhibit P-gp activity and Darunavir is a P-gp substrate. Different batches were prepared using high pressure homogenization technique by varying tween 80 concentrations. Darunavir was dissolved in soyabean oil (containing sodium oleate). Egg lecithin was dispersed in

distilled water to prepare aqueous phase. Oil phase was homogenized into aqueous phase using Homogenizer (Ultraturrax) at 8000 rpm for 20 min and then emulsion was cooled and volume made up with distilled water. Globule size, zeta potential, drug entrapment, creaming volume upon centrifugation and short term stability (for 30 days at 4ºC and 20 ºC) was measured for each batch for optimization. Batch **DNE-3** having globule size 209.5 nm, zeta potential of -41.1 mV, 93% drug entrapment efficiency, 98 % creaming volume was selected as optimized formulation. It also remained stable at 4ºC for a period of 1 month with a non-significant change in globule size and zeta potential.

ATZ loaded SLNs were prepared by emulsification- solvent evaporation technique. Optimized values of various parameters obtained during preparation of placebo nanoparticles were used and ATZ loaded SLNs were formulated using various solid lipids, varying ratio of drug: solid lipid and changing sodium oleate concentration. HCO gave highest drug entrapment of up to 78 % at drug: lipid ratio of 1:8 (Batch ALN-9). So HCO was used for further optimization and various batches were prepared using HPH technique using 2 cycles (1^{st} at 10000 kpa and 2^{nd} at 15000 kpa). Optimized batch **ALN-23** contained 1:10 drug: lipid ratio and 6% v/v sodium oleate concentration. It had particle size of 190.1 nm and % drug entrapment of 94.65 %. It was lyophilized using sucrose and trehalose as cryoprotectant to optimize its concentration. Trehalose at 60 mg/ml formulation was optimized based on minimal increment in particle size and drug content upon lyophilization. Peptide was grafted on finalized ATZ loaded nanoparticles as per procedure done for Pept-Dar-SLN.

All three selected SLNs loaded with Darunavir (Dar-SLN1, Dar-SLN2 and Dar-SLN3) were characterized for various parameters. Dar-SLN1 had mean particle size of 93.28 ± 3.62 nm, PDI of 0.191 ± 0.01, zeta potential of -48.82 ± 2.14 mV and entrapment efficiency of 89.09 ± 2.4%. Dar-SLN2 had mean particle size, PDI, zeta potential and entrapment efficiency of 189.45 ± 2.10 nm, 0.115 ± 0.024, -50.1 ± 1.17 mV and 90.10 ± 1.15 % respectively while for Dar-SLN3, the values obtained were 527.62 ± 2.15 nm, 0.114 ± 0.031, -49.23 ± 2.28 mV and 88.13 ± 2.26 % respectively. Drug loading for Dar-SLN1, Dar-SLN2 and Dar-SLN3 were estimated as 12.92 ± 2.12, 13.06 ± 1.18 and 12.87 ± 1.67 % w/w. TEM and FE-SEM images of all three SLNs demonstrated discrete and

round structures without aggregation. DSC studies were performed for pure Darunavir, HCO, physical mixture of Darunavir: HCO (1:0.5, 1:1 and 1:6), placebo SLNs and lyophilized form of Dar-SLN2 in order to characterize the physical state of drug in the nanoparticles. Darunavir showed sharp endothermic peak at 71.58ºC corresponding to its melting point while lipid showed two peaks at 76.51ºC and 85.19ºC due to its two isoforms. As drug: lipid ratio was increased, the drug peak was found to merge with lipid peak (76.51ºC) and at ratio of 1:6, the thermogram showed only two peaks at 75.21ºC and 84.06ºC. These same peaks were observed in placebo and drug loaded SLNs indicating a homogenous distribution of drug in lipid matrix. In FT-IR study, the spectrum of physical mixture (drug + lipid) and lyophilized formulation (Dar-SLN2) showed characteristic peaks of both drug and lipid indicating compatibility of drug with excipients of formulation. *In-vitro* drug release study was performed for all 3 SLNs and plain drug suspension (prepared using 0.4% w/v methyl cellulose) using dialysis membrane for 2 hr in SGF followed by 10 hr in SIF. Samples were withdrawn at predetermined time intervals and analyzed by developed UV method. The results indicated insignificant difference in release profile of all 3 SLN formulations. Plain drug suspension showed incomplete release of only 11.5 % in 8 hr while in case of SLN formulations, there was 15-17% drug release by end of 12 hr. This study do not incorporate the transcellular uptake of SLNs from M-cells of intestinal membrane, lipid lysis intracellularly upon SLN uptake and effect of SLNs' mucoadhesion property that results in increase in residence time in GIT. So, the *in-vivo* study only can predict the correct fate of SLNs.

Upon grafting of peptide to surface of Darunavir loaded SLNs (Pept-Dar-SLNs), particle size increased insignificantly from 189.45 nm to 195.11 ± 1.53 nm (PDI of 0.211 ± 0.03). Zeta potential increased significantly from -50.1 mV to -35.45 ± 1.10 mV due to peptide grafting. SDS-PAGE study qualitatively confirmed presence of peptide in Pept-Dar-SLN. % peptide conjugation in Pept-Dar-SLN was found to be 73.12 % as quantified by Lowry's method. *In-vitro* release demonstrated nonsignificant change upon peptide grafting in SLNs.

Optimized Darunavir loaded nanoemulsion (DNE-3) was characterized for various parameters. Its TEM image revealed discrete and round structures without aggregation. In-vitro drug release of DNE-3 was performed using dialysis bag technique for 2 hr in SGF followed by 10 hr in SIF. The results indicated sustained release of 95 % in 12 hr. The drug release was higher compared to plain drug suspension indicating greater solubilization of Darunavir in presence of lipid. The drug release was found to follow Higuchi model (R^2 = 0.9960) indicating diffusion based release from lipid matrix.

Upon peptide grafting to optimized ATZ loaded SLNs (ALN-23), the resulting formulation (Pept-ATZ-SLN) showed nonsignificant increase in particle size (from 190.1 ± 2.45 nm to 194.65 ± 1.15 nm) and a significant change in zeta potential (-42.63 ± 2.46 to -33.64 ± 2.17 mV). % EE and drug loading of Pept-ATZ-SLN were estimated to be 94.26 ± 2.12 % and 8.0 ± 0.4% w/w. It's TEM and FE-SEM images confirmed discrete and round structures without aggregation. DSC thermogram of pure ATZ showed a sharp endothermic peak at 198.79ºC corresponding to its melting point. Absence of drug peak in DSC thermogram of physical mixture and lyophilized nanoparticles showed that drug got solubilized in the lipid matrices completely. The FT-IR spectrum of physical mixture and lyophilized formulation showed characteristic peaks of both drug and lipid indicating compatibility of drug with excipients. The presence of peptide on SLNs was qualitatively confirmed by SDS-PAGE and % peptide conjugation was found to be 76.37% (estimated by Lowry's assay method). ALN-23 and Pept-ATZ-SLNs released about 20-21 % ATZ *in-vitro* in 12 hr indicating a sustained release of ATZ from SLNs. This sustained release is favorable since nanoparticles can be taken up by M-cells and then the drug release can occur.

The cytotoxicity of excipients in the formulation of SLNs (placebo and drug loaded nanoparticles) was evaluated by MTT dye reduction assay in human epithelial colorectal adenocarcinoma (Caco-2) cell line. The results showed 94% cell viability at all concentrations used irresptive of drug loading indicating the non-cytotoxicity of excipients used in formulation of SLNs up to the high concentration of 10 µg/ml Darunavir equivalent formulations. The mechanisms involved in the uptake of nanoparticles (Dar-SLN2 and ALN-23) from Caco-2 cells were investigated.

Chloropromazine, Amiloride and Nystatin were used as specific inhibitor or clathrin-, caveole- mediated endocytosis and macropinocytosis respectively and amount of drugs taken up by the cells in presence of these inhibitors were quantified by HPLC methods developed. The results suggested that uptake of both drugs was decreased in presence of Chlorpromazine and Amiloride (and not in presence of Nystatin) indicating that Caco-2 uptake of SLNs was mediated via clathrin and caveole mediated endocytosis and not via macropinocytosis. Highest uptake was found by caveole mediated endocytosis supported by the literature that anionic nanoparticles majorly use this pathway. Intestinal permeability study in Caco-2 cell line revealed that permeability of Darunavir and ATZ increased by 4.04 and 2.52 fold in comparison to Darunavir plain drug suspension and ATZ plain drug suspension respectively. The peptide grafted nanoparticles were tested for binding affinity to $CD4^+$ expressing cells (Molt-4 cell line) and non- $CD4^+$ expressing cells (Caco-2) qualitatively by confocal microscopy using coumarin-6 as lipophilic dye loaded in nanoparticles. The images obtained showed higher fluorescence obtained using peptide grafted SLNs in Molt-4 cell line in comparison to non-peptide grafted SLNs indicating the greater binding of peptide grafted SLNs to $CD4^+$ expressing cells. This would lead to enhanced exposure of drug to the HIV host cells that exclusively consist of $CD4^+$ receptors.

In-vivo pharmacokinetic studies in rats were performed for Dar-SLN1, Dar-SLN2, Dar-SLN3 and Pep-Dar-SLN for 24 hr and compared with plain Darunavir suspension and marketed tablet (DARUVIR-300 mg). All samples for study were administered at dose of 40mg/kg. The plasma samples obtained were quantified by LCMS using developed method. Non-compartmental analysis was performed using Kinetica software (version 5.0.11, Thermofischer scientific). The results suggested significant improvement in bioavailability of Darunavir (by 469.47 %, 481.35 % and 253.45 %) for Dar-SLN1, Dar-SLN2 and Dar-SLN3 respectively) upon nanoparticle formulation in comparison to Darunavir suspension. Highest bioavailability was obtained using Dar-SLN2 and a non-significant difference was found between pharmacokinetics of Dar-SLN1 and Dar-SLN2. Thus, SLNs of size ~100 nm and ~200 nm gives enhanced bioavailability. So, nanoparticles having size ~200 nm could be most appropriate for enhancing bioavailability of Darunavir with possible reasons being the enhanced absorption and

lymphatic uptake of nanoparticles. *In-situ* absorption in gastrointestinal tract (stomach and intestine) of Darunavir from suspension and optimized SLNs (Dar-SLN2) was studied. Results depicted enhanced plasma concentration and AUC_{0-t} after intragastric and intraduodenal administration of SLNs in comparison to plain drug suspension. In addition, the AUC of Dar-SLN2 after intragastric administration was only 11.8 % of the AUC after intraduodenal administration indicating that the absorption of SLNs mainly occurred in the intestine. The plasma concentration of Darunavir in rats after oral administration of SLNs and peptide grafted SLNs showed no significant difference ($P>0.5$). It indicated that the grafting of peptide had no effect on its pharmacokinetic profile. Lymphatic transport of Dar-SLN2 was studied upon intraduodenal administration to rats at dose of 40mg/kg. Cycloheximide was used to block the lymphatic route and plasma concentrations were measured at predetermined time intervals and analyzed using developed LCMS method. The C_{max} and AUC_{0-t} of Darunavir was significantly reduced by 55.78% ($P<0.05$) and 59.15% ($P<0.05$) respectively in rats treated with cycloheximide which could be attributed to the blockage of intestinal lymphatic transport of cycloheximide. Thus, lymphatic transport pathway played an important role in intestinal transport of SLNs into the systemic circulation. Biodistribution of Darunavir upon administration of Darunavir suspension, Dar-SLN2 and Pept-Dar-SLN was studied in male wistar rats at 1, 4, 8 and 24 hr of administration (dose 40 mg/kg). Dar-SLN2 showed a higher distribution of Darunavir than from plain drug suspension in virtually all investigated tissues except liver. The order of Darunavir AUC from highest to lowest for Dar-SLN2 formulation was as follows: intestine > stomach > spleen > Liver > kidney > brain > lung > heart. The clearance of Darunavir from nanoparticles was slower than that given in suspension as indicated by a gradual increase in concentrations in kidney up to 8 hr while in suspension form; it showed clearance in the first hr. Major HIV reservoir organs namely, spleen and brain showing higher Darunavir concentration upon nanoparticle administration indicate the superiority of nanoparticulate systems in increasing the accumulation of drug in various target organs. SLN formulation also increased the drug accumulation in brain and a decreased concentration in liver (drug metabolism site) is of a great therapeutic benefit. There was significant difference between the drug concentrations in spleen and liver for peptide grafted and non-peptide

grafted nanoparticles which is likely due to the presence of peptide on nanoparticle surface leading to higher binding with T-cells present in liver and spleen. In other organs, no statistical differences were observed between distributions of both the nanoparticles. Peyer's patch (PP) region of intestine showed 4.12 fold higher Darunavir concentrations in comparison to non-PP region upon administration of Dar-SLN2. It is hypothesized that nanoparticles because of their smaller size would have an efficient disposition via PP to other lymphatic organs such as mesenteric lymph nodes and to spleen.

In-vivo pharmacokinetics of Darunavir loaded nanoemulsion (DNE-3) was studied in male wistar rats upon administration at dose of 40 mg/kg. In comparison to pure Darunavir and marketed tablet, DNE-3 showed significantly higher plasma drug profiles. C_{max} of DNE-3 (231.89 ± 13.22 ng/ml) was approximately 1.99 fold higher than suspension form (116.58 ± 7.56 ng/ml). Higher T_{max} (1 hr) reflected the higher solubility of Darunavir in oil phase leading to slower drug release in outer phase compared to suspension form (T_{max} = 0.5 hr). Bioavailability was enhanced by 223 % relative to suspension form. The possible reasons might be the higher solubilization of drug eliminating dissolution step in GI media, lymphatic transport through intestinal transcellular pathways and Tween 80's inhibitory action on P-gp (Darunavir being P-gp substrate) and property of soyabean oil in enhancing lipoprotein synthesis and subsequent lymphatic absorption. Biodistribution pattern of Darunavir to different organs upon lipid nanoemulsion administration was studied. The order of Darunavir AUC from highest to lowest for DNE-3 is as follows: stomach > intestine > liver > spleen > kidney > brain > lung > heart. Brain uptake of Darunavir for DNE-3 was observed to be 2.65 fold higher than that for suspension and even 1.09 fold significantly higher than that for Darunavir SLNs (Dar-SLN2). Endocytosis and transcytosis may also be possible mechanisms for entry of DNEs into brain because of their lipophilic nature. Another reason could be the P-gp inhibitory effect of Tween-80 present in formulation.

In-vivo pharmacokinetics of peptide grafted Atazanavir sulfate loaded SLNs (Pept-Dar-SLN), Atazanavir sulfate loaded SLNs (ALN-23) and ATZ suspension was studied in male wistar rats upon administration at dose of 7 mg/kg. ALN-23 showed significant enhancement in the plasma concentration in comparison to plain drug suspension. C_{max},

AUC_{0-t} and relative bioavailability for ALN-23 increased by 2.71 and 2.12 fold and 200 % respectively in comparison to plain drug suspension. The grafting of peptide on the nanoparticles surface lead to a no significant change in its pharmacokinetic profile as indicated by non-significant difference in their plasma profiles. Biodistribution pattern of Atazanavir in to different organs upon suspension, peptide grafted and non-peptide grafted nanoparticle administration was studied. There was significant improvement ($P<0.05$) in tissue accumulation of Atazanavir into different organs upon SLN administration in comparison to plain drug suspension along with significant difference ($P<0.05$) found between the drug concentrations in spleen and liver for peptide grafted and non-peptide grafted nanoparticles due to presence of T-cell binding peptide. In comparison to plain drug suspension, nanoparticle formulations showed higher drug accumulation in spleen, heart, brain and lung which are considered as anatomical reservoirs sites for HIV.

Stability studies for optimized formulation of Darunavir loaded SLNs (Dar-SLN2), peptide grafted Darunavir loaded SLNs (Pept-Dar-SLN), Nanoemulsion containing Darunavir (DNE-3) and Optimized Peptide grafted Atazanavir sulfate loaded SLNs (Pept-ATZ-SLN) were performed according to International Conference on Harmonization (ICH) Q1A (R2) Guidelines 2003. For nanoparticle formulations, comparative stability studies were carried out at 25 ± 2ºC/ 60±5 % RH and at 5 ± 3ºC for 6 months. For nanoemulsion formulation, study was performed at 5 ± 3ºC for 6 months. Freshly prepared (lyophilized samples for nanoparticles) were filled into type-1 tubular glass vials and stored at above mentioned conditions. The content of vials was tested for different parameters at 0, 1, 2, 3 and 6 months. For nanoparticle formulations (Dar-SLN2, Pept-Dar-SLN, ALN-23 and Pept-ATZ-SLN), there was insignificant change ($P>0.05$) in % drug retained, particle size, zeta potential and water content after six months at both storage conditions. Peptide assay (for Pept-Dar-SLN and Pept-ATZ-SLN) decreased significantly upon storage of at 25ºC indicating its instability, while it remained stable at 5 ± 3ºC. So, Dar-SLN2 and ALN-23 can be stored at both conditions while Pept-Dar-SLN and Pept-ATZ-SLN should be stored at 5 ± 3ºC only. DNE-3 remained stable with no significant change ($P>0.05$) in its evaluated parameters (% assay, % drug retained,

globule size, PDI, Zeta potential and creaming volume) for a period of 6 months. This indicated that the prepared nanoemulsion is stable at 5 ± 3ºC.

9.2 Conclusions

Darunavir loaded solid lipid nanoparticles as well as nanoemulsion and Atazanavir sulfate loaded solid lipid nanoparticles were successfully developed. The optimum size of the SLNs was found to be 200 nm for enhancing bioavailability. The SLN formulations of both Drugs were found to significantly increase the oral bioavailability compared to their plain drug suspensions. The optimized SLN formulations of both the drugs were found to passively target the drugs to the lymphatic system.

Peptide having affinity for HIV host cells was successfully grafted on the surface of Darunavir loaded SLNs and Atazanavir loaded SLNs. The resulting formulation gave increased binding with the HIV host cells and thus could be promising carrier in active targeting of the drugs to the HIV reservoir. Nanoemulsion of Darunavir was also found to enhance the bioavailability, which was higher than the plain drug suspension but lower than that of SLNs. The biodistribution studies indicated that the SLN and the nanoemulsion formulations of Darunavir led to its higher distribution to viral reservoir organs i.e brain, spleen and heart compared to the plain drug suspension. However, nanoemulsion formulation was found to have a better targeting efficiency to brain compared to the SLN formulation probably because of the presence of Tween-80 which is P-gp inhibitor. The non-peptide grafted SLNs were found to be stable at 25± 2ºC/ 60 ±5 % RH and 5± 3ºC while peptide grafted SLNs and Darunavir nanoemulsion were stable at 5ºC. Thus the present research work led to a development of formulations for bioavailability enhancement of protease inhibitors and their targeting to HIV reservoirs. However, extended research involving pharmacodynamic studies could yield a clearer picture of the efficiency of the formulation in reduction of viral loads.