CHAPTER 7:

SUMMARY & CONCLUSION
7.1. Summary

An estimated 60 million people are infected with human immunodeficiency type-1 (HIV-1) worldwide. The majority of infected people live in the developing world with limited treatment resources. Antiretroviral (ARV) therapy has significantly reduced HIV-1 disease morbidity and improved life expectancy. However, the economics of drug treatment, treatment failures due to the development of resistance, and limited global access has prevented world-wide utility of ARV therapy. Dosing regimens that require multiple daily dosing with diet considerations and ARV side effects have compromised the achievement of long-term HIV-1 suppression in infected patients. Additionally, the use of ARV requires a concerted level of commitment from the patient to prevent treatment failure due to resistance.

ARV drug therapy has contributed significantly to improved patient/disease management, its current use is associated with several disadvantages and inconveniences to the HIV/AIDS patient. Many ARV drugs undergo extensive first pass metabolism and gastrointestinal degradation leading to low and erratic bioavailability. The half-life for several ARV drugs is short, which then requires frequent administration of doses leading to decreased patient compliance. A major limitation is that HIV is localised in certain inaccessible compartments of the body such as the CNS, the lymphatic system and within the macrophages. The severe side effects associated with ARV therapy can therefore be attributed to the subsequent large doses essential for achieving a therapeutic effect, due to the inadequate drug concentrations at the site of action, and/or the poor bioavailability of several ARV drugs. These drugs also suffer from physico-chemical problems such as poor solubility that may lead to formulation difficulties.

According to the biopharmaceutical classification system guidance by FDA, efavirenz (EFV) comes under a class II category drug, i.e. it has low solubility and high intestinal permeability. It is a crystalline lipophilic solid with an aqueous solubility of 3-9 μg/ml and with a low intrinsic dissolution rate of 0.037 mg/cm²/min. Hence, it has very low bioavailability. To achieve effective therapy against viral diseases for orally administered drugs, it is essential that the drug should be adequately and consistently absorbed. Therefore, the recommended dose of EFV in adults is 600 mg q.d. The
frequent administration of several drugs in relatively high doses is a main cause of patient incompliance. The reason for this is very low solubility of EFV hinders its administration, absorption and biodistribution. Thus, there is need to have some innovative formulation approach to enhance the bioavailability and for site-specific drug delivery. The objective of studies was to develop stable nanosuspension (NS) and self-microemulsifying drug delivery system (SMEDDS) of EFV for improvement of oral bioavailability by improving its solubility, dissolution and absorption properties. Another objective was to develop EFV loaded mannose (MN) incorporated PLGA nanoparticles (MN-PLGA NPs) for site-specific delivery to macrophages.

Present first study has been undertaken to develop NS of EFV, by media milling method, with improved oral bioavailability. Formulation of NS requires a careful selection of stabilizers. Steric and electrostatic stabilizers are needed to stabilize the nanoparticles against inter-particle forces and prevent them from aggregating. Steric stabilization is often combined with electrostatic stabilization for additional repulsive contribution. Different pharmaceutical excipients including povidone (PVP K30), and poloxamers (188 and 407) as steric stabilizer and sodium lauryl sulphate (SLS) as anionic electrostatic stabilizer were used in an effort to develop stable EFV loaded NS. In order to obtain best formulation of NS, the relationship between dependent and independent variable must be understood. Design of experiment can serve as an efficient and economical method of obtaining the necessary information to understand relationship between variables. Box-Behnken design, one of response surface method (RSM) design, was applied to optimize the NS formulation. The independent variables for the present study were the following: concentration of drug ($X_1$), concentration of polymer ($X_2$), concentration of surfactant ($X_3$) and milling time ($X_4$). The dependent variables included mean particle size (MPS) and zeta potential (ZP). NSs were subsequently transformed to dry powder by lyophilization, to enhance the stability of EFV. The physicochemical properties of NSs in terms of MPS, polydispersity index (PDI), and ZP before and after lyophilisation were investigated. Dissolution velocity and saturation solubility are generally performed using official pharmacopoeia methods. Corresponding physical properties of the prepared EFV NS were characterized by differential scanning calorimetry (DSC), X-ray diffraction (XRD), scanning electronic microscopy (SEM) and transmission
electron microscopy (TEM). The chemical stability of NS was assessed by determining percentage of EFV present in the formulations stored at different temperatures (4-8°C and 25°C) during a period of 6 months. *In situ* intestinal permeability and *in vitro* parallel artificial membrane permeability assay (PAMPA) study were carried out to assess permeability of EFV in NS. Finally, oral bioavailability of NS was evaluated in rabbits and compared with standard EFV and marketed EFV formulation (MF). We hypothesized that NS formulation of EFV might lead to improved oral bioavailability due to enhanced solubility, dissolution and, thus absorption.

Present second study has been undertaken to develop and characterize S-SMEDDS formulation of EFV, and the physicochemical characteristics were evaluated *in vitro*, *in situ* and *in vivo*. Formulation of L-SMEDDS requires a careful selection of oil, surfactant and cosurfactant. Selection of excipient was optimized using solubility, phase diagram and self-emulsification property. The application of a mixture experimental design has been demonstrated to be an efficient and satisfactory method for optimization of the formulation and to acquire the necessary information to understand the relationship between independent variables and dependent variables in a formulation. Experimental mixture design using Design-Expert® software was applied to optimize L-SMEDDS that contain a minimum amount of surfactant, a maximum amount of lipid, and possess minimum droplet size (MDS) and maximum % transmittance (% T). Based on the results of ternary phase diagrams and self-emulsification studies, captex 500 as oil ($X_1$), tween 20 as surfactant ($X_2$) and transcutol HP as cosurfactant ($X_3$) was selected for components of SMEDDS. The range of each component was selected as follows: $10\% \leq X_1 \leq 40\%$, $30\% \leq X_2 \leq 60\%$, $20\% \leq X_3 \leq 50\%$. The optimized L-SMEDDS formulation was converted into free flowing powder by adsorbing onto Aerosil 200 used as a solid carrier. Optimized SMEDDS formulation was characterized for various physicochemical parameters (like droplets size and size distribution, ZP, dilution studies, thermodynamic stability studies, morphology and stability studies). The morphology of solid SMEDDS (S-SMEDDS) and droplet size/distribution of EFV microemulsion were observed by SEM and TEM, respectively. Solid state characterization of S-SMEDDS performed by DSC and XRD. The release profile of L- and S-SMEDDS from capsules were evaluated using paddle dissolution apparatus in water containing 1.0% w/v SLS and
compared the release of EFV from a MF. *In situ* absorption property in rat intestine and *in vivo* oral absorption in rabbit was performed with L- and S-SMEDDDS and compared with a marketed formulation (MF) of EFV. S-SMEDDDS show good potential to improve oral bioavailability for the delivery of EFV.

Present third study has been undertaken to develop EFV loaded PLGA nanoparticles (NPs) and their formulation parameters were statistically optimized using $3^2$ factorial designs. PLGA NPs were prepared using nanoprecipitation method. The formulation was prepared by dissolving PLGA and EFV in organic solvent. This organic phase was added into aqueous phase contains surfactant with continuous stirring on magnetic stirrer at room temperature. Stirring was continued for 3-4 h to allow complete evaporation of organic solvent. Finally, traces of organic solvent were eliminated under reduced pressure in rotary flask evaporator at 40°C for 30 min. Preliminary experiments were carried out by varying one parameter at a time, while keeping other constant, so that effect of various parameters could be evaluated. For Preliminary experiments, various parameters like type of organic phase, type of surfactant, ratio of organic phase to aqueous phase and concentration of PLGA were varied and the effect on particle size of PLGA nanoparticles was studied. The parameters were optimized to obtain nano-ranged particles with narrow size distribution. The effect of drug : polymer ratio and surfactant (Poloxamer 188) concentration on particle size was assessed by using Design-Expert® software. The factor levels are evenly spaced and coded for low, medium and high settings, as $-1$, $0$ and $+1$. Dependent variables were MPS ($Y_1$) and % entrapment efficiency (% EE) ($Y_2$). Incorporation of mannose (MN) in PLGA was carried out and EFV loaded PLGA-MN NPs was prepared. NPs were subsequently transformed to dry powder by lyophilization, to enhance the stability of EFV. The optimized formulations were characterized for particle size, % EE, DSC and TEM. In vitro diffusion study of EFV loaded PLGA and PLGA-MN NPs were performed in 1% SLS in water, selected as the drug release medium. The chemical stability of NPs were assessed by determining percentage of EFV present in the formulations stored at different temperatures (4-8°C and 25°C) during a period of 3 months. Isolated rat peritoneal macrophages are used for *in vitro* uptake study of EFV loaded PLGA and PLGA-MN NPs. The effect of EFV, EFV loaded PLGA NPs and PLGA-MN NPs on cell proliferation was determined by MTT based colorimetric assay. Fluorescence microscopy was
performed to study the qualitative uptake of prepared NPs by macrophage cells. The 6-coumarin was used as a fluorescent marker and loaded into PLGA-NPs and PLGA-MN NPs instead of EFV. Biodistribution of EFV loaded PLGA and PLGA-MN NPs in organs was evaluated in albino rats until 24 hours after intraperitoneal injection and amount of EFV present in liver, spleen, lung, kidney and brain at different time intervals were determined.

HPLC analytical method were developed and validated to estimate EFV in various developed formulations (NS, SMEDDS, PLGA NPs), in vitro and in vivo studies. The chromatographic separation was performed using a Phenomenex Hypersil C4 (100 mm × 4.6 mm i.d., 5 μm particle size) column. Separation was achieved using a mobile phase consisting of acetonitrile and 100 mM ammonium acetate buffer pH 7.0 in the ratio of 45:55 (v/v), pumped at a flow rate of 1 ml/min. The eluent was monitored using UV detector at a wavelength of 247 nm. The column was maintained at 40°C and an injection volume of 20 μL was used. Method was validated for system suitability, accuracy, precision, robustness. All validation parameters meet the acceptance criteria as per ICH guideline. Extraction of EFV from plasma and tissue homogenates was carried out using Tert-butyl methyl ether and analyzed using validated HPLC method.

7.2. Conclusion

A media milling method using zirconium oxide beads was successfully employed to produce stable EFV NS. The results obtained in this study demonstrate that the particle size can be influenced by parameters, such as drug concentration, type and concentration of stabilizers. Efficient particle size reduction by nanogrinding was achieved by using excipients that provide proper wetting and physical stabilization (steric and electrostatic) of the practically water-insoluble drug, EFV. The combination of PVP K30–SLS stabilizer system was most suitable and optimized by the use of the Box-Behnken design to produce NSs with maximal particle size reduction. Lyophilization of the NSs with trehalose yielded nanopowders that were re-dispersed completely in the water. XRD and DSC data revealed that the crystalline state of EFV was not altered through operations, and shall be of great importance when considering long-term stability of EFV formulation. SEM images exhibited
distinct differences in the morphological structure of nanoparticles influenced by the stabilizers. The NS was physically and chemically stable over 6 months. The physical mixture of the drug and stabilizer did not significantly improve the dissolution of the drug suggests that the increased dissolution rate for the NS is primarily due to the reduction of the particle size. Significant enhancement in the saturation solubility of EFV in NS form was observed as compared to standard EFV. The in vitro transport study in PAMPA model demonstrated that NS was successful in enhancing the permeation of EFV. The results of in situ absorption of EFV in rat intestine suggested that NS played an important role in absorption enhancing effect. Pharmacokinetic evaluation clearly showed that the EFV NS exhibited improved pharmacokinetic properties compared to the MF. Oral bioavailability of EFV in rabbits resulted from NS was increased by 2.19-fold compared with the MF. The media milling method is easy to apply and needs only simple equipment and, thus, is a promising method for preparing drug NSs. Results of this study lead to the conclusion that NS approach is effective in preparing EFV formulations with enhanced dissolution velocity and oral bioavailability attributed to better wettability, increased saturation solubility and surface area, reduced particle size and decreased diffusion layer thickness. Moreover, NS may give added value by allowing a reduction in either the dose or its frequency of administration.

Solubility evaluation, pseudoternary phase diagram and self-emulsification test were carried out to select excipients of SMEDDS. Composition of EFV loaded SMEDDS was optimized using factorial design. Optimal SMEDDS contains captex 500 as oil phase, tween 20 as a surfactant and transcutol HP as cosurfactant, in the ratio of 25:50:25 %w/w, formulates SMEDDS with lower droplet size (30.4 nm), PDI (0.126), and ZP (-19.9 mV) values. The L-SMEDDS converted into S-SMEDDS using Aerosil 200 as a solid carrier. Both DSC measurements and X-ray diffraction analysis suggested that EFV in the S-SMEDDS may be in the molecular dispersion state. Following self-emulsification in water the droplet size distribution of the S-SMEDDS was nearly same to the L-SMEDDS, and the in vitro dissolution performance was similar for L- and S-SMEDDS both significantly higher than the MF. The L- and S-SMEDDS were physically and chemically stable over 6 months. The in vitro transport study in PAMPA model demonstrated that L- and S-SMEDDS was successful in enhancing the permeation of EFV. The results of in situ absorption of EFV in rat
intestine suggested that SMEDDS played an important role in absorption enhancing effect. Pharmacokinetic evaluation clearly showed that the EFV loaded L- and S-SMEDDS exhibited improved pharmacokinetic properties compared to the MF. The oral bioavailability of EFV from S-SMEDDS was 2.16-fold higher than the MF and no significant difference compared with the L-SMEDDS. Our results illustrated the potential use of S-SMEDDS to dispense poorly water soluble drug by oral route.

EFV loaded PLGA NPs prepared by the nanoprecipitation method had high % EE, spherical shape, regular surface and particle size less than 200 nm. The application of a 3² factorial design proved to be a useful tool for the optimization of EFV loaded PLGA-NPs. The analysis of the obtained results led to the polynomial equations obtained by multiple regression described adequately the influence of the selected variables (drug:polymer ratio and surfactant concentration) at three levels on the responses. According to the studied factors, the selected optimum formulation was that prepared with 7.5:50, drug:polymer ratio and 0.5 %w/, surfactant concentration. Incorporation of MN in PLGA can be used as a successful strategy to enhance the uptake of PLGA-MN NPs by macrophages. In vitro drug release study showed 50.31 ± 1.39 % EFV release from PLGA NPs and 45.49 ± 1.56 % EFV release from PLGA-MN NPs in 5 days. The NPs formulations were stable over 3 months. DSC thermograms indicated that EFV was dispersed as amorphous state in the NPs. The NPs penetrate macrophages and do not cause toxicity to these cells by MTT assay. Higher uptake in peritoneal macrophages was observed with PLGA-MN NPs than PLGA NPs. Administration of PLGA-MN NPs resulted in a significantly higher concentration of EFV in liver, lung, spleen, kidney and brain as compared to PLGA NPs. These nanoparticles sustained the release of EFV and may be used to reduce the frequency of administration and dose-dependent side-effects, reducing the chances of dose dumping and increasing the patient compliance.