Chapter 6

Preparation and characterisations of SLN

6.1. Introduction:

Lipid nanoparticles are an alternative drug delivery system to emulsions, liposome and polymeric nanoparticles. (Muller RH et al 1999) The inherent property of the solid nature of lipids and the ability to form smaller particles as well as their stability has popularized their use in drug delivery.(Muller RH et al 1999) Lipid nanoparticles are usually aqueous dispersions of solid lipids or dry powders obtained by lyophilization (Lim SJ et al 2002) or spray drying.(Freitas C,et al 1998) Lipid nanoparticles overcome the membrane stability and drug-leaching problems associated with liposomes and emulsions, (Magenheim B et al 1993) and the biodegradation and toxicity problems of polymeric nanoparticles, (Muller RH et al., 1997) and facilitate prolonged drug release.(Zur Muhlen A et al., 1998) Lipid nanoparticles are prepared from biocompatible lipids and possess excellent biodegradability and low toxicity. (Muller RH et al., 1997) (Muller RH et al 1996)

Lipid nanoparticles have received great attention as drug carriers in recent years. A striking advantage of lipid nanoparticles is the feasibility of large-scale production by a high pressure homogenization technique. Several reports are available on formulation, characterization, (Schwarz C et al., 1999) (Unruh T et al., 2001) sterilization, (Cavalli R et al., 1997) in vitro degradation, and lipid recrystallization behavioral studies by various techniques (Siekmann B et al., 1994) like differential scanning calorimetry [DSC], (Westesen K et al., 1997) small-angle and wide-angle x-ray diffractometry (Bunjes H et al 2001) and on their in vitro drug release potential.(Westesen K et al., 1997) Extensive work by Bunjes and coworkers (Bunjes H et al., 2000, 2002, 2003) reports on crystalline properties of lipids and their recrystallization patterns during nanoparticles preparation, and the influence of nanoparticle size on recrystallization pattern. Recent reviews by Mehnert and Mader (Mehnert W et al., 2001) and by Muller and coworkers (Muller RH et al., 2000) provide extensive information on nanoparticle preparation, characterization, and drug release properties. To date, the majority of studies reported the application of lipid nanoparticles for parenteral administration (Zara GP et al., 1999) (Lakkireddy at al, 2004). There are few studies reported for peroral administration of drugs for the improvement of bioavailability. Of interest is that despite the advantages of lipid nanoparticles with respect to their ease of preparation, stability, and drug incorporation and release potential, only a few studies are available on their use for the improvement of bioavailability of poorly soluble drugs, (Penkler L et al, 1999) (Yang S, Zhu et al., 1999)

6.2 Instruments:

High Pressure Homogenizer (Avestin, Canada). Remi High Speed Mechanical Stirrer (Remi Scientific equipment, Bombay), Single Pan Electronic Balance (Precisa 205 ASCS), Remi Magnetic Stirrer (Remi Scientific equipment, Bombay). Sigma 3K 30 refrigerated laboratory centrifuge (Sigma Laboratory, GmBH). Particle Size Analyzer (Malvern Instruments, UK). U.V. Spectrophotometer (Shimadzu, Japan). Lyophilizer / Vacuum pump (Heto vaccubrand, India). Differential Scanning Calorimeter (DSC – 60, Shimadzu, Japan), Scanning Electron Microscope (JSM 56 10 LV SEM, JEOL DATUM LTD, Japan). Optical microscope (OPTIK, Olympus B 201).

6.3 Materials:

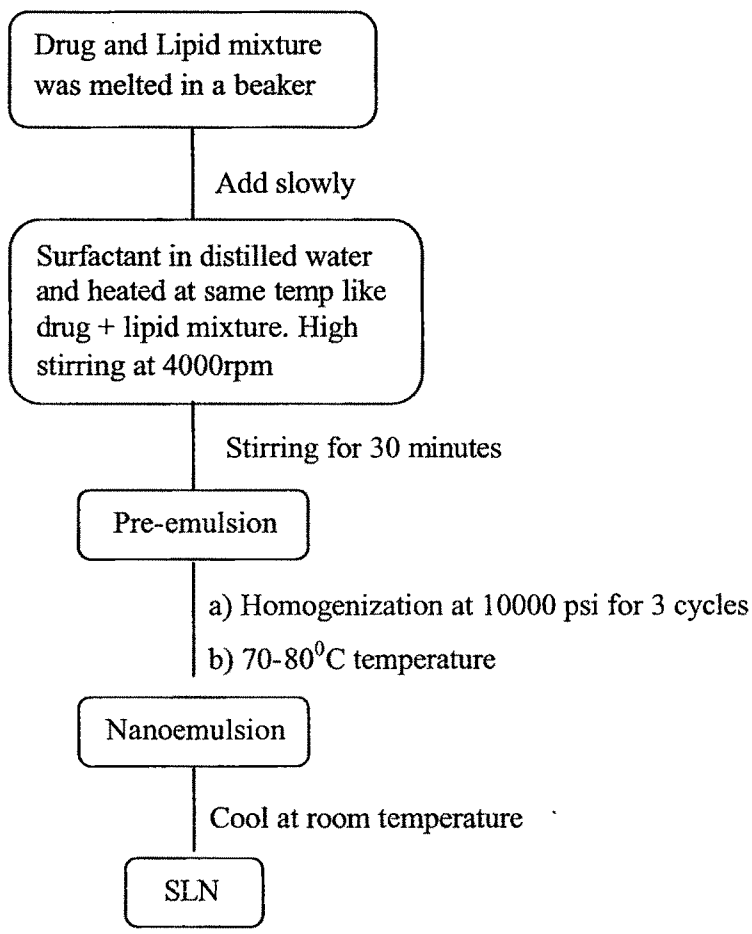
Pluronic F64 (poloxamer 188) was obtained as gift sample from Sun Pharma Advance Research Center (SPARC) Baroda. Propylene glycol was purchased from S. D. Fine chemicals, India. Sodium deoxycholate (SDC) was purchased from Loba Chemie, India. Sucrose AR was purchased from Suvidha Laboratories, India, Tween 80, Polyethylene Glycol (PEG) 400 was purchased from Qualigens, India. Ethanol (Suvidha lab, India) was taken from Pharmacy dept with proper authorization. Steaic acid, Glyceryl monostearate, and Glyceryl tristearate was purchased from National Chemicals, India. Glyceryl distearate (Precirol ATO 5) was obtained as gift sample from colorcon asia Pvt ltd., India (Mfg: Gattefosse, france). Cellophane membrane (12000 daltons, Kalpana Traders, India). Methanol (AR grade) – was purchased from Qualligens, India. Ethanol (Suvidha lab, India) was taken from Pharmacy dept, MS University of Baroda after authorization. Sucrose AR, Lactose AR was purchased from Suvidha laboratories, India.

6.4. Method of SLN preparation:

Solid Lipid Nanoparticles (SLN) dispersions were prepared by the High Pressure Homogenization (HPH) technique. The drug, lipid and surfactant were separately weighed in different ratios in a beaker. Hydrophilic surfactants (Sodium deoxycholate, Polaxomer 188 or their combinations) were separately weighed in another beaker and dissolved in distilled water. The drug and lipid mixture was heated till complete melting of all ingredients. Simultaneously, the aqueous surfactant containing phase was also heated to the same temperature as the melt. The drug containing lipid melt was then added to the hot aqueous phase under high speed stirring using a Remi High Speed Mechanical Stirrer (Remi Scientific equipment, Bombay) to form an initial pre-emulsion. This pre-emulsion was subsequently homogenized in a heated High Pressure Homogenizer (Avestin, Canada) maintained at 70-80°C in a water bath. The homogenization was carried out at high

pressures of about 10000 psi for three cycles, wherein the pre-emulsion is pushed through a narrow gap (in the range of a few microns). The fluid accelerates on a very short distance to a very high velocity (over1000 Km/h). Very high shear stress and cavitations forces disrupt the particles down to the submicron range, giving a hot nano-emulsion which contains liquid lipid droplets. This nano-emulsion was allowed to cool down to room temperature which resulted in the re-crystallization of the lipid back to the solid state giving an SLN dispersion containing drug entrapped solid lipid nanoparticles suspended in an aqueous medium.(Figure 6.1)

Figure 6.1: Flow chart for the preparation of SLN:



6.4.1. Acyclovir:

For acyclovir SLN preparation stearic acid (SA), glyceryl mono-stearate (GMS) and glyceryl di-stearate (GDS) was taken as lipid. Polaxomer 188 and sodium deoxycholate (SDC) and 1:1 ratio of Polaxomer 188 and SDC was taken as surfactant. Amount of acyclovir was kept constant for all the batches and that was 10mg. Taguchi orthogonal experimental design (Taguchi et al, 1993) was used to optimize the combination of four independent variables type of lipid, concentration of lipid, type of surfactant and concentration of surfactant to achieve maximum drug entrapment and optimum particle size. It resulted into the optimized combinations of independent variables only in nine experiments recorded in Table 6.1. Each experiment was repeated thrice and optimized batch was repeated six times on six different days to ascertain reproducibility as shown in Table 6.6. The concentration of lipids was varied from 3-5% and concentration of surfactant was varied from 1-3%with respect to the total aqueous phase. All the batches were prepared under similar conditions keeping the all process parameters constant. 25ml distilled water taken as aqueous phase. Homogenization pressure was fixed at 10,000psi and process continued for 3cycles.

Table 6.1: Coded units of Taguchi orthogonal experimental design L_9 (3^4) for preparation of acy - SLN.

	Levels		
Independent variables	**1**	**2**	**3**
A: Type of Lipid	SA	GDS	GMS
B: Concentration of lipid (%)	4	3	5
C: Type of surfactant	Polaxomer 188	SDC	Polaxomer 188: SDC (1:1)
D: Concentration of Surfactant (%)	2	1	3

6.4.2 Efavirenz:

Similarly for efavirenz SLN preparation three different lipids, two different surfactants and their mixture was taken. Glyceryl monostearate (GMS), glyceryl di-stearate (GDS) and glyceryl tri-stearate (GTS) was taken as lipid. Polaxomer 188 and sodium deoxycholate (SDC) and 1:1 ratio of Polaxomer 188 and SDC was taken as surfactant. Like acyclovir SLN, the quantity of efavirenz was kept constant for all the batches and that was 10mg. To optimize the combination of four independent variables like type of lipid, concentration of lipid, type of surfactant and concentration

of surfactant to achieve maximum drug entrapment and optimum particle size, Taguchi orthogonal experimental design (Taguchi et al, 1993) was used. It resulted into the optimized combinations of independent variables only in nine experiments recorded in Table 6.2. Each experiment was repeated thrice and optimized batch was repeated six times on six different days to ascertain reproducibility as shown in Table 6.6. The concentration of lipids was varied from 3-5% and concentration of surfactant was varied from 1-3% with respect to the total aqueous phase. All the batches were prepared under similar conditions keeping the all process parameters constant. 25ml distilled water taken as aqueous phase. Homogenization pressure was fixed at 10,000psi and process continued for 3cycles.

Table 6.2 Coded units of Taguchi orthogonal experimental design I_9 (3^4) for preparation of Efa - SLN.

	Levels		
Independent variables	**1**	**2**	**3**
A : Type of Lipid	GMS	GDS	GTS
B : Concentration of lipid (%)	4	3	5
C : Type of surfactant	Polaxomer 188	SDC	Polaxomer 188: SDC (1:1)
D : Concentration of Surfactant (%)	2	1	3

6.5. Characterizations of SLN:

6.5. 1. Particle size measurements

The size analysis of nanoparticles was performed by laser diffraction using a Malvern Hydro 2000SM particle size analyzer (Malvern Instruments, Worcestershire, UK). The nano-particulate dispersion obtained was added to the sample dispersion unit containing stirrer and stirred in order to minimize the inter-particle interactions; the laser obscuration range was maintained between 10% and 20%. The instrument was set to measure the sample 3 times at a rate of 3000 snaps (or counts) per second. The sample was counted 3 times and average volume mean diameter was obtained.

6.5.2. Estimation of drug entrapment efficiency (DEE):

The nanoparticles in dispersion were aggregated by adding 0.1 mL of 10 mg/mL protamine sulfate solution, and the dispersion was centrifuged to obtain the pellet (Lakkireddy et al, 2004). The

supernatant was decanted and the pellet was washed with distilled water and lyophilized after the addition of 2 parts by weight sucrose with respect to total lipid content of the formulation. Twenty five milligrams of lyophilized powder was dissolved in a suitable solvent and analyzed in a UV-visible spectrophotometer (Shimadzu, Japan).

6.5.2.1. Acyclovir:

25mg of the lyophilized powder was taken in a 25ml volumetric flask. To it ethanol was added to dissolve the drug and the volume was made up to the mark by with ethanol. The resulted solution was further diluted with ethanol, if necessary and the absorbance was measured at 236.5nm against ethanol as blank. The analysis was carries out in triplicates and the mean absorbance's were considered for further calculations. The concentration of drug was calculated from the calibration curve, Y= 0.0744 x + 0.053 equation.

6.5.2.2. Efavirenz:

25mg of the lyophilized powder was taken in a 25ml volumetric flask. To it methanol was added to dissolve the drug and the volume was made up to the mark by with methanol. The resulted solution was further diluted with methanol, if necessary and the absorbance was measured at 247nm against methanol as blank. The analysis was carries out in triplicates and the mean absorbance's were considered for further calculations. The concentration of drug was calculated from the calibration curve, Y= 0.0501 x +0.0159 equation.

6.5.3. Lyophilization of nanoparticles dispersions

The amount of drug entrapped inside Solid Lipid Nanoparticles was found out by centrifugation of the SLN dispersions at 20,000 rpm for 1 hour in a High Speed Cooling Centrifuge (Sigma, GmBH) after addition of 0.1 mL of 10 mg/mL protamine sulfate solution into the SLN dispersion The supernatant containing the un-entrapped drug and excess surfactant was discarded and the sediment was taken in glass vials. Sucrose was weighed in a quantity equivalent to double the amount of lipid concentration, dissolved in about 1 ml distilled water and added to the sediment as a cryoprotectant. The mixture was then kept at freezing temperatures of about –40°C overnight and then lyophilized for 24 hours in a Lyophilizer / Vacuum pump (Heto vaccubrand, Denmark).

Optimization of homogenization pressure:

For optimizing the homogenization pressure, the optimum formulations (Batch No- Acy-SLN-opt and Ef-SLN-opt) and process (three homogenization cycles) which gave the smallest particle sizes were selected. The same formulations were prepared again at three different homogenization pressures viz. 5000, 10000 and 15000 psi and particle size measurements were conducted. The optimized pressure was the one at which the particle size was found to be the least

6.5.5. Optimization of number of homogenization cycles

For optimizing the homogenization cycle number, the optimum formulations which gave the smallest particle sizes were selected. The same formulations were prepared again by homogenizing them for one to four cycles at a homogenization pressure of 5,000, 10,000 and 15,000 psi and measuring the particle sizes after each cycle. The optimized cycle number was the one at which the particle size was found to be the minimum.

6.5.6. Optimization of drug loading:

In order to find out the maximum drug loading capacity of SLN, different known quantity of drug was added in to the lipid mixture, keeping all other formulation and process parameters constant similar to optimum formulation (Acy-SLN-opt and Ef-SLN-opt). For acyclovir 10mg-30mg and for efavirenz 10-50mg drug was incorporated and check the particle size and drug entrapment.

6.5.7. Differential Scanning Calorimeter study (DSC):

Differential Scanning Calorimetry (DSC) studies were conducted for optimum batches having minimum particle size and maximum entrapment efficiency. These included studies on pure drug, bulk lipid, SLN dispersions. The main objective of these studies was to determine the melting behavior of lipids in the SLN dispersions, and possible various polymorphic modifications that the lipid may assume during the process of hot melt homogenization and subsequent cooling of the nano-emulsions.

6.5.8. Scanning electron microscopy (SEM)

Scanning Electron Microscopic (SEM) studies were done for Solid Lipid Nanoparticles. The aim was to study the particle shape, size and surface characteristics. The nanodispersion formed was spray dried using a spray drier (JISL Instruments, Mumbai, India) after the addition of 2 parts by

weight lactose monohydrate with respect to the total lipid content in the formulation. Optimum condition for spray drying was tabulated in Table 6.3.

Table 6.3. Optimum parameter for spray drying of acy-SLN and Ef-SLN.

Spray drying parameters		Optimum condition
Inlet air pressure	:	2.5 Kg/cm^2
Aspiration volume	:	50 cu ft/min
Flow rate	:	2 ml/min
Inlet temperature	:	70^{0}C
Outlet temperature	:	40^{0}C

The powder nanoparticles were stuck to a brass stub with double-sided adhesive tape. The stub was fixed into a sample holder and placed in the vacuum chamber of a Jeol JSM 1560 LV SEM (Jeol, Peabody, MA) and observed under low vacuum (10$^-$3 torr). For the imaging of Solid Lipid Nanoparticles, three viewing fields were selected at different magnifications. The magnification giving the best resolution was selected. The SEM images are as shown in Figure 6.14 and 6.15.

6.5.9. Zeta Potential Measurement

Zeta potential of acy-SLN-opt and Ef-SLN-opt was measured in a Malvern Zetasizer 3000 HSA (Malvern Instruments). The nanoparticles were dispersed in water and 0.1N HCl and sonicate for 30second to avoid agglomeration. Samples were injected into the cylindrical couvettes and results were recorded. Before putting the fresh sample couvettes was washed with the methanol and rinsed using the sample to be measured before each experiment. Zeta potentials were calculated from the mean electrophoretic mobility by applying the Smoluchowski equation. The results are the means of 5 determinations ± standard deviation.

6.5.10. Preparation of membrane for release study

For studying the in vitro release pattern of drug from SLN dispersions, an in vitro method was developed. A Dialysis membrane having pore size 2.4 nm, molecular weight cutoff between 12,000 –14,000 was used. The membrane was soaked overnight in distilled water, cleaned the next morning, and then used for the experimental work.

6.5.11. In vitro diffusion studies:

The SLN dispersions were accurately pipette out (1ml) and were packed in the diffusion membrane prepared earlier. This formed the donor compartment, which was dipped in to a receptor compartment containig 25ml of distilled water or 1% SLS solution for acyclovir and efavirenz respectively.. A continuous stirring of the receptor for compartment was achieved by using a magnetic stirrer (Remi instruments, India). Samples of 3 ml were then periodically withdrawn from the receptor compartment and analyzed for drug content by UV spectroscopy as described previously. The amount withdrawn was subsequently replaced each time. For in-vitro diffusion study optimum formulation was compared with different trial batches.

6.5.12. Data analysis

6.5.12.1. Percent Drug Diffusion

The percentage of drug diffused was determined by the formula

%drug diffused = CrVr / CdVd X 100

Cr = Concentration of drug in the receptor compartment

Vr = Volume of the receptor compartment

Cd = Concentration of drug in the donor compartment

Vd = Volume of the donor compartment

6.5.12.2. Kinetics of release

The order of drug release was determined by plotting graphically percent cumulative drug release versus Time (Figure 6.17 and 6.19) and Percent drug release versus time (Figure 6.16 and 6.18).

Mean steady state flux

The flux across the cellophane membrane (J) was calculated using the formulae

$J = V (dc / dt)$

Where V = Volume of receptor compartment

dc / dt = Rate of change of concentration with time and was the slope of the drug release versus time curve.

6.5.13. Permeation data analysis

Absorption data was plotted as mean cumulative amount of drug diffused as a function of time. J is flux (steady-state rate of penetration) in $\mu g\ cm^{-2}\ min^{-1}$ and was calculated from the slope of the linear portion of the cumulative amount permeated through the membrane per unit area versus time plot (Ceschel,G.C et al., 2000).

Permeability coefficient of the drug through the membrane (P) was determined using following equation (Zhang, H. et al 1996)

$P = J/Cd$

Where, J is flux calculated at the steady time and Cd is drug concentration on the surface of the mucosa.

6.5.14. The physical stability of SLN in dispersions

From the stability aspect, the nanoparticles have to disperse stably in an aqueous medium and not aggregate during storage. The change in SLN size and drug entrapment (DE) as a function of time was studied. Dispersions were stored at 4 °C in a refrigerator and at room temperature approx. 25 ^{0}C (in amber colour bottle at dark and clear glass vial under normal daylight) and the particle size and DE was measured repeatedly during a period of six months.

6.6. Results and Discussion:

6.6.1. Preparation of SLN

6.6.1.1. Acyclovir:

High-pressure homogenization was reported to be the most reliable technique for the preparation of lipid nanoparticles with smaller size.(Mehnert W et al., 2001) Very high shear stress and cavitations forces produced during homogenization disrupt the particles down to submicron range. Another advantage of this method is that it avoids the use of organic solvents. Nine batches of Acy-SLN were prepared by high pressure homogenization technique using Taguchi orthogonal experimental design [$L_9(3^4)$] (Table 6.2) varying four independent variables, type of lipid (A), concentration of lipid molar ratio (B), Type of surfactant (C) and concentration of surfactant (D) at three levels. The particle size and DEE (dependent variables) of prepared batches were determined and recorded in Table 6.4. Taguchi orthogonal experimental designs [L_9 (3^4)] offer the possibility of investigating four independent variables at three levels after performing only nine experiments (Taguchi, G. Taguchi Method: Design of Experiments; Quality Engineering Series, Vol. 4; Konishi, S., Ed.). The selection of factors and levels in the design would be based on the results of a preliminary investigation. Depending on the therapeutic application, obtaining a stable suspension with small monodisperse particle size requires information on the effects that formulation and production variables have on the Acy-SLNs properties. Taguchi's signal to noise ratio was used for finding the optimum levels. Since further optimization of the factors could not be possible by making use of Taguchi's signal to noise ratio values as there was no degree of freedom available for estimation of effect of factors. The concept ANOVA was further used to find the optimum levels of the factors.

The mean diameters of Acy-SLNs determined by Malvern particle Size analyzer are shown in Table 6.4, and analysis of variance (ANOVA) of the orthogonal experimental design is presented in Table 6.5. The type and concentration of lipid, concentration of surfactants had a statistically significant ($P<0.001$) influence on the particle size of Acy-SLNs. The mean particle size was decrease in case of GDS which was followed by GMS and stearic acid. When concentration of lipid was 4%, the mean diameter of Acy-SLNs was smallest. The type of different surfactant showed only a slight influence on the size of resultant Acy –SLNs but concentrations of surfactant have significant impact on particle size. This can be further simplified and optimum values of the factors be obtained by the concept of ANOVA which was presented in the Table 6.5.

Table 6.4: Experimental design for acyclovir SLN and their corresponding results.

Batch No	*Independent variables*				*Particle size (μm)*			*% DEE*
	A	B	C	D	Trial 1	Trial 2	Trial 3	
Acy-SLN01	1	1	1	1	0.203	0.224	0.186	54.23 ± 2.56
Acy-SLN02	1	2	2	2	3.654	4.652	2.656	ND
Acy-SLN03	1	3	3	3	5.94	8.252	6.584	ND
Acy-SLN04	2	1	2	3	0.221	0.198	0.242	72.52 ± 3.12
Acy-SLN05	2	2	3	1	0.229	0.245	0.205	68.42 ± 2.24
Acy-SLN06	2	3	1	2	0.254	0.264	0.248	76.46 ± 3.82
Acy-SLN07	3	1	3	2	0.237	0.189	0.242	58.64 ± 4.35
Acy-SLN08	3	2	1	3	8.178	6.158	7.284	ND
Acy-SLN09	3	3	2	1	1.046	1.684	2.654	ND

DEE= drug entrapment efficiency

Table 6.5: Analysis of Variance (ANOVA) for Acy-SLN

Source of variation	DF	SS	MSS	F-ratio	P-Value
A	2	64.574	32.287	16.82	0.00008
B	2	63.195	31.597	16.47	0.00009
C	2	2.827	1.414	0.74	0.49261
D	2	89.252	44.626	23.25	0.00001
Error	18	3.055	0.0170	-	
Total	26	222.904	-	-	

DF: Degree of freedom, SS: Sum square, MSS: mean ism square.

From the ANOVA table it was clearly evident that Factor A, B, D was found statistically significant. The effect of different factors was presented in Figure 6.2, from which it can be concluded that optimum batch should consist of A_2 (type of lipid was GDS), B_1 (concentration of lipid was 4%), C_2 (type of surfactant was SDC) and D_1 (concentration of surfactant was 2%)

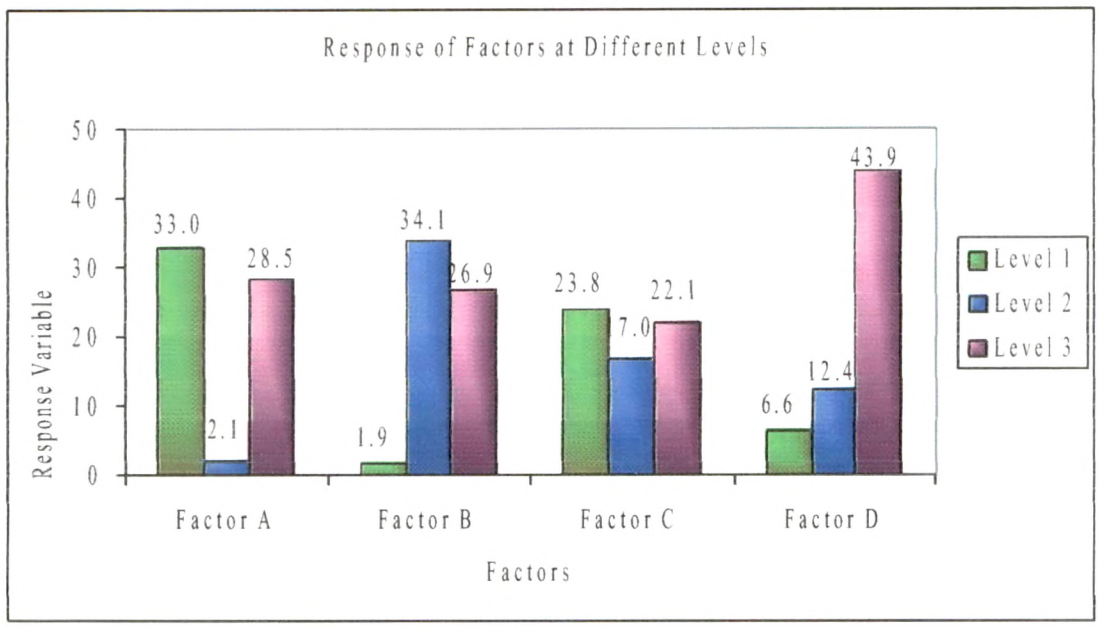


Figure 6.2: Response of factors at different level for Acy-SLN

Legend: Factor A-Type of lipid, Factor B: concentration of lipid, Factor C: type of surfactant, Factor D: concentration of surfactant

Reproducibility of optimized batch was checked by preparing Acy-SLN (Acy-SLN-opt) using the following formula (%w/w): Acyclovir 0.16g, GDS (4gm), SDC (2gm) and distilled water to 100gm results are present in Table 6.6. Total six batches were prepared using the optimum composition at six different days. No significant differences ($p < 0.01$) were observed within and among the batches with respect to their particle size.

Table 6.6: Reproducibility of the optimum batch for Acy- SLN

Batch No	Particle Size (nm)	% DEE
Acy-SLN-opt1	221	68.6
Acy-SLN-opt2	226	72.5
Acy-SLN-opt3	212	66.5
Acy-SLN-opt4	194	78.5
Acy-SLN-opt5	242	68.6
Acy-SLN-opt6	208	69.5
Average ± SD	**217 ± 16.47**	**70.7 ± 4.28**

6.6.1.2 Efavirenz:

Nine batches of Ef-SLN were prepared by high pressure homogenization technique using Taguchi orthogonal experimental design [$L_9(3^4)$] (Table 6.2) varying four independent variables, type of lipid (A), concentration of lipid molar ratio (B), Type of surfactant (C) and concentration of surfactant (D) at three levels. The particle size and PDE (dependent variables) of prepared batches were determined and recorded in Table 6.7. Taguchi's signal to noise ratio was used for finding the optimum levels. Since further optimization of the factors could not be possible by making use of Taguchi's signal to noise ratio values as there was no degree of freedom available for estimation of effect of factors. The concept ANOVA was further used to find the optimum levels of the factors.

The selection of factors and levels in the design would be based on the results of a preliminary investigation. Depending on the therapeutic application, obtaining a stable suspension with small mono-disperse particle size requires information on the effects that formulation and production variables have on the Efa-SLNs properties.

The mean diameters of Efa-SLNs determined by Malvern particle Size analyzer are shown in Table 6.7 and analysis of variance (ANOVA) of the orthogonal experimental design is presented in Table 6.8. A substantial high drug entrapment (98.24%) and optimal particle size (244nm) of SLN (batch Ef-SLN09) achieved at 3 level of A (GTS), 3 level of B (5%), 2 level of C (SDC) and 1 level of D (2%). Similar entrapment (97.56) and particle size (259nm) was also achieved for Ef-SLN08 where 3 level of A (GTS), 2 level of B (3%), 1 level of C (Polaxomer 188) and 3 level of D (3%) The results (dependable variables) of Taguchi orthogonal experimental design revealed that glyceryl tristearate (GTS) influenced DEE of Efa-SLN maximum, followed by GDS and GMS respectively. This is consistent with the findings that chemical and physical structure of solid lipid matrix determines the loading efficiency of drug in the SLN (Muller R H et al 2000). This can be further simplified and optimum values of the factors be obtained by the concept of ANOVA which was presented in the Table 6.8.

The different type and concentration of lipid as well as surfactants had a statistically significant ($P<0.001$) influence on the particle size of Efa-SLNs. The mean particle size was decreased in case of GTS which was followed by GDS and GMS. With an increasing concentration of lipid, the mean diameter of Efa-SLNs decreased significantly. The different type and concentrations of surfactant have significant impact on particle size. When combination of surfactants (Polaxomer 188: SDC, 1:1) was used in the SLN preparation, best results obtained. It was also observed that, the greater the concentration of surfactant, lesser particle size.

Table 6.7: Experimental design for efavirenz SLN and their corresponding results.

Batch No	*Independent variables*				*Particle size (μm)*			*%DEE*
	A	B	C	D	Trial 1	Trial 2	Trial 3	
Ef-SLN01	1	1	1	1	56.23	42.25	65.35	ND
Ef-SLN02	1	2	2	2	19.41	27.56	16.58	ND
Ef-SLN03	1	3	3	3	0.221	0.198	0.242	89.67
Ef-SLN04	2	1	2	3	0.222	0.254	0.168	85.64
Ef-SLN05	2	2	3	1	0.636	0.946	1.458	88.65
Ef-SLN06	2	3	1	2	0.195	0.224	0.186	96.65
Ef-SLN07	3	1	3	2	0.495	0.465	0.594	89.64
Ef-SLN08	3	2	1	3	0.265	0.229	0.284	97.56
Ef-SLN09	3	3	2	1	0.255	0.215	0.264	98.24

DEE= drug entrapment efficiency

Table 6.8: Analysis of Variance (ANOVA) for Efa-SLN

Source of variation	DF	SS	MSS	F-ratio	P-Value
A	2	3735.02	1867.51	27.50	0.0000
B	2	1516.256	758.128	11.16	0.0007
C	2	1458.538	729.269	1074	0.0008
D	2	1541.276	770.638	11.35	0.0006
Error	18	336.992	18.72	-	
Total	26	8688.08	-	-	

DF: Degree of freedom, SS: Sum square, MSS: mean sum square.

From the ANOVA table it was clearly evident that all the Factors A, B, C and D was found statistically significant. The effect of different factors was presented in Figure 6.3, from which it can be concluded that optimum batch should consist of A_3 (type of lipid was GTS), B_3

(concentration of lipid was 5%), C_3 (type of surfactant was Polaxomer 188: SDC, 1:1) and D_3 (concentration of surfactant was 3%)

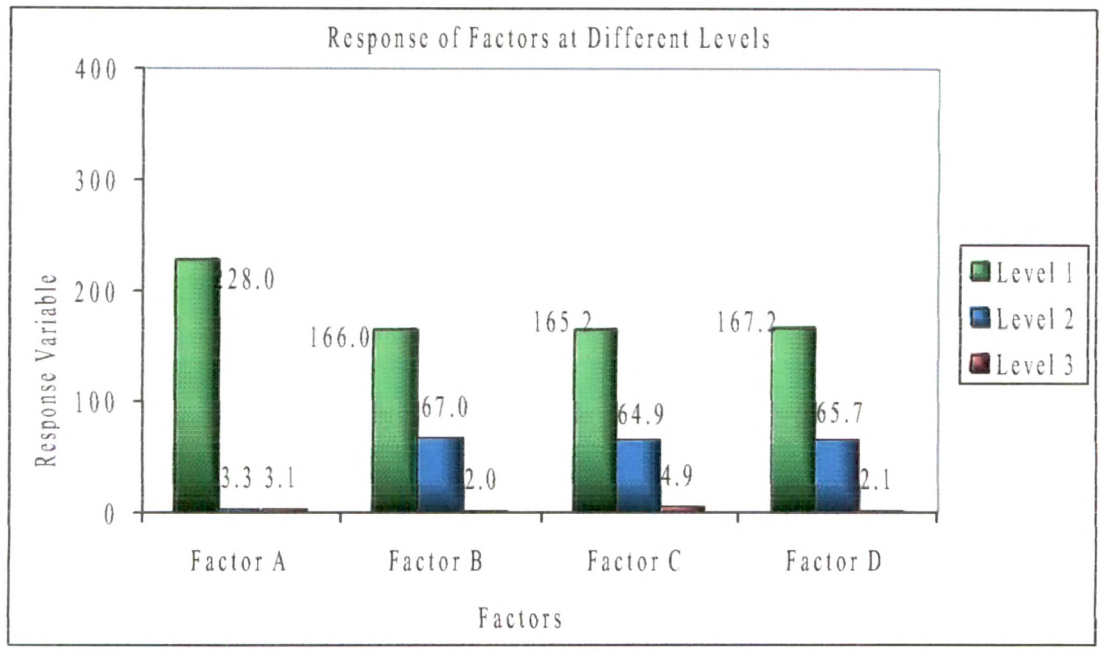


Figure 6.3: Response of factors at different level for Efa-SLN

Legend: Factor A-Type of lipid, Factor B: concentration of lipid, Factor C: type of surfactant, Factor D: concentration of surfactant

Reproducibility of optimized batch was checked by preparing Efa-SLN (Efa-SLN-opt) using the following formula (%w/w): Efavirenz 0.16g, GTS (5gm), 1:1 mixture of Polaxomer 188 and SDC (3gm) and distilled water to 100gm. (Table 6.9) Total six batches were prepared using the optimum composition at six different days. No significant differences ($p < 0.01$) were observed within and among the batches with respect to their particle size and DEE.

Table 6.9: Reproducibility of the optimum batch for Efa-SLN.

Batch No	Particle Size (nm)	% DEE
Efa -SLN-opt1	192	965
Efa -SLN-opt2	185	98.2
Efa -SLN-opt3	186	94.6
Efa -SLN-opt4	218	98.1
Efa -SLN-opt5	206	97.6
Efa -SLN-opt6	221	98.6
Average ± SD	201.33 ± 15.97	97.4 ± 1.41

6.6.2. Particle size analysis and Drug Entrapment Efficiency (DEE)

The results of particle size measurements and DEE of optimized formulations were as shown in Figure 6.4 to Figure 6.7 and Table 6.6 and Table 6.9. The results were plotted as the volume percentage of the particles having a given size versus the particle size in microns.

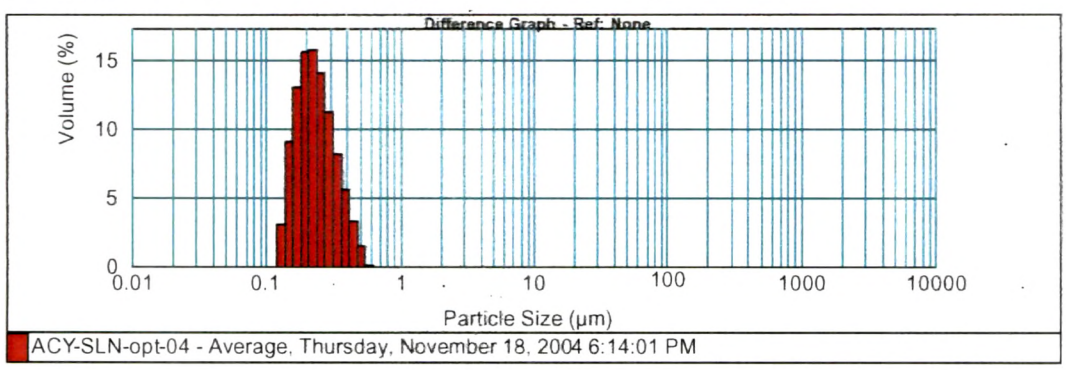


Figure 6.4: Particle size distribution of the acyclovir SLN (Batch No: ACY- SLN-opt04)

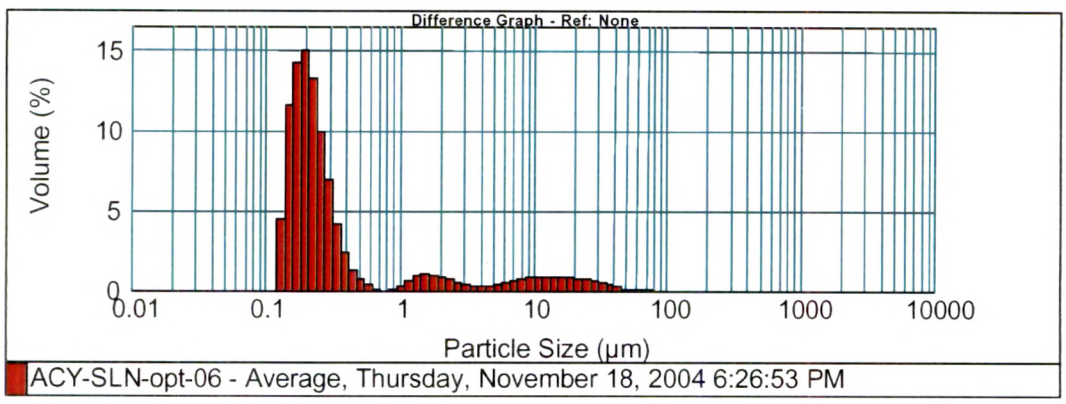


Figure 6.5: Particle size distribution of the acyclovir SLN (Batch No: ACY- SLN-opt-06)

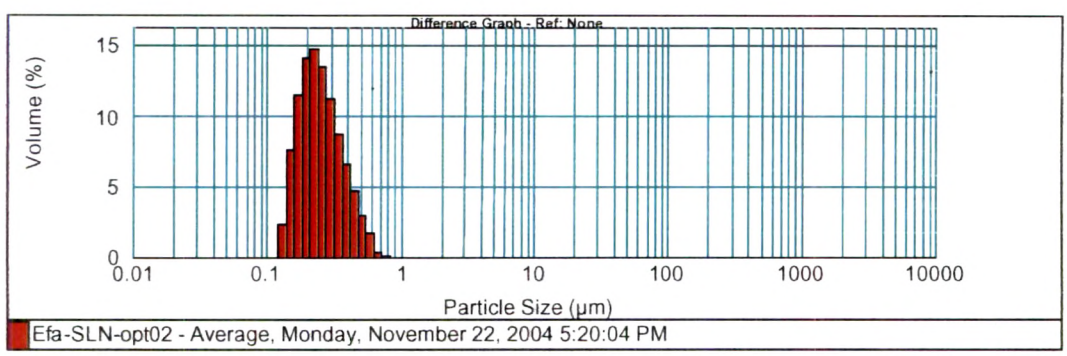


Figure 6.6: Particle size distribution of the efavirenz SLN (Batch No- Ef-SLN-opt02)

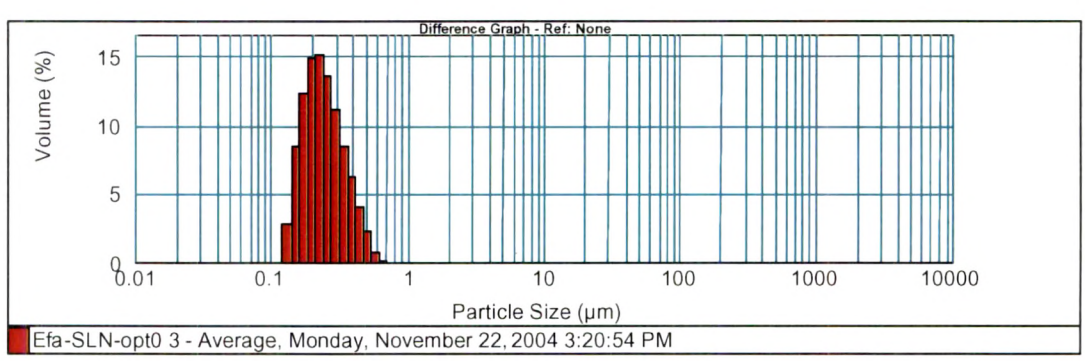


Figure 6.7: Particle size distribution of the efavirenz SLN (Batch No- Ef-SLN-opt03)

6.6.3 Optimization of homogenization pressure:

Homogenization pressures at different number of cycles, and mean particle diameter at each pressure and cycle number was recorded. Homogenization pressure was found to have significant impact on the particle size of the Acy-SLN-opt and Ef-SLN-opt (Table 6.10). Homogenization pressures above 10 000 psi did not show much decrease in mean particle diameter, while low pressures resulted in higher mean particle size. Decrease in particle size during homogenization was associated with pushing liquid with high pressure through a narrow gap, accelerating the fluid on a very short distance to very high velocity. Very high shear stress and cavitation forces disrupt the particles down to the submicron range (Mehnert W et al., 2001) (ZurMuhlen A et al., 1996). The optimum homogenization pressure was found to 10000 psi, 3cycles (Table 6.10).

In an additional study with the same formulations it was found that excessively high homogenization pressures of above 20,000 psi caused an increase in particle size instead of decreasing it. This could be attributed to particle coalescence which occurs as a result of the higher kinetic energy imparted to the particles during the homogenization process.

6.6.4. Optimization of number of homogenization cycles

Homogenization of hot emulsion was performed at various homogenization pressure and number of cycles. The results showed that particle size is largest after a single homogenization cycle. As the number of homogenization cycles increase, the size gradually reduces. This was because during homogenization, the dispersion is pushed at high pressure through a narrow gap (in the range of a few microns). So, with increasing number of cycles, the polydispersity of the SLN dispersions is reduced. The optimized number of homogenization cycles was homogenization of the dispersions was 10,000 psi for three cycles. Although the homogenization at 10 000 psi for 4 cycles resulted in a decrease of 4 nm mean diameter (for acy-SLN) compared with that of 3 cycles, the latter was considered optimum because the reduction in particle size after that was not significant.

Table 6.10: Effect of homogenization cycle on particle size (μm) of Acy-SLN and Efa-SLN

Homogenization pressure (psi)	No of homogenization cycle	Mean particle diameter	
		Acy-SLN	Efa-SLN
5000	1	1.848 (0.045)	1.465 (0.034)
5000	2	1.054 (0.022)	0.954 (0.023)
5000	3	0.788 (0.014)	0.654 (0.016)
5000	4	0.464 (0.012)	0.524 (0.008)
10000	1	1.264 (0.014)	1.164 (0.022)
10000	2	0.564 (0.009)	0.412 (0.009)
10000	**3**	**0.217 (0.016)**	**0.201 (0.015)**
10000	4	0.221 (0.006)	0.196 (0.008)
15000	1	0.812 (0.012)	0.746 (0.014)
15000	2	0.385 (0.009)	0.286 (0.009)
15000	3	0.216 (0.006)	0.198 (0.008)
15000	4	0.204 (0.007)	0.194 (0.009)

6.6.5. Optimization of drug loading:

It was observed drug loading does not having any significant affect on the particle size but have significant effect on the entrapment efficiency after certain concentration of drug.

6.6.5.1. Acyclovir:

The effect of drug load very slightly increases the particle size but drastically decreases the DEE after 20mg of drug loading. Particle size slightly increases to 264nm as compared to 238 when drug load was increased to 30mg from 20mg. But DEE was drastically decreases and it comes to 56.67% from 65.55% when drug load was increased from 20 to 30mg (Table 6.11). Upto 20mg of drug load, there was no substantial change in particle size or DEE which proves that drug can be loaded upto 20mg. Small increase in particle size proves that drug was solubilised into the lipid core which was surrounded by the surfactant layer. But decreasing DEE may be due to the less solubility of drug in the lipid phase. Drug was precipitate out or not entrapped into the lipid core. Similar to the earlier report, (Westesen K, Bunjes, 1997) the crystallization of the melted triglycerides very often causes drug expulsion from the lipid resulting in low drug entrapment. According to Westesen and

coworkers, (Westesen K, Bunjes et al, 1997) the drug-loading capacity of the lipid carriers is limited owing to the generally low solubilization capacity of the molten lipids for many poorly water-soluble drugs, thus implying that entrapment efficiency is dependent on the solubility of drug in the lipid portion.

Table 6.11: Effect of drug loading on the particle size (nm) and drug entrapment efficiency (%DEE)

Drug load	Acyclovir		Efavirenz	
(mg)	Particle size ± SD	DEE ± SD	Particle size ± SD	DEE ± SD
10	217 ± 16	70.7 ± 4.28	201 ± 15	97.4 ± 1.41
20	238 ± 09	65.55 ± 5.97	217 ± 08	98.08 ±2.65
30	264 ± 12	56.67 ± 3.52	262 ± 12	99.67 ± 1.72
40	ND	ND	265 ± 09	98.23 ± 1.61
50	ND	ND	250 ± 10	90.84 ± 3.69

6.6.5.2. Efavirenz:

It was also observed from the Table 6.11 that loading of efavirenz was neither altered the particle size nor DEE significantly. It was also observed that upto 40mg of efavirenz loading DEE was found satisfactory (98.23%) but further increase of drug load decreased DEE significantly (90.84%). According to Westesen and coworkers, (Westesen K, Bunjes, 1997) the drug-loading capacity of the lipid carriers is limited owing to the generally low solubilization capacity of the molten lipids for many poorly water-soluble drugs, thus implying that entrapment efficiency is dependent on the solubility of drug in the lipid portion. But in case of efavirenz, the higher DEE may be due to the high solubility of efavirenz into the lipid core and mixture of Polaxomer 188 and SDC. In this case, the drug was particularly associated with the polaxomer portion and the expulsion of drug due to modified crystallization is unlikely. According to Bunjes and coworkers (Bunjes H, Drechsler et al, 2001) the crystallization habits of tristearin nanoparticles also vary with the quantity of drug incorporated. May be the above-described differences are responsible for the observed higher entrapment efficiencies of efavirenz nanoparticles. However, we did not perform the recrystallization studies of these nanoparticles and may need further such investigations to support the maximum entrapment efficiency of drug observed in our case.

So from the above observations, further characterization of SLN was carried out keeping drug load at 20mg and 40mg for acyclovir and efavirenz respectively.

6.6.6. Differential Scanning Calorimetry (DSC)

6.6.6.1. Acyclovir:

The DSC heating and cooling curve of acyclovir (Figure 6.8.) showed a melting endotherm for the drug at 215°C. This peak was absent in the thermo-rams of acyclovir baded SLN (Figure 6.11). This clearly indicated that the drug is present in the amorphous state after entrapment within the SLN, as the melting of the drug is not observed as a distinct, sharp transition. Also the melting point of the lipid had increased from 57°C to 60°C by the incorporation of the drug due to the increased lattice defects resulting from drug incorporation, which in turn reduced the degree of crystallinity.

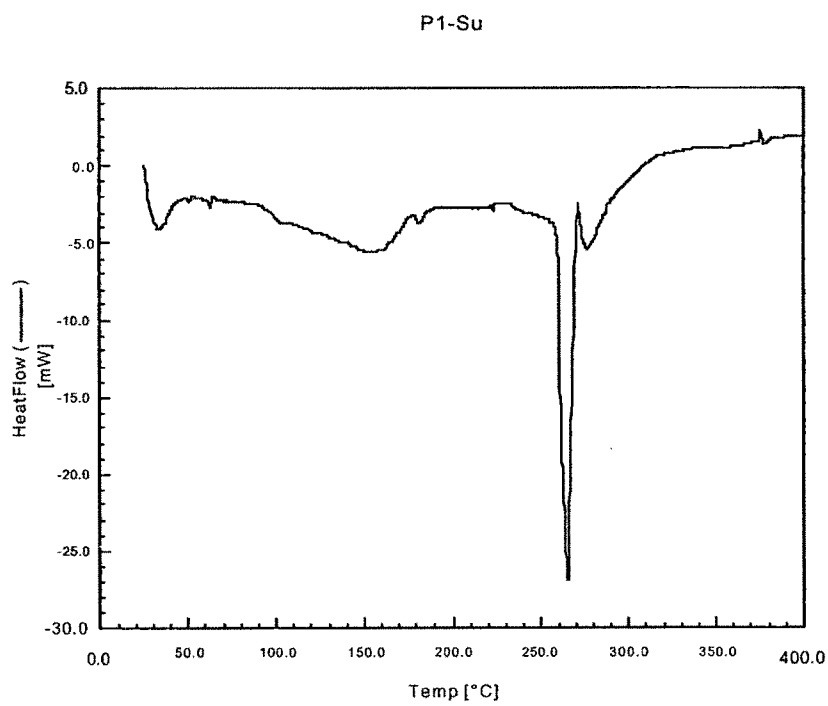


Figure 6.8: DSC thermogram of pure acyclovir

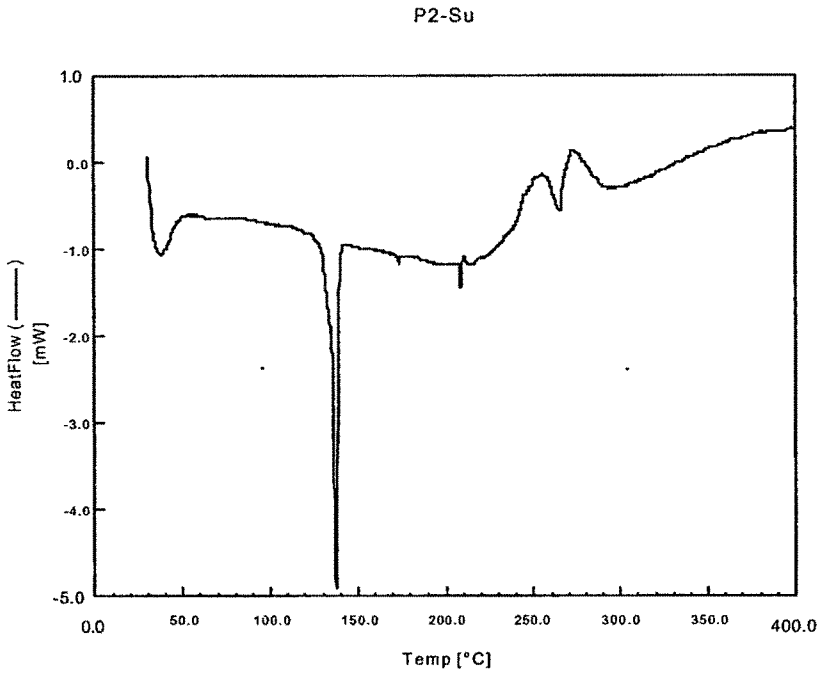


Figure 6.9: DSC thermogram of pure efavirenz

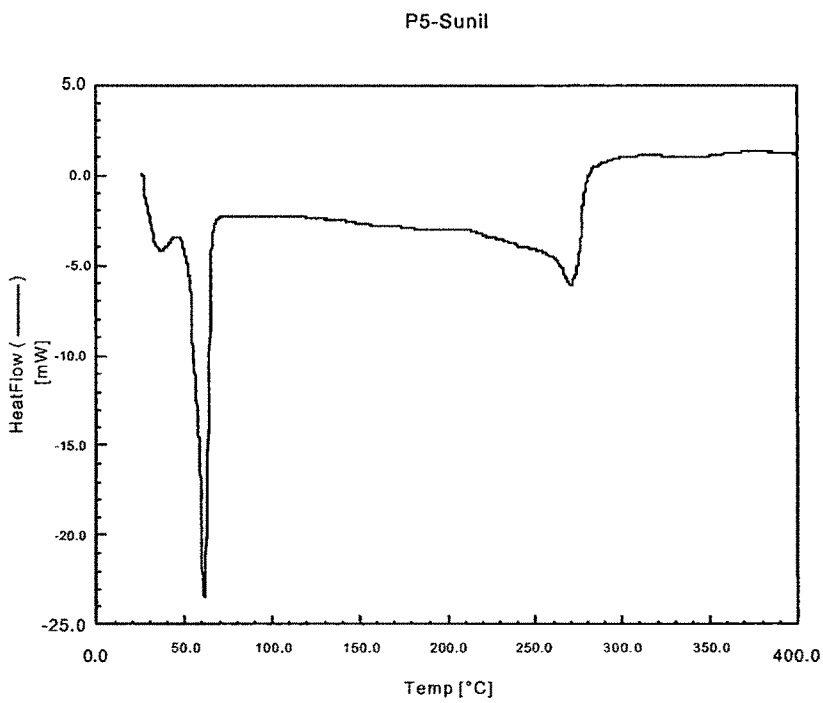


Figure 6.10: DSC thermogram lipid (GDS)

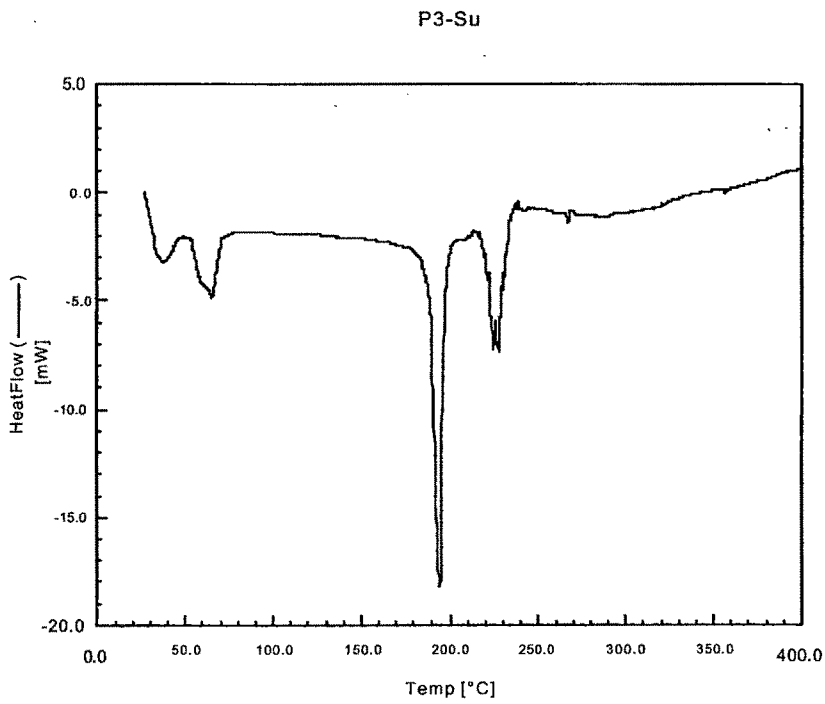


Figure 6.11: DSC thermogram of acyclovir loaded SLN

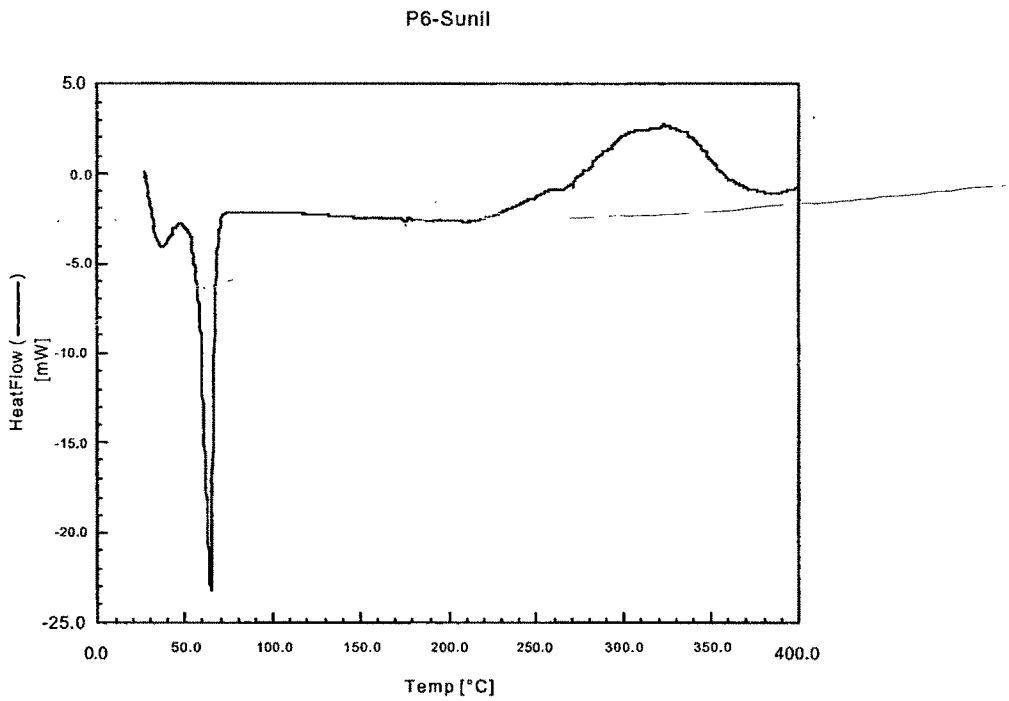


Figure 6.12: DSC thermogram of lipid (GTS)

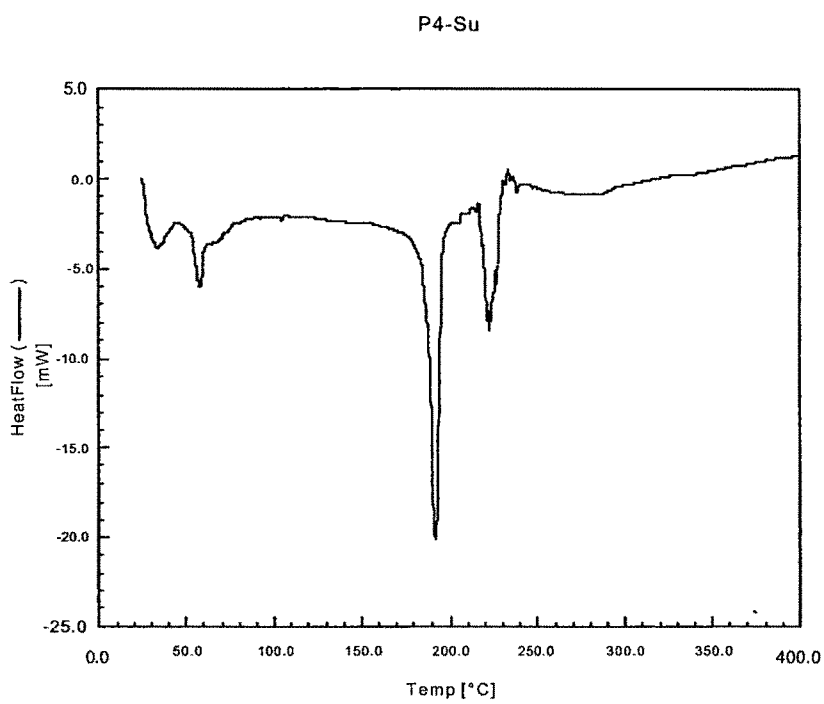


Figure 6.13: DSC thermogram of efavirenz loaded SLN

6.6.6.2. Efavirenz

The DSC heating and cooling curve of efavirenz (Figure 6.9.) showed a melting endotherm for the drug at 140°C. But in efavirenz loaded SLN, the similar peak was absent (Figure 6.11). This clearly indicated that the drug is present in the amorphous state after entrapment within the SLN, as the melting of the drug is not observed as a distinct, sharp transition. Also the melting point of the lipid had increased from 57°C to 60°C by the incorporation of the drug due to the increased lattice defects resulting from drug incorporation, which in turn reduced the degree of crystallinity.

6.6.7. Scanning electron microscopy (SEM)

It was found that the SLN had a smooth surface and were spherical in shape. Also, there was a complete absence of any other colloidal species like liposome or micelles. According to Westesen and Siekmann (Westesen K et al., 1997) the lipids prefer to crystallize in the platelet form; hence the shape of nanoparticles very often differs from a spherical form. Similar observation was observed for Acy-SLN-opt but for Efa-SLN-opt, the SEM of spray-dried nanoparticles shows spherical nature (Figure 6.14 and Figure 6.15). The differences in the results may be attributed to the differences in composition used for the nanoparticle preparation.

The Efa-SLN contains 5% w/w lipid content stabilized by 3% w/w of 1:1 mixture of SDC and polaxomer 188. Increase in the lipid content from 5% to 10% has been reported to cause the formation of particles with broader distribution including microparticles also. (Siekmann B et al., 1994) Bunjes et al reported the differences in crystallization of GTS when surfactants of different type and chain lengths were used. (Bunjes et al 2002) These differences were attributed to the influence of head group and chain lengths of surfactants on the crystallization temperature of GTS. Incorporation of lactose for spray drying of nanoparticle dispersion may also be responsible for the formation of spherical nanoparticles due to their surface coverage by lactose.

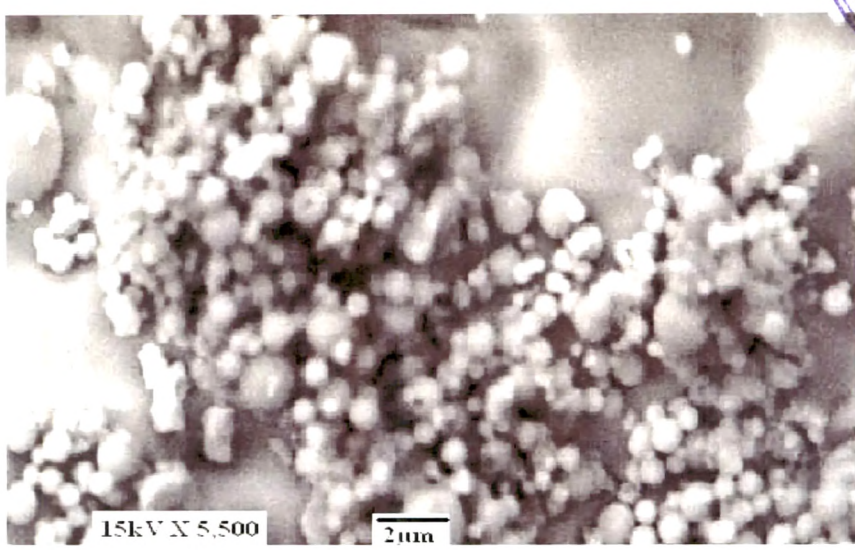


Figure 6.14: Scanning Electron Micrograph of acyclovir loaded SLN (Acy-SLN-opt)

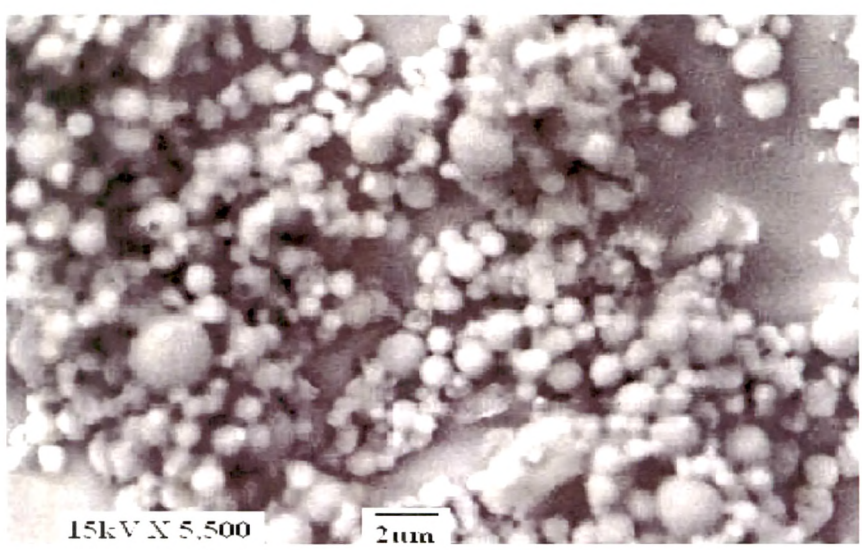


Figure 6.15: Scanning Electron Micrograph of efavirenz loaded SLN.(Efa-SLN-opt)

6.6.8. Zeta Potential Measurement

SLN are stabilizing not only by forming a mechanical barrier of lipid and surfactants but also by producing an electrical (electrostatic) barrier or surface charge. The electrical surface charge of the droplets is produced by the ionization of interfacial film-forming components. The surface potential

and the resulting zeta-potential of emulsion droplets will depend on the extent of ionization of surfactants. (S. Benita et al 1986) Summary of zeta potential was presented in Table 6.12. All the SLN formulation shows a negative surface charge when dispersion was made in water. It was also shown that zeta potential value of Acy- SLN-opt was -45.5mV, when dispersion was made in water. But dispersion in 0.1N HCl shows a positive zeta potential and which was 13.9mV. But in case of Efa-SLN=opt, zeta potential value was -20.5mV in aqueous dispersion and 11.5mV in 0.1N HCl dispersion where combination of SDS and Polaxomer was used as surfactants.

The SLN dispersion in 0.1N HCl showed high positive surface charge which suggests the possibility of better adherence to the intestinal mucosa which is negatively charged. This may also increase the bioavailability of poorly absorbable drugs incorporated in it, by increasing the residence time into the stomach as well as in intestine. (T. Gershanik et al 1998)

Table 6.12: Summary of zeta potential of different SLN dispersion in water and in 0.1N HCl

Batch No	Zeta Potential (mV) ± S.D	
	Water	0.1N HCl
Acy-SLN-opt	-45.5 ± 2.4	13.9 ± 2.1
Efa-SLN-opt	-20.5 ± 2.1	11.5 ± 2.8

6.6.9. In-vitro diffusion:

6.6.9.1. Acyclovir:

From Table 6.13 and figure 6.16 it was clear that all the SLN formulation having some initial burst release followed by sustained release. But for Acy-SLN01 and Acy-SLN04, maximum drug was released in 4hour, and then substantial quantity of drug not diffused further. It can be postulating that; acyclovir was entrapped on the surface of the SLN rather than depositing in the core. Similar observation was also made by several researchers, where they found initial burst release is due to presence of the drug enriched shell outside the SLN. (A. Zur et al., 1998) (R.H. Muller et al, 1994). But in case of Acy-SLN07 and Acy-SLN-opt, after initial burst, there was sustained release of the drug. Acy-SLN-opt show a gradual increase of percentage diffusion. After 24hour of diffusion study 67.95% of acyclovir gets diffused in this case. Here the acyclovir was located at the SLN core which was surrounded by lipid layer. Percentage cumulative drug released vs vt curve shows a straight line with regression coefficient (R^2) (Table 6.16) greater than 0.94, suggesting that SLN formulation follows Higuchi kinetics. Table 6.16 summarizes the mean flux rate (J) and permeation

constant (P) of all the SLN formulation. Formulations exhibited higher flux value (J) as well as higher permeation coefficient (P) across the membrane compared to the other formulation, which may be due to either higher release rate from the formulation or higher retention of the drug inside the membrane resulting in lower concentration in the receptor compartment following absorption. Formulation Acy-SLN-opt shows higher flux rate and higher permeation constant compare to the other SLN formulation.

Table 6.13: In-vitro diffusion of acyclovir from SLN

	% Diffusion			
Time (Hr)	Acy-SLN01	Acy-SLN04	Acy-SLN06	Acy-SLN-opt
0.5	17.71	18.8	26.69	21.12
1	18.86	19.07	36.73	22.44
2	22.17	22.97	43.79	23.08
3	42.45	43.4	42.62	24.78
4	56.45	60.14	45.17	28.76
6	59.47	58.54	46.32	33.12
8	62.24	58.19	48.46	36.32
12	62.20	60.84	52.13	43.95
24	61.24	63.8	60.93	67.95

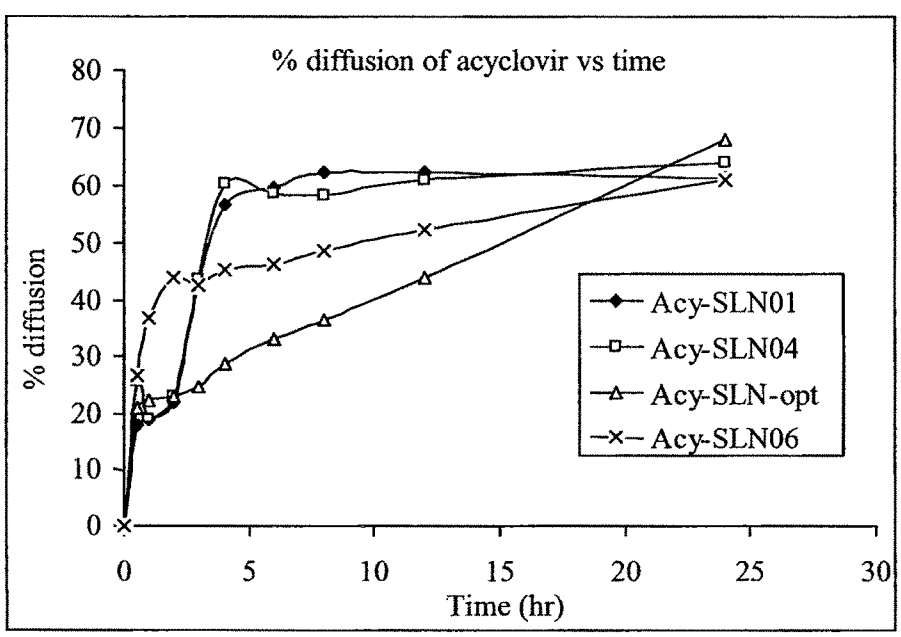


Figure 6.16: In-vitro diffusion of acyclovir from SLN formulations.

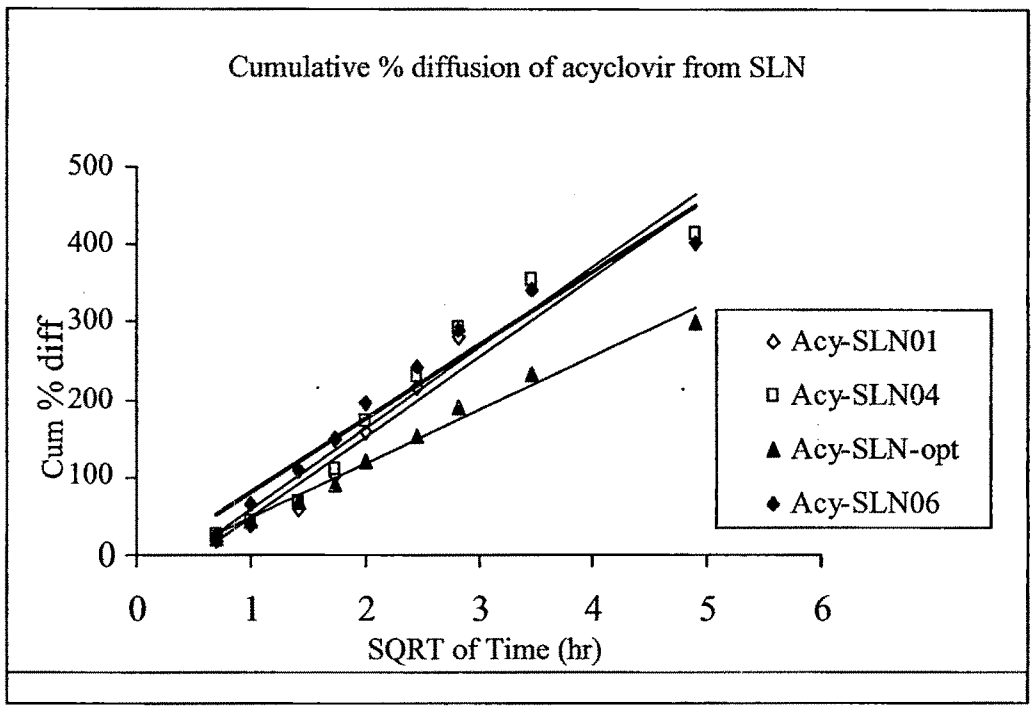


Figure 6.17: Cumulative diffusion of acyclovir from different SLN formulation

Table 6.14: Various diffusion parameter of acyclovir through dialysis bag

Batch No	J(μg cm^{-2} min^{-1})	P (cm min^{-1})	R^2 of Higuchi equation.
Acy-SLN01	9.4901	0.00949	0.9504
Acy-SLN04	5.8183	0.003094	0.9487
Acy-SLN06	12.032	0.012032	0.9514
Acy-SLN-opt	**15.381**	**0.015381**	**0.9861**

6.6.9.2. Efavirenz:

From Table 6.15 and Figure 6.18 it was clear that all the SLN formulations having some initial burst release followed by sustained release, similar to acyclovir SLN. Unlike acyclo vir SLN, in the entire cases drug gets sustained release through out the study. It can be postulating that; efavirenz was entrapped on the core of the SLN which was further surrounded by surfactant layer rather than adhered to the surface of SLN. It was also observed from the Table 6.15 that, % diffusion of drug hindered by increasing the lipid concentration irrespective of the type and concentration of surfactant. As in case Ef -SLN 04 and Ef-SLN06; after 24 hour of diffusion study 89.05% of drugs

gets released from Efa-SLN04 (lipid concentration was 4%) in compare to 37.54% from Efa-SLN-06 (lipid concentration ws 5%).

Percentage cumulatie drug released vs. vt curve shows a straight line with regression coefficient (R^2) (Table 6.16) greter than 0.98, suggesting that SLN formulation follows Higuchi kinetics. Table 6.16 summarizs the mean flux rate (J) and permeation constant (P) of all the SLN formulation. Formulations exhibited higher flux value (J) as well as higher permeation coefficient (P) across the membrane compared to the other formulation, which may be due to either higher release rate from the formulation or higher retention of the drug inside the membrane resulting in lower concentration in the receptor compartment following absorption. Formulation Efa-SLN-opt showing higher flux rate and higher permeation constant compare to other formulation.

Table 6.15: *In-vitro* diffusion of efavirenz from different SLN formulations.

Time (hr)	% Diffusion				
	Efa-SLN03	Efa-SLN04	Efa-SLN06	Efa-SLN08	Efa-SLN-opt
0.5	23.38	7.62	6.99	8.29	16.35
1	27.76	11.42	10.96	10.27	17.79
2	29.94	16.37	12.32	13.28	19.02
3	31.58	23.29	12.8	14.17	20.05
4	34.83	25.39	14.24	15.88	22.85
6	37.83	27.47	16.08	17.31	30.77
8	42.38	32.45	18.82	18.47	35.01
12	56.63	60.74	30.41	28.31	52.64
24	80.83	89.05	37.54	41.84	102.53

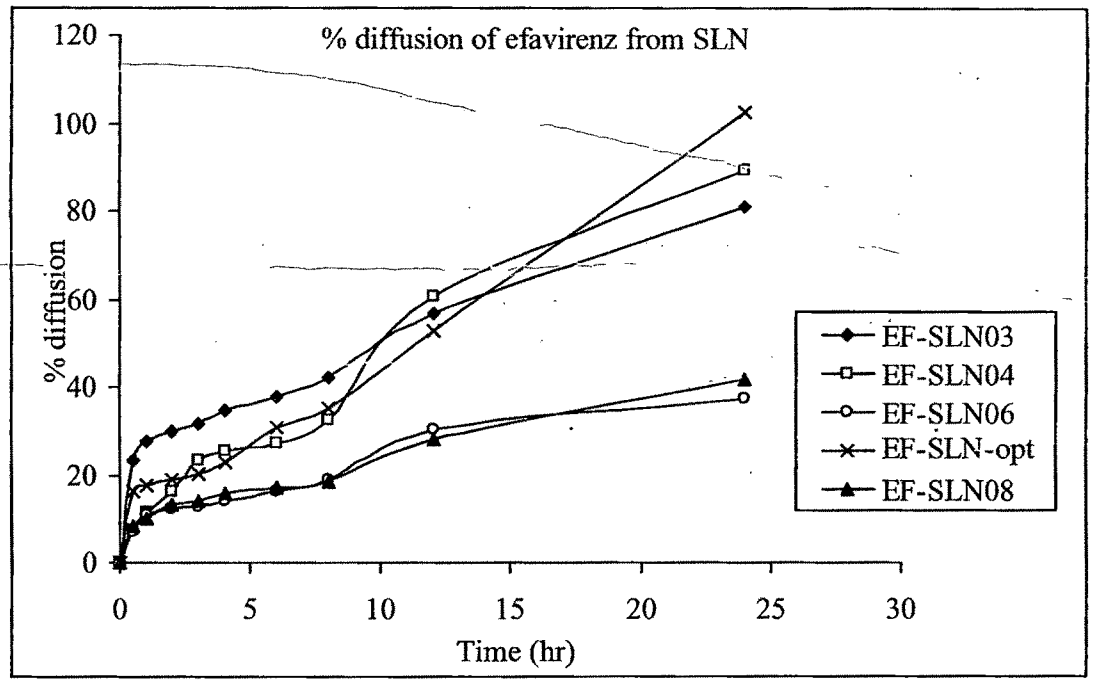


Figure 6.18: *In-vitro* diffusion of efavirenz from different SLN formulations.

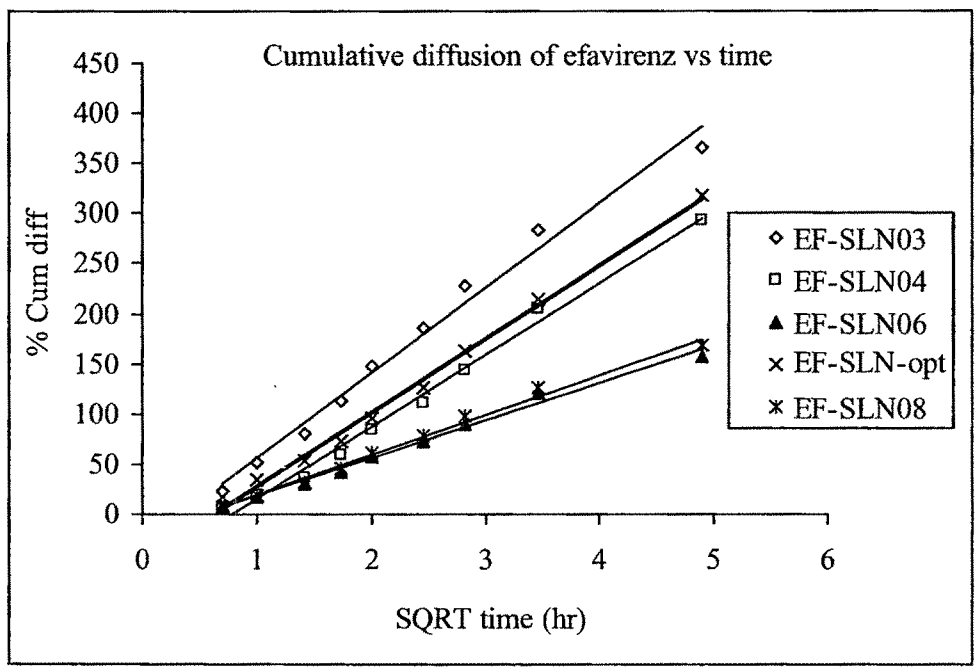


Figure 6.19: Cumulative diffusion of efavirenz from different SLN formulations

Table 6.16: Various diffusion parameter of efavirenz through dialysis bag

Batch No	J($\mu g cm^{-2} min^{-1}$)	P ($cm\ min^{-1}$)	R^2 of Higuchi equation.
Ef-SLN03	34796	0.002320	0.9861
Ef-SLN04	31019	0.002068	0.9916
Ef-SLN06	10843	0.001084	0.9916
Ef-SLN08	11216	0.001122	0.9918
Ef-SLN-opt	**13317**	**0.013317**	**0.9952**

6.6.10. Stability study:

After 6month of storage of at 4^0C, increase in size of SLN ranged from208 nm to 259nm for acyclovir and from 185 to 238nm for efavirenz. After similar period of storage, SLN at 25^0C (dark, amber color bottle) showed an increase of particle size from 208 to 302nm and from 185 to 312 nm for acyclovir and efavirenz respectively (Table 6.17). But when SLN was kept at 25^0C under normal daylight, after 1month of storage it showed a tremendous increase in particle size and which are deposited at the bottom of the glass vial. Particle sizes increased to 2.462µm and 3.245µm for acyclovir and efavirenz respectively. Drug entrapment efficiency of SLN was lowered by 1.2% & 3.4% for acyclovir and 0.6% &1.8% for efavirenz after 6months of storage at 4^0C and 25^0C (at dark) respectively. Drug entrapment efficiency was not carried out for sample storage for 1 month under daylight. Transitions of dispersed lipid from metastable forms to stable form might occur slowly on storage due to small particle size and the presence of emulsifier that may lead to drug expulsion from solid lipid nanoparticles (W. Mehnert et al., 2001) (K. Westesen et al., 1993) (H. Bunjes et al., 1995). Therefore lowered entrapment efficiency observed on storage may be due to drug expulsion during lipid modification.

Table 6.17: Effect of time of storage on particle size and drug entrapment (DE) of SLNs

Batch no	Storage condition	Particle size (nm)			%DE	
		Zero day	1month	6month	Zero day	6month
Acy-SLN-opt6	4^0C	208	222	259	69.5	68.3
Acy-SLN-opt6	25^0C, at dark	208	254	302	69.5	66.1
Acy-SLN-opt6	25^0C, at daylight	208	2462	ND	69.5	ND
Efa-SLN-opt2	4^0C	185	205	238	98.2	97.6
Efa-SLN-opt2	25^0C, at dark	185	232	312	98.2	96.4
Efa-SLN-opt2	25^0C, at daylight	185	3245	ND	98.2	ND

From the particle size measurements of SLN stored at different temperatures, it could be found that the particle agglomeration process was accelerated with increasing storage temperatures. This could be explained by the destabilization of physically critical SLN dispersions as a consequence of input of energy provided by the successively higher temperatures. This energy input increases the kinetic energy of the particles and favor collision of the particles.

Another reason that could be allocated to the increase in particle sizes is that the surfactant film on the particle surfaces might change its performance with temperature. There are constant changes in polymorphic modifications of the lipid particles with time, which results in an increase in the particle surface area due to preferred formation of platelet shaped particles characteristic of the β-modification. The surfactant molecules can no longer give sufficient coverage to the newer surfaces formed which results in particle growth. Thus it was concluded that the optimum temperature of storage of SLN dispersions of all the three lipids was 4°C

The particle size analysis of SLN dispersions stored under different light conditions indicated that storage of the dispersion in dark had minimum effect on particle agglomeration. Daylight caused particle growth in the lipid dispersions very rapidly. So the gelation process is obviously slower when the sample was kept in amber color bottle and protected from light. Storage of the dispersions in dark could not stop the gelation process completely. Slight increase of particle size was observed, but it was negligible in comparison to samples stored under daylight.

An explanation for such type of behavior is that light radiation has a destabilizing effect on SLN dispersions. Greater the intensity of light, faster the particle growth and gelation occurs. High energy radiations of the white light from the sun (UV radiations, short wavelengths) increase the kinetic energy of the particles in much the same way as high temperature does, which leads to a increased particle collision and growth. As the particles grow in size, the larger lipid particles can attract towards each other due to the loss of electrostatic repulsion and form a network like structure possibly promoted by the surfactant (i.e. gel formation properties of poloxamer, bridging).

From the stability study it may conclude that SLNs are stable for 6months only when preserved under refrigerator or in normal temperature (25^0C) protected for light.

References:

A. zur Muhlen, C. Schwarz, W. Mehnert, Solid lipid nanoparticles for controlled drug delivery—drug release and release mechanism, *Eur. J. Pharm. Biopharm.* 45 (1998) 149–155.

A. zur Muhlen, W. Mehnert, Drug release and release mechanism of prednisolone loaded solid lipid nanoparticles, *Pharmazie* 53 (1998) 552.

Ahlin P & Kristl J, Optimization of procedure parameters and physical stability of solid lipid nanoparticles in dispersions, Acta Pharm, 48 (1998) 257).

Bunjes H, Drechsler M, Koch MHJ, Westesen K. Incorporation of the model drug Ubidecarenone into the solid lipid nanoparticles. Pharm Res.2001;18:287-293.

Bunjes H, Koch MHJ, Westesen K. Effect of surfactants on the crystallization and polymorphism of lipid nanoparticles. Prog Colloid Polym Sci. 2002;121:7-10.

Bunjes H, Koch MHJ, Westesen K. Influence of emulsifiers on the crystallization of solid lipid nanoparticles. J Pharm Sci. 2003;92:1509- 1520.

Bunjes H, Koch MHJ, Westesen K. Effect of particle size on colloidal solid triglycerides. Langmuir. 2000;16:5234-5241.

Cavalli R, Caputo O, Carlotti ME, Trotta M, Scarnecchia C, Gasco MR. Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles. Int J Pharm. 1997;148:47-54.

Ceschel,G.C., Maffei,P., Moretti,M. D. L, Demontis, S., and Peana, A. T., In vitro permeation through porcine buccal mucosa of Salvia desoleana Atzei & Picci essential oil from topical formlations. *Int. J. Pharm.,* 195(2) :171-177,2000.

Freitas C, Muller RH. Spray-drying of solid lipid nanoparticles (SLN™). Eur J Pharm Biopharm. 1998;46:145-151.

H. Bunjes K. Westesen, Do nanoparticles prepared from lipids solids at room temperature always possess a solid lipid matrix? Int. J. Pharm. 115 (1995) 129– 131

K. Westesen, B. Siekmann, M.H.J. Koch, Investigations on the physical state of the lipid nanoparticles by synchrotron radiation X-ray diffraction, Int. J. Pharm. 93 (1993) 189– 199.

Lakkireddy Harivardhan Reddy, Rakesh Kumar Sharma, Krishna Chuttani, Anil Kumar Mishra, and Rayasa Ramachandra Murthy. Etoposide-incorporated Tripalmitin Nanoparticles With Different

Surface Charge: Formulation, Characterization, Radiolabeling, and Biodistribution Studies. The AAPS Journal 2004; 6 (3) Article 23

Lim SJ, Kim CK. Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-trans retinoic acid. Int J Pharm. 2002;243:135-146.

Mehnert W and Mader K, Solid lipid nanoparticles production, characterization and applications, Adv Drug Del Rev, 47 (2001) 165

Muller R H, Mader K & Gohla S, Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art, Eur J Pharm Biopharm, 50 (2000) 161

Mehnert W, Mader K. Solid lipid nanoparticles-production, characterization and applications. Adv Drug Del Rev. 2001;47:165-196.

Muller RH, Olbrich C. Solid lipid nanoparticles: Phagocytic uptake, in vitro cytotoxicity and in vitro biodegradation. Drugs Made Ger.1999;42: 49-53.

Magenheim B, Levy MY, Benita S. A new in vitro technique for evaluation of drug release profile from colloidal carriers-ultrafiltration technique at low pressure. Int J Pharm. 1993;94:115-123.

Muller RH, Ruhl D, Runge S, Schulze-Foster K, Mehnert W. Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant. Pharm Res. 1997;14:458-462.

Muller RH, Maassen S, Weyhers H, Mehnert W. Phagocytic uptake and cytotoxicity of solid lipid nanoparticles (SLN) sterically stabilized with Poloxamine 908 and Poloxamer 407. J Drug Target. 1996;4:161-170.

Mehnert W, Mader K. Solid lipid nanoparticles-production, characterization and applications. Adv Drug Del Rev. 2001;47:165-196.

Muller RH, Mader K, Gohla S. Solid lipid nanoparticles for controlled drug delivery-a review of the state of the art. Eur J Pharm Biopharm. 2000; 50: 161-177.

Penkler L, Muller RH, Runge SA, Ravelli V. Pharmaceutical cyclosporine formulation with improved biopharmaceutical properties, improved physical quality and greater stability, and method for producing said formulation. WO 99/56733. 1999.

R.H. Muller, C. Schwarz, A. zur Mu¨hlen, W. Mehnert, Incorporation of lipophilic drugs and drug release profiles of solid lipid nanoparticles (SLN), Proc. Int. Symp. Control. Rel. Bioact. Mater. 21 (1994) 146–147.

Siekmann B & Westensen K, Sub-micron sized parenteral carrier systems based on solid lipid, Pharm Pharmacol Lett, 1(1992)123

Schwarz C, Mehnert W. Solid lipid nanoparticles (SLN) for controlled drug delivery. II. Drug incorporation and physicochemical characterization. J Microencapsul. 1999;16:205-213.

Siekmann B, Westesen S. Thermoanalysis of the recrystallization process of melt-homogenized glyceride nanoparticles. Colloids Surf B Biointerfaces. 1994;3:159-175.

Siekmann B, Westesen K. Melt-homogenized solid lipid nanoparticles stabilized by the nonionic surfactant tyloxapol. I. Preparation and particle size determination. Pharm Pharmacol Lett. 1994;3:194-197.

S.Benita, D. Friedman, M. Weinstock, Int J Pharm 30(1986) 47-55.

T. Gershanik, S. Benzeno, S. Benita, Pharm. Res. 15 (1998) 863-869.

Taguchi G, Taguchi Method: Design of Experiment, Quality Engineering Series Vol 4 (American Supplier Institute Inc, Dearborn) edited by Konishi S, 1993.

Unruh T, Bunjes H, Westesen K, Koch MHJ. Investigations on the melting behaviour of triglyceride nanoparticles. Colloid Polym Sci. 2001;279:398-403.

Westesen K, Bunjes H, Koch MHJ. Physicochemical characterization of lipid nanoparticles and evaluation of their drug loading capacity and sustained release potential. J Control Release. 1997;48:223-236.

Westesen K, Siekmann B. Investigations on the gel formation of phospholipids stabilized solid lipid nanoparticles. Int J Pharm. 1997;151:35-45.

W. Mehnert, K. Mader, Solid lipid nanoparticles production, characterization and applications, Adv. Drug Deliv. Rev. 47 (2001) 165– 196.

Yang S, Zhu J, Lu Y, Liang B, Yang C. Body distribution of camptothecin solid lipid nanoparticles after oral administration. Pharm Res. 1999;16:751-757.

ZurMuhlen A, Feste Lipid-Nanopartikel mit prolongierter Wirkstoffliberation: Herstellung, Langzeitstabilitat, Charakterisierung, Freisetzungsverhalten und mechanismen, Ph.D. Thesis, Free University of Berlin,1996

Zur Muhlen A, Schwarz C, Mehnert W. Solid lipid nanoparticles (SLN) for controlled drug delivery-Drug release and release mechanism. Eur J Pharm Biopharm. 1998;45:149-155.

Zara GP, Cavalli R, Fundaro A, Bargoni A, Caputo O, Gasco MR.Pharmacokinetics of doxorubicin incorporated in solid lipid nanospheres (SLN). Pharm Res. 1999;40:281-286.

Zhang, H., and Robinson,J.R., In vitro methods for measuring permeability of the oral mucosa, in : Rathbone, M.J.(eds), Oral Mucosal Drug Delivery, Marcel Dekker, New York, pp. 90-93,1996.