## 4.1 Introduction

Emulsification-solvent evaporation technique was used for preparation of Darunavir loaded solid lipid nanoparticles (SLNs) and Atazanavir sulfate loaded SLNs. Both the drugs being lipophilic in nature were able to dissolve in organic solvent and subsequently homogenized into aqueous surfactant solution. The globule size of internal phase (organic solvent) determined the final particle size. The size of globules at this step is large so probe sonication was done to reduce the size followed by evaporation of organic solvent. As organic solvent evaporates, drug and lipid solidifies into particulate form.

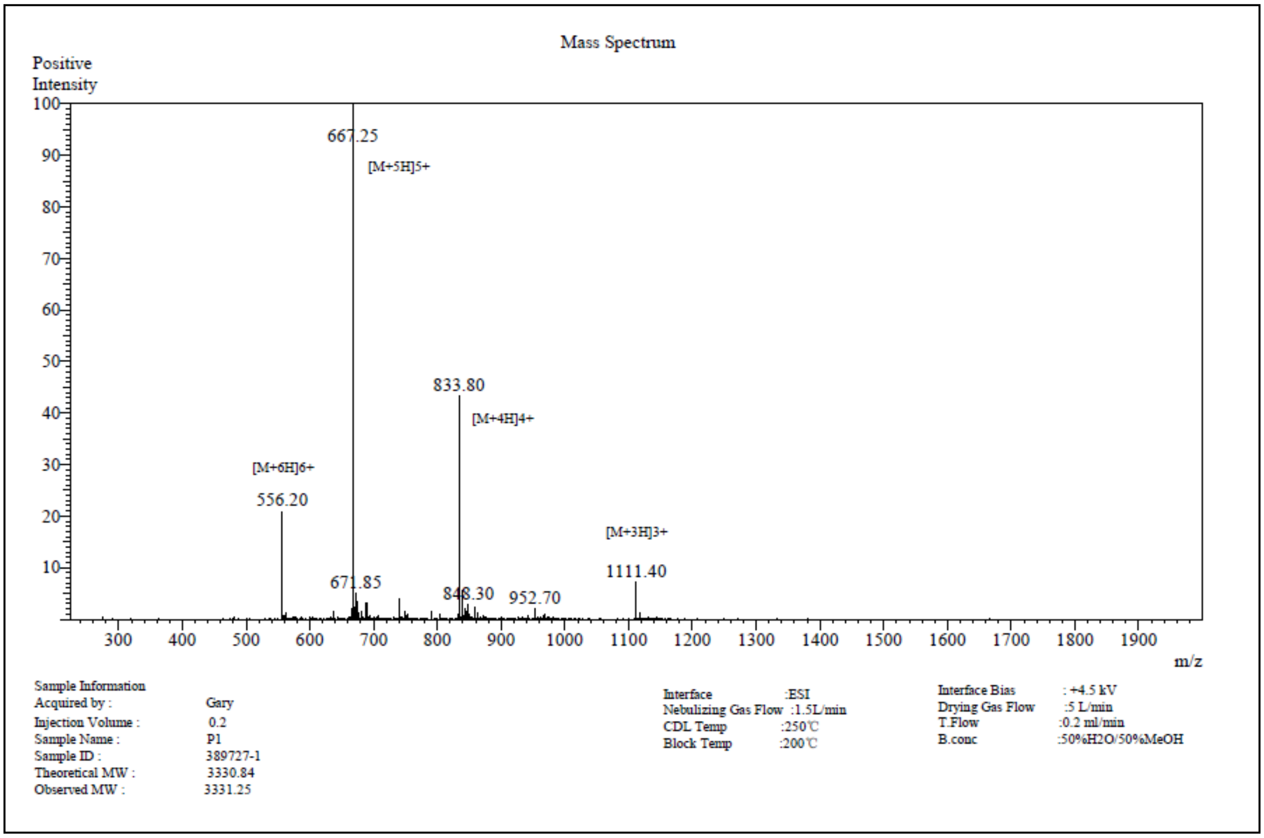
The melting point range of Darunavir was found to be 73-76ºC which was close to the melting point range of different solid lipids. Hence, the initial solubility study of drug which is usually done by melting of lipids and subsequent addition of drug was not possible. So, placebo nanoparticles were prepared during which various parameters were optimized including Ultraturrax speed, homogenization time, ratio of organic and aqueous phase, sonication cycle, suitable organic solvent and suitable surfactant. Using these optimized values of parameters for placebo nanoparticles, SLNs loaded with Darunavir and Atazanavir sulfate individually were formulated using various solid lipids. The suitable solid lipid was selected based on lipid giving greater entrapment of drug. High pressure homogenizer (HPH) was used for formulation since industrial production of SLNs is more feasible using this technique.

Three different sized nanoparticles (~100 nm, ~200 nm and ~500 nm) for Darunavir were formulated and the optimized size showing enhanced bioavailability was selected (based on results of *in-vivo* pharmacokinetic studies). The optimum sized SLNs of Darunavir was selected for ligand (peptide) grafting in order to enhance the binding of nanoparticles to the HIV host cells. The optimum size obtained for Darunavir was chosen to prepare Atazanavir sulfate loaded SLNs.

Nanoemulsion formulation of Darunavir was also prepared. Tween 80, a widely used surfactant is reported to inhibit P-gp efflux activity and Darunavir is a P-gp substrate. Hence, tween-80 was taken as surfactant for preparation of nanoemulsion. Oil was selected based on solubility of Darunavir tested in different oils.

### Selection of Peptide

Peptide sequence CARRPKFYRAPYVKNHPNVWGPWVAYGP was selected that is reported to bind selectively to CD4 molecules (1) and thus able to bind to the T-cells containing $CD4^+$ receptors. The peptide was obtained as synthesized by Genscript (Piscataway, NJ) limited. It is 28 AA with the molecular weight of 3331.25 Da and purity of 94.6 %. It was obtained as a white lyophilized powder. The mass spectrum of the peptide is shown in Figure 4.1.



Sample Information
Acquired by : Gary
Injection Volume : 0.2
Sample Name : P1
Sample ID : 389727-1
Theoretical MW : 3330.84
Observed MW : 3331.25

Interface :ESI
Nebulizing Gas Flow :1.5L/min
CDL Temp :250℃
Block Temp :200℃

Interface Bias : +4.5 kV
Drying Gas Flow :5 L/min
T.Flow :0.2 ml/min
B.conc :50%H2O/50%MeOH

**Figure 4.1 Mass spectrum of peptide**

## 4.2 Materials

Darunavir and Atazanavir sulfate were kindly gifted by Matrix laboratories, Hyderabad. Glyceryl behenate (Compritol® 888 ATO) and Glyceryl palmitostearate

(Precirol® ATO 5) were gifted by Gattefosse (Germany). Trimyristin (Dynasan® 114) and Tripalmitin (Dynasan® 116) were gifted by Sasol Germany GmbH (Hamburg, Germany). Hydrogenated castor oil (HCO) was obtained as gift sample from Meril Life Sciences Pvt. Ltd. (Gujarat, India). DSPE-Mal-m PEG 2000 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (poly ethylene glycol) 2000]) was purchased from Avanti polar lipids (Alabaster, AL). Peptide sequence CARRPKFYRAPYVKN HPNVWGPWVAYGP was obtained from Genscript (Piscataway, NJ). Pluronic F68 was obtained from Fischer scientific and polyvinyl alcohol, sodium hydroxide, oleic acid and tween 80 were purchased from S.D. Fine chem Ltd. (Mumbai, India). Dichloromethane, chloroform and methanol were purchased from spectrochem (Mumbai, India). Sucrose and trehalose dihydrate were obtained from Himedia Lab. (Mumbai, India). Soyabean oil was obtained from Fluka (Mumbai, India). Egg lecithin was gifted by Lipoid GmbH (Germany). Medium chain triglyceride (Miglyol 812) and Glyceryl Mono-, Di-, Tri-hexanoate (Imwitor 642) were obtained from Sasol Germany GmbH (Hamburg, Germany). Glyceryl monocaprylate (Capmul MCM), propylene glycol dicaprylocaprate (Captex 200) and Glycerol monooleates (Peceol) were obtained from Abitec corporation (USA). Caprylocaproyl macrogol-8 glycerides (Labrasol) and caprylic/capric triglyceride (Labrafac- lipophile WL 1349) was procured from Gattefosee (Mumbai, India). Distilled water was prepared in the laboratory. All other chemicals and reagents were of analytical grade and used without further purification.

## 4.3 Methods

### 4.3.1 Formulation development of Darunavir loaded solid lipid nanoparticles

#### 4.3.1.1 Formulation of placebo (without drug) solid lipid nanoparticles

Placebo solid lipid nanoparticles were prepared using emulsification-solvent evaporation technique with few modifications (2). Briefly, solid lipid (0.6 %w/v of aqueous phase) was dissolved in organic solvent and injected through needle in a beaker containing aqueous surfactant solution kept under homogenization (Ultraturrax, IKA T25). Upon homogenization, the emulsion formed was sonicated using probe sonicator (Labsonic®M, Sartorius) followed by evaporation of organic solvent at room temperature for 6 hr until complete evaporation. The schematic representation of the preparation of placebo solid lipid nanoparticles is shown in Figure 4.2.

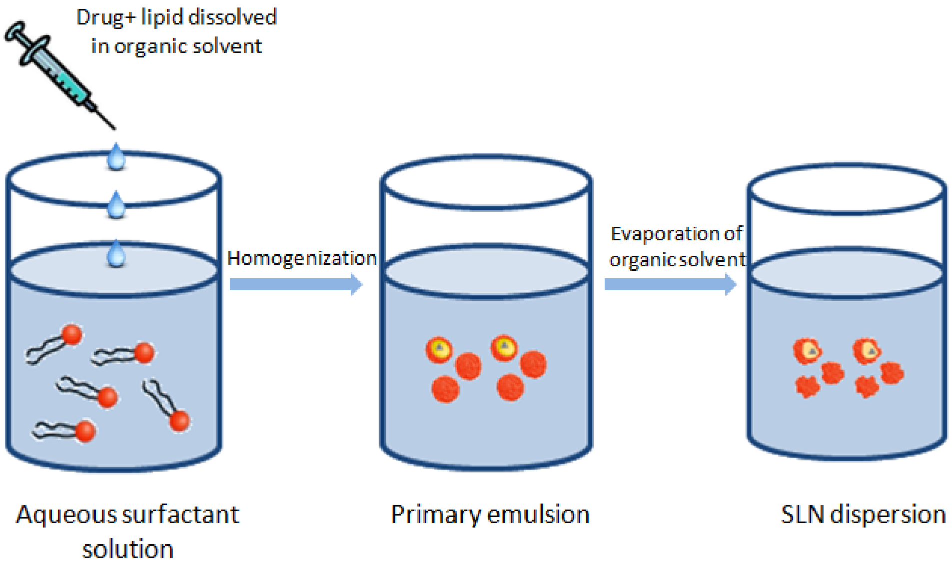


**Figure 4.2 Diagrammatic representation of preparation of solid lipid nanoparticles**

## Preliminary optimization of parameters:

In preliminary optimization, the possible parameters influencing the formulation of nanoparticles and its size were identified and optimized. The parameters studied were ultraturrax speed and homogenization time, ratio of aqueous and organic phase, sonication cycle, type of organic solvent and type of surfactant.

### a. Ultraturrax speed and homogenization time

Ultraturrax speed ranging from 5000-15000 rpm was taken with varying time (10-15 min). The constant parameters selected were glyceryl behenate as solid lipid, Pluronic F68 (1 %w/v) as surfactant, dichloromethane as organic solvent, 1:0.1 ratio of aqueous phase: organic phase and 0.7sec x 70% amplitude x 2 min sonication cycle. The particle size and polydispersibility index (PDI) for each batch was estimated using Malvern zetasizer.

### b. Ratio of aqueous and organic phase

The ratio of aqueous phase: organic phase was varied and the nanoparticles were prepared using the above procedure. The ultraturrax speed of 12 000 rpm and homogenization time of 15 min was kept constant based on their optimization. Other constant parameters were glyceryl behenate as solid lipid, pluronic F68 (1% w/v) as surfactant, dichloromethane as organic solvent and 0.7sec x 70% amplitude x 2 min sonication cycle. The particle size and PDI for each batch were measured using Malvern zetasizer.

**c. Sonication cycle**

Different batches were prepared using varying sonication cycle and the batches were measured for particle size and PDI. Optimized values of ultraturrax speed, homogenization cycle and ratio of aqueous phase: organic phase were taken. Dichloromethane was taken as organic solvent, glyceryl behenate as solid lipid and pluronic F68 (1 %w/v) as surfactant.

**d. Type of organic solvent**

Two solvents- dichloromethane and chloroform were taken as organic solvent and particle size and PDI were measured. Above optimized parameter values were taken as constant.

**e. Type of surfactant**

Three different types of surfactants- pluronic F68 (poloxamer 188), polyvinyl alcohol (PVA) and sodium oleate were taken. For above parameters, optimized values were used. Different batches were prepared using six different solid lipids in increasing concentration of each surfactant individually. Upon formulation of nanoparticles, initial visual observation was done to check aggregation of particles. The particle size and PDI were measured through Malvern zetasizer.

#### 4.3.1.2 Formulation of Darunavir loaded solid lipid nanoparticles

#### A) Formulation procedure:

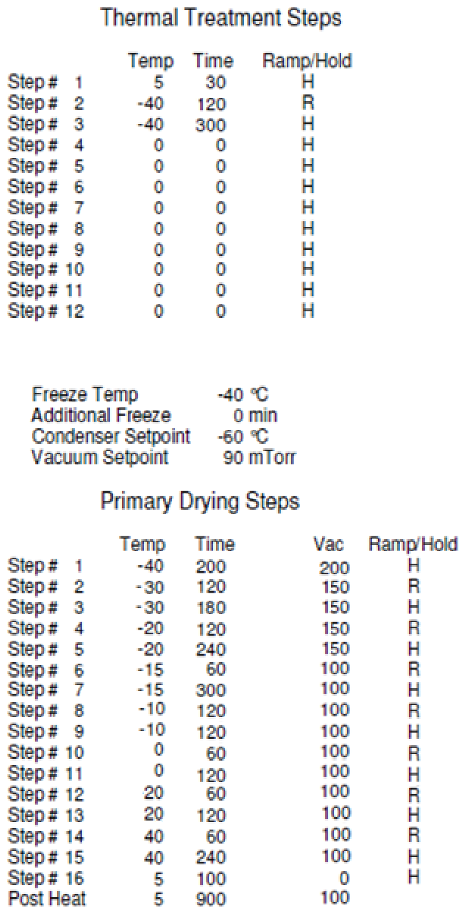
Darunavir loaded solid lipid nanoparticles were prepared by emulsification-solvent evaporation technique as done for placebo nanoparticles with few modifications. Briefly, Darunavir and various solid lipids were accurately weighed and dissolved in dichloromethane to form organic phase. Sodium oleate was added to distilled water to form aqueous phase (10 ml). Organic phase was homogenized into aqueous phase using homogenizer (Ultraturrax, IKA-T25) at 12 000 rpm for 15 min. Ratio of organic to aqueous phase was 1:10. The primary emulsion formed was then sonicated using probe sonicator at cycle of 0.7sec x 70% amplitude for 3 min followed by evaporation of organic solvent at room temperature for 6 hr until complete evaporation of solvent. The nanoparticles dispersion was centrifuged at 10 000 rpm, for 10 min at 4ºC using sigma centrifuge (3K30, Osterode, Germany) to settle the unentrapped drug and the supernatant containing nanoparticles was collected. Different batches were prepared using different solid lipids, increasing solid content (amount of drug and solid lipid per ml of aqueous suspension) and increasing concentration of sodium oleate. The particle size and PDI were measured and entrapment efficiency was estimated. The solid lipid that gave high drug entrapment and particle size less than 100 nm was selected. It is difficult to formulate nanoparticles have size ~100 nm in comparison to nanoparticles with size ~200 nm and ~500 nm. So, 100 nm size was selected at this stage for optimization.

### B) Scale up by high pressure homogenizer:

Further optimization was done through emulsification by ultraturrax followed by high pressure homogenization technique (3). Darunavir (80mg) and hydrogenated castor oil (HCO) were accurately weighed and dissolved in dichloromethane to form organic phase. Sodium oleate was added to distilled water to form aqueous phase (20ml). The organic phase was homogenized into aqueous phase using homogenizer at 12 000 rpm for 15 min. The primary emulsion so formed was passed through high pressure homogenizer (Emulsiflex C5, Avestin, Canada) in 3 cycles along with intermittent cooling in ice bath. First cycle was performed at 10 000 kpa while second and third cycle at 15 000 kpa. Organic solvent was evaporated at room temperature for 6 hr until complete evaporation. The nanoparticles suspension was centrifuged at 10 000 rpm for 10 min at 4ºC using sigma centrifuge to settle down unentrapped drug. The supernatant containing solid lipid nanoparticles was collected. Different batches were prepared using varying ratio of drug: lipid and surfactant concentration. For each batch particle size, PDI and entrapment efficiency were estimated.

### C) Lyophilization of optimized Darunavir loaded nanoparticles and optimization of cryoprotectant:

The optimized nanoparticles formulations (Dar-SLN1, Dar-SLN2 and Dar-SLN3) were lyophilized using Virtis lyophilizer (Virtis-Advantage plus, USA). Two cryoprotectants- sucrose and trehalose at different concentrations (30, 50 and 70 mg/ml of nanoparticles dispersion) were tried and the cryoprotectant which showed minimum increment in particle size was selected. 2 ml of nanoparticle dispersion + cryoprotectant were taken in 10 ml glass vials (Schott, USA), half stoppered with grey bromobutyl slotted rubber stoppers and kept on the shelf of the lyophilizer. The nanoparticles were frozen at -40ºC and dried under vacuum for various time periods to optimize the particle characteristics of final powder. Complete lyocycle describing freezing time, primary and secondary drying time, ramp and hold duration as well as vacuum level are given below in Figure 4.3. The lyophilized samples were then characterized for particle size, drug content and residual water content.



Thermal Treatment Steps

| | Temp | Time | Ramp/Hold |
|---|---|---|---|
| Step # 1 | 5 | 30 | H |
| Step # 2 | -40 | 120 | R |
| Step # 3 | -40 | 300 | H |
| Step # 4 | 0 | 0 | H |
| Step # 5 | 0 | 0 | H |
| Step # 6 | 0 | 0 | H |
| Step # 7 | 0 | 0 | H |
| Step # 8 | 0 | 0 | H |
| Step # 9 | 0 | 0 | H |
| Step # 10 | 0 | 0 | H |
| Step # 11 | 0 | 0 | H |
| Step # 12 | 0 | 0 | H |

| | |
|---|---|
| Freeze Temp | -40 °C |
| Additional Freeze | 0 min |
| Condenser Setpoint | -60 °C |
| Vacuum Setpoint | 90 mTorr |

Primary Drying Steps

| | Temp | Time | Vac | Ramp/Hold |
|---|---|---|---|---|
| Step # 1 | -40 | 200 | 200 | H |
| Step # 2 | -30 | 120 | 150 | R |
| Step # 3 | -30 | 180 | 150 | H |
| Step # 4 | -20 | 120 | 150 | R |
| Step # 5 | -20 | 240 | 150 | H |
| Step # 6 | -15 | 60 | 100 | R |
| Step # 7 | -15 | 300 | 100 | H |
| Step # 8 | -10 | 120 | 100 | R |
| Step # 9 | -10 | 120 | 100 | H |
| Step # 10 | 0 | 60 | 100 | R |
| Step # 11 | 0 | 120 | 100 | H |
| Step # 12 | 20 | 60 | 100 | R |
| Step # 13 | 20 | 120 | 100 | H |
| Step # 14 | 40 | 60 | 100 | R |
| Step # 15 | 40 | 240 | 100 | H |
| Step # 16 | 5 | 100 | 0 | H |
| Post Heat | 5 | 900 | 100 | |

**Figure 4.3 Thermal cycle for freeze drying**

### 4.3.1.3 Evaluation parameters

a. Particle size and particle size distribution (PDI)

The particle size of each sample was determined using Malvern zetasizer (Nano ZS, malvern instrument, UK). The samples were diluted 10 times with filtered distilled water to avoid multi-scattering phenomena and placed in disposable sizing cuvette. Each determination was repeated in triplicate and the results were expressed as mean value ± SD (standard deviation). The lyophilized samples were rehydrated with distilled water and diluted appropriately before analysis.

b. % Entrapment efficiency (EE)

The nanoparticle formulations were filled in cellulose dialysis bag (MWCO 12000 Da, Sigma, Germany) and kept for dialysis on magnetic stirrer (50 rpm; Remi, Mumbai, India) against double distilled water under sink conditions for 10 min to remove any unentrapped drug from the formulation (4). After dialysis, the nanoparticles were dissolved using methanol: dichloromethane (7:3) solvent mixture, diluted suitably and analyzed by developed UV method (chapter 3). % EE was calculated using following formulae:

$$\% EE = \frac{amount\ of\ entrapped\ drug}{total\ amount\ of\ drug\ added}\ x\ 100$$

c. Residual water content

The residual water content of lyophilized formulations was estimated using Karl-Fischer titration method (5) in which pyridine free reagent was standardized with addition and determination of known quantity of water (250 mg). Firstly, 40 ml of anhydrous methanol was added into the titration vessel and titrated with reagent to remove any residual water in methanol. Following this, samples were added and water content was determined by titrating slowly with reagent.

d. Drug content estimation of lyophilized samples

For estimation of drug content, the rehydrated nanoparticles were dissolved in methanol: dichloromethane (7:3) and amount of drug present was estimated using developed UV method in chapter 3. Percentage drug content was calculated using following formula.

$$\%\, drug\ content = \frac{amount\ of\ drug\ estimated\ after\ lyophilization}{Initial\ drug\ present\ before\ lyophilization}\ x\ 100$$

### 4.3.2 Formulation development of peptide grafted solid lipid nanoparticles of Darunavir

From the *in-vivo* pharmacokinetics results, Dar-SLN2 was obtained as the optimized batch (having particle size ~200 nm). Hence, this formulation was selected for peptide grafting in order to enhance binding of nanoparticles to HIV host cells. Peptide that has been reported to selectively bind to CD4 molecules was selected. DSPE-Mal-m PEG 2000 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (poly ethylene glycol) 2000]) contain a maleimide linker attached to the head group of the lipid, which allows for covalent binding to antibodies or peptides with a terminal cysteine amino acid (of peptide) to form thioether linkage. Covalent coupling of lipid to peptide occur optimally at pH 6.5.

#### 4.3.2.1 Formulation procedure

The peptide was grafted to the surface of nanoparticles by covalent coupling through thioether linkage (6). The optimized formulation Dar-SLN2 was prepared. DSPE-Mal-m PEG was taken in organic phase during preparation. Then, for peptide coupling to preformed SLNs, 100µg of peptide and SLNs equivalent to 1 µM of lipid (molecular weight of lipid: 939.50) were combined in 100 µL of buffer (0.9% NaCl, 10mM $NaHCO_3$) at pH 6.5 at room temperature and were agitated for 8 hr in an incubator (Obritek, Scigenics, India) at 50 rpm. To remove any unbound peptide, nanoparticles were dialyzed against 1L of buffer. The binding of peptide to SLNs is illustrated in Figure 4.4. The peptides get coupled to nanoparticles at pH 6.5 through covalent binding of cysteine SH to the maleimide on the lipid, forming a stable thioether linkage. The formulation was lyophilized using Virtis lyophilizer as per procedure mentioned in section 4.3.1.2. Trehalose at 50 mg/ml of the nanoparticles dispersion was added as cryoprotectant.

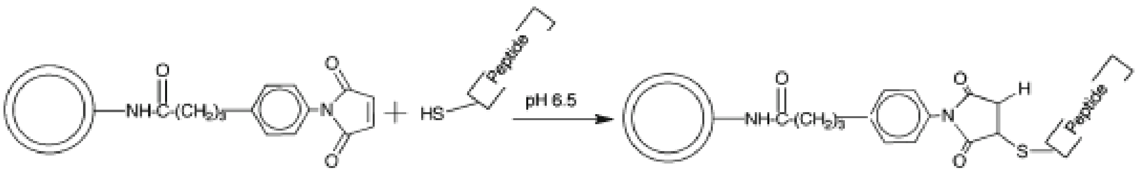


**Figure 4.4 Schematic representation of peptide coupling to nanoparticles**

### 4.3.3 Formulation development of Nanoemulsion of Darunavir

#### 4.3.3.1 Assessment of solubility of Darunavir in different oils and surfactant

The solubility of Darunavir in different oils and surfactant was determined by shake flask method (7). An excess amount of Darunavir was added to 1.5 ml of each sample in an eppendorf and shaken at 100 rpm at 25°C for 24 hr. The samples were then centrifuged at 10 000 rpm for 10 min to settle undissolved drug. An aliquot of the supernatant (filtered by membrane filter 0.45 µm) was properly diluted with methanol: DCM (7:3) and Darunavir concentration was assayed spectrophotometrically.

#### 4.3.3.2 Formulation of nanoemulsion of Darunavir

Nanoemulsion of Darunavir was prepared using high pressure homogenization technique (8). Aqueous phase was prepared by dispersing egg lecithin in distilled water with continuous stirring. Organic phase was prepared by adding tween 80 into soyabean oil. The drug was dissolved in this oil and surfactant mixture with shaking.

The aqueous phase and oil phase was heated at 55-60°C with stirring. Oil phase was homogenized into aqueous phase using homogenizer (Ultraturrax, IKA-T23) at 8000 rpm for 20 min at 55-60°C. The emulsion was cooled to 20-30°C and the volume was made up to 25ml with distilled water. Emulsion was transferred to high pressure homogenization tank and temperature was maintained between 20-25°C during passes through high pressure homogenizer. Emulsion was passed from HPH at 15 000 psi during 1st and 20 000 psi during 3-5 passes. The various batches prepared with varying concentration of tween 80 are given below in Table 4.1.

**Table 4.1 Composition of various batches of nanoemulsion of Darunavir**

| Ingredient | Composition (% w/v) | | | | |
|---|---|---|---|---|---|
| | **DNE-1** | **DNE-2** | **DNE-3** | **DNE-4** | **DNE-5** |
| Darunavir (% w/v) | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| Soyabean oil (% w/v) | 8 | 8 | 8 | 8 | 8 |
| Tween 80 (% w/v) | 0.5 | 0.75 | 1 | 1.25 | 1.5 |
| Egg lecithin (% w/v) | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 |
| Distilled water | q.s | q.s | q.s | q.s | q.s |

#### 4.3.3.3 Evaluation of nanoemulsion of Darunavir

The following parameters were evaluated for each batch in order to optimize the formulation (9).

a. Globule size, PDI and zeta potential: Globule size, PDI and zeta potential of nanoemulsion formulation was done by Malvern zetasizer after proper dilution.
b. Total drug content and % entrapment efficiency: For total drug content, aliquot of formulation was dissolved in methanol: dichloromethane (7:3) and analyzed by UV spectrophotometry using developed calibration plot. For % EE, the nanoemulsions were centrifuged at 5 000 rpm for 10 min (to remove free drug) and supernatant analyzed by UV spectrophotometry. The nanoemulsion formulations were filled in cellulose dialysis bag (MWCO 12000 Da, Sigma, Germany) and dialyzed with magnetic stirring (50 rpm; Remi, Mumbai, India) against double distilled water under sink conditions for 10 min to remove any unentrapped drug from the formulation (4). After dialysis, the nanoemulsion was dissolved using methanol: dichloromethane (7:3) solvent mixture, diluted suitably and analyzed by developed UV method (chapter 3). EE was calculated using following formulae.

$$\% EE = \frac{amount\ of\ entrapped\ drug}{total\ amount\ of\ drug\ added}\ x\ 100$$

c. Centrifugal stress: 1 ml aliquot of nanoemulsion was centrifuged at 15 000 rpm for 10 min. The creaming volume was calculated as follows:

Creaming volume (C) = 100 * (Vt-Vs)/ Vt
Vt: total volume of sample
Vs: Volume of lower phase layer

d. Short term stability: Short term stability was done at 4°C and 25°C of various formulation batches for optimization. The study was done over 30 days with intermittent checking of the various parameters. The nanoemulsions were visually observed for creaming, coalescence, phase separation and/or precipitation. Globule size and zeta potential were also measured.

### 4.3.4 Formulation development of peptide grafted solid lipid nanoparticles of Atazanavir sulfate

#### 4.3.4.1 Formulation of solid lipid nanoparticles of Atazanavir sulfate

Optimized values of different process parameters like ultraturrax speed and time, sonication cycle etc. which were optimized during formulation of placebo nanoparticles (section 4.3.1.1) were used in following procedure.

**Formulation procedure for SLNs of Atazanavir sulfate:**

Atazanazavir sulfate (ATZ) loaded solid lipid nanoparticles were prepared by emulsification-solvent evaporation technique with few modifications (2). Briefly, ATZ (5 mg) and various solid lipids were accurately weighed and dissolved in dichloromethane (1 ml) to form organic phase. Sodium oleate was added to distilled water to form aqueous phase (10 ml). Organic phase was homogenized into aqueous phase using homogenizer (Ultraturrax, IKA-T25) at 12 000 rpm for 15 min. Ratio of organic to aqueous phase was 1:10. The primary emulsion formed was then sonicated using probe sonicator at cycle of 0.7sec x 70% amplitude for 3 min followed by evaporation of organic solvent at room temperature for 6 hr until complete evaporation of solvent. The nanoparticles suspension was centrifuged at 10 000 rpm, 10 min at 4ºC using sigma centrifuge (3K30, osterode, Germany) to settle the unentrapped drug and the supernatant containing nanoparticles was collected. Different batches were prepared using different solid lipids, increasing solid content (amount of drug and solid lipid per ml of aqueous suspension) and increasing concentration of sodium oleate. The particle size and PDI were measured and entrapment efficiency was estimated. The solid lipid that gave high drug entrapment was selected.

### Scale up by high pressure homogenizer:

ATZ (5mg) and hydrogenated castor oil (HCO) were accurately weighed and dissolved in dichloromethane to form organic phase. Sodium oleate was added to distilled water to form aqueous phase (10ml). The organic phase was homogenized into aqueous phase using homogenizer at 12 000 rpm for 15 min. The primary emulsion so formed was passed through high pressure homogenizer (Emulsiflex C5, Avestin, Canada) in 2 cycles along with intermittent cooling in ice bath. First cycle was performed at 10 000 kpa while second at 15 000 kpa. Organic solvent was evaporated until complete evaporation. The nanoparticles suspension was centrifuged at 10 000 rpm for 10 min at 4ºC using sigma centrifuge to settle down unentrapped drug. The supernatant containing solid lipid nanoparticles was collected. Different batches were prepared using varying ratio of drug: lipid and surfactant concentration. For each batch, particle size, PDI and entrapment efficiency were estimated.

### Lyophilization of optimized ATZ loaded nanoparticles and optimization of cryoprotectant:

The optimized nanoparticles formulation (ALN-23) was lyophilized using virtis lyophilizer (Virtis-Advantage plus, USA). Two cryoprotectants- sucrose and trehalose at different concentrations (30, 50 and 70 mg/ml of nanoparticles suspension) were tried and the cryoprotectant which showed minimum increment in particle size was selected. 2 ml of nanoparticle suspensions were taken in 10 ml glass vials, half stoppered with grey bromobutyl slotted rubber stoppers and kept on the shelf of the lyophilizer. The nanoparticles were frozen at -40ºC and dried under vacuum for various time periods to optimize the particle characteristics of final powder. Complete lyocycle describing freezing time, primary and secondary drying time, ramp and hold duration as well as vacuum level were as per lyophilization of Darunavir loaded nanoparticles (section

4.3.1.2). The lyophilized samples were then characterized for particle size, drug content and moisture content as per procedure mentioned in same section.

### 4.3.4.2 Grafting of peptide to optimized SLNs of Atazanavir sulfate

The peptide (sequence CARRPKFYRAPYVKNHPNVWGPWVAYGP) that selectively binds to HIV host CD4$^+$ T cells was conjugated to developed solid lipid nanoparticles of ATZ (ALN-23). The peptide was attached as per procedure mentioned in section 4.3.2.1 and lyophilized using Vertis lyophilizer. Trehalose (60 mg/ml) was added as cryoprotectant. After ligand attachment the formulation was characterized for particle size, zeta potential, qualitative and quantitative estimation of bound peptide to nanoparticles.

## 4.4 Results and discussion

### 4.4.1 Formulation development of Darunavir loaded solid lipid nanoparticles

### 4.4.1.1 Formulation of placebo (without drug) solid lipid nanoparticles

The objective was to formulate three different sized nanoparticles- about 100 nm, about 200 nm and about 500 nm. Hence, initial aim was to formulate nanoparticles with size less than 100 nm which is in fact difficult to prepare as compared to other two sizes. For this, the following process parameters and formulation parameters were initially optimized such that desirable particle size near to 100 nm was obtainable.

#### a. Ultraturrax speed and homogenization time

Emulsification is one of the key factors in the process of nanoparticles preparation because an insufficient dispersion of phases results in larger particle with wide size distribution. The final size of nanoparticles depends on the globule size in entire emulsification process. Thus, the effect of change in speed and time of ultraturrax in the preparation of placebo solid lipid nanoparticles was investigated and results obtained are given in Table 4.2. It can be seen that on increasing speed of ultraturrax up to 12 000 rpm and 15 min of homogenization time, there was significant decrease in particle size. PDI also decreased significantly. Further increase in speed of ultraturrax led to increase in particle size. Homogenization time dictates the total energy input to the preparation process. The medium value of duration appears to be advantageous because prolonged duration would lead to cubosome destruction (10). Thus, 12 000 rpm as speed of ultraturrax and 15 min time for homogenization was selected as optimized value.

**Table 4.2 Effect of ultraturrax speed and homogenization on particle size and PDI of placebo nanoparticles**

| Batch no. | Ultraturrax speed (rpm) | Homogenization time (min) | Particle size (nm) | PDI |
|---|---|---|---|---|
| BN-1 | 5000 | 10 | 444 ± 6.12 | 0.278 ± 0.03 |
| BN-2 | 10 000 | 10 | 388 ± 3.14 | 0.152 ± 0.04 |
| BN-3 | 10 000 | 15 | 368 ± 4.15 | 0.148 ± 0.02 |
| **BN-4** | **12 000** | **15** | **353 ± 2.12** | **0.111 ± 0.06** |
| BN-5 | 15 000 | 10 | 444 ± 4.14 | 0.258 ± 0.05 |

*Values are represented as mean ± SD, n=3.

**b. Ratio of aqueous and organic phase**

Ratio of aqueous and organic phases was varied to optimize their influence on particle size of nanoparticles. The results are shown in Table 4.3. Least particle size of 353 nm was found when ratio of two phases was 1: 0.1 (aqueous: organic phase). As the amount of organic phase is increased compared to aqueous phase, the particle size was increased with greater PDI possibly due to higher agglomeration of globules in a larger space. Thus, this ratio was selected and used for further formulation.

**Table 4.3 Effect of ratio of aqueous phase: organic phase on particle size and PDI of placebo nanoparticles**

| Batch no. | Aqueous phase : Organic ratio | Particle size (nm) | PDI |
|---|---|---|---|
| **BN-6** | **1: 0.1** | **353 ± 4.14** | **0.111 ± 0.02** |
| BN-7 | 1: 0.15 | 411 ± 5.16 | 0.155 ± 0.03 |
| BN-8 | 1: 0.2 | 423 ± 4.15 | 0.215 ± 0.05 |

*Values are represented as mean ± SD, n=3.

### c. Sonication cycle

Sonication cycle was found to greatly influence size of nanoparticles. The results (Table 4.4) shows that an increase in sonication amplitude or time (up to 3 min sonication at 0.7 sec, 70 % amplitude) led to reduction in particle size which might be due to high energy waves leading to rapid dispersion of organic phase (containing solid lipid) into aqueous phase resulting into smaller globule size of emulsion that is responsible for final particle size of nanoparticles. However, an optimal sonication cycle of 0.7 sec, 70 % amplitude and 3 min gave least particle size and further increase in time led to increase particle size probably due to re-aggregation because of extremely high kinetic energy. Similar results were reported by Siddiqui et. al (11) indicating that an optimal value of sonication cycle is required for desired particle size. Hence, an optimum value of cycle as in batch BN-12 was selected as final sonication cycle for use in further formulation development.

**Table 4.4 Effect of sonication cycle on particle size and PDI of placebo nanoparticles**

| **Batch no.** | **Sonication cycle (sec x amplitude x min)** | **Particle size (nm)** | **PDI** |
|---|---|---|---|
| BN- 9 | 0.6 x 60 x 2 | 391 ± 3.13 | 0.132 ± 0.042 |
| BN-10 | 0.6 x 60 x 4 | 380 ± 3.14 | 0.158 ± 0.03 |
| BN-11 | 0.7 x 70 x 2 | 353 ± 4.14 | 0.111 ± 0.02 |
| **BN-12** | **0.7 x 70 x 3** | **322 ± 2.12** | **0.156 ± 0.03** |
| BN-13 | 0.7 x 70 x 5 | 415 ± 3.18 | 0.432 ± 0.05 |

*Values are represented as mean ± SD, n=3.

### d. Type of organic solvent

Suitable organic solvent was selected on the basis of particle size and PDI. Two organic solvents- dichloromethane and chloroform were screened since both these solvents were able to dissolve Darunavir and solid lipid. The results are shown in

Table 4.5. There was insignificant difference (P>0.5) in particle sizes upon use of either chloroform or dichloromethane. However, chloroform is reported to be more toxic and volatile (12) compared to dichloromethane and hence later was selected as final organic solvent for further formulation development.

**Table 4.5 Effect of type of organic solvent on particle size and PDI of placebo nanoparticles**

| **Batch no.** | **Organic solvent** | **Particle size (nm)** | **PDI** |
|---|---|---|---|
| BN-14 | Chloroform | 345 ± 2.15 | 0.232 ± 0.04 |
| **BN-15** | **Dichloromethane** | **322 ± 2.14** | **0.156 ± 0.05** |

*Values are represented as mean ± SD, n=3.

### e. Type of surfactant

Before lipid selection, appropriate surfactant that gives minimum particle size (near to 100 nm) was chosen since preparation of 100 nm particles is more difficult in comparison to bigger particles. For this three different surfactants were tried for formulation of placebo solid lipid nanoparticles. Keeping the concentration of glyceryl behenate constant, the amount of surfactant was increased gradually to study their effect on particle size. The results obtained are shown in Table 4.6, Table 4.7 and Table 4.8. The concentration of surfactant that gives smaller particles in glyceryl behenate nanoparticles was selected for other solid lipids in order to compare particle size at the same surfactant concentration. With the use of Pluronic F68 (Table 4.6) and polyvinyl alcohol (Table 4.7), the particle sizes of nanoparticles were in range of 262-432 nm and 337-502 nm respectively in various solid lipids used. Use of sodium oleate as surfactant resulted in lesser particle sizes (138-229 nm) with all the solid lipids in comparison to other two surfactants. The use of polyvinyl alcohol (PVA) led to higher particle size in comparison to pluronic F68 and sodium oleate irrespective of the type of solid lipid used. A study done by Kwon et al. indicated that increase in PVA concentration increases the viscosity of aqueous solution that results in decreased net shear stress and subsequent increase in particle size (13). Nanoparticles prepared using sodium oleate as stabilizer gave smaller particles in comparison to other two surfactants and hence it was selected as surfactant for further optimization.

**Table 4.6 Effect of pluronic F68 on particle size and PDI of solid lipid nanoparticles**

| **Batch no.** | **Glyceryl behenate concentration (% w/v)** | **Pluronic F68 concentration (% w/v)** | **Initial observation for aggregation (+ presence, - absence)** | **Particle size (nm)** | **PDI** |
|---|---|---|---|---|---|
| BN-16 | 0.6 | 0.2 | + | - | - |
| BN-17 | 0.6 | 0.4 | + | - | - |
| BN-18 | 0.6 | 0.8 | - | 312 ± 1.89 | 0.345±0.02 |
| BN-19 | 0.6 | 1.2 | - | 350 ± 2.19 | 0.324±0.06 |
| BN-20 | 0.6 | 1.6 | - | 305 ± 2.12 | 0.301±0.05 |
| BN-21 | 0.6 | 2.0 | - | 265 ± 1.88 | 0.124±0.03 |
| | **Solid lipid (0.6 % w/v), pluronic F68 (2 % w/v)** | | | | |
| BN-22 | Glyceryl palmitostearate | | | 380 ± 3.14 | 0.230±0.06 |
| BN-23 | Hydrogenated castor oil | | | 262 ± 2.18 | 0.143±0.03 |
| BN-24 | Trimyristin | | | 283 ± 2.12 | 0.189±0.02 |
| BN-25 | Tripalmitin | | | 432 ± 3.18 | 0.323±0.06 |

*Values are represented as mean ± SD, n=3.

**Table 4.7 Effect of polyvinyl alcohol on particle size and PDI of solid lipid nanoparticles**

| **Batch no.** | **Glyceryl behenate concentration (% w/v)** | **Polyvinyl alcohol concentration (% w/v)** | **Initial observation for aggregation (+ presence, -** | **Particle size (nm)** | **PDI** |
|---|---|---|---|---|---|

| | | | absence) | | |
|---|---|---|---|---|---|
| BN-26 | 0.6 | 0.1 | + | 521 ± 6.16 | 0.601±0.01 |
| BN-27 | 0.6 | 0.2 | - | 380 ± 3.12 | 0.344±0.03 |
| BN-28 | 0.6 | 0.3 | - | 637 ± 4.15 | 0.432±0.06 |
| BN-29 | 0.6 | 0.4 | - | 502 ± 4.18 | 0.274±0.03 |
| | **Solid lipid (0.6 % w/v), PVA (0.2 % w/v)** | | | | |
| BN-30 | Glyceryl palmitostearate | | | 412 ± 3.14 | 0.300±0.01 |
| BN-31 | Hydrogenated castor oil | | | 337 ± 2.15 | 0.332±0.02 |
| BN-32 | Trimyristin | | | 375 ± 2.16 | 0.305± 0.02 |
| BN-33 | Tripalmitin | | | 382 ± 2.16 | 0.350±0.04 |

*Values are represented as mean ± SD, n=3.

**Table 4.8 Effect of sodium oleate on particle size and PDI of solid lipid nanoparticles**

| **Batch no.** | **Glyceryl behenate concentration (% w/v)** | **Sodium oleate concentration (% v/v)** | **Initial observation for aggregation (+ presence, - absence)** | **Particle size (nm)** | **PDI** |
|---|---|---|---|---|---|
| BN-34 | 0.6 | 0.2 | + | - | - |
| BN-35 | 0.6 | 0.4 | + | - | - |
| BN-36 | 0.6 | 0.6 | - | 245 ± 1.14 | 0.212±0.01 |
| BN-37 | 0.6 | 0.7 | - | 238 ± 1.25 | 0.220±0.01 |
| BN-38 | 0.6 | 0.8 | - | 232 ± 1.34 | 0.232±0.06 |
| BN-39 | 0.6 | 0.9 | - | 210 ± 2.21 | 0.210±0.03 |
| | **Solid lipid (0.6 % w/v), sodium oleate (0.6 % v/v)** | | | | |

| | | | |
|---|---|---|---|
| BN-40 | Glyceryl palmitostearate | 229 ± 2.18 | 0.250±0.06 |
| BN-41 | Hydrogenated castor oil | 164 ± 2.15 | 0.236±0.03 |
| BN-42 | Trimyristin | 138 ± 3.12 | 0.177±0.09 |
| BN-43 | Tripalmitin | 163 ± 3.16 | 0.137±0.08 |

*Values are represented as mean ± SD, n=3.

#### 4.4.1.2 Formulation of Darunavir loaded solid lipid nanoparticles

Darunavir loaded SLNs were formulated using various solid lipids to select the suitable lipid. Drug: lipid ratio was varied in order to study the effect of concentration of various lipids on drug entrapment into nanoparticles. The results are shown in Table 4.9. It can be seen that on increasing lipid concentration, there was increase in drug entrapment irrespective of the type of solid lipid. This is because of the availability of higher amount of lipid to form matrix and entrap the drug. Similarly, on increasing surfactant concentration (DLN-3 and DLN-4), the particle size of nanoparticles decreased significantly due to availability of higher surfactant molecules on nanoparticles surface and hence more stabilization of nanoparticles in the aqueous medium. On increasing solid content (drug + lipid), the dispersion viscosity also increases that result into higher surface tension and thus larger particles were observed. Highest entrapment (up to 90 %) was observed when hydrogenated castor oil (HCO) was taken as solid lipid and particle size less than 100 nm was also obtained with the use of same lipid. Hence, HCO was selected as final solid lipid.

**Table 4.9 Effect of various formulation parameters on particle size, PDI and drug entrapment efficiency of Darunavir loaded solid lipid nanoparticles**

| Batch no. | Drug: lipid ratio | Total solid content (drug + lipid) (mg) | Concentration of sodium oleate (%v/v) | Particle size (nm) | PDI | Entrapment efficiency (%) |
|---|---|---|---|---|---|---|
| Glyceryl behenate | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| DLN-1 | 1:2 | 30 | 0.7 | 148 ± 1.18 | 0.390 ± 0.01 | 58.12 ± 2.15 |
| DLN-2 | 1:2 | 48 | 0.7 | 181 ± 1.16 | 0.305 ± 0.01 | 59.23 ± 2.34 |
| DLN-3 | 1:4 | 50 | 0.7 | 161 ± 2.15 | 0.300 ± 0.03 | 74.23 ± 3.18 |
| DLN-4 | 1:4 | 50 | 1 | 135 ± 2.14 | 0.270 ± 0.04 | 74.90 ± 2.14 |
| DLN-5 | 1:6 | 70 | 1.0 | 180 ± 3.15 | 0.249 ± 0.02 | 76.14 ± 1.15 |
| DLN-6 | 1:6 | 70 | 1.2 | 163 ± 3.58 | 0.243 ± 0.02 | 76.51 ± 1.01 |
| Glyceryl palmitostearate | | | | | | |
| DLN-7 | 1:2 | 30 | 0.7 | 151 ± 2.10 | 0.106 ± 0.03 | 58.14 ± 2.18 |
| DLN-8 | 1:4 | 50 | 1.0 | 142 ± 2.15 | 0.132 ± 0.02 | 65.10 ± 2.21 |
| DLN-9 | 1:6 | 70 | 1.2 | 134 ± 3.11 | 0.143 ± 0.02 | 72.32 ± 3.13 |
| **Hydrogenated castor oil** | | | | | | |
| DLN-10 | 1:2 | 30 | 0.7 | 131 ± 2.15 | 0.125 ± 0.03 | 64.6 ± 2.15 |
| DLN-11 | 1:4 | 50 | 1.0 | 99 ± 1.14 | 0.171 ± 0.02 | 89.72 ± 2.18 |
| DLN-12 | 1:6 | 70 | 1.2 | 126 ± 2.18 | 0.149 ±0.02 | 91.4 ± 2.42 |
| Trimyristin | | | | | | |
| DLN-13 | 1:2 | 30 | 0.7 | 122 ± 3.12 | 0.216 ± 0.03 | 68.10 ± 3.12 |
| DLN-14 | 1:4 | 50 | 1.0 | 122 ± 2.18 | 0.173 ± 0.04 | 72.15 ± 2.10 |
| DLN-15 | 1:6 | 70 | 1.2 | 115 ± 2.25 | 0.137 ± 0.08 | 74.21 ± 1.01 |
| Tripalmitin | | | | | | |
| DLN-16 | 1:2 | 30 | 0.7 | 104 ± 2.18 | 0.183 ± 0.03 | 42.25 ± 3.12 |
| DLN-17 | 1:4 | 50 | 1.0 | 128 ± 2.10 | 0.148 ± 0.04 | 54.10 ± 3.10 |
| DLN-18 | 1:6 | 70 | 1.2 | 136 ± 2.15 | 0.160 ± 0.03 | 60.52 ± 2.12 |

*Values are represented as mean ± SD, n=3.

**Scale up by high pressure homogenizer (HPH):**

Upon selection of HCO as lipid, further optimization of lipid and surfactant concentration was done along with the scale up of the formulation by high pressure homogenizer. Various batches were prepared using this technique by varying drug: lipid ratio and surfactant concentration. Preliminary batches using HPH technique inferred a requirement of 3%- 4% v/v sodium oleate concentrations since the drug loading was also increased in comparison to earlier batches during selection of lipid. The results obtained are shown in Table 4.10.

**Table 4.10 Effect of lipid and surfactant concentration on particle size, PDI and drug entrapment efficiency of Darunavir loaded solid lipid nanoparticles**

| Batch no. | Drug: lipid ratio | Concentration of sodium oleate (%v/v) | Particle size (nm) | PDI | Entrapment efficiency (%) |
|---|---|---|---|---|---|
| DLN-19 | 1:4 | 3 | 89.99 ± 4.22 | 0.156 ± 0.11 | 74.89 ± 3.1 |
| DLN-20 | 1:4 | 3.5 | 69.94 ± 3.12 | 0.205 ± 0.14 | 89.33 ± 3.3 |
| DLN-21 | 1:4 | 4 | 78.07 ± 4.16 | 0.209 ±0.13 | 89.79 ± 4.1 |
| DLN-22 | 1:5 | 3 | 100.3 ± 3.75 | 0.208 ± 0.03 | 81.21 ± 2.3 |
| DLN-23 | 1:5 | 3.5 | 78.68 ± 3.71 | 0.189 ± 0.11 | 85.26 ± 1.5 |
| DLN-24 | 1:5 | 4 | 81.16 ± 4.33 | 0.210 ± 0.07 | 86.32 ± 2.4 |
| DLN-25 | 1:6 | 3 | 115 ± 3.10 | 0.228 ± 0.04 | 88.18 ± 1.6 |
| **DLN-26** | **1:6** | **3.5** | **93.28 ± 3.62** | **0.191 ± 0.10** | **89.09 ± 2.4** |
| DLN-27 | 1:6 | 4 | 94.98 ± 4.63 | 0.241 ± 0.13 | 90.15 ± 1.6 |

*Values are represented as mean ± SD, n=3.

High drug entrapment of up to 90% was obtained in batches having 1:6 drug: lipid ratio (DLN-25, DLN-26 and DLN-27). Particle size less than 100 nm was observed in batches with sodium oleate concentration of 3.5 and 4 % v/v. Lower surfactant concentration batch DLN-26 was selected as final formulation (Dar-SLN1) having particle size less than 100 nm. This formulation was obtained upon 3 cycles of HPH. Further upon decreasing the number of cycles to two and one, formulations having particle size near to 200 nm (Dar-SLN2) and 500 nm (Dar-SLN3) were obtained. Thus, the results of all three final formulations in terms of particle size and drug entrapment are given in Table 4.11.

**Table 4.11 Final optimized Darunavir loaded solid lipid nanoparticles**

| **Batch no.** | **Composition (% of aqueous phase)** | **Parameter** | **Particle size (nm)** | **PDI** | **Entrapment efficiency (%)** |
|---|---|---|---|---|---|
| DLN-26 **(Dar-SLN1)** | Darunavir- 0.4% w/v<br>HCO- 2.4 % w/v<br>Sodium oleate- 3.5% v/v<br>Distilled water- q.s. (upto 20 ml) | 3 cycle of HPH | 93.28 ± 3.62 | 0.191 ± 0.10 | 89.09 ± 2.4 |
| DLN-28 **(Dar-SLN2)** | | 2 cycles of HPH | 189.45 ± 2.10 | 0.115 ± 0.024 | 90.10 ± 1.15 |
| DLN-29 **(Dar-SLN3)** | | 1 cycle of HP | 527.62 ± 2.15 | 0.114 ± 0.03 | 88.13 ± 2.26 |

*Values are represented as mean ± SD, n=3.

**Lyophilization of optimized Darunavir loaded nanoparticles and optimization of cryoprotectant:**

**Freeze drying (lyophilization) is most suitable way of removing water from nanoparticles suspension. Cryoprotectants are required to be added during this process to keep nanoparticles deaggregated. Hence, two cryoprotectants (sucrose and trehalose) were added at different ratios and optimum concentration of cryoprotectant which showed minimum increase in particle size of nanoparticle formulation was determined. Figure 4.5 shows the thermal kinetic of products in comparison with shelf during the cycle of lyophilization. The results are shown in**

Table 4.12.

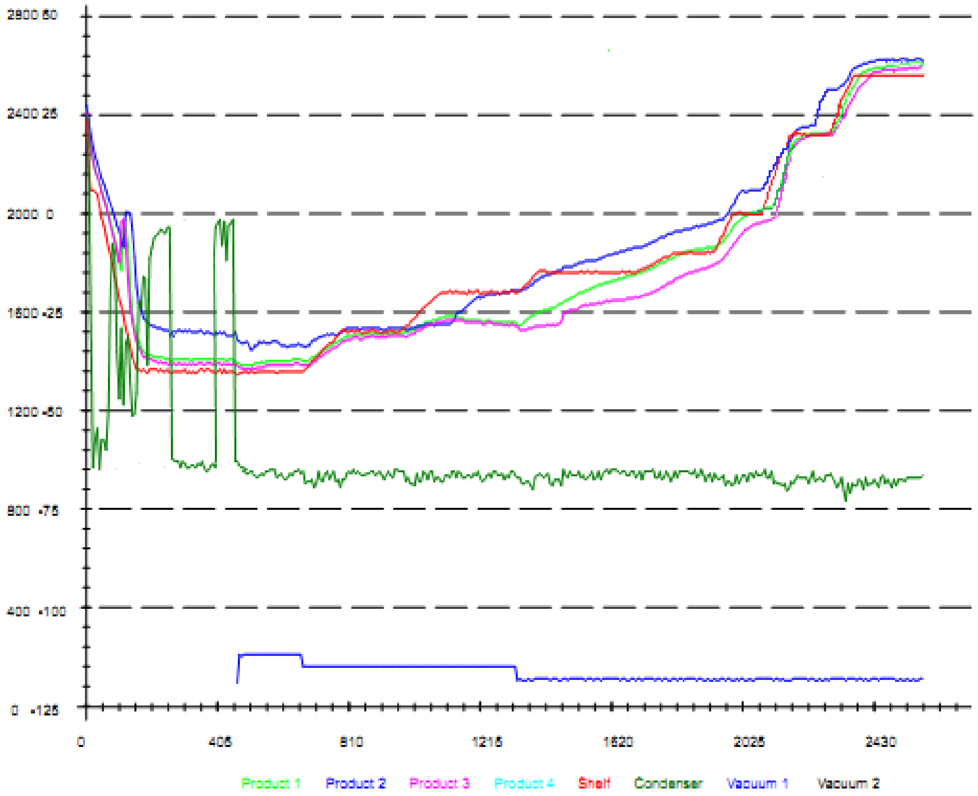


**Figure 4.5 Thermal graph of freeze drying cycle for lyophilization of Darunavir loaded SLNs**

**Table 4.12 Results of cryoprotectant effect on the optimized Darunavir loaded solid lipid nanoparticles**

| **Batch no.** | **Cryo-protectant** | **Cryo-protectant** | **Water content** | **Particle size before lyo-** | **After lyophilization** | |
|---|---|---|---|---|---|---|
| | | | | | **Particle size** | **% drug** |

| | | **concentration (mg/ml of formulation)** | **(% w/w of lyophilized powder)** | **philization** | | **content** |
|---|---|---|---|---|---|---|
| **Dar-SLN1** | | | | | | |
| DLN-30 | Sucrose | 30 | 1.81±0.15 | 92.52±2.9 | 150.2 ± 6.3 | 90.2 ± 1.7 |
| DLN-31 | Sucrose | 50 | 1.72±0.16 | | 120.5 ± 4.2 | 95.4 ± 4.1 |
| DLN-32 | Sucrose | 70 | 1.77±0.12 | | 124.7 ± 2.7 | 93.4 ± 3.2 |
| DLN-33 | Trehalose | 30 | 1.73±0.16 | | 120.3 ± 3.5 | 94.2 ± 3.1 |
| **DLN-34** | **Trehalose** | **50** | **1.65±0.10** | | **99.2 ± 4.5** | **99.5 ± 0.2** |
| DLN-35 | Trehalose | 70 | 1.53±0.13 | | 102.4 ± 3.2 | 98.4 ± 1.2 |
| **Dar-SLN2** | | | | | | |
| DLN-36 | Sucrose | 30 | 1.76±0.11 | 188.64± 2.5 | 224.4 ± 3.2 | 89.4 ± 3.7 |
| DLN-37 | Sucrose | 50 | 1.60±0.18 | | 216.7 ± 3.2 | 96.3 ± 2.3 |
| DLN-38 | Sucrose | 70 | 1.82±0.12 | | 191.4 ± 3.2 | 93.2 ± 4.3 |
| DLN-39 | Trehalose | 30 | 1.71±0.13 | | 215.7 ± 3.9 | 90.2 ± 4.1 |
| **DLN-40** | **Trehalose** | **50** | **1.77±0.18** | | **193.5 ± 4.1** | **99.1 ± 0.4** |
| DLN-41 | Trehalose | 70 | 1.76±0.11 | | 195.3 ± 2.2 | 98.4 ± 1.1 |
| **Dar-SLN3** | | | | | | |
| DLN-42 | Sucrose | 30 | 1.71±0.10 | 528.12±3.2 | 580.4 ± 5.2 | 84.3 ± 4.8 |
| DLN-43 | Sucrose | 50 | 1.93±0.15 | | 551.5 ± 5.4 | 94.2 ± 1.7 |
| DLN-44 | Sucrose | 70 | 1.81±0.18 | | 556 ± 3.8 | 92.5 ± 1.2 |
| DLN-45 | Trehalose | 30 | 1.65±0.10 | | 560.7 ± 5.3 | 88.7 ± 3.1 |
| **DLN-46** | **Trehalose** | **50** | **1.77±0.09** | | **533.8 ± 4.6** | **98.2 ± 0.5** |

| | | | | | | |
|---|---|---|---|---|---|---|
| DLN-47 | Trehalose | 70 | 1.72±0.15 | | 536.7 ± 5.4 | 96.3 ± 1.6 |

*Values are represented as mean ± SD, n=3.

The lyophilization cycle for 42 hr provided the good physical properties of cake with good dispersibility. After lyophilization, the lyophilized product had moisture content of less than 2 %. Trehalose as cryoprotectant was able to preserve the particle size of all three nanoparticles formulations at 50 mg/ml concentration. Moreover, in the same samples, the drug content was also found to be near 99 % in all formulations. Hence, trehalose at 50 mg/ml concentration was selected as optimized cryoprotectant.

### 4.4.2 Formulation development of peptide grafted solid lipid nanoparticles of Darunavir (Pept-Dar-SLN)

The peptide (sequence CARRPKFYRAPYVKNHPNVWGPWVAYGP) that selectively binds to HIV host $CD4^+$ T cells was conjugated to developed solid lipid nanoparticles of Darunavir (Dar-SLN2). The coupling occurred covalently at pH 6.5 to the terminal cysteine amino acid of peptide.

### 4.4.3 Formulation development of nanoemulsion of Darunavir

#### 4.4.3.1 Assessment of solubility of Darunavir in various oils and surfactant

Results of solubility study of Darunavir in various oils and surfactant is shown in Table 4.13. Soyabean oil showed highest solubility and thus was selected as oil for further development of formulation.

**Table 4.13 Results of solubility study of Darunavir in various oils/surfactants**

| Sr. No | Oil/Surfactant | Solubility (mg/g) |
|---|---|---|
| 1 | Soyabean oil | 55.3 mg |
| 2 | Medium chain triglyceride | 12.42 mg |
| 3 | Glycerol monooleate | 22.36 |
| 4 | Glyceryl monocaprylate | 39.56 |

| | | |
|---|---|---|
| 5 | Caprylocaproyl macrogol-8 glycerides | 15.12 |
| 6 | Propylene glycol dicaprylocaprate | 4.88 |
| 7 | Carylic/ capric triglyceride | 2.45 |
| 8 | Glyceryl Mono-, Di-, Tri-hexanoate | 1.55 |
| 9 | Soyabean oil + Tween 80 (1: 0.1) | 73.27 mg |

### 4.4.3.2 Formulation of nanoemulsion of Darunavir

The results of evaluation parameters of various batches of nanoemulsion are shown in Table 4.14. All the batches gave globule size in range of 190 to 235 nm and negative zeta potential in range of -37mV to -45mV. Centrifugal stress is a rapid way to check the stability of nanoemulsions. It is reported that the emulsion with higher creaming values have better stability to centrifugal stress (14). Higher creaming values from 98-99% were observed with DNE-3, 4 and 5 batches compared to DNE-1 and DNE-2 batch indicating their more stability.

**Table 4.14 Results of evaluation parameters of various batches of nanoemulsion of Darunavir**

| Batch no. | Globule size (nm) | PDI | Zeta potential (mV) | Total drug content (mg) | % EE | Creaming volume % |
|---|---|---|---|---|---|---|
| DNE-1 | 233.1 ± 2.34 | 0.308 ± 0.02 | -37.1 ±1.47 | 193.6 ± 1.2 | 85.05 ± 2.3 | 86.12 ± 0.1 |
| DNE-2 | 225.3 ± 1.67 | 0.311 ± 0.01 | -38.2 ± 2.36 | 191.6 ± 1.26 | 90.55 ± 3.2 | 92.5 ± 0.5 |
| DNE-3 | 209.5 ± 2.36 | 0.150 ± 0.03 | -41.1 ±1.46 | 190.8 ± 2.16 | 93.11 ± 1.2 | 98.2 ± 0.3 |
| DNE-4 | 204.6 ± 1.46 | 0.111 ± 0.02 | -42.3 ± 2.47 | 190.4 ± 1.16 | 94.52 ± 1.9 | 99.1 ± 0.5 |
| DNE-5 | 196.5 ± 1.80 | 0.106 ± 0.03 | -44.9 ± 1.37 | 191.2 ± 1.72 | 95.11 ± 2.5 | 99.12 ± 0.3 |

*Values are represented as mean ± SD, n=3.

The results of short term stability of the nanoemulsion are shown in Table 4.15 and Table 4.16. For all batches, phase separation was observed at 25ºC while there was no phase separation when the batches were stored at 4ºC indicating higher stability of nanoemulsion at this temperature. The globule size of batches DNE-1 and DNE-2 (containing lesser tween 80 concentration) increased greatly upon storage as compared to other batches. There was insignificant difference in zeta potential values obtained in batches DNE-3, DNE-4 and DNE-5 upon storing at 4ºC and a significant decrease in zeta potential values in batches DNE-1 and DNE-2. The overall result suggested that batches DNE-3, DNE-4 and DNE-5 were stable at 4ºC for 1 month. Thus, among these batches DNE 3-5, DNE-3 batch was selected as optimized batch (containing lower tween-80 concentration) and its further evaluation was performed.

**Table 4.15 Results of globule size of different batches of nanoemulsion upon storage at 4ºC and 25 ºC.**

| **Batch No.** | **Temp. (°C)** | **Globule size (nm)** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Day 0** | **Day 1** | **Day 5** | **Day 12** | **Day 20** | **Day 30** |
| DNE-1 | 4°C | 233.1 ±2.34 | 233.8 ± 2.10 | 247.3 ± 1.27 | 246.3 ± 2.55 | 261.3 ± 3.10 | 274.1 ± 3.26 |
| | 25°C | | 332.3 ± 2.45 | 744.1 ± 4.26 | Phase separation | - | - |
| DNE-2 | 4°C | 225.3 ±1.67 | 226.3 ± 2.15 | 231.6 ± 1.27 | 244.5 ± 2.13 | 252.3 ± 2.26 | 267.3 ± 2.37 |
| | 25°C | | 313.6 ± 3.17 | 412.6 ± 3.27 | 815.3 ± 9.27 | 2556.3 ± 17.35 | Phase separation |
| **DNE-3** | **4°C** | **209.5 ±2.36** | **210.2 ± 1.36** | **210.2 ± 1.20** | **210.3 ± 1.78** | **210.3 ± 2.17** | **211.5 ± 1.79** |
| | 25°C | | 256.3 ± 1.69 | 514.7 ± 2.29 | 811.2 ± 3.28 | 1996.2 ± 13.27 | Phase |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | | separation |
| DNE-4 | 4°C | 204.6 ±1.46 | 204.3 ± 1.98 | 204.5 ± 1.50 | 205.6 ± 2.28 | 205.4 ± 2.75 | 207.3 ± 2.54 |
| | 25°C | | 311.3 ± 3.26 | 613.3 ± 10.20 | 1141.6 ± 13.28 | 2001.3 ± 21.27 | Phase separation |
| DNE-5 | 4°C | 196.5 ±1.80 | 196.2 ± 1.17 | 195.4 ± 1.37 | 198.4 ± 2.11 | 200.2 ± 2.10 | 201.2 ± 2.18 |
| | 25°C | | 258.9 ± 2.37 | 413.6 ± 5.89 | 711.8 ± 8.27 | 1396.3 ± 16.78 | Phase separation |

*Values are represented as mean ± SD, n=3.

**Table 4.16 Results of zeta potential of different batches of nanoemulsion upon storage at 4ºC and 25ºC.**

| **Batch No.** | **Temp. (°C)** | **Zeta potential (mV)** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Day 0** | **Day 1** | **Day 5** | **Day 12** | **Day 20** | **Day 30** |
| DNE-1 | 4°C | -37.1 ±1.47 | -37.5 ± 1.14 | -38.8 ± 1.52 | -39.3 ± 1.10 | -37.3 ± 1.18 | -42.4 ± 1.36 |
| | 25°C | | -41.6 ± 1.20 | -44.5 ± 1.15 | - | - | - |
| DNE-2 | 4°C | -38.2 ±2.36 | -44.2 ± 1.52 | -44.3 ± 1.26 | -46.5 ± 1.20 | -48.2 ± 2.17 | -48.3 ± 2.65 |
| | 25°C | | -43.8 ± 1.86 | -46.8 ± 2.37 | -44.6 ± 2.75 | -54.3 ± 3.19 | - |
| **DNE-3** | **4°C** | **-41.1 ±1.46** | **-42.6 ± 1.26** | **-42.5 ± 1.37** | **-42.4 ± 1.28** | **-43.3 ± 1.30** | **-42.5 ± 1.18** |
| | 25°C | | -41.0 ± 1.10 | -47.8 ± 1.19 | -45.8 ± 1.29 | -51.6 ± 2.27 | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| DNE-4 | 4°C | -42.3 ±2.47 | -44.8 ± 1.48 | -45.2 ± 2.18 | -45.3 ± 2.16 | -45.1 ± 2.15 | -45.8 ± 2.16 |
| | 25°C | | -43.7 ± 1.17 | -45.3 ± 1.73 | -49.6 ± 2.17 | -53.2 ± 2.57 | - |
| DNE-5 | 4°C | -44.9 ±1.37 | -44.6 ± 1.16 | -45.1 ± 1.27 | -45.5 ± 1.38 | -46.3 ± 2.17 | -45.2 ± 2.15 |
| | 25°C | | -44.5 ± 1.37 | -46.3 ± 2.16 | -48.3 ± 2.17 | -52.6 ± 3.27 | - |

*Values are represented as mean ± SD, n=3.

### 4.4.4 Formulation development of peptide grafted Atazanavir sulfate loaded solid lipid nanoparticles

Atazanavir sulfate loaded solid lipid nanoparticles were prepared using emulsification solvent evaporation and the effect of various formulation parameters on particle size and drug entrapment of nanoparticles was studied. The results are shown in Table 4.17. From the results it can be seen that when glyceryl behenate and tripalmitin were taken as solid lipids, the entrapment of drug was only up to 50 %. Highest entrapment up to 70 % was observed when HCO was taken as solid lipid. In addition, particle size was also less than 200 nm in these batches (ALN-7, 8 and 9). Hence HCO was selected as solid lipid and further scale up was performed.

**Table 4.17 Effect of various formulation parameters on particle size, PDI and drug entrapment efficiency of Atazanavir sulfate loaded solid lipid nanoparticles**

| Batch no. | Drug: lipid ratio | Concentration of sodium oleate (%v/v) | Particle size (nm) | PDI | Entrapment efficiency (%) |
|---|---|---|---|---|---|
| Glyceryl behenate | | | | | |
| ALN-1 | 1:2 | 0.7 | 132 ± 2.15 | 0.283 ± 0.04 | 35.87 ± 4.82 |
| ALN-2 | 1:5 | 1.2 | 192 ± 1.72 | 0.274 ± 0.04 | 46.18 ± 3.31 |

| | | | | | |
|---|---|---|---|---|---|
| ALN-3 | 1:8 | 1.5 | 235 ± 5.21 | 0.311 ± 0.08 | 51.59 ± 3.67 |
| Glyceryl palmitostearate | | | | | |
| ALN-4 | 1:2 | 0.7 | 168 ± 2.90 | 0.258 ± 0.04 | 42.32 ± 1.15 |
| ALN-5 | 1:5 | 1.2 | 186 ± 1.75 | 0.174 ± 0.08 | 52.38 ± 3.14 |
| ALN-6 | 1:8 | 1.5 | 234 ± 2.73 | 0.312 ± 0.07 | 68.13 ± 2.17 |
| **Hydrogenated castor oil** | | | | | |
| ALN-7 | 1:2 | 0.7 | 98 ± 1.15 | 0.163 ± 0.07 | 44.4 ± 3.12 |
| ALN-8 | 1:5 | 1.2 | 151 ± 3.21 | 0.175 ± 0.05 | 59.41 ± 3.14 |
| ALN-9 | 1:8 | 1.5 | 178 ± 3.17 | 0.111 ±0.05 | 78.90 ± 2.93 |
| Trimyristin | | | | | |
| ALN-10 | 1:2 | 0.7 | 132 ± 1.84 | 0.173 ± 0.03 | 41.17 ± 4.6 |
| ALN-11 | 1:5 | 1.2 | 162 ± 1.52 | 0.216 ± 0.03 | 47.85 ± 3.15 |
| ALN-12 | 1:8 | 1.5 | 189 ± 3.14 | 0.278 ± 0.06 | 64.18 ± 2.17 |
| Tripalmitin | | | | | |
| ALN-13 | 1:2 | 0.7 | 142 ± 1.18 | 0.284 ± 0.05 | 33.15 ± 3.31 |
| ALN-14 | 1:5 | 1.2 | 201 ± 2.31 | 0.273 ± 0.07 | 41.17 ± 3.63 |
| ALN-15 | 1:8 | 1.5 | 221 ± 2.65 | 0.155 ± 0.04 | 47.26 ± 3.62 |

*Values are represented as mean ± SD, n=3.

### Scale up by high pressure homogenizer (HPH):

Upon selection of HCO as lipid, further optimization of lipid and surfactant concentration was done along with the scale up of the formulation by high pressure homogenizer. Various batches were prepared using this technique by varying drug: lipid ratio and surfactant concentration. The results obtained are shown below in Table 4.18.

**Table 4.18 Effect of lipid and surfactant concentration on particle size, PDI and drug entrapment efficiency of ATZ loaded solid lipid nanoparticles**

| **Batch no.** | **Drug: lipid ratio** | **Concentration of sodium oleate (% v/v)** | **Particle size (nm)** | **PDI** | **Entrapment efficiency (%)** |
|---|---|---|---|---|---|
| ALN-16 | 1:6 | 4 | 175.2 ± 4.21 | 0.638 ± 0.04 | 62.39 ± 1.23 |
| ALN-17 | 1:6 | 5 | 170.7 ± 2.11 | 0.729 ± 0.06 | 65.16 ± 2.34 |
| ALN-18 | 1:6 | 6 | 162.3 ± 3.12 | 0.844 ± 0.08 | 69.15 ± 2.33 |
| ALN-19 | 1:8 | 4 | 182.14 ± 2.78 | 0.529 ± 0.05 | 71.38 ± 2.27 |
| ALN-20 | 1:8 | 5 | 171.6 ± 2.67 | 0.720 ± 0.07 | 76.17 ± 1.28 |
| ALN-21 | 1:8 | 6 | 171.34 ± 3.16 | 0.825 ± 0.08 | 79.23 ± 2.65 |
| ALN-22 | 1:10 | 4 | 198.4 ± 3.98 | 0.628 ± 0.06 | 83.19 ± 2.73 |
| **ALN-23** | **1:10** | **5** | **190.1 ± 2.45** | **0.534 ± 0.06** | **94.26 ± 2.12** |
| ALN-24 | 1:10 | 6 | 186.3 ± 1.16 | 0.517 ± 0.07 | 94.65 ± 1.19 |

*Values are represented as mean ± SD, n=3.

High drug entrapment of up to 94 % was obtained in batches having 1:10 drug: lipid ratio (ALN-23 and ALN-24). With sodium oleate, particle size less than 200 nm was observed in all batches with sodium oleate concentration of 5 and 6 % v/v. Selecting lower surfactant concentration, the batch ALN-23 (with 5 % v/v sodium oleate) was selected as final formulation.

### Lyophilization of optimized ATZ loaded nanoparticles and optimization of cryoprotectant:

Two cryoprotectants (sucrose and trehalose) were added at different ratios in the nanoparticles dispersion to find out suitable cryoprotectant and optimum concentration of cryoprotectant which showed minimum increase in particle size of nanoparticle formulation. Figure 4.6 shows the thermal kinetic of products in comparison with shelf during the cycle of lyophilization. Particle size, drug content and moisture content were estimated and the results are shown in Table 4.19.

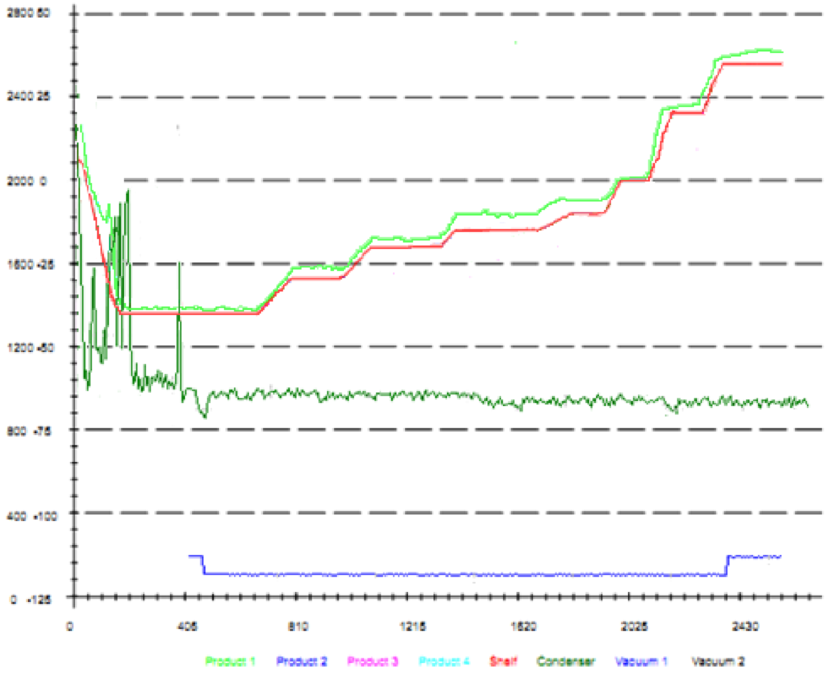


**Figure 4.6 Thermal graph of freeze drying cycle for lyophilization of ATZ loaded SLNs**

**Table 4.19 Results of cryoprotectant effect on the optimized ATZ loaded solid lipid nanoparticles**

| **Batch no.** | **Cryo-protectant** | **Cryo-protectant concentration (mg/ml)** | **Moisture content** | **Particle size before lyo-philization** | **After lyophilization** | |
|---|---|---|---|---|---|---|
| | | | | | **Particle size** | **% drug content** |
| ALN-25 | Sucrose | 50 | 1.63 | 190.1 ± 2.45 | 254.4 ± 2.6 | 90.2 ± 3.4 |
| ALN-26 | Sucrose | 60 | 1.25 | | 235.8 ± 3.8 | 91.1 ± 3.6 |
| ALN-27 | Sucrose | 70 | 1.39 | | 233.4 ± 4.2 | 92.3 ± 3.3 |
| ALN-28 | Trehalose | 50 | 1.43 | | 214.5 ± 2.6 | 94.2 ± 3.2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **ALN-29** | **Trehalose** | **60** | **1.14** | | **194.3 ± 3.2** | **99.3 ± 0.2** |
| ALN-30 | Trehalose | 70 | 1.21 | | 194.2 ± 3.4 | 98.3 ± 2.4 |

*Values are represented as mean ± SD, n=3.

The results show that there was significant increase in particle size of nanoparticles when sucrose was used as cryoprotectant. Trehalose was able to preserve the particle size upon lyophilization. Batches ALN-29 and ALN-30 were found to have sufficient trehalose concentration since their drug content were near to 99 % and non significant change in particle size. Its moisture content was also less than 2 %. Thus, Batch ALN-29 containing 60 mg/ml trehalose was taken as finalized formulation.

## 4.5 References

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