International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page:www.iscientific.org/Journal.html

Formulation, Statistical Optimization, and *In vivo* Evaluation of Darunavir loaded Solid lipid Nanoparticles (SLNs)

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Abstract

Darunavir loaded SLN were prepared by using hot homogenization technique followed by ultra-sonication method and 3^2 full factorial design has been employed. The statistical optimization reduced the number of experiments that were carried out for obtaining formulations with desired properties. The derived polynomial equations, response and contour plots helped in predicting the values of selected independent variables for preparation of optimum SLN with desired properties. Solid lipid nanoparticles showed minimum particles size, optimum zeta potential and higher %EE. This experimental design revealed that Phospholipon 90H based formulation showed better result compared to Sphingomyelin, Soya Phosphatidyl Ethanolamine based formulations which could be due to maximum carbon chain length and high phase transition temperature. The drug excipients characterization parameters reveal that there is no drug-excipients interaction.

Keywords: Darunavir, SLNs, zeta potential

Full length article **Corresponding Author*, e-mail: yprasanth.mpharm@gmail.com

1. Introduction

It is seen mostly that the *in vitro* data do not co-relate with those obtained *in vivo* and the main reason for this happens to be insufficient or poor absorption, rapid metabolism and elimination [1], e.g. peptide drugs, distribution of the drug to accompanying tissues (cancer drugs), low aqueous solubility of drugs, high fluctuation in plasma levels of drug which is due to unpredictable bioavailability after paroral administration and effect of presence of food on plasma levels [2-5]. A promising strategy to overcome the aforementioned problems encompasses development of suitable drug carrier systems with potential of releasing the active compound according to the specific requirements of the undergoing therapy. Solid lipid nanoparticles (SLN) not only combine the advantages of colloidal drug carrier systems such as liposomes, polymeric nanoparticles and emulsions but also avoid drawbacks associated with these systems [6-7].Darunavir, a non-peptide protease inhibitor, suffers from poor oral bioavailability (37%) as it acts as a substrate for polyglycoprotein (PgP) which causes efflux of the absorbed drug back into the intestinal lumen and a substrate for cyp3A metabolism [8]. The bioavailability of darunavir can be increased to 82% by co-administering ritonavir, which is a potent cyp3A inhibitor.

The present work attempts to improve bioavailability of darunavir by formulation as lipid nanoparticulate, as these have been reported to improve oral bioavailability of drugs prone to PgP efflux and CYP-mediated first-pass metabolism [9]. A lipid mixture, melting at temperature less than that of darunavir's melting point, was used to formulate the SLN. It is believed that the SLN would be taken up by the lymphatic system owing to the lipid carrier and the lipid matrix, bypass the hepatic metabolism, and reduce the PgP efflux [10]. The novelty of the work lies in successful preparation and characterization of a non-lipid, temperature degradable anti-HIV drug into a SLN carrier and demonstration of improved permeability of the same.

2. Materials and Methods

2.1. Materials

Darunavir was received as a kind gift sample from Lupin Research Park, Pune, Sphingomyelin, Soya Phosphatidyl Ethanolamine, Phospholipon 90H, Tween 20, Span 20, PEG 200, PEG 400, Propylene glycol were obtained as gift sample from Merck, Capmul MCM, Captex 200, Abitec Group, Labrafac LipophileWL1349, Labrasol, Labrafil gift sample from Gattefosse, France.

2.2. Methods

2.2.1. Appearance and melting point

The organoleptic characteristics like colour, odour and textures were observed by sensory organs. The melting point was determined using capillary fusion method where a small amount of drug was filled in a capillary sealed from one side and kept inverted. The temperature at which drug started liquefy was recorded and compared with literature value and shown in table1.

2.2.2. Solubility study of the drug

The solubility study of Darunavir was carried out in various solvents. Accurately weighed 20 mg of drug was added to screw capped vials containing 10 ml of solvent. The vials were kept in a water bath shaker at 37±0.5 ℃ and shaken for 24 h. The mixtures were then filtered through millipore filter membrane of pore size 0.45 µm, diluted and drug was analyzed using UV spectrometer.

2.2.3. Preparation of Standard solution of Darunavir

Working standard of DRN 10mg was accurately weighed and transferred into 10ml volumetric flask, containing 5ml of ethanol and it was ultra-sonicated for 10 min and diluted up to the mark with further quantity of ethanol to get a concentration of 1000 µg/ml.

2.2.4. Determination of λ_{max} of Darunavir

Aliquots prepared from working standard in increasing order were scanned in the wavelength of 200-400. The λ_{max} was found at 262nm. The calibration curve was constructed, the regression equation was calculated, and regression coefficient (r^2) was found 0.999. This equation was used for the estimation of Darunavir.

2.2.5. Selection of oils, surfactants and co-surfactants

The lipids, surfactants and cosurfactants were selected based on solubility of the drug. The study was carried out by taking 2 ml of selected lipid (Corn oil, Olive oil, Soyabean Oil, Peanut oil, Sesame oil, Labrafac Lipophilewl1349, Capmul MCM, Ethyl oleate, Sphingomyelin, Soya Phosphatidyl Ethanolamine and Phospholipon 90H) / surfactant (Span 80, Tween 80, Tween 20, Span 20, Labrasol, Cremophor EL, Labrafil) / cosurfactant (Poloxamer 407, PEG 200, PEG 400, Propylene Glycol) in glass vial containing excess amount of drug. The mixtures were mixed manually for 30 min in order to facilitate proper mixing of drug with the vehicles. The vials were sonicated for 2 h and kept in water bath for 48 h for equilibration.

2.2.6. Preparation of solid lipid nanoparticles

Darunavir had good solubility in various lipids. Out of which the better solubility was shown by Labrafac Lipophilewl1349, Capmul MCM and Ethyl oleate. Hence, drug loaded SLNs were prepared with the above mentioned lipids usinghot homogenization technique followed by ultra-sonication method. Darunavir, Phospholipids and Tween 80 were heated above the melting temperature of lipid around60°C and mixed rapidly with glass rod in hot molten condition. Poloxamer188 dissolved in water heated to equal temperature and was added to the molten lipid phase and homogenization was carried out. Hot homogenization was carried out for 3 minutes at 5000 rpm in order to get coarse emulsion. Finally, the obtained pre-emulsion was subjected to ultra sonication.

2.2.7. Optimization of ultra-sonication time

Ultra-sonication was carried out with the help of ultrasonic homogenizer. Ultra-sonication was carried out for different time intervals 5, 10, 15, 20 and 25minutes.With the help of Zetasizer NanoZS average particle size of prepared SLN was measured.

2.2.8. Factorial design

A 3^2 randomized full factorial design was used in this study and 2 factors were evaluated, each at 3 levels, experimental trials were performed at all 9 possible combinations. Amount of Poloxamer 188(X_1) and Tween 80(X_2) were selected as two independent variables which were varied at three levels, low level (-1), medium level (0), high level (+1). Amount of drug Darunavir (100mg), Phospholipids (400mg) concentrations and dispersion medium (water20ml) were kept constant. Particle size (Y1), zeta potential (Y2) and entrapment efficiency (Y3) were selected as dependent variables. Values of variables and formulation codes are shown in the Tables 1, 2.

2.2.9. Optimization of surfactant and co-surfactant

The optimization of surfactant and co-surfactant was done by using 3^2 factorial design. Poloxamer 188 was chosen as independent factor (X_1) and taken at three different concentrations 100 mg, 150 mg and 200 mg. Tween 80 was selected as independent factor (X_2) and taken at three different concentrations 100 mg, 150 mg and 200 mg. Quantity of drug (100 mg), Phospholipids (400 mg) and the final volume of SLN (20 ml) were kept constant. The particle size (Y_1), zeta potential (Y_2) and % entrapment efficiency (Y_3) were selected as three dependent factors.

2.2.10. Determination of particle size distribution polydispersity index (PDI),and zeta potential of SLN

The particle size distribution, polydispersity index, and zeta potential of Darunavir loaded SLN were measured using a Malvern Zetasizer. About 100µL of the prepared SLN, dispersion was diluted to5mL with double distilled water and analyzed with Zetasizer. Photon correlation spectroscopy is the most widely used technique for measurement of particle size and zetapotential. The principle of dynamic light scattering at a scattering angle of 90 degrees is used to measure particle size.

2.2.11. Determination of percentage entrapment efficiency (%EE)

The percentage of drug entrapped in the lipid is determined by measuring the concentration of the drug in the aqueous phase by ultra-filtration method using centrisart devices. Centrisart consist of filter membrane (Molecular weight cut off 20,000 Daltons) at the base of sample recovery chamber. About1ml of undiluted sample is placed in the outer chamber on the top of the sample holder. The unit is centrifuged at 3,500 rpm for 15-20 min. The solid lipid nanoparticles along with the encapsulated drug remaining the outer chamber and the aqueous phase is moved into the sample recovery chamber through membrane.

$$(\%)\ EE = [(C_d - C)/C_d] * 100$$

Where, Cd is the concentration of total drug and C is the concentration of un-entrapped drug.

2.2.12. Statistical analysis of the data and optimization

Response surface modelling and evaluation of the quality of fit of the model for the current study were performed employing Design Expert® 12 software trial version. Polynomial models including linear, interaction and quadratic terms were generated for all the response variables using multiple linear regression analysis (MLRA). A second order polynomial equation that describes the effect of independent factors on the response is expressed in the following forms:

- **Linear model:**

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2$$

- **2FI (interaction) model:**

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2$$

- **Quadratic model:**

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2$$

Where, Y is the dependent variable, β_0 is the arithmetic mean response of the 9 runs, and β_1& $\beta_2$are the estimated coefficients for the factors X_1&X_2respectively. The main effect (X_1& X_2) represents the average result of changing one factor at a time from its low to high value. The interaction term (X_1X_2) shows how the response changes when two factors were changed simultaneously. The polynomial terms (X_1X_1, X_2X_2) are included to investigate nonlinearity. The equation enables the study of the effects of each factor and their interaction over the considered responses. The polynomial equation was used to draw conclusions after considering the magnitude of coefficients and the mathematical sign it carries, i.e., positive or negative. A positive sign signifies a synergistic effect, whereas a negative sign stands for an antagonistic effect. The best fitting mathematical model was selected based on the comparisons of statistical parameters which include the coefficient of variation (CV), the coefficient of determination (R2), adjusted coefficient of determination (adjusted R^2) and the predicted residual sum of square (PRESS), provided by Design Expert software. Among them, PRESS indicates how well the model fits the data and for the chosen model, it should be small relative to the other models under consideration. Level of significance was considered at $p<0.05$. Mathematical relationships in the form of polynomial equations are generated using multiple linear regression analysis (MLRA) and used to find out the relative influence of each factor on the response. Analysis of variance (ANOVA) for the responses was performed to identify significant effect of factors on responses and the model parameters were obtained. The relationship between the dependent and independent variables was further elucidated using contour and response surface plots. These plots are very useful to study of the effects of two factors on the response at one time and predict the responses of dependent variables at the intermediate levels of independent variables. Subsequently, a numerical optimization technique by the desirability and graphical optimization technique by the overlay plot approach were used to generate the new formulation with the desired responses. An optimized formulation was developed by setting constraints (goals) on the dependent and independent variables.

2.2.13. Compatibility Study

Interaction between the drug, oil, surfactant, and co-surfactant were studied by FT-IR. The blank KBr pellets were prepared, onto which oil, surfactant and co-surfactant were dropped individually and it was pressed with another blank KBr pellet using hydraulic press. The pure drug was mixed with KBr in the ratio of 1:3 and punched in a hydraulic press at 5–6-ton load. The prepared pellets were scanned from 4000 to 400 cm^{-1} using FT-IR spectrophotometer (FT-IR 8400 S, Shimadzu). The FT-IR spectra of the physical mixture were compared with the spectra of pure drug, phospholipid, surfactant, and co-surfactant. Differential scanning calorimetry (DSC)was performed using a Differential scanning calorimeter (DSC 220C, Seiko, Japan) at a heating rate of 10^0 C/min from 30 to 300^0 C in nitrogen atmosphere.

2.2.14. In vitro drug release studies from SLN

The *in vitro* release studies of Darunavir loaded solid lipid nanoparticles were carried out by using modified Franz diffusion cell. Dialysis membrane having pore size 2.4 nm with molecular weight cut off 10,000 Daltons was used. Membrane was soaked in double distilled water for 12 hours before mounting in Franz diffusion cell. Darunavir loaded 2 ml of SLN dispersion equivalent to 30 mg was applied to the donor compartment. In addition, the receptor compartment was filled with 12 ml of dialysis medium of 0.1N HCl. Samples (100 µL) were withdrawn from receiver compartment through side tube at regular time intervals and the same was replaced with fresh dialysis medium maintained at same temperature. In the similar way pure drug equivalent to 4 mg was also added to the 2 ml of distilled water and release studies were performed for comparison.

2.2.15. Release kinetics

The analysis of drug release kinetics and mechanism from a pharmaceutical dosage form is an

important parameter but requires complicated mathematical treatment. The order of drug release from SLNs was described by using zero order or first order kinetics. The mechanism of drug release was studied by using Higuchi diffusion model and Hixon-Crowell erosion model.

2.3. Pharmacokinetics of the Optimized Formulation Animals

Pharmacokinetic evaluation of SLNs were conducted in male Wistar rats weighing between 200 and 250 g. Animals were inbred at Animal Research Facility. (In which place, JNTU or kakinadaetc – location missing) Animals were housed in polypropylene cages provided with sterile husk and under controlled temperature (23 ± 3 °C) and humidity conditions.

2.3.1. Study Design

Animals were divided into 2 groups (group I, group II) with six animals in each group. All the animals were fasted overnight before experimentation. Group I animals were dosed with a suspension of marketed formulation of darunavir (Daruvir, 50 mg/kg/10 ml) orally. Moreover, group II animals were dosed with optimized SLNs (batch I) of darunavir (50 mg/kg/10 mL) by oral route. Approximately 0.25 ml of blood was withdrawn from each animal at different time points and collected into tubes with an anti-coagulant. All the samples were centrifuged, and plasma was separated. Drug concentration in all the samples were estimated using HPLC bioanalytical method.

2.3.2. Extraction of Darunavir from Rat Plasma Samples

A simple bioanalytical method was developed for the extraction and quantification of darunavir in plasma samples. Protein precipitation method was used for the extraction of darunavir from plasma samples. Briefly, to 50 µl of plasma, 20 µl of internal standard (darunavir, 200 µg/ml) and 150 µl of chilled methanol were added to precipitate the samples. Furthermore, all samples were vortexed, centrifuged at 15,000 rpm, 4°C for 10 min.

3. Results and discussion

The results of the present study are given in tables 1-17 and figures 1-10.

3.1. Determination of λ_{max}

From the fig 1, it was found that λ_{max} of Darunavir at 262 nm. Standard plot that was plotted helps in understanding the wavelength at which drug absorbs. The table 3 details the relationship between concentration and absorbance at 262nm. Figure 2 explains the standard plot of Darunavir in 0.1N HCl and table shows the Statistical parameters for standard curve of Darunavir in 0.1N HCl. The calibration curve of Darunavir was prepared using 0.1N HCl. Accurately weighed 50 mg of drug was dissolved in 50 ml 0.1N HCl to obtain concentration of 1mg/ml. 1 ml of prepared solution was further diluted 10 times to obtain stock solution of 100 µg/ml. From the secondary stock solution 1ml, 2ml, 3ml, 4ml and 5ml were taken separately and diluted to 10ml separately with 0.1N HCl to get 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml concentrations respectively.

3.2. Optimization of ultra-sonication time on particle size

Average particle size of Labrafac Lipophilewl1349, Capmul MCM and Ethyl oleate based SLN were prepared with ultra-sonication time of 5, 10, 15, 20 and 25minutes and are shown in Table 5 and Fig 3. Sonication time has influenced particle size. As the sonication time increased the particle, size was decreased up to 20 minutes and further decrease in particle size was not observed at 25minutes.

3.3. Percent drug content (%)

The percentage drug content of the SLN formulations varied between 95.5%±0.6 to 102.4%±0.6 respectively as shown in Tables 6-8 and Fig 5. Hence, all the formulations were within the standard limits(90% to 110%). This indicated uniform distribution of drug in eachSLNformulationandtherewasnowastageofthedrugduring preparationofSLN.

3.4. Particle size distribution (nm), zetapotential (mV) and polydispersity index (PDI)

The mean particle sizes were in the range of 101±0.6 to 213.3±0.1nm, 101±0.6 to 207±0.1nm and 105±0.3to167±0.9 nm for Labrafac Lipophile, Capmul MCM and Ethyl oleate based formulations respectively as shown in Tables and in Figsafter 20 min time of ultra-sonication time. The polydispersity index (PDI) was in therangeof0.181±0.3 to0.439±0.7. Zeta potential values of SLN ranged from 22.3±0.5to-45.0±4.0mV, -18.22±1.0to-48.3±0.1 mV, -20.2±0.2 to-44.3±0.8mVforLabrafac Lipophile, Capmul MCM and Ethyl oleate based formulations respectively. The zetapotential value was found to be >±30mV for almost all the formulations prepared. For any liquid dosage form, surface charge is essential for its stability. Zeta potential value>±30mV is essential for effective stability and to inhibit aggregation of particles. As the poloxamer118 concentration increased particle size was decreased. In three formulations, optimum size was obtained at 200 mg of poloxamer 118 concentrations. The low polydispersity index for all the formulations indicated the homogeneity of the particle size. The formulations showed negative zetapotential since solid lipid nano particles have negative charge on their surface as shown in Tables 6-8.

3.5. Determination of percent entrapment efficiency (%EE)

The percent entrapment efficiency of SLN was determined after separating entrapped and unentrapped drug by ultra-filtration. The percent entrapment efficiency varied from 49.3% to 97.7% for all the formulations as shown in Tables 6-8 and in Fig 5. Highest entrapment efficiency of 97.7% was observed for Ethyl oleate based SLN. The lowest entrapment efficiency was observed when the independentvariables poloxamer 188 (X1) and Tween 80 (X2) were at 100 mgand 150 mg concentrations for all the SLN formulations prepared with three different phospholipids. The highest entrapment efficiency was observed when the independent variables poloxamer188(X1) and Tween 80 (X2) were at higher level (200mg)

concentrations for all the SLN formulations prepared with three different phospholipids. There is no difference in entrapment efficiency, among three phospholipids. This could be due to maximum carbon chain length in the three phospholipids.

3.6. Statistical analysis of the data and optimization

In the current study, three Phospholipids namely Labrafac Lipophile, Capmul MCM and Ethyl oleate were selected for the preparation of SLN using fixed concentration of 400 mg. From the preliminary study, it was found that poloxamer 188 and Tween 80 has strong effect on physico-chemical properties of solid lipid nanoparticles such as particle size, entrapment efficiency, stability and invitro behavior. Amount of poloxamer 188 (X1) and amount of Tween 80 (X2) were selected as independent factors for preparation of solid lipid nanoparticles respectively as shown in tables 9-11. Particle size, zetapotential and % entrapment efficiency was considered as the 3dependentfactors.All the responses observed for nine runs (3 different bases of solid lipid nanoparticles) were simultaneously fitted to linear, interaction and quadratic models using Design Expert software trial version 12. The comparative values of R2, adjustedR2, predicted R2, PRESS, SD, %CV at significant p values ($P<0.05$), are given in Table 9-11. A suitable polynomial model for describing the data was selected based on coefficient of determination(R2) and PRESS values. The three responses Y1, Y2, Y3for the three different bases of SLN were independent, for SLN prepared with Labrafac Lipophile, Capmul MCM the responses Y1, Y2 and Y3 followed linear model whereas Ethyl oleate based SLN response Y1 and Y3 followed linear model and Y2 followed quadratic model. The fitted polynomial equations relating the responses are given inTable12. Model parameters obtained from analysis of variance (ANOVA)for the responses Y1-Y3 of three different bases of SLN are shown in Table 13. These parameters were used to construct the models that describe the effect of the on the responses(Y1-Y3). From the ANOVA data, the F value of three different bases of SLN i.e. Labrafac Lipophile, Capmul MCM and Ethyl oleate were given in the tables given below. The P value was less than 0.05 for the response factors indicated that the models aresignificant. For the Labrafac Lipophile based SLN, the response observation for particlesizeX1,X2, were found to be non-significant terms, the response observation for zeta potential X1, X2, X1X2 were found to benon-significant terms response observation for % EE X1 was found to be non-significant term and X2 was found to be significant term. For the Capmul MCM based SLN, the response observation for particle size X1,X2, X22, X1X2 were found to be significant terms, X12 found to be non-significant term, the response observation for zeta potential X1, X12, X1X2 were found to be significant terms. X2, X22, were found be non-significant terms response observation for % EE X1 was found tobenon-significanttermandX2wasfoundtobesignificantterm.For the Ethyl oleate based SLN, the response observation for particle size X1, X2, were found to be non-significant terms, the response observation for zetapotential X1, X2, X1X2were found to be significant terms response observation for %EE X1was found to be non-significant term and X2was found to be significant term. From the Table12three different bases of SLN "PredR- Squared" value of all three responses are not in reasonable agreement with the "AdjR-Square" value.

3.7. Optimization

To optimize 3responses with different targets, a multi-criteria decision approach, like a numerical optimization technique by the desirability function and graphical optimization technique by the overlay plot were used. The optimized formulation was obtained by applying constraints (goals) on dependent (response) and independent variables (factors). Optimum formulation was selected based on the criteria of minimum particle size, higher zeta potential and maximum % EE. Various feasibility and grid searches were executed to establish the optimum formulation by plotting desirability function response plot and overlay plot, where one solution was found with a desirability of 1.0. The recommended quantities of poloxamer188 and Tween 80 werecalculatedbytheDesignExpert12 software trial version. Optimization was carried out by both numerical optimization and graphical optimization techniques. The three different bases of SLN desirability and overlay plots are shown respectively in Fig 8. The desirability function was found to be higher for the optimized formula indicating the suitability of the formulations. The optimized SLN contain 200 mg of poloxamer 188 and 200 mg of Tween 80 for all the three different phospholipids. Predicted model formulations were found matching with the optimized SNF9, SPF9 andSHF9 formulations.

Table 1. Variables in 3^2 factorial designs

Independent variable	**Levels**		
	Low(mg)	**Medium (mg)**	**High(mg)**
X_1: Poloxamer 188	100.00	150.00	200.00
X_2: Tween 80	100.00	150.00	200.00

Table 2. Compositions of Dorunavir loaded SLN

Concentration (µg/ml)	Absorbance (nm)
0	0
10	0.202±0.33
20	0.412±0.19
30	0.607±0.15
40	0.811±0.16
50	0.999±0.35

Data is expressed as mean ±SD (n=3)

Table 3. Calibration curve of Darunavir in 0.1N HCl

Formulation	Poloxamer 188 (mg)	Tween 80 (mg)
	X1	X2
F1 (-1, -1)	100	100
F2 (-1, 0)	100	150
F3 (-1, +1)	100	200
F4 (0, -1)	150	100
F5 (0, 0)	150	150
F6 (0, +1)	150	200
F7 (+1, -1)	200	100
F8 (+1, 0)	200	150
F9 (+1, +1)	200	200

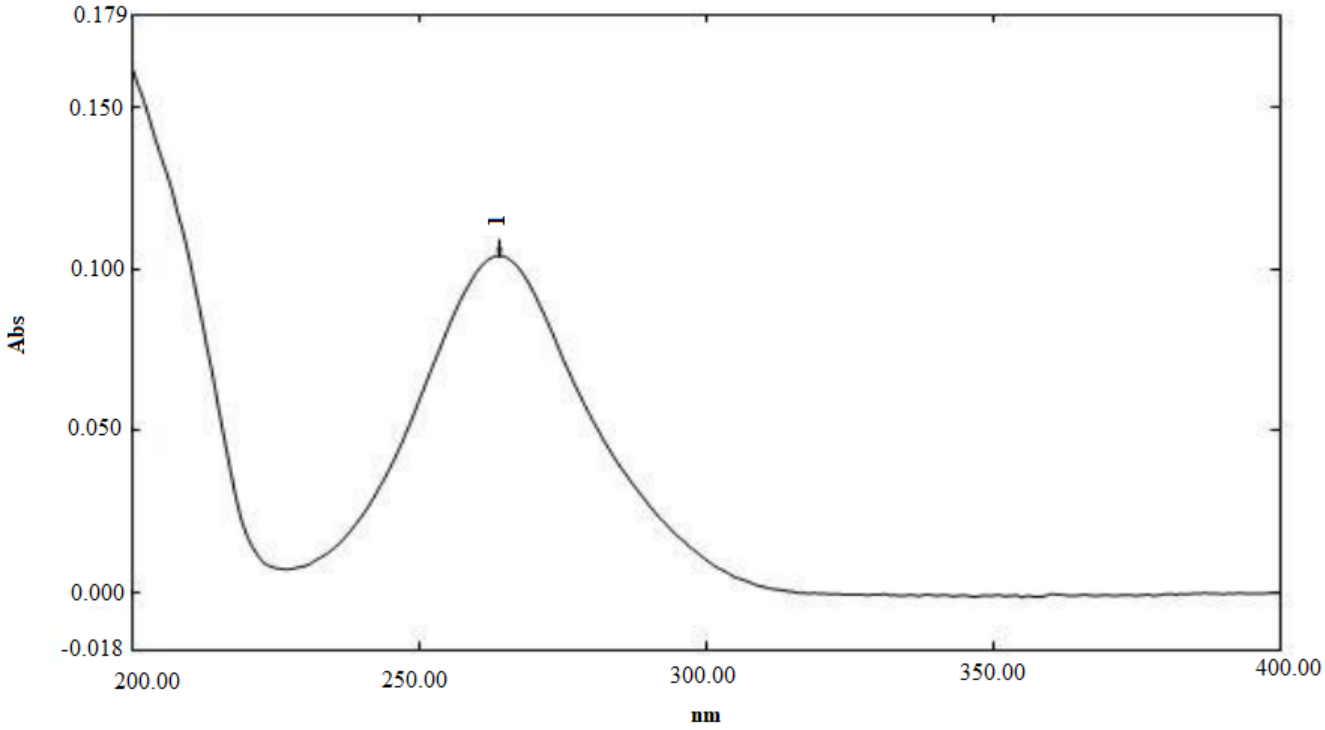


Figure 1. UV Spectra of Darunavir at 262 nm

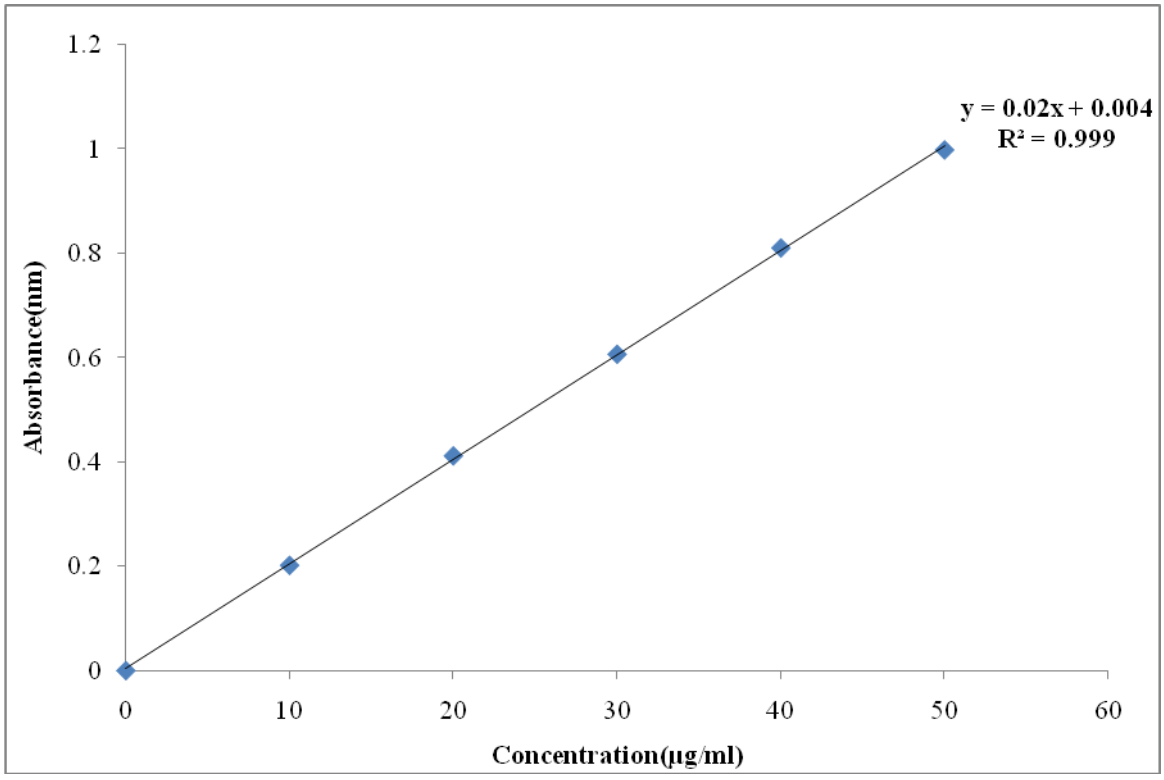


Figure 2. Standard plot of Darunavir in 0.1N HCl.

Table 4. Statistical parameters for standard curves of Darunavir in 0.1N HCl

S. No	Parameter	0.1 N HCl
1.	λ_{max} (nm)	262
2.	Linearity range (µg/ml)	0-50
3.	Regression equation	y=0.02x-0.004
4.	Intercept	0.004
5.	Slope	0.02
6.	Correlation coefficient (R^2)	0.9998

Table 5. Effect of sonication time on particle size(nm)(n=3)

Sonication time (min)	Labrafac Lipophilewl1349 SLN		Capmul MCM SLN		Ethyl oleate SLN	
	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
5	213.3±0.1	0.395±0.5	207±0.1	0.242±0.1	167±0.9	0.323±0.7
10	183.1±0.8	0.353±0.3	168±0.5	0.215±0.5	144±0.6	0.255±0.3
15	152±0.21	0.301±0.7	135±0.57	0.209±0.7	123±0.4	0.196±0.5
20	112±0.24	0.315±0.1	121±0.24	0.181±0.3	112±0.7	0.354±0.6
25	101±0.6	0.439±0.7	101±0.6	0.233±0.6	105±0.3	0.281±0.33

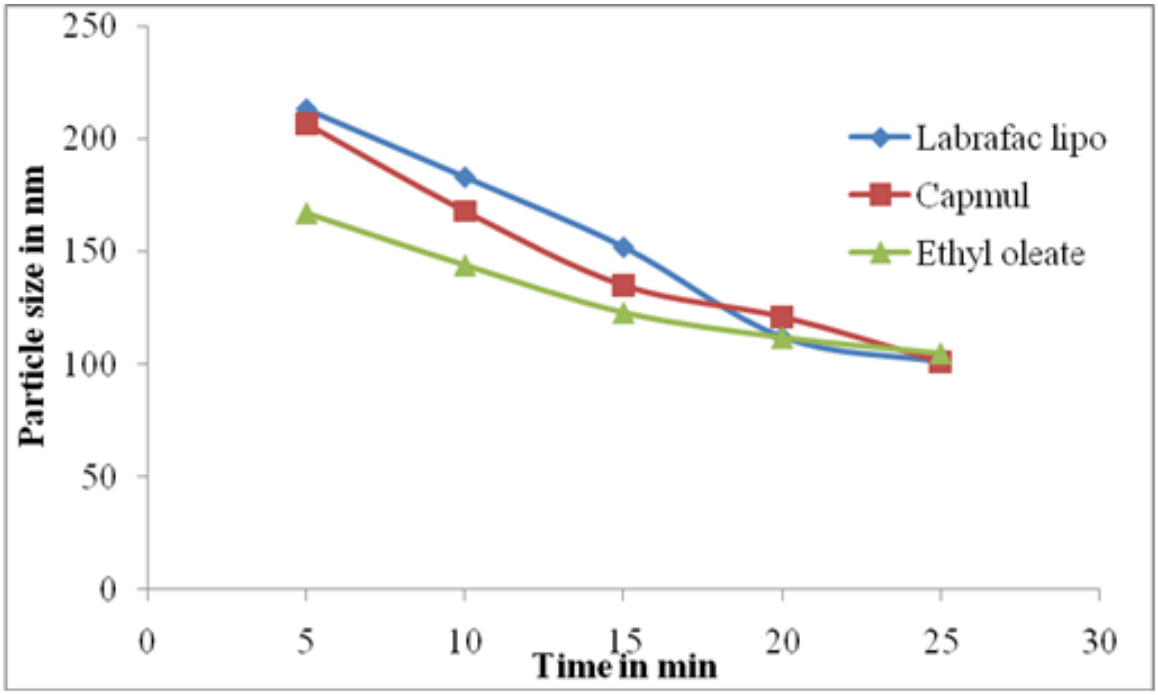


Figure 3. Relationship between Sonication times (min) Vs Particle Size (nm)

Table 6. Characterization of SLN prepared with Labrafac Lipophile

Batch	% Drug content	Size(nm)	Zetapotential(mV)	PDI	%EE
LF1	98.1±0.7	157.8±0.7	-25.5±0.6	0.229±0.4	60.7±0.9
LF2	97.0±0.3	112.3±0.8	-25.9±0.2	0.181±0.3	49.3±0.5
LF3	100.9±0.6	126.8±0.3	-47.5±3.9	0.256±0.7	76.9±0.5
LF4	99.8±0.3	198.3±0.6	-39.5±0.6	0.284±0.1	49.3±0.3
LF5	99.5±0.5	99.3±0.6	-29.5±0.6	0.199±0.5	73.5±0.6
LF6	99.7±0.4	99.5±0.8	-34.7±0.5	0.431±0.6	78.3±0.9
LF7	102.3±0.9	103.3±0.9	-36.7±0.2	0.220±0.7	63.6±0.5
LF8	99.7±0.4	105.8±0.6	-33.3±0.9	0.208±0.6	55.3±0.9
LF9	101.3±0.6	98.5±0.2	-31.5±0.6	0.216±0.4	96.3±0.4

Table 7. Characterization of SLN prepared with Capmul MCM

Batch	% Drug content	Size(nm)	Zetapotential(mV)	PDI	%EE
CF1	98.3±0.5	212.9±0.7	-18.9±0.6	0.185±0.6	59.6±0.7
CF2	99.3±0.4	135.2±0.4	-23.6±0.2	0.322±0.5	56.3±0.4
CF3	99.4±0.6	105.9±0.5	-36.3±0.9	0.339±0.6	72.9±3.3
CF4	99.5±0.5	169.1±0.9	-28.5±3.3	0.269±0.9	55.9±0.7
CF5	99.7±0.4	119.1±0.5	-20.8±0.7	0.249±0.7	74.9±0.4
CF6	99.9±0.5	105.4±0.7	-23.5±0.9	0.305±0.5	75.9±0.6
CF7	94.6±0.4	137.3±0.8	-48.9±0.9	0.376±0.6	61.9±0.5
CF8	99.3±0.9	119.6±0.9	-45.7±0.38	0.274±0.9	52.1±0.9
CF9	99.6±0.5	93.9±0.5	-30.7±0.8	0.185±0.7	97.6±0.2

Table 8. Characterization of SLN prepared with ethyl oleate

Batch	% Drug content	Size(nm)	Zetapotential(mV)	PDI	%EE
EF1	99.0±0.6	195.6±0.5	-23.5±0.6	0.319±0.6	62.6±0.8
EF2	99.6±0.5	207.8±0.6	-20.3±0.4	0.365±0.4	54.8±0.7
EF3	101.7±0.6	214.8±0.4	-43.9±3.0	0.269±0.6	79.8±0.6
EF4	99.0±0.7	136.3±0.9	-24.9±0.6	0.266±0.9	55.7±0.9
EF5	99.5±0.5	215.9±0.5	-39.5±0.9	0.309±0.7	79.9±0.6
EF6	99.1±0.7	171.9±0.6	-35.9±0.6	0.329±0.4	82.7±0.7
EF7	98.9±0.5	165.9±0.2	-36.9±1.4	0.199±0.1	67.9±0.4
EF8	99.7±0.4	131.9±0.7	-39.6±2.9	0.309±0.7	59.3±0.5
EF9	99.5±0.6	96.9±0.5	-30.9±0.7	0.271±0.9	97.7±2.3

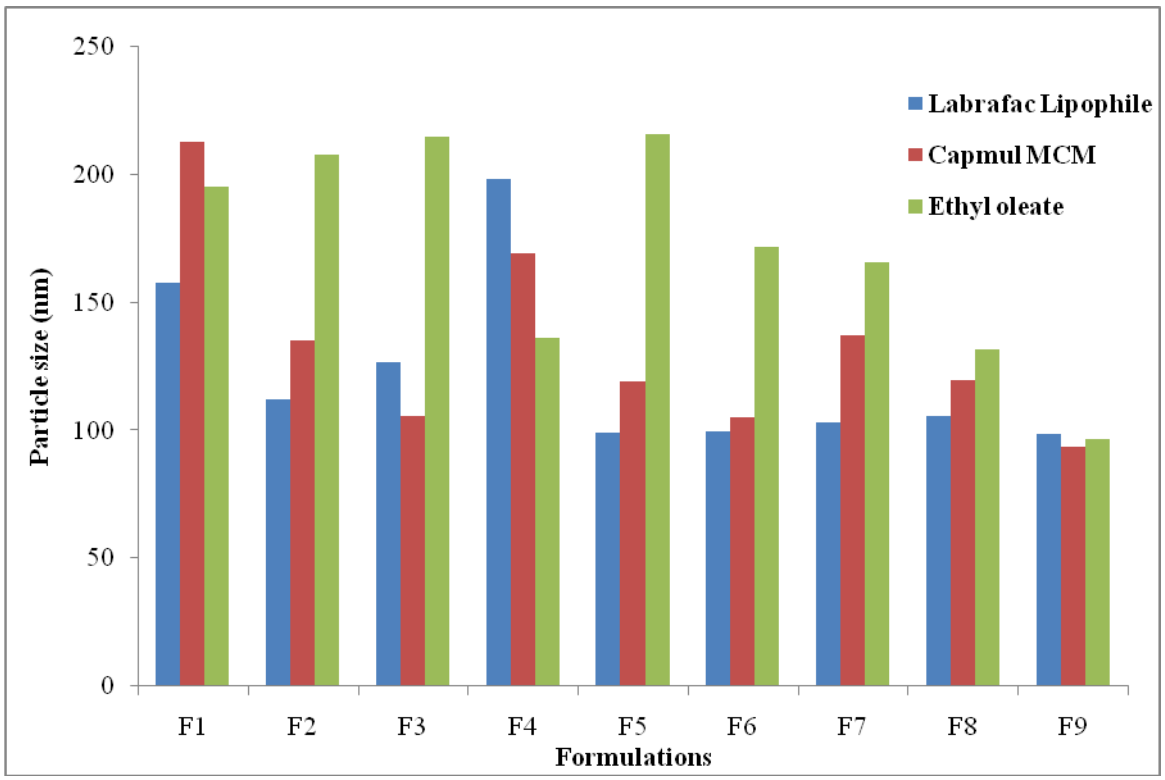


Figure 4. Comparison of particle size of SLNs prepared by different Phospholipids

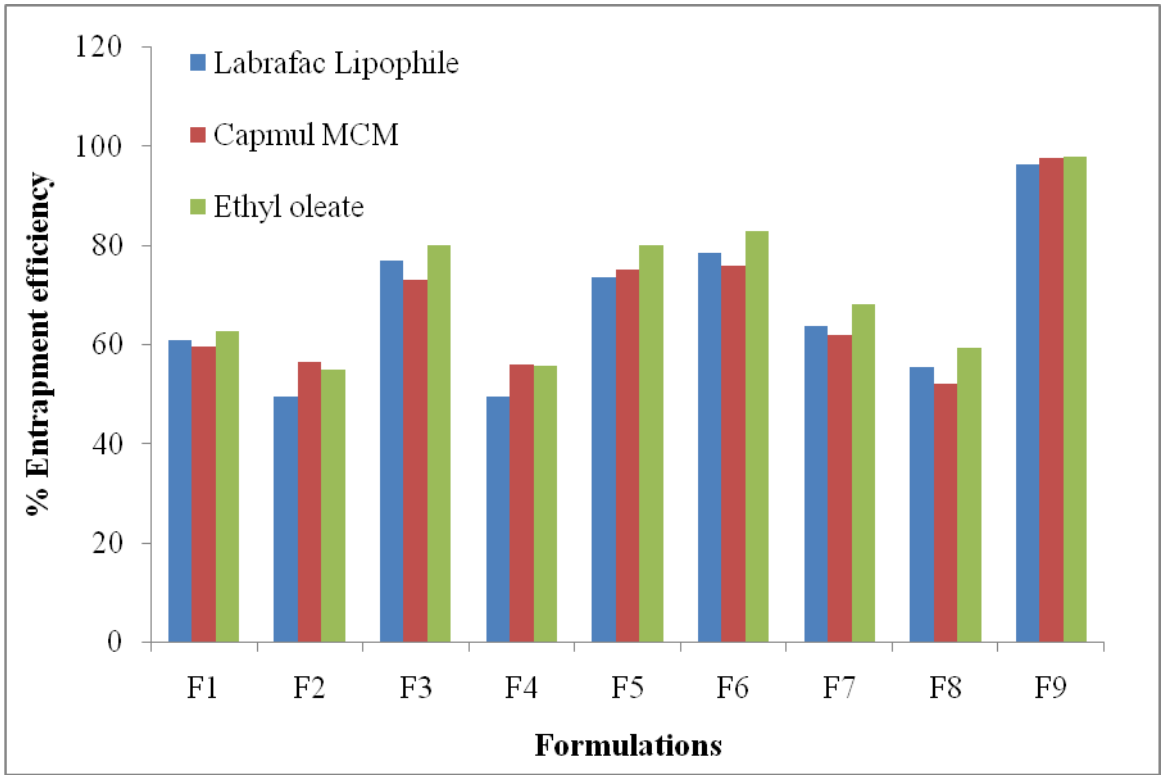


Figure 5. Comparative % EE of SLN formulations

Table 9. Observed responses of Darunavir loaded Labrafac Lipophile based SLN

Batch	Poloxamer 188	Tween 80	Size(nm)	Zeta potential (mV)	%EE
DLF1	-1	-1	156.3±0.4	-26.1±0.4	59.3±0.6
DLF2	-1	0	111.4±0.5	-22.4±0.3	47.3±0.9
DLF3	-1	+1	125.3±0.7	-29.5±3.8	75.9±0.8
DLF4	0	-1	210.3±0.9	-35.2±0.4	53.1±0.2
DLF5	0	0	101.1±0.6	-30.3±0.6	74.2±0.2
DLF6	0	+1	102.4±0.7	-34.1±0.7	78.5±0.4
DLF7	+1	-1	103.9±0.6	-37.1±0.2	65.1±0.9
DLF8	+1	0	107.9±0.1	-36.2±0.4	54.0±0.8
DLF9	+1	+1	98.4±0.4	-40.3±0.7	94.1±0.7

Table 10. Observed responses of Darunavir loaded Capmul MCMSLN

Batch	Poloxamer 407	Cremophor EL	Size (nm)	Zeta potential (mV)	%EE
DEF1	-1	-1	193.8±0.6	-23.6±0.9	62.4±0.7
DEF2	-1	0	208.5±0.1	-20.7±0.2	54.8±0.5
DEF3	-1	+1	213.7±0.9	-35.3±3.1	80.5±1.1
DEF4	0	-1	139.5±0.8	-24.3±0.7	56.4±0.7
DEF5	0	0	213.7±0.3	-39.3±1.0	78.5±1.1
DEF6	0	+1	170.9±0.5	-35.3±0.6	82.9±2.1
DEF7	+1	-1	167.5±0.6	-36.6±1.7	67.1±0.5
DEF8	+1	0	131.9±0.4	-38.5±2.2	58.9±0.7
DEF9	+1	+1	96.5±0.7	-44.3±0.7	97.5±0.8

Table 11. Observed responses of Darunavir loaded Ethyl oleate SLN

Batch	Poloxamer 188	Tween 80	Size(nm)	Zeta potential (mV)	%EE
DCF1	-1	-1	212.7±0.6	-18.0±1.5	53.9±0.6
DCF2	-1	0	133.6±0.4	-23.6±0.5	51.3±0.4
DCF3	-1	+1	104.7±0.9	-34.1±3.0	71.2±11
DCF4	0	-1	164.3±0.7	-26.4±1.3	55.1±0.5
DCF5	0	0	112.9±0.2	-19.2±0.5	69.2±0.7
DCF6	0	+1	102.7±0.6	-23.0±0.1	75.3±0.6
DCF7	+1	-1	139.1±0.5	-33.6±0.2	61.3±0.4
DCF8	+1	0	116.3±0.1	-25.3±1.1	52.2±0.1
DCF9	+1	+1	93.5±0.6	-41.9±1.7	95.3±0.6

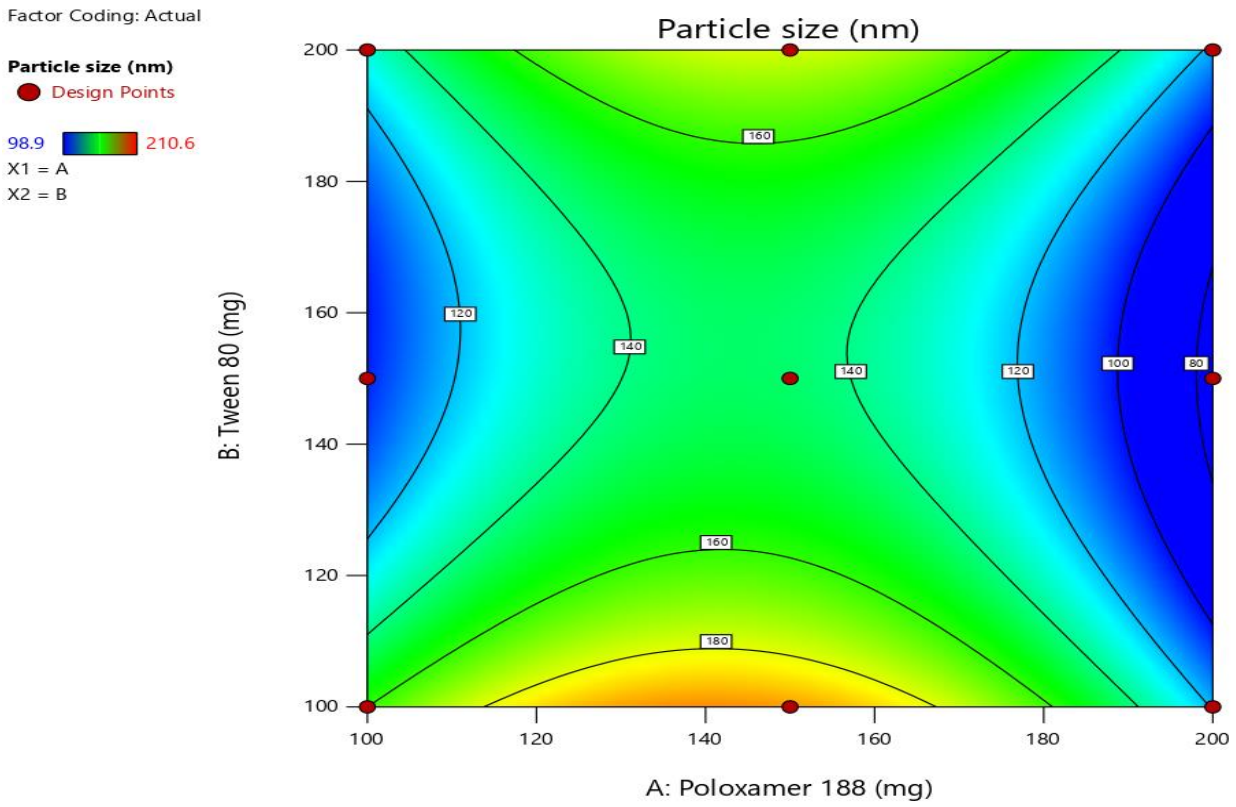
Table 12. Regression analysis summary of Labrafac Lipophile Based SLN for responses of Y1, Y2 and Y3

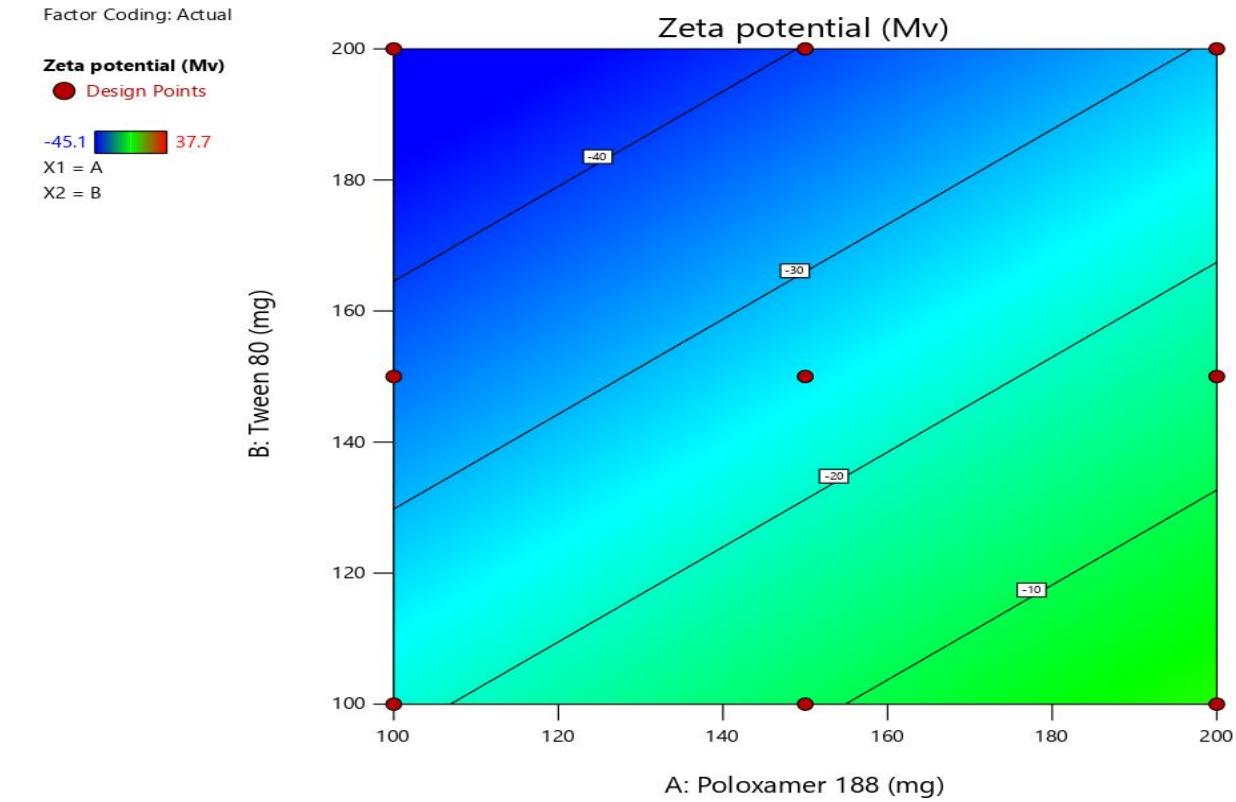
Model R^2 Adjusted R^2	R^2	Adjusted R^2	Predicted R^2	PRESS	S.D	F-value	p-value	Remarks
Response Y_1 (Particle size(nm)=+116.33-13.41*A-24.33*B								
Linear	0.3833	0.2427	-0.1643	14246.9	29.03	2.57	0.1164	**Suggested**
Interactive	0.3957	0.1685	-0.6552	20248.5	30.4	5.21	0.0416	
Quadratic	0.6409	0.3409	-2.3535	41017.1	27.08	2.33	0.2101	
Response Y_2 (Zeta Potential mV)=-30.31-2.15*A-1.87*B+6.55*AB								
Linear	0.1268	-0.0673	-1.1830	837.4	6.10	4.33	0.5433	**Suggested**
Interactive	0.570	0.4097	-0.5535	595.9	4.54	24.69	<0.001	
Quadratic	0.741	0.5261	-1.6651	1022.4	4.07	6.37	0.2183	
Response Y_3Entrapment efficiency (%)= +15.50+0.10663*A+0.23667*B								
Linear	0.5466	0.4459	0.1473	1690.0	9.99	2.39	0.0285	**Suggested**
Interactive	0.6873	0.1400	-35.966	73263.1	12.45	3.21	0.9453	
Quadratic	0.6784	0.4103	-1.1821	21	4324.668	5.23	0.4036	

Table 13. Model parameters (ANOVA) for the dependent responses of the Labrafac Lipophile based SLN

SS: Sum of squares; Df: Degrees of freedom; MS: Mean sum of squares

Source	SS	df	MS	p-value	Significance
Response Y_1					
Model	4648.2	2	2324.1	0.1164	NS
X_1	1134.3	1	1134.3	0.2758	NS
X_2	3513.8	1	3513.8	0.715	NS
Residual	7584.3	9	842.7		
Total	12232.5	11			
Response Y_2					
Model	218.9	3	72.9	0.0676	NS
X1	27.7	1	27.7	0.2792	NS
X2	20.9	1	20.9	0.3431	NS
X1X2	170.3	1	170.3	0.0206	NS
Residual	170.3	1	20.5		
Total	164.6	8			
Response Y_3					
Model	1083.3	2	541.6	0.0285	S
X1	170.6	1	171.6	0.2235	NS
X2	912.6	1	912.6	0.0144	S
Residual	898.5	9	99.8		
Total	1981.8	11			


Factor Coding: Actual
Particle size (nm)
Design Points
98.9
210.6
X1 = A
X2 = B
Particle size (nm)
B: Tween 80 (mg)
A: Poloxamer 188 (mg)
200
180
160
140
120
100
160
120
140
140
120
100
80
160
180


Factor Coding: Actual
Zeta potential (Mv)
Design Points
-45.1
37.7
X1 = A
X2 = B
Zeta potential (Mv)
B: Tween 80 (mg)
A: Poloxamer 188 (mg)
200
180
160
140
120
100
-40
-30
-20
-10

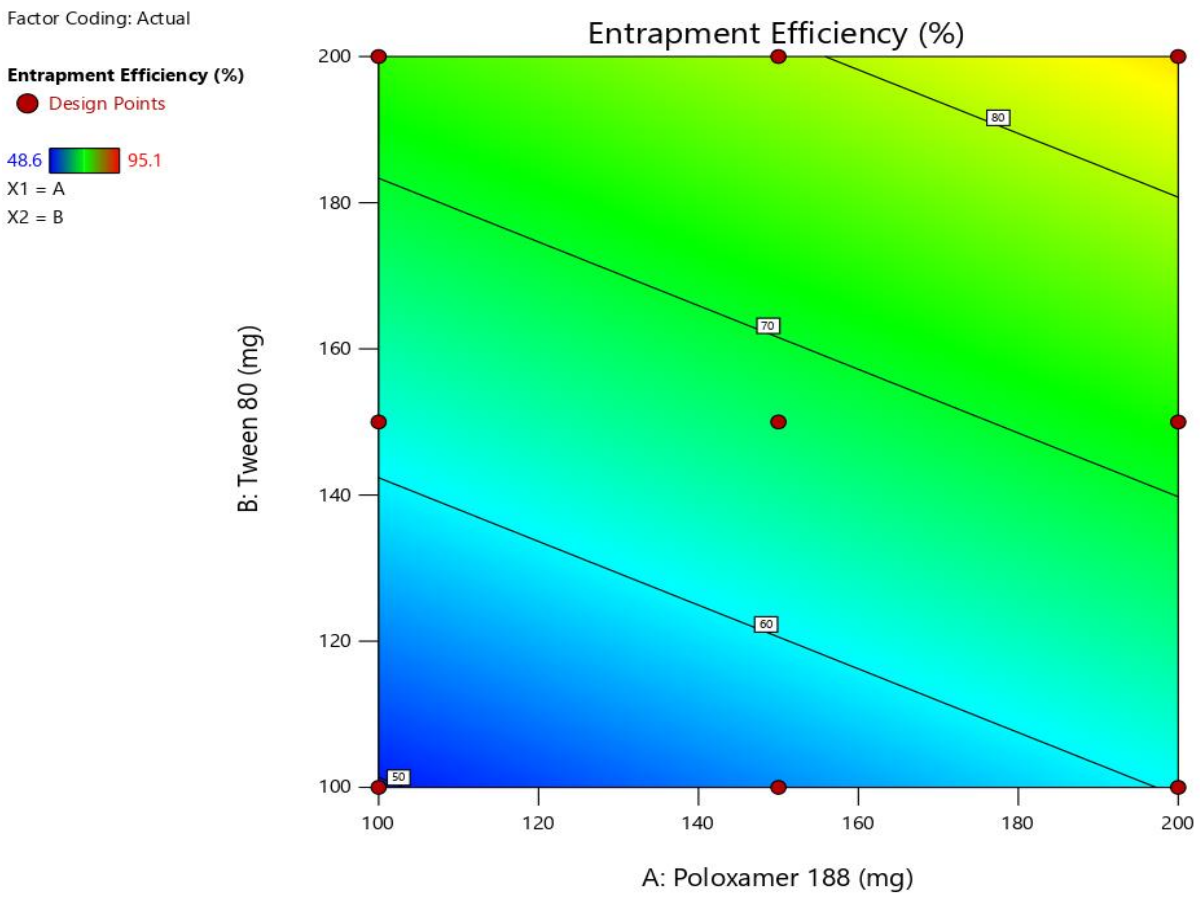
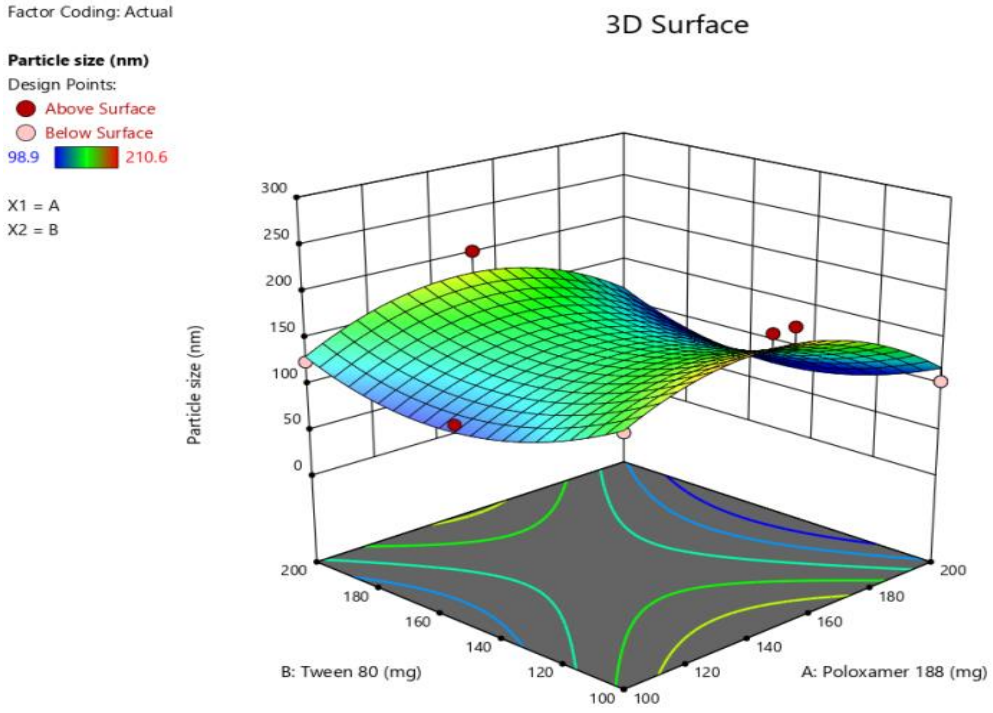
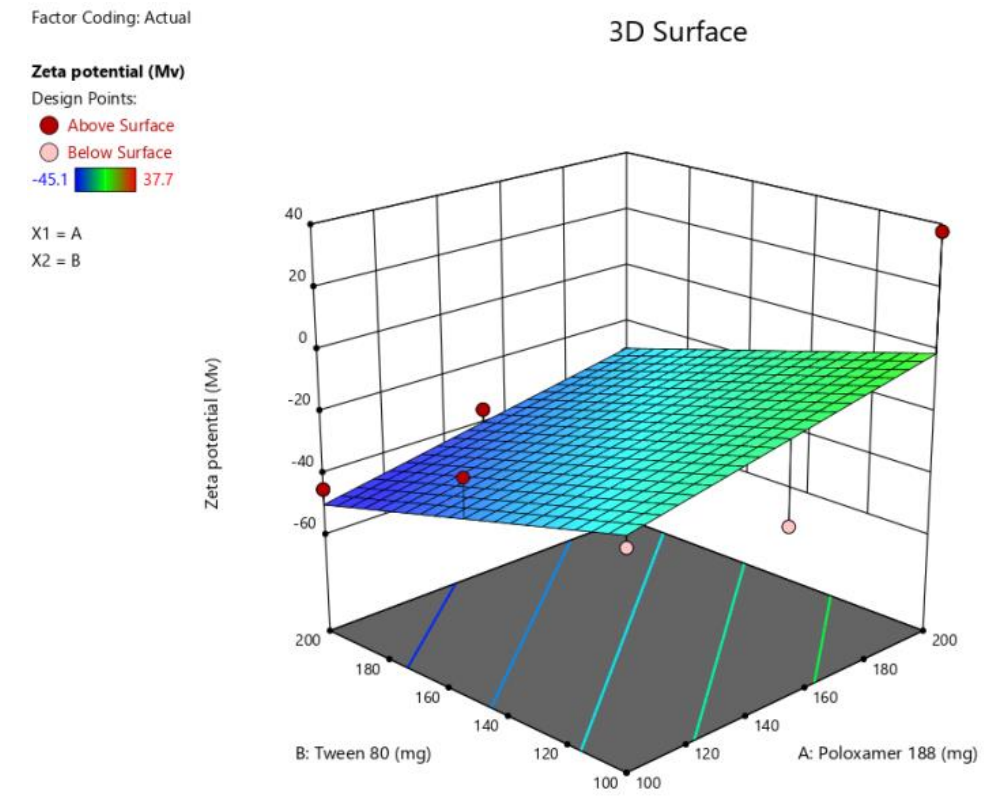


Figure 6. Contour plots for the effects of poloxamer 188 (X1) and Tween 80 (X2) on particle size (Y1), zeta potential (Y2) and %entrapment efficiency (Y3) in Labrafac Lipophile based SLN





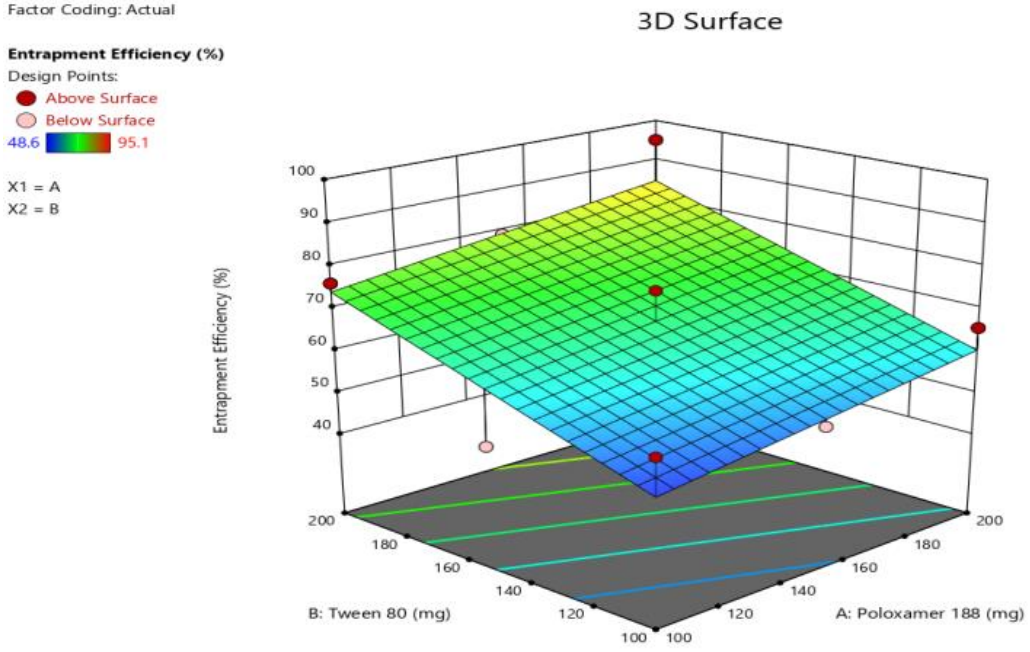


Figure 7. Response surface plots for the effects of poloxamer 188 (X1) and Tween 80 (X2) on particle size (Y1), Zeta potential (Y2) and % entrapment efficiency (Y3) in Labrafac Lipophile based SLN

Table 14. Optimized formulation of SLNs prepared.

Base	Poloxamer 188	Tween 80
Labrafac Lipophile	200mg	200mg
Capmul MCM	200mg	200mg
Ethyl oleate	200mg	200mg

Table 15. Appearance and melting point of Darunavir

Drug	Parameter	Reported	Observed
Darunavir	Appearance	White to off white	White to off white
	Odour	None	None
	Melting point	98-100^{0}c	99^{0}c

From the above results obtained the observed values have not shown any deviations from the reported values.

Table 16. Solubility of drug in various solvents

Solvent	Darunavir Solubility
DMSO	10mg/ml
Ethanol	5 mg/ml
Water	0.15mg/ml

It was found that Darunavir is having highest solubility in DMSO.

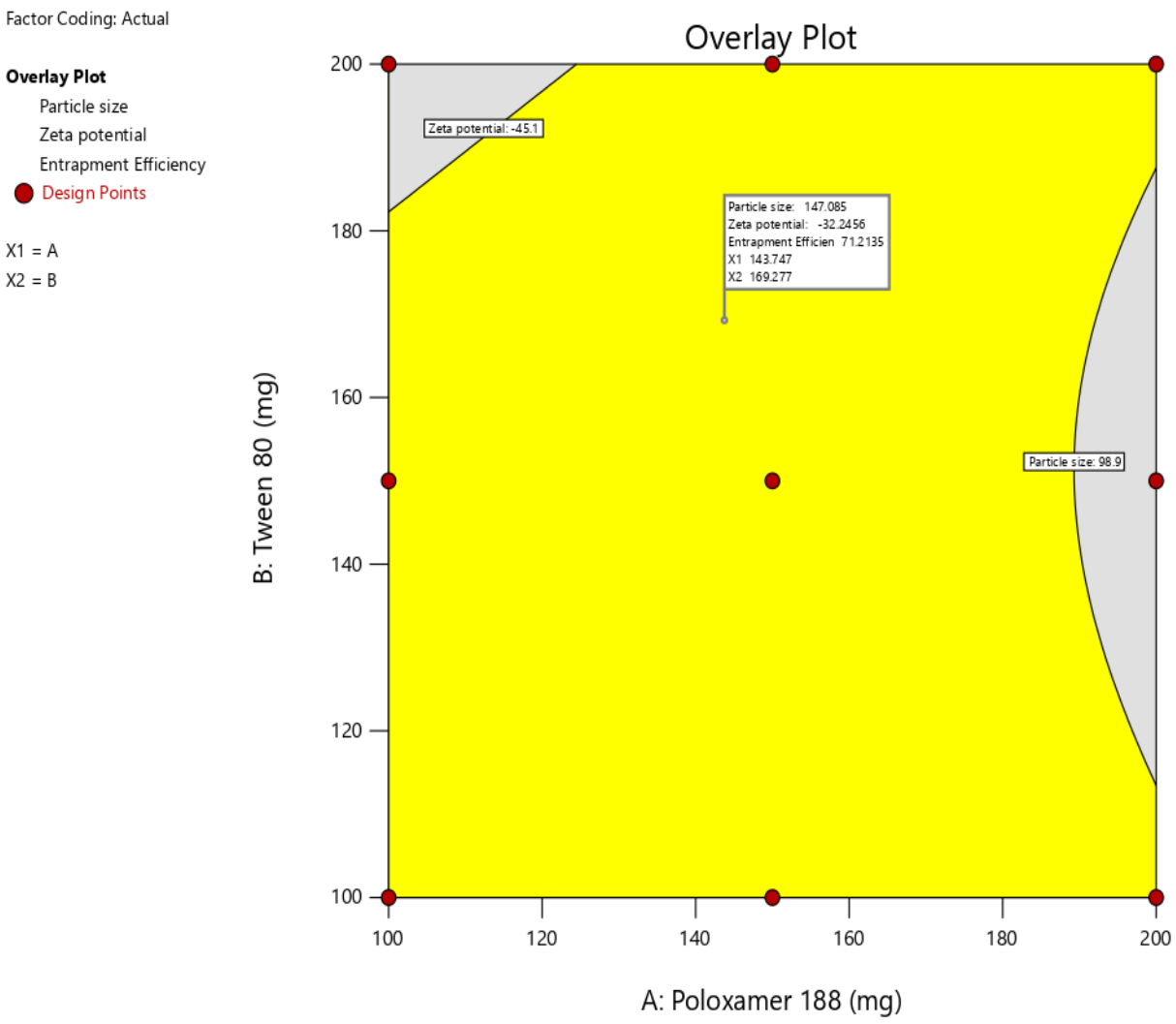


Figure 8. Desirability and overlay plots of Labrafac Lipophile based SLN

Table 17. Formulation, statistical optimization and in vivo evaluation of DARUNAVIR loaded solid lipid nanoparticles

Parameters	Reference	DLF9
C_{max}(ng/ml)	143.1±0.32	300.51±0.02
T_{max}(hr)	0.5	1
AUC_{0-t}(ng h/ml)	414.3±0.32	1988.64±0.51
$AUC_{t-\infty}$(ng h/ml)	429.3±0.09	2060±0.01

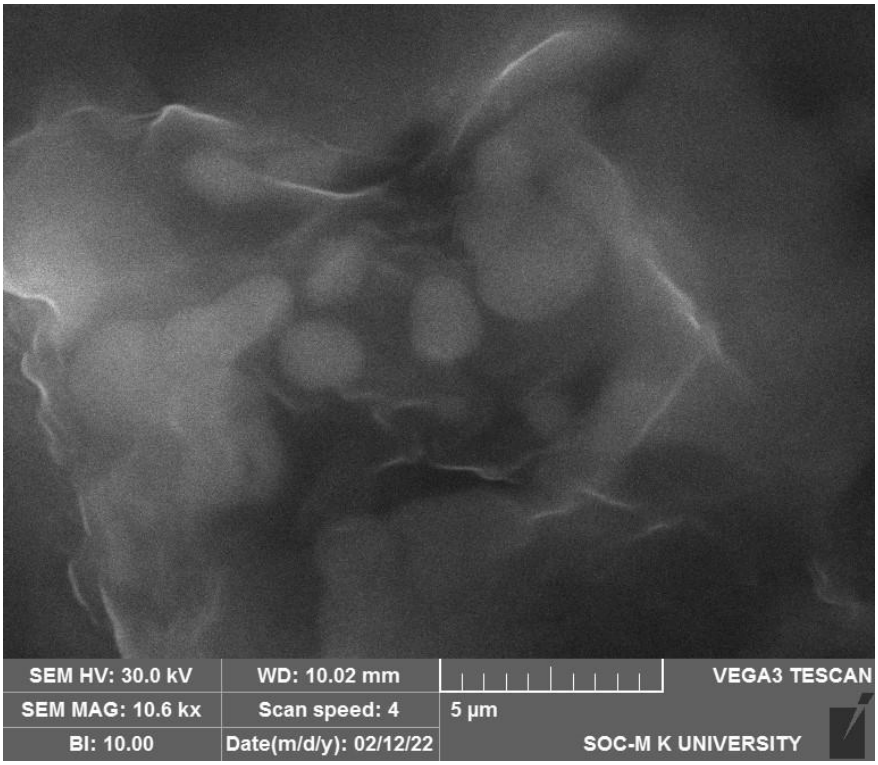
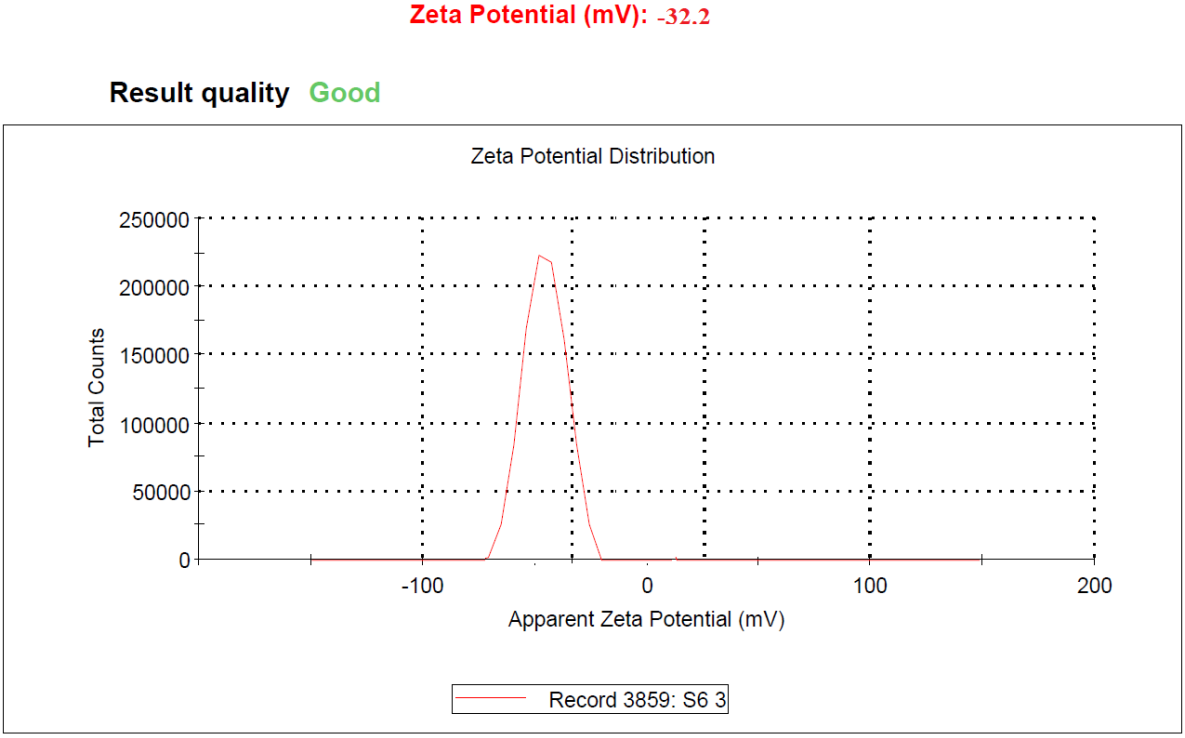


Figure 9. SEM OF Labrafac Lipophile based DLF9 formulation



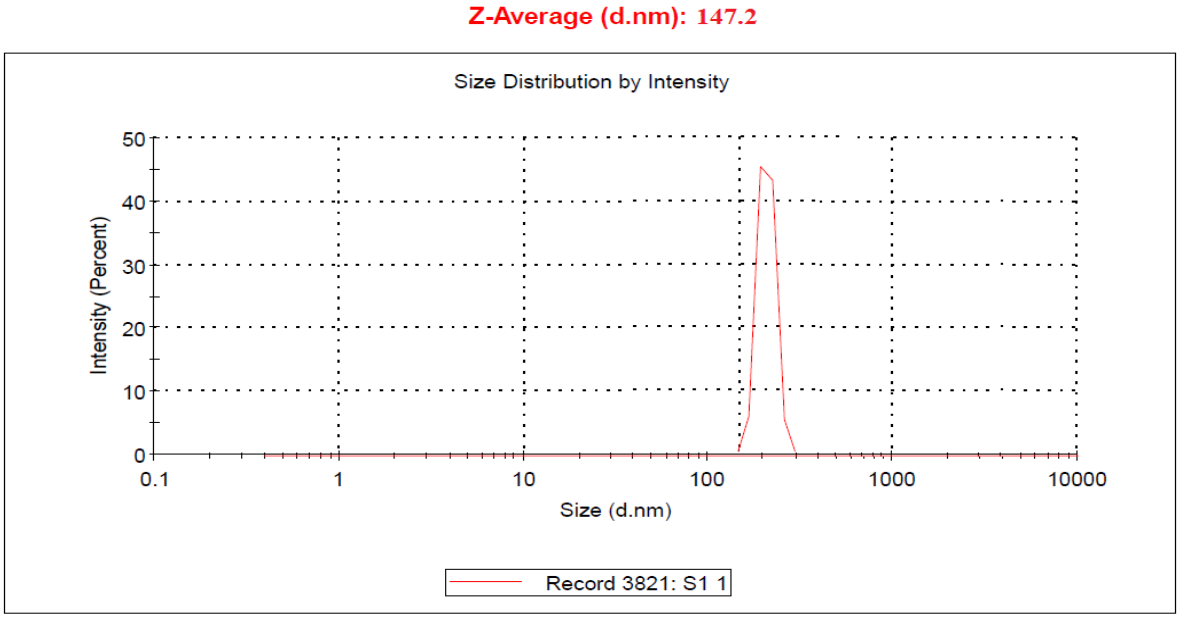


Figure 10. Zeta potential and particle size of optimized formulation

From the above Table 14, the optimized SLN contain 200 mg of poloxamer 188 and 200 mg of Tween 80 for Labrafac, Lipophile, Capmul MCM, and Ethyl oleate based SLN formulations. 400 mg of Darunavir and 400 mg of phospholipids were kept constant in all the formulations. The prepared optimized formulations were found to be of good quality fulfilling all the requirements of nanoparticles.

3.8. SEM

SEM for the Labrafac Lipophile based DLF9 formulation was performed to elucidate the surface morphology as shown in Figure 9. The SLN obtained were in nanometer-size with well-defined periphery at 10.6KX magnification. The size of the solid lipid nanoparticles was found to be in agreement with the Malvern Zetasizer particles size distribution for the selected sample.

3.9. Zeta potential and Particle size

The optimized formulation DLF9 was taken and studied for Zeta potential and Particle size it was observed that the zeta potential was -32.2 mV and particle size was found to be 147.2 nm. This was similar to overlay plot obtained for Labrafac Lipophile. Hence, this was considered for further *in vivo* studies (Figure 10).

3.10. Pharmacokinetic evaluation of Darunavir SLNs

The pharmacokinetic parameters including C_{max}, T_{max}, AUC_{0-t} $AUC_{0-\infty}$ and plasma half-life ($t_{1/2}$) were analyzed by Kinetica software 5.0 version and the results obtained are given in Table V. There was a significant increase in C_{max}($P<0.05$) upon nanoparticle administration in comparison to reference. There were 2.1-fold increase in $C_{max}$of DLF9 respectively in comparison to reference. Similarly, there was significant increase in $AUC_{0-\infty}$ for SLN formulations ($P<0.05$) in comparison to reference. The $AUC_{0-\infty}$ increased by 4.8 foldfor DLF9 respectively relative to reference.

4. Conclusions

From the experimental results obtained, it was observed that DLF9 was best formulation consisting of 200 mg of poloxamer 188 and 200 mg of Tween 80 and comprises of Labrafac Lipophile base. The formed SLNs were having enhanced bioavailability, and this can be proved through the 2.1 fold increase in C_{max}, $AUC_{0-\infty}$ increased by 4.8 fold when compared to reference. The method used for preparation of Darunavir loaded SLN were hot homogenization technique followed by ultra-sonication method. Factorial design was the statistical method used for optimization and this reduced the number of experiments that were carried out for obtaining formulations with

required properties. The polynomial equations obtained response and contour plots helped in understanding the values of selected independent variables for preparation of optimum SLN with desired properties. Optimized Solid lipid nanoparticles is having minimum particles size, optimum zeta potential and higher percentage of entrapment efficiency. Thus, by using experimental design and optimization techniques, darunavir loaded solid lipid nanoparticles with enhanced bioavailability was successfully developed.

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