1 EXPERIMENTAL: TRIAMCINOLONE ACETONIDE

1.1 MATERIALS

Triamcinolone acetonide (TA) was purchased from Sigma (St. Louis, EUA). The lipids, dipalmitoylphosphatidylcholine (DPPC) and cholesterol were obtained as gift sample from Lipoid KG (Ludwigshafen, Germany). Glyceryl monostearate (GMS) was purchased from Loba chemie, Mumbai, India. Nile red was purchased from Sigma Aldrich, USA. Chloroform (HPLC Grade) and Methanol (HPLC Grade) were purchased from Merck, Mumbai, India. Potassium dihydrogen phosphate, sodium hydroxide and all other analytical reagents were obtained from S.D. fine-chem limited, Baroda, India. All the reagents used were of analytical grade. Cellulose dialysis tubing (Molecular weight cut of 14000; pore size 0.4nm) and membrane filter of pore size 0.2 μm were purchased from Himedia Lab, Mumbai, India. Distilled water used in the study was filtered using 0.22-lm nylon filter (Nylon N66 membrane filters 47 mm, Rankem, Bangalore, India).

1.2 EQUIPMENTS

Analytical weighing balance (Shimadzu, Switzerland)

Rotary Evaporator with thermostatically controlled water bath (Superfit Equipments, India)

Probe Sonicator (Labsoic ®M, Sartorius Ltd, Mumbai, India)

Ultrasonic Bath Sonicator (Ultrasonics Selec, Vetra, Italy)

Particle size Analyzer (Zeta sizer Nano series, Malvern Instruments, UK)

Cyclomixer (Spinix, Mumbai)

Shimadzu 1601 UV-VIS Spectrophotometer (Shimadzu, Kyoto, Japan)

High Pressure Liquid Chromatography (Shimadzu, Kyoto, Japan)

Lyophilizer (Heto Drywinner, Denmark)

Differential Scanning Calorimeter (Mettler Toledo DSC 822e, Japan)

Transmission Electron Microscope (Morgagni, FEI Company, USA)

1.3 METHODS

1.3.1 Preparation of triamcinolone acetonide emulsomes

Various batches of triamcinolone acetonide (TA) loaded emulsomes were prepared by thin film hydration method as described by (Amselem et al., 1994) with slight modification as per laboratory set up. The size and entrapment efficiency of emulsomes was monitored and recorded at each stage of sonication by particle size Analyzer (Zeta sizer Nano series, Malvern Instruments, UK).

For ocular distribution studies, dye loaded emulsomes were prepared by replacing the drug with Nile Red in the aforementioned procedure. Nile red loaded emulsomes were then separated from free Nile red by centrifugation and finally these seperated emulsomes were characterized for particle size and utilized immediately in experiments.

1.3.2 Preliminary optimization of process parameters:

Preliminary optimization of process parameters for TA emulsomes was performed as per the reported methods and protocols given in section 4.3.2 of chapter 4. Following preliminary parameters were selected for the preparation of TA emulsomes (Table 5.1):

Table 5.1 Selection of preliminary process parameters for TA emulsomes

Process Parameters	Levels
Vaccum	350 mm Hg
Speed of rotation	120 rpm
Hydration time	2 hr
Speed of rotation during hydration	70 rpm

DPPC: GMS ratio- 1:1, Lipid: drug ratio- 20:1 and Sonication time-6 Min

1.3.3 Optimization by Box Behnken Design

Quantitative aspects of the effects and relationships among various formulation variables of emulsomes were investigated using Response Surface Methodology (RSM).

Box-Behnken Design with a total of 17 experimental runs was selected to optimize the various process parameters at three levels (low, medium, and high, coded as –1, 0, and +1). DPPC: GMS ratio (A), Lipid: drug ratio (B) and sonication time (C) were taken as independent variables and their effect was studied on size (Y_1) and % entrapment (Y_2) which were taken as dependent variables. The Design-Expert software (version 8.0.7,1 State-Ease Inc., Minneapolis, USA) was used for design of experiment and analysis of second-order model and for drawing of three dimensional response surface and contour plots. The optimized batch was selected on the basis of desirability criteria. Following formula was used to calculate the % prediction error:

$$\% \text{ Prediction error} = \frac{\text{Actual value} - \text{predicted value}}{\text{Actual value}} \times 100$$

The level and code of variables considered in this study are shown in Table 5.2.

Table 5.2 Variables in Box Behnken Design for triamcinolone acetonide emulsomes

Independent Variables	Units	Coded Values			Response (Y1)	Response (Y2)
		-1	0	1		
DPPC: GMS ratio (A)	Weight ratio	0.5: 1	1:1	1.5: 1	Particle Size (nm)	Entrapment Efficiency (%)
lipid: drug ratio (B)	Weight ratio	10:1	20:1	30:1		
Sonication time (C)	Minutes	3	6	9		

1.3.4 Selection of cryoprotectant for lyophilization of emulsomes

The optimized TA loaded emulsomes was lyophilized using lyophilizer (Heto Drywinner, Germany) as per the method described in section 4.3.4 of chapter 4.

1.3.5 Characterization of emulsomes

1.3.5.1 Determination of particle Size (PS) and Zeta Potential (ζ)

Mean PS and ζ of traimcinolone acetonide loaded emulsomes was determined by using Photon Correlation Spectroscopy with a Malvern Zetasizer NanoZS (Malvern Instruments, Malvern,UK). For PS and ζ , analysis (n = 3) was carried out for 100 s and 60s resp. at room temperature by keeping angle of detection at 90̊.

1.3.5.2 Entrapment Efficiency (EE)

The entrapment efficiency of triamcinolone acetonide in emulsomes was determined by centrifugation technique (Araujo et al., 2011). Finally, entrapment efficiency was calculated by the following formula:

$$\%\, EE = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug added to emulsome formulation}} \times 100$$

In lyophilized TA emulsomes, drug content was determined by dissolving 2mg of obtained lyophilized powder in chloroform: methanol (1:9) and the samples were then analyzed by UV spectrophotometer at 239 nm, after suitable dilutions.

1.3.5.3 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry studies of drug, lipids and lyophilized emulsomes (without cryoprotectant) were carried out (DSC-60, Shimadzu, Japan) in order to define the physical state of drug in emulsomes and possibility of interaction between the drug and excipients within the vesicles. Each sample was sealed in standard aluminum pans with lids and purged with air at a flow rate of 40 ml/min. Temperature ramp speed was set at 20ºC /min, and the heat flow was recorded in the range of 30–300 ºC under inert nitrogen atmosphere. Thermograms were taken for TA, GMS, DPPC and lyophilized TA emulsomes (without cryoprotectant).

1.3.5.4 Transmission electron microscopy (TEM)

TEM analysis of the prepared formulation was carried out to understand the morphology of emulsomes. A drop of emulsomes containing 0.01% of phosphotungstic acid was placed on a carbon film coated on a copper grid. TEM studies were performed at 100 kV using, Morgagni Transmission Electron Microscope 268 (D) (FEI Company,

USA). The copper grid was fixed into sample holder and placed in vacuum chamber of the transmission electron microscope and observed under low vacuum, and TEM images were recorded.

1.3.5.5 *In vitro* release study

In vitro release of TA from emulsomes was evaluated by the dialysis bag diffusion technique reported by Suen et al., 2013. All the experiments were performed in triplicate, and the average values were taken. TA solution (350µg/ml) prepared in PBS (pH 7.4) was used as a control.

1.3.5.6 *Ex vivo* study

Triamcinolone acetonide loaded emulsomes were evaluated for corneal permeation characteristics using the isolated goat cornea model as per method described in section 4.3.5.6 of chapter 4. 500 µl (drug equivalent to 87.5µg) of formulation was placed over the cornea and 500µl of aliquot sample was withdrawn at the end of every hour and analyzed for drug content by previously described HPLC method.

The apparent permeation coefficient (Papp, cm/s) of TA was determined by reported method of Shen et al., 2007

$$Papp\ Q = \frac{\Delta Q}{\Delta t} X \frac{1}{AC0} X \frac{1}{60} X\ 10{,}000{,}00$$

Where, CD_0 is the initial concentration of drug in the donor compartment, and A is the area of the cornea. For the calculation of the apparent permeation coefficient in the present study, A was determined as 0.941 ± 0.26 cm^2. $\Delta\ Q\ /\ \Delta\ t$ is the steady-state rate of drug permeation across the intact cornea, as obtained from the slope of the straight line relating corneal permeability to time.

1.3.5.7 Stability studies

The stability studies were performed for the lyophilized TA loaded emulsomes. The samples were kept in transparent glass vials and stored at refrigerated conditions (2-8°C) and at room temperature (25-30°C). At different time points the samples were withdrawn and analyzed for particle size and drug content study.

1.4 RESULTS AND DISCUSSION

1.4.1 Optimization of Emulsomes

1.4.1.1 Box Behnken Design

Various batches of TA emulsomes were prepared according to Box Behnken Design by varying three independent variables DPPC: GMS ratio (A), lipid: drug ratio (B) and sonication time (C). The design matrix of the variables in the coded units along with the results of response variables (size and EE) obtained from each batch is shown in Table 5.3.

Obtained data of dependent variables (size and entrapment efficiency) were subjected to multiple regression analysis to yield a second- order polynomial equation (full model), using Design Expert software. This second-order polynomial model helps in relating the responses to selected variables. The data of PS and EE were fitted into equation (1):

$$Y=\beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} AA + \beta_{22} BB + \beta_{33} CC \ldots (1)$$

where Y represents the measured responses (dependent variable), A,B and C were the coded values of independent variables, β_0 is the intercept coefficient, β1, β2 and β3 are the linear coefficients, β11, β22 and β33 are the squared coefficients, and β12, β13 and β23 are the interaction coefficients.

Table 5.3 Box Behnken design matrix of triamcinolone acetonide loaded emulsomes

Std.	Run	DPPC:GMS ratio (A)	Lipid:drug Ratio (B)	Sonication Time (C)	Size (nm) Y1	Entrapment Efficiency (%) Y2
10	1	0	1	-1	141.01±2.98	80.17±3.47
3	2	-1	1	0	127.66±4.32	67.10±4.66
5	3	-1	0	-1	129.65±4.98	69.52±4.01
7	4	-1	0	1	116.05±6.55	57.91±3.98
11	5	0	-1	1	112.74±6.34	56.83±5.54
17	6	0	0	0	134.14±3.98	72.92±5.01
2	7	1	-1	0	121.64±5.76	60.81±6.12

14	8	0	0	0	134.54±3.76	72.82±4.89
9	9	0	-1	-1	131.93±3.65	68.80±2.89
13	10	0	0	0	134.44±4.12	72.51±4.34
8	11	1	0	1	129.64±2.47	64.22±3.12
16	12	0	0	0	134.14±3.12	72.54±3.98
4	13	1	1	0	137.95±3.54	75.91±5.00
12	14	0	1	1	136.39±2.19	68.70±5.12
15	15	0	0	0	134.74±4.43	72.91±4.12
6	16	1	0	-1	136.85±3.22	76.92±4.89
1	17	-1	-1	0	110.74±7.01	58.11±6.12

Finally, two equations were obtained for PS and EE:

$Y_1=134.40+5.12A+8.43B-5.51C-0.15AB+1.85AC+4.02BC-6.25A^2-3.65B^2+0.15C^2$... (2)

$Y_2=72.74+3.15A+5.92B-5.97C+1.53AB-0.27AC-0.13BC-4.37A^2-2.89B^2-1.23C^2$.... (3)

Positive and negative sign in front of the terms indicates synergistic and antagonistic effect, respectively. The results of ANOVA of the second-order

polynomial equation are given in Tables 5.4 and 5.5 for PS and EE, respectively.

The statistical model was checked by *F*-test, and the analysis of variance (ANOVA) for the response surface quadratic model. In Table 5.4 and 5.5, the Model *F*-values of 1536.63 and 643.91 for PS and EE, respectively implies that the model is highly significant. There was only a 0.01% chance that Model *F*-values this large (for PS and EE) could occur due to noise.

Table 5.4 ANOVA for the response surface quadratic polynomial model for size

Response	1	Size				
ANOVA for Response Surface Quadratic Model						
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		Mean	F	p-value	
Source	Squares	Df	Square	Value	Prob > F	
Model	1332.63	9	148.0706	1536.62	< 0.0001	Significant
A-PPC:GMS Ratio	209.920	1	209.920	2178.48	< 0.0001	

B-Lipid:Drug Ratio	569.025	1	569.025	5905.15	< 0.0001	
C-Sonication Time	243.211	1	243.211	2523.97	< 0.0001	
AB	0.09302	1	0.09302	0.96538	0.3586	
AC	13.6530	1	13.6530	141.686	< 0.0001	
BC	64.6416	1	64.6416	670.829	< 0.0001	
A^2	164.473	1	164.473	1706.85	< 0.0001	
B^2	56.1716	1	56.1716	582.930	< 0.0001	
C^2	0.09160	1	0.09160	0.95064	0.3620	
Residual	0.67452	7	0.09636			
Lack of Fit	0.40252	3	0.13417	1.97316	0.2602	not significant
Pure Error	0.272	4	0.068			
Cor Total	1333.30	16				

R^2=0.9995; adjusted-R^2=0.9988; predicted-R^2=0.9949 and Adequate precision=126.149.

Table 5.5 ANOVA for the response surface quadratic polynomial model for entrapment efficieny

Response	2	Entrapment Efficiency			
ANOVA for Response Surface Quadratic Model					
Analysis of variance table [Partial sum of squares - Type III]					
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	786.9278	9	87.43643	643.9126	< 0.0001	significant
A-DPPC:GMS Ratio	79.50605	1	79.50605	585.5105	< 0.0001	
B-Lipid:Drug Ratio	280.0161	1	280.0161	2062.137	< 0.0001	
C-Sonication Time	285.0078	1	285.0078	2098.898	< 0.0001	
AB	9.333025	1	9.333025	68.73167	< 0.0001	
AC	0.297025	1	0.297025	2.187396	0.1827	
BC	0.0625	1	0.0625	0.460272	0.5193	
A^2	80.408	1	80.408	592.1528	< 0.0001	
B^2	35.10592	1	35.10592	258.5323	< 0.0001	

C^2	6.344237	1	6.344237	46.72119	0.0002	
Residual	0.950525	7	0.135789			
Lack of Fit	0.789925	3	0.263308	6.558115	0.0504	not significant
Pure Error	0.1606	4	0.04015			
Cor Total	787.8784	16				

R^2=0.9988; adjusted-R^2=0.9972; predicted-R^2=0.9836 and Adeq. Precision=84.105.

1.4.1.2 Response surface plots

Three-dimensional response surface plots for PS and EE were generated by the Design Expert software and are presented in Figs. 5.1 and 5.2, for TA emulsomes respectively.

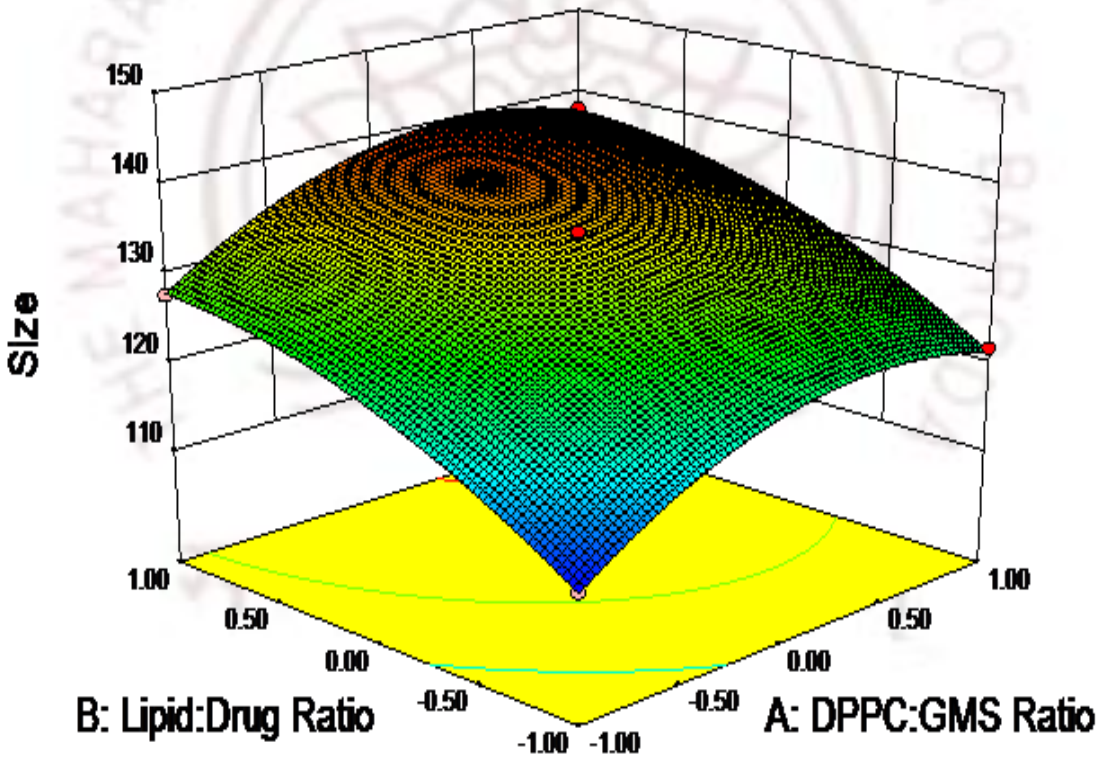


Fig 1.1 Three-dimensional surface plot for size of DPPC: GMS ratio vs lipid:drug ratio

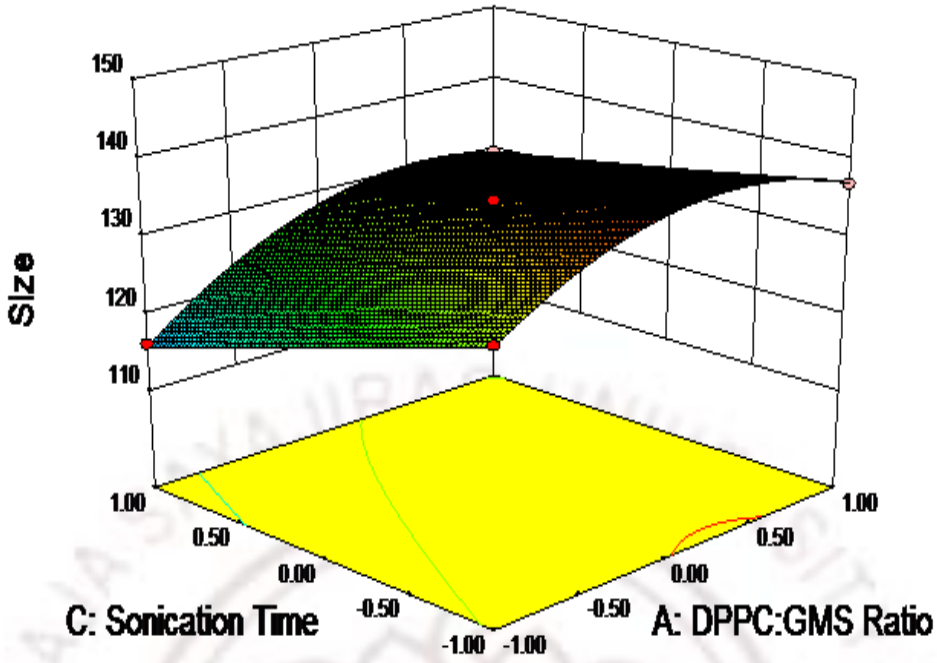


Fig 1.2 Three-dimensional surface plot for size of DPPC: GMS ratio vs sonication time

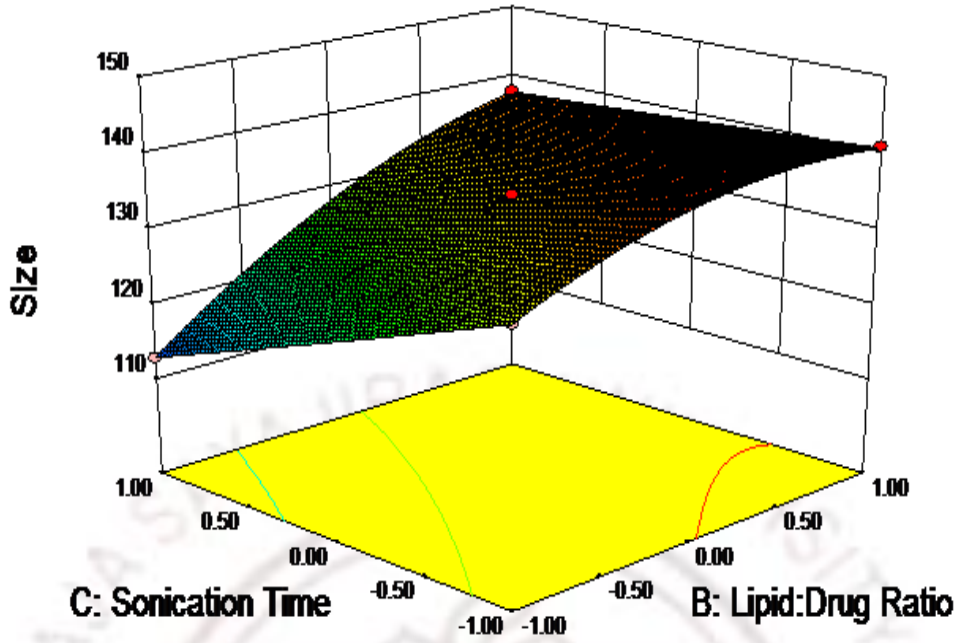
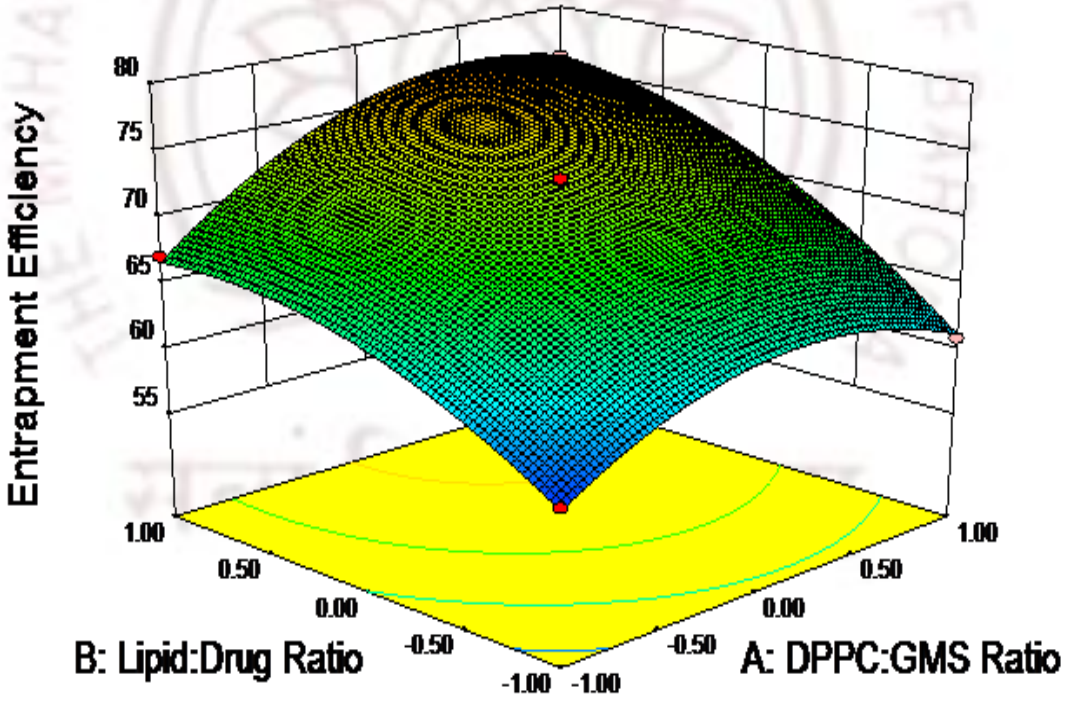


Fig 1.3 Three-dimensional surface plot for size of lipid: drug ratio vs sonication



time

Fig 1.4 Three-dimensional surface plot for entrapment efficiency of DPPC: GMS ratio vs lipid:drug ratio

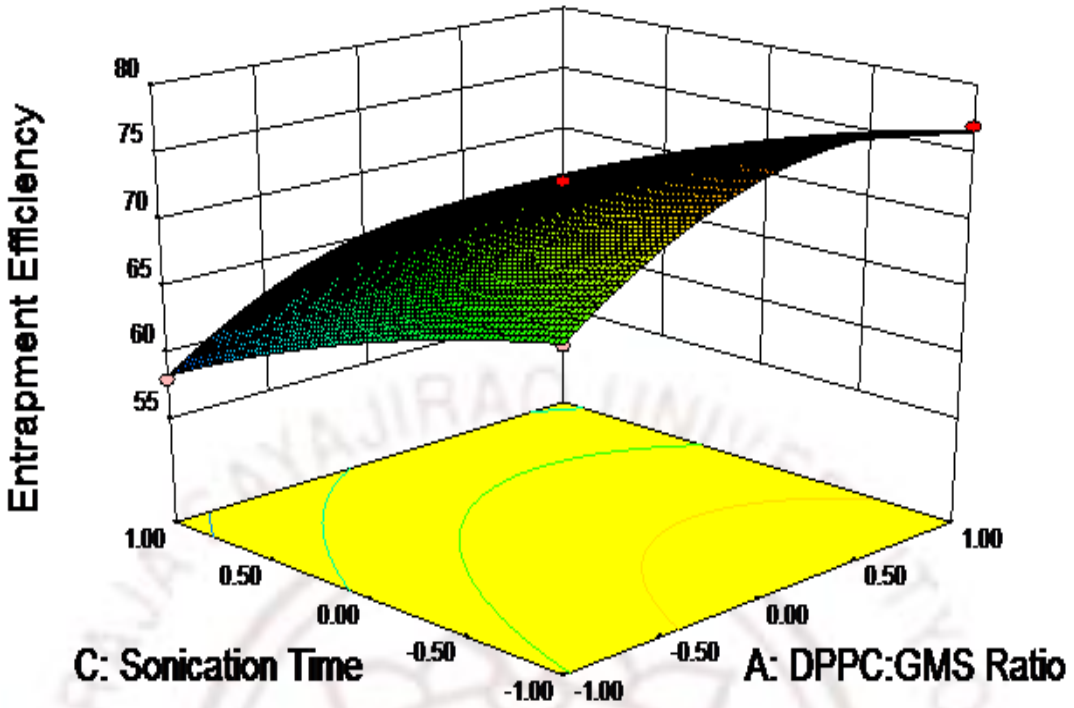


Fig 1.5 Three-dimensional surface plot for entrapment efficiency of DPPC: GMS ratio vs sonication time

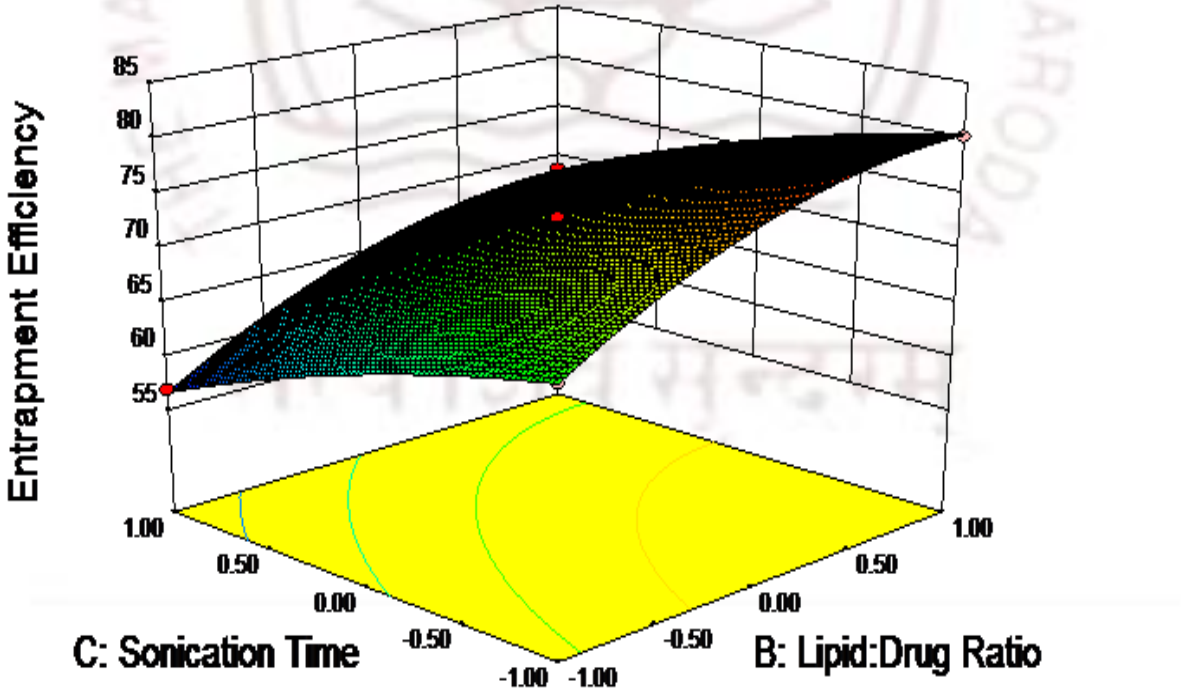


Fig 1.6 Three-dimensional surface plot for entrapment efficiency of lipid:drug ratio vs sonication time

1.4.1.3 Contour plots

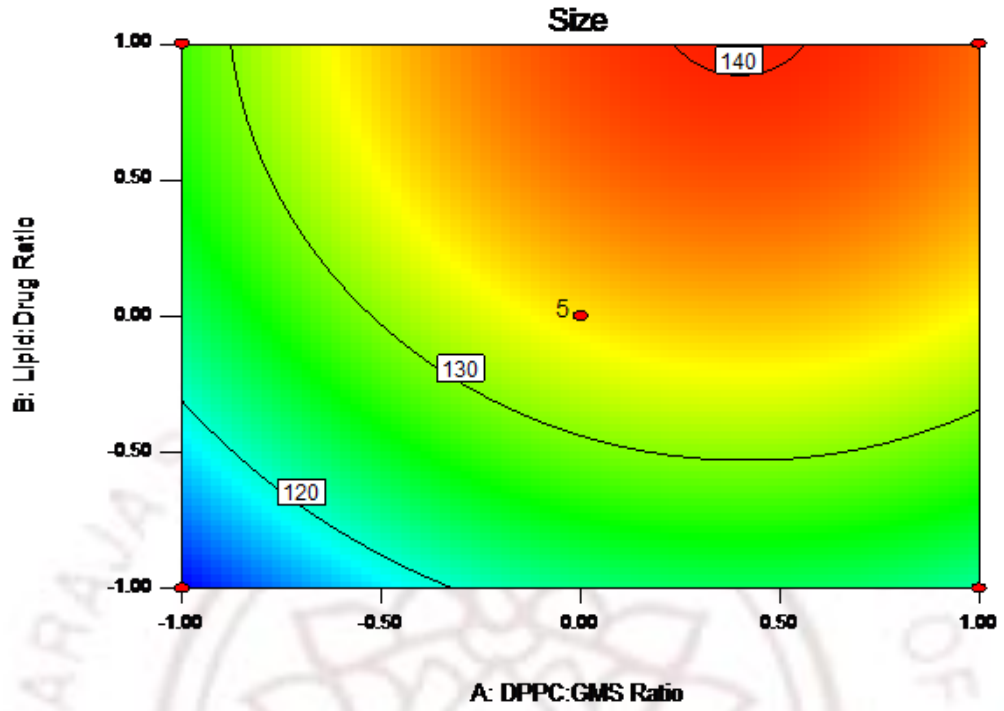


Fig 1.7 Contour plot for size of DPPC: GMS ratio vs lipid: drug ratio

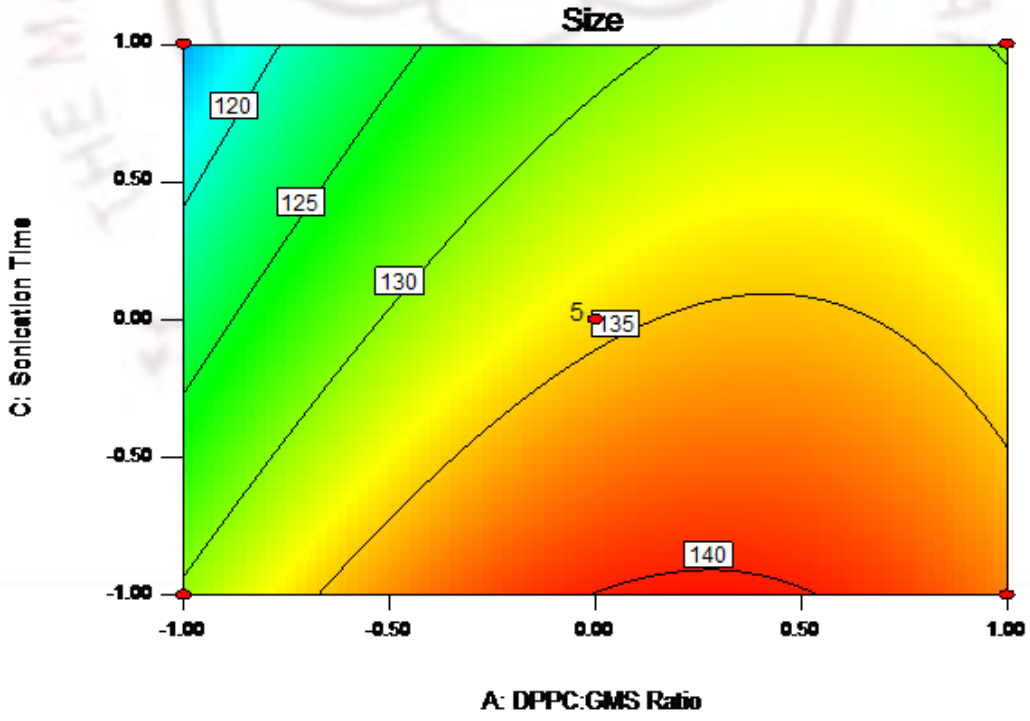


Fig 1.8 Contour plot for size of DPPC: GMS ratio vs sonication time

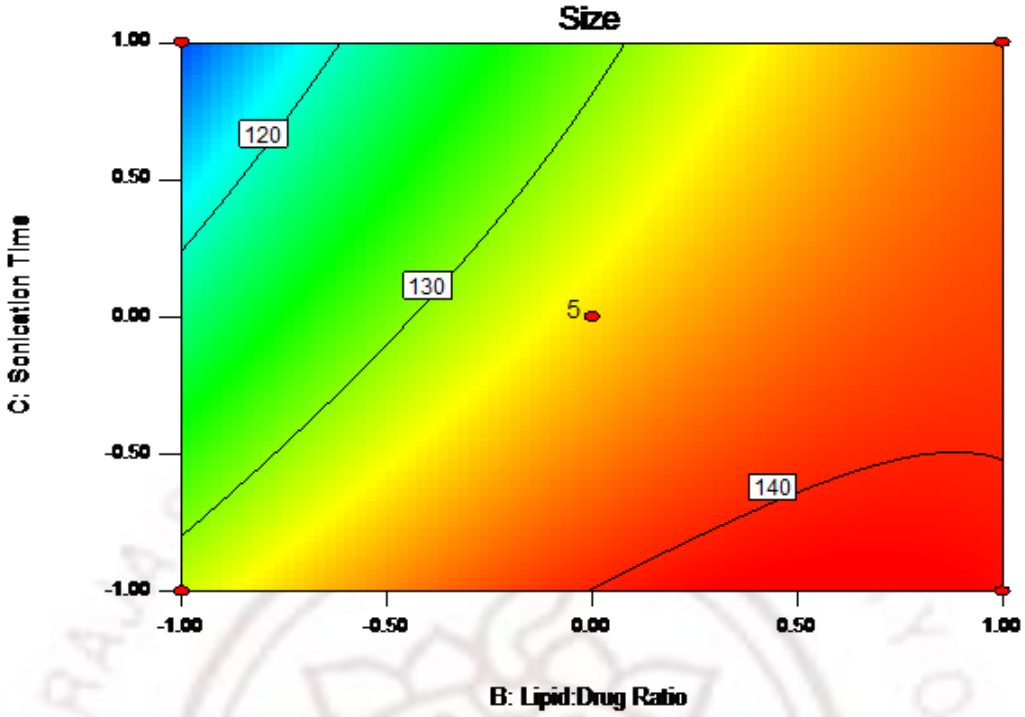


Fig 1.9 Contour plot for size of lipid:drug ratio vs sonication time

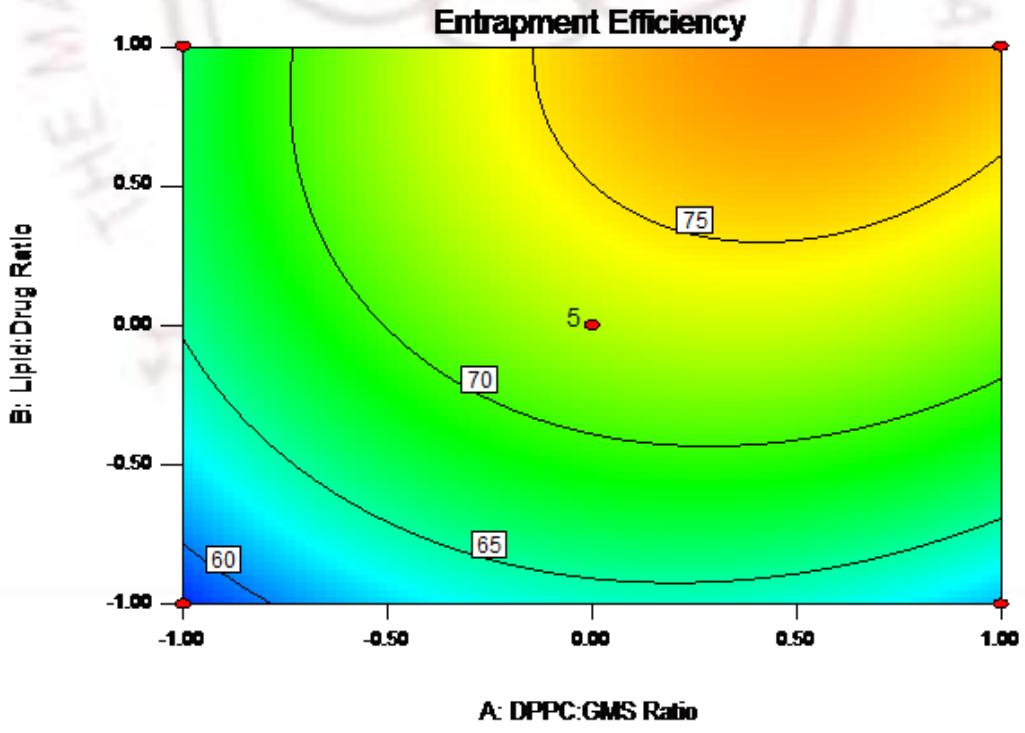


Fig 1.10 Contour plot for entrapment efficiency of DPPC: GMS ratio vs lipid: drug ratio

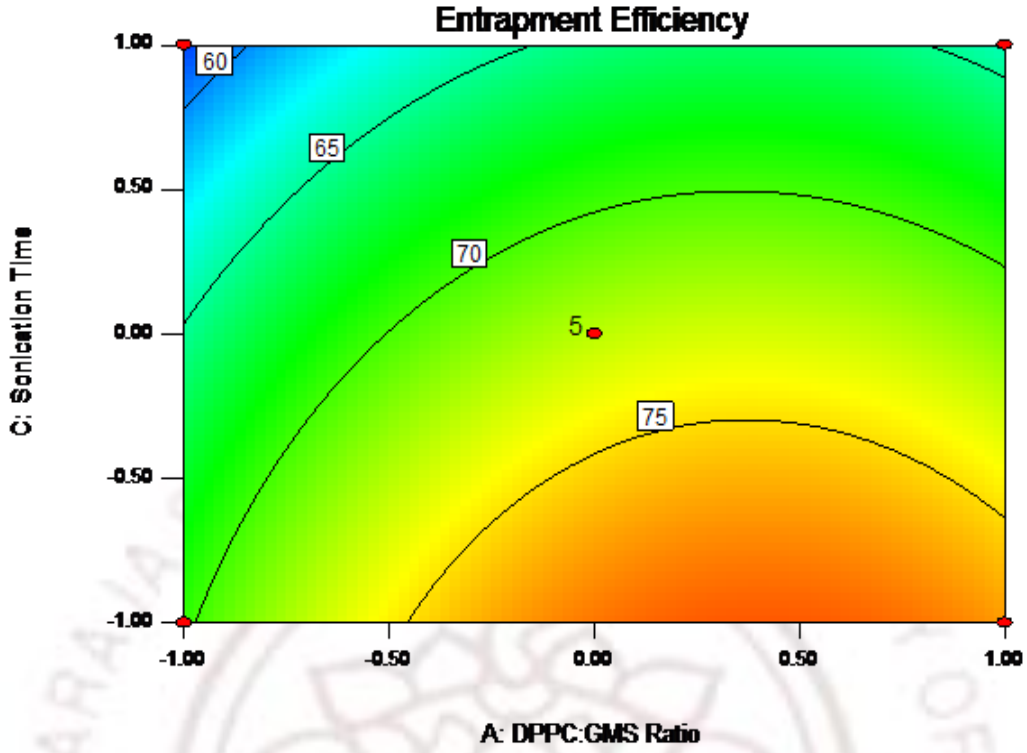


Fig 1.11 Contour plot for entrapment efficiency of DPPC: GMS ratio vs sonication time

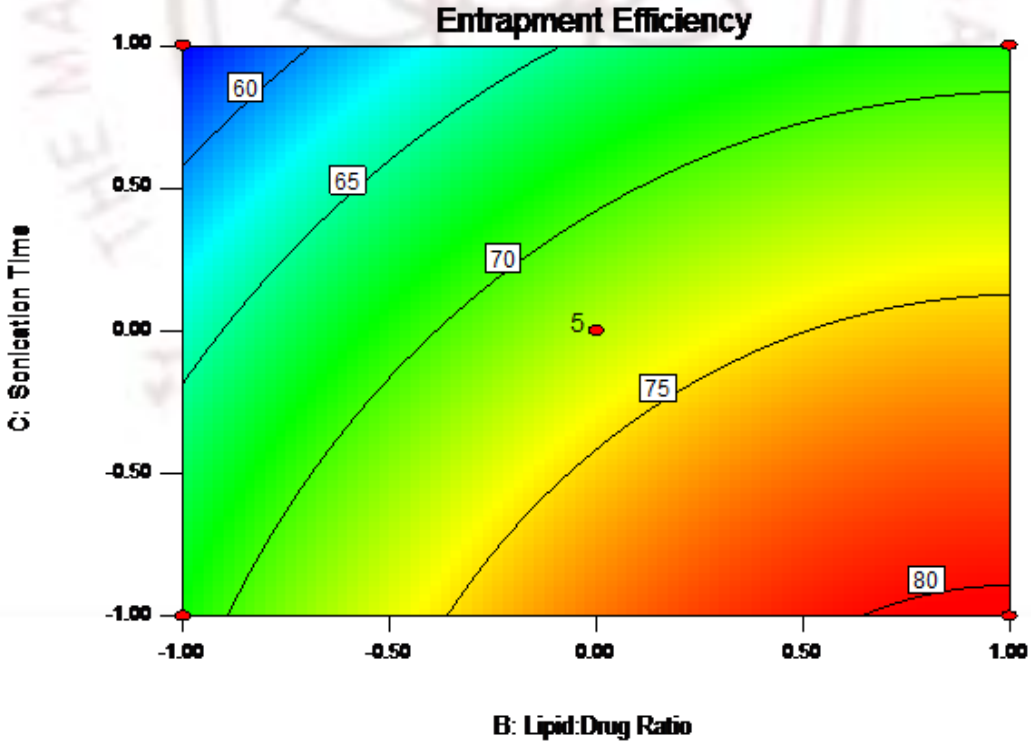


Fig 1.12 Contour plot for entrapment efficiency of lipid: drug ratio vs sonication time

The plots are shown in Figs. 5.7 to 5.12. Two parameters of each model were plotted at any one time on the X and Y axes with the yield in Z axis.

1.4.1.4 Selection of optimized batch

The prepared batches of TA loaded emulsomes, showed a wide variation from 110.74±7.01 to 141.01±2.98nm and 58.11±6.12 to 80.17±3.47% for PS and EE, respectively (Table 5.3). Moreover, as PS and EE have to be considered simultaneously, the selection of optimized batch was more difficult as the batch with smallest particle size 110.74±7.01 nm exhibited EE of only 58.11±6.12% (A=-1, B=-1, C=0) while the batch with maximum EE 80.17±3.47% have particle size of 141.01±2.98 nm (A=0, B=1, C=-1). Thus, the optimized batch was selected based on overall desirability factor calculated by Design Expert Software.

The results of dependent variables from the software were found to be 128.338± 4.19 nm for particle size and 68.868 ± 3.95 for % EE at these levels which is as per our desired criteria. The calculated desirability factor for offered formulations was 0.628 and indicates suitability of the designed factorial model.

Using these parameters i.e., A=-0.50, B=-0.20,C=0, a batch of TA loaded emulsomes was prepared, which was found to have the particle size (Y1) of 131.17± 3.17 nm, and % EE (Y2) of 70.56 ± 4.19 %.

Predicted error was calculated by using the following formula:

$$\text{Predicted Error\%} = \frac{\text{Observed Value} - \text{Predicted Value}}{\text{Predicted Value}} \times 100$$

Table 5.6 Observed and Predicted response variables of TA emulsomes

	Size (nm) Y1	Entrapment Efficiency (%) Y2
Observed Value	131.17± 3.17	70.56 ± 4.19
Predicted Value	128.34± 3.04	68.87 ± 3.95
Predicted Error (%) [a]	2.20	2.45

The lower values of % prediction error 2.20 % for (Y1) and 2.45 % for (Y2) indicate the reliability of developed mathematical models (Table 5.6).

1.4.2 Selection of cryoprotectant for lyophilization of emulsomes

The optimized batch of TA emulsomes was lyophilized using different ratios of sucrose; mannitol and trehalose dehydrate in order to find out optimum ratio of drug: cryoprotectant which showed minimum increase in particle size. Thus, this batch was considered for stability studies. Particle size of optimized batch after lyophilisation is shown in Fig. 5.13.

Table 5.7 Effect of different cryoprotectant ratios on particle size and redispersibility of TA emulsomes

Cryoprotectant Name and Ratio	Particle size before lyophilisation (Si)	Particle size after lyophilisation (Sf)	Redispersibility (Sf/Si)
Sucrose (1:3)	128.34± 4.19 nm	143.81±3.19 nm	1.12
Sucrose (1:5)		155.20±4.11 nm	1.20
Mannitol (1:3)		239.12±3.59 nm	1.86
Mannitol (1:5)		254.68±4.12 nm	1.98
Trehalose dehydrate (1:3)		229.16±4.29 nm	1.78
Trehalose dehydrate (1:5)		241.91±3.74 nm	1.88

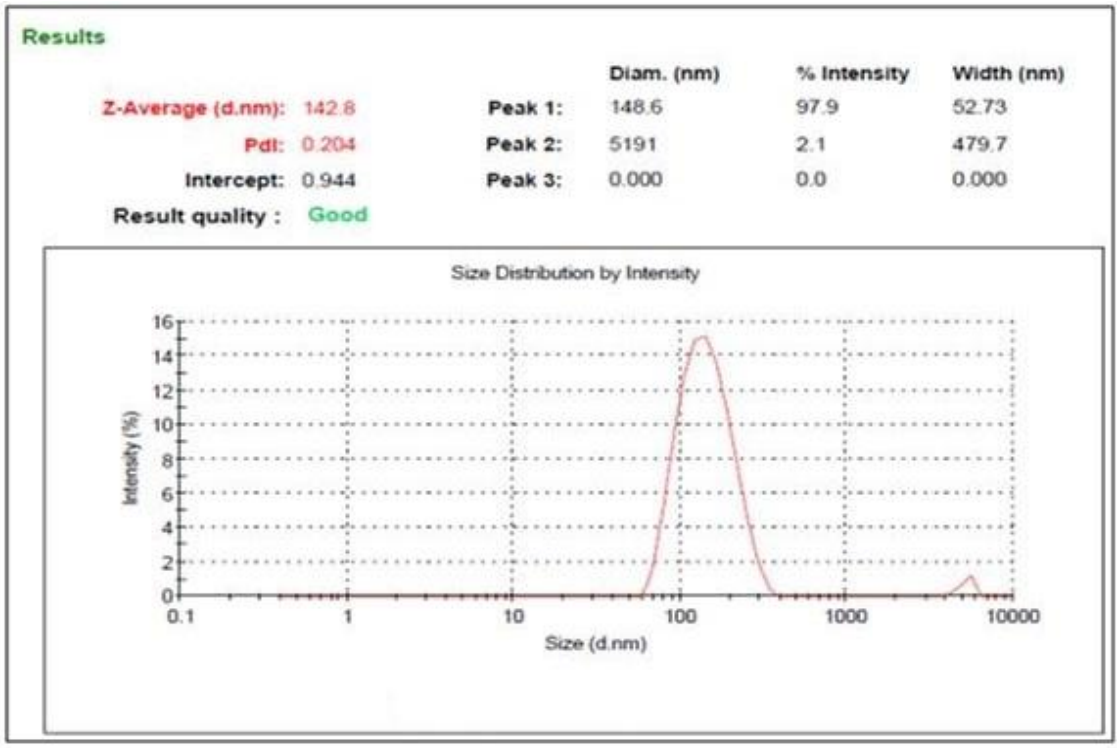


Fig 1.13 Particle size of lyophilized TA loaded emulsomes

1.4.3 Characterization of emulsomes:

1.4.3.1 Determination of particle size, ζ and entrapment efficiency

Mean particle sizes of various batches of emulsomes were in the range of 110.74±7.01 to 141.01±2.98nm. Optimized batch had mean particle size of 131.17± 3.17 nm and poly dispersity index (PDI) of 0.198 and zeta potential value of -24.2 ± 2.12 mV.

Fig. 5.14 and 5.15 shows the particle size distribution and zeta potential of optimized batch of TA emulsomes.

Fig 1.14 Graph showing average particle Size of TA loaded emulsomes

Fig 1.15 Zeta potential of TA emulsomes

For ocular distribution studies, fluorescent dye loaded emulsomes were prepared by incorporating Nile red in emulsomes instead of TA. The mean particle size of Nile red loaded emulsomes was around .

1.4.3.2 Determination of Entrapment efficiency:

Entrapment efficiencies of the prepared batches were in the range of 58.11±6.12 to 80.17±3.47%. Optimized batch had entrapment efficiency of 71.56 ± 4.19 %.

1.4.3.3 Differential Scanning Calorimetry

DSC thermograms of DPPC, GMS, TA and lyophilized TA loaded emulsomes are shown in Fig. 5.16. Since the melting point of TA has been recorded at approximately 293 ◦C, DSC analysis was run heating the sample from 25 ◦C to 300 ◦C. DSC thermogram shows peaks of a) DPPC at 42.50º C corresponding to its glass transition temperature (Tg) b) GMS at 63.70 º C and c) pure TA at 290.09 ºC which corresponds with the melting point around 300˚C, and is indicative of a crystalline anhydrous state of TA. While, thermogram of lyophilized TA loaded emulsomes showed only one peak at 41.20ºC indicating minor shifts in peak positions of DPPC. Absence of characteristic melting endothermic peak of TA in lyophilized emulsomes indicates that TA is present in amorphous form and is molecularly dispersed in lipid matrix of emulsomes.

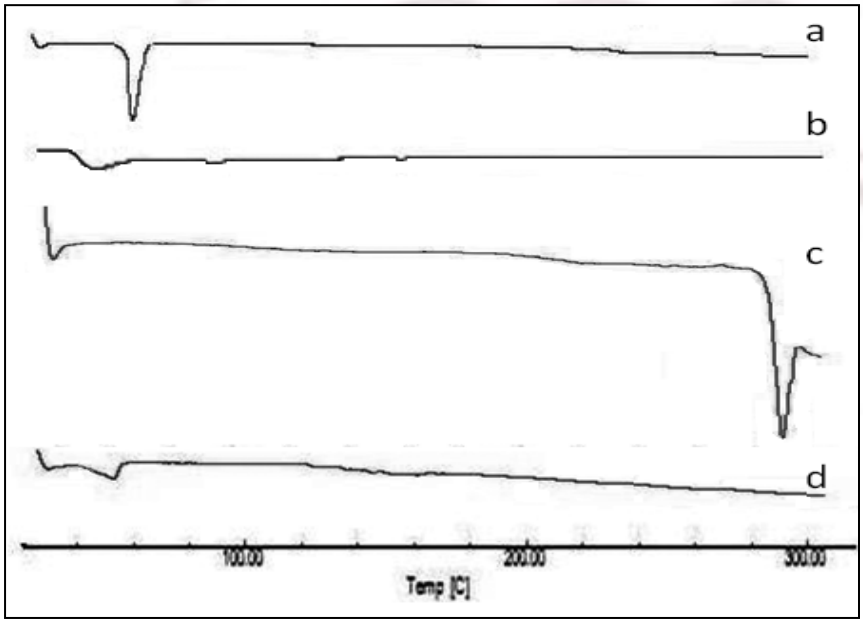


Fig 1.16 DSC thermogram of a) DPPC, b) GMS, c) triamcinolone acetonide and lyophilized TA emulsomes

3.4 Transmission Electron Microscopy

TEM image of TA emulsomes is shown in Fig. 5.17 which reveals discrete, nearly round shaped emulsomes. The mean diameters of emulsomes were in the range of 100- 200 nm.

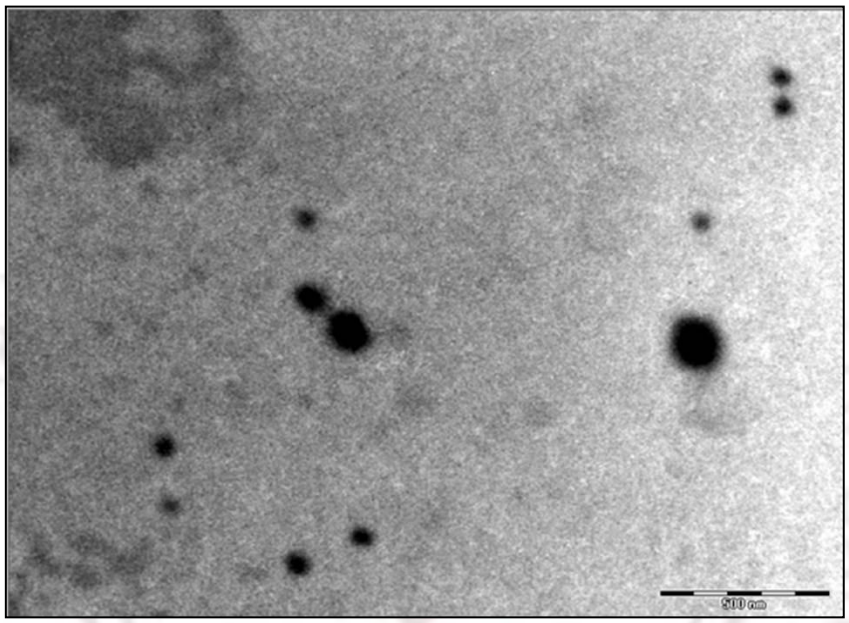


Fig 1.17 TEM image of triamcinolone acetonide loaded emulsomes

1.4.3.4 *In vitro* drug release studies

The *in vitro* release of triamcinolone acetonide from the emulsomes was studied by dialysis tube method. Fig. 5.18 shows the percentage release of TA in 0.1 M PBS (pH 7.4) vs time for 24 hrs.

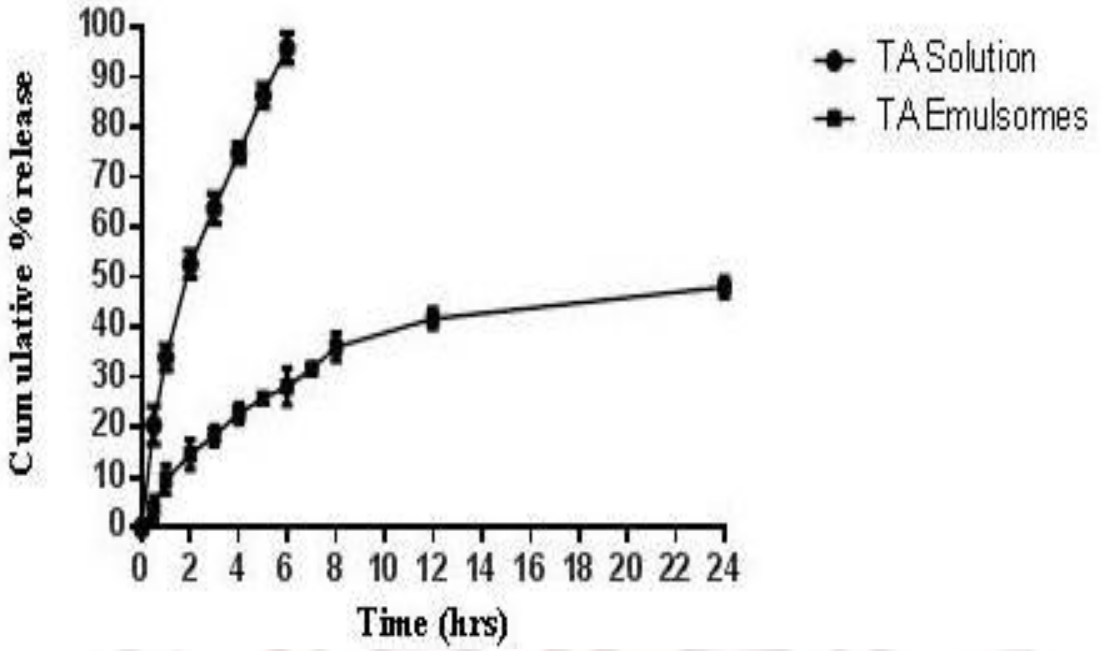


Fig 1.18 Release profile of triamcinolone acetonide from triamcinolone acetonide solution and triamcinolone acetonide loaded emulsomes

1.4.3.5 *Ex vivo* studies

Table 5.8 compares the results of *ex vivo* corneal permeation of triamcinolone acetonide from the emulsomes with triamcinolone acetonide solution across isolated goat cornea. As compared to TA solution, higher corneal permeation was observed in case of TA emulsomes.

Table 5.8 *Ex vivo* permeation of triamcinolone acetonide from emulsomes and aqueous solution

S. No.	Formulations	Corneal Permeation (cm/s)
1	TA loaded emulsomes	3.84±0.82
2	TA solution	2.39± 0.60

1.4.3.6 Stability Studies

The data of stability studies of lyophilized TA emulsomes at 2-8°C and at 25-30ºC are shown in Table 5.9. It was observed that at 2-8°C and 25-30ºC conditions both; there

was no significant change in either particle size or the drug content of lyophilized TA emulsomes.

Table 5.9 Stability profile of lyophilized TA loaded emulsomes at 2-8º C and 25-30ºC

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