1 EXPERIMENTAL: GANCICLOVIR EMULSOMES

1.1 MATERIALS

Ganciclovir was obtained as a gift sample from Ranbaxy (super speciality) Pvt. Ltd., Gurgaon, India. 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. Sephadex G-50, and sodium fluorescein were purchased from Sigma (St. Louis, MO, 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. Sucrose, Mannitol, D (+) Trehalose dehydrate, 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-µm 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)

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 ganciclovir emulsomes

Various batches of ganciclovir loaded emulsomes were prepared by thin film hydration method as described by Amselem et al., 1994 with slight modification as per laboratory set up (Fig. 4.1). 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 sodium fluorescein in the aforementioned procedure.

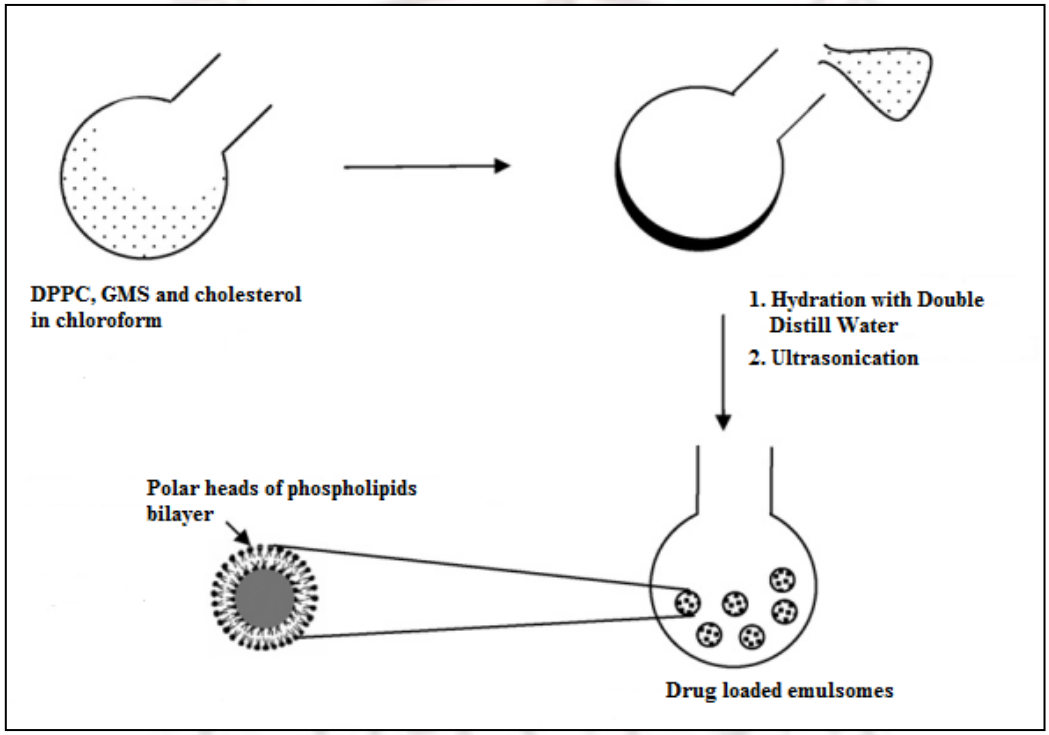

Fig 1.1 Schematic representation of emulsomes preparation by thin film hydration method

1.3.2 Preliminary optimization of process parameters

During preparation of emulsomes, process variables, such as selection of lipids, vacuum conditions for dry film formation, speed of rotation of flask during film formation and hydration, were optimized for desired results. The effect of one variable was studied at a time keeping other variables constant.

Table 4.2 Selection of preliminary process parameters

Process Parameters	Level
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- 15:1 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 8.0.4, 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. % prediction error of the prepared batch was calculated in order to evaluate the reliability of developed mathematical models (Sharma et al., 2012). 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 4.3.

Table 4. 3 Variables in Box Behnken Design of ganciclovir 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	15:1	20:1		
Sonication time (C)	Minutes	3	6	9		

1.3.4 Selection of cryoprotectant for lyophilization of emulsomes

The optimized GCV loaded emulsome formulation was lyophilized using lyophilizer (Heto Drywinner, Germany). Different cryoprotectants (Sucrose, Mannitol and Trehalose dehydrate) in different ratio (1:3 w/w and 1:5w/w) were tried, to select the cryoprotectant which showed minimum increment in particle size.

Results of particle size and redispersibility of lyophilized batches are reported in Table 4.8.

1.3.5 Characterization of emulsomes

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

Mean PS of ganciclovir loaded emulsomes was determined by using dynamic light scattering mehod (Zetasizer Nano ZS, Malvern, Worcestershire, UK). The ζ of emulsomes was measured using the laser Doppler method (Zetasizer Nano ZS). Each batch was analyzed in triplicate. For PS and ζ, analysis 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)

EE of ganciclovir loaded emulsomes was determined by employing size exclusion chromatography as described by Fry (1998).

Finally, entrapment efficiency was calculated by the following formula:

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

Mass balance was checked by adding the drug present in entrapped and unentrapped portions.

In lyophilized GCV emulsomes, drug content was determined by dissolving 2mg of obtained lyophilized powder in chloroform: methanol (2:8) and the samples were then analyzed by UV spectrophotometer at 254 nm, after suitable dilutions.

1.3.5.3 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry studies of drug, lipids and lyophilized emulsomes 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. Samples were 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 GCV, GMS, DPPC and lyophilized GCV emulsomes.

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 GCV from emulsomes was evaluated by the dialysis bag diffusion technique reported by Fujisawa et al. (Fujisawa et al., 2012). The samples were measured for amount of GCV released using UV method described in section 3.6.1.2 of analytical methods. All the experiments were performed in triplicate, and the average values were taken. Plain GCV solution (200µg/ml) prepared in PBS (pH 7.4) was used as a control.

1.3.5.6 *Ex vivo* study

Ganciclovir loaded emulsomes were evaluated for corneal permeation characteristics using the isolated goat cornea model (Yadav & Ahuja, 2010).

The apparent permeation coefficient (Papp, cm/s) of GCV was determined by reported method of Shen and Tu, 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 0.851 ± 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

Lipid based formulations are more susceptible to degradation, hydrolysis and drug leakage, especially as liquid dosage form. Thus, stability studies were performed for the lyophilized GCV loaded emulsomes. The lyophilized samples were kept in glass vials and stored at refrigerated conditions (2-8 °C) and at room temperature (25- 30°C). At different time points, the stored samples were withdrawn and analyzed for particle size and drug content.

1.4 RESULTS AND DISCUSSION

1.4.1 Selection of Optimized Batch

1.4.1.1 Box Behnken Design

Various batches of GCV 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 4.4.

Table 4. 4 Box Behnken design matrix of ganciclovir loaded emulsomes

Std.	Run	DPPC:GMS ratio (A)	Lipid:drug Ratio (B)	Sonication Time (C)	Size (nm) Y1	Entrapment Efficiency (%) Y2
6	1	1	0	-1	132.6± 4.12	42.9±4.01
9	2	0	-1	-1	123.2±3.01	30.8±2.16
14	3	0	0	0	127.9±2.94	37.9±3.21
16	4	0	0	0	127.7±1.54	37.8±2.97
17	5	0	0	0	127.5±2.01	37.5±3.01
10	6	0	1	-1	138.7±5.04	45.1±3.76
3	7	-1	1	0	123.9±3.01	28.1±3.87
5	8	-1	0	-1	119.9±6.78	32.5±1.60
12	9	0	1	1	133.1±5.13	33.7±2.45
11	10	0	-1	1	112.9±5.12	21.8±4.02
7	11	-1	0	1	111.8±6.34	22.9±4.94
4	12	1	1	0	137.0±3.13	40.9±3.07
8	13	1	0	1	122.8±3.01	31.27±2.53
13	14	0	0	0	127.4±2.18	37.5±3.05
2	15	1	-1	0	118.8±3.08	25.8±4.05
15	16	0	0	0	127.9±3.12	37.9±2.98
1	17	-1	-1	0	106.9±6.05	19.1±4.82

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):

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

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 4.5 ANOVA for the response surface quadratic polynomial model for PS

Response	1	PS					
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	1204.1	9	133.79	993.08	< 0.0001	Significant	
A-DPPC:GMS ratio	297.44	1	297.44	2207.79	< 0.0001		
B-Lipid:Drug ratio	624.1	1	624.1	4632.56	< 0.0001		
C-Sonication Time	141.12	1	141.12	1047.49	< 0.0001		
AB	0.35	1	0.35	2.63	0.149		
AC	0.65	1	0.65	4.81	0.0644		
BC	5.27	1	5.27	39.1	0.0004		
A^2	131.51	1	131.51	976.18	< 0.0001		
B^2	0.65	1	0.65	4.85	0.0636		
C^2	0.36	1	0.36	2.7	0.1446		
Residual	0.94	7	0.13				
Lack of Fit	0.74	3	0.25	4.71	0.0842	not significant	
Pure Error	0.21	4	0.052				
Cor Total	1205.04	16					

R^2=0.9992; adjusted-R^2=0.9982; predicted-R^2=0.9900 and Adequate recision=113.007.

Finally, two equations were obtained for PS and EE:

$Y_1=127.68+6.1A+8.83B-4.2C+0.3AB-0.4AC+ 1.15\ BC-5.59A^2-0.39\ B^2-0.29\ C^2$ ... (2)

$Y_2=39.72+4.75\ A+6.29B-5.24C+1.52AB-0.57AC-0.6BC-4.88A^2-4.36B^2-0.51C^2$.. (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 4.5 and 4.6 for PS and EE, respectively.

Table 4.6 ANOVA for the response surface quadratic polynomial model for entrapment efficiency

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	923.16	9	102.57	725.07	< 0.0001	Significant	
A-DPPC:GMS ratio	180.6	1	180.6	1276.58	< 0.0001		
B-Lipid:Drug ratio	316.26	1	316.26	2235.57	< 0.0001		
C-Sonication Time	219.35	1	219.35	1550.5	< 0.0001		
AB	9.3	1	9.3	65.76	< 0.0001		
AC	1.31	1	1.31	9.27	0.0187		
BC	1.44	1	1.44	10.18	0.0153		
A^2	100.43	1	100.43	709.88	< 0.0001		
B^2	80.09	1	80.09	566.11	< 0.0001		
C^2	1.09	1	1.09	7.7	0.0275		
Residual	0.99	7	0.14				
Lack of Fit	0.82	3	0.27	6.53	0.0508	not significant	
Pure Error	0.17	4	0.042				
Cor Total	924.15	16					

R^2=0.9989; adjusted-R^2=0.9976; predicted-R^2=0.9855 and Adequate precision = 90. 173

1.4.1.2 Response surface plots

Three-dimensional response surface plots for PS and EE of GCV emulsomes were generated by the Design Expert software and are presented in Fig. 4.2 to 4.7, respectively.

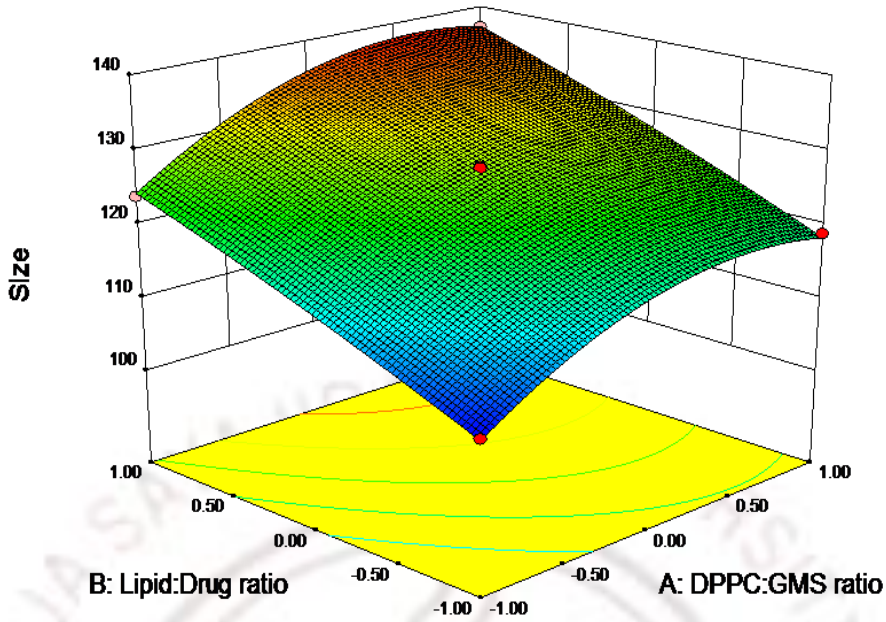

Fig 1.2 Three-dimensional surface plot for size of DPPC: GMS ratio vs lipid:drug ratio

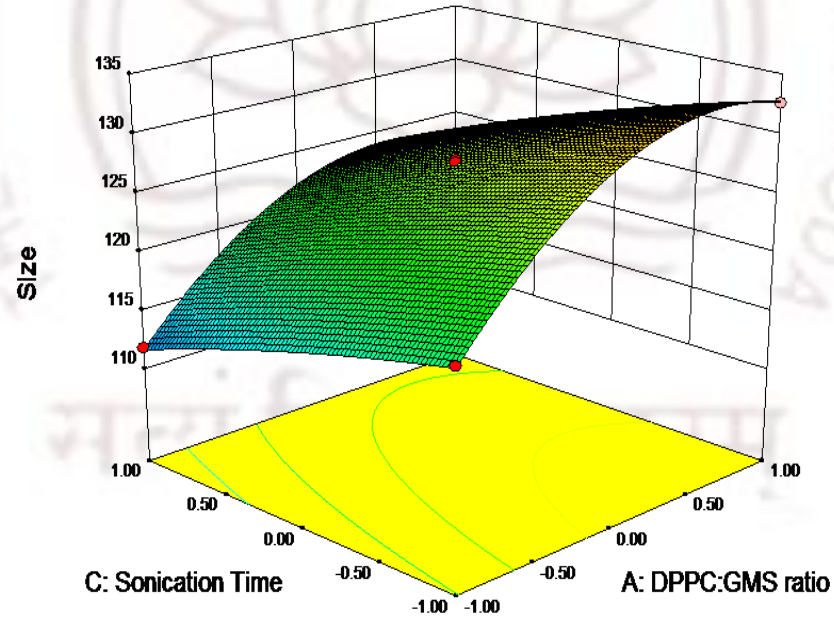

Fig 1.3 Three-dimensional surface plot for size of DPPC: GMS ratio vs sonication time

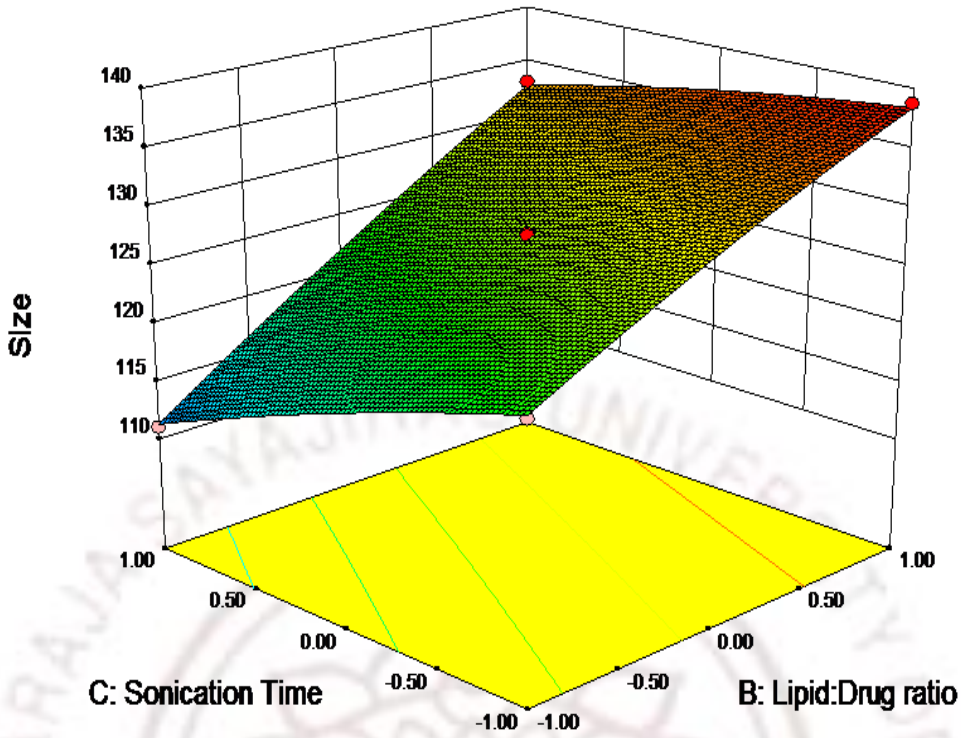

Fig 1.4 Three-dimensional surface plot for size of lipid: drug ratio vs sonication time

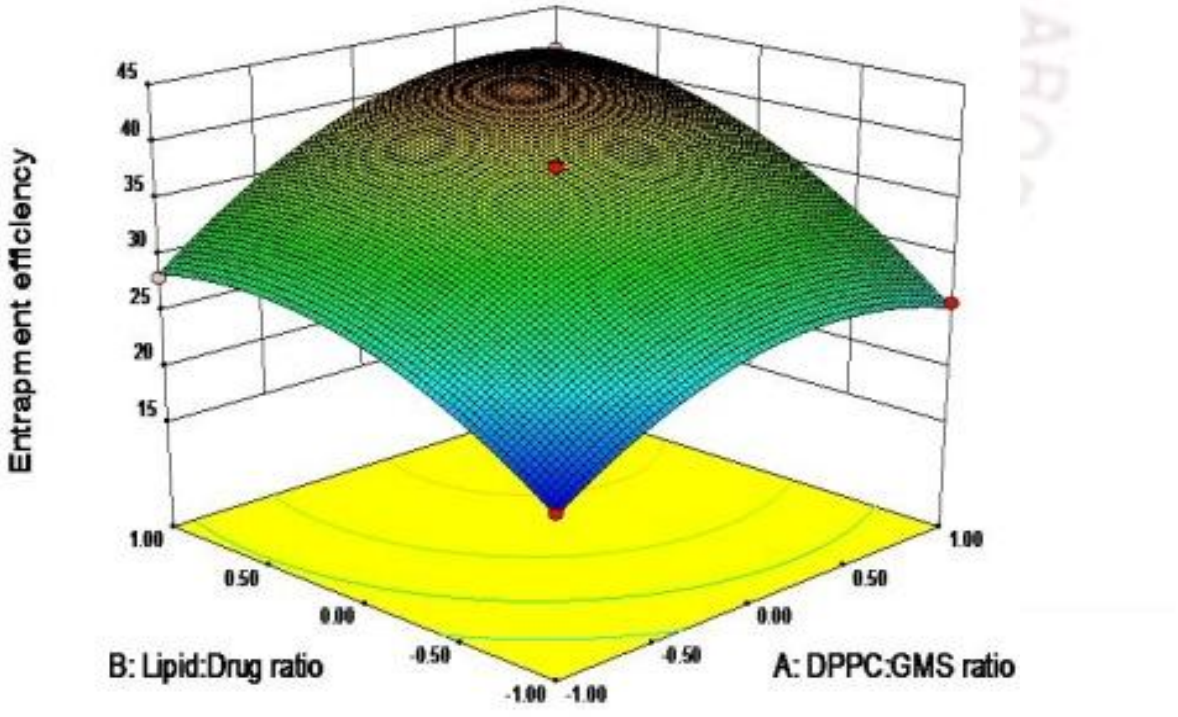

Fig 1.5 Three-dimensional surface plot for entrapment efficiency of DPPC: GMS ratio vs lipid:drug ratio

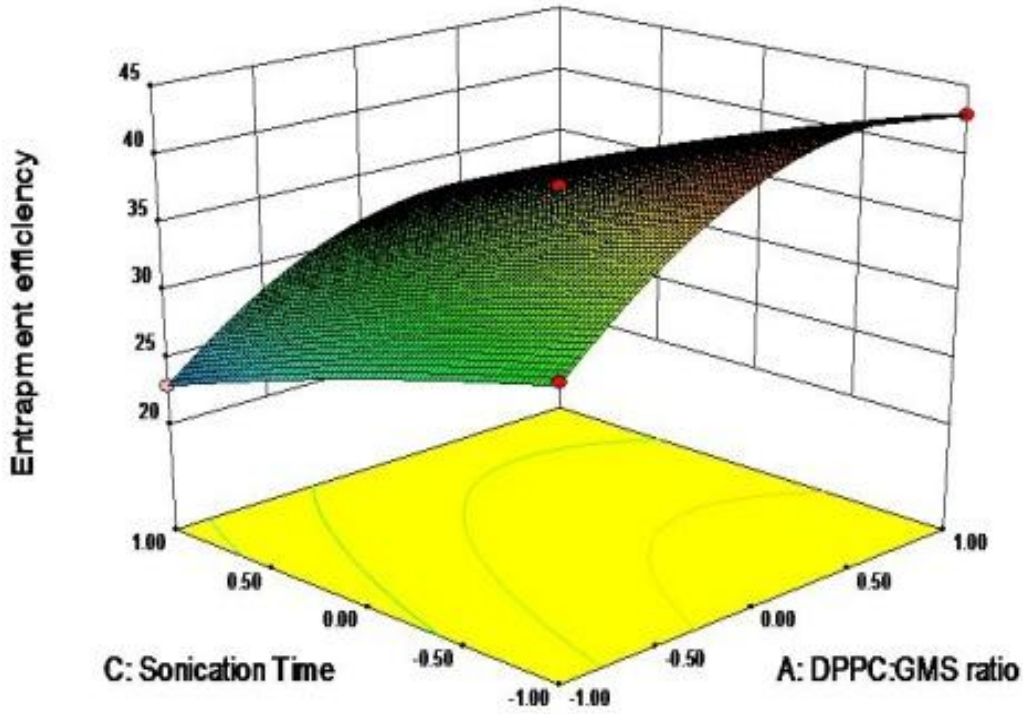

Fig 1.6 Three-dimensional surface plot for entrapment efficiency of DPPC: GMS ratio vs sonication time

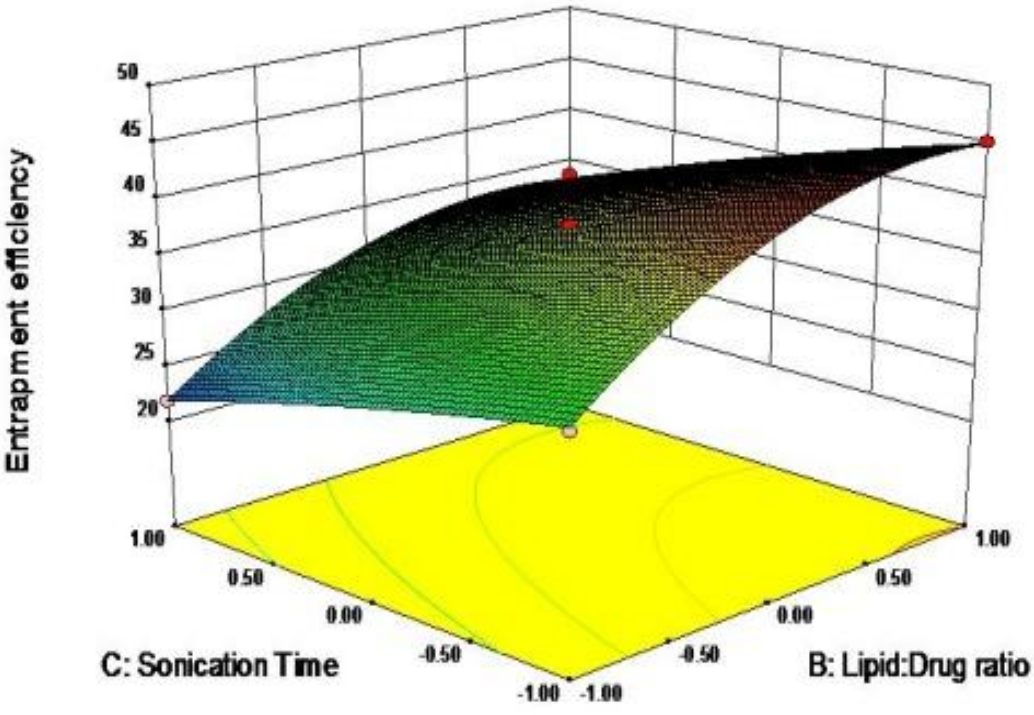

Fig 1.7 Three-dimensional surface plot for entrapment efficiency of lipid:drug ratio vs sonication time

1.4.1.3 Contour plots

The shape of the contour plots (circular or elliptical) indicates whether the mutual interactions between variables are significant or not. A circular contour plot indicates that the interactions between related variables are negligible. An elliptical contour plot indicates that the interactions between related variables are significant (Murlidhar et al., 2001). The plots are shown in Fig. 4.8 to 4.13. Two parameters of each model were plotted at any one time on the X and Y axes with the yield in Z axis. The other one remaining parameters set at their center point values (i.e. DPPC: GMS ratio of 1:1, lipid:drug ratio of 15:1 , and sonication time of 6) automatically by the software to make each plot.

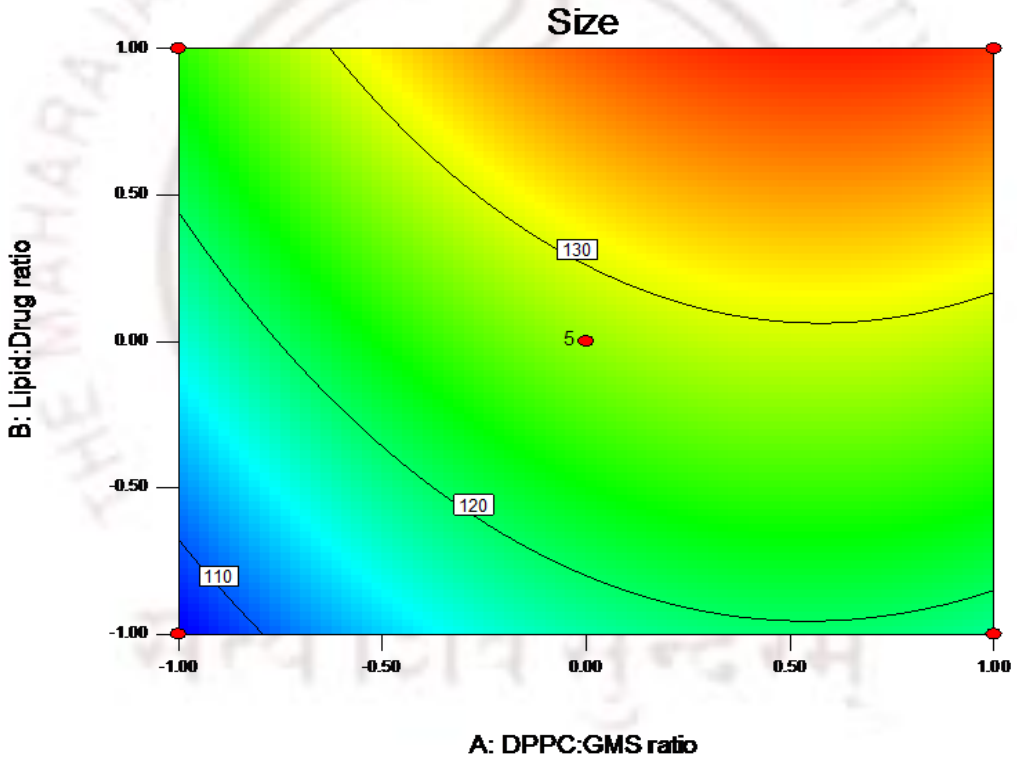

Fig 1.8 Contour plot for size of DPPC: GMS ratio vs lipid: drug ratio

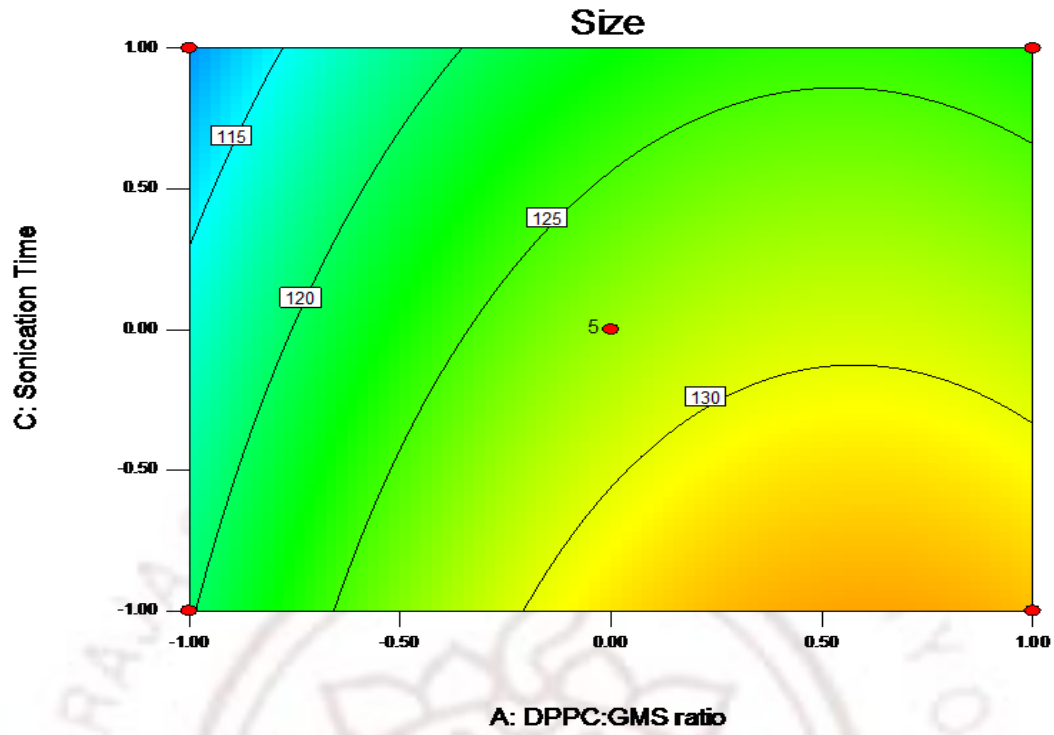

Fig 1.9 Contour plot for size of DPPC: GMS ratio vs sonication time

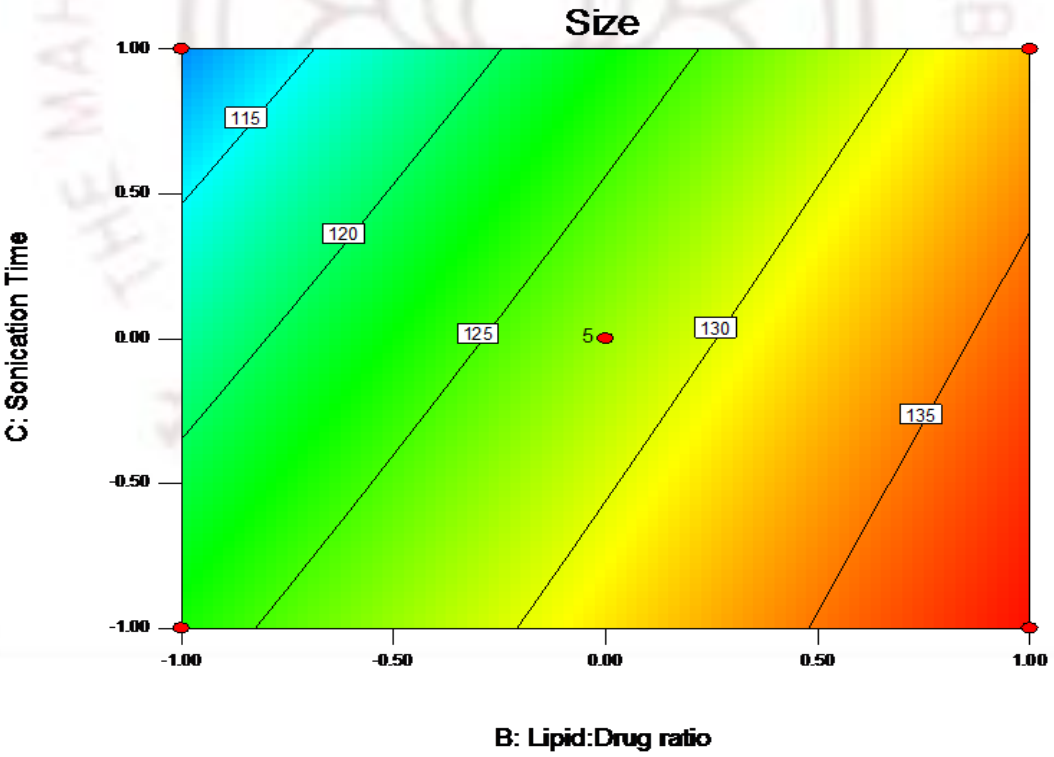

Fig 1.10 Contour plot for size of lipid:drug ratio vs sonication time

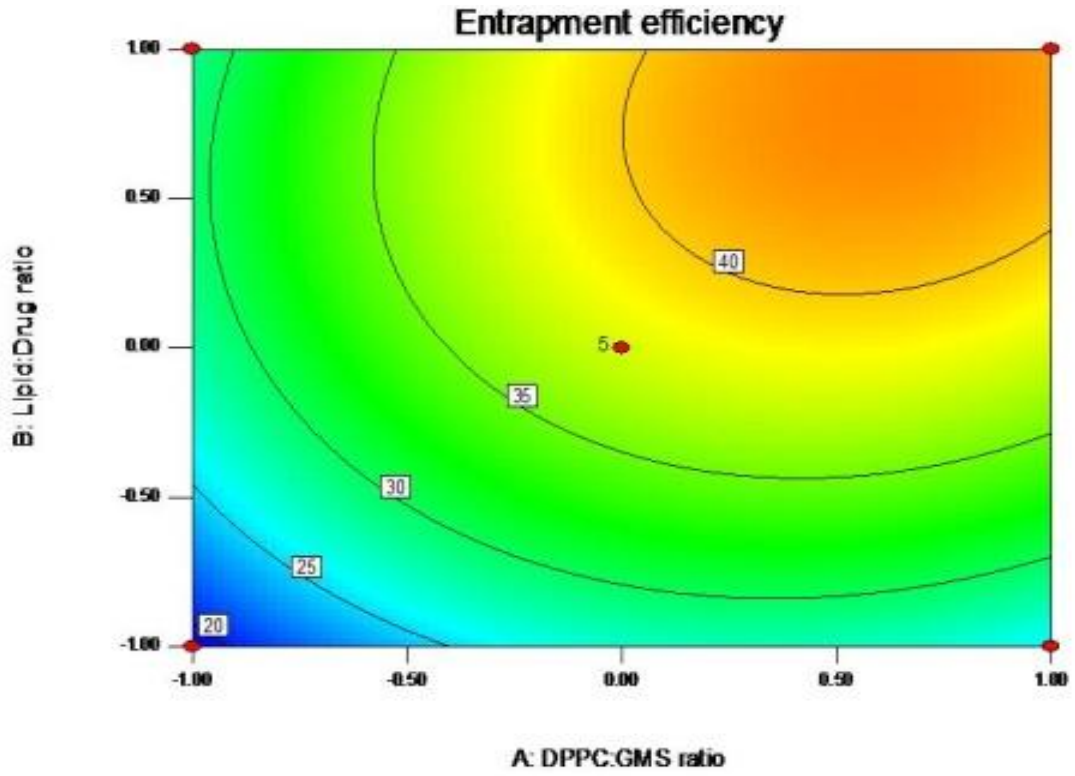

Fig 1.11 Contour plots for entrapment efficiency of DPPC: GMS ratio vs lipid:drug ratio

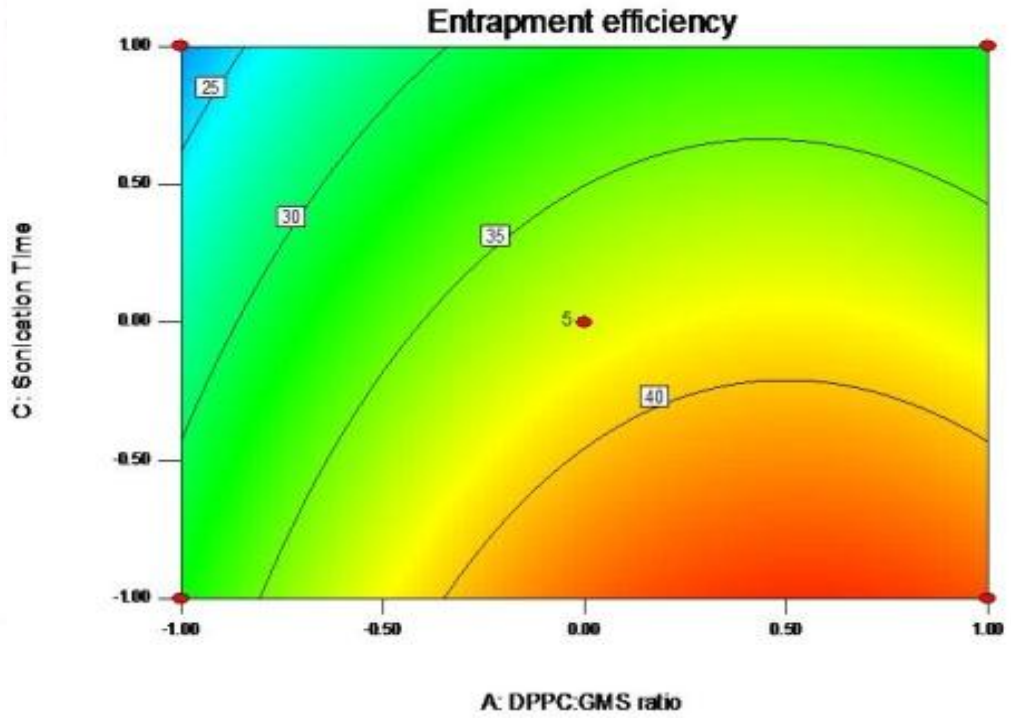

Fig 1.12 Contour plots for entrapment efficiency of DPPC: GMS ratio vs sonication time

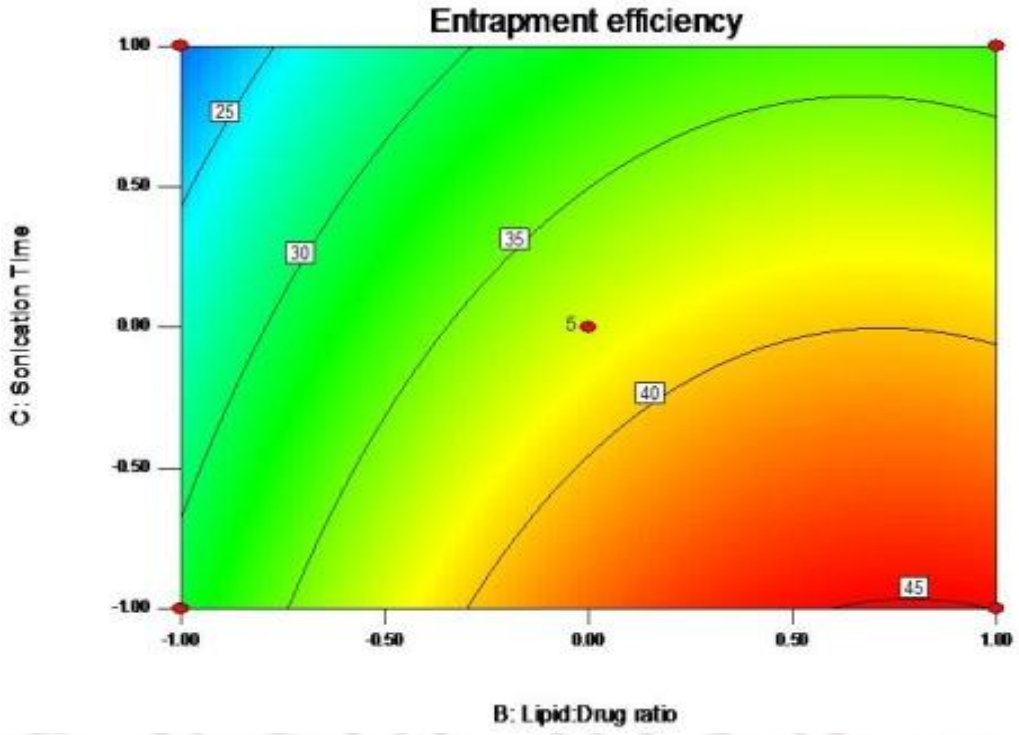

Fig 1.13 Contour plots for entrapment efficiency of lipid:drug ratio vs sonication time

1.4.1.4 Selection of optimized batch

The prepared batches of GCV loaded emulsomes, showed a wide variation from 106.9±6.05 to 138.7±5.04 nm and 19.1±5.12 to 45.1±3.76 % for PS and EE, respectively (Table 4.2). 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 106.9±6.05 nm exhibited EE of only 19.1±5.12 % (A=-1, B=-1, C=0) while the batch with maximum EE 45.1±3.76 % had particle size of 138.7±5.04 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 129.34± 3.04 nm for particle size and 38.96 ± 2.95 for % EE at these levels which is as per our desired criteria. The calculated desirability factor for offered formulations was 0.748, which was near to 1 and indicated suitability of the designed factorial model. Using these

parameters i.e., A=0.48, B=0,C=0, a batch of GCV loaded emulsomes was prepared. 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 4.7 Observed and Predicted response variables of GCV emulsomes

	Size (nm) Y1	Entrapment Efficiency (%) Y2
Observed Value	131.04± 4.87	39.42 ± 3.09
Predicted Value	129.34± 3.04	38.96± 2.95
Predicted Error (%)	1.31	1.18

The lower values of % prediction error 1.31 % for (Y1) and 1.18 % for (Y2) indicate the reliability of developed mathematical models (Sharma et al., 2012).

1.4.2 Selection of cryoprotectants for lyophilization of emulsomes

Freeze-drying is a well-established approach to increase the chemical and physical stability of colloidal systems over extended time periods.

Table 4.8 Effect of different cryoprotectants and their ratios on particle size and redispersibility of GCV emulsomes

Cryoprotectant Name and Ratio	Particle size before lyophilisation (Si)	Particle size after lyophilisation (Sf)	Redispersibility (Sf/Si)
Sucrose (1:3)	129.31± 3.04 nm	142.53±3.56 nm	1.10
Sucrose (1:5)		151.23 ± 6.97 nm	1.16
Mannitol (1:3)		232.74±4.12 nm	1.79
Mannitol (1:5)		257.27±4.67 nm	1.98
Trehalose dehydrate (1:3)		226.32±5.87 nm	1.75
Trehalose dehydrate (1:5)		236.49±4.56 nm	1.82

The optimized batch of GCV 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. The redispersibility of the freeze-dried formulations and particle size of the emulsomes before and after freeze-drying was measured and recorded in Table 4.8.

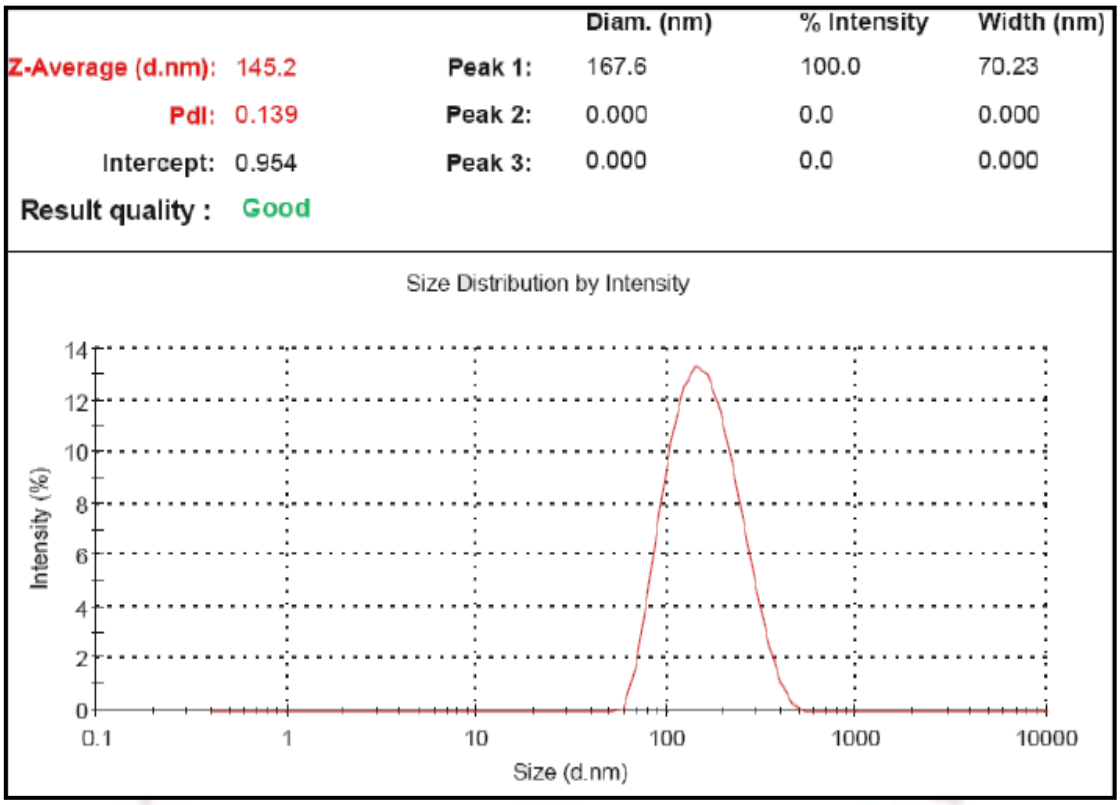

Fig 1.14 Average particle size of lyophilized GCV loaded emulsomes

1.4.3 Characterization of emulsomes

1.4.3.1 Determination of particle size (PS) and Zeta potential (ζ)

Mean particle sizes of various batches of emulsomes were in the range of 106.9±6.05 to 138.7±5.04 nm. Optimized batch had mean particle size of 129.34± 3.04 nm and poly dispersity index (PDI) of 0.139 and zeta potential of -19.9± 3.15 mV.

Fig 4.15 and 4.16 shows the particle size distribution and zeta potential of optimized batch of GCV emulsomes.

Fig 1.15 Particle Size distribution of GCV emulsomes

Fig 1.16 Zeta potential of GCV emulsomes

For ocular distribution studies, dye loaded emulsomes were prepared by incorporating sodium fluorescein in emulsomes instead of GCV. Sodium fluorescein was chosen as a fluorescent marker because of its hydrophilic nature which is similar to that of GCV.

1.4.3.2 Determination of Entrapment efficiency

Entrapment efficiencies of the prepared batches were in the range of 19.1±5.12 to 45.1±3.76 %. Optimized batch has entrapment efficiency of 38.96 ± 2.95 %.

1.4.3.3 Differential Scanning Calorimetry

DSC is useful in the investigation of the thermal properties of drug delivery carriers, providing information about the physicochemical state of drug inside the drug delivery systems. In the absence of any interaction, the thermogram of a formulation will show patterns corresponding to those of the individual components. In the event that interaction occurs, there may be appearance of one or more new peaks an or the disappearance of one or more peaks corresponding to those of the components (Nanjwade et al., 2009).

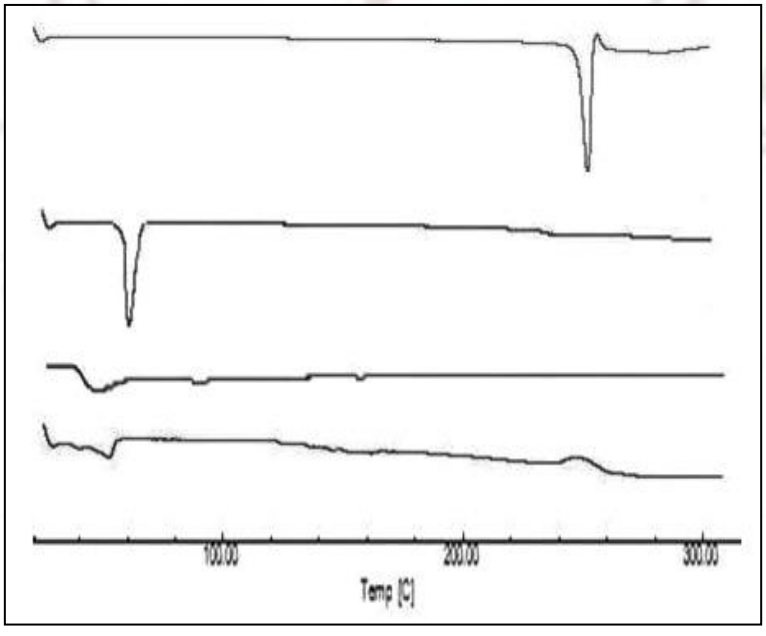

Fig 1.17 DSC thermogram of a) ganciclovir, b) GMS, c) DPPC and d) lyophilized GCV emulsomes

1.4.3.4 Transmission Electron Microscopy

TEM image of GCV emulsomes is shown in Figure 4.18 which reveals discrete, nearly spherical shaped emulsomes with appearance of phospholipid layer around solid lipid core. The mean diameters of emulsomes were in the range of 100- 200 nm, which is in accordance of the results obtained from particle size analysis.

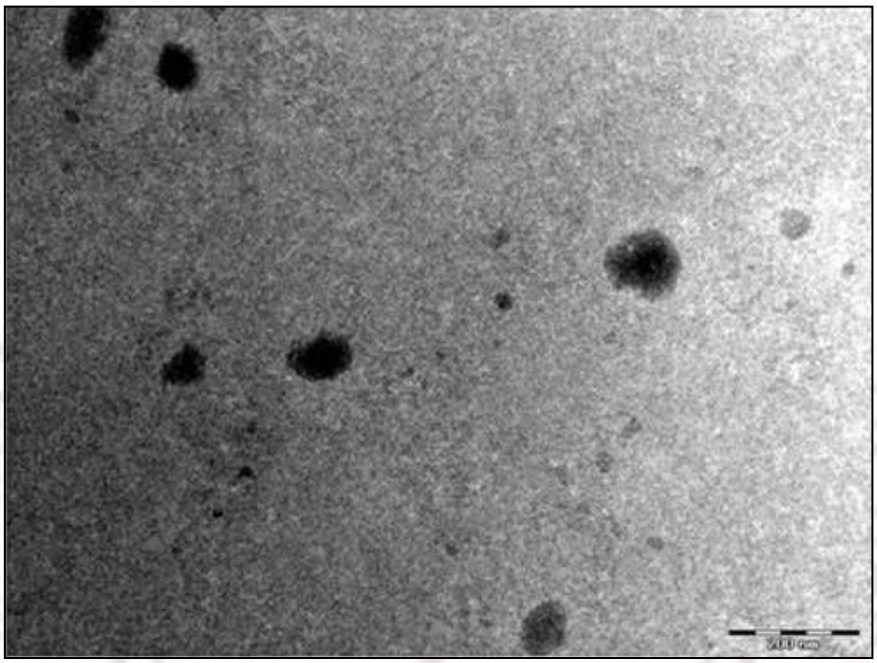

Fig 1.18 TEM image of ganciclovir loaded emulsomes

1.4.3.5 *In vitro* drug release studies

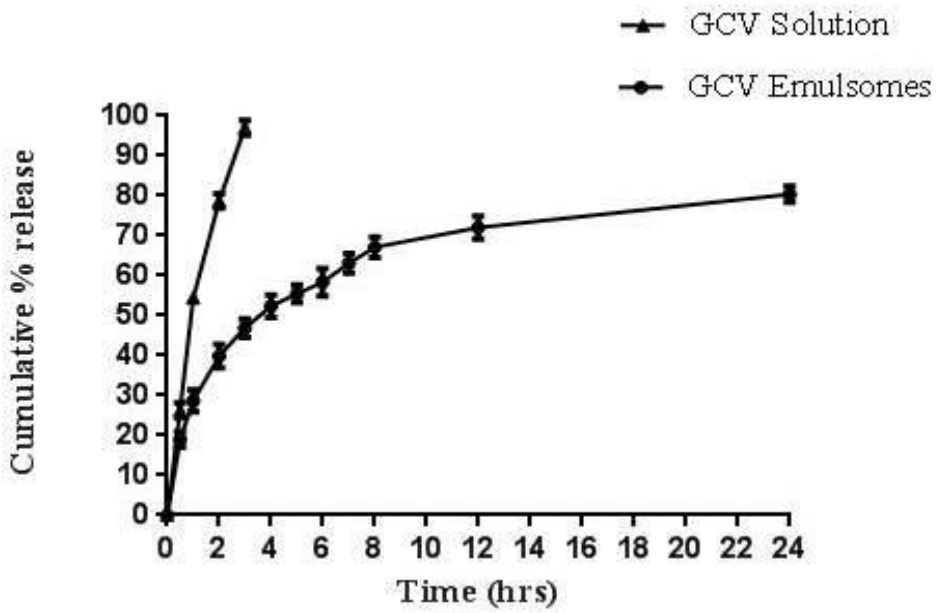

Fig 1.19 Release profile of ganciclovir solution and ganciclovir loaded emulsomes (n=3)

The *in vitro* release profiles of ganciclovir solution and ganciclovir loaded emulsomes was investigated in phosphate buffer saline at 37 ^{2}C ± 2 ºC (Fig. 4.19).

1.4.3.6 *Ex vivo* studies

Table 4.9 compares the results of *ex vivo* corneal permeation of ganciclovir from the emulsomes with ganciclovir solution across isolated goat cornea. The measured Papps values were 3.24±0.65 cm/s and 1.29± 0.72cm/s in case of GCV emulsomes and GCV solution, resp. Significant difference was observed in between the two, higher corneal permeation was observed in case of GCV emulsomes.

Table 4.9 *Ex vivo* permeation of ganciclovir from emulsomes and aqueous solution

S. No.	Formulations	Corneal Permeation (cm/s)
1	Ganciclovir loaded emulsomes	3.24 ± 0.65
2	Ganciclovir solution	1.29 ± 0.72

1.4.3.7 Stability Studies

No significant change in either particle size or the drug content was observed in lyophilized GCV emulsomes at 2-8°C and 25-30ºC as compared to initial batch, suggesting that the dried lyophilized emulsomes should be stored at 2-8 ºC or 25-30ºC to maintain their storage stability.

Table 4.10 Stability profile of lyophilized GCV loaded emulsomes at 2-8º C and 25-30 °C

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