1 EXPERIMENTAL: TRIAMCINOLONE ACETONIDE NANOPARTICLES

1.1 MATERIALS

Triamcinolone acetonide (TA) was purchased from Sigma (St. Louis, EUA). Chitosan (medium molecular weight) was purchased from Sigma Aldrich (St. Louis, MO). Lipoid S100 was kindly supplied by Lipoid AG (Ludwigshafen, Germany). HPLC grade chloroform, ethanol (96%) and methanol were purchased from Merck, Mumbai, India. Potassium dihydrogen phosphate, sodium hydroxide, sodium chloride and all other analytical reagents were obtained from S.D. fine-chem limited, Baroda, India. Cellulose dialysis tubing (Molecular weight cut of 12-14000; 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, India).

1.2 EQUIPMENTS

Analytical weighing balance (Shimadzu, Switzerland)

High speed magnetic stirrer (Remi, MS500, Remi equipments, Mumbai, India)

Ultrasonic Bath Sonicator (Ultrasonics Selec, Vetra, Italy)

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

UV-VIS Spectrophotometer (Shimadzu 1600, Japan)

High Pressure Liquid Chromatograph (Shimadzu Corporation, Japan)

Lyophilizer (Heto Drywinner, Denmark)

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

Transmission Electron Microscope (Morgagni, FEI Company, USA)

^{1}H Nuclear Magnetic Resonance (Bruker Instruments, USA)

Gel Permeation Chromatography (Shimadzu LC-10 A HPLC/GPC system, Japan)

Cyclomixer (Spinix, Tarsons, India)

1.3 METHODS

1.3.1 Preparation of Chitosan hydrochloride salt

Chitosan hydrochloride salt (CS HCl) was prepared from medium molecular weight chitosan purchased from Sigma Aldrich (St. Louis, MO) by reported method of Signini et al., 1999 as per the protocol and procedure described in section 6.3.1 of chapter 6.

1.3.2 Preparation of triamcinolone acetonide nanoparticles

Various batches of triamcinolone acetonide loaded lecithin/chitosan hydrochloride nanoparticles (TA loaded lecithin/ CS HCl NPs) were prepared by reported method of Sonvico et al (Sonvico et al., 2006).

For ocular distribution studies, dye loaded nanoparticles were prepared by replacing the drug with Nile red in the aforementioned procedure.

1.3.3 3^2 Factorial Design

Various batches of TA loaded lecithin/CS HCl nanoparticles were prepared based on the 3^2 factorial design. The independent variables taken were lecithin: chitosan ratio (A) and lecithin: TA ratio (B). Particle size (Y1) and encapsulation efficiency (Y2) were taken as dependent variables.

Quantitative aspects of the effects and relationships among various formulation variables of nanoparticles were investigated using 3^2 factorial designs. 3^2 factorial design with a total of 9 experimental runs was selected to optimize the various process parameters at three levels (low, medium, and high, coded as –1, 0, and +1). lecithin: chitosan ratio (A) and lecithin: TA ratio (B) were taken as independent variables and their effect was studied on size (Y_1) and % encapsulation efficiency (Y_2) which were taken as dependent variables. The Design-Expert software (version 9.0.0.7, 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 7.1.

Table 1.1 Variables in 3^2 factorial design for TA loaded lecithin/ chitosan hydrochloride nanoparticles

Independent Variables	Units	Coded Values			Response (Y1)	Response (Y2)
		-1	0	1		
lecithin: chitosan HCl ratio (A)	w/w	5:1	10:1	20:1	Particle Size (nm)	Encapsulation Efficiency (%)
lecithin: TA ratio (B)	w/w	5:1	10:1	20:1		

1.3.4 Selection of cryoprotectant for lyophilization of nanoparticles

The optimized TA loaded lecithin/CS HCl nanoparticles formulation was lyophilized using lyophilizer (Heto Drywinner, Germany) as per the method described in section 4.3.4 of chapter 4.

1.4 CHARACTERIZATION

1.4.1 Characterization of nanoparticles

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

Mean PS and ζ of triamcinolone acetonide loaded CS HCl nanoparticles 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 90º.

1.4.1.2 Encapsulation Efficiency (EE)

The prepared nanoparticles were separated from the free drug using a Sephadex G-50 minicolumn centrifugation technique for measurement of entrapment efficiency (Sorensen et al., 1977; Tan et al., 2007.

Ffinally, encapsulation efficiency was calculated by the following formula:

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

In lyophilized TA nanoparticles, 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.

1.4.1.3 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry studies of TA, excipients and lyophilized nanoparticles were carried out (DSC-60, Shimadzu, Japan) in order to define the physical state of drug in nanoparticles and possibility of interaction between the drug and excipients within the nanoparticles

1.4.1.4 Transmission electron microscopy (TEM)

TEM analysis of the prepared formulation was carried out to understand themorphology of nanoparticles. A drop of nanoparticles containing 0.01% of phosphotungstic acid was placed on a carbon film coated on a copper grid. TEM studies were performed at 1000 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.

1.4.1.5 *In vitro* release study

In vitro release of TA from nanoparticles was evaluated by the dialysis bag diffusion technique reported by Suen et al. (Suen et al., 2013). The samples were measured for amount of TA released using HPLC method described in section 3.6.3.2 of chapter 3. All the experiments were performed in triplicate, and the average values were taken. TA solution prepared in PBS (pH 7.4) was used as a control.

1.4.1.6 *Ex vivo* study

TA loaded lecithin/ CS HCl nanoparticles 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.

The apparent permeation coefficient (Papp, cm/s) of GCV 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 0.821 ± 0.22 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.

1.4.1.7 *In vivo* Precorneal Retention Studies

The drug concentration in the precorneal area after instillation in rabbits was determined (as per the protocol and procedure described in section 4.3.5.7 of chapter 4) in order to evaluate the precorneal retention of TA loaded lecithin/ CS HCl nanoparticles, compared with TA aqueous solution. Here, the rabbits were given an instillation of 150µl of samples into the lower conjunctival sac of both eyes and evaluated by HPLC methods as described in Section 3.6.3.2 of chapter 3.

1.4.1.8 Stability studies

The stability studies were performed for the lyophilized triamcinolone acetonide loaded CS HCl nanoparticles. The samples were kept in transparent glass vials and stored at refrigerated conditions (5-8°C) and at room temperature (25-30°C). At different time points the samples were withdrawn and checked for particle size and drug content.

1.5 RESULTS AND DISCUSSION

1.5.1 Preparation and optimization of TA loaded lecithin/ CS HCl nanoparticles

1.5.1.1 Preparation of lecithin/ CS HCl nanoparticles

Lecithin/ CS HCl nanoparticles were prepared by supra-molecular self-organizing interaction of negative lipid material lecithin and positively charged polysaccharide chitosan by the method described by Sonvico et al., 2006.

1.5.1.2 Optimization of nanoparticles

1.5.1.3 3^2 Factorial Design

Various batches of TA loaded lecithin/ CS HCl nanoparticles were prepared according to 3^2 Design by varying two independent variables lecithin: chitosan ratio (A) and lecithin: TA ratio (B). 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 7.2.

Table 1.2 3^2 Factorial design matrix of TA loaded lecithin/ CS HCl nanoparticles

Std.	Run	Lecithin: chitosan ratio (w/w) A	Lecithin: TA ratio (w/w) B	Size (nm) Y1	Encapsulation Efficiency (%) Y2
5	1	-1	1	140.15 ±3.01	57.34 ±2.89
3	2	-1	-1	150.93 ± 3.83	31.32 ± 3.93
4	3	0	-1	134.85 ±2.87	35.12 ±2.43
8	4	1	-1	127.67 ±3.98	42.58 ±3.12
2	5	0	0	129.82 ±2.45	57.67 ±2.87
6	6	1	0	124.34 ±2.65	65.98 ±4.13
1	7	0	0	129.12 ±3.21	60.12 ±2.87
10	8	-1	0	146.23 ±4.12	57.56 ±3.12
9	9	1	1	119.22 ±4.19	69.67 ± 2.92
7	10	0	1	125.12 ±2.98	61.54 ±3.04
11	11	0	0	129.18 ±2.67	60.34 ±2.67

Obtained data of dependent variables (size and encapsulation 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):

$Y=\beta_0 + \beta_1 A + \beta_2 B + \beta_{12} AB + \beta_{11} AA + \beta_{22} BB$ ..... (1)

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

Finally, two equations were obtained for PS and EE:

$Y_1=129.67-11.01A-4.83B+0.58AB + 5.17A^2 -0.13 B^2$ ..... (2)

$Y_2=59.48+5.33 A+13.26B-0.27AB-2.13A^2-11.31B^2$ ..... (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 7.3 and 7.4 for PS and EE, respectively.

Table 1.3 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	940.96	5	188.19	601.34	< 0.0001	Significant
A-A	727.76	1	727.76	2325.48	< 0.0001	
B-B	139.78	1	139.78	446.65	< 0.0001	
AB	1.36	1	1.36	4.34	0.0918	
A^2	67.78	1	67.78	216.59	< 0.0001	
B^2	0.041	1	0.041	0.13	0.7319	

Residual	1.56	5	0.31				
Lack of Fit	1.26	3	0.42	2.80	0.2742	not significant	
Pure Error	0.30	2	0.15				
Cor Total	942.52	10					

R^2=0.9983; adjusted-R^2=0.9967; predicted-R^2=0.9903 and Adequate precision=76.677

Table 1.4 ANOVA for the response surface quadratic polynomial model for encapsulation efficiency

Response	2	Encapsulation 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	1551.27	5	310.25	185.75	< 0.0001	Significant	
A-A	170.77	1	170.77	102.24	0.0002		
B-B	1054.17	1	1054.17	631.13	< 0.0001		
AB	0.29	1	0.29	0.17	0.6961		
A^2	11.52	1	11.52	6.90	0.0468		
B^2	323.92	1	323.92	193.93	< 0.0001		
Residual	8.35	5	1.67				
Lack of Fit	3.96	3	1.32	0.60	0.6737	not significant	
Pure Error	4.39	2	2.20				
Cor Total	1559.63	10					

R^2=0.9946; adjusted-R^2=0.9893; predicted-R^2=0.9683 and Adequate precision=38.952

1.5.1.3.1 Response surface plots

Three-dimensional response surface plots for PS and EE were generated by the Design Expert software and are presented in Figs. 7.1 and 7.2, for triamcinolone acetonide loaded lecithin/ CS nanoparticles respectively.

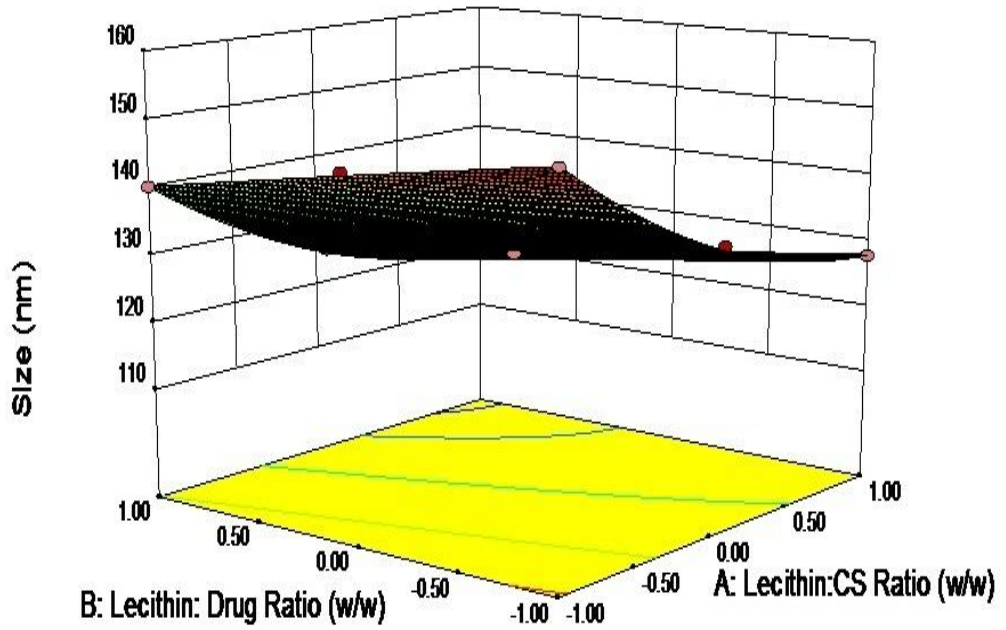

Fig 1.1 Three-dimensional surface plot of lecithin: CS ratio vs lecithin: drug ratio for size

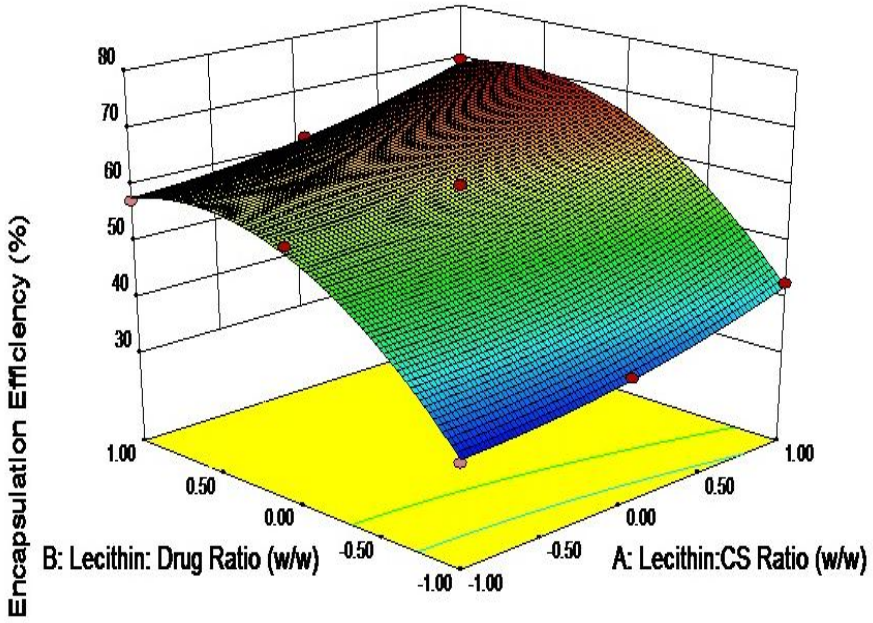

Fig 1.2 Three-dimensional surface plot of lecithin: CS ratio vs lecithin: drug ratio for encapsulation efficiency

1.5.1.3.2 Contour plots

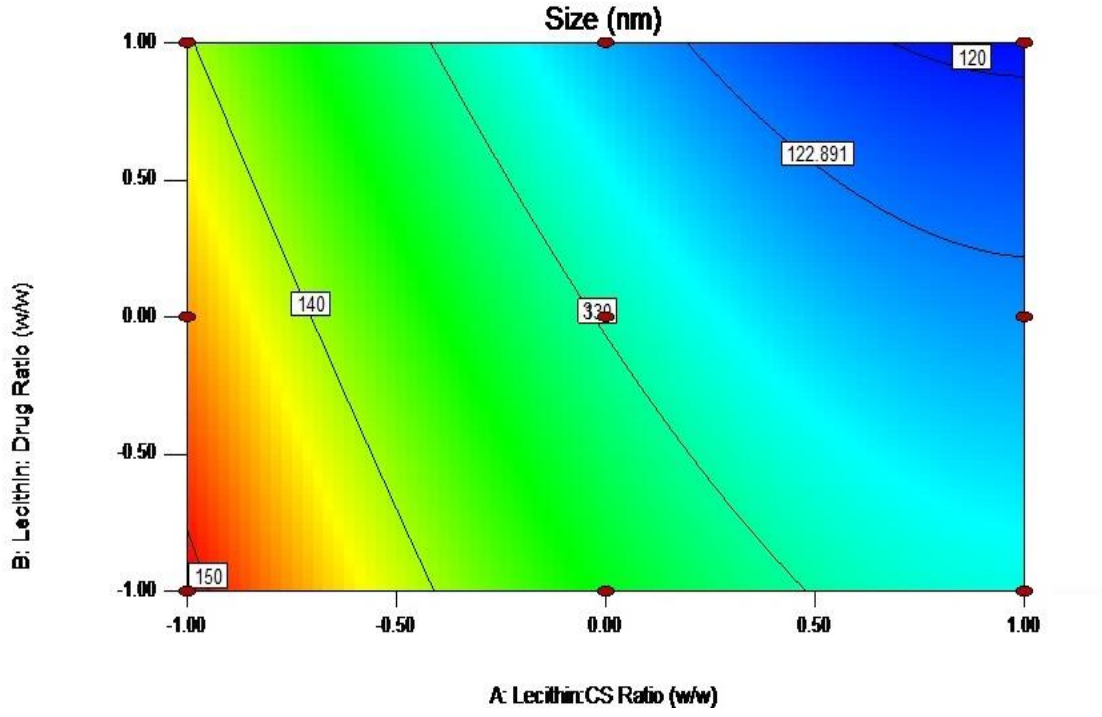

Fig 1.3 Contour plot of lecithin: CS ratio vs lecithin: drug ratio for size

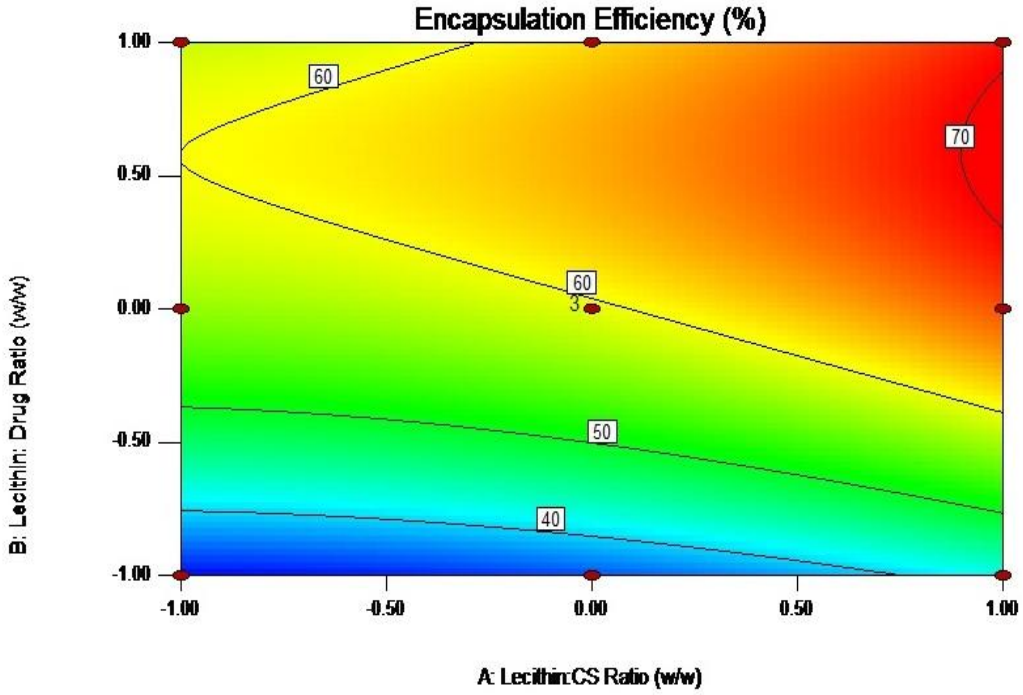

Fig 1.4 Contour plot of lecithin: CS ratio vs lecithin: drug ratio for encapsulation efficiency

Fig. 7.3 shows the contour plot for the response of lecithin: CS ratio vs lecithin: drug ratio to the acquired size. Fig. 7.4 shows the contour plot for the response of lecithin: CS ratio vs lecithin: drug ratio to the acquired encapsulation efficiency.

1.5.1.3.3 Selection of optimized batch

The prepared batches of TA loaded CS/HA nanoparticles, showed a wide variation from 119.22±4.19 to 150.93±3.83nm and 31.32±3.93 to 69.67±2.92 % for PS and EE, respectively (Table 7.2). The optimized batch was selected based on overall desirability factor calculated by Design Expert Software. The optimum formulation was selected by setting the criteria of maximum EE and minimum particle size.

The results of dependent variables from the software were found to be 119.73± 4.22 nm for particle size and 69.67 ± 2.94 for % EE at these levels which is as per our desired criteria. The calculated desirability factor for offered formulations was 0.992, which was near to 1 and indicates suitability of the designed factorial model.

Using these parameters i.e., A=1 and B=0.94, a batch of TA loaded lecithin/ CS nanoparticles was prepared, which was found to have the particle size (Y1) of 121.32± 3.41nm, and % EE (Y2) of 67.12 ± 2.16 %. 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 1.5 Observed and Predicted response variables of TA loaded lecithin/ CS nanoparticles

The lower values of % prediction error -2.78 % for (Y1) and -3.66 % for (Y2) indicate the reliability of developed mathematical models.

1.5.2 Selection of cryoprotectant for lyophilization of emulsomes

The optimized batch of TA loaded CS HCl nanoparticles 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.

Table 1.6 Effect of different cryoprotectant ratios on particle size and redispersibility of TA loaded lecithin/ CS HCl nanoparticles

Cryoprotectant Name and Ratio	Particle size before lyophilisation (Si)	Particle size after lyophilisation (Sf)	Redispersibility (Sf/Si)
Sucrose (1:3)	119.22 ±4.19 nm	145.21±4.11 nm	1.21
Sucrose (1:5)		149.55±4.14 nm	1.25
Mannitol (1:3)		155.17±3.15 nm	1.30
Mannitol (1:5)		163.25±3.13 nm	1.36
Trehalose dehydrate (1:3)		133.8±2.19 nm	1.12
Trehalose dehydrate (1:5)		140.19±3.63 nm	1.17

Fig 1.5 Average particle size of lyophilized TA loaded lecithin/ CS nanoparticles

1.5.3 Characterization of nanoparticles

1.5.3.1 Determination of particle size, ζ and entrapment efficiency

The sizes of the various batches of lecithin/ CS nanoparticles were in the range of 119.22± 4.19 to 150.93± 3.83nm. The optimized nanoparticles batch prepared with 20:1 lecithin: CS ratio had an average PS diameter of 116.40± 3.41 and narrow size distribution having a poly dispersity index of 0.185.

Fig. 7.6 shows the average particle size and particle size distribution of optimized TA loaded lecithin/ CS nanoparticles batch.

Fig 1.6 Particle Size distribution of TA loaded lecithin/ CS nanoparticles

Fig 1.7 Zeta potential of TA loaded lecithin/ CS nanoparticles

Charge on the surface of nanoparticles varied with the concentration of CS HCl in lecithin/ CS HCl nanoparticles. Nanoparticles had positive zeta potential values. For ocular distribution studies, dye loaded emulsomes were prepared by incorporating Nile red in nanoparticles instead of TA.

1.5.3.2 Differential Scanning Calorimetry

DSC analysis was performed by heating the samples from 25 ºC to 300 ºC as the melting point of TA has been recorded at 290.09 ºC

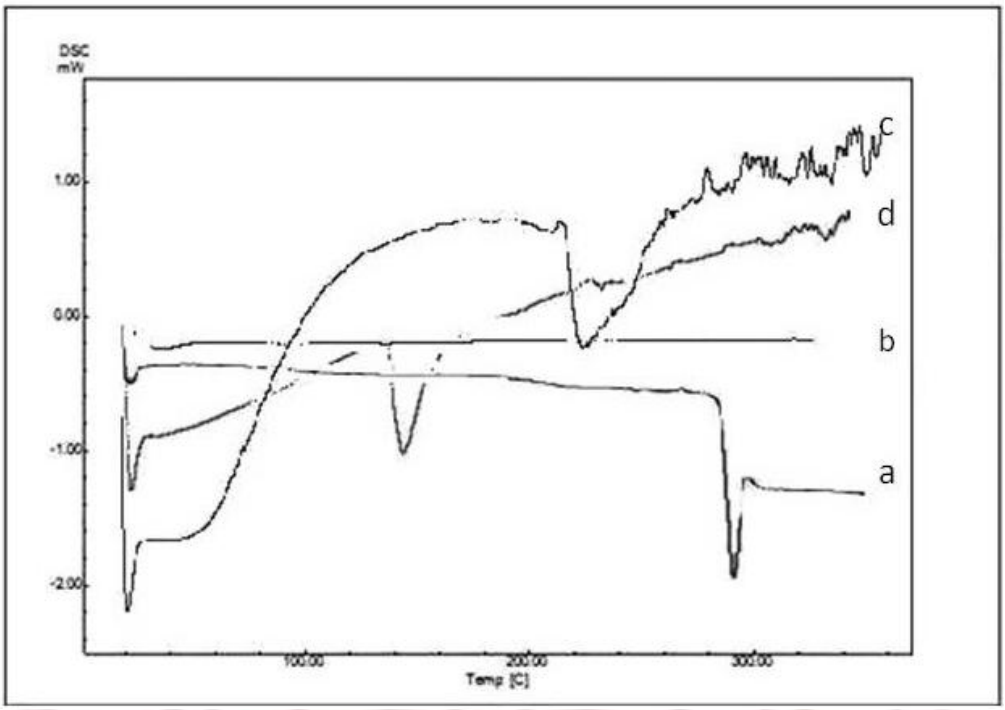

Fig 1.8 DSC thermogram of Triamcinolone acetonide

1.5.3.3 Transmission Electron Microscopy

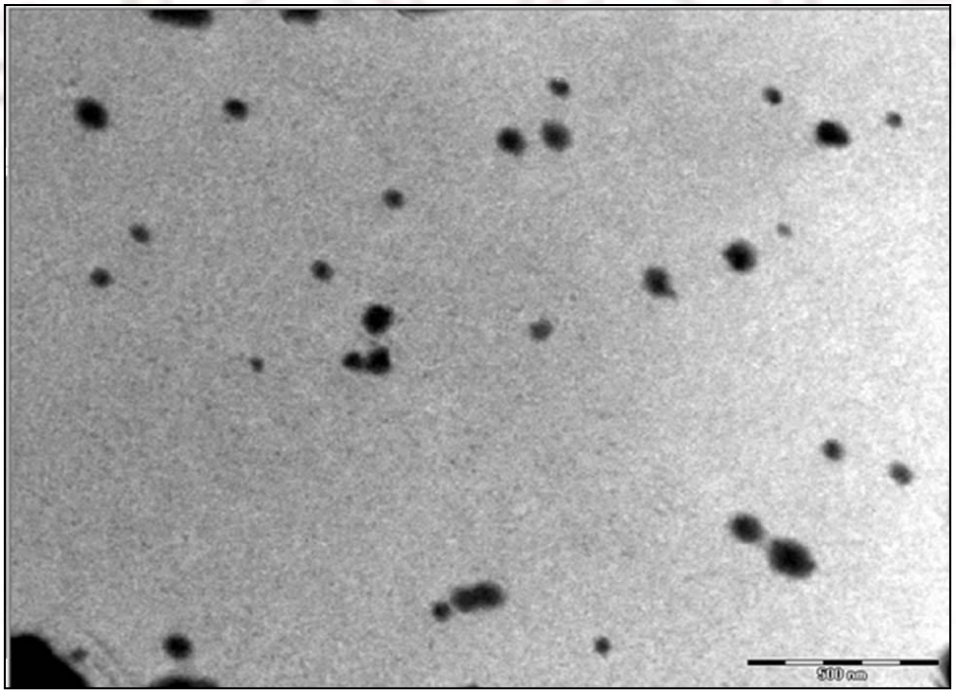

Fig 1.9 TEM image of TA loaded lecithin/ CS nanoparticles

The shape of the TA lecithin/ CS HCl nanoparticles was investigated using transmission electron microscopy (TEM). Lecithin/ CS HCl nanoparticles were seen to be distinct, spherical particles (Fig. 7.9). No aggregation was observed between nanoparticles indicating possible stabilization of the nanoformulation by positive surface charges.

1.5.3.4 *In vitro* drug release studies

The *in vitro* release of TA from its aqueous solution and lecithin/ CS HCl nanoparticles was investigated in phosphate buffer saline at 37 ◦C ± 2 ºC.

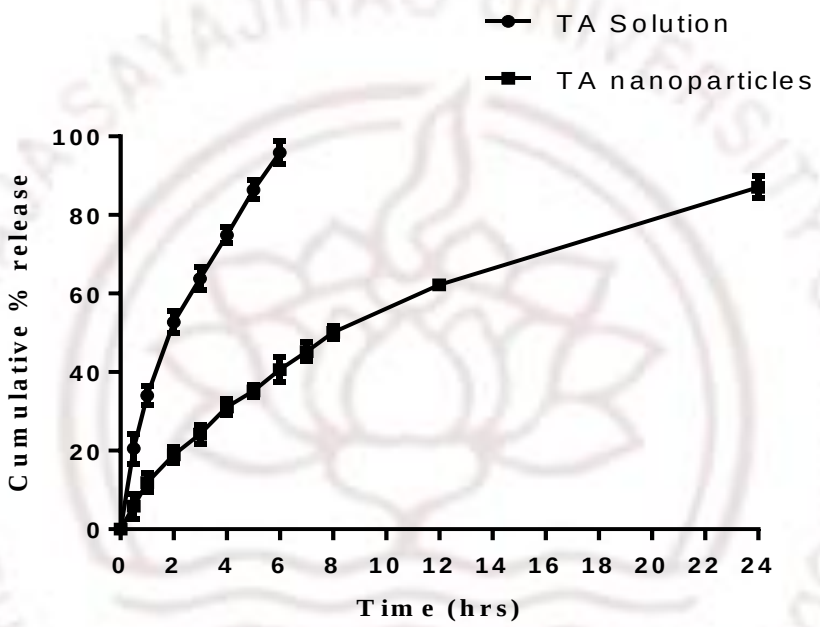

Fig 1.10 Release profile of TA from TA solution and TA loaded lecithin/ CS HCl nanoparticles

1.5.3.5 *Ex vivo* studies

Table 7.7 compares the results of *ex vivo* corneal permeation of triamcinolone acetonide from the nanoparticles with triamcinolone acetonide solution across isolated goat cornea. As compared to TA solution, higher corneal permeation was observed in case of TA nanoparticles.

Table 1.7 *Ex vivo* permeation of triamcinolone acetonide from nanoparticles and aqueous solution

S. No.	Formulations	Corneal Permeation (cm/s)
1	TA loaded nanoparticles	2.97±0.79
2	TA solution	2.39± 0.60

1.5.3.6 Stability Studies

The data of stability studies of lyophilized TA loaded lecithin/ CS nanoparticles at refrigerated conditions (2-8°C) and at room temperature (25-30 ºC) are shown in Table 7.8. It was observed that at refrigerated conditions, TA nanoparticles lyophilized in presence of Trehalose dihydrate displayed superior stability compared to room temperature, for upto three months.

Table 1.8 Stability profile of lyophilized TA loaded nanoparticles at 2-8° C and 25-30 °C

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