Chapter 4 Formulation and development: Docetaxel

4.0 MATERIALS AND METHODS

4.1 Materials

Docetaxel Trihydrate (DTX) was obtained as gift sample from Sun Pharma Advanced Research Centre (SPARC), Vadodara, India. Human Serum Albumin (HSA) fraction V, purity 96–99%, 65,000 Da was purchased from Sigma Aldrich, Germany. Chloroform was purchased from H. B. Chemicals, Vadodara, India. Double distilled water used in the study was filtered using 0.22-µm nylon filter (Nylon N66 membrane filters 47 mm, 60-125 µm thickness, Rankem, India). Cellulose dialysis tubing (Molecular weight cut of 12000) and membrane filter of pore size 0.2 µm were purchased from HiMedia Lab, Mumbai, India. All other chemicals and reagents used in this study were of analytical grade.

4.2 Equipments

- Digital Analytical Balance (Shimadzu SCS, Switzerland)
- Probe Sonicator (LabsonicM, Sartorius Ltd, Mumbai, India)
- High Pressure Homogenizer (HPH, Emulsiflex C5, Avestin, Canada).
- High Speed Magnetic Stirrer (Remi, MS500, Remi Equipments, Mumbai, India)
- High Speed Centrifuge (Sigma 3K30, Germany)
- Lyophilizer (Heto, Vaccubrand, Denmark)
- Particle Size Analyzer 3000 HS (Zeta Sizer Nano Series, Malvern Instruments, UK)
- Differential Scanning Calorimeter (DSC, Mettler Toledo DSC 822e, Japan)
- X-ray diffraction (XRD, Philips-PW-1050, Netherland)
- Transmission Electron Microscope (TEM, Philips, Technai 20, Holland)

4.3 Preparation of DTX HSA Nanoparticles (DTX-HSA-NPs) by HPH

Briefly, 10-30 mg of DTX [0.5-1.5 mg DTX/mL of HSA solution] was dissolved in required volume of chloroform [0.4-0.8 mL] and added slowly into 20.0 mL of human serum albumin solution [2.5 -7.5 mg/mL in water] with simultaneous probe sonication to form a crude emulsion. Crude emulsion was then transferred into a HPH (1) and the emulsification was performed at 10000-20000 PSI and recycled for different number of cycles [4-10 cycles] (2). The resulting system was transferred into a rotary evaporator, and the chloroform was rapidly removed at 40 °C under reduced

pressure. The dispersion was further lyophilized without any cryoprotectant (3). Finally, the solid cake of DTX-HSA-NPs was obtained.

4.4 Preliminary Optimization of Parameters

In preliminary optimization the formulation and process parameters influencing the formation of NPs were identified and optimized (4).

4.4.1 Optimization of Formulation Parameters

Formulation parameters such as DTX:HSA ratio and concentration of DTX were optimized for desired results. Effect of one variable was studied at a time keeping other variables constant.

4.4.1.1 DTX:HSA ratio

Optimized on the basis of particle size and percent drug entrapment of the NPs formed at different DTX:HSA ratio. Numbers of trials were carried out at different DTX:HSA ratio [1:3 to 1:8] to optimize the ratio forming uniform NPs.

4.4.1.2 Concentration of DTX

Optimized on the basis of particle size and percent entrapment of DTX in NPs formed at different concentration of DTX in the formulation. Numbers of trials were carried out at different concentration of DTX (0.5-2.5 mg/mL) to optimize the concentration forming uniform and stable NPs.

4.4.2 Optimization of Process Parameters

Process parameter optimization such as sonication energy, pressure during HPH and number of HPH cycles were optimized for desired results. Effect of one variable was studied at a time keeping other variables constant.

4.4.2.1 Sonication energy

The effect of various levels of sonication energy on crude emulsion formation was determined by application of sonication for 300 second at 0.6 Duty cycle at different amplitude (40, 60 and 80%).

4.4.2.2 Pressure of HPH

Pressure of HPH was optimized on the basis of pressure required to form nano sized particles with maximum uniformity and drug entrapment. Numbers of trials were

carried out at different pressure of HPH (5000-25000 PSI) to optimize the pressure required to from uniform NPs.

4.4.2.3 Number of HPH cycles

Optimized on the basis of number of HPH cycles required to form NPs with maximum uniformity and drug entrapment. Number of trials was carried out at different number of HPH cycles [4-10 cycles] to optimize the number of HPH cycles required to from uniform NPs.

4.5 Optimization of DTX-HSA-NPs by Box Behnken Design (BBD)

In response surface methodology (RSM) (5), the experimentation is completed before the optimization takes place and this methodology also called as simultaneous optimization. In RSM methods, one or more selected experimental responses are recorded for a set of experiments carried out in a systematic way to predict the optimum and the interaction effects (6). In this study, a BBD was used to optimize the formulation of albumin NPs. A specially made design, the BBD requires only three levels for each factor i.e., –1, 0, and +1 (7). The BBDs are also popularly used for response surface optimization of drug delivery systems (8). The BBD is rotatable (or nearly rotatable), and the treatment combinations are located at the midpoints of edges and the center of the experimental domain, as showed in **Figure 4.1** (7).

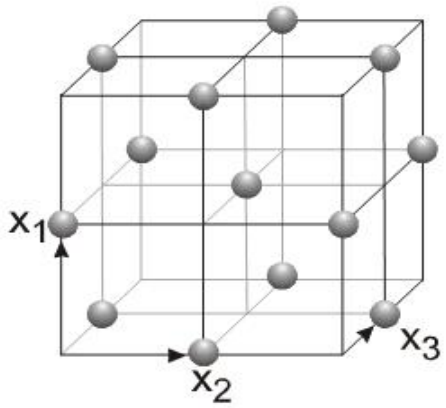

Figure 4. 1 Diagrammatic representation of a BBD for three factors

Preliminary studies were undertaken to decide levels of formulation and process parameters in experimental design having significant and interaction effect. After preliminary optimization of formulation and process parameters the mathematical model was developed for optimization of NPs by BBD in order to find out the adequate

conditions required to prepare albumin NPs of desired characteristics (9). RSM combined with BBD was used to generate the relationship between independent variables and dependent variables (10). A three-factor, three-level BBD with three replicates at the center point was selected to build response surface models. The effects of formulation variables on the NPs characteristics and optimization procedure were examined by BBD. The design and statistical analysis were performed by Design Expert® Software (Version 8.0.7.1, Stat-Ease Inc, Minneapolis, USA) for design of experiments (DOE).

Based on the preliminary experiments, concentration of drug (mg/mL) (X_1), concentration of albumin (mg/mL) (X_2) and Homogenization Pressure (PSI) (X_3) were selected as independent variables and particle size (nm) (Y_1), zeta potential (mV) (Y_2) and percentage entrapment efficiency (% EE) (Y_3) were selected as dependent variables. For three factors, the BBD offers advantage in requiring a fewer number of runs over three level full factorial designs. In full factorial designs increase in number of factors increases the number of trial runs exponentially, such as 3^3=27, but with BBD, optimization can be completed with 15 experiments (12 factorial and 3 center points). Factors that might affect the designed characteristic of nanoparticle formulation were varied over three levels given in **Table 4.1** and formulations were arranged according to a BBD given in **Table 4.2.**

Table 4. 1 Factors and levels of factors studied in a BBD

Independent Variable (Factors)	Coded Levels		
	-1 (Low)	0 (Medium)	+1 (High)
Amount of drug (mg/mL) (X_1)	0.5	1.0	1.5
Amount of albumin (mg/mL) (X_2)	2.5	5.0	7.5
Homogenization Pressure (PSI) (X_3)	10000	15000	20000

Table 4. 2 BBD Experimental Layout

Formulation Code	Run	Coded Level (Independent variables)		
		X_1	X_2	X_3
F 1	14	-1	-1	0
F 2	15	1	-1	0
F 3	9	-1	1	0
F 4	5	1	1	0
F 5	4	-1	0	-1
F 6	6	1	0	-1
F 7	13	-1	0	1
F 8	7	1	0	1
F 9	10	0	-1	-1
F10	2	0	1	-1
F11	11	0	-1	1
F12	8	0	1	1
F13	3	0	0	0
F14	12	0	0	0
F15	1	0	0	0

4.5.1 Optimization Data Analysis

Various RSM computations for the current optimization study were performed and polynomial models including interaction and quadratic terms were generated for the response variables using multiple regression analysis (MLRA) approach (6). A second degree polynomial model for this response is as follows:

$$\mathbf{Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X^2_1 + \beta_{22}X^2_2 + \beta_{33}X^2_3 \ (1)}$$

Where Y = measured response associated with each factor level combination; β_0 = intercept; β_1 to β_{33} are regression coefficients computed from the observed experimental values of Y from experimental runs; and X_1, X_2 and X_3 are the coded levels of independent variables. The polynomial equation was used to draw conclusions after considering the magnitude of coefficients and the mathematical sign it carries, i.e.

positive sign indicates the synergistic effect, whereas a negative sign indicates the antagonistic effect. By using this equation, it is possible to evaluate appropriately the linear, quadratic and interactive effects of the independent variables on the responses. The statistical analysis of the data through regression model and plotting the response surface graphs were carried out using Design-Expert. Analysis of variance (ANOVA) evaluated the significance of each coefficient by p-value (less than 0.05) through Fisher's test and it helps to choose the significant model (11). The predicted values and the experimental parameters were evaluated by the correlation coefficient and the adjusted correlation coefficient. In order to find the optimized formulations, in all experimental regions the numerical searches were employed by considering the constraints in which the particle size is in its minimum level, zeta potential between (-20 to -30.8) and % EE is in its maximum level. The experimental responses were compared with the predicted values (obtained from the equation) to evaluate precision of the model.

4.5.2 Response Surface Plots

Response surface plots were used as a function of two factors at a time maintaining all other factors at fixed levels to understand main and interaction effects of two variables (12). These plots were obtained by calculating the values taken by one factor where the second varies (from -1 to 1 for instance) with constraint of a given Y value. The yield values for different levels of variables can also be predicted from the respective response surface plots.

4.5.3 Validation of Optimization Methodology (Check Point Analysis)

Validation of the optimization methodology (13) is a very crucial step that tells about the prognostic ability of the model studied. So the generated polynomials are tested for their predictive abilities. The predicted values are compared with that of the observed experimental data and the percentage residual error is determined. Various new batches of DTX-HSA-NPs were prepared experimentally by taking the amounts of the independent variables (X_1 and X_2) selected from the different regions of the experimental domain and evaluated according to the standard operating conditions. Each batch was prepared three times and mean values were determined. Difference in the predicted and mean values of experimentally obtained particle size, zeta potential and % EE was compared.

4.5.4 Desirability Criteria

For simultaneous optimization of particle size, zeta potential and % EE, desirability function was applied and total desirability was calculated using Design Expert software. The desirability lies between 0 and 1 and it represents the closeness of a response to its ideal value. If both the quality characteristics reach their ideal values, the individual desirability is 1. Consequently, the total desirability is also 1. Our optimization criteria included minimum particle size, zeta potential in the range -20 to -30.8 and maximum % EE.

4.6 Lyophilization of DTX-HSA-NPs

The optimized nanoparticulate formulation was lyophilized without any cryoprotectant (3). Nanoparticulate suspension (3 ml) was dispensed in 10 ml semi-stoppered vials with rubber closures and frozen for 24 h at -70 °C. Thereafter, the vials were lyophilized (24 h) in lyophilizer. Finally, glass vials were sealed and stored until being re-hydrated. Lyophilized NPs were re-dispersed in exactly the same volume of distilled water as before lyophilization and were evaluated for particle size and drug content.

4.7 Characterization of DTX-HSA-NPs

4.7.1 Particle Size

The size analysis and polydispersity index of the NPs were determined using a Malvern Zetasizer. Each sample was diluted ten times with filtered double distilled water to avoid multi-scattering phenomena and placed in disposable sizing cuvette. Polydispersity index was noted to determine the narrowness of particle size distribution. Analysis was performed in triplicate and the results were expressed as mean ± SD.

4.7.2 Zeta Potential

Zeta potential was also measured using a Malvern Zetasizer at 25°C. Each sample was suitably diluted 10 times with filtered double distilled water and placed in a disposable zeta cell. The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and there is no tendency to flocculate.

However, if the particles have low zeta potential values then there is no force to prevent the particles coming together and flocculating. The electrophoretic mobility (µm/sec) was converted to zeta potential by in-built software using Helmholtz-Smoluchowski equation.

4.7.3 Percent Entrapment Efficiency (% EE)

For the determination of % EE, the nanoparticulate dispersion was centrifuged at 3000 RPM (RCF: 855g), for 10 minutes using centrifuge (Sigma, 3K30, Germany). The amount of entrapped DTX in nanoparticle in the supernatant was determined by HPLC. The HPLC system comprised of a pump and UV Vis detector with a C18 column (Particle size 10 µm, 4.6 mm x 250 mm) that was used to elute DTX. The mobile phase was a mixture Acetonitrile: Water (60:40). The flow rate was kept 1.0 mL/min with detection at 229 nm wavelength. 1 ml of supernatant was dissolved in methanol: water (50:50) from which 20 µL supernatant was injected into the HPLC system. The peak area of DTX was recorded and the concentration of DTX was calculated from a standard curve. The % EE was estimated by calculating amount of drug entrapped in NPs with respect to total amount of drug added during preparation of formulation.

4.7.4 Differential Scanning Calorimetry

DSC analysis was carried out using a Differential Scanning Calorimeter at a heating rate of 10°C per minute in the range of 30°C to 300°C under inert nitrogen atmosphere at a flow rate of 40 ml/min. DSC can be used to determine the nature and speciation of crystallinity within nanoparticles through the measurement of glass and melting point temperatures and their associated enthalpies. DSC thermogram was recorded for DTX, HSA, DTX-HSA physical mixture and DTX-HSA-NPs.

4.7.5 X-Ray Diffractometry (XRD)

XRD patterns were obtained by using an X-Ray diffractometer with a horizontal goniometer. The samples were placed in the sample holder and scanned at a rate of 1° per minute in scanning range of 2θ from 6° to 70°. XRD study was carried out for DTX, lyophilized blank NPs, physical mixture of DTX and blank NPs and DTX-HSA-NPs.

4.7.6 Transmission Electron Microscopy (TEM)

TEM is useful since it gives idea of its internal structure, morphology and allows particles much smaller than 1 µm to be measured. In present work, TEM analysis was performed by Philips Technai 20 instrument. NPs were dispersed in distilled water and drop of redispersed nanoparticle was placed on carbon coated copper grid. This copper grid was fixed into sample holder and placed in vacuum chamber of transmission electron microscope and observed under low vacuum.

4.7.7 *In-vitro* drug release

The *in vitro* drug release was carried out by dialysis bag technique (14). A dialysis membrane MWCO 12000 was used for *in vitro* release studies. 1 ml of formulation (Plain drug dispersion and DTX-HSA-NPs) was placed in dialysis bag. Bag was sealed at both ends and placed in a beaker containing 20 ml of receptor medium (PBS {pH 7.4}: methanol {7:3}) maintained at 37 ℃. Samples (1 ml) were withdrawn from the receptor compartment at predetermined time intervals, and the volume was replenished with same volume of diffusion medium. Addition of diffusion medium to the receptor compartment was performed with great care to avoid trapping air beneath the diffusion membrane. The samples were diluted and analyzed by HPLC at 229 nm. The % drug release was calculated. Graph of % drug release vs. time was plotted. The drug release studies were performed in triplicate.

4.7.8 Hemolysis

The *in vitro* hemolysis assay was performed to evaluate the hemoglobin release in the plasma (as an indicator of red blood cell lysis) following formulation exposure and it provide information about the toxicity of the drug, its metabolite, or an excipient in the formulation. Blood was freshly drawn from male Sprague Dawley (SD) rat by retro-orbital sinus puncture. Briefly, freshly collected rat blood was washed three times with PBS solution (pH 7.4) by centrifugation at 3000 rpm for 5 minutes. The red blood cell suspension was diluted with saline to obtain a 2% suspension (v/v). Various formulations in phosphate buffer were added into the RBC suspension. After incubation at 37℃ for 1 hour, samples were centrifuged at 3000 rpm for 10 minutes to remove non-lysed RBC. The supernatants were collected and analyzed for hemoglobin content by spectrophotometric detection at 416 nm. The degree of hemolysis was determined by the following equation:

$$\text{Hemolysis (\%)} = (Abs - Abs_0)/(Abs_{100} - Abs_0) \times 100$$

Where Abs, Abs_{100}, and Abs_0 are the absorbencies of the samples, a solution of 100 % hemolysis, and a solution of 0 % hemolysis, respectively

4.7.9 Stability Studies

The stability of DTX-HSA-NPs in terms of drug content and particle size distribution was monitored for 6 months at 2-8 ℃ and room temperature (25 ℃). Periodically, samples were withdrawn and the particle size and DTX content was determined.

4.8 Results

4.8.1 Preliminary Optimization

4.8.1.1 Optimization of Formulation Parameters:

DTX:HSA ratio: The effect of DTX:HSA ratio on particle size and % EE of NPs was observed. Results indicate that at 1:4 DTX:HSA ratio the particle size of NPs was minimum and % EE was low. At 1:5 DTX:HSA ratio the particle size of NPs was minimum and % EE was highest. As the DTX to HSA ratio increased from 1:5 to 1:8, there was increase in particle size of nanoparticle and decrease in % EE was observed. Results of particle size, PDI and % EE obtained at different DTX:HSA ratios are given in **Table 4.3.**

Concentration of DTX: The effect of various concentration of DTX on particle size and stability of nanoparticle was observed. Results indicate that at lower concentration of drug (0.5 mg/ml), the particle size of NPs was somewhat large. At intermediate concentration of drug (1 mg/ml), the particle size of NPs was minimum and stable nanoparticle was formed. As the concentration of drug increased from 1.5 to 2 mg/ml, there was increase in particle size of nanoparticle and unstable nanoparticle was observed. Results are given in **Table 4.4.**

Table 4. 3 Particle size, PDI and % EE at different DTX: HSA ratios.

Drug :HSA Ratio	Particle Size of NPs(nm)* (Mean ± SD)	PDI* (Mean ± SD)	% EE* (Mean ± SD)
1:4	285±4.87	0.347±0.065	54±1.456
1:5	156±5.35	0.213±0.045	68±1.834
1:6	340±5.97	0.356±0.057	50±1.231
1:7	643±11.87	0.623±0.121	39±1.114
1:8	754±14.65	0.767±0.137	26±0.678

* The experiment was performed in triplicate (n=3)

Table 4. 4 Particle size, PDI and stability results at different drug concentration

Concentration of Drug (mg/mL)	Particle size NPs(nm)* (Mean ± SD)	PDI* (Mean ± SD)	Observation
0.5	305±4.65	0.342±0.062	Stable NPs
1	154± 6.54	0.271±0.051	Stable NPs
1.5	412±5.83	0.435±0.065	Stable NPs
2	576±7.85	0.562±0.076	Unstable NPs

* The experiment was performed in triplicate (n=3)

4.8.1.2 Optimization of Process Parameters:

Sonication energy: The effect of various levels of sonication energy on crude emulsion formation was determined by application of sonication for 300 second at 0.6 cycle time at different amplitudes. It was observed that at 40% amplitude emulsion was not formed. At 60% and 80% amplitude stable emulsion was formed. So, the optimized amplitude was 60% based on minimum amplitude required to form stable crude emulsion.

Pressure of HPH: The effect of homogenization pressure on particle size of nanoparticle was determined by application of different level of homogenization pressure and the particle size of prepared NPs was observed. Results indicate that particle size of nanoparticle prepared at lower pressure and higher pressure (5000 PSI and 25000 PSI) was very large and PDI was also very high. At intermediate pressure (15000 PSI) the particle size of nanoparticle was found optimum for i.v. administration with good PDI given in **Table 4.5.**

Table 4. 5 Particle size and PDI at different Homogenization pressure

Pressure (PSI)	Particle size NPs (nm)* (Mean ± SD)	PDI* (Mean ± SD)
5000	1567± 55.67	0.714±0.184
15000	162±14.72	0.235±0.035
25000	427±18.15	0.478±0.056

* The experiment was performed in triplicate (n=3)

Number of HPH cycles: The effect of number of HPH cycles on particle size of NPs was determined by application of different number of HPH cycles. Numbers of trials were carried out at 15000 PSI pressure of HPH to find optimum no. of cycles to form uniform NPs. Results indicated that by applying 4 HPH cycles at 15000 PSI the formed NPs have very large particle size. As the no. of cycles increases from 5 to 7 there was gradually decrease in the particle size of NPs. At 8 HPH cycles the formed NPs have 153 ± 5.42nm particle size with 0.216 ± 0.002 PDI. Further as the no. of cycles increases there was gradually increase in the particle size of NPs. Results are given in **Table 4.6.** Conventionally it would be expected that as the no. of HPH cycles increases, the particle size should decrease. However result demonstrates that the particle size passes through a minimum size at an intermediate no. of cycles and then increases at higher no. of cycles. This effect is described as “over-processing” which is caused by increase in nanoparticle coalescence at higher no. of cycles.

Table 4. 6 Particle size and PDI at different no. of HPH cycle

No. of Cycles (15000 PSI)	Particle size NPs (nm)* (Mean ± SD)	PDI* (Mean ± SD)
4	1150±75.66	0.967±0.021
5	867±31.21	0.867±0.018
6	425±7.15	0.627±0.013
7	275±4.45	0.367±0.007
8	153 ± 5.42	0.216±0.002
9	387±6.14	0.357±0.005
10	534±7.24	0.469±0.008

* The experiment was performed in triplicate (n=3)

4.8.2 Optimization of DTX-HSA-NPs by BBD

Total 15 batches of DTX-HSA-NPs were prepared as BBD by varying the three independent variables and evaluated for particle size in nm (Y_1), zeta potential in mV (Y_2) and entrapment efficiency in % (Y_3). Results of analysis are given in **Table 4.7** and the coded and actual values of formulation parameters are given in **Table 4.1**. The quality of formulation can be improved by optimizing the formulation systematically.

Table 4. 7 Particle size, zeta potential and % EE results of DTX-HSA-NPs by BBD

Formulation Code	Run	Coded Level (Independent variables)			Responses (Dependent variables)		
		X_1	X_2	X_3	Y1 (nm)	Y2 (-mV)	Y3 (%)
F 1	14	-1	-1	0	330.2	19.5	52.3
F 2	15	1	-1	0	390.6	15.7	38.6
F 3	9	-1	1	0	486.8	28.4	64.6
F 4	5	1	1	0	570.7	26.7	61.3
F 5	4	-1	0	-1	250.5	16.2	58.9
F 6	6	1	0	-1	342.6	14.6	55.6
F 7	13	-1	0	1	258.4	22.4	59.8
F 8	7	1	0	1	296.8	15.4	54.7
F 9	10	0	-1	-1	320.4	15.2	30.2
F10	2	0	1	-1	515.6	28.6	53.4
F11	11	0	-1	1	320.5	17.6	26.4
F12	8	0	1	1	442.8	30.8	43.8
F13	3	0	0	0	150.0	23.6	70.5
F14	12	0	0	0	153.9	21.6	69.7
F15	1	0	0	0	152.6	22.8	68.8

On the basis of the results obtained in the preliminary screening studies, the high and low level independent variables were selected. In order to investigate the factors systematically, a BBD was employed for optimization of DTX-HSA-NPs. The particle size (nm), zeta potential (mV) and entrapment efficiency (%) for the 15 batches (F1 to F15) showed a wide variation 150.0-570.7 nm, -30.8 to -14.6 and 26.4 - 70.5 %

respectively as given in **Table 4.7**. Optimization results of analysis for particle size (nm), zeta potential (mV) and entrapment efficiency (%) are given below.

4.8.2.1 Statistical Analysis of Particle Size (Response 1)

Sequential p-value, lack of fit p-value, adjusted R^2, predicted R^2 values and suggested model are given in the **Table 4.8.**

Table 4. 8 Summary of ANOVA results (Particle Size) for Different Models

Source	Sequential p-value	Lack of Fit p-value	Adjusted R-Squared	Predicted R-Squared	Model Suggested
Linear	0.3119	0.0002	0.0676	-0.1683	----
2FI	0.9912	0.0001	-0.2661	-1.1686	----
Quadratic	< 0.0001	0.0512	0.9973	0.9852	Suggested
Cubic	0.0512		0.9998		Aliased*

*The Cubic Model and higher are aliased. This shows that the predicted responses would be confounded by the other factors implying that the predicted response would give the wrong idea of the actual response.

Table 4.8 suggested the best model to fit the experimental results of particle size of NPs is quadratic model and results of ANOVA analysis of the suggested quadratic model given in **Table 4.9.**

Table 4. 9 ANOVA for Response Surface Quadratic Model (Particle Size)

Source	Sum of Squares	df	Mean Square	FValue	p-value Prob > F	
Model	2.408E+005	9	26759.93	583.89	< 0.0001	Significant
X_1- Drug	*9439.38*	*1*	*9439.38*	*205.96*	*< 0.0001*	
X_2-Albumin	*53497.21*	*1*	*53497.21*	*1167.29*	*< 0.0001*	
X_3-HP	*1529.05*	*1*	*1529.05*	*33.36*	*0.0022*	
$X_1 X_2$	*138.06*	*1*	*138.06*	*3.01*	*0.1431*	
$X_1 X_3$	*720.92*	*1*	*720.92*	*15.73*	*0.0107*	
$X_2 X_3$	*1328.60*	*1*	*1328.60*	*28.99*	*0.0030*	
X_1^2	*29794.26*	*1*	*29794.26*	*650.10*	*< 0.0001*	
X_2^2	*1.515E+005*	*1*	*1.515E+005*	*3306.24*	*< 0.0001*	

X_3^2	*7503.25*	*1*	*7503.25*	*163.72*	*< 0.0001*	
Residual	229.15	5	45.83			
Lack of Fit	*221.27*	*3*	*73.76*	*18.70*	*0.0512*	**Not**
Pure Error	*7.89*	*2*	*3.94*			**significant**
Cor Total	2.411E+005	14				
Std. Dev.	6.77		**R-Squared**	0.9990		
Mean	332.16		**Adj R-Squared**	0.9973		
C.V. %	2.04		**Pred R-Squared**	0.9852		
PRESS	3557.98		**Adeq Precision**	74.972		

The Model F-value of 583.89 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. *In this case amount of drug, amount of albumin, homogenization pressure, interation between amount of drug and homogenization pressure, interation between amount of albumin and homogenization pressure as well as amount of drug, amount of albumin and homogenization pressure having quadartic effect and are significant model terms.* Term *interaction between amount of drug and amount of albumin* (term X_1*X_2) having values greater than 0.1000 indicate the model term is not significant. The "Lack of Fit F-value" of 18.70 implies there is a 5.12% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good because we want the model to be fit for our data. The "Pred R-Squared" of 0.9852 is in reasonable agreement with the "Adj R-Squared" of 0.9973."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable and our ratio of 74.972 indicates an adequate signal.

Final Polynomial Equation in Terms of Coded Factors for Particle Size

$$= +152.17$$
$$+34.35*X_1 +81.78 * X_2 -13.83* X_3$$
$$+5.87*X_1*X_2 -13.43*X_1*X_3 -18.22*X_2*X_3$$
$$+89.83 * X_1^2 +202.58 * X_2^2 +45.08 * X_3^2$$

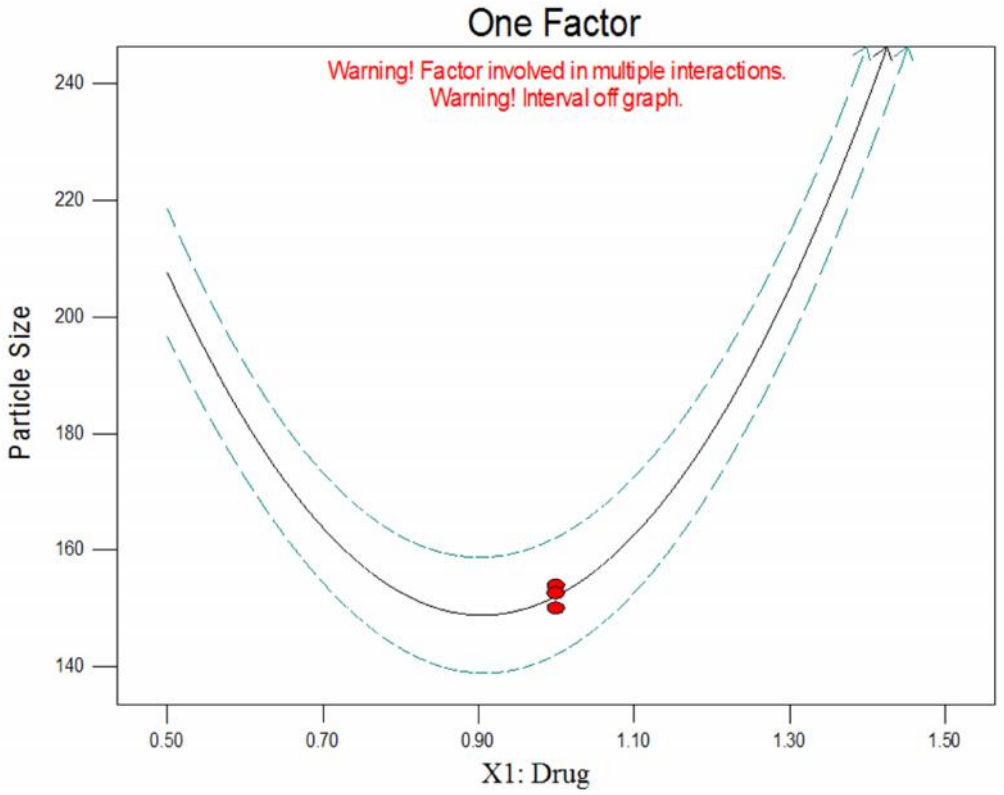

Figure 4. 2 Effect of Drug on Particle Size

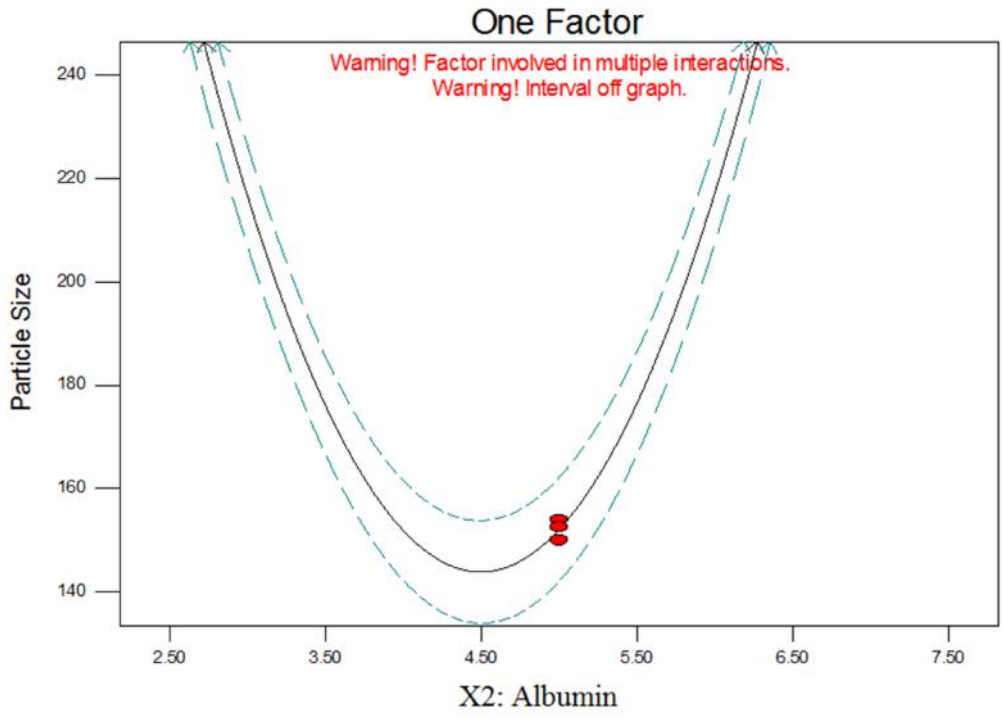

Figure 4. 3 Effect of Albumin on Particle Size

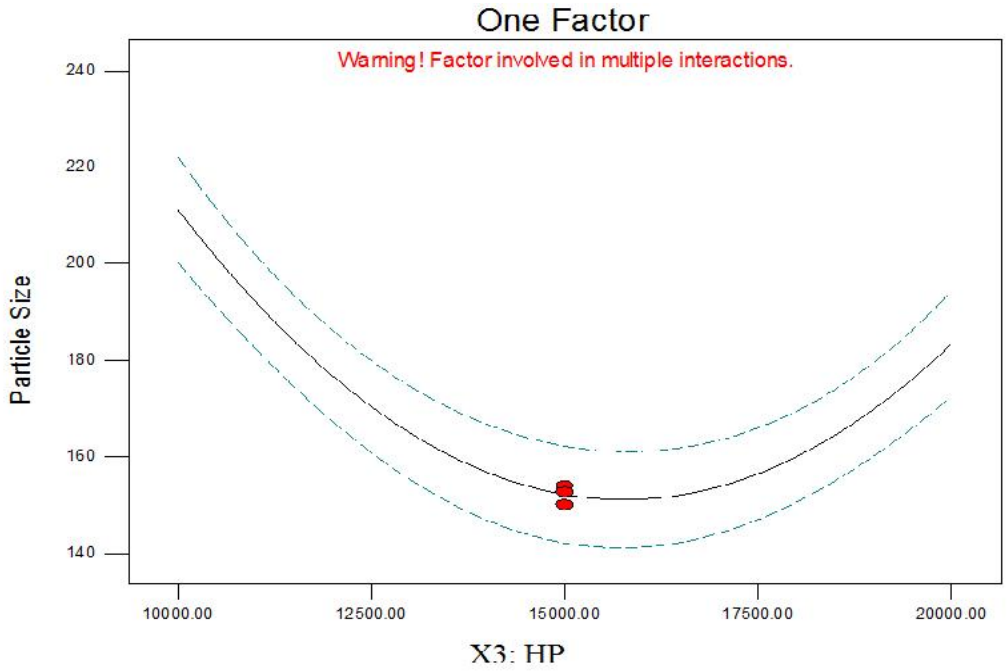

Figure 4. 4 Effect of HP on Particle Size

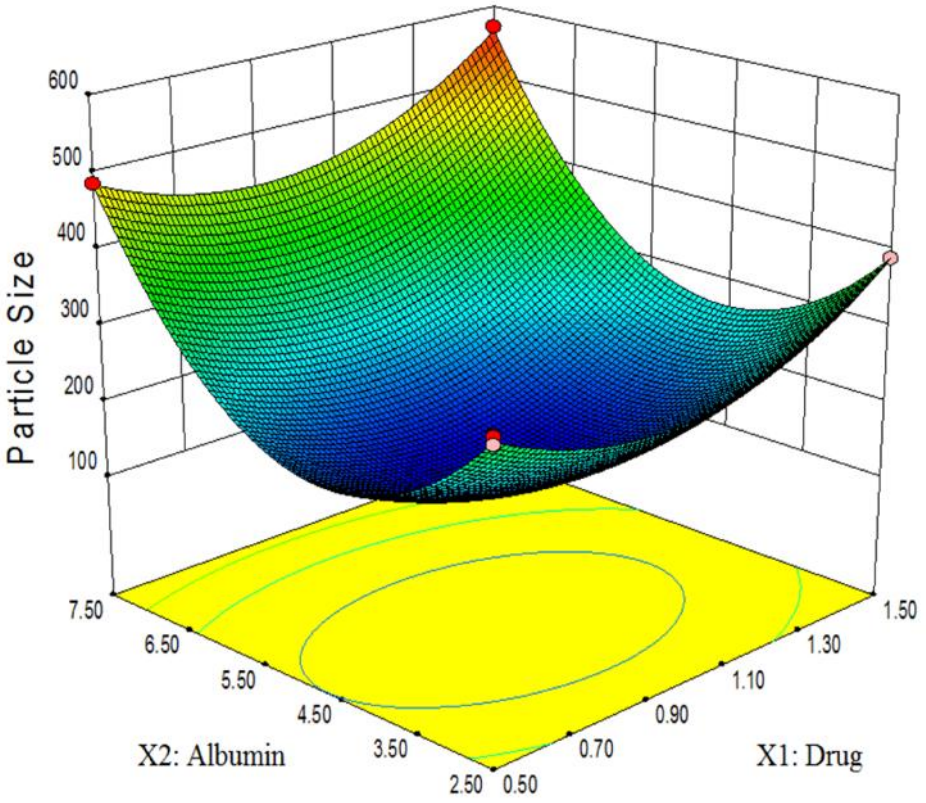

Figure 4. 5 Response surface showing combined effect of drug and albumin on particle size.

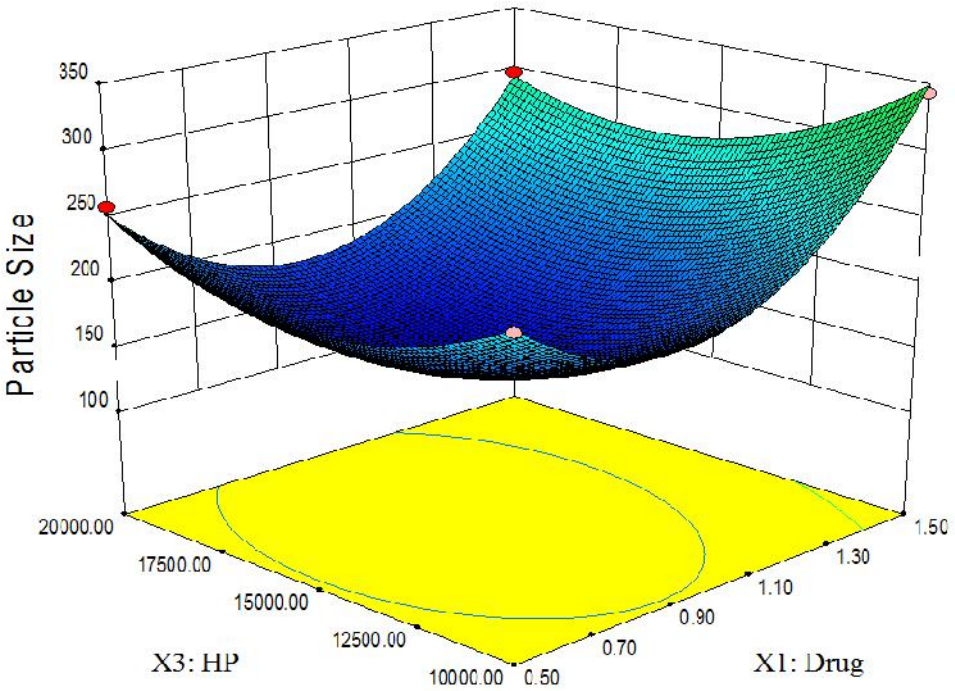

Figure 4. 6 Response Surface Showing Combined Effect of Drug and HP on Particle Size.

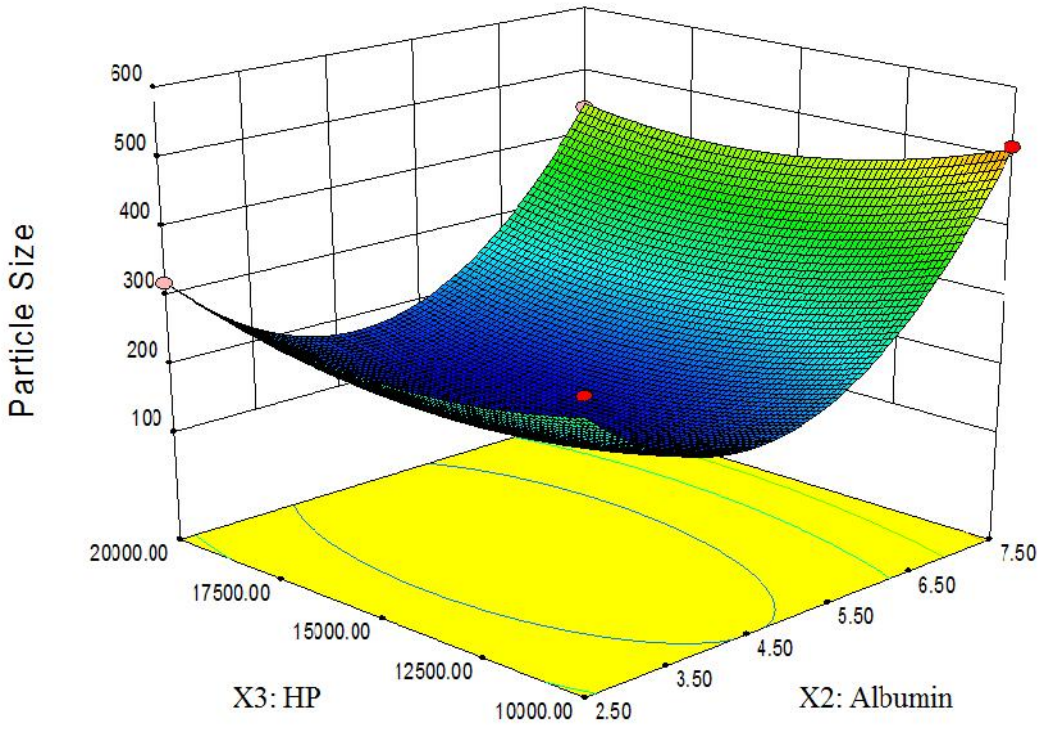

Figure 4. 7 Response Surface Showing Combined Effect of Albumin and HP on Particle Size.

One factor effect plots shown in **Figure 4.2, Figure 4.3** and **Figure 4.4** for factors X_1, X_2, and X_3 respectively shows that all three factor have quadratic effect (curvilinear plot) on the particle size of NPs. Two-factor response surface plots shown in **Figure 4.5, Figure 4.6** and **Figure 4.7** for factors X_1X_2, X_1, X_3 and X_2X_3 shows the same effect of X_3 over other factors X_1 and X_2. From response surface plots it was found that all three factors were important in deciding particle size of the final formulation i.e. all the factors have some influence on the particle size. This was further confirmed by observing p value which proved that all the factors have significant influence on size. Additionally it was seen that all factors in lower quantity gave higher size and there was continuous reduction in size till concentration of drug 1 mg/mL, concentration of albumin 5.0 mg/mL and Homogenization Pressure 15000 PSI. Again when factors exceeded above mentioned limit there was gradual increase in size may be due to increased concentration of albumin and drug.

4.8.2.2 Statistical Analysis of Zeta Potential (Response 2)

Sequential p-value, lack of fit p-value, adjusted R^2, predicted R^2 values and suggested model are given in the **Table 4.10.**

Table 4. 10 Summary of ANOVA results (Zeta Potential) for Different Models

Source	Sequential p-value	Lack of Fit p-value	Adjusted R-Squared	Predicted R-Squared	Model Suggested
Linear	0.0015	0.0667	0.6675	0.4572	
2FI	0.8799	0.0486	0.5777	-0.2152	
Quadratic	0.0048	0.2607	0.9389	0.7056	Suggested
Cubic	0.2607		0.9721		Aliased*

*The Cubic Model and higher are aliased. This shows that the predicted responses would be confounded by the other factors implying that the predicted response would give the wrong idea of the actual response.

Table 4.10 suggested the best model to fit the experimental results of Zeta Potential of NPs is quadratic model and results of ANOVA analysis of the suggested quadratic model given in **Table 4.11.**

Table 4. 11 ANOVA for Response Surface Quadratic Model (Zeta Potential)

Source	Sum of Squares	df	Mean Square	FValue	p-value Prob > F	
Model	413.04	9	45.89	24.91	0.0012	Significant
X_1- Drug	*24.85*	*1*	*24.85*	*13.49*	*0.0144*	
X_2-Albumin	*270.28*	*1*	*270.28*	*146.69*	*< 0.0001*	
X_3-HP	*16.82*	*1*	*16.82*	*9.13*	*0.0294*	
$X_1 X_2$	*1.10*	*1*	*1.10*	*0.60*	*0.4742*	
$X_1 X_3$	*7.29*	*1*	*7.29*	*3.96*	*0.1034*	
$X_2 X_3$	*1.000E-002*	*1*	*1.000E-002*	*5.427E-003*	*0.9441*	
X_1^2	*32.41*	*1*	*32.41*	*17.59*	*0.0085*	
X_2^2	*31.86*	*1*	*31.86*	*17.29*	*0.0088*	
X_3^2	*22.85*	*1*	*22.85*	*12.40*	*0.0169*	
Residual	9.21	5	1.84			
Lack of Fit	*7.53*	*3*	*2.51*	*2.99*	*0.2607*	**Not significant**
Pure Error	*1.68*	*2*	*0.84*			
Cor Total	422.26	14				
Std. Dev.	1.36		**R-Squared**	0.9782		
Mean	21.26		**Adj R-Squared**	0.9389		
C.V. %	6.38		**Pred R-Squared**	0.7056		
PRESS	124.30		**Adeq Precision**	14.245		

The Model F-value of 24.91 implies the model is significant. There is only a 0.12% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. *In this case amount of drug, amount of albumin, homogenization pressure as well as amount of drug, amount of albumin and homogenization pressure having quadartic effect and are significant model terms.* Term *interation between amount of drug & amount of albumin, amount of drug & homogenization pressure and amount of albumin & homogenization pressure* having values greater than 0.1000 indicate the model term are not significant. The "Lack of Fit F-value" of 2.99 implies the Lack of Fit is not significant relative to the pure error. There is a 26.07% chance that a "Lack of Fit F-value" this large could occur

due to noise. Non-significant lack of fit is good because we want the model to be fit for our data. The "Pred R-Squared" of 0.7056 is not as close to the "Adj R-Squared" of 0.9389 as one might normally expect. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio of 14.245 indicates an adequate signal.

Final Polynomial Equation in Terms of Coded Factors for Zeta Potential

$$= +22.60$$
$$-1.76 * X1 +5.81 * X2 +1.45 * X3$$
$$+0.53 * X1*X2-1.35 * X1*X3 -0.050 * X2*X3$$
$$-2.96 * X1^2 +2.94 * X2^2 -2.49 * X3^{2\backslash}$$

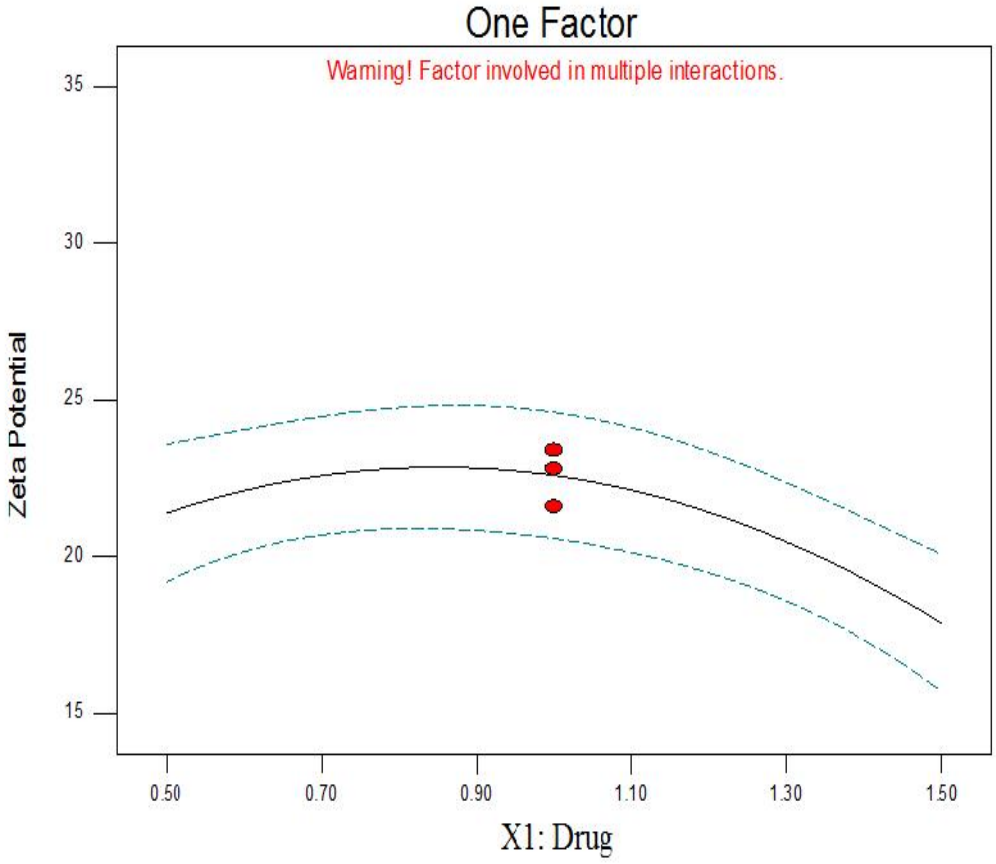

Figure 4. 8 Effect of Drug on Zeta Potential

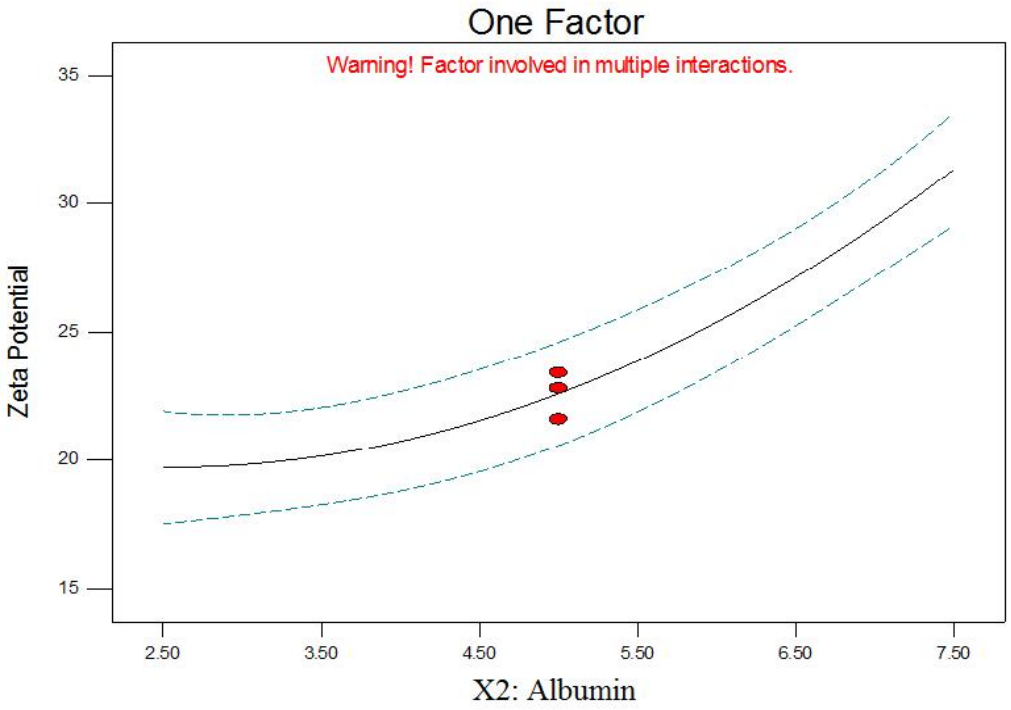

Figure 4. 9 Effect of Albumin on Zeta Potential

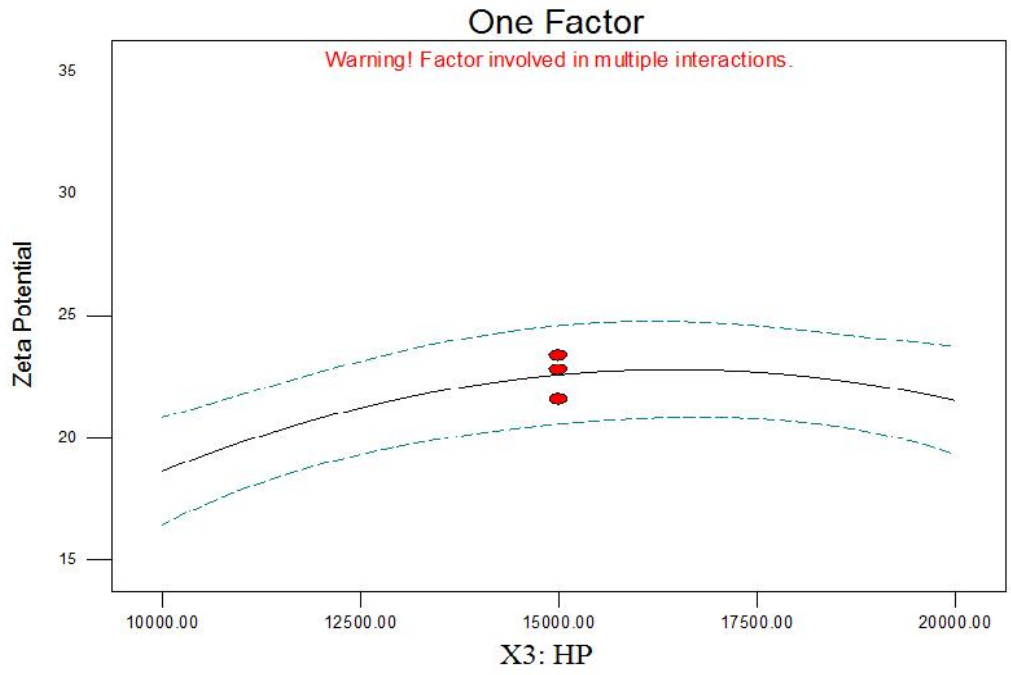

Figure 4. 10 Effect of HP on Zeta Potential

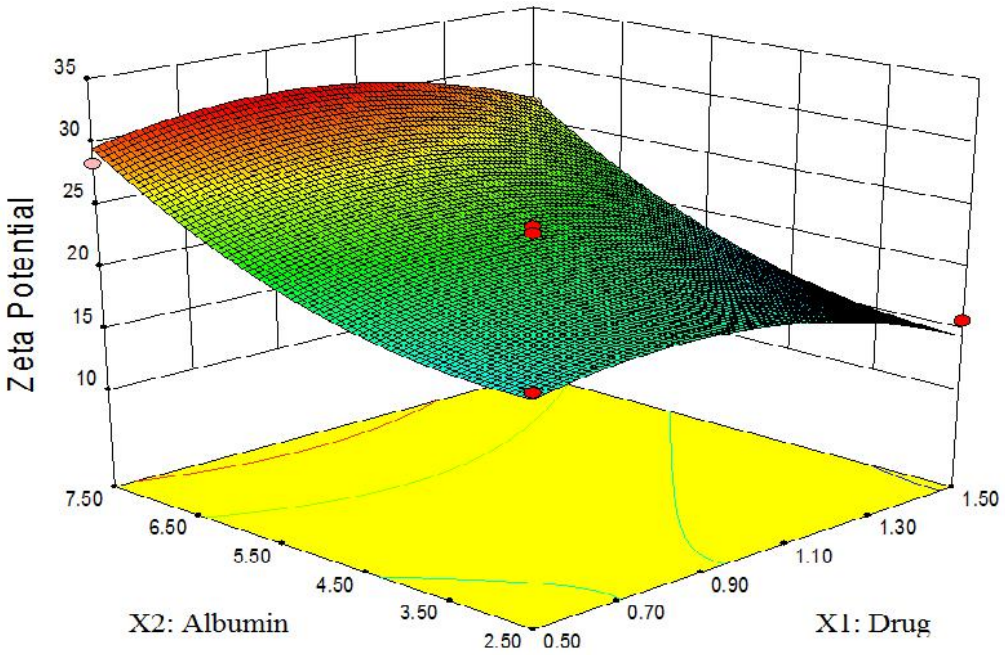

Figure 4. 11 Response Surface Showing Combined Effect of Drug and Albumin on Zeta Potential.

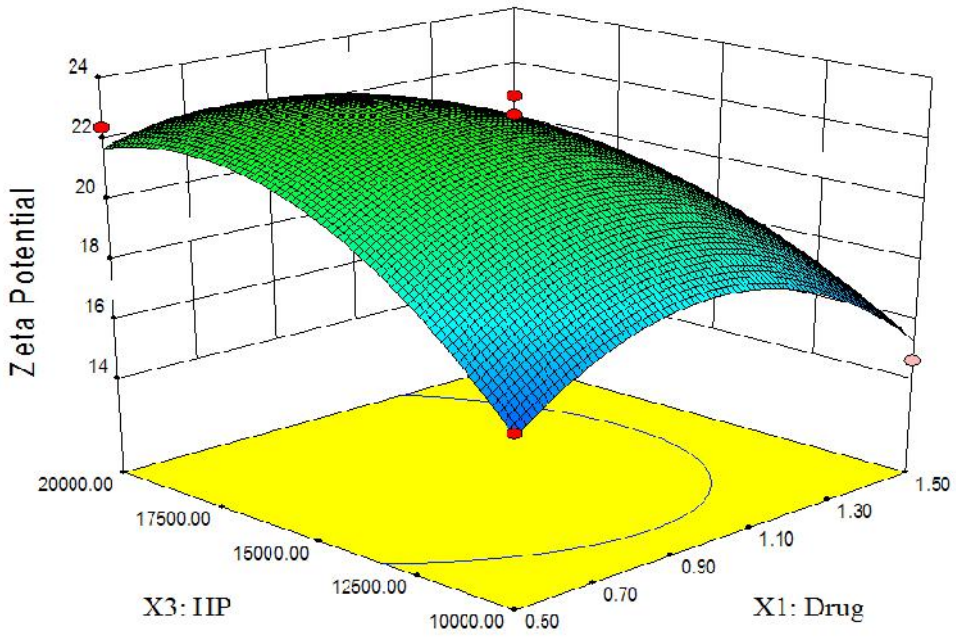

Figure 4. 12 Response Surface Showing Combined Effect of Drug and HP on Zeta Potential.

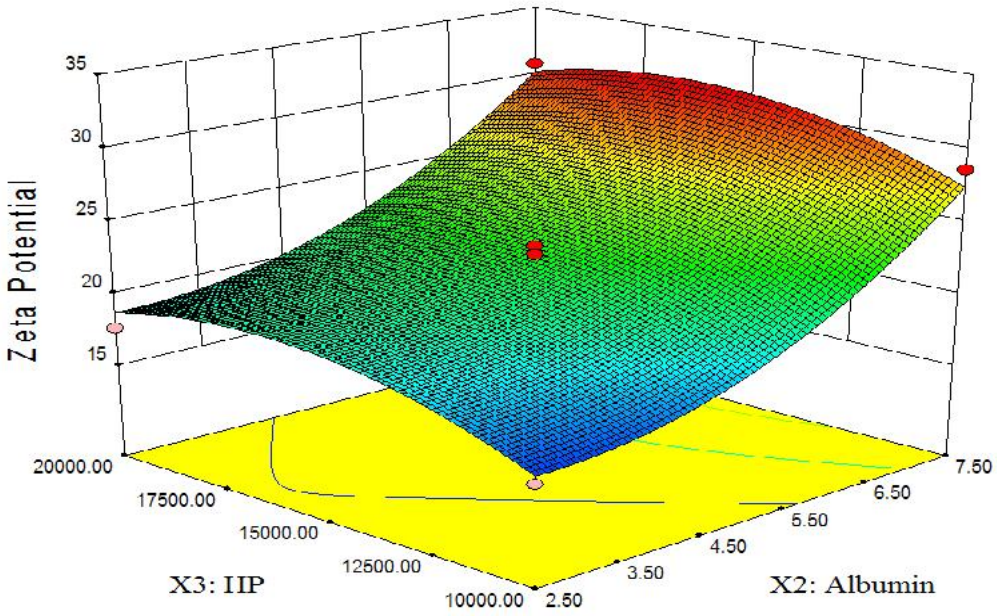

Figure 4. 13 Response Surface Showing Combined Effect of Albumin and HP on Zeta Potential.

One factor effect plots shown in **Figure 4.8, Figure 4.9** and **Figure 4.10** for factors X_1, X_2, and X_3 respectively shows that all three factor have quadratic effect (curvilinear plot) on the Zeta Potential of NPs. Two-factor response surface plots shown in **Figure 4.11, Figure 4.12** and **Figure 4.13** for factors X_1X_2, X_1, X_3 and X_2X_3 shows the same effect of X_3 over other factors X_1 and X_2. From response surface plots it was found that drug concentration and homogenization pressure had little effect on zeta potential but still a particular trend was observed for change in zeta potential with change in these factors. There was minor increase in zeta potential with increase in these factors which after attaining a particular value showed gradual reduction. Furthermore, zeta potential was found to be directly proportional to albumin concentration i.e. as albumin concentration increased there was rise in zeta potential due to net negative on albumin molecule. Increased zeta potential may enhance the stability of the formulation as it restricts the aggregation of particles.

4.8.2.3 Statistical Analysis of % EE (Response 3)

Sequential p-value, lack of fit p-value, adjusted R^2, predicted R^2 values and suggested model are given in the **Table 4.12.**

Table 4. 12 Summary of ANOVA results (% EE) for Different Models

Source	Sequential p-value	Lack of Fit p-value	Adjusted R-Squared	Predicted R-Squared	Model Suggested
Linear	0.2351	0.0036	0.1219	-0.1962	
2FI	0.9822	0.0024	-0.1833	-1.4181	
Quadratic	0.0001	0.0580	0.9606	0.7827	Suggested
Cubic	0.0580		0.9962		Aliased*

*The Cubic Model and higher are aliased. This shows that the predicted responses would be confounded by the other factors implying that the predicted response would give the wrong idea of the actual response.

Table 4.12 suggested the best model to fit the experimental results of Zeta Potential of NPs is quadratic model and results of ANOVA analysis of the suggested quadratic model given in **Table 4.13.**

Table 4. 13 ANOVA for Response Surface Quadratic Model (% EE)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2599.59	9	288.84	38.97	0.0004	Significant
X_1- Drug	80.65	1	80.65	10.88	0.0215	
X_2-Albumin	714.42	1	714.42	96.40	0.0002	
X_3-HP	22.45	1	22.45	3.03	0.1423	
$X_1 X_2$	27.04	1	27.04	3.65	0.1144	
$X_1 X_3$	0.81	1	0.81	0.11	0.7544	
$X_2 X_3$	8.41	1	8.41	1.13	0.3355	
X_1^2	10.26	1	10.26	1.38	0.2924	
X_2^2	1083.88	1	1083.88	146.25	< 0.0001	
X_3^2	732.33	1	732.33	98.81	0.0002	
Residual	37.06	5	7.41			
Lack of Fit	35.61	3	11.87	16.41	0.0580	**Not significant**
Pure Error	1.45	2	0.72			
Cor Total	2636.65	14				

Std. Dev.	2.72	**R-Squared**	0.9859
Mean	53.91	**Adj R-Squared**	0.9606
C.V. %	5.05	**Pred R-Squared**	0.7827
PRESS	573.02	**Adeq Precision**	18.396

The Model F-value of 38.97 implies the model is significant. There is only a 0.04% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. *In this case amount of drug, amount of albumin as well as amount of albumin and homogenization pressure having quadartic effect and are significant model terms.* Term *homogenization pressure, interation between amount of drug & amount of albumin, amount of drug & homogenization pressure, amount of albumin & homogenization pressure and quadartic effect of amount of drug* having values greater than 0.1000 indicate the model term are not significant. The "Lack of Fit F-value" of 16.41 implies there is a 5.80% chance that a "Lack of Fit F-value" this large could occur due to noise. The "Pred R-Squared" of 0.7827 is in reasonable agreement with the "Adj R-Squared" of 0.9606. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio of 18.396 indicates an adequate signal.

Final Polynomial Equation in Terms of Coded Factors for Entrapment Efficiency

$$= +69.67$$
$$-3.18 * X_1 + 9.45 * X_2 - 1.68 * X_3$$
$$+2.60 * X_1 * X_2 - 0.45 * X_1 * X_3 - 1.45 * X_2 * X_3$$
$$+1.67 * X_1^2 - 17.13 * X_2^2 - 14.08 * X_3^2$$

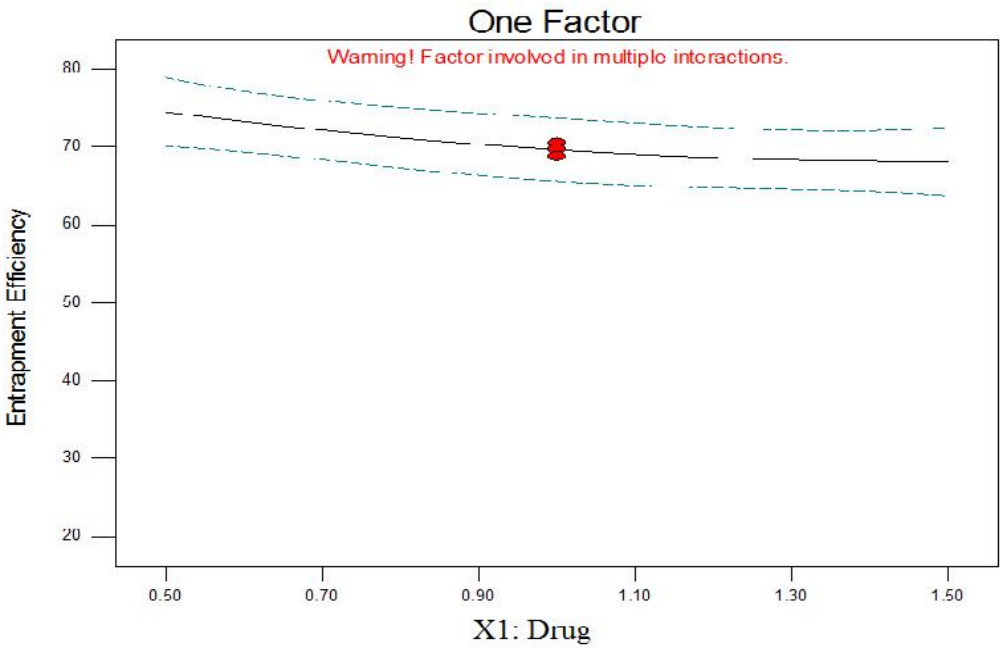

Figure 4. 14 Effect of Drug on % EE

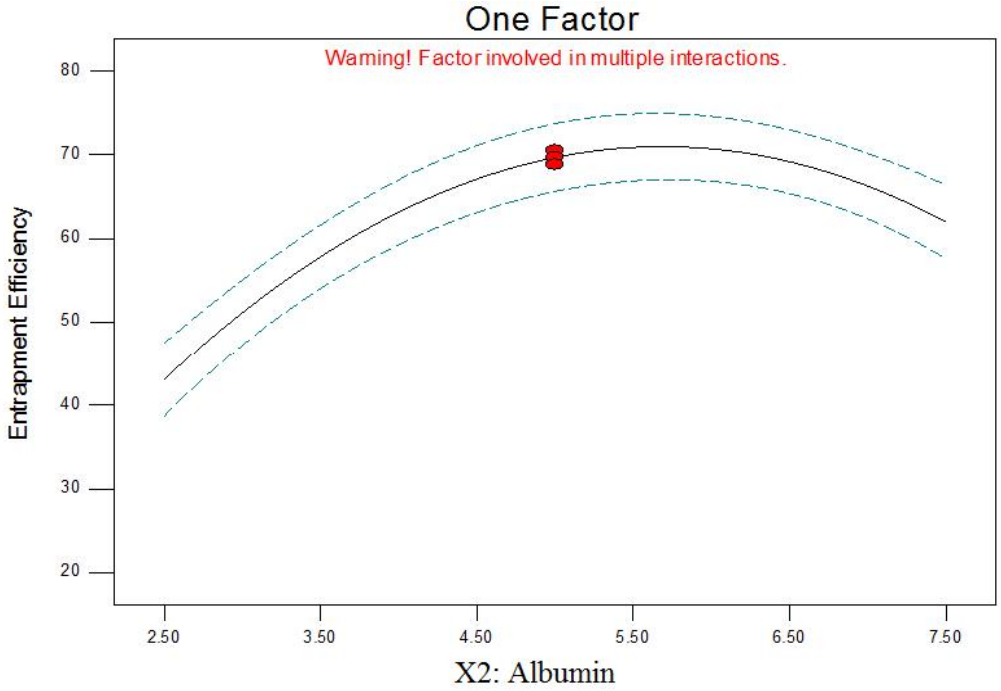

Figure 4. 15 Effect of Albumin on % EE

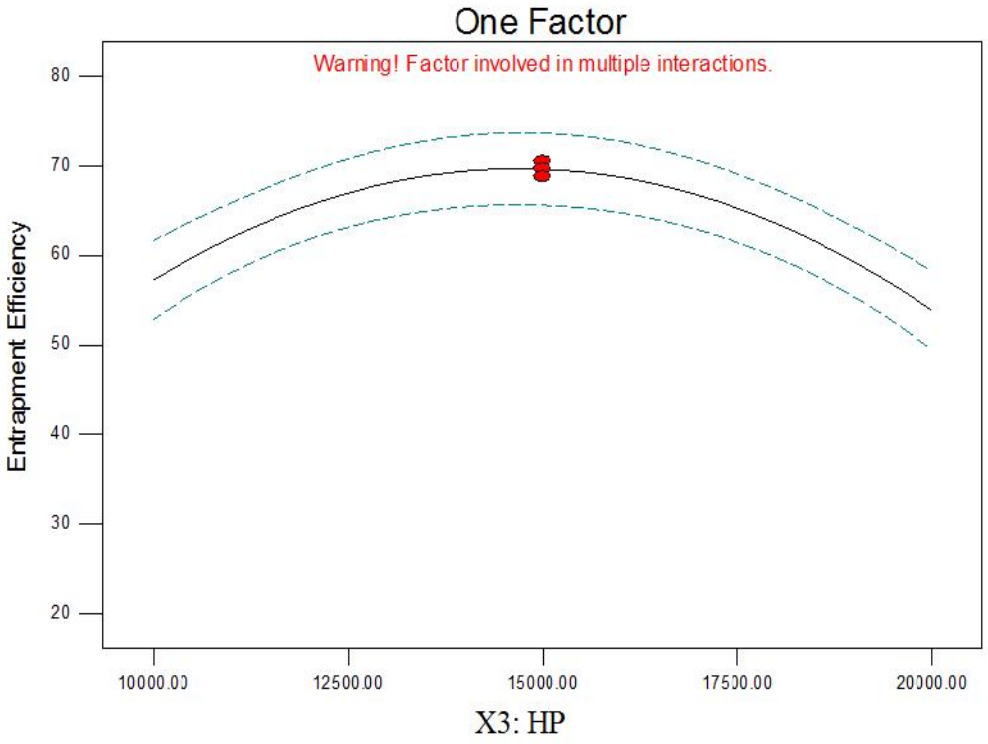

Figure 4. 16 Effect of HP on % EE

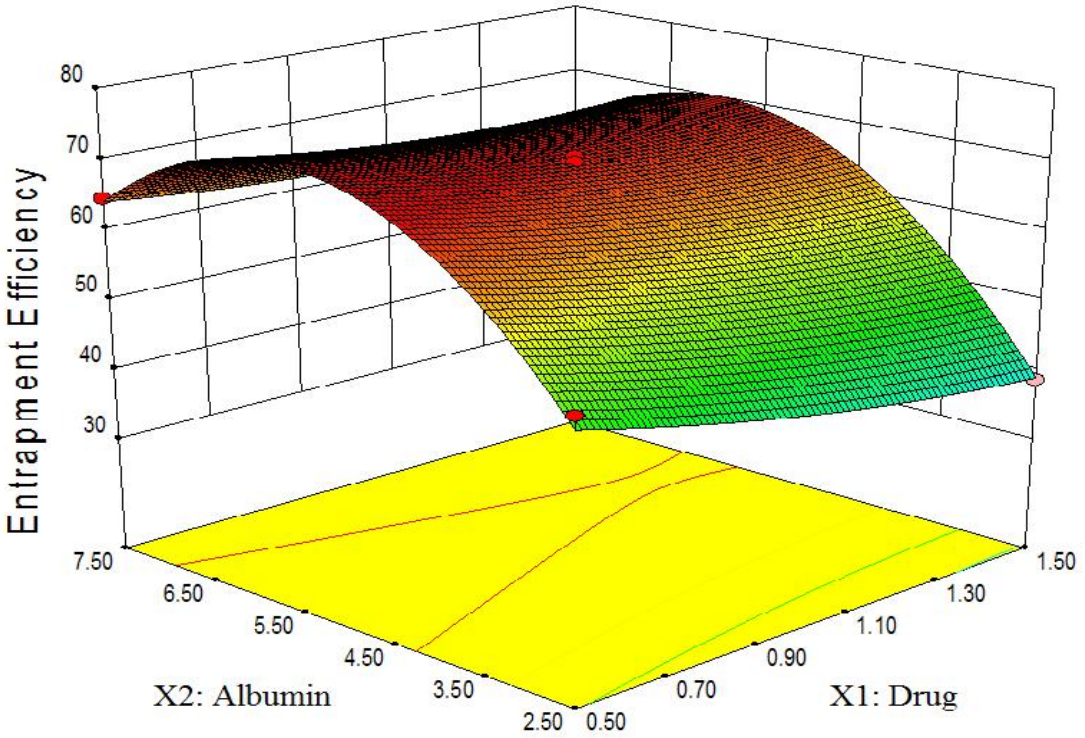

Figure 4. 17 Response Surface Showing Combined Effect of Drug and Albumin on % EE.

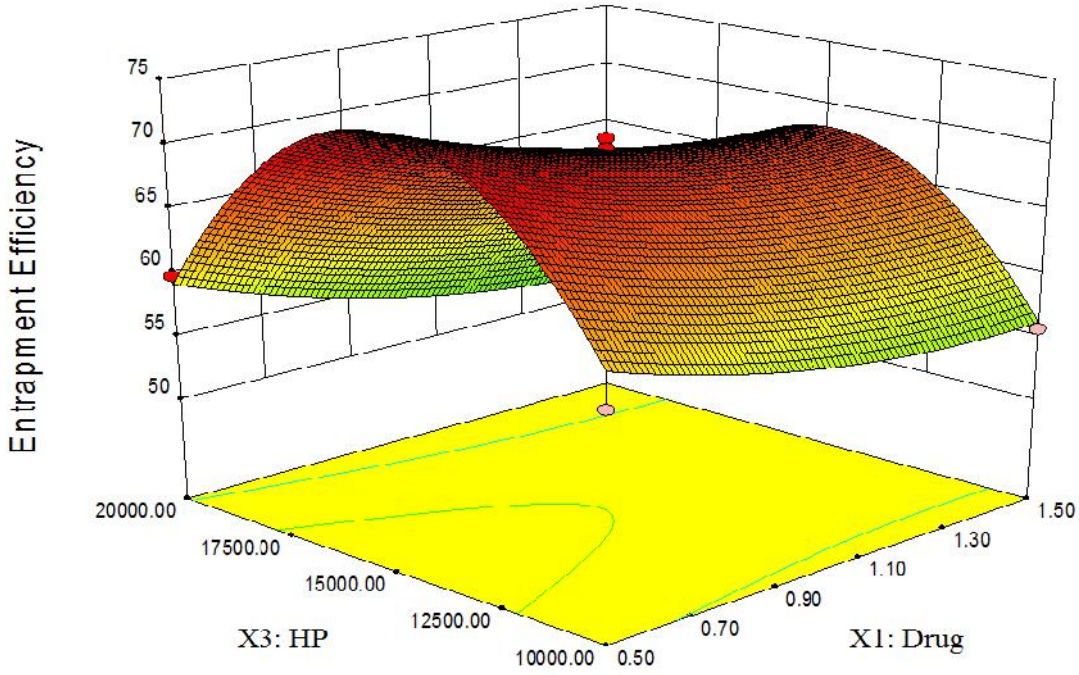

Figure 4. 18 Response Surface Showing Combined Effect of Drug and HP on % EE.

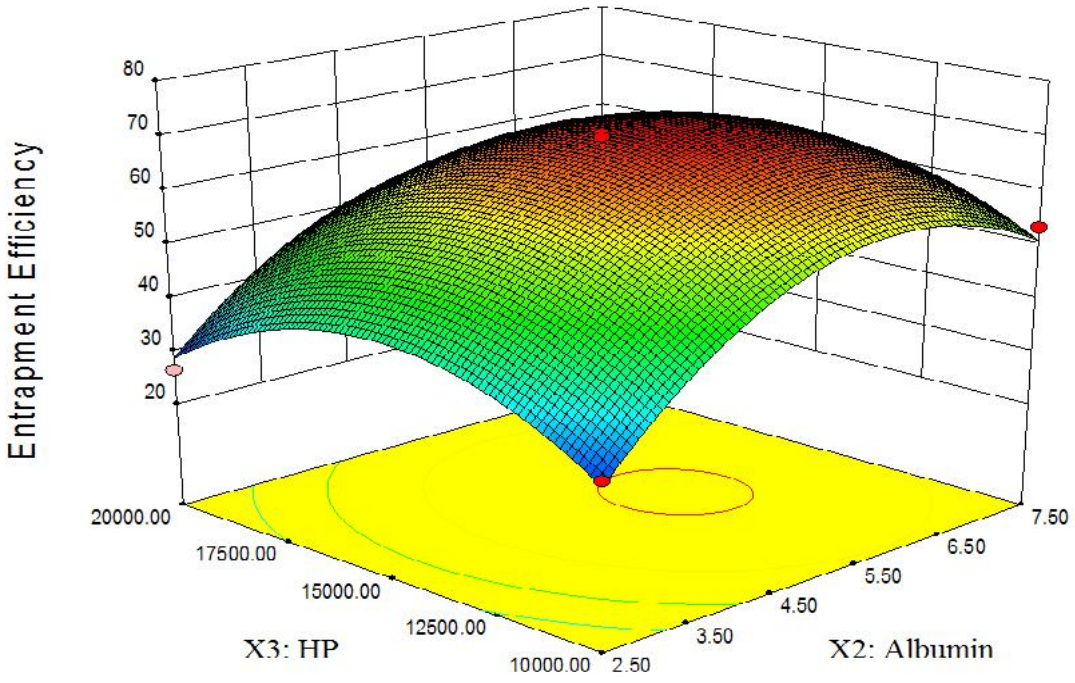

Figure 4. 19 Response Surface Showing Combined Effect of Albumin and HP on % EE.

One factor effect plots shown in **Figure 4.14, Figure 4.15** and **Figure 4.16** for factors X_1, X_2, and X_3 respectively shows that increase in factor X_1 linear decrease in % EE was observed while X_2, and X_3 factors have quadratic effect (curvilinear plot) on the % EE of NPs. Two-factor response surface plots shown in **Figure 4.17, Figure 4.18** and **Figure 4.19** for factors X_1X_2, X_1,X_3 and X_2X_3 shows the same effect of X_3 over other factors X_1 and X_2. From response surface plots it was found that albumin concentration and homogenization pressure showed quadratic effect on % entrapment efficiency i.e. there was gradual increase in % EE with rise in quantity of these factors up to certain value which later resulted in reduction of % EE when these factors exceeded optimum value. There was minor change in % EE with increase in drug concentration but still there was little reduction in % EE with rise in drug concentration. Higher % EE is preferable as higher dose can be incorporated in lower volume and thus can reduce the cost related to formulation. Reduction of % EE at higher albumin concentration might be due to inability of albumin to maintain drug in entrapped state at higher drug concentration or homogenization pressure.

The data clearly indicate that the results of response variables are strongly dependent on the selected independent variables. The equation can be used to obtain

estimates of the responses. Factor X_1 (amount of drug) has a positive effect on particle size indicated by the positive signs of coefficient X_1 (+34.35) whereas negative effect on zeta potential and entrapment efficiency indicated by the negative signs of coefficient X_1 (-1.76 and -3.18 respectively). Factor X_2 (amount of albumin) shows positive effect on particle size, zeta potential as well as on entrapment efficiency indicated by the positive sign of coefficient X_2 (+81.78, 5.81 and +9.45 respectively). Factor X_3 (homogenization pressure) shows positive effect on zeta potential indicated by the positive signs of coefficient X_1 (+1.45) whereas negative effect on particle size and entrapment efficiency as shown by the negative sign of coefficient X_3 (-13.83 and -1.68 respectively). Similarly, effects of different interaction terms such as X_1X_2, X_1X_3 and X_2X_3 on response variables can be seen from the signs and values of X_1X_2, X_1X_3 and X_2X_3 respectively. X_1^2, X_2^2, X_3^2terms are second order terms and are useful to estimate non linearity of response. Desirability plots for optimized formulations are presented in **Figure 4.20.**

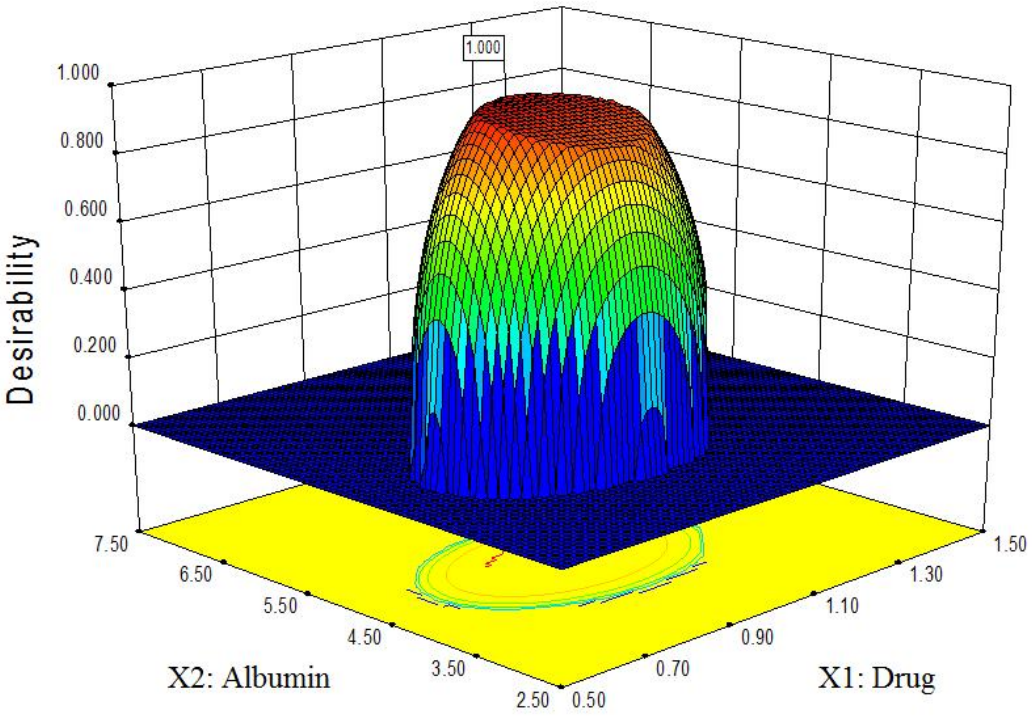

Figure 4. 20 Desirability plot for optimized formulations

The polynomial equations form excellent fit to the experimental data and are highly statistically valid. The criteria for selection of suitable feasible region was primarily based upon minimum particle size (less than 150 nm), zeta potential in the

range of -20 to -30.8 mV and the highest possible values of % EE (>70.5%). Composition of optimized batches and comparison of the observed responses with that of the predicted responses along with percentage error is listed in **Table 4.11.**

The optimum values of the variables were obtained by graphical and numerical analyses using the Design- Expert® software and based on the criterion of desirability. Afterwards, new batches of NPs with the predicted levels of formulation factors were prepared to confirm the validity of the optimization procedure. **Table 4.11** demonstrates that the observed values of a new batch were mostly similar with predicted values. % prediction error of 1.177 % for mean particle size, 3.077 for zeta potential and 0.073 % for encapsulation efficiency was observed.

Table 4. 14 ANOVA for Response Surface Quadratic Model (% EE)

Optimized Formulation Composition	**Drug (0.83 mg), Albumin (4.92 mg), Homogenization Pressure (15078.87 PSI)**		
Response	**Particle Size (nm)**	**Zeta Potential (-mV)**	**% EE**
Predicted value	148.254	22.701	70.598
SD	6.769	1.357	2.722
SE Mean	3.786	0.759	1.522
95% CI low	138.521	20.749	66.684
95% CI high	157.987	24.652	74.512
Observed value	150.0	23.40	70.65
% error	1.177	3.077	0.073

4.8.3 Characterization

4.8.3.1 Particle sizes, zeta potential and encapsulation efficiency

The particle sizes, zeta potential and encapsulation efficiency of optimized DTX-HSA-NPs was found 150 ± 3.52 nm, -23.60 ± 1.34 mV and 70.65± 2.27% respectively. Results of particle size and zeta potential are shown in **Figure 4.21** and **Figure 4.22** respectively

		Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm): 150.0	Peak 1:	176.5	100.0	64.37
PdI: 0.157	Peak 2:	0.000	0.0	0.000
Intercept: 0.957	Peak 3:	0.000	0.0	0.000
Result quality : Good				

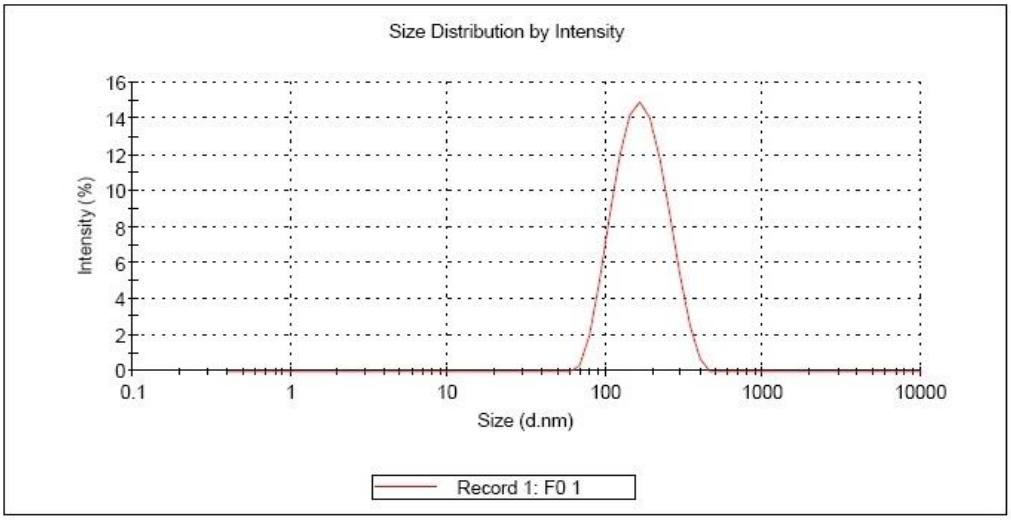

Figure 4. 21 Particle size distributions of optimized DTX-HSA-NPs

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -23.6	Peak 1:	-23.6	100.0	4.02
Zeta Deviation (mV): 4.02	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0808	Peak 3:	0.00	0.0	0.00
Result quality : Good				

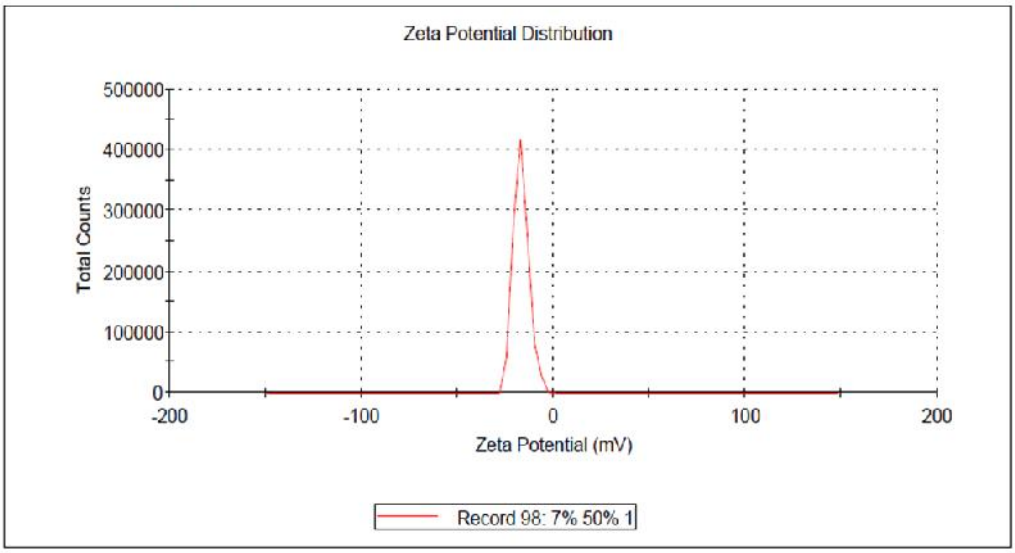

Figure 4. 22 Zeta potential of optimized DTX-HSA-NPs

4.8.3.2 Transmission Electron Microscopy (TEM)

TEM imaging of optimized DTX-HSA-NPs exhibit a spherical shape without aggregation as shown in **Figure 4.23.** The diameter of NPs were in the range of 140-200nm similar to particle size results obtained by Malvern Zetasizer.

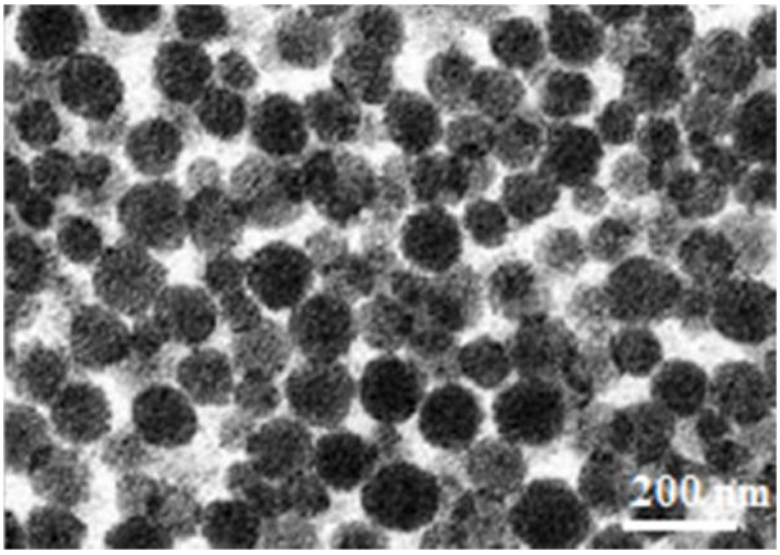

Figure 4. 23 TEM images of DTX-HSA-NPs

4.8.3.3 X-ray powder diffraction results

The X-ray powder diffraction results of optimized DTX-HSA-NPs are shown in **Figure 4.24.** Pure DTX powder showed strong peak typical of a crystalline sample. The two most intensive DTX peaks were located at 8.8, 11.0, 13.9 and 17.7°respectively. In contrast to pure DTX, blank NPs showed broad humps typical of amorphous material. Mixture of DTX and blank NPs also showed majorly the broad humps of blank NPs, but in addition the peaks at 8.8, 11.0, 13.9 and 17.7° from DTX were visible. DTX-HSA-NPs showed an identical result to blank NPs indicating no crystalline characteristic of DTX exists in the NPs.

4.8.3.4 Differential scanning calorimetry (DSC)

The DSC curves of DTX and human serum albumin showed a melting endotherm at 200ºC **(Figure 4.25 A)** and at 70ºC **(Figure 4.25 B)** respectively. In DSC thermogram of physical mixture (DTX and HSA) **(Figure 4.25 C),** a melting endotherm for DTX and HSA were observed and there was no shifting of melting endotherm compared to melting endotherm of individual components which indicates compatibility between HSA and DTX in formulation while the melting endotherm for DTX was not observed in thermogram of lyophilized nanoparticulate formulation

(Figure 4.25 D). Therefore, it could be concluded that DTX in the NPs was in an amorphous or disordered crystalline phase of a molecular dispersion or a solid solution state in the polymer matrix after the production.

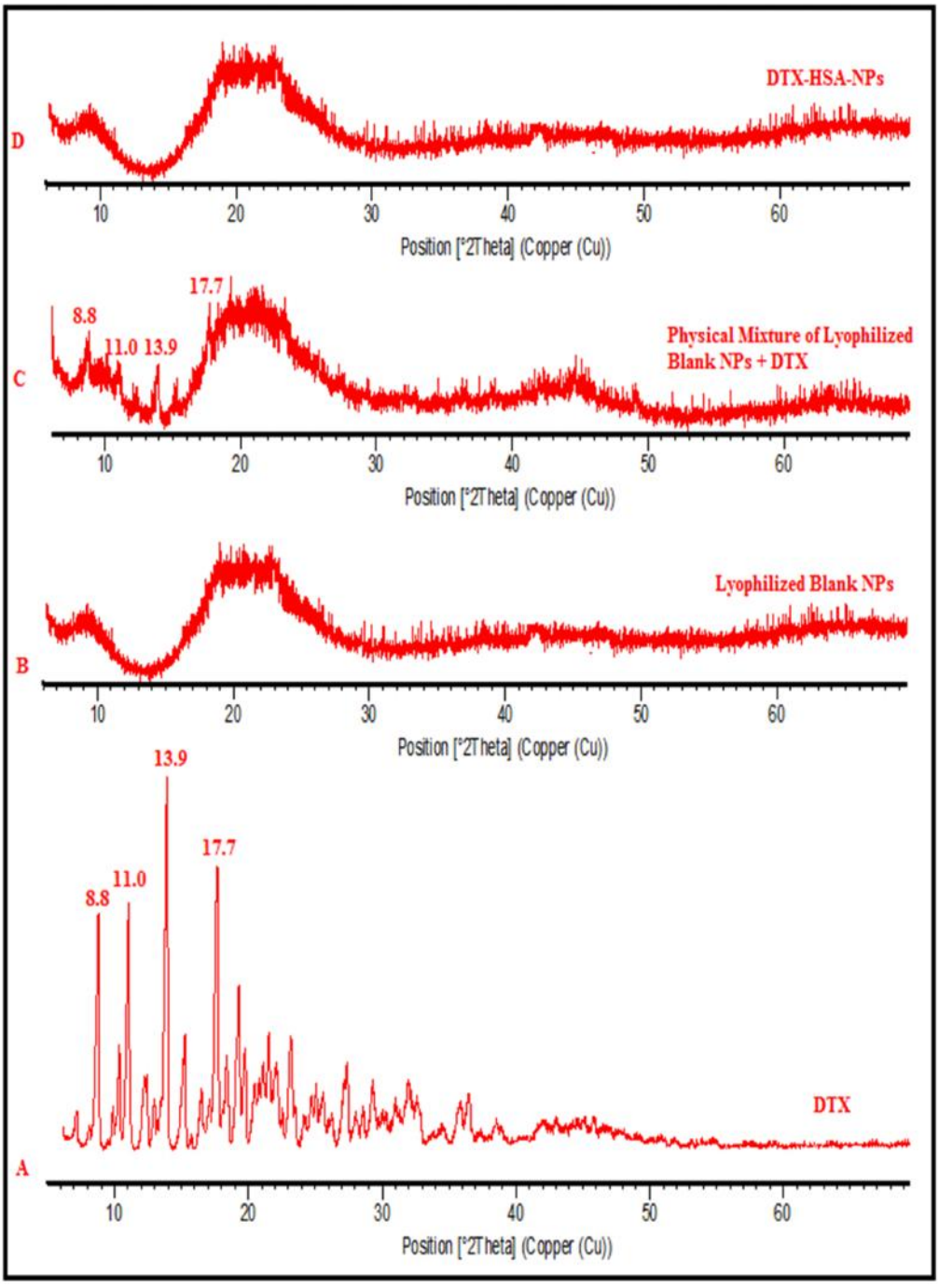

Figure 4. 24 XRD spectra of pure DTX (A), Lyophilized blank NPs (B), Physical mixture of DTX and blank NPs (C) and DTX-HSA-NPs (D)

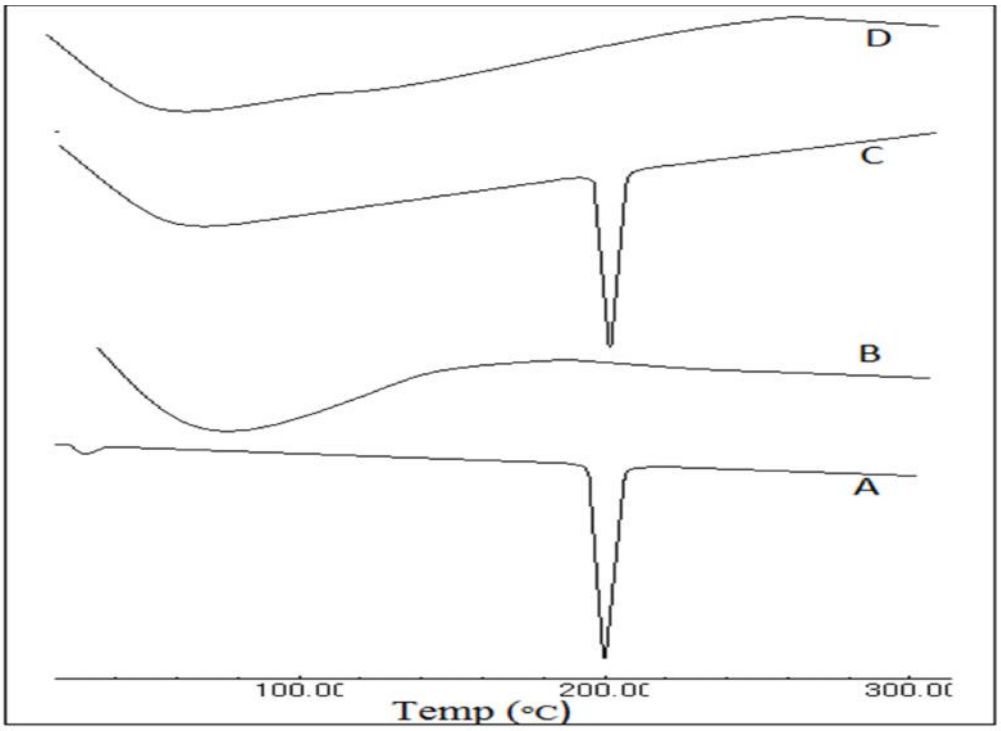

Figure 4. 25 DSC thermogram of DTX (A), Human serum albumin, (B) Physical mixture of DTX and human serum albumin (C) and DTX-HSA-NPs (D)

4.8.3.5 In vitro drug release

In vitro drug release studies were carried out for both plain DTX solution and for DTX-HSA-NPs by diffusion method for 24 h and results were compared by different kinetic models. The results of in-vitro drug release studies have shown that after the end of 8 h 96.84±1.132 % of the drug was released from plain drug dispersion compared to 43.78±1.532 % from DTX-HSA-NPs while at the end of 24 h 67.86±1.54 % of the drug was released from the DTX-HSA-NPs indicating prolonged release of drug from DTX-HSA-NPs. After application of different drug release kinetics models, it was observed that the DTX-HSA-NPs followed the Korsmeyer Peppas model because R^2 value (0.9885) which was nearer to 1 and having n value of 0.5721 indicating that drug transport mechanism is non-Fickian transport. *In vitro* drug release data for plain DTX solution and DTX-HSA-NPs given in **Table 4.15** and Drug release pattern shown in **Figure 4.26**.

Table 4. 15 *In-Vitro* drug release data for plain DTX solution and DTX-HSA-NPs.

Time (h)	Cumulative% Drug Release(Mean ± SD)*	
	Plain drug dispersion	DTX-HSA-NPs
0.5	16.2±0.215	7.56±0.458
1	29.47±0.967	12.67±0.788
2	43.12±1.312	19.93±0.987
4	75.36±1.415	28.54±1.312
6	85.34±1.115	32.46±1.423
8	96.84±1.132	43.78±1.532
12	--	54.69±1.143
24	--	67.86±1.540

* The experiment was performed in triplicate (n=3)

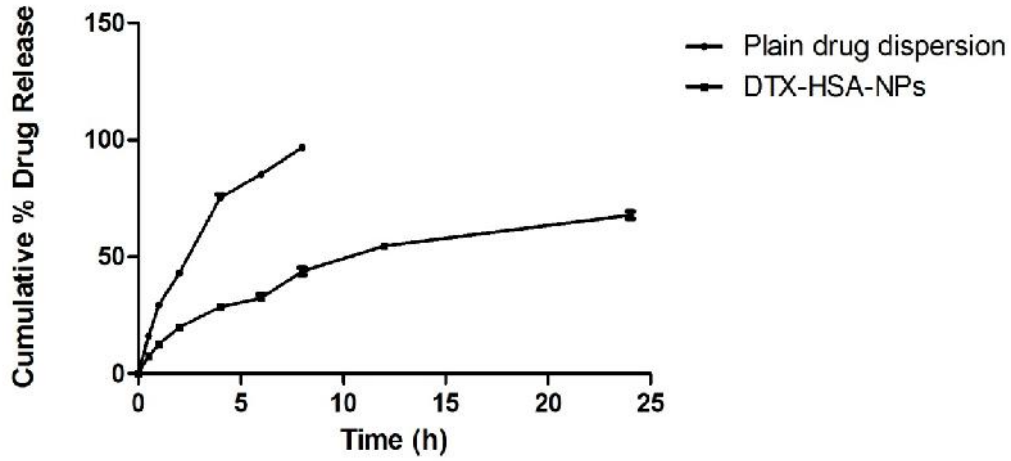

Figure 4. 26 *In Vitro* drug release pattern

4.8.3.6 Hemolysis study

The concentration range of DTX studied was between 0.01 and 1 mg/mL which was the highest possible concentration range for Tween-80 /ethanol system of DTX. It was observed that DTX-HSA-NPs were significantly less hemolytic activity than that of the Taxotere ($P<0.01$). As the concentration of DTX increased, hemolysis of Taxotere was detected at a concentration only above 0.01 mg/mL, while the hemolytic

activity of DTX-HSA-NPs was negligible up to 1 mg/mL. At a concentration of 1 mg/mL, hemolysis by Taxotere reached 90.2%. The main component in Taxotere is Tween 80 which has been reported to interact with cell membrane of RBC and cause significant hemolytic activity. Therefore it can be concluded that DTX-HSA-NPs were more hemocompatible compared to Taxotere.

4.8.3.7 Stability of DTX-HSA-NPs

Stability study results of DTX-HSA-NPs for drug content and particle size are given in **Table 4.13** and comparative changes in particle size with temperature during stability shown in **Figure 4.26.** It was observed that DTX-HSA-NPs were stable over the period of 6 months. The DTX-HSA-NPs showed physical stability for the period of 6 months at 2-8°C. The drug content at room temperature was found to decrease during storage and the particle size was also increased above 200 nm, which was not desirable. Hence, Room Temperature is not suitable for storage of DTX-HSA-NPs while storage at 2 -8°C no significant difference was observed in the particle size and drug content of NPs after 6 months at refrigerated conditions indicating its suitability for storage at 2 - 8°C.

Table 4. 16 Stability data of DTX-HSA-NPs at different temperature conditions.

Temperature Condition	Sampling Time (days)	Particle size (nm.)* Mean ±SD	% Assay* Mean ± SD
Initial	0	150.0 ± 3.52	99.82±0.089
Room Temperature	15	158.7 ± 5.93	99.43±0.012
	30	160.5 ± 6.23	99.11±0.056
	45	168.0 ± 5.88	98.78±0.064
	60	185.0 ± 4.75	98.56±0.032
	90	210.0 ± 4.96	98.22±0.057
	180	220.0 ± 3.45	97.68±0.056
2-8îC	15	152.1 ± 3.84	99.78±0.054
	30	155.6 ± 4.45	99.68±0.014
	45	159.6 ± 3.96	99.55±0.079
	60	163.4 ± 4.25	99.46±0.098
	90	164.5 ± 3.24	99.38±0.028
	180	168.3 ± 2.34	99.12±0.013

* The experiment was performed in triplicate (n=3)

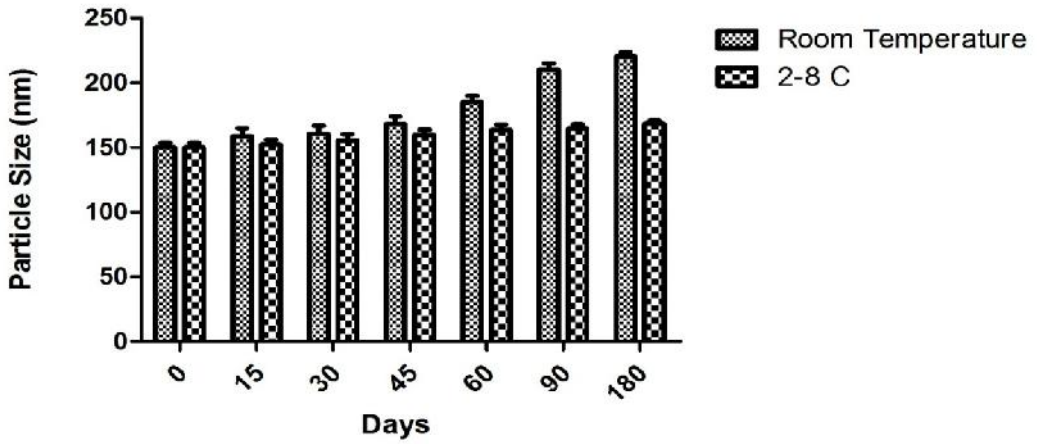

Figure 4. 27 Comparative changes in particle size with temperature during stability.

4.9 Discussion

DTX-HSA-NPs were successfully prepared by novel high-pressure homogenization technique (Nab Technology). The principle of the nanoparticle formation was crosslinking of protein (HSA) as result of exposure to high shear condition in a high pressure homogenizer. High pressure homogenization produces cavitation in the liquid that causes tremendous local heating due to high shear condition and results in the formation of superoxide ions which is capable of crosslinking the protein. In which a new crosslinking disulfide bond could be formed by disrupting existing disulfide bond or by oxidizing the sulfhydryl residues (15). The NPs were further converted into the powder form by lyophilization at suitable temperature condition and time profile. In lyophilization albumin itself acts as a cryoprotectant.

DTX as such present in needle shape crystals and the presence of crystals in a drug formulation for intravenous injection is obviously detrimental due to potential blockage of capillaries. It is also known that with the increase of drug loading in a formulation the tendency of crystallization also increases. X-ray powder diffraction and DSC analysis were performed to investigate the physical states of the drug in nanoparticles and it is very important aspect that could influence the *in vitro* and in vivo release of the drug from systems. From the XRD results of different samples it was concluded DTX in albumin nanoparticles was present in an amorphous or disordered crystalline phase of a molecular dispersion, or a solid solution state in the albumin matrix after the production. In DSC analysis the DTX-HSA-NPs showed an

identical result to blank NPs indicating no crystalline characteristic of DTX exists in the NPs. TEM imaging of optimized DTX-HSA-NPs exhibit a spherical shape without aggregation. The diameters of NPs were in the range of 140-200 nm similar to particle size results obtained by Malvern Zetasizer.

In vitro drug release studies were carried out for both plain DTX solution and for DTX-HSA-NPs by diffusion method for 24 h and results were compared by different kinetic models. The results of *in-vitro* drug release studies have shown that at the end of 8 h, 96.84±1.132 % of the drug was released from plain drug dispersion compared to 43.78±1.532 % from DTX-HSA-NPs while at the end of 24 h, 67.86±1.54 % of the drug was released from the DTX-HSA-NPs indicating prolonged release of drug from DTX-HSA-NPs. After application of different drug release kinetics models, it was observed that the DTX-HSA-NPs followed the Korsmeyer Peppas model because R^2 value (0.9885) which was nearer to 1 and having n value of 0.5721 indicating that anomalous transport is the drug transport mechanism. Hemolysis study revealed that DTX-HSA-NPs were more hemocompatible compared to Taxotere. Stability study results of DTX-HSA-NPs for drug content and particle size were evaluated and it was observed that DTX-HSA-NPs were stable over the period of 6 months at 2-8°C. Based on these results it was concluded that optimized DTX-HSA-NPs are suitable for further studies.

4.10 References

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