5.1 Materials

Temozolomide (TMZ) was obtained as a gift sample from Cipla Ltd., Mumbai (India). Bovine serum albumin (BSA), acetic acid, sodium acetate, sodium hydroxide, and dialysis membrane (12000 Da cut-off) and gluteraldehyde were purchased from Himedia (India). Ethanol, hydrochloric acid, glacial acetic acid and phosphoric acid were purchased from Spectrochem Pvt Ltd, Mumbai (India). Trehalose was purchased from SD Fine chemicals, Mumbai (India). HPLC grade acetonitrile and methanol were purchased from Renkem (India). Disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, sodium acetate, sodium hydroxide, potassium sulphate, Tris-hydrochloride and dipotassium EDTA dihydrate were purchased from SD fine chemicals Pvt. Ltd., Mumbai, India. All other chemicals and solvents used were of analytical grade.

5.2 Equipments

- pH meter (Lab India Pvt. Ltd. India)
- Digital analytical balance (ATX224 Shimadzu, Japan)
- UV-Visible spectrophotometer (1800 Shimadzu, Japan)
- Cooling centrifuge (Remi equipment Pvt Ltd, India)
- Magnetic stirrer (Remi sci. Equipment, India)
- Deep freezer (EIE Inst. Ltd, Ahmedabad)
- Zetasizer (Nano ZS, Malvern ltd., UK)
- Differential Scanning Calorimeter (DSC-60-Shimadzu Corporation , Japan)
- Infrared Spectrophotometer (IR Affinity -1S,Shimadzu , Japan)
- Lyophilizer (Advantage 2.0 Bench Top Freeze Dryer/ Lyophilizer, SP Scientific, USA)

5.3 Methods

5.3.1 Preparation of temozolomide loaded BSA nanoparticles (TNPs)

TMZ loaded albumin nanoparticles (TNPs) were prepared by desolvation method as reported earlier with slight modification (1). Briefly, BSA (16.67 mg/ml) was dissolved in double distilled water (pH was adjusted to 5.7 ± 0.2 using dilute acetic acid) under magnetic stirring. TMZ was

added in the albumin solution and incubated in it for 2 h under continuous stirring. For preparation of nanoparticles, ethanol (organic phase) was added drop wise in the aqueous phase at a constant flow rate of 1 ml/min under constant magnetic stirring (rpm = 700). Then gluteraldehyde (GA) (8 % w/v) was added for hardening of the prepared nanoparticles The nanoparticles were then separated by centrifugation at 16000 rpm for 30 min and washed three times with ethanol to remove excess GA. The prepared nanoparticles were freeze dried using trehalose (5% w/w) as cryoprotectant. Placebo albumin nanoparticles (PNPs) were prepared with the same method.

5.3.2 Preliminary optimization of process and formulation variables

Various process and formulation variables like drug to polymer ratio, polymer concentration, aqueous to organic ratio, crosslinker (gluteraldehyde) concentration, rate of addition of organic phase, effect of stirring speed, effect of pH, etc were screened and optimized using one factor at a time (OFAT) analysis.

5.3.2.1 Effect of BSA concentration

The effect of BSA concentration on quality attributes like particle size and PDI of nanoparticles were assessed by formulating different batches of PNPs (placebo BSA NPs) using different albumin concentration (10 mg/ml, 20 mg/ml, 30 mg/ml and 40 mg/ml).

5.3.2.2 Effect of solvent as aqueous phase

The effect of solvent as aqueous phase was assessed by formulating different batches of BSA NPs using different solvents (distilled water, sodium acetate buffer, phosphate buffer, citro phosphate buffer, distilled water having pH 5.7 ± 0.2 adjusted with dilute acetic acid) for dissolving BSA and TMZ and the effect was studied on the basis of particle size, PDI and percent entrapment efficiency (% EE) of TMZ in the NPs (TNPs).

5.3.2.3 Effect of pH of albumin solution

The effect of pH of the aqueous phase on particle size, PDI and %EE of TNPs was also studied by varying the pH in the range of 4.0 to 9.0. For varying the pH of the aqueous phase either

different buffer as mentioned above were used or pH was adjusted with 0.1 N HCl / 0.1 N NaOH.

5.3.2.4 Effect of aqueous to organic phase ratio

The effect of aqueous to organic phase ratio on quality attributes like particle size, PDI and %EE of BSA NPs were studied by varying the ratio from 1:1 to 1:3.

5.3.2.5 Effect of organic solvent

Different organic solvents like ethanol, acetone and IPA were selected as organic phase and NPs were prepared. The effect of solvent was studied on the basis of particle size, PDI and % EE of NPs.

5.3.2.6 Effect of gluteraldehyde concentration

The gluteraldehyde (GA) was utilized as cross linker in BSA NPs preparation. To study the effect of GA, different concentrations (0.2 µl/mg of BSA - 0.98 µl/mg of BSA) of GA were used to prepare the NPs and the effect was analysed on the basis of particle size, PDI and %EE of formulations.

5.3.2.7 Effect of rate of addition of organic phase

To study the effect of rate of addition of organic phase on the quality attributes like particle size, PDI and %EE, different batches were prepared by varying rate of addition from 0.25 ml/min to 2.0 ml/ min.

5.3.2.8 Effect of polymer to drug ratio

Various TMZ loaded BSA NPs (TNPs) batches were prepared by varying the polymer to drug ratio (3.33: 1 to 10:1) and its effect on particle size, PDI and % entrapment efficiency was observed to assess the effect of polymer to drug ratio.

5.3.3 Optimization of TNPs using experimental design

TNPs were optimized using Box-Behnken response surface design (BBD) (2). Three independent variables (polymer: drug ratio (A), Organic phase volume (B) and GA amount (C)) at two different levels were taken and their effect on four dependent variables (particle size, PDI, zeta potential and percent entrapment efficiency) were observed. BBD design matrix was generated by SAS JMP software (13 version). According to the design matrix, 15 different TNPs batches were formulated and effect of variables was studied.

5.3.4 Establishment and analysis of design space

Analysis of design space was done using overlay plot and desirability curve. The overlay plot helps in visual examination of the design space. An overlay plot represents the area (which represents the different combination of factors/independent variables) in which there is most likely chance to obtain the desired responses. An overlay plot for optimized formulation is a type of contour plots which is plotted with respect to the obtained desirability curve. The desirability curve is plotted on the basis of selected response/desired response after analysis of experimental design. The desirability curve represents the value of independent variables which in combination will give the desired response according to the response of experimental design. Analysis of design space was done for TNPs. For analysis of design space the optimized batch as predicted by software was formulated and the obtained response was compared with the predicted response and the error was calculated (2).

5.3.5 Lyophilization of albumin nanoparticles

The albumin nanoparticles (PNPs and TNPs) aqueous dispersion with 5 % w/w cryoprotectant (trehalose) was frozen in a refrigerator at –70°C for 12 hours. Then the samples were lyophilized using a lab freeze-dryer (Advantage 2.0 Bench Top Freeze Dryer/ Lyophilizer, SP Scientific, USA). The freeze-drying was conducted for 48 hours. After this, the vials were sealed with rubber caps (3).

5.3.6 Characterization of albumin nanoparticles

5.3.6.1 Particle size and PDI determination

The particle size and PDI of developed albumin nanoparticles (PNPs and TNPs) were determined by photon correlation spectroscopy (PCS) with a Malvern Zetasizer (Nano ZS, Malvern ltd., UK). The measurement using PCS is based on the light scattering phenomena in which the statistical intensity fluctuations of the scattered light from the particles in the measuring cell are measured. Prior to the measurements, all samples were diluted with double distilled water to produce a suitable scattering intensity. The z-average and PDI values were obtained using disposable polystyrene cells having 10 mm diameter cells at 25°C, which were equilibrating for 120 seconds. Refractive index (RI), for size measurement of NPs dispersion, was set as RI = 1.330 (abs = 0.01). All measurements were performed in triplicate at 25°C (1).

5.3.6.2 Zeta potential determination

The zeta potential, reflecting the electric charge on the particle surface and indicating the physical stability of colloidal systems, of developed albumin nanoparticles (PNPs and TNPs) were measured by determining the electrophoretic mobility using the Malvern Zetasizer (Nano ZS, Malvern ltd., UK). The measurements were performed after diluting in samples with double-distilled water. Zeta potential was measured using Dip cell with applying field strength 20 V/cm and the average of the zeta potential was given from 30 runs. Smoluchowski approximation was used to calculate zeta potential from the electrophoretic mobility. All measurements were performed in triplicate at 25°C (1).

5.3.6.3 DSC analysis

DSC analysis of developed albumin nanoparticles (PNPs and TNPs) were carried out using a Differential Scanning Calorimeter (DSC-60, Shimadzu, Japan) at a heating rate of 10°C per minute in the range of 30°C to 250°C under inert nitrogen atmosphere at a flow rate of 40 ml/min (4).

5.3.6.4 FTIR analysis

FTIR spectrum of developed albumin nanoparticles (PNPs and TNPs) were measured with a FTIR spectrophotometer (IR Affinity -1S (Shimadzu, Japan) in range 400–4000 cm^{-1} using a resolution of 4 cm^{-1} (4).

5.3.6.5 XRD analysis

X-ray diffraction patterns of developed albumin nanoparticles (PNPs and TNPs) were obtained using X-ray diffractometer (RigakuUltima IV; Japan) in which Cu-Kα line used as a source of radiation by operating at the voltage 40 kV and the current applied was 40 mA. Both samples were measured in the 2θ angle range between 5°-50° with a scanning rate of 3°/min and a step size of 0.02° (4)

5.3.7 Evaluation of albumin nanoparticles

5.3.7.1 Estimation of entrapment efficiency and drug loading

TMZ entrapment in the TNPs was determined indirectly by measuring the amount of free TMZ in the supernatant using UV–visible spectrophotometer (Shimadzu UV-1700 at 330 nm (4). Then percentage entrapment efficiency (% EE) and drug loading (% DL) was determined using the formula:

$$\% EE = \frac{(\text{Total drug} - \text{Free drug})}{\text{Total drug}} \times 100 \quad \text{.....Equation 5.1}$$

$$\% DL = \frac{\text{Entrapped drug}}{\text{Total weight of nanoparticles}} \times 100 \quad \text{.....Equation 5.2}$$

5.3.7.*2* *In-vitro* drug release

The *in-vitro* drug release studies were carried out using dialysis bag method at 37 °C under mild stirring (50 rpm) (5). Pure TMZ and TNPs (equivalent to 5 mg drug) were taken into dialysis bags (MWCO = 12000), and were immersed into beakers containing 30 ml sodium acetate buffer (pH 5.5 ± 0.2). At predetermined period, 1.0 ml of sample was withdrawn and same quantity of

fresh buffer was added into the beaker to maintain sink condition. The amount of TMZ released was determined using UV spectrophotometer at 330 nm.

5.3.8 Stability studies

The stability of the lyophilized TNPs was investigated by storing samples at refrigerated condition (4°C) and at room temperature (25°C ±2°C) for 3 months. At regular time interval of 1 month, samples were withdrawn and particle size, assay and zeta potential were determined (6,7).

5.3.9 Statistical Data Analysis

Results are given as mean ± SD. Statistical significance was tested by two-tailed Student's t test or one-way ANOVA. Statistical significance was set at $P < 0.05$.

5.4 Results and discussion

5.4.1 Preliminary optimization of process and formulation variables

Albumin nanoparticles (PNPs and TNPs) were prepared by desolvation method. Preliminary investigation was done to assess the effect of various factors on desired response. Optimization was done on the basis of OVAT (one variable at a time) analysis. The aim was to optimize the process in such a way to obtain TNPs with minimum particle size and higher entrapment efficiency of TMZ along with to study the influence of process and formulation variables on quality attributes of TNPs like particle size, PDI, zeta potential and %EE.

5.4.1.1 Effect of BSA concentration

Different albumin concentration 10mg/ml, 20 mg/ml, 30mg/ml and 40mg/ml was tried to formulate placebo BSA NPs (PNPs) and results are summarized in table 5.1 and figure 5.1. The results indicated positive effect of BSA concentration on particle size and PDI of PNPs which means as the concentration of BSA increased, particle size and PDI of PNPs also increased. With albumin concentration 10mg/ml and 20 mg/ml, smaller particles (less than 200 nm) and homogeneous dispersion with PDI (less than 0.3) were obtained. So this range was selected as

albumin concentration. The obtained results were also correlated with previously reported literature (8)

Table 5.1: Effect of albumin concentration

Albumin conc. (mg/ml)	Particle size (nm)	PDI
10	145.6 ± 1.5	0.259 ± 0.003
20	160.3 ± 1.67	0.261 ± 0.007
30	220.1 ± 2.35	0.301 ± 0.012
40	214.7 ± 2.87	0.614 ± 0.025

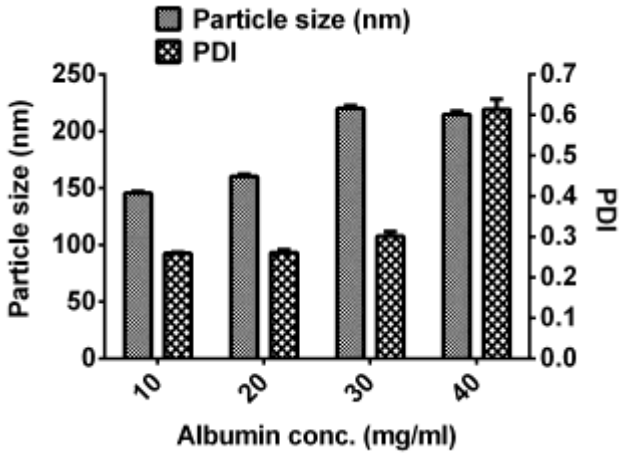

Figure 5.1: Optimization of BSA concentration

5.4.1.2 Effect of solvent and pH of aqueous phase

The influence of the pH of aqueous phase and type of solvent as aqueous phase was studied as it affects the stability of NPs and TMZ both. As discussed earlier, TMZ showed pH dependent stability so that it is necessary to maintain the pH in the acidic range. Apart from this, pH also affects the zeta potential of albumin NPs. When the pH of the aqueous phase was adjusted to 3, zeta potential of the NPs was positive while in case of pH range 5-11, the zeta potential shifted towards negative side (9). Apart from this, TMZ also showed time dependent stability in different solvents (as discussed in previous chapter). So selection of the solvent should be in such a way that provides longer stability of drug in it. As per the stability studies of TMZ, different

solvents were selected for the studies and pH was also selected on the basis of the stability data of drug. Although available literature reported that albumin based nanoparticles showed lesser particle size in alkaline pH (7-11) (9) but this pH range was not suitable for us as drug degraded in the alkaline pH so it was not considered in the optimization process. The influence of different solvents along with pH is summarized in table 5.2 and figure 5.2 and results indicated that in various buffer based solvents, precipitation occurred or large aggregated particles formed which may be due to the precipitation of the buffer components in the presence of organic solvent. In case of distilled water, particle size of TNPs was lesser but % EE was only 41.25% ± 2.90 which may be due to degradation of the drug in neutral to alkaline pH. So for increasing the % EE of TMZ in TNPs, pH of distilled water was adjusted to 5.7 ± 0.2 with dilute acetic acid and its effect was studied. The results indicated increase in particle size as well as % EE (63.50 ± 2.87) of TMZ which may be due to stability of TMZ in acidic condition. Although size was higher but can be controlled by varying other variables.

Table 5.2: Effect of solvent and pH of aqueous phase

Solvent and pH	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)
NaCl (10 mmol)	308.5 ± 2.5	0.238 ± 0.012	-18.0 ± 1.5	28.20 ± 2.7
Distilled water	130.3 ± 1.9	0.189 ± 0.009	-35.7 ± 0.8	41.25 ± 2.9
Sodium acetate buffer (pH 5.0)	Precipitation occurred			
Sodium acetate buffer (pH 5.5)	Precipitation occurred			
Phosphate buffer (pH 5.6)	Precipitation occurred			
Citro phosphate buffer (pH 6.5)	Precipitation occurred			
Water (5.7±0.2 pH adjusted with dilute acetic acid)	222.9 ± 1.67	0.217 ± 0.015	-33.9 ± 1.1	63.50 ± 2.9

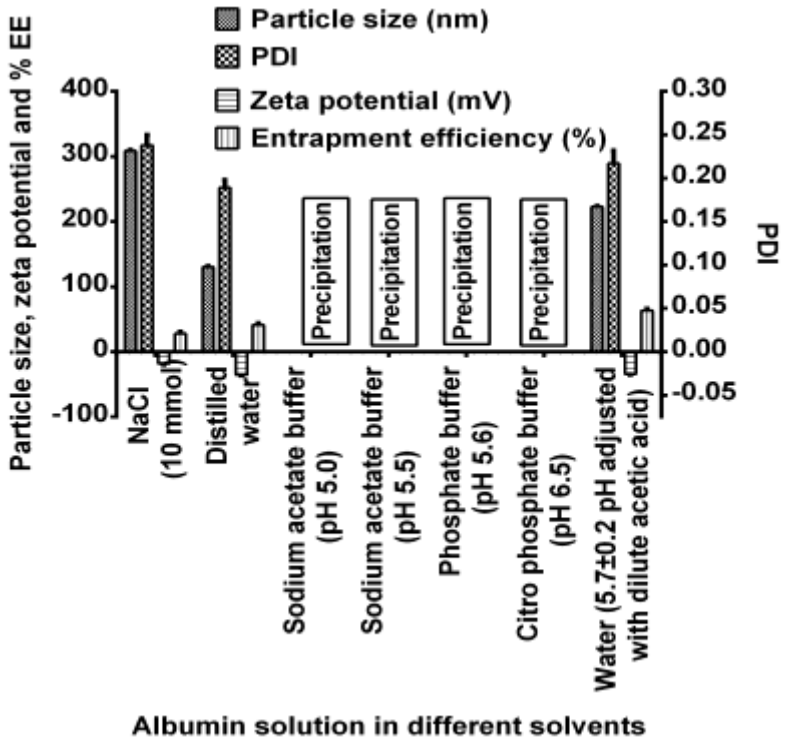

Figure 5.2: Optimization of solvent and pH of aqueous phase

5.4.1.3 Effect of aqueous to organic phase ratio

Various TNPs batches were prepared by varying the aqueous to organic phase ratio (1:1 to 1:3) and its effect on particle size, PDI, zeta potential and % entrapment efficiency was observed (table 5.3 and figure 5.3). With 1:3 aqueous to organic phase ratio, smaller particle size (224.3± 2.9 nm) and lesser PDI (0.341± 0.025) was obtained with 73.2 ± 1.3 % entrapment of TMZ in BSA NPs. Zeta potential value (-26.1 mV± 1.7) also showed the stability of batch. So this was selected as optimum aqueous to organic phase ratio. This may be correlated to the fact that formation of nanoparticles is dependent on the volume of organic solvent. If volume of organic phase is decreased, the amount of organic phase will not be sufficient for desolvation of the protein, and cause delayed precipitation and nucleation leading to formation of large aggregated particles with wide size distribution. On increasing the volume of organic solvent, smaller particles with narrow size distribution were obtained and further increase in volume of organic

solvent did not affect size but only increased the number of particles and affect size distribution. In case of EE, this slow nucleation gives enough time for drug molecule to settle down in polymer matrix leading to increase in EE (10)

Table 5.3: Effect of aqueous to organic phase ratio

Aqueous: organic ratio	Organic phase volume (ml)	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)
1:1	3	Particles not formed			
1:2	6	219.6 ± 1.9	0.243 ± 0.013	-27.3 ± 1.3	67.6 ± 1.5
1:2.5	7.5	227.7 ± 2.5	0.330 ± 0.021	-28.7 ± 1.5	66.1 ± 2.9
1:3	9	224.3 ± 2.9	0.341 ± 0.025	-26.1 ± 1.7	73.2 ± 1.3

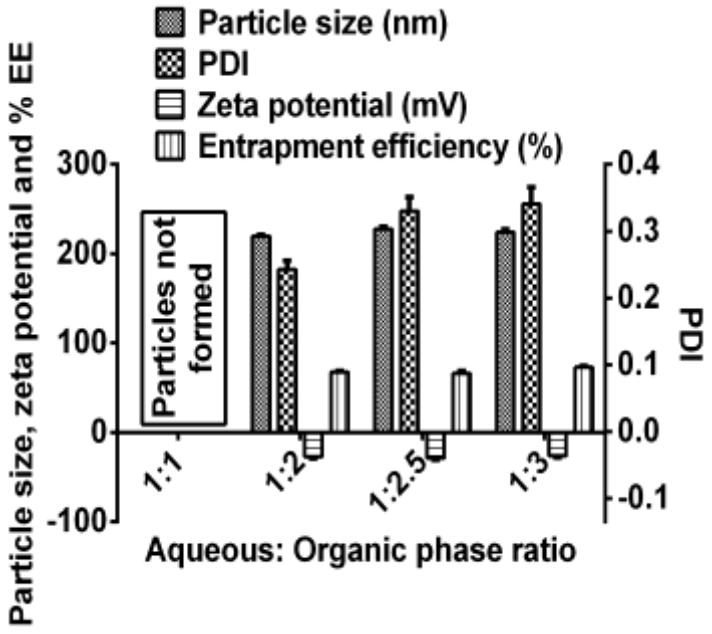

Figure 5.3: Optimization of aqueous: organic phase ratio

5.4.1.4 Effect of organic solvent

On the basis of stability of TMZ, three organic solvents (ethanol, acetone and IPA) were selected as desolvating agent and their effect on particle size and PDI were studied. The results (Table 5.4 and Figure 5.4) indicated that with ethanol, smaller particle size and more homogeneous dispersion of nanoparticles formed along with higher % EE so this was selected as organic phase.

Table 5.4: Effect of organic solvent

Organic solvent	Particle size (nm)	PDI	Zeta potential (mV)	% EE
Ethanol	209.8 ± 1.5	0.086 ± 0.014	-27.7 ± 1.1	65.1 ± 2.5
Acetone	388.7± 2.9	0.302 ± 0.021	- 29.5 ± 1.6	35.7 ± 3.1
IPA	Precipitation occurred			

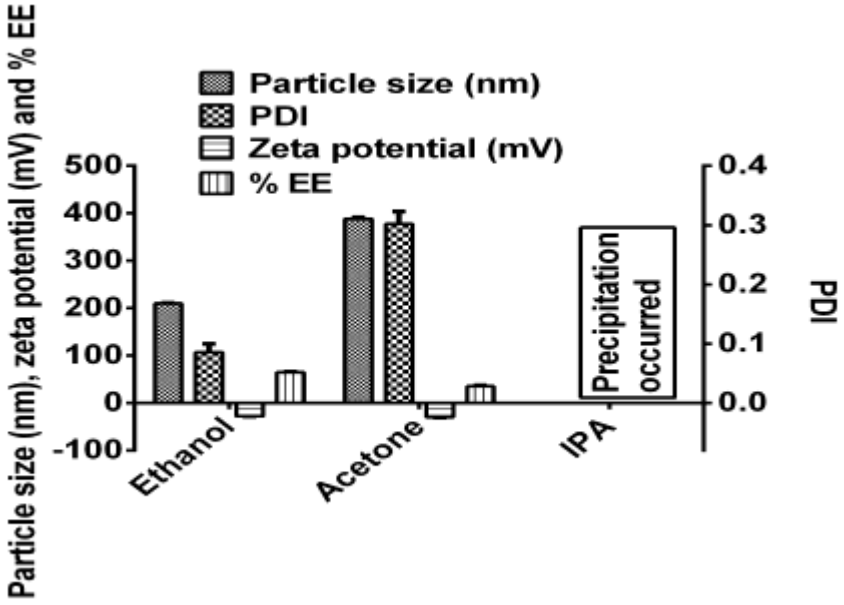

Figure 5.4: Selection of organic solvent as desolvating agent

5.4.1.5 Effect of gluteraldehyde concentration

Different amount to gluteraldehyde (GA) was used to cross link the TNPs and results are summarized in table 5.5 and Figure 5.5. On the basis of obtained results, 0.58 µl of GA/mg BSA was found to be optimum as it showed highest EE (77.3 ± 2.3) of TMZ in BSA NPs. Particle size, PDI and zeta potential was found to be 199.6 ± 3.1 nm, 0.265 ± 0.021 and -27.1 ± 1.70 mV respectively. Although the particle size is more but on the basis of higher EE 0.58 µl/mg BSA was selected. The obtained results may be correlated with the fact that in presence of higher amount of GA, the cross linking of nanoparticles becomes faster leading to inter particulate crosslinking causing aggregation and hence increase in particle size. The agglomeration formed sometimes displays porous natures through which drug leakage might take place leading to decrease in EE (1,10).

Table 5.5: Effect of gluteraldehyde concentration

GA concentration	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)
0.2 µl/mg BSA	140.3 ± 2.5	0.228 ± 0.011	-20.4 ± 2.5	75.5 ± 1.5
0.39 µl/mg BSA	172.7 ± 2.7	0.363 ± 0.015	-31.9 ± 1.9	71.4 ± 1.9
0.58 µl/mg BSA	199.6 ± 3.1	0.265 ± 0.021	-27.1 ± 1.7	77.3 ± 2.3
0.96 µl/mg BSA	207.7 ± 3.7	0.282 ± 0.029	-27.7 ± 2.1	66.3 ± 1.9

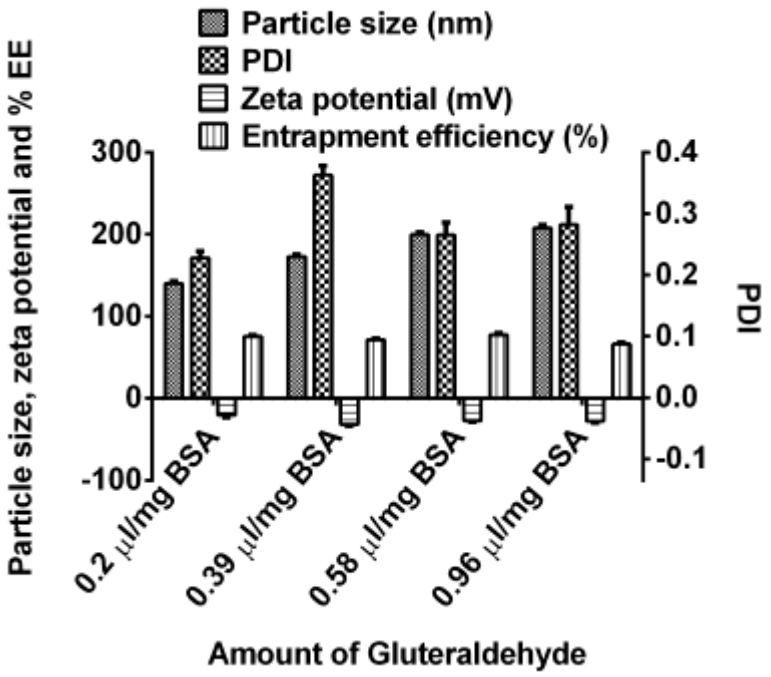

Figure 5.5: Optimization of Gluteraldehyde amount

5.4.1.6 Effect of rate of addition of organic phase

Different TNPs batches were prepared by varying the rate of addition of organic phase and its effect on particle size, PD, zeta potential and % EE was observed (table 5.6 and figure 5.6). The results indicated that after increasing the rate of addition of organic phase, particle size and EE was increased but PDI and zeta potential decreased. The increase in the particle size might be due to the fast desolvation process that leads to the larger aggregates and precipitation of albumin (17). Increase in EE with increase in rate of addition of organic phase was also accordance with reported literature (11). The results of zeta potential was also in accordance with previously reported literature (9).

Table 5.6: Effect of rate of addition of organic phase

Rate of addition of organic phase (ml/min)	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)
0.5	169.7 ± 2.5	0.344 ± 0.019	-31.60 ± 1.50	62.84 ± 2.50
1.0	199.6 ± 2.9	0.265 ± 0.011	-27.10 ± 1.90	77.31 ± 2.90

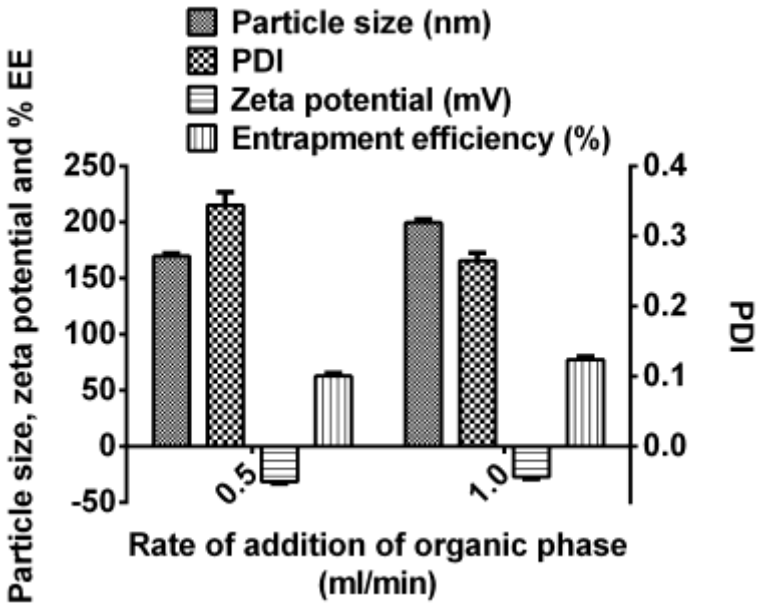

Figure 5.6: Optimization of rate of addition of organic phase

5.4.1.7 Effect of polymer to drug ratio

Various TNPs batches were prepared by varying the polymer to drug ratio (3.33:1 to 10:1) and its effect on particle size, PDI, zeta potential and % entrapment efficiency was observed (figure 5.7). With 5:1 polymer to drug ratio smaller particle size (139.3 ± 1.3 nm) and lesser PDI (0.2 ± 0.017) was obtained but % entrapment was also decreased (64.8± 1.5). Higher % EE (76.2 % ± 2.5 %) of TMZ with 7.5:1 was observed. So this was selected as optimum polymer to drug ratio.

Particle size and PDI was further optimized by varying different parameters. The obtained results may be correlated with the fact that in case of higher polymer: drug ratio, quantity of drug was less with respect to amount of polymer due to which the encapsulation of drug inside polymer matrix was more efficient leading to high drug entrapment efficiency. With increasing the amount of drug (lower polymer: drug), the amount of polymer is less than amount of drug and the saturation of polymer takes place leading to less loading of drug inside polymer matrix (5,10).

Table 5.7: Effect of polymer to drug ratio

Polymer: Drug ratio	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)
10:1	222.9 ± 1.5	0.217 ± 0.013	-33.9 ± 1.5	63.5± 1.9
9:1	224.3 ± 1.9	0.341 ± 0.015	-26.1 ± 2.1	73.2± 2.3
7.5:1	210.3 ± 2.1	0.251 ± 0.011	-31.0 ± 1.9	76.2± 2.5
6.7:1	171.4 ± 1.5	0.337 ± 0.016	-29.6 ± 2.3	65.8± 1.9
5:1	139.3 ± 1.3	0.200 ± 0.017	-27.6 ± 2.5	64.8± 1.5
3.33:1	160.3 ± 1.9	0.211 ± 0.019	-33.3 ± 1.2	52.1± 2.7

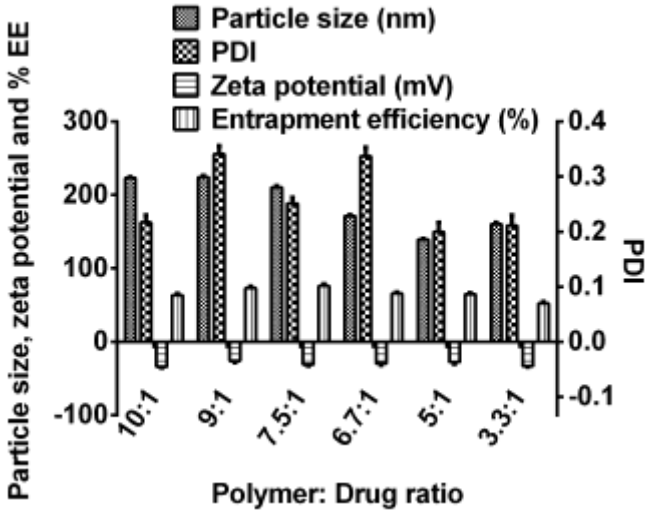

Figure 5.7: Optimization of polymer: drug ratio

The results of preliminary optimization in term of constant variables and varying variables for further optimization are summarized in table 5.8

Table 5.8: Working range of different variables after preliminary optimization

Sr. No.	Name of variable	Range/ Constant value
1.	BSA Concentration	16.67 mg/ml
2.	Aqueous phase	Water (5.7±0.2 pH adjusted with dilute acetic acid)
3.	Organic phase	Ethanol
4.	polymer to drug ratio	10:1 – 5:1
5.	Aqueous. : Organic phase ratio	1:2 – 1:3
6.	Organic phase volume	6 – 9 ml
7.	Amount of GA	0.2 µl/mg BSA - 0.58 µl/mg BSA
8.	Rate of addition of organic phase	1 ml/min

5.4.2 Optimization of TNPs

TNPs were optimized by BBD (SAS JMP software). Preliminary investigation was done to assess the effect of various factors on desired response. Based on preliminary investigation, the upper and lower limits for BBD were selected. According to the BBD design matrix, different batches were prepared and results are summarized in Table 5.9.

Table 5.9: Optimization of TNPs using BBD

Polymer : Drug ratio* (A)	Organic phase volume (ml)* (B)	Gluteraldehyde (GA) (µl)* (C)	Particle size (nm)	PDI	ZP (mV)	% EE
7.5	6	30	269.6 ± 1.1	0.379± 0.009	-18.5 ± 1.1	63.5 ± 1.7
5	9	20	260.5 ± 0.9	0.619 ± 0.011	-30.9 ± 1.7	59.4 ± 1.5
5	7.5	10	194.8 ± 1.3	0.254 ± 0.013	-28.7 ± 1.3	69.6 ± 1.3
7.5	6	10	220.2 ± 1.2	0.319 ±0.011	-20.5 ± 0.9	65.4 ± 0.9
7.5	9	10	166.9 ± 1.8	0.252 ± 0.017	-31.9 ± 1.3	68.3 ± 1.4
5	6	20	267.4 ± 0.9	0.391 ± 0.008	-24.6 ± 1.5	64.8 ± 1.6
10	6	20	216 ± 1.4	0.215 ± 0.008	-21.8 ± 1.2	60.9 ± 1.5
7.5	7.5	20	230.1 ± 1.1	0.386 ± 0.013	-23.4 ± 1.1	59.6 ± 1.3
7.5	9	30	210.6 ± 1.7	0.356 ± 0.011	-23.4 ± 1.1	64.4 ± 1.5
7.5	7.5	20	230.1 ± 1.5	0.386 ± 0.009	-23.4 ± 1.2	59.6 ± 1.7
7.5	7.5	20	230.1 ± 1.5	0.386 ± 0.009	-23.4 ± 1.2	59.6 ± 1.7
10	9	20	183.2 ± 1.4	0.258 ± 0.012	-22.4 ± 0.9	74.4 ± 0.9
10	7.5	30	236.4 ± 1.3	0.228 ± 0.013	-23.9 ± 0.8	67.5 ± 1.1
10	7.5	10	184.5 ± 1.6	0.224 ± 0.007	-20.1 ± 1.1	73.6 ± 1.3
5	7.5	30	277.3 ± 1.5	0.371 ± 0.011	-22.9 ± 0.7	61.0 ± 1.5

* Polymer conc. = 50 mg, A = 1:5, 1:7.5 and 1:10 represented by 10 mg drug, 6.67 mg drug and 5 mg drug respectively , C = 0.2 µl/ mg of BSA, 0.4 µl/ mg of BSA and 0.8 µl/ mg of BSA represented by 10, 20 and 30 respectively. Data is represented as mean ± SD (n= 3)

5.4.2.1 Variables effect on particle size

The obtained particle size (Table 5.9) varied from 166.9 ± 1.8 nm to 277.3 ± 1.5 nm which indicated combined effect of variables on particle size of TNPs. Half normal plots (figure. 5.8A) showed that C was having most significant effect as compared to A and B on particle size.

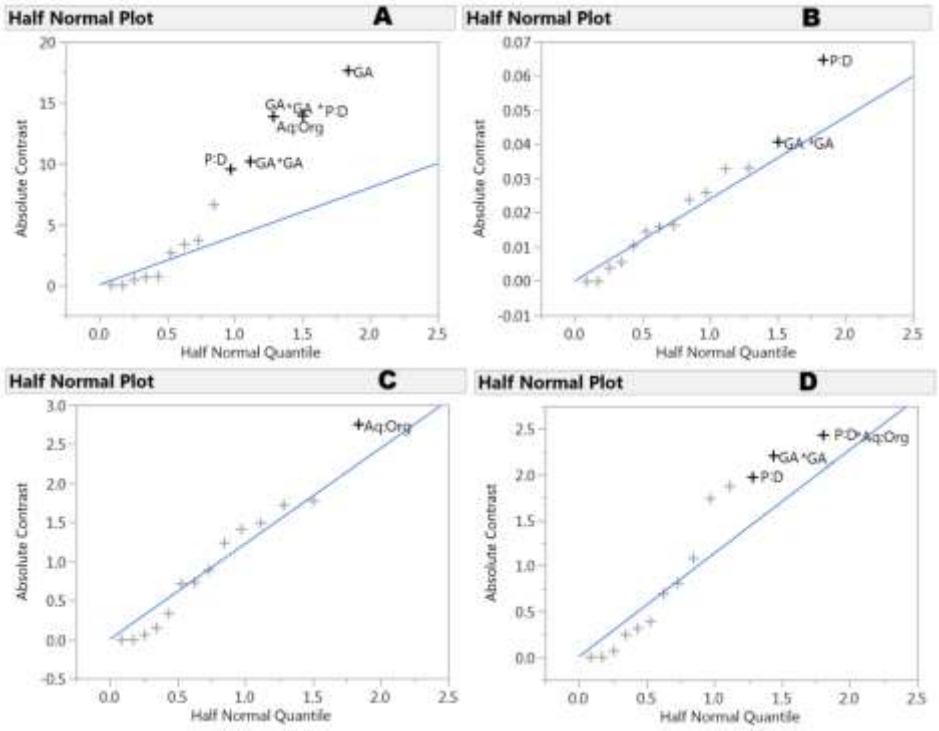

Figure 5.8: Half normal plot obtained for Particle size (A), PDI (B), Zeta potential (C) and % entrapment efficiency (D) of TNPs

The R^2 value of particle size was found to be 0.90 (figure. 5.9A). Among various variables, C (p = 0.0056) showed maximum effect on particle size followed by B (p =0.0130) and A (p = 0.0376). The p value of overall analysis was <0.001 which denoted statistical significance (figure. 5.10A). The obtained results from effect analysis of critical variables showed both positive and negative effect of factors on response. The estimate for C was found to be 28.4375 which demonstrated that an increase in the value of C will lead to increase in particle size and vice versa. Increase in particle size with increased amount of C was also reported earlier (7). This increase in particle size with increasing quantity of C can be attributed to the fact that in presence of higher amount of C, the cross linking of nanoparticles becomes faster leading to inter particulate crosslinking causing aggregation and hence increase in particle size. In case of A, parameter estimate was -22.4875 which means an increase in value of A will cause decrease in particle size and vice versa. In case of higher A, quantity of drug was less with respect to amount of polymer due to which the encapsulation of drug within the polymer matrix was more efficient

leading to high drug entrapment efficiency. With increasing amount of drug (lower polymer : drug), the amount of polymer is insufficient to accommodate the drug and the saturation of polymer takes place leading to less loading of drug inside the polymer matrix (12). Parameters estimate for B was also found to be negative (-19) indicating that an increase in organic phase will cause decrease in particle size. This may be correlated to the fact that formation of nanoparticles is dependent on the volume of organic solvent. If volume of organic phase is decreased, the amount of organic phase will not be sufficient for desolvation of the protein, and there will be delayed precipitation and nucleation leading to formation of large aggregated particles with wide size distribution. On increasing the volume of organic solvent, smaller particles with narrow size distribution were obtained and after certain limit, further increase in volume of organic solvent did not affect size (only increase number of particles) but affect size distribution (9). The interaction between other independent variables was also found to be negative.

The ANOVA results confirmed that the selected design model was significant with p value < 0.05. Visual representation of obtained results can be seen in the contour plots shown in figure. 5.11. It can be observed that increase in A led to decrease in particle size which is in accordance with the effect analysis of variables on particle size and effect model obtained from the design. In case of B, decrease in particle size can be observed with increase in organic phase volume. In case of C, increase in value of C led to increase in particle size as explained earlier.

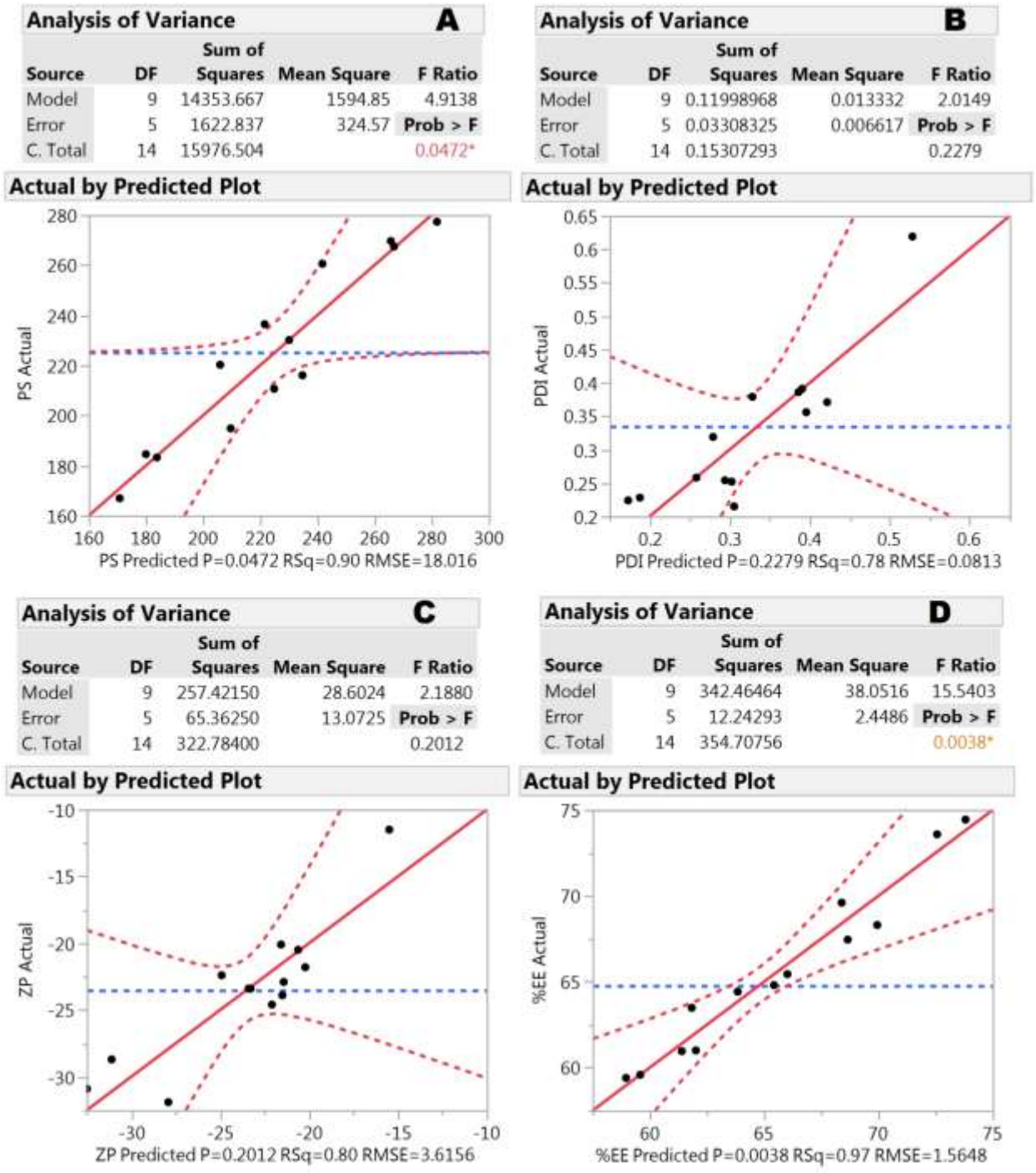

Figure 5.9: Statistical analysis for particle size (A), PDI (B), zeta potential (C) and % entrapment efficiency (D) of TNPs

Parameter Estimates **A**

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	230.1	10.4014	22.12	<.0001*
P:D(5,10)	-22.4875	6.369532	-3.53	0.0167*
Org P vol (6,9)	-19	6.369532	-2.98	0.0307*
GA(10,30)	28.4375	6.369532	4.46	0.0066*
P:D*Org P vol	-6.475	9.007878	-0.72	0.5044
P:D*GA	-7.65	9.007878	-0.85	0.4345
Org P vol*GA	-1.425	9.007878	-0.16	0.8805
P:D*P:D	4.05	9.375697	0.43	0.6838
Org P vol*Org P vol	-2.375	9.375697	-0.25	0.8101
GA*GA	-10.9	9.375697	-1.16	0.2975

Parameter Estimates **B**

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.386	0.046963	8.22	0.0004*
P:D(5,10)	-0.08875	0.028759	-3.09	0.0273*
Org P vol (6,9)	0.022625	0.028759	0.79	0.4671
GA(10,30)	0.035625	0.028759	1.24	0.2704
P:D*Org P vol	-0.04625	0.040671	-1.14	0.3070
P:D*GA	-0.02825	0.040671	-0.69	0.5183
Org P vol*GA	0.011	0.040671	0.27	0.7976
P:D*P:D	-0.03625	0.042332	-0.86	0.4309
Org P vol*Org P vol	0.021	0.042332	0.50	0.6409
GA*GA	-0.0805	0.042332	-1.90	0.1156

Parameter Estimates **C**

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-23.4	2.087463	-11.21	<.0001*
P:D(5,10)	2.3625	1.278305	1.85	0.1238
Org P vol (6,9)	-3.775	1.278305	-2.95	0.0318*
GA(10,30)	2.4375	1.278305	1.91	0.1149
P:D*Org P vol	1.425	1.807796	0.79	0.4663
P:D*GA	-2.4	1.807796	-1.33	0.2417
Org P vol*GA	-0.125	1.807796	-0.07	0.9476
P:D*P:D	-1.8	1.881613	-0.96	0.3827
Org P vol*Org P vol	0.275	1.881613	0.15	0.8895
GA*GA	1.3	1.881613	0.69	0.5204

Parameter Estimates **D**

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	59.58	0.903435	65.95	<.0001*
P:D(5,10)	2.70375	0.553239	4.89	0.0045*
Org P vol (6,9)	1.485	0.553239	2.68	0.0436*
GA(10,30)	-2.57125	0.553239	-4.65	0.0056*
P:D*Org P vol	4.72	0.782398	6.03	0.0018*
P:D*GA	0.6175	0.782398	0.79	0.4657
Org P vol*GA	-0.48	0.782398	-0.61	0.5664
P:D*P:D	3.90875	0.814345	4.80	0.0049*
Org P vol*Org P vol	1.41125	0.814345	1.73	0.1436
GA*GA	4.41875	0.814345	5.43	0.0029*

Figure 5.10: Parameter estimates for particle size (A), PDI (B), zeta potential (C) and % entrapment efficiency (D) of TNPs

5.4.2.2 Variables effect on PDI

The obtained PDI varied from 0.215 ± 0.008 to 0.619 ± 0.011 nm (Table 5.9). Half normal plots show that A has significant effect on PDI (figure 5.8B). The R^2 value for PDI was 0.78 (figure. 5.9B). Among all variables affecting PDI, A (p = 0.0267) demonstrated maximum effect on PDI while C and B were insignificant. The p = 0.0004 for overall effect analysis indicated no significance (figure 5.10B). The obtained results indicated that the factors did not have significant effect on response which was similar to previously reported work (11). The estimate for C, A and B was found to be 0.03562, -0.08875 and 0.02262 respectively. The p value (0.2279) for ANOVA also indicated that the model was not significant.

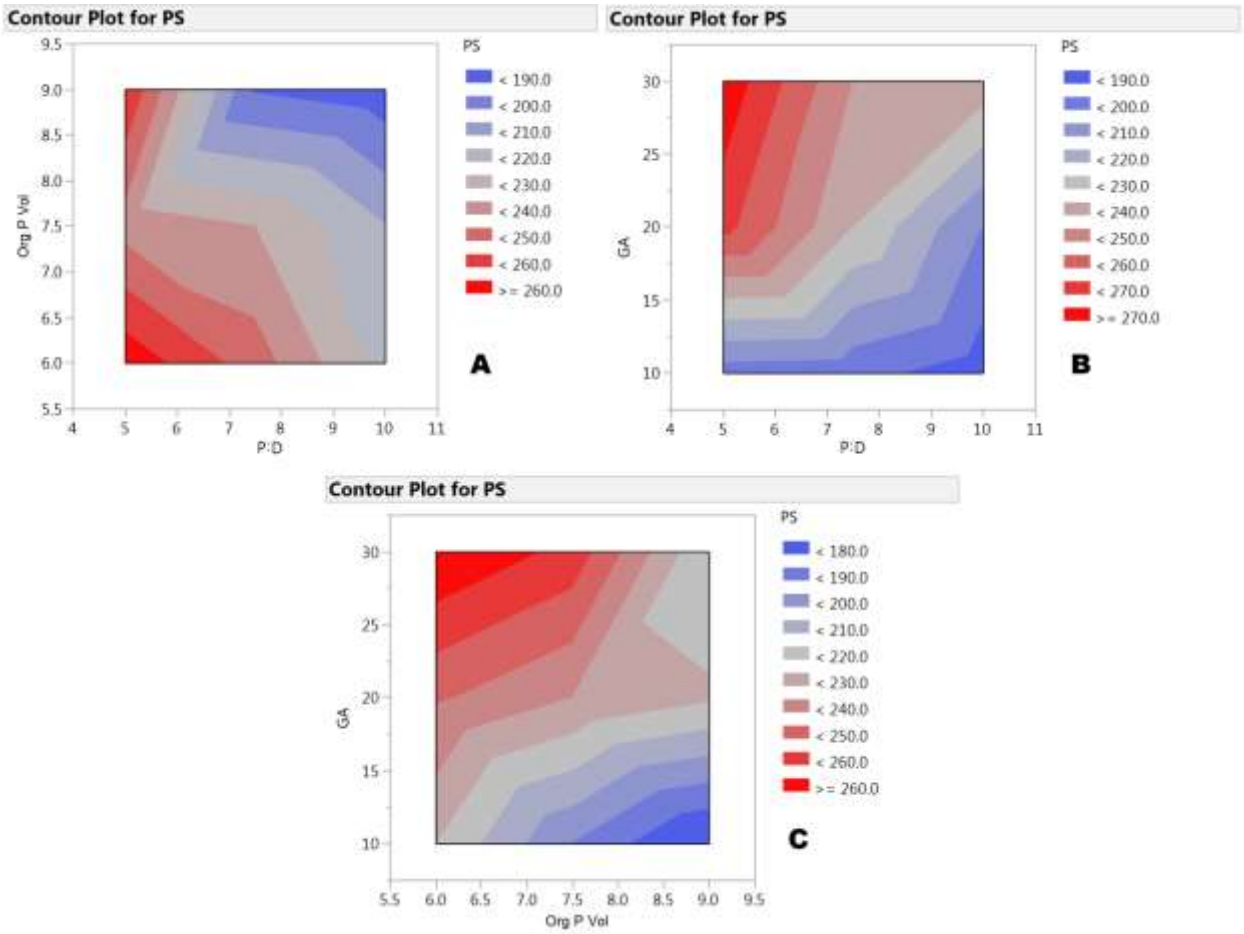

Figure 5.11: Contour plots showing (A) effect of Organic phase volume and polymer : drug ratio on particle size; (B) effect of GA and polymer : drug ratio on particle size; (C) effect of GA and organic phase volme on particle size of TNPs

5.4.2.3 Effect of independent variables on zeta potential

Zeta potential varied from -18.5 mV ± 1.1 to -30.9 ± 1.3 mV (Table 5.9). Half normal plots show that B had significant effect on zeta potential (figure. 5.8C).The R^2 value for zeta potential was 0.80 (figure 5.9C). Among all variables affecting zeta potential, B (p value = 0.0445) demonstrated maximum effect on zeta potential while C and A were insignificant as indicated by their p values 0.1464 and 0.1560 respectively. The p value < 0.001 of overall effect analysis was significant (figure. 5.10C). The obtained results indicated that the factors did not have significant effect on response. The estimate for C, A and B was found to be 2.4375, 2.3625 and -3.775

respectively. The p value = 0.2012 of ANOVA indicates that the model was not significant. The results were similar to previous reports (15).

5.4.2.4 Effect of independent variables on % entrapment efficiency

The % entrapment efficiency varied from 59.40% ± 1.5 to 74.44% ± 0.9 (Table 5.9). The results indicate combined effect of variables on EE of TNPs. Half normal plots show that A and interaction of A and B had most significant effect as compared to C and B on EE (figure 5.8D).

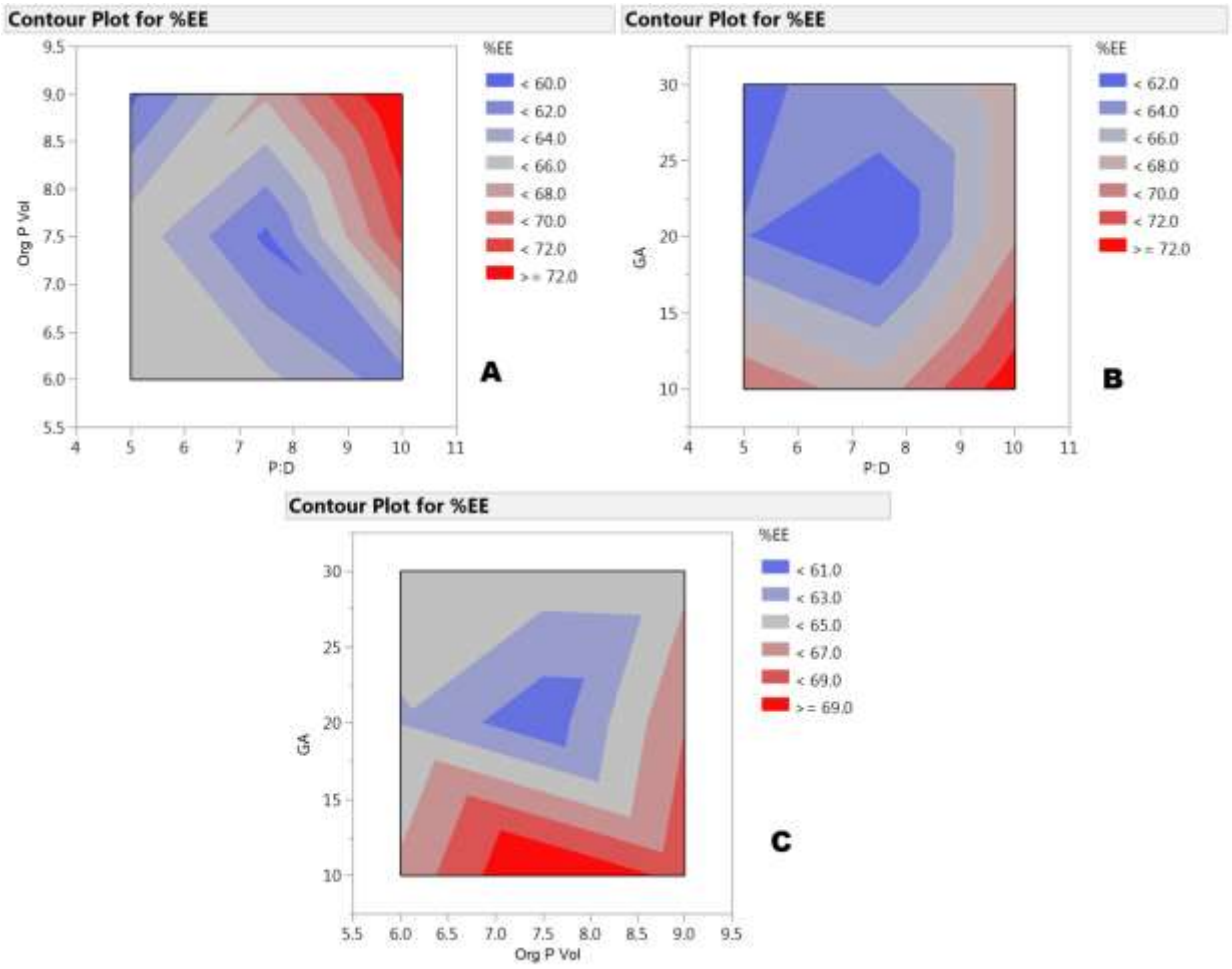

Figure 5.12: Contour plots showing (A) effect of organic phase volume and polymer : drug ratio on % entrapment efficiency; (B) effect of GA and polymer : drug ratio on % entrapment efficiency; (C) effect of GA and organic phase volume on % entrapment efficiency of TNPs

The R^2 value of EE was 0.97 (figure 5.9D). Among all variables, A (p value = 0.0995) demonstrated maximum effect on EE. Overall effect analysis of variables was also found to be significant (p value < 0.001 (figure 5.10D). The obtained results of effect analysis for critical variables suggested that the independent variables had both positive and negative effect on response i.e., there was increase in response value with increase in value of one independent variable while the response value decreased with increase in value of another independent variables. The positive estimate for A demonstrated that an increase in the value of A will lead to increase in EE and vice versa. In case of C, parameter estimate was -2.57125 which means an increase in value of C will cause decrease in EE and vice versa. Parameters estimate for B was 1.485 which indicates that increase in value of B will cause increase in EE. The interaction between A* B and A* C was positive while interaction between B * C was negative. The p value (0.0038) of ANOVA indicates that the model was significant. Positive correlation of factor A with EE can be seen in the counter plots (figure 5.12) which means increase in A would lead to increase in EE. In case of B, similar positive correlation can be seen while in case of C negative correlation can be seen which means increase in value of C led to decrease in EE. The obtained results were similar to previous reports (9,11). The effect of A has been explained above with respect to polymer saturation in which the matrix volume of polymeric nanoparticles are not able to encapsulate total amount of drug added in the formulation, although the drug loading efficiency might remain constant. The decrease in EE with increase in C can be attributed to the fact that increasing amount of C leads to increase in particle size due to inter-particle cross-linking. The agglomeration formed sometimes displays porous natures through which drug leakage might take place leading to decrease in EE (9) whereas it is quite opposite in case of B. Here, increase in ratio causes slow nucleation which leads to agglomeration and increase in particle size. But this slow nucleation gives enough time for drug molecule to settle down in polymer matrix leading to increase in EE.

5.4.3 Establishment and analysis of design space

SAS JMP software was used to generate desirability plot after selecting the upper and lower desired values of responses. According to these values software generated values of variables that would aid to accomplish the desired response. A composite desirability of 0.892 was

obtained (figure 5.13) that indicates if the TNPs are fabricated according to the obtained values of variables, the chance to obtain desired value of response is 89.2%.

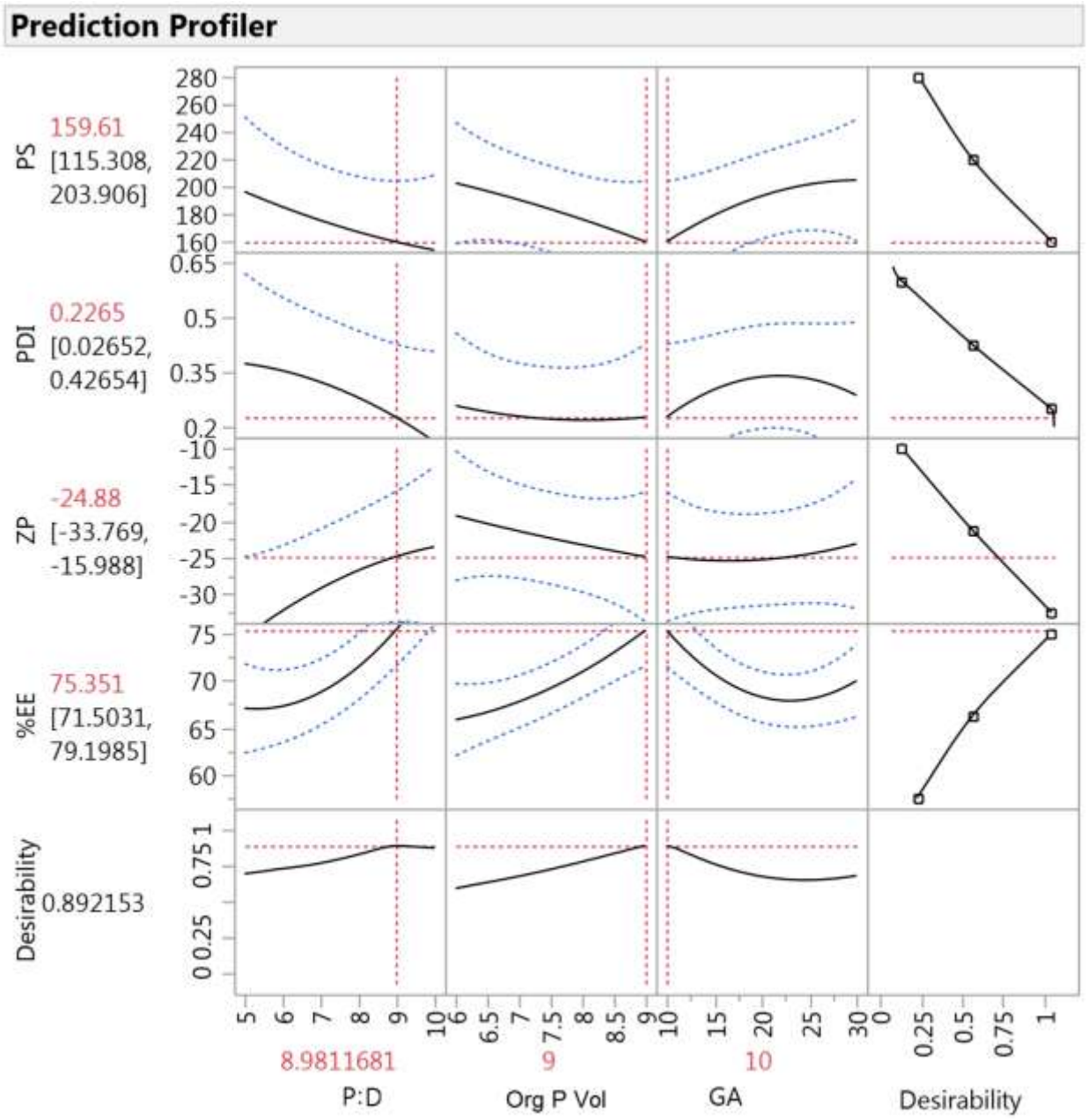

Figure 5.13: Desirability plot obtained of TNPs

The desirability plot suitability was assessed by preparing TNPs of recommended batch and comparing its characteristics with predicted responses (Table 5.10).

Table 5.10: Predicted and obtained value for desirability plot for preparation of TNPs

Independent variables	**Values**	
Polymer : drug ratio (A)	8.981	
Organic phase volume (ml) (B)	9	
GA (µl) (C)	10	
Response	**Predicted**	**Obtained**
Particle size (nm)	159.61	158.6
PDI	0.227	0.158
Zeta potential	-24.88	-27.7
% entrapment efficiency	75.35	70.96

* Polymer = 50 mg

The obtained results indicate the suitability of the predicted desirability plot for optimized formulation. Results obtained for particle size, PDI and % EE were almost similar to that of the predicted values. The combined effect of factors on particle size, PDI, zeta potential and % entrapment efficiency of TNPs is shown in figure. 5.14.

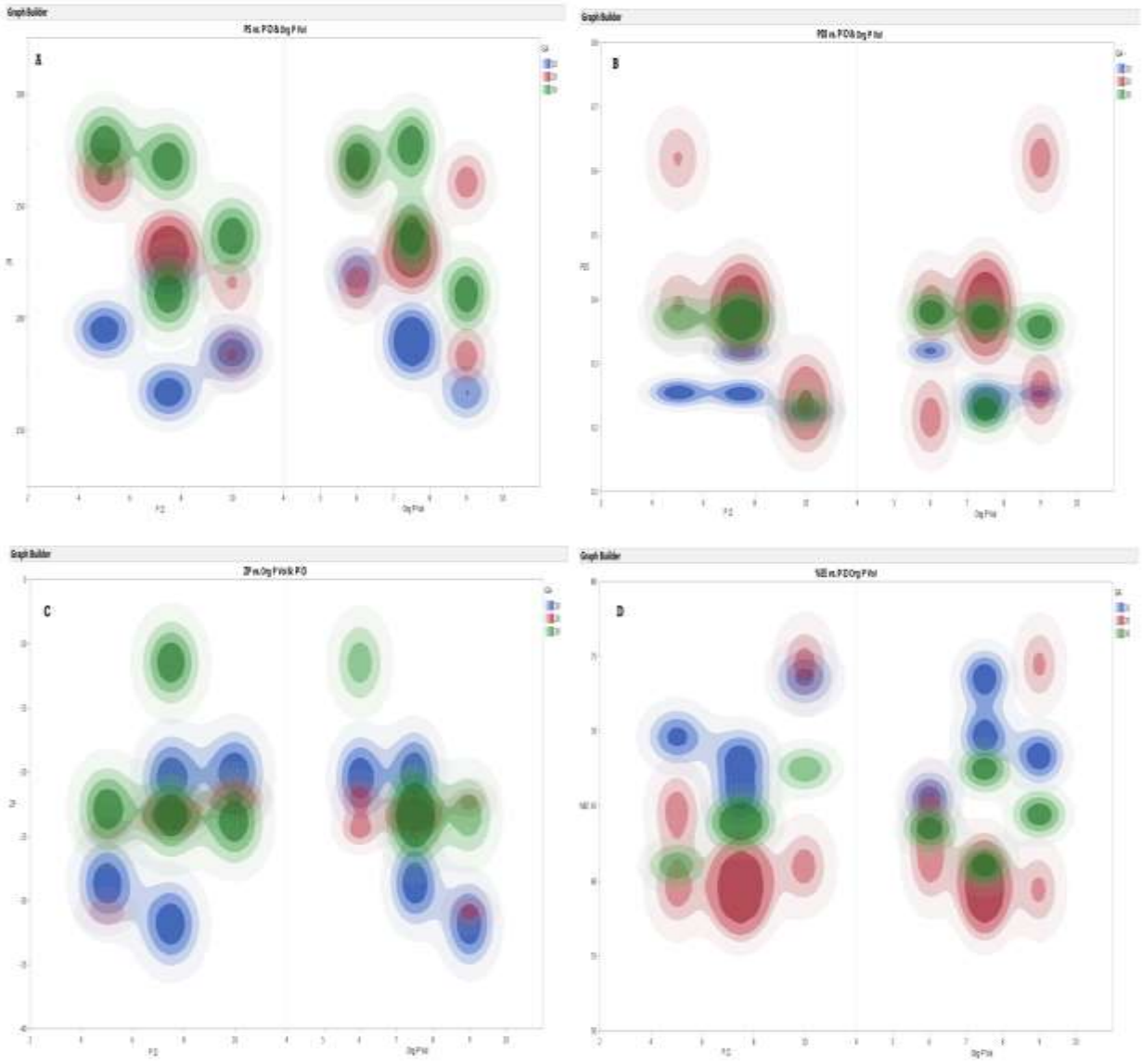

Figure 5.14: Visual representation summarizing theeffect of various independent variables on particle size (A), PDI (B), zeta potential (C) and % entrapment efficiency (D) of TNPs

5.4.4 Characterization of albumin nanoparticles

5.4.4.1 Particle size and PDI determination

Particle size and PDI of TNPs were found to be 158.8 ± 1.87 nm and 0.124 ± 0.012 respectively (figure 5.15).

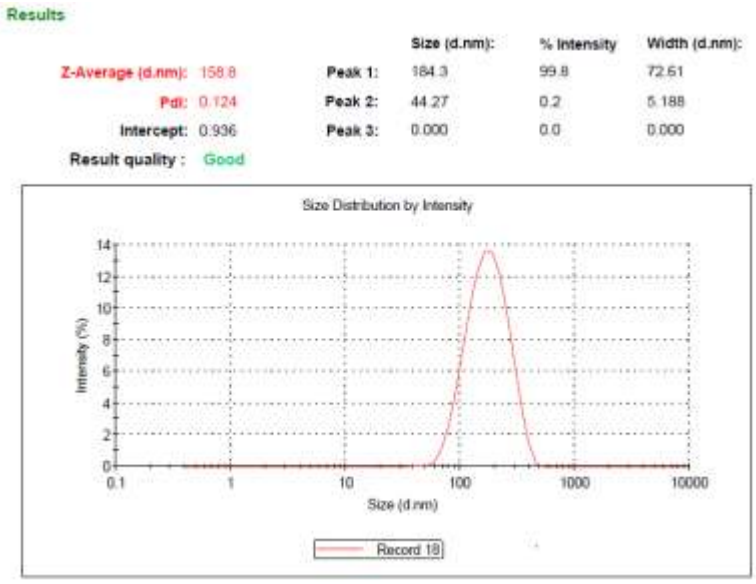

Figure 5.15 Particle size and PDI of optimized TNPs

5.4.4.2 Zeta potential determination

Zeta potential of TNPs was observed as -27.7± 1.1 mV (figure 5.16). Above pH 4.3, BSA showed negative zeta potential thus TNPs also showed negative zeta potential. The value of zeta potential of TNPs indicated good colloidal stability.

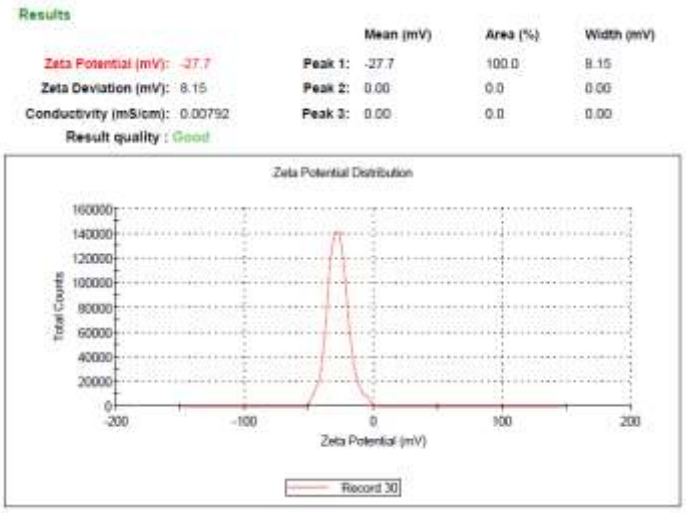

Figure 5.16 Zeta potential of optimized TNPs

5.4.4.3 DSC analysis

DSC thermograms of BSA, TMZ, PNPs and TNPs are shown in figure 5.17. The drug (TMZ) showed a sharp exothermic peak at 208.81^{o}C which indicates its crystalline nature of TMZ. Thermo gram of TNPs did not show any exothermic peak of TMZ which may be due to encapsulation of TMZ in the NPs in form of molecular dispersion (4).

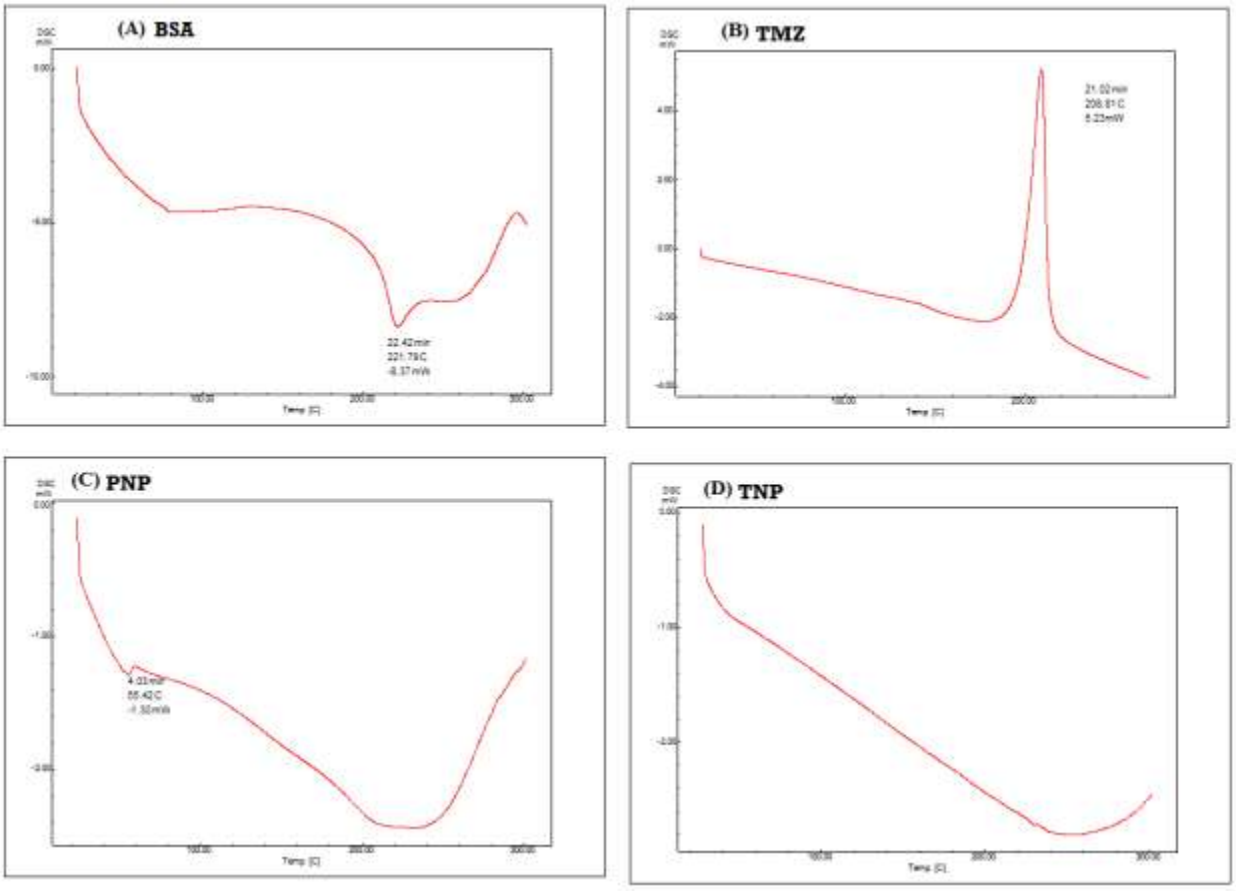

Figure 5.17: DSC thermograms of (A) BSA, (B) TMZ, (C) PNPs and (D) TNPs

5.4.4.4 FTIR analysis

The FTIR spectrum of PNPs and TNPs are shown in figure 5.18 and all characteristic peaks are summarized in table 5.11. The FTIR spectrum of PNPs indicated that all characteristic peaks of BSA were present with lesser intensity as compared to pure BSA. In case of TNPs, all characteristic peaks of BSA and TMZ were present but the intensity of TMZ characteristic peaks were decreased which indicates that TMZ was present in TNPs (4,10).

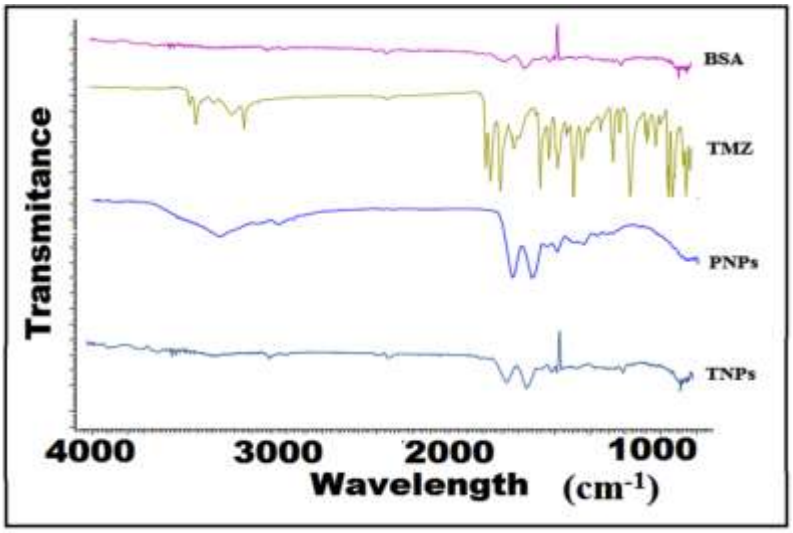

Figure 5.18: FTIR spectrum of BSA, TMZ, PNPs and TNPs

Table 5.11: Summary of absorption peaks of FTIR spectrum of TMZ, BSA, PNPs and TNPs

Functional moiety	Observed absorption peak ($cm^{-1)}$	Functional group
TMZ	3419.79 and 3385.07	–N-H stretch for amines
	3113.11, 3184.48 and 3284.77	=C-H- (alkene) stretch
	1757.15, 1730.15 and1674.21	–C=O stretch contributed by aldehydes, ketones or amide group
	1598.99 and 1674.21	–C=C- (Alkene) stretch
	1360cm to 1180	-C-N stretch from amines
BSA	3277.06	3277.06
	2873 -2950	-C-H and -C-H methoxy stretching vibration
	1641.42	-C=O stretching vibrations of amide
	1535.34	-N–H bending -C–N stretching vibration of amide
PNPs	1791.87, 1639.49	-C=O stretching vibrations of amide
	1535.34	-N–H bending -C–N stretching vibration of amide
TNPs	1791.84 and 1641.42	-C=O stretching vibration of amide
	1534.34	-N–H bending -C–N stretching vibration of amide

5.4.4.5 XRD analysis

X-ray diffractograms (XRD) of pure TMZ, BSA, PNPs and TNPs are shown in figure 5.19. TMZ showed characteristic sharp and intense peaks at 2θ values of 10.7°, 14.64°, 26.4° and 28.2° indicative of highly crystalline nature of drug. XRD pattern of BSA did not show any sharp peaks representing their amorphous nature. The hollow pattern of PNPs indicated that the formed nanoparticles were amorphous. XRD pattern of TNPs were also similar to blank nanoparticles (PNPs) indicating the probability of most of the drug getting entrapped in the polymeric matrix of the nanoparticles as sharp peaks of TMZ were not seen in the drug loaded nanoparticles (4).

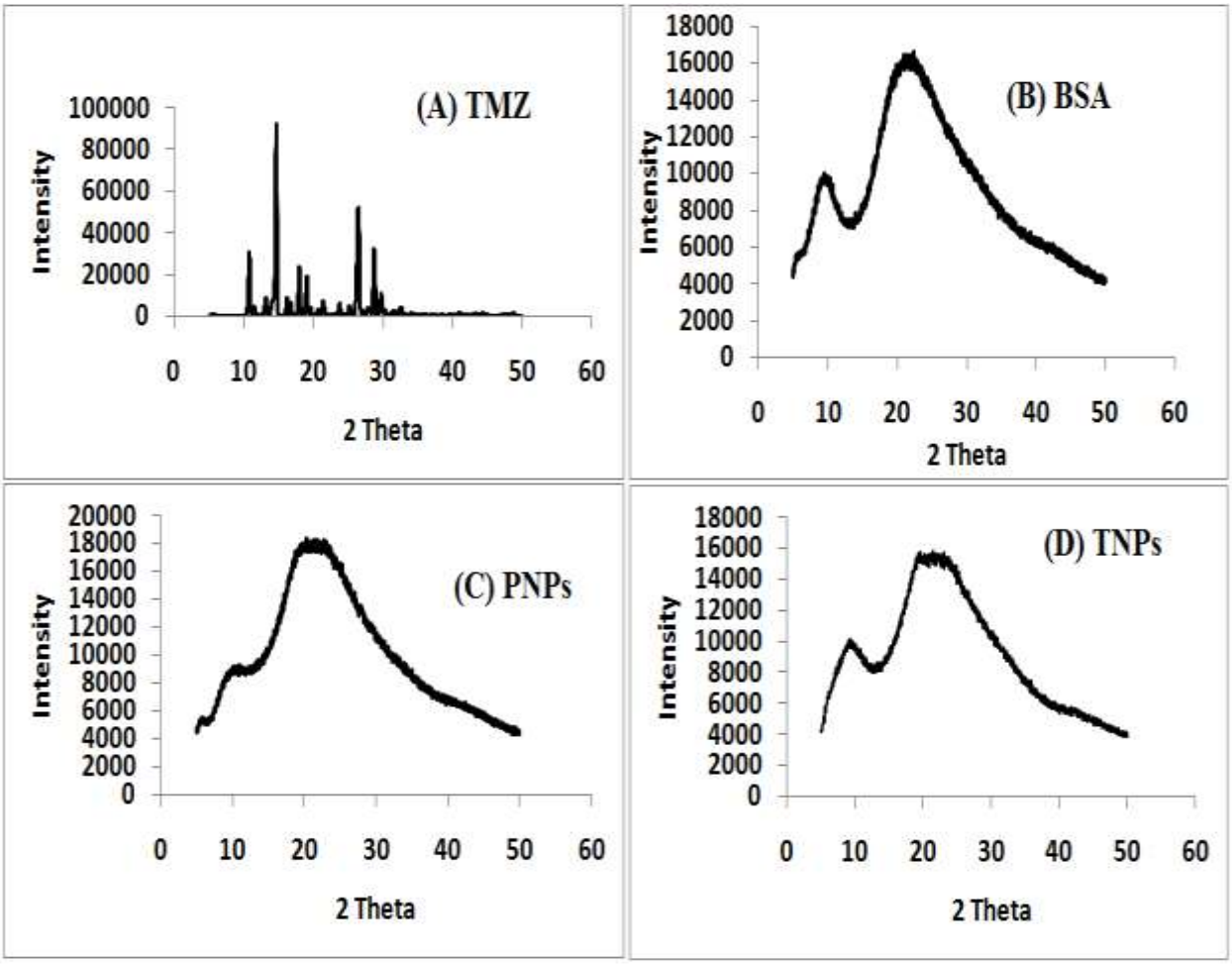

Figure 5.19: XRD diffractograms of (A) BSA, (B) TMZ, (C) PNPs and (D) TNPs

5.4.5 Evaluation of albumin nanoparticles

5.4.5.1 Estimation of entrapment efficiency and drug loading

Percent entrapment efficiency of final optimized TNPs was found to be 70.96 % ± 2.57 % while percent drug loading was found to be 9.94 % ± 1.87 %.

5.4.5.2 *In-vitro* drug release

The results of cumulative *in-vitro* drug release (% CDR) from pure TMZ and TNPs dispersion (figure 5.20) indicated almost 100 % drug release of pure TMZ within 1.5 h while only 43.81 % ± 1.30 % drug was released after 2 h from TNPs. From TNPs 56.68 % ± 1.48 % and 61.12 % ± 1.56 % drug release was observed after 24 h and 48 h respectively. A biphasic release pattern of TMZ was observed from developed TNPs characterized by an initial rapid release within half an hour, followed by slower and sustained release. The slower and sustained release behavior of drug may be due to slow diffusion of drug from entrapped BSA while initial rapid release may be due to release of drug present in the periphery of nanoparticles.

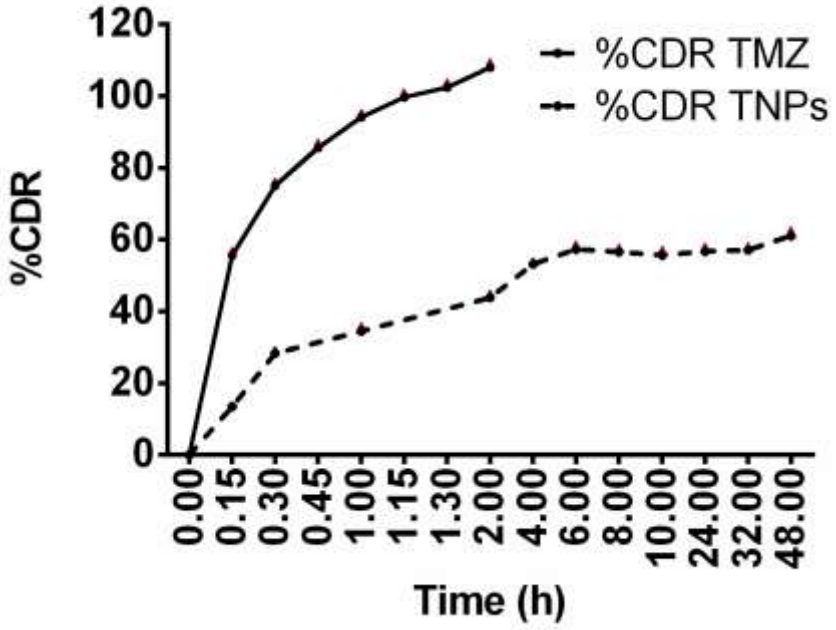

Figure 5.20: In vitro drug release study of TMZ and TNPs in sodium acetate buffer pH 5.5

5.4.6 Stability studies

The results of stability studies (Table 5.12) indicated that prepared TNPs were stable at both refrigerated condition and room temperature (25°C) for three months as no significant change in particle size, assay and zeta potential was observed.

Table 5.12: Stability studies of lyophilized TNPs

Time (month)	Particle size (nm)		Assay		Zeta potential (mV)	
	At refrigerated condition (4 °C)	At room temperature (25°C ±2°C and 65% ±5% relative humidity)	At refrigerated condition (4 °C)	At room temperature (25°C ±2°C and 65% ±5% relative humidity)	At refrigerated condition (4 °C)	At room temperature (25°C ±2°C and 65% ±5% relative humidity)
0	158.6± 1.87	158.6± 1.87	100.0% ± 2.57%	100.0% ± 2.57%	-27.7± 1.1	-27.7± 1.1
1	158.3 ± 2.58	159.1 ± 2.93	99.46% ± 2.89%	98.48% ± 3.01%	-26.3 ± 1.1	-26.1 ± 1.2
2	159.1 ± 3.37	159.5 ± 4.29	98.45% ± 3.23%	96.56% ± 3.95%	-26.1 ± 1.2	-25.7± 1.3
3	159.7 ± 5.51	159.9 ± 6.47	97.69% ± 3.77%	96.05% ± 4.97%	-25.7± 1.3	-24.2 ± 1.5

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