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RESEARCH ARTICLE

Formulation Characterization and Optimization of Chitosan Nanoparticles Loaded with Sorafenib Tosylate

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ABSTRACT

Chitosan is a biocompatible natural polymer used extensively for fabrication of nanoparticles with the aim of safe and efficient drug delivery. Sorafenib, is an orally active bi-aryl urea compound that inhibits cell proliferation by multikinase inhibition and reported to be effective in the treatment of hepatocellular carcinoma. The clinical application of this drug is limited by its decreased bioavailability. Use of biocompatible nanoparticles as drug delivery system is a possible alternative to avoid this limitation and with this objective the present investigation concerns with the development, characterization and optimization of chitosan nanoparticles loaded with sorafenib tosylate based on 2^3 full factorial design experimentation. Three independent process factors namely concentration of chitosan, amount of drug added and the pH were selected for the study. Relationship and effect of these factors on two responses, encapsulation efficiency and percentage yield, were used to characterize and optimize the nanoparticle formulation. The significance of combinations were analysed by ANOVA and p values. Production of discrete nanoparticles below 20nm size was confirmed by performing TEM analysis. Contour plots and three dimensional surface response plots were drawn to evaluate the interaction of independent variable on the chosen dependent variables. Maximum encapsulation efficiency of 30.16% was obtained for nanoparticles prepared with a combination of 1.25 mg/ml chitosan concentration, pH 4.5 and 10 mg of sorafenib tosylate.

KEYWORDS

Chitosan, nanoparticles, sorafenib tosylate, factorial design, TEM, HPLC, DSC

INTRODUCTION

Chitosan is a naturally derived polysaccharide obtained by partial alkaline deacetylation of chitin. It has been extensively used as a polymer for fabrication of drug delivery systems for many drugs taking advantage of its excellent biocompatibility and biodegradability[1,2]. Presence of hydroxyl and amino groups in chitosan molecule are represented in figure 1.

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Chitosan has been used as an effective carrier for a variety of drugs like hormones, nutraceuticals, genes, and anticancer drugs[3-7]. Chitosan is reported to be safe and interacts with polyanions to form colloidal complexes[8-10]. One of the most popular and established methods to prepare chitosan nanoparticles is by ionotropic gelation with a polyanion usually sodium tripolyphosphate[11].

Hepatocellular carcinoma is one of the most prevalent cancers in the world and third most cause of cancer related death. Sorafenib is an oral

multikinase inhibitor effective for the treatment of hepatocellular carcinoma[12].

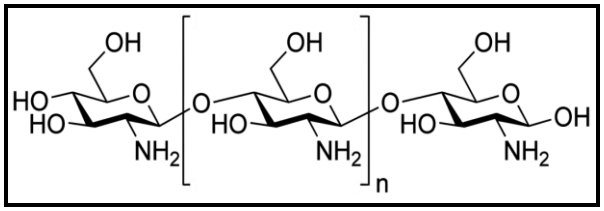

Figure 1: Chemical Structure of Chitosan

It targets Raf/MEK/ERK signalling at the level of Raf serine/threonine kinases and exerts antiangiogenic effect through the vascular endothelial growth factor (VEGF) receptor -2/-3, and platelet- derived growth factor (PDGF) receptor-β tyrosine kinases[13,14]. Sorafenib is considered to be the only drug that produces significant improvement in patients with advanced hepatocellular carcinoma in terms of overall survival[15]. Poor bioavailability and undesirable side effects including hand-foot skin reactions, diarrhoea, fatigue etc of sorafenib, are reported as the limiting factors to its successful therapeutic application[16,17]. Improvement in therapeutic efficacy of this drug through application of suitable drug delivery system would be beneficial to the patients with hepatocellular carcinoma. Nanoparticles have been widely used as carriers for many drugs to achieve targeted delivery in hepatocellular carcinoma and recent development suggest potent application of particle assisted drug delivery in the management of this disease[18-20].

Optimization of nanoparticle formulation based on statistical design is an efficient and economic approach to accomplish essential information pertaining to the relationship between the process variables and their effect on selected responses[21-23].

The objective of this study was to investigate the utility of a 2^3 full factorial design to optimize the process parameters of chitosan nanoparticles formulation encapsulated with sorafenib tosylate. Encapsulation efficiency of each batches of formulations were evaluated by application of HPLC method and statistical analysis of data done using Design Expert software (DX9). DSC analysis of nanoparticles and morphological characterization by TEM were also performed.

MATERIAL AND METHODS

Chitosan was purchased from Sigma Aldrich, USA. Sorafenib Tosylate was a gift from Cipla Ltd India. All the other chemicals used in the experiment were of standard analytical grade and purchased from Merck specialties Pvt Ltd. India.

2^3 Factorial Design (Full)

To optimize the processing factors in the formulation of sorafenib tosylate loaded chitosan nanoparticles a statistical based 2^3 full factorial design was applied in this study. Primary objective of the study design is to identify the significance of various processing factors like concentration of chitosan solution (A), the amount of drug (sorafenib tosylate) added (B) and pH (C) and their effect on selected responses like encapsulation Efficiency (%) (R1), and yield (%) (R2). Optimization of processing factors to accomplish optimal value of responses was made based on the results of statistical evaluation of these parameters. Selected processing factors and their levels are given in Table 1.

Table 1: Factorial design (2^3 full) of batches of nanoparticles with three selected processing factors and their levels

Batch Name	Factor A Concentration of Chitosan (mg/ml)	Factor B Amount of Drug (mg)	Factor C pH
A	1.25	25	4.5
B	1.25	25	3.5
C	1.25	10	4.5
D	1.25	10	3.5
E	0.75	25	4.5
F	0.75	25	3.5
G	0.75	10	4.5
H	0.75	10	3.5

Preparation of Chitosan Nanoparticles Loaded with Sorafenib Tosylate

Chitosan nanoparticles were prepared by ionic gelation method as reported earlier, with some modification[24]. In this method positively charged amino group of chitosan interacts with negatively charged tripolyphosphate resulting in gelation and to form nanoparticles. Briefly, 100 ml chitosan solution was prepared in 1 % (v/v) acetic acid in deionized water, placed in a 250ml beaker and stirred overnight using a magnetic stirrer (ANM, India) at 1500 rpm. Adjusted the pH using 0.1N hydrochloric acid solution, incorporated sorafenib tosylate and 100µl of tween 80 in to the chitosan solution and continued stirring for another one hour. 100 ml of Tripolyphosphate solution (1mg/ml) concentration, prepared in 0.9%(w/v) sodium chloride, was added drop wise from a burette, at a rate of 12ml/hour in to the chitosan solution while maintained stirring at 1500 rpm. After complete addition of tripolyphosphate solution continued stirring for another 15 minutes. The reaction mixture was allowed to stand for one hour and the formed nanoparticles were separated by centrifugation at 12000 rpm for 30 minutes at 4° C. Decanted the supernatant, washed the nanoparticles three times with de-ionized water and freeze dried.

Characterization

Transmission Electron Microscopy (TEM) Analysis

A well dispersed suspension of nanoparticle formulation was prepared and a drop was layered on the grid and allowed to dry overnight. The grid was loaded and the sample was viewed under a Hitachi H-7650 transmission electron microscope at an accelerating voltage of 80 kV.

Differential Scanning Calorimetry (DSC)

Thermal behaviour of the nanoparticles was analyzed using differential scanning calorimetry. About 1-5mg of sample was transferred to Tzero aluminium pan and scanned in the temperature range from 25-400°C at a heating rate of 10°C/min. Inert atmosphere was maintained by purging nitrogen gas at a rate of 50ml/min. Differential Scanning calorimetry was performed for all batches using DSC Q20 V24.4 Build 116 instrument and module DSC standard cell FC.

High Performance Liquid Chromatography (HPLC)

HPLC method was used to analyze drug content in the nanoparticles using Shimadzu LC 2010A HT at UV max 260 nm and column Enable C18 G 5µm(250 x 4.6 mm). Mobile phase used was Acetonitrile: disodium Phosphate 55:45 v/v (buffer pH = 4 adjusted with phosphoric acid) degassed and filtered (0.45µ Millipore) at a flow rate of 1.0ml/min. Column temperature was maintained at ambient condition.

Preparation of Standard Calibration Curve

Standard calibration curve was prepared as reported earlier[25]. Briefly, a stock solution of sorafenib tosylate of 1mg/ml concentration was prepared by dissolving 25mg in 25ml of mobile phase in a 25ml standard flask and sonicated for 25 minutes in a bath sonicator and filtered through 0.45µ membrane filter. Standard solutions of different concentrations were prepared by spiking required volume of stock solution into standard flasks (25ml) and added mobile phase to produce 100, 50, 25, 12.5, 6.25, and 3.125µg/ml solutions. Aliquot of 20µl from each standard solution was injected into the analytical column and the resultant peak area was measured.

Evaluation of Encapsulation Efficiency (EE %) and Yield (%)

The drug content in each batch of formulations was analyzed by extracting 5mg of drug loaded nanoparticles with 5ml of mobile phase for 24 hours in 5ml volumetric flasks. Then the flasks were placed in a bath sonicator and sonicated for 25 minutes. The solution was filtered using a syringe adaptor filter unit (millex vv 0.1µm Durapore PVDF membrane). A volume of 500µl filtrate was collected for HPLC analysis. The encapsulation efficiency EE (%) (R1) and yield (%) (R2) were calculated as follows.

$$R1 \quad EE\ (\%) = \frac{\text{Wt. of drug (mg) in total wt of batch X100}}{\text{Wt of drug added in batch (mg)}}$$

R2 Yield (%) = Wt. of dried nanoparticles (mg) X 100

Wt. of chitosan employed (mg)

RESULTS AND DISCUSSION

Transmission Electron Microscopy (TEM) Analysis

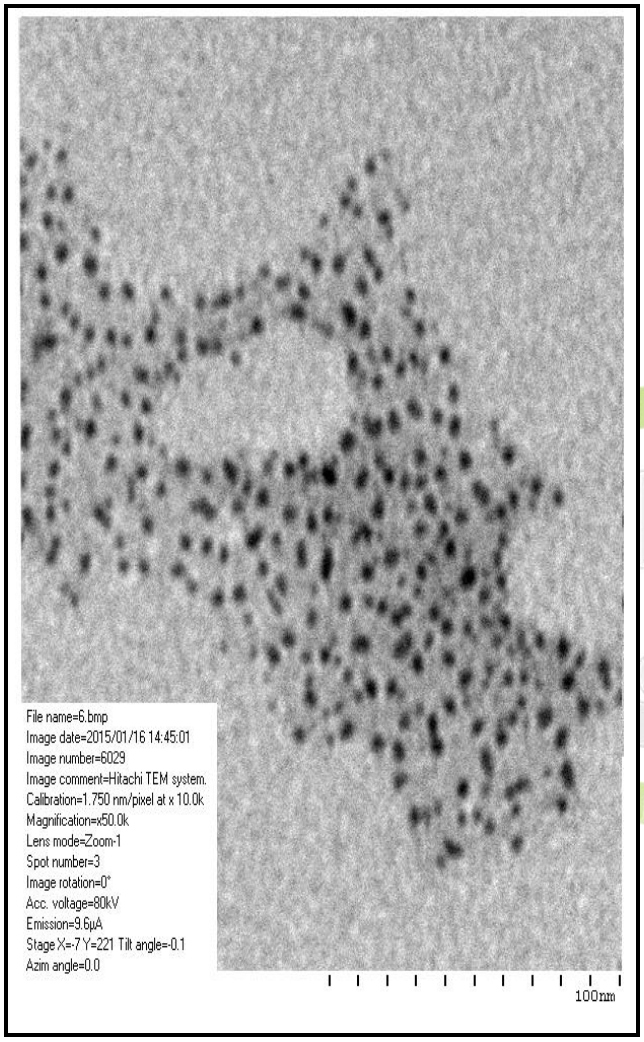

Figure 2: TEM Image of Chitosan Nanoparticles

Morphology of nanoparticles was examined by TEM analysis. It reveals that the nanoprticles are obviously discrete and almost spherical in shape. Micrograph shown in Figure 2 confirms that the sizes of nanoparticles are below 20nm. The absence of agglomeration may be due to the presence of saline and tween80.

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry was performed for all batches of nanoparticles.

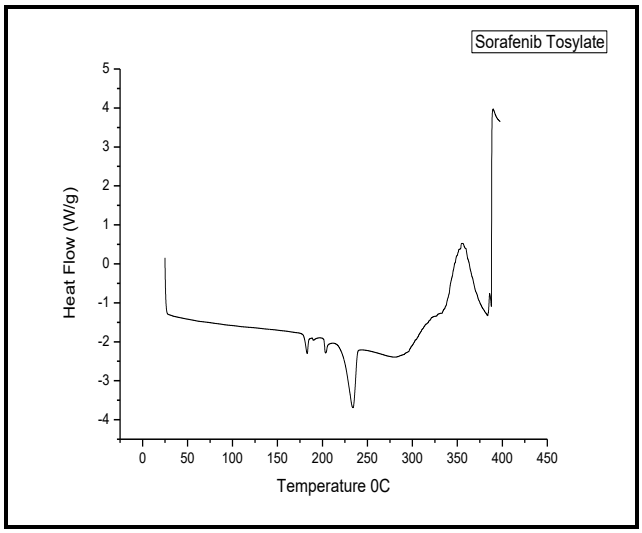

Figure 3a: DSC Curve of Sorafenib Tosylate

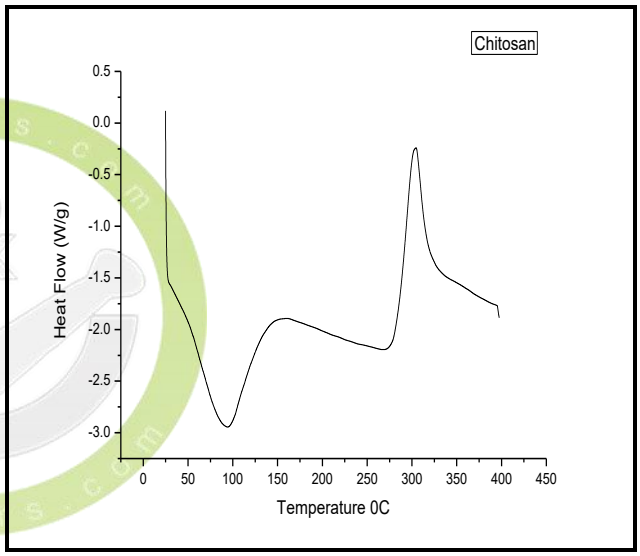

Figure 3b: DSC Curve of Chitosan

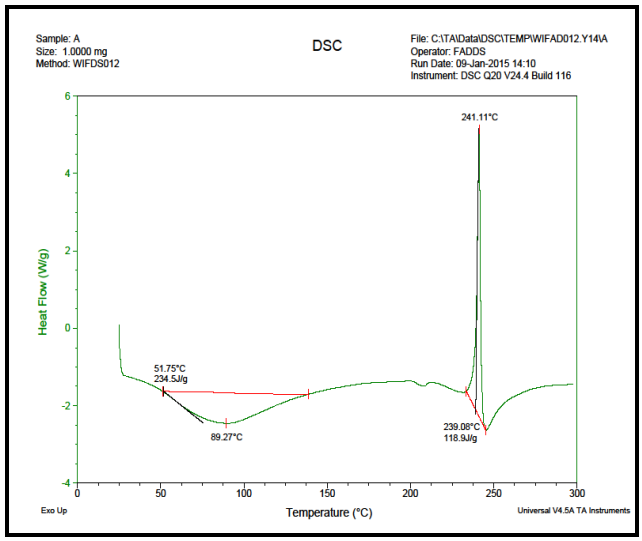

Figure 3c: DSC Curve of Batch A

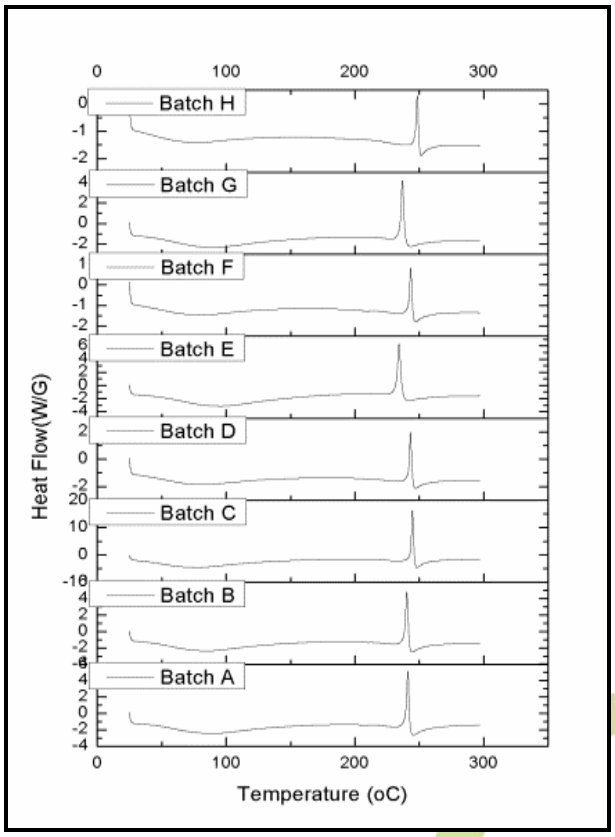

Figure 3d: DSC Curves of Eight Batches

Figure 3a shows the thermogram of sorafenib tosylate. Endothermic peak around 220-235ºC indicates melting. Figure 3b represents thermogram of chitosan, wide endothermic peak at around 75 -100ºC attributed to desorption of water and the exothermic peak from 275-320ºC represents degradation. Thermogram obtained for Batch A is given in Figure 3c. DSC curves of all the eight batches are compared in Figure 3d and arranged starting from bottom. DSC thermogram analysis of all samples shows a common endothermic peak at around 75-100ºC which was due to desorption of water molecule from chitosan nanoparticles. This result is in agreement with reports by Othayoth R et al. and Sarmento B et al[26,27]. All batches present similar peaks at this temperature range and indicate that change in process factors did not influence this behaviour of samples.

High Performance Liquid Chromatography (HPLC)

HPLC analysis of sorafenib tosylate has presented retention time with a single peak at about 17 minutes. A typical chromatogram of sorafenib tosylate is shown in figure 4. A calibration curve was prepared as explained with an equation Y= 285525x – 130287, R2= 0.9994.

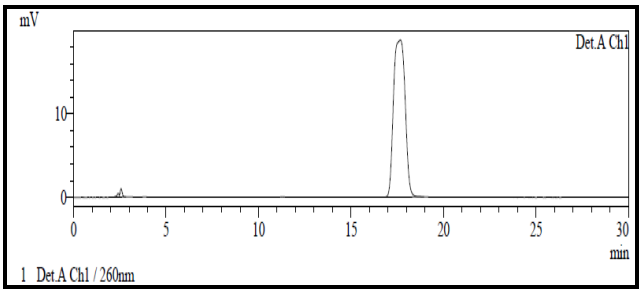

Figure: 4 Chromatogram of Sorafenib Tosylate

For determination of the drug content, 5mg of Sorafenib tosylate loaded chitosan nanoparticles were extracted with the solvent system and filtered aliquot of solution was analyzed with HPLC. Results obtained and calculations of response are tabulated in table 2.

Table 2: HPLC Analysis of All Batches of Nanoparticles and Results of Responses R1& R2

Batch	Area	Conc^{n} µg/ml	R1 EE (%)	R2 Yield (%)
A	3448086	12.53261	13.0156	170.32
B	6845431	24.43120	18.2954	182.72
C	4535836	16.34235	30.1653	177.20
D	3446601	12.52741	24.6836	170.24
E	4026402	14.55806	6.8007	174.40
F	10352190	36.71299	19.4651	180.27
G	3396246	12.35105	13.3460	155.60
H	2531807	9.323506	10.6568	152.40

Pertaining to response R1, the encapsulation efficiency (%), batch C was presented with the highest value of 30.1653 followed by batches D and F. Here batches C and D were prepared with high value for factor A and amount of drug added was 10mg. The lowest value for this response was given by batch H.

Table 3: Analysis of Variance [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	386.06	4	96.52	10.52	0.0412	Significant
A-Concentration of Chitosan	161.02	1	161.02	17.55	0.0248	
B-Amount of Drug	56.58	1	56.58	6.17	0.0890	
AB	83.21	1	83.21	9.07	0.0571	
BC	85.25	1	85.25	9.29	0.0555	
Residual	27.52	3	9.17			
Cor Total	413.58	7				

For the response R2, yield (%), best result was expressed by batch B, followed by F and C. Batch H indicated the lowest value of 152.4%.

Statistical Model Evaluation

A statistical evaluation of selected factors and their levels with the resultant responses were analyzed using Design Expert software (DX9) as follows. A 2^3 full factorial design was used for formulation of batches.

Response (R1), Encapsulation Efficiency (%)

ANOVA, Table 3, shows that model is significant as the p value is below 0.05. Also a high significance predicted for factor A.

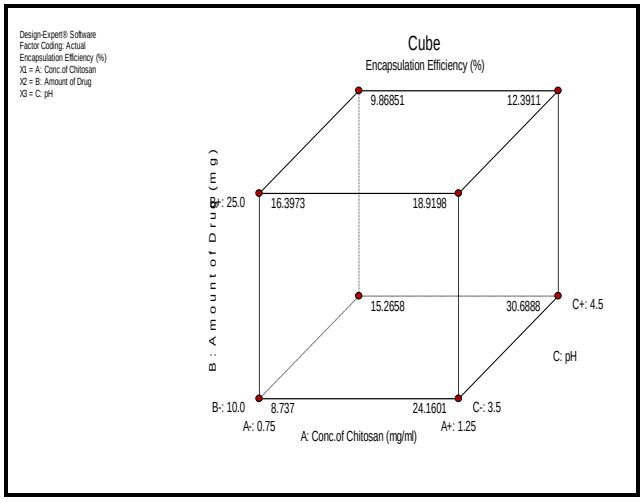

Figure 5a: Cube (R1)

The cube represented in Figure 5a shows that a maximum EE (%) of 30.6888 is observed at high level for factor A & C and low level for factor B.

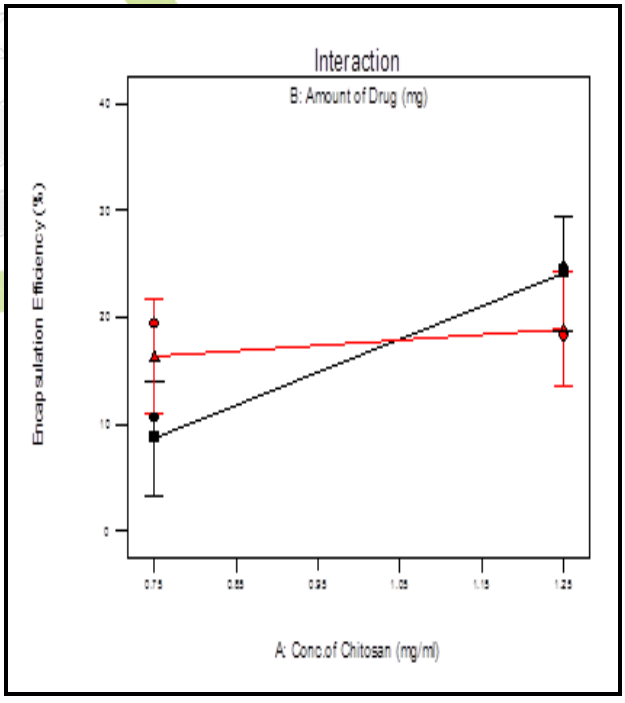

Figure 5b: Interaction (R1) at pH = 3.5, Δ-Amount drug 25 mg, □-amount of drug 10 mg.

At pH=3.5, shown in Figure 5b, a high value of factor B have small effect on EE (%) when concentration of chitosan increased from 0.75mg/ml to 1.25mg/ml.

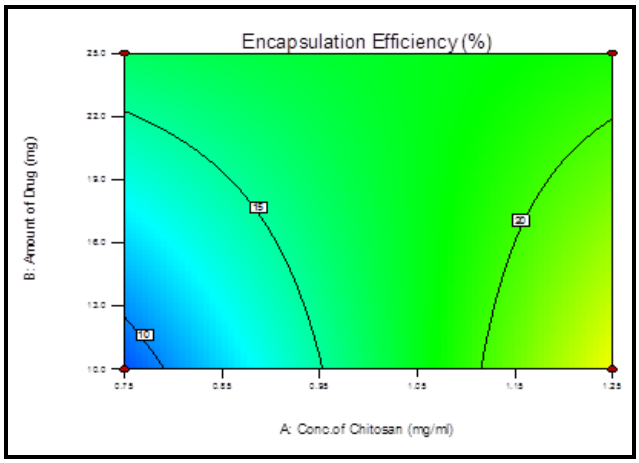

Figure 5c: Contour Plot (R1)

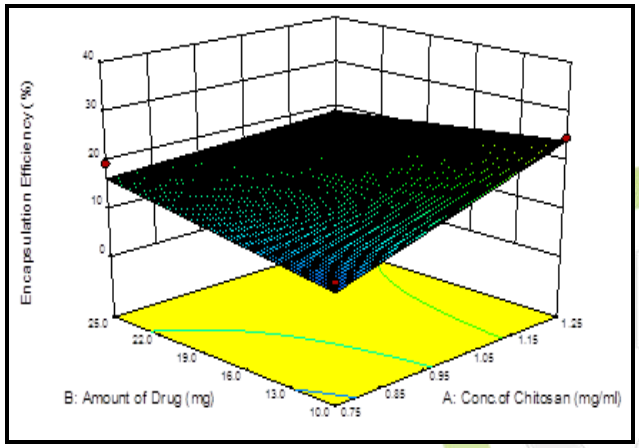

Figure: 5d Surface plot (R1)

At pH 3.5 contour and 3D surface plots, figure 5c and 5d respectively, shows higher EE (%) towards A+ and B- corner, predicting a high EE (%) with factor A at 1.25mg/ml and B at 10mg.

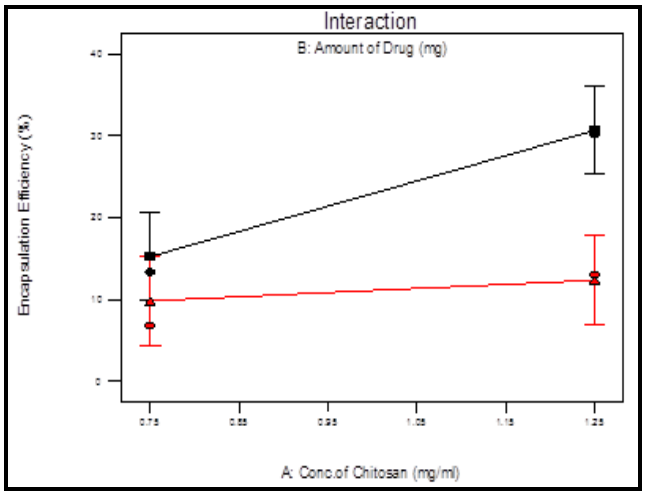

Figure: 5e Interaction (R1) at pH=4.5, Δ- Amount of Drug 25 mg, □- Amount of Drug 10 mg

Interaction at pH=4.5, figure 5e, shows that there is a higher change in encapsulation efficiency (%) when factor B is kept at 10mg and the factor A increased from 0.75 to 1.25mg/ml.

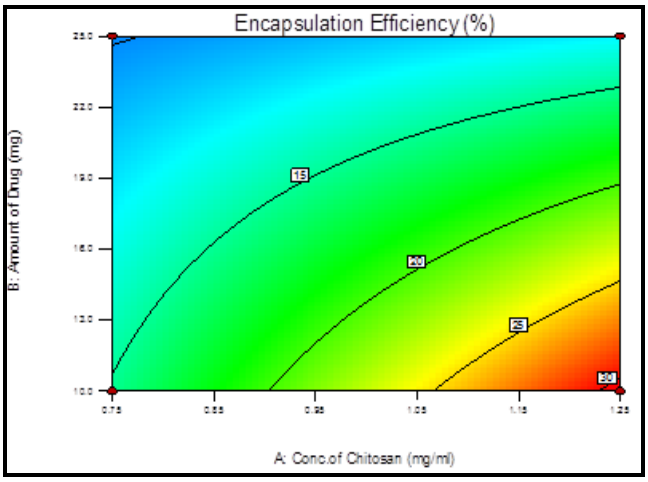

Figure 5f: Contour plot (R1)

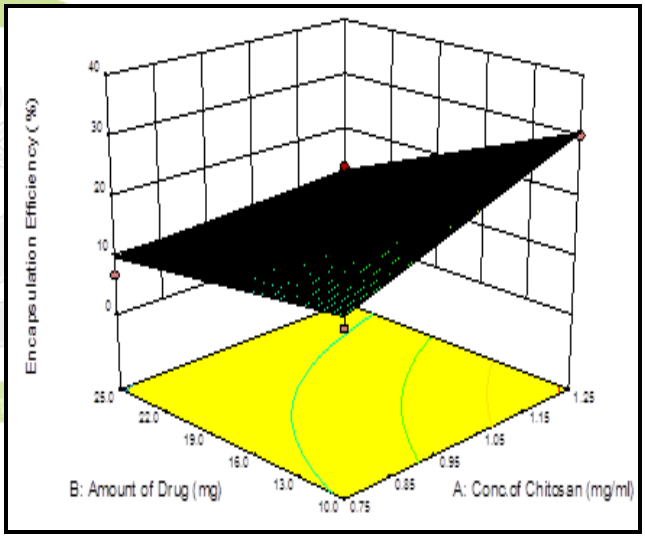

Figure 5g: Surface plot (R1)

Contour plot and the 3D surface plot obtained at pH 4.5, figure 5f and 5g respectively, also indicates that maximum value for encapsulation efficiency (%) can be obtained by maintaining level of factors A+(at 1.25mg/ml), and B- (at 10mg).

Response (R2), Yield (%)

ANOVA gives a model p value of 0.0105 and is significant (Table 4). All factors A, B, AB and BC are also significant.

Table 4: Analysis of variance [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	832.02	4	208.01	27.83	0.0105	Significant
A-Concentration of chitosan	178.73	1	178.73	23.92	0.0164	
B-Amount of Drug	341.48	1	341.48	45.69	0.0066	
AB	210.81	1	210.81	28.21	0.0130	
BC	101.01	1	101.01	13.52	0.0348	
Residual	22.42	3	7.47			
Cor Total	854.45	7				

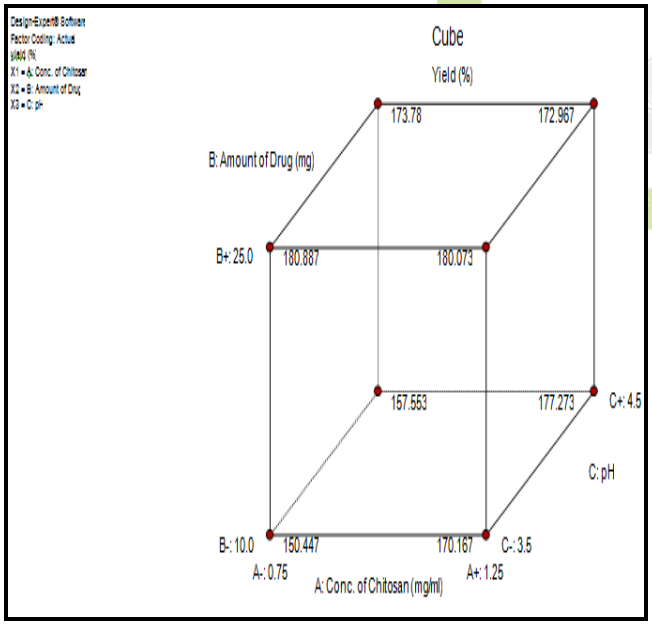

Figure: 6a Cube (R2)

Figure 6a shows that highest yield (%) was obtained at A-B+C-and at A+B+C-. But it is better to reduce the amount of drug while maintaining yield%. A reasonably high value for the yield (%) of 177.273 was attained at A+, B- & C+.

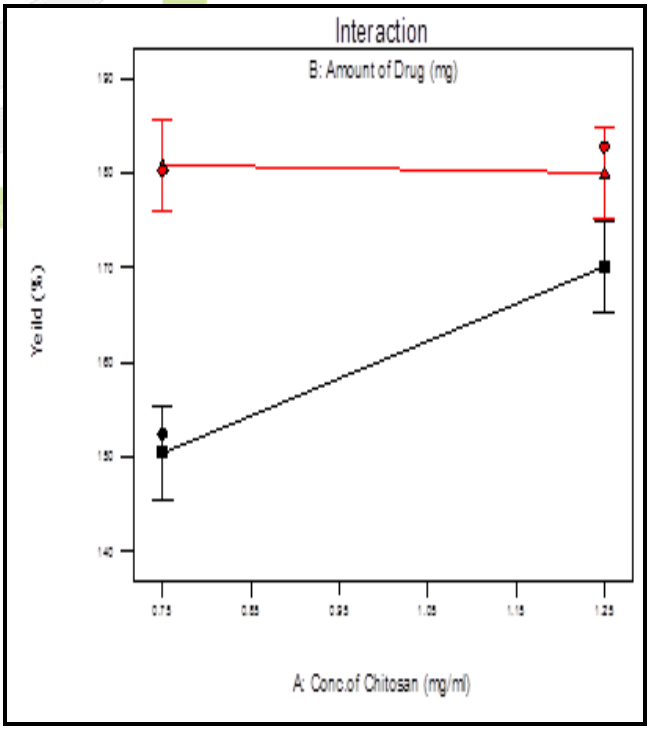

Figure: 6b Interaction (R2) at pH=3.5, Δ- Amount of Drug 25 mg, □- Amount of Drug 10 mg

It is evident from interaction at pH=3.5, figure 6b, spread of points at right side is less than at the left i.e., effect of amount of drug is less significant at higher concentration of chitosan. Factor B at high level has little effect on yield (%) as there is change in chitosan concentration. When factor B maintained at 10mg there is an increase in yield (%) as the concentration of chitosan is increased.

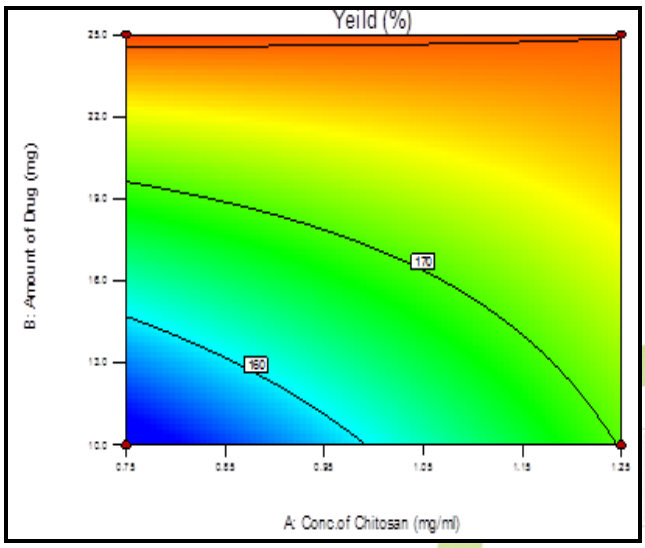

Figure: 6c Contour plot (R2)

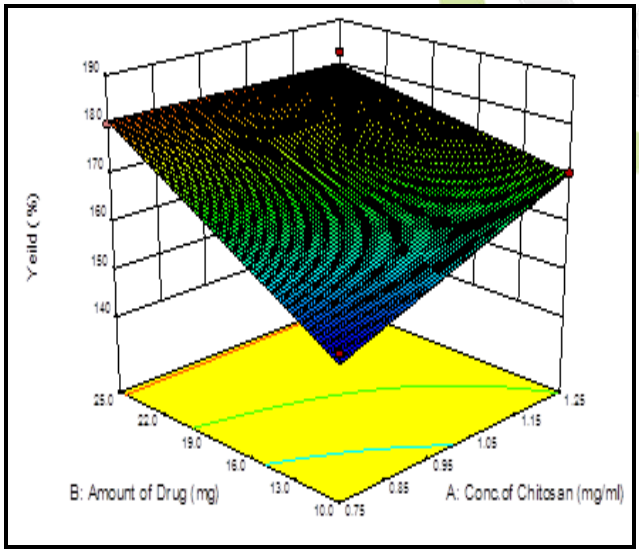

Figure: 6d Surface plot (R2)

At pH= 3.5 contour and 3D surface plots, figure 6c and 6d respectively, shows that yield (%) of above 180 can be obtained by keeping factor A at a range from 0.75 to1.25 mg/ml and factor B at 25mg.

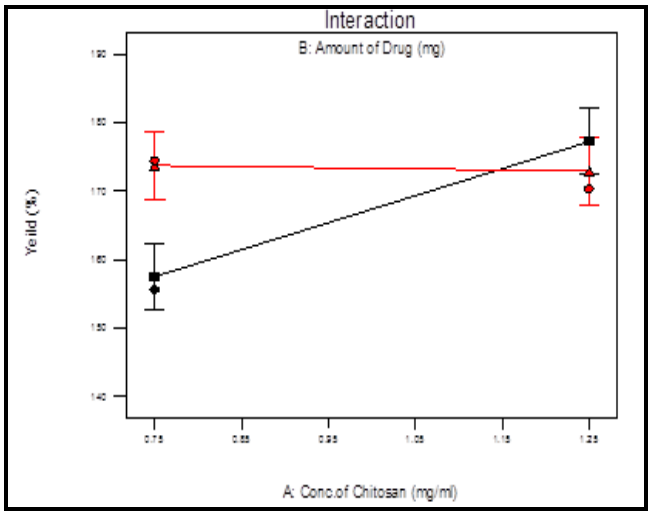

Figure: 6e Interaction(R2) at pH = 4.5, Δ- Amount of Drug 25 mg, □- Amount of Drug 10 mg

Figure 6e, Interaction at pH= 4.5, shows an increase in Yield (%) when factor B kept at 10mg and factor A, increased to 1.25mg/ml

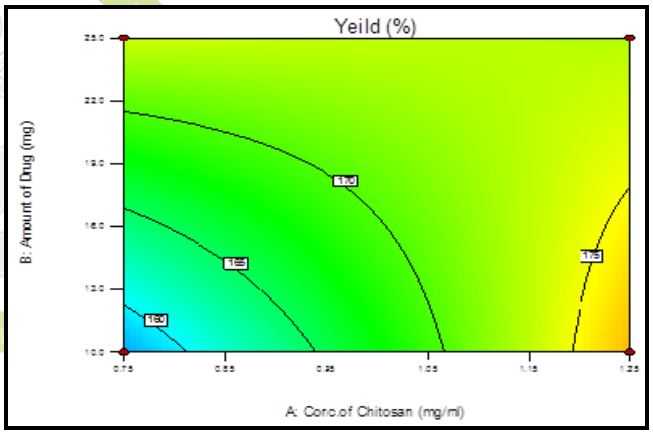

Figure 6f: Contour plot (R2)

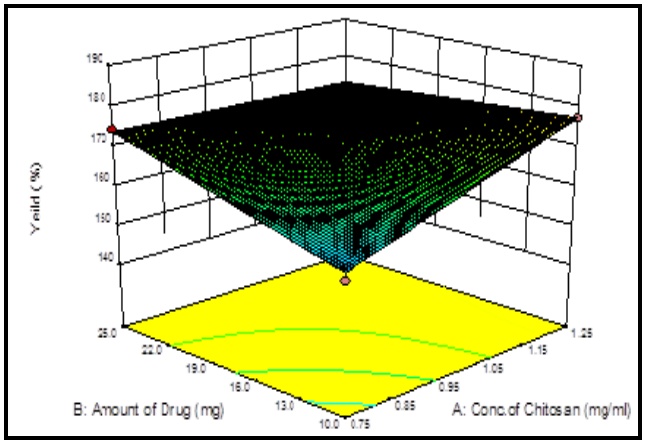

Figure 6g: Surface plot (R2)

Both contour plot and 3D surface plot, figure 6f and 6g, obtained at pH 4.5 points to a high yield (%) when factor A is at 1.25mg/ml and factor B at10mg.

Optimization

Table: 5 show the criteria of optimization. The effects of independent process variables on selected responses were analyzed with the objective to maximize encapsulation efficiency (%) as well as the yield (%).

Table: 5 Criteria for Optimization of Chitosan Nanoparticles Loaded with Sorafenib Tosylate.

		Criteria
Factor A	Concentration of Chitosan	In range
Factor B	Amount of drug	minimize
Factor C	pH	In range
Response 1	Encapsulation Efficiency (%)	maximize
Response 2	Yield (%)	maximize

It was observed that high encapsulation efficiency (%) obtained at pH 3.5 as well as at 4.5 when the concentration of chitosan kept at 1.25 mg/ml and the amount of drug at 10mg. Increase in amount of drug did not resulted in corresponding change in encapsulation efficiency (%).

Yield (%) was highest when the amount of drug maintained at 25 mg and pH at 3.5, for all range of chitosan concentrations. But the analysis also revealed that a comparable high value of yield (%) was achieved at pH 4.5, when amount of drug at kept 10 mg and concentration of chitosan at 1.25 mg/ml.

After evaluation of process variables and their effects on responses, to accomplish the criteria envisaged in optimization process, the optimum level of factors were selected as concentration of chitosan at 1.25 mg/ml, pH 4.5, and amount of drug at 10 mg.

CONCLUSION

Chitosan nanoparticles loaded with sorafenib tosylate were prepared successfully and the process parameters were optimized using 2^3 full factorial design. HPLC method was employed to determine the drug content. Encapsulation efficiency (%) and the yield (%) of all the eight batches were calculated and compared. Maximum encapsulation efficiency (%) of 30.1653 with a yield (%) of 177.2 was acquired for batch C nanoparticles loaded with sorafenib tosylate. Size of the nanoparticles was analyzed by TEM micrograph and characterization was done by DSC thermograms.

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