ORIGINAL ARTICLE

Preparation, Characterization and Optimization of Carvedilol Loaded Chitosan Nanoparticles by Applying Taguchi Orthogonal Array Design

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Abstract

Aim: The aim of the study was to develop and optimize carvedilol (CAR) loaded chitosan (CS) nanoparticles (NPs) to enhance its oral bioavailability and comparing it with marketed tablets. **Materials and Methods:** The NPs were prepared by ionic gelation technique and evaluated for particle size, zeta potential (ZP), entrapment efficiency, *in vitro* release kinetics, and stability profile. **Results and Discussion:** The results indicated stable CAR-CS NPs with the value of ZP to be +32 mV ± 2 mV, the small particle size of 79.23 nm and high entrapment efficiency of 72.56%. The optimization of the formulation by Taguchi orthogonal array design exhibited $C_3T_5R_{11}S_{20}$ as the optimized batch with CS concentration of 0.3% w/v, tripolyphosphate (TPP) concentration of 0.5% w/v, CS: TPP volume ratio of 1:1 v/v and a stirring speed of 2000 rpm. The *in vitro* release study revealed sustained release of drug for 72 h with 74.84% cumulative drug release. **Conclusion:** The promising results from the study revealed the applicability of Taguchi orthogonal array design in identifying the critical parameters in the formulation of CAR loaded CS NPs.

Key words: Carvedilol, chitosan, ionic gelation technique, nanoparticles, Taguchi orthogonal array

INTRODUCTION

In the last few years, the main impediment allied with the development of drug formulations of new drug entities is poor solubility and poor permeability of lead moiety.[1] Despite important success in the discovery of new drug molecules, there are a number of drugs whose clinical development failed due to poor solubility, inadequate bioavailability, and other poor biopharmaceutical properties. It is estimated that more than 40% of the new chemical entities are poorly water soluble in nature.[2,3] The major task in the development of these drugs is the improvement in solubility, thereby enhancing oral bioavailability, where the conventional approach presents problems such as over or under medication and poor patient compliance. The most frequently applied nanotechnology-based strategies in the development of delivery systems are polymeric nanoparticles (NPs), solid lipid NPs, liposomes, nanoemulsions, nanosuspension, dendrimers, micelles, and so forth, which provide controlled, sustained, and targeted drug delivery.[4] The NPs based delivery systems present a significant approach for enhancing saturation solubility, absorption rate, and oral bioavailability.[5]

Chitosan (CS) is extensively been studied as the polymer in the preparation of NPs. It is a naturally occurring cationic polysaccharide, derived by deacetylation of chitin, a copolymer consisting combined units of glucosamine and N-acetyl glucosamine. The basis of CS NPs is the electrostatic interaction between the amine group of CS and a negatively charged group of polyanion such as sodium tripolyphosphate (TPP). The nanoparticulate formulation containing CS is reported to be stable, permeable and therapeutically active.[6-8]

Carvedilol (CAR) is a third-generation antihypertensive agent, with non-selective β-blocking and selective α-adrenergic

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blocking and vasodilating activity.[9] It is highly lipophilic with an oral bioavailability of only 25-35% as it exhibits high first pass metabolism. The high lipophilicity and poor water solubility render it a drug of Class II in biopharmaceutical classification system.[10] Reports showed that several numbers of conventional dosage forms - such as solid dispersion, micronization, and inclusion complexation - have been employed to enhance the solubility of CAR.[11-14] These approaches have limited scope to the success of these dosage forms mainly depends on particular properties of drug molecule such as the ability to ionize, oil solubility, molecular size and structure to fit into the hydrophobic cavities.[15]

NPs are assumed as an effective strategy to enhance the oral bioavailability of lipophilic drugs such as valsartan, isradipine, telmisartan, and felodipinec.[16-22] Kumar *et al.* have shown enhancement in oral bioavailability of valsartan by formulating its NPs. The NPs were optimized and developed by full factorial design.[23] A study of *in vitro* and *in vivo* drug release of nifedipine NPs also showed improved patient compliance by decreasing frequency of administration. The NPs have also shown increased antihypertensive activity owing to its improved oral bioavailability and sustained action.[24] NPs enable ease of administration and penetration through the capillary and epithelial membrane, thereby permitting an effective delivery of lipophilic drugs. The objective of this study was to design nanoparticulate formulation of CS containing CAR to enhance its bioavailability by application of Taguchi design. The Taguchi design with L_9 type of robust orthogonal array was applied to optimize the experimental conditions. The CAR loaded CS NPs were prepared by ionic gelation method using TPP as a cross-linking agent. The effect of CS concentration, TPP concentration, CS to TPP volume ratio and stirring speed on the drug entrapment efficiency and particle size was evaluated.

MATERIALS AND METHODS

Materials

CS was obtained as gift sample from Central Institute of Fisheries Technology (Cochin, India). Sodium TPP was procured from Loba Chemie Pvt. Ltd. (Mumbai, India). CAR was a gift sample from Piramal Enterprises Ltd. (Pithampur, Indore, India). All other chemicals and reagents were of analytical grade.

Preparation of NPs

NPs were prepared by ionic gelation method according to the procedure originally proposed by Calvo *et al.*[25] Briefly, CS was dissolved in 1% (v/v) acetic acid solution to make up CS concentrations of 0.1%, 0.2%, 0.3% w/v. Tween 80 (0.5% [v/v]) was added to CS solution as a resuspending agent, to prevent particle aggregation, then CS solutions were maintained at pH 4.5-4.8 with 0.1 M sodium hydroxide. Further, TPP was dissolved in distilled water to maintain TPP solutions of 0.25%, 0.50%, 0.75% w/v. All solutions were filtered using 0.2 µ filter. Prepared CS solutions were added dropwise into the TPP solutions in different CS:TPP volume ratios of 1:1, 1:1.5, 1:4 (v/v) under magnetic stirring at room temperature. This lead to the formation of CS NPs spontaneously via TPP induced ionic gelation mechanism. Finally, NPs were collected by centrifugation at 20,000 rpm for 20 min at 20°C using cooling centrifuge (REMI CPR-24 Plus, Remi Elektrotechnick, India). The supernatant was subjected to the determination of free CAR by ultraviolet (UV) spectrophotometer (UV 1700, Shimadzu, Japan).

Taguchi orthogonal array design

The optimization of the NPs formulation was carried out by Taguchi orthogonal array design. Based on the number of factors and their levels as shown in Table 1, L_9 type orthogonal array design was employed for statistical optimization of formulations for investigating the effects of major factors on responses. Design expert® Software (Stat-Ease, Inc., Version 9.0.6.2 Trial 2016) was used for optimization wherein the concentration of CS as well as TPP, was varied so as to identify their optimum concentrations for the development of NPs formulation to obtain reduced particle sized, maximum entrapment efficiency and to induce crosslinking between TPP and CS. Similarly, the effect of CS: TPP volume ratio and the stirring speed were also studied on particle size and entrapment efficiency. The optimum conditions with optimal

Table 1: Variables, factors and their levels used in L_9 Taguchi orthogonal array design

Variables	Units	Levels		
		–1	0	+1
Independent variables				
X_1				
Concentration of CS	%	0.1	0.2	0.3
X_2				
Concentration of TPP	%	0.25	0.50	0.75
X_3				
CS: TPP volume ratio	ml	1:1	1:1.5	1:4
X_4				
Stirring speed	rpm	1000	1500	2000
Constraints				
Dependent variables				
Y_1				
Particle size	nm	Minimize		
Y_2				
Drug entrapment efficiency	%	Maximize		

CS: Chitosan, TPP: Tripolyphosphate

desirability were determined with the minimum possible effect of the noise factor.

According to the orthogonal L_9 array, nine formulations were prepared to optimize the formulation parameters as per the Taguchi's design [Table 2]. The statistical analysis of the results using analysis of variance was performed to determine the factors which had a paramount influence on particle size and entrapment efficiency.

Characterization of NPs

Transmission electron microscopy (TEM)

The morphology of NPs was observed under TEM (Morgagni 268D TEM instrument, AIIMS, New Delhi). The diluted and filtered sample was plunged on the 200 mesh carbon coated copper grids and was allowed to dry completely in the air. After drying, sample grid was loaded onto a specimen holder and viewed under a TEM.

Drug-excipient compatibility studies by differential scanning calorimeter (DSC)

The NPs and drug powder were subjected to previously calibrated DSC (DSC-60, Shimadzu Corporation, Japan). The sample was sealed hermetically in an aluminum pan and subjected to nitrogen gas at a flow rate of 50 ml/min. The thermograms were obtained at scanning temperature range of 50-250°C at a heating rate of 10°C/min. DSC thermograms were recorded for CS, CAR, and CAR-CS NPs.

Measurement of particle size, polydispersity index (PDI), and zeta potential (ZP) of NPs

Particle size, PDI, and ZP of NPs were determined through dynamic light scattering analysis with Malvern Zetasizer Nano S (Malvern, UK). About 100 µL of the prepared NPs dispersion was diluted to 5 ml with double distilled water and analyzed with zeta sizer. The analysis was performed in triplicate at a temperature of 25°C.

Determination of entrapment efficiency

The entrapment efficiency of the nanoparticulate formulation was determined in triplicate using UV spectrophotometer. The NPs were separated from the aqueous medium (containing unentrapped CAR) by centrifugation at 25,000 rpm for 30 min (REMI CPR-24 Plus, Remi Elektrotechnick, India). The supernatant was diluted with an appropriate amount of 0.1 N HCl and analyzed for the amount of unentrapped drug by UV-Visible spectrophotometer (Shimadzu 1700, Japan) at 285 nm.

The percentage drug encapsulated was determined by following the formula:

$$\text{Entrapment efficiency } (\%) = \frac{\text{Total drug } (\text{mg}) - \text{free drug } (\text{mg})}{\text{Totaldrug } (\text{mg})} \times 100 \quad (1)$$

In vitro drug release studies

The *in-vitro* drug release of NPs was studied using dialysis membrane (Himedia, India) with a pore size of 2.4 nm and molecular weight cut-off between 12,000 and 14,000 in phosphate buffer saline (PBS) pH 7.4 at 37 ± 2°C. Dialysis membrane was soaked overnight in double distilled water before the release studies. The drug-loaded NPs were placed into a dialysis bag and were suspended in a beaker containing PBS under magnetic stirring while maintaining perfect sink condition. Aliquot samples were withdrawn periodically and replaced with fresh dissolution medium in the same volume. The amount of drug released was analyzed spectrophotometrically at 285 nm for CAR. For comparative purpose, the *in vitro* drug release study was also performed for the marketed formulation using USP paddle type dissolution apparatus.

Accelerated stability studies

CAR loaded NPs were subjected to a stability testing for 3 months as per International Conference on Harmonisation

Table 2: Formulations and results of CAR-CS NPs by Taguchi orthogonal array design

Formulation code	CS concentration (%, w/v)	TPPconcentration (%, w/v)	CS:TPP volume ratio (ml)	Stirring speed (rpm)	Particle size (nm)	Entrapment efficiency (%)
$C_3T_5R_{11}S_{20}$	0.3	0.50	1:1	2000	79.23	72.56
$C_2T_7R_{11}S_{15}$	0.2	0.75	1:1	1500	125.95	65.25
$C_3T_2R_{14}S_{15}$	0.3	0.25	1:4	1500	130.23	70.15
$C_1T_5R_{115}S_{15}$	0.1	0.50	1:1.5	1500	122.23	57.28
$C_3T_7R_{115}S_{10}$	0.3	0.75	1:1.5	1000	260.67	71.92
$C_2T_5R_{14}S_{10}$	0.2	0.50	1:4	1000	255.83	63.56
$C_2T_2R_{115}S_{20}$	0.2	0.25	1:1.5	2000	76.85	64.16
$C_1T_7R_{14}S_{20}$	0.1	0.75	1:4	2000	70.56	56.15
$C_1T_2R_{11}S_{10}$	0.1	0.25	1:1	1000	255.05	58.64

CS: Chitosan, NP: Nanoparticles, CAR: Carvedilol, TPP: Tripolyphosphate

Q1A (R^2) guidelines. Freshly prepared NPs were transferred to 5 ml glass vials sealed with plastic caps and were kept in stability chamber (Remi SC-12 Plus, Remi Instruments Ltd., Mumbai, India) maintained at 25 ± 2°C/60 ± 5% RH for a period of total 3-month. The formulations were monitored for changes in particle size, ZP, and entrapment efficiency.

RESULTS AND DISCUSSION

Experimental design and statistical analysis

In this study, CAR-CS NPs were prepared by ionic gelation method using polymer (CS), a cross-linking agent (sodium TPP) and resuspending agent (tween 80). A Taguchi orthogonal array design was adopted to select the optimal conditions with the factors having the utmost influence on the response variables. The experimental results from analysis of variance are found to be significant and the values of *F* value, R^2, predicted R^2, and adjusted R^2 for each response is as shown in Table 3. The graphs clearly depict trend of each factor in relation to different levels which were found to be in good agreement with experimental results. The results of the statistical analysis reveal that changing the concentration of CS from 0.1% to 0.3% have a shown slight increase in the particle size [Figure 1a], which could be due to increasing in viscosity of CS solution, which thereby decreases the shear capacity of the stirrer and inversely affects the particle size. Furthermore, by changing the stirring speed at different levels, showed a decrease in the particle size significantly [Figure 1b] that could be attributed to formation smaller size emulsion droplets at high speed. Change in the concentration of CS has shown a remarkable increase in the entrapment efficiency. This enhancement is attributed to increasing in viscosity and ionic gel formation at high levels of CS that resists the diffusion of the drug into the external phase [Figure 2a]. Moreover, changing the CS to TPP volume ratio from 1:1 to 1:4 have revealed a decrease in the drug entrapment which suggests insufficient cross-linking occurs at the high level of CS [Figure 2b]. The higher value of CS:TPP mass ratio lowers the cross-linking density of polymer and electrostatic interaction between the drug and polymer.

Table 3: Values of R^2, adjusted R^2 and predicted R^2 for responses

Values	Particle size	Drug entrapment efficiency
R^2	0.9999	0.9986
Adjusted R^2	0.9997	0.9971
Predicted R^2	0.9992	0.9927

The selection of optimum formulation of CAR loaded NPs was based on the criteria of attaining the minimum value of particle size and the maximum value of entrapment efficiency, by applying L_9 Taguchi design using Design expert® Software (Stat-Ease, Inc., Version 9.0.6.2 Trial 2016). The formulation $C_3T_5R_{11}S_{20}$ have shown minimum particle size of 79.23 nm and maximum drug entrapment 72.56 [Table 4]. These obtained experimental values of particle size (79.23 nm) and entrapment efficiency (72.56%) of NPs were found in agreement with the predicted value of particle size (79.30 nm) and entrapment efficiency (72.62%), respectively. Thus, these results showed that the Taguchi design could successfully be applied to predict the best condition for preparing the CS NPs as well as suggesting that the optimized formulation was reliable and rational.

Particle size analysis by TEM

The structural morphology of NPs was examined by TEM. TEM image showed that the optimized formulation is nearly spherical in shape and a smooth surface distributed throughout the sample [Figure 3]. TEM images also revealed that NPs were without aggregation.

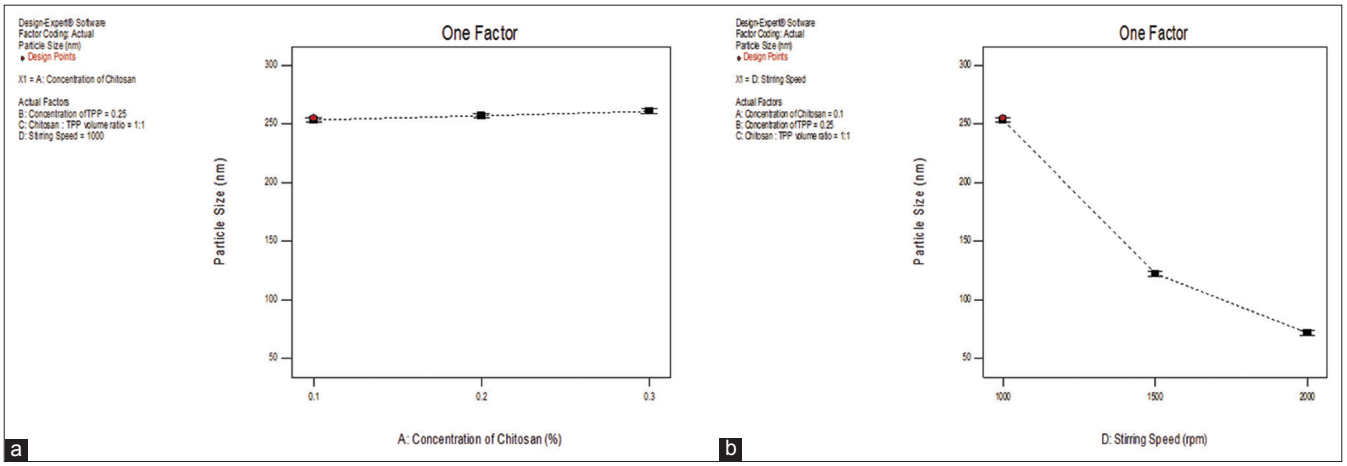

Figure 1: Effect of different variables on particle size (a) concentration of chitosan and (b) stirring speed

Table 4: Predicted versus actual responses obtained for the formulations

Formulation code	Particle size (nm)		Entrapment efficiency (%)	
	Actual value	Predicted value	Actual value	Predicted value
$C_3T_5R_{11}S_{20}$	79.23	79.30	72.56	72.62
$C_2T_7R_{11}S_{15}$	125.95	126.06	65.25	65.40
$C_3T_2R_{14}S_{15}$	130.23	129.89	70.15	70.42
$C_1T_5R_{115}S_{15}$	122.23	122.46	57.28	57.40
$C_3T_7R_{115}S_{10}$	260.67	260.94	71.92	71.59
$C_2T_5R_{14}S_{10}$	255.83	257.10	63.56	63.20
$C_2T_2R_{11}5S_{20}$	76.85	75.47	64.16	64.37
$C_1T_7R_{14}S_{20}$	70.56	71.87	56.15	56.24
$C_1T_2R_{11}S_{10}$	255.05	253.51	58.64	58.43

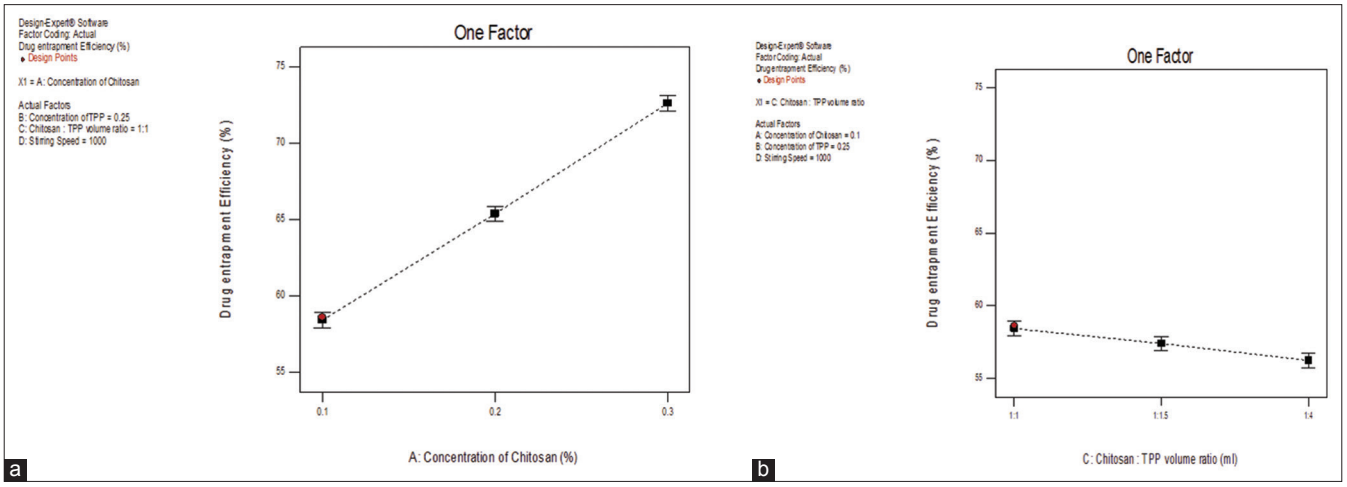

Figure 2: Effect of different variables on drug entrapment efficiency (a) Concentration of chitosan (CS), (b) CS: Tripolyphosphate volume ratio

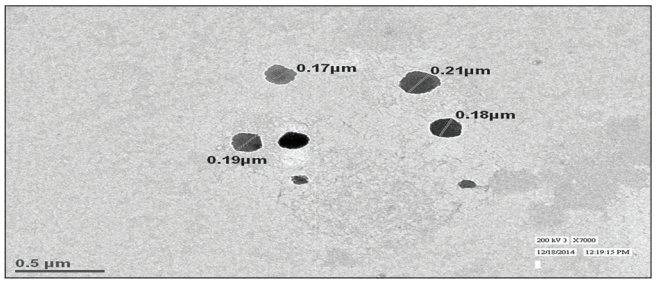

Figure 3: Transmission electron microscopic image of nanoparticles

Drug-excipient compatibility studies

DSC is an important technique in the investigation of thermal properties of a drug delivery system as it provides qualitative as well as quantitative data of the physicochemical state of the drug in the system. DSC thermograms of CS, CAR, and CAR-CS NPs are shown in Figure 4. The pure drug, CAR, showed a sharp endothermic peak at 119°C corresponding to its melting temperature. CS showed broad endothermic peaks at 102°C corresponding to its glass transition temperature. CAR-CS NPs showed both broad and sharp endothermic peaks at 102°C and 119°C which corresponds to CS and CAR, respectively, which predicts that the drug is homogenously dispersed in polymer matrix. These thermograms suggest that there was no interaction between drug and polymer.

Particle size, PDI and ZP of NPs

The average particle size of the optimized batch ($C_3T_5R_{11}S_{20}$) of NPs was found to be 79.23 nm. Particle size along with ZP (ζ) is the critical factor that affects the biological performance of CS NPs. The ZP of CAR-CS NPs were found to be + 32.5 mV ± 2 mV, which indicate the physical stability of the formulation [Figure 5]. The ZP also tends to affect particle stability and mucoadhesivity. The more the values of ZP, higher is the stability, as it prevents aggregation of particles. This high positive value of ZP was attributed to the positively charged CS because of protonation of amino groups on CS in the presence of acetic acid. The higher the positive ZP value, higher is the mucoadhesion with mucin which contains negatively charged sialic acid and sulfonic acid residues. Therefore, the ZP value was found to be in suitable agreement for rendering the NPs formulation

physically stable. Particle size was found to be varied in the range of 70.56 nm ($C_1T_7R_{14}S_{20}$) to 260.67 nm ($C_3T_7R_{115}S_{10}$) as shown in Table 2. The CAR-CS NPs exhibited relatively narrow particle size distribution as indicated by relatively low PDI value of 0.423 [Figure 6]. Low PDI values also indicate the relatively homogenous nature of the dispersion.

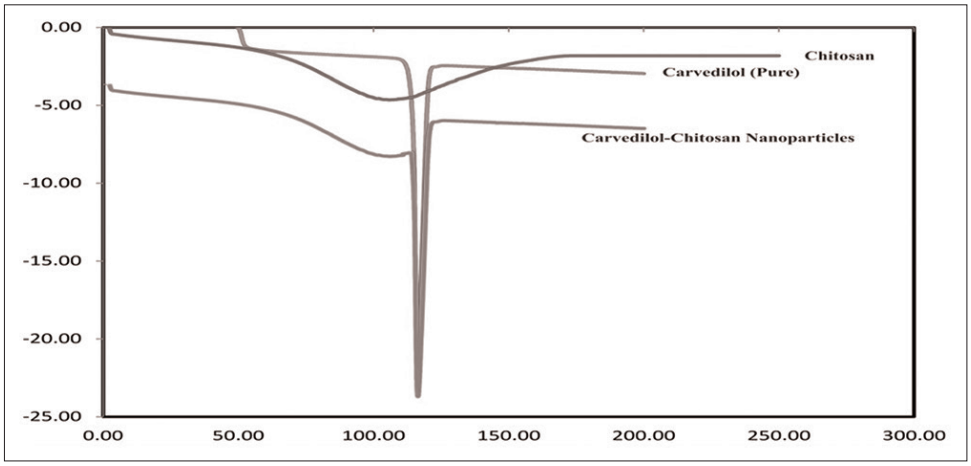

Figure 4: Differential scanning calorimeter thermogram of chitosan (CS), carvedilol (CAR) and CAR CS nanoparticles

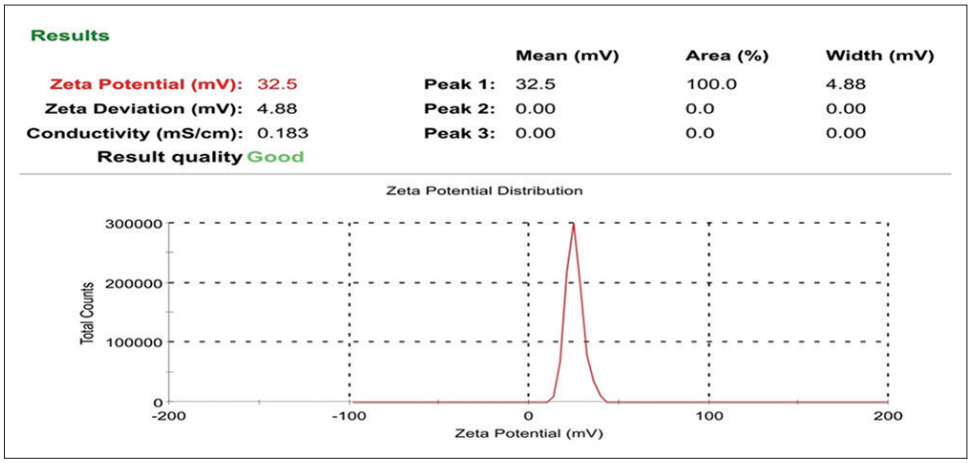

Figure 5: Zeta potential (ζ) of carvedilol loaded chitosan nanoparticles

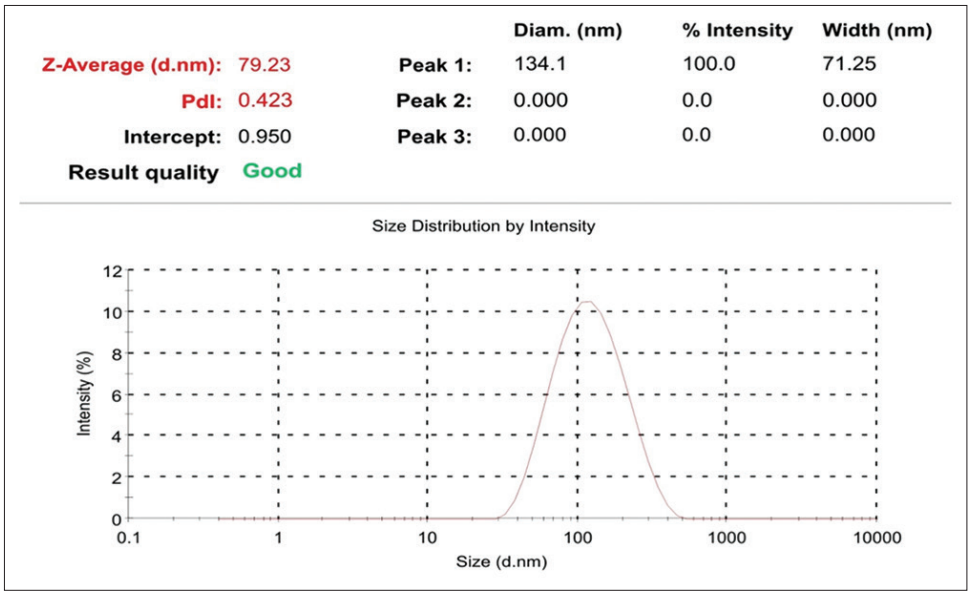

Figure 6: Average particle size and polydispersity index of nanoparticles

Entrapment efficiency

The entrapment efficiency acts as an important factor influencing the drug release, as well as the overall efficacy of the formulation. All the formulations were analyzed for entrapment efficiency using UV-visible spectrophotometer (Shimadzu 1700, Japan) at 285 nm, and the results are shown in Table 2. From the results obtained, all formulations showed good entrapment efficiency ranging from 56.15% ($C_1T_7R_{14}S_{20}$) to 72.56% ($C_3T_5R_{11}S_{20}$).

In vitro drug release

The *in vitro* drug release studies were carried out for CAR-CS NPs and marketed formulation in PBS 7.4 at 37 ± 2°C [Figure 7]. The drug release profile of CAR-CS NPs showed biphasic release pattern with an initial burst release in the first 2 h followed by a controlled release over a period of 72-h, and cumulative percentage of drug released was obtained to be 74.84%. The initial burst release was attributed to dissolution and diffusion of the poorly entrapped drug in the system, whereas slow and continuous release was due to diffusion of the drug from the polymer matrix. The marketed formulation showed 28.67% of the cumulative percentage of drug release that indicated a significant difference in the dissolution rate of the conventional formulation as compared to the CAR-CS NPs. The drug release data revealed remarkable improvement in the dissolution rate and better control over the release of drug from CAR-CS NPs. To know the drug release kinetics of NPs of CAR, the data were treated according to zero order kinetic model (cumulative % drug release vs. time), first order kinetic model (log of cumulative % drug remaining vs. time), Higuchi model (cumulative % drug release vs. square root of time), and Korsmeyer-Peppas model (log of cumulative % drug release vs. log of time). The results of release kinetics study are summarized in Table 5, and plots are shown in Figure 8. According to the highest correlation (R^2) value, it is evident that the optimized formulation of CS NPs follows the Higuchi model.

Accelerated stability studies

Stability studies were conducted in triplicate for optimized formulation ($C_3T_5R_{11}S_{20}$) which showed variations in particle size, ZP, and drug entrapment during the 3 months of storage [Table 6]. The particle size was increased slightly from 89.23 ± 7.7 to 99.16 ± 6.70 nm during stability studies, due to aggregation of small NPs together leading to increasing in

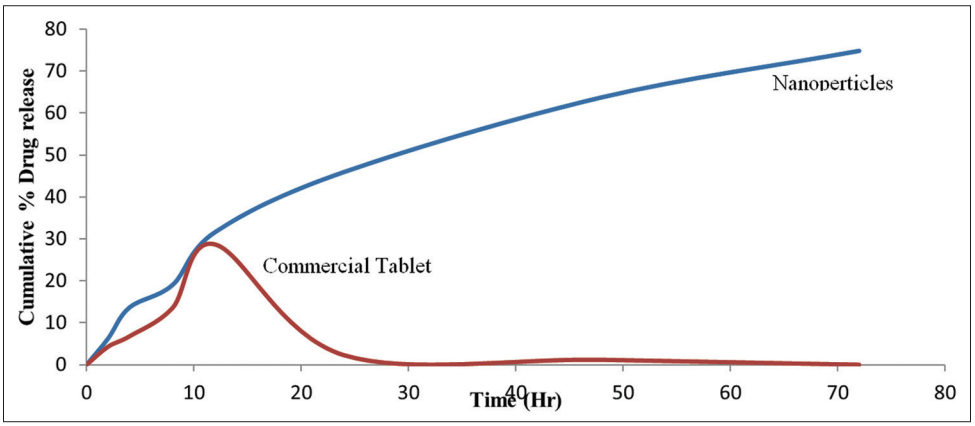

Figure 7: *In vitro* drug release profile of carvedilol loaded chitosan nanoparticles and marketed formulation

Table 5: Release kinetics data of optimized batch of NPs by applying various mathematical models

Formulation	R^2				
	Zero-order	First-order	Higuchi	Korsmeyer_Peppas	"*N*" value
$C_3T_5R_{11}S_{20}$	0.9138	0.9869	0.9906	0.9722	0.67

NP: Nanoparticles

Table 6: Stability data of CS NPs

Stability parameter	Test period			
	0 month	1 month	2 months	3 months
Particle size (nm)	89.23±7.7	93.47±5.58	97.25±7.56	99.16±6.70
ZP (mV)	+32.3±1.2	+31.0±1.2	+29.6±0.9	+28.8±1.6
Drug entrapment (%)	72.56±0.33	70.22±0.23	68.47±1.38	65.54±2.41

ZP: Zeta potential, CS: Chitosan, NP: Nanoparticles

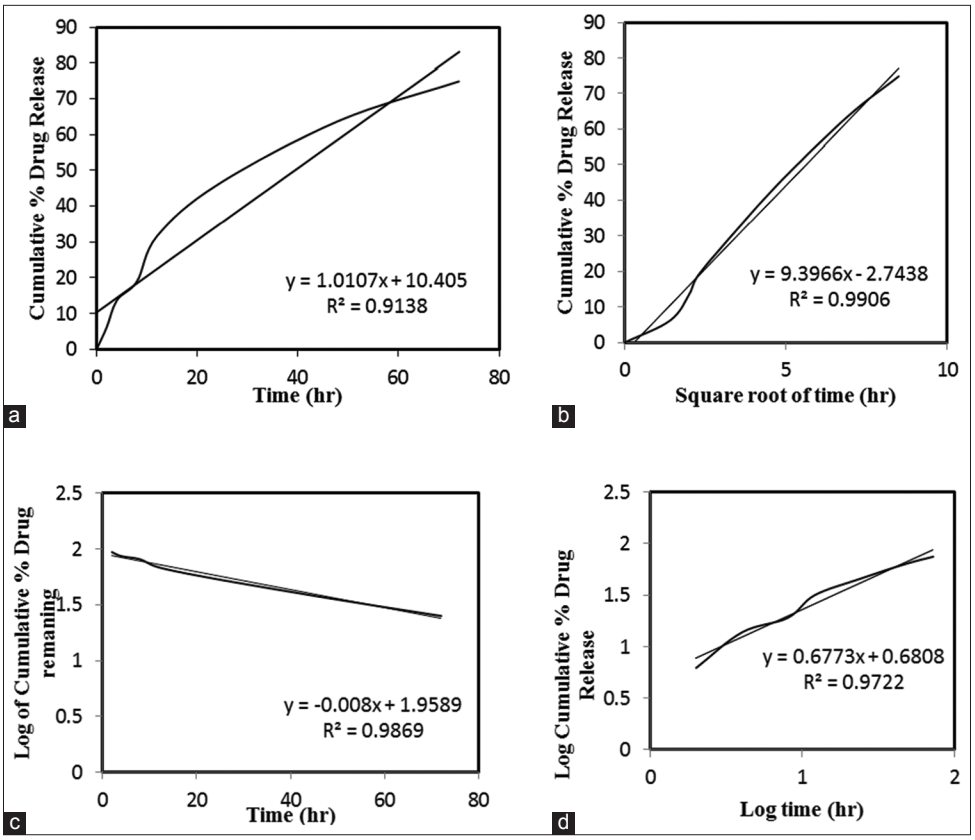

Figure 8: Drug release kinetics plots: (a) Zero order plot, (b) Higuchi plot, (c) first order plot and (d) Korsmeyer–Peppas plot

size of NPs. The ZP of the optimized batch after 3 months (+32.3 ± 1.2 to +28.8 ± 1.6 mV) indicated that the drug was retained within the NPs throughout the stability period. There was a very slight decrease of the drug entrapment (72.56 ± 0.33 to 65.54 ± 2.41) which may be due to the expulsion of drug from polymer matrix during storage. The obtained results indicated no significant change in the particle size, ZP, and drug entrapment during 3 months of storage that ensured the stability of NPs.

CONCLUSION

The major challenge in the formulation development is the poor aqueous solubility of the new chemical entity or existing drug molecules. The formulation of these molecules by the application of conventional approaches is difficult and associated with several pharmacological or therapeutical performance issues. The NPs provide a promising approach for enhancing solubility and oral bioavailability of water insoluble drugs. In this study, stable NPs were formulated using ionic gelation technique and optimized formulation ($C_3T_5R_{11}S_{20}$) has shown optimal parameters with minimal particle size and maximum entrapment efficiency. The *in vitro* drug release study revealed that cumulative percentage of drug released was obtained to be 74.84% for CAR loaded NPs whereas 28.67% from the conventional formulation. This increased dissolution of CAR from NPs is attributed to its increased solubility, which therefore may result in enhanced oral bioavailability of CAR. In conclusion, formulation of CS NPs could be an effective strategy for enhancing oral bioavailability of CAR and other lipophilic drugs on further *in vivo* pharmacokinetics and pharmacodynamics studies.

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