ORIGINAL ARTICLE

Enhanced antibacterial effect by antibiotic-loaded nanoparticles: A new strategy for battling bacterial resistance against *Neisseria gonorrhoeae*

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Abstract

Introduction: The recent reoccurrence of gonorrhea in the developed countries has been followed by rise and spread of gonococcal resistance to fluoroquinolones. The current research is aimed to prolong the contact time of antibiotics to the surface of the micro-organisms through the formulation of antibiotic-loaded nanoparticles. **Methods:** Ionotropic gelation method was used for the preparation of ciprofloxacin-loaded chitosan nanoparticles. The effect of various factors, including concentration of chitosan, sodium tripolyphosphate (TPP) on the different parameters such as entrapment efficiency, loading efficiency, and drug release was studied. Surface morphology, particle size, and antimicrobial studies, that is, zone of inhibition and minimum inhibitory concentration were also assessed. **Results:** The particles prepared under optimal condition of 1.77% Chitosan and 0.2% Sodium TPP had 96.23nm with polydispersivity index 1. In these particles with 1:1.5 Ciprofloxacin hydrochloride to chitosan, the entrapment efficiency, and loading efficiency were found to be 74.59% and 78.95%, respectively, with *in vitro* drug release of 10.23% in 24 h. **Conclusion:** The developed ciprofloxacin hydrochloride-loaded chitosan nanoparticles were found to exhibit effective antimicrobial activity against *Neisseria gonorrhoeae*, which showed high resistance toward conventional dosage forms.

Key words: Ciprofloxacin hydrochloride, Chitosan nanoparticle, Ionotropic gelation method, Microbial resistance, *Neisseria gonorrhoeae*

INTRODUCTION

Bacterial infections are one of the major source of chronic infections along with mortality. In view of fact of cost effectiveness and powerful outcomes, antibiotics have been preferred for the treatment of bacterial infections.[1] Wide spread use and prescribing habits of antibiotics led to the exposure of multidrug resistant bacterial strains.[1,2] More than 70% of bacteria causing infections are resistant at least to one of the drugs commonly used for the treatment.[3]

Gonorrhea, the urethritis is the second most common sexually transmitted infection caused by Gram-negative diplococcus bacteria *Neisseria gonorrhoeae*[4-7] and has more affinity toward the lower urogenital tract mucosa contrast to the risk of progressing into an ascending infection of the upper genital tract leading to complications such as ectopic pregnancy, pelvic inflammatory disease, infertility in women, and epididymitis in men.[7,8]

The World Health Organization (WHO) has estimated that 78 million cases worldwide occurred among adults. Among them, 35.2 million cases occurred in the WHO Western Pacific Region, 11.4 million in the American

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region, 4.5 million in the Eastern Mediterranean Region, and 11.4 million in the African Region.[4,5,8] In the 1980s, gonorrhea became resistant toward penicillin and tetracycline. During Gonococcal Isolate Surveillance Project, fluoroquinolones were used to treat 39% of the patients affected from gonococcal infection.[9] *Neisseria gonorrhoeae* resistance to Ciprofloxacin was first isolated in 1998 and showed remarkable increase in the ciprofloxacin prevalence in the past 3 year's, that is, 66.7% in 2001, 100% in 2002, and 95.2% in 2003.[10] In 2009, European survey of gonococcal resistance among 1366 strains, collected from 17 countries, showed 63% of all *N. gonorrhoeae* strains resistance toward Ciprofloxacin.[11]

The resistance mechanisms of antibiotic in gonococci involve the decrease approach of the antibiotics to the target site as well as the alteration of the target site itself. Diminished permeability of the cell envelope caused by changes in porin proteins limits the entry of antibiotics to the target site. One of the major mechanisms adapted by *N. gonorrhoeae* to resist antimicrobial agents is the interpretation of multidrug efflux pumps responsible for active exports of antibiotics from the cell which, consequently, eliminates the antibiotics before the interaction with the target. Therefore, modification or removal of the target site of the antibiotic results in decrease affinity of the antibiotics.[12]

Nanotechnology system concerned with the understanding and control of manners within the range of 1–100 nm[13] where by characterized by unique physicochemical properties including ultra-small size, high surface/volume ratio, and unique ability to interact with biological systems such as host cells and micro-organism further more modified structurally.[14] Above-mentioned features control the problem associated with the conventional antimicrobial therapies, there by enhancing solubility and stability of the drug, improving cellular internalization, targeted drug delivery, controlled, and sustained release as well as prolonged systemic circulation as compared to free drugs, along with the reduction of the side effect.[15] Most nanoparticles overcome at least one of the most common resistance mechanisms including the restriction of formation of biofilm and the retardation of the bacterial membranes.[16]

Conventional dosage forms are resistance toward *Neisseria gonorrhoeae*. In this study, porin protein porB present in *Neisseria gonorrhoeae* is selected as a target. PorB one of the main outer membrane proteins responsible for antibiotic resistance is a voltage gated pore facilitates exchange of ions and also allows the access of small nutrients required for bacterial viability.[17] Hence, this study aimed to prolong the contact time of antibiotics to the surface of the micro-organisms through the formulation of antibiotic-loaded nanoparticles, leading to the leakage of the bacterial cells resulting cell death.

MATERIALS AND METHODS

Materials

Ciprofloxacin hydrochloride was obtained as a gift sample from Karnataka antibiotics pharmaceuticals limited (Bangalore). Low molecular weight Chitosan and Sodium Tripolyphosphate (TPP) were procured from Yarrow chemicals Private Limited (Mumbai, India). Glacial acetic acid was purchased from Bharat Scientific World (Bangalore).

Methods

Fourier-transform infrared (FT-IR) spectroscopy

During preparation, the drug and the polymer may interact as they will be in close contact with each other which would affect the stability of the drug. The pure drug, polymer, drug-polymers combinations, and the formulation were exposed to FT-IR studies. The drug and/or polymer in 1:1 ratio were taken. In the sample holder of the instrument, the mixtures were placed and the spectra were run from 4000 cm^{-1} to 1000 cm^{-1} wave number. FT-IR spectrum obtained for the pure drug was compared to the FT-IR spectrum of drug with polymer.[18]

Drug-Polymer Compatibility Study by Differential Scanning Calorimetry (DSC) Thermal Analysis

DSC experiments were carried out in an order to characterize the physical state of the drugs. The drug sample (30 mg) and 1:1 drug: polymer ratio in physical state were placed in aluminum pans and thematically sealed. The heating rate was 10°C/min using nitrogen as the purge gas. Using Indium, the instrument was calibrated for temperature. In addition, for enthalpy calibration, Indium was sealed in aluminum pans with sealed empty pan as a reference.

Preparation of Ciprofloxacin Hydrochloride Nanoparticles

Nanoparticles of ciprofloxacin hydrochloride were prepared using ionotropic gelation method. Different concentrations of chitosan solution were prepared by dissolving chitosan in concentration of 0.1% acetic acid solution [Table 1]. To the above solution, constant concentration of the drug was added and was homogenized at 6000 rpm for 10 min [Table 2].

Table 1: Factors and levels used for optimization of ciprofloxacin hydrochloride loaded chitosan nanoparticles

Variable factors	Level		Optimized level (CPL10)
	−1	1	
Chitosan (A)	0.6	3	1.77
TPP (B)	0.1	0.3	0.2

Table 2: Composition of ciprofloxacin hydrochloride loaded Chitosan nanoparticle formulations

F. code	Compositions (drug: polymer)	Ratios	Concentration of TPP (%)	RPM
CPL1	Ciprofloxacin HCl: Chitosan	1:0.6	0.1	6000
CPL2	Ciprofloxacin HCl: Chitosan	1:0.8		
CPL3	Ciprofloxacin HCl: Chitosan	1:1		
CPL4	Ciprofloxacin HCl: Chitosan	1:1.2	0.2	8000
CPL5	Ciprofloxacin HCl: Chitosan	1:1.4		
CPL6	Ciprofloxacin HCl: Chitosan	1:1.6		
CPL7	Ciprofloxacin HCl: Chitosan	1:1.8	0.3	10,000
CPL8	Ciprofloxacin HCl: Chitosan	1:2		
CPL9	Ciprofloxacin HCl: Chitosan	1:3		

Sodium TPP solution prepared in distilled water was added to the above mixture dropwise with the help of syringe. Furthermore, the resulting suspension was subsequently centrifuged at 6000; 8000; and 10000 rpm for 10 min. The formed pellet was then dried using vacuum oven.[19]

Evaluation of Ciprofloxacin Hydrochloride-Loaded Chitosan Nanoparticles

Drug entrapment efficiency

The entrapment efficiency of the prepared nanoparticles was determined first by separating nanoparticles from the aqueous medium. The aqueous medium was then ultra-centrifuged at different rpm, that is, 6000 rpm for 10 min (CPL1, CPL2, and CPL3), 8000 rpm for 10 min (CPL4, CPL5, CPL6, and CPL10), and 10000 rpm for 10 min (CPL7, CPL8, and CPL9).The amount of free Ciprofloxacin Hydrochloride present in the supernatant solution was measured by UV-Vis spectrophotometer at 271 nm.[20] The entrapment efficiency of nanoparticles was calculated using following equation:

$$EE\% = \frac{\text{Total amount of drug loaded} - \text{free drug in supernatant}}{\text{Total amount of drug loaded}} \times 100$$

Determination of Drug Content and Drug loading Capacity (Loading Efficiency)

The drug content was determined by dissolving 50 mg of nanoparticles in 10 mL of purified water. The suspension was, then, sonicated gently for 10 min. The sample was then centrifuged at 3000 rpm for 3 h. The concentration of the ciprofloxacin hydrochloride was determined in the supernatant at 271 nm.[21] Drug content and loading efficiency were calculated using following equation:

$$\text{Drug content} = \frac{\text{Drug weight in nanoparticles}}{\text{Total weight of nanoparticles}} \times 100$$

$$\text{Loading efficiency} = \frac{\text{Drug remained in the nanoparticles}}{\text{Feeding weight of drug}} \times 100$$

In Vitro Drug Release

Release study was carried out using dialysis method. The *in vitro* drug release study was performed using USP type II dissolution test apparatus. Dialysis membrane was soaked in pH 7.4 phosphate buffered saline (PBS) before the test. Accurately weighed (100 mg) nanoparticle was placed in a dialysis membrane, tied at both the ends, and then immersed in 900 mL dissolution medium for 24 h at 50 rpm. The temperature of the whole setup was maintained at 37°C ± 1°C. 7.4 pH PBS was used as dissolution medium. Two mL of sample was withdrawn at regular time interval (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, 18, and 24 h) to determine the concentration of the drug and equal volume of fresh pre-warmed PBS pH = 7.4 solution was replaced to maintain the sink condition. Samples were then analyzed spectrophotometrically at 271 nm.[19,20]

Release Kinetics Study

The kinetic models were used for the interpretation of the drug release data. To analyze the mechanism of the drug release rate kinetics of the dosage form, the obtained data were fitted into different mathematical models such as zero order release kinetics, first order release kinetics, Higuchi diffusion equation, and Korsmeyer–Peppa's exponential equation. The equation with the high regression coefficient (r^2) will be the best fit of release data. Zero order release equation describes that the dissolution rate is independent of the concentration of the dissolved species. First order equation describes the system, where the release rate depends on the concentration of the dissolving species. The Higuchi equation describes the release from the system, where the solid drug is dispersed in an insoluble matrix and the release rate of drug is related to the rate of drug diffusion. For Korsmeyer–Peppa's equation, $n = 0.5$ indicates pure fickian diffusion, $n = 0.5–1$ indicates anomalous non-fickian diffusion and $n = 1$ indicates zero order release.[22,23]

Experimental Design and Optimization

According to the results obtained from the previous studies, the independent variables such as chitosan concentration and the TPP concentration would significantly affect the loading efficiency, entrapment efficiency, and drug release profile of the nanoparticles. The Central Composite design using Design-Expert software (version 11.1.2.0, Stat-Ease) was conducted to figure out the effect of these variables on the properties of nanoparticles in a fewer experimental runs. The effect of two variables including chitosan concentration from 0.6% to 3% and the TPP concentration from 0.1–0.3% on nanoparticle properties such as loading efficiency (%), entrapment efficiency (%), and ciprofloxacin hydrochloride release in 24 h was investigated in 9 runs. Quantitative variables were studied at two experimental levels −1 and +1. ANOVA was used for evaluation of the significance of independent variables on responses.[24]

Characterization of Ciprofloxacin Hydrochloride-Loaded Chitosan Nanoparticle

X-ray diffraction (XRD)

XRD diffraction was performed to confirm the crystalline and amorphous nature of the drug in the nanoparticles. XRD pattern of drug and Chitosan nanoparticles containing ciprofloxacin hydrochloride was determined by X-ray diffractometer and data collection was performed using Cu anode and the voltage was 45 Kv and current 30 mA. The scanned angle was set at 2θ.

Scanning Electron Microscopy (SEM)

The shape and surface characteristics of the nanoparticles were observed by SEM. The nanoparticles was thinly sprinkled onto a metal stub and vacuum coated with a thin layer of gold in an argon atmosphere. The SEM analysis of the nanoparticles was carried out by QUNTA-200 FEI using analytical scanning electron microscope. The nanoparticles were viewed at an accelerating voltage of 20KV.

Particle Size Analysis

Laser scattering method was used to measure the particle size distributions using a Malvern Mastersizer-S. To obtain, an obstruction between 10% and 20% approximately 50 mL of suspension was diluted with 500 mL of pure deionized water. Distributions based on volume fraction were calculated with refractive index of 1.610 for ITZ and were reported by listing D10, D50, and D90 which correspond to the diameters at which the cumulative sample volume was 10%, 50%, and 90%, respectively. The span was used to describe the polydispersivity index which was defined by,

$$\text{Span} = \frac{\text{D90} - \text{D10}}{\text{D50}}$$

In Vitro Antimicrobial Efficiency

Neisseria gonorrhoeae suspension was prepared and grown on Peptone Broth and cultures were incubated for 24–48 h at 37°C. The cell suspensions of all the cultures were adjusted to 1-2×10^5 cells/mL.

Well Diffusion Technique

The antibiotic-resistant profile of the sample was determined by well diffusion technique to determine the zone of inhibition. *Neisseria gonorrhoeae* (100 µL) was inoculated on Nutrient agar plates (90 mm). Ciprofloxacin hydrochloride-loaded chitosan nanoparticle (25 µL), standard Ciprofloxacin Hydrochloride (25 µL) and Control (25 µL) were added to the 5mm well on agar plate containing *Neisseria gonorrhoeae*. The plate treated with *Neisseria gonorrhoeae* was incubated at 37°C for 24–48 h. The plates were then observed for zone of inhibition around the wells.

Broth Dilution Technique

Mix 90µL test compounds of different test concentration with 10µL inoculum in 96 well plate in triplicate. Mix 90µL Peptone broth without drug with 10µL inoculum. Treated cultures were incubated at 37°C. The test plates were observed after 24–48 h and optical density (OD) at 590 nm was measured in plate reader. MIC was determined as Minimum concentration of drug giving 50% inhibition of OD as compared with control.

Accelerated Stability Studies

The effect of the temperature and humidity on the optimized formulation of the Ciprofloxacin Hydrochloride nanoparticles was evaluated for 3 months under different condition of storage. The prepared nanoparticles were filled into an amber colored glass bottle which was then flushed with nitrogen gas before air-tight closure with a plastic cap. Nanoparticles were then stored at a freezing temperature (5°C ± 3°C), room temperature (25°C ± 2°C/60% ± 5% RH), and 40°C ± 2°C/75% ± 5% RH as per ICH guidelines. The nanoparticles were evaluated for entrapment efficiency and drug release. The percentage of drug release was assessed by ultraviolet analysis at 271 nm after proper dilution.[25]

RESULTS

Fourier-Transform Infrared (FT-IR) Spectroscopy

The FT-IR peaks of the pure Ciprofloxacin Hydrochloride, Chitosan, TPP, and physical mixture are presented in Figure 1. In the FT-IR spectra of Ciprofloxacin Hydrochloride, a peak at 1045.81 cm^{-1} was assigned to C-F group and the another characteristics peak at 1342.48 cm^{-1} and

1309.56 cm^{-1} were related to the C-N stretching. The peak at 1270.22 cm^{-1} attributed to the vibration of the O-H group related to the presence of carboxylic acid. A characteristic band at 1622.98 cm^{-1} indicated N-H bending. The peaks at 1705.48 cm^{-1} indicated carbonyl (C=O) stretching. A band at 3099.94 cm^{-1} signified alkenes and aromatic C-H stretching. A characteristic peak at 3370.33 cm^{-1} was related to stretch and a band at 3527.55 cm^{-1} indicated O-H stretching vibration.

Drug-Polymer Compatibility Study by DSC Thermal Analysis

The DSC thermogram of pure Ciprofloxacin Hydrochloride showed a sharp endothermic peak at 141.08°C. In the Chitosan thermogram, a sharp endothermic peak at 56.36°C and an exothermic peak at 288.62°C were in accordance with the literature.[26] Characteristics peak of chitosan and Ciprofloxacin Hydrochloride was joint together and seen in the DSC curves of drug-loaded nanoparticles [Figure 2].

Evaluation of Ciprofloxacin Hydrochloride-Loaded Chitosan Nanoparticle

Drug entrapment efficiency

In this section, different drug: polymer ratio (W: W) ranged from 1:0.6 to 1:3 was considered. The average percent of drug entrapment efficiency of various nanoparticles formulation is shown in Figure 3. The drug entrapment efficiency of optimized formula CPL10 was found to be 74.59%. This formulation with drug: polymer ratio 1:1.77 and TPP 0.2% was selected for further analysis.

Determination of Drug Content and Drug Loading Capacity (Loading Efficiency)

The drug content of formulation CPL1-CPL9 was found increasing from 11.48% to 55.64%, as illustrated in Table 3. The loading efficiency of the Ciprofloxacin Hydrochloride increased from 48.17% to 90.97% by increasing the TPP concentration from 0.1% to 0.3% and the concentration of Chitosan from 0.6% to 3%. The drug content and the loading efficiency of optimized formula CPL10 {Ciprofloxacin Hydrochloride: Chitosan (1:1.77) and TPP (0.2%)} were found to be 33.03% and 78.95%, respectively.

In Vitro Drug Release Studies

The release profile of ciprofloxacin hydrochloride from nanoparticles formulation in PBS is shown in Figure 4. Release profile of formulation showed an initial burst after 2 h. Released drug from nanoparticles was little and <16% drug was released through 24 h. The drug release of optimized formula CPL 10 was found to be 10.23% in 24h.

Release Kinetics Study

The drug release data were fitted into different models such as zero order, first order, Higuchi equation, and Korsmeyer–Peppas [Table 4]. Based on the highest regression coefficient

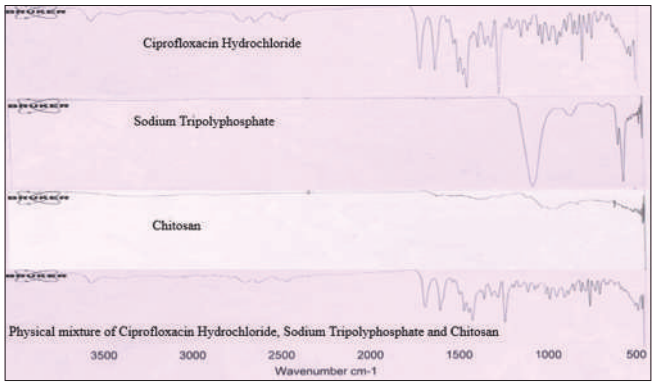

Figure 1: FT-IR of ciprofloxacin hydrochloride, sodium tripolyphosphate, physical mixture of ciprofloxacin hydrochloride, sodium tripolyphosphate, and Chitosan

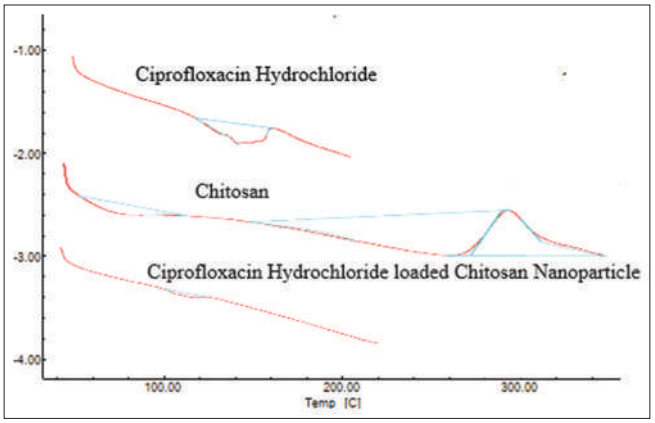

Figure 2: DSC of ciprofloxacin hydrochloride, chitosan, and ciprofloxacin hydrochloride-loaded chitosan nanoparticles

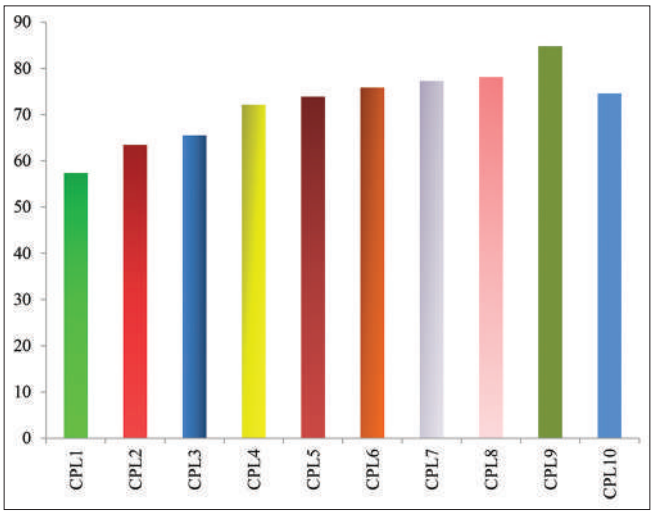

Figure 3: Average percent drug entrapment efficiency of different nanoparticle formulations. Data are represented as mean ± standard deviations (n = 3)

value (r^2), the best fit release data for optimized formulation were found to be the first order (0.9795). The results also demonstrated very close and above 0.9 r^2 value of Higuchi and Korsmeyer–Peppas model and the n values lied between 0.5 and 1.

Experimental Design and Optimization

The results of ANOVA flaunted that the model was significant for all dependent variables [Table 5 and Figures 5-7]. Regression analysis was carried out to determine the regression coefficients. All the independent variables (factors) were found to be significant for all R1, R2, and R3 response variables. 2FI (two factor interaction) was found to be significant for all responses. Hence, the above result indicated that the two factors (concentration of Chitosan and concentration of TPP) play an important role in the formulation of ciprofloxacin hydrochloride-loaded Chitosan nanoparticles.

Table 3: Percentage drug content and loading efficiency of formulations*

Formulation code	Drug content in 50 mg of nanoparticles		Loading efficiency (% w/w)
	Theoretical (%)	Experimental (%)	
CPL1	11.9167	11.48253±0.02	48.178±0.02
CPL2	11.5000	11.1344±0.05	48.410±0.05
CPL3	11.2500	11.5298±0.09	51.244±0.09
CPL4	21.0833	31.45767±0.07	74.603±0.07
CPL5	20.9643	31.85397±0.04	75.971±0.04
CPL6	20.8750	33.8022± 0.03	80.963±0.03
CPL7	30.8056	54.29771±0.08	88.129±0.08
CPL8	30.7500	55.5672± 0.06	90.353±0.06
CPL9	30.5833	55.64427±0.09	90.972±0.09
CPL10	28.0269	33.03±0.06	78.95±0.06

*Data represented as mean±standard deviation (n = 3)

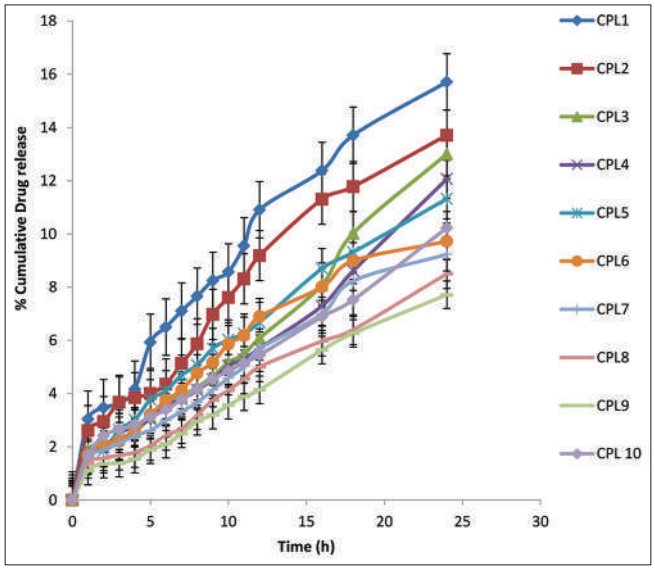

Figure 4: *In vitro* drug release profile of prepared formulations. Data are represented as mean ± standard deviation (n = 3)

According to the statistical analysis of loading efficiency, the following equation was provided by Design-Expert software:

Loading efficiency = +4.57414 + 0.126461*CHITOSAN + 281.99913*TPP − 0.414854* CHITOSAN*TPP

The equations provided by Design-Expert software for entrapment efficiency are presented as follows:

Entrapment efficiency = + 37.83264 + 0.099380*CHITOSAN + 93.30451*TPP − 0.247682*CHITOSAN*TPP

According to the statistical analysis of *In vitro* drug release, the following equation was provided by Design-Expert software:

In vitro drug release = + 20.97785 - 0.025505*CHITOSAN − 28.37568*TPP + 0.062439*CHITOSAN*TPP

The preparation recipes of optimized formulations are shown in Table 6. The optimized formulation was prepared according to the predicted model and evaluated for the responses. All the selected parameters (variables) have showed values of Prob > F < 0.05 which indicates that all model terms are significant.

Characterization of Ciprofloxacin Hydrochloride-Loaded Chitosan Nanoparticle X-ray

Diffraction (XRD)

XRD a non-destructive technique used for the characterization of crystalline or amorphous materials.XRD pattern of Ciprofloxacin Hydrochloride and Ciprofloxacin Hydrochloride loaded Chitosan nanoparticles [Figure 8] exposed many diffraction bands at 2θ scattered angles of 8–30°.

Scanning Electron Microscopy (SEM)

SEM study revealed that the surface morphology of the optimized formulation CPL10 was found to be spherical and smooth surface with solid dense structure [Figure 9].

Particle Size Analysis

The particle size of the optimized formula CPL10 was found to be 96.23 nm with polydispersivity index 1 [Figure 10] and was selected for further analysis.

Table 4: Curve fitting of all formulations

Formulation code	Zero order	First order	Higuchi model	Korsmeyer-Peppas	*n*-value
CPL1	0.9761	0.9807	0.9741	0.9502	0.5879
CPL2	0.9741	0.9764	0.9468	0.9182	0.5826
CPL3	0.9813	0.9769	0.8928	0.906	0.6307
CPL4	0.9836	0.979	0.9006	0.984	0.8474
CPL5	0.9915	0.9937	0.9773	0.9726	0.6386
CPL6	0.9671	0.9699	0.9706	0.9599	0.6257
CPL7	0.9853	0.9853	0.9385	0.9314	0.607
CPL8	0.9879	0.9882	0.9362	0.9048	0.6305
CPL9	0.9923	0.9921	0.9357	0.924	0.6719
CPL10	0.9779	0.9795	0.9401	0.9526	0.5467

Table 5: A summary of analysis of variance for models and independent variables

Response	Model	Model intercept	*P*-value		
			Model	A	B
Loading efficiency	Quadratic	80.54	< 0.0001	0.0172	0.0210
Entrapment efficiency	Quadratic	78.92	< 0.0001	0.0001	0.1513
Drug release 24 h	Quadratic	9.44	0.0005	0.0118	0.9729

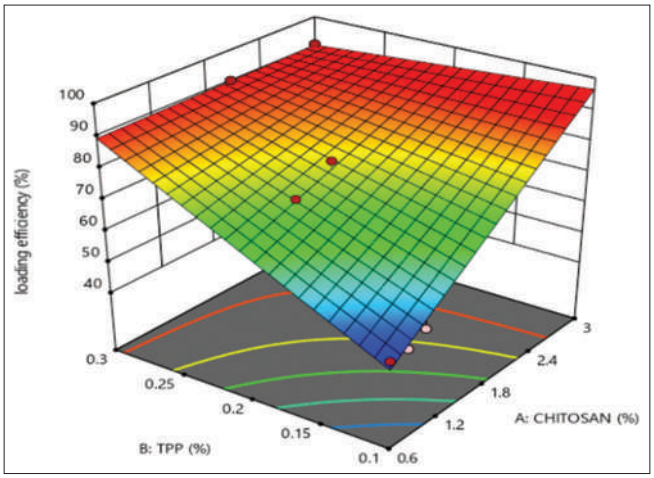

Figure 5: 3D graph of loading efficiency

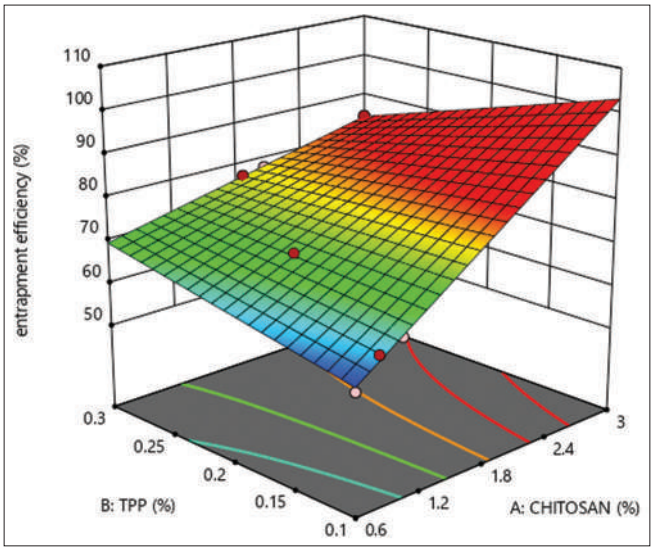

Figure 6: 3D Graph of Entrapment Efficiency

In Vitro Antimicrobial Efficiency

The well diffusion technique revealed that the pure drug (2.5 µg) yielded 17 mm clear zone surrounding the disk, whereas prepared formulations (10 µg, 5 µg, and 1 µg) disks produced 27 mm, 25 mm, and 20 mm, respectively [Figure 11]. This confirmed that the prepared formulation CPL10 shows better zone of inhibition than the pure drug solution. Chitosan itself has antimicrobial activity and chitosan in nanosize exhibits higher affinity for bacterial cells which may be probably responsible for its higher antimicrobial activity.

The MIC50 values for pure drug and prepared formulation CPL10, from broth dilution technique for *Neisseria gonorrhoeae* isolates, were found to be 0.5 µg/mL and 0.62 µg/mL, respectively [Table 7 and Figure 12].

Accelerated Stability Studies

The stability of ciprofloxacin hydrochloride-loaded chitosan nanoparticles was studied to monitor the effect of storage condition on their physiochemical characteristics and percentage of drug release. Nanoparticles were stored for 3 months at a freezing temperature (5°C ± 3°C), room temperature (25°C ± 2°C/60% ± 5% RH), and 40°C ± 2°C/75% ± 5% RH. The nanoparticles were evaluated for particle size, entrapment

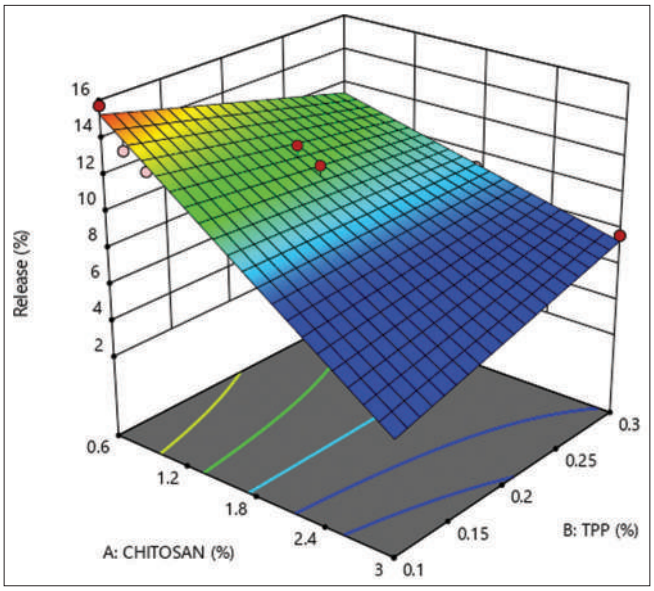

Figure 7: 3D graph of drug release 24 h

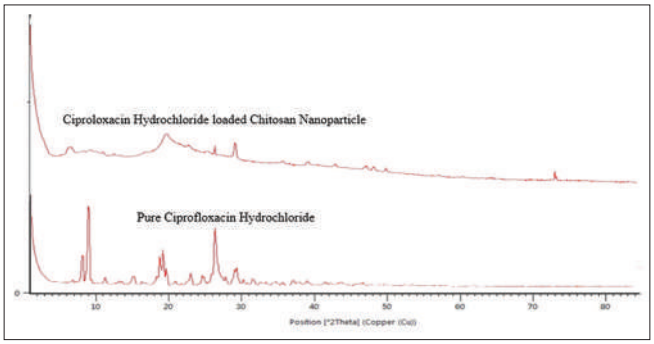

Figure 8: XRD pattern of ciprofloxacin hydrochloride and ciprofloxacin hydrochloride loaded nanoparticles

efficiency, and drug release. The results unveiled some changes in the physiochemical characteristics of the formulation.

DISCUSSION

Chitosan one of the natural mucoadhesive polymers with antibacterial activity selected for the preparation of nanoparticles by ionic gelation method containing ciprofloxacin hydrochloride to evaluate the possible changes in the potency of antibacterial agent. In this study, various concentrations of Chitosan and Sodium TPP were explored to get the lowest particle size. The particles prepared, under optimal condition of 1.5% Chitosan concentration and 0.2% TPP concentration, homogenizer rate at 6000 rpm, and centrifuged at 8000 rpm has 96.23 nm.[19] The FTIR spectra of Ciprofloxacin Hydrochloride, Sodium TPP, Chitosan and physical mixture of Ciprofloxacin Hydrochloride, Sodium TPP, and Chitosan [Figure 1] showed very minor changes in the peaks, which hence, can be concluded that drug can be entrapped in the nanoparticles without owing any kind of functional group interaction.[27] The DSC thermogram

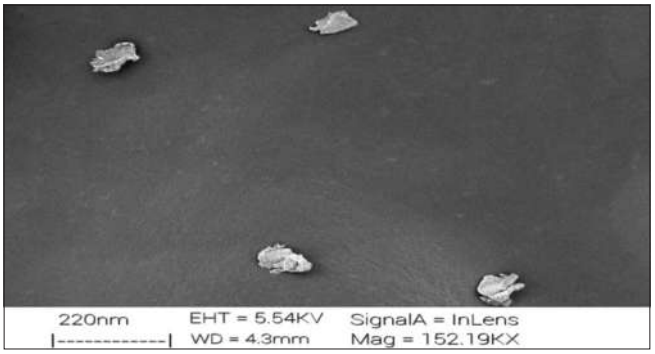

Figure 9: SEM micrograph of ciprofloxacin hydrochloride loaded Chitosan nanoparticles

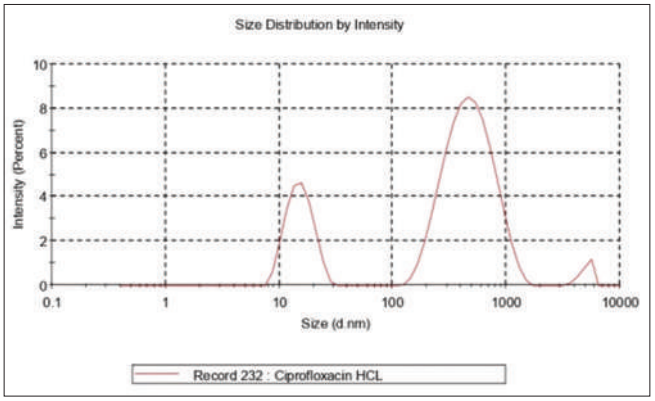

Figure 10: Particle size of optimized formula CPL10

of ciprofloxacin hydrochloride-loaded chitosan showed the endothermic peak at 139.4°C. Thus, it could be concluded that ciprofloxacin hydrochloride was entrapped in an amorphous or molecular dispersion state in the polymer matrix as well as there is no interaction between Chitosan, Sodium TPP, and ciprofloxacin hydrochloride.[26] The encapsulation efficiency was 74.59%. The results of entrapment efficiency revealed that drug entrapment efficiency depends on the polymer concentration. Increase in the concentration of polymer increases the drug entrapment efficiency. Decrease in ratio of Chitosan/TPP decreases the drug entrapment efficiency giving more compact solid-matrix structure that led to increasing amount of nanoparticle formation resulting in enhanced nanoencapsulation.[28,29] Furthermore, the loading capacity on nanoparticle increases with the increasing in concentration of polymer.[29] Release profile of nanoparticles showed an initial burst after 2 h, so that could be attributed to the dissolution of drug molecules in the release medium adsorbed to the surface of nanoparticles. The percentage of drug release was decreased with increased concentration of the polymer used. The cross-linking agent also affected the release of drug from the formulation resulting in the polymer density and reduction of the macromolecular chain mobility. This forms more stable and rigid nanoparticles which cause decrease in drug release. During drug release study, it was found that the release of the drug from the nanoparticle depends on the concentration of chitosan. High concentration of chitosan showed the slow release of

Table 6: Formulation table for the optimized formula CPL10

Formulation code	Compositions (drug and polymer)	Ratios	Concentration of tripolyphosphate (%)	RPM
CPL10	Ciprofloxacin: Chitosan	1:1.5	0.2	8000

RPM

Table 7: Minimum Inhibitory activity of standard (ciprofloxacin hydrochloride) and sample (ciprofloxacin hydrochloride loaded Chitosan nanoparticle) against *Neisseria gonorrhoeae*

Neisseria gonorrhoeae					
Standard (ciprofloxacin hydrochloride)			**Sample (ciprofloxacin hydrochloride loaded Chitosan nanoparticles)**		
Concentration (µg/ml)	**OD**	**% Inhibition**	**Concentration (µg/mL)**	**OD**	**% inhibition**
0	0.56	0	0	0.53	0
0.125	0.43	24.33	0.15	0.46	13.21
0.25	0.35	37.22	0.31	0.34	35.85
0.5	0.27	51.31	0.62	0.26	50.94
1	0.22	60.44	1.25	0.2	62.26
2	0.17	70.26	2.5	0.14	73.58
4	0.12	79.49	5	0.1	81.13
8	0.09	84.2	10	0.07	86.79
MIC	0.5 µg/mL		MIC	0.62 µg/mL	

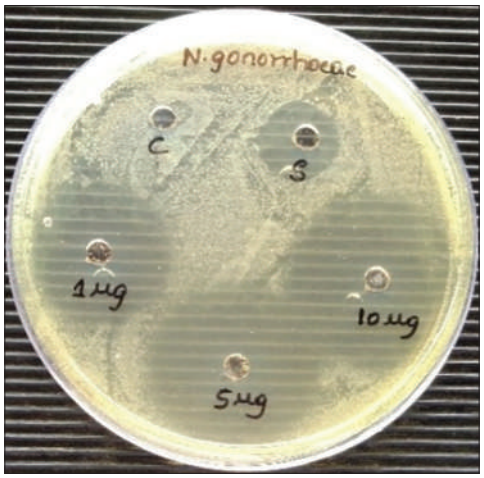

Figure 11: Determination of zone of inhibition of sample against Neisseria gonorrhoeae S-standard (ciprofloxacin hydrochloride); C–control

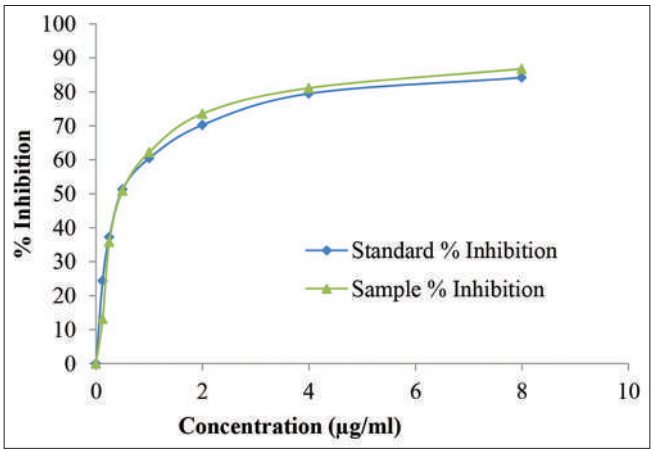

Figure 12: Minimum inhibitory of standard and sample against Neisseria gonorrhoeae

the drug from nanoparticles due to the higher cross linking. Due to high concentration of polymer, the release of drug from the nanoparticle was retarded.[19,30,31] The n values lied between 0.5 and 1 which indicated anomalous non-fickian diffusion, that is, the increased diffusivity of drug from the matrix by solvent-induced relaxation of the polymers.[22,23,32] Diffraction bands shown in ciprofloxacin hydrochloride XRD were decreased in the ciprofloxacin hydrochloride-loaded chitosan nanoparticle formulation. This decreases the crystalline nature of the drug. The intensity indicates the increased amorphous nature of drug in nanoparticle form.[27] Numerous studies have suggested enhanced efficiency of antibacterial agents loaded into the polymeric nanoparticles such finding includes many factors including: drug penetration into the cell of bacteria, better delivery of drug to the site of action as well as higher stability of drug encapsulated into the nanoparticles. In the present study, the potency of ciprofloxacin hydrochloride-loaded Chitosan nanoparticles against *N. gonorrhoeae* was increased. This confirmed that the prepared formulation CPL10 shows better zone of inhibition than the pure drug solution. Chitosan itself has antimicrobial activity and chitosan in nanosize exhibits higher affinity for bacterial cells which may be probably responsible for its higher antimicrobial activity. The slow release of ciprofloxacin hydrochloride from the ciprofloxacin hydrochloride-loaded chitosan nanoparticle would permit an increase in the time period between the administrations as well as decrease in the side effects of the drug. *Neisseria gonorrhoeae,* Gram-negative bacteria, has negative charge on the cell wall leading to more adsorption of Chitosan

Table 8: Accelerated stability studies of optimized formulation CPL10

Temperature (°C/%RH)	Duration (months)	Entrapment efficiency (%)	%CDR at 24 h
Freeze temperature (5°C±3°C)	Initial	78.95±0.09	10.23±0.05
	1	78.85±0.10	10.22±0.15
	2	78.75±0.11	10.09±0.16
	3	78.69±0.06	9.99±0.06
Room temperature (25°C±2°C/60%±5% RH)	Initial	78.95±0.12	10.23±0.09
	1	76.36±0.16	9.86±0.10
	2	74.1±0.07	8.12±0.11
	3	71.63±0.08	7.69±0.13
40°C±2°C/75%±5%RH	Initial	78.95±0.15	10.23±0.06
	1	74.42±0.19	8.65±0.09
	2	70.69±0.06	6.96±0.13
	3	68.53±0.07	5.12±0.14

favoring better inhibitory effect.[19] It was concluded that the formulation stored at 5°C ± 3°C was more stable as compared to those stored at 25°C ± 2°C/60% ± 5% RH and 40°C ± 2°C/75% ± 5% RH [Table 8]. The entrapment efficiency as well as the release of the drug was reduced at 25°C ± 2°C/60% ± 5% RH and 40°C ± 2°C/75% ± 5% RH. This may be due to heat sensitive effect of Ciprofloxacin Hydrochloride when exposed to high temperature for longer time. On that basis, freezing temperature 5°C ± 3°C was concluded to be the optimum storage condition for nanoparticles.[25]

CONCLUSION

In this study, ciprofloxacin hydrochloride-loaded chitosan nanoparticles were prepared and characterized. Thus formed, antibiotic-loaded Chitosan nanoparticles were found to exhibit effective antimicrobial activity against *Neisseria gonorrhoeae*, which showed high resistance toward conventional dosage forms. Hence, this study highlights the need for the formulation of antibiotic loaded chitosan nanoparticles as a new strategy to overcome *Neisseria gonorrhoeae* associated multidrug resistance problems.

REFERENCES

1. Wang L, Hu C, Shao L. The antimicrobial activity of nanoparticles: Present situation and prospects for the future. Int J Nanomed 2017;12:1227.
2. Kumar M, Curtis A, Hoskins C. Application of nanoparticle technologies in the combat against anti-microbial resistance. Pharmaceutics 2018;10:11.
3. Ranghar S, Sirohi P, Verma P, Agarwal V. Nanoparticle-based drug delivery systems: Promising approaches against infections. Braz Arch Bio Technol 2014;57:209-22.
4. Zhu W, Tomberg J, Knilans KJ, Anderson JE, McKinnon KP, Sempowski JD, *et al*. Properly folded and functional PorB from *Neisseria gonorrhoeae* inhibits dendritic cell stimulation of $CD4^+$ T cell proliferation. J Biol Chem 2018;293:11218-29.
5. Wi T, Lahra MM, Ndowa F, Bala M, Dillon JA, Ramon-Pardo P, *et al*. Antimicrobial resistance in *Neisseria gonorrhoeae*: Global surveillance and a call for international collaborative action. PLoS Med 2017;14:e1002344.
6. Dan M. The use of fluoroquinolones in gonorrhoea: The increasing problem of resistance. Expert Opin Pharmacother 2004;5:829-54.
7. Skerlev M, Čulav-Košćak I. Gonorrhea: New challenges. Clin Dermatol 2014;32:275-81.
8. Khazaei T, Barlow JT, Schoepp NG, Ismagilov RF. RNA markers enable phenotypic test of antibiotic susceptibility in *Neisseria gonorrhoeae* after 10 minutes of ciprofloxacin exposure. Sci Rep 2018;8:11606.
9. Newman LM, Wang SA, Ohye RG, O'Connor N, Lee MV, Weinstock HS. The epidemiology of fluoroquinolone-resistant *Neisseria gonorrhoeae* in Hawaii, 2001. Clin Infect Dis 2004;38:649-54.
10. Hsueh PR, Tseng SP, Teng LJ, Ho SW. High prevalence of ciprofloxacin-resistant *Neisseria gonorrhoeae* in Northern Taiwan. Clin Infect Dis 2005;40:188-92.
11. Pedersen ML, Poulsen P, Berthelsen L, Nørgaard C, Hoffmann S, Jensen JS. Rapid spread of *Neisseria gonorrhoeae* ciprofloxacin resistance due to a newly introduced resistant strain in Nuuk, Greenland, 2012–2015: A community-based prospective cohort study. BMJ Open 2016;6:e011998.
12. Patel AL, Chaudhry U, Sachdev D, Sachdeva PN, Bala M, Saluja D. An insight into the drug resistance profile and mechanism of drug resistance in *Neisseria gonorrhoeae*. Indian J Med Res 2011;134:419-31.
13. Ismail NS, Gopinath SC. Enhanced antibacterial effect by antibiotic loaded starch nanoparticle. J Assoc Arab Univ Basic Appl Sci 2017;24:136-40.

14. Zhang L, Pornpattananangkul D, Hu CM, Huang CM. Development of nanoparticles for antimicrobial drug delivery. Curr Med Chem 2010;17:585-94.
15. Parisi OI, Scrivano L, Sinicropi MS, Puoci F. Polymeric nanoparticle constructs as devices for antibacterial therapy. Curr Opin Pharmacol 2017;36:72-7.
16. Rudramurthy GR, Swamy MK, Sinniah UR, Ghasemzadeh A. Nanoparticles: Alternatives against drug-resistant pathogenic microbes. Molecules 2016;21:836.
17. Chen A, Seifert HS. Structure-function studies of the *Neisseria gonorrhoeae* major outer membrane porin. Infect Immun 2013;81:4383-91.
18. Kasgavada P, Swapnil M, Formulation and evaluation of fast dissolving tablet of Ciprofloxacin. Int J Adv Pharm 2016;5:52-60.
19. Sobhani Z, Samani SM, Montaseri H, Khezri E. Nanoparticles of chitosan loaded ciprofloxacin: Fabrication and antimicrobial activity. Adv Pharm Bull 2017;7:427-432.
20. Sreelola VU, Sailaja AK, Pharmacy M. Preparation and characterisation of ibuprofen loaded polymeric nanoparticles by solvent evaporation technique. Int J Pharm Pharm Sci 2014;6:416-21.
21. Singh K, Verma S, Prasad S. Novel formulation development and evaluation of nanoparticles based *in situ* gelling system for the ophthalmic delivery of ciprofloxacin hydrochloride. Int J Drug Deliv Technol 2015;5:126-31.
22. Barzegar-Jalali M. Kinetic analysis of drug release from nanoparticles. J Pharm Pharm Sci 2008;11:167-77.
23. Siepmann J, Peppas NA. Modeling of drug release from delivery systems based on hydroxypropy lmethylcellulose (HPMC). Adv Drug Deliv Rev 2012;64:163-74.
24. Farhangi M, Kobarfard F, Mahboubi A, Vatanara A, Mortazavi SA. Preparation of an optimized ciprofloxacin-loaded chitosan nanomicelle with enhanced antibacterial activity. Drug Dev Ind Pharm 2018;44:1273-84.
25. Mathew B, Rangapriya M, Rajendra N. Evaluation and stability study of Rifampicin loaded chitosan nanoparticles. World J Pharm Pharm Sci 2014;3:609-24.
26. Kahdestani SA, Shahriari MH, Abdouss M. Synthesis and characterization of chitosan nanoparticles containing teicoplanin using sol-gel. Polym Bull 2021;78:1133-48.
27. Singh K, Mishra A, Singh A. Synthesis characterization and *in vitro* release study of ciprofloxacin-loaded chitosan nanoparticles. BioNanoSci 2018;8:229-36.
28. Hou D, Gui R, Hu S, Huang Y, Feng Z, Ping Q. Preparation and characterization of novel drug-inserted-montmorillonite chitosan carriers for ocular drug delivery. Adv Nano 2015;4:1-14.
29. Shi Y, Wan A, Shi Y, Zhang Y, Chen Y. Experimental and mathematical studies on the drug release properties of aspirin loaded chitosan nanoparticles. Bio Med Res Int 2014;2014:613619.
30. Farhangi M, Kobarfard F, Mahboubi A, Vatanara A, Mortazavu SA. Preparation of an optimized ciprofloxacin-loaded chitosan nanomicelle with enhanced antibacterial activity. Drug Dev Ind Pharm 2018;44:1273-84.
31. Mahmoudzadeh M, Fassihi A, Emami J, Davies NM, Dorkoosh F. Physicochemical, pharmaceutical and biological approaches toward designing optimized and efficient hydrophobically modified chitosan-based polymeric micelles as a nanocarriers system for targeted delivery of anticancer drugs. J Drug Target 2013;21;1-17.
32. Constantin M, Voicu V, Anuta V, Andra T, Celia C, Paolino D, *et al*. Mathematical modeling of release kinetics from supramolecular drug delivery systems. Pharmaceutics 2019;11:2-45.

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