RESEARCH ARTICLE

Acyclovir-loaded chitosan nanoparticles for ocular delivery

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The topical application of acyclovir as eye ointment remains a concern for effective management of various ocular viral diseases owing to poor ocular drug bioavailability. Hence the present study was aimed to develop and evaluate nanosphere colloidal suspension containing acyclovir as potential ophthalmic drug delivery system. The acyclovir-loaded chitosan nanoparticles were prepared by ionic gelation of chitosan with tripolyphosphate anions. The nanoparticles were characterized by scanning electron microcopy, zeta potential analyser, differential scanning calorimetry (DSC) and Fourier transform infrared Spectroscopy. All the prepared formulations resulted in nano-range size particles (200-495 nm) and displayed spherical smooth morphology with zeta potential (+36.7 to +42.3 mV). The encapsulation efficiency and loading capacity were 56-80% and 10-25%, respectively. The acyclovir-loaded chitosan nanoparticles displayed more crystallinity than acyclovir. The *in vitro* diffusion profile of acyclovir from the nanoparticles showed a sustained release of the drug over a period of 24 hrs. Kinetic release profiles of acyclovir from nanoparticles appeared to fit best with Higuchi model with zero order and the non-Fickian diffusion was superior phenomenon. Thus the results suggest that acyclovir-loaded chitosan nanoparticle suspension appears to be promising enough for effective management of ocular viral infections.

Key words: *Acyclovir, chitosan, ionic gelation method, ocular delivery nanoparticles*

INTRODUCTION

Acyclovir is an antiviral drug with a significant and highly specific activity against herpes viruses and is widely used in the treatment of various ocular viral diseases.[1-4] The topical application of acyclovir, now commercially available as ointments and drops, is limited by the low corneal penetration of the drug, poor ocular drug bioavailability, pulse drug entry, systemic exposure due to the nasolacrimal duct drainage and poor entrance to the posterior segments of the eye due to the lens-iris diaphragm. Hence development of acyclovir-loaded chitosan nanoparticles was undertaken for effective ocular delivery of the drug with improved bioavailability.

Many attempts have been made to improve the ocular bioavailability and the therapeutic effectiveness of acyclovir, e.g., chemical modification of the drug[5] and its incorporation into colloidal systems such as liposomes or nanoparticles.[6] Nanoparticles have been used as ophthalmic delivery systems because they are able to penetrate into the corneal or conjunctival tissue by an endocytotic mechanism.[7] Further nanoparticles owing to their polymeric nature present some important advantages such as high storage stability, controlled release of the encapsulated drug and a prolonged residence time in the precorneal area, particularly in the case of ocular inflammation and/or infection.[8]

Among the mucoadhesive polymers investigated until now, the cationic polymer chitosan has attracted a great deal of attention because of its unique properties, such as acceptable biocompatibility,[9] biodegradability and ability to enhance the paracelluar transport of drugs.[10] Besides, the cornea and conjunctiva have a negative charge; use of the cationic polymer chitosan will interact intimately with these extraocular structures, which would increase the concentration and residence time of the associated drug. Moreover, chitosan has recently been proposed as a material with a good potential for ocular drug delivery.

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Previous study on acyclovir-loaded poly d, l lactic acid (PLA) nanosphere for ocular drug delivery indicated that both types of PLA nanospheres were able to increase the aqueous levels of acyclovir and improve the pharmacokinetics profile, but the efficacy of the PEG-coated nanosphere was significantly higher than that of the simple PLA ones.[11] The potential of chitosan nanoparticles for ocular drug delivery and their interactions with ocular mucosa *in vivo* and also toxicity in conjunctival cell cultures was studied and it was reported that the chitosan nanoparticles are able to interact and remain associated to the ocular mucosa for extended periods of time, thus being promising carriers for enhancing and controlling the release of drugs to the ocular surface.[12] Similar conclusion has been proposed that chitosan nanoparticles readily penetrate conjunctival epithelial cells and were well-tolerated by the ocular surface tissues of the rabbits and further stated that chitosan nanoparticles hold promise as a drug delivery system for the ocular mucosa.[13] A recent study on the effect of acyclovir-loaded chitosan nanoparticles in rabbit's eye indicated that chitosan nanoparticles facilitated absorption of acyclovir compared to market preparations.[14] However, literature research indicates that the role of acyclovir concentration on nanoparticles has not been studied in detail and hence the present study was attempted to demonstrate the influence of acyclovir concentration on the physicochemical characteristics and release profile of the chitosan nanoparticles.

MATERIALS AND METHODS

Materials

Acyclovir was obtained as a gift sample from Micro labs (Hosur, India). Chitosan (degree of deacetylation of 85%; intrinsic viscosity,1390 ml/g in 0.30 M acetic acid/0.2 M sodium acetate solution and viscometric molecular weight, 4.08×10^5 Da) was obtained as gift sample from Central Institute of Fisheries Technology (Cochin, India). Sodium tripolyphosphate (STPP) was purchased from S.D. Fine Chemicals Ltd (Mumbai, India) and Tween-80 was supplied by Loba Chemie Pvt Ltd (Mumbai, India). Ultra pure water was purchased from Himedia Ltd (Mumbai, India). All other reagents and solvents used were of analytical grade.

Methods

Preparation of acyclovir-loaded chitosan nanoparticles

Chitosan nanoparticles were prepared according to the procedure first reported by Calvo *et al*,[15] based on the ionic gelation of chitosan with STPP anions. Chitosan nanoparticles were prepared by ionic gelation of chitosan solution with STPP (0.25%) prepared in the presence of Tween-80 (0.5%) as a resuspending agent to prevent aggregation, at ambient temperature while stirring. Two hundred and fifty milligrams of chitosan and acyclovir at various concentrations (25, 50, 75, 100, 125 mg/10 ml) were dissolved in acetic acid in aqueous solution under magnetic stirring at room temperature for 45 mins in the presence of Tween-80 (0.5%). Ten milliliters TPP aqueous solution was added to 10 ml chitosan solution and the mixture was sonicated (Sonicater, Bandelin Sono plus Model HD 2070, Bandelin electronic, Germany) for 3 mins. The nanosuspensions were cold centrifuged at 12000 g in a glucose bed for 30 mins using Hitachi centrifuge (Hitachi koki USA Ltd, USA). The supernatant liquid was analyzed by spectrophotometer to calculate the percent drug entrapment and drug loading. Chitosan nanoparticles separated from suspension were dried by a freeze dryer (Labconco, Kansas city, USA) and lyophilized at 0.4 mbar and -40°C for 5 hrs using glucose and lactose (1:2).The lyophilized nanoparticles were stored in a desiccator at 4°C. The lyophilized nanoparticles were resuspended in pH 7.4 phosphate buffer and submitted to characterization experiments.

Evaluation of acyclovir-loaded chitosan nanoparticles

Fourier transform infrared spectroscopy

The fourier transform infrared spectroscopy (FTIR) spectra of acyclovir, chitosan and acyclovir-loaded chitosan nanoparticles were determined by using Perkin Elmer RX1 model. The pellets were prepared by gently mixing of 1 mg sample with 200 mg potassium bromide at high compaction pressure. The pellets thus prepared were scanned at a resolution of 4 cm $^{-}$1 from 450 to 4000 cm^{-1}.

Differential scanning calorimetry

Differential scanning calorimetric (DSC) curve of pure acyclovir, chitosan and acyclovir-loaded chitosan nanoparticles measurement were carried out by using a thermal analysis instrument (DSC DA 60 Shimadzu, Japan) equipped with a liquid nitrogen subambient accessory. 2-6 mg samples were accurately weighed in aluminum pans thematically sealed and heated at a rate of 10°C/min in a 30-300°C under nitrogen flow of 40 ml/min.

Particle size, polydispersity and zeta potential of nanoparticles

The particle size, polydispersity and zeta potential of nanoparticles were measured by Photon Correlated Spectoscopy (BI MAS, Multiangle sizing option on Zetaplus, Brookhaven Instruments, Holtsville, NY, USA) using dyanamic light scattering principles. The samples were diluted with pH 7.4 phosphate buffer and placed in eletrophoretic cell and measured in the automatic mode.

Scanning electron microscopy

The scanning electron microscopy (SEM) (JEOL MODEL JSM 6400, Tokyo) was used to characterize the surface morphology of nanoparticles. The nanoparticles were mounted directly on the SEM stub, using doublesided, sticking tape and coated with platinum and scanned in a high vacuum chamber with a focused electron beam. Secondary electrons, emitted from the samples were detected and the image formed.

Acyclovir encapsulation efficiency and loading capacity of the nanoparticles

The encapsulation efficiency and loading capacity of the nanoparticles were determined by the separation

of nanoparticles from the aqueous medium containing non-associated acyclovir by cold centrifugation (Hitachi Centrifuge) at 12000 g for 30 minutes. The amount of free acyclovir in the supernatant was measured by UV-Visible Spectrophotometer (Perkin Elmer Lambda 25) at 253 nm.

The acyclovir encapsulation efficiency (EE) and loading capacity (LC) of the nanoparticles was calculated as follows.

$$\text{Encapsulation efficiency} = \frac{\text{Total amount of acyclovir - Free acyclovir}}{\text{Weight of nanoparticles}} \times 100$$

$$\text{Loading capacity} = \frac{\text{Total amount of acyclovir – Free acyclovir}}{\text{Total amount of acyclovir}} \times 100$$

In vitro release of acyclovir from the nanoparticles

The *in vitro* release profile of acyclovir from nanoparticles was performed on the optimized formulation that showed the least size of the nanoparticles (F5). Nanoparticles equivalent to 2 mg of acyclovir nanoparticles were redispersed in 10 ml 7.4 phosphate buffer solution and placed in a dialysis membrane bag with a molecular cutoff of 5 kDa which acts as a donor compartment, tied and placed into 10 ml 7.4 phosphate buffer solution which acts as a receptor compartment. The entire system was kept at 37°C with continuous magnetic stirring. At appropriate time intervals (1, 2, 3, 4…24 hrs), 1 ml of the release medium was removed and 1 ml fresh 7.4 phosphate buffer solution was added into the system. The amount of acyclovir in the release medium was evaluated by UV-visible spectrophotometer at 253 nm.

Release kinetics

In order to understand the mechanism and kinetics of drug release, the results of the *in vitro* drug release study were fitted to various kinetics equations like zero order (%cumulative drug release vs time), first order (log %cumulative drug remaining vs time), Higuchi matrix (%cumulative drug release vs square root of time).[16] In order to define a model which will represent a better fit for the formulation, drug release data were further analyzed by Peppas equation, $Mt/M\infty = ktn$, where Mt is the amount of drug released at time t and $M\infty$ is the amount released at ∞, $Mt/M\infty$ is the fraction of drug released at time t, k is the kinetic constant and n is the diffusional exponent, a measure of the primary mechanism of drug release. R^2 values were calculated for the linear curves obtained by regression analysis of the above plots.

RESULTS

FTIR Spectroscopy

There are three characterization peaks of chitosan at 2883.87 cm^{-1} of ν (OH), 1095.41 cm^{-1} of ν (C O C) and 1654.47 cm^{-1} of ν (NH_2). The spectrum of chitosan-STPP is different from that of chitosan matrix. In chitosan-STPP the characteristic peak of chitosan at 3424.62 cm^{-1} becomes wider, indicating that hydrogen bonding is enhanced. In chitosan-TPP, the 1654.47 cm^{-1} peak of $–NH_2$ bending vibration shifts to 1085.82 cm^{-1} and a new sharp peak at 2108.55 cm^{-1} appears. These findings propose that there is linkage between phosphoric and ammonium ion. Compared with the spectrum of acyclovir, the spectrum of acyclovir-loaded chitosan nanoparticles showed that the absorption peak of 3184.15 cm^{-1} (amino group absorption peak) disappeared and a new sharp peak of 2873.04 cm^{-1} (linkage between hydroxyl group of acyclovir and amino group of chitosan) appeared. The results indicate electrostatic interactions between hydroxyl ethoxy methyl group of acyclovir and amino groups of chitosan.

DSC

Acyclovir showed characteristic endothermic peaks at 121.06°C, 150.48°C and 254.07°C. Chitosan showed a broad peak at 102.81°C. The physical mixture of acyclovir and chitosan showed characteristic peaks at 121.06°C, 150.48°C and 254.07°C. The thermogram of acyclovir-loaded chitosan nanoparticles exhibited all characteristic peaks of acyclovir, thus indicating that there was no change in the crystallinty of acyclovir. However, the broad peak of chitosan has disappeared in the thermogram of acyclovir nanoparticles. The absence of characteristic peak of chitosan at 102.81°C in acyclovir-loaded chitosan nanoparticles may be due to an interaction between acyclovir and chitosan.

Particle size, polydispersity and zeta potential of acyclovir-loaded chitosan nanoparticles

The particle size, polydispersity and zeta potential of acyclovir-loaded chitosan nanoparticles (F1– F5) are shown in Table 1. The maximum size of nanoparticles was observed in F1 (495±05nm) as compared to other formulations and the least size was seen in F5 (200±30nm). The size of the nanoparticles varied with the acyclovir concentration [Figure 1]. The range of polydispersity was 0.16-0.37 and the values decreased as the concentration of acyclovir increased. The zeta potential values ranged from +36.7 to +42.3 mV

Table 1: Mean particle size, polydispersity index, zeta potential, encapsulation efficiency and loading capacity

Formula code	Mean particle size (nm)	Polydispersity index ((μ2)/Γ²)	Zeta potential (mV)	Encapsulation efficiency (%)	Loading capacity (%)
F1	495±05	0.37	+ 42.3±1.3	80.00	10.00
F2	450±28	0.29	+40.2±1.2	74.00	16.81
F3	368±12	0.24	+38.1±1.4	66.66	20.83
F4	298±25	0.18	+37.5±1.1	60.00	23.07
F5	200±30	0.16	+36.7±1.5	56.00	25.00

and the values decreased as the concentration of acyclovir increased [Figure 2]. Zeta potential above +30 mV indicating that the formulations are stable.

SEM

The morphological characters of acyclovir-loaded chitosan nanoparticles (F5) was shown in Figure 3: Acyclovir-loaded chitosan nanoparticles have shown spherical shape.

Encapsulation efficiency and loading capacity of the nanoparticles

Table 1 shows the results of encapsulation efficiency and loading capacity of the acyclovir-loaded chitosan nanoparticles. The encapsulation efficiency was maximum with the lower drug concentration (F1) and minimum with the higher drug concentration (F5). The encapsulation efficiency ranged from 56 to 80% [Figure 4]. Conversely the loading capacity of nanoparticles increased as the concentration of the drug increased. The loading capacity ranged from 10 to 25% [Figure 5].

***In vitro* release of acyclovir from the nanoparticles**

Figure 6 displayed the release profile of acyclovir from chitosan nanoparticles. The diffusion study was performed on the formulation (F5) that showed the least particle size (200±30 nm). The release pattern demonstrated a very slow release of drug at each point of time from nanoparticles. There was an initial phase of rapid release of acyclovir followed by a more gradual release over a period of 24 hrs.

Release kinetics

The *in vitro* release profile was analyzed by various kinetic models. The kinetic models used were zero order, first order, Higuchi and Korsemeyer Peppas equation. The releases constant were calculated from the slope of the respective plots. Higher correlation was observed in the Higuchi equation. For planery geometry, the value of $n=0.5$ indicates a Fickian diffusion mechanism, for $0.5<n<1.0$, indicates anomalous (non-Fickian) and $n=1$ implies class II transport. Both dissolution and diffusion profile of the drug from the nanoparticles showed proper fitting to Higuchi plot with zero order release kinetics and indicated non-Fickian diffusion mechanism for the release of the drug from the nanoparticles.

DISCUSSION

The results of the present investigation demonstrated the potential use of chitosan nanoparticles for effective delivery of acyclovir for treating various ocular viral diseases. Drug delivery system for the ocular surface must overcome important physical barriers to reach the target cells. Different colloidal systems have been developed to solve these problems.[17] Among them chitosan-based systems are

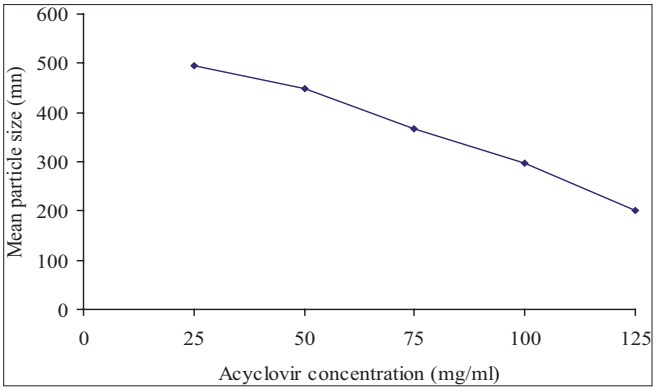

Figure 1: Mean particle size of acyclovir-loaded chitosan nanoparticles

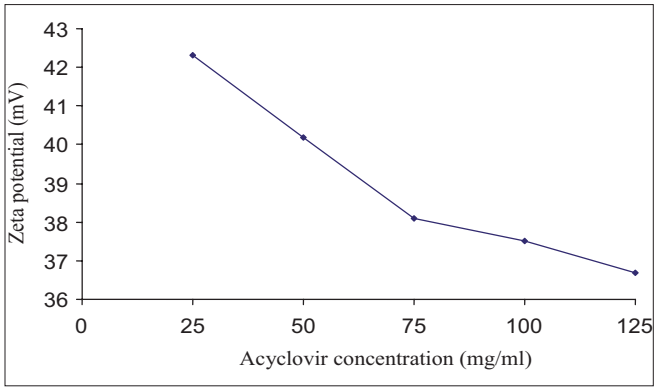

Figure 2: Zeta potential of acyclovir-loaded chitosan nanoparticles

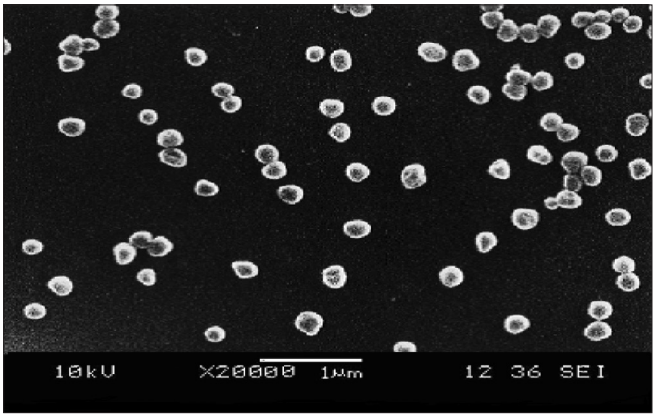

Figure 3: SEM Photograph of acyclovir-loaded chitosan nanoparticles (F5)

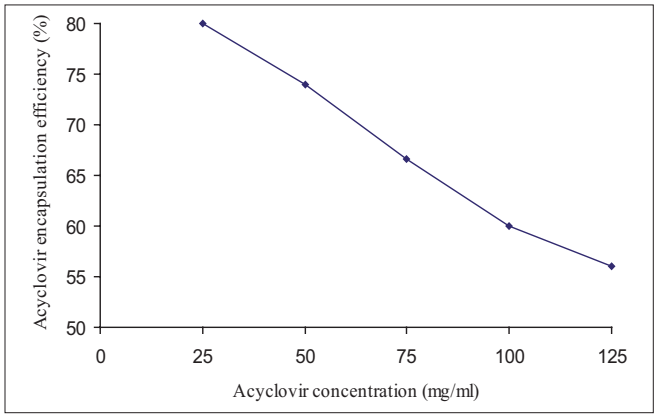

Figure 4: Encapsulation efficiency of acyclovir nanoparticles

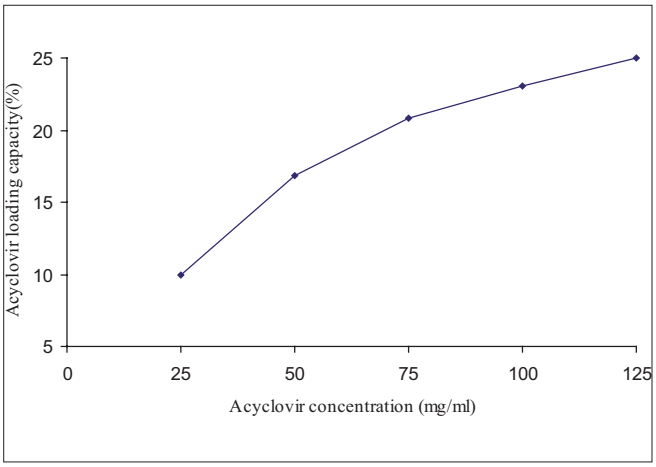

Figure 5: Loading capacity of acyclovir nanoparticles

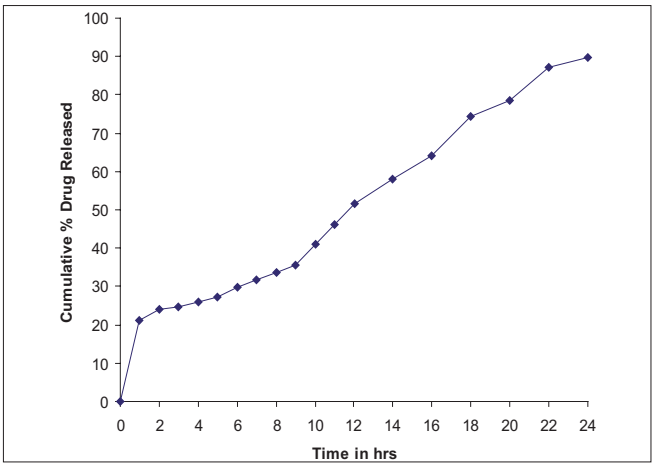

Figure 6: Acyclovir release from acyclovir-loaded chitosan nanoparticles (F5)

acknowledged to be more suitable for ocular pathway, based on the favorable biological characteristics of chitosan.[18,19] Several studies have shown that nanoparticles can transport across epithelia more readily than microparticles.[20] Moreover chitosan nanoparticles can be easily prepared under mild conditions, besides incorporating macromolecular bioactive compounds. This characteristic is extremely beneficial for drugs, proteins, genes or hydrophobic molecules that are poorly transported across epithelia.

Among the various methods developed for preparation of nanoparticles, ionic gelation method is simple to operate and also to optimize the required particle size of the drug that can penetrate the ocular surface and hence this method was followed in the study. Previously it has been reported that the particle size is dependent on the chitosan concentration, the minimum size corresponding to the lowest chitosan concentration.[21] However, reports are scanty on the role of acyclovir concentration and hence the present study attempted to demonstrate the influence of the acyclovir concentration on the physicochemical characteristics and release profile of the acyclovir-loaded chitosan nanoparticles.

The presence of a non-ionic surfactant is very important for the so-called "long-term" stability[22] of the nanosphere colloidal suspension, which is determined by the adsorption of hydrophilic macromolecules on the nanosphere surface, thus increasing the steric repulsion between particles. The presence of hydrophilic macromolecules on the surface of nanosphere leads to a change of the surface properties (zeta potential) of the colloidal carrier. In particular, the zeta potential of colloidal nanosphere is significantly reduced by coating with non-ionic surfactants.[23] Considering these factors, the non-ionic surfactant Tween-80 (0.5%) was used to stabilize the formulation.

The SEM of the acyclovir-loaded chitosan nanoparticles showed that the nanoparticles have a solid dense structure with smooth spherical shape [Figure 3]. In consistent with previous findings[24] a significant reduction of nanoparticle mean size was observed in the formulation (F5) with lowest concentration of chitosan relative to drug concentration [Table 1]. Previously it has been reported that the particle size of cyclosporin A-loaded chitosan nanoparticles is dependent upon chitosan concentration, the minimum size corresponding to the lowest chitosan concentration.[8]

Earlier it has also been demonstrated that the particle size of ammonium glycyrrhizinate-loaded chitosan nanoparticles significantly increased as the concentration of ammonium glycyrrhizinate increased.[24] Interestingly such an observation was absent in our findings wherein the particle size of acyclovir-loaded nanoparticles decreased as the concentration of acyclovir increased [Figure 1]. Although the exact mechanism for the diagonally opposite effect with respect to nanoparticle's size related to drug concentration is not clearly understood, it can be considered that the size of the nanoparticles appears partly dependant on the association between acyclovir and chitosan or on the increased solubility of low molecular weight chitosan that may aid in the colloidal stability of nanoparticles in solution.[25]

The range of polydispersity was 0.16-0.37 and the values decreased as the concentration of acyclovir increased. Polydispersity indicates the degree of non-uniformity of the particle size. Obviously a low polydispersity indicates more uniformity in size distribution.

The zeta potential of nanoparticles is commonly used to characterize the surface charge property of nanoparticles. It reflects the electrical potential of particles and is influenced by the composition of the particle and the medium in which it is dispersed. Nanoparticles with a zeta potential above (+/-) 30 mV have been shown to be stable in suspension, as the surface charge prevents aggregation of the particles. The zeta potential can also be used to determine whether a charged active material is encapsulated within the centre of the *nanocapsule* or adsorbed onto the surface. The zeta potential

of acyclovir-loaded chitosan nanoparticles ranged from +36.7 to +42.3 mV [Table 1]. The present study demonstrated decrease in zeta potential as the concentration of acyclovir increased [Figure 2] in consistent with earlier findings on the ammonium glycyrrhizinate-loaded chitosan nanoparticles.[24]

As shown in Table 1 the encapsulation efficiency and loading capacity of the acyclovir-loaded chitosan nanoparticles were affected by initial acyclovir concentration in the chitosan solution and the amount of acyclovir incorporated. The loading capacity of the nanoparticles ranged from 10 to 25% and the loading capacity of nanoparticles increased as the concentration of drug increased [Figure 5]. The encapsulation efficiency of the nanoparticles ranged from 56 to 80%. The increase of acyclovir concentration leads to a decrease of encapsulation efficiency [Figure 4] and an enhancement of loading capacity, possibly due to effect of the chain length of chitosan as longer chains of high molecular weight chitosan can entrap greater amount of drug when gelated with TPP as observed in the previous study.[24] The failure to increase encapsulation efficiency proportionate with increase in acyclovir concentration may be due to shorter chains low molecular weight chitosan used in the present study.

With regard to the diffusion of acyclovir from chitosan nanoparticles the drug leakage was monitored for 24 hrs. The acyclovir release profile from chitosan nanoparticles is characterized by an rapid initial burst released (52.46% release in 12 hrs and 90.70% release in 24 hrs) followed by a sustained release of the drug over a period of 24 hrs [Figure 6]. The release involves two different mechanisms of drug molecules diffusion and polymer matrix degradation.[25] The burst release of drug is associated with those drug molecules dispersing close to the microsphere surface, which easily diffuse in the initial incubation time. The initial rapid release can be due to the burst effect resulting from the release of the drug encapsulated near the nanosphere surface and thereafter the slow release of acyclovir from the chitosan nanoparticles is possibly the consequence of the release of the drug fraction encapsulated in the core of the nanospheres and also due to strong association between the drug and polymer through electrostatic interaction between the hydroxyl ethoxy groups of acyclovir and the amino groups of chitosan as shown by FTIR spectra.[26] Therefore, the rapid dissolution process suggests that the release medium penetrates into the particles due to the hydrophilic nature of chitosan, and dissolves the entrappedacyclovir. In addition, the nanoparticles with huge specific surface area can adsorb acyclovir, so the first burst release is also possibly due to the part of acyclovir desorbed from nanoparticle surface. Besides the crystallinity of acyclovir has not been affected as evident from DSC curve and this characteristic may also play a role in sustained release of drug from the nanoparticles. The profile of the drug from the nanoparticles showed fitting with Higuchi plot with zero order release kinetics and indicated non-Fickian diffusion mechanism for the release of the drug from the nanoparticles.

The improved interaction of chitosan-loaded nanoparticles with the cornea and the conjunctiva could be found in the mucoadhesive properties of chitosan[27] or it is due to the electrostatic interaction between the positively charged chitosan nanoparticles and the negatively charged corneal and conjunctival cells[28] that is the major force responsible for the prolonged residence of the drug. In consistent with these observations and also based on the results of the present study we propose that chitosan nanoparticles may be beneficial in improving the corneal permeation, contact time and bioavailability of acyclovir for the treatment of ocular viral infections.

CONCLUSIONS

Chitosan nanoparticles had shown an excellent capacity for the association of acyclovir. The mean particle size, morphological characteristics and surface property of the nanoparticles appear to depend on concentration of acyclovir loaded in chitosan nanoparticles. The release profile of acyclovir from nanoparticles has shown a sustained release following zero order kinetic with non-Fickian diffusion mechanism. The results demonstrated the effective use of acyclovir-loaded chitosan nanoparticles as a controlled release preparation for treatment of ocular viral infections.

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