International Journal of Pharmaceutics 432 (2012) 105–112

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Mucoadhesive nanoparticles for prolonged ocular delivery of natamycin: *In vitro* and pharmacokinetics studies

R.S. Bhatta[a,*], H. Chandasana[b], Y.S. Chhonker[a], C. Rathi[b], D. Kumar[a], K. Mitra[c], P.K. Shukla[d]

[a] *Pharmacokinetics and Metabolism Division, CSIR-Central Drug Research Institute, Lucknow, 226001, India*
[b] *Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Raebareli, 229010, India*
[c] *Electron Microscopy Division, CSIR-Central Drug Research Institute, Lucknow, 226001, India*
[d] *Medical Mycology Dept., Division of Fermentation Technology, CSIR-Central Drug Research Institute, Lucknow, 226001, India*

ARTICLE INFO

Article history:
Received 24 January 2012
Received in revised form 13 April 2012
Accepted 23 April 2012
Available online 28 April 2012

Keywords:
Natamycin
Nanoparticles
Chitosan
Lecithin
Ocular pharmacokinetic

ABSTRACT

The aim of this study was to prepare natamycin encapsulated lecithin/chitosan mucoadhesive nanoparticles (NPs) for prolonged ocular application. These NPs were characterized by their mean particle size 213 nm, encapsulation efficiency 73.57%, with a theoretical drug loading 5.09% and zeta potential +43. *In vitro* release exhibited a biphasic drug release profile with initial burst followed by a very slow drug release. The MIC_{90} and zone of inhibition of NPs showed similar antifungal activity as compared to marketed suspension and free natamycin against *Candida albicans* and *Aspergillus fumigates.* The ocular pharmacokinetics of NPs and marketed formulation were evaluated in *NZ* rabbits. The NPs exhibit significant mucin adhesion. The $AUC_{(0-\infty)}$ was increased up to 1.47 fold and clearance was decreased up to 7.4-fold as compared to marketed suspension. The PK–PD and pharmacokinetic simulation was carried out to estimate optimum dosing regimen for good efficacy. Thus, lecithin/chitosan NPs could be considered useful approach aiming to prolong ocular residence and reduce dosing frequency.

1. Introduction

According to the World Health Organization (WHO), corneal diseases are major cause of vision loss and blindness, second only to cataract (Whitcher et al., 2001). Corneal fungal infection is frequently caused by species of *Fusarium, Aspergillus, Curvularia*, and *Candida*. It has serious consequences even blindness and needs immediate medical intervention (Asbell and Stenson, 1982; Shukla et al., 2008).

Natamycin (NAT), a polyne antifungal drug, has been considered as the drug of choice. The NAT is only available antifungal drug that has been approved for filamentous FK by the U.S. Food and Drug Administration. The current NAT formulation and dosage regimen consists of 5% (w/v) ophthalmic suspension instilled in the conjunctival sac at hourly or two-hourly intervals for several days. The therapy is maintained for at least 4–6 weeks to get complete relief (Mathews and Kuriakose, 1995; Shukla et al., 2008). Only 1–5% of the applied drug is available to the ocular tissue (Bourlais et al., 1998; Davies, 2000; Nagarwal et al., 2009). The poor bioavailability of suspension eye drop is due to poor permeability of cornea, tear turnover, nasolacrimal drainage effect and metabolic degradation. Thus, marketed eye drops are often less effective and require frequent application; a major proportion of the drug eliminated through conjuctiva to nasal passage tear flow, may reach systemic circulation or gastro-intestinal track, causing undesirable side effects and loss of drug activity. The frequent dosing schedule as described above is often difficult to achieve, resulting in sub-optimal drug concentration causing treatment failure and emergence of drug resistant fungal infection. The detailed ocular pharmacokinetic and rational PK–PD based dosage regimen design has not been reported in literature.

Various ocular drug delivery as hydrogels, micro-particles, NPs, liposomes, insert, in situ gelling system and other colloidal systems, as well as solid inserts and shields, or surgically applied polymeric implants have been proposed for prolonging the release of drugs and enhancing corneal bioavailability (Bourlais et al., 1998; Choy et al., 2008; Gupta et al., 2000; Ibrahim et al., 2010; Joshi, 1994; Kawakami et al., 2001; Wadhwa et al., 2010; Zimmer et al., 1994). However such specific formulation designed for ocular application of NAT has not been reported in literature. Present efforts in ocular drug delivery are focused on prolonging the contact of NAT with the ocular surface, prolonging the release of drugs, maintaining drug concentration above MIC and enhance corneal availability. To meet the specific requirements of ocular delivery, cationic polyelectrolyte chitosan (CS) and lecithin were chosen as constitutive materials. There are few reports in the literature on the use of CS for ocular drug delivery significantly increased the ocular bioavailability (Alexandridis and Alan Hatton, 1995; Calvo et al., 1997; Chetoni

* Corresponding author. Tel.: +91 522 2612411 18x4277; fax: +91 522 2623405.
E-mail address: rabi.cdri@gmail.com (R.S. Bhatta).

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijpharm.2012.04.060

et al., 2000; de Campos et al., 2004; Jiao, 2008; Pepic et al., 2010). The CS exhibits several favorable biological properties like biodegradability, non-toxicity, biocompatibility and mucoadhesiveness that make it an interesting polymer for use in pharmaceutical formulations (Enriquez de Salamanca et al., 2006; Felt et al., 1999; Gratieri et al., 2011; Smart, 2005). Lecithin is a natural mix of phospholipids and is considered a safe and biocompatible excipient (Batzri and Korn, 1973; Senyigit et al., 2011). Lecithin being lipophilic in nature may result in higher lipophilic drug loading and sustains release.

The aim of present study was to prepare sustain release mucoadhesive lecithin/chitosan (L/C) NPs as a new drug delivery for NAT ocular instillation. Clinically NAT is administered as suspension, which has a drawback of rapid clearance from eye. We developed and characterized NAT loaded NPs as an attempt for improving its availability in ocular tissues and improve efficiency to treat fungal keritiitis (FK). The applicability of NPs was determined by evaluating pharmacokinetic and PK–PD indices of NPs with clinically applied and widely used commercially available Natamet® suspension (NTM). We also report the ocular pharmacokinetic profile and rational pharmacokinetic based ocular dosage regimen design of NAT in preclinical *NZ* rabbit model.

2. Materials and methods

2.1. Materials

Natamycin was a kind gift from Cipla Ltd. (Mumbai, India). Lecithin (Phospholipon 50) was generous gift from Lipoid AG (Ludwigshafen, Germany). Low molecular weight CS (specifications: MW 50–190 KDa, deacetylation degree 75–85%, viscosity 20–300 cP) and mucin (type II: partially purified, from porcine stomach), MOPS-buffer and glutamine were obtained from Sigma, USA. Natamycin ophthalmic suspension USP Natamet® (NTM) was purchased from local pharmacy store. Calibrated glass capillary (microcaps) of 10 μL was obtained from Dummond Scientific Co., USA. Ultrapure water (18.2 MΩcm) was obtained from a Milli-Q PLUS PF water purification system. All other analytical grade reagents and salts were obtained from standard commercial suppliers.

2.2. Preparation of NPs

NAT-loaded NPs with varying L/C ratio (20:1, 10:1 and 5:1, w/w) was prepared by ionic gelation method (Sonvico et al., 2006). Briefly, Lecithin was dissolved in methanol at a concentration of 2.5% (w/v) containing 0.2% (w/v) of NAT. The CS was solubilized in aqueous 1% (v/v) acetic acid at a concentration of 1% (w/v). The NPs suspensions were obtained by injection of 4 mL methanolic lecithin/NAT solution (syringe inner needle diameter 0.75 mm) into 46 mL of water-diluted CS solutions that were magnetically stirred at 1000 rpm. In all preparations, the NAT to CS weight ratio was kept constant and all systems presented an acidic pH value (4.5 ± 0.05). Blank NPs (without drug) were prepared following the same procedure. The NPs suspension was centrifuged at 3000 × *g* for 30 min in order to separate the possible NAT/polymer precipitated in the preparation process. Further supernatant was ultracentrifuged at 100,000 × *g* for 1 h in order to separate the NPs.

2.3. Physico-chemical characterization of NPs

2.3.1. Particle size and zeta potential

NPs size distribution and zeta potential were determined using Photon Correlation Spectroscopy with a Zetasizer 3000 (Malvern Instruments, Malvern, UK). The size distribution analysis was performed at a scattering angle of 90° and at a temperature of 25 °C. The samples appropriately diluted with ultrapure water, whereas zeta potential was measured by electrophoretic light scattering using a disposable zeta cuvette. All measurements were performed in triplicate and results are presented as mean ± SD.

2.3.2. Fourier transforms infra-red spectroscopy (FT-IR)

FT-IR spectra of freeze-dried NPs were obtained with a PerkinElmer 1600 spectrophotometer using the potassium bromide (KBr) disk technique. FT-IR spectra of NAT loaded NPs and their pure components were compared for evaluation drug–excipient and excipient–excipient chemical interaction.

2.3.3. Entrapment efficiency (EE) and drug loading (DL) capacity

Quantitative determination of NAT from NPs was conducted by HPLC assay. The separation was achieved on Spheri-5, Cyano column (30 mm × 4.6 mm, 5 μm) with a mobile phase consisting of acetonitrile: 10 mM sodium acetate buffer (25:75, v/v) at a flow rate of 1.0 mL/min. The detection wavelength was 303 nm. The retention time of NAT and amphotericin B (IS) were 5.33 and 12.5 min, respectively. The calibration curves were designed over the range 0.3–20.0 μg/mL (r^2 = 0.999). The HPLC method was validated as per ICH guidelines (ICH and Guidelines, 2005). In order to separate the entrapped NAT from free drug, freshly prepared NPs were subjected to ultracentrifugation (BECKMAN COULTER, ultracentrifuge, USA) 36,000 rpm at 4 °C for 1 h. The supernatant containing the free drug was withdrawn for HPLC analysis as described above. Total amount of drug incorporated in the NPs was determined by polymer disruption with acidic methanol followed by HPLC quantitation. All analyses were performed in triplicate.

The EE and DL were determined using the following equations:

$$\text{EE (\%, w/w)} = \frac{(\text{Total amount of drug} - \text{Amount of drug in solution})}{\text{Total amount of drug used}} \times 100$$

$$\text{DL (\%, w/w)} = \frac{(\text{Total amount of drug} - \text{Amount of drug in solution})}{\text{Total amount of formulation components}} \times 100$$

2.3.4. Morphology of NPs

Morphological evaluation of the NPs was performed using negative staining transmission electron microscopy technique. Around 6 μL of the suspension was applied to glow discharged pioloform coated Cu grids and allowed to adsorb for 2 min. After blotting off excess solution, the grids were stained with 1% phosphotungstic acid pH 7.0. The grids were observed under a FEI Tecnai 12 TWIN transmission electron microscope equipped with a MegaView II CCD camera and analyzed using analySIS software at 80 kV.

2.3.5. Stability of NPs

The stability of NPs in different storage conditions was evaluated. The samples were sealed in closed amber-colored glass vials and stored at 4 ± 2 °C and 25 ± 2 °C for a period of 60 days. The vials kept away from exposure to direct light to avoid photo degradation and at predefined intervals aliquots of samples were withdrawn for particle size, zeta potential and encapsulation efficiency.

2.3.6. Mucoadhesive evaluation: zeta potential and turbidimetry evaluation

In this study, the mucoadhesive properties of NPs were evaluated by measuring the changes of zeta potential on interaction with negatively charged mucin (de Campos et al., 2004). The NPs were incubated at 35 °C in 0.1% (w/w) aqueous mucin dispersion. The zeta potential of the NPs were measured upto 6 h during incubation. The alteration of zeta potential of the NPs indicates interaction with mucin.

Turbidity of NPs spiked mucin aqueous dispersion was compared with native mucin at 650 nm by UV–vis spectrophotometer (Thermo Scientific, USA) (Yoncheva et al., 2009). The accurately weighed NPs (10 mg) were added to 10 mL aqueous mucin dispersion (0.1%, w/v) and stirred at 200 rpm. The increase in turbidity of mucin NPs dispersion indicated mucoadhesive property.

2.4. In vitro studies of NPs

2.4.1. In vitro drug release from NPs

The release of NAT from NPs was assessed using a dialysis bag under sink condition for 7 h. Samples of the NPs (1.0 mL) containing 1.0 mg NAT, were enclosed in dialysis bags (cellulose membrane, mw cut-off 12400, Sigma), were incubated in 30 mL of phosphate-buffer solution (PBS), pH 7.4 at 37 °C under mild agitation in a water bath. In order to increase its NAT solubility and maintain sink condition, 1.0% (v/v) tween 80 was added in to dissolution medium. Aliquots (1.0 mL) were collected from the vials at predetermined intervals and replaced with equal volume of fresh buffer medium to maintain sink conditions. The amount of the drug in the receiving solution, was analyzed by HPLC as described above. Clinically used preparation (NTM) was evaluated by same dissolution condition and compared with the NPs for their sustain release potential. All release experiments were done in triplicate.

2.4.2. In vitro antifungal activity

The NPs, NTM, and free NAT in DMSO were examined for antifungal activity by testing against *Candida albicans* (MTCC 183) and *A. fumigatus* (Patient isolated) by minimum inhibitory concentration (MIC_{90}) and by disk diffusion (DD) method. These procedures were adapted directly from NCCLS protocols (NCCLS, 2003).

2.4.2.1. MIC assays. The influence of the NAT encapsulated NPs on the antifungal activity was evaluated in terms of MIC_{90}. Sample tests were performed in 96-well plates using serial dilutions of NAT in by adding known concentration of the NPs suspensions to RPMI growth medium with L-glutamine and MOPS-buffer, pH 7.0 for a total volume of 1 mL in each well. The final concentrations of NPs used in each line of wells were range of 0.39–50 μg/mL. Some wells served as the growth control and sterility check. Cell suspensions of *C. albicans* and *A. fumigatus* were prepared in RPMI 1640 medium and adjusted to give final inoculums concentration of 1.0×10^3, -5.0×10^3 and 0.4×10^4, -5.0×10^4 CFU/mL, respectively. The fungus suspension (100 μL) was added to each well, resulting in the desired final drug concentration and inoculums size. The microplates were incubated at 35 °C for 24 h (*C. albicans*) or 48 h (*A. fumigatus*). A solvent control, organism control and medium control were performed simultaneously to check the growth inhibiting activity, organisms and sterility of broth medium.

2.4.2.2. Disk diffusion (DD) assay. The agar-based DD assay is an alternative way to check antifungal susceptibility. The disks of NPs, NTM and free NAT in DMSO were prepared by pipetting appropriate volumes of stock solutions onto the sterile blank disks (Himedia Laboratories), to make 10, 25 and 50 μg/disks. The disks were dried. A layer of nutrient agar (20 mL) seeded with the test microorganism (0.2 mL) was allowed to solidify in the petri plate. These disks were placed on the solidified agar layer with the help of a sterile forcep. After keeping Petri plates at room temperature for 4 h, the plates were incubated at 37 °C for 24 h. The diameter of the zone of inhibition was measured by an antifungal zone finder.

2.5. In vivo studies

The animal studies were carried out as per the approval and guidelines of the Institutional Animal Ethics Committee (IAEC). Male *NZ*-rabbits weighing 2.0–2.5 kg were used in the studies.

2.5.1. Ocular irritation studies

The ocular irritancy and damaging effects of the NPs and NTM were evaluated according to a modified Draize test (Wilhelmus, 2001). Test substance (20 μL) was instilled directly to the cornea in the right eye every 30 min for 6 h (12 treatments). Left eye served as control and were treated with distilled water. At the end of the treatment, six observations at 12 h intervals were carried out to evaluate the ocular tissues. The congestion, swelling, discharge, and redness of the conjunctiva were graded on a scale from 0 to 3, 0 to 4, 0 to 3 and 0 to 3 respectively. Irritation and corneal opacity were graded on a scale from 0 to 4.

2.5.2. In vivo ocular retention and pharmacokinetics studies

The ocular pharmacokinetics of NAT administered as NPs and NTM was evaluated at dose strength of 1% (w/v) and 5% (w/v), respectively. During the experiments the rabbits were placed in restraining cage, and their heads and eyes movements were not restricted. Briefly, the rabbits were given an instillation of 20 μL of each formulation into the lower conjunctival sac of left cornea using a micropipette without actually touching the eyes and irritating the corneal surface. Food and water intake were free during the study. The rabbit lachrymal fluid (10 μL) was withdrawn from the conjunctival sac by calibrated glass capillary at 5, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min post dose. They were stored in micro-centrifuge tubes at −20 °C until analysis. The amount of NAT in lachrymal fluid was determined by previously reported a highly sensitive LC-MS/MS method which required only ~10–20 μL of lachrymal sample (Bhatta et al., 2010).

2.5.3. Pharmacokinetics/pharmacodynamic (PK/PD) indices

The PK/PD indices are recognized as key factor in determining the selection of treatment and dosage regimen for antifungal agent such as NAT, exhibiting concentration dependent killing. Although the standard parameter of antifungal activity (MIC) is useful, it does not provide information about *in vivo* effective concentrations in tissues. Based on the C_{max} and $AUC_{(0-24)}$ values obtained form NPs and NTM in tears following a single topical ocular administration to rabbits, the resulting PK/PD indices C_{max}/MIC_{90}, $AUC_{(0-24)}/MIC_{90}$ ratios and $T > MIC$ were calculated using the MIC_{90} values.

For effective antimicrobial activity C_{max}/MIC_{90} and $AUC_{(0-24)}/MIC_{90}$ should be higher than 10 and 125 respectively. If regimens with shorter dosing intervals are more effective, then the time that plasma concentrations remain above the MIC ($T > MIC$) is considered to be more critical than C_{max}/MIC_{90} or AUC/MIC_{90} for dosing efficacy (Andes et al., 2003; Lewis, 2007; Venisse et al., 2008).

2.6. Statistical, pharmacokinetic data analysis and dose simulation

Statistical data analysis was performed using the student *t*-test with $p < 0.05$ as the minimal level of significance. Ocular pharmacokinetic parameters of NAT in lachrymal fluid were derived from tear concentration–time profile using WinNonlin software Ver

5.1. Simulation of lachrymal concentration–time profile at different dosing interval was evaluated by principle of superimposition using Microsoft excel software. The NAT concentration at several time intervals was estimated graphically from concentration–time profile. The subsequent dosing was based on the time where the lachrymal concentration was maintained twice the MIC_{90} i.e. 3.12 μg/mL.

3. Results and discussion

3.1. Preparation and characterization of NPs

The NPs were characterized in term of size, zeta-potential and drug loading capacity (Table 1). The lecithin/chitosan ratio has significant impact on particle size, encapsulation efficiency and zeta potential. The increase in the lecithin content in the NPs resulted in significant decrease in particle size, zeta potential and increase in encapsulation efficiency. However insignificant difference in polydispersity index was observed, indicating uniform particle shapes. The increase in encapsulation efficiency was may be due to higher amount of partition of lipophilic NAT to lecithin core, and decrease in zeta potential was due to increase in negative potential with increasing lecithin concentration. The decreasing positive charge results in low affinity for negatively charged mucin for mucoadhesion. To counter the balance between encapsulation efficiency and zeta potential L/C ratio of 10:1 was found to be appropriate with particle size of 213 nm, 73.57% encapsulation efficiency with a theoretical drug loading of about 5.09%, polydispersity index of 0.186 and more importantly zeta potential of +43 for mucoadhesion. Particle size is an important physical property of NPs directly affecting the cellular uptake capabilities and ultimately biodistribution. The ophthalmic administration of particles of higher size can result in an irritation, discomfort and high ocular clearance. In general, the upper size limit for carriers for ophthalmic administration is about 5–10 μm (Ali and Lehmussaari, 2006).

The TEM images (Fig. 1) showed that NPs were roughly spherical and sub-spherical in shape, suggesting possible stabilization by positive surface charges.

Drug–excipient and excipient–excipient interactions have been studied by FT-IR (Fig. 2). The characteristic FT-IR band of CS due to $-NH_2$ scissoring vibration at 1591 cm^{-1} was not present in NPs

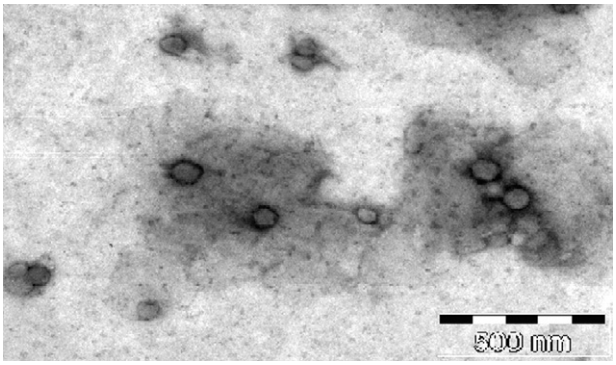

Fig. 1. Representative TEM image of NAT loaded NPs (L/C weight ratio of 10:1). Size bar 500 nm.

spectra. This is may be due to the ionic interaction of the amino groups of CS with the phosphate groups of lecithin. The absorption band of the phosphate group of lecithin shifted from 1245 cm^{-1} in the lecithin to 1216 cm^{-1} in the NPs sample. This was indicative to the fact that ionic interactions between phosphate groups of lecithin and amino groups of CS have taken place. On the contrary; the typical absorption peak of the CS amino group was still present at 1648 cm^{-1}. Moreover, the stretching of the carbonyl groups of the fatty acids the absorption band at 1737 cm^{-1} in the NPs spectrum.

In the compatibility study of NAT and formulation component alone, and NAT loaded NPs, characteristic peaks of NAT in FTIR spectra were obtained, that is NH_3^+ stretching around 3289 cm^{-1}, CH=CH at 1575 cm^{-1}, and —CH (1440–1500 cm^{-1}). This infers two facts: first, the FTIR curve obtained is similar to that of pure drug and excipients confirming that there is no interaction between formulation component and NAT.

3.2. Stability

The physicochemical stability of NPs at 4 ± 2 °C and 25 ± 2 °C for 30 and 60 days showed no evidence of aggregation or precipitation, indicating good stability of the NPs (Table 2). At 4 and 25 °C after 60 days, insignificant change in the particle size and zeta potential

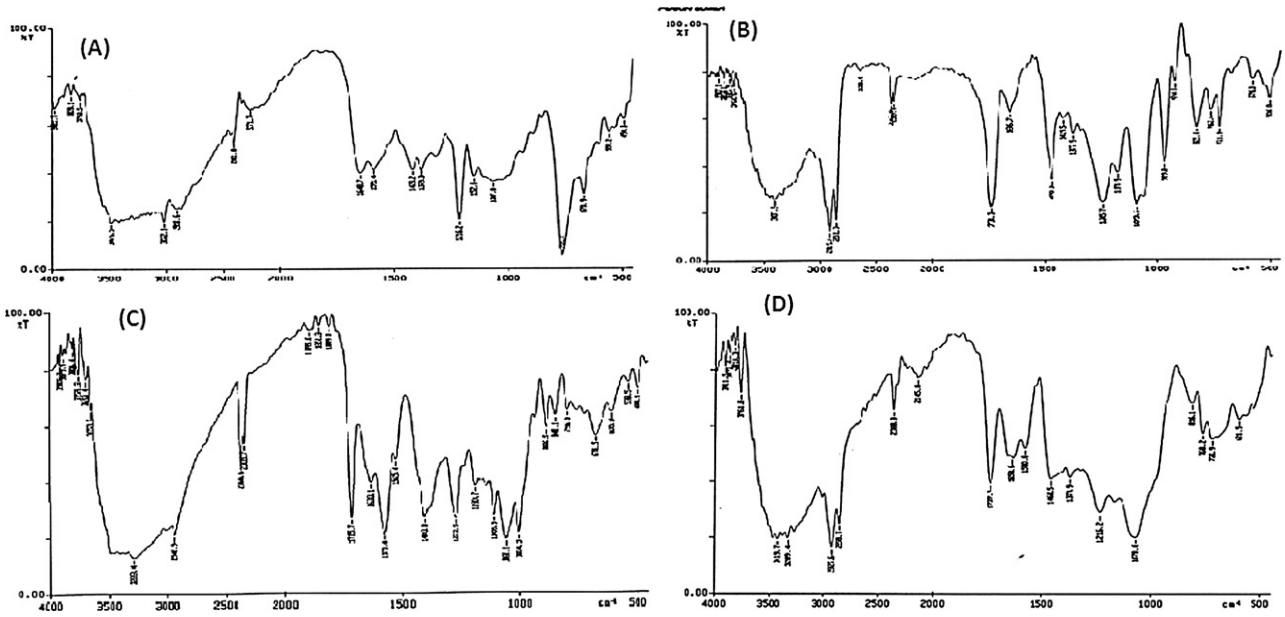

Fig. 2. FT-IR spectra of (A) chitosan, (B) Lecithin, (C) NAT, (D) NAT loaded NPs.

Table 1
Physico-chemical characterization of drug-loaded NPs with different L/C ratio.

L/C (w/w)	Particle size (nm)	PDI	Zeta potential (mV)	EE (%)
5/1	284.96 ± 4.02	0.225 ± 0.02	50.06 ± 2.31	60.06 ± 1.25
10/1	213.83 ± 2.02^{+}	0.186 ± 0.03*	43.83 ± 1.80^{+}	73.57 ± 0.62^{+}
20/1	191.83 ± 2.02^{+}	0.190 ± 0.02*	33.23 ± 1.75^{+}	78.29 ± 0.50^{+}

*Not significant different. $^{+}$Significant different, $P > 0.05$. All data represent mean ± SD, $n = 3$.

Table 2
NAT loaded NPs (L/C 10:1) stability at 30 and 60 days in different storage conditions.

Temperature	Particle size (nm)	Zeta potential (mV)		EE (%)		
	30 days	60 days	30 days	60 days	30 days	60 days
25 °C	215.16 ± 4.8*	217.85 ± 4.2* 22	42.96 ± 2.7*	42.19 + 1.1*	71.26 ± 0.7^{+}	68.09 ± 2.7^{+}
4 °C	214.73 ± 2.2*	215.66 ± 5.3*	43.36 ± 1.5*	42.59 + 2.3*	72.13 ± 0.9*	70.06 ± 1.3^{+}

*Not significant different from 0 day (Table 1). $^{+}$Significant different from 0 day (Table 1). $P > 0.05$. All data represent mean ± SD ($n = 3$).

was observed. However encapsulation efficiency was significantly decreased probably due to leaching of NAT from the NPs.

3.3. Mucoadhesive

The electrostatic interaction is the most expectable mucoadhesive mechanism. The decrease in zeta potential for a suspension of NPs before and after incubation with mucin supports this observation (Fig. 3). This is may be due to interaction of negatively charged sialic groups of mucin with positively charged surface layer of NPs. The surface charge of NPs reduced after 6 h incubation with mucin. This reduction could be attributed to the ionic interaction between the negatively charged mucin particles and NPs. Therefore, it can be concluded that the NPs are able to interact with mucin due to ionic interaction.

Turbidity of NPs/mucin aqueous dispersions was examined aiming to obtain preliminary information about the mucoadhesiveness. The absorbance of the mucin-free dispersions of NPs did not significantly deviate from zero (0.053–0.065). Changes in the turbidity of NPs/mucin dispersions should be considered as an indication for an eventual interaction between NPs and mucin, and not due to the motion of particles. The turbidity of NPs/mucin dispersions was higher than the turbidity of mucin dispersion itself (Fig. 4).

3.4. In vitro release

In vitro drug release of NPs and NTM in the simulated ocular circumstances (37 °C, pH 7.4) was studied (Fig. 5). The NPs showed a two-step release pattern: one initial burst release about 10–15.0% of NAT followed by a second slow-release phase. An initial burst release is beneficial in terms of antifungal activity as it helps to achieve the therapeutic concentration of drug in minimal time followed by constant release to maintain sustained and controlled release of the drug. The burst release was mainly due to desorption and diffusion of the drug from the surface. It was supposed that the smaller particles possessed more specific surface area and the drug delivery process would be facilitated. The NAT released from NPs was ~41.23% in 2 h and ~64.22% in 7 h.

Evaluation of the release profiles of NTM showed that almost all the NAT was released within 2 h after start of the study, suggesting that the developed NPs can be used as an important platform for sustained drug release.

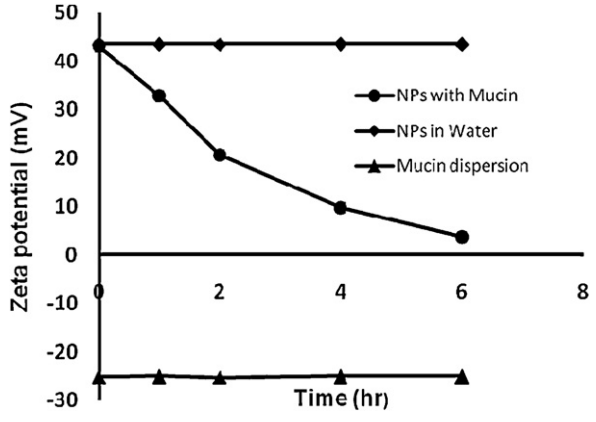

Fig. 3. Estimation of the zeta-potential of the NPs during incubation in 0.1% aqueous mucin dispersion. The charge of NPs in mucin free aqueous dispersion was used as a reference (mean ± SD, $n = 3$).

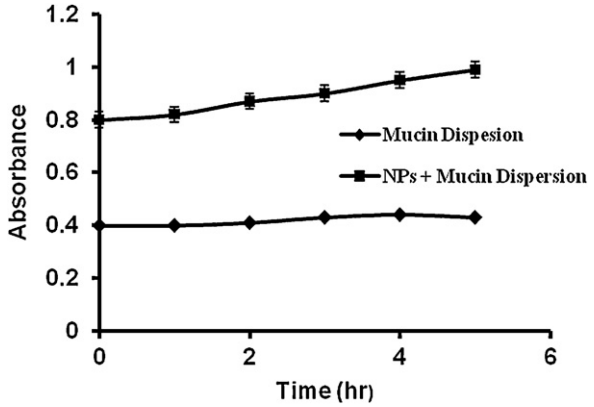

Fig. 4. Estimation of the interaction between NPs and mucin by turbidimetric assay (mean ± SD, $n = 3$).

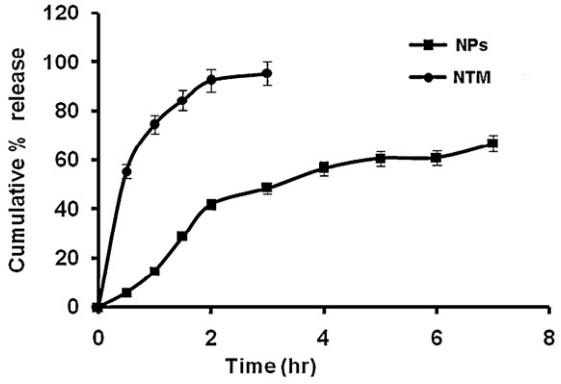

Fig. 5. *In vitro* release profiles of the optimized NPs (L/C 10:1, w/w) and NTM.

Table 3
In vitro antifungal susceptibility by MIC_{90} and disk diffusion method, each value represents the mean ± SD ($n = 3$).

Fungal strain	Test item	MIC_{90} (μg/ml)	Zone diameter in mm		
			10 μg	25 μg	50 μg
Candida albicans (MTCC 183)	NAT	3.12	10.19 ± 0.72	14.36 ± 0.61	21.36 ± 0.69
	NPs	3.12	8.25 ± 0.52	11.40 ± 0.36	14.88 ± 0.46
	NTM	3.12	8.26 ± 0.33	10.41 ± 0.15	14.07 ± 0.99
Aspergillus fumigates (Isolated from patient)	NAT	1.56	12.33 ± 0.20	16.28 ± 0.65	25.79 ± 0.23
	NPs	1.56	9.18 ± 0.16	13.66 ± 0.77	15.54 ± 0.28
	NTM	1.56	8.63 ± 0.35	12.25 ± 0.64	15.17 ± 0.51

Table 4
Pharmacokinetic estimation of NAT in precorneal region of *NZ* rabbits after topical instillation of NPs and NTM, each value represents the mean ± SD ($n = 3$).

PK parameters	Units	NPs (1%, w/v)	NTM (5%, w/v)	*P* value
$AUC_{0-\infty}$	min μg/mL	13348.90 ± 1293.48	9047.35 ± 132.15	0.0046
$t_{1/2}$	min	56.04 ± 3.74	10.85 ± 1.39	<0.0001
C_{max}	μg/mL	164.94 ± 7.87	584.56 ± 81.08	0.0009
Cl	mL/min	0.015 ± 0.0015	0.111 ± 0.0015	<0.0001
MRT	min	80.85 ± 5.39	15.66 ± 2.00	<0.0001

3.5. *In vitro* antifungal activity NPs

In order to assess the antifungal activity of NPs and compare it with NTM and free NAT in DMSO, the MIC_{90} and zone of inhibition was determined (Table 3). The MIC_{90} of NPs showed similar antifungal activity as NTM and free NAT against *C. albicans* and *Aspergillus fumigates*. The zone of inhibition by the NPs as compared to marketed suspension was almost similar or higher. Results revealed antifungal activity of developed NPs formulation was comparable with marketed ophthalmic suspension.

3.6. Ocular irritation evaluation

The *in vivo* results showed no sign of irritation or damaging effects in the cornea, conjunctiva or iris. The scores for conjunctiva swelling and discharge were always grade zero. Iris hyperemia and corneal opacity scores were grade zero at all observations. Therefore, the potential clinical interest of the L/C NPs is supported because of the absence of irritant effects *in vivo*.

3.7. *In vivo* precorneal retention, ocular pharmacokinetics and PK–PD indices

Pharmacokinetic studies was designed to evaluate the potential of L/C NPs in the terms of prolong residence at the target site (cornea), optimal therapeutic concentration at cornea with low unit dose and higher antifungal efficiency compared to NTM.

The ocular pharmacokinetic of NAT administered as marketed suspension (NTM 5%, w/v) was compared with NPs 1% (w/v) formulation in *NZ* rabbit (Fig. 6 and Table 4). The dose volume for both the formulation was same, but strength was different. This disparity in the dose strength was selected to evaluate dose lowering potential of NPs and its efficient use. Another objective was to achieve an optimum C_{max} (not very high as compared to NTM) so as to reduce the naso-lacrimal elimination of NAT, avoiding any adverse effect on repeated administration. In comparison to the marketed suspension, the NPs formulation exhibits significant enhancement of $AUC_{(0-\infty)}$ (~1.47-fold) and clearance was significantly decreased (~7.4-fold). MRT of NPs were significantly higher than NTM. Thus, the positively charged NPs can provide a binding force to the eye surface. Nevertheless, the bioadhesion of CS is not exclusively determined by the positive charge. It could also be promoted by the presence of free $-NH_2$ and $-OH$ groups of CS molecules which form hydrogen bonds to the mucin of eye surface.

Table 5
The estimated PK–PD indices of NPs and NTM after single ocular instillation in *NZ* rabbit.

PK/PD indices	Units	NPs (1%, w/v)	NTM (5%, w/v)
C_{max}/MIC	unitless	52.28	182.67
AUC/MIC	min	417.15	253.14
AUC above MIC	min μg/mL	12340.99	8018.63
T above MIC	min	227.76	161.98

For efficient antifungal activity, drug concentration has to be maintained above MIC for a required period. High drug concentration for a short period of time may not have good efficacy due to unavailability of drug for at least three life cycles of microbes at the target site. Both *in vitro* MIC and pharmacokinetic parameters evaluated as PK–PD indices, which is used for predicting antifungal therapeutic efficacy. PK–PD indices C_{max}/MIC and AUIC of both the formulations were greater than 10 and 125, indicating optimal therapeutic effectiveness at single dose. The C_{max} of NPs was significantly lower than market preparation however it was ~52.28 fold higher than MIC (C_{max}/MIC > 10, Table 5). The drug may be non-toxic at minimum effective concentration, increase in concentration upon topical administration may induce irritation or toxic response. The NTM exhibits high tear concentration (Fig. 6) and high clearance, which may results in greater amount of drug reaching naso-lacrimal region, thus a possibility of higher systemic or gastrointestinal adverse effect as compared to NPs. The total NAT available above the MIC (AUC above MIC) by NPs was 1.53 fold higher than NTM. The time above MIC by NAT-NPs was 1.4 fold higher than NTM. Thus overall there is a possibility of 1/5th

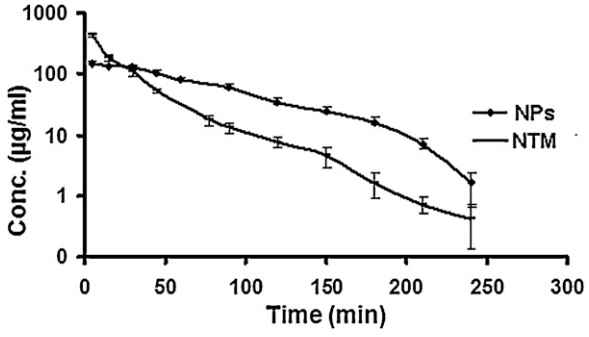

Fig. 6. Tear concentration–time profile of NAT administered as NPs and NTM suspension (mean ± SD, $n = 3$).

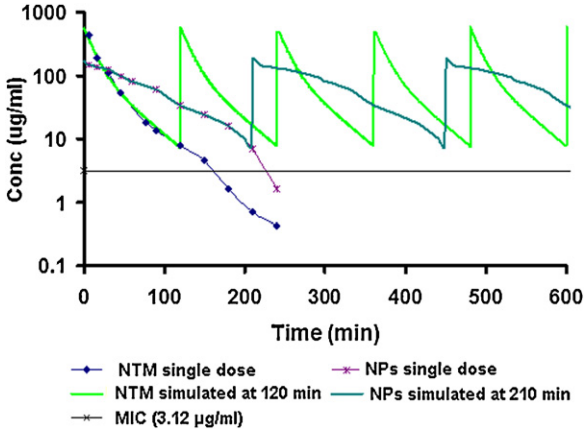

Fig. 7. Simulated ocular concentration time-profile of NAT for 10 h at a dosing interval of 120 min for NTM and 210 min for NPs. Horizontal line represent MIC_{90} value for relevant ophthalmic pathogens.

reduction in ocular loading dose with 1.4 fold reduction in dosing frequency as compared to NTM formulation.

The PK/PD indices estimates of both NPs and NTM indicate clinical effectives. However the AUC/MIC, AUC above the MIC and *T* above the MIC of NPs were higher than NTM, indicating the better efficacy. The reduced dose of NAT by NPs than NTM has resulted in low C_{max} of NPs. However, 1% (w/v) NPs dose was sufficient for optimum efficacy (as C_{max}/MIC > 10) and may also results in low ocular and nasso-lachrymal toxicity. Using the best fit model parameters from this PK model, ocular concentration–time profiles for different formulations of NAT as a function of time were simulated with every 120 min (2 h) for NTM (5%w/v) and 210 min (~3.5 h) for NPs (1%w/v) dosing regimens.

Simulation shows NTM dosing interval should not exceed 150 min (2.5 h) whereas NPs can be administered within 225 min (3.75 h). The ideal dosing interval which could maintain approximate two times the MIC_{90} by NTM and NPs was 120 min and 210 min. The representative concentration–time simulation for 10 h period shows three-instillation of NPs as compared to six-instillation of suspension preparation (Fig. 7) is needed for effective treatment. Thus in conclusion NPs significantly reduce the dose and dosing frequency by maintaining the NAT level above MIC for a prolong period for better efficacy and patient compliance.

4. Conclusion

In the present study, we report the first evidence of NAT loaded-nanoparticle consisting of Lecithin and CS to enhance its precorneal retention, sustain release, high ocular availability at reduce dose and dosing frequency. The NPs obtained can be considered as a self-organized structure, result of the electrostatic interaction of the polycation CS and lecithin, due to the presence of negatively charged components in the lipid mixture. The matrix formed by ionic interactions between lecithin and CS provided both sufficient EE and prominent surface charge-dependent mucoadhesive properties. No ocular damage or clinically abnormal signs in the cornea, conjunctiva or iris were observed. The study also reported ocular pharmacokinetic profile of NAT, which could be utilized for further dosage regimen and formulation design.

This new formulation is a viable alternative to conventional ophthalmic suspension by virtue of its ability to sustain the drug release, for its ease of administration because of reduced dosing frequency resulting in better patient compliance. However, further studies are required for evaluating clinical efficacy.

Acknowledgements

The authors are thankful to Director, CSIR-CDRI, for providing facilities and infrastructure for the study. Authors Y.S.C. and D.K. are also thankful to the Indian Council of Medical Research and MoeS, respectively for fellowship. CSIR-CDRI communication number is 8244.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2012.04.060.

References

Alexandridis, P., Alan Hatton, T., 1995. Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer surfactants in aqueous solutions and at interfaces: thermodynamics, structure, dynamics, and modeling. Colloid Surf. A 96, 1–46.
Ali, Y., Lehmussaari, K., 2006. Industrial perspective in ocular drug delivery. Adv. Drug Deliv. Rev. 58, 1258–1268.
Andes, D., Marchillo, K., Stamstad, T., Conklin, R., 2003. In vivo pharmacokinetics and pharmacodynamics of a new triazole, voriconazole, in a murine candidiasis model. Antimicrob. Agents Chemother. 47, 3165–3169.
Asbell, P., Stenson, S., 1982. Ulcerative keratitis. Survey of 30 years' laboratory experience. Arch. Ophthalmol. 100, 77–80.
Batzri, S., Korn, E.D., 1973. Single bilayer liposomes prepared without sonication. Biochim. Biophys. Acta 298, 1015–1019.
Bhatta, R.S., Chandasana, H., Rathi, C., Kumar, D., Chhonker, Y.S., Jain, G.K., 2010. Bioanalytical method development and validation of natamycin in rabbit tears and its application to ocular pharmacokinetic studies. J. Pharm. Biomed. Anal. 54, 1096–1100.
Bourlais, C.L., Acar, L., Zia, H., Sado, P.A., Needham, T., Leverge, R., 1998. Ophthalmic drug delivery systems–recent advances. Prog. Retin. Eye Res. 17, 33–58.
Calvo, P., Remuñán-López, C., Vila-Jato, J., Alonso, M., 1997. Development of positively charged colloidal drug carriers: Chitosan-coated polyester nanocapsules and submicron-emulsions. Colloid Polym. Sci. 275, 46–53.
Chetoni, P., Panichi, L., Burgalassi, S., Benelli, U., Saettone, M.F., 2000. Pharmacokinetics and anti-inflammatory activity in rabbits of a novel indomethacin ophthalmic solution. J. Ocul. Pharmacol. Ther. 16, 363–372.
Choy, Y.B., Park, J.-H., McCarey, B.E., Edelhauser, H.F., Prausnitz, M.R., 2008. Mucoadhesive microdiscs engineered for ophthalmic drug delivery: effect of particle geometry and formulation on preocular residence time. Invest. Ophthalmol. Vis. Sci. 49, 4808–4815.
Davies, N.M., 2000. Biopharmaceutical considerations in topical ocular drug delivery. Clin. Exp. Pharmacol. Physiol. 27, 558–562.
de Campos, A.M., Diebold, Y., Carvalho, E.L., Sanchez, A., Alonso, M.J., 2004. Chitosan nanoparticles as new ocular drug delivery systems: in vitro stability, in vivo fate, and cellular toxicity. Pharm. Res. 21, 803–810.
Enriquez de Salamanca, A., Diebold, Y., Calonge, M., Garcia-Vazquez, C., Callejo, S., Vila, A., Alonso, M.J., 2006. Chitosan nanoparticles as a potential drug delivery system for the ocular surface: toxicity, uptake mechanism and in vivo tolerance. Invest. Ophthalmol. Vis. Sci. 47, 1416–1425.
Felt, O., Furrer, P., Mayer, J.M., Plazonnet, B., Buri, P., Gurny, R., 1999. Topical use of chitosan in ophthalmology: tolerance assessment and evaluation of precorneal retention. Int. J. Pharm. 180, 185–193.
Gratieri, T., Gelfuso, G.M., de Freitas, O., Rocha, E.M., Lopez, R.F., 2011. Enhancing and sustaining the topical ocular delivery of fluconazole using chitosan solution and poloxamer/chitosan in situ forming gel. Eur. J. Pharm. Biopharm. 79, 320–327.
Gupta, A.K., Madan, S., Majumdar, D.K., Maitra, A., 2000. Ketorolac entrapped in polymeric micelles: preparation, characterisation and ocular anti-inflammatory studies. Int. J. Pharm. 209, 1–14.
Ibrahim, H.K., El-Leithy, I.S., Makky, A.A., 2010. Mucoadhesive nanoparticles as carrier systems for prolonged ocular delivery of gatifloxacin/prednisolone bitherapy. Mol. Pharm. 7, 576–585.
ICH, Guidelines, 2005. International Conference of Harmonization, Q2 (R1) Validation of Analytical Procedures: Text and Methodology.
Jiao, J., 2008. Polyoxyethylated nonionic surfactants and their applications in topical ocular drug delivery. Adv. Drug Deliv. Rev. 60, 1663–1673.
Joshi, A., 1994. Microparticulates for ophthalmic drug delivery. J. Ocul. Pharmacol. 10, 29–45.
Kawakami, S., Nishida, K., Mukai, T., Yamamura, K., Nakamura, J., Sakaeda, T., Nakashima, M., Sasaki, H., 2001. Controlled release and ocular absorption of tilisolol utilizing ophthalmic insert-incorporated lipophilic prodrugs. J. Control. Release 76, 255–263.
Lewis, R.E., 2007. Pharmacodynamic implications for use of antifungal agents. Curr. Opin. Pharmacol. 7, 491–497.

Mathews, M.S., Kuriakose, T., 1995. Keratitis due to Cephaliophora irregularis Thaxter. J. Med. Vet. Mycol. 33, 359–360.
Nagarwal, R.C., Kant, S., Singh, P.N., Maiti, P., Pandit, J.K., 2009. Polymeric nanoparticulate system: a potential approach for ocular drug delivery. J. Control. Release 136, 2–13.
NCCLS, 2003. NationalCommittee for Clinical Laboratory Standards, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically-sixth edition: approved Standard M7-A6, USA.
Pepic, I., Hafner, A., Lovric, J., Pirkic, B., Filipovic-Grcic, J., 2010. A nonionic surfactant/chitosan micelle system in an innovative eye drop formulation. J. Pharm. Sci. 99, 4317–4325.
Senyigit, T., Sonvico, F., Barbieri, S., Ozer, O., Santi, P., Colombo, P., 2011. Lecithin/chitosan nanoparticles of clobetasol-17-propionate capable of accumulation in pig skin. J. Control. Release 142, 368–373.
Shukla, P.K., Kumar, M., Keshava, G.B., 2008. Mycotic keratitis: an overview of diagnosis and therapy. Mycoses 51, 183–199.
Smart, J.D., 2005. The basics and underlying mechanisms of mucoadhesion. Adv. Drug Deliv. Rev. 57, 1556–1568.
Sonvico, F., Cagnani, A., Rossi, A., Motta, S., Di Bari, M.T., Cavatorta, F., Alonso, M.J., Deriu, A., Colombo, P., 2006. Formation of self-organized nanoparticles by lecithin/chitosan ionic interaction. Int. J. Pharm. 324, 67–73.
Venisse, N., Gregoire, N., Marliat, M., Couet, W., 2008. Mechanism-based pharmacokinetic-pharmacodynamic models of in vitro fungistatic and fungicidal effects against *Candida albicans*. Antimicrob. Agents Chemother. 52, 937–943.
Wadhwa, S., Paliwal, R., Paliwal, S.R., Vyas, S.P., 2010. Hyaluronic acid modified chitosan nanoparticles for effective management of glaucoma: development, characterization, and evaluation. J. Drug Target. 18, 292–302.
Whitcher, J.P., Srinivasan, M., Upadhyay, M.P., 2001. Corneal blindness: a global perspective. World Health Organ 79, 214–221.
Wilhelmus, K.R., 2001. The Draize eye test. Surv. Ophthalmol. 45, 493–515.
Yoncheva, K., Vandervoort, J., Ludwig, A., 2009. Development of mucoadhesive poly(lactide-co-glycolide) nanoparticles for ocular application. Pharm. Dev. Technol. 16, 29–35.
Zimmer, A.K., Zerbe, H., Kreuter, J., 1994. Evaluation of pilocarpine-loaded albumin particles as drug delivery systems for controlled delivery in the eye I. In vitro and in vivo characterisation. J. Control. Release 32, 57–70.