

Exploring the potential of complex-vesicle based niosomal ocular system loaded with azithromycin: Development of *in situ* gel and *ex vivo* characterization

Nida Akhtar¹, Rahul Kumar Singh¹, Kamla Pathak^{2*}

¹Department of Pharmaceutics, Rajiv Academy for Pharmacy, P.O. Chhatikara, Mathura- 281001, Uttar Pradesh, India

²Department of Pharmaceutics, Pharmacy College Saifai, Uttar Pradesh University of Medical Sciences, Saifai, Etawah-206130, Uttar Pradesh, India

Received: Feb13, 2017, Revised: Mar14, 2017, Accepted: Apr20, 2017

Abstract

Bacterial conjunctivitis characterized as pink eye referred as an inflammation of an eye caused by the enlargement of blood vessels present in conjunctiva, resulting in a red or bloodshot appearance of the eyes. Topical ocular delivery is found to be useful in treating conjunctivitis, but to maintain an effective drug concentration at a site of action in order to achieve desired pharmacological action is highly challenging. Thus, keeping in mind this limitation niosomal carrier was designed to provide localized drug delivery with enhanced residence time. Thus, the present investigation was targeted to explore the utility of niosomes loaded with azithromycin- β -CD complex. Azithromycin- β -CD complex was prepared and niosomes containing this complex were developed based on 3² full factorial design using ether injection method and characterized. Optimized niosomal formulation (NF2) was selected on the basis of minimum vesicle size (306 ± 3.05 nm), polydispersity index (0.115 ± 5.51), maximum zeta potential (45.3 ± 0.25 mv), entrapment efficiency (78.17 ± 1.81 %) and % CDR (73.09 ± 2.10). Optimized formulation was then formulated in the form of *in situ* gel (temperature sensitive) and evaluated. Optimized formulation [*in situ* gel (NG-5)] was found to exhibit superior *in vitro* drug release profile in comparison to Zithromax® eye drop. Better *in-vitro* mucoadhesive strength was observed and formulation was found to be non-irritant to the sclera surface. Thus, it can be put into conclusion that temperature-sensitive niosomal *in situ* ocular gel possessed increased residence time and provide localized drug delivery effective for the treatment of bacterial conjunctivitis.

Keywords: β -CD, conjunctivitis, eye, penetration, residence time, vesicular carrier

Pharm Biomed Res 2017; 3(1): 22-33

Introduction

Azithromycin is commercially available as eye drops in a dose of 0.5 % w/v. The major limitation of the conventional formulations is enhanced precorneal drainage, because of which frequent instillation is needed. Use of high drug concentration is also required which usually leads to pulse kinetics pattern of drug release. The efficacy of azithromycin as an antibacterial agent in ophthalmic delivery is limited by poor solubility and limited permeability. Due to limited solubility and permeability, the ocular bioavailability gets reduced (1). A large portion of drug when administered using eye drops is reported to undergo loss by lachrymation, nasolachrymal drainage, tear turnover and tear dilution. These losses resulted in poor ocular bioavailability. The delivery of drug to ocular region basically depends upon the type of disease to be managed i.e. intraocular infection and extraocular infection. Extraocular related infection commonly includes conjunctivitis, keratoconjunctivitis, corneal ulcers, etc. Extraocular bacterial infection requires an antibiotic treatment regimen, as treatment with antibacterial agent reduces the chances of complications associated with chronic conditions such as corneal perforations if remain untreated (2).

The success of an antibacterial therapy depends upon achieving and maintaining the therapeutic drug concentration at optimum site of action for longer duration of time. Major limitation in maintaining extraocular infection is the lack of ability of the delivery system to provide long term therapeutic concentration in the extraocular region without compromising the intraocular region (3). The most suitable treatment of extraocular bacterial infection is topical therapy but has limited potential due to poor bioavailability at the site of action and due to constraints in ocular delivery (4). To conquer the problems associated with topical therapy of azithromycin, many novel delivery systems have been designed including suspension (5), films (6), ointments (7). These systems have drawbacks of blurred vision, no true sustained effect, may cause allergies with selective diseases, and poor patient compliance.

Hence, present research is being undertaken to make use of β -cyclodextrin complex encapsulated in vesicular system. Complexation of drug with β - cyclodextrin will improve the solubility of azithromycin by forming complex with the drug (8). Drug delivered through the vesicles on the other hand will prolong drug delivery and provide SR/CR at the desired corneal region (9), by minimizing the systemic drainage and

improving the residence time. Thus, resulting in improved ocular bioavailability (10). The advantage of vesicular system does not only reside in providing prolonged effect at corneal region but also to prevent enzymatic drug metabolism at the site of action. Thus, they are examined as drug delivery carriers that can modify extent and rate of drug absorption, are chemically stable with an ability to improvise the drug performance (11). Selection of *in situ* gel is beneficial as it is based on the theory of phase transition in response to the environmental changes like pH triggered, temperature induced and ion induced systems. This system has the advantage of ease of administration as it can be instilled in sol form which converts to gel in response to the physiological condition encountered at the site of application. Thus, an enhanced retention can be assured with such delivery system because of gel formulation (12). Thus, the research is aimed to make use of vesicular *in situ* gel for delivery of azithromycin via ocular route that will exert dual approach of vesicles and *in situ* gel. Niosomes control the drug release, modify its distribution pattern as well as targeted the drug delivery to the desired site. They are expected to improvise local drug levels at desired site. Effective contact time improvise drug's bioavailability. Ocular drug delivery via niosomes modifies the rate and extent of absorption, which consequently results in reduced intraocular pressure. Thereby, providing controlled delivery. *In situ* gelling systems have main benefits of transporting reproducible and optimum amount, easy administration and promoting retention at precorneal site. Hence, β -cyclodextrin-niosomal *in situ* gel will improvise ocular bioavailability by enhancing the precorneal residence time.

Materials and methods

Materials

Azithromycin was gifted from Akums Pvt. Ltd., Haridwar, India as a free sample. Poloxamer 407 (Pluronic F127) was obtained from BASF Corporation, New Jersey, USA. β -cyclodextrin, sodium dihydrogen phosphate, cholesterol, span 60 were procured from S.D. Fine Chem. Limited, Mumbai, India. Cetyl pyridinium chloride monohydrate and carbopol 934P were purchased from Central Drug House, New Delhi, India. Tetrahydrofuran (THF), sodium chloride, methanol, and sodium hydroxide were obtained from Qualigens Fine Chem. Pvt. Ltd., Mumbai, India. Himedia Dialysis membrane 150 was procured from HiMedia Laboratories, Mumbai, India.

Methods

Preparation of complex

Kneading method

Drug and β -cyclodextrin were used in the molar ratios of 1:1 in order to form a complex. For this, β -cyclodextrin was triturated

with mortar and pestle using small quantities of water to prepare a homogeneous paste. Drug was allowed to add in this and the mixture was grounded for 6 h. The paste obtained was a dried complex, which was then pulverized and sieved (using sieve no. 60).

Evaluation of complex

Scanning electron microscopy (SEM)

Scanning electron micrographs of both azithromycin (ATZ) and ATZ- β -CD complex were taken using instrument scanning electron microscope (JEOL 5400, Jeol, Tokyo, Japan). Particles coated with thin gold layer under an argon atmosphere by sputter coater unit (VG Microtech, West Sussex, UK) were examined. The coating time was 5–6 min. Surface morphology was studied at an acceleration voltage of 4 kV.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry was conducted for both ATZ and ATZ- β -CD complex using differential scanning calorimeter DSC60 controlled by TA-60 WS software (Shimadzu, Kyoto, Japan, 0-300 °C. For measurements, 5 mg samples were taken and study was performed at a heating rate of 10 °C/min over 0-300 °C range. An inert atmosphere was created using nitrogen at a flow rate of 10 mL/min.

Preliminary studies

Optimization of niosomes

Preliminary studies were conducted on blank niosomes to optimize the method of preparation, type of surfactant and ratio of cholesterol and surfactant. To prepare niosomes, three methods were used (ether injection method, thin film hydration method and hand shaking method) in order to optimize an appropriate method. For the selection of optimum type of non-ionic surfactant, niosomes were developed by adding surfactants like Span 60, Span 80 and Span 20. Further, optimum ratio of cholesterol and surfactant was selected among several ratios (1:2, 2:3, 7:8, 8:7). Optimization was done on the basis of photomicrographs taken using photomicrograph (HICON, New Delhi, India) at 40x.

Equilibrium solubility

Excess drug added to distilled water (0.05 %w/v; 20 mL) in a conical flask (37 °C \pm 0.5 °C) stirred for a period of 72 h in a water bath shaker (HICON®, New Delhi, India). Additionally, in several flasks, excess amount of drug mixed with β -cyclodextrin, cholesterol and span 60 was taken. Samples were filtered (nylon filter disc (0.22 μ)), diluted and analyzed spectrophotometrically at 196 nm.

Phase solubility

Phase solubility analysis was conducted at 25 °C as per the methodology depicted by Higuchi and Connors (13). Excess drug amount added to distilled water containing β-cyclodextrin was shaken in water bath shaker at 100 rpm (0.559 × g) for 72 h. Samples were filtered and analyzed against the blank prepared by using different concentrations of β-cyclodextrin in distilled water spectrophotometrically at 196 nm. Stability constants were determined. Complexation efficiency (C.E) and apparent stability constant (K_s) were determined from the slope of the phase solubility diagram as per the following equations:

$$K = \text{Slope} / S_0 (1 - \text{Slope})$$

(Eq.1)

$$\text{Complexation efficiency (CE)} = \text{Slope} / (1 - \text{Slope})$$

(Eq.2)

Where, S₀: intrinsic drug solubility

Preparation of composite niosomes

Composite niosomes of azithromycin were prepared based on 3² full-factorial design as per Table 1. In total nine formulations were formulated using ether injection method. Surfactant and cholesterol dissolved in ether were mixed with methanol in a ratio of 4:1. To this ATZ-β-CD complex was then added. The mixture was then injected with the help of 14-gauge needle in phosphate buffer solution (pH 7.4) at 60-65 °C and stirred continuously. Ether and methanol evaporated at this temperature, leaving behind suspension of niosomes.

Purification of composite niosomes

Composite niosomes were purified using dialysis membrane technique to remove the free drug. Hi-media dialysis membrane 150 (MWCO 12-14 kDa) kept in saline solution for 2 h was used. Drug-loaded vesicles were placed in a dialysis bag dipped in 200 mL of phosphate buffer pH 6.8 and undergo stirring using magnetic stirrer at 500 rpm (13.98 × g). Five mL of sample was withdrawn under sink conditions and analyzed spectrophotometrically. Purification time was optimized using statistical paired-t test at 5% level of significance.

Characterization of composite niosomes

Vesicle size, zeta potential and polydispersity index

All the prepared niosomal formulations were subjected to evaluation for vesicle size, zeta potential and polydispersity index determined by dynamic light scattering based on laser diffraction using Malvern Zetasizer (Malvern Co., UK) at 25°C. Samples were diluted using deionized water and filtered through millipore 0.45µm pore size membrane (Milex®, Millipore Co., USA).

Entrapment efficiency

Centrifuged purified formulation using centrifuge (R-4 C, Remi centrifuge, Vasai, India, 1 h at 4,000 rpm (894.0 × g)) was used to evaluate entrapment efficiency. The sediment formed was then lysed by methanol. After filtration, the sample was assayed to determine the entrapment efficiency by following Eq. 3.

$$\% \text{ Entrapment efficiency} = (W_a - (W_s + W_p) / W_a) \text{ (Eq.3)}$$

Where,

W_a= amount of drug added in the system;

W_s= amount of drug in the supernatant after centrifugation;

W_p= amount of drug in sediment.

In vitro drug release

Dialysis method was used to carry out in vitro drug release analysis. Niosomal suspension placed in a dialysis bag, kept in 200 mL phosphate buffer, pH 7.4 was stirred at 100 rpm. Withdrawn samples were analyzed spectrophotometrically at 215 nm under sink conditions.

Validation of experimental design

Polynomial equation was generated using Design Expert Software version 9.0.6.2 (Stat-Ease, Minneapolis, USA Inc.) for dependent variable. Extra design check point formulations were developed to validate the model and characterized for percent entrapment efficiency. The findings were then compared with

Table 1 3² Factorial design for the development of ATZ-β-CD complex encapsulated composite niosomes

Formulati on code	Cetylpyridinium chloride (% w/v)	Cholesterol (% w/v)	Span 60 (% w/v)	Di-ethyl ether: methanol
NF1	0.1	0.1	0.1	4:1
NF2	0.1	0.1	0.5	4:1
NF3	0.1	0.1	1	4:1
NF4	0.1	0.5	0.1	4:1
NF5	0.1	0.5	0.5	4:1
NF6	0.1	0.5	1	4:1
NF7	0.1	1	0.1	4:1
NF8	0.1	1	0.5	4:1
NF9	0.1	1	1	4:1

the predicted value and evaluated statistically using pooled t- test at 5 % level of significance.

Selection of optimized formulation

Optimized formulation was selected based on highest desirability value and loaded into suitable gel base.

Preparation of in situ gel

Gel based formulations were prepared by cold method. Niosomal pellets were added to presoaked carbopol 934 P and stirred at room temperature (25 °C). The dispersion was then cooled at 4 °C by keep in a refrigerator. Polaxamer 407 was added slowly as per the composition (Table 2) to the above dispersion under continuous stirring (thermostatically controlled magnetic stirrer (S.M. Scientific instruments Pvt Ltd., New Delhi, India)), maintaining the temperature at 4 °C. Suspensions were then stored in a refrigerator overnight to get clear solutions. Thereafter, formulations were stored in a refrigerator so that they remain in sol form.

Characterization of in situ gel

Clarity, pH and percent drug content

Clarity of the developed gel based formulations was determined before and after gelation by visual examination of the formulations under light alternatively against white and black background. pH of the developed gel formulations was determined using Digital pH meter model 111 E (HICON®, New Delhi, India). For drug content, weighed amount (50 mg) of ATZ loaded ocular niosomal in situ gel formulations were diluted using phosphate buffer, pH, 7.4. The resultant dispersion was vortexed using Vortex shaker (HICON®, New Delhi, India) and shaken for 10 min. The volume was made up to 5 mL with phosphate buffer pH, 7.4 and analyzed spectrophotometrically at 215 nm. All readings were taken in triplicate.

Table 2 Composition of azithromycin (ATZ) loaded composite niosomal in situ gels

Formulation Code	Polaxamer 407 (%w/v)	Carbopol 934P (%w/v)	ATZ loaded composite niosomes (% w/v)
NG1	12	2	0.5
NG2	14	2	0.5
NG3	16	2	0.5
NG4	18	2	0.5
NG5	20	2	0.5

In vitro gelation

In vitro gelling efficiency was determined using a transparent vial containing 10 mL of niosomal gelling solution and a

magnetic bar was placed in a low-temperature water bath (HICON®, New Delhi, India). A thermometer with an accuracy rating of 0.1 °C was immersed in the niosomal gelling solution. The solution was heated at the rate of 1 °C/1–2 min with constant stirring of 5 rpm. The temperature at which the magnetic bar completely stopped movement because of gelation was regarded as the phase transition temperature. Each study was done in triplicate. Gelling time is the time for first detection of gelation was also recorded.

Viscosity

The rheological characteristics of in situ gelling system was evaluated by Brookfield viscometer DV- II+ pro (Brookfield Engineering Laboratories, Inc, MA) coupled with T spindle S-96 at physiological condition i.e. 37 ± 2 °C.

Ex vivo permeability

Ex vivo permeability study of all ATZ- β -CD complex loaded niosomal gel (NG1-NG5) was carried out using fabricated Franz diffusion cell consisting of donor and receptor compartment separated by dialysis membrane of pore size 0.22 μ m (Himedia Ltd., Mumbai, India). Gel based formulations equivalent to 1 % w/v of ATZ - β -CD complex loaded niosomes placed in donor compartment and receptor compartment was filled with phosphate buffer, pH 7.4 and constantly stirred using magnetic stirrer. Temperature was kept constant at 37 ± 1.0 °C in order to mimic body conditions. One mL sample was withdrawn through the sampling port of the diffusion cell at 1, 2, 3, 4, 5, 6, 7 and 8 h time intervals and analyzed spectrophotometrically at 215 nm. An equal volume of fresh phosphate buffer, pH 7.4 was added into the receptor compartment after each sampling to maintain the sink conditions.

Selection and characterization of optimized gel formulation

Optimized gel formulation was selected on the basis of formulation that showed clarity, maximum gelling capacity along with optimum viscosity, maximum drug content and minimum ex- vivo permeation. Optimized formulation was then characterized for in vitro mucoadhesion, and histological analysis.

In vitro mucoadhesion

Mucoadhesive strength was determined as per the method given by Qi et al., (14). The sclera was obtained from goat eye collected from local butcher shop and washed with normal buffer saline and kept in freshly prepared Ringer's salt solution (37 °C, 15 min). Excised scleral tissue was attached to the under surface of pan of balance and equilibrated. Optimized in situ gel formulation was placed in sample holder placed on water bath at

37°C. pH of sample was adjusted to 7.4. Equilibrium of balance was disturbed by putting a weight of 5.0 gm on the left pan of balance to assure complete contact between gel surface and scleral tissue. The weight was then removed and water was added drop wise to the beaker placed on right pan with the help of burette. Water was added continuously until the gel and corneal tissue got detached. The mucoadhesive force was determined by calculating detachment stress (dynes/cm^2), which gives the value of minimal force required to detach cornea from the gel.

$$\text{Detachment stress} = m \times \frac{g}{A}$$

(Eq.4)

Where,

m= the weight added to balance in gram.

g = acceleration due to gravity taken as 980 cm/sec^2

A = area of exposed tissue.

Comparative in vitro release analysis

In vitro release study of optimized niosomes loaded in situ gel, niosomes, and azithromycin eye drop (Zithromax; Allembic Pharmaceuticals, Vadodara, India) was performed using dialysis bag method. Sample were placed in dialysis bag and kept in a 200 mL of phosphate buffer pH, 7.4, stirred at 100 rpm using magnetic stirrer ($37 \pm 2 \text{ }^\circ\text{C}$). Samples were drawn and analyzed spectrophotometrically under sink conditions.

Histological analysis

Histological analysis was performed to evaluate the effect of formulations on scleral integrity. Collected whole eye balls of goat were transported immediately to the laboratory in normal buffered saline in cold condition within 1h of slaughtering. Rinsed sclera from excised eyes was incubated for 30 min in optimized NG5 formulation at $34 \text{ }^\circ\text{C}$, phosphate buffer saline (PBS), pH 7.4 as negative control and 75% isopropyl alcohol in PBS as positive control was taken as reference. The sclera was washed with PBS, pH 7.4 and immediately fixed with 10 % v/v formalin solution for 24 h. The sclera was dehydrated with ethyl alcohol and xylene, put in melted paraffin, and solidified in block forms. Cross sections ($< 1\text{mm}$) were observed under microscopy for any histological changes after staining with hematoxylin and eosin (15).

Results

Preparation and evaluation of complex

Drug- β -cyclodextrin complex was prepared by kneading method and undergo characterization for surface morphology and DSC.

Scanning electron microscopy

Scanning electron microscopy is thought to be a mode to study the morphological changes at surface. Scanning electron microphotographs of azithromycin and ATZ- β -CD complex are depicted in Figure 1. ATZ was appeared as regular three dimensional crystals (Figure 1A). A major morphological change has been observed in the complex formation. However, the original morphological diagnosis of the materials diminished and was unable to undergo differentiation. Crystalline structure of drug has perished (Figure 1B) and the amorphous agglomerate is formed after complex formation. These modifications in shape of the particles in binary complexes indicated the presence of new solid phase.

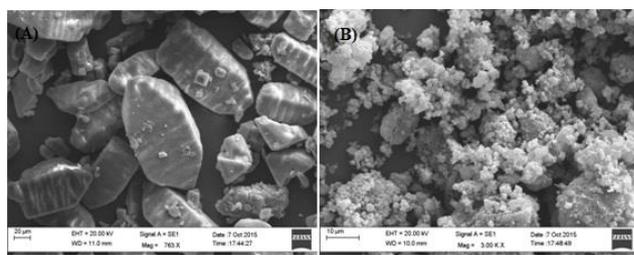


Figure 1 SEM photomicrograph of (A) Azithromycin (ATZ), (B) ATZ- β -CD complex

Differential scanning calorimetry

DSC was conducted to trace any form of drug- excipient interaction. Figure 2A depicted sharp endothermic peak at $110.54 \text{ }^\circ\text{C}$ (due to ATZ) because of the melting of drug whereas broad endothermic peak at $272.79 \text{ }^\circ\text{C}$ showed thermal decomposition of drug. In case of complex formation (ATZ- β -CD) (Figure 2B), peak of drug was not observed at $110.54 \text{ }^\circ\text{C}$. Thus, suggesting the fact that ATZ was dispersed molecularly in complex and exothermic peak at $353.53 \text{ }^\circ\text{C}$ had proved the thermal decomposition of complex form.

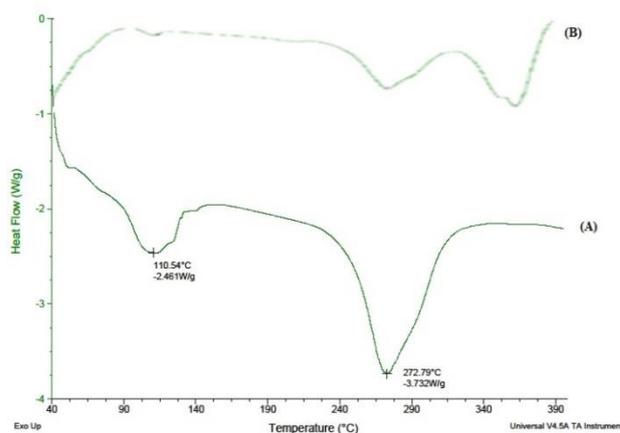


Figure 2 DSC thermogram of (A) Azithromycin (ATZ), (B) ATZ- β -CD

Preliminary optimization studies on blank composite niosomes

Optimization of niosomes

Blank niosomes were visualized and the photomicrographs revealed that thin film hydration method vesicles were irregular in size/shape and possessed undefined structures while vesicles formation could not be facilitated by hand shaking method (Figure 3 (I) A). Niosomes prepared by ether injection method (Figure 3(I) C) were spherical in shape with structure uniformity.

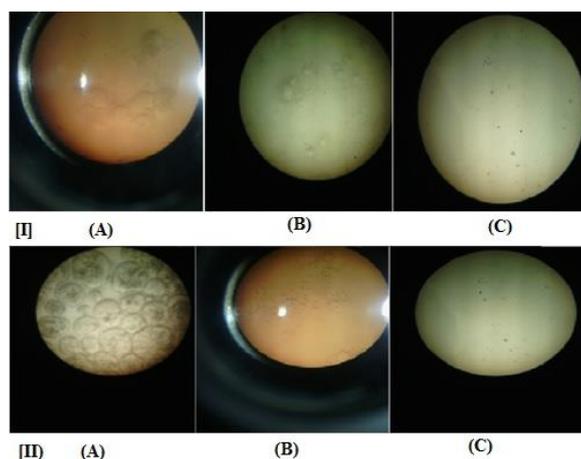


Figure 3 Photomicrographs of blank niosomes prepared by [I] (A) Hand shaking method, (B) Thin film hydration method, (C) Ether injection method; [II] (A) Span 20; (B) Span 80; (C) Span 60

Thus, ether injection method was optimized for preparing niosomes. Next, to select appropriate surfactant among several added surfactants, span 60 was selected (Figure 3(II) C). As, it was observed that niosomes prepared using span 60 were uniform in shape with rigidity and firmness in their structure (Figure 3(II) C). Whereas vesicles did not form in case of span 20 (Figure 3(II) A) and in presence of span 80 vesicles were non-uniform size and shape (Figure 3(II) B). Thus, span 60 was selected as an optimum surfactant. Further, appropriate ratio of span 60 and cholesterol was selected by changing the ratio at 1:1, 3:2, 7:8 and 8:7. Out of these ratios 1:1 selected optimum for preparation of niosomes because most of the vesicles were uniform in shape and of similar size in comparison with other ratios prepared niosomes. Thus, optimum ratio of span 60 and cholesterol was identified to be 1:1.

Equilibrium solubility

Solubility of drug in distilled water was determined as 0.0851 mg/ml. Addition of β -cyclodextrin and span 60, depicted the percent solubility enhancement as 75.30 and 42.32, respectively (Table 3). β -cyclodextrin improves the solubility of azithromycin by forming soluble complexes with drug by enclosing the drug in its hydrophobic cavity (16) whereas span

60 increased the solubility due to its surfactant nature. Hydrophilic head and hydrophobic tail in its structure decrease the surface tension in aqueous media and facilitate solubilization. Methanol as a solvent depicted a solubility enhancement due to their cosolvent action. Thus, it may be put into conclusion that the excipients used in formulating composite niosomes, represents solubility enhancement and hence higher drug entrapment. On the basis of these findings, composite niosomes were developed.

Phase solubility

Phase solubility study was performed to optimize the levels of β -cyclodextrin for experimental design as well to find stoichiometric ratio of β -cyclodextrin and azithromycin in order to prepare binary complex to explore its utility as a carrier. Azithromycin in presence of β -cyclodextrin indicated increased drug solubility on increasing the concentration of β -cyclodextrin. Phase solubility profile was plotted using apparent equilibrium concentration or solubility of drug against increasing concentration of β -cyclodextrin (Figure 4). Apparent drug solubility enhanced linearly with β -cyclodextrin over the entire concentration range. Linearity was considered as the characteristic of AL- type system that suggested the formation of a complex (water soluble).

Table 3 Solubility data of azithromycin in distilled water in the presence of excipients

Sample composition	Strength of excipients (%w/v)	Solubility (mg/ml)	% solubility Enhancement
D	0.05	0.0851	-
D + SP-60	0.05+0.5	0.147	42.32±1.08
D + CHO	0.05+0.5	0.104	18.33±0.98
D + MeOH	0.05+20	0.529	83.91±1.15
D + β -CD	0.05+0.5	0.346	75.30±1.34
D + SP-60+CHO+MeOH+ β -CD	0.05+0.5+0.5+20+0.5	1.077	92.10±1.01

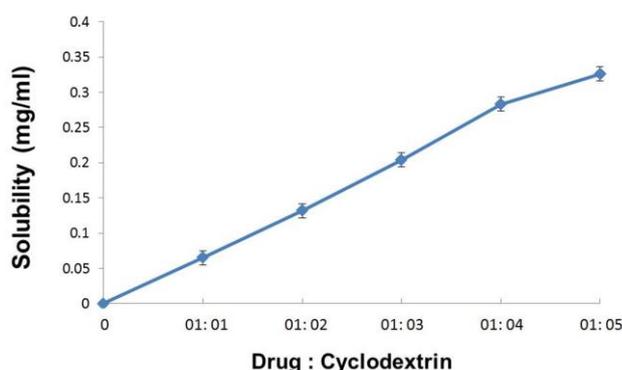


Figure 4 Phase solubility diagram with varying ratio of drug and cyclodextrin

Further, 0.896 obtained as a slope value of the complex, indicated the formation of inclusion complex (1:1). K1:1 was observed as 168 M^{-1} . The value of inclusion complex suggested that the complex formed was very less stable which may increase the process of dissociation of drug. Phase solubility curve did not confirm the formation of complex. These curves however, depicted that the increasing concentration of solubilizer influence the drug solubility (17). Based on these results, β -cyclodextrin-azithromycin ratio was selected as 1:1 for designing composite niosomes.

Preparation and evaluation of niosomes

Niosomes were developed by ether injection method as described earlier according to the formulation design as given in table 1 and evaluated.

Vesicles Size, Zeta Potential and Polydispersity index (PDI)

Vesicle size of ATZ- β -CD loaded niosomes varied from 306 ± 3.05 to 2212 ± 4.35 nm with PDI value ranging from $0.115 \pm 5.51 - 0.677 \pm 0.02$. Di cetyl pyridinium chloride containing NFs (NF1-NF9) showed positive zeta potential values of 24.2 ± 0.23 to 45.3 ± 0.25 mV due to hydrophilic cationic nature of it.

Entrapment efficiency and in vitro drug release

Percent entrapment efficiency was observed to be $30.07 \pm 5.09 - 78.17 \pm 1.81$ as shown in Table 4. *In vitro* release study was conducted to correlate the functioning of a system with its *in vivo* effectiveness. *In vitro* drug release (Figure 5) was found to be varied from 23.95 ± 2.30 to 76.28 ± 2.22 %. Higher drug release was observed in NF1 formulation (76.28 ± 2.22 %).

Validation of Experimental Design

Design Expert Software version 9.0.6.2 (Stat-Ease, Minneapolis, USA Inc.) was used to validate 3^2 factorial design. The developed model was described using polynomial equation. This can be given as follows:

$$\% \text{ Entrapment efficiency} = + 84.18 + 4.78x_1 - 2.10x_2 \quad (\text{Eq.5})$$

The presence of positive sign indicated the positive effect of cholesterol (x_1) on percent entrapment efficiency. Negative sign in numerical values showed the inhibitory effect of span 60 (x_2) on the response. Extra design check point formulation (NF10) showed percent entrapment efficiency to be 76.59 ± 1.06 %.

Selection of optimized formulation

Formulation NF2 showed maximum desirability (equal to 0.807). Also, exhibit optimum percent cumulative drug release (73.09 ± 2.11 %), entrapment efficiency (78.17 ± 1.81 %) with optimum particle size (306 ± 3.05 nm) and optimum PDI value 0.218 ± 5.51 . Thus, NF2 was considered as best among all the formulations. Optimized formulation was further incorporated into gel and characterization.

Preparation and characterization of in situ gel

Optimized ATZ loaded niosomal formulation NF2 was incorporated into temperature controlled *in situ* gels prepared by cold method. Gels were prepared with the aim to achieve more controlled release of niosomes from gelling system and increase ocular residence time.

Table 4 Pharmacotechnical characterization of ATZ- β -CD loaded composite niosomes (NF1-NF9)

Formulation code	Vesicle size (nm)	Zeta potential (mV)	Polydispersity index	% Entrapment efficiency	% CDR	Kinetic model	r ²
NF1	1258 ± 7.21	38.5 ± 0.43	0.115 ± 5.51	75.51 ± 2.40	76.28 ± 2.22	Higuchi	0.9972
NF2	306 ± 3.05	45.3 ± 0.25	0.218 ± 5.51	78.17 ± 1.81	73.09 ± 2.1	Higuchi	0.9948
NF3	714 ± 3.60	32.6 ± 0.20	0.279 ± 3.79	74.36 ± 0.07	54.82 ± 1.79	Higuchi	0.9877
NF4	2212 ± 4.35	30.0 ± 0.20	0.555 ± 0.02	68.84 ± 0.52	43.47 ± 0.91	Higuchi	0.9875
NF5	877 ± 4.16	41.4 ± 0.23	0.647 ± 0.03	65.80 ± 0.25	53.64 ± 1.33	Korsmeyer and Peppas	0.9986
NF6	553 ± 3.51	35.4 ± 0.20	0.677 ± 0.02	63.65 ± 0.69	34.91 ± 1.55	Higuchi	0.9610
NF7	876 ± 4.35	32.7 ± 0.057	0.577 ± 0.02	59.23 ± 1.14	36.10 ± 6.8	Higuchi	0.9759
NF8	847 ± 2.51	28.4 ± 0.35	0.653 ± 0.04	41.28 ± 4.12	23.95 ± 2.30	Korsmeyer and Peppas	0.9934
NF9	2041 ± 3.51	24.2 ± 0.23	0.662 ± 0.04	30.07 ± 5.09	27.55 ± 1.26	Korsmeyer and Peppas	0.9944
NF10*	634 ± 3.60	46.9 ± 0.81	0.566 ± 0.01	76.59 ± 1.06	61.065 ± 2.67	First	0.9884

NF10*= Extra design check point formulation.

Clarity, pH and drug content

All the formulations were found to be clear, there was no residue matter present which can harm sclera tissue and affect the syringeability of the formulations. pH of all formulations before gelation was found to be towards acidic side (6.30 ± 0.03 to 6.83 ± 0.03) and after gelation it changes (6.28 ± 0.01 to 7.21 ± 0.02) (Table 5). These changes confirmed the ability of conversion from sol to gel on ocular instillation (15).

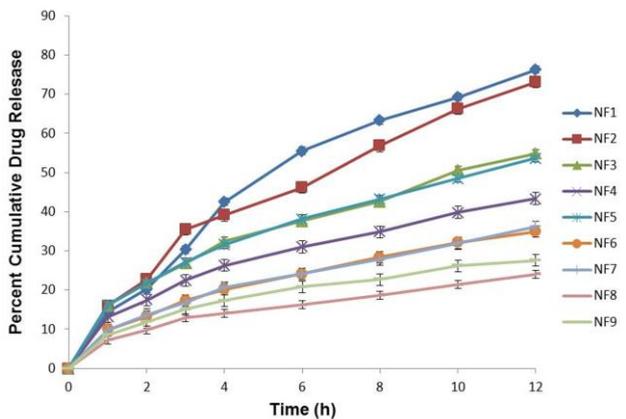


Figure 5 *In vitro* drug release profiles in phosphate buffer, pH 7.4

Further, pH range of 6.8 to 7.4 is thought to be acceptable and safe for ocular delivery. Drug content lies in the range from 63.44 ± 0.68 to 80.79 ± 1.04 %.

In vitro gelation and viscosity

Gelling temperature lies in between 28.33 ± 0.57 - $32.00 \pm .0.0^\circ\text{C}$ for all the formulations (Table 5). A gelling temperature is recorded to be optimum in the range of 25°C to 37°C . Viscosity of all the formulations lies in between 38×10^3 - 45×10^3 cps.

Ex-vivo permeability

Ex vivo permeation study of *in situ* ocular gels was performed to evaluate the role of niosomes in improvising permeation across the sclera. % CDP varied widely between 37.07 ± 2.06 to 51.34 ± 0.89 .

Selection and characterization of optimized formulation

NG5 possessing highest drug content, maximum gelling capacity, and lowest permeation capacity was found to be best among all the formulations in order to exert localize effect in ocular delivery. Optimized formulation was then characterized for *in vitro* mucoadhesion and histological study.

In vitro mucoadhesion and comparative *in vitro* release analysis

Mucoadhesive strength of niosomal *in situ* gel (NG5) was found to be 3462.67 dynes/cm² which was approximately 23 folds greater than ocular shear force (150 dynes/cm²). *In vitro*

release profile of NG5 was compared with that of NF2, Zithromax® eyedrop and subjected to kinetic analysis. The role of niosomes and *in situ* gel in improvising the release through sclera was demonstrated. NG5 was able to sustain the drug release and achieved 65.45 ± 0.87 % in 8 h (Figure 6) that was comparable with CDR of NF2 that achieved 67.32 ± 1.27

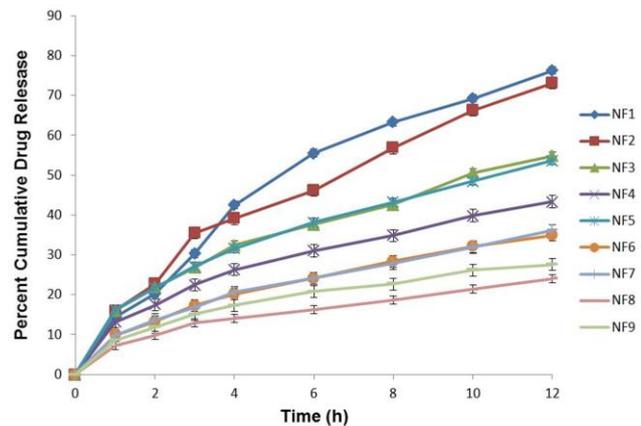


Figure 6 Comparative *in vitro* release of azithromycin from optimized niosomal formulations, Niosomal *in situ* gel, and marketed formulation in phosphate buffer, pH 7.4

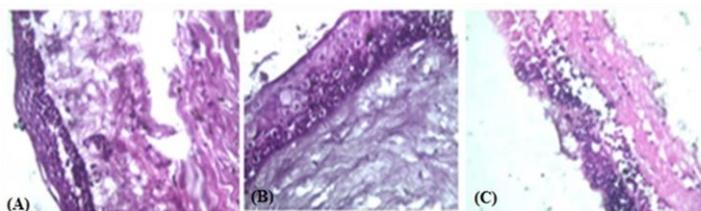
% in the same time period. Release of Zithromax® (marketed preparation) showed instantaneous release of drug as drug present in solution form. Thus, maximum drug released within 2 h and only 54 % of drug was released from marketed solution. So, on comparing the release of Zithromax® with NG5, *in situ* gel follows zero order release. Zero order release was followed by gel as drug release undergoes retardation when gel forms and follows more constant release of drug. On the other hand, marketed preparation releases the drug in an uncontrolled order and release maximum drug within in 2 h. In case of NF2, niosomes showed better release of drug because it follows sustained release of drug. Whereas *in situ* gel based NG5 formulation was found to be best among all three formulation in case of retaining and release manner of drug.

Histological analysis

Effect of optimized *in situ* gel formulation (NG5) on the structural features of scleral tissue was evaluated (Figure 7). After incubating scleral cross-section in 75% isopropyl alcohol (Figure 7 (C)), narrow intercellular spaces widened with deformation. Further, superficial epithelial cells undergo distortion and got detached from the tissue assembly. Incubation of sclera with NG5 (Figure 7 (B)) did not reveal any destructive effect on corneal epithelium, stromal structure. Thus, maintained the corneal integrity. The above results showed by optimized formulation were found to be similar to the effect shown after incubating scleral tissue in PBS, pH 7.4 (Figure 7(A)).

Table 5 Characterization of composite niosomal loaded *in situ* gel (NG1-NG5)

Formulation Code	Temperature (Celsius)	Gellingtime (seconds)	pH (sol)	pH (gel)	Percent Drug content	% CDP
NG1	28.33 ± 0.58	74 ± 1.22	6.83 ± 0.03	6.90 ± 0.01	63.44 ± 0.69	51.34 ± 0.89
NG2	31.66 ± 1.07	40 ± 2.00	6.30 ± 0.03	7.18 ± 0.04	65.73 ± 0.68	48.07 ± 0.90
NG3	32.00 ± 0.00	33 ± 1.52	6.34 ± 0.01	7.21 ± 0.02	75.16 ± 0.80	46.46 ± 0.27
NG4	30.66 ± 1.15	22 ± 1.00	6.40 ± 0.01	6.41 ± 0.02	76.38 ± 2.07	38.68 ± 1.52
NG5	32.00 ± 0.00	16 ± 0.57	6.40 ± 0.03	6.28 ± 0.005	80.796 ± 1.04	37.07 ± 2.06

**Figure 7** Histological cross section of excised goat sclera, stained with hematoxylin-eosin after incubation in (A) PBS, pH 7.4 (negative control); (B) NG5; (C) 75% isopropyl alcohol (positive control).

Discussion

Niosomes loaded with azithromycin- β -CD complex were developed based on 3^2 full factorial design using either injection method. All the nine formulations were then evaluated for vesicles size, zeta potential, polydispersity index (PDI), percent entrapment efficiency, and percent cumulative drug release.

Formulation NF1 to NF3 containing least amount of cholesterol showed minimum particle size. This can be revealed based on the concentration of cholesterol. As amphipathic feature of cholesterol is an important parameter to understand the effect of increasing cholesterol content on average increment in particle size of niosomes. Cholesterol can be embedded into bilayer membrane by orienting its hydrophilic head towards the aqueous surface and aliphatic chain arranged parallel to the hydrocarbon chains in centre of bilayer (18). At low concentration of cholesterol, it can be expected that cholesterol get arranged surfactant monomers in close packing with increasing curvature and thus, minimizing the particle size (as observed in NF1 to NF3). However, upon increasing the cholesterol content, with its known lipophilic nature ($\log P$ 7.02), it would have enhanced the hydrophobicity of the bilayer membrane and disturbed the vesicular membrane, thus, improvised the radius to provide a more thermodynamic stable form, which is finally found in average particle size of NF4 to NF6. Similarly, in case of NF7 to NF9, increment in radius was observed but in higher amount because of the presence of higher cholesterol concentration.

PDI is depicted to be the measurement of homogeneity which dictates the distribution of vesicles. A value of less than 0.3 PDI value depicts the homogenous particle distribution (19). PDI value was found to be minimum from NF1 to NF3 (0.115 ± 5.507 to 0.279 ± 3.785) among niosomal vesicles which

signifies the homogenous nature of first three formulation and higher PDI value as found in NF4 to NF9 defined the heterogeneous nature formulation which might be due to aggregation of the particle. Zeta potential values highlight the particle surface charge that defines the degree of repulsion between the adjacent particles. Its value largely affected the stability of nanoparticles through the electrostatic repulsion between the particles. Zeta potential of -30 to +30 mV signifies that a system is stable (20, 21).

The zeta potential values of ATZ- β -CD conferred physical stability to the system and positive value indicates the stability of niosomes by electrostatic repulsive forces (22). Zeta potential values also depicted that NFs maintains their charge even in the presence of drug molecules thus, suggesting that drug molecules are uniformly dispersed within the niosomes (23). Further, depiction of entrapment efficiency highlighted that niosomal formulations containing optimum concentration of cholesterol showed highest entrapment as shown in formulations (NF1-NF3). This can be attributed to the presence of cholesterol which exhibited cement leaking quality. As cholesterol concentration increased from 0.1 - 0.5% w/v (in NF4-NF6), entrapment efficiency decreased from 68.84 ± 0.5 to 63.65 ± 0.69 %. Beyond a level of 0.5 % w/v, disruption of regular bilayer structure occurred that led to drug loss (24). As observed in concentration of 0.5 to 1% w/v in (NF7- NF9), drug entrapment efficiency decreased from 59.23 ± 1.14 to 30.07 ± 5.09 %. Thus, with increased cholesterol concentration, entrapment efficiency decreased because of cholesterol intercalation in bilayers. Also, higher amount of cholesterol, for packing space within the bilayer may compete with the drug. As 0.1% w/v of cholesterol present in NF1-NF3, these formulations showed better entrapment efficiency in

comparison with NF4 to NF6 because less drug entrapped in niosomes as drug compete with cholesterol for entrapment in bilayers. Similarly, least drug entrapped in NF7-NF9 because of highest concentration of cholesterol in all these formulations. So, upon increasing the concentration of cholesterol, stability and bilayer hydrophobicity increased and permeability got reduced. This may also led to an efficient hydrophobic drug trapping into bilayers (25). The findings of *in vitro* drug release demonstrated that upon increasing the cholesterol concentration, entrapment efficiency decreased, thus, percent drug release also decreased. As drug release dependent on the entrapment efficiency (24).

Formulations containing least amount of cholesterol showed higher percent of drug release because of higher drug entrapment. Reduced concentration of cholesterol resulted in the reduced membrane permeability that resulted in lesser drug elution via vesicles (26). Data of *in vitro* release was then subjected to kinetic analysis to understand the mechanism of release. *In vitro* drug release via niosomes was found to follow Higuchi model kinetics. After evaluating all the parameters, experimental design was then subjected to validation using Design Expert Software version 9.0.6.2 (Stat-Ease, Minneapolis, USA Inc.). Transformed polynomial equation indicated no significant difference ($p > 0.05$) between the two values (experimental and predicted value (79.40 %)) upon evaluation using pooled t- test. Thus, it was concluded that design has been validated. Thus, based on maximum desirability (equal to 0.807), formulation NF2 was selected as an optimized formulation and developed in the form of *in situ* gel. Developed gels were then evaluated for clarity, pH, drug content, *in vitro* gelation

For the development of ocular *in situ* gel, clarity, pH and gelling capacity become the important prerequisite parameters. Thus, the formed gel should be clear and should not cause blurring of vision. As for a successful *in situ* gelling system, pH of the solution should be such that it allows instillation of liquid solution easily. This further will transform from sol to gel on coming in contact with ocular physiological conditions and could not cause any irritation in ocular area (25). However, at lower value of gelling temperature (less than 25 °C), gel formation occurs at room temperature. This might led to several problems in handling, manufacturing, and administration. At higher values of more than 37 °C, gel would not form at the temperature of ocular cavity so result in rapid ocular clearance of administered drug because it remain in sol form (27). When temperature is increased, the number of vesicles formed increases due to negative coefficient of solubility of niosomes.

Also, the niosomes packed so closely, rendering the solution immobile and thus, the gel is formed. Results revealed that the

gelling temperature decreased with increase in the concentration of poloxamer 407 because of the development of larger number of the vesicles that occupy larger volume at lower temperature (27, 28). Recently, gelation mechanism on the basis of particle association and interaction has been suggested by several authors. Also, conformation modifications in methyl group orientation in the side chains of poly (oxypropylene) that constitute the core with water removal have also been suggested to contribute to the gelation process (24).

The mucoadhesive agent carbopol 934 NF also causes lowering of gelling temperature. It was due to its property to undergo binding with poly (ethylene) oxide chains present in poloxamer 407 molecule, thus promoting dehydration and cause an increase in entanglement of adjacent molecule with more extensive intermolecular H-bonding and so enhance the association (29). Immediate gelling increased the residence time and improvised the bioavailability of drug. Residence time at the sclera surface is affected by the viscosity of ophthalmic formulation. Hence, viscosity is considered an important parameter for increasing the residence time of ophthalmic formulation upon instillation into cul-de-sac. This property will help in predicting the *in vivo* behaviour of niosomal dispersion (30). With respect to ocular physiology, the range of shear rate was from 0.03 to 0.14 s⁻¹ during inter blinking period of 4250- 28500 s⁻¹. Thus, the viscosity of formulation should not be such that it may disturb the pseudoplastic behavior of tear film in the eye (30).

From the results, it was found that high viscosity values were observed in all formulations having optimum amount of carbopol 934NF as it is a viscosity enhancing polymer. So, it helps in increasing the ocular residence time. Percent cumulative drug permeation was found to be less than 60 % because of high amount of poloxamer 407. It was found that presence of poloxamer 407 in gels reduced the release rate of drug. Thus, permeation decreased slightly due to reduction in dimension of water channels. As poloxamer 407 comprises of micelles in aqueous phase in very large amount, the incorporated niosomes may be released by diffusion through gel matrix.

Niosomal permeation may also be affected by the viscosity of gel, aqueous channel size and niosomal distribution. Because of this reason, niosomal gel permeation goes on decreasing from NG1 to NG5. Also, increase in poloxamer cause slight increase in viscosity and decrease in release and thus decrease in the permeation of niosomes was observed. Thus, the minimum permeation showed by NG5 which consists of 20 % w/v poloxamer and maximum permeation showed by NG1 consist of 12 % w/v of poloxamer 407. Among all the gels, NG5 was selected as an optimized gel based formulation

which was then characterized for *in vitro* mucoadhesion and histological study. The results of *in vitro* mucoadhesion revealed that variable amount of carbopol 934 NF affected the mucoadhesive strength. The mucoadhesive strength depends on wetting and swelling of carbopol 934 NF that permitted proper contact with scleral tissue. Interaction of carbopol 934NF (bioadhesive in nature) chains with sclera tissue leads to entanglement and weak chemical bonds formed between the entangled chains. Carboxyl group present in carbopol 934 NF exhibits hydrogen bonding which reveals sol to gel transition as the temperature rises above 28°C. The formation of hydrogen bonding resulting in strengthening the network between polymer and sclera tissue. The results signified the presence of mucoadhesive force to resist the shear force at the time of reflex blinking.

Thus, it can be put into conclusion that the formulation might showed desirable residence in the scleral area. Niosomal based formulation NF2 showed better release of drug in comparison to marketed preparation because it follows sustained release of drug. This was due to the fact that drug release occurred through niosomes in a controlled manner for a selective time period (31). The release profile obtained defined the greater controlled release ability of developed niosomal *in situ* gel as compared to NF2. Decrease in drug release from the gel matrix was observed in the niosomal *in situ* gel in comparison to niosomes and marketed formulation. Finally, proven the fact that marketed preparation did not showed controlled release and did not possessed retaining capacity. Thus, NG5 formulation was found to be best among all three formulation in case of retaining and release manner of drug. Further, results of histological analysis revealed the safety of niosomal *in-situ* gel via ocular route.

Conclusion

ATZ- β -CD complex loaded niosomal *in situ* gel of (NG5) is depicted to be safe and effective alternative for ocular delivery that overcomes the limitations of conventional therapy. Also minimizes systemic side effects associated with the oral delivery of ATZ. The prepared system proved to have better retention ability and exhibit localized delivery. Thus, it can be put into conclusion that delivery via complex-vesicle based system exhibited potential to carry hydrophobic drug to scleral segment of the eye and capable of treating bacterial conjunctivitis.

Author's contribution

All the authors have contributed substantially to the design, performance, analysis, or reporting of the work. Dr. Kamla Pathak has contributed in drafting, editing and revising the manuscript. Mr. Rahul Kumar Singh has carried out the bench

work, laboratory analysis and Ms. Nida Akhtar has conducted the literature survey, collected the data and drafted the manuscript.

Conflict of interest

Authors declare no conflict of interest.

References

1. Lin HH, Ko SM, Hsu LR, Tsai YH. The preparation of norfloxacin loaded liposomes and their *in vitro* evaluation in pig's eye. *J Pharm Pharmacol* 1996;48:801-5.
2. Tarabishy AB, Jeng BH. Bacterial conjunctivitis: a review for internists. *Cleveland Clinic J Med* 2008;75:507-12.
3. Alvarez-Lorenzo C, Yanez F, Barreiro-Iglesias R, Concheiro A. Imprinted soft contact lenses as norfloxacin delivery systems. *J Controlled Release* 2006;113:236-44.
4. Grateri T, Gelfuso GM, Freitas O, Rocha EM, Lopez RF. Enhancing and sustaining the topical ocular delivery of fluconazole using chitosan solution and poloxamer/chitosan *in situ* forming gel. *Eur J Pharm Biopharm* 2011;79:320-27.
5. Utani CA. Update and critical appraisal of the use of topical azithromycin ophthalmic 1 % solution in the treatment of ocular infections. *Clin Ophthalmol* 2011;5:801-09.
6. Gilhotra RM, Nagpal K, Mishra DN. Azithromycin novel drug delivery system for ocular application. *Int J Pharm Investig* 2011;1:22-8.
7. Kumarasen C. Development of novel ocusert contain norfloxacin and *in vitro* evaluation. *J Pharm Res* 2011;4:393-5.
8. Challa R, Ahuja A, Ali J, Khar RK. Cyclodextrins in drug delivery: an updated review. *AAPS PharmSciTech* 2005;6:329-57.
9. Kute SD, Sakore SC, Chakraborty BS. Formulation approaches in ocular drug delivery system. *Int J Pharm Tech* 2010;2:118-45.
10. Akhtar N. Vesicular ocular drug delivery system, preclinical and clinical perspective of drugs delivered via niosomes. *Int J Biopharm* 2013;4:38-48.
11. Abdelbary G, El-gendy N. Niosome-encapsulated gentamicin for ophthalmic controlled drug delivery. *AAPS PharmSciTech* 2008;9:740-7.
12. Liu Z, Li J, Nie S, Liu H, Ding P, Pan W. Study of an alginate/HPMC based *in situ* gelling ophthalmic delivery system for gatifloxacin. *Int J Pharm* 2006;315:12-17.
13. Higuchi T, Connors KA. Phase-solubility techniques. *Adv Anal Chem Instrum* 1965;4:117-210.
14. Qi H, Chen W, Huang C, Li L, Chen C, Li W, et al. Development of a poloxamer analogs/carbopol-based *in situ* gelling and mucoadhesive ophthalmic delivery system for puerarin. *Int J Pharm* 2007;337:178-87.
15. Jaiswal M, Kumar M, Pathak K. Zero order delivery of itraconazole via polymeric micelles incorporated in *in situ* ocular gel for the management of fungal keratitis. *Colloids Surf B Biointerfaces* 2015;130:23-30.
16. Loftsson T, Jarto P, Masson M, Jarvinen T. Cyclodextrin in drug delivery. *Expert Opin Drug Deliv* 2005;2:335-51.
17. Akhtar N, Arkvanshi S, Bhattacharya SS, Verma A, Pathak K. Preparation and evaluation of a buflomedil hydrochloride Niosomal patch for transdermal delivery. *J Liposome Res* 2015;25:191-201.
18. Gan Q, Wang T. Chitosan nanoparticles as protein delivery carrier systematic examination of fabrication conditions for efficient loading and release. *Coll Surf B Biointerf* 2007;59:24-34.
19. Agnihotri SM, Vavia PR. Diclofenac- loaded biopolymeric nanosuspensions for ophthalmic applications. *Nanomedicine* 2009;5:90-5.
20. Chhabra G, Chuttani K, Mishra AK, Pathak K. Design and evaluation of nanoemulsion drug delivery system of amlodipine besilate for improvement of oral bioavailability. *Drug Develop Ind Pharm* 2011;37:907-16.
21. Pignatello R, Bucolo C, Ferrara P, Maltese A, Puleo A, Puglisi

- G. Eudragit RS100 nanosuspension for the ophthalmic controlled delivery of ibuprofen. *Eur J Pharm Sci* 2002;16:53-61.
22. Shirsand SB, Para MS, Kumar ND, Kanani KM, Keerthy D. Formulation and evaluation of ketoconazole niosomal gel drug delivery system. *Int J Pharm Investing* 2012;2:201-7.
 23. Tamizharasi S, Dubey A, Rathi V, Rathi JC. Development and characterization of niosomal drug delivery of gliclazide. *J Young Pharm* 2009;1:205-9.
 24. Uchegbu IF, Florence AT. Non-ionic surfactant vesicles (niosomes): Physical and pharmaceutical chemistry. *Adv Coll Interf Sci* 1995;58:1-55.
 25. Maurya SD, Prajapati SK, Gupta AK, Saxena GK, Dhakar RC. Formulation development and evaluation of ethosome of stavudine. *Ind J Pharm Edu* 2010;44:102-8.
 26. Srividya B, Cardoza RM, Amin PD. Sustained ophthalmic delivery of ofloxacin from a pH triggered in situ gelling system. *J Controlled Rel* 2001;73:205-11.
 27. Singh J, Chhabra G, Pathak K. Development of acetazolamide-loaded, pH-triggered polymeric nanoparticulate in situ gel for sustained ocular delivery: in vitro. ex vivo evaluation and pharmacodynamic study. *Drug Dev Ind Pharm* 2014;40:1223-32.
 28. Riccia EJ, Lunardi LO, Nanclares DMA, Marchetti JM. Sustained release of lidocaine from polaxame 407 gels. *Int J Pharm* 2005;288:235-44.
 29. Majithiya RJ, Ghosh PK, Umerethia ML, Murthy RSR. Thermoreversible-mucoadhesive gel for nasal delivery of sumatriptan. *AAPS PharmSciTech* 2006;7:80-6.
 30. Choy Y, Park J, McCarev B, Edelhauser HF, Prausnitz M. Mucoadhesive microdiscs engineered for ophthalmic drug delivery: Effect of particle geometry and formulation on precorneal residence time. *Investig Ophthalmol Visual Sci* 2008;49:4808-49.
 31. Moghddam SRM, Ahad A, Aqil M, Imam SS, Sultana Y. Formulation and optimization of niosomes for topical diacerein delivery using 3-factor, 3-level Box-Behnken design for the management of psoriasis. *Mat Sci Eng C* 2016;69:789-97.