

Investigating nanostructured liquid crystalline particles as prospective ocular delivery vehicle for tobramycin sulfate: *Ex vivo* and *in vivo* studies

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ABSTRACT

Tobramycin remains the anchor drug for bacterial keratitis treatment and management; however, unlike other aminoglycosides, it does not pass through the gastrointestinal tract. The aim of the current investigation was to formulate tobramycin-loaded nanostructured liquid crystalline particles as an ophthalmic drug delivery system to ameliorate its preocular residence duration and ophthalmic bioavailability. Tobramycin cubosomes were fabricated by liquid-lipid monoolein, water, and poloxamer 407 as a stabilizer. Corneal penetration studies exhibited that the apparent permeation coefficient of tobramycin cubosomes was nearly 3.6-fold greater than marketed tobramycin eye drops. Ocular *in vivo* analysis performed in rabbits' eyes manifested that the intensity of bacterial keratitis was reduced on day 3, and on day 5, the manifestations were considerably mitigated with tobramycin cubosomes as compared to marketed eye drops. Pharmacokinetic study of rabbit aqueous humor demonstrated that the area under curve and the peak concentration of optimized cubosomes were 3.1-fold and 3.3-fold, respectively, which was significantly higher than marketed eye drops. Moreover, histopathological studies illustrated the existence of normal ocular structures, thus indicating that there was no damage to the corneal epithelium or stromal layer. Consequently, the results acquired demonstrated that tobramycin-loaded cubosomal formulation could be a propitious lipid-based nanodelivery system that would enhance retention time and corneal permeability contrast to commercial eye drops.

Key words: Bacterial keratitis, cubosomes, hen's egg chorioallantoic membrane test, nanostructured liquid crystalline particles, ocular pharmacokinetic studies

INTRODUCTION

Tobramycin sulfate (TS) is a hydrophilic cationic aminoglycoside derived from cultures of *Streptomyces*

tenebrarius. Different bacterial infections, particularly Gram-negative microbes, namely strains of *Pseudomonas*, are treated with it. Similar to all aminoglycosides, tobramycin cannot be administered through the oral route as it is not absorbed through the digestive tract and is delivered through the intravenous route, intramuscular route, and ocular route. Tobramycin acts by targeting specific aminoglycoside receptors on 30S and 50S bacterial ribosomes and averting the formation of 70S

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complex. Consequently, mRNA cannot be translated into protein that corroborate apoptosis and necrosis.^[1]

Bacterial keratitis can be elucidated as a sight-menacing visual infection that could be an acute or chronic, transitory, or recurrent infection. It involves any part of the cornea such as marginal or central. Signs and symptoms of keratitis include eye redness, pain, excessive tearing, eye discharge, blurred vision, photophobia, decreased vision, and irritation. 80% of cases of bacterial keratitis are due to the species of *Pseudomonas*, *Staphylococcus*, and *Streptococcus*. The majority of cases of keratitis are related to diseases of ocular surface and ocular trauma. However, extensive use of contact lenses has escalated the prevalence of contact lens-related keratitis. These aspects might alter the ocular surface defense mechanism and allow bacteria to invade the corneal membrane. These factors cause the breakdown of corneal epithelium which allows penetration of bacteria.^[2]

Cubosomes are well-defined, three-dimensional structures that consist of two discrete, continuous, and nonoverlapping hydrophilic nanodomains partitioned by lipid bilayer. They predominantly consist of lipids such as glyceryl monooleate (GMO), water, and stabilizers. Where GMO forms liquid-crystalline cubic phase in excess of water, which partitions two networks of water-channels. Owing to the distinctive anatomy of GMO, cubical phases can amalgamate and provide controlled drug release of diverse polarities and molecular weight. When congregated into cubosomal formulation, they imitate the anatomy of the biological layer which allows cubosomes to the exceedingly lipophilic cornea. Moreover, cubosomes are organized in cubical honeycombed structure permitting them to retain for prolonged duration on corneal membrane, as compared to the spherical vesicles analogues that offers increased duration to drug-loaded molecules to penetrate corneal surface. Cubosomal formulation can be fragmented and disseminated to formulate particulate dispersion that is thermodynamically stable for a prolonged duration. They have the capability to encapsulate hydrophobic, hydrophilic, and amphiphilic molecules. Cubosomes can incorporate high drug payloads because the cubic structure has a high internal surface area.^[3]

Consequently, in the current investigation, tobramycin cubosomes were developed for increased drug targeting, ameliorated therapeutic effect, increased permeation, curtailed dosing frequency, protract precorneal residence span, and enhanced stability. Therefore, the potential of formulated cubosomes to act as a topical carrier was characterized by corneal permeability studies, *in vivo*, ocular pharmacokinetics, and histopathological studies in rabbits and compared with the commercial formulation.

MATERIALS AND METHODS

Materials

TS was procured from Mankind Pharma Ltd., New Delhi, India. GMO was purchased from Otto Chemie., Mumbai, Maharashtra, India. Poloxamer 407 was procured from S.D. Fine-Chem Pvt. Ltd., Mumbai, Maharashtra, India.

Methodology

Preparation of tobramycin sulfate-loaded cubosomes

Cubosomes were formulated by fragmentation of GMO and surfactant bulk cubic phase gels. First, GMO (2.5% w/w) and poloxamer 407 (1.5% w/w) were completely liquidized at 60°C on a hot water bath, and subsequently, TS (0.3%) was intermixed under perpetual agitation. Water was incorporated steadily and the concoction was vortexed. After 48 h, the isotropic cuboidal gel was formulated at room temperature. About 20 ml water was added to disarrange the cubic gel under mechanical stirring. Finally, the formulation was subjected to high-pressure homogenization to form cubosomal formulation. TS cubosomal formulation was stored at room temperature.^[4]

Characterization of tobramycin-loaded nanostructured liquid crystalline particles

Ex vivo transcorneal permeation studies

Transcorneal penetration assessment was executed utilizing extracted rabbit corneal membrane and modified Franz diffusion cell. Rabbits were sacrificed and corneas were cautiously dissected and cleaned with saline solution. Isolated corneas were secured in the middle of the donor compartment and receptor compartment, with epithelium aligned toward the donor compartment. TS cubosomes and tobramycin-marketed eye drops (Tobrex) were positioned in the donor chamber, and stimulated tear fluid (STF) was incorporated in the receptor compartment. The temperature of STF was kept at 37°C ± 0.5°C with continual magnetic stirring. From the receptor chamber, samples were extracted at prearranged interims and refilled with an identical amount of STF.^[5] The samples were analyzed utilizing High performance liquid chromatography (HPLC) analysis at 210 nm. The apparent permeability coefficient (P_{app}) ($\mu\text{g}/\text{s}/\text{cm}^2$) and steady state flux (J_{ss}) ($\mu\text{g}/\text{cm}^2/\text{h}$) were determined using these expressions:

$$J_{SS} = \frac{dQ}{A \times dt}$$

$$P_{app} = \frac{J}{C_0}$$

Where Q is the total volume of drug permeated, A is the area of the exposed cornea, and C_0 is the initial concentration of drug in the donor compartment.

Ocular tolerance test

For evaluation of the ophthalmic irritant potential of

the TS-loaded cubosomes, the hen's egg chorioallantoic membrane (HET-CAM) analysis was performed. Fertilized eggs of 50–60 g in weight were procured from the poultry farm and CAM was created in them. The eggs were incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $55\% \pm 5\%$ relative humidity for 3 days. On day 3, 3 ml of the albumin was withdrawn by utilizing sterile strategies and the aperture was sealed. A window was cut on the equatorial side of the eggs on the 10th day. Thereafter, 0.9% normal saline as negative control, 0.1 N sodium hydroxide as positive control, and TS-loaded cubosomes were introduced on the membrane of CAM. At predetermined interludes, CAM was perceived for vascular impairments, and grading was done: no evident hemorrhage = 0 (nonirritant), noticeable discoloration of membrane = 1 (mild-irritant), vesicles covered moderately due to hemorrhage = 2 (fairly irritant), and vesicles covered entirely due to hemorrhage = 3 (severely irritant).^[6]

Isotonicity evaluation

Isotonicity analysis was executed by the hemolytic method. TS cubosomal formulation was merged with a few droplets of mice blood and was assessed under an inverted microscope at $\times 45$. The integrity and shape of red blood cells were observed with the formulation and compared with the observations using 0.9% NaCl (isotonic solution), hypotonic suspension (3% w/v), and hypertonic suspension (0.45% w/v NaCl).

In vivo studies

All experimental protocols and animal analyses were conducted in accordance with the standard guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The experimental procedure was approved by the Institutional Animal Ethics Committee (1327/PO/ReBi/S/10/CPCSEA).

For *in vivo* investigation, the eyes of the rabbits were inoculated with keratitis. In the present examination, male New Zealand albino rabbits weighed 2–3 kg with no indication of ocular deformity, swelling, and redness were used. Rabbits were classified into three categories and the respective category comprised four rabbits. Group I (diseased control) was instilled with sterile saline solution, Group II (standard) was treated with Tobrex (0.3% w/v) four times a day, whereas Group III received 50 μl of TS cubosomes twice a day.

Pseudomonas aeruginosa was cultured aerobically in 10 ml of brain–heart infusion broth (HiMedia, India) for 18 h at 37°C . Microorganisms were accumulated and centrifuged at 6000 rpm for 15 min. Subsequently washed with phosphate-buffered saline (PBS) having pH 7.4 and then suspended in 2 ml PBS. All three groups of rabbits were inoculated with *P. aeruginosa* to induce keratitis. The middle segment of the corneal membrane was intrastromally infected with 50 μl *P. aeruginosa* suspension (containing nearly 10^7 – 10^8

colony-forming units) utilizing a sterilized 29-gauge (G) needle.^[7] Following 24 h of inoculation, bacterial keratitis was established, and then, topical treatment was initiated. The eyes of rabbits were scrutinized for clinical symptoms of keratitis for instance lacrimal secretion, redness, corneal ulceration, ocular mucus discharge, and inflammation of eyelids. The assessment was done from 0 to 4, and the rating was designated as 0 = none, 1 = minimal, 2 = moderate, 3 = acute, and 4 = grievous. The assessment of clinical indications was executed at interims of 0, 24, 72, and 120 h.^[8] Relative examination of developed tobramycin cubosomes with tobramycin-marketed eye drops (Tobrex) was carried out and the treatment outcomes were compared.

Ocular pharmacokinetic studies

The rabbits were split into two categories: Group I received commercial Tobrex eye drops (0.3%) and Group II administered with TS cubosomes. The rabbits were anesthetized with sodium pentobarbital (30 mg/kg) introduced into marginal ear artery. One group received 50 μL commercial eye drops, and other cubosomal formulation, subsequently, aqueous humor was withdrawn with 1 ml hypodermic insulin needle affixed with a 26G needle after 0.25, 0.5, 1, 2, 3, 4, 5, and 6 h. The samples of aqueous humor were accumulated in Eppendorf and protein was precipitated by vortexing with 100 μl 6% perchloric acid-methanol solution. Precipitated protein was withdrawn by centrifugation at 5000 rpm for 15 min and drug concentration was ascertained by HPLC. The pharmacokinetic parameters were estimated from graphs employing the linear trapezoidal rule.^[9]

Corneal toxicity studies (histopathology studies)

Histopathological evaluation was executed to validate outcomes acquired from *in vivo* analysis on TS cubosomal formulation and furthermore to inspect anatomy of the cornea. For euthanization of rabbits, sodium pentobarbital was injected, and subsequently, eye balls were extracted. The corneal membranes were removed and inoculated with TS cubosomes (test sample), phosphate buffer saline (negative control) and sodium dodecyl sulfate (SDS) 0.1% w/v (positive control). Thereafter, corneal membranes were rinsed with PBS. Corneal membranes were instantly instantaneously fixed with formalin (8% v/v). Cross-sections were taken and stained by using eosin and hematoxylin and the modifications were viewed through a microscope.

RESULTS AND DISCUSSION

Ex vivo transcorneal permeation studies

P_{app} of TS cubosomal and Tobrex eye drops was $7.85 \pm 0.32 \times 10^6$ and $2.19 \pm 0.47 \times 10^6$ $\mu\text{g}/\text{s}/\text{cm}^2$, respectively. Jss was $2.62 \pm 0.48 \times 10^3$ and $0.71 \pm 0.23 \times 10^3$ $\mu\text{g}/\text{cm}^2/\text{h}$. In contrast to tobramycin eye drops, TS cubosomes exhibited approximately 3.6-fold enhancement in P_{app} and 3.7-fold enhancement in a Jss. Increased permeability of the

developed cubosomes could be owed to their bioadhesive nature. Cubosomes mimic the anatomy of biological surface which allows cubo-nanoformulations to bond with the exceedingly lipophilic cornea. Moreover, they are organized in a cubical honeycombed structure permitting them to remain for an extended span on the cornea.^[10] In addition, poloxamer could have led to interference in narrow barriers of ocular surface epithelium. Transcorneal permeation profiles of tobramycin cubosomal formulation and marketed eye drops are depicted in Figure 1.

Ocular tolerance test

The HET-CAM estimates the potential of the test substance to cause clotting, hemorrhage, hyperemia, or impairment in blood vessels as depicted in Figure 2. The results obtained were compared with that of normal saline which is practically nonirritant. A grading of 0 was attained with normal saline through the study period. However, the test preparation was also completely nonirritant, and the observed mean score was 0. Positive control revealed hemorrhage and clotting that established acute irritating nature, and the observed mean score was 3. From these results, it can be said that TS cubosomes were potentially nonirritant to the ocular surface owing to the nonimmunogenic and biocompatible nature of cubosomal formulation.^[11]

Isotonicity evaluation

Hypotonic and hypertonic suspension has a propensity to irritate and cause impairment to the ocular surface. The results revealed that prepared TS cubosomes were isotonic with blood and hence lachrymal fluid. The droplets of mice blood merged with hypertonic dispersion appeared to have contract, whereas those treated with hypotonic dispersion appeared to have bulged. However, the blood cells treated with isotonic solution, marketed preparation, and test preparation were unaffected and did not show any signs of shrinkage or swelling. Hence, it was deduced that the formulation did not affect the tone of the cells, i.e., cells were not shrunken or swollen. Thus, the prepared formulation was able to achieve isotonicity.

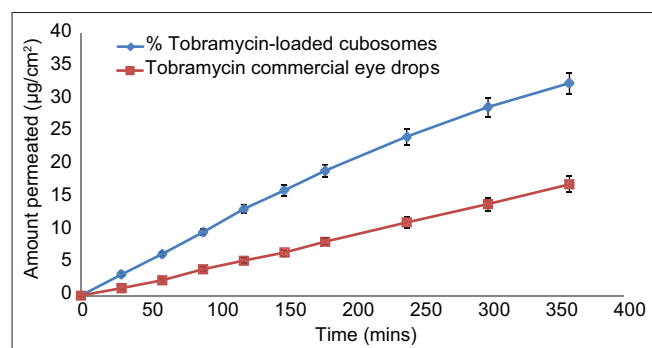


Figure 1: Transcorneal permeation profiles of tobramycin sulfate cubosomes and tobramycin commercial eye drops using excised rabbit corneas. All the observations were carried out in triplicate and mean \pm standard deviation

In vivo studies

In vivo examinations were executed for the estimation of ocular topical therapy in rabbits as shown in Figure 3. Results indicated that following 120 h, the grading of the conjunctival redness in Group II was 1.465 ± 0.51 and in Group III was 0.08 ± 0.0 , which was substantially low. Lacrimal secretion and ocular mucus discharge in Group II were 1.642 ± 0.85 and 1.336 ± 0.78 ; on the contrary, in Group III, it was 0.06 ± 0.0 and 0.09 ± 0.0 .^[12] The severity of bacterial keratitis was alleviated following a period of 72 h by the TS cubosomal formulation, and following 120 h, the manifestations were remarkably mitigated. Comparison of scores obtained with TS cubosomes indicated revealed that the formulation was effective in alleviating manifestations of keratitis with the advantage of lesser frequency of administration compared to eye drops.^[13] Scores of TS cubosomal formulation and Tobrex eye drops are compared as depicted in Table 1. The results obtained were due to enhanced precorneal residence duration of the drug on the corneal membrane. Minimized dosage was achieved by TS cubosomal formulation contrast to commercial eye drops.

Ocular pharmacokinetic studies

To assess the volume of drugs in aqueous humor, TS cubosomes and Tobrex eye drops were analyzed for *in vivo* ocular pharmacokinetic evaluation. Area under curve value of TS cubosomes was 473.41 ± 0.54 $\mu\text{g min/ml}$ and tobramycin eye drops was 152.51 ± 0.61 $\mu\text{g min/ml}$, which was approximately 3.1 times greater. However, C_{max} of tobramycin cubosomes was 4.86 ± 0.48 $\mu\text{g/ml}$ and tobramycin eye drops was 1.48 ± 0.86 $\mu\text{g/ml}$, which was about 3.3 times greater ($P < 0.01$). The time to peak concentration (T_{max}) of cubosomes was 2.1-fold higher and mean residence time was nearly 2.5-fold higher than tobramycin eye drops.^[14] The results obtained could be owed to the bioadhesive nature of GMO that could fuse cubosomes with the corneal surface and conjunctival sac, and due to this reason, transcorneal residence time of tobramycin was increased. The ameliorated ocular bioavailability could be ascribed to three aspects, namely, ocular contact duration, increased

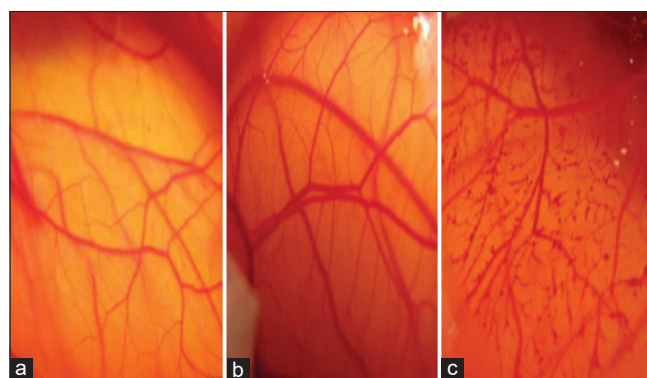


Figure 2: Photographs illustrating (a) normal saline (negative control), (b) tobramycin-loaded cubosomes, and (c) sodium hydroxide (positive control)

Table 1: Scoring of parameters of keratitis in rabbit's eyes

Parameters	0 h	24 h	72 h	120 h
Clinical scores in Group II - Tobramycin commercial eye drops				
Conjunctival redness	3.538±1.18	3.127±0.26	2.813±0.67	1.465±0.51
Lacrimal secretion	3.671±0.65	2.551±0.48	2.101±0.73	1.642±0.85
Ocular mucus discharge	3.184±1.26	2.327±0.54	1.858±0.42	1.336±0.78
Corneal ulceration	2.831±0.85	1.834±0.39	1.174±0.56	0.861±0.46
Inflammation of eyelids	2.592±1.17	1.693±0.85	1.265±0.78	0.711±0.08
Clinical scores in Group III - Tobramycin-cubosomes				
Conjunctival redness	3.357±0.57	2.806±0.68	1.262±0.76	0.08±0.0
Lacrimal secretion	3.292±1.42	1.273±0.42	0.533±0.51	0.06±0.0
Ocular mucus discharge	3.637±0.59	2.118±0.76	0.796±0.82	0.09±0.0
Corneal ulceration	2.622±1.28	0.521±0.12	0.035±0.67	0.00±0.0
Inflammation of eyelids	2.745±0.92	0.613±0.31	0.121±0.14	0.00±0.0

Data expressed as mean values±SD (n=4). SD: Standard deviation

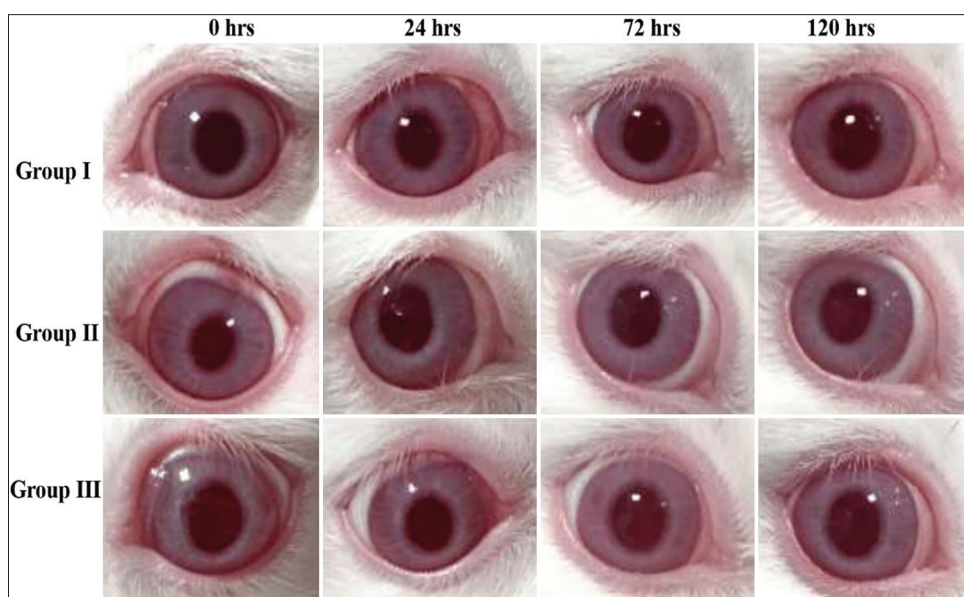


Figure 3: Photographs of rabbit eyes during treatment at different time intervals. The photograph of each group was the representative of four rabbits per group

drug-loading ability, and high drug permeability through the corneal membrane. Results exhibited that cubosomes exhibited prolonged residence time which enhances the ocular contact time. Such novel delivery systems are beneficial than conventional dosage forms because of enhanced solubility and bioavailability.^[15] Pharmacokinetic parameters are tabulated in Table 2 and concentration–time profiles of drug in the aqueous humor are depicted in the graphical form shown in Figure 4.

Corneal toxicity studies (histopathology studies)

Corneal cross-sections were incubated with optimized TS cubosomes, SDS, and PBS. Corneal samples treated with SDS (positive control) exhibited impairment of corneal epithelium. Impaired cells were visibly noticeable with remarkable inflammation of corneal epithelium. However, test formulation and normal saline illustrated the existence of normal ocular structures and no corneal toxicity.

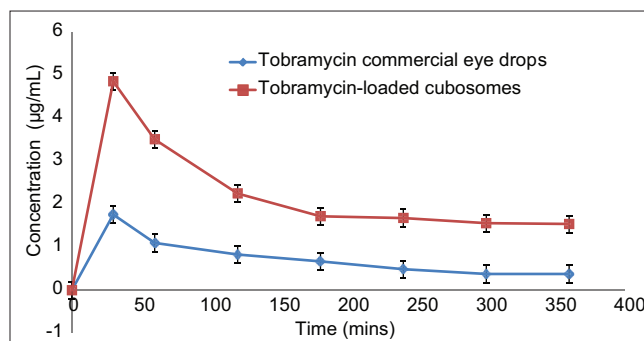


Figure 4: Concentration–time profiles of tobramycin in aqueous humor after topical delivery of tobramycin eye drops and tobramycin cubosomes (n = 4, mean ± standard deviation)

Consequently, it was corroborated that there was no damage to the corneal epithelium or stromal layer, thus implying the safety of the developed cubosomes. Figure 5 illustrates the

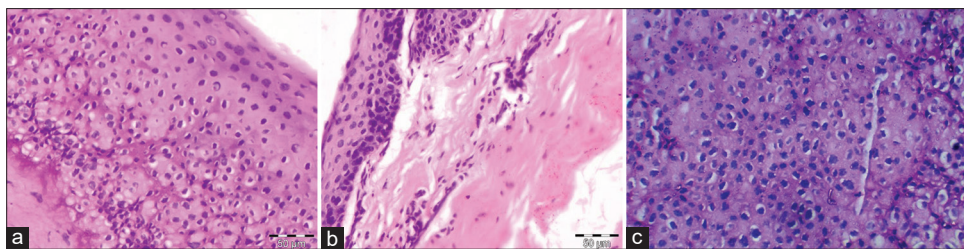


Figure 5: Histological sections of rabbit cornea (a) negative control, (b) positive control, and (c) test sample (tobramycin sulfate-loaded cubosomes)

Table 2: Pharmacokinetic parameters of tobramycin eye drops and tobramycin cubosomes in aqueous humor following topical delivery

Parameters	Tobramycin eye drops	Tobramycin cubosomes
AUC ₍₀₋₃₆₀₎ ($\mu\text{g min/ml}$)	164.51 \pm 0.61	473.41 \pm 0.54
C _{max} ($\mu\text{g/ml}$)	1.76 \pm 0.86	4.86 \pm 0.48
T _{max} (min)	28.5 \pm 0.26	60.27 \pm 0.73
Mean residence time (min)	57.32 \pm 0.93	141.5 \pm 0.52

Data expressed as mean values \pm SD (n=4). AUC: Area under curve, C_{max}: Peak concentration, T_{max}: Time to peak concentration, SD: Standard deviation

histopathological images for the negative control, positive control, and test formulation.

CONCLUSIONS

In the current investigation, cubosomes were formulated utilizing GMO, water, and surfactant poloxamer 407 by fabrication technique. HET-CAM estimated that test preparation was completely nonirritant to the ocular surface owing to the nonimmunogenic and biocompatible nature of cubosomal formulation. *Ex vivo* permeation assessment demonstrated that the cubical honeycombed structure of cubosomes permitted them to remain for an extended span on the cornea. However, *in vivo* examination illustrated that the developed formulation was effective in alleviating manifestations of keratitis with the advantage of lesser frequency of administration compared to eye drops. It was deduced for pharmacokinetic studies that cubosomal formulation was effective in extending the retention time and metabolic rate of tobramycin in the aqueous humor and releasing drugs in a sustained manner. Histological sections illustrated the existence of normal ocular structures and no corneal toxicity. Therefore, it could be presumed that TS cubosomal formulation is a prospective substitute to commercial formulations.

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Conflicts of interest

There are no conflicts of interest.

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