

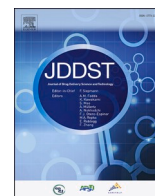
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Moxifloxacin loaded microspheres-composed gel for controlled release and enhanced penetration in ocular tissues: *In vitro*, *ex vivo* and *in vivo* proof of concept

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ABSTRACT

Microspheres-enriched ocular gels are proposed in this work for the loading of a model quinolone antibiotic of broad-spectrum - moxifloxacin hydrochloride (MXF) - as an approach to achieve a controlled release profile and enhance drug's bioavailability in ocular milieu. MXF is a synthetic fluoroquinolone antibacterial agent used for treatment of ocular diseases caused by strains of aerobic gram positive and gram-negative bacteria, besides others. MXF-loaded Eudragit RS microspheres were prepared by emulsion solvent evaporation method, as previously described by us. Optimized microspheres were characterized and then formulated in an ocular gel. Hydrophilic polymeric base HPMC K100 was used as gelling agent. Ocular gel enriched with microspheres was evaluated for its physicochemical parameters, *in vitro* drug release, *ex vivo* corneal and scleral permeation and bioadhesion, *in vivo* primary eye irritation and *in vivo* efficacy study. The developed MXF microspheres dispersed in an ocular gel showed an *in vitro* drug release over a period 12 h, improved corneal and scleral penetration and increased therapeutic efficacy with a reduced administration frequency which may contribute to increase patient's compliance to ocular treatment.

1. Introduction

Ocular infections, such as conjunctivitis, constitute a serious health problem worldwide with antimicrobial resistance being a major challenge in modern medicine [1]. The inflammation of conjunctiva (the membrane that covers the sclera and lines the inside of the eyelids) – or conjunctivitis - can result either from gram positive (e.g., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Streptococcus pneumoniae*, *Streptococcus viridans* group) or gram negative (e.g., *Acinetobacter* species, *Haemophilus influenzae*) bacteria, from viral infections (e.g., adenoviruses, herpes virus), or from allergic reactions from e.g. pollen, animal dander and dust mites or even

chemicals. As happens with systemic infections, local antimicrobial resistance in ocular isolates has been on the rise over the past two decades [2,3].

In this research field, 4th generation fluoroquinolones accomplish the ideal ophthalmic anti-infective drug properties since they exhibit broad-spectrum activity against gram-positive, gram-negative, and atypical bacterial species [4]. Amongst the new antibiotics generations, moxifloxacin hydrochloride (MXF) shows a broad antibacterial spectrum against several key ocular pathogens and can be administered topically to the eye [4–6]. MXF is known to inhibit topoisomerase II (DNA gyrase) and topoisomerase IV. These latter are enzymes involved in the replication, transcription, partitioning and repair of bacterial

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DNA. In ophthalmology MXF is commonly used for treatment of conjunctivitis and is also being used to treat bacterial endophthalmitis which is a serious complication of ocular surgery and of eye trauma. Currently, MXF is available in the form of eye drops, which are conventional dosage forms exhibiting the poor bioavailability of drug at the site of action, resulting in a short duration of ocular therapeutic effect and therefore demanding a frequent dosing regimen. In ocular drug delivery, the physiological constraints imposed by the protective mechanisms of the eye (e.g., lacrimation, tear dilution, tear turn over, nasolacrimal drainage and solution drainage) lead to poor absorption of drugs with very small fractions of instilled dose penetrating the cornea and reaching the intraocular tissues [7–9].

Drug solution drainage away from the precorneal area has been shown to be the most significant factor in reducing the contact time of the drug with cornea and consequently compromise the ocular bioavailability of topical dosage forms. In case of ophthalmic administration of conventional dosage forms is further made inefficient by tear turn over. Due to these factors, typically only less than 5% of drug reaches the aqueous humor [10]. To increase the contact time of drug with the ocular surfaces and thus to increase ocular bioavailability, various ophthalmic dosage forms, such as viscous solutions, ointments or polymeric inserts, have been used [11]. Though contact time is increased with these dosage forms, they also suffer from disadvantages such as blurred vision (viscous solutions, ointments) and patient discomfort (ocular inserts) [11].

These shortcomings have led researchers to seek other approaches to increase the bioavailability by controlling the release of the drugs on the ocular tissues over extended time. Polymeric microparticles have shown to possess unique properties as drug delivery systems able to encapsulate both water-insoluble and water-soluble agents, elicit their efficacy and provide an extended release [12]. Besides, they can also be tailored-made to show mucoadhesive properties and fine-tune the release profile of the payload [13]. Among different materials used to formulate ocular microspheres, Eudragit is one of the most widely used due to their biocompatibility [14,15]. Specifically, Eudragit RS, is a widely used polymer for sustained-release microencapsulated dosage forms. Eudragit RS has been investigated as a suitable polymer to prolong the ophthalmic delivery of drugs and increase their ocular penetration [16,17]. Therefore, this polymer represents a suitable candidate for topical ocular administration. In addition, to potentiate the mucoadhesiveness of the drug delivery systems, one of the most patient-comfortable approaches are the mucoadhesive gels. Eudragit microparticles can be dispersed in mucoadhesive gels that will increase the contact time of the drug with the first layers of the eye.

The aim of this work was to develop a controlled release delivery system for ophthalmic administration of a model fluoroquinolone (MXF) and based on microspheres that will act as drug reservoir, entrapped in a polymeric mucoadhesive gel base, to be an alternative to conventional eye drops for administration of MXF. In this system, the microsphere delivery system would act as a matrix drug reservoir to control the release of the payload while the polymeric gel would permit ocular administration and prolonged retention in the conjunctival sac. For this purpose, Eudragit RS was used for the encapsulation of MXF to prepare drug-loaded microspheres. Optimized MXF-loaded Eudragit RS microspheres were then incorporated into hydroxypropyl methyl cellulose (HPMC) K100 mucoadhesive polymeric gel base to prepare a controlled release ocular gel. Ocular gel enriched with MXF microspheres was then evaluated for its physicochemical parameters, *in vitro* drug release, *ex vivo* corneal and scleral permeation and bioadhesion, *in vivo* primary eye irritation and *in vivo* efficacy study.

2. Materials and methods

2.1. Materials

The quinolone antibiotic moxifloxacin hydrochloride (MXF) was

obtained as a gift from Ciron Drugs & Pharmaceuticals Pvt. Ltd. (Maharashtra, Mumbai, India). The polyacrylate polymer Eudragit®RS was obtained as a gift from Degussa India Pvt. Ltd. (Chennai, Tamil Nadu, India) and hydroxypropyl methyl cellulose polymer HPMC K®100 M was gift sample from Signet Excipients Private Limited (Mumbai, India). If not otherwise stated, all remaining reagents were of analytical grade and obtained from Sigma-Aldrich Chemicals Private Limited (Bangalore, India).

2.2. Animals

New Zealand White strain of rabbits weighing in size range of 1.8–2.0 kg were used for the primary ocular irritation study and efficacy study. The rabbits were housed separately in each cage under temperature condition of 20–24 °C and RH 50–60%. The animals were fed with conventional laboratory diet and with potable water. The experimental protocols were approved by the Institutional Animal Ethics Committee of C. U. Shah College of Pharmacy, SNDT Women's University, Mumbai and conducted according to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

2.3. Preparation of MXF loaded eudragit microspheres

Eudragit RS microspheres loaded with MXF were prepared by emulsification solvent evaporation method, as previously described by us [9,18], after optimizing the formulation by a 3² factorial design study [18]. Briefly, Eudragit RS was dissolved in acetone by sonication for 10 min and MXF was dissolved in methanol. MXF solution was added dropwise to the polymer solution. The obtained solution containing MXF and Eudragit RS was added to a mixture of 0.2% Span 80 in light liquid paraffin (160 mL) to obtain an emulsion. The stirring of this emulsion was done at speed of 2500 rpm until the complete evaporation of the organic solvents. MXF-loaded Eudragit RS microspheres were obtained and collected by vacuum filtration, washed with n-hexane and dried. Microspheres were then carefully sieved, weighed, labeled and stored in air-tight container which were finally stored in a desiccator [19].

2.4. Mean particle size

The mean particle size of MXF-loaded Eudragit RS microspheres was determined by optical microscopy (Nexcope NE910, Bresser GmbH, Rhede, Germany) [20]. For each formulation, at least 200 microspheres were observed, checked for their appearance and their mean size was determined.

2.5. Loading capacity

To determine the loading capacity, 20 mg of dried microspheres were dissolved in 10 ml methanol by sonicating it for 10 min (USC100T ultrasonic bath, VWR, Radnor, Pennsylvania, USA). MXF was quantified at λ 294 nm using an UV-spectrophotometer V-700 (Jasco UK Limited, Yorkshire, UK), against a standard calibration curve of the drug in methanol ($Y = 0.1238x + 0.0031, R^2 = 0.9989$).

2.6. Preparation of ocular gel containing MXF loaded eudragit microspheres

Gelling agent HPMC was dispersed in distilled water with continuous stirring under overhead stirrer. Gelling agent was then allowed to soak for about 4 h and then MXF loaded microspheres equivalent to required dose (0.5%) were dispersed in gel base under overhead stirring and allowed to stir until the microspheres get uniformly distributed throughout gel base [21]. The formulation was then sterilized by autoclaving at 121 °C and 15 p.s.i. for 15 minutes. The sterilized formulations were then filled in aluminum tubes under the laminar air flow.

Ocular gel was optimized by modifying the percentage of gelling agent used and examining the physicochemical properties.

2.7. Evaluation of the ocular gel containing MXF loaded microspheres

The developed ophthalmic gel enriched with MXF loaded microspheres was evaluated for color, odor, appearance and pH. In addition, the total drug content of the ocular gel was determined by using a validated UV spectrophotometric method. The ocular gel containing MXF loaded microspheres was taken in volumetric flask containing methanol. The solution was then sonicated for 20 min followed by filtration and the absorbance was measured, as described in section 2.5. Spreadability index of the prepared ocular gel was determined by using spreadability test apparatus. Spreadability test apparatus consists of two glass plates of width and length 10×20 cm. One of glass plate is fixed on to a wooden block, while other plate is free to slide onto the former one. One end of the mounted plate is tightly attached to a pan meant for holding the weight [22]. The spreadability index (SI) was determined as follows:

$$SI = \frac{\text{Time take for plate to slide down (s)}}{\text{Weight of sample applied on the plate (g)}}$$

2.8. In vitro drug release studies

In vitro drug release from the prepared ocular gel containing MXF-loaded microspheres was determined by using specially designed Franz diffusion cells (Orchid Scientific, Nashik, India). Diffusion media used was phosphate buffer pH 7.4. Same procedure was followed as discussed in the *in vitro* release studies of microspheres [23,24].

2.9. Rheological studies of the ocular gel containing MXF loaded microspheres

The rheology of the ocular gel was determined by Brookfield Cone/Plate Rheometer (Sunjay Biotech Solutions Pvt. Ltd., Mumbai, India). Rheogram shows the plot of shear stress verses shear strain. The sample was placed at the center of the plate, which was then raised into position under the cone. The cone was driven by variable-speed motor and sample was sheared in a narrow gap between the stationary plate and rotating cone. Rate of shear varied accordingly and the viscous traction or torque produced on the cone was read on the indicator scale [25,26].

2.10. Microbial assay method

Quantification of MXF was done by the agar diffusion test employing the cup-plate technique [27]. Two organisms, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228, which are susceptible to MXF were selected. The amount of MXF penetrated in the sclera and cornea was estimated by using a calibration plot. The standard solution was obtained by homogenizing the isolated sheep sclera or cornea in 5 ml of dimethyl sulfoxide. This homogenized solution was used as a solvent for preparation of stock solution of MXF. Stock solution was further diluted with the homogenized solution to obtain concentrations in the range of 50–250 µg/ml. Four cups were made using sterile borer in each plate of sterile nutrient agar previously seeded with *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228. To each cup different concentrations of MXF were added in sterile condition. The plates were kept for incubation for 24 h at $37 \pm 0.5^\circ\text{C}$. After the incubation period the zones of inhibition were compared. For all the microorganisms the test was carried out by triplicate.

2.11. Ex vivo bioadhesion study

Ex vivo bioadhesion study of developed ocular gel was performed using freshly excised sheep cornea and sclera obtained from a local slaughterhouse in Mumbai (India) [28]. A special apparatus fabricated using modified analytical balance was used to test the bioadhesion of ocular gel. The working of double beam physical balance formed the basis of the bioadhesion fabricated test apparatus. Bioadhesion was determined regarding the weight in g required for detachment from cornea or sclera. This weight in g was expressed in the form of detachment stress (DS) (dyne/cm²) using Equation (1):

$$DS = m \times g \times a \quad (\text{Eq. 1})$$

where “*m*” is the mass (grams), “*a*” is the area of exposed tissue (cm²) and “*g*” is the acceleration due to gravity (9.8000 m/s²).

2.12. Ex vivo penetration study

Ex vivo penetration of MXF from the developed ocular gel was determined both in sheep scleral and corneal tissue obtained from a local slaughterhouse in Mumbai (India). A validated microbial assay method was used for estimation of MXF in cornea and sclera. *Ex vivo* penetration of ocular gel containing microspheres was determined by using modified Franz diffusion cell apparatus [29]. Ocular gel equivalent to a drug dose was applied on the top of the sheep sclera and cornea mounted on the diffusion cell. Water at 37°C was circulated through the water jacket surrounding the receptor cell and Teflon coated magnetic stir bar kept at bottom of the receptor cell created a homogeneous receptor volume. Phosphate buffer pH 7.4 was used as diffusion media. Sample of receptor media was withdrawn at predetermined time intervals (1, 2, 4, 6, 8, 24 h) and the amount of MXF was evaluated. Sclera and cornea were dismantled from the cell at the end of 24 h and the tissues were homogenized with 5 ml of dimethyl sulfoxide for 5 min. Extract containing homogenized scleral and corneal tissues was filtered through Whatman filter paper and subjected to microbial assay for quantification of MXF penetrated.

2.13. In vivo primary ocular irritation study

The developed controlled release ocular gel of MXF was subjected to Draize irritation test on rabbits to check eye irritation of developed formulation. New Zealand White strain of rabbits weighing in size range of 1.8–2.0 kg were selected for the study. Both the eyes of experimental animals provisionally selected for testing were examined within 24 h before stating the test. Animals showing eye irritation, ocular defects and pre-existing corneal injury were not used. The test substance was applied in a single dose to one eye of the experimental animal; the untreated eye serves as a control. 0.5 g of sample were placed in the conjunctival sac of one eye of each animal after gently pulling the lower lid away from the eyeball. The lids were then gently held together for about 1 s in order to prevent loss of material. The other eye which remains untreated served as control. The degree of eye irritation was evaluated by scoring lesion of conjunctiva, cornea and iris at specific intervals. Rabbit eyes were examined at 1, 6, 8, 24, 48 and 72 h after application of test substance [10]. The grade of ocular reaction was recorded at each examination and photographs of the intact rabbit eye at each timepoint were taken.

2.14. In vivo efficacy study

Staphylococcal conjunctivitis is one of the most frequent ocular conditions. MXF, a fluoroquinolone antibacterial agent is widely used for conjunctivitis. The developed controlled release ocular gel containing MXF loaded microspheres was evaluated for their antimicrobial efficacy using staphylococcal conjunctival model in rabbits [19]. Efficacy of the

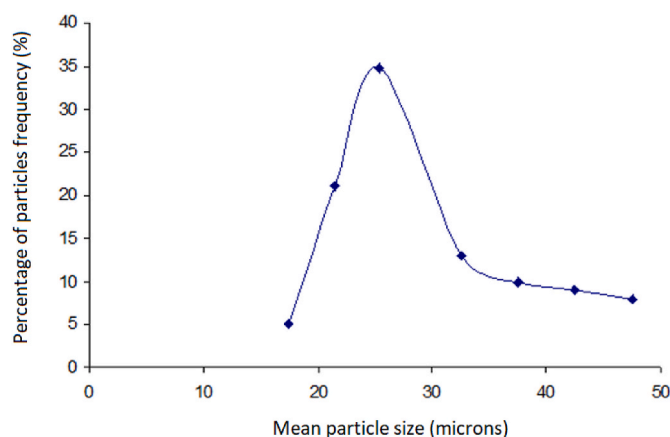


Fig. 1. Particle size distribution of the optimized moxifloxacin microspheres.

Table 1

Characterization and evaluation of ocular gel containing MXF-loaded microspheres.

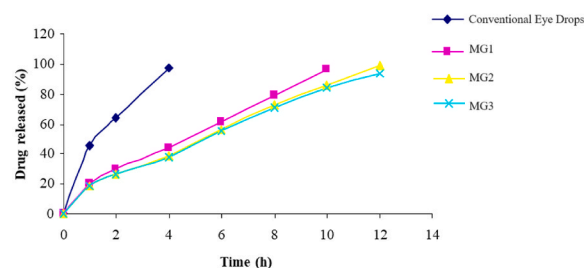
Parameter	Formulation code		
	MG1 6% HPMC (w/v)	MG2 7% HPMC (w/v)	MG3 8% HPMC (w/v)
Color	Pale yellow	Pale yellow	Pale yellow
Odor	Odorless	Odorless	Odorless
Appearance	Translucent	Translucent	Translucent
Visual observation	Stable	Stable	Stable
pH	7.3	7.4	7.2
Spredability index (sec/g)	2.52	2.18	1.52
Total drug content (%)	96.19	98.99	97.10

developed controlled release ocular gel was compared with commercially available conventional MXF eye drops. Conjunctivitis was induced in one eye of all the experimental animals, by instillation of 0.1 ml normal saline suspension containing 10^6 organisms of *Staphylococcus aureus* per ml. Observations on the degree of severity of conjunctivitis were noted 24 h after ocular instillation of the Staphylococcal suspension. Treatments were given to all infected rabbits (except negative control group) 24 h after the induction of conjunctivitis daily for five days. Four types of treatments were given to the rabbits. Group I received gel dispersed MXF loaded microspheres once a day whereas Group II received gel dispersed MXF loaded microspheres twice a day. Conventional eye drops were given to Group III and Group IV in which Group III received the conventional eye drops for once a day while Group IV received the conventional eye drops more frequently (four times a day). During the treatment, eyes were examined daily by grading the ocular lesions viz. cornea, iris, conjunctivae, chemosis, lacrimation or mucopurulent discharge and photophobia and the observations were recorded.

3. Result and discussion

A 3^2 factorial design was previously reported by us to achieve the optimal MXF loaded Eudragit microspheres [18], which were found to be free flowing and pale-yellow colored particles. From the optical microscopy analysis, all the microspheres showed a smooth surface and spherical shape and a normal size distribution with the mean particle size of 25.2 μm , thus suitable for ophthalmic administration avoiding irritation [30,31]. Particle size distribution of the optimized microspheres is depicted in Fig. 1. In our previous study, we showed by scanning electron microscopy images, that the optimized microspheres were spherical with a smooth surface [18], in accordance with Eudragit

A



B

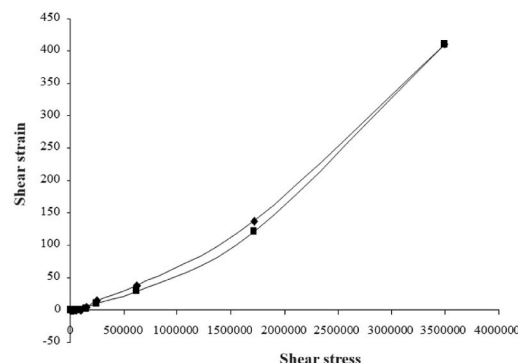


Fig. 2. A) Comparative *in vitro* drug release of conventional eye drops and ocular gels containing MXF loaded microspheres with increasing concentration of gelling agent, 6% (MG1), 7% (MG2) and 8% (MG3) B) Rheogram of MXF-loaded microspheres ocular gel (MG2).

microspheres developed by other authors [32]. The mean particle size of around 25 μm was also confirmed which was same as obtained from these optical microscope studies (Fig. 1).

HPMC was used to disperse the freshly prepared microspheres in order to develop the ocular gel. Three different concentrations of HPMC were assessed, as shown in Table 1. Lajri and Ravindranath reported the use of up to 5% of HPMC K100 for the development of *in situ* ocular gels [33]. Our purpose was to develop a semi-solid formulation in which microspheres loading MXF could be dispersed, thus we tested HPMC concentrations slightly above 5%, namely, 6%, 7% and 8% (w/v). The developed ocular gel containing MXF loaded microspheres was pale-yellow, odorless, stable and translucent, in the three concentrations assessed. Moreover, pH was close to neutrality and total drug content was more than 95 % in all the formulations. At high HPMC concentration, a lower value of the spreadability index was recorded, attributed to its higher viscosity. Therefore, an optimized HPMC concentration of 7% was chosen, provided that it resulted in the higher drug content, while keeping a suitable spreadability index and a neutral pH.

A comparative *in vitro* drug release was carried out for conventional MXF eye drops and the ocular gels with the three HPMC concentrations where the MXF microspheres were dispersed in order to evaluate the influence of HPMC amount on the release profile (Fig. 2A). As HPMC concentration was increased, the rate of drug release decreased [19]. The $t_{90\%}$ was increased from 9.10 h at 6% HPMC concentration to 10.8h at 7% and 11 h at 8% HPMC concentration. Conventional MXF eye drops showed a fast release with the $t_{10\%}$ of 6 min, $t_{50\%}$ of 1.2 h and $t_{90\%}$ of 3.70 h and 97.4% drug was released at the end of 4 h. Although no lag-time was recorded for any of the three formulations, it is clear that microspheres delayed the release of MXF when compared to the conventional eye drops. By the end of the second hour, almost 70% of the drug was released from the eye drops, whereas only about 30% from MG2 and MG3 formulations. Due to the small difference between 7% and 8% HPMC concentration, and provided that MG2 showed the highest drug content (Table 1), 7% HPMC was chosen for further studies. Rheogram of MXF-loaded microspheres containing HPMC gel (MG2) shows the plot of shear stress verses shear strain (Fig. 2B). From the

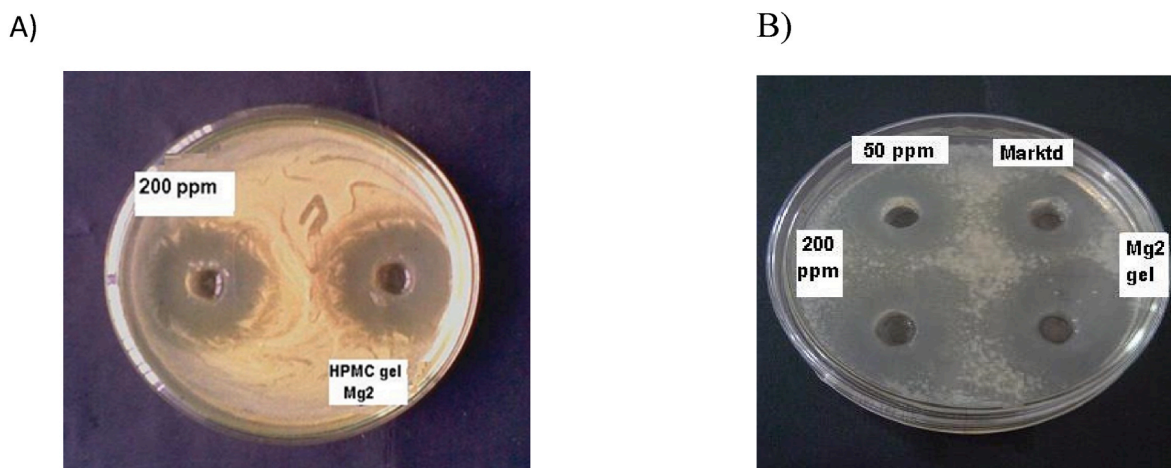


Fig. 3. Antimicrobial activity of MXF microspheres dispersed in HPMC gel against A) *Staphylococcus aureus*, B) *Staphylococcus epidermidis*.

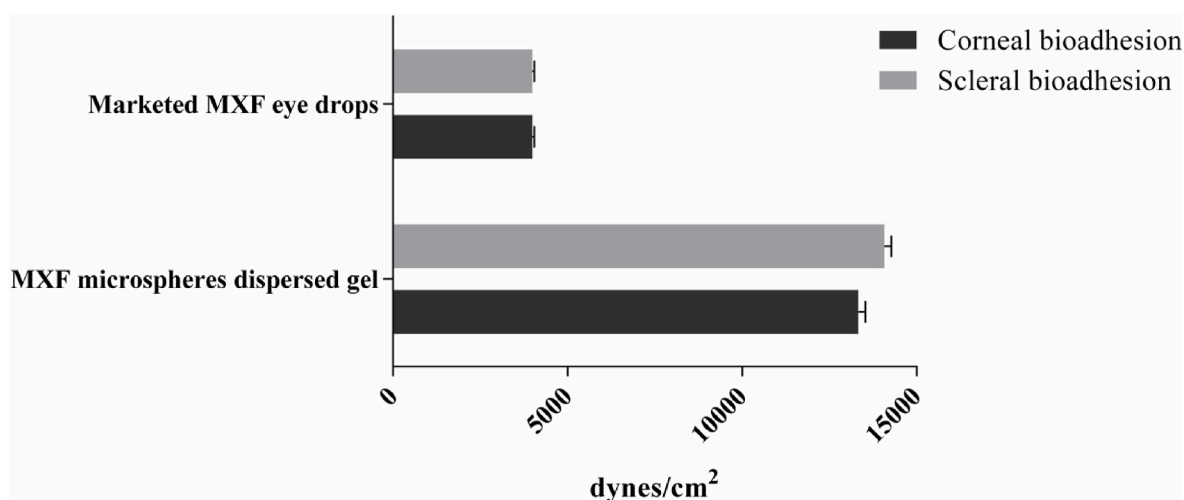


Fig. 4. Comparative corneal and scleral bioadhesion study of developed MXF microspheres dispersed in HPMC gel with the marketed eye drop formulation.

rheological study, it is observed that developed ocular gel showed a thixotropic behavior, where down curve was displaced to the left of the up curve, showing that the material has lower consistency at any one rate of shear than it had on the up curve.

This thixotropic behaviour means that the apparent viscosity decreases under shear stress, followed by a gradual recovery when the stress is removed [34]. Moreover, the hysteresis loop was formed between the two curves. This indicates breakdown of structure (shear thinning systems i.e., pseudoplastic) that does not reform immediately when the stress is removed or reduced. Thus, thixotropy, or time dependent flow, occurs because the gels require the finite time to rebuild its original structure that breaks down during continuous shear measurement. This allows easy spreading on the ocular surface by blinking and transformation into a viscous fluid by favoring a prolonged ocular retention time [35].

To evaluate the antimicrobial activity of MXF microspheres dispersed in HPMC gel (MG2), two gram-positive strains have been selected. *Staphylococcus aureus* is the most dangerous *staphylococcus* strain capable of infecting external eye tissues and is one of the primary causes of acute conjunctivitis. *Staphylococcus epidermidis*, although commensal of the skin microbiota and healthy conjunctiva, it may be one of the causes of conjunctivitis, keratitis and blepharitis [36]. After the incubation period of 24 h at $37 \pm 0.5^\circ\text{C}$, the zones of inhibition were compared (Fig. 3). The test against *Staphylococcus aureus* (A) compares MXF-loaded microspheres (200 ppm) and the HPMC gel (MG2), whereas

the test against *Staphylococcus epidermidis* (B) compares the marketed formulation with the MXF-loaded microspheres (50 ppm and 200 ppm) and the HPMC gel (MG2). The enhanced inhibition zones with the presence of microspheres loading MXF are clearly a sign of antimicrobial activity of the developed formulation. From the results shown in Fig. 3, one can confirm that particles retain the bioactivity of the drug.

Ex vivo bioadhesion study of developed formulations was performed using freshly excised sheep cornea and sclera. Bioadhesion was determined in the form of weight in g required for detachment from cornea or sclera. This weight in g expressed in the form of detachment stress dyne/cm² using equation (1) and, as can be observed in Fig. 4, the developed controlled release ocular gel containing MXF loaded microspheres showed both higher corneal and scleral bioadhesive strength than the marketed MFX eye drops. Detachment stress values (corneal and scleral 13337.37 and 14081.59 dyn/cm², respectively) are in accordance with other authors developing ocular gelling systems for topical delivery [37].

Ex vivo penetration of MXF from the developed ocular gel (MG2) was determined both in sclera and cornea of sheep. A validated microbial assay method was used for estimation of MXF in cornea and sclera. The method showed good linearity from 0 to 300 µg/ml with the R^2 of 0.9890. The method showed low interday variation and good repeatability with RSD less than 2%. The amount of MXF penetrated in the sclera and cornea was estimated by using a calibration plot. The developed ocular gel containing microspheres showed higher corneal

Table 2*Ex vivo* corneal and scleral penetration study.

Formulation	Drug remaining on cornea (μg)	Drug penetrated in cornea (μg)	Drug diffused in receptor media (mg)	Drug remaining on sclera (μg)	Drug penetrated in sclera (μg)	Drug diffused in receptor media (mg)
HPMC Gel containing microspheres	217.89 \pm 0.08	213.81 \pm 0.08	4.57 \pm 0.09	219.5 \pm 0.05	218.07 \pm 0.044	4.59 \pm 0.06
Conventional eye drops	–	55.44 \pm 0.09	4.92 \pm 0.02	–	56.81 \pm 0.07	4.86 \pm 0.03

Table 3

Summary of the treatments and healing of each animal group in Staphylococcal conjunctivitis model.

Group	Treatment	Treatment Schedule	Healing process
Group I	MXF microspheres dispersed in HPMC gel (MG2)	Once Daily-5 days	Moderate recovery by third day; Moderate to marked recovery by fifth day
Group II	MXF microspheres dispersed in HPMC gel (MG2)	Twice Daily-5 days	Marked recovery by third day
Group III	MXF Eye Drops	Once Daily-5 days	None to minimal recovery by fifth day
Group IV	MXF Eye Drops	4 times Daily-5 days	Moderate recovery by third day; Moderate to marked recovery by fifth day
Group V	Negative control	No treatment	None
Group VI	Positive control	No treatment	None

and scleral drug accumulation of 213.81 μg and 218.07 μg , respectively, which was found to be 3.85 and 3.83 times higher than the conventional MXF eye drop (Table 2) and thus would have higher ocular penetration targeting potential as compared to conventional eye drops. Besides the dynamic barriers (e.g., tear dilution, efflux pumps, lymphatic clearance, choroidal and conjunctival blood flow [38]), drug penetration through ocular tissues is hindered by several static barriers that include corneal and scleral tissues. Scleral permeability is strongly dependent on the

molecular weight of the drug, being smaller molecules of sizes up to 70 kDa more permeable than larger ones. MXF is a synthetic 8-methoxyfluroquinolone antibacterial agent with a diazabicyclononyl ring at C7 position, with a 401.431 g/mol [39], thus posing no constraints provided that its hydrophilic character also contributes to an ease in penetration through the sclera, as it consists of porous spaces within a collagen aqueous network [38]. It is thus expected that the mucoadhesive properties of HPMC gels and the drug reservoir offered by the microspheres will contribute to increase in the residence time of the system onto the ocular tissues, generating a modified release profile (i.e., controlled release as shown in Fig. 2A), and thereby contributing to increased penetration of the drug through cornea and sclera. One need however to bear in mind that the proposed formulation is intended for the treatment of conjunctiva, thus its targeting is to the external tissues.

Irritation study was carried out using rabbits and irritation phenomena was written and plotted. The optimized ocular gel containing MXF loaded microspheres did not show any signs of irritation in cornea, iris and conjunctiva, there was no chemosis or swelling in eyelid. The treated eye was comparable to the eye which was not given any treatment and is therefore safe for ophthalmic administration [40]. The *in vivo* efficacy of the developed controlled release ocular gel was compared with conventional MXF eye drops in a staphylococcal conjunctival model in rabbits (Table 3). In case of treatment with developed controlled release ocular gel containing MXF loaded microspheres, Group I (once a day administration of ocular gel for 5 days) showed moderate recovery by 3rd day whereas moderate to marked recovery by 5th day. However, Group II receiving ocular gel twice a day for 5 days, showed good results with the marked recovery by 3rd day

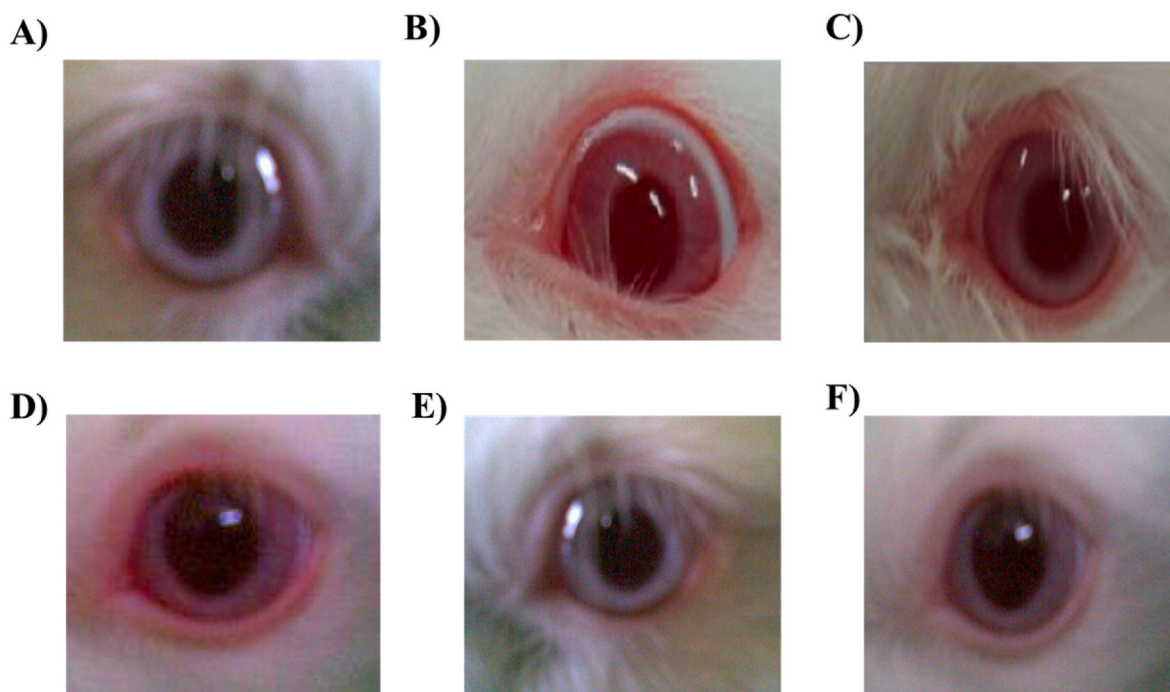


Fig. 5. *In vivo* therapeutic efficacy assessment. A) Negative control, B) Positive control (eye with conjunctivitis), C) Rabbit eye with the minimal healing, D) Rabbit eye with the moderate healing, E) Eye with complete recovery after MXF microspheres dispersed in HPMC ocular gel treatment twice daily for three days, F) Treatment after eye drop marketed four times a day for five days.

whereas in case of treatment with conventional eye drops, Group III receiving conventional eye drops once a day for 5 days showed only none to minimal recovery by 5th day while Group IV receiving conventional eye drops four times a day for 5 days showed moderate recovery by 3rd day and moderate to marked recovery by 5th day.

Thus, the developed ocular gel containing MXF loaded microspheres with once-a-day administration is comparable in efficacy to MXF eye drops having four times a day administration. Therefore, both formulations required 5 days treatment to clear conjunctivitis. However, twice a day administration of developed ocular gel showed better efficacy with improved recovery from conjunctival infections within three days. The *in vivo* therapeutic efficacy is shown in Fig. 5.

4. Conclusions

Microspheres loaded controlled release ocular gel of MXF have been successfully prepared. Microspheres incorporated in ocular gel were spherical in shape, smooth surface with high drug loading and mean particle size of 25.2 μm , which was found to be optimal for ophthalmic drug delivery. Microspheres loaded gels were pale yellow in color, odorless and translucent in appearance. Gels showed prolonged retention in eye with controlled drug release over a period of 12 h. The developed controlled release ocular gel showed higher corneal and scleral penetration of 213.81 μg and 218.07 μg respectively which was found to be 3.85 and 3.83 times higher than conventional eye drop. *In vivo* studies have shown that the ophthalmic controlled release gels containing MFX microspheres, for the first time formulated with 7% of HPMC, required much less frequent administration for fewer number of days as compared to conventional eye drops for recovery from conjunctivitis due to staphylococcal infection. Thus, ophthalmic MXF formulation with the controlled drug release, higher eye penetration, better efficacy, reduced frequency of administration and improved patient compliance have been successfully developed.

Ethics issues

The experimental protocols were approved by the Institutional Animal Ethics Committee of C. U. Shah College of Pharmacy, SNDT Women's University, Mumbai and conducted according to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Credit author statement

All authors have actively contributed to the conception and design of the study, data acquisition, analysis and interpretation, drafting the article and revising it critically for important intellectual content, and have approved the final version to be submitted.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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