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Effect of Formulation Factors on In Vitro Permeation of Moxifloxacin From Aqueous Drops Through Excised Goat, Sheep, and Buffalo Corneas

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ABSTRACT

The purpose of this investigation was to evaluate the effect of formulation factors on in vitro permeation of moxifloxacin from aqueous drop through freshly excised goat, sheep, and buffalo corneas. Aqueous isotonic ophthalmic solutions of moxifloxacin hydrochloride of different concentrations (pH 7.2) or 0.5% (wt/vol) solutions of different pH or 0.5% solutions (pH 7.2) containing different preservatives were made. Permeation characteristics of drug were evaluated by putting 1 mL formulation on freshly excised cornea (0.50 cm^2) fixed between donor and receptor compartments of an all-glass modified Franz diffusion cell and measuring the drug permeated in the receptor (containing 10 mL bicarbonate ringer at 37°C under stirring) by spectrophotometry at 291 nm, after 120 minutes. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test. Increase in drug concentration in the formulation resulted in an increase in the quantity permeated but a decrease in percentage permeation. Increase in pH of the solution from 5.5 to 7.2 increased drug permeation, indicating pH-dependent transport. Compared with control formulation, moxifloxacin 0.5% (wt/vol) solution (pH 7.2) containing disodium edetate (EDTA) (0.01% wt/vol) produced significantly ($P < .05$) higher permeation with all the corneas. Formulation with benzyl alcohol significantly ($P < .05$) increased permeation with buffalo cornea compared with its control. Presence of benzalkonium chloride (BAK) (0.01% wt/vol) and EDTA (0.01% wt/vol) in the formulation increased permeation to the maximum with all the corneas. The results suggest that moxifloxacin 0.5% ophthalmic solution (pH 7.2) containing BAK (0.01%) and EDTA (0.01%) provides increased in vitro ocular availability through goat, sheep, and buffalo corneas.

KEYWORDS: moxifloxacin, concentration, pH, preservative, cornea, permeation.

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INTRODUCTION

The antibiotics commonly used topically for treating ocular infections have been tetracycline, chloramphenicol, gentamicin, tobramycin, and erythromycin. After the introduction of fluoroquinolones, ocular preparations of these antimicrobial agents such as ciprofloxacin, norfloxacin, ofloxacin, and levofloxacin became available to control various eye infections caused by *Staphylococcus aureus, Streptococcus pneumoniae, Hemophilus species*, and others. Fluoroquinolones are bactericides, which inhibit bacterial DNA replication by inhibiting topoisomerase and DNA gyrases.[1]

Moxifloxacin is a fourth-generation fluoroquinolone with a methoxy group in the C-8 position and a bulky C-7 side chain. This fourth-generation fluoroquinolone has in vitro activity similar to that of ciprofloxacin and ofloxacin against gram-negative bacteria but enhanced activity against gram-positive bacteria including *S aureus*.[2-4] The fourth-generation fluoroquinolones, gatifloxacin and moxifloxacin, have been reported to have increased susceptibility to *S aureus* (isolated from clinical cases of keratitis) compared with second- and third-generation fluoroquinolones such as ciprofloxacin, levofloxacin, or ofloxacin.[5] In experimental staphylococcal keratitis model in rabbits, moxifloxacin has demonstrated greater effectiveness than ciprofloxacin or levofloxacin.[6] Experiments in rabbits suggest that surgical prophylaxis with topical 0.5% moxifloxacin could be effective for prevention of bacterial endophthalmitis.[7,8] Moxifloxacin is available as hydrochloride salt, which is water soluble. A tablet dosage form of moxifloxacin is available in the Indian market. Recently, aqueous ocular drop formulations have been launched. Most of the permeation studies reported have used rabbit cornea. Animal Ethics Committees are putting restrictions on experiments with rabbit cornea. Thus, it appears reasonable to look for alternate mammalian corneas, especially from those animals that are slaughtered every day for meat (eg, goat, sheep, buffalo). In addition, such a study would also help in the development of veterinary ophthalmic formulation of the drug as goat, sheep, and buffalo constitute the bulk of the cattle population in the Indian subcontinent.

Accordingly, the purpose of this investigation was to study the effect of formulation factors such as concentration of drug, pH, and presence of preservatives in aqueous drop on

in vitro permeation of moxifloxacin through excised goat, sheep, and buffalo corneas. To avoid biological variation, attempts were also made to evaluate the permeation characteristics of moxifloxacin from control and optimized formulation through freshly excised paired corneas of each species.

MATERIALS AND METHODS

Moxifloxacin hydrochloride (purity 99.97% on anhydrous basis) was obtained from Ranbaxy Laboratories (Gurgaon, India) as a gift. Preservatives were received from Central Drug House (New Delhi, India). All other chemicals used were of analytical reagent grade. Fresh whole eyeballs of goat or sheep or buffalo were obtained from butcher's shop (Ambedkar Nagar, New Delhi, India) within one hour of slaughtering of the animal. The method of dissection of cornea and the apparatus used in permeation studies were the same as published elsewhere.[9]

Permeation Experiment

Freshly excised cornea was fixed between clamped donor and receptor compartments of an all-glass modified Franz diffusion cell in such a way that its epithelial surface faced the donor compartment. The corneal area available for diffusion was 0.50 cm^2. The receptor compartment was filled with 10 mL freshly prepared bicarbonate ringer solution (pH 7.2), and all air bubbles were expelled from the compartment. An aliquot (1 mL) of test solution was placed on the cornea and the opening of the donor cell was sealed with a glass cover slip; receptor fluid was kept at 37°C with constant stirring using a Teflon-coated magnetic stir bead. Permeation study was continued for 120 minutes, and samples were withdrawn from receptor and analyzed for moxifloxacin content by measuring absorbance at 291 nm in a spectrophotometer (1601 Shimadzu, Kyoto, Japan). Results were expressed as amount permeated and percentage permeation or in vitro ocular availability. The permeation (%) or in vitro ocular availability was calculated as follows:

$$\textit{Permeation}\ (\%) = \textit{Amount of drug permeated in receptor} \,/\, \textit{Initial amount of drug in donor} \times 100. \quad (1)$$

At the end of the experiment, each cornea (freed from adhering sclera) was weighed, soaked in 1-mL methanol, dried overnight at 90°C, and reweighed. From the difference in weights, corneal hydration was calculated. Permeation characteristics of moxifloxacin from control and optimized formulations were also evaluated through freshly excised paired goat, buffalo, and sheep corneas. Statistical calculations were done by one-way ANOVA followed by Dunnett's test. Paired *t*-test was used for studies with paired cornea. A *P* value less than .05 was considered as criterion for significance.

Preparation of Test Solutions

Moxifloxacin ophthalmic solutions of increasing concentration of pH 7.2

Required amount of moxifloxacin hydrochloride was dissolved in sufficient distilled water; sodium chloride was added to make final solution isotonic; pH of the solution was adjusted to 7.2 using 0.1N NaOH and 0.1N HCl; and final volume was made up to 100 mL with distilled water, to have solutions of 0.1, 0.2, 0.3, 0.4, and 0.5% (wt/vol) concentrations.

Moxifloxacin ophthalmic solutions (0.5% wt/vol) of different pH

Moxifloxacin hydrochloride (0.5 g) was dissolved in sufficient distilled water; sodium chloride was added to make the final solution isotonic; pH of the solution was adjusted to 5.5 or 6.0 or 6.5 or 7.0 or 7.2 using 0.1N HCl and 0.1N NaOH; and final volume was made up to 100 mL with distilled water to have solutions of different pH.

Moxifloxacin ophthalmic solutions (0.5% wt/vol, pH 7.2) containing preservatives

Moxifloxacin hydrochloride (0.5 g) was dissolved in sufficient distilled water; sodium chloride was added to make the final solution isotonic; and pH of the solution was adjusted to 7.2. To this solution benzalkonium chloride (BAK 0.01% wt/vol) or benzyl alcohol (BA, 0.05% wt/vol) or thiomersal (THM, 0.005% wt/vol) or phenyl mercuric acetate (PMA, 0.002% wt/vol) or phenyl mercuric nitrate (PMN, 0.002% wt/vol) or disodium edetate (EDTA 0.01% wt/vol) or a combination of BAK (0.01% wt/vol) and EDTA (0.01% wt/vol) was added; and the final volume of each solution was made up to 100 mL with distilled water.

Measurement of Surface Tension

Formulation containing surfactant can emulsify corneal epithelium and help in quicker partitioning of the drug in the epithelium. To explore any possible relationship between surface tension of formulation and corneal penetration, surface tension of each moxifloxacin ophthalmic solution (0.5% wt/vol, pH 7.2) containing preservative was measured by a stalagmometer.

Table 1. Effect of Concentration of Moxifloxacin in Aqueous Solution (pH 7.2) on Permeation of Drug Through Excised Goat, Sheep, and Buffalo Corneas*

Conc (% wt/vol)	Amount Permeated (mg) (120 minutes)			Permeation (%) (120 minutes)			Corneal Hydration (%)		
	Goat	Sheep	Buffalo	Goat	Sheep	Buffalo	Goat	Sheep	Buffalo
0.1	0.016 ± 0.0003	0.015 ± 0.0009	0.012 ± 0.0003	1.6	1.50	1.2	79.92 ± 0.577	79.66 ± 0.333	79.00 ± 0.00
0.2	0.020 ± 0.0007	0.016 ± 0.0007	0.015 ± 0.0003†	1.0	0.80	0.75	80.00 ± 0.000	79.43 ± 0.297	78.89 ± 0.187
0.3	0.023 ± 0.0018†	0.023 ± 0.0007†	0.022 ± 0.001†	0.77	0.77	0.73	79.41 ± 0.391	79.93 ± 0.105	79.59 ± 0.303
0.4	0.028 ± 0.0009†	0.029 ± 0.0006†	0.025 ± 0.0006†	0.70	0.73	0.63	79.85 ± 0.178	79.89 ± 0.11	76.63 ± 0.709
0.5	0.037 ± 0.0013†	0.035 ± 0.0017†	0.027 ± 0.0003†	0.74	0.70	0.54	78.82 ± 0.129	80.02 ± 0.02	78.50 ± 0.583

*Conc indicates concentration. Values are mean ± SE of 3 corneas in each group.

†Statistically significant ($P < .05$) compared with solution of 0.1% concentration, as determined by one-way ANOVA followed by Dunnett's test.

Measurement of Partition Coefficient

Equal volumes of moxifloxacin ophthalmic solution (0.5% wt/vol, pH 7.2) with EDTA or BAK + EDTA or without the additives (control) and butanol were shaken for 3 hours at room temperature in a mechanical shaker at 200 rpm (Adolf Kuhner, Basel, Switzerland). The experiment was done with duplicate samples of each formulation. The concentration of drug in each phase was analyzed and the partition coefficient was calculated. The result was expressed as mean ± SE.

Results and Discussion

Permeation data of moxifloxacin from ophthalmic solutions of increasing concentrations through excised corneas are shown in Table 1. Increase in drug concentration in the aqueous drops resulted in increase in amount of drug permeated but decrease in percentage permeation or in vitro ocular availability. Cornea has 3 layers, namely, epithelium (lipophilic), stroma (hydrophilic), and endothelium (less lipophilic than epithelium). Thus, to penetrate the cornea the drug must have solubility in both lipid and water. A lipid-soluble drug having no or poor aqueous solubility will not penetrate beyond corneal epithelium to any significant extent, whereas a water-soluble drug having no lipid solubility will not be able to cross epithelium unless assisted by a carrier. In other words, the drug should have a desired partition coefficient for corneal penetration. A drug will first partition into the corneal epithelium and saturate the same; from there it will partition through hydrophilic stroma and relatively lipophilic endothelium. Permeation studies with ketorolac and pilocarpine have revealed that corneal epithelium acts as a reservoir for drug accumulation and continuous delivery of drug to aqueous humor.[10-12] Thus, in the permeation study, only the drug

Table 2. Effect of pH of Moxifloxacin Aqueous Solution (0.5% wt/vol) on Permeation of Drug Through Excised Goat, Sheep, and Buffalo Corneas*

pH	Amount Permeated (mg) (120 minutes)			Permeation (%) (120 minutes)			Corneal Hydration (%)		
	Goat	Sheep	Buffalo	Goat	Sheep	Buffalo	Goat	Sheep	Buffalo
5.5	0.031 ± 0.0015	0.022 ± 0.0003	0.017 ± 0.0003	0.62	0.44	0.34	80.53 ± 0.236	78.63 ± 0.331	80.34 ± 0.338
6.0	0.034 ± 0.0015	0.026 ± 0.0007	0.021 ± 0.0006†	0.68	0.52	0.42	80.22 ± 0.387	80.00 ± 0.00	80.02 ± 0.629
6.5	0.035 ± 0.0007	0.030 ± 0.0007†	0.025 ± 0.0007†	0.70	0.60	0.50	79.73 ± 0.263	78.66 ± 0.882	78.59 ± 0.338
7.0	0.036 ± 0.0006†	0.033 ± 0.0012†	0.026 ± 0.0009†	0.72	0.66	0.52	79.25 ± 0.748	78.99 ± 0.363	77.06 ± 0.035
7.2	0.037 ± 0.0013†	0.035 ± 0.0017†	0.027 ± 0.0003†	0.74	0.70	0.54	78.82 ± 0.129	80.02 ± 0.02	78.50 ± 0.583

*Values are mean ± SE of 3 corneas in each group.

†Statistically significant ($P < .05$) compared with solution of pH 5.5, as determined by one-way ANOVA followed by Dunnett's test.

Table 3. Effect of Preservative on Permeation of Moxifloxacin From 0.5% Aqueous Solution (pH 7.2) Through Excised Goat Cornea*

Preservative	Surface Tension (dyne/cm)	Amount Permeated (mg) (120 minutes)	Permeation (%) (120 minutes)	Corneal Hydration (%)
None (Control)	70.24	0.037 ± 0.0013	0.74	78.82 ± 0.129
BAK	53.60	0.044 ± 0.0028	0.88	80.27 ± 1.504
EDTA	68.93	0.059 ± 0.0006†	1.18	80.68 ± 0.188
BAK+EDTA	45.68	0.070 ± 0.0017†	1.40	80.99 ± 0.297
BA	64.15	0.039 ± 0.0003	0.78	79.62 ± 0.207
THM	69.12	0.045 ± 0.0009	0.90	80.35 ± 0.324
PMA	68.26	0.030 ± 0.0044	0.60	78.75 ± 0.375
PMN	68.45	0.037 ± 0.0015	0.74	80.55 ± 0.431

*BAK indicates benzalkonium chloride; EDTA, disodium edetate; BA, benzyl alcohol; THM, thiomersal; PMA, phenyl mercuric acetate; and PMN, phenyl mercuric nitrate. Values are mean ± SE of 3 corneas in each group.
†Statistically significant ($P < .05$) compared with control, as determined by one-way ANOVA followed by Dunnett's test.

present in the epithelium would be able to partition through the stroma and endothelium to the receptor. Increase in drug concentration in donor does not result in proportionate increase in amount of drug permeated (Table 1). As a result, increase in drug concentration in donor would decrease the percentage permeation or in vitro ocular availability. Decreased in vitro ocular availability with increase in drug concentration in drops has been reported for anionic drugs such as ibuprofen and flurbiprofen.[13,14] The corneal hydration level of normal mammalian cornea is between 75% and 80%.[15] Increase in drug concentration, however, did not affect corneal hydration, which remained in the normal range of 75% to 80%.

Table 2 shows the effect of pH of formulation on permeation of moxifloxacin through excised corneas. Increase in pH of the solution from 5.5 to 7.2 increased drug permeation, indicating a pH-dependent transport of moxifloxacin. Moxifloxacin, being a basic drug, will be in unionized form as the pH of the formulation is shifted toward neutrality resulting in increased permeation. Another explanation for increased permeation of moxifloxacin at physiological pH of tears (ie, 7.2) could be because cornea contains both positively and negatively charged groups whose magnitude and polarity depend on the degree of protonation. At pH above the isoelectric point (pI = 3.2), the cornea carries a net negative charge and is selectively permeable to cations.[16] It is important to mention that levofloxacin transport across excised rabbit cornea has also been reported to be pH-dependent.[17] Corneal transport of moxifloxacin at pH 5.5 indicates that the ionized form of drug also can permeate through cornea.

The effects of preservatives on permeation of moxifloxacin through excised corneas are shown in Tables 3, 4, and 5. Moxifloxacin, 0.5% aqueous drop (pH 7.2), containing EDTA (0.01% wt/vol) produced significantly ($P < .05$) higher permeation, compared with control formulation containing no preservative, with all the corneas. Formulation with BA showed significant ($P < .05$) increase in permeation through buffalo cornea compared with its control. The formulation had lower surface tension than

Table 4. Effect of Preservative on Permeation of Moxifloxacin From 0.5% Aqueous Solution (pH 7.2) Through Excised Sheep Cornea*

Preservative	Surface Tension (dyne/cm)	Amount Permeated (mg) (120 minutes)	Permeation (%) (120 minutes)	Corneal Hydration (%)
None (Control)	70.24	0.035 ± 0.0017	0.70	80.02 ± 0.02
BAK	53.60	0.040 ± 0.0007	0.80	80.20 ± 0.239
EDTA	68.93	0.047 ± 0.0012†	0.94	80.14 ± 0.330
BAK+EDTA	45.68	0.063 ± 0.0025†	1.26	80.01 ± 0.023
BA	64.15	0.040 ± 0.0015	0.80	79.25 ± 0.634
THM	69.12	0.038 ± 0.001	0.76	80.85 ± 0.522
PMA	68.26	0.032 ± 0.0021	0.64	79.65 ± 0.369
PMN	68.45	0.037 ± 0.0009	0.74	79.36 ± 0.305

*BAK indicates benzalkonium chloride; EDTA, disodium edetate; BA, benzyl alcohol; THM, thiomersal; PMA, phenyl mercuric acetate; and PMN, phenyl mercuric nitrate. Values are mean ± SE of 3 corneas in each group.
†Statistically significant ($P < .05$) compared with control, as determined by one-way ANOVA followed by Dunnett's test.

Table 5. Effect of Preservative on Permeation of Moxifloxacin From 0.5% Aqueous Solution (pH 7.2) Through Excised Buffalo Cornea*

Preservative	Surface Tension (dyne/cm)	Amount Permeated (mg) (120 minutes)	Permeation (%) (120 minutes)	Corneal Hydration (%)
None (Control)	70.24	0.027 ± 0.0003	0.54	78.51 ± 0.582
BAK	53.60	0.032 ± 0.0017	0.64	80.36 ± 0.321
EDTA	68.93	0.037 ± 0.0006†	0.74	80.38 ± 0.357
BAK+EDTA	45.68	0.062 ± 0.0019†	1.24	80.40 ± 0.270
BA	64.15	0.038 ± 0.001†	0.76	79.05 ± 0.682
THM	69.12	0.031 ± 0.0024	0.62	79.24 ± 0.65
PMA	68.26	0.025 ± 0.0006	0.50	79.81 ± 0.53
PMN	68.45	0.027 ± 0.0006	0.54	80.11 ± 0.22

*BAK indicates benzalkonium chloride; EDTA, disodium edetate; BA, benzyl alcohol; THM, thiomersal; PMA, phenyl mercuric acetate; and PMN, phenyl mercuric nitrate. Values are mean ± SE of 3 corneas in each group.

†Statistically significant ($P < .05$) compared with control, as determined by one-way ANOVA followed by Dunnett's test.

control formulation. The addition of BAK (0.01% wt/vol), a cationic surfactant, in the formulation reduced surface tension substantially (53.60 dyne/cm), but the increase in permeation was insignificant. Similarly, formulation-containing THM showed marginal increase in permeation, while those with PMA and PMN did not have any effect on permeation. Combined presence of BAK and EDTA in the formulation, however, provided maximum permeation of the drug through all the corneas. Partitioning experiment indicated partition coefficient of moxifloxacin in butanol/moxifloxacin drop with EDTA system as 1.7 ± 0.005 against a partition coefficient of 1.02 ± 0.02 in butanol/control formulation system. Thus, EDTA increases the partitioning of moxifloxacin in lipid phase. It appears that EDTA, being anionic in nature, interacts with cationic moxifloxacin to form a more lipid-soluble ion pair, which increases the permeation through cornea. In addition, EDTA, a known calcium-chelating agent, has been shown to act on cell junctions by interfering with calcium ions and altering intercellular integrity. EDTA also disrupts plasma membrane and consequently increases intercellular permeability.[18] EDTA has been reported to increase corneal absorption of various drugs through intact corneas.[19,20]

The partition coefficient of moxifloxacin in butanol/moxifloxacin drop with BAK and EDTA system was 1.98 ± 0.014 and formulation with BAK+EDTA had least surface tension (45.68 dyne/cm). The increased permeation with formulation with BAK and EDTA appears to be caused by emulsification of corneal epithelium and increased lipid solubility of moxifloxacin. The formulation increased corneal hydration with goat cornea (80.99%), indicating slight corneal damage. Since the corneal hydration is below 83%, the damage appears to be reversible.[21] Permeation characteristics of drug from optimized formulation containing BAK and EDTA (each 0.01% wt/vol) and control formulation (without BAK and EDTA) were evaluated using paired corneas of goat, sheep, and buffalo and the results are shown in Table 6. By paired cornea we mean that from a single animal, one cornea was treated with optimized formulation containing BAK and EDTA, while the other cornea was treated with control formulation containing no additive. This procedure was adopted to minimize biological variation. The results indicate that formulation containing BAK and EDTA increased the permeation of moxifloxacin through all the mammalian corneas compared with the control formulation, where the permeation enhancing effect

Table 6. Relative Permeation Characteristics of Moxifloxacin From Control and Optimized Formulation Through Excised Goat, Sheep, and Buffalo Corneas (Paired)*

		Control Formulation (No additive)			Optimized Formulation (BAK+EDTA)		
Animal	Thickness of the Cornea (mm)	Amount Permeated (mg) (120 minutes)	Permeation (%) (120 minutes)	Corneal Hydration (%)	Amount Permeated (mg) (120 minutes)	Permeation (%) (120 minutes)	Corneal Hydration (%)
Goat	0.70 ± 0.0033	0.038 ± 0.0009	0.76	78.92 ± 0.795	0.070 ± 0.0009†	1.40	79.37 ± 0.693
Sheep	0.90 ± 0.0033	0.036 ± 0.0006	0.72	78.88 ± 0.628	0.067 ± 0.0009†	1.34	80.36 ± 0.317
Buffalo	1.15 ± 0.0033	0.025 ± 0.0017	0.50	78.65 ± 0.847	0.067 ± 0.0041†	1.34	79.71 ± 0.664

*Values are mean ± SE of 3 corneas in each group.

†Statistically significant ($P < .05$) compared with control, as determined by paired *t*-test.

was maximal with buffalo cornea. Corneal hydration obtained with goat and buffalo corneas was less than 80%, whereas with sheep cornea the hydration was marginally higher than 80%. Thus, the formulation could be considered to cause no substantial damage to cornea. Permeability of the control formulation was least with buffalo cornea, which had maximum thickness (1.15 mm) followed by sheep and goat corneas. The usual concentration of BAK used in topical eye drops is 0.01%. Some strains of *Pseudomonas aeruginosa* have been found that are resistant to BAK and, in fact, can be grown in concentrated solution of this agent. The acquired resistance could be eliminated by the presence of EDTA in the solution. The use of EDTA where it is compatible is recommended in concentrations of 0.01% to 0.1%.[22] In our study, EDTA at 0.01% concentration increased the permeation of drug and thus became an obvious choice. Since it can potentiate the antibacterial activity of BAK against *P aeruginosa*, BAK and EDTA combination appeared promising. EDTA concentration was kept at minimum (0.01%) to have minimum corneal damage. The formulation increased permeation through all the mammalian corneas without causing any substantial damage.

CONCLUSION

It can be concluded from the present studies that increase in concentration of moxifloxacin in aqueous drop causes a disproportionate increase in permeation. Corneal transport of moxifloxacin is pH dependent, having maximum transport at physiological pH of tears (ie, 7.2). Moxifloxacin, 0.5% wt/vol aqueous drop (pH 7.2), containing BAK (0.01% wt/vol) and EDTA (0.01% wt/vol) provides maximum in vitro ocular availability through goat, sheep, and buffalo corneas.

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