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Effect of Formulation Factors on *In Vitro* Permeation of Diclofenac from Experimental and Marketed Aqueous Eye Drops through Excised Goat Cornea

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The effect of formulation factors on permeation of diclofenac from some experimental and marketed aqueous eye drops through excised goat cornea was evaluated. Raising the pH of formulation from 6.0 to 8.0 or diclofenac concentration from 0.05 to 0.15% (w/v) or adjusting tonicity with mannitol or addition of viscolizing agent decreased apparent permeability coefficient (Papp). Formulation (pH 7.4) containing sodium metabisulfite or EDTA or combination of methyl and propyl paraben showed significantly ($p<0.05$) higher Papp whereas benzalkonium chloride (BAC) had no effect and sorbic acid (SA) had reduced permeation. Surprisingly marketed drops containing BAC or SA, showed significantly ($p<0.05$) higher Papp and decreased in the order of Difen>Voveran>NSAID>Dicol>Diclolab. Lower pH (7.1-7.3) and surface tension of drops indicating presence of surfactant, could mediate increased permeation and presence of buffer could cause irritation on *in vivo* instillation. The marketed formulations showed corneal hydration > 83% suggesting corneal damaging potential.

Key words——diclofenac; pH; preservative; tonicity/viscosity modifier

INTRODUCTION

Topical therapy with corticosteroids is most commonly used in the management of ocular inflammations but their use is associated with increase in intraocular pressure, cataract formation, and risk of infection.[1] Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin,[2] flurbiprofen,[3] ketorolac[4] and diclofenac,[5] have been found to be viable alternative to corticosteroids in the management of ocular inflammation. Diclofenac and ketorolac were found neither to attenuate the antiviral activity of cefdovir nor to facilitate the adenoviral replication.[6,7] Diclofenac was found to be more potent than indomethacin and dexamethasone in inhibition of endothelial PGE_2 synthesis induced by calcium ionophore A23187 or lipopolysaccharide (LPS) of *Salmonella typhimurium*.[8] Diclofenac, a phenyl acetic acid NSAID, is available as sodium salt. Aqueous diclofenac sodium (0.1% w/v) solution is applied topically in the eye for the management of pain in corneal epithelial defects following surgery or accidental trauma, treatment of postoperative ocular inflammations, chronic non-infectious inflammations, and prevention of intra-operative miosis during cataract surgery and for symptomatic relief of seasonal allergic conjunctivitis.[9] About 90% of the dose applied topically in the eye from such solutions is lost due to pre-corneal losses. The absorption of drugs from the eye is dependent upon the complex interplay of physiological, physiochemical and formulation factors. Formulation of an optimal ophthalmic dosage form requires a balancing act between the ocular irritation, corneal permeation and stability.[10] One of the approaches to enhance the ocular absorption of drugs is manipulation of formulation parameters like use of viscosity modifiers, bioadhesives and penetration enhancers.[11]

Diclofenac sodium is a weakly acidic drug (pKa= 4.2), with a very low aqueous solubility.[12] Commercially available solutions of diclofenac sodium employ solubilizers like polyoxyethylene castor oil (POC) to enhance the solubility. In one of the study n-octenylsuccinate starch has been suggested as an alternative solubilizer and was found to give better permeation across excised porcine cornea than the polyoxyethylene solubilized diclofenac sodium solutions.[13] Acidified solutions (pH 6.5) of diclofenac complexed with cyclodextrins to increase the solubility were reported to permeate better through porcine

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cornea than the neutral (pH 7.0) diclofenac solutions.[14] Diclofenac solutions formulated using polydisperse carrier solution (Sophisen) showed better tolerance and sustained release of diclofenac.[15] The purpose of the present study was to evaluate the effect of formulation factors like concentration of diclofenac, pH, presence of preservative and tonicity/viscosity modifiers in ophthalmic solution, on permeation characteristics of diclofenac through freshly excised goat cornea. The study also aimed at evaluating the corneal permeation characteristics of diclofenac from some marketed eye drop formulations.

EXPERIMENTAL

Materials Diclofenac sodium (purity 98.58%) was obtained as a gift sample from Dabur Research Foundation (Ghaziabad, India). High performance liquid chromatography (HPLC) grade acetonitrile, glacial acetic acid, triethylamine and water were purchased from Qualigens Fine Chemicals (Mumbai, India). All other chemicals purchased were of analytical grade and were used as received. Fresh whole eye balls of goat were obtained from local butcher shop (Hisar, India). Commercially available brands of diclofenac ophthalmic solutions namely NSAID (Syntho Pharmaceuticals, Lucknow, India), Difen (Optica Pharmaceuticals, Yamunanagar, India), Diclolab (Laborate Pharmaceuticals, Panipat, India), Dicol (Ind-swift Ltd., Chandigarh, India) and Voveran Ophtha (Novartis India Ltd., Mumbai, India) were purchased from the local market. The apparatus used in permeation studies was same as published elsewhere.[16]

Preparation of Test Formulations

Diclofenac ophthalmic solutions of different pH Diclofenac sodium solution of 0.02% (w/v, pH 6.0), 0.05% (w/v, pH 6.5) and 0.1% (w/v, pH 7.0, 7.4 and 8.0) concentrations were made in isotonic phosphate buffer (0.0667M USP).

Diclofenac ophthalmic solutions of increasing concentration of pH 7.4 Required quantity of diclofenac sodium was dissolved in 100 ml of 0.0667M isotonic phosphate buffer (pH 7.4) to have 0.05, 0.1 and 0.15% (w/v) concentrations.

Diclofenac ophthalmic solutions 0.1% (w/v) containing different tonicity modifier Diclofenac sodium 0.1% (w/v) solution in 0.0667M phosphate buffer (pH 7.4) made isotonic with either of sodium chloride or mannitol or glucose, was prepared.

Diclofenac ophthalmic solutions 0.1% (w/v, pH 7.4) containing preservative Diclofenac sodium 0.1% (w/v) solution in isotonic phosphate buffer (0.0667 M, pH 7.4) containing either benzalkonium chloride (BAC 0.002%, w/v) or sorbic acid (SA 0.2%, w/v) or benzyl alcohol (BA 0.5%, v/v) or phenyl mercuric acetate (PMA 0.002%, w/v) or phenyl mercuric nitrate (PMN 0.002%, w/v) or thiomersal (THM 0.005%, w/v) or sodium metabisulphite (SMS 0.2% ,w/v) or disodium edetate (EDTA 0.01%, w/v) or combination of benzalkonium chloride (BAC 0.002%, w/v) and disodium edetate (EDTA 0.01%, w/v) or combination of methyl paraben (MP 0.02%, w/v) and propyl paraben (PP 0.01%, w/v) was made.

Diclofenac ophthalmic solutions (0.1%, w/v) containing viscolizing agent Diclofenac sodium 0.1% (w/v) solution in 0.0667M isotonic phosphate buffer (pH 7.4), containing either methyl cellulose (MC 0.5%, w/v) or hydroxypropyl methylcellulose (HPMC 0.1%, w/v) or hydroxypropyl cellulose, low viscosity grade (HPC-L 0.1%, w/v) or polyvinyl alcohol (PVA 1.4%, w/v) was made.

Viscosity Measurement Viscosity of diclofenac 0.1% (w/v) ophthalmic solution containing viscolizing agent was measured using an Ostwald viscometer

Surface Tension Measurement Surface tension of selected formulations was measured by using a stalagmometer.

***In Vitro* Titration of Ophthalmic Solutions** Ten ml of selected formulation was titrated with 0.1 N NaOH to a final pH of 7.4.

Transcorneal Permeation Studies Whole eye ball of goat was transported from the local butcher shop to the laboratory in cold (4℃) normal saline within 1 hour of slaughtering of the animal. The cornea was carefully excised along with 2 to 4 mm of surrounding scleral tissue and was washed with cold normal saline till the washing was free from proteins. Isolated cornea was mounted by sandwiching surrounding scleral tissue between clamped donor and receptor compartments of an all glass modified Franz diffusion cell[16] in such a way that its epithelial surface faced the donor compartment. The corneal area available for diffusion was 0.95 cm^2. The receptor compartment was filled with 11 ml of freshly prepared bicarbonate ringer (pH 7.4). An aliquot (1 ml) of test formulation was placed on the cornea and opening of the donor compartment was sealed with a glass cover slip,

while the receptor fluid was maintained at 35°C with constant stirring, using a Teflon-coated magnetic stir bead. One ml sample was withdrawn from the receptor compartment at various time intervals upto 120 min and was analyzed for diclofenac content using reversed phase high performance liquid chromatography (HPLC). Each sample withdrawn was replaced with equal volume of bicarbonate ringer. At the end of the experiment, each cornea (freed from sclera) was weighed, soaked in 1-ml methanol, dried overnight at 90°C and reweighed. From the difference in weights corneal hydration was calculated.

Calculation of Apparent Permeability Coefficient Apparent permeability coefficient was calculated using the following equation:

$$Papp = \frac{\Delta Q}{\Delta t} \cdot \frac{1}{(A \cdot Co \cdot 60)} \qquad (1)$$

Where, $\Delta Q/\Delta t$ (μg/min) is the flux across the corneal tissue. A is the area of diffusion (cm^2), Co ($\mu g/cm^3$) is the initial concentration of drug in donor compartment, and 60 is taken as the factor to convert minute into second. The flux across the cornea was obtained from the slope of the regression line obtained from the linear part of the curve between the amount permeated (Q) Vs time (t) plot.

HPLC Analysis Analysis of permeation samples was carried out by injecting 20 μl of the solution, spiked with ketorolac tromethamine as internal standard into a chromatographic system equipped with 600 pump controllers (Waters), 2487 dual λ absorbance detector (Waters), and 7725i Rheodyne injector. The resolution of diclofenac was achieved using acetonitrile : water : acetic acid : triethylamine (60 : 38.25 : 1.65 : 0.10) at a flow rate of 1 ml/min, as the mobile phase in an isocratic run through Spherisorb (Waters) C 18, 5 μ (250×4.6 mm *id*) column. The eluant was monitored for diclofenac at 276 nm. The retention time and the lowest limit of quantification of diclofenac were 5.6 min and 0.4 μg/ml, respectively.

Statistical Analysis Statistical calculations were done by 1-way analysis of variance (ANOVA) followed by Dunnett's test. A p value<0.05 was considered significant.

RESULTS AND DISCUSSION

Effect of pH of formulation on corneal permeation of diclofenac is shown in Table 1 and Fig. 1. Increase in pH of diclofenac formulation from pH 6.0 to 8.0 resulted in significant decrease ($p<0.05$) in apparent permeability coefficient (Papp). Diclofenac ophthalmic solution is commercially available as 0.1% (w/v) solution having pH between 7.0 and 7.3. At pH 6.0, 0.99% of diclofenac (a weak acid, pKa=4.2) remains in unionized form while at pH 8.0, the percentage unionized drug reduces to 0.0099%. Higher pH of the formulation, thus, decreases the fraction unionized and Papp. Another explanation of reduced Papp of diclofenac at higher pH 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 thereby becomes less permeable to negatively charged species or anion.[17] However there was a decrease in the cumulative permeation of drug at low pH (Fig. 1), because the solubility of

Table 1. Effect of pH on Corneal Permeation of Diclofenac

Formulation pH	Papp* (cm/sec×10^6)	Relative Papp	Corneal hydration* (%)
6.0	21.3±1.25	1.0	81.7±0.39
6.5	17.6±0.52	0.82	81.5±0.66
7.0	14.1±0.95†	0.66	81.4±0.75
7.4	9.20±1.51†	0.43	80.7±0.57
8.0	5.23±1.09†	0.24	81.8±0.24

* Values are Mean±S.D. ($n=3$), † Statistically significant ($p<0.05$) compared with solution of pH 6.0, as determined by 1-way ANOVA followed by Dunnett's test.

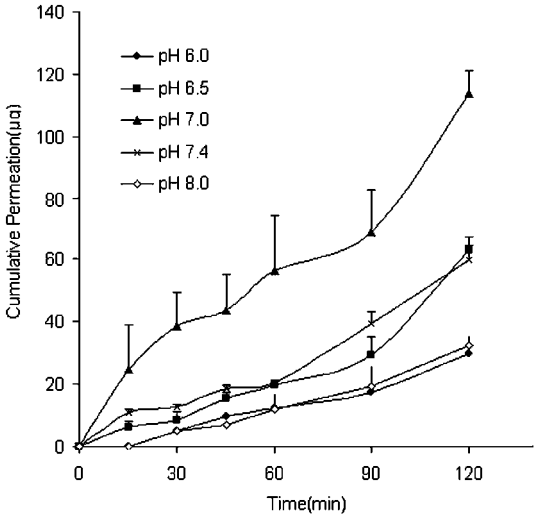

Fig. 1. Effect of pH on *In Vitro* Transcorneal Permeation of Diclofenac
Values are mean±S.D. ($n=3$).

diclofenac decreases on reduction of pH of formulation, as a result formulations of pH 6.5 and 6.0 had diclofenac concentrations of 0.05% (w/v) and 0.02 % (w/v), respectively. Thus reduced concentration of drug in donor at low pH reduces the cumulative permeation. Topical application of diclofenac produces annoying sensations like burning and intense stinging.[15] Formulations having a lower pH will further increase the ocular irritation potential of diclofenac. Permeation of drug from formulations of pH 6.0 and 8.0 had a lag phase of 30 minute (Fig. 1).

Table 2 and Fig. 2 show the effect of concentration of diclofenac in ophthalmic solution (pH 7.4) on corneal permeation. As the concentration of diclofenac was decreased from 0.1% to 0.05% (w/v), no significant change in Papp was observed, while a significant ($p<0.05$) decrease in Papp was observed when the concentration of diclofenac was increased from 0.1% to 0.15% (w/v).Permeation of drug from 0.05% (w/v) concentration showed a lag phase of 30 minute (Fig. 2).

Sodium chloride, glucose and mannitol were employed to adjust the tonicity of diclofenac sodium 0.1 % (w/v) formulations. The results (Table 2 and Fig. 2) show that maximum Papp was obtained, when sodium chloride was used to adjust the tonicity. Use of glucose reduced the permeation, but there was a significant decrease ($p<0.05$) in corneal permeation of diclofenac when mannitol was used as the tonicity modifier.

Diclofenac has a tendency to precipitate in a crystalline form in spite of the fact that the concentration is below the limit of saturation.[18] Considering the same, pH of diclofenac 0.1% (w/v) ophthalmic solution was kept at 7.4 where diclofenac, (a weak acid, pKa=4.2), would have maximum solubility due to increased ionization. Table 3 summarizes the effect of different preservatives on corneal permeation of diclofenac. The results reveal that there was a significant increase ($p<0.05$) in Papp of drug with the use of combination of MP and PP, while there was a significant decrease ($p<0.05$) with the use of SA, BA, PMA, THM and BAC/EDTA as preservatives. There was no significant difference in the permeation

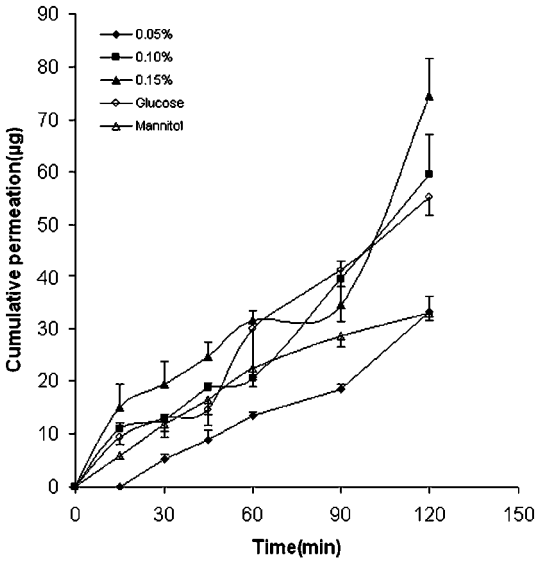

Fig. 2. Effect of Drug Concentration and Tonicity Modifier on *In Vitro* Transcorneal Permeation of Diclofenac
Values are mean±S.D. ($n=3$).

Table 2. Effect of Concentration and Tonicity Modifiers on Corneal Permeation of Diclofenac

Concentration (% wt/vol)	Papp* (cm/sec×10⁶)	Relative Papp	Corneal hydration* (%)
0.05	10.2 ±0.62	1.11	79.6 ±0.69
0.10	9.20±1.51	1.00	80.7 ±0.57
0.15	6.49±0.28†	0.70	81.4 ±0.47
0.10[a)]	8.60±0.55	0.93	80.6 ±0.64
0.10[b)]	4.07±0.36†	0.44	81.04±0.46

*Values are mean±S.D. ($n=3$), *a*) Tonicity adjusted with glucose, *b*) Tonicity adjusted with mannitol. †Statistically significant ($p<0.05$) compared with solution of 0.1% (wt/vol) containing sodium chloride as tonicity modifier, as determined by 1-way ANOVA followed by Dunnett's test.

Table 3. Effect of Preservatives on Corneal Permeation of Diclofenac

Formulation	Papp* (cm/sec×10⁶)	Relative Papp	Corneal hydration* (%)
None (Control)	9.20±1.51	1.00	80.7 ±0.57
MP/PP	11.5 ±0.68†	1.25	80.7 ±0.17
SA	2.53±0.78†	0.27	80.8 ±0.61
BAC	8.60±1.39	0.93	80.6 ±0.68
BAC/EDTA	5.63±1.04†	0.61	80.7 ±0.43
PMN	9.06±2.78	0.98	80.2 ±0.85
PMA	2.87±0.37†	0.31	81.04±0.56
THM	6.06±0.08†	0.65	80.3 ±0.59
BA	5.74±1.11†	0.62	81.2 ±0.36
SMS	19.7 ±1.99†	2.15	80.07±0.45
EDTA	15.3 ±3.16†	1.66	82.9 ±0.66

*Values are mean±S.D. ($n=3$). †Statistically significant ($p<0.05$) compared with solution containing no preservative, as determined by 1-way ANOVA followed by Dunnett's test.

of diclofenac from control solution and solution containing BAC or PMN. Combination of MP and PP has been reported to enhance the corneal permeation of insulin, a peptide.[19] SMS is used as antioxidant and has also got preservative action. Addition of SMS or EDTA to diclofenac ophthalmic solution 0.1% (w/v) was also found to significantly increase the permeation. BAC (0.01% w/v) has earlier been reported to enhance the corneal permeation of anionic drugs like ibuprofen, flurbiprofen and ketorolac[20,21] though BAC is incompatible with ibuprofen and flurbiprofen aqueous solution. The formation of more lipid soluble ion pair and disruption of corneal epithelium has been proposed as the underlying mechanism of BAC promoted corneal permeation. However, the addition of BAC (0.01%, w/v) to diclofenac 0.1% (w/v) ophthalmic solution produces a cloudy solution due to strong cation-anion interaction, necessitating the use of lowest concentration of BAC (*i.e.,* 0.002%, w/v) which was too small to increase permeation. As a result there was no significant difference in corneal permeation of drug from BAC preserved and control solutions. EDTA has been used in combination with BAC to supplement its antibacterial action.[21] EDTA, alone, could increase the permeation but combination of BAC and EDTA significantly reduced the corneal permeation of diclofenac. This is contrary to earlier reports where the combination of both has been found to enhance the corneal permeation of anionic drug like ketorolac.[21] The physicochemical properties of drug, appears to affect the permeation enhancing effect of formulation additives.

Table 4 and Fig. 3 show the effect of viscolizing agents on corneal permeation of diclofenac. The results show that there was a significant decrease in Papp with the use of MC, HPC-L and PVA as the viscolizing agents, while the use of HPMC showed least reduction in Papp. Even though the viscosity of PVA containing solution was much less compared with HPMC or MC, it gave lesser permeability. The use of viscolizing agents was associated with the increase in lag times because of slower diffusion of drug from viscous solutions. It is generally believed that the inclusion of a viscosity-increasing agent in an ophthalmic solution will increase ocular bioavailability by prolonging contact time of drug in the eye. The results of the *in vitro* study indicate reduced permeability of drug in presence of viscolizing agents which could be explained by Stoke's-Einstein relation which describes diffusion coefficient as inversely related to

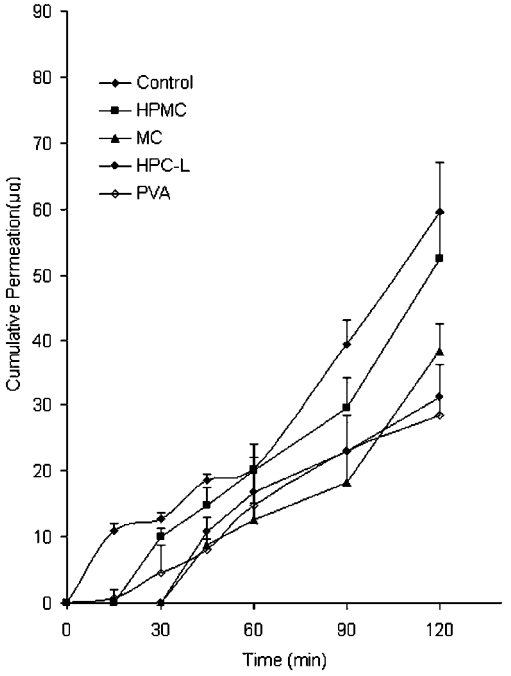

Fig. 3. Effect of Viscolizing Agents on *In Vitro* Transcorneal Permeation of Diclofenac
Values are mean±S.D. ($n=3$).

Table 4. Effect of Viscolizing Agents on Corneal Permeation of Diclofenac

Viscolizing agent	Papp* (cm/sec×10^6)	Relative Papp	Viscosity (cps)	Corneal hydration* (%)
None (Control)	9.20±1.51	1.00	0.897	80.7±0.57
MC	6.59±0.89†	0.71	13.6	79.8±0.43
HPMC	7.97±0.94	0.86	12.1	80.4±0.63
HPC-L	4.59±0.12†	0.49	9.12	80.9±0.73
PVA	4.91±1.02†	0.53	2.048	79.8±0.14

*Values are mean±S.D. ($n=3$). †Statistically significant ($p<0.05$) compared with solution containing no viscolizer, as determined by 1-way ANOVA followed by Dunnett's test.

viscosity.

Permeation characteristics of diclofenac from some marketed eye drop formulations are shown in Table 5. Difen showed maximum Papp followed by Voveran, NSAID, Dicol and Diclolab. Difen contained BAC (0.02%) and had a solution pH of 7.1. Lower pH of the formulation could be partially responsible for increased permeation. Diclofenac 0.1% (w/v) solution containing BAC (0.02%) produces a cloudy solution that has a surface tension of 43.3 dynes/cm whereas Difen was a clear solution and the formulation had a surface tension of 28.9 dynes/cm, suggesting the presence of additional surfactant (besides BAC), which could be responsible for increased permeation. Voveran contained SA (0.2%) and had a solution pH of 7.1. The surface tension of the solution was 38.5 dynes/cm. Diclofenac 0.1% (w/v) solution containing SA (0.2%) has a surface tension of 66.2 dynes/cm which suggests the presence of a surfactant in Voveran too, and the same could contribute towards increased permeation, in addition to lower pH-induced increase in permeation. NSAID also contained BAC (0.02%) and the formulation had a surface tension of 38.9 dynes/cm indicating likely presence of an additional surfactant. Similarly surface tensions of Dicol (which contained SA) and Diclolab (which did not contain any preservative as per label claim) support the presence of surfactant in the formulations. Thus, presence of surfactant in the marketed eye drop formulations appears to be responsible for increased permeation of diclofenac. The normal cornea has a hydration level of 75—80%.[22] In an earlier study[23] it has been reported that 83—92% hydration level *i.e.*, 3—7 percent units or more above the 'normal' value, denotes damage of the epithelium and/or endothelium. All the marketed formulations showed corneal hydration more than 83% being maximum with Difen (87.7%), indicating corneal damaging potential. Since corneal hydration beyond 83% could result in irreversible damage of cornea, caution is needed while using marketed diclofenac eye drop formulation like Difen. *In vitro* titration of the formulations with 0.1 N NaOH to pH 7.4 showed alkali consuming capacity by all the formulations indicating presence of buffer (Table 5). On *in vivo* instillation to the eye each drop will be titrated by tears to physiological pH (*i.e.*, 7.4) which will cause irritation and influx of tears resulting in loss of drug from conjunctival sac. Diclolab consumed least alkali suggesting least buffer capacity and irritating potential which may increase the *in vivo* ocular absorption prospect of diclofenac from the formulation. However, further studies *in vivo* are needed to comment more in this respect.

CONCLUSIONS

On the basis of present studies it can be concluded that raising the pH of the formulation from 6.0 to 8.0 or the drug concentration from 0.05 to 0.15% (w/v) or adjusting tonicity with mannitol or addition of viscolizing agent to diclofenac ophthalmic solution, formulated in phosphate buffer, reduces apparent permeability coefficient through goat cornea. Presence of sodium metabisulfite or EDTA or combination of methyl and propyl paraben in 0.1% (w/v) diclofenac ophthalmic solution, formulated in isotonic phosphate buffer (pH 7.4), favours permeation. Among the marketed eye drops Difen showed highest permeation followed by Voveran, NSAID, Dicol and Diclolab. All the formulations contained surfactant and thereby could damage the cornea. Hence caution is needed while using the formulations.

Table 5. Comparative Corneal Permeation of Diclofenac from Commercial Eye Drops

Eye drops	Papp* (cm/sec×10⁶)	Relative Papp	Corneal* hydration (%)	pH	Preservative[a)]	Titre value (ml)	ST* (dyne/cm)
Control	9.20±1.51	1.00	80.7±0.57	7.4	None	—	68.5±0.20
NSAID	22.2 ±5.64†	2.41	84.1±1.07	7.2	BAC	0.65	38.9±0.30
DIFEN	29.9 ±3.63†	3.25	87.7±0.43	7.1	BAC	0.60	28.9±0.50
DICOL	16.5 ±2.84†	1.80	84.5±0.47	7.1	SA	0.70	55.8±0.21
DICLOLAB	13.2 ±1.87†	1.43	84.7±1.57	7.3	None	0.35	56.5±0.50
VOVERAN	23.7 ±4.17†	2.58	85.8±0.45	7.1	SA	0.55	38.5±0.21

*ST indicates surface tension. Values are mean±S.D. ($n=3$). †Statistically significant ($p<0.05$) compared with control solution, as determined by 1-way ANOVA followed by Dunnett's test. *a*) as per label.

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