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IN VITRO RABBIT CORNEAL PERMEABILITY STUDY OF KETOROLAC, TROMETHAMINE, A NON-STEROIDAL ANTI-INFLAMMATORY AGENT

Roger Cherng-Chyi Fu* and Deborah M. Lidgate
Institute of Pharmaceutical Science
Syntex Research, Palo Alto, CA 94304

ABSTRACT

In vitro rabbit corneal studies utilizing ketorolac tromethamine were designed to 1) determine its permeability at different pH levels; 2) study the effects of two commonly used ophthalmic preservatives on corneal permeability; and 3) study the corneal penetration effects through ion pairing of the anionic ketorolac with positively charged counter ions. The results indicate that ketorolac shows a pH dependent penetration; it also exhibits penetration in both the unionized as well as ionized form. Results of the preservative evaluation shows that benzalkonium chloride enhances penetration while Thimerosal has no effect. Evaluation of the counter ions paired with ketorolac gave penetration rates in the following descending, relative, order: quaternary ammonium compounds > guanidino compounds > aliphatic amines > alkali metal.

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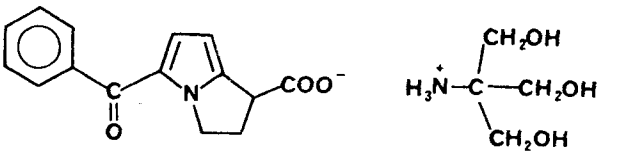
INTRODUCTION

Ketorolac, ±5-(benzoyl)-3H-1,2-dihydropyrrolo-[1,2a]-pyrrole-1-carboxylic acid, Tromethamine (I) is a non-steroidal compound which possesses potent analgesic and anti-inflammatory activity[1]. Ketorolac is effective in inhibiting corneal neovascularization resulting from silver nitrate cauterization; it is also effective in blocking arachadonic acid induced increases in intraocular pressure[2]. Ketorolac tromethamine is currently being developed as a post-surgical ocular anti-inflammatory agent. The carboxylic acid group of



(I)

ketorolac has an apparent pKa of 3.54[3] thus it exists as an anion at the ocular physiological pH of 7.4. It is generally believed that the ionized form of a drug penetrates the cornea poorly. With this in mind, experiments were performed to: (i) determine the permeability of ketorolac at different pH levels; (ii) study the effects of the most commonly used ophthalmic preservatives (thimerosal, benzalkonium chloride) on the permeability of ketorolac; and (iii) study the involvement of ion pairing between an

anionic drug and a positively charged counter ion on corneal penetration.

Because in vivo bioavailability studies, are complicated by lacrimation and eyelid action, compounded with the inability to obtain samples successively from a given eye, an in vitro corneal diffusion model was established. This model allows for convenient monitoring of drug penetration through the corneal membrane at chosen time intervals.

EXPERIMENTAL

Materials - Ketorolac free acid and the tromethamine salt were obtained from and synthesized by the Institute of Organic Chemistry, Syntex Corporation. ^{14}C-glycerol was obtained from New England Nuclear having a specific activity of 15.76 mCi/mmole and a radiochemical purity of 98%. Adenosine, arginine, guanidine, dodecyl-, tetradecyl-, and hexadecyltrimethyl ammonium bromide were obtained from Sigma Chemical Company. Aquasol LSC scintillation cocktail came from NEN Research Products. All chemicals were reagent grade and were used without further purification.

Apparatus - A modified Franz diffusion cell consisting of a glass receptor cell and a teflon donor cell was

used for the penetration experiments. The receptor cell held an internal receptor volume of 8.0 ml and a side arm allowed sampling of the receptor phase. The donor cell was recessed to accommodate the corneal curvature and was clamped onto the top of the glass cell. A 0.3 ml volume of donor solution was placed on top of the cornea, and evaporation of this donor solution was diminished by sealing a glass cover slip over the opening of the donor cell with silicon grease. To insure corneal curvature throughout the course of the experiment, a 1.0 ml latex bulb was placed over the sampling port of the glass diffusion cell. By so doing, enough pressure was exerted under the cornea to maintain a curved, wrinkle-free membrane. Water at 37°C was circulated through the water jacket surrounding the receptor volume, and a magnetic stir bar placed in the bottom of the receptor cell created a homogeneous receptor volume.

Cornea Preparation - Because rabbits were used in conjunction with other departments, the availability of species shifted. Hence, cornea were obtained from two species of rabbit; replicate experiments of a given compound, however, were performed on the same species. The two species used were: New Zealand white and Dutch Belted rabbits. The rabbits were sacrificed with a

lethal dose of phenobarbital. The cornea were carefully removed along with 2-4 mm of surrounding scleral tissue. These were then placed in a freshly prepared solution of balance base buffer[4] containing 0.133% adenosine; the addition of adenosine helped maintain the integrity of the corneal membrane[5].

Experimental Procedure - Fresh cornea were mounted by sandwiching the surrounding scleral tissue between the top and bottom of the teflon donor cell; this whole unit was then clamped onto the glass receptor cell. The receptor volume was filled with degassed adenosine-balance base buffer solution; all air bubbles were expelled from beneath the cornea by inverting the entire diffusion cell then allowing the bubbles to travel out of the sampling port. After the magnetic stir bar was turned to the "on" position and the 1.0 ml bulb placed over the sampling port, a 0.3 ml aliquot of donor solution was placed on top of the cornea. At 10, 20, 30, 45, 60, 80, 100 and 120 minutes, a 0.3 ml sample was withdrawn from the receptor volume through the sampling port; the receptor volume was then replenished with 0.3 ml of degassed adenosine balance base buffer. The withdrawn sample was prepared for quantitative analysis.

Preparation of Test Solutions -

1. For studies involving the effect of pH on ketorolac

permeability, the following two solutions were prepared. To an aqueous mixture of 0.35% ketorolac acid and 0.79% sodium chloride, 1N sodium hydroxide solution was added dropwise to dissolve the ketorolac. The solution was then divided into two parts and 1N sodium hydroxide was again used to adjust the pH of the two solutions to 5.4 and 7.4, respectively. The resulting solutions had a tonicity value equivalent to a 0.9% sodium chloride solution.

2. For studying the preservative effects, the following solutions were made and adjusted to a pH of 7.4 using 1N sodium hydroxide solution:

   (a) 0.5%[6] ketorolac trometnamine salt with 0.01% benzalkonium chloride in water;

   (b) 0.5% ketorolac tromethamine salt with 0.0025% thimerosal in water.

3. For studying ketorolac permeability in the presence of a counter ion, the solutions listed below were prepared and adjusted to a pH of 7.4. The amount of counter ion added to each solution was determined on a molar ratio basis; the molar ratio of ketorolac to counter ion was 1:1.2.

(a) 0.35% ketorolac acid and 0.08% NaOH dissolved in water;

(b) 0.35% ketorolac acid and 1.7% tromethamine in water;

(c) (i) 0.35% ketorolac acid and 0.26% Arginine in water;

(ii) 0.35% ketorolac acid and 0.14% Guanidine HCl in water;

(d) (i) 0.5% ketorolac, tromethamine salt, with 0.42% dodecyltrimethyl ammonium bromide in water;

(ii) 0.5% ketorolac, tromethamine salt, with 0.46% tetradecyltrimethyl ammonium bromide in water;

(iii) 0.5% ketorolac, tromethamine salt, with 0.50% hexadecyltrimethyl ammonium bromide in water;

^{14}C-glycerol penetration studies through rabbit cornea

To monitor the corneal integrity throughout the course of the permeability studies and to assess the extent to which the change in ketorolac cornea permeability is due to corneal membrane alteration, a ^{14}C-glycerol penetration study was performed. Non-ionized ^{14}C-glycerol was incorporated into the above testing formulations, and its penetration was monitored. To a

2.0 ml aliquot of each test solution, including an aliquot of balance base buffer, 20 µl of the ^{14}C-glycerol (15.76 mCi/mmole) was added. Following the experimental procedure as outlined above, 0.3 ml of receptor volume was collected at 15 minute intervals until the two hour time point. Each aliquot was placed in a scintillation vial with 10 ml of Aquasol; the samples were vortexed then placed in a Beckman LS 8100 Scintillation Counter for radioactivity counting.

Analytical Method - Quantitation of ketorolac was performed using HPLC methodology. The mobile phase was composed of methanol, water and acetic acid (60:39.9:0.1). The equipment included: a Spectra-Physics SP8700 solvent delivery system; a Waters WISP 710B autoinjector; a Schoeffel detector SF769; a Spectra-Physics SP4100 Computing Integrator; and a reverse-phase Whatman Partisil ODS-3, 10 µ column. The injection volume was 100 µl; the flow rate of the mobile phase was 2.0 ml/min; and the absorbance wavelength used was 254 nm. A 100 µl aliquot of each sample was diluted with 100 µl of mobile phase and 50 µl of 1.0 N acetic acid. Three solutions of known ketorolac concentration were used as external standards; the three standards were run after every 10-15 samples in order to insure reliable and accurate quantitation.

Partition Coefficient - A known amount of ketorolac combined with a known amount of specific counter ion were equilibrated at room temperature between equal volumes of mutually saturated octanol and water. Each preparation underwent 24 hours of continuous, gentle shaking; aliquots from each phase were withdrawn for HPLC assay of ketorolac. The partition coefficients represent the ratio of ketorolac distribution between the two respective phases: octanol and water.

RESULTS AND DISCUSSION

Each experiment was performed simultaneously with a matched control; that is, from a single rabbit, one cornea was treated with the solution of interest while the other cornea served as a control and was treated accordingly. The control solutions for each set of experiments are listed in the Tables. Because of the large variability attributable to biological membranes, experimental results can only fairly be compared to their matched control values.

I. Rabbit Cornea Penetration of ^{14}C-glycerol

In order to evaluate the integrity of the corneal membrane after exposure to the various ketorolac solutions, the cornea penetration of the marker ^{14}C-glycerol in these solutions was determined and the

TABLE I

Results of Corneal Penetration Studies of ^{14}C-glycerol in Various Ketorolac Ophthalmic Vehicles

Preparations	^{14}C-Glycerol Penetration	
	% of Initial Dose (60 min)	% of Initial Dose (120 min)
Balance Base Buffer	3.85	10.18
Isotonic Saline at pH 7.4	3.04	8.93
Isotonic Saline at pH 5.4	3.12	10.33
With Thimerosal	3.34	12.80
With Benzalkonium Chloride	5.18	15.56
Counter Ion Na^+	4.34	10.53
Counter Ion Tromethamine	1.74	8.53
Counter Ion Arginine	2.91	10.21
Counter Ion Guanidine	3.62	15.29
Counter Ion C-12*	13.86	26.67
Counter Ion C-14*	12.33	21.95
Counter Ion C-16*	10.44	19.44

*C-12, C-14, and C-16 refer to dodecyl-, tetradecyl-, and hexadecyltrimethyl ammonium ion respectively.

results are given in Table I. In these studies ^{14}C-glycerol in balance base buffer containing adenosine served as the control. As such, a review of the data shows no real deviation from the control values except for the quaternary ammonium compounds and guanidine (to a lesser degree).

TABLE II

pH Effects of Ketorolac Permeability through Rabbit[a] Cornea

	pH 7.4	pH 5.4
Cumulative mgs[b] 60 min	0.0072 ± 0.0008(2)	0.0199 ± 0.0010(2)
Cumulative mgs 120 min	0.0167 ± 0.0023(2)	0.0504 ± 0.0156 (2)
Flux (mgs/hour/cornea)	$2.1 \times 10^{-4} \pm 4.5 \times 10^{-5}$	$5.8 \times 10^{-4} \pm 2.4 \times 10^{-5}$

[a]Rabbit Species used: Dutch Belted.
[b]The values are given as: mean ± S.E.M. (n).

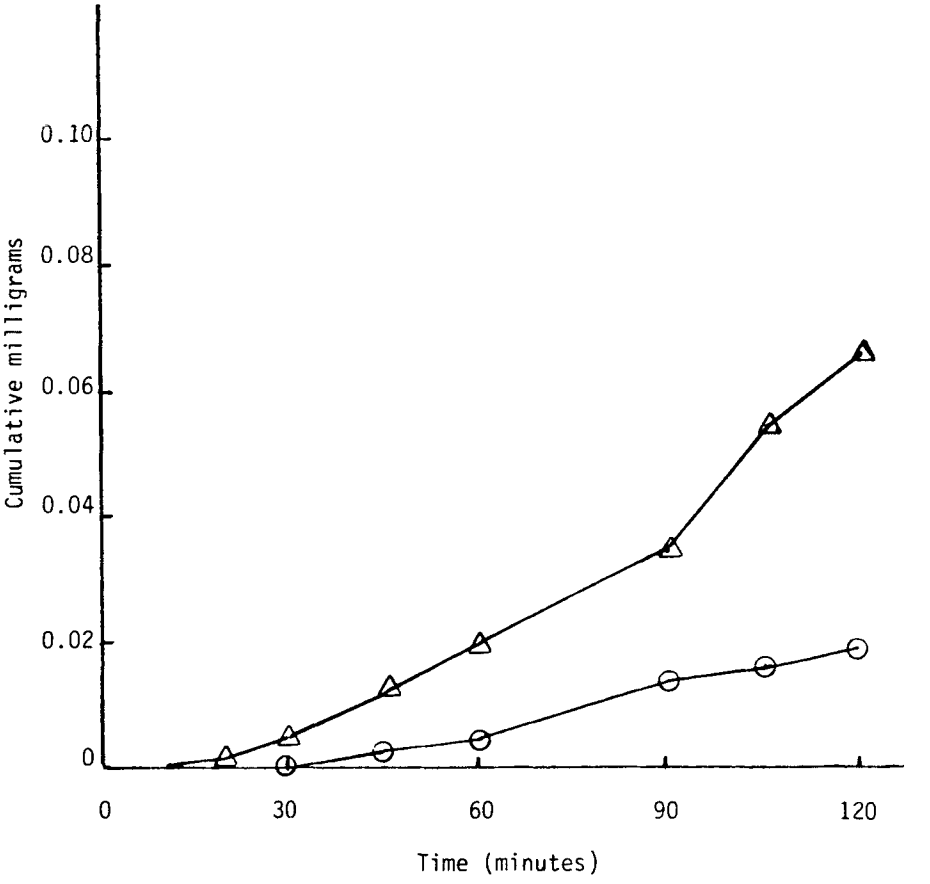


Figure 1. Ketorolac permeability compared at two pH levels (○ pH 7.4; △ pH 5.4).

II. Effects of pH on Ketorolac Corneal Permeability

Transcorneal permeability was evaluated at two pH levels. The rationale for choosing these pH levels was that pH 5.4 is the lowest possible pH at which a 0.35% ketorolac solution will remain clear, and pH 7.4 represents the ocular physiological pH. The permeability profile of ketorolac is presented in Table II and Figure 1. The ratio of the nonionized carboxylic

acid form of the drug to the anion form of the drug at pH 5.4 ± 0.2 and 7.4 ± 0.2 are approximately $1/10^2$ and $1/10^4$, respectively. The pH at 5.4 provides a 100 fold increase in the amount of the nonionized carboxylic acid form prevalent in solution. The permeability data for the two pH levels show a 3-fold increase in ketorolac penetration at pH 5.4 indicating that the increased acid form more readily penetrates the cornea. ^{14}C-glycerol, the marker, in comparison, shows no difference in corneal permeability under the two pH conditions; this would indicate that a low pH at 5.4 is not detrimental to the cornea membrane. An increased ketorolac penetration, then, cannot be attributed to membrane abberations. Considering that at pH 7.4 only approximately 1 out of 10^4 molecules of ketorolac is nonionized, a considerably large, relative, amount of ketorolac is found to have penetrated the corneal membrane. This finding indicates that both ionized and nonionized forms of ketorolac pass through the corneal barrier. Nogami[7], et.al., demonstrated that both ionized and unionized forms of sulfonamide can pass through isolated intestinal membranes; likewise, Swarbrick[8], et.al., showed that chromone-2-carboxylic acids permeate human skin in both the ionized and unionized species. Further evidence of possible ionized drug movement has been documented for cromolyn sodium[9].

The relationship between the penetration of the two species of ketorolac can be demonstrated mathematically. The steady state flux (J) is expressed as: $J = P \cdot C$ where P represents the permeability coefficient and C is the concentration of the permeating species. Assuming two species are present (i.e., ionized and nonionized) the flux expression at each pH value is given by: $J_{pH\ value} = J_{HA} + J_{A^-}$ where J_{HA} is the flux of the nonionized species and J_{A^-} is the flux term for the ionized species. A ratio of the two equations and a substition for the permeability coefficient and concentration terms renders the following equation:

$$\frac{J_1}{J_2} = \frac{P_{HA}[C_{HA}]_1 + P_{A^-}[C_{A^-}]_1}{P_{HA}[C_{HA}]_2 + P_{A^-}[C_{A^-}]_2}$$

$$= \frac{\frac{P_{HA}}{P_{A^-}}[C_{HA}]_1 + [C_{A^-}]_1}{\frac{P_{HA}}{P_{A^-}}[C_{HA}]_2 + [C_{A^-}]_2}$$

Where J_1, J_2 = flux at pH 7.4 and 5.4, respectively

$[C_{HA}]_1$, $[C_{A^-}]_1$ = concentration at pH 7.4

$[C_{HA}]_2$, $[C_{A^-}]_2$ = concentration at pH 5.4

Applying this equation to the <u>in vitro</u> corneal data yields a P_{HA}/P_{A^-} ratio of 207, when J_1/J_2 is

TABLE III

Preservative Effects on Ketorolac Permeability Through Rabbit[a] Cornea

	Benzalkonium Chloride	Thimerosal
60 min Cumulative mgs[b]	0.0273 ± 0.0056 (3)	0.0107 ± 0.0048 (3)
Matched Control[c]	0.0081 ± 0.0008 (3)	0.0164 ± 0.0068 (3)
120 min Cumulative Mgs	0.1003 ± 0.0221 (3)	0.0484 ± 0.0115 (3)
Matched Control	0.0270 ± 0.0015 (3)	0.0507 ± 0.0117 (3)

[a]Rabbit Species used: New Zealand White.
[b]The values are given as: mean ± S.E.M. (n).
[c]The matched control solution for these experiments was ketorolac tromethamine salt in water at pH 7.4 without preservative.

equal to our empirical finding of about 1/3. Had permeation of the ionized species been insignificant, the theoretical value of 1/100 for J_1/J_2 should have been realized. But the increased permeability observed in our experiments strongly indicates that permeation of ionized ketorolac through excised rabbit cornea is possible and cannot be assumed to be negligible.

III. Preservative Effects

The two most commonly used preservatives in

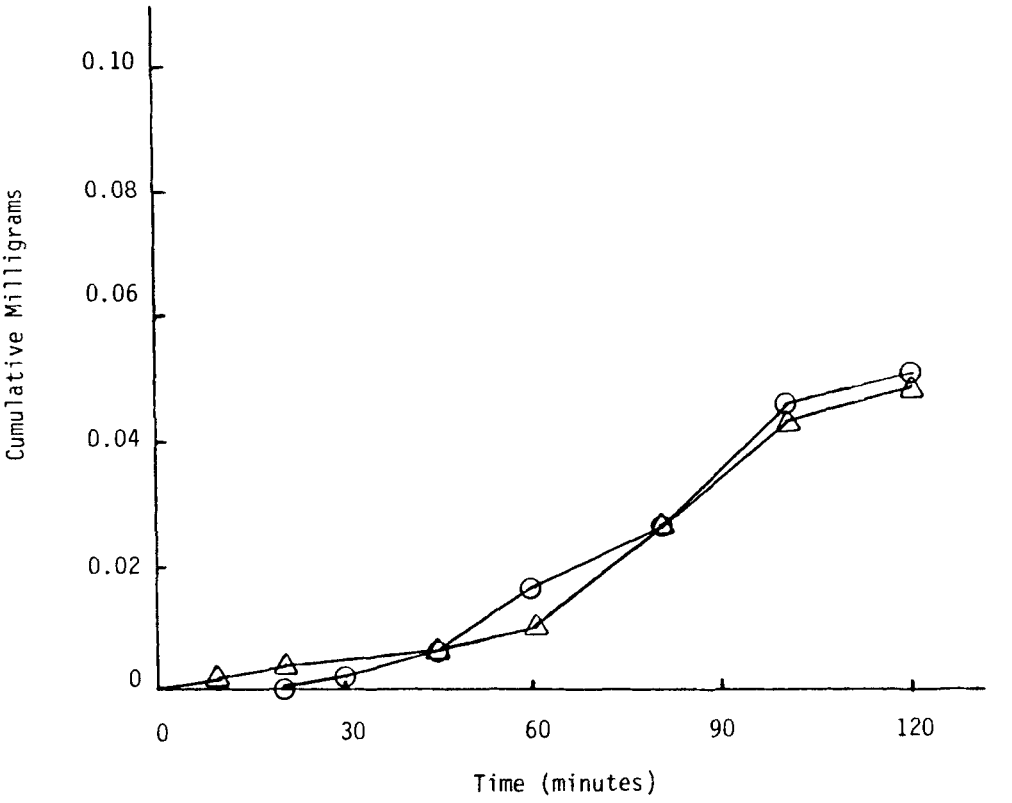


Figure 2. Permeability profiles of Ketorolac tromethamine with and without the presence of Thimerosal;
(○ no Thimerosal; △ with Thimerosal).

ophthalmic preparations, 0.01% benzalkonium chloride and 0.0025% thimerosal, were studied for their effects on the permeability of ketorolac through rabbit cornea. Table III and Figures 2 and 3 summarize the penetration of Ketorolac in the presence of these preservatives. The results of these experiments show that benzalkonium chloride gives a 2-3 fold increase in penetration of ketorolac when compared to the penetration of ketorolac

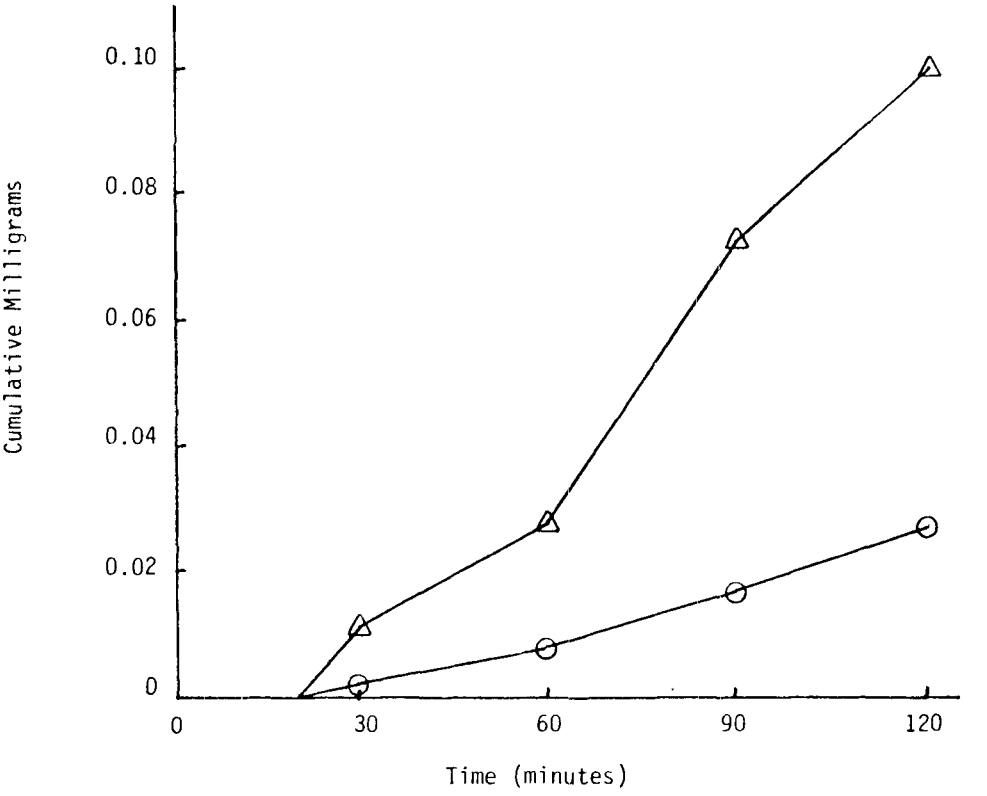


Figure 3. Permeability profiles of Ketorolac tromethamine with and without the presence of Benzalkonium Chloride (BAC); (○ no BAC; △with BAC).

alone; in contrast, thimerosal exhibits no enhancement of ketorolac.

In general, when animal corneas are exposed to benzalkonium chloride an increase in solute permeability is observed for both in vivo and in vitro testing[10,11,12,13]. The 2-3 fold increase seen for ketorolac penetration through the cornea is consistent

with those increases reported in the literature. Two mechanisms, possibly working in tandem, may account for the increased penetration: (i) benzalkonium chloride disrupts the integrity of the epithelial membrane; and (ii) the possible formation of a more lipid soluble ion pair between ketorolac and benzalkonium chloride may enhance penetration. Because benzalkonium chloride appears to increase glycerol penetration (1.35x over the control), membrane disruption is suspect. This penetration increase observed for glycerol, however, is not totally sufficient to account for the comparatively larger increase in ketorolac penetration (3x over the control). The difference is attributed to ion pairing formation.

IV. Counter Ion Effects

As demonstrated above, ionized drug molecules tend to permeate mucous tissue at a reduced rate. Schanker[14] suggests that organic ions can diffuse across the blood barrier in the gut in the form of a less polar complex. This study involves application of this approach by investigating the altered absorption characteristics of an anionic drug (Ketorolac) upon ion-association with a cation. Evaluation of ion-association and increased lipophilicity of the resulting complex were made by comparing the change in the

TABLE IV

Partition Coefficients

Chemical Form in Which Ketorolac Was Used	Counter Ion Added	Partitition Coefficient (mg in octanol/mg in Water)
Free Acid	Na^+	0.0251 ± 0.0060
Tromethamine Salt	-	0.0490 ± 0.0028
Free Acid	Arginine	0.0335 ± 0.0035
Free Acid	Guanidine	0.0733 ± 0.0106
Tromethamine Salt	C-12*	0.8684 ± 0.0141
Tromethamine Salt	C-14	0.8946 ± 0.0340
Tromethamine Salt	C-16	1.0470 ± 0.2713

*C-12, C-14 and C-16 refer to dodecyl-, tetradecyl-, and hexadecyltrimethyl ammonium ion respectively.

octanol/water partioning behavior as well as the degree of permeability through corneal membrane. The types of cationic counter ions used are categorized as follows: alkali metal ion, aliphatic amine, amino acid (arginine), stronger base (guanidine) and quaternary ammonium compounds.

Table IV gives the partition coefficients of the solutions prepared for studying the counter ion effect. This study was performed in order to determine the compounds' ability to partition into hydrophobic octanol. As shown in Table IV, the partition coefficient for Ketorolac sodium salt is extremely small

TABLE V

Summary of Cumulative Milligrams of Ketorolac Penetrating the Rabbit[1] Cornea in the Presence of a Counterion

I. Aliphatic Amine	Cumulative mgs 60 min	Matched control[2]	Cumulative mgs 120 min	Matched control
a. with Tromethamine	0.0171 ± 0.0088(4)	0.0057 ± 0.006(4)	0.0568 ± 0.0101(7)	0.0407 ± 0.0082(7)
II. Guanidino Compounds	**Cumulative mgs after 60 min**	**Matched control[2]**	**Cumulative mgs after 120 min**	**Matched control**
a. with Arginine	0.0146 ± 0.0047(6)	0.0056 ± 0.0027(6)	0.0608 ± 0.0116(7)	0.0388 ± 0.0074(7)
b. with Guanidine	0.0080 ± 0.0051(5)	0.0043 ± 0.0031(5)	0.0491 ± 0.0093(5)	0.0278 ± 0.0038(5)

III. Quaternary ammonium compounds[3] mgs after 60 min	Cumulative mgs 60 min	Cumulative mgs 120 min
a. With dodecyltrimethyl ammonium bromide	0.0775 ± 0.0074(4)	0.1684 ± 0.0163(4)
b. With tetradecyltrimethyl ammonium bromide	0.0377 ± 0.0042(3)	0.0958 ± 0.0059(3)
c. With hexadecyltrimethyl ammonium bromide	0.0435 ± 0.0090(3)	0.0890 ± 0.0145(3)
d. Average penetration with Na^+ as the counterion (for comparison purposes).	0.0052 ± 0.0008(15)	0.0358 ± 0.0070(19)

[1]All rabbits used for these experiments were New Zealand White.
[2]The matched control value represents the milligrams of Ketorolac penetrating the cornea in the presence of sodium salt; for each ribbit, one cornea was dosed with the Ketorolac-Na^+ control while the remaining cornea was dosed with a Ketorolac-counterion complex.
[3]The quaternary ammonium compounds were compared, experimentally, to themselves; the matched control solution was simply another quaternary ammonium compound. For example, the dodecyl - group was placed on one cornea and the hexadecyl-group was placed on the matched cornea. An average value for sodium as a counterion is given for the sake of comparison.

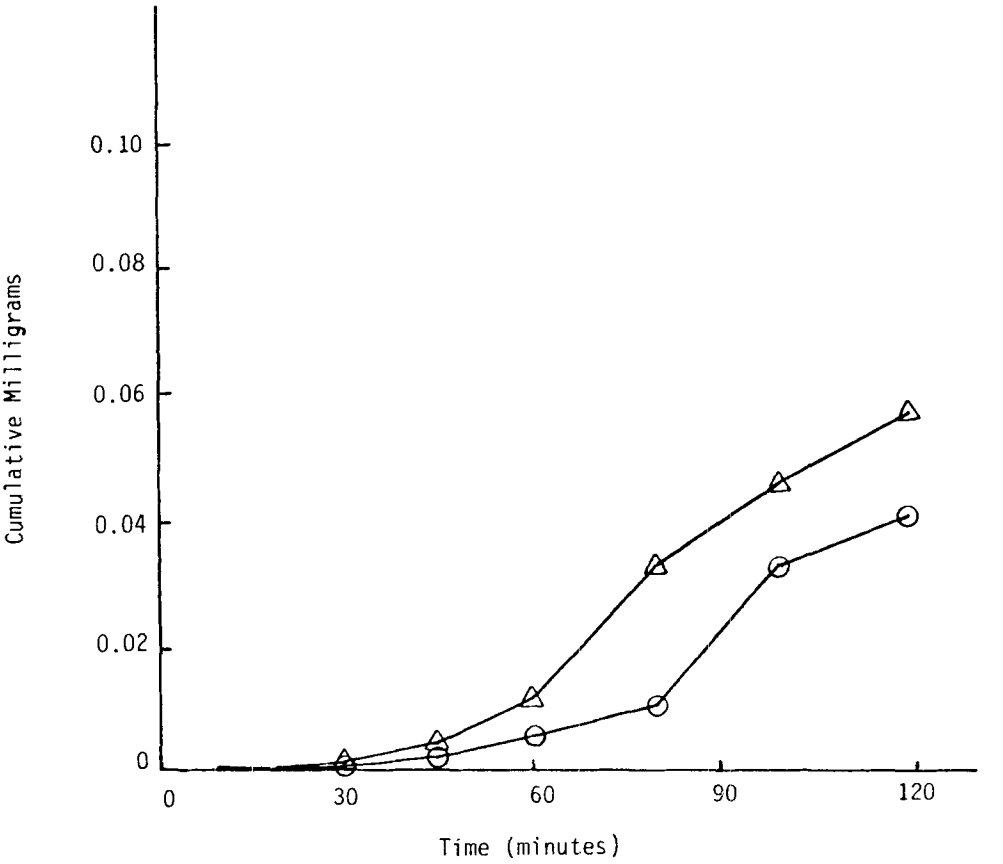


Figure 4. Permeability comparison of Ketorolac Tromethamine salt (△) to Ketorolac free acid with the addition of sodium ion (○).

at pH 7.4. However, it is increased when tromethamine is presented as the counter ion. Likewise, further increases are seen in the presence of guanidine (3 fold increase) and the alkyl quaternary ammonium compounds (32 to 42 fold increase). The increases observed in the octanol/water partition coefficient are indicative of formation of a more lipid soluble ion pair complex between ketorolac and the counter ion.

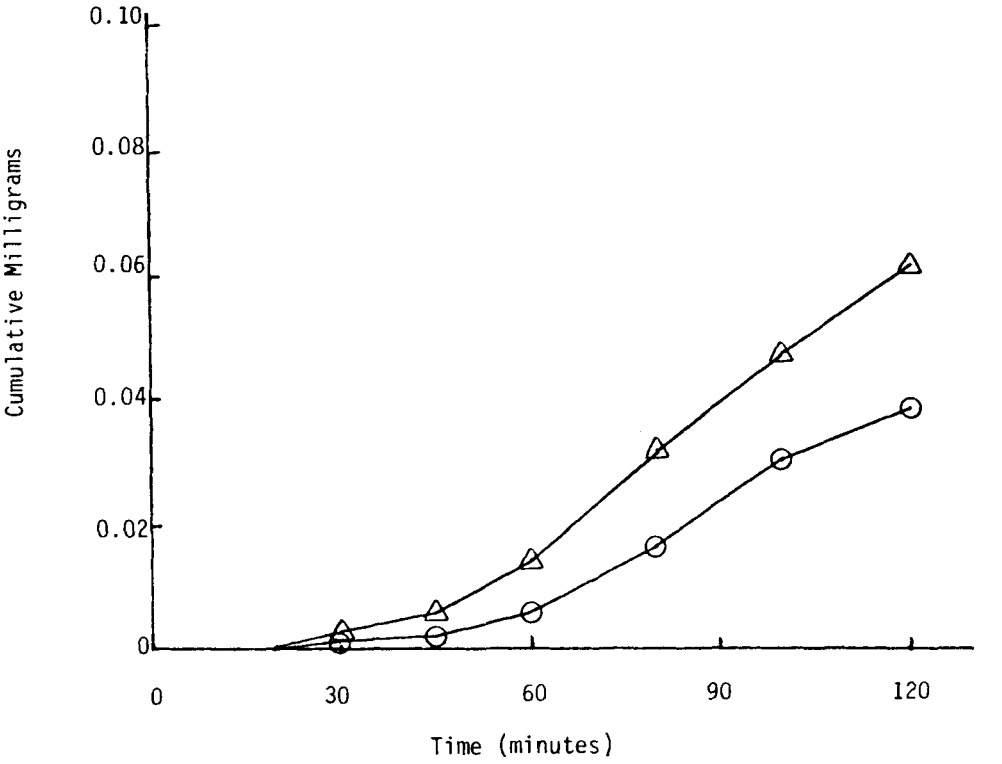


Figure 5. Ketorolac permeability comparison in the presence of Arginine and Sodium ion;
(○ sodium ion; △ Arginine).

The profiles of penetration for the above compounds through the corneal membrane are presented in Table V and Figures 4-7. The data shows that the presence of counter ion affects the rate of ketorolac's permeability, and in general, the trend correlates well with that seen for the octanol/water partitioning ability. When the quaternary ammonium compounds were present as counter ions, ketorolac's octanol/water

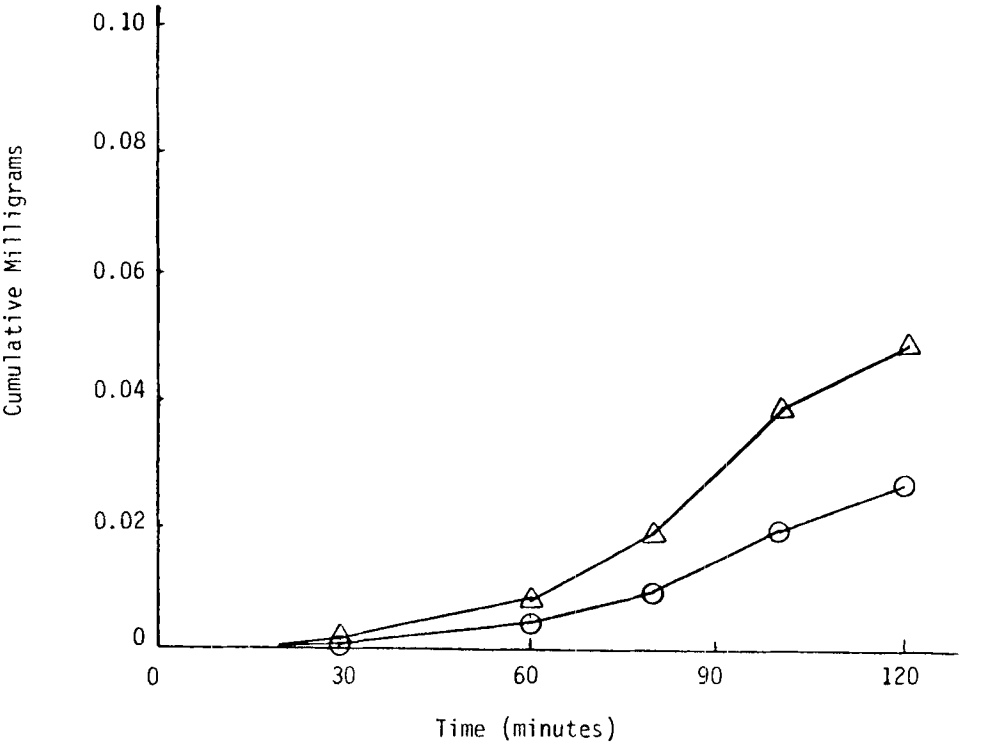


Figure 6. Ketorolac permeability comparison in the presence of Guanidine and sodium ion;
(○ sodium ion; △ Guanidine).

partitioning ability was dramatically increased; penetration through corneal membrane was also increased 8-16 fold. As can be noted from the ^{14}C-glycerol penetration data, the increase in ketorolac is not entirely due to formation of a more lipophilic complex. Rather, as much as 25% of the enhancement can be accounted for by membrane alterations caused by the quaternary ammonium compounds. As the chain length of the quaternary ammonium compounds increase, the

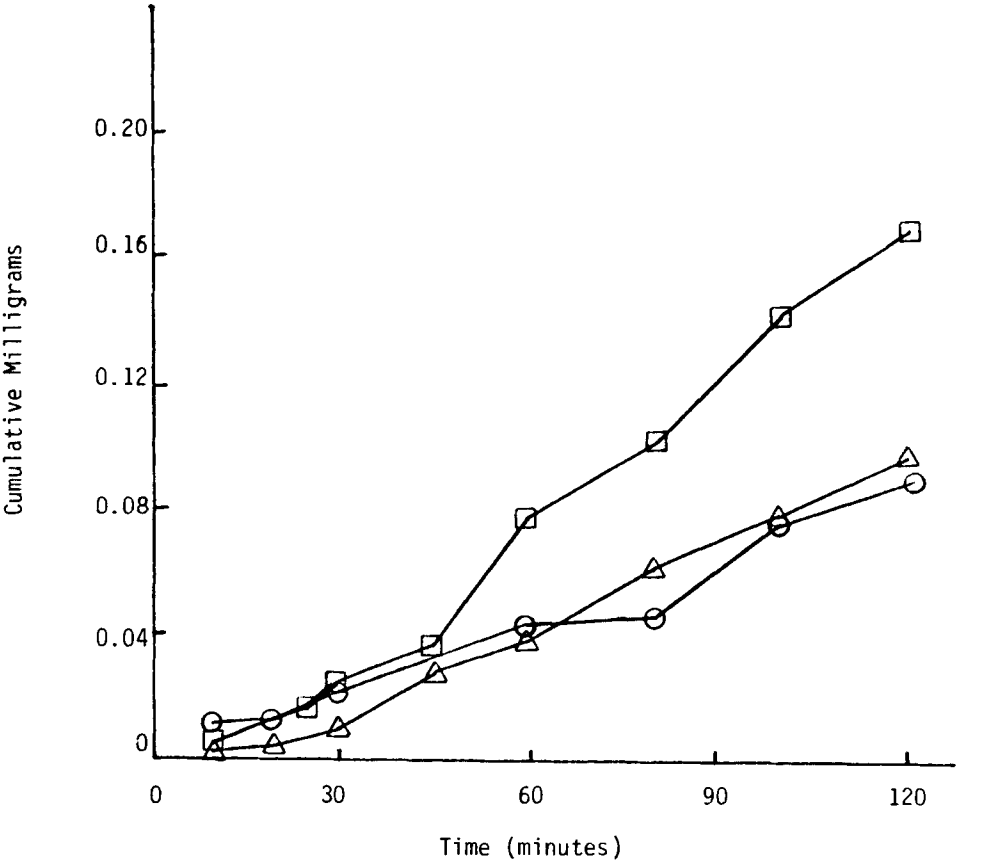


Figure 7. Penetration of the various quaternary ammonium compounds; (○ hexadecyl-, △ tetradecyl-, □ dodecyl-trimethylammonium bromide).

octanol/water partioning ability of ketorolac also increases; in contrast, however, the penetration through corneal membrane is found to decrease. This observation can in part be explained by the parallel penetration trend seen for ^{14}C-glycerol; glycerol penetration also decreases with increasing chain length indicating a pattern in corneal membrane disruption. Other factors which may be involved in this phenomenon are: (1) the

hydrophobic/ hydrophilic balance of the penetrant as well as (2) the possibility of steric involvement between the membrane and the increasing size of the ketorolac - counter ion complex.

The addition of tromethamine and guanidine as counter ions also produced an increase in ketorolac penetration over the sodium ion control. The formation of a more lipid soluble complex is enhanced because of the presence of increasingly stronger bases with increasingly larger association constants.

The addition of arginine resulted in an increase in ketorolac ocular penetration, yet its effect on the partition coefficient was very minimal. Arginine is present as a zwitterion at pH 7.4; consequently, a ketorolac-arginine complex would have a net negative charge. This fact might account for the relatively low partition coefficient but counters the mechanism of enhanced membrane penetration through formation of a less polar compound. Frimmer[15], et.al. describes enhanced compound permeability through capilliaries in the presence of various amino acids, including arginine. Because arginine produces enhanced ketorolac ocular permeability, with no apparent membrane damage, an alternate mechanism of diffusion must be at work.

Arginine appears to offer several benefits as a potential component in ophthalmic preparations; in addition to its role as a penetration enhancer, arginine has been used successfully to diminish eye irritation as a side effect of the drug Indomethacin[16].

CONCLUSION

The results of the *in vitro* corneal penetration studies lead to several observations. Ketorolac shows the ability to penetrate through the corneal membrane, and it does so in both the unionized as well as ionized form. The degree to which ketorolac will penetrate the corneal membrane can be influenced by the vehicle environment with alteration to the pH, addition of a preservative, or addition of a counter ion. The *in vitro* permeability data is supported by, and correlates with the octanol/water partitioning studies.

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2. Unpublished data from the Preformulation Department of Syntex Research. Private communication with D. Johnson.

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4. Balance base buffer contains 0.57% NaCl, 0.361% $NaHCO_3$, 0.04% KCl, 0.023% K_2HPO4, 0.007% $MgSO_4$, and 0.08% $CaCl_2$ in water and is adjusted to pH 7.4.

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