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CORNEAL PERMEABILITY OF KETOROLAC TROMETHAMINE WHEN FORMULATED WITH TOBRAMYCIN

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ABSTRACT

In vitro rabbit corneal penetration studies were designed to determine the effect tobramycin (an antibiotic) has on the diffusion of ketorolac tromethamine (a nonsteroidal anti-inflammatory compound). Evaluation was performed in two vehicle solutions: (i) a simple sodium chloride vehicle and (ii) a suitable ophthalmic formulation. Quantitation of both ketorolac tromethamine and tobramycin were performed to determine the corneal penetration of each drug. Tobramycin was found to penetrate rabbit cornea to a limited extent. Also,

tobramycin proved neither to impede nor enhance ketorolac's corneal diffusion. Both compounds showed greater penetration in an ophthalmic formulation, presumably due to the effects of the preservative, benzalkonium chloride (a quaternary ammonium compound) -- known for disrupting corneal integrity.

INTRODUCTION

Tobramycin is a water soluble aminoglycosidic antibiotic (Figure 1) having a broad spectrum of action against both gram negative and gram positive bacteria. Aminoglycoside antibiotics are useful in treating ocular infections and are used prophylactically before and after ocular surgery.[1] Tobramycin (a polycationic and highly polar compound) demonstrates greater effectiveness and fewer side effects when compared with gentamicin[1,2] or chloramphenicol[3].

Ketorolac, ±5-(benzoyl)-3H-1,2-dihydropyrrolo-[1,2a]-pyrrole-1-carboxylic acid, is a potent analgesic and anti-inflammatory compound; it has been shown previously[4] to penetrate rabbit cornea in both the ionized and unionized forms. The water soluble tromethamine salt of ketorolac is currently being developed for treatment of post-surgical ocular inflammation. Ketorolac, with a pKa of 3.54, is negatively charged at physiological pH and thus, can ion-pair with cations present in solution.

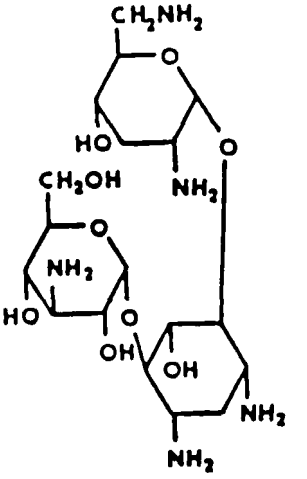

Figure 1: Tobramycin

Potential development of a combination product containing both tobramycin and ketorolac tromethamine led to the current study. In vitro rabbit corneal penetration of ketorolac was evaluated in the presence of tobramycin. The underlying question is whether tobramycin alters penetration of ketorolac through rabbit cornea.

Two sets of studies were performed to evaluate tobramycin's effect on ketorolac penetration. In the first study, the effect of adding tobramycin was evaluated in a simple ketorolac/saline solution. The second study was designed to evaluate tobramycin and ketorolac in a suitable ophthalmic vehicle containing excipients for enhanced stability. This latter formulation was not used for all studies because it contains benzalkonium chloride and edetate disodium;

benzalkonium chloride has been shown to disrupt the integrity of the cornea's epithelial membrane,[5,6,7,8] and edetate disodium can produce broadening of intercellular spaces in both the epithelium and endothelium.[9,10] The effect of these excipients would result in increased solute permeability through the cornea.

EXPERIMENTAL

Materials - Ketorolac tromethamine was obtained from the Institute of Organic Chemistry, Syntex Corporation. Tobramycin was received from Sigma having a potency of 935 µg/mg. ^{14}C-glycerol (15.76 mCi/mmole) was obtained from NEN with a radiochemical purity of 98%. All other chemicals were reagent grade and used as received.

Apparatus - A modified Franz diffusion cell consisting of an 8.0 mL glass receptor cell along with a teflon donor cell were used for the penetration experiments (Figure 2). A side arm allowed sampling of the receptor phase. The donor cell was recessed to accommodate corneal curvature. A 0.3 mL volume of donor solution was placed on the epithelial side of the cornea, and evaporation of this donor solution was diminished by sealing a glass coverslip over the opening of the donor cell with silicon grease. To ensure 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

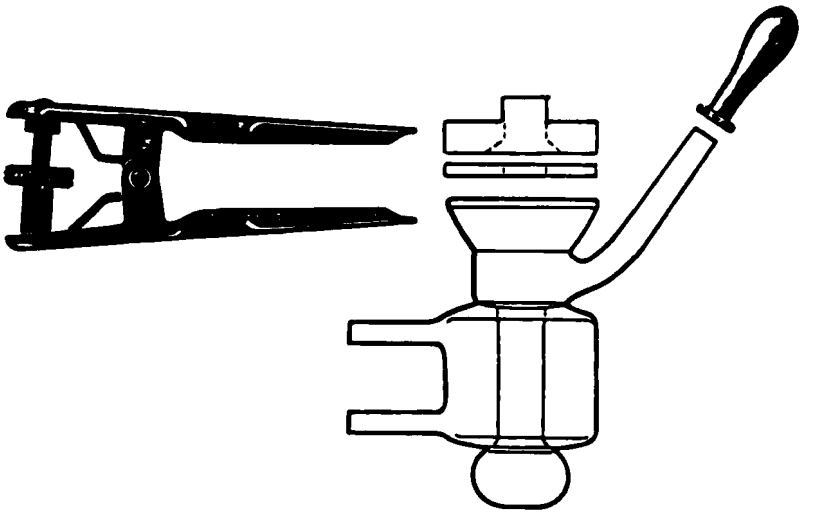

Figure 2: Diffusion Apparatus

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 cell. A magnetic stir bar placed in the bottom of the receptor cell maintained homogeneity within the receptor solution.

Cornea Preparation - New Zealand white rabbits weighing 3.5 to 4 kg were used for the studies. Rabbits were sacrificed by rapid injection of 1.25 mL/kg of T-61 Euthanasia Solution (American Hoechst Corp., Animal Health Division, Somerville, NJ) into a marginal ear vein. The cornea were carefully removed along with 2 - 4 mm of surrounding scleral tissue then placed in a buffer containing: 0.57% sodium chloride, 0.361%

sodium bicarbonate, 0.04% potassium chloride, 0.023% potassium phosphate dibasic, 0.007% magnesium sulfate, 0.08% calcium chloride, and 0.133% adenosine in water, adjusted to pH 7.4. This buffer was used as receptor solution for all studies; its selection was based on the ability to maintain corneal integrity[11] throughout the diffusion studies.

Experimental Procedure - A fresh cornea was placed between the top and bottom of the teflon donor cell; this unit was then clamped onto the glass receptor cell. The receptor cell was filled with sterile, degassed buffer solution; all air bubbles were expelled from beneath the cornea by inverting the entire diffusion cell and allowing bubbles to travel out the sampling port. After donor solution was placed on the cornea, a 0.3 mL sample of receptor solution was collected at the following time points: 15, 30, 45, 60 and 120 minutes. The 0.3 mL aliquot was replaced at each time point with fresh buffer solution.

Preparation of Test Solutions - 1. To determine ketorolac corneal diffusion in the presence of tobramycin, and to determine a dose effect, a saline vehicle was utilized to avoid potential complications by excipients. The following solutions were isotonic and prepared at pH 7.4:

(a) 0.5% ketorolac tromethamine,
0.79% sodium chloride,
purified water;

(b) (a) with 0.15% tobramycin;

(c) (a) with 0.30% tobramycin;

(d) (a) with 0.60% tobramycin.

2. To evaluate whether 0.30% tobramycin (a clinically acceptable and efficacious concentration) has an effect on ketorolac corneal diffusion when administered in a more complex vehicle, an isotonic solution at pH 7.4 was made which contained the following:

(a) 0.5% ketorolac tromethamine,
0.79% sodium chloride,
edetate disodium,
benzalkonium chloride,
purified water;

(b) (a) with 0.30% tobramycin.

^{14}C-glycerol Penetration - To monitor corneal integrity throughout the course of the permeability studies, ^{14}C-glycerol penetration was evaluated. Nonionized ^{14}C-glycerol was incorporated into selected test solutions (1a and d, above). For controls, two additional isotonic test solutions were made at pH 7.4:

(1) phosphate buffered saline;

(2) 0.6% Tobramycin in phosphate buffered saline.

To a 2.0 mL aliquot of each test solution, 10 µL of ^{14}C-glycerol was added. At designated time intervals, 0.3 mL of

receptor solution was sampled for scintillation counting (Beckman model LS 8100).

Analytical Methods - 1. Quantitation of ketorolac was performed by HPLC. The mobile phase was composed of methanol, water and glacial acetic acid (65:34:1). The equipment included: a Spectra - Physics 8440 UV/Vis detector; a Spectra - Physics 4270 integrator; a Spectra - Physics 8700 solvent delivery system; a Dynatech autosampler; and a Whatman Partisil ODS 3, 10 micron column. The mobile phase flow rate was 1.0 mL/min; the sample injection volume was 50 µL; and the absorbance wavelength was 254 nm. A 100 µL aliquot of each sample was diluted with 150 µL of mobile phase.

2. Quantitation of tobramycin was performed using the Syva EMIT Tobramycin assay kit. The assay is an enzyme immunoassay intended to quantitatively analyze tobramycin in human serum or plasma; the limit of detection is 1.0 µg/mL. The assay is based on competition for antibody sites between free drug in sample and drug labeled with glucose-6-phosphate dehydrogenase (G-6-P-DH). Since G-6-P-DH activity decreases upon binding with antibody, tobramycin concentration can be measured in terms of enzyme activity. Active enzyme converts oxidized nicotinamide adenine dinucleotide (NAD) to NADH. This conversion results in an absorbance change that is measured

spectrophotometrically. The Syva EMIT Tobramycin Reagent kit contained the following:

(a) Reagent A - Antibody/Substrate Reagent
containing: - sheep antibodies reactive to tobramycin
- glucose-6-phosphate
- nicotinamide adenine dinucleotide (NAD)
- Tris buffer;

(b) Reagent B - Enzyme Reagent
containing: - tobramycin labeled with G-6-P-DH
- Tris buffer;

(c) Buffer - Tris buffer;

(d) Calibrators - 0,1,2,4,8,16 μg/mL tobramycin in human serum.

RESULTS AND DISCUSSION

Each experiment was performed with matched controls; that is, from a single rabbit, one cornea was treated with a ketorolac (control) solution, and the other cornea was treated with the ketorolac and tobramycin solution. Each test solution containing tobramycin was evaluated in triplicate. For the study using the simple isotonic vehicle, data for nine control corneas were generated. The ketorolac penetration profile for all these cornea is shown in Figure 3. Since these were control cornea, each is from a different rabbit; hence, the deviation shown at each time point gives an indication of both the

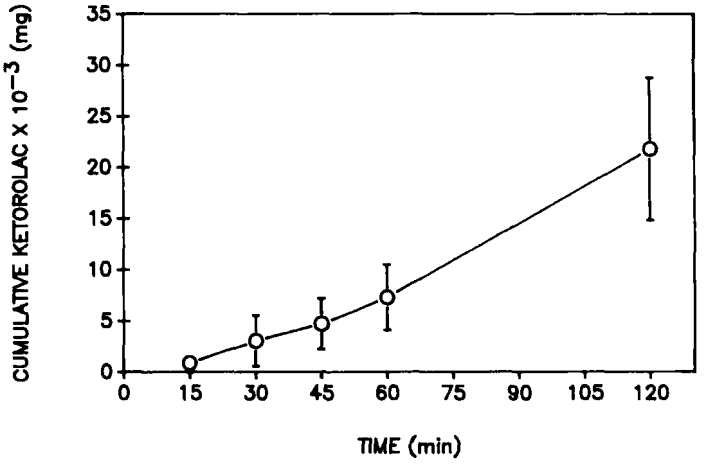

Figure 3: Diffusion data for ketorolac tromethamine control corneas (each point represents the mean ± S.D. for n = 9)

biological as well as experimental deviation inherent to this type of study.

An indication of corneal integrity throughout the course of these studies was determined by penetration of ^{14}C-glycerol. Changes in the permeability profile of ^{14}C-glycerol can be attributed to corneal alteration or damage[9]. Select vehicles were chosen to evaluate whether corneal damage could be attributed to a particular compound or combination. The ophthalmic formulation containing benzalkonium chloride was not evaluated; the presence of benzalkonium chloride has previously demonstrated elevated ^{14}C-glycerol penetration.[4] With phosphate buffered saline serving as control, a two or

TABLE I

^{14}C-Glycerol Penetration Through Rabbit Cornea

Preparation	Percent of Initial Counts per Minute at 60 min	at 120 min
Phosphate Buffered Saline	2.10	7.36
Ketorolac Tromethamine in Saline	2.47	8.60
Tobramycin (0.6%) in Phosphate Buffered Saline	1.83	7.08
Ketorolac Tromethamine and Tobramycin (0.6%) in Saline	2.01	6.03

three-fold increase in ^{14}C-glycerol penetration would indicate substantial corneal alteration. Table I shows that ^{14}C-glycerol penetration in a solution containing ketorolac tromethamine, or 0.6% tobramycin, or their combination, does not differ from its penetration in buffer, alone. These results suggest that corneal integrity is not altered by ketorolac tromethamine or tobramycin.

Figures 4, 5 and 6 compare the average total milligrams of ketorolac penetrating the cornea at each time point for the simple solutions containing ketorolac alone and solutions containing either 0.15%, 0.30% or 0.60% tobramycin,

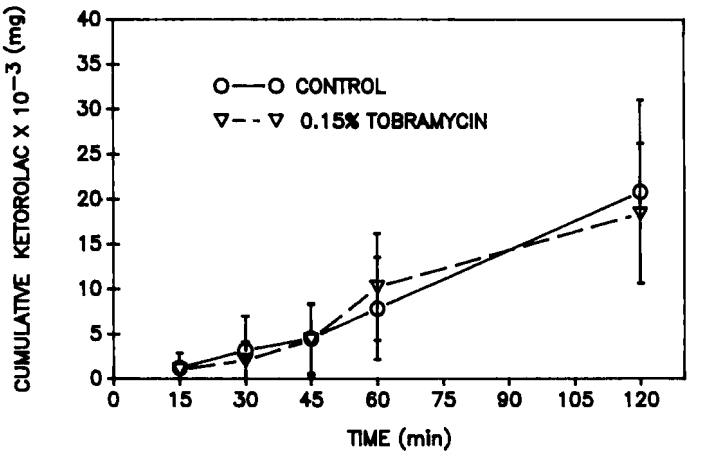

Figure 4: Comparison of ketorolac penetration for a simple ketorolac solution (control) and the same containing 0.15% tobramycin (each point represents the mean ± S.D. for n = 3)

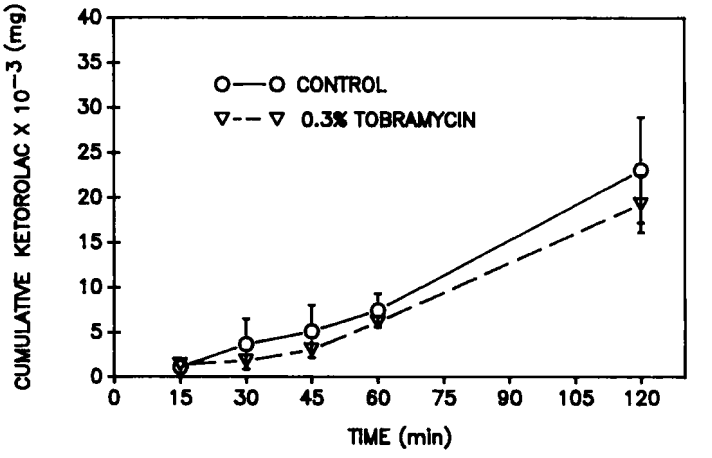

Figure 5: Comparison of ketorolac penetration for a simple ketorolac solution (control) and the same containing 0.30% tobramycin (each point represents the mean ± S.D. for n = 3)

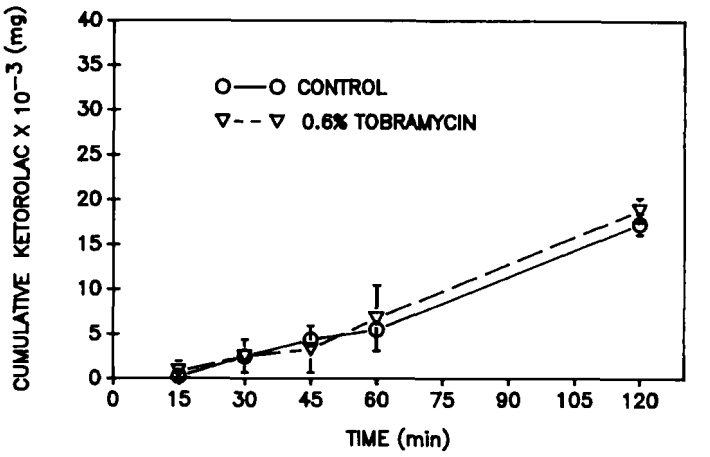

Figure 6: Comparison of ketorolac penetration for a simple ketorolac solution (control) and the same containing 0.60% tobramycin (each point represents the mean ± S.D. for n = 3)

respectively. In all cases, the solutions containing tobramycin were equivalent to the control solution.

Figure 7 likewise compares the average total milligrams of ketorolac penetrating the cornea at each time point for the ophthalmic formulation with and without 0.30% tobramycin. Again, the test solution and the control solution were equivalent. Studies with the formulation demonstrated that after 60 minutes, there occurs a two to three fold increase in ketorolac diffusion. This enhanced penetration led to the pronounced alteration in curve shape, apparent in Figure 7. Enhanced penetration seen with this formulation can be attributed to: (i) disruption of the integrity of the corneal

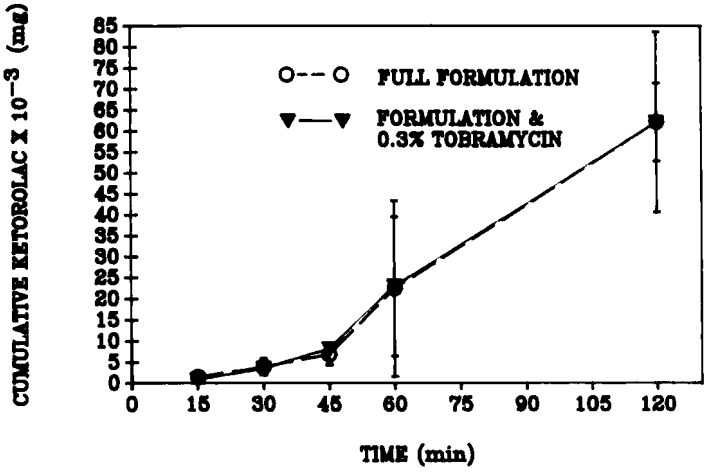

Figure 7: Comparison of ketorolac penetration for the ketorolac ophthalmic solution and the same containing 0.30% tobramycin (each point represents the mean ± S.D. for n = 3)

epithelium by benzalkonium chloride; (ii) expansion of epithelial intercellular spaces by edetate disodium; and/or (iii) formation of a more lipid soluble complex between benzalkonium chloride and ketorolac. Evidence suggesting this latter mechanism has been shown previously[4], and the data presented here is consistent with those findings.

Because the Syva EMIT immunoassay is intended for tobramycin quantitation in human serum, concern arose as to whether tobramycin could be detected in the buffer system used for the penetration studies; also, the possibility existed that ketorolac might interfere with the tobramycin assay. Evaluation of the assay limits showed that accurate quantitation of

TABLE II

Tobramycin Content in Receptor Solution after Two Hours of Diffusion Study

I. Isotonic Solution

0.15% Tobramycin in donor solution[1]

(1) ND
(2) 1.1 µg/mL
(3) ND

0.30% Tobramycin in donor solution

(1) 1.3 µg/mL
(2) < 1.0 µg/mL
(3) 1.1 µg/mL

0.60% Tobramycin in donor solution

(1) < 1.0 µg/mL
(2) ND
(3) 3.1 µg/mL

II. Ophthalmic Solution

0.30% Tobramycin

(1) 4.0 µg/mL
(2) 3.8 µg/mL
(3) 3.9 µg/mL

ND = not detectable

[1]The remainder of the donor solution contains 0.5% ketorolac tromethamine and 0.79% sodium chloride in water.

tobramycin was possible in the buffer of interest, and the levels of ketorolac present, even after two hours (about 0.01 mg/mL), did not interfere with that quantitation. Table II shows the levels of tobramycin present for each sample after two hours. As with ketorolac the same trend occurs: comparatively low tobramycin levels were observed for the saline solution comparisons, whereas a three to four fold increase in tobramycin penetration was seen in the ophthalmic formulation.

Unlike ketorolac quantitation, tobramycin was only evaluated in the 120 min samples due to the lower limit of detection for the EMIT assay (1 μg/mL). Even at 120 minutes, several samples demonstrated tobramycin values bordering on that lower limit of detection. Samples giving very low, or near background enzyme activity are reported as "not detectable"; those samples giving enzyme activity close to the 1.0 μg/mL calibrator are reported as "< 1 μg/mL".

These studies have shown that tobramycin does not interfere with the penetration of ketorolac through rabbit cornea. Tobramycin itself appears to penetrate the cornea to some extent. The solution containing benzalkonium chloride and edetate disodium leads to greater penetration of both ketorolac and tobramycin.

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