Ocular Disposition of Aminozolamide in the Rabbit Eye

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We have previously determined that 6-amino-2-benzothiazolesulfonamide (aminozolamide) significantly lowers IOP in rabbits and, more importantly, in ocular hypertensive human subjects. Results from in vitro experiments established that both the inhibitory activity of aminozolamide against carbonic anhydrase B as well as the penetration rate across excised rabbit corneas were equivalent to ethoxzolamide. Consequently, we have investigated the ocular disposition of aminozolamide to explain its activity when instilled topically to the eye. A constant concentration of 67.4 μg/ml of drug was applied for 90 min to the left eye of anesthetized rabbits. Drug and metabolite were measured in both aqueous humor and iris/ciliary body over time. The metabolite was collected and purified. Identification using mass spectroscopy, high pressure liquid chromatography (HPLC) and fluorescence scans indicated that the metabolite was 6-acetamido-2-benzothiazolesulfonamide. Relatively high levels of metabolite were identified in the cornea and iris/ciliary body but were much lower in aqueous humor. Tissue concentrations over time for the metabolite in iris/ciliary body were approximately 2-fold higher than levels of metabolite measured in aqueous humor. When compared to drug levels measured in either aqueous humor or iris/ciliary body, metabolite levels in these respective tissues were much higher. It is hypothesized that topical activity is a consequence of both metabolite retention in the iris/ciliary body as well as inhibition of 99+% of carbonic anhydrase. Both of these events must occur over a sufficient time period to effect a significant lowering of IOP. Invest Ophthalmol Vis Sci 28:1373-1382, 1987

The development of new drugs for systemic use focuses upon the optimization of chemical structure and pharmacological effect, the latter of which relates to therapeutic efficacy. Although this approach quite often leads to the development of very effective drugs, it does not address certain fundamental pharmacokinetic processes which may be responsible for the clinical failure of a new drug.

Until recent years, the introduction of new drugs for ophthalmic use has depended upon the identification of candidates which were originally designed for the systemic route of administration.1—3 With the introduction of the Ocusert®4 and dipivefrin,5 it has become apparent that levels of drug within the eye can be altered for the benefit of therapy by controlling the corneal penetration rate. However, an assumption cannot be made that enhanced corneal penetration is the only pharmacokinetic process of concern and that other processes can be ignored.

In previous studies, Eller et al6—8 reported on the physicochemical and pharmacokinetic properties of carbonic anhydrase inhibitors related in structure to ethoxzolamide. One such analog, 6-hydroxyethoxy-2-benzothiazolesulfonamide, was shown to significantly lower intraocular pressure in rabbits from a single instillation.6 This latter effect was particularly interesting since the corneal penetration of the hydroxyethoxy analog was less than ethoxzolamide, yet in vitro carbonic anhydrase inhibition of both compounds was equivalent.7,8

In this report we describe the ocular disposition of 6-amino-2-benzothiazolesulfonamide (aminozolamide) in the albino rabbit. Its physicochemical and pharmacological properties are similar to the 6-hydroxyethoxy analog and, in fact, it has been shown to significantly lower IOP when 50 μl of 3% gel was instilled in the eyes of ocular hypertensive patients.5 We are also proposing an explanation other than ocular penetration to account for the activity of aminozolamide when dosed topically.

Materials and Methods

Materials

The methods by which aminozolamide was synthesized, purified and structurally confirmed have been described elsewhere.10 Other chemicals used in the study were analytical or research grade and were pur-
chased. New Zealand white rabbits of either sex, without observable eye defects, weighing 1.5 to 2.2 kg and 2 to 3 months of age were purchased for the study. All technics performed on the rabbits conformed to the ARVO Resolution on the Use of Animals in Research. Aminozolamide (micronized) was administered to rabbits in a single dose as a 3% gel (Carbopol 934P; B. F. Goodrich, Cleveland, OH) or 3% suspension. The gel preparation was sterile and came from the same lot used in the human clinical studies. The suspension vehicle contained a phosphate buffer (pH 7.2), 0.01% benzalkonium chloride and sufficient NaCl to make the preparation isotonic. In a separate series of experiments designed to study constant rate infusion, an isotonic buffered (pH 7.65) solution containing 67.4 µg/mL of aminozolamide was used.

IOP Measurements

IOP was measured with a pneumatonograph (Digi-labs Model 30D, Cambridge, MA) using topically applied 0.5% proparacaine hydrochloride for anesthesia. The IOP was measured in both eyes before drug application, at 30 min and then at every hour after instillation through 12 hr and at 24 hr. High pressures were induced in the right eye of normal rabbits by a single anterior chamber injection of chymotrypsin (150 units, 1:5000 dilution). In approximately 1 month IOP had stabilized at about 40 mm Hg. The pneumatonograph (Model 30D, Digilab Inc., Cambridge, MA) was calibrated to produce consistent readings but was not adjusted for rabbit IOP.

Chromatographic Assay Conditions

The HPLC system consisted of a solvent delivery pump (Model 6000A, Waters Associates, Milford, MA), a syringe-loading sample injector (Model 7125, Rheodyne Inc., Cotati, CA) fitted with a 100 µL loop (Cat. No. 724, Rheodyne Inc.), a reverse-phase di-phenyl column (10 µm, 25 cm, ES Industries, Marlton, NJ) and a diphenyl guard column (10 µm, ES Industries). The mobile phase consisted of 80/20 1% acetic acid/methanol; the flow rate was 1.5 ml/min. Chromatograms were recorded on a chart recorder (Omniscribe Model A5211-1, Houston Instruments, Austin, TX) operating at 0.5 cm/min. Aminozolamide had a retention time of 7.5 min and the internal standard, 6-hydroxy-2-benzothiazolesulfonamidine, had a retention time of 9 min. A metabolite was detected at 15 min.

Metabolite Recovery and Identification

HPLC chromatograms obtained from either aqueous humor or iris/ciliary body samples showed complete separation of drug and metabolite from extraneous peaks. The eluate from the HPLC fraction representing the metabolite was collected from iris/ciliary body and aqueous humor samples used in the kinetic experiments. Rabbit eyes which were used in the pharmacokinetic experiments were also used to extract 3–4 µg of metabolite. The eluate was injected onto the HPLC column for further purification after which the metabolite fraction was again collected. It was immediately frozen and stored until a large enough quantity had been obtained. Evaporation of the collected eluate was conducted under vacuum with an applied temperature of 35°C (Buchi Rotavapor R110, Buchi Laboratories, Switzerland) until only a dry residue remained. The residue was reconstituted with 2 ml of methanol, dried with nitrogen gas and identified using mass (R10-10C Nermag, Division of Delsi, Inc., Fairfield, NJ), fluorescence (SPF-500C Spectrofluorometer, SLM Instruments, Inc., Urbana, IL), and UV (8450A UV-VIS, Hewlett-Packard, Palo Alto, CA) spectroscopy.

Constant Rate Infusion and Tissue Extractions

Rabbits were anesthetized with 90 mg/kg of ketamine (Vetalar®, Parke-Davis, Morris Plains, NJ) given by intramuscular injection 10 min prior to the start of the experiment. A plastic well has been designed and used for topical administration to the rabbit eye. The base of the device was lightly coated with silicone grease and placed over the sclera, leaving the cornea exposed. The area and volume in contact with the cornea was 0.503 cm and 0.7 ml, respectively. An isotonic solution containing 67.4 µg/ml of aminozolamide and buffered at pH 7.65 was added to the well at time zero. Rabbits were sacrificed periodically through 425 min. At each time period aqueous humor and iris/ciliary body were removed and extracted for drug and metabolite analysis. Drug solution remained in contact with the cornea through 90 min, at which time the well was removed and the eye rinsed of residual drug with normal saline. Prior to removal the drug solution in the well was replenished every 10 min with fresh solution in order to maintain a constant concentration on the cornea. Tissue samples removed beyond 90 min represented the time necessary for the elimination of drug from the eye.

Two hundred microliters of aqueous humor and 0.3 ml of pH 3.0 phosphate buffer were quantitatively transferred to a syringe-loaded extraction column (Bond Elut PH 1 ml, Analytichem International, Harbor City, CA) attached to a vacuum manifold (Vac Elut, Analytichem International). The columns had been prerinsed first with methanol then with distilled water. Within 2 min the aqueous
humor mixture had settled onto the column and a vacuum was applied to draw the solution through the column. With the vacuum left on, 1.5 ml of distilled water was also forced through the column to clear it of aqueous humor components. The drug was retained by the column. The vacuum was then turned off and collection tubes (#30 A16076 Analytichem Int.) were inserted into the manifold. The column was extracted three times using 300 µl of methanol. The eluant was warmed (ReactiTherm, Pierce Chemical Co., Rockford, IL) to 40°C and dried under a gentle stream of nitrogen gas. The contents of the collection tubes were reconstituted to 100 µl with 50/50 water/methanol, vortexed for 2 min and injected onto the HPLC column for analysis. Samples of aqueous humor containing known concentrations of drug as well as aqueous humor devoid of drug were also carried through this procedure.

Once removed, the iris/ciliary body (average weight = 35 mg) was homogenized in centrifuge tubes (Potter-Elvehjem tissue grinder) at 90 rpm for 2 min with 0.2 ml 1 M pH 9 sodium carbonate solution. Ethyl acetate in a volume of 0.6 ml was then added to the mixture and the contents vortexed for 2 min. The organic phase, containing drug, was separated by centrifugation and transferred to a small vial. Two additional 0.6 ml portions of ethyl acetate was used to further extract the tissue homogenate. The ethyl acetate portions were combined and evaporated to dryness with nitrogen gas. To the residue was added 100 µl of 50/50 of water/methanol; the solution was then mixed thoroughly and injected onto the HPLC column. Tissue samples without and with known concentrations of drug were treated in the same manner to validate the assay procedure.

Carbonic Anhydrase Inhibition

Inhibition of carbonic anhydrase B (Sigma Lot No. 103F-9405, Sigma Chemical Co., St. Louis, MO) by aminozolamide, ethoxzolamide and 6-acetamido-2-benzothiazolesulfonamide was determined using initial rate kinetic analysis of the ester hydrolysis of p-nitrophenyl acetate (Sigma). This reaction is known to parallel the CO₂ hydration of carbonic anhydrase. The isoenzyme B was chosen because of its availability and because it exists along with isoenzyme C in the rabbit ciliary body; both isoenzymes are inhibited by 5 × 10⁻⁵ M acetzolamide. Carbonic anhydrase activity was determined continuously by measuring the appearance of p-nitrophenolate ion at 400 nm. Reactions were conducted in temperature-controlled cells at 25°C over 1.5–2 min. The assay mixture consisted of 2 ml final volume and contained 0.02 M sodium phosphate at pH 7.5, the appropriate concentration of substrate (40, 80, 160 or 320 µM) in 40 µl of acetonitrile, and inhibitor (0–3 µM) in 200 µl of distilled water.

The reaction was initiated by the addition of carbonic anhydrase (final concentration = 0.1 mg/ml). Both the substrate and carbonic anhydrase were kept in an ice bath until ready for use. Any non-enzymatic rate of hydrolysis of the substrate was determined without carbonic anhydrase present in the mixture. This rate was always less than 25% of the enzyme-dependent rate of hydrolysis.

The type of inhibition was determined at various concentrations of inhibitor from a plot of 1/velocity versus 1/substrate concentration. Slopes of the regression lines from the double reciprocal plots were obtained from the data and plotted versus the corresponding inhibitor concentrations for the purpose of understanding the inhibitor-enzyme interaction. A graphical analysis of the % enzyme activity remaining versus the log inhibitor concentrations permitted a determination of the concentration of inhibitor sufficient to reduce the enzymatic reaction velocity by one-half (IC₅₀).

IOP Measurements

Topical administration of 50 µl of aminozolamide to the pressure-induced rabbit eye produced a significant decline in IOP when compared to baseline measurements. To test the contribution of the gel vehicle, 50 µl of a 3% micronized suspension (isotonic and buffered at pH 7) was administered to the same group of rabbits. These results, summarized in Table 1 and Figure 1, show a steady decline in IOP through 10–12 hr for the gel and 8 hr for the suspension at which time the measurements were discontinued. At 24 hr IOP for the gel results had returned to baseline in the control eye but not in the treated eye. Measurements were taken 48 hr later following administration of either gel or suspension and were found to have returned to baseline. The control eye showed a significant decline for either the gel or suspension dosage forms. This decline likely represents systemic absorption, however; a total dose of only 1.5 mg was administered. In a previous study in which we administered ethoxzolamide intravenously to normal rabbits, a dose of 2–6 mg/kg (about 5–15 mg/rabbit) was required to produce a perceptible decline in IOP.

The relative potency of the drug is best illustrated by the fact that the decline in IOP in the treated eye from baseline reached a maximum at 10 hr for the gel (−16.7, 26.5 mm Hg) which was nearly equivalent to the baseline IOP obtained for the control eye (26 mm Hg, see Table 1). Although the decline in IOP is not as great as achieved with the gel over the first 6 hr, the
Table 1. Effect of 3% aminozolamide gel and suspension applied to the hypertensive* eye of rabbits

<table>
<thead>
<tr>
<th>Dosed eye</th>
<th>No. of rabbits</th>
<th>Baseline</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>24</th>
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<tbody>
<tr>
<td>Hypertensive†</td>
<td>6</td>
<td>43.2</td>
<td>37.3</td>
<td>35.7</td>
<td>36.3</td>
<td>35.0</td>
<td>33.8</td>
<td>33.6</td>
<td>30.8</td>
<td>30.5</td>
<td>29.8</td>
<td>28.7</td>
<td>26.5</td>
<td>27.5</td>
<td>27.3</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.2)</td>
<td>(7.1)</td>
<td>(7.3)</td>
<td>(7.3)</td>
<td>(7.7)</td>
<td>(5.6)</td>
<td>(5.2)</td>
<td>(4.8)</td>
<td>(5.7)</td>
<td>(5.9)</td>
<td>(6.6)</td>
<td>(6.9)</td>
<td>(7.0)</td>
<td>(9.0)</td>
<td>(8.0)</td>
</tr>
<tr>
<td>Normotensive§</td>
<td>6</td>
<td>26.0</td>
<td>23.0</td>
<td>21.7</td>
<td>22.3</td>
<td>22.5</td>
<td>23.2</td>
<td>21.7</td>
<td>21.7</td>
<td>22.0</td>
<td>21.8</td>
<td>22.7</td>
<td>25.7</td>
<td>25.0</td>
<td>23.2</td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.7)</td>
<td>(3.5)</td>
<td>(3.3)</td>
<td>(4.2)</td>
<td>(4.0)</td>
<td>(4.3)</td>
<td>(3.8)</td>
<td>(4.8)</td>
<td>(3.7)</td>
<td>(3.8)</td>
<td>(4.5)</td>
<td>(5.6)</td>
<td>(4.9)</td>
<td>(4.0)</td>
<td>(3.0)</td>
</tr>
</tbody>
</table>

Suspension

| Hypertensive† | 9              | 43.7     | 42.7 | 40.2| 38.8| 37.2| 35.6| 34.9| 32.9| 29.4| 27.7 | —   | —   | —   | —   | —   |
|              |                | (7.35)   | (5.7) | (5.9)| (5.7)| (5.1)| (6.1)| (5.5) | (5.0)| (8.6)| (6.7) | —   | —   | —   | —   | —   |
| Normotensive§ | 9              | 25.1     | 25.7 | 24.1| 22.9| 22.4| 21.8| 22.0| 21.2| 21.1| 22.0 | —   | —   | —   | —   | —   |
|              |                | (2.7)    | (3.0)| (4.2)| (3.5)| (3.2)| (2.4)| (2.2)| (2.5)| (2.8)| (3.2)| —   | —   | —   | —   | —   |

*High-pressure-induced with the injection of alpha-chymotrypsin (150 units, 1:5000 dilution).
†Compared to the baseline value, IOPs (except at t = 0.5 hr) are statistically different (P < .05) at 1–3 hr and highly different (P < .01) from 4–24 hr.
§1 standard deviation.
§Compared to the baseline value, IOPs at 1 and 5–8 hr are statistically significant (P < .05).

suspension nevertheless showed a significant drop in IOP as well as a long-lasting effect, indicating that at 3% the drug can be considered very effective in lowering IOP. The results obtained with the gel dosage form were not unlike the results obtained in ocular hypertensive patients.⁹

Metabolite Identification

Mass spectral scans of the aqueous humor and iris/ciliary body extract representing metabolite were identical to scans of 6-acetamido-2-benzothiazolesulfonamide. Also, the ultraviolet absorption spectra of solutions containing metabolite at different pHs, as well as fluorescence scans of excitation and emission versus wavelength, confirmed that acetylation had occurred on the 6-amino functionality and not on the sulfonamide nitrogen. The extracted metabolite and the 6-acetamido analog showed identical fluorescence intensities which were about 1/100 of aminozolamide at equal concentrations. An amino functionality attached to benzene fluoresces intensely unless one or both of the hydrogens are displaced.¹⁶

Figure 2 gives the structure of aminozolamide and its metabolite.

We are not aware of any other finding of N-acetylation in the rabbit eye or the eye of other species. However, N-acetyltransferase, the enzyme that is probably responsible for catalyzing the transfer of an acetyl group from acetyl CoA to aminozolamide, has been found in rabbit liver.¹⁷ In general, N-acetyltransferase is commonly known to acetylate primary amino groups adjacent to aromatic rings, particularly sulfonamides, in many animal species, including man.¹⁸ Specifically, the rabbit is considered a high acetylator of sulfonamides, whereas man is intermediate and the monkey (and dog) are low acetylators.¹⁸
Ocular Pharmacokinetics

The aqueous humor and iris/ciliary body drug levels resulting from topical infusion of aminozolamide are shown in Figure 3 (A and B).

The concentrations of drug in the iris/ciliary body are relatively constant and low throughout 260 min. It is likely that these levels remain low because of a rapid conversion of drug to metabolite. These results also show a lack of distribution equilibrium between aqueous humor and iris/ciliary body for aminozolamide through this time period.

Figure 3 (C and D) gives the results for the metabolite measured in aqueous humor and iris/ciliary body tissue over time. It is interesting to note that metabolite levels in aqueous humor and particularly iris/ciliary body are higher than drug levels in these tissues. This would suggest that metabolite is formed in iris/ciliary body tissue. The half-life representing elimination of drug and metabolite from aqueous humor was similar and measured 69 and 56 min, respectively; however, no apparent exponential decline was observed for drug or metabolite from iris/ciliary body through 420 min. The loss by bulk flow of aqueous humor is approximately 1.5% of the volume of the anterior chamber per minute (t 1/2 ~ 1 hr). Therefore, the half-life of the drug and metabolite from aqueous humor suggests that elimination occurs predominantly by bulk flow. However, a much slower loss rate is occurring from the iris/ciliary body tissue.

Figure 3D shows that metabolite concentrations could be detected in the iris/ciliary body through 420 min.
After a short lag time of approximately 12 min, the results of Figure 3 (C and D) show a rapid formation of metabolite. In fact, after removal of the constant concentration of aminozolamide from the cornea, metabolite concentrations continue to increase for an additional 90 min in aqueous humor and 30 min in the iris/ciliary body tissue. These results can occur if metabolite is formed in high concentrations in the cornea and the release rate from the cornea is relatively slow. As a consequence a relatively low concentration of metabolite could be released into aqueous humor over time, even though aminozolamide was no longer in contact with the epithelial surface of the cornea.

An additional experiment was conducted in order to determine if metabolite formation occurred in the cornea. Aminozolamide was again applied to the eye of anesthetized rabbits in a constant concentration of 67.4 mcg/ml for 90 min as described previously. Corneal samples as well as iris/ciliary body and aqueous humor were excised and analyzed for drug and metabolite. Measurements were made at 10 and 90 min (n = 8 at each time interval) only. Table 2 shows the high concentration of metabolite found in the cornea at 10 and 90 min. Metabolite to drug ratios equaled approximately one-third at both sampling times. However, at 10 min no metabolite could be detected in aqueous humor or iris/ciliary body, suggesting that metabolite formed in the cornea was released very slowly into aqueous humor at early times. The results for aqueous humor and iris/ciliary body agreed with previous results shown in Figure 3.

Carbonic Anhydrase Inhibition

From Figure 4 (A, B and C) it is not obvious that each of the inhibitors exhibit noncompetitive inhibition. However, for a given ratio of inhibitor to carbonic anhydrase concentration, a change in substrate concentration does not alter the degree of inhibition. These results are indicative of a classical noncompe-

| **Table 2. Cornea concentrations of aminozolamide and metabolite following constant application of 67.4 µg/ml of aminozolamide to the rabbit eye over 10 and 90 min** |
|-----------------|-----------------|-----------------|
|                  | 10 min          | 90 min          |
| Aminozolamide    | 4.14 (1.50)*    | 16.6 (6.80)     |
| Metabolite       | 1.60 (0.29)     | 5.16 (1.46)     |

* Mean ± SD (n = 8).

Further analysis revealed that by reploting the slopes of the reciprocal plot versus the corresponding inhibitor concentration, the resulting
curve (Fig. 5 [A and B]) was not linear but curvilinear, particularly at the upper inhibitor concentration of 2-3 μM. A nonlinear replot such as Figure 5 indicates that two or more inhibitor binding sites exist for the enzyme under the conditions of the experiment. Figures 5A and B differ in the manner in which the slopes, or K/V values, were obtained from the initial rate versus concentration of substrate at each inhibitor concentration. Figure 5A used slopes determined by linear regression of the data in Figure 4. A nonlinear estimate of the enzyme inhibition data at each inhibitor concentration was used to obtain K/V in constructing Figure 5B. With the exception of ethoxzolamide in Figure 5A at the highest inhibitor concentration, the slopes from the Lineweaver-Burk relationships when plotted versus the inhibitor concentration were identical for each inhibitor. The difference at 3 μM for ethoxzolamide in Figure 5A would suggest a difference only in the affinity of inhibitor for the binding site with the lowest affinity. At 3 μM a greater inhibitor activity occurs with ethoxzolamide (Fig. 5A). Therefore, it could be considered a slightly better inhibitor, but at 3 μM only. The results shown in Figure 5B indicate no difference exists between the inhibitor at any inhibitor concentration. The % inhibition in Table 3 are averages of four determinations at each inhibitor concentration. The standard deviations represent random variability. At 3 μM the % inhibition for ethoxzolamide is 96.4%, but slightly less for aminozolamide and the 6-acetamido analog, or 94.1 and 93.2 respectively. These small differences are not the determining factor in topical activity, especially since the differences predict ethoxzolamide as the more active inhibitor.

Table 3. Summary of inhibition of carbonic anhydrase B* for aminozolamide, 6-acetamide analog and ethoxzolamide at each inhibitor concentration

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor concentration (μM)</th>
<th>% Inhibition ± SD†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminozolamide</td>
<td>0.50</td>
<td>19.8 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>35.9 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>44.1 ± 2.1</td>
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<tr>
<td></td>
<td>1.50</td>
<td>53.3 ± 2.5</td>
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<tr>
<td></td>
<td>2.00</td>
<td>68.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>85.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>94.1 ± 0.2</td>
</tr>
<tr>
<td>6-Acetamido analogue</td>
<td>0.50</td>
<td>14.3 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>30.8 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>40.7 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
<td>49.1 ± 1.5</td>
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<td></td>
<td>2.00</td>
<td>60.1 ± 1.8</td>
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<td></td>
<td>2.50</td>
<td>77.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>93.2 ± 0.9</td>
</tr>
<tr>
<td>Ethoxzolamide</td>
<td>0.50</td>
<td>15.5 ± 6.7</td>
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<td></td>
<td>1.00</td>
<td>36.1 ± 2.0</td>
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<td></td>
<td>1.25</td>
<td>41.4 ± 2.7</td>
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<td>1.50</td>
<td>49.9 ± 2.7</td>
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<td>2.00</td>
<td>65.2 ± 0.5</td>
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<tr>
<td></td>
<td>2.50</td>
<td>83.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>96.4 ± 0.5</td>
</tr>
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* Concentration = 0.1 mg/mL.
† n = 4.

Fig. 5. Replot of the slopes of the enzyme inhibition data versus the corresponding inhibitor concentrations. Key: (A) Slopes obtained from linear regression of Lineweaver-Burk plots (Fig. 4A, B and C), and (B) Slopes (K/V) obtained from nonlinear regression analysis of Michaelis-Menten equation.

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was concluded from standard experiments where enzyme and inhibitor were preincubated for up to 90 min, diluted into a standard assay mixture, and the rate of p-nitrophenyl acetate hydrolysis determined.21

Discussion

Since ethoxzolamide is lipophilic and not very water soluble at physiological pH, its maximum penetration rate is relatively low. As a result high aqueous humor levels cannot be attained. Therefore, our first approach was to optimize corneal penetration through molecular modification.

In our previous studies, we designed, synthesized and tested 2-benzothiazolesulfonamide, which was equally lipophilic and 19 times more soluble at physiological pH when compared to ethoxzolamide. As expected, it showed a greater potential for an increased corneal penetration rate and yielded aqueous humor levels that were 16 times higher than ethoxzolamide.8 However, when 2-benzothiazolesulfonamide was instilled in the rabbit eye as either a 1% gel or 1% suspension formulation, it did not lower IOP.22

The maximum attainable penetration rate (MPR) can be calculated by multiplying solubility times the in vitro corneal permeability coefficient. In previous studies,8 MPR values correlated very well to steady state aqueous humor levels. Table 4 lists these values for aminozolamide, its metabolite (6-acetamido analog) and a few other carbonic anhydrase inhibitors. However, MPR did not predict topical activity. Both aminozolamide and the 6-hydroxyethoxy analog penetrated the rabbit cornea at approximately equal rates to ethoxzolamide, yet only aminozolamide and the 6-hydroxyethoxy analogs reduced IOP in rabbit6,22 and monkey eyes.23 It must be concluded that high corneal penetrability does not necessarily predict topical activity with regard to lowering IOP for our carbonic anhydrase inhibitors.

Other 6-substituted 2-benzothiazolesulfonamides, namely, 7-amino-6-ethoxy and 4,7-dimethyl-6-amino analogs, are equally potent in inhibiting carbonic anhydrase in vitro when compared to ethoxzolamide. They also possess good, albeit not optimal corneal penetration. However, they do not significantly lower IOP in the pressure-induced (laser) monkey eye.23 These results have forced us to reassess our original hypothesis which focused upon optimizing corneal penetration. Based upon the similarity of the slopes for each inhibitor shown in Figure 5 (A and B), differences in inhibition potency do not explain the topical activity of aminozolamide. Consequently, neither activity nor corneal penetration can account for the lowering IOP from topical application of aminozolamide.

From aminozolamide and particularly metabolite concentrations in iris/ciliary body at steady state (Fig. 3), we can estimate that approximately 8 μM of metabolite but only 1 μM of drug is present. However, the concentration of both drug and metabolite at the site of action is likely much higher. Our concentration values are an average of the combined tissues and fluids within the iris/ciliary body and probably do not reflect the actual concentration at the active site, the nonpigmented epithelial cells of the ciliary processes. Only 67.4 μg/ml was applied to the eye in the topical infusion studies, which is 25% of the drug’s solubility at physiological pH; on a proportional basis 36 μM could be attained theoretically at the drug’s solubility. Nevertheless, if we assume that at least 9 μM of inhibitor(s) and 0.34 μM24 of carbonic anhydrase are present in the ciliary body, a ratio of 26.5 would result. From the results presented in Table 3, it is not unreasonable to assume that sufficient inhibitor
could reach the active site and lower IOP. Since the in vitro experiments may not accurately reflect the in vivo conditions for the hydration of CO₂, these estimates must be considered an approximation at best.

It is reasonable to assume that metabolite and not drug is responsible for a lowering of IOP. However, when 6-acetamido-2-benzothiazolesulfonamide was administered topically to the normotensive rabbit eye, or the laser-induced high-pressure monkey eye, no significant decrease in IOP was observed. We believe that the long residence time of the metabolite in the rabbit eye, and particularly in the iris/ciliary body is primarily responsible for lowering IOP.

Our results show that metabolite is formed within the cornea as well as within the iris/ciliary body tissue, the latter in sufficient concentration (along with drug) to inhibit 99+ % of carbonic anhydrase. Retention of the inhibitor is an important requirement in order to effect a significant reduction of aqueous humor secretion and hence a lowering of IOP. This is hypothesized because of the rapid turnover of carbonic anhydrase. If inhibitor was not retained at the site of carbonic anhydrase, elimination of drug out of the eye by bulk flow would be rapid (t 1/2 = 1 hr) so that a lowering of IOP would be relatively small and of short duration. These results have been observed for trifluoromethazolamide by Maren et al., and for other 6-substituted 2-benzothiazolesulfonamides. It is possible that an equilibrium relationship exists between two enzyme systems, carbonic anhydrase and N-acetyltransferase, for substrates and inhibitors. The resulting retention that occurs from the presumed binding of the active species is believed to permit accumulation. However, at this time we cannot discern if the two enzymes systems and/or some other tissue components are responsible. Nevertheless, since a lowering of IOP occurs, it is assumed that sufficient free metabolite (and drug) is available to bind to carbonic anhydrase (presumably by a shift in equilibrium) and inhibit its enzyme activity.

Although metabolite is formed within the cornea, its rate of distribution to and subsequent uptake by the active site may contribute only marginally in maintaining a drop in IOP in comparison to metabolite, which is formed and retained at the active site. This hypothesis would explain why topical application of the metabolite did not lower IOP in rabbit or monkey eyes.

Key words: aminozolamide, ocular pharmacokinetics, IOP, metabolite, rabbit

References


