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# Corneal Penetration Behavior of β-Blocking Agents III: *In Vitro–In Vivo* Correlations

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**Abstract** □ Aqueous humor levels were determined over time after the topical administration to rabbit eyes of 1% isotonic buffered (pH 7.3) solutions of three β-blocking agents, acebutolol hydrochloride, timolol maleate, and bufuralol hydrochloride (arranged in order of increasing lipophilicity). Corneal permeability coefficients, determined from a previous *in vitro* study, were inversely related to the observed time to peak for the three drugs, as expected. Two of the drugs, bufuralol and timolol, did not give the expected rank order for $C_{max}$ and AUC, which could result from differences in distribution and/or elimination processes. Aqueous boundary layers were postulated for *in vivo* corneal permeability which suggested that bufuralol and timolol may have nearly identical effective permeability coefficients *in vivo*.

**Keyphrases** □ Permeability—acebutolol, timolol, bufuralol, excised rabbit corneas, pharmacokinetics, *in vitro–in vivo* correlations □ Acebutolol—corneal permeability in rabbits, pharmacokinetics, *in vitro–in vivo* correlations □ Timolol—corneal permeability in rabbits, pharmacokinetics, *in vitro–in vivo* correlations □ Bufuralol—corneal permeability in rabbits, pharmacokinetics, *in vitro–in vivo* correlations

In a previous report (1) the penetration behavior of 12 β-blocking agents measured across excised rabbit corneas was correlated with partitioning, which varied over a fourfold logarithmic range. Optimal penetration (log permeability coefficient) reached a maximum at a log distribution coefficient (octanol–buffer, pH 7.65) of ~2–3. Subsequent results (2) showed that a plateau was reached because the stroma, and to a lesser extent the endothelium, became the rate-controlling barrier for the most lipophilic compounds, while the epithelium acted as a rate-determining barrier for the hydrophilic compounds.

The purpose of this study was to determine if the corneal permeability coefficients of three compounds ranging widely in lipophilicity could be correlated with parameters obtained from the aqueous humor–time profile. The three drugs (in descending lipophilic order: bufuralol, timolol, and acebutolol) were administered as 1% isotonic, buffered (pH 7.3) solutions.

## EXPERIMENTAL

**Reagents and Materials**—Isotonic, buffered (pH 7.3), 1% w/v solutions of acebutolol hydrochloride, timolol maleate, and bufuralol hydrochloride were prepared separately[1]. The reagents used for aqueous humor extraction and subsequent high-performance liquid chromatographic (HPL) assay were reagent- or UV spectrophotometry-grade chemicals. New Zealand White rabbits, 2 months of age and of either sex, weighing 1.6–2.0 kg were used for the experiments.

**Topical Administration and Aqueous Humor Sampling**—The rabbits were administered drug with their heads in an upright position while resting in a restraining box. The rabbits were returned to their cages when the sampling interval was >1 hr. A 50-μl volume was instilled onto the cornea of each eye while the lower lid was gently pulled away from the eye globe to form a pocket. The lower eyelid was held against the upper lid for 20 sec after instillation. Second and third instillations were given 2 and 4 min after the first application. The multiple-dose regimen was designed to give aqueous humor concentrations above the sensitivity of the assay. This especially applies to acebutolol hydrochloride, since its permeability was found to be the lowest.

At various postinstillation times, rabbits were sacrificed by a rapid injection of ~25 ml of air into the marginal ear vein. Each cornea was then quickly rinsed with 1 ml of normal saline solution to get rid of residual drug. The aqueous humor samples were withdrawn by puncture with a 26-gauge 0.95-cm needle attached to a 0.5-ml disposable syringe[2] through the corneal–scleral junction into the anterior chamber. The same syringe was used for the opposite eye of each rabbit in order to pool the aqueous humor of both eyes.

The sampling times for each drug are listed in Table I; each value represents an average of 4–12 rabbit eyes. The aqueous humor samples were left in the syringes and were assayed within a few hours. Although rabbit aqueous humor sample volumes varied from animal to animal (ranging from 0.25 to 0.35 ml), a constant volume of sample was used in the assay for each drug.

**Extraction and Analyses**—A mixer[3] was used to facilitate the mixing and extraction. In 10-ml, glass centrifuge tubes, aqueous humor samples of 0.25 ml were mixed with 0.1 ml of 0.5 *N* NaOH, extracted with 2.0 ml of methylene chloride, and centrifuged. After discarding the aqueous layer, the organic phase was extracted with 1.0 ml of 0.05 *N* sulfuric acid. The acidic aqueous phase was used for HPLC assay of acebutolol.

A 0.30-ml volume of aqueous humor sample was mixed with 0.1 ml of 1 *N* NaOH and extracted with 5 ml of heptane containing 4% isoamyl alcohol in a 10-ml glass centrifuge tube. No centrifugation was necessary

[1] 1% Acebutolol (as hydrochloride salt) contained the following vehicle ingredients: 0.184 g of $NaH_2PO_4 \cdot H_2O$, 0.758 g of $Na_2HPO_4$, and 0.288 g of NaCl/100 ml of solution. 1% Timolol (as maleate salt) contained the following vehicle ingredients: 0.947 g of $Na_2HPO_4$, 0.265 g of NaOH, and 0.332 g of NaCl/100 ml of solution. 1% Bufuralol (as hydrochloride salt) contained the following vehicle ingredients: 0.184 g of $NaH_2PO_4 \cdot H_2O$, 0.758 g of $Na_2HPO_4$, and 0.242 g of NaCl (1.0 g bufuralol hydrochloride)/100 ml of solution.

[2] Glaspack B-D, sterile disposable glass syringe; Becton, Dickinson, and Co., Rutherford, N.J.

[3] Vortex genie mixer, S8223; Scientific Products.

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**Table I—Aqueous Humor Concentrations of Acebutolol, Timolol, and Bufuralol after Multiple Instillations in Rabbit Eyes of 50 μl of an Isotonic, Buffered (pH 7.3), 1% Solution at 0, 2, and 4 min [a]**

| Time, min | Aqueous Humor Concentration, μg/ml | | |
|---|---|---|---|
| | Acebutolol | Timolol | Bufuralol |
| 7 | — | — | 20.6 (3.35) |
| 10 | 0.023 (0.015) | 11.3 (0.46) | 22.9 (4.22) |
| 20 | 0.270 (0.014) | — | 18.3 (2.71) |
| 25 | — | 30.7 (3.31) | — |
| 30 | 0.380 (0.053) | — | 7.39 (3.96) |
| 40 | — | 16.2 (6.26) | 4.15 (1.25) |
| 55 | 0.910 (0.203) | — | 1.15 (0.57) |
| 60 | — | 8.67 (1.02) | — |
| 85 | 1.260 (0.280) | — | 0.33 (0.11) |
| 120 | 1.120 (0.180) | 4.05 (1.08) | 0.084 (0.028) |
| 180 | 0.590 (0.150) | 1.66 (1.06) | 0.063 (0.014) |

[a] Values in parentheses represent one standard deviation.

as the two layers separated completely. A 4-ml volume of the heptane layer was transferred to 1.0 ml of 0.1 *N* HCl in another 10-ml glass tube and mixed. The acidic aqueous layer was then assayed for timolol by HPLC.

Aqueous humor samples of 0.25 ml from bufuralol-treated rabbits were placed into a 10-ml glass centrifuge tube, alkalinized with 0.1 ml of 0.5 *N* NaOH, and extracted with 0.5 ml of heptane containing 1.5% isoamyl alcohol. After centrifugation, 0.4 ml of the heptane layer was transferred to a 4-ml glass vial, extracted with 0.5 ml of 0.1 *N* HCl; and then assayed by HPLC for bufuralol concentration.

Aqueous humor blanks obtained from control rabbits were spiked with various quantities of each drug and extracted by the methods described above. The slopes of the calibration curves were used for calculation of drug concentrations in the unknown aqueous humor samples.

The HPLC system[4] was equipped with an injector[5] consisting of different-sized loops ranging from 50 to 200 μl, which enabled the injection of an accurate sample volume. Each sample was divided so that two injections could be made and the results averaged. The mobile phase for

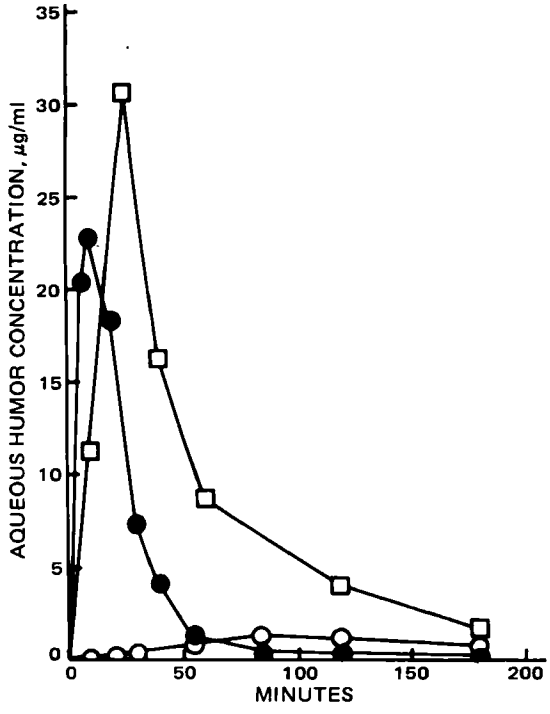


**Figure 1**—*Aqueous humor concentration–time profiles for three β-blocking agents following multiple instillations in rabbit eyes of 50 μl of an isotonic, buffered (pH 7.3), 1% solution at 0, 2, and 4 min. Key: (□) timolol; (●) bufuralol; (○) acebutolol.*

[4] M-6000A solvent delivery system, Model 440 absorbance detector, μ-Bondapak C18 (acebutolol and timolol) and μ-Bondapak CN (bufuralol) columns, Waters Associates, Milford, MA 01575; Omniscribe Model 5211-1 recorder, Houston Instruments, Austin, Tex.

[5] Model 7125 injector; Rheodyne, Cotati, CA 94928.

**Table II—Aqueous Humor Concentration in Comparison with the Excised Corneal Permeability Coefficient and Distribution Coefficient [a]**

| Drug | $t_p$[b], min | $C_{max}$[b], μg/ml | AUC[c], min·μg/ml | $P_T$[d], $10^{-6}$ cm/sec | Log DC[e] (octanol–buffer) |
|---|---|---|---|---|---|
| Bufuralol | 10 | 22.86 | 603 | 57.00 | 2.31 |
| Timolol | 25 | 30.65 | 1525 | 11.70 | 0.34 |
| Acebutolol | 85 | 1.26 | 176 | 0.85 | 0.20 |

[a] 50 μl of a 1%, isotonic, buffered (p 7.3) solution was topically administered to rabbit eyes at 0, 2, and 4 min. [b] Time to peak ($t_p$) and peak concentration ($C_{max}$). [c] Area under the curve up to last sampling point (180 min). [d] Intrinsic corneal permeability coefficient obtained from *in vitro* permeability experiments (1, 2). [e] Log distribution coefficient between octanol and buffer (pH 7.65) (1, 2).

acebutolol and bufuralol consisted of methanol and 1.5% acetic acid in deaerated, deionized water adjusted to pH 4 with sodium hydroxide (3:7 and 7:18, respectively). For timolol the mobile phase contained 42% methanol and 58% 0.005 *M* heptanesulfonic acid in 1% acetic acid solution adjusted to pH 4. For all three drugs the flow rate was 2.0 ml/min (1). The assay sensitivity for acebutolol and bufuralol was 25 ng/ml; the assay sensitivity for timolol was 50 ng/ml.

**Permeability and Distribution Coefficients**—The procedures for determining these coefficients, as well as the reported values for each drug used in this study, were reported in the previous papers (1, 2). Both coefficients were determined at pH 7.65.

## RESULTS AND DISCUSSION

Following the topical administration of 50 μl of isotonic, buffered (pH 7.3) 1% drug solutions to rabbit eyes at 0, 2, and 4 min; the aqueous humor concentrations at various times were measured. Table I and Fig. 1 list the results obtained for acebutolol, timolol, and bufuralol.

Table II lists the peak concentration ($C_{max}$), time to peak ($t_p$), the area under the aqueous humor–time curve through 180 min (AUC), excised corneal permeability coefficient ($P_T$), and the log distribution coefficient (log DC). A correlation exists between $t_p$ and $P_T$ (and DC). Excluding elimination considerations, the more slowly the drug penetrates, the greater $t_p$ becomes; theoretically, this will occur as the lipophilicity of

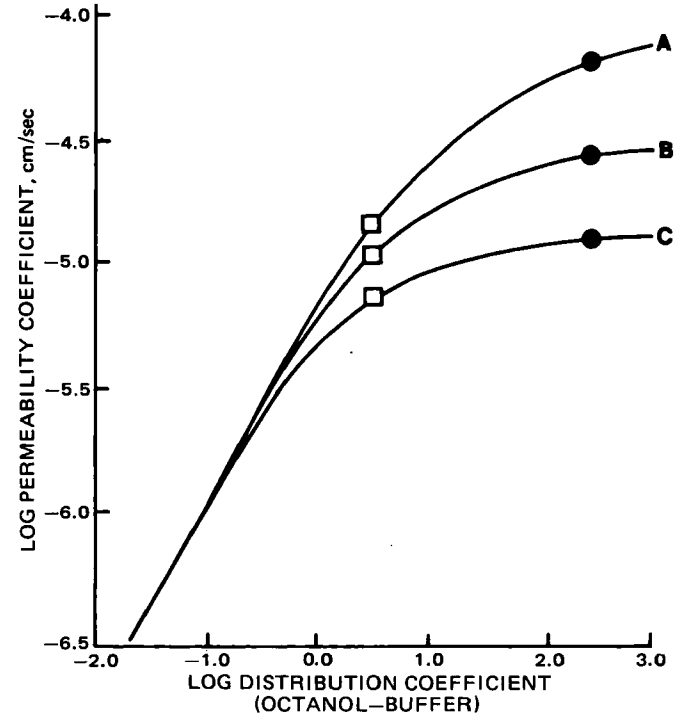


**Figure 2**—*Simulated curves for the log–log plot of permeability coefficient* versus *distribution coefficient (octanol–buffer, pH 7.65) for intact cornea in the presence of various postulated thicknesses of aqueous humor diffusional layers. Key: (A) none, (B) 0.15 cm, (C) 0.45 cm; (●) bufuralol; (□) timolol.*

the drug decreases. Acebutolol has the lowest cornea permeability coefficient and, thus, has the longest $t_p$ (85 min). Bufuralol has the shortest $t_p$ (10 min), whereas timolol has an intermediate $t_p$ of 25 min. Acebutolol has the lowest $C_{max}$ and AUC; these results are expected for a hydrophilic drug which does not rapidly penetrate the cornea. Although bufuralol is much more lipophilic than timolol, its $C_{max}$ and AUC are actually less than the values reported for timolol (Table II).

These drugs differ structurally and therefore could vary not only in penetration, but also in distribution, metabolism, and excretion processes. Consequently, $C_{max}$, $t_p$, and AUC may not necessarily correlate perfectly to the permeability coefficient. The $t_p$ value is perhaps the parameter most likely to show a perfect correlation. This is reasoned from the work of Makoid and Robinson (3), who determined the ophthalmic pharmacokinetics of pilocarpine topically applied to the rabbit eye. From their work an equation was developed for $t_p$:

$$t_p = \frac{\ln \frac{k_{10}}{k_{23}}}{k_{10} - k_{23}} \qquad \text{(Eq. 1)}$$

where $k_{10}$ is the precorneal loss rate constant and $k_{23}$ is the loss rate constant from cornea to aqueous humor. The permeability coefficient would be directly related to $k_{23}$. Equation 1 assumes that $k_{10}$ is much larger than uptake into the epithelium of the cornea from the precorneal area. This assumption applies to most, if not all, ophthalmic drugs, since $k_{10}$ is a function of scleral absorption as well as drainage rate, the latter being relatively large for aqueous solutions. The drainage rate would be expected to be the same for each $\beta$-blocking agent at the same pH and osmolarity.

Both $C_{max}$ and AUC are a function of distribution to corneal tissue and elimination from the eye in addition to penetration. A smaller AUC or $C_{max}$ may result from a larger volume of distribution within the eye and/or from a more rapid elimination from the aqueous humor. For drugs with large differences in $P_T$, such that this factor predominates, perfect correlations between $P_T$ and $C_{max}$ or AUC may be more likely. However, if aqueous boundary layers are significant this may not be true. Aqueous boundary layers have been shown to play an important role in intestinal absorption. For high lipophilic compounds, the absorption rate assumes a plateau with a maximal rate; intestinal absorption is limited by the rate of diffusion through the aqueous diffusional barrier adjacent to the mucous membrane.

Aside from disposition considerations, drug permeation across the cornea *in vivo* may be significantly hindered by the diffusional layers on both sides of the cornea. The aqueous boundary layer adjacent to the endothelium and within the anterior chamber may be large. The physiological aqueous volume of the rabbit is 287 $\mu$l with a turnover rate of ~1%/min (4, 5). It is difficult to estimate the effective diffusional layer thickness in aqueous humor that would exist in the presence of this turnover rate. It is possible that a turnover rate of 1% would have a negligible mixing effect on the aqueous humor compared with the stirring in the modified perfusion chamber. For example purposes let us assume that the entire anterior chamber volume is a barrier. Since the aqueous diffusional layer represents the entire sampling volume for drug analysis, only half of the aqueous humor thickness can be used as the diffusional layer. As a result, the diffusional layer is estimated to be ~0.15 cm[6].

In the precorneal region the thickness of the tear film ($6–7 \times 10^{-4}$ cm) could also serve as an aqueous boundary layer (6). Although blinking of the eyelid mixes the drug with the tear film, it may also reduce the size of an aqueous boundary layer to below the thickness of the tear film. Regardless, it is probably small and not significant in size compared with the potential aqueous barrier in the anterior chamber.

To show the effect of a postulated *in vivo* aqueous boundary layer in addition to corneal layer resistances, curves were simulated for the log–log plot of permeability coefficient *versus* distribution coefficient. These curves[7] are shown in Fig. 2 for intact cornea in the presence of postulated aqueous boundary layers of 0, 0.15, and 0.45 cm. As the aqueous boundary layer increases, the plateau region in curves B and C occur at a lower distribution coefficient. Also, the maximum log permeability coefficient is greatly reduced. Therefore, if we consider these additional boundary resistances, which may exist *in vivo*, it is likely that the log $P_T$[8] *versus* log DC curve would form a plateau at lower log DC values than calculated from the *in vitro* experiments (1, 2). This suggests that timolol and bufuralol may have nearly identical effective permeability coefficients *in vivo*. Although the results shown in Fig. 2 may not explain the lack of a perfect correlation between the *in vitro* permeability and distribution coefficients for two of the drugs, it should be realized that another prodrug more lipophilic than timolol may not increase the penetration rate.

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[6] The anterior chamber can be assumed crudely to represent a cylinder with a radius $r$, a height $h$, and a volume $V$ equal to 287 $\mu$l or ~0.3 cm$^3$. Since $V = \pi r^2 h$, $h$ is calculated to be 0.15 cm.

[7] The curves in Fig. 2 were generated from Eq. 20 in Ref. 2 with the additional consideration of an $R_{aq}$ = 0.15 and 0.45. Equation 20 in Ref. 2 equates the permeability coefficient to the sum of the reciprocal of the resistances of each boundary layer.

[8] $P_T$ represents the intrinsic permeability coefficient for excised corneas for which the *in vitro* $R_{aq}$ was substracted.