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Age-related differences in ophthalmic drug disposition III. Corneal permeability of pilocarpine in rabbits

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Summary

In the two previous papers in this series, age-related differences in ocular tissue size and protein binding were investigated relative to the distribution of drug within the eye. The present paper deals with another potentially important determinant of ocular drug disposition, namely corneal permeability. An in vitro technique was utilized to study the corneal permeability of pilocarpine in rabbits of two different ages. The effect of pH on pilocarpine transport was also examined. Results indicate that the transport of ionized pilocarpine contributes significantly to the overall corneal permeation of pilocarpine. There appears to be no difference in the transport characteristics in corneas of the two ages as far as ionized pilocarpine is concerned. However, the corneas of the younger animals are more permeable to non-ionized pilocarpine than those of 60-day-old rabbits. These results suggest that different pathways or layers in the cornea are rate-limiting for the transport of ionized and unionized pilocarpine. Furthermore, the significant transport of ionized species observed in these studies indicates that the pH-partition hypothesis is not totally adequate to explain the corneal transport of pilocarpine in this particular system.

Introduction

In the formulation or the evaluation of a rational therapeutic approach in ophthalmology, the age of the patient may be an important consideration. Normal

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adult doses, when administered topically to the eyes of small children or infants, can lead to significant side-effects (Hoefnagel, 1961; Gray, 1979). This is primarily a result of the fact that typically 90% or more of such a dose is available to be absorbed systemically (Friedman and Patton, 1976; Patton and Francoeur, 1978). In view of the considerably smaller body size of a child versus an adult, potentially harmful blood levels of an ophthalmic drug may subsequently be achieved.

In the two previous papers in this series (Miller and Patton, 1981; Miller and Patton, 1982), the rabbit has served as an animal model for the study of age-related factors which affect ocular drug disposition. While the human and rabbit eye are very similar, both anatomical and physiological differences have been well documented (Prince, 1964; Wolff, 1976). This makes the correlation of experimental data between man and rabbits much easier. The studies described herein were done using rabbits of two ages, 20- and 60-days-old. The size and degree of development of the rabbit eye at these ages roughly correspond to that of a neonate and 3-year-old child, respectively (Prince, 1964).

Previous studies (Miller and Patton, 1981; Friedman and Patton, 1976) in this laboratory have demonstrated that significantly different concentrations of pilocarpine are attained in the aqueous humor and other intraocular tissues when identical doses are administered topically to 20- and 60-day-old rabbits. In addition, more pilocarpine reached the aqueous humor in animals of both ages when the pH of the instilled solution was increased from 6.0 to 7.2. The reason(s) for these differences could be the age- and/or pH-dependence on the part of a number of factors.

Recently, the obvious influence of the size difference in the ocular tissues of rabbits of the two ages was examined (Miller and Patton, 1981). The results indicated that size alone could not account for all of the observed differences. Protein binding in the eye was also investigated as a function of age (Miller and Patton, 1982). Age-related differences in binding were observed for pilocarpine in both secondary aqueous humor and blood plasma. The binding interaction of pilocarpine in normal aqueous humor, however, was determined to be insignificant relative to the overall pharmacokinetics in the rabbit eye.

It is well recognized that the cornea is a major route for the intraocular penetration of most topically applied ophthalmic drugs (Doane et al., 1978). Consequently, age- and/or pH-related differences in corneal permeability could substantially influence the absorption and hence, the ocular bioavailability of pilocarpine in rabbits. The objective in this study was to characterize the corneal permeability of pilocarpine as a function of age and pH.

Materials and Methods

Materials

Pilocarpine nitrate (Sigma Chemicals, St. Louis, MO), 1-octanesulfonic acid sodium salt (Eastman Kodak, Rochester, NY) and sodium pentobarbital (Abbott Laboratories, Chicago, IL) were all obtained commercially and used without further purification. The methanol and tetrahydrofuran (Fisher Scientific, Fair Lawn, NJ)

were HPLC grade. The water used in the preparation of solutions and chromatography was first treated by reverse-osmosis. Subsequently, it was passed through one charcoal adsorber and 3 ion-exchange columns (IWT, Rockford, IL). After acidification with sulfuric acid the water was then distilled in a glass apparatus with potassium permanganate. All other chemicals used were of at least reagent grade.

The rabbits (Small Stock Industries, Pea Ridge, AK) used in these experiments were white, New Zealand males. At the time of use, their ages ranged between either 17 and 23 or between 55 and 65 days old. No restrictions were placed on water or food prior to their use.

Solution preparation

Solutions of pilocarpine nitrate were 1/2% (w/v) and were prepared in 0.0667 M sodium phosphate buffer. All pilocarpine and buffer solutions were made isotonic with the addition of sodium chloride. The solutions were adjusted to a final pH of either 6.0, 6.6 or 7.2. Pilocarpine solutions were prepared fresh for each experiment.

Pilocarpine analysis

Analysis of pilocarpine was accomplished using high-pressure liquid chromatography. A UV detector with a 214 nm Zn source (LDC, Riviera Beach, FL), a solvent pump (Altex, Berkeley, CA), a 50 μl fixed-loop injector (Rheodyne Cotati, CA) and integrator (Shimadzu, Columbia, MD) were used for the analysis. The analytical column (Waters, C-18, 30 cm $\times$ 3.9 mm i.d., 10 μm particle size, Milford, MA) was coupled with a guard column (Rheodyne, C-18, 3 cm $\times$ 4.6 mm i.d., 5 μm particle size, Cotati, CA), with the entire system operating under ambient conditions.

The mobile phase consisted of 3 mM 1-octane sodium sulphonate and 0.5% (w/v) potassium dihydrogen orthophosphate. The methanol and tetrahydrofuran concentrations were 24% (v/v) and 2% (v/v), respectively. Prior to the addition of organic modifiers to the mobile phase, the pH was adjusted to 2.5 with orthophosphoric acid. The mobile phase was filtered and degassed just prior to use. During the analysis, the flow rate was maintained at 1.4 ml/min.

This chromatographic system distinguished between pilocarpine and its hydrolysis product, pilocarpic acid. As little as 10 ng of either species could be detected.

Apparatus

To measure corneal permeability an apparatus was adapted from an in vitro system previously developed (Mosher and Mikkelson, 1979). The excellent agreement with results obtained from in situ corneal uptake studies (Francoeur and Patton, 1979; Olejnik et al., 1981) indicate that this in vitro system can, in some cases, be successfully utilized to measure corneal permeability. It is recognized that one must consider the question of corneal integrity when this structure is isolated and removed from the intact animal. That potential problem will be addressed in the Discussion section.

The glass diffusion cells (Fig. 1) were constructed from 25 ml erlenmeyer flasks (N. Erway, Oregon, WI). The end of the 1 cm sidearm projection on each half-cell

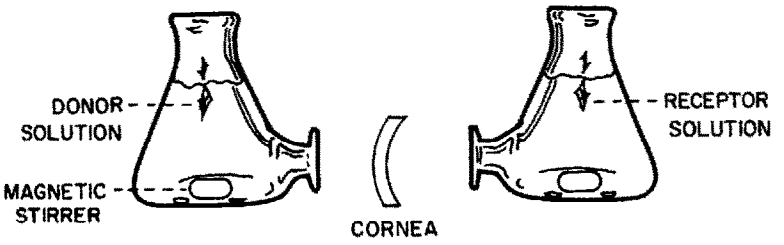

Fig. 1. In vitro diffusion cells used to measure corneal permeability.

had a ground-glass finish with a circular opening in the middle. The cross-sectional surface area of this opening was 0.28 cm^2. This left approximately a 5 mm ground-glass margin around the entire edge of the opening.

Corneal mounting procedure

Rabbits were sacrificed with an overdose of pentobarbital. The entire globe was surgically removed intact from the animal's eyesocket. The cornea was positioned on the donor half-cell such that the epithelial surface was centered in the sidearm opening. A thin bead of cyanoacrylic glue (Superglue, Cleveland, OH) was placed around the cornea–ground-glass junction. Slight positive finger pressure on the back of the globe prevented the glue from occluding the sidearm opening. After allowing a few minutes for drying, a small incision was made on the sclera approximately 2–3 mm away from the limbus. This incision was then extended around the entire perimeter of the eyeball leaving about a 3 mm scleral flap attached to the cornea. The remaining intraocular parts were removed exposing the corneal endothelium. The receptor half-cell was positioned symmetrically with respect to the donor cell facing the endothelial surface. The cells were then secured together by means of a ball and socket, pinch-type clamp (A.H. Thomas, Philadelphia, PA). This procedure prevented any leaks and allowed the natural curvature of the cornea to be maintained during the course of the experiment.

Sampling

After the cornea was securely mounted, 10 ml of buffer was added to the receptor cell. Likewise, 10 ml of the 1/2% (w/v) pilocarpine solution (same pH) was transferred to the donor half-cell. Both sides were capped with teflon-lined stoppers to prevent evaporation. The entire apparatus was placed in a water bath thermostated at 32.5°C. The donor and receptor solutions were mixed vigorously with magnetic stirbars. Aliquots of 100 μl were withdrawn from the receptor solution at 10, 20, 30, 40, 50, 60, 70, 80 and 90 min. Each sample was replaced in the receptor solution with 100 μl of buffer. Samples were analyzed by HPLC as described before and revealed only intact pilocarpine.

Results

Fick's first law can be stated as follows:

$$J = -D\left(\frac{dC}{dx}\right) \tag{1}$$

where J is the diffusive flux of material through a fixed reference plane of unit area. The magnitude D, is the diffusivity or diffusion coefficient, and dC/dx is the concentration gradient.

From Fick's second law dC/dx, in a membrane of finite thickness, can be shown to be constant with time such that Fick's first law can be rewritten:

$$J = \frac{-D}{h}(C_i - C_o) \tag{2}$$

where C_i and C_o are the concentration of diffusant at the membrane boundaries. The thickness of the barrier is represented by h.

The concentration of diffusant in the donor and receptor phases (C_D and C_R) external to the membrane can normally be related to the boundary concentrations by an equilibrium distribution coefficient, K, such that:

$$\frac{C_i}{C_D} = \frac{C_o}{C_R} = K \tag{3}$$

For most biological membranes h is only known approximately and K cannot be determined directly. Thus it has become customary, for practical purposes, to express Fick's law using a permeability coefficient, P:

$$J = -P(C_D - C_R) \tag{4}$$

At steady-state, under sink conditions (i.e., $C_R \cong 0$), the flux is constant with time such that:

$$J = \frac{V_R}{A}\left(\frac{dC_R}{dt}\right) \tag{5}$$

where V_R is the volume of the receptor solution and dC_R/dt is the rate of change of its concentration. The steady-state flux, J, can then be determined by plotting C_R vs time and evaluating the slope of the linear portion (Fig. 2). Since $J = -P \cdot C_D$, the apparent membrane permeability can be calculated if C_D changes insignificantly over the course of an experiment.

In considering the diffusion of an ionizable diffusant such as pilocarpine, it may be useful to distinguish between the fluxes of the ionized and neutral species. The total flux may be viewed as having two components:

$$J_{total} = J_i + J_u \tag{6}$$

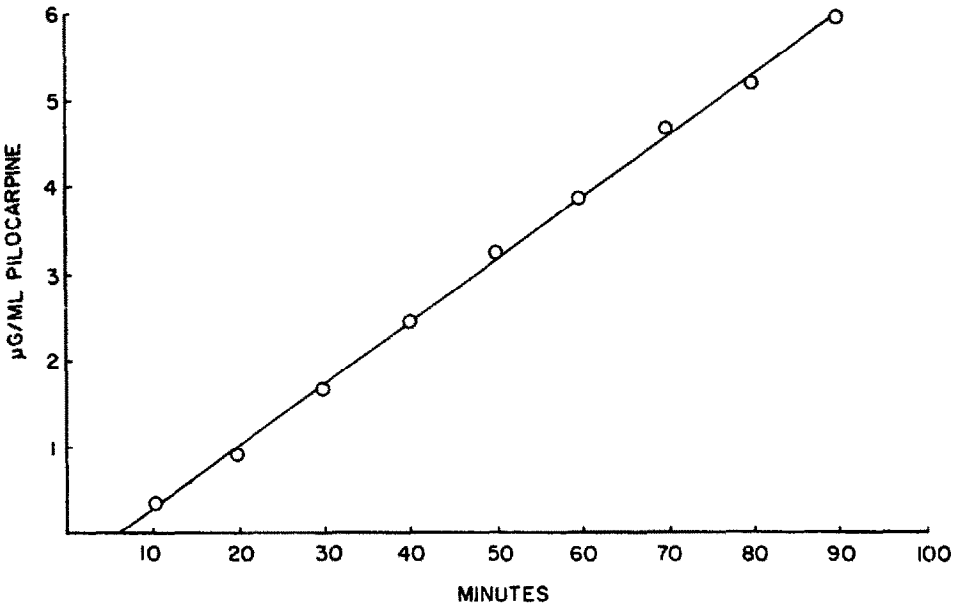

Fig. 2. Typical plot of the pilocarpine concentration in the receptor solution versus time.

where J_i is the flux of ionized pilocarpine and J_u is the flux of neutral pilocarpine. At steady-state $J = P \cdot C_D$ and Eqn. 6 becomes

$$P \cdot C_D = P_i C_i + P_u C_u \tag{7}$$

Since $C_u = f_u C_D$ where f_u is the fraction of pilocarpine unionized in the donor solution, the above equation can be rewritten and rearranged:

$$P = P_i + (P_u - P_i) \cdot f_u \tag{8}$$

The cornea in this treatment is viewed as a homogeneous membrane. Anatomically, the cornea contains several distinct barriers that offer varying degrees of resistance toward drug transport. For purposes of comparison, however, the effects of these barriers have been combined into a single resistance or permeability term. For the present no attempt has been made to separate the component parts of this lumped permeability coefficient into resistances which physically describe the different structural layers of the cornea.

The steady-state fluxes measured at 3 different pHs (6.0, 6.6, 7.2) for both 20- and 60-day-old rabbit corneas are listed in Table 1. Within both age groups the flux increased as the pH of receptor and donor solutions were raised. At each particular pH the flux measured across the 20-day-old corneas exceeded that of the 60-day-old corneas. However, a statistically significant difference was seen only at the highest pH (7.2).

The fraction of pilocarpine in a solution that is unionized at a particular pH can be calculated by means of the Henderson-Hasselbalch equation (Niebergall, 1975).

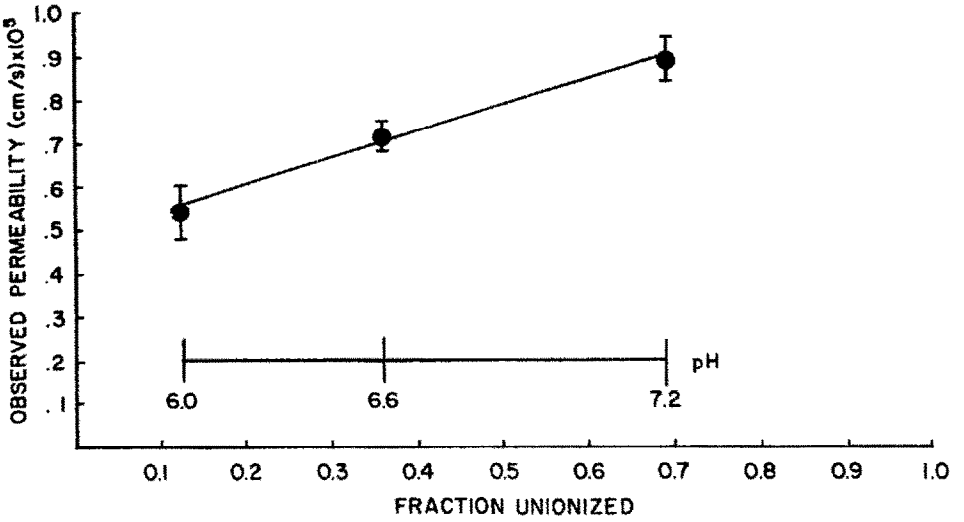

Fig. 4. Corneal permeability of 60-day-old rabbit corneas as a function of the fraction (f_u) of pilocarpine unionized in the donor solution.

TABLE 2

THE CORNEAL PERMEABILITY FOR UNIONIZED AND IONIZED PILOCARPINE IN 20- AND 60-DAY-OLD RABBITS.

	Permeability ($cm \cdot s^{-1}$)$\times 10^5$	
	20-day	60-day
P_u [a]	1.40	1.09
P_i [a]	0.447	0.475

[a] Calculated from Eqn. 8.

unionized components. This calculation is summarized in Table 2 for the experiments in both ages of rabbits. The permeability associated with ionized pilocarpine (P_i) appears to be very similar for the 20- and the 60-day corneas. In the older corneas, however, the transport of ionized pilocarpine represents a larger proportion of the overall observed permeability.

Discussion

During the course of any given diffusional run, the condition or integrity of the isolated cornea was not directly ascertained. Some stromal swelling did occur as evidenced by the partial clouding of the cornea, usually about 40 min into the experiment. However, the repeated linearity of the steady-state plots (see Fig. 2) in well over 37 separate experiments strongly suggests that the integrity of the rate-limiting barrier(s) in the cornea was not compromised. The fact that stromal swelling

did occur, with no deviation from linearity, also implies that the serial resistance of the stroma is negligible in the overall transcorneal permeation of pilocarpine. This further supports the notion that the corneal epithelium is probably the rate-limiting barrier for the intraocular penetration of pilocarpine (Mishima, 1981). Drugs having more significant stromal resistance, would require more direct monitoring of the condition of the cornea, specifically, the hydration level of the stroma. It must also be kept in mind that excessive swelling of the cornea could lead to disruption of the endo- and epithelial cell layers. Again, the linearity of the data tacitly suggests that this is not a problem in these studies. It is possible that, if all corneal swelling had occurred prior to sampling, linear plots could still be obtained. However, as yet unpublished results from this laboratory show that in this medium, the isolated cornea swells to 5–10% of its final value initially. The cornea then continues to swell for a period of 3–4 h. Therefore, if endo- and/or epithelial damage as a result of swelling were a problem in these studies, we would not have observed linearity as swelling continued throughout the time course of the studies. Furthermore, as mentioned in the Experimental section, results obtained with the system used in these studies compare very favorably with those obtained with an in situ corneal perfusion system (Francoeur and Patton, 1979; Olejnik et al., 1981).

The results clearly indicate that the corneal permeability of pilocarpine depends on the age of the rabbit. All other factors being equal, the permeability differences due to age can probably be attributed to structural variations between the two ages. This effect may simply be the result of changes in the overall corneal thickness or may, in fact reflect more subtle differences in one or more of the corneal layers. While the rate and extent of change in the overall size and shape of the rabbit cornea is well documented (Prince, 1964), little data is available as regards to the postnatal development of its various components. Without this information and additional permeability studies on the individual corneal layers, it is not possible to hypothesize which structural units or subunit are responsible for these age-related differences.

The results also demonstrate that the pH of the solution in contact with the cornea controls the transport of pilocarpine. Within both age groups a significant fraction of the total pilocarpine transported is in the ionized form. This fraction is proportionally greater in the 60-day-old rabbits.

Generally, drug molecules which are ionized do not have favorable free energies for transfer into most biological membranes. Under these conditions the transport of an ionizable drug such as pilocarpine should be largely determined by the fraction of drug that is unionized in the solution bathing the membrane. Additionally the permeability coefficient of the ionized species, P_i, should be nearly zero. This is, in fact, the essence of the so-called pH-partition hypothesis (Shore et al., 1957). The transport of both ionized and unionized pilocarpine observed in these studies, suggest that in this particular biological system the pH-partition hypothesis may not be strictly applicable.

Looking at the pH- and age-related effects together on pilocarpine transport, it appears that it is primarily the unionized species that is sensitive to the structural development of the rabbit cornea. Differences in the permeation of pilocarpine between 20- and 60-day-old corneas are not significant at lower pHs (6.0 and 6.6).

However, at the higher pH (7.2), where most of the drug is unionized, a greater permeability is observed for the 20-day-old corneas. These facts imply one of two possibilities. Either the transport of ionized and unionized pilocarpine is rate-limited by different structures or layers in the cornea, or the transport of the two species is rate-limited by the same layer and occurs by a different pathway or mechanism. In view of the insensitivity of the transport to stromal swelling and literature reports (Mishima, 1981) that the cornea epithelium is rate-limiting for pilocarpine, the latter possibility appears the most likely.

In summary, changes in corneal permeability do occur with age and have the potential to alter ocular drug bioavailability. Such bioavailability differences do exist in different age animals (Friedman and Patton, 1976) and corneal permeability differences are but one of a complex set of factors contributing to this phenomenon. The previous two papers in this series (Miller and Patton, 1981; Miller and Patton, 1982) addressed the influence of size and drug–protein interactions in contributing to age-related differences in ocular bioavailability. Subsequent papers will consider other precorneal and intraocular disposition factors.

Acknowledgement

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