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Disposition of pilocarpine in the pigmented rabbit eye

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Summary

The disposition of pilocarpine in the pigmented rabbit eye following instillation of a pilocarpine solution was studied using radiotracer techniques. The results suggest that a topically applied pilocarpine solution was removed as efficiently from the tear chamber of the pigmented rabbit as from that of albino rabbit. Nevertheless, a larger fraction of the applied dose was recovered in the pigmented rabbit eye, possibly due to the combined effect of improved corneal permeability, metabolism of the drug in the cornea, and metabolism and binding of the drug in the iris-ciliary body. Based on the area under the drug concentration vs time curve, about 10 times as much pilocarpine was found in the iris-ciliary body of the pigmented than the albino rabbit. Despite this drug accumulation in the iris-ciliary body, which presumably derived its drug supply from the aqueous humor, the pilocarpine concentration in the aqueous humor of the pigmented rabbit was virtually indistinguishable from that in the albino rabbit.

Introduction

The albino rabbit is commonly used in ocular drug penetration studies, in part because extensive information about its ocular biochemistry and physiology has been accumulated. Nonetheless, because of the lack of iris and ciliary body pigmentation, the albino rabbit would be a poor experimental animal to use for ocular pharmacokinetic studies with drugs that demonstrate high binding potential. In this case, the pigmented rabbit would be a more suitable animal to use. Previous ocular drug studies employing pigmented rabbits (Harris and Galin, 1971; Obianwu and

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Rand, 1965; Salazar and Patil, 1976; Salazar, Shimada and Patil, 1976; Seidehamel et al., 1970; Sherman, 1977; Yoshida and Mishima, 1975) have focused on the single aspect of drug–pigment interaction influence on the reduction of pharmacological response to a variety of autonomic drugs without exploring the distribution patterns of these drugs in the precorneal area and intraocular tissues. The inevitable result is that any interrelationships that may exist among these processes are unknown. The present study was initiated to obtain preliminary data on the concentration of pilocarpine in the cornea, aqueous humor and iris-ciliary body of pigmented rabbits. These data were then compared with those from previous work employing albino rabbits to generate a working profile of drug disposition in the pigmented rabbit eye.

Materials and methods

Materials

Tritiated pilocarpine alkaloid, spec. act. 4.165 Ci $mmol^{-1}$, was obtained commercially [1] and was purified by vacuum evaporation immediately prior to each run as previously described (Chrai and Robinson, 1974). All other chemicals used were either reagent or analytical grade and were used as received. Male, dark iride rabbits [2], weighing between 1.8 and 2.4 kg, were used throughout the studies. They were fed a regular diet with no restrictions on food or water consumed.

Methods

Preparation of 0.01 M pilocarpine solutions. A 0.01 M pilocarpine solution, prepared in isotonic Sorensen's phosphate buffer at pH of 6.24 (Sieg and Robinson, 1976), was spiked with radiolabeled pilocarpine prior to each experiment. It was determined that 0.25 mCi of the tritiated pilocarpine per ml of final solution was sufficient to ensure good counting efficiency, i.e., 150,000–175,000 cpm μl^{-1} (Sieg, 1977).

Aqueous humor drug concentration vs time profile. The basic experimental techniques used for instilling solutions and for monitoring aqueous humor drug levels after topical dosing were described previously (Sieg and Robinson, 1975). Unanesthetized animals were used and a standard 25-μl dose was instilled.

Corneal drug concentration vs time profile. Immediately after the aqueous humor was aspirated from the anterior chamber, the eyes was proptosed and secured with a hemostat behind the globe. A single incision was made with a scalpel at the corneal margin and the entire cornea was excised. Each cornea was carefully rinsed in saline to remove excess radioactivity and blotted on tissue. It was then placed into tared combustion cones [3] and the wet weight was determined using an analytical balance. A tissue oxidizer [4] was used to burn each cornea, and samples were counted in

[1] New England Nuclear, Boston, MA.
[2] Klubertanz, Edgerton, WI.
[3] Combusto-Cone, Packard Instruments, Downers Grove, IL.
[4] Model 306, Packard Instruments, Downers Grove, IL.

plastic vials[5] using a commercial liquid scintillation solution[6] and scintillation spectrometer[7]. After suitable corrections, the final count for each sample was converted to μg of total pilocarpine, i.e. intact pilocarpine plus the metabolite pilocarpic acid, per gram of cornea.

Iris and ciliary body drug concentration vs time profile. Immediately after the cornea was excised, the entire iris and ciliary body were extracted intact and care was exercised to ensure that they were free from choroidal adhesions. The tissues were then treated in a manner identical to the cornea. After suitable corrections, the final count for each sample was converted to μg of total pilocarpine per gram of iris and ciliary body.

Throughout the studies the surgical procedures were performed within 5 min of sacrificing the rabbit so that errors due to redistribution of drug during the time required to obtain samples were minimized.

Influence of instilled concentration on drug concentration in the cornea, aqueous humor, and iris-ciliary body at 20 min post-instillation. Pilocarpine solutions of 0.005 M, 0.02 M and 0.04 M were prepared in the same manner as the 0.01 M solution. Unanesthetized animals were used in all experiments and a standard 25-μl dose was instilled. Cornea, aqueous humor and iris-ciliary body samples were obtained and analyzed as described earlier in this report.

Identification of pilocarpine and pilocarpic acid. Procedures for preparation and identification of pilocarpic acid were reported earlier (Lee et al., 1980). Both pilocarpine and pilocarpic acid were identified in biological samples as previously described (Lee et al., 1980). Briefly, pilocarpine and pilocarpic acid were extracted from the cornea and iris-ciliary body using chloroform. The extract was evaporated to dryness under a stream of nitrogen, the residue was reconstituted in 100 μl of chloroform and a 60 μl volume of this solution was applied to Whatman linear LKD preadsorbent silica gel TLC plates[8]. Aqueous humor samples, 60 μl each, were applied directly to the TLC plates. Such plates were developed in *n*-butanol saturated with 14.8 M NH_4OH and allowed to air-dry. Two-centimeter sections were scraped off each plate, and the sections were transferred to scintillation cocktails[9] for eventual scintillation counting. The approximate R_f value of pilocarpine on these plates was 0.67, whereas the value for pilocarpic acid was 0.33.

Results

Figs. 1–3 display the time course of total pilocarpine concentration in the cornea, aqueous humor and iris-ciliary body from 5 to 120 min post-instillation of a 0.01 M pilocarpine solution in the pigmented rabbit eye. For comparison purposes the

[5] The Vial, Research Products International, Elk Grove Village, IL.
[6] Monophase-40, Packard Instruments, Downers Grove, IL.
[7] Model 2002, Packard Instruments, Downers Grove, IL.
[8] Pierce Chemicals, Rockford, IL.
[9] Aquasol, New England Nuclear, Boston, MA.

albino rabbit data from previous studies, conducted under similar conditions as the present study, are included (Sieg and Robinson, 1976; Makoid, 1977). The pigmented rabbits employed in the present study were of the same age and weight as the albino rabbits.

Fig. 1 shows that peak concentration of pilocarpine is reached in the cornea within 5 min of instillation of the dose, suggesting that a parallel loss process as efficient as that in the albino rabbit is operative in the precorneal area. Beyond 5 min, biphasic decline of drug concentration is evident, a behavior resembling that seen in the albino rabbit. The first phase is associated with an apparent first-order rate constant of 0.044 min^{-1} and the second phase with an apparent first-order rate constant of 0.017 min^{-1}.

Fig. 2 shows that the concentration of pilocarpine in the aqueous humor reaches a peak within 30 min of solution instillation, as compared to the peak time of 20 min seen in the albino rabbit. One-compartment pharmacokinetic analysis of the data yields an apparent transcorneal permeation rate constant of 0.10 min^{-1} for the pigmented rabbit.

Fig. 3 shows that relative to the albino rabbit, the time at which peak pilocarpine concentration is achieved in the iris-ciliary body of the pigmented rabbit is markedly prolonged, approximately 90 min post-instillation of solution. As expected, the peak

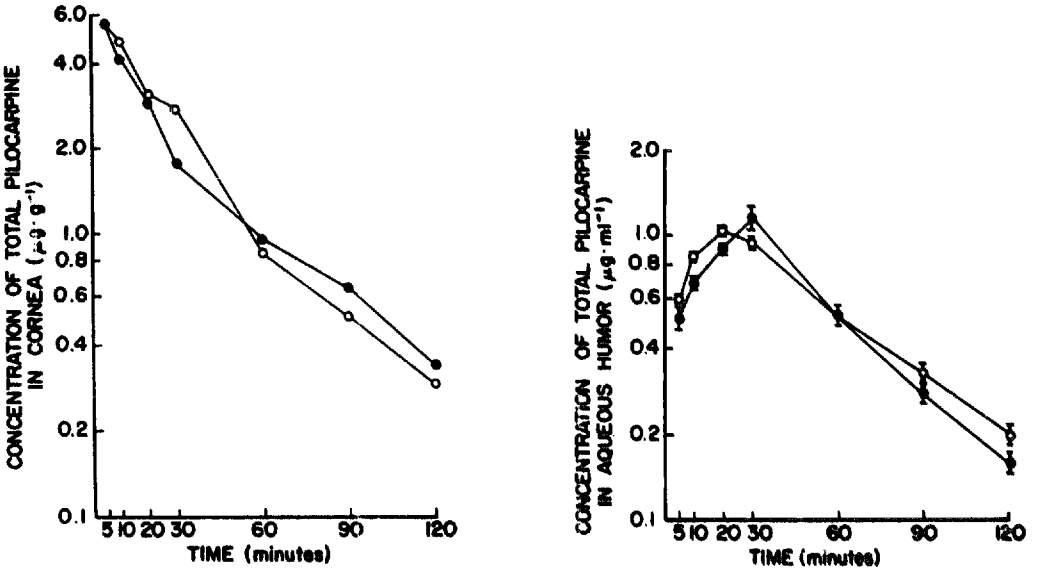


Fig. 1. Concentration of total pilocarpine in the cornea of albino (○) and pigmented rabbits (●) following topical instillation of a 0.01 M pilocarpine solution. Between 6 and 10 eyes were used for each time point. Error bars are omitted for the sake of clarity. Albino rabbit data (Sieg and Robinson, 1976) are included for comparison purposes.

Fig. 2. Concentration of total pilocarpine in the aqueous humor of albino (○) and pigmented rabbits (●) following topical instillation of a 0.01 M pilocarpine solution. Between 6 and 10 eyes were used for each time point. The error bars represent standard error of the mean. Albino rabbit data (Sieg and Robinson, 1976) are included for comparison purposes.

pilocarpine concentration is higher in the iris-ciliary body of the pigmented rabbit as compared to the albino.

To determine whether the concentration of pilocarpine instilled, 0.01 M, provides

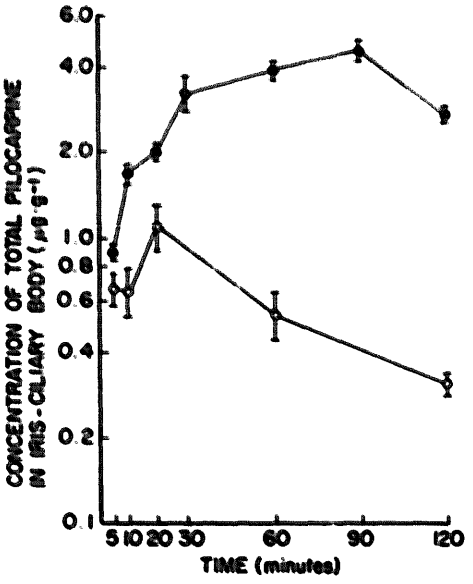


Fig. 3. Concentration of total pilocarpine in the iris and ciliary body of albino (○) and pigmented rabbits (●) following topical instillation of a 0.01 M pilocarpine solution. Between 6 and 10 eyes were used for each time point. The error bars represent standard error of the mean. Albino rabbit (Makoid, 1977) are included for comparison purposes.

sufficient pilocarpine to cause saturation of the binding sites in the pigment of the iris-ciliary body, the drug concentration in this tissue, as well as in the cornea and aqueous humor, at 20 min after instilling 25 μl doses of pilocarpine solutions with strengths ranging from 0.005 M to 0.04 M was determined. The data, presented in

TABLE I

TOTAL PILOCARPINE CONCENTRATION IN THE CORNEA, AQUEOUS HUMOR, AND IRIS-CILIARY BODY AT 20 MIN AFTER INSTILLATION OF 25-μl DOSES OF VARIOUS CONCENTRATIONS OF PILOCARPINE

Tissues	Instilled pilocarpine concentration			
	0.005 M	0.01 M	0.02 M	0.04 M
Cornea ($\mu g \cdot g^{-1}$)	2.31 (0.11) [a,b]	2.84 (0.19)	13.41 (0.44)	21.16 (0.87)
Aqueous humor ($\mu g \cdot ml^{-1}$)	0.52 (0.04)	0.91 (0.04)	3.02 (0.17)	4.92 (0.21)
Iris-ciliary body ($\mu g \cdot g^{-1}$)	2.20 (0.08)	2.04 (0.14)	12.78 (0.60)	23.77 (0.70)

[a] Numbers in parentheses represent standard error of the mean.
[b] An average of 8 eyes were used for each instilled concentration.

TABLE 2

LOGARITHMIC CORRELATION OF OCULAR TISSUE CONCENTRATION OF TOTAL PILOCARPINE AT 20 MIN POST-DOSING WITH INSTILLED CONCENTRATION

Tissue	Equation		R^2
Cornea	$\log[C]^a = (1.18 \pm 0.26) \log[S] - (2.31 \pm 0.48)$	0.956	0.915
Aqueous humor	$\log[AH]^a = (0.97 \pm 0.21)^c \log[S]^b - (3.36 \pm 0.40)$	0.956	0.914
Iris-ciliary body	$\log[I]^a = (1.30 \pm 0.36) \log[S] - (2.14 \pm 0.67)$	0.932	0.869

[a] [AH] represents concentration of pilocarpine in aqueous humor expressed in molar quantities; [C], cornea; and [I], iris-ciliary body. (A density of 1 $g \cdot cm^{-3}$ was assumed for the cornea and iris-ciliary body.)

[b] [S] represents instilled pilocarpine concentation expressed in molar quantities.

[c] The figures within parentheses represent the 95% confidence interval on the regression coefficient.

Table 1, are linearized by correlating with instilled concentration on a logarithmic basis (Chrai and Robinson, 1974). The results, which are displayed in Table 2, show that the instilled concentration and intraocular pilocarpine concentration are related logarithmically with a proportionality constant of about one. At least for the aqueous humor, this result is in accord with that obtained in albino rabbits by Chrai and Robinson (1974) when they varied the instilled concentration over 6 orders of magnitude.

Discussion

It is now known (Lee et al., 1980; Lee et al., 1982) that compared to the albino rabbit, the pigmented rabbit possesses two times greater esterase activity in the cornea and iris-ciliary body and tends to metabolize pilocarpine between one and two orders of magnitude greater. Therefore, the composite of concentration–time profiles in the cornea, aqueous humor and iris-ciliary body, shown in Fig. 4, is in actuality a plot of concentration of total pilocarpine—intact drug plus metabolite—against time. Using published data on the metabolism of pilocarpine (Lee et al., 1980), the data are replotted to yield the profiles for intact pilocarpine and pilocarpic acid shown in Figs. 5 and 6, respectively. Thus, the concentration of intact pilocarpine and pilocarpic acid is the highest in the iris-ciliary body and the lowest in the aqueous humor. Unlike the cornea and aqueous humor, in which pilocarpine and pilocarpic acid attain a peak concentration at essentially the same time, pilocarpine concentration in the iris-ciliary body reaches a peak at least 30 min sooner than pilocarpic acid. The remaining discussion will refer to total pilocarpine, i.e. pilocarpine plus pilocarpic acid, simply because the total amount of drug present in the eye is of concern.

Except for its behavior in the iris-ciliary body, the total pilocarpine concentration in the aqueous humor and cornea, over a period of 120 min, in the pigmented rabbit

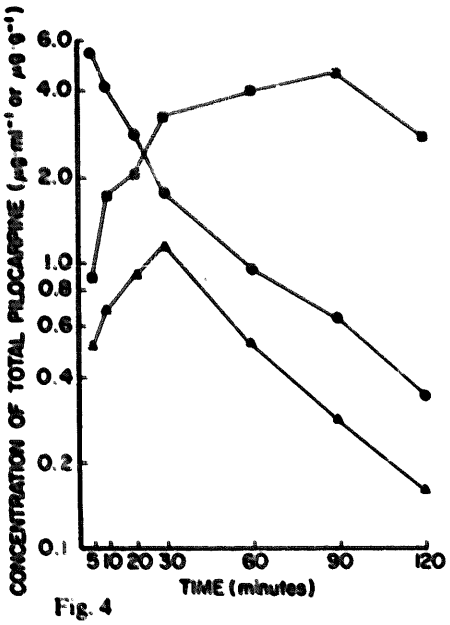


Fig. 4

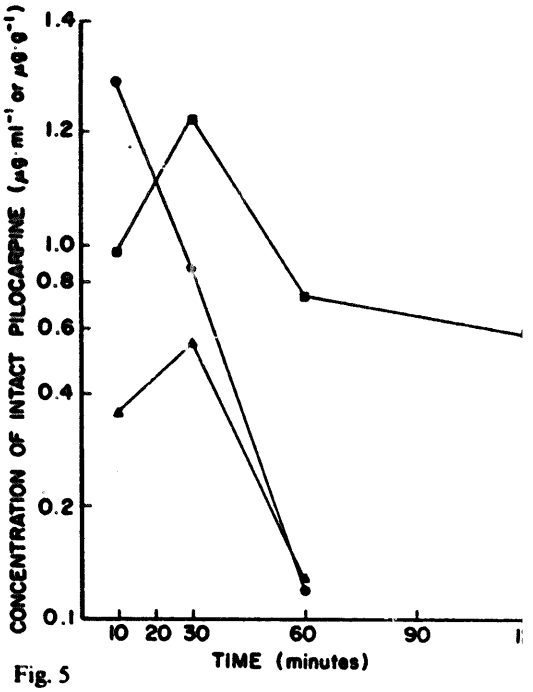


Fig. 5

Fig. 4. Concentration of total pilocarpine in the cornea (●), aqueous humor (▲), and iris-ciliary body (■) of pigmented rabbits following topical instillation of its solution. Total pilocarpine refers to intact pilocarpine plus its metabolite pilocarpic acid. Concentration is expressed as $\mu g \cdot ml^{-1}$ for aqueous humor and as $\mu g \cdot g^{-1}$ for cornea and iris-ciliary body. Between 6 and 10 eyes were used for each time point. Error bars are omitted for the sake of clarity.

Fig. 5. Concentration of intact pilocarpine in the cornea (●), aqueous humor (▲), and iris-ciliary body (■) of pigmented rabbits following topical instillation of its solution. It is expressed as $\mu g \cdot ml^{-1}$ for aqueous humor and as $\mu g \cdot g^{-1}$ for cornea and iris-ciliary body. Between 6 and 10 eyes were used for each time point. Error bars are omitted for the sake of clarity. Profile for total pilocarpine is presented in Fig. 4.

is rather similar to that in the albino rabbit. However, because the wet weight of the pigmented rabbit cornea is about 1.5 times that of the albino rabbit (unpublished data), the *amount* of total pilocarpine in the pigmented rabbit cornea is actually higher than that in the albino rabbit. This apparent increase in drug uptake by the pigmented rabbit cornea may be attributed to the combined influence of a larger permeation rate constant and a somewhat smaller precorneal parallel loss rate constant. That peak drug concentration is still attained in the cornea within 5 min of solution instillation favors an increase in permeation constant as the dominant cause. However, this increased permeation rate may not be reflecting, just reduced resistance to permeation. Indeed, there is not a priori reason to expect a more facile penetration in the pigmented rabbit on the basis of anatomy. An alternate explanation is that metabolism of pilocarpine in the cornea would continuously lower, or at least maintain, a concentration gradient for drug permeation. This then should indirectly contribute to increased drug uptake.

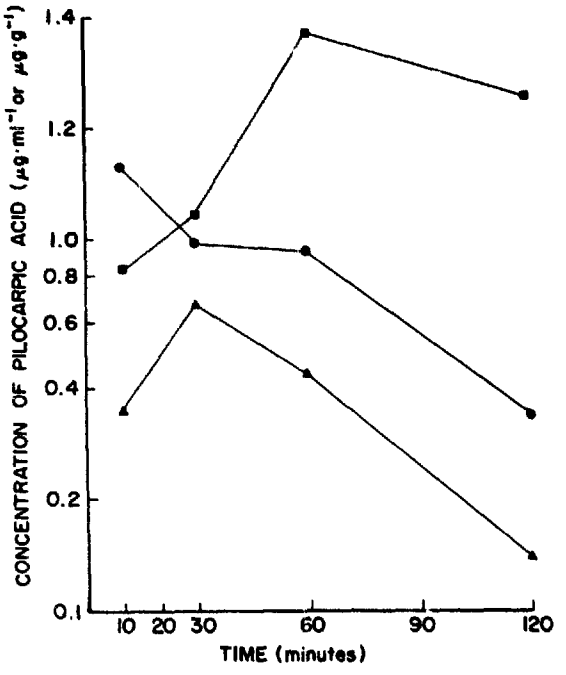


Fig. 6. Concentration of pilocarpic acid in the cornea (●), aqueous humor (▲), and iris-ciliary body (■) of pigmented rabbits following topical instillation of its solution. It is expressed as $\mu g \cdot ml^{-1}$ for aqueous humor and $\mu g \cdot g^{-1}$ for cornea and iris-ciliary body. Between 6 and 10 eyes were used for each time point. Error bars are omitted for the sake of clarity. Profile for total pilocarpine is presented in Figure 4.

As expected, the amount of pilocarpine recovered in the iris-ciliary body is larger in the pigmented than in the albino rabbit. This has generally been attributed to binding of the drug to the pigments, believed to be melanin (Larsson et al., 1977; Valenzuela et al., 1977). Indeed, Kloog et al. (1979) have identified in both the cat and pigmented rabbit irides, high capacity, low affinity binding sites for [^{3}H]N-methyl-1,4-piperidyl benzilate (a highly potent and specific muscarinic antagonist); these binding sites do not exist in the albino rabbit. It can be shown that as a result of drug–pigment binding the rate constant governing the decline of pilocarpine concentration in the iris-ciliary body is reduced by a factor of $1 + ([P_T]/K_D)$ (Silhavy et al., 1975), where $[P_T]$ is the total pigment concentration and K_d the dissociation constant of the drug–pigment complex. This reduced elimination rate constant, in turn, contributes to drug accumulation. Based on the AUC from zero to 120 min for the iris-ciliary body, approximately 10 times as much total drug accumulated in this tissue of the pigmented rabbit as the albino rabbit. Table 3 further compares the amount of total pilocarpine in the iris-ciliary body in the two breeds of rabbits at selected times following solution instillation.

That the pharmacological response is actually reduced in patients with heavily pigmented irides in spite of drug accumulation (Seidehamel et al., 1970; Harris and Galin, 1971; Melikian et al., 1971) implies that the amount of free-drug available to act on the receptors must be reduced. The underlying mechanisms are unknown. It

TABLE 3

RATIO OF AMOUNT OF TOTAL PILOCARPINE IN THE IRIS AND CILIARY BODY AT SELECTED TIMES IN ALBINO AND PIGMENTED RABBITS

Time (min)	Ratio [a,b]
10	5.5
20	3.8
60	13.9
120	18.0

[a] Ratio is defined as ratio of μg pilocarpine in iris-ciliary body of pigmented rabbits to that in albino rabbits.

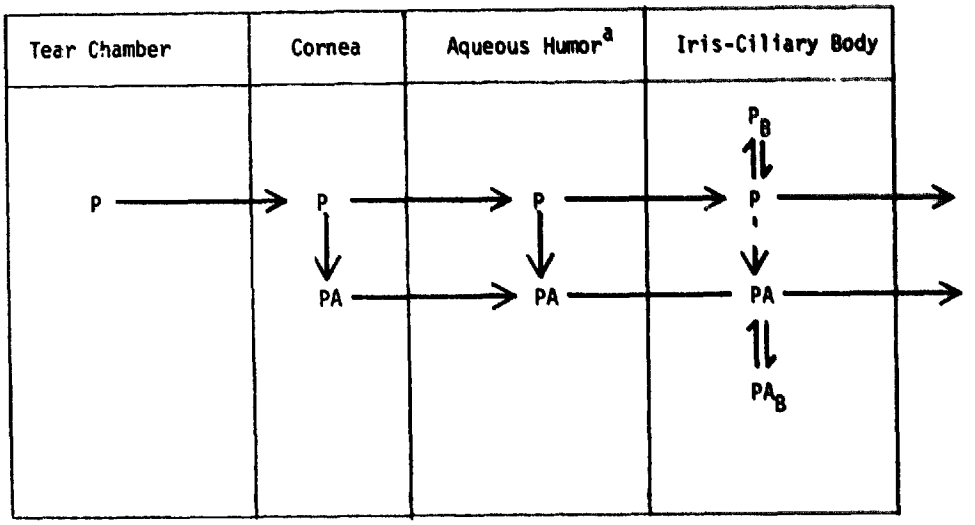
[b] Data on albino rabbits are those of Makoid (1977).

can be speculated that the same amount of pilocarpine reaches the iris-ciliary body of both the albino and pigmented rabbit, but because of the opportunity for binding to the pigments present only in the pigmented rabbit, the free-drug concentration in the iris-ciliary body of the pigmented rabbit is reduced. The in vitro incubation data of Lyons and Krohn (1974) tend to refute this possibility however. These investigators have demonstrated that under identical incubation conditions only 10% of the pilocarpine in the incubation medium is being taken up by the iris-ciliary body of the pigmented rabbit, in contrast to the 5% observed in the albino rabbit. In any event, the reduction in free-drug concentration in the iris-ciliary body accompanying binding of the drug by the pigments requires a corresponding increase in instilled concentration. In fact, analysis of miosis–time data led Yoshida and Mishima (1975) to predict an approximately 3-fold increase in the least effective instilled concentration for individuals with dark irides over those with light ones. Likewise, Harris and Galin (1971) noted that an approximately 8-fold increase in instilled concentration was necessary to obtain the same degree of intraocular pressure lowering in patients with brown irides relative to those with blue.

This binding picture is further complicated by ongoing metabolism of pilocarpine in the iris-ciliary body. Thus, as shown in Scheme I, the total radioactivity detected in this tissue conceivably can be due to both drug and metabolite, in both free and bound form. As in the cornea, this metabolism may promote drug uptake by the pigment, thereby contributing to a further reduction in free-drug concentration in the iris-ciliary body.

Surprisingly, in spite of the apparent increase in uptake of drug by the iris-ciliary body in the pigmented rabbit, the aqueous humor concentration–time profile is virtually indistinguishable between the albino and pigmented rabbit. Conceivably this is achieved by a compensatory increase in uptake of drug into the cornea which subsequently is being made available to the aqueous humor. Because of this finding, caution must be exercised in evaluating aqueous humor drug concentration. In fact, were the aqueous humor the only compartment with its drug concentration assayed, one may be inclined to extrapolate from the albino rabbit data a pilocarpine

Scheme I. Hypothetical Profile for the Disposition of Pilocarpine in the Pigmented Rabbit Eye.



[a]Both P and PA are distributed to intraocular tissues other than the iris-ciliary body.

Key: P = pilocarpine, PA = pilocarpine acid, P_B = pigment-bound pilocarpine

PA_B = pigment-bound pilocarpic acid.

concentration in the iris-ciliary body of the pigmented rabbit being similar to that in the albino rabbit.

In summary, to topically applied dose of pilocarpine in the pigmented rabbit appears to be subject to precorneal parallel loss factors as efficient as those in the albino rabbit. More of the dose is absorbed, however, possibly due to a combined effect of increased membrane permeability and drug metabolism in the cornea. The most striking difference in the behavior of pilocarpine in pigmented and albino rabbits is the accumulation of drug in the iris-ciliary body of the pigmented rabbit. This possibly is due to a combined effect of metabolism of the drug and binding of the drug and its metabolite to the pigments in this tissue. In a future report we will present the in vitro and in vivo metabolite profile of pilocarpine in the pigmented rabbit, including the corneal permeation and intraocular disposition of its metabolite, pilocarpic acid.

Acknowledgement

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