Table III—Apparent Permeability Coefficient, P_{app}, Determined for the Vaginal Absorption of the Alcohols

Monkey	$P_{app} \times 10^4$, cm/sec		
	Methanol	1-Butanol	1-Octanol
774	0.80	1.44	2.18
775	1.16	1.77	3.52
778	0.75	0.96	2.85

metrical surface area of the cell used, V is the total volume of the drug solution, and P_{app} is the apparent permeability coefficient. The ratio of the surface area to the volume depended upon the cell employed.

The results of some typical P_{app} determinations are summarized in Table III. As can be seen, the values increased from methanol through octanol for all monkeys used. This finding is in qualitative accord with the results obtained in the rabbit vaginal absorption studies. Furthermore, the P_{app} values for the monkey were approximately of the same order of magnitude as those obtained with the rabbit system (2). However, since these experiments were carried out with little regard for the menstrual cycle effects, the results in Table III mainly represent a demonstration of the feasibility of the experiments. A rather systematic study of the menstrual cycle effects, that is, the influence of the phase of the cycle and the influence of the alkyl chain length as a function of the menstrual cycle, will be reported[9] (7).

In separate experiments, as was found in the rabbit studies, there were no significant losses of the alcohols in the perfusion system through, for example, adsorption when the rib-cage cell was bypassed.

[9] Presented at the APhA Academy of Pharmaceutical Sciences, New Orleans meeting, Apr. 1976 (abstract 54).

In conclusion, this study has shown that a relatively simple procedure involving perfusion and a special cell inserted into the vagina may be used to carry out vaginal membrane permeability experiments in the rhesus monkey. The procedure should be well suited for determining vaginal membrane permeability coefficients over a wide range of conditions, especially as a function of the menstrual cycle employing, wherever possible, the particular monkey as its own control.

REFERENCES

(1) T. Yotsuyanagi, A. Molokhia, S. Hwang, N. F. H. Ho, G. L. Flynn, and W. I. Higuchi, *J. Pharm. Sci.*, **64**, 71 (1975).

(2) S. Hwang, E. Owada, T. Yotsuyanagi, L. Suhardja, N. F. H. Ho, G. L. Flynn, and W. I. Higuchi, *ibid.*, **65**, 1574 (1976).

(3) S. Hwang, E. Owada, L. Suhardja, N. F. H. Ho, G. L. Flynn, and W. I. Higuchi, *ibid.*, in press.

(4) *Ibid.*, in press.

(5) L. P. Bengtsson, *Acta Endocrinol., Suppl.*, **13**, 11 (1953).

(6) C. G. Hartman, *Endocrinology*, **25**, 670 (1939).

(7) E. Owada, C. R. Behl, Y. Kurono, S. S. Hwang, G. L. Flynn, N. F. H. Ho, and W. I. Higuchi, *Obstet. Gynecol.*, in press.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 12, 1976, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48109.*

Accepted for publication March 31, 1976.

Supported by the National Institute of Child Health and Human Development, Bethesda, Md. (Contract NO1-HD-3-2740).

The authors thank Dr. T. J. Roseman, Dr. K. T. Kirton, and Dr. F. H. Schadewald for helpful comments on the feasibility of the reported method.

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Aqueous Chamber Drug Distribution Volume Measurement in Rabbits

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Abstract □ A method was developed for aqueous chamber drug distribution volume measurement in the albino rabbit, and the apparent volume of distribution was determined for inulin, pilocarpine alkaloid, and 1-hexanoic acid. The method consists of injecting a suitable concentration of drug, in an appropriate volume of fluid, into the anterior chamber of the eye and monitoring the decline in drug concentration as a function of time by periodic sampling of the aqueous humor. Graphical analysis of the resulting data yields both the apparent volume of distribution and the turnover rate constant of the aqueous humor. The technique does traumatize the eye, causing formation of plasmoid aqueous, which does not interfere with the apparent drug distribution volume measurement or the determination of aqueous humor turnover. Inulin was used to determine the physiological aqueous volume, 287 μl, in good agreement with literature values. The turnover rate constant was 0.016 min^{-1}, also in good agreement with literature values. The apparent volume of distribution for pilocarpine alkaloid was 575 μl in albino eyes and 760 μl in pigmented irides; for 1-hexanoic acid in albino eyes, it was 760 μl. For pilocarpine alkaloid, literature citations on the fraction of dose absorbed have been based on an assumed apparent volume of distribution of 250–300 μl. Therefore, a factor of two error has been introduced when using albino eyes and a factor of almost three has been introduced when using pigmented eyes. The implication of the apparent volume of distribution for pilocarpine in its ocular disposition is discussed, as is the unexpected observation that pilocarpine alkaloid apparently inhibits formation of plasmoid aqueous and follows one-compartment kinetics during these studies. It is shown that the one-compartment kinetics for pilocarpine are due to its biological activity in the aqueous chamber.

Keyphrases □ Drug distribution—aqueous chamber drug volume, inulin, pilocarpine, and 1-hexanoic acid, rabbit eyes □ Inulin—aqueous chamber volume of distribution, rabbit eyes □ Pilocarpine—aqueous chamber volume of distribution, rabbit eyes □ 1-Hexanoic acid—aqueous chamber volume of distribution, rabbit eyes □ Ocular volumes of distribution—aqueous chamber, pilocarpine, inulin, and 1-hexanoic acid, rabbit eyes

To make meaningful quantitative statements about the bioavailability of an ophthalmic preparation, it is necessary to know the apparent volume of distribution for that drug in the eye. At present, the ophthalmic literature contains statements on the bioavailability of topically applied ocular drugs that are based, at best, only on an approximation to the actual aqueous humor volume, ignoring all other factors, and, at worst, on poorly controlled clinical studies

(1–4). The present study was designed to establish procedures for obtaining ocular volumes of distribution in the anterior and posterior chambers of the eye, to provide an accurate value for aqueous humor volume, and to determine the apparent volume of distribution of some typical ophthalmic drugs.

BACKGROUND

The apparent volume of distribution for ocular drugs can be used to provide insight on drug disposition if a physiological basis for the actual volume is provided, *i.e.*, if the ocular tissue volume is known. Significant deviations from the physiological volume for a determined apparent volume of distribution would be helpful in a mechanistic understanding of a drug's disposition.

Reported values for the physiological volume of the aqueous humor in rabbits have been derived primarily from paracentesis studies where the anterior chamber was aspirated and the volume was determined. Such values, however, may be in error since they may not measure the aqueous humor in the posterior chamber or fluid adhering to the walls of the anterior chamber. The aqueous humor contained in and on these structures is not measurable *via* normal aspiration techniques. Moreover, the iris and ciliary body are relatively porous structures containing aqueous humor that probably will not be removed *via* paracentesis.

Apparently, there are no reports on the apparent volumes of distribution for ocular drugs. For situations requiring an apparent volume of distribution, *e.g.*, determination of the fraction of dose absorbed, it is common practice to assume a 250–300-μl volume, irrespective of the drug under study.

Without an accurate value for the apparent volume of distribution of a drug in the eye, it is still possible to make statements regarding the percent of an applied dose that crosses the cornea to the aqueous humor. However, these values are only estimates and do not consider many parameters that can affect the bioavailability of a particular drug, *e.g.*, protein binding, partitioning into tissues, and poor mixing. By determining a precise value for the apparent distribution volume, it is possible to calculate accurately the fraction of dose absorbed. Armed with this information, it is possible to modify the size of the dose, the frequency of dosing, the vehicle for the dose, *etc.*, and to gauge the improvement in drug bioavailability due to these manipulations. In addition, the apparent volume of distribution for a drug can be used as an initial probe in the design of mechanistic studies of drug disposition.

An additional parameter, important to the disposition of a drug in the aqueous chamber, is aqueous humor turnover, since drug can be rapidly eliminated by the inflow and outflow of the aqueous humor. Removal of drug *via* this route is influenced by distribution equilibria of the drug in the aqueous chambers. Hence, the terminal elimination portion of the aqueous humor drug concentration–time profile can be used to estimate the importance of aqueous humor turnover, as well as distribution, on drug elimination.

Physiological turnover rates have been determined by various invasive and noninvasive techniques in several animal species (5–9). The results derived from the present method compare well with previously reported values. The salient feature of the described method is that it allows calculation of both the rate of aqueous humor turnover and the apparent volume of distribution for the drug from a single experimental technique. Other methods, both invasive and noninvasive, do not have the capability of determining the apparent volume of distribution, since they are mainly either continuous perfusions of the anterior chamber or intravenous injections of tracer material that appears in the eye after penetration of the blood–aqueous barrier *via* the animal's circulatory system (5–14). Thus, these methods do not provide for extrapolating drug concentration–time data to zero time, which is a necessary feature in developing a procedure to obtain the apparent volume of distribution for a drug.

EXPERIMENTAL

Materials—Male, albino, and mixed breed dark iride rabbits[1], 1.8–2.4 kg, were maintained on a regular diet with no restrictions on food or water intake.

^{14}C-Inulin[2] dry powder, specific activity 2.47 μCi/mg, was used as received. ^{3}H-Pilocarpine alkaloid[2] alcoholic solution, specific activity 2.4 mCi/mg, was purified by vacuum distillation (15) prior to use. The purification of pilocarpine was carried out immediately before each experimental run to minimize tritium exchange with the solvent. ^{14}C-1-Hexanoic acid[3], sodium salt aqueous solution, specific activity 10 mCi/mmole, was used as received. ^{14}C-Inulin and ^{14}C-1-hexanoic acid were 99% chromatographically pure. All other chemicals were either reagent or analytical grade.

Methods—*Preparation of ^{14}C-Inulin Solution*—^{14}C-Inulin was dissolved in water so that a concentration of 2×10^{-5} *M* was obtained. Since this concentration represents the limit of aqueous solubility for inulin, no "cold" (nonlabeled) inulin was added to the solution. This concentration corresponds to 0.1 μg of inulin/μl of solution. In the study employing an inulin–pilocarpine solution, radioactive inulin, 2×10^{-5} *M*, was added to a 1×10^{-4} *M* cold pilocarpine solution.

Preparation of ^{3}H-Pilocarpine Solution—Earlier work established a peak aqueous humor drug level of approximately 1×10^{-4} *M* from topical application of a 1×10^{-2} *M* solution of pilocarpine (16). Since the present study involved direct injection of drug into the anterior chamber, 1×10^{-4} *M* pilocarpine was used. The alcoholic solution of ^{3}H-pilocarpine alkaloid was evaporated to dryness. To the evaporated ^{3}H-pilocarpine was added 5 ml of 1×10^{-4} *M* cold pilocarpine alkaloid in aqueous solution. The addition of ^{3}H-pilocarpine alkaloid was small enough to have no appreciable effect on the molarity of the final solution. The solution concentration corresponded to 2.08 μg of pilocarpine alkaloid/μl.

Preparation of ^{14}C-1-Hexanoic Acid Solution—From the aqueous solution of the sodium salt, 50-μl samples were diluted to 3 ml with water to produce a solution that was 1.67×10^{-4} *M* in ^{14}C-1-hexanoic acid. Therefore, the solution for injection corresponded to 1.94×10^{-2} μg of hexanoic acid/μl.

Aqueous Humor Drug Concentration–Time Profile—Rabbits were anesthetized by intraperitoneal injection of pentobarbital sodium solution followed by phenobarbital sodium solution (17). Due to variations in response to the anesthetic doses, some animals had to be titrated with additional anesthetic until no head movements and/or blink responses were present. Also, for time points beyond 30 min, an intravenous infusion of a phenobarbital sodium solution was administered periodically to maintain anesthesia.

Anesthetized animals were then placed in wooden restraining boxes which maintained the normal upright posture for the animal. About 10 min prior to dosing, each eye received 1 drop of 0.5% tetracaine hydrochloride solution[4]. Due to the manipulations required, each rabbit was used for two different time points. For example, the left eye was dosed 10 min after the right eye so that at the end of 20 min the right eye yielded a 20-min point while the left eye yielded a 10-min point. To eliminate any right to left or left to right bias, the next rabbit was dosed so that the left eye yielded the 20-min point while the right eye yielded the 10-min point. For the later time points of 60 min and beyond, the same time point could be obtained from both eyes since the entire procedure of refilling the syringes, injecting the drug solution, and positioning the delivery apparatus on the wooden blocks took approximately 1 min, and this period introduced a potential error of only 1.67% in a 60-min time point. Moreover, this error was averaged out by reversing the order of dosing in the next rabbit.

Once the anesthetized animal was positioned in the restraining box and the topical anesthetic was applied, measured volumes of drug solution (5, 10, or 20 μl) were accurately administered to each rabbit eye *via* a microliter syringe[5]. The syringe was fitted with a modified vein infusion set[6] prefilled with the drug solution. The modification consisted of shortening the tubing length from the normal 30 cm to approximately 3 cm. This change was made to minimize drug solution loss and to make the delivery apparatus more manageable. Once the needle of the infusion set had punctured the cornea and was in position in the anterior chamber, the appropriate dose was delivered from the syringe. To prevent leakage of the aqueous humor fluid, the 3-cm tubing was then clamped off with Kelly forceps and the tubing was cut just behind the clamp. The clamp was then supported on a wooden block so that its weight would not pull on the punctured cornea and produce leakage at the entrance point of the needle into the cornea.

The clamp assured no loss of aqueous humor due to positive pressure outflow caused by the intraocular pressure. The small amount of tubing

[1] Klubertanz, Edgerton, Wis.
[2] New England Nuclear, Boston, Mass.
[3] ICN, Chemical and Radioisotope Division, Irvine, Calif.
[4] Pontocaine, Winthrop Laboratories, New York, N.Y.
[5] Hamilton 705, Hamilton Co., Reno, Nev.
[6] Miniset, Travenol Laboratories, Deerfield, Ill.

Table I—Pharmacokinetic Parameters Obtained *via* Anterior Chamber Injection of Various Drugs

Drug	Dose, μl	Amount of Drug per Dose, μg	C_0, μg/ml	Apparent V_d, μl	Turnover Rate Constant, k, min^{-1}
Inulin[a]	5	0.5	1.75	286	0.018
	10	1.0	3.40	294	0.011
	20	2.0	7.15	280	0.020
				Average 287	Average 0.016
Pilocarpine[b]	10	0.2083	0.36	579	0.060
	20	0.4165	0.73	571	0.058
				Average 575	Average 0.059
Pilocarpine[c]	10	0.2083	0.27	760	0.066
1-Hexanoic acid[a]	10	0.1937	0.26	745	0.019
	20	0.3874	0.50	775	0.015
				Average 760	Average 0.017

[a] Analyzed by apparent two-compartment open model. The turnover rate constant was determined from the terminal portion of the line. [b] Analyzed by apparent one-compartment open model. [c] In dark iride rabbits. The turnover rate constant was determined from the initial portion of the line.

remaining, 1.5 cm, and the supported clamp allowed the rabbit some head movement without danger of dislodging the needle.

At the end of the designated time periods, 5, 10, 15, 20, 30, and 60 min, the rabbit was sacrificed by rapid marginal ear vein injection of an overdose of phenobarbital sodium. The 90- and 120-min time points were obtained only with the ^{14}C-inulin at the 20-μl volume. The aqueous humor was aspirated from each eye using a 1-ml tuberculin syringe fitted with a 27-gauge, 1.27-cm needle. Aqueous humor samples, 100 μl, were quantitatively transferred to scintillation counting vials[7] containing 5 ml of a scintillation cocktail[8]. Samples were then stored in the dark at room temperature for a minimum of 48 hr prior to counting to eliminate error due to chemiluminescence. Ten-minute counts of each sample were made using a liquid scintillation spectrometer[9]. The final count for each sample, corrected for background, was then converted to a microgram of drug per milliliter of aqueous humor basis by standardization techniques to facilitate analysis and interpretation of results.

A minimum of 10 eyes was used for each data point, with the exception of the 60-min point for pilocarpine in albino rabbits where four eyes were used; no animal was used more than once. Mean values and standard errors of the mean were determined from the pooled data at each time point. The concentration–time profiles were least-squares fit to the one- or two-compartment model.

RESULTS

Inulin has been employed in the past to measure renal clearance values. Since it does not readily penetrate tissues and is not bound to protein to any measurable extent, it was considered ideal for such clearance measurements because it is eliminated solely by glomerular filtration. These same properties make inulin an attractive probe for measuring the physiological aqueous humor volume. In addition, tissues surrounding the aqueous chamber, *i.e.*, iris and ciliary body, are porous and should allow free movement of the relatively small inulin molecule so that all resident aqueous humor is detected. These assumptions are borne out by experimentation. Assay of ocular tissues surrounding the anterior chamber, *i.e.*, cornea, ciliary body, iris, vitreous humor, and lens, shows that inulin is not absorbed and/or adsorbed onto these tissues. Thus, the only means of exit for inulin from the anterior chamber is by normal aqueous humor drainage.

Figure 1 shows the aqueous humor inulin concentration–time profile for three different injected volumes (5, 10, and 20 μl). Different volumes were employed to determine the influence of instilled volumes on the apparent volume of distribution and apparent aqueous humor turnover as well as to check on the proposed mechanism for inulin removal, *i.e.*, to explain the shape of the drug concentration–time profile. The drug concentration–time profiles were analyzed as an apparent two-compartment open model[10], with the initial portion of the curve attributed to an effect due to injection of a volume above that of the normal resident volume and the terminal portion due to normal aqueous humor turnover.

An analogy can be made between this system and instilling a volume of drug into the precorneal portion of the eye (18). In the precorneal portion, an instilled solution is diluted by resident tears, the tear–drug solution then rapidly drains from the eye until the volume is back to the normal resident volume, and, finally, tear turnover accounts for most of the remaining drug removal. In the aqueous chamber, the injected inulin mixes with resident aqueous humor and drains from this area until a normal volume is achieved, at which point aqueous humor turnover is responsible for additional removal. Thus, the initial portion of the inulin profile is attributed to rapid removal of drug simply because a volume has been injected. The increasing slopes of these lines with an increasing volume support this proposal. The terminal portion of the profile corresponds to aqueous humor turnover. Since no distribution of inulin into aqueous chamber tissues occurs, the terminal slope can be directly related to aqueous humor turnover.

After graphical analysis and extrapolation to time zero, the volume of distribution of the aqueous humor can be calculated in the usual manner. These values for the three volumes are presented in Table I along with the turnover rate constants obtained from the terminal portion of the curves. The physiological volume of distribution, *i.e.*, true aqueous humor volume, and the turnover rate constant derived from these measurements agree very well with those reported for rabbits using other techniques (5, 19).

The problem of plasmoid aqueous formation due to trauma has been well documented for the rabbit (20–22). Any trauma such as inflammation or puncture of the globe of the eye leads to a breakdown on the blood–

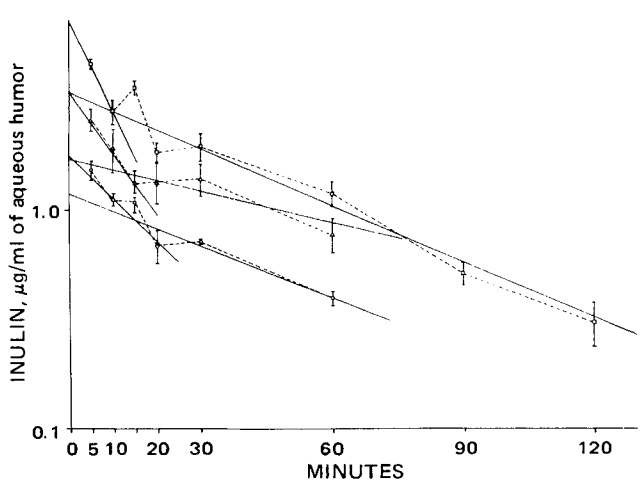

Figure 1—*Aqueous humor drug concentrations following intracameral injection of 2 × 10^{-5} M inulin solutions in albino rabbits. Error bars represent the standard error of the mean. Where no error bars are drawn, the standard error was smaller than the symbol used. Solid lines are best-fit lines. Dotted lines connect the mean value at each time point. Key: ○, 5 μl injected; △, 10 μl injected; and □, 20 μl injected.*

[7] Mini-vial, Research Products International Corp., Elk Grove Village, Ill.
[8] Aquasol.
[9] Packard model 2002, Packard Instrument Co., Downers Grove, Ill.
[10] The term compartment is used in a very loose sense throughout this article. With inulin, only the aqueous chamber is involved, *i.e.*, one compartment, and the biphasic concentration–time profile is simply a fast volume-dependent removal of drug followed by a slower removal.

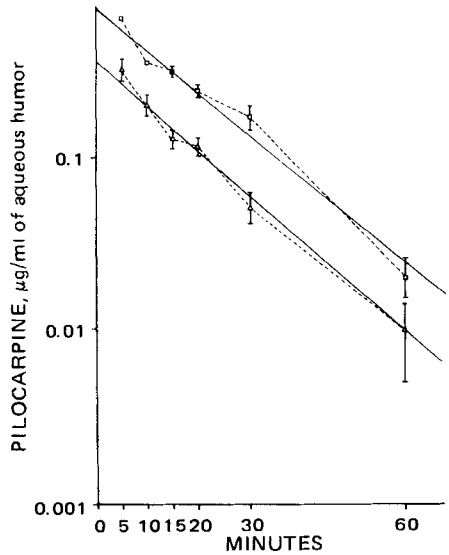

Figure 2—*Aqueous humor drug concentrations following intracameral injection of 1 × 10^{-4} M pilocarpine solutions in albino rabbits. Error bars represent the standard error of the mean. Where no error bars are drawn, the standard error was smaller than the symbol used. Solid lines are best-fit lines. Dotted lines connect the mean value at each time point. Key: △, 10 μl injected; and □, 20 μl injected.*

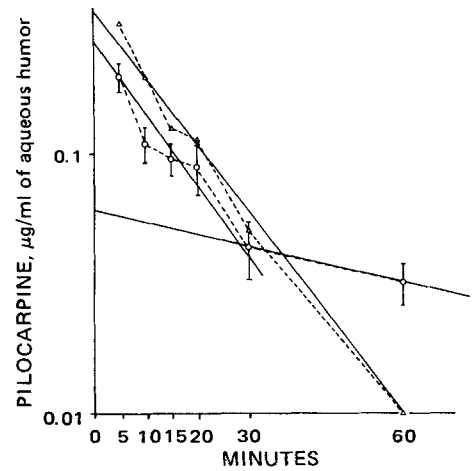

Figure 3—*Comparison of aqueous humor drug concentrations following intracameral injection of 1 × 10^{-4} M pilocarpine solutions in albino and pigmented iride rabbits. Error bars represent the standard error of the mean. Error bars were deleted from the albino rabbit data to facilitate comparisons. Solid lines are best-fit lines. Dotted lines connect the mean value at each time point. Key: △, 10 μl injected in albino rabbits; and ○, 10 μl injected in pigmented rabbits.*

aqueous barrier with a concomitant rise in the protein content of the aqueous humor. In the inulin study, plasmoid formation was visually observed at the 30-min time point and beyond. Plasmoid formation results in an extremely viscous aqueous humor, which is difficult to aspirate into the tuberculin syringe. It appears as a transparent semisolid, gel-like substance which undergoes shear thinning with repeated uptake and discharge through the syringe needle.

Formation of plasmoid aqueous in the rabbit occurs with very mild trauma, whereas the human eye seems much more resistant to its formation (23). Since plasmoid aqueous does not appear to affect vision, at least in the human eye, and the calculated turnover rate constant of the aqueous humor from the present study agrees well with that of normal aqueous humor in rabbits, its formation does not appear to be a cause for concern in the present study. Apparently, the rabbit eye makes all necessary accommodative changes to compensate for the more viscous plasmoid aqueous. Plasmoid aqueous formation is, of course, a reversible process; in a relatively short period, *i.e.*, a few hours, a normal aqueous humor is again formed.

Figure 2 shows the aqueous humor drug concentration–time profile for pilocarpine, and Table I shows the apparent volume of distribution and turnover rate constant for pilocarpine derived from graphical analysis of the data. From the data in Table I, it is clear that the apparent volume of distribution for pilocarpine is twice that of the physiological volume and the turnover rate constant is significantly greater than the normal value. The larger volume of distribution can be attributed to the fact that pilocarpine is somewhat protein bound, can and does penetrate ocular tissues, and may be bound to tissue surfaces (4, 24–27)[11].

Because protein contained in the aqueous humor leaves the eye during the normal removal of the aqueous humor, the present method is unable to discern whether or not a drug is highly bound to protein, as can be done with the traditional drug volume of distribution in the blood. However, the method can discern tissue penetration and binding of drugs to ocular tissue surfaces, as was shown clearly in the study of pilocarpine in dark iride rabbits (Fig. 3). The apparent volume of distribution for pilocarpine was 760 μl, considerably larger than the 575 μl determined in albino rabbits. For comparative purposes, Fig. 3 includes the albino rabbit data for pilocarpine. The decline in drug concentration between pigmented and nonpigmented animals was parallel. However, the 60-min point was considerably different between the pigmented and nonpigmented animals. No explanation for this difference is offered, but it is important to recognize that the data from albino rabbits are based on only four eyes with a large standard error and that the radioactive counts at this level are uncomfortably low. Thus, we have less confidence in this point as compared to others.

The shape of the drug concentration–time profile for pilocarpine was considerably different than that of inulin. A biphasic curve was not observed over the time course of the study, and the slope of the line yielded a rate constant considerably greater than physiological turnover of the aqueous humor. A suggested explanation for this behavior is related to the pharmacological effect of pilocarpine. It is well known that pilocarpine exerts various pharmacological effects in the eye, such as decreased anterior chamber depth (28) and an influence on aqueous humor turnover (9, 29–30). These effects were absent with inulin as well as with 1-hexanoic acid, and both of these compounds showed the expected biphasic appearance. In support of the idea that this finding is not an artifact of the present technique, it was observed that the 1st hr or so beyond the peak of the aqueous humor pilocarpine–time profile, from topically applied drug, also showed an increased rate of removal over and above physiological turnover[11].

As an alternative explanation for the data, it is possible that the apparent one-compartment profile for pilocarpine represents a nonsteady-state distribution into other tissues, which would invalidate this approach for pilocarpine apparent volume of distribution measurement. However, other data[11] do not support this thesis; therefore, a pharmacological effect is offered as a reasonable explanation. As added support for a pharmacological effect explanation, a study was undertaken where inulin and pilocarpine were injected together and the decline in inulin concentration was monitored as a function of time. If a pharmacological effect from pilocarpine occurs, it would be expected that the decline in inulin concentration, when an inulin–pilocarpine solution is injected, would be more rapid and perhaps follow different kinetics than when inulin is administered by itself.

Figure 4 shows the results of this study as well as the data for inulin and pilocarpine. Inulin did indeed leave the aqueous chamber at a more rapid rate when pilocarpine was present. Although there are insufficient data to draw a firm conclusion, it appears that the inulin–pilocarpine data closely parallel the pilocarpine elimination line, and it is considerably different than the inulin line by itself.

When pilocarpine was injected into the anterior chamber, no plasmoid aqueous humor was observed up to 60 min postinjection while with inulin and 1-hexanoic acid, a plasmoid aqueous formed readily.

Figure 5 presents the aqueous humor drug concentration–time profile for 1-hexanoic acid. This compound, of course, is not used in ophthalmic therapy. However, because it is a medium chain-length fatty acid, it can be expected to penetrate tissues readily and to have a relatively large apparent volume of distribution. As indicated in Table I, its apparent volume of distribution was approximately 2.5 times larger than the true physiological volume of distribution obtained in the inulin study.

Graphical analysis of the 1-hexanoic acid data yielded an apparent two-compartment open model with the same volume effect initially as that shown for inulin. The break points in the curves for the two volumes followed the same pattern established for inulin, and this finding lends credibility to the volume effect argument.

[11] M. C. Makoid, University of Wisconsin, Madison, Wis., unpublished data.

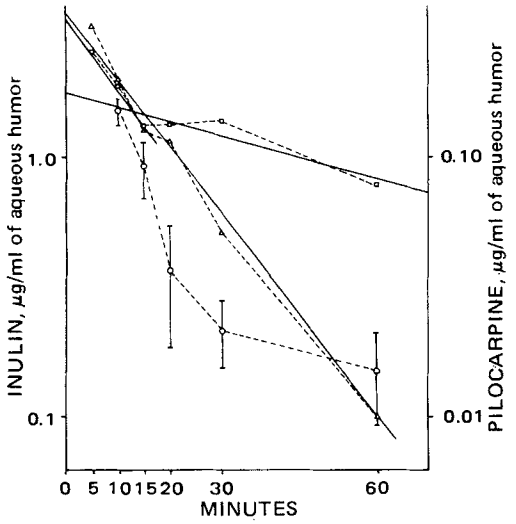

Figure 4—*Comparison of aqueous humor drug concentrations following intracameral injection of 10 μl of 2 × 10^{-5} M inulin solutions (□), 1 × 10^{-4} M pilocarpine solutions (△), or 2 × 10^{-5} M inulin in 1 × 10^{-4} M cold pilocarpine solutions (○) in albino rabbits. Error bars represent the standard error of the mean. Error bars were deleted from the 2 × 10^{-5} M inulin and from the 1 × 10^{-4} M pilocarpine data to facilitate comparisons. Solid lines are best-fit lines. Dotted lines connect the mean value at each time point.*

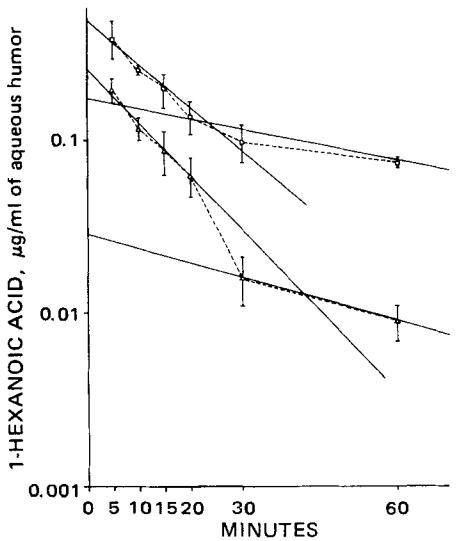

Figure 5—*Aqueous humor drug concentrations following intracameral injection of 1.67 × 10^{-4} M 1-hexanoic acid solutions in albino rabbits. Error bars represent the standard error of the mean. Where no error bars are drawn, the standard error was smaller than the symbol used. Solid lines are best-fit lines. Dotted lines connect the mean value at each time point. Key: △, 10 μl injected; and □, 20 μl injected.*

Another feature of the apparent two-compartment open model used to describe the inulin and 1-hexanoic acid data is that the turnover rate constant obtained after curve stripping of the initial phase of the curves, *i.e.*, that portion attributed to the volume effect, agrees reasonably well with the turnover rate constant found for pilocarpine in that study. This result indicates that the volume effect is qualitatively similar to the pharmacological effect produced by pilocarpine.

DISCUSSION

A primary consideration of this study was the development of a technique for systematically measuring the apparent volume of distribution of ophthalmic drugs. The method itself is obviously unrealistic for human use, since it involves puncturing the cornea and direct injection of drug solution into the anterior chamber. However, the insights gained from animal studies are useful in providing initial estimates of ocular behavior in humans. For example, since the rabbit eye is similar in size and gross morphology to the human eye, a drug's apparent volume of distribution measured in rabbits is expected to approximate that of human eyes.

The described method has limitations. As mentioned previously, protein binding will not be detected *per se*, because the movement of drug bound to protein will be indistinguishable from free drug movement. Both bound and free drug leave the chamber *via* normal aqueous humor drainage, and the present technique cannot distinguish free and bound drug.

A second limitation is that the drug must be in an aqueous vehicle. Since it is injected into a small confined aqueous milieu, an oil-soluble drug in an oily vehicle could precipitate in the anterior chamber and block the aqueous humor drainage pathways or the oil vehicle could cause a physical blockade to drainage.

Based on the previous discussion, relative to the effect of injected volume on the pharmacokinetic profile, it is clear that there should be constraints on the volume employed. Too large a volume may result in possible tissue damage so that an upper limit is placed on the accommodative mechanism of the rabbit eye. A small volume is limited by the reproducibility of repetitive injections. It is suggested that the 10-μl volume be used on a routine basis, because the 5-μl volume requires considerable skill for reproducibility and the 20-μl volume might generate damage to the eye, particularly if small rabbits are employed. However, if solubility problems or assay limitations are encountered, the 20-μl volume can be employed; in fact, a 30-μl volume can be used. Studies using 30 μl were also conducted for inulin, and the results (not reported here) from these studies are consistent with the reported volumes.

One concern with the proposed experimental technique was that having the test animal under general anesthesia throughout the experiment might decrease the rate of aqueous humor formation and removal (6, 31). Results, namely that physiological turnover rates of the aqueous humor were determined from the inulin and 1-hexanoic acid data, indicate that this reduction or stoppage does not occur in the rabbit under anesthesia.

Several additional observations made during this study require further comment. As noted earlier, the trauma of intracameral injection and increased volumes in the chamber due to injection caused the formation of plasmoid aqueous within 30 min postinjection of inulin and 1-hexanoic acid. At first glance, it would seem likely that this more viscous, proteinaceous aqueous humor should not leave the anterior chamber very easily, thus leading to a slow turnover rate. This is obviously not the case. Two possible mechanisms can explain this finding. Either the rabbit's trabecular meshwork expands to accommodate the plasmoid aqueous or a microdiffusion effect removes the more fluid portions of the plasmoid, leaving behind a "matrix" of highly viscous protein plasmoid aqueous which is subsequently removed at a slower rate.

If increased accommodation is the method of plasmoid aqueous removal, this process is easily reversible since, upon removal of the cause of plasmoid aqueous, the rabbit aqueous humor returns to normal within a matter of a few hours (32). The accommodation must be throughout the anterior and posterior chambers because, not only does the viscous plasmoid leave at a normal rate, it must form and enter the posterior chamber at a normal rate to maintain a constant turnover rate (20, 32).

The second possibility, a microdiffusion effect, would involve formation and destruction of a protein matrix with relatively free flow of the aqueous humor in and through this matrix. If this microdiffusion mechanism is operative, it presents an interesting form of sustained release for any highly protein-bound drug. The drug would be bound to the protein matrix, which should prolong its residence time in the aqueous chamber. These possible mechanisms for plasmoid aqueous removal are interesting and require additional work. However, the most important factor relative to the proposed technique is that plasmoid aqueous formation does not interfere with the apparent volume of distribution determination.

The pilocarpine and 1-hexanoic acid data were intriguing from several points of view. As noted in Table I, the apparent volume of distribution for pilocarpine in the aqueous humor was 575 μl, and the concentration–time data fit a monoexponential curve with an apparent first-order rate constant of 0.059 min^{-1}. The apparent volume of distribution was twice the physiological volume, which was attributed to sorption of the drug into and onto tissues lining the aqueous chamber (4, 24–27)[11]. Since published studies on the fraction of dose absorbed for pilocarpine have relied on an assumed volume of distribution of 250–300 μl, these values probably are off by a factor of two for albino rabbits and by a factor of almost three for pigmented rabbits. The more important point is that other drugs, as illustrated by 1-hexanoic acid, could have much larger

apparent volumes of distribution and be off by factors of three, four, or greater.

In defense of the proposed mechanism for the increased turnover rate constant due to pilocarpine, *i.e.*, a pharmacological effect, a comparison of these results in rabbits with those obtained in humans (33) for topically applied pilocarpine is in order. Yoshida and Mishima (33) found that the rate constant for normal aqueous turnover in humans was 0.7 hr^{-1} while that following topical dosing with pilocarpine was 0.27 hr^{-1}. In the present study, the normal aqueous turnover in rabbits was 0.72 hr^{-1} while the turnover following pilocarpine injection was 4.2 hr^{-1}. It would appear that these data are in conflict. However, it should be kept in mind that topical dosing leads to a reservoir effect in the corneal epithelium (34) so that, at later times, the turnover of pilocarpine appears slower than it actually is. As pilocarpine leaves the aqueous chamber, more drug is being fed in to maintain a level of drug in the aqueous chamber that falls less rapidly than would occur if the reservoir was absent. Application of drug through direct injection bypasses the reservoir effect of the corneal epithelium and generates a more rapid removal of the aqueous humor, in keeping with what one would intuitively expect for a glaucoma drug. Over the time course of these studies, pilocarpine apparently is unable to penetrate the corneal epithelium from direct injection so there is no reservoir effect. Recent evidence (4, 24)[11] demonstrates that pilocarpine disposition from the aqueous humor is quite complex and does not follow a simple one- or two-compartment model.

From the results of this pilocarpine study in albino and pigmented animals, it is clear that the method can discern binding. Thus, the earlier noted differences in pilocarpine uptake in heavily pigmented animal eyes (4, 25) as well as humans (26) have been borne out in an *in vivo* experiment and support the need for more drug to treat glaucoma in heavily pigmented eyes.

1-Hexanoic acid data were biphasic, with the terminal portion of the curve having an apparent rate constant of 0.017 min^{-1}, in agreement with normal aqueous humor turnover. One would intuitively expect that the elimination of a drug that distributes extensively into aqueous chamber tissues would be influenced by this distribution and that, therefore, the terminal portion of the 1-hexanoic acid curve should probably not correspond to normal turnover. One can speculate that drug taken up by tissue is removed from the aqueous chamber by absorption into the general circulation or perhaps that the drug is irreversibly bound to tissue or in some manner degraded.

REFERENCES

(1) H. K. Von Muller, O. Hockwin, and O. Kleifeld, *Graefes Arch. Ophthalmol.*, **162,** 107 (1960).

(2) J. E. Harris, in "Symposium on Ocular Therapy," J. H. Leopold, Ed., C. V. Mosby, St. Louis, Mo., 1968, p. 99.

(3) C. F. Asseff, R. L. Weisman, S. M. Podos, and B. Becker, *Am. J. Ophthalmol.*, **75,** 212 (1973).

(4) R. Lazare and M. Horlington, *Exp. Eye Res.*, **21,** 281 (1975).

(5) V. E. Kinsey and D. V. N. Reddy, in "The Rabbit in Eye Research," J. H. Prince, Ed., Charles C Thomas, Springfield, Ill., 1964, p. 235.

(6) S. J. Cevario and F. J. Macri, *Invest. Ophthalmol.*, **13,** 384 (1974).

(7) F. J. Macri and J. O'Rourke, *Arch. Ophthalmol.*, **83,** 741 (1970).

(8) F. J. Macri, R. Dixon, and D. P. Rall, *Invest. Ophthalmol.*, **5,** 386 (1966).

(9) J. Edwards, V. L. Hallman, and E. S. Perkins, *Exp. Eye Res.*, **6,** 316 (1967).

(10) F. J. Macri, *Arch. Ophthalmol.*, **78,** 629 (1967).

(11) W. W. Oppelt, *Invest. Ophthalmol.*, **6,** 76 (1967).

(12) F. J. Marci, R. L. Dixon, and D. P. Rall, *ibid.*, **4,** 927 (1965).

(13) J. K. Lauber, J. E. Boyd, and T. A. S. Boyd, *Exp. Eye Res.*, **13,** 77 (1972).

(14) E. Barany and V. E. Kinsey, *Am. J. Ophthalmol.*, **32,** 177 (1949).

(15) S. S. Chrai and J. R. Robinson, *ibid.*, **77,** 735 (1974).

(16) T. F. Patton and J. R. Robinson, *J. Pharm. Sci.*, **64,** 1312 (1975).

(17) T. J. Mikkelson, S. S. Chrai, and J. R. Robinson, *ibid.*, **62,** 1648 (1973).

(18) S. S. Chrai, T. F. Patton, A. Mehta, and J. R. Robinson, *ibid.*, **62,** 1112 (1973).

(19) D. M. Maurice, *Am. J. Ophthalmol.*, **47,** 361 (1958).

(20) W. G. Unger, D. F. Cole, and B. Hammond, *Exp. Eye Res.*, **20,** 255 (1975).

(21) M. de Vincentiis, *J. Physiol.*, **146,** 252 (1959).

(22) H. Davson and C. B. Weld, *Am. J. Physiol.*, **134,** 1 (1941).

(23) F. H. Adler, "Physiology of the Eye," C. V. Mosby Co., St. Louis, Mo., 1965, pp. 96–143.

(24) L. Sendelbeck, D. Moore, and J. Urquhart, *Am. J. Ophthalmol.*, **80,** 274 (1975).

(25) J. S. Lyons and D. L. Krohn, *ibid.*, **75,** 885 (1973).

(26) L. S. Harris and M. A. Galin, *ibid.*, **72,** 923 (1971).

(27) D. A. Newsome and R. Stern, *ibid.*, **77,** 918 (1974).

(28) D. H. Abramson, D. J. Coleman, M. Forbes, and L. A. Franzen, *Arch. Ophthalmol.*, **87,** 615 (1972).

(29) P. P. Ellis, *Surv. Ophthalmol.*, **16,** 165 (1971).

(30) L. S. Harris, T. W. Mittag, and M. A. Galin, *Arch. Ophthalmol.*, **86,** 1 (1971).

(31) B. Becker, T. Krupin, and S. M. Podos, *Am. J. Ophthalmol.*, **70,** 686 (1970).

(32) H. Davson, in "The Eye," vol. I, 2nd ed., Academic, London, England, 1969, pp. 67–186.

(33) S. Yoshida and S. Mishima, *Jpn. J. Ophthalmol.*, **19,** 121 (1975).

(34) J. W. Sieg and J. R. Robinson, *J. Pharm. Sci.*, **65,** 1816 (1976).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 26, 1976, from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706.*

Accepted for publication April 15, 1976.

Supported by Allergan Pharmaceuticals, Irvine, CA 92664, and Grant EY-01332 from the National Institutes of Health.

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