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AGE-RELATED DIFFERENCES IN OPHTHALMIC DRUG DISPOSITION II: DRUG–PROTEIN INTERACTIONS OF PILOCARPINE AND CHLORAMPHENICOL

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ABSTRACT

Although drug–protein interactions have been shown to be important in the transport of drugs into and through the eye, age-related differences in ocular drug–protein binding have not been explored. This study was designed to investigate protein binding characteristics of pilocarpine and chloramphenicol in aqueous humour and plasma from rabbits of different ages. The method of equilibrium dialysis was used to quantitate drug–protein interactions. Younger animals showed decreased binding of chloramphenicol in plasma when compared to older rabbits. On the other hand, the interactions of both drugs with aqueous humour proteins were more extensive in younger rabbits. Age-related differences in drug–protein binding were not directly related to the total protein contents of the respective biological fluids. These findings suggest possible age-related differences in the relative protein fractions contained in aqueous humour and plasma, or qualitative differences in the binding capacity of the proteins involved.

KEY WORDS Pilocarpine Chloramphenicol Drug–protein interactions Age-related Rabbits

INTRODUCTION

Numerous factors can influence ocular drug bioavailability. These have been reviewed[1, 2] and include drug-specific properties as well as drug-independent phenomena. Age-related differences in the ocular bioavailability of topically administered drugs have been observed in rabbits.[3] Such observations have important implications from both a therapeutic and toxicity viewpoint.[4, 5] This paper is the second in a series dealing with age-related differences in ophthalmic drug disposition. The first paper addressed the effect of size on the intraocular tissue distribution of pilocarpine.[6] This present paper, describes age-related differences in ocular drug–protein interactions.[6]

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The binding of drugs to proteins present in ocular fluids and tissues has been shown to influence their transport into and through the eye.[7] Age-related differences in the binding of drugs to ocular proteins have not been reported. This study was designed to investigate protein binding characteristics of pilocarpine and chloramphenicol in aqueous humour and plasma from rabbits of different ages. Two age categories of rabbits were used in the study; younger rabbits being approximately 20-days of age and older animals being about 60-days old. Equilibrium dialysis was used to quantitate drug–protein interactions.

Younger rabbits showed decreased binding of pilocarpine but comparable binding of chloramphenicol in plasma, when compared with older animals. On the other hand, the interactions of both drugs with aqueous humour proteins were more extensive in younger rabbits. In most instances, differences in drug–protein interactions did not correlate with differences in the total protein content of the various biological fluids.

Results suggest that the developmental state of the blood–aqueous barrier may influence the extent of drug binding to proteins present in aqueous humour. In addition, age-related differences in drug binding to plasma proteins may influence the penetration of systemically administered drugs into the eye.

EXPERIMENTAL

Materials

Male, New Zealand white rabbits were obtained from small Stock Industries, Inc., Pea Ridge, AR. Prior to experimentation, the animals were housed in standard animal cages with no restrictions on food or water intake. Whenever possible, rabbits used were either 20- or 60-days old. Owing to the difficulty of obtaining a sufficient number of animals of these exact ages, a range of ages around these two were used. Reference to 20-day old rabbits implies the use of rabbits 17 to 23-days of age. Similarly, 60-day old rabbits were between 56- and 65-days of age.

Pentobarbital sodium (Nembutal®) was obtained from Abbott Laboratories, North Chicago, IL. Heparin sodium (Lipo–Hepin®) 5000 U.S.P. units ml^{-1} was obtained from Riker Laboratories, Northridge, CA. Tetracaine hydrochloride ophthalmic solution U.S.P. (Pontocaine® hydrochloride) was obtained from Winthrop Laboratories, New York, NY. Coomassie brilliant blue, chloramphenicol, and pilocarpine nitrate were obtained from Sigma Chemical Co., St. Louis, MO. Rabbit albumin 4× crystallized was obtained from ICN Pharmaceuticals, Cleveland, OH.

Radiolabelled [^{3}H(G)]-pilocarpine, [dichloroacetyl-1,2-^{14}C]-chloramphenicol and Aquasol™ were obtained from New England Nuclear, Boston, MA. Silica gel G plates were obtained from Analtech, Newark, DE. Dialysis tubing was obtained from Union Carbide, Chicago, IL. Water used in these

studies was deionized and charcoal filtered. All other chemicals used were of analytical or reagent grade.

Methods

Collection of biological fluids. Rabbits were anaesthetized with ether and blood was collected, via cardiac puncture, into heparinized syringes. the sample was transferred to polystyrene centrifuge tubes and centrifuged at 2500 rev-min^{-1} for 15 min. The plasma was removed from the sample and the red cells discarded. Plasma samples were either used immediately or quick-frozen (dry ice/acetone bath) and stored in glass vials at $-5°$.

Primary (normal) aqueous humour samples were obtained from animals under pentobarbital anaesthesia approximately 2 min after a single drop of tetracaine hydrochloride had been applied topically to the eye. Secondary aqueous humour samples were obtained from animals immediately after sacrifice with an overdose of pentobarbital. All aqueous humour samples were obtained by making a single puncture at the limbus, using a $27G \times \frac{1}{2}$ in needle attached to a 1 ml tuberculin syringe. The tip of the needle was placed in the centre of the anterior chamber and the aqueous humour was aspirated by gently pulling back on the plunger. Aqueous humour samples not used immediately were frozen and stored as described for the plasma samples. Rabbits were used only once for collection of biological fluids and samples obtained from several animals were pooled.

Preparation of radiolabelled drug solutions. Tritiated pilocarpine was obtained as the alkaloid in ethanol solution and was evaporated from ethanol several times prior to use to remove any solvent that had become labelled via tritium exchange.[8] The specific activity of the pilocarpine varied from 4·1 to 10·0 Ci $mmol^{-1}$ depending on the lot received. In all cases the radiochemical purity was reported by the manufacturer to be greater than 97 per cent and this was verified by the following thin-layer chromatography (TLC) procedure.

Approximately 3 µl of ^{3}H-pilocarpine, as received from the manufacturer, was spotted on silica gel G plates. The plates were developed in 1 : 1, methanol : chloroform,[9] radiochromatographically scanned (Packard Model 7230, Downer's Grove, IL), and the chromatograms were integrated (Packard Model 7240). For all lots received, the experimentally determined radiochemical purity was in agreement with that reported by the manufacturer.

Pilocarpine nitrate solutions were prepared in isotonic Sorensen's phosphate buffer,[10] pH 7·38. Sufficient labelled pilocarpine was added to the 'cold' solutions to ensure good counting efficiency. The final molarity of the solution was calculated based on the contribution of both labelled and unlabelled pilocarpine. All solutions were prepared on the day of use, and any unused portion was discarded.

Radiolabelled ^{14}C-chloramphenicol was obtained in ethanol and was evaporated from ethanol two times prior to use. Specific activity was

43·2 mCi mmol^{-1} and radiochemical purity was 98·5 per cent as reported by the manufacturer. Experimentally, the radiochemical purity was determined to be 97 per cent using a TLC procedure which, except for the mobile phase, was similar to that described above for pilocarpine. TLC plates spotted with chloramphenicol were developed in benzene : methanol : acetic acid, 45 : 8 : 4.

Chloramphenicol solutions were prepared in a modified Palitzsch borate buffer[11] at pH 7·36. Sufficient labelled chloramphenicol was added to the 'cold' solutions to ensure good counting efficiency and the final molarity of the solutions were determined based on the amount of 'hot' and 'cold' chloramphenicol added.

Total protein assays of rabbit plasma and aqueous humour. Rabbit aqueous humour and plasma samples were assayed for their total protein content by a modification of the method of Bradford.[12] The method involves the binding of coomassie brilliant blue (CBB) to protein which causes a bathochromic shift in the absorption maximum of the dye from 465 nm to 595 nm. The increase in absorbance at 595 nm is monitored via visible spectrophotometry.

The protein reagent was prepared by dissolving 100 mg of CBB in 50 ml of methanol. After dissolving the dye, 100 ml of 85 per cent (w/v) phosphoric acid was added. The resulting solution was brought to a final volume of 1·0 litre with water. The reagent was filtered through Whatman No. 1 paper and stored in an amber glass bottle at room temperature. The reagent was standardized using rabbit serum albumin (RSA) as described below. Reagent solutions kept for a period of time longer than two weeks were restandardized before use.[13]

A series of RSA solutions, ranging in concentration from 0·5 to 5·0 mg ml^{-1}, were prepared in water. A 10 µl sample was placed in a 12 × 100 mm test tube and 100 µl of phosphate buffer (pH 7·38) were added. The solution was vortexed for approximately 5 s. Five millilitres of protein reagent were added, the solution was revortexed, and then allowed to set at room temperature for 10 min.

The solution was transferred to a 1 cm pathlength, disposable plastic cuvette and the absorbance at 595 nm was determined via visible spectrophotometry (Model-20, Hitachi, Ltd., Tokyo, Japan). Absorbance readings were corrected using a regent blank consisting of 100 µl of buffer and 5 ml of reagent.

It has been reported[12] that the protein–dye complex adsorbs onto the sides of quartz cuvettes but the problem was not encountered when using glass cuvettes. Adsorption of the complex onto the plastic cuvettes used in these studies was also noticed, therefore, cuvettes were disposed of after a single use. Absorbance readings at 595 nm using a set of eight different cuvettes filled with phosphate buffer varied by less than 0·3 per cent. Therefore, it was felt that the error introduced by the use of separate cuvettes for each reading was within tolerable limits.

Over the concentration range of RSA solutions prepared, plots of µg of protein vs. O.D. 595 nm obeyed Beer's law. Between individual reagent solutions, the slope of the line obtained from such plots varied somewhat.

Generally, however, the slope (absorbtivity) was about 48·5 ml mg^{-1} with coefficients of determination in all cases being greater than 0·98.

Before assaying for total protein content, the plasma samples were diluted with phosphate buffer such that the final concentration of total protein would be within the range of concentrations used in the preparation of the standard curve. The method of dilution (i.e. the final protein concentration after dilution) did not affect the results of the assay. Ten microlitres of diluted plasma were assayed as outlined in the procedure for the standard curve. A series of controls were also run to verify that heparin, used as an anti-coagulant in the collection of blood samples did not interfere with the protein assay.

Primary aqueous humour samples were assayed using 10 µl of the sample without dilution. Aqueous humour obtained from the rabbits post-mortem were generally too high in protein content to assay undiluted. Therefore, 2 µl of these samples were added to 8 µl of phosphate buffer before following the above described procedure.

Equilibrium dialysis. In all experiments, an acrylic, multi-cavity microdialysis cell (Bel-Art Products, Pequannock, NJ) of 1 ml half-cell capacity was used. The protein and drug compartments were separated by a sheet of cellulose dialysis tubing which had been cut open on one edge to obtain a single thickness. Prior to use, the dialysis tubing was washed by soaking it in deionized water and having deionized water also run through it for 6 h. The tubing was then stored in deionized water at 5° until used.

The half-cell chambers were filled using a microlitre syringe, placing the protein solution on one side of the membrane and the drug solution on the other. During dialysis the chambers were sealed by Teflon screws placed in the filling chambers. The cell was attached to an overhead stirring motor, immersed in water bath at 33° and rotated at 35 rev min^{-1} for 4 h. Results of preliminary experiments indicated that 3 h was a sufficient length of time to obtain an equilibration between the drug and protein solutions.

At the end of the experiments, the solution in the 'drug side' of the cell was removed using a disposable pasteur pipette and transferred to a glass screw-cap vial. A minimum of 5 aliquots of this solution were then transferred to individual, polyethylene mini-vials containing 5 ml of pre-refrigerated Aquasol®. Prior to counting, samples were stored in the dark for 24 h to minimize photoluminescence. Samples were counted for 5 min in a liquid scintillation spectrometer (Model LS-7000, Beckman Instruments, Irvine, CA). the counts obtained from each aliquot were corrected for background radiation and averaged for each individual cell. The drug concentration (free) was calculated on the basis of standards prepared from the initial stock solution. The equilibrium concentration and fraction of drug bound was obtained by difference.[14]

Three control experiments were also run. These consisted of dialysing: (1) buffer against buffer; (2) drug solution against buffer; and (3) protein solution against buffer. The controls indicated that neither pilocarpine nor chloramphe-

nicol was lost from the system due to adsorption onto the cell and/or membrane. In addition, there was no interference in the determination of drug concentration due to components of the membrane or protein solutions.

Plasma experiments were conducted by dialysing 1 ml of drug solution against an equal volume of plasma. In the aqueous humour binding studies 500 μl of drug solution was dialysed against an equal volume of aqueous humour. Preliminary binding studies with plasma indicated that the volume of fluids used in the study did not affect the experimental results. Results of these experiments suggested, that for the dialysis cell used, a minimum of 200 μl of solution in each half-cell was required to obtain reproducible results. Preliminary studies also indicated that freezing the biological fluids had no affect on their drug-binding behaviour. Therefore, the results of studies using fresh and frozen samples have been combined.

RESULTS

Total protein content of rabbit plasma and aqueous humour

To fully quantitate drug–protein interaction, the type and amount of protein to which drug binds must be identified. In addition, the association or affinity constant(s) a drug may have for a particular type of protein must be determined. None the less, determination of the total protein content of a biological fluid or tissue can aid in the preliminary characterization of drug–protein interactions. Therefore, although it was recognized that both of the biological fluids used were heterogeneous with respect to their protein composition, it was felt that a determination of the total protein content of these fluids might provide some initial insight relative to their drug-binding behaviour.

The total protein content of plasma and aqueous humour was determined in samples obtained from both ages of animals. The results of these studies are listed in Table 1. For both biological fluids, age-related differences in the total protein content were statistically significant. The total protein content of plasma obtained from 60-day old rabbits was slightly greater than that obtained from 20-day old rabbits. Unlike plasma, however, the protein content of both types of aqueous humour was higher in samples obtained from 20-day old rabbits than those 60-days of age. The protein content of primary aqueous humour showed considerable animal-to-animal variation, the coefficient of variation (C.V.) being about 64 per cent in both ages of rabbits. When the protein content of secondary aqueous humour was considered, however, the C.V. dropped to approximately 10 per cent. This variation was similar to the animal-to-animal variation observed in plasma protein content, the C.V. for plasma protein being approximately 10–15 per cent in each age category.

It is well established,[15–17] that ocular trauma and death result in a rapid breakdown of the blood–aqueous barrier, which permits a flux of protein into

the anterior chamber. Aqueous humour samples taken immediately after death of the rabbits contained a 3- to 6-fold higher concentration of protein than aqueous humour sampled while the animal was under general anaesthesia. The protein content of aqueous humour obtained post-mortem, however, was still 3 to 6 times less than that observed in the plasma.

Table 1. Total protein content of rabbit plasma and aqueous humour

Age*	Concentration (%)	n†	p‡
Plasma			
20	4·11 (0·21)§	9	<0·05
60	4·78 (0·21)	7	
Normal aqueous humour			
20	0·38 (0·05)	28	<0·001
60	0·15 (0·02)	17	
Secondary aqueous humour			
20	1·35 (0·13)	20	<0·001
60	0·78 (0·07)	21	

*Age is given in days.
†This number refers to the number of determinations: in plasma n represents samples taken from individual animals; in ocular fluids n represents samples obtained from individual eyes.
‡Probability of age-related differences in concentration based on a two-tailed weighted t-test ($\alpha = 0{\cdot}05$).
§Numbers in parentheses refer to the standard error of the mean.

It is possible that trauma, incurred during the sampling procedure, may account for some of the variability of the protein content of normal aqueous humour. It has been reported that pentobarbital (used for anaesthesia) can influence the permeability of the blood–aqueous barrier.[18] However, Anjou and Krakau have reported that pentobarbital induced anaesthesia does not appear to alter the protein content of aqueous humour in rabbits.[19]

In this laboratory, some preliminary investigations were undertaken to determine what effect, if any, pentobarbital anaesthesia had on the protein content of aqueous humour. In these studies, the protein content of aqueous humour obtained from 20-day old rabbits under general anaesthesia (pentobarbital) and local anaesthesia (tetracaine), was compared to the protein content of aqueous humour sampled under only local anaesthesia (tetracaine). When the results of these studies were analysed by a t-test, it was found that there was no significant difference in the protein content of aqueous humour obtained from rabbits under different anaesthetic conditions. Owing to the difficulty of obtaining aqueous humour from 60-day old rabbits using only a topical, local anaesthetic, all primary aqueous humour samples were subsequently collected as described in the 'Methods' section.

Additional, preliminary studies utilizing polyacrylamide gel electrophoresis were undertaken to assess qualitative differences in the proteins present in

aqueous humour and plasma between rabbits of different ages. The results of these investigations indicated that for plasma, the number and type (i.e. relative position) of protein bands present appeared to be the same in both ages of rabbits. The bands themselves appeared somewhat denser in plasma samples taken from 60-day old rabbits as compared to plasma from 20-day old rabbits, thereby indicating differences in the total amounts of the various proteins present in the two plasma samples. However, with the aqueous humour samples, the amount of protein present appeared to be slightly greater in samples from 20-day old rabbits than those 60-days of age and there appeared to be a greater number and proportion of high molecular weight proteins in the aqueous humour from the 20-day old rabbits.

Interaction of chloramphenicol and pilocarpine with rabbit plasma

The interaction of chloramphenicol and plasma proteins was investigated over a thousand-fold range of initial drug concentration ($1 \times 10^{-7} M$ to $1 \times 10^{-4} M$). Studies were conducted in plasma obtained from both 20-day old and 60-day old rabbits and the results of these are listed in Table 2.

Table 2. Binding of chloramphenicol in rabbit plasma

Initial concentration (M)	Final concentration* (M)	Fraction bound	n†
20-day old			
$1{\cdot}00 \times 10^{-7}$	$6{\cdot}21 \times 10^{-8}$ [2·11%]‡	0·39 (0·010)§	13
$1{\cdot}00 \times 10^{-6}$	$6{\cdot}35 \times 10^{-7}$ [1·71%]	0·43 (0·012)	5
$1{\cdot}00 \times 10^{-4}$	$6{\cdot}24 \times 10^{-5}$ [0·91%]	0·40 (0·006)	7
60-day old			
$1{\cdot}00 \times 10^{-7}$	$6{\cdot}03 \times 10^{-8}$ [3·12%]	0·34 (0·014)	14
$1{\cdot}00 \times 10^{-6}$	$6{\cdot}19 \times 10^{-7}$ [3·44%]	0·38 (0·016)	13
$1{\cdot}00 \times 10^{-4}$	$6{\cdot}16 \times 10^{-5}$ [1·66%]	0·38 (0·009)	7

* Final concentration refers to the average based on all determinations.
† This number refers to the number of determinations.
‡ Numbers in brackets refer to the coefficient of variation for the final concentration.
§ Numbers in parentheses refer to the standard error of the mean.

From the tabulated data, it can be seen that the binding of chloramphenicol was slightly greater in plasma samples obtained from 20-day old rabbits than those obtained from rabbits 60-days of age. Within an age group, slight

differences in binding were observed at all 3 initial concentrations. None the less, in each case, the results of *t*-tests indicated that the age-related difference in the mean fraction bound for any initial concentration was not statistically significant.

The binding of pilocarpine in plasma obtained from both ages of rabbits was investigated over a hundred-fold range of initial concentration. Unlike chloramphenicol, pilocarpine binding in plasma showed marked age-related differences as noted in Table 3. Although the binding of pilocarpine was considerably greater in plasma obtained from 60-day old rabbits than plasma from 20-day old rabbits, within an age group, the binding of pilocarpine was essentially constant over the drug concentration range investigated.

Table 3. Binding of pilocarpine in rabbit plasma

Initial concentration (*M*)	Final concentration* (*M*)	Fraction bound	*n*†
20-day old			
$1{\cdot}12 \times 10^{-6}$	$5{\cdot}50 \times 10^{-7}$ [8·31%]‡	0·10 (0·025)§	11
$1{\cdot}01 \times 10^{-5}$	$5{\cdot}43 \times 10^{-6}$ [1·39%]	0·14 (0·010)	7
$1{\cdot}00 \times 10^{-4}$	$5{\cdot}28 \times 10^{-5}$ [3·73%]	0·11 (0·019)	12
60-day old			
$1{\cdot}12 \times 10^{-6}$	$6{\cdot}62 \times 10^{-7}$ [6·14%]	0·30 (0·039)	7
$1{\cdot}01 \times 10^{-5}$	$5{\cdot}77 \times 10^{-6}$ [4·57%]	0·24 (0·031)	7
$1{\cdot}00 \times 10^{-4}$	$5{\cdot}79 \times 10^{-5}$ [4·93%]	0·27 (0·021)	16

* Final concentration refers to the average based on all determinations.
† This number refers to the number of determinations.
‡ Numbers in brackets refer to the coefficient of variation for the final concentration.
§ Numbers in parentheses refer to the standard error of the mean.

Interaction of chloramphenicol and pilocarpine with rabbits aqueous humour

Owing to the fact that, for both chloramphenicol and pilocarpine, the fraction of drug bound remained relatively constant over a wide range of initial drug concentrations, the binding of these drugs in aqueous humour was studied at only one initial concentration. For both drugs, the concentration chosen was $1{\cdot}00 \times 10^{-6}$ *M*. This is a concentration comparable to aqueous humour levels of pilocarpine achieved after topical dosing.

The extent of interaction, as indicated by the fraction of drug bound, of both chloramphenicol and pilocarpine with proteins in the aqueous humour is listed in Table 4. These results are from studies in which the binding of chloramphenicol and pilocarpine was investigated in aqueous humour obtained immediately after sacrifice of the rabbits. Studies were also conducted using aqueous humour obtained from anaesthetized rabbits. However, there was no detectable interaction of either chloramphenicol or pilocarpine with normal aqueous humour from rabbits of either age.

For chloramphenicol, binding in the secondary aqueous humour was considerably lower than that observed in plasma for both ages of rabbits. The same was true for the binding of pilocarpine in secondary aqueous humour of 60-day old rabbits. The binding of pilocarpine in secondary aqueous humour obtained from 20-day old rabbits, however, was comparable to the binding observed for pilocarpine in the plasma. Age-related differences in the binding of either drug in secondary aqueous humour were not significant as determined from the results of *t*-tests.

Table 4. Binding of chloramphenicol and pilocarpine in secondary aqueous humour

Drug*	Final concentration† (*M*)	Fraction bound	*n*‡
20-day			
Chloramphenicol	$5{\cdot}43 \times 10^{-7}$ [3·03%]§	0·16 (0·025)‖	5
Pilocarpine	$5{\cdot}36 \times 10^{-7}$ [3·41%]	0·13 (0·023)	7
60-day			
Chloramphenicol	$5{\cdot}26 \times 10^{-7}$ [4·10%]	0·097 (0·039)	4
Pilocarpine	$5{\cdot}23 \times 10^{-7}$ [2·31%]	0·086 (0·015)	8

* In all experiments the initial concentration was $1{\cdot}00 \times 10^{-6}$ *M*.
† Final concentration refers to the average based on all determinations.
‡ This number refers to the number of determinations.
§ Numbers in brackets refer to the coefficient of variation for the final concentration.
‖ Numbers in parentheses refer to the standard error of the mean.

DISCUSSION

Drug–Protein interaction in plasma

As listed in Table 1, the total protein content of plasma was determined to be 4·11 and 4·78 per cent for 20- and 60-day old rabbits, respectively. It has been reported,[20] that the protein content of serum obtained from rabbits 2–3-months

old averages 4·66 per cent, in reasonable agreement with the plasma protein determinations reported herein.

Table 2 lists the results of the studies in which the binding of chloramphenicol in plasma was investigated. From this table it can be determined that, for both ages of rabbits, the fraction of chloramphenicol bound in plasma remained relatively constant over the range of initial drug concentrations studied. Although a slightly greater extent of binding was observed in the younger rabbits, binding differences observed between the two ages of rabbits were not significantly different. In addition, the binding behaviour of chloramphenicol in plasma does not appear to correlate with the total protein content determined for this biological fluid. Although the total protein content of plasma was lower in 20-day old rabbits than those 60-days of age, the interaction of chloramphenicol with plasma appeared to be more extensive in the 20-day old rabbits.

Table 3 lists the results from studies in which the interactions of pilocarpine and rabbit plasmas were investigated. As with chloramphenicol, within an age group, the binding of pilocarpine in rabbit plasma remained relatively constant over the drug concentration range investigated, as evidenced by only small changes in the fraction of drug bound. In contrast to chloramphenicol, however, there was a marked difference in the binding behaviour of pilocarpine in plasma obtained from the two ages of rabbits. The binding of pilocarpine in plasma from 20-day old rabbits was significantly less than the binding observed in plasma from 60-day old rabbits.

The difference in pilocarpine-plasma binding between 20-day old and 60-day old rabbits was greater than the relative difference in the total protein content of plasma, from these two ages of rabbits. More specifically, depending on the initial drug concentration, the ratios for fraction bound (60/20) ranged between 1·72 and 3·03, whereas the total plasma protein ratio was only 1·16.

The results of the investigations of the interactions of chloramphenicol and pilocarpine with plasma obtained from 20-day old and 60-day old rabbits indicate that the binding of chloramphenicol in plasma was greater than that observed for pilocarpine. For chloramphenicol, there did not appear to be any age-dependency in the extent of drug–protein interaction. In addition, there was no direct correlation between the fraction of chloramphenicol bound in the two plasmas and their total protein contents. For pilocarpine, there was a marked difference in drug binding in plasma obtained from the two ages of rabbits, with binding in the 60-day old rabbits being more extensive than that observed in plasma from 20-day old rabbits. Age-related differences in pilocarpine-plasma binding also appeared to be greater than the difference observed in the total protein content of plasma obtained from 20-day old and 60-day old rabbits.

These results indicate that age-related differences in the binding of drugs to rabbit plasma proteins do exist. Furthermore, age-related differences in drug binding do not appear to correlate with age-related differences in the total plasma protein content. To better understand the changes that occur in drug–protein interactions, as a function of age, it appears necessary to identify

and quantitate the specific protein(s) to which these drugs bind. It would also be useful to determine whether endogeneous compounds may be interacting with these proteins, to cause qualitative protein differences which alter their drug binding capacity. Age-related differences in drug binding to plasma proteins may be particularly important when the developmental state of the blood–aqueous barrier is considered.

Drug–Protein interaction in aqueous humour

For primary aqueous humour, the total protein contents, determined in these studies, were higher than values generally reported for this ocular fluid, the reported values being in the range of 10–85 mg per cent.[19, 21] Since both the 20- and 60-day old rabbits used in these studies can be considered as immature animals, the higher aqueous humour protein content determined in these two ages of rabbits, in both cases, might reflect incompletely developed blood–aqueous barriers.[22–24]. Preliminary gel electrophoresis studies also suggest age-related differences in the blood–aqueous barrier between 20- and 60-day old rabbits.

The interaction of chloramphenicol and pilocarpine with secondary aqueous humour was investigated and the results of these studies are listed in Table 4. For both chloramphenicol and pilocarpine, there was no statistically significant difference in aqueous humour drug binding between the two age groups of rabbits. For both drugs, however, the trend was toward a greater extent of binding in aqueous humour from the 20-day old rabbits. Drug binding differences in the aqueous humour, between ages, appeared to be more closely parallel differences in the total protein content of this fluid, than binding studies with plasma. For chloramphenicol, the fraction bound ratio (20/60) was 1·65 and for pilocarpine the ratio was 1·51. The ratio of the total protein content of this fluid was 1·73; all 3 ratios being quite similar.

As indicated above, there was no detectable interaction of either chloramphenicol or pilocarpine with primary aqueous humour from rabbits of either age. The apparent lack of interaction of sulfisoxazole and methylprednisolone with primary aqueous humour has also been reported.[7] It appears, that for all of these drugs, the protein concentration in primary aqueous humour is too low for any drug protein interactions to be significant.

Detectable interactions of methylprednisolone, pilocarpine and sulfisoxazole have been reported[7] to occur with plasmoid aqueous humour, however. Plasmoid aqueous humour was obtained by sampling the anterior chamber 1 to 4 h after initial paracentesis. In the present studies, drug–protein interactions were also detectable for chloramphenicol and pilocarpine with aqueous humour obtained post-mortem. Mikkelson *et al.*[7] reported that the fraction of pilocarpine bound in plasmoid aqueous humour was about 0·4 when the initial drug concentration was of the order of 10^{-7} *M*. In the present studies, the interaction of pilocarpine with aqueous humour obtained immediately after sacrifice of the rabbits appeared to be less extensive, the fraction of drug bound for the two

ages of rabbits averaging about 0·1. The apparent discrepancy between the two observations may be attributable to either the higher initial concentration of drug used in these studies (10^{-6} *M*) or to a difference in the protein content of the aqueous humour obtained by the two methods of sampling. Since Mikkelson *et al.* did not determine the protein content of plasmoid aqueous humour, a direct comparison cannot be made.

A rather surprising result of the drug-binding studies was the observation that for pilocarpine, in 20-day old rabbits, the fraction of drug bound in secondary aqueous humour and plasma was virtually identical, despite a 3-fold difference in the total protein content of these two fluids. Such an observation may suggest that the specific proteins which pilocarpine interacts with are present in a difference proportion between the plasma and aqueous humour, than the ratio for total protein content of these fluids.

It has been reported that, in rabbits, the relative proportions of albumin and globulin in the anterior aqueous are approximately the same as in the plasma or serum.[25] The results reported herein, though, suggest that this may not be true for all ages of rabbits. Since aqueous humour is, at least in part, a filtrate of blood, these observations suggest age-related alterations in the permeability characteristics of the blood–aqueous barrier which may selectively allow certain drug-binding constituents of blood to pass into the eye while excluding others. Such differences may be particularly important for systemically administered drugs, as an incompletely developed blood–aqueous barrier might even allow drugs which are protein-bound to cross into the eye. This observation should be considered from a toxicity viewpoint, owing to the fact that many drugs have the potential to cause adverse ocular side effects.[26]

SUMMARY

Results of these studies indicate that age-related differences in drug binding to proteins present in rabbit aqueous humour and plasma do exist. However, the observed differences in drug–protein binding did not correlate with differences in the total protein contents of the biological fluids as discussed earlier. Drug–protein interactions of chloramphenicol and pilocarpine in normal aqueous humour are insignificant. Such interactions may become important, however, in pathological conditions of the eye which cause an abnormal flux of protein into the anterior chamber.[27] For both drugs, binding to plasma protein appeared to be linear over the concentration ranges investigated, as evidenced by the fraction of drug bound remaining relatively constant. Although the aqueous humour binding data is somewhat limited, results indicate that for both ages of rabbits, there is little correlation between aqueous humour and plasma protein binding of drugs. Drug–protein interactions may be influenced by the selectivity of the blood–aqueous barrier. Alternatively, the presence of endogeneous compounds in either biological fluid may alter the drug-binding capacity of its proteins. Age-related differences in drug binding to plasma

proteins may be particularly important when considered together with the developmental state of the blood–aqueous barrier.

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REFERENCES

1. T. F. Patton, in *Ophthalmic Drug Delivery Systems*, J. R. Robinson (Ed.), American Pharmaceutical Association, Washington, 1980, pp. 28–54.
2. S. P. Eriksen, in *Ophthalmic Drug Delivery Systems*, J. R. Robinson (Ed.), American Pharmaceutical Association, Washington, pp. 55–70.
3. T. S. Friedman and T. F. Patton, *J. Pharm. Sci.*, **65**, 1095 (1977).
4. T. F. Patton, *J. Pediatr. Ophthalmol.*, **14**, 254 (1977).
5. T. F. Patton and M. Francoeur, *Am. J. Ophthalmol.*, **85**, 225 (1978).
6. S. C. Miller and T. F. Patton, *Biopharm. drug dispos.*, **2**, 215 (1981).
7. T. J. Mikkelson, S. S. Chrai and J. R. Robinson, *J. Pharm. Sci.*, **62**, 1648 (1973).
8. S. S. Chrai and J. R. Robinson, *Am. J. Ophthalmol.*, **77**, 735 (1974).
9. P. H. Chung, T. F. Chin and J. L. Lach, *J. Pharm. Sci.*, **59**, 1300 (1970).
10. Remington's Pharmaceutical Sciences, 14th ed, Mack Publishing Co., Easton, PA 1970, p. 1554.
11. G. E. Schumaker (Ed.), *Am. J. Hosp. Pharm.*, **23**, 628 (1966).
12. M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
13. T. Spector, *Anal. Biochem.*, **86**, 142 (1978).
14. H. Kurz, H. Trunk and B. Weitz, *Arzneim. Forsch.*, **27**, 1373 (1977).
15. E. Bengtsson, *Acta Ophthalmol.* (Suppl), **130**, 3 (1977).
16. E. L. Howe and V. K. Cruse, *Arch. Ophthalmol.*, **96**, 1668 (1978).
17. M. H. Kottow and L. J. Seligman, *Am. J. Ophthalmol.*, **85**, 392 (1978).
18. A. Giardini and B. Bagolini, *Ann di ottal. e. clin. ocul.*, **80**, 25 (1954). [Abstr. *Am. J. Ophthalmol.*, **38**, 746 (1954)].
19. C. I. N. Anjou and C. E. T. Krakau, *Acta Ophthalmol.*, **39**, 95 (1961).
20. R. E. Kuhlman and H. E. Kaufman, *Arch. Ophthalmol.*, **63**, 41 (1960).
21. V. E. Kinsey and D. V. N. Reddy, in *The Rabbit in Eye Research*, C. C. Thomas, Springfield, MO, 1964, p. 223.
22. V. E. Kinsey and B. Jackson, *Am. J. Ophthalmol.*, **32**, 374 (1949).
23. V. E. Kinsey and M. B. Williamson, *Am. J. Ophthalmol.*, **32**, 509 (1949).
24. R. Uusitalo, J. Stjernschantz and A. Palkama, *Acta Ophthalmol.*, **54**, 17 (1976).
25. L. von Sallmann and D. H. Moore, *Arch. Ophthalmol.*, **40**, 278 (1948).
26. C. G. Mason, *J. Toxicol. Environ. Health*, **2**, 977 (1977).
27. J. P. Dernouchamps and J. Michiels, *Exp. Eye Res.*, **25**, 25 (1977).