Analysis of Theophylline by Automated Multidimensional High-Performance Liquid Chromatography Involving Direct Plasma Injection

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Received November 23, 1981, from the *Drug Metabolism Department, Riker Laboratories, Inc., Subsidiary of 3M, St. Paul, MN 55144.* Accepted for publication April 20, 1982.

Abstract □ A procedure of direct injection of whole plasma for the analysis of theophylline by an automated multidimensional high-performance liquid chromatographic (HPLC) technique is described. The procedure requires as little as 30 μl of plasma sample and has a linear range of 0.25–30 μg/ml. Unknown plasma samples, after the addition of internal standard, are directly injected into the HPLC system. The chromatographic procedure is fully automated, thus the attention and time required from the analyst is reduced to a minimum. About 70 samples can be analyzed per day with ~3 hr of analyst time.

Keyphrases □ Theophylline—analysis by automated multidimensional high-performance liquid chromatography involving direct plasma injection □ High-performance liquid chromatography—automated multidimensional analysis of theophylline involving direct plasma injection □ Direct plasma injection—analysis of theophylline by automated multidimensional high-performance liquid chromatography

Multidimensional high-performance liquid chromatography (HPLC) has been in use for quite some time. However, the majority of the applications do not involve the direct injection of plasma samples (1–5).

The purpose of the present investigation was to test the concept of analyzing drugs in plasma by the multidimensional column chromatography technique and to determine if the technique could be completely automated. The test compound in this case was theophylline. After the investigation was completed, an abstract outlining the feasibility of the concept for theophylline was published (6). In the present report, the successful application of a fully automated multidimensional HPLC technique for the routine analysis of theopnylline is described.

BACKGROUND

Monitoring plasma levels of drugs in clinical management of patients or studying pharmacokinetic parameters for a marketed or developmental drug both require the quantitative determination of the drug in biological fluids, most often in plasma. Generally used procedures require the separation of the drug from the plasma proteins by a solvent extraction or protein precipitation technique prior to quantitation. With the recently available gel permeation columns designed to separate water soluble polymers, it is possible to employ a gel permeation column in place of the normal extraction procedure for the purpose of separating the drug from the plasma proteins. Thus, a coupled-column or multidimensional column chromatography technique can be used. In this technique, the drug is first separated from the plasma proteins on a gel permeation column based on molecular size difference, then the drug is selectively transferred *via* a switching valve onto a reverse-phase column where, on the basis of partition characteristics, it is further separated from any endogenous material with a similar range of molecular weight. The first step eliminates the plasma proteins, and the second step separates and quantitates small molecular weight substances.

EXPERIMENTAL

Reagents and Apparatus—The tetrahydrofuran was distilled in glass[1], and the methanol was suitable for liquid chromatographic use[2]. All chemicals were analytical reagent grade. All HPLC analyses were performed using a liquid chromatograph equipped with two solvent delivery systems[3], an automatic liquid sampler[4], and a detector[5] with a 280-nm filter. Peak heights were measured by an integrator[6]. The two columns used were a gel permeation column[7] and a 25-cm × 4.6-mm i.d. reverse-phase column[8]. A 4-port switching valve with air actuator[9] was placed in line, and the valve switching was controlled by the integrator through an external relay. The integrator was programmed to turn the valve at a certain time during each sample run. Figure 1 shows the arrangement of apparatus.

Standard Solutions—Standard solutions of theophylline and β-hydroxyethyl theophylline[10] were prepared by diluting 400-μg/ml stock solutions of each compound. Concentrations used were 0.3, 0.6, 1.8, 4.2, 9.0, 18.0, and 36.0 μg of theophylline/ml for the theophylline standard and 8.0 μg of β-hydroxyethyl theophylline/ml for the internal standard. All solutions were made in 0.05 *M* sodium phosphate buffer, pH 7.0, and were kept refrigerated.

Mobile Phase—Mobile phase A consisted of 0.05 *M* sodium phosphate buffer, pH 7.0. The phosphate buffer was prepared from 0.1 *M* dibasic sodium phosphate and 0.1 *M* monobasic sodium phosphate. Mobile phase B was prepared by mixing 70 ml of methanol, 10 ml of tetrahydrofuran, 92 ml of 0.1 *M* sodium acetate buffer pH 5.0, and 828 ml of distilled water. The final composition was methanol–tetrahydrofuran–0.01 *M* sodium acetate buffer pH 5.0 (7:1:92, v/v/v). This mobile phase was reported previously for the analysis of theophylline by HPLC (7). The mobile

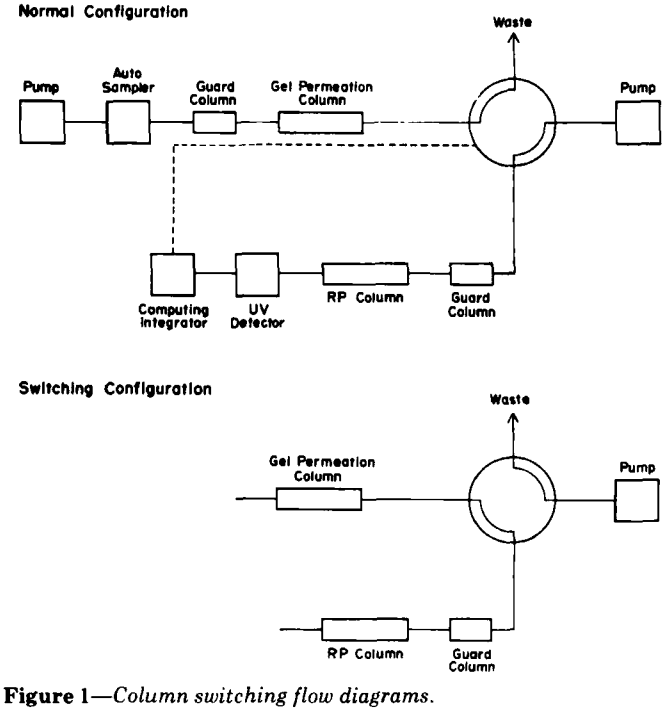

Figure 1—*Column switching flow diagrams.*

[1] Burdick-Jackson.
[2] Omnisolv, MCB.
[3] Model 6000, Waters Associates.
[4] Model 710A, Waters Associates.
[5] Model 440, Waters Associates.
[6] Model 4100, Spectra-Physics.
[7] I-125, Waters Associates.
[8] Ultrasphere-ODS, Altex, Inc.
[9] Helical Drive Air Actuator, Valco Instruments Co.
[10] Pierce Chemical Company.

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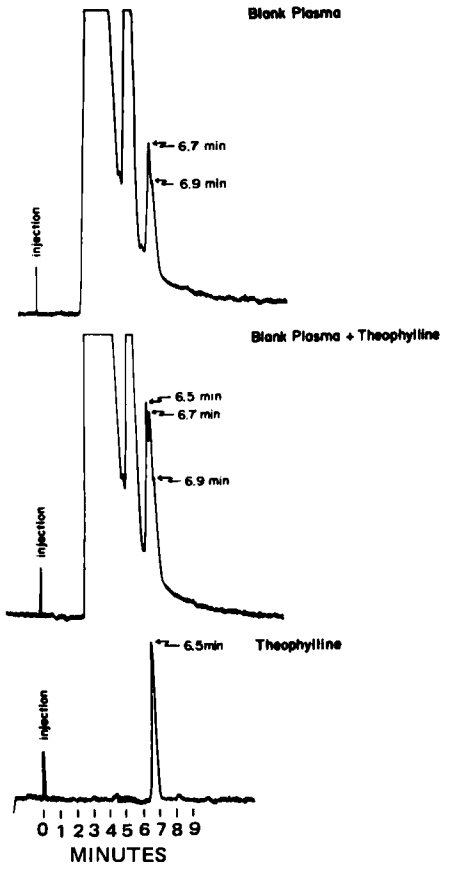

Figure 2—*Separation of theophylline from human plasma proteins on the gel permeation column.*

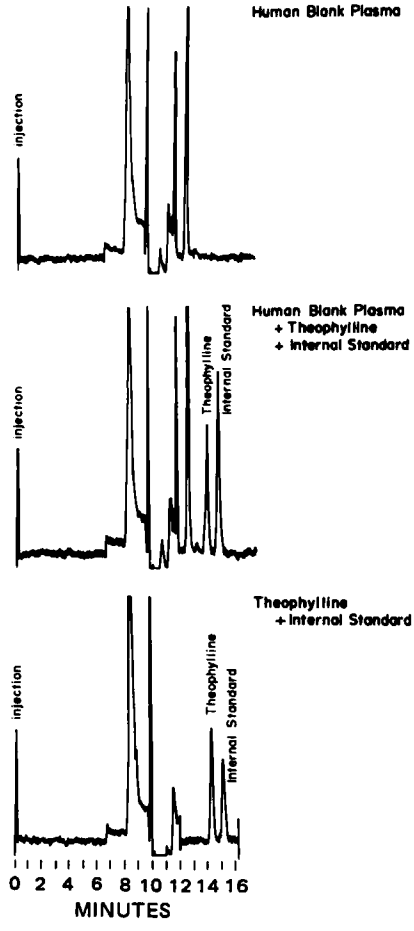

Figure 3—*Determination of theophylline in human plasma by a multidimensional technique.*

phases were filtered through a membrane filter[11] (pore size 0.45 μm) and deoxygenated prior to use.

Sample Preparation and Injection—Plasma, internal standard, and phosphate buffer (0.05 *M*, pH 7.0) were mixed in the ratio 30:25:25 (v/v/v). The minimum volume of plasma used was 30 μl. Eight theophylline standards were prepared by mixing blank plasma, internal standard, and the theophylline standard in the same ratio as above. The standard concentrations were 0, 0.25, 0.5, 1.5, 3.5, 7.5, 15, and 30 μg/ml plasma. To prevent overloading the gel permeation column, no more than 53 μl of the mixture (equal to 20 μl plasma) was injected into the liquid chromatograph. If duplicate injections are desired, the volumes for mixing should be doubled.

At 0 min, the valve was positioned so that mobile phase A flowed through the gel permeation column to the waste. Simultaneously, mobile phase B flowed through the reverse-phase column to the UV detector. The sample was injected onto the gel permeation column which separated the plasma proteins from the lower molecular weight materials, including theophylline. At the beginning of the theophylline peak the valve was switched so that effluent from the gel permeation column was directed onto the reverse-phase column. Mobile phase B then flowed directly to waste. The theophylline was concentrated at the head of the reverse-phase column by the aqueous mobile phase A. At 2 min after the switching, the valve was turned back to the original position. Mobile phase B was then flowing through the reverse-phase column where further separation of theophylline and the other lower molecular weight materials occurred. Actual switching times can vary by ±0.4 min, depending upon the retention time of theophylline on different gel permeation columns.

Method of Calculation—The construction of a standard curve, and subsequent calibration of unknown samples with the standard curve, was fully automated and was performed by a computing integrator. The integrator collected the peak height ratios (theophylline–internal standard) from the standards during a run, and the slope (A) and intercept (B) for the least-squares line between the peak height ratios (*x*-axis) and the theophylline concentrations (*y*-axis) were determined. The slope and intercept were automatically entered into the integrator's memory. For the unknown samples, the integrator determined the peak height ratio for each sample, retrieved the slope and intercept from the memory, and calculated the theophylline concentration in the following manner: theophylline concentration = peak height ratio × A + B. The final result of each unknown is reported in micrograms per milliliter.

[11] Millipore Corp.

RESULTS AND DISCUSSION

Determination of Valve-Switching Time—The purpose of the multidimensional technique was to separate the plasma proteins from the drug. This was accomplished through the switching valve. Thus, the time and duration that the valve is switched become a critical part of the overall procedure. An early switch will result in the transfer of plasma proteins onto the reverse-phase column and the rapid deterioration of that column. A late switch will result in the loss of the drug to waste. The duration of the time the valve was in the switching configuration controlled the quantitative transfer of the drug onto the reverse-phase column. For each drug studied and each gel permeation column used, the time and duration of the valve switching were predetermined. Figure 2 shows a set of typical chromatographic profiles of the gel permeation column alone. The majority of the UV-absorptive materials were eluted before 6 min. The theophylline started eluting at 6.3 min and peaked at 6.5 min; thus 6 min after the injection was chosen as the switching time of the valve. The peaks at 6.7 and 6.9 min from the blank plasma were also transferred onto the reverse-phase column, but were separated from theophylline and the internal standard and constitute no interference with the assay. Based on experimental results for theophylline, it was found that if the valve remained in the switching configuration for at least 2 min, a quantitative transfer of theophylline onto the reverse-phase column resulted.

Separation—The separation of theophylline and internal standard from endogenous materials in the plasma by the multidimensional technique is shown in Fig. 3. Baseline separation was achieved between theophylline and the internal standard. The retention times were 14.3 and 15.1 min, respectively, under the experimental conditions. There were

Table I—Intraday Precision and Accuracy

Sample Concentration[a], μg/ml	Mean ± *SD*, μg/ml	*RSD*, %	Relative Error, %
0.38	0.35 ± 0.01	2.9	−9.2
2.5	2.48 ± 0.05	2.0	1.0
11.3	11.35 ± 0.26	2.3	0.4
22.5	22.75 ± 0.70	3.1	1.1

[a] Four samples were used at each concentration.

two major peaks from the plasma which were also transferred onto the reverse-phase column during the switch; their retention times were 12 and 12.8 min, respectively, and constituted no interference with theophylline and the internal standard. The results clearly indicated that for theophylline, adaptation of an existing HPLC separation (7) to the multidimensional technique presented no chromatographic problems.

The application of the multidimensional technique did not prolong the elution time in comparison with the reported retention times of theophylline and the internal standard with the use of the same mobile phase and the same reverse-phase column alone; 15 and 17.5 min were reported for theophylline and the internal standard, respectively (7). The reason that this elution process with a rather long elution time was chosen for the multidimensional technique was that the procedure provided better specificity than most of the other HPLC methods for theophylline. The caffeine metabolite, 1,7-dimethylxanthine, was also separated from theophylline. The other major peaks seen in the chromatogram were due to the change of pressure and mobile phase on the reverse-phase column at the valve-switching step of the procedure. For repeated injections, the next injection was made before the last sample was completely eluted from the system. Under the experimental conditions, a sample was injected every 11 min. About 70 samples can be analyzed per day with ~3 hr of analyst time. This procedure is ideal for maximum utilization of the liquid chromatographic equipment for overnight runs with minimum requirement of an analyst's time for sample preparation.

Calibration Curve and Linearity—Daily calibration curves were prepared by spiking blank human plasma with theophylline standards resulting in final concentrations of 0, 0.25, 1.5, 3.5, 7.5, 15, and 30 μg/ml. The theophylline concentrations and the peak height ratios for theophylline and internal standard were fitted by the computing integrator to a straight line by the least-squares method. The mathematical representation of the line was y (theophylline concentration) = 0.2186x (response ratio) + 0.0182. The correlation coefficient was 0.9999. A linear relationship was demonstrated between response and theophylline concentration over a range of 0.25–30 μg/ml. The relationship was not tested beyond 30 μg/ml. At the 0.25 μg/ml concentration level, the response was 3–5 times the noise level of the system.

Precision and Accuracy—Intraday precision and accuracy were established by spiking blank human plasma in quadruplicate with theophylline at four concentration levels: 0.38, 2.5, 11.3, and 22.5 μg/ml. These spiked samples were analyzed by the procedure outlined in *Experimental*. The results are shown in Table I. The intraday precision, expressed as the relative standard deviation, was 2.9, 2.0, 2.3, and 3.1% for 0.38, 2.5, 11.3, and 22.5 μg/ml levels, respectively. The accuracy, expressed as relative error, was −9.2, 1.0, 0.4, and 1.1% for the four concentrations. The intraday precision and accuracy were excellent and certainly adequate for the monitoring of theophylline levels in clinical therapeutic settings and for more research-oriented pharmacokinetic studies. The interday precision was established by analyzing seven theophylline standards, 0.25, 0.5, 1.5, 3.5, 7.5, 15.0, and 30.0 μg/ml plasma, five separate times over an 8-month period. During this time, different

Table II—Interday Precision

Sample Concentration[a], μg/ml	Mean ± *SD*, μg/ml	*RSD*, %
0.25	0.31 ± 0.04	12.9
0.5	0.58 ± 0.11	19.0
1.5	1.52 ± 0.04	2.6
3.5	3.52 ± 0.08	2.3
7.5	7.44 ± 0.18	2.4
15.0	14.68 ± 0.24	1.6
30.0	30.16 ± 0.17	0.6

[a] Five samples were used at each concentration.

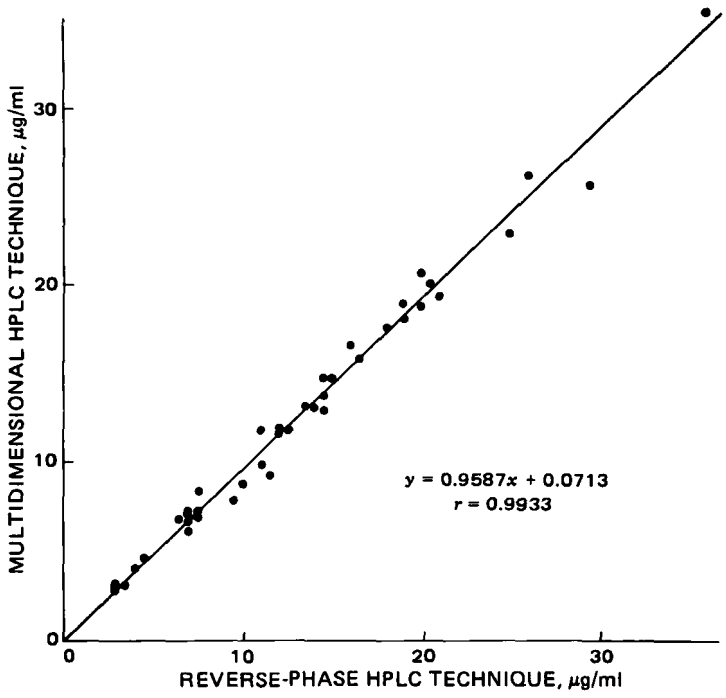

Figure 4—*Comparison of the multidimensional HPLC technique with an established reverse-phase HPLC method.*

theophylline standard solutions, gel permeation columns, and reverse-phase columns were used. The results are tabulated in Table II. Excellent day-to-day reproducibility over the concentration range of 1.5–30.0 μg/ml of plasma was demonstrated with the relative standard deviation ≤2.6% in each case. At lower concentrations, 0.25 and 0.5 μg/ml of plasma, larger relative standard deviations were obtained.

Comparison with Other Methods—Plasma samples were obtained from a local hospital. These samples were initially analyzed by the hospital using an HPLC method (8), and were reanalyzed using the multidimensional technique. Both results are presented in Fig. 4 and exhibit excellent correlation (slope = 0.96, r = 0.99) between methods for these 42 samples over a concentration range of 2.9–36 μg/ml.

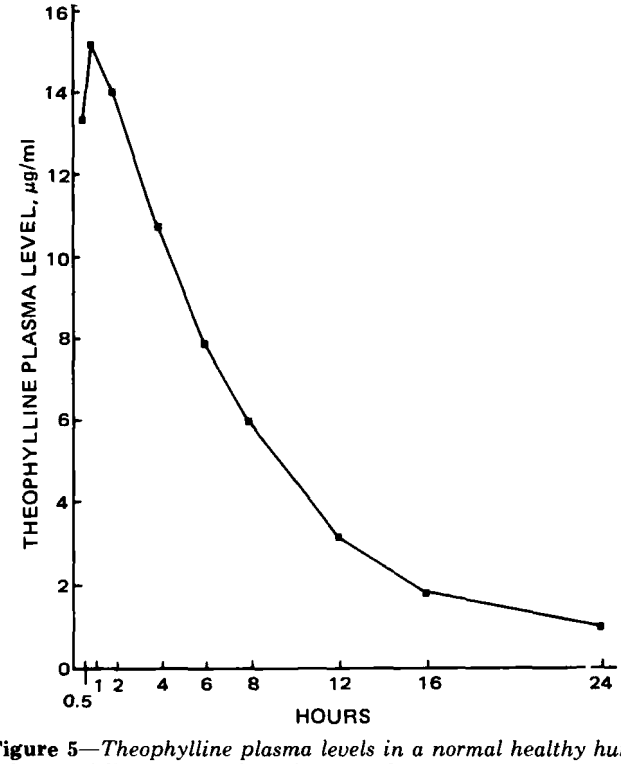

Figure 5—*Theophylline plasma levels in a normal healthy human volunteer following a single oral 500-mg dose.*

Interferences—Dietary xanthines, theophylline and caffeine metabolites, and several drugs that could be administered with theophylline in a therapeutic situation, were tested for interference. The compounds that were tested and found not to interfere with the assay procedure are: acetaminophen, ampicillin, caffeine, ceforamide, cephalothin sodium, cephloridine, corticosterone, dilantin, 1,3-dimethyluric acid, 1,7-dimethylxanthine, ephedrine, hypoxanthine, isoproterenol, 1-methyluric acid, 3-methyluric acid, 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, phenobarbital, theobromine, uric acid, and xanthine.

Applicability—This method has been used in the evaluation of new theophylline formulations in dogs and in bioavailability studies of theophylline formulations in healthy human volunteers. Figure 5 is a typical theophylline plasma level–time profile following the oral administration of a 500-mg theophylline dose to a human volunteer.

It has been demonstrated that theophylline can be efficiently analyzed by the automatic multidimensional HPLC technique, thus, totally eliminating the extraction step. With the use of a computing integrator (as described in this report) to automate the data reduction step, the total analysis of plasma theophylline has been reduced to manually mixing the unknown plasma sample with the internal standard solution in a small glass tube of the autosampler; the rest of the procedure is performed automatically by instruments.

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ACKNOWLEDGMENTS

The results were presented in part at the Pittsburg Conference and Exposition on Analytical Chemistry and Applied Spectroscopy, Atlantic City, N.J., March 1981.

The authors thank Sue Price, Toxicology Laboratories, St. Paul Ramsey Medical Center for providing patient plasma samples for cross checking theophylline methods, and Jeanne Fox for her critical review of the manuscript.

Esterase Activities in Adult Rabbit Eyes

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Received January 11, 1982, from the *School of Pharmacy, University of Southern California, Los Angeles, CA 90033.* Accepted for publication April 14, 1982.

Abstract □ The rational design of prodrugs to improve the therapeutic efficacy of existing drugs would be expedited if the nature of the *in vivo* enzymatic conditions that regenerate the drugs from their prodrugs is known. Using albino and pigmented rabbits as models, this research seeks to delineate the esterase activities in their corneas, irises, ciliary bodies, and aqueous humor, which are intimately involved in the disposition of drugs from topical dosing. This was achieved by monitoring the hydrolysis kinetics of α-naphthyl acetate, both in the presence and absence of esterase inhibitors, upon incubation with aqueous humor and homogenates of cornea, iris, and ciliary body. It was found that in both breeds of rabbits, esterase activity was the highest in the iris–ciliary body followed by the cornea and then the aqueous humor, and that multiple esterases probably existed in the aqueous humor and the ocular tissues studied. However, the esterase activity in the cornea and iris–ciliary body of the pigmented rabbit was greater when compared with the albino rabbit. Based on these results, drugs and prodrugs containing ester linkages can undergo varying extents of esterase-mediated hydrolysis while permeating the cornea and upon entering the aqueous humor, iris, and ciliary body. Moreover, in view of the differences in esterase activity that exist between the albino and pigmented rabbits, it would be necessary to employ both breeds of rabbits in evaluating the rate and extent to which ocular ester prodrugs would be converted to their parent compounds.

Keyphrases □ Esterase activities—adult rabbit eyes, disposition, hydrolysis kinetics □ Eyes, adult rabbits—esterase activities, disposition, hydrolysis kinetics □ Disposition—esterase activities in adult rabbit eyes, hydrolysis kinetics □ Kinetics, hydrolysis—esterase activities in adult rabbit eyes, disposition

Until recently, there have been few reports on drug metabolism in the eye (1). Invariably, they are concerned with polycyclic aromatic compounds entering the uveal circulation and reaching the photoreceptor cells through the choriocapillaries. Because of its dual capability to terminate the pharmacological activities of inherently active drugs and to transform inactive drugs to their active moieties, drug metabolism in the eye is an important aspect of drug action. In view of the necessarily close association between esterase activities in the various segments of the eye and the extent of metabolic alterations of drugs containing ester linkages, knowledge of esterase activities in the eye would allow a first estimation of the clinical efficacy of such drugs as pilocarpine, atropine, and dipivefrin.

During the past 5 years, the prodrug approach (2) of preparing bioreversible derivatives of existing drugs has been successfully extended to the topical ophthalmic drug epinephrine (3). In principle, several other topical ophthalmic drugs that are currently available should also benefit from this approach, since they, like epinephrine, contain functional groups amenable to ester prodrug derivatization.

For these ester prodrugs to be useful clinically, esterase activity must be available in the ocular tissues to regenerate the parent compounds. Several investigators (4–10) have evidence to support the presence of esterases predominantly in innervated ocular tissues. They include the cornea, iris–ciliary body, retina, and optic nerve. However, the level of esterase activities in each tissue and the biochemical properties of these esterases remain to be determined. It is reasonable to expect that when such information is available, the rational design of ester prodrugs would follow.

The first step in the eventual characterization of esterases present in the various ocular tissues of both albino and pigmented rabbits is the determination of esterase activities in their corneas, irises, ciliary bodies, and aqueous humor, and this is the subject of this report. The rationale for studying esterases in both albino and pigmented rabbit

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