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High-performance chromatographic determination of pilocarpine and pilocarpic acid in ocular tissues

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Summary

High-performance liquid chromatographic (HPLC) procedures were developed for determination of pilocarpine and its major metabolite, pilocarpic acid, in ocular tissues. Chromatographic separation was carried out on an octadecyl reverse-phase column. The mobile phase consisted of 97% (v/v) water, 3% (v/v) methanol, and 5% (w/v) potassium dihydrogen phosphate. At a flow rate of 1.0 ml/min and by monitoring UV absorbance at 215 nm, the retention times for pilocarpine and pilocarpic acid are approximately 18 and 10 min, respectively. In addition, a method to extract pilocarpine and pilocarpic acid from ocular tissues prior to chromatography has been developed and applied to routine tissue drug analysis. This assay method is able to detect 20 ng of compound and is shown to be linear in the range 1–50 μg/ml. With this level of sensitivity, tissues from three eyes need to be pooled, extracted, and assayed in order to monitor pilocarpine and pilocarpic acid ocular disposition.

Introduction

Pilocarpine is an important drug for the treatment of glaucoma, and although there has been a systematic examination of its ocular disposition in the albino rabbit

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(Chrai et al., 1974; Sieg et al., 1976; Patton et al., 1976; Makoid et al., 1976; Lee et al., 1979; Conrad, 1980), there have been few reports on ocular metabolism, due in large measure to analytical limitations. It has been reported that anterior segment metabolism of pilocarpine in albino rabbits is rather modest (Sendelbeck et al., 1974). However, Lee et al. (1980) found that the rate and extent of corneal metabolism was two orders of magnitude greater in the pigmented rabbit than in the albino rabbit. The major metabolite was identified as pilocarpic acid. These in vivo metabolism studies utilized tritiated pilocarpine in conjunction with thin-layer chromatography and liquid scintillation to quantitate levels of pilocarpine and pilocarpic acid. A major drawback to this technique is that the tritium label is non-specific and in the case of pilocarpine very labile (Chrai et al., 1974). The purpose of this paper is to describe an HPLC procedure to quantitate pilocarpine and pilocarpic acid in ocular tissues.

There have been several reports in the recent literature describing HPLC methods to separate pilocarpine and pilocarpic acid. All of these techniques are suitable to measure stability of pilocarpine in aqueous solution but have not been extended to biological tissues, which requires workup of the tissue sample and requires detection of greatly reduced drug concentrations. For example, Noordham et al. (1978, 1981) reported the separation of pilocarpine, isopilocarpine, pilocarpic acid, and isopilocarpic acid on a reversed-phase column using both a refractive index detector and UV absorbance at 215 nm. The principal drawback to this method is that the retention time for resolving the 4 compounds is approximately 25 min. Kennedy et al. (1981) reported an HPLC method which is basically a modification of Noordham's approach and utilizes a temperature-controlled column at 40 °C. This increase in temperature reduces the retention time to about 15 min. Dunn et al. (1981) report an HPLC technique utilizing a normal phase column and UV detection at 220 nm to determine pilocarpine and isopilocarpine. However, this method was unable to detect pilocarpic acid. Mitra et al. (1980) were able to increase the sensitivity of detection of pilocarpine by forming *p*-nitrobenzyl bromide derivatives which were subsequently separated by ion-pair chromatography. However, there was no mention of detection or estimation of pilocarpic acid.

To date there have been no reports of an HPLC method for the detection of pilocarpine and its metabolite(s) in biological samples. This paper presents a modification of the HPLC method described by Noordham et al. (1978) adapted to the detection of pilocarpine and pilocarpic acid in ocular tissues.

Materials and Methods

Isopilocarpine (free base) was prepared by dissolving 20 mg of isopilocarpine nitrate (Aldrich Chem., Milwaukee, WI, U.S.A.) in 25 ml of 0.3 M potassium bicarbonate. Twenty-five ml of methylene chloride were added to the solution and shaken in a separatory funnel. The organic phase was collected and evaporated, and the residue reconstituted in deuterated chloroform for NMR (Varian, Palo Alto, CA, U.S.A.) analysis. The free base form of pilocarpine (J.T. Baker Chem., NJ, U.S.A.)

was also dissolved in deuterated chloroform for NMR analysis. Pilocarpic acid and isopilocarpic acid were prepared by the method of Repta et al. (1971). The pilocarpic and isopilocarpic acids were then neutralized with 0.1 N aqueous hydrochloric acid; lyophilized (FTS Systems, NY, U.S.A.); reconstituted in deuterated water (Aldrich Chem., WI, U.S.A.); and subjected to NMR analysis. Identity of the pilocarpic and isopilocarpic acids were confirmed by subsequent chromatography and observing that the NMR spectra obtained for the four compounds were identical to those obtained by previous workers (Nunes et al., 1974).

The HPLC system consisted of a solvent pump (Varian, CA, U.S.A.); a fixed volume (20 μl) sample injection valve (Rheodyne, CT, U.S.A.), a 5 μm particle size reversed-phase octadecyl column equipped with precolumn (Perkin Elmer, CT, U.S.A.); and a variable UV detector (Perkin-Elmer, CT, U.S.A.) set at 215 nm. All chromatograms were recorded at a chart speed of 20 cm/h.

Mobile phase consisted of 97% (v/v) doubly distilled water, 3% (v/v) methanol, and 5% potassium dihydrogen orthophosphate. There was no pH adjustment. The flow rate was 1.0 ml/min, and an external standard curve method was used to quantitate pilocarpine and pilocarpic acid utilizing peak heights.

Standard solutions were prepared by diluting stock aqueous solutions of pilocarpine nitrate (J.T. Baker Chem., NJ, U.S.A.) and pilocarpic acid to yield 5 standard solutions ranging in concentration from 1–50 μg/ml. These standard solutions were prepared and used just prior to the assay of a set of samples. To determine the accuracy and precision of the determination of pilocarpine and pilocarpic acid from aqueous solution, known various quantities of pilocarpine and pilocarpic acid in aqueous solution were injected into the HPLC system in triplicate, and the concentrations were determined using the constructed standard curves.

To evaluate the percent recovery from ocular tissue homogenates, male adult, mixed-breed rabbits (Klubertanz, WI, U.S.A.), 2.0–2.5 kg, were sacrificed by rapid injection of a lethal dose of sodium pentobarbital into a marginal ear vein. Aqueous humor was aspirated (200 μl) followed by excision of the entire cornea and the iris-ciliary body. Each tissue was transferred to a holding vial containing 1 ml of water and 100 μl of a 1% solution of either pilocarpine or pilocarpic acid and allowed to soak for approximately 24 h. Each tissue was then rinsed with distilled water before placement into a second holding vial. The remaining solution, including the rinse water, was assayed for pilocarpine or pilocarpic acid and the amount of compound taken up by the tissue determined by subtraction. The tissue was homogenized (Virtis, NY, U.S.A.) in 2 ml of doubly distilled water at 4°C. Following addition of 20 μl of a 10% solution of trichloroacetic acid, the homogenate was centrifuged (Becton Dickinson, NJ, U.S.A.) at approximately 5000 *g*. The supernatant was pipetted into a culture tube and subjected to lyophilization. The resulting residue was then reconstituted in 0.5 ml of doubly distilled water, and 4 ml of hexane was added followed by vortexing (Scientific Industries, NY, U.S.A.) for 1 min, and the hexane phase was discarded. Ten μl of a 10% solution of Na_2CO_3 was then added to adjust the pH to approximately 9, followed by 4 ml of chloroform to extract pilocarpine with subsequent vortexing for 1 min. Preliminary experiments showed that pilocarpine was optimally extracted from aqueous solution at pH 9

using chloroform. Occasionally, a gel formed following addition of chloroform and vortexing. In these cases, it was necessary to centrifuge the samples at approximately 5000 *g* to obtain a clear separation. The organic phase was then transferred to a holding tube and the extraction repeated. To the remaining aqueous phase was added 200 μl of 0.1 N HCl to adjust the pH to approximately 4 followed by 4 ml of *n*-butanol to extract pilocarpic acid. Preliminary experiments indicated that pilocarpic acid was optimally extracted from aqueous solution at pH 4 using *n*-butanol. The mixture was then vortexed for 1 min and the 2 phases allowed to separate. The organic phase was then transferred to the same holding tube. The combined extracts were dried under nitrogen at 40 °C, reconstituted in 60 μl of doubly distilled water, and assayed for pilocarpine and pilocarpic acid content.

Aqueous humor was treated in a similar manner except no equilibration time was needed, i.e. varying concentrations of pilocarpine and pilocarpic acid solution were added directly. It was also not necessary to add trichloroacetic acid solution or to lyophilize the samples. In order to quantify the percent recovery from the biological samples, aqueous standard solutions of pilocarpine and pilocarpic acid were used to construct the external standard curve. The use of aqueous standards was justified by results of preliminary experiments which indicated that aqueous solutions of pilocarpine or pilocarpic acid and aqueous solutions of pilocarpine or pilocarpic acid of identical concentration added to the dried extracts of the various ocular tissues yielded chromatograms in which the peak heights were the same, within 1%.

To obtain preliminary results of ocular disposition of pilocarpine in the aqueous humor of the pigmented rabbit, using this technique, animals were dosed with 25 μl of a 5×10^{-2} M solution of pilocarpine nitrate in Sorensen's buffer, pH 6.24 (Sieg and Robinson, 1976). At appropriate times, rabbits were sacrificed by rapid injection of sodium pentobarbital into a marginal ear vein. Aqueous humor was aspirated from the anterior chamber of the eye, and 200 μl was quickly transferred to a holding vial immersed in a dry-ice–isopropanol bath until sample workup began. Aqueous humor samples were then assayed as described above.

Results and Discussion

Fig. 1 shows a chromatogram of an aqueous solution of pilocarpic acid, isopilocarpic acid, isopilocarpine nitrate, and pilocarpine nitrate with retention times of 10.2, 13.8, 15.9, and 17.7 min, respectively. The order of elution of the four compounds differs from that in the method reported by Noordham et al. (1979). In Noordham's method, the pH of the mobile phase was adjusted to 2 using phosphoric acid. At this pH, the relative polarity of the 4 compounds are altered such that the order of elution is isopilocarpine, pilocarpine, pilocarpic acid, and isopilocarpic acid. Moreover, lowering the pH of the mobile phase to 2 resulted in an incomplete resolution of the pilocarpine and pilocarpic acid peaks. However, the mobile phase used in the present report resulted in an excellent separation of pilocarpine and pilocarpic acid. The use of 5% (w/v) potassium dihydrogen phosphate in the mobile phase resulted in superior resolution of the four compounds and decreased tailing of the peaks.

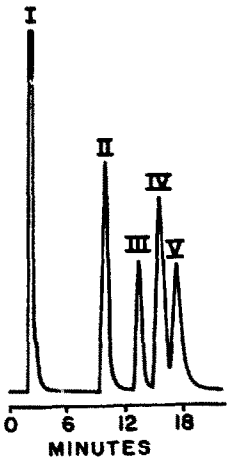

Fig. 1. Chromatograms of pilocarpic acid (II), isopilocarpic acid (III), isopilocarpine (IV), and pilocarpine (V) in aqueous solution. Peak I is due to nitrate ion.

For purposes of determining pilocarpic acid in ocular tissues, an external standard solution of pilocarpic acid is required. However, the hydrolysis of pilocarpine resulted in two products corresponding to both pilocarpic acid and isopilocarpic acid based on retention times. This observation is consistent with the work of Bundgaard et al. (1982), who also found that hydrolysis of pilocarpine resulted in two products and supports the position that hydrolysis of pilocarpine does not result in a pure solution of pilocarpic acid as assumed in several reports (Repta et al., 1972; Noordham et al., 1978 and 1981; Kennedy et al., 1981). The hydrolysis of isopilocarpine, however, resulted in a nearly pure solution (97%) of isopilocarpic acid with about 3% pilocarpic acid. Having an almost pure standard of isopilocarpic acid, the composition of a hydrolyzed solution of pilocarpine, under the reaction conditions described, was found to be 83.5% pilocarpic acid and 16.5% isopilocarpic

TABLE 1

ACCURACY AND PRECISION OF ASSAY FOR PILOCARPINE AND PILOCARPIC ACID (3 REPLICATES AT EACH CONCENTRATION). S.D. IS STANDARD DEVIATION

Theoretical conc. (μg/ml)	Experimental conc. (μg/ml)		Coefficient of variation (%)
	Mean	S.D.	
Pilocarpine			
5	4.72	0.40	8.5
20	20.5	0.19	9.3
35	34.6	0.69	2.0
45	45.1	0.90	2.0
Pilocarpic acid			
7	8.4	0.32	3.8
21	23.2	0.38	1.6
35	32.0	0.61	1.9
42	43.0	1.21	2.8

acid. Therefore to determine the concentration of pilocarpic acid in ocular samples, corrected standard solutions were required. Pilocarpic acid and isopilocarpic acid, in identical concentrations, give essentially the same peak height within ±2%.

The results of experiments designed to assess the accuracy and precision of this assay for pilocarpine and pilocarpic acid are shown in Table 1. The coefficient of variation for pilocarpine ranged from 2.0% (45 μg/ml) to 8.5% (5 μg/ml) and for pilocarpic acid ranged from 1.6% (21 μg/ml) to 3.8% (7 μg/ml).

Table 2 summarizes the results obtained in the in vitro determination of percent recovery for pilocarpine and pilocarpic acid from aqueous humor, cornea, and iris-ciliary body. It was not possible to control the amount of pilocarpine and pilocarpic acid taken up by the tissue; and, therefore, the reported percent recoveries are an average of single determinations of various concentrations. Preliminary experiments indicated that the extraction efficiency of both pilocarpine and pilocarpic acid from aqueous solution was constant over a wide range of initial concentrations.

External standard curves for pilocarpine and pilocarpic acid were prepared to determine the concentration of these two compounds in ocular tissues by measuring peak heights. The standard curves were found to be linear over the concentration range used, i.e. 1.0–50 μg/ml. Typical standard curves constructed prior to a set of daily assays are described by the following equations which were obtained by linear regression:

$$y = 0.08x - 0.02 \qquad r^2 = 0.9996 \tag{1}$$

and

$$y = 1.49x + 0.05 \qquad r^2 = 0.9997 \tag{2}$$

for pilocarpine and pilocarpic acid, respectively. The day-to-day variability of the standard curves for pilocarpine and pilocarpic acid is illustrated in Eqns. 3 and 4, respectively. These equations present the mean slope and mean intercept with the corresponding standard deviations of 6 standard curves, each of which was prepared prior to a daily set of assays.

$$y = 0.067\ (\pm 0.008)x - 0.003\ (\pm 0.02) \tag{3}$$

TABLE 2

PERCENT RECOVERY OF PILOCARPINE AND PILOCARPIC ACID FROM OCULAR TISSUES [a]

	Aqueous humor	Cornea	Iris/ciliary body
Pilocarpine	96.8 ± 2.1 [b]	81.3 ± 6.4	79 ± 8.2
Pilocarpic acid	93.4 ± 3.2	78.6 ± 7.2	76 ± 5.8

[a] Number of determinations ≥ 7.

[b] ± standard deviation.

Fig. 2.

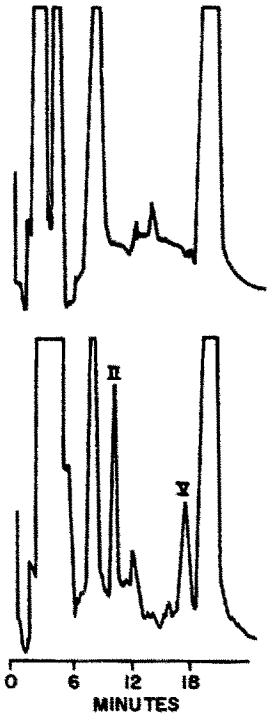

Fig. 3.

Fig. 4.

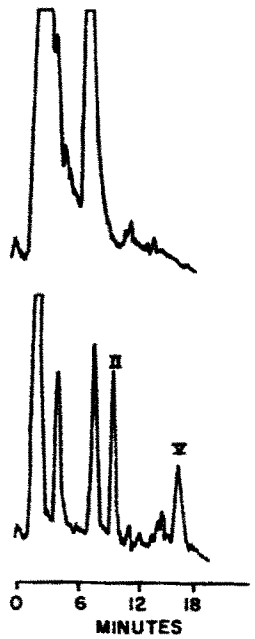

Fig. 2. Chromatograms of pilocarpic acid (II) and pilocarpine (V) in aqueous humor. Top chromatogram is aqueous humor blank; bottom is spiked aqueous humor sample.

Fig. 3. Chromatogams of pilocarpic acid (II) and pilocarpine (V) in cornea sample. Top chromatogram is cornea sample blank; bottom is spiked cornea sample.

Fig. 4. Chromatograms of pilocarpic acid (II) and pilocarpine (V) in iris-ciliary body sample. Top chromatogram is iris-ciliary body blank; bottom is spiked iris-ciliary body sample.

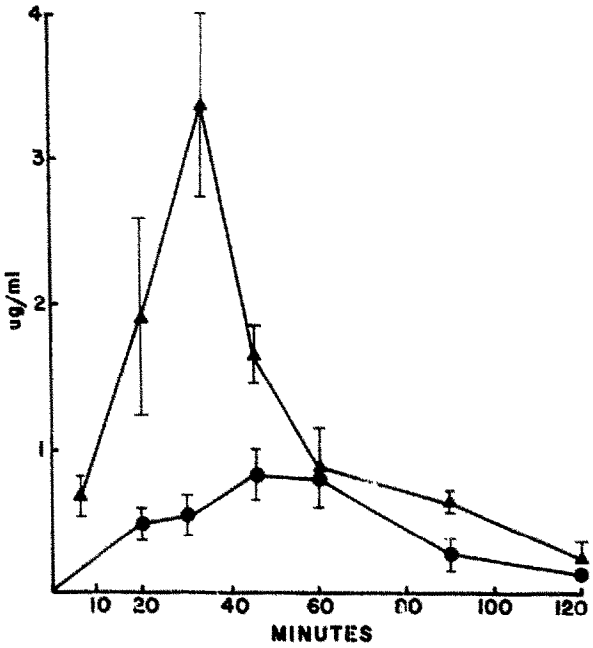

Fig. 5. Aqueous humor concentration of pilocarpine (▲) and pilocarpic acid (●) versus time profile following topical administration of 25 µl of 5×10^{-2} M pilocarpine nitrate to the eye. Error bars represent standard error of the mean; $n \geq 7$.

$$y = 1.51\ (\pm 0.005)x + 0.035\ (\pm 0.047) \quad (4)$$

The sensitivity of the assay was determined by analyzing progressively lower concentrations and was found to be 20 ng for pilocarpine and 10 ng for pilocarpic acid for a signal : noise ratio of 2 : 1. Because of this relatively low sensitivity, it was necessary to pool ocular samples for actual in vivo application. Pooling 3 samples was found to suffice.

Figs. 2–4 show the chromatograms of aqueous humor, cornea and iris-ciliary body samples, respectively, following sample workup, containing no drug or metabolite and spiked with pilocarpine and pilocarpic acid. These chromatograms indicate no interferring peaks from endogenous compounds as well as an excellent separation. The abundance of peaks due to endogenous compounds in these chromatograms is due to the final extraction with *n*-butanol. In the case of pilocarpine disposition studies in the albino rabbit where metabolism of pilocarpine is negligible, the analyst is interested in quantitating pilocarpine only. Therefore, extraction with only chloroform is permissable, and the resulting chromatograms are essentially free of peaks due to endogenous substances.

Fig. 5 shows concentration versus time profiles of pilocarpine and its metabolite, pilocarpic acid, in the aqueous humor of pigmented rabbits obtained using the present assay technique. Although a more complete description of pilocarpine will appear in a subsequent publication, neither isopilocarpine or isopilocarpic acid were detected in ocular tissues in these preliminary studies.

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