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Analysis of Pilocarpine and Isopilocarpine in Ophthalmic Solutions by Normal-Phase High-Performance Liquid Chromatography

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Abstract □ A normal-phase high-performance liquid chromatographic separation for pilocarpine and isopilocarpine was developed which is suitable for the routine analysis of ophthalmic preparations. The method utilizes a column packed with 5-μm silica with a mobile phase of hexane–2% ammonium hydroxide in 2-propanol (70:30). Peak detection is by UV at 220 nm. It is suggested that a partition separation mechanism is involved rather than adsorption. A separation factor (α) of 1.17 was obtained with a relative separation (R_s) of 2.13. Column lifetimes were typically 6–8 months with daily use. Several standard pilocarpine–isopilocarpine mixtures and five commercially available ophthalmic solutions from different manufacturers were analyzed. The method was specific for pilocarpine and isopilocarpine in the presence of each other and pilocarpic acid and is a significant improvement over the nonspecific colorimetric methods of analysis.

Keyphrases □ Pilocarpine—high-performance liquid chromatographic analysis in the presence of isopilocarpine, ophthalmic solutions □ Isopilocarpine—high-performance liquid chromatographic analysis in the presence of pilocarpine, ophthalmic solutions □ High-performance liquid chromatography—separation of pilocarpine and isopilocarpine, ophthalmic solutions

Pilocarpine, a widely used alkaloid, possesses several important pharmacological properties. For example, it is a miotic and lowers intraocular pressure. The major ophthalmic application of pilocarpine has been for the treatment of glaucoma. The structure of pilocarpine is well established and contains both an imidazole and a lactone.

In aqueous solution, pilocarpine (I) decomposes through two major pathways, which are both base catalyzed (Scheme I). The lactone ring can undergo hydrolysis, resulting in the formation of pilocarpic acid (II), or epimerization can occur at the α-carbon to form isopilocarpine (III). Both pilocarpic acid and isopilocarpine are essentially pharmacologically inactive (1, 2).

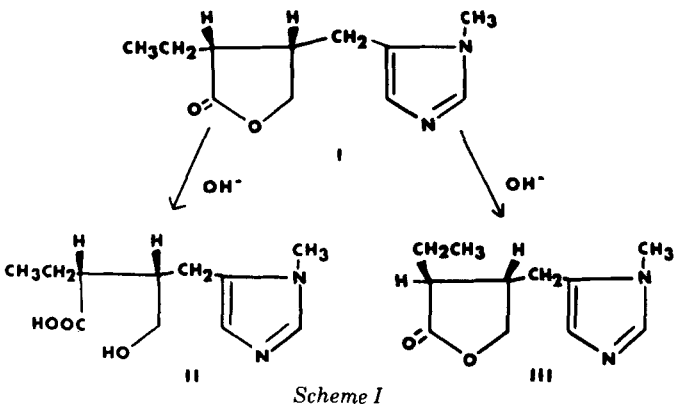
BACKGROUND

Analytical methods to measure the extent of pilocarpine degradation in ophthalmic medications have been published, but most cannot differentiate between pilocarpine and isopilocarpine. For instance, the USP method for determining pilocarpine is a colorimetric assay based on the formation of hydroxamic acid (3). Since the moiety responsible for producing color is an intact lactone ring, the assay can distinguish between pilocarpine and pilocarpic acid but not between pilocarpine and isopilocarpine (4). This flaw is serious since recent studies indicated that the primary degradation product of pilocarpine is not pilocarpic acid but its diastereomer, isopilocarpine (4).

Several techniques have been investigated to produce an analytical separation of pilocarpine and isopilocarpine. Some of the published methods include TLC (5), NMR (6–9), IR spectroscopy (10), GLC (8, 11), and polarimetry (12, 13). However, most of these separations are not suitable for development into a routine analysis. The reported TLC separation is questionable and could not be duplicated by one investigator (10); the NMR analysis requires an expensive 100-MHz instrument (10); IR analysis gives poor results when one diastereomer predominates (10), which is usually the case in ophthalmic solutions; and GLC requires derivatization prior to analysis, which makes multiple-sample runs too time consuming. Of these techniques, only polarimetry seems to have practical value. In fact, a UV–optical rotation analysis has been used successfully by this laboratory for several years[1].

The UV–optical rotation method requires elaborate sample preparation and is tedious in actual practice. An analytical procedure such as high-performance liquid chromatography (HPLC), which minimizes sample preparation and retains a high separation capability, would be highly desirable, and several HPLC separations of pilocarpine and isopilocarpine have been reported. Initially, ion-exchange columns packed with cation-exchange resins[2] were used with UV peak detection at 217 nm (4). Unfortunately, these HPLC systems gave variable peak retention times and generally produced erratic results (10, 14).

Khalil (15) reported an HPLC analysis for pilocarpine using an octa-



Scheme I

[1] See B. S. Scott, D. L. Dunn, and E. D. Dorsey, *J. Pharm. Sci.*, in press.
[2] Aminex A-7 (7–11 μm), Bio-Rad Laboratories.

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Table I—Pilocarpine Hydrochloride Analysis of Standard Mixtures

Pilocarpine Hydrochloride, mg/ml	Isopilocarpine Nitrate, mg/ml	Pilocarpine Peak Height, cm	Peak Height/ Concentration, cm/mg/ml	Pilocarpine Hydrochloride Concentration Found[a], mg/ml	Recovery, %
1.12	0.080	8.95	7.99	1.12	100.0
1.12	0.080	9.00	8.04	1.13	100.9
1.36	0.080	10.95	8.05	1.37	100.7
1.36	0.080	11.00	8.09	1.38	101.5
1.60	0.080	12.85	8.03	1.61	100.6
1.60	0.080	12.85	8.03	1.61	100.6
1.84	0.080	14.65	7.96	1.84	100.0
1.84	0.080	14.65	7.96	1.84	100.0
2.08	0.080	16.75	8.05	2.10	101.1
2.08	0.080	16.75	8.05	2.10	101.1

[a] A standard containing 1.60 mg of pilocarpine hydrochloride/ml was used to calculate these concentrations.

Table II—Isopilocarpine Nitrate Analysis of Standard Mixtures

Pilocarpine Hydrochloride, mg/ml	Isopilocarpine Nitrate, mg/ml	Isopilocarpine Peak Height, cm	Peak Height/ Concentration, cm/mg/ml	Isopilocarpine Nitrate Concentration Found[a], mg/ml	Recovery, %
1.61	0.0367	2.45	66.75	0.0367	100.0
1.61	0.0367	2.45	66.75	0.0367	100.0
1.61	0.0735	4.90	66.67	0.0735	100.0
1.61	0.0735	4.80	65.31	0.0720	98.0
1.61	0.1102	7.25	65.79	0.1087	98.6
1.61	0.1102	7.25	65.79	0.1087	98.6
1.61	0.1470	9.80	66.67	0.1469	99.9
1.61	0.1470	9.70	65.99	0.1454	98.9
1.61	0.1837	12.30	66.96	0.1844	100.4

[a] A standard containing 0.1102 mg of isopilocarpine nitrate/ml was used to calculate these concentrations.

decylsilane[3] and cyanopropylsilane[4] column in series. However, no separation of isopilocarpine was demonstrated, and UV peak detection was at 254 nm. Since neither pilocarpine nor isopilocarpine absorb appreciably at 254 nm, this procedure seems questionable. More recently, a system using a noncommercial octadecylsilane column[5] with refractive index detection was reported (14). With commercially available columns and UV detection at 215 nm, the separation between pilocarpine and isopilocarpine was insufficient for a good analytical procedure.

An HPLC separation for pilocarpine and isopilocarpine is discussed here; a commercially available silica column[6] with UV peak detection at 220 nm is used. This normal-phase system was satisfactory for the routine analysis of pilocarpine or isopilocarpine in ophthalmic solutions. Data are included to demonstrate that the procedure gives a linear response and is specific. The actual analysis of five ophthalmic products from different manufacturers is presented, and several methods of pilocarpine analysis are compared.

EXPERIMENTAL

Reagents and Solvents—Pilocarpine hydrochloride USP and isopilocarpine nitrate[7] (reagent grade) were used without further purification. Hexane[8] was UV grade; 2-propanol[8], methanol[8], and ammonium hydroxide were reagent grade.

Equipment—A liquid chromatograph equipped with a high-pressure pump[9], a variable-wavelength UV detector[10], a fixed-loop injector[11], and a strip-chart recorder[12] was used with a 25-cm × 4.6-mm i.d. column packed with 5-μm silica[6]. No precolumn was used.

Mobile Phase—Concentrated ammonium hydroxide (10.0 ml) was diluted to 500 ml with 2-propanol. This 2% ammonium hydroxide solution (300 ml) was mixed with hexane (700 ml) and filtered through a 0.5-μm filter[13] before use.

HPLC Analysis Conditions—The mobile phase was pumped through the column at a flow rate of 2.0 ml/min. UV detection was at 220 nm, and the attenuation used for isopilocarpine analysis was four times more sensitive than for pilocarpine analysis. The injection size was 10 μl for pilocarpine and 20 μl for isopilocarpine. The plate count for the silica column, determined at a chart speed of 1.27 cm/min with a 1.60-mg/ml pilocarpine hydrochloride solution, was 8000 plates/m[14]. After completion of the analysis, hexane was pumped through the silica column prior to storage.

Preparation of Standard Solutions—The following methanol dilutions were made with a pilocarpine hydrochloride or an isopilocarpine nitrate stock solution:

1. Pilocarpine standard curve—five samples were prepared over the concentration range of 1.12–2.08 mg of pilocarpine hydrochloride/ml. Each solution also contained 0.080 mg of isopilocarpine nitrate/ml.
2. Isopilocarpine standard curve—five samples were prepared over the concentration range of 0.037–0.184 mg of isopilocarpine nitrate/ml. Each solution also contained 1.61 mg of pilocarpine hydrochloride/ml.

Preparation of Commercially Available Ophthalmic Products—Aliquots of each sample were pipetted accurately into separate volumetric flasks and diluted with methanol to obtain a final concentration of ~1.6 mg of pilocarpine hydrochloride/ml. The resulting solutions were injected directly into the liquid chromatograph. The concentration of pilocarpine or isopilocarpine then was determined by comparing the sample peak height with the peak height of a pilocarpine or isopilocarpine standard, respectively.

RESULTS AND DISCUSSION

Standard solutions containing pilocarpine and isopilocarpine in the ratio of about 19:1 were prepared and analyzed (Tables I and II). This pilocarpine to isopilocarpine ratio corresponds to what would be expected from the analysis of an actual ophthalmic solution. By using the normal-phase HPLC system described under *Experimental*, pilocarpine eluted at ~16 min and isopilocarpine eluted at ~20 min.

Pilocarpine and isopilocarpine had capacity factors (K') of 12.07 and 14.13, respectively[15]. These factors correspond to a separation factor (α)

[3] μBondapak C_{18} (10 μm), Waters Associates.
[4] μBondapak CN (10 μm), Waters Associates.
[5] The packing material was LiChrosorb RP-18 (10 μm), E. Merck.
[6] Si 60 Hibar (5 μm), E. Merck.
[7] Aldrich Chemical Co.
[8] Burdick & Jackson "Distilled in Glass".
[9] M-6000A, Waters Associates.
[10] Model SF 770, Schoeffel Instruments.
[11] Model 710A, Rheodyne.
[12] Omniscribe model A5111-1, Houston Instruments.
[13] Fluoropore, Millipore Corp.

[14] Theoretical plates per meter (N) = 16 $(T/T_w)^2(100/L)$, where T is the retention time of pilocarpine, T_w is the peak width of pilocarpine measured along the baseline, and L is the column length in centimeters (16).
[15] An injection of carbon tetrachloride was used to determine the elution time of a nonretained sample (*i.e.*, T_0).

Table III—Determination of Pilocarpine and Isopilocarpine in Various Commercial Pilocarpine Ophthalmic Solutions

Manufacturer	Concentration, Salt	Pilocarpine Analysis[a], % HPLC	Pilocarpine Analysis[a], % USP[b]	Pilocarpine Analysis[a], % UV–Optical Rotation[c]	Isopilocarpine Analysis[a], % HPLC	Isopilocarpine Analysis[a], % UV–Optical Rotation[c]	pH
1	2%, HCl	101, 100	100, 98	99, 98	0.7, 0.8	0.0, 0.8	3.75
2	2%, HCl	98, 99	99, 99	97, 99	0.9, 1.0	0.0, 1.0	3.63
3	2%, HNO_3	94, 95	127, 128[d]	105, 108[d]	1.2, 1.2	0.0, 4.0	4.50
4	1%, HCl	94, 96	95, 96	96, 95	1.4, 1.4	2.7, 4.4	4.22
5	2%, HCl	102, 101	103, 104	99, 100	3.7, 3.6	3.7, 3.9	4.16

[a] Percent labeled amount of pilocarpine salt. [b] The current USP method for pilocarpine analysis is reaction with hydroxylamine to form hydroxamic acid and subsequent colorimetric analysis. [c] B. S. Scott, D. L. Dunn, and E. D. Dorsey, *J. Pharm. Sci.*, in press. [d] This sample contained polyvinyl alcohol, which caused noticeable positive interference when analyzed by the USP and UV–optical rotation methods.

of 1.17. Since α is ≤ 1.20, this separation can be classified as "moderately difficult" using Snyder and Kirkland's terminology (16). This finding is not surprising since only subtle polarity differences exist between the two diastereomers. A relative separation (R_s) of 2.13 was calculated[16], which means a quantitative separation suitable for an analytical procedure was obtained (16). Typical chromatograms are given in Figs. 1 and 2.

The average recoveries obtained for the analysis of pilocarpine and isopilocarpine standards were 100.7 and 99.4%, respectively. These values indicate that the method is specific for each diastereomer in the presence of the other. In addition, a plot of concentration *versus* peak height gave a linear curve that intercepted the origin. A least-squares analysis of the data in Tables I and II yielded a correlation coefficient of 0.9997 and a y intercept of 0.02 cm for the pilocarpine analysis. For the isopilocarpine analysis, the correlation coefficient was 0.9998 and the y intercept was −0.04 cm. The use of peak areas instead of peak heights to measure response might possibly produce more accurate results. However, a suitable integrator was not available, and peak height measurements gave acceptable recoveries and standard curve statistics for a routine analysis.

A pilocarpic acid sample was prepared using a previously described method (14, 15). Pilocarpine hydrochloride was subjected to basic hydrolysis, neutralized, and injected directly into the liquid chromatograph. No peak for pilocarpic acid was observed after 60 min. Therefore, analysis of carpic acids (*i.e.*, pilocarpic acid and isopilocarpic acid) requires another technique, such as UV spectroscopy, which can determine the total imidazole present (*i.e.*, pilocarpine, isopilocarpine, pilocarpic acid, and isopilocarpic acid). After pilocarpine and isopilocarpine analysis, total carpic acids then may be found by taking the difference.

Five different manufacturers's commercially available pilocarpine ophthalmic solutions were analyzed for pilocarpine and isopilocarpine content (Table III). These solutions contained excipients usually found in ophthalmic formulations, such as benzalkonium chloride, chlorobutanol, edetate disodium, sodium acetate, citric acid, boric acid, menthol, camphor, and hydroxypropyl methylcellulose. The USP colorimetric analysis for pilocarpine was compared to the normal-phase HPLC analysis and the UV–optical rotation analysis. This latter method is based on the difference in optical rotation shown by pilocarpine and isopilocarpine, and details will be published elsewhere[1].

Excipient interference was noticed in only one sample, which contained polyvinyl alcohol (Manufacturer 3, Table III). Accurate analysis of this sample was difficult using the USP method. Less interference was noted with the UV–optical rotation method, but the HPLC analysis seemed to be completely unaffected. With this one exception, all three pilocarpine analyses gave similar results. The isopilocarpine analysis by HPLC ranged from 0.7 to 3.7% of the labeled pilocarpine concentration. In general, there appeared to be a higher isopilocarpine content in more basic solutions. This relationship was observed previously (10).

Attempts to improve the HPLC analysis by increasing resolution and decreasing the retention time were unsuccessful. Mobile phases containing other alcohols, such as methanol, ethanol, or 2-methyl-1-propanol, did not give a comparable separation. Since the silica columns used daily for this analysis had quite remarkable lifetimes (6–8 months with daily use) considering the mobile phase contained 30% 2-propanol, the separation probably is due to a partition mechanism rather than adsorption. 2-Propanol molecules theoretically could be adsorbed on the polar silica sites to form a stationary organic phase. Pilocarpine and isopilocarpine then could be partitioned differently between the mobile phase and the stationary organic phase. This process would explain why other alcohols were not as satisfactory as 2-propanol.

Silica columns containing 10-μm particles instead of 5-μm particles can be used for pilocarpine analysis but not isopilocarpine. In general, 10-μm columns operate at much lower pressures than 5-μm columns. This operation allows higher flow rates and shorter retention times. Unfortunately, a corresponding decrease in resolution also is observed.

During initial development of the analysis, UV peak detection was at 230 nm but was changed to 215 nm because of a corresponding increase in peak height of 100%. Detection was finally increased to 220 nm to reduce noise and to stabilize the baseline, causing only a 5% decrease in peak

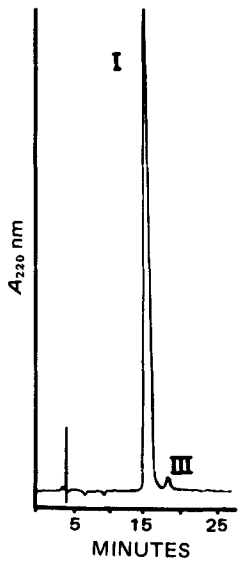

Figure 1—*Typical chromatogram for pilocarpine (I) analysis in a 1.60-mg/ml pilocarpine hydrochloride solution containing ~3% isopilocarpine nitrate (III).*

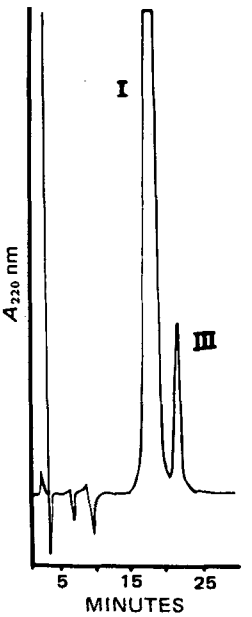

Figure 2—*Typical chromatogram for isopilocarpine (III) analysis in a 1.60-mg/ml pilocarpine hydrochloride (I) solution containing ~3% isopilocarpine nitrate.*

[16] $R_s = 2(T_2 - T_1)/(T_{w1} + T_{w2})$, where T_1 is the retention time of pilocarpine, T_2 is the retention time of isopilocarpine, T_{w1} is the peak width of pilocarpine measured along the baseline, and T_{w2} is the peak width of isopilocarpine measured along the baseline (16).

height. Since the nitrate ion absorbs at this wavelength, an injection of isopilocarpine nitrate solution gave an extra peak at ~40–50 min after injection, presumably due to ammonium nitrate elution. This peak can be avoided, if desired, by first passing the isopilocarpine nitrate sample through an ion-exchange column to replace the nitrate ion with a UV inactive ion.

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Effect of Dosing Volume on Intramuscular Absorption Rate of Aminoglycosides

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Abstract □ The Loo–Riegelman method was applied to serum amikacin level data after intravenous and intramuscular administration. Intramuscular amikacin absorption can be described by first-order kinetics, but the absorption rate constant decreased from 1.95 hr^{-1} at a 125-mg dose to 1.00 hr^{-1} at a 750-mg dose. This rate change apparently is a physical phenomenon due to differing dosing volumes at different doses and attendant changes in the surface area to volume ratio at the injection site. Amikacin absorption rates on intramuscular injection can be maximized by giving several smaller injections rather than a single larger injection. This phenomenon should be generally observed with aminoglycoside antibiotics and could be partly responsible for reported variations in absorption rate and the poor predictability of serum concentrations.

Keyphrases □ Aminoglycosides—effect of dosing volume on intramuscular absorption rate □ Absorption, intramuscular—aminoglycosides, dosing volume effects □ Amikacin—intramuscular absorption rates, dosing volume effects

In a review of the clinical pharmacokinetics of aminoglycoside antibiotics, Pechere and Dugal (1) referred to reports of wide intramuscular absorption rate variations within and between studies and antibiotics. They commented: "In view of the fact that rates of absorption vary widely, nomograms based on equations which make use of a universal absorption rate constant should be critically examined. This, incidentally, may be partly responsible for the poor predictability of serum aminoglycoside concentrations noted by some authors." According to Greenblatt and Koch-Weser (2), nonlinear intramuscular absorption may be fairly common because local and systemic factors that influence the absorption rate rarely remain constant while absorption is taking place.

In this study, available intravenous and intramuscular amikacin data were used to estimate intramuscular absorption kinetics of an aminoglycoside antibiotic. Since the data were derived from separate studies, mean values were used for the pharmacokinetic parameters and intramuscular serum levels. A crossover intravenous–intramuscular amikacin study at 125- and 500-mg doses was reported, but no estimates of the intramuscular absorption kinetics were made (3).

EXPERIMENTAL

The data analyzed were drawn from three Phase I amikacin clinical investigations (Studies 1, 2, and 7). The subjects in all three studies were normal, healthy adult males ranging from 21 to 40 years and from 60 to 90 kg.

In Study 7, 7.5 mg/kg was administered intravenously as 5-, 30-, and 60-min infusions. Six subjects received both the 30- and 60-min infusions, according to a balanced crossover design, into an antecubital vein using an infusion pump[1]. Six other subjects received the 5-min infusion as a push injection into an antecubital vein.

In Studies 1 and 2, amikacin was administered into the right or left superior lateral gluteal quadrant. In Study 1, 12 subjects each received 250- and 500-mg doses; some results were previously published (4). In Study 2, three subjects each received 125-, 250-, 500-, or 750-mg doses.

Blood samples were collected for serum preparation, and serum samples were analyzed for amikacin content using a standard cup plate bioassay (5) with *Bacillus subtilis* (ATCC 6633) as the bioassay organism. The minimum quantitative sensitivity of the assay was 0.06 μg/ml. Typically, an analysis of variance of the regression slope of the standard response line (zone diameter *versus* log concentration) yielded $F(1, 3) = 240$.

Mean postinfusion intravenous data were fitted to the biexponential equation for an open, two-compartment model of drug distribution with central compartment elimination only:

$$C = A' \exp(-\alpha t') + B' \exp(-\beta t') \qquad \text{(Eq. 1)}$$

[1] Model 940, Harvard Instrument Co., South Natick, Mass.

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