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Analysis of Pilocarpine and Isopilocarpine in Ophthalmic Solutions by UV Spectrophotometry–Polarimetry

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Abstract □ An improved analytical method was developed that simultaneously quantitates pilocarpine and isopilocarpine in the presence of each other and pilocarpic acid. Pilocarpine and isopilocarpine are first separated from any pilocarpic acid present in the sample by eluting with water-washed chloroform through a column packed with acid-washed diatomaceous earth. The concentrations of pilocarpine and isopilocarpine then are determined by a combination of UV spectrophotometric and polarimetric measurements. UV absorbance is measured at the absorption maximum (215 nm), and optical rotation is measured at the 254-nm line of mercury. Standard curve and standard recovery data are presented. The method is applicable to several commercially available ophathalmic solutions of pilocarpine and is compared to both the USP colorimetric method and a high-performance liquid chromatographic method.

Keyphrases □ Pilocarpine—analysis in the presence of isopilocarpine and pilocarpic acid, ophthalmic solutions □ Isopilocarpine—analysis in the presence of pilocarpine and pilocarpic acid, ophthalmic solutions □ UV spectrophotometry—analysis of pilocarpine and isopilocarpine, ophthalmic solutions □ Polarimetry—analysis of pilocarpine and isopilocarpine, ophthalmic solutions

Pilocarpine is an alkaloid used in the treatment of glaucoma to lower intraocular pressure. It has been reported to isomerize into isopilocarpine or to form pilocarpic acid reversibly in basic solution (1, 2). Both processes result in a loss of pharmacological activity (3, 4).

BACKGROUND

Several satisfactory nonspecific methods have been developed for the analysis of pilocarpine (5–11), but only recently have numerous specific analytical methods been reported (1, 2, 12–17). Two procedures combine colorimetry and polarimetry (1, 12); the first measures a chromium complex of pilocarpine (5), and the second measures an iron complex of the hydroxamic acid of pilocarpine (8). While both methods are theoretically sound in that the colorimetric methods should quantitate total pilocarpine and isopilocarpine and the polarimetric measurements should determine the amount of pilocarpine, these methods are not widely used for routine analysis.

One specific NMR spectrometry method, requiring a 100-MHz instrument, distinguishes between pilocarpine, isopilocarpine, pilocarpic acid, isopilocarpic acid, and pilocarpate and isopilocarpate ions (2). However, this procedure is not designed for multiple analyses and requires expensive instrumentation and reagents. A GLC method that separates pilocarpine and isopilocarpine was also described, but it involves a tedious derivatization of pilocarpine prior to analysis (13). Several specific high-performance liquid chromatographic (HPLC) methods for the quantitation of pilocarpine and isopilocarpine were reported (14–17) and were discussed previously (18).

The present report describes a specific procedure involving a combination of column chromatography, UV spectrophotometry, and polarimetry to analyze for pilocarpine and isopilocarpine in the presence of each other and pilocarpic acid. Validation data for the procedure and data resulting from the analysis of commercially available ophthalmic solutions by this and two other analytical methods are presented.

EXPERIMENTAL

Reagents—USP reference standard pilocarpine nitrate, USP grade pilocarpine hydrochloride[1], and ACS reagent grade isopilocarpine nitrate[2], hydrochloric acid, dibasic potassium phosphate, monobasic potassium phosphate, water-washed chloroform, and acid-washed diatomaceous earth[3] were used.

Solutions—The following were used: 0.1 *M* HCl and pH 5.8 potassium phosphate buffer (prepared by mixing one volume of 1 *M* dibasic potassium phosphate with nine volumes of 1 *M* monobasic potassium phosphate and adjusting to pH 5.8 with the appropriate potassium phosphate solution).

Equipment—A UV spectrophotometer[4], polarimeter[5], and 60-MHz NMR instrument[6] were used.

Optical Rotation Measurements—Stock solutions of pilocarpine hydrochloride and isopilocarpine nitrate were prepared in 0.1 *M* HCl. From these stock solutions, a series of solutions was prepared in concentrations ranging from 0.17 to 1.70 mg/ml of pilocarpine or isopilo-

[1] Quimitra S. A., Merck.
[2] Aldrich Chemical Co.
[3] Celite 545, Johns-Manville Product Corp.
[4] Beckman model ACTA CV with matched 1-cm quartz cells.
[5] Perkin-Elmer model 241 MC with a 100 × 4-mm i.d. cell.
[6] Varian model EM360A.

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carpine. Several solutions containing both pilocarpine and isopilocarpine were also prepared. The pilocarpine and isopilocarpine concentrations of these solutions varied inversely at intervals of 20% and ranged from 0.00 to 1.70 mg/ml. The total concentration was kept at a constant 1.70 mg/ml.

The optical rotation of each solution was measured as will be described under *Procedure*.

Standard Preparation—Approximately 100 mg of pilocarpine hydrochloride or the pilocarpine nitrate equivalent was accurately weighed into a 10.0-ml volumetric flask. The standard was dissolved and diluted to volume with purified water. After mixing, a 2.0-ml aliquot was pipetted into a 250-ml beaker.

Commercial Product Preparation—Sufficient sample to contain the equivalent of 20 mg of pilocarpine hydrochloride was pipetted into a 250-ml beaker.

Standard Curve Preparation—Aqueous stock solutions of pilocarpine and isopilocarpine were prepared similarly to those already described, and appropriate volumes were pipetted into 250-ml beakers. The resulting concentrations of the optical rotation solutions ranged from 0.35 to 1.04 mg/ml for the pilocarpine standard curve.

The total pilocarpine–isopilocarpine concentration of the combined solutions was 0.68 mg/ml. Again, the pilocarpine and isopilocarpine concentrations varied inversely (at intervals of 25%).

Standard Addition Sample Preparation—A series of pilocarpine nitrate samples containing ~17 mg as pilocarpine free base was prepared. Isopilocarpine nitrate was added to the pilocarpine solutions in intervals of 2%. The amount of isopilocarpine in the standard addition samples ranged from 2 to 10%.

Procedure—Six milliliters of pH 5.8 buffer was added to each standard and sample in the 250-ml beakers and thoroughly mixed. Approximately 12 g of acid-washed diatomaceous earth was added to each beaker and mixed well by stirring with a spatula. The contents of each beaker were transferred into separate glass chromatographic columns containing a pledget of glass wool at the bottom. Each beaker was then dry washed with ~3 g of acid-washed diatomaceous earth, which was also transferred to the columns. Another pledget of glass wool was placed at the top of each column. The contents of the columns were packed by gentle tapping, and the carpines were eluted with 200 ml of water-washed chloroform into separate 250-ml separators containing 25.0 ml of 0.1 *M* HCl. The separators were then vigorously shaken for 1 min.

The optical rotations of the acid layers and a 0.1 *M* HCl blank were measured at 254 nm, using a mercury vapor lamp. The optical rotation of the blank was subtracted from all other optical rotation measurements. Two milliliters of each optical rotation solution was then accurately diluted to 100.0 ml with 0.1 *M* HCl. The UV absorbance of these solutions was measured at the peak maximum (215 nm) by scanning from 245 to 205 nm after the spectrophotometer was zeroed at 215 nm with a 0.1 *M* HCl blank. Pilocarpine and isopilocarpine concentrations, as percent label pilocarpine, were calculated using the following:

$$C_p = (R/a_p - 0.165A/e_p)/0.835 \quad \text{(Eq. 1a)}$$

$$\%\ \text{label pilocarpoine} = 100\ C_p/C_t \quad \text{(Eq. 1b)}$$

$$\text{isopilocarpine (expressed as \% label pilocarpine)} = 100\ (AC_{std}/A_{std}C_t) - \%\ \text{label pilocarpine} \quad \text{(Eq. 1c)}$$

where all concentrations are those of the optical rotation solutions, in milligrams per milliliter, and:

C_p = calculated pilocarpine concentration in sample
R = degrees of optical rotation of sample
$a_p = R_{std}/C_{std}$
R_{std} = degrees of optical rotation of standard
C_{std} = pilocarpine concentration in standard
A = absorbance of sample
$e_p = A_{std}/C_{std}$
A_{std} = absorbance of standard
C_t = theoretical pilocarpine concentration in sample

RESULTS AND DISCUSSION

Optical Rotation—Pilocarpine and isopilocarpine both have the same molar absorptivity at their absorbance maximum of 215 nm, *i.e.*, $\log \epsilon = 3.77$ (19). Although both compounds rotate light in a positive manner in aqueous solution at 254 nm, pilocarpine exhibits a larger optical rotation than isopilcarpine. These two physical characteristics form the basis for the present analytical method.

Table I—Analysis of Isopilocarpine at Levels Commonly Found in Pilocarpine Ophthalmic Solutions

Theoretical Pilocarpine, %	Experimental Pilocarpine, %	Theoretical Isopilocarpine, %	Experimental Isopilocarpine, %
98.0	97.9, 98.0	2.0	2.2, 3.1
96.0	95.7, 95.3	4.0	4.0, 4.5
94.4	94.1, 93.9	5.6	6.2, 6.6
92.3	92.3, 90.9	7.7	7.9, 8.1
90.5	90.6, 90.6	9.5	9.6, 8.6

Three sets of aqueous standards, containing either pilocarpine, isopilocarpine, or a mixture of both, were prepared, and the optical rotations were measured. A least-squares analysis of the optical rotation *versus* concentration gave a correlation coefficient for pilocarpine of 1.0000 and a y intercept of −0.0018°. The relative standard deviation was 1.01%, and the slope was 1.330°/mg/ml. The correlation coefficient for the isopilocarpine data was 0.9993, and the y intercept was 0.0012°. The relative standard deviation was 2.07%, and the slope was 0.219°/mg/ml. The optical rotations of the pilocarpine and isopilocarpine solutions were additive, and the observed optical rotations of the pilocarpine–isopilocarpine mixtures agreed well with predicted values (99.2 ± 0.9%)[7].

Calculations—Equations for determining the pilocarpine and isopilocarpine content of the solutions were derived based on the UV and optical rotation measurements of pilocarpine and isopilocarpine standards. The derivations are as follows:

$$R = R_p + R_i \quad \text{(Eq. 2a)}$$

$$R = a_pC_p + a_iC_i \quad \text{(Eq. 2b)}$$

At a constant temperature and wavelength:

$$ya_p = a_i \quad \text{(Eq. 3)}$$

From the slopes of the pilocarpine and isopilocarpine optical rotation curves:

$$a_p = 1.330°/\text{mg/ml} \quad \text{(Eq. 4a)}$$

$$a_i = 0.219°/\text{mg/ml} \quad \text{(Eq. 4b)}$$

So:

$$y = a_i/a_p = 0.219/1.330 = 0.165 \quad \text{(Eq. 5)}$$

Thus:

$$R = a_p(C_p + 0.165C_i) \quad \text{(Eq. 6)}$$

Since:

$$A = A_p + A_i \quad \text{(Eq. 7)}$$

And then:

$$A = e_pC_p + e_iC_i \quad \text{(Eq. 8)}$$

Since:

$$e_p = e_i \quad \text{(Eq. 9)}$$

$$A = e_p(C_p + C_i) \quad \text{(Eq. 10)}$$

$$C_i = (A/e_p) - C_p \quad \text{(Eq. 11)}$$

So:

$$R = a_p[C_p + 0.165(A/e_p - C_p)] \quad \text{(Eq. 12a)}$$

$$R = a_p(C_p + 0.165A/e_p - 0.165C_p) \quad \text{(Eq. 12b)}$$

$$R = a_p[C_p(1 - 0.165) + 0.165A/e_p] \quad \text{(Eq. 12c)}$$

Therefore:

$$C_p = (R/a_p - 0.165A/e_p)/0.835 \quad \text{(Eq. 13)}$$

and:

$$\%\ \text{label pilocarpine} = 100\ C_p/C_t \quad \text{(Eq. 14)}$$

Isopilocarpine, expressed as percent label pilocarpine, equals the concentration of total carpine as determined by UV analysis after elution

[7] Mean ± *SD*.

Table II—Determination of Pilocarpine and Isopilocarpine in Commercially Available Pilocarpine Ophthalmic Solutions

Sample Concentration as Hydrochloride, %	Pilocarpine Analysis[a], %			Isopilocarpine Analysis[a], %	
	UV–Polarimetry	HPLC[b]	USP[c]	UV–Polarimetry	HPLC[c]
2	97, 99	98, 99	99, 99	0.0, 1.0	0.9, 1.0
1	96, 95	94, 96	95, 96	2.7, 4.4	1.4, 1.4
2	99, 100	102, 101	103, 104	3.7, 3.9	3.7, 3.6
2	99, 98	101, 100	100, 98	0.0, 0.8	0.7, 0.8

[a] Percent label as pilocarpine salt. [b] Reference 18. [c] The USP procedure is the colorimetric method described in Ref. 8.

from the column, minus the pilocarpine concentration as already determined:

isopilocarpine, as % label pilocarpine

$$= 100\,(AC_{std}/A_{std}C_t) - \%\text{ label pilocarpine} \quad \text{(Eq. 15)}$$

where all concentrations are those of the optical rotation solutions, in milligrams per milliliter, subscripts p refer to pilocarpine, subscripts i refer to isopilocarpine, and:

R = degrees of optical rotation
$a = R/C$
C = sample concentration
$e = A/C$
A = absorbance
C_{std} = pilocarpine concentration in standard
A_{std} = absorbance of standard
C_t = theoretical pilocarpine concentration in sample.

The pilocarpic acid concentration can then be calculated by subtracting the total carpine concentration from the total imidazole concentration, which can be obtained without chromatographic separation from the direct UV comparison of sample and standard dilutions. All concentrations in this calculation are expressed as percent label pilocarpine.

Analysis of Pilocarpine and Isopilocarpine—Pilocarpine standard solutions with concentrations of 0.007–0.021 mg/ml (0.34–1.04 mg/ml for the optical rotation concentrations) were analyzed by this procedure. A least-squares analysis of the absorbance plotted *versus* the concentration gave a correlation coefficient of 0.9996 and a y intercept of 0.014 absorbance unit. The relative standard deviation was 2.16%. The average log ϵ was 3.77, which agreed well with the published value (19). The correlation coefficient for the optical rotation plotted *versus* concentration was 0.9999 with a y intercept of −0.0071°, and the relative standard deviation was 0.48%. Complete elution of pilocarpine from the column was indicated by the good agreement of the observed optical rotation values with the predicted optical rotation values (102.0 ± 0.5%)[7].

A standard curve containing known mixtures of pilocarpine and isopilocarpine was evaluated using USP reference standard pilocarpine nitrate as the standard. The concentration of pilocarpine and isopilocarpine ranged inversely from 0 to 100% at intervals of 25%. The results showed that the method was specific for pilocarpine and isopilocarpine in the presence of each other; the experimental values agreed within 3% of the theoretical percent label.

A series of pilocarpine samples was analyzed after making standard additions of isopilocarpine ranging in concentration from 2 to 10% of the amount of pilocarpine present. The results showed that isopilocarpine can be analyzed accurately at levels commonly found in pilocarpine ophthalmic solutions. The results for pilocarpine and isopilocarpine were within 1% of the theoretical amount present (Table I).

Eluting the pilocarpine solutions through an acid-washed diatomaceous earth column with chloroform leaves methylcellulose (7) and most other interfering excipients of commercially available ophthalmic solutions, as well as pilocarpic and isopilocarpic acids (carpic acids) and pilocarpate and isopilocarpate ions (carpate ions), on the column. The pH 5.8 phosphate buffer prevents pilocarpine protonation, which, in turn, ensures that it will not be retained by the column. The retention of carpic acids and carpate ions by the column was confirmed by analyzing a sodium hydroxide-hydrolyzed solution of pilocarpine hydrochloride by NMR spectrometry (2) and the described UV–optical rotation method. This method detected 13% pilocarpine and 49% isopilocarpine, equal to 62% total carpines. NMR analysis detected 60% total carpines (pilocarpine plus isopilocarpine) and 40% total carpate ions. The results from these two methods were consistent and confirmed that carpic acids and carpate ions are not eluted from the column.

Analysis of Commercially Available Pilocarpine Solutions—Commercially available pilocarpine ophthalmic solutions were analyzed by the described method, a normal-phase HPLC method (18), and the USP colorimetric method (9). These solutions contained excipients commonly found in ophthalmic formulations such as benzalkonium chloride, chlorobutanol, disodium edetate, sodium acetate, citric acid, boric acid, menthol, camphor, and hydroxypropyl methylcellulose. Overall, the results of the UV–polarimetric method were in good agreement with the results from the HPLC and USP colorimetric methods. With the described procedure, the total pilocarpine and isopilocarpine content of the formulations approximately equaled the value obtained by the nonspecific USP colorimetric method, as expected (Table II). Thus, this method exhibits specificity and appears to be an improved analytical procedure for pilocarpine in ophthalmic solutions.

The only inconsistent results among the three methods were obtained on a 2% pilocarpine nitrate formulation containing polyvinyl alcohol and chlorobutanol. Average pilocarpine percent label values of 106, 94, and 127% were obtained for the UV–polarimetry, HPLC, and USP methods, respectively. The UV–polarimetry and HPLC methods produced average results for isopilocarpine of 2.0 and 1.2% label, respectively.

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