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Simultaneous Determination of Pilocarpine and Isopilocarpine in Pharmaceutical Preparations by Liquid Chromatography

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Abstract □ A liquid chromatographic assay method is described for the simultaneous determination of pilocarpine and isopilocarpine in pharmaceutical preparations. The method involves separation of the isomers on a high-resolution ion-exchange column, followed by detection of pilocarpine and isopilocarpine by UV absorption in the 217-nm region. The specificity of the method is such that pilocarpine and isopilocarpine can be assayed separately in the presence of one another and in the presence of the excipients commonly found in commercial pilocarpine solutions. Its sensitivity for isopilocarpine is at least 25 ppm (independent of the concentration of pilocarpine). In contrast, USP methods lack both specificity and sensitivity. It is concluded that this method is applicable to the routine analysis of commercial pilocarpine preparations and is an improvement over the official methods.

Keyphrases □ Pilocarpine—chromatographic separation and UV analysis, pharmaceutical preparations, in presence of isopilocarpine, compared to USP methods □ Isopilocarpine—chromatographic separation and UV analysis, pharmaceutical preparations, in presence of pilocarpine, compared to USP methods □ Chromatography—separation of pilocarpine and isopilocarpine in pharmaceutical preparations □ UV analysis—pilocarpine and isopilocarpine in pharmaceutical preparations

It is almost a century since the pilocarpine alkaloid was isolated from the plant *Pilocarpus microphyllus* Stapf (1), and its highly effective therapeutic application for glaucoma (2) treatment was soon realized. After the isolation of pilocarpine, its structure was well defined. However, the isolated alkaloid contains an inactive stereoisomer, which is not purely an artifact formed during the isolation process but occurs in the natural plant (3).

The common isomer is isopilocarpine, differing from pilocarpine only in the position of the ethyl substitution at the α-carbon on the lactone ring. Isopilocarpine has been reported to be therapeutically inactive (4). A literature review revealed only one paper reporting the determination of isopilocarpine (5). Isopilocarpine is thermodynamically more stable than pilocarpine in most environments (5) and readily forms from pilocarpine upon heating or in neutral or alkaline solutions (4, 6).

DISCUSSION

The lactone ring in pilocarpine is very labile (7); even under mild conditions, it can be opened, forming another inactive product, pilocarpic acid. While pilocarpic acid can be detected by the official methods (8–10), neither isomerization to isopilocarpine nor differentiation from pilocarpine in pharmaceutical preparations has been reported until now.

The official methods—optical rotation for the drug substance and colorimetric methods (9) for pharmaceutical preparations—lack selectivity and specificity. These methods do not discriminate isopilocarpine. Although formulations containing isopilocarpine have a lower optical rotation than those with pure pilocarpine, this method is not sensitive enough for isopilocarpine determination and is not applicable to pharmaceutical formulations. Additionally, a decrease in the optical rotation does not show which degradation products are formed. The colorimetric test is specific for pilocarpine so long as the lactone ring is intact; it differentiates pilocarpine from pilocarpic acid, its decomposition product.

It was observed in this study that the primary degradation product of pilocarpine was not pilocarpic acid but its isomer, isopilocarpine. The experiments showed that isomerization occurred fairly easily at elevated temperatures. Isomerization is known to proceed with increasing pH as well as with buffer effect (11, 12). Since the commercially available ophthalmic solutions analyzed were mostly in buffered solution and had variable pH values, some isomerization probably occurred.

The goal of this study was to develop a suitable, sensitive method that would improve the specificity of the official method. The proposed method is based on chromatographic separation of isopilocarpine and pilocarpine in the presence of various commonly used excipients in aqueous solutions. Isopilocarpine and pilocarpine are detected by their UV absorption at about 217 nm where their absorbances are identical. The method can be adopted for pilocarpine and isopilocarpine determination in commercial ophthalmic formulations, even where extremely high sensitivities are desired. In routine analysis, the proposed method has a sensitivity of 25 ppm for either pilocarpine or isopilocarpine in pharmaceutical formulations.

EXPERIMENTAL

Reagents and Solutions—The following were used: pilocarpine nitrate USP, isopilocarpine nitrate[1] (reagent grade), tromethamine[2] (ultrapure grade), cation-exchange resin[3], and reagent grade isopropanol, sulfuric acid, hydrochloric acid, methylene chloride, and ether.

Chromatography—For the elution method, the resin was placed in a glass column[4] (6 mm i.d. × 10 cm) packed by the slurry technique; the mobile phase was used to prepare the slurry. The column was washed thoroughly with the mobile phase until an optimum stable baseline was obtained.

A 0.2 *M* tromethamine buffer solution was prepared in distilled water. Then 5% isopropanol was added, and the pH was adjusted to 9 with sulfuric acid.

The flow rate during the elution was adjusted to 0.4 ml/min, and a pressure of 200 ± 100 psi was maintained. As conditioning proceeds, a slight increase in pressure and resolution may be observed.

A liquid chromatograph equipped with a suitable high or low pressure pump[5], a detector[6], and a recorder[7] were used. The detector was a variable wavelength spectrophotometer equipped with 8-μl flowcells of 1-cm pathlength. A pH meter[8] was used also.

Other column materials, sizes, and flow rates may be used so long as adequate resolution is obtained; a column "bed volume" of 2.8–3.0 cm^3 is normally required for separation. The assay time can be reduced by utilizing a larger bore or a shorter column or by changing the flow rates and the pressure.

Standard Preparation—USP pilocarpine nitrate reference standard and isopilocarpine nitrate (reagent grade) were accurately weighed into separate volumetric flasks and then dissolved and diluted to volume with water to obtain solutions containing 3–4 mg/ml of pilocarpine. To prepare a standard calibration curve, the two solutions were mixed accurately and proportionally, thus providing various concentrations of isopilocarpine. Fifty microliters was then injected by means of an injection loop on the column, and the chromatogram was recorded.

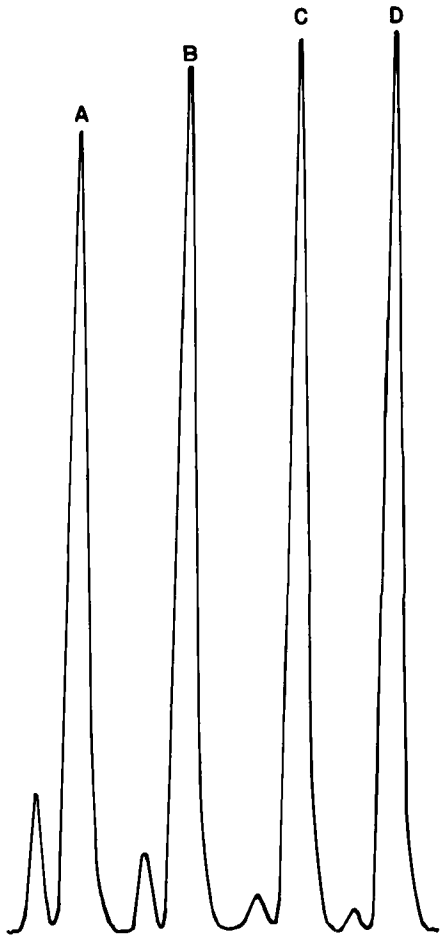

Figure 1—*Synthetic mixtures of pilocarpine adulterated with (A) 10, (B) 5, (C) 2, and (D) 1% isopilocarpine. Retention times are 45 and 38 min, respectively.*

Preparation of Commercial Products—Aliquots of commercially used ophthalmic solutions were accurately pipetted into separate containers and diluted with water to obtain about 3–4 mg of pilocarpine/ml. These solutions (50 μl) were injected in the same manner as the standard preparation, and the chromatograms were recorded.

Ocular Therapeutic Systems Containing Pilocarpine—In an ocular therapeutic system[9], pilocarpine is surrounded by a rate-controlling polymer membrane. Therefore, an extraction procedure was employed to liberate the pilocarpine. One or two systems were placed in a small centrifuge tube in 1 ml of methylene chloride to dissolve the polymer membrane. Then 3.0 ml of 0.1 *N* sulfuric acid was added, and the pilocarpine was extracted for a few minutes. Fifty microliters from the aqueous phase was withdrawn and injected on the column, and the chromatogram was recorded.

RESULTS

A suitable method that shows specificity and sensitivity for the determination of a pure substance and/or pharmaceutical preparation has always been a primary requirement of analytical chemistry. The various pilocarpine methods in the literature all lack specificity. Under certain mild conditions, pilocarpine undergoes isomerization. The existing methods are unable to detect the isomerized product, which is a main degradation product not only of the drug substance but of commercial products as well. Therefore, an attempt was made to develop a technique that would allow the determination of pilocarpine in the presence of isopilocarpine and vice versa.

[1] Aldrich Chemical Co.
[2] Tris buffer solution, Aldrich Chemical Co.
[3] Aminex A-7, sulfonated styrene divinylbenzene copolymer with 8% divinyl benzene cross-linking, particle size of 7–11 μm, Bio-Rad Laboratories.
[4] Chromatronic.
[5] Water Associates model M-6000.
[6] Schoeffel model 770.
[7] Texas Instrument Inc.
[8] Radiometer model 26.
[9] Ocusert, Alza Corp.

Table I—Determination of Total Pilocarpine and Isopilocarpine in a Mixture by USP Colorimetric Method[a]

Isopilocarpine–Pilocarpine Ratio	Total Isopilocarpine and Pilocarpine Weight, mg	Total Pilocarpine Found, mg	Recovery, %
Pure isopilocarpine	2.26	1.82 ± 0.02	80.5
1:4	2.05	1.99 ± 0.02	97.1
1:1	2.07	1.81 ± 0.02	87.5

[a] All tests were performed in duplicate.

Various concentrations of pilocarpine and isopilocarpine standard solutions were chromatographed using the recommended column and buffer solution. Figure 1 shows a fairly good resolution and linear relation between the two compounds.

To illustrate the validity of the proposed method, Fig. 2 demonstrates the separation of isopilocarpine from pilocarpine in a sample and the absence of isopilocarpine in USP pilocarpine nitrate reference standard. It is evident from these curves that sufficient resolution is obtained with the specified conditions; the method is adequate for the quantitative determination of pilocarpine in the presence of isopilocarpine.

To demonstrate that the official method is not specific enough for the determination of isopilocarpine, pure isopilocarpine and isopilocarpine in a mixture with pilocarpine were analyzed by the official colorimetric method. The reaction rate of hydroxylamine hydrochloride with isopilocarpine was slower than with pilocarpine. When using the official method, only 80% of the isopilocarpine was recovered. The recovery was not improved with an increased reaction time; but when the concentration of the reagent was doubled, the isopilocarpine was fully recovered. The data obtained on pure isopilocarpine and on mixtures with pilocarpine by the official method are summarized in Table I. One can see from this table that the total recovery of pilocarpines increased significantly with the increase of pilocarpine.

The data collected in Table II clearly show that the official method does not differentiate between the pilocarpine isomers and that the liquid chromatographic method shows significant differences in pilocarpine and isopilocarpine content. To prove the efficacy of the proposed method, several commercial ophthalmic solutions with the manufacturer's information (such as the name, formulation, and content) were analyzed for pilocarpine by the official colorimetric method and simultaneously for pilocarpine and isopilocarpine by the chromatographic method (Table III). The pH of these solutions was determined using a combination glass electrode.

It was observed that some ophthalmic formulations (C and D) could not be analyzed by the official method and gave very high assays; no significant interferences were observed with the chromatographic method. When the USP method was modified so that the extraction solvent was changed from ether to methylene chloride, normal results were obtained, indicating that the interfering substances were removed by methylene chloride but not by ether.

To date, no substance including pilocarpic acid has been found to interfere with the new method. Furthermore, since the ophthal-

Table II—Determination of Pilocarpine Content in Synthetic Mixtures with Isopilocarpine by the Official and Liquid Chromatographic Methods

	Pilocarpine and Isopilocarpine Assay[a]		
		Chromatographic	
Solution	USP	Pilocarpine	Isopilocarpine
49.7% pilocarpine and 50.3% isopilocarpine	90.0% ± 1.0	49.4% ± 0.4	50.6% ± 0.4
74.7% pilocarpine and 25.3% isopilocarpine	95.2% ± 0.9	74.8% ± 0.3	25.2% ± 0.4

[a] Average from duplicate determinations.

Table III—Comparison of USP and Liquid Chromatographic Methods for the Determination of Pilocarpine and Isopilocarpine in Commercial Products

					USP Method			Liquid Chromatographic Method			
Manufacturer	Salt	Pilocarpine Declared, mg/ml	pH	Expiration Date	Extracting Solvent	Total Pilocarpine Found, mg/ml	Recovery, %	Isopilocarpine, mg/ml	Pilocarpine, mg/ml	Recovery, %	Isopilocarpine in Total Pilocarpine, %
A	HNO_3	20	5.3	—	Ether	13.6	68.0	3.6	10.7	71.5	25.2
B	HCl	20	4.2	2/78	Ether	20.3	101.5	0.5	19.7	101.0	2.5
C	HNO_3	10	4.8	7/77	Ether	20.3	203.0	0.1	11.3	114.0	0.9
C		10	4.8		Methylene chloride	10.3	103.0				
D	HNO_3	40	4.6	6/77	Ether	49.7	124.3	0.5	43.8	110.0	1.1
D		40	4.6		Methylene chloride	44.3	110.8				
E	HCl	5	4.5	—	Ether	4.9	98.0	0.1	4.7	96.0	2.1
F	HCl	10	4.4	—	Ether	10.1	101.0	0.4	10.1	105.0	3.8
G	HCl	40	4.5	5/78	Ether	39.7	99.3	0.9	39.0	99.8	2.3
Ocular system[a]	Base	5.0	—	5/76	Methylene chloride	4.86	97.2	0.05	4.84	97.8	1.0

[a] Milligrams per system.

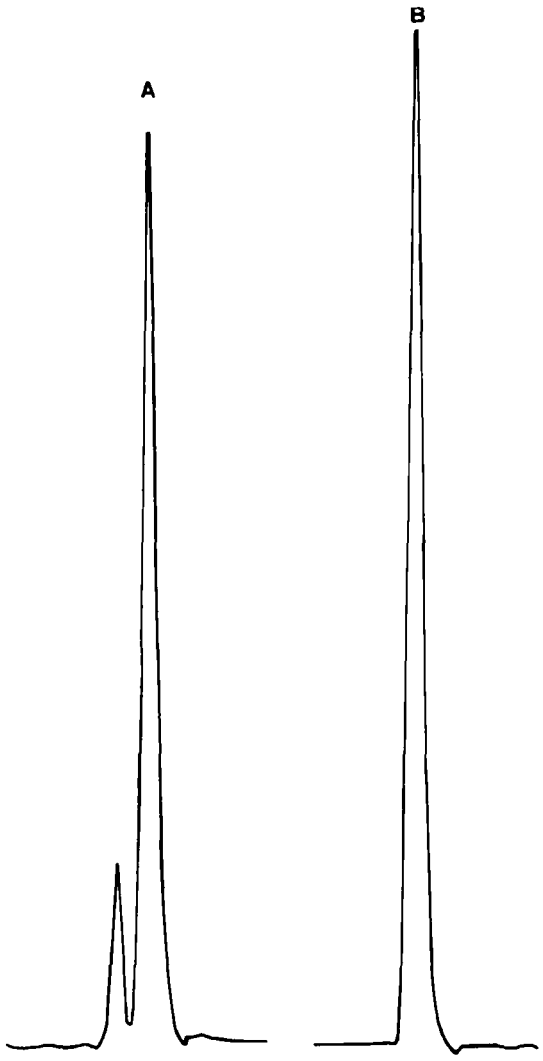

Figure 2—*Comparison of a commercial sample containing isopilocarpine (A) to USP pilocarpine nitrate reference standard (B).*

mic solutions can be injected directly onto the column, the analysis is simpler than with the USP method, a great advantage in everyday routine assay. Table III clearly indicates that the proportion of isomer tends to be higher when pH values are higher.

From Tables I–III, it is obvious that the official method does not discriminate between pilocarpine and isopilocarpine content. It is also evident that the official method is not suitable for total isopilocarpine and pilocarpine determination because of reaction rate differences between the two compounds.

A further study was carried out to fill the gap caused by the official method and to separate the two compounds quantitatively. The official method was compared with the liquid chromatographic method for the analysis of mixtures where the ratio of pilocarpine and isopilocarpine varied. These observations have already been mentioned. However, the official method was unable to differentiate between the two pilocarpine isomers and, unfortunately, its values depended on isopilocarpine concentration. The liquid chromatographic method distinguished the pilocarpine from the isopilocarpine with true and accurate results.

The pilocarpine and isopilocarpine values presented so far in this paper were based on ophthalmic solutions where the buffer effect and the storage conditions greatly affected the isomerization. Data are now presented for an ocular therapeutic system where the alkaloid was not in aqueous buffered solutions but in a semisolid form, enclosed by rate-controlling polymeric membranes. Accelerated stability studies showed that isomerization took place in this system with increasing temperature and time. Since the rate of delivery of the active drug was in the microgram range and this amount regulated the effectiveness of the product, the determination of its inactive degradation product was a critical problem from the analytical standpoint. The linearity and stability of pilocarpine and isopilocarpine were demonstrated in the figures. A plot of these values showed that concentrations of pilocarpine in the 0–100-μg range resulted in a straight line that followed Beer's law. A similar plot was obtained for isopilocarpine in the region of 0–6 μg. The extraction procedure was necessary to eliminate the excess polymer which ruins the column in continuous assays, consequently increasing the pressure and diminishing the resolution.

The method described in this paper was applied to ophthalmic solutions and ocular therapeutic systems. However, with a slight modification, this method can be applied to other pilocarpine formulations. The method has been used in these laboratories as a routine test with great success and accuracy.

SUMMARY AND CONCLUSION

A novel and specific method for the simultaneous determination of pilocarpine and isopilocarpine in various pharmaceutical formulations is presented. The procedure is based on a liquid chromatographic separation, followed by spectrophotometric analysis. The chromophoric groups of pilocarpine and isopilocarpine are the same; the absorbances of the two compounds in the range of measurement are similar. This similarity makes possible the quantitative determination of pilocarpine relative to isopilocarpine. The measurement can be performed by peak areas or by peak heights from the same chromatogram.

The proposed method is applicable for pure substances and/or pharmaceutical preparations containing various excipients. The method is sensitive at the microgram level required for low dose formulations. The specificity of the proposed method provides great improvement over the official and other published methods for pilocarpine analysis and is convenient for routine use. The limits of detection are approximately 0.1 μg of isopilocarpine in the presence of 100 μg of pilocarpine or vice versa.

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