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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR PILOCARPINE IN AQUEOUS HUMOR

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SUMMARY

A high-performance liquid chromatographic assay for pilocarpine has been developed for the determination of pilocarpine in aqueous humor. A structurally similar internal standard is used, and pilocarpine is separated from isopilocarpine under the chromatographic conditions used. A 100-μl sample is mixed with an aliquot of internal standard at pH 8.3 and extracted with methylene chloride. The extract is evaporated to dryness and the alkaloids are quaternized with *p*-nitrobenzyl bromide. Following the quaternization, the sample is evaporated to dryness, washed and diluted with a mobile phase—triethylamine mixture and analyzed by high-performance liquid chromatography using a reversed-phase octadecylsilane column with detection at a wavelength of 254 nm. This is a highly sensitive, reproducible and selective assay for measuring pilocarpine at physiological levels in individual aqueous humor samples.

INTRODUCTION

The widespread use of pilocarpine in clinical ophthalmology has stimulated the development of many assay techniques to determine the levels and distribution of the drug after topical application in the eye. Several methodologies have been employed including radioactive isotope techniques, spectrophotometry, polarography, and chromatographic assays [1–5]. Wide disparity in results has occurred with the different techniques. Often sensitive methods have not differentiated between pilocarpine and inactive degradation products [2, 3]. Hydrolysis and epimerization studies of Nunes and Brochmann-Hanssen [6] demonstrated the importance of considering these degradation products. Additionally, most techniques are not sensitive enough to measure concentrations achieved in individual aqueous humor samples.

With the development of high-performance liquid chromatography (HPLC)

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and gas—liquid chromatography it has become possible to separate the epimer of pilocarpine with sufficient sensitivity to measure the drug in small-volume biological samples [4, 5, 7]. The HPLC assay for pilocarpine described by Mitra et al. [7] did not give separation of the epimers in our hands. A serious shortcoming of the procedure is the lack of an internal standard.

The present paper describes an HPLC assay for pilocarpine which has proved reproducible and sufficiently sensitive to measure low concentrations of the drug in individual aqueous humor samples. The technique incorporates the derivatization on the imidazole ring of pilocarpine with *p*-nitrobenzyl bromide to increase sensitivity [7]. Excellent separation of pilocarpine and its epimer is achieved using ion-pair chromatography. Pilocarpic acids are not extracted at the pH used. A structurally similar internal standard is used.

EXPERIMENTAL

Chemicals

Pilocarpine nitrate and triethylamine were obtained from Sigma (St. Louis, MO, U.S.A.). A commercially available pilocarpine solution (Isoptocarpine, Alcon Labs.) was used for administration to laboratory animals. Commercial preparations of therapeutic agents tested for drug interference were obtained from the University Hospital Pharmacy. Isopilocarpine nitrate and *p*-nitrobenzyl bromide were obtained from Aldrich (Milwaukee, WI, U.S.A.). *p*-Nitrobenzyl bromide was recrystallized from ethanol—water before use. Pilosine was obtained from ICN Pharmaceuticals (Plainview, NY, U.S.A.) and recrystallized from ethanol. 1-Octanesulfonic acid, sodium salt, was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Methylene chloride, cyclohexane, and diethyl ether without preservative, distilled-in-glass grade, were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals were reagent grade. Water was double-distilled. Aqueous humor and all solutions were filtered through a 0.45-μm Millipore filter prior to use. Centrifuge tubes and ampules used in the derivatization procedure were silanized with dimethyldichlorosilane.

Working solutions

An aqueous stock solution containing pilocarpine nitrate at 66 μg/ml was used to prepare a series of standard solutions of the drug in normal rabbit aqueous humor at 0.25, 0.50, 1.0, 2.5, 5.1, 7.6, 8.5, 9.2 and 10.1 μg/ml free base concentrations. Standard solutions of the epimer were prepared from an aqueous stock solution containing 13 μg/ml isopilocarpine nitrate. The working internal standard solution contained pilosine at a concentration of 21 μg/ml in water. Potassium bicarbonate was prepared at a concentration of 0.3 *M*, pH 8.3. The derivatizing solution was *p*-nitrobenzyl bromide, 0.5 mg/ml, in acetonitrile.

Chromatography

The Beckman (Anaheim, CA, U.S.A.) Model 330 isocratic system consisting of a Model 110 A pump, Model 210 sample injection valve and Model 153 detector for ultraviolet (UV) detection at 254 nm was used in conjunction with

a Heathkit strip-chart recorder. An Altex-Beckman Ultrasphere reversed-phase octadecylsilane (RP-ODS) column, particle size 5 μm, 25 cm × 4.5 mm I.D. was used. The mobile phase was pumped at 1.3 ml/min. Column-to-column variation in initial backpressure required occasional adjustment in the flow-rate. The column temperature was maintained at 25–26°C. The mobile phase was prepared by adding 48 ml of 0.25 *M* glycine–hydrochloric acid buffer, pH 2.1, and 20 ml of 0.1 *M* 1-octanesulfonic acid to 1 l of water. The resulting solution was mixed with 440 ml of isopropyl alcohol containing 1.2 ml triethylamine, followed by the addition of water to a final volume of 2 l.

Assay procedure

A 100-μl aliquot of the standard solution or aqueous humor was placed in a 12-ml conical test tube. A 30-μl aliquot of working internal standard solution was added, followed by 600 μl of potassium bicarbonate solution. The mixture was swirl-mixed immediately; 1 ml methylene chloride was added. The sample was mixed for 1 min by swirl-mixing, and centrifuged for 5 min at 300 *g*. The aqueous layer was aspirated and discarded. The organic layer was transferred to an ampule and evaporated to dryness under nitrogen. A 100-μl aliquot of derivatizing solution was added to the dry sample. The ampule was sealed under a nitrogen atmosphere and mixed. The sample was derivatized by placing the ampule in a 40°C oil bath for 40 h. Following derivatization, the ampule was cooled to room temperature and frozen at −40°C until chromatography was performed.

Prior to chromatographic analysis, the ampule was opened and diluted with 500 μl of acetonitrile. The diluted sample was mixed thoroughly and a 200–300 μl aliquot was removed and evaporated to dryness under nitrogen. The dried sample was treated with 0.4 ml of diethyl ether, the tube was swirl-mixed for 30 sec and the diethyl ether was discarded. This washing procedure was performed six times with diethyl ether followed by three times with cyclohexane. The sample was then redissolved in 32 μl of a solution containing mobile phase and 2 m*M* triethylamine (96:4), swirl-mixed, and a 20-μl sample was injected into the HPLC system. Aqueous controls were routinely used.

Milligram quantities of pilocarpine, isopilocarpine and pilosine derivatives were prepared as described by Mitra et al. [7] and used in preliminary work to establish retention times and chromatographic conditions.

Stability of derivatives

Pure derivatized standards of pilocarpine, isopilocarpine and pilosine were dissolved in acetonitrile at a concentration of 6 μg/ml and frozen at −40°C. Ten samples at a concentration of 2.5 μg/ml were carried through the assay procedure and frozen in the unopened ampules. The ampules were opened and the samples assayed as described. Peak height ratios were determined.

Standard curve

Standard curves were constructed by analyzing a series of aqueous humor samples containing known amounts of pilocarpine free base in a concentration range of 0.25 to 10.1 μg/ml.

Precision

Within-day and between-day variability were determined by analyzing ten replicate samples containing pilocarpine at a concentration of 2.5 μg/ml.

Specificity and interference studies

Samples of rabbit aqueous humor, obtained from animals not treated with pilocarpine, were analyzed without the addition of internal standard to identify potential interference by endogenous components.

Interference by therapeutic agents frequently used with pilocarpine or often encountered in our patient population was evaluated. Drugs tested included methazolamide, acetazolamide, diazepam, acetaminophen, caffeine, echothiophate iodide, epinephrine·HCl, ketamine·HCl, pentobarbital, carbachol and timolol maleate. Drugs were tested at a 5 μg/ml concentration except for diazepam, which was studied at a 1 μg/ml concentration.

Animal studies

New Zealand white male rabbits weighing approx. 2 kg were given a 50-μl dose of 4% pilocarpine topically in the right eye. Rabbits were sedated with pentobarbital. At 30 min, aqueous humor was aspirated and frozen at −40°C for subsequent assay.

Column maintenance

The column was washed 30 min with water at a flow-rate of 1.0 ml/min, and 30 min with acetonitrile at a flow-rate of 1.0 ml/min at the end of each day. The column was equilibrated overnight with mobile phase at a flow-rate of 0.5 ml/min. At the end of the week the column was washed with water for 1 h at a flow-rate of 1 ml/min and stored in methanol.

RESULTS

Under the chromatographic conditions used, the retention times of pilocarpine, isopilocarpine and the internal standard were 11.2, 12.2 and 18.4 min, respectively. The resolution factor (R_s) was 1.62 for the two epimers. A separation factor (α) of 1.07 was calculated. Some variability from HPLC column to column was observed, but adequate resolution of pilocarpine and isopilocarpine was always possible. Fig. 1. is a chromatogram of the standards.

Pure standards of derivatized isopilocarpine and pilocarpine, dissolved in acetonitrile at 6 μg/ml concentration, were found to be stable when stored at −40°C for four months. Samples carried through the assay procedure and frozen in unopened ampules were found to be stable for two weeks after derivatization. Ten samples containing pilocarpine at a concentration of 2.5 μg/ml were found to contain 2.8 μg/ml pilocarpine, coefficient of variation (C.V.) 7.3%, after storage for thirteen days at −40°C. Derivatized samples stored for more than two weeks at −40°C had a C.V. of 15.6%. Pilocarpine in aqueous humor samples frozen without sample preparation appeared stable for several months.

Analysis of a series of aqueous humor samples containing known amounts of pilocarpine yielded a standard curve in which the concentration of the drug

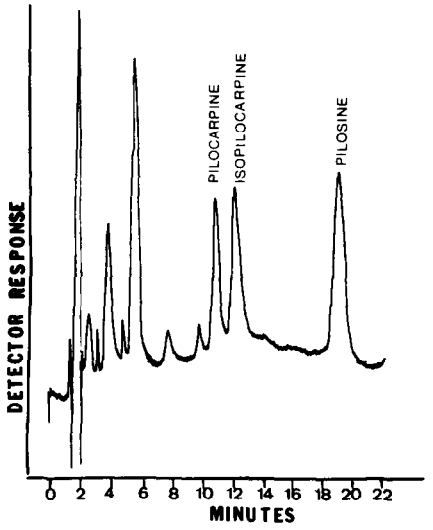

Fig. 1. Chromatogram of a mixture of pilocarpine, isopilocarpine and pilosine standards carried through the assay procedure.

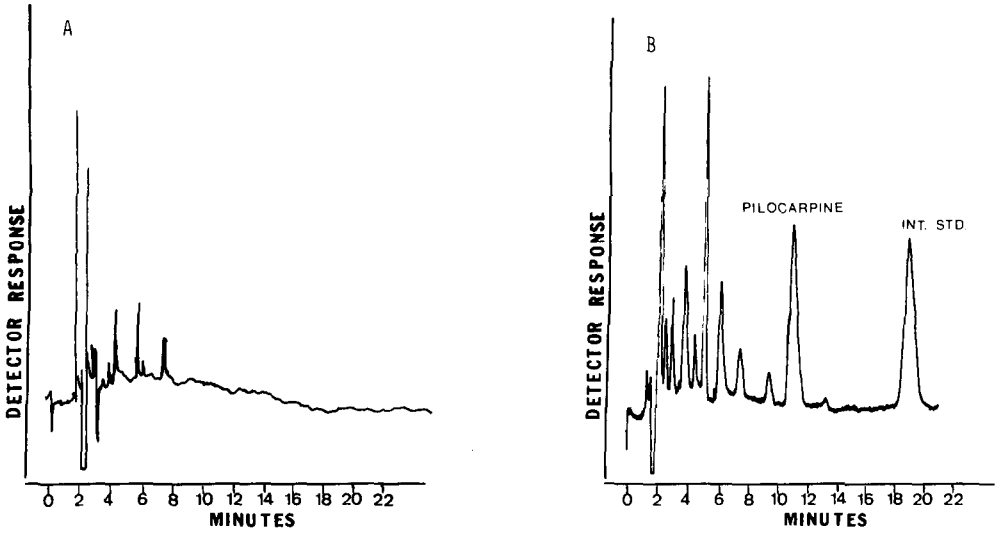

Fig. 2. Chromatograms obtained by analysis of rabbit aqueous humor: (A) obtained from animals not treated with pilocarpine, and without addition of internal standard; (B) 30 min after topical administration of 50 μl of 4% pilocarpine. Concentration: 5.75 μg/ml.

was linearly related to the pilocarpine:internal standard peak height ratios. The data fit the equation of a straight line: peak height ratio = 0.2198[pilocarpine] + 0.0043. Least-square analysis yielded a coefficient of correlation (r) of 0.9984. The assay was developed for a concentration range of 0.25—10.1 μg/ml. The lowest concentration studied was 0.25 μg/ml. Preliminary experiments (data not shown) indicated that the range of linearity may be extended well beyond the 10 μg/ml concentration. Curves constructed from pilocarpine in water were essentially identical to those from aqueous humor. The variability

observed from both sources was no greater than that observed from injection to injection, therefore, aqueous controls were routinely used.

The within-day analysis of ten replicate samples containing 2.5 μg/ml pilocarpine gave a mean drug concentration of 2.48 μg/ml with a C.V. of 7.1%, while between-day results were 2.3 μg/ml, C.V. 7.9%.

No interference by endogenous compounds was found when aqueous humor from normal rabbits was analyzed (Fig. 2A). Methazolamide, acetazolamide, diazepam, acetaminophen, caffeine, echothiophate iodide, epinephrine·HCl, ketamine·HCl and carbachol, did not interfere with the assay. Timolol maleate showed a small peak at the pilocarpine retention time. Pentobarbital, used as the sedating agent, did not interfere.

A 50-μl dose of 4% pilocarpine was given topically to four unsedated young rabbits. At 30 min after administration, the aqueous humor was found to contain pilocarpine in a concentration range of 5—8 μg/ml. Fig. 2B was obtained by analyzing rabbit aqueous humor following topical administration of pilocarpine.

DISCUSSION

Pilocarpine may epimerize to isopilocarpine under certain conditions, and since isopilocarpine is essentially pharmacologically inactive, separation of the two compounds during analysis is important [6, 8, 9]. We were unable to obtain the desired separation using the procedure described by Mitra et al. [7]. One broad peak was obtained with no resolution. This may have been due to the differences in the RP-ODS columns used. We retained the derivatization procedure to obtain the desired sensitivity and turned to other solvents for separation. Acetonitrile -water (75:25) with millimolar ion-pairing agent gave excellent separation of the epimers, but the internal standard was eluted with isopilocarpine. The chromatographic conditions used in the present procedure provide good separation of pilocarpine and isopilocarpine. However, to effect good separation it is necessary to inject the sample in a mobile phase—triethylamine mixture. Triethylamine decreases tailing of the peaks and improves separation of the epimers. Increased concentrations of ion-pairing agent, iso-

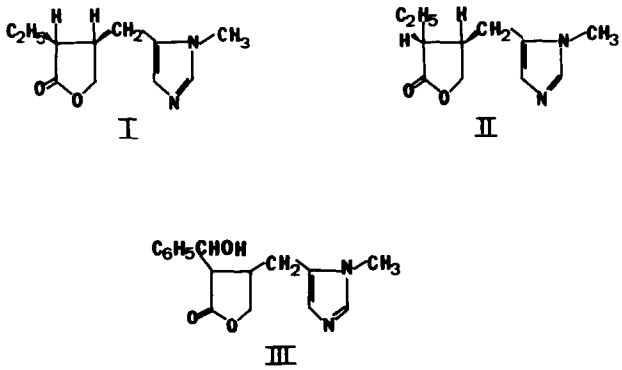

Fig. 3. Structures of the epimers pilocarpine (I) and isopilocarpine (II), and the internal standard, pilosine (III).

propyl alcohol, buffer or triethylamine in the mobile phase decreases the retention times at the expense of resolution.

Pilosine was chosen for the internal standard because it closely resembles pilocarpine in chemical structure (Fig. 3), is extracted and derivatized similarly to the compounds under study and is commercially available. Pilosine is unlikely to epimerize during the procedure [10].

The extraction procedure was also patterned after Mitra et al. [7] but utilized only a single extraction. This was shown to provide significantly cleaner extracts although there was some compromise in extraction efficiency. We found that approx. 85% of the pilocarpine was recovered with one extraction. The derivatization of the compounds of interest with *p*-nitrobenzyl bromide was extended from 24 h [7] to 40 h, since it was found that pilosine required a longer reaction time. The washing procedure with diethyl ether and cyclohexane was included to remove contaminants from the samples.

No isopilocarpine was found in the rabbit aqueous humor after administration of pilocarpine. For this reason a sample of commercial pilocarpine was diluted to 10 μg/ml free base and assayed. The commercial preparation was found to contain 10.9 μg/ml pilocarpine and no detectable isopilocarpine. This finding is similar to those of Drake et al. [11] and Noordam et al. [12].

In the chosen concentration range, one-third to one-half of the derivatized sample was used for each injection. This permitted a repeat injection if necessary. At pilocarpine concentrations of 0.5 μg/ml or less, the entire derivatized sample was required for accurate quantification.

Column temperatures and pressure as well as complete equilibration of the column proved critical in obtaining reproducible retention times. Extensive column washing was necessary to extend column life.

The stability studies indicate that aqueous humor samples may be frozen at −40°C for several months prior to sample preparation. Derivatized samples should be assayed within two weeks after derivatization. The purified standards, made up in acetonitrile, were stable for several months at −40°C. Pure, dried, derivatized standards of pilocarpine and isopilocarpine broke down completely after six months storage in vacuum dessicator at room temperature.

Drugs tested for potential interference were antiglaucoma drugs commonly used with pilocarpine; also diazepam, acetaminophen and caffeine. Only timolol showed a peak which could interfere in the assay. It was not established if this peak were due to the drug itself or a constituent of the commercial solution. Steroids were not tested as they would be removed in the diethyl ether wash. Pilocarpic acids are not extracted into methylene chloride at the pH used. Ketamine and pentobarbital were also tested for interference since they are routinely used in animal work in our laboratory.

The concentration of pilocarpine found in the rabbit aqueous humor was similar to that found by Chrai and Robinson [2] following a dose of 25 μl of 0.1 *M* pilocarpine with an assay using tritium-labeling.

A considerable number of assays for pilocarpine exist for the determination of the drug in commercial preparations. A need existed, however, for a highly sensitive and selective assay for the drug at physiological concentrations. While our assay is elaborate, it is selective, sensitive and reproducible. It is applicable to the study of pilocarpine distribution in the animal eye and to the analysis of pilocarpine in individual human aqueous humor samples.

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