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High-performance liquid chromatographic determination of pilocarpine hydrochloride and its degradation products using a β-cyclodextrin column

Kent D. Sternitzke, Tony Y. Fan and Danny L. Dunn*

Analytical Chemistry, Alcon Laboratories, Inc., 6201 South Freeway, Fort Worth, TX 76134-2099 (USA)

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method for the determination of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid was developed. A β-cyclodextrin column achieved the separation in less than 10 min. Baseline resolution of all four compounds permitted the determination of each degradation product in the presence of pilocarpine. The calibration graphs for each compound were linear, and pilocarpine degradation products could be determined without a correction for the ultraviolet detector response using a pilocarpine standard. A comparison of β-cyclodextrin separation with the USP HPLC method demonstrated similar results for pilocarpine contents in several commercial ophthalmic formulations.

INTRODUCTION

Pilocarpine hydrochloride is a miotic (cholinergic) used to control intraocular pressure [1]. Because of its widespread use for the treatment of glaucoma, much effort has been made to develop methods to determine this drug and its degradation products.

The degradation routes of pilocarpine are shown in Fig. 1. Pilocarpine (**I**) can undergo degradation by either epimerization to isopilocarpine (**II**) or hydrolysis to pilocarpic acid (**III**) [2]. Isopilocarpine can degrade further to isopilocarpic acid (**IV**). Competition between the degradation routes is dependent on both pH and temperature [3–7]. Because all three degradation products show decreased cholinergic activity, it is desirable to determine pilocarpine in the presence of the degradation products in ophthalmic formulations. Determination of the degradation products is also often desirable to acquire information concerning product stability and demonstrate material balance.

A variety of methods have been applied to the determination of pilocarpine in ophthalmic solutions, including ^{13}C NMR [6,7], spectrophotometry and polarimetry [8,9] and gas chromatography [10]. However, high-performance liquid chromatography [HPLC] has proved to be the most sensitive and selective technique for the accurate determination of pilocarpine and small amounts of degradation products [11–20].

Many studies have dealt with the HPLC of pilocarpine, utilizing a wide selection of stationary and mobile phases. Several methods involved octadecylsilane (C_{18}) columns with aqueous mobile phase systems [11–15]. Although some separation was attained, baseline resolution was usually not achieved, and some of the methods suffered from a high back-pressure, short column lifetime, long run times and irreproducibility. Silica columns with an aqueous phosphate buffer–methanol mobile phase have achieved some resolution of the carpines, but not sufficient to determine small amounts of isopilocarpine [16]. The use of a phenyl column resolved the carpines [17,18], but the resolution and column lifetimes were observed to be inconsistent in our

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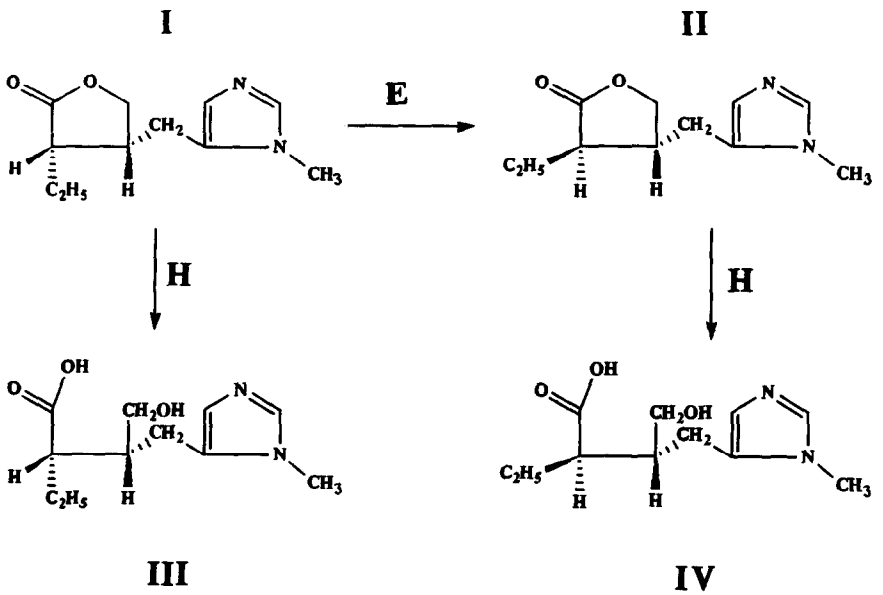

Fig. 1. Degradation reactions of pilocarpine. **I**, pilocarpine; **II**, isopilocarpine; **III**, pilocarpic acid; **IV**, isopilocarpic acid. Reactions: epimerization; H = hydrolysis.

laboratory. A recently published analysis on a cyano column gave an excellent separation, but required a mobile phase pH of 2.5 [19].

The present USP method for pilocarpine ophthalmic solution is a normal-phase system, utilizing a silica column and a mobile phase consisting of ammonia in isopropyl alcohol and hexane [20]. Although baseline resolution of the carpines is attained and column lifetimes are long, determination of the carpic acids is impossible owing to their complete retention on the HPLC column. It would be desirable to have a method that combines high resolution, determination of carpic acids and good column lifetime.

In this paper we present an improved HPLC method for the analysis of ophthalmic formulations containing pilocarpine and each of its degradation products using a β-cyclodextrin column. Baseline resolution of all four compounds is achieved in less than 10 min. Calibration graphs are given for each compound. Pilocarpine and its degradation products were determined in several commercial ophthalmic formulations and the results compared with those of the USP method.

EXPERIMENTAL

Reagents

Sodium sulfate, ammonium sulfate, pilocarpine hydrochloride and isopilocarpine nitrate were obtained from Aldrich (Milwaukee, WI, USA), phosphoric acid from J. T. Baker (Phillipsburg, NJ, USA) and HPLC-grade triethylamine from Fisher Scientific (Pittsburgh, PA, USA). Isopilocarpine hydrochloride, acquired from Inland Alkaloid (Tipton, IN, USA) was found to be a mixture of pilocarpine hydrochloride and isopilocarpine hydrochloride (42:58), and this mixture was used for evaluating chromatographic resolution. All chemicals except triethylamine were of analytical reagent grade and used as received.

Pilocarpic acid and isopilocarpic acid were prepared by dissolving the Inland Alkaloid mixture in 1 *M* sodium hydroxide solution at room temperature in a manner similar to that of Repta and Higuchi [21]. In alkaline solution, hydrolysis opens the γ-lactone ring of the carpines [16,22]. Conversion of the carpines (**I** and **II**) to the carpic acids (**III** and **IV**) was rapid (less than 1 min) and complete, as no

carpine peaks were observed in subsequent chromatograms.

Chromatography

Separations were performed isocratically on a 25 × 0.46 cm I.D. β-cyclodextrin (5 μm) column (Cyclobond I; Rainin, Woburn, MA, USA). Occasionally new β-cyclodextrin columns received from Rainin would not give baseline resolution of pilocarpine and isopilocarpine and could not be used for analytical work. The mobile phase was prepared by dissolving 40 g of ammonium sulfate and 20 ml of triethylamine in 1000 ml of water; the pH was adjusted to 4.0 by the addition of phosphoric acid. The flow-rate of a Waters (Milford, MA, USA) Model 510 HPLC pump was set at 1.0 ml/min, resulting in a back-pressure of 100 bar. AWaters WISP 710B autosampler was used to make 10-μl injections. Output from a Waters Model 486 variable-wavelength ultraviolet detector, set at 214 nm, was connected to a Waters Model 746 electronic integrator for recording data.

All dilutions to prepare samples and standards for injection were made with mobile phase. Because of its acidic pH and high ionic strength, the mobile phase was capable of dispersing a variety of ophthalmic vehicles. For instance, an ophthalmic gel could be dissolved in mobile phase without the use of additional reagents to disperse the gel matrix, thus saving a step in sample preparation. Final concentrations of pilocarpine were about 0.04 mg/ml, as this gave a reasonable signal-to-background ratio without overloading the column. At this concentration, several injections of pilocarpine were needed to equilibrate the column prior to quantitative analysis.

RESULTS AND DISCUSSION

Preliminary development

Although some separation occurred on α- and γ-cyclodextrin columns, the resolutions were not comparable to those observed on a β-cyclodextrin column for any of the mobile phases tested. Only β-cyclodextrin columns achieved baseline separation of the carpines. For instance, with the mobile phase described under Experimental, the resolution of pilocarpine and isopilocarpine was found to be 0.59, 2.25 and 0.26 on α-, β- and γ-cyclodextrin columns, respectively.

Mobile phase optimization with the β-cyclodextrin columns included adjustment of pH, ionic strength and column modifier concentration. Minimum use of organic mobile phase modifiers, such as methanol or acetonitrile, was necessary to increase retention with a concomitant increase in the separation of pilocarpine and isopilocarpine. However, tailing was severe, as is common for amine compounds in reversed-phase HPLC systems with a high aqueous content. High concentrations of triethylamine column modifier decreased tailing, but the tailing remained too excessive for baseline resolution of the carpines until the mobile phase ionic strength was increased by the addition of sodium or ammonium sulfate. The resulting decrease in tailing was accompanied by a comparatively small increase in retention time. Therefore, it was possible to attain greater resolutions with higher salt concentrations.

Chromatographic resolution

Fig. 2 presents a chromatogram of pilocarpine and its three degradation products, with the ionic strength mobile phase used for the remainder of the work presented here. The peaks in this chromatogram exhibit good symmetry, as measured by calculation of tailing factors according to the US Phar-

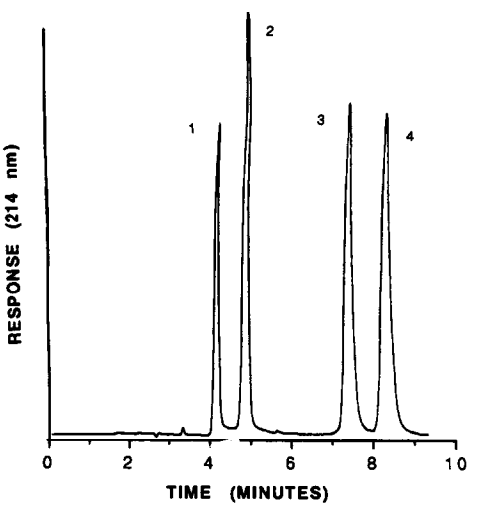

Fig. 2. Chromatogram demonstrating the separation of pilocarpine and its degradation products on β-cyclodextrin. Peaks: 1 = pilocarpic acid; 2 = isopilocarpic acid; 3 = pilocarpine; 4 = isopilocarpine.

macopeia [23]. Tailing was calculated as 1.40 for pilocarpine, 1.14 for isopilocarpine and less than 1.10 for both of the carpic acids.

Chromatographic resolution is critical in pilocarpine analysis because there is usually a small amount of degradation products in the presence of a much larger amount of pilocarpine. The resolution between pilocarpine and isopilocarpine calculated according to the US Pharmacopeia [23] was 2.25, whereas that between pilocarpic acid and isopilocarpic acid was 2.53. This is superior to any previously reported HPLC method and is sufficient to determine each species without interference.

Dynamic range

A five-point calibration graph generated for pilocarpine exhibited linear behaviour over the range 25–150% of 0.04 mg/ml, the final dilution concentration used for samples. For the calibration line, $R^2 = 0.9992$ and y-intercept = 0.34% of the maximum response. Hence the use of a single-point is justified. A set of ten standard replicates near 0.04 mg/ml had a relative standard deviation of 0.62%. This level of precision is sufficient to justify 0.04 mg/ml as a suitable concentration for a standard.

Stability samples of pilocarpine ophthalmic solution usually contain isopilocarpine at concentrations less than 10% of the labeled pilocarpine. In order to test the accuracy of this method for determining the isopilocarpine content of such samples, solutions were prepared with pilocarpine present at 0.04 mg/ml and isopilocarpine present at 2%, 6% and 10% of this concentration. The resulting calibration line for isopilocarpine had $R^2 = 0.9998$ and y-intercept = 0.15% of the maximum response. The chromatographic peak for pilocarpine does not appear to interfere with that of isopilocarpine.

Pilocarpic acid and isopilocarpic acid elute several minutes before pilocarpine and are baseline resolved from each ohter. Calibration graphs were prepared for each acid at concentrations of 1%, 3% and 5% of the 0.04 mg/ml concentration used for pilocarpine. These concentrations are typical for partially degraded ophthalmic solutions. A three-point calibration line for pilocarpic acid had $R^2 = 0.9975$ and y-intercept = 3.1% of the maximum response and for isopilocarpic acid $R^2 = 0.9997$ and y-intercept = 2.1% of the maximum response. This degree of linearity at low concentrations suggests that the acids do not interfere with each other

TABLE I

CHROMATOGRAPHIC ANALYSIS OF COMMERCIAL PILOCARPINE OPHTHALMIC FORMULATIONS: COMPARISON OF ANALYSIS ON A β-CYCLODEXTRIN COLUMN AND THE USP METHOD

Sample[a]	% of label claim				
	Pilocarpic acid[b]	Isopilocarpic acid[b]	Pilocarpine[b]	Isopilocarpine[b]	Pilocarpine[c]
Ophthalmic gel	5.7	–	105	1.5	106
Ophthalmic solutions:					
Manufacturer 1					
Lot 1	1.7	–	101	0.9	102
Lot 2	1.7	–	102	0.9	103
Lot 3	0.8	–	102	0.6	101
Manufacturer 2	2.2	–	96	0.3	96
Manufacturer 3	4.4	0.2	99	0.8	96
Manufacturer 4	5.4	–	101	–	98
Manufacturer 5	–[d]	–	95	–	94

[a] 10-μl injections, two injections each of two independently prepared replicates per lot. Results are stated in % of label claim for pilocarpine.

[b] HPLC analysis on a β-cyclodextrin column.

[c] USP HPLC analysis: silica gel colum; mobile phase, *n*-hexane–2% ammonia hydroxide in isopropyl alcohol (70:30). HPLC analysis of gel preceded by digestion in lead acetate.

[d] Interference from epinephrine prevented the determination of pilocarpic acid.

and are not interfered with by pilocarpine. A small amount of interference was caused by unidentified peaks eluting near to the solvent front in some of the ophthalmic formulations tested.

Analysis of ophthalmic gels and solutions

The method was compared with the USP method in the analysis of five commercially available pilocarpine ophthalmic solutions and a pilocarpine ophthalmic gel. The ophthalmic solutions selected were 2% in pilocarpine hydrochloride or nitrate in aqueous vehicles. The gel, Pilopine HS Gel (Alcon Labs., Fort Worth, TX, USA), contains 4% pilocarpine hydrochloride in a Carbomer 940 (BF Goodrich, Cleveland, OH, USA) vehicle. As stated previously, direct dilution with mobile phase was sufficient to prepare either formulation for HPLC analysis.

The samples were diluted with mobile phase to obtain a concentration of about 0.04 mg/ml pilocarpine and analyzed by the HPLC method reported here. These samples had previously been analyzed by the USP HPLC method with analysis of the gel preceded by digestion in lead acetate. The results are presented in Table I. The two methods are comparable for pilocarpine determination, but the present method also permits the determination of all three degradation products. Pilocarpine standards can be used to determine all four analytes, and a correction for the ultraviolet response at 214 nm was found to be unnecessary as the ratio of the molar absorptivities ($\varepsilon^{214}_{\text{carpic acid}}/\varepsilon^{214}_{\text{carpine}}$) was measured as unity.

Detection limits

Detection limits were calculated for each species. For the carpic acids, the signal-to-noise ratio was 3 when the concentrations were *ca.* 0.2% of a 0.04 mg/ml pilocarpine injection. The detection limit for isopilocarpine was dictated by the degree of separation from pilocarpine. The isopilocarpine signal became an indistinguishable shoulder on the pilocarpine peak when the isopilocarpine concentration was about 0.3% that of pilocarpine. These detection limits demonstrate satisfactory levels of sensitivity for most analyses.

CONCLUSIONS

The HPLC method presented here is an improvement on existing methods for the determination of pilocarpine hydrochloride in ophthalmic formulations. The determination of pilocarpine in aged ophthalmic samples gave results comparable to those obtained by the current USP HPLC method. However, separation on a β-cyclodextrin column is superior because complete determination of degradation products is also possible. Isopilocarpine, pilocarpic acid and isopilocarpic acid all gave well resolved peaks and a linear response in the presence of a 100-fold larger concentration of pilocarpine. The β-cyclodextrin HPLC method appears to be accurate and sensitive enough for the routine determination of pilocarpine and its degradation products in ophthalmic samples.

Complete separations are obtained in less than 10 min on a β-cyclodextrin column, compared with *ca.* 15 min on a phenyl column [17], 16 min on a cyano column [19], 18 min on a C_{18} column [12] and 7 min with the USP method on a 10-cm silica column (16 min on a 25-cm column [20]). The slight increase in time for analysis on a β-cyclodextrin column over the USP method is compensated for by the ability to determine the carpic acids.

A possible disadvantage of this method is the high salt concentration in the mobile phase, requiring thorough rinsing of the HPLC system after use to avoid damage to pump seals. The β-cyclodextrin HPLC column used here was found to have a lifetime of several months or more than 1000 injections with no precolumn. This lifetime is longer than that of the phenyl columns used for pilocarpine determination, but shorter than that of the silica columns used in the USP method. It was found that the use of ammonium sulfate in place of sodium sulfate increased the column lifetime with no effect on retention times and resolution, possibly owing to the less aggressive impurities in ammonium salts [24].

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