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Short communication

Improved high-performance liquid chromatographic determination of pilocarpine and its degradation products in ophthalmic solutions Importance of octadecylsilane column choice

Tony Y. Fan*, G. Michael Wall, Kent Sternitzke, Lynn Bass, Angela B. Morton, Erik Muegge

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

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Abstract

An improved high-performance liquid chromatographic (HPLC) method for the determination of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid in ophthalmic solutions was developed. The new method was adapted from the USP HPLC method for pilocarpine base and has been shown to give superior resolution of pilocarpine related substances compared to any previously reported HPLC technique. However, evaluation of several brands of C_{18} (octadecylsilane, ODS) columns revealed significant column-to-column variability: only two (YMC Pack ODS-AM and Supelco LC-18-DB) out of eight columns tested were capable of baseline resolution of these analytes. Additionally, optimization of the diluent was needed to prevent any significant interconversion of the carpic acids to their respective carpines. Analyses for pilocarpine and its degradation products in several commercial ophthalmic formulations were performed to demonstrate the precision, accuracy and general applicability of the method. The preparation of a stable resolution test solution is also described.

Keywords: Ophthalmic solutions; Stationary phases, LC; Pharmaceutical analysis; Pilocarpine; Isopilocarpine

1. Introduction

Pilocarpine hydrochloride is a miotic agent used to control intraocular pressure [1]. Because of its widespread use for the treatment of glaucoma and its facile degradation profile in solution (eye drops), much effort has been made to develop methods to determine this drug and its degradation products. Pilocarpine can undergo degradation by either epimerization or hydrolysis, see Fig. 1 [2]. The epimerization of pilocarpine yields isopilocarpine and the hydrolysis yields pilocarpic acid. Isopilocarpine can degrade further to isopilocarpic acid. Competition between the degradation routes is dependent on both pH and temperature [3–7].

A variety of methods have been applied to the determination of pilocarpine in ophthalmic solutions, including spectrophotometry and polarimetry [8,9] and gas chromatography [10]. However, high-performance liquid chromatography (HPLC), utilizing a wide selection of stationary and mobile phases, has proved to be the most desirable, sensitive and selective technique for the accurate determination of degradation products [11–26].

*Corresponding author.

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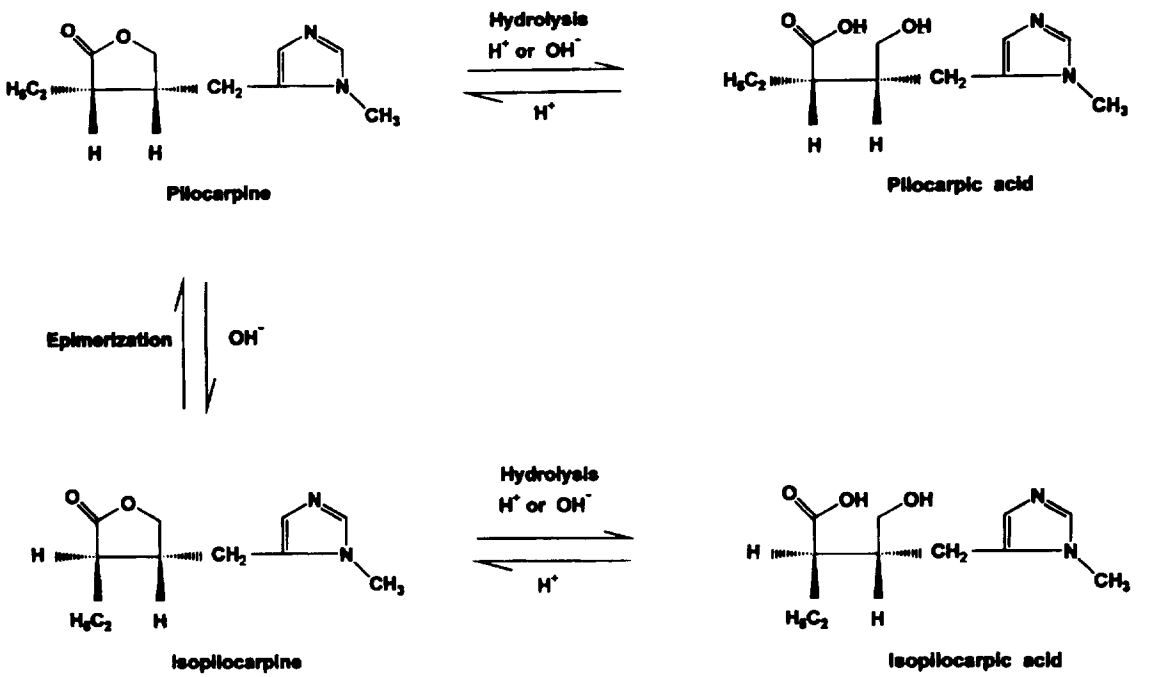

Fig. 1. Degradation scheme for pilocarpine.

Several methods employing octadecylsilane (ODS, C_{18}) columns with aqueous mobile phase systems [11–15] provided some separation but suffered from high back-pressure, short column lifetime, long run times and/or irreproducibility. Normal-phase methods (silica columns) have proven deficient for the resolution of related substances: some are inadequate in determining small amounts of isopilocarpine [16,24], while the current USP method for pilocarpine ophthalmic solution [20,25] completely retains the carpic acids. Phenyl columns have been used to resolve the carpines [17,18,23], but the resolution and column lifetimes were observed to be inconsistent in our laboratory. A cyano-column gave excellent separation, but the acidic mobile phase [19] shortened column lifetime. A β-cyclodextrin column method provided baseline resolution of all four compounds with a run time of less than 10 min [26]. However, this method frequently failed in our laboratory because of variability in the quality of analytical columns from the manufacturer.

The current USP method for pilocarpine base is a reversed-phase system, utilizing a 3-μm octadecylsilane column [25]. In our laboratory, that method has presented several problems. Frequently, ODS columns have failed the system suitability requirement for resolution (resolution between any two adjacent peaks $\geq$1.2) and the 3-μm columns often suffered from high back pressure and short column lifetime.

Resolution is extremely important in the analysis of pilocarpine degradation products because there are usually small amounts of degradation products in the presence of a much larger amount of pilocarpine. Recently in our laboratory, the current USP method for pilocarpine base has been adapted to ophthalmic formulations with particular attention to the column selection and modifications to the sample diluent (critical for inhibiting degradation), injection volume and the sample concentration. This paper presents an improved HPLC method, based on the USP HPLC method for pilocarpine base, for the analysis of ophthalmic formulations containing pilocarpine and each of its degradation products. A practical and stable solution for testing resolution is also described. Baseline resolution of all four compounds was achieved in less than 20 min. Pilocarpine and its degradation products were determined in several

commercial ophthalmic formulations illustrating the usefulness of the method.

2. Experimental

2.1. Reagents

Triethylamine was obtained from Aldrich (Milwaukee, WI, USA), sodium hydroxide (50% solution), ammonium hydroxide, phosphoric acid from J.T. Baker (Phillipsburg, NJ, USA) and HPLC grade methanol from EM Science (Gibbstown, NJ, USA). All chemicals except methanol were of analytical reagent grade and all were used as received. Pilocarpine (Vegetex-Extratos, Merck, Brazil) and isopilocarpine (Aldrich) were purchased from commercial sources and certified as in-house analytical reference standards.

2.2. Sample diluent, standard and sample solutions

The sample diluent (USP specifies water only) was prepared by mixing 13.5 ml of concentrated phosphoric acid, 3 ml of triethylamine and water to a total volume of 1000 ml. The pH was adjusted to 5.0 by the addition of 50% sodium hydroxide. All standard and sample solutions were prepared by dilution with the sample diluent to a concentration of 0.2 mg ml^{-1} pilocarpine hydrochloride (USP specifies 0.03 mg ml^{-1}). A dilute standard solution containing 0.008 mg ml^{-1} pilocarpine was also prepared in sample diluent to quantitate the degradation products.

2.3. Resolution test solution

Pilocarpic acid and isopilocarpic acid are not commercially available. Therefore, a resolution test solution containing these two compounds was generated in situ in a manner similar to that of Repta and Higuchi [27]. The USP method for the preparation of the resolution test solution (system suitability preparation) is relatively lengthy including a 1-h reflux [25]. An easier and equally effective method for the preparation of the resolution test solution was developed in our laboratory. The resolution test solution was prepared by mixing 5 ml of a 1 mg ml^{-1} pilocarpine hydrochloride aqueous solution with 100 μl of concentrated ammonium hydroxide and the mixture was heated for 30 min in an oven at ca. 90°C. The mixture was allowed to cool to room temperature, then diluted to 25 ml with sample diluent. Conversion of the pilocarpines to pilocarpic acids was rapid and complete with minimum pilocarpine left in the solution. Then 3 ml of this degraded mixture was spiked with 5 ml of the 1 mg ml^{-1} pilocarpine hydrochloride aqueous solution and this mixture was diluted with sample diluent to a total volume of 25 ml.

2.4. Chromatography

Separations were routinely performed isocratically on a YMC Pack ODS-AM, 15×0.46 cm I.D., 5 μm, octadecylsilane column (Wilmington, NC, USA) using a Waters LC Module I liquid chromatographic system (Milford, MA, USA) coupled to a Spectra-Physics ChromJet Integrator (San Jose, CA, USA). The buffer solution was prepared by mixing 13.5 ml of phosphoric acid, 3 ml of triethylamine and water to a total volume of 1000 ml. The pH was adjusted to 3.0 by the addition of 50% sodium hydroxide. The mobile phase was prepared by mixing 980 ml of the buffer solution with 20 ml of methanol and filtered through a 0.45-μm filter before use. All separations were performed at ambient temperatures (24–26°C). The flow-rate was 1.0 ml min^{-1}, resulting in a back-pressure of about 1000 psi. The injection volume was 20 μl and the detection wavelength was 214 nm. Other brand columns presented in Table 1 were evaluated under the same conditions for the separation of pilocarpine and its degradation products.

3. Results and discussion

Experimentation in our laboratory showed that the USP method was highly susceptible to tailing of pilocarpine leading to resolution problems due primarily to column-to-column variability. During preliminary method development, eight octadecylsilane columns were evaluated and only two gave acceptable results, YMC Pack ODS-AM and Supelco LC-

Table 1
Number of theoretical plates and resolution factors for various columns

Column	Theoretical plates (N) plates per column for pilocarpine	Resolution (R_s) pilocarpine/ pilocarpic acid
Supelco LC-18-DB 15×0.46 cm I.D.	5000	2.33
YMC Pack ODS-AM 15×0.46 cm I.D.	6600	4.20
Phenomenex Spherisorb ODS(2) 15×0.46 cm I.D.	5000	1.68
Waters Symmetry C_{18} 15×0.39 mm I.D	4800	2.16
Phenomenex Spherisorb ODS(1) 15×0.46 cm I.D.	2200	0
Waters Nova-Pak C_{18} 15×0.39 cm I.D.	2700	1.23
MAC MOD Zorbax Rx-C_{18} 15×0.46 cm I.D.	700	0.57
Whatman PartiSphere C_{18} 12.5×0.46 cm I.D.	500	0

18-DB. Both of these columns provided baseline separation and minimal tailing from pilocarpine. Chromatograms are shown in Fig. 2. Although the USP mobile phase composition was sufficient for the analysis, diluting the samples in the mobile phase as recommended lead to the interconversion of the carpic acids to their respective carpines. Experimentation with the pH of the diluent showed pH 5 to be optimal.

3.1. Resolution and specificity

Fig. 2 presents chromatograms of pilocarpine and its three degradation products using various columns with the chromatographic conditions described in section 2. Best separation was obtained with a YMC Pack ODS-AM column. The peaks in this chromatogram exhibited good symmetry, as measured by calculation of tailing factors according to the USP [21]. Tailing was calculated as 1.08 for isopilocarpine, 1.10 for pilocarpine, 1.12 for pilocarpic acid and 1.14 for isopilocarpic acid. Specificity was demonstrated by preparing blank solutions in the same manner as the samples but without the presence of pilocarpine. The preparations were then forcibly degraded separately using heat, acid, base and peroxide. No interference was observed.

The resolution between pilocarpine and isopilocarpine calculated according to the USP [21] was 3.18, whereas that between pilocarpine and pilocarpic acid was 4.20. These values are superior to any previously reported HPLC method in the literature.

The resolution test solution was found to be stable for at least two months and can be used as long as there is sufficient quantity of each component to determine resolution criteria.

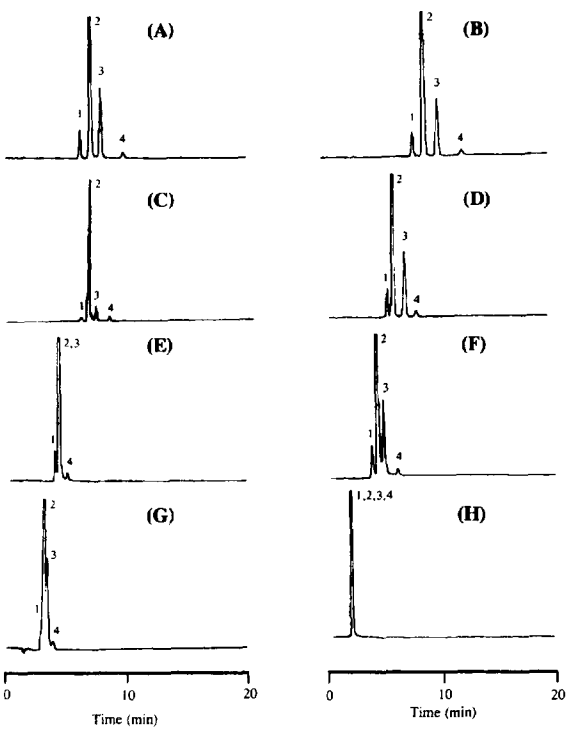

Fig. 2. Chromatograms of resolution test solution demonstrating the separation of pilocarpine and its degradation products on various ODS columns. Peaks: 1=isopilocarpine; 2=pilocarpine; 3=pilocarpic acid; 4=isopilocarpic acid. Total run time: 30 min. Column A: Supelco LC-18-DB, 3 μm, 15×0.46 cm I.D.; Column B: YMC Pack ODS-AM, 5 μm, 15×0.46 cm I.D.; Column C: Phenomenex Spherisorb ODS (2), 3 μm, 15×0.46 cm I.D.; Column D: Waters Symmetry C_{18}, 5 μm, 15×0.39 cm I.D.; Column E: Phenomenex Spherisorb ODS (1), 5 μm, 15×0.46 cm I.D.; Column F: Waters Nova-Pak C_{18}, 5 μm, 15×0.39 cm I.D.; Column G: Zorbax Rx C_{18}, 5 μm, 15×0.46 cm I.D.; Column H: Whatman, Partiaphere, C_{18}, 12.5×0.46 cm I.D.

3.2. Optimization of diluent

Various pH buffers were evaluated to find a pH at which the rate of interconversion between the carpines and carpic acids was minimal. Our laboratory initially had difficulties in determining precision and accuracy for the carpic acid degradation products. A study was done to evaluate seven different pH buffers used as sample diluent, ranging from pH 3 to 7. It was found that at pH 3, isopilocarpic acid was completely converted to isopilocarpine in 18 h and pilocarpic acid also showed some conversion to pilocarpine. At pH 7, the conversion was at minimum but a skewed peak shape of pilocarpine was observed. The best overall results with minimal conversion was found using a pH 5 buffer as sample diluent and the precision and accuracy were excellent.

3.3. Linearity and precision of the method

A five-point calibration curve, in duplicate, generated for pilocarpine exhibited excellent linearity and a y-intercept value was -1.2% (of the median points of the calibration curve), which justified the use of a single-point standard for the quantitation of pilocarpine (Table 2). A set of ten standard replicates near 0.2 mg ml^{-1} pilocarpine·HCl gave a relative standard deviation of 0.12%. This level of precision was sufficient to justify 0.2 mg ml^{-1} pilocarpine·HCl as a suitable concentration for the standard.

Pilocarpic acid and isopilocarpic acid curves were prepared from stock solutions of the acids prepared by degrading pilocarpine with ammonium hydroxide and heat. The resulting stock solution was a mixture of the two acids with a small amounts of the carpines remaining. Concentrations of the acids were determined versus a pilocarpine standard assuming equal response to pilocarpine. Dilutions were made to obtain three-point curves for each degradation product, with isopilocarpine ranging from about 2 to 7.2%, pilocarpic acid ranging from about 2.6 to 8% and isopilocarpic acid ranging from about 1 to 4% of a normal product dilution of 0.2 mg ml^{-1} pilocarpine·HCl. The final sample dilutions were made in 0.2 mg ml^{-1} pilocarpine·HCl to demonstrate that pilocarpine did not interfere with the response of either carpic acid (Table 2). The recoveries for each of the degradation products were quantitated against a 0.008 mg ml^{-1} pilocarpine dilute standard de-

Table 2
Performance characteristics of the method

Compound		Range (mg ml^{-1})	r^2	Intercept (%)	R.S.D. (%)	Mean recovery (%)
Pilocarpine	Vehicle Standard Curve 1 ($n=10$)	0.100–0.299	0.99981	−0.47	0.76	100.2
	Vehicle Standard Curve 2 ($n=10$)	0.100–0.299	0.99974	−2.30	1.62	98.3
	Vehicle Standard Curve 3 ($n=6$)	0.100–0.300	0.99988	−0.04	0.59	100.0
	Vehicle Standard Replicates 1 ($n=8$)	0.199			0.25	99.8
	Vehicle Standard Replicates 2 ($n=8$)	0.199			0.15	99.1
	Vehicle Standard Replicates 3 ($n=6$)	0.200			0.10	100.1
Isopilocarpine[a]	Vehicle Standard Curve 1 ($n=8$)	0.004–0.018	0.99965	−0.12	1.35	98.1
	Vehicle Standard Curve 2 ($n=6$)	0.004–0.012	0.99972	−1.96	1.72	100.5
	Vehicle Standard Curve 3 ($n=6$)	0.005–0.013	0.99997	−2.18	0.96	100.3
	Vehicle Standard Replicates 1 ($n=8$)	0.005			0.55	101.2
	Vehicle Standard Replicates 2 ($n=6$)	0.009			0.18	103.9
Pilocarpic acid[a]	Vehicle Standard Curve 1 ($n=6$)	0.010–0.021	0.99993	−3.81	1.43	105.4
	Vehicle Standard Curve 2 ($n=8$)	0.005–0.032	0.99998	−3.59	2.58	102.0
	Vehicle Standard Replicates 1 ($n=6$)	0.016			0.17	107.2
	Vehicle Standard Replicates 2 ($n=8$)	0.013			0.36	102.3
Isopilocarpic acid[a]	Vehicle Standard Curve 1 ($n=6$)	0.005–0.010	0.99946	−3.23	1.43	106.2
	Vehicle Standard Curve 2 ($n=8$)	0.003–0.015	0.99989	−1.59	2.35	103.7
	Vehicle Standard Replicates 1 ($n=6$)	0.007			0.35	109.0
	Vehicle Standard Replicates 2 ($n=8$)	0.006			1.21	102.7

[a] In the presence of 0.2 mg ml^{-1} pilocarpine.

scribed in Section 2. The accuracy of the method was performed by spiking appropriate amounts of each compound into a pilocarpine vehicle (Table 2).

3.4. Column selectivity: column to column variability

Several octadecylsilane columns (all either 0.39 or 0.46 cm I.D. and 12.5 or 15 cm in length) from different manufacturers were evaluated using the resolution test solution under the chromatographic conditions listed in the Experimental section. The results are presented in Fig. 2 and Table 1. Several ODS columns exhibited reasonably good separation while the others exhibited very little retention under the same chromatographic conditions. Best separation was achieved with the conditions listed in the Experimental section with regular end-capped octadecylsilane columns. While there was some improvement in resolution by increasing the column length (from 12.5 to 15 cm) and decreasing the particle size (from 6 to 3 μm), the most significant factor appeared to be the type of column (i.e., column manufacturer). The YMC Pack ODS-AM column presented the least tailing of all ODS columns tested. The number of theoretical plates and resolution between the closest two adjacent peaks for the eight octadecylsilane columns are shown in Table 1.

3.5. Limits of detection

Detection limits were estimated to be about 0.1% of a normal product dilution for each compound isopilocarpine, pilocarpic acid and isopilocarpic acid in the presence of 0.2 mg ml^{-1} pilocarpine·HCl. The detection limits of the related substances were the lowest concentrations that were found to give a reproducibly quantifiable peak area.

3.6. Analysis of ophthalmic solutions

Commercial ophthalmic aqueous solutions ranging in concentration from 1 to 4% pilocarpine hydrochloride were analyzed to demonstrate the usefulness of this method. Each sample was analyzed by the HPLC method reported here. The results are presented in Table 3.

4. Conclusions

The HPLC method presented here shows a significant improvement in resolution over existing methods for the determination of pilocarpine and its related substances in ophthalmic formulations. Complete separations were obtained in less than 20 min on a YMC Pack ODS-AM column, compared with about 15 min on a phenyl column [17], 16 min on a

Table 3
Chromatographic analysis of commercial pilocarpine ophthalmic formulations

Product, % pilocarpine, (Vendor)	Pilocarpine (% label)	Isopilocarpine (% label)[a]	Pilocarpic acid (% label)[a]	Isopilocarpic acid (% label)[a]
IsoptoCarpine, 2%, (Alcon)	107%	1.6%	4.9%	<0.1%
IsoptoCarpine, 1%, (Alcon)	104%	1.6%	5.7%	<0.1%
Timpilo 4, 4%, (Merck, Sharp and Dohme-Chibert)	112%	0.5%	3.4%	<0.1%
AKARPINE, 2%, (Akorn)	106%	1.2%	4.2%	<0.1%
PILOKAIR, 2%, (Pharmafair)	100%	1.7%	8.2%	<0.1%
Pilocarpine HCl Ophthalmic Solution, USP, 2%, (Geneva Generics)	100%	1.4%	4.6%	<0.1%
Piloptic-2, 2%, (Optopics)	104%	1.6%	4.2%	<0.1%
Pilocarpine HCl Ophthalmic Solution, USP, 2%, (Aligen)	103%	0.3%	4.7%	<0.1%
Pilocar, 2%, (Iolab)	100%	0.4%	1.8%	<0.1%
Pilocarpine HCl Ophthalmic Solution, 1%, (Goldline)	102%	0.6%	6.6%	<0.1%
Pilocarpine HCl Ophthalmic Solution, 1%, (Rugby)	107%	0.4%	6.1%	<0.1%

[a] Based on % label pilocarpine

cyano column [19], 10 min on a β-cyclodextrin column [22], 18 min on 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 analysis time was justified by the superior resolution of pilocarpine and its degradation products.

Octadecylsilane column choice was found to be critical for this pilocarpine assay: only three out of eight columns were found to be acceptable for this assay, based on resolution factors, and two (YMC Pack ODS-AM and Supelco LC-18-DB) were clearly superior. Though other instances of column-to-column variability have been reported in the literature [28–31] and observed by this laboratory, this was by far the most significant.

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. This method appears to be accurate, specific, sensitive and general enough for the routine determination of pilocarpine and its degradation products in ophthalmic samples and would be useful as a routine release or stability assay for pilocarpine raw material or pilocarpine drug products. Finally, a stable and practical resolution solution is described in which the carpic acids were generated in situ.

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