Note

Reversed-phase high-performance liquid chromatographic separation for pilocarpine and isopilocarpine using radial compression columns

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(Received March 8th, 1983)

The alkaloid pilocarpine is frequently used in ophthalmology to treat glaucoma because of its ability to lower intraocular pressure. In aqueous formulations pilocarpine has been observed to isomerize at the α-carbon to form isopilocarpine (Fig. 1). The rate of this isomerization is dependent upon pH and temperature[1]. Since isopilocarpine is pharmacologically inactive[2,3], a specific analysis for pilocarpine in the presence of isopilocarpine is highly desirable.

Because of a high separation capability and ease of use, several high-performance liquid chromatographic (HPLC) procedures have been reported. Originally, a separation for pilocarpine and isopilocarpine was described using Aminex A-7 cation-exchange resin with peak detection at 217 nm in the ultraviolet (UV)[4]. Later this system was reported to produce erratic results[2]. Khalil[5] used a μBondapak C_{18} column in series with a μBondapak CN column using detection at 254 nm to analyze for pilocarpine. Later investigators noted that the borate buffer (pH 9.2)–tetrahydrofuran (70:30) mobile phase gradually dissolved the column packing material[6].

More recently, several HPLC systems have been described that provide adequate separation between pilocarpine and isopilocarpine, and in addition, were used for the analysis of actual ophthalmic samples. Noordam and co-workers[7,8] and
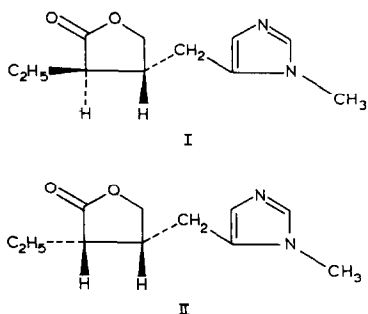

Fig. 1. The structures of pilocarpine (I) and isopilocarpine (II).

O'Donnell and co-workers[9,10] have published essentially the same reversed-phase procedure which uses a water–methanol (97:3) mobile phase containing 5% monobasic potassium phosphate at pH 2.5 with a 10-μm RP-18 column. At first, refractive index detection was used, but finally both groups used UV detection at 215–216 nm. Dunn et al.[11] developed a normal-phase system using a mobile phase of 70:30 hexane–2% ammonium hydroxide in 2-propanol with a 5-μm Si60 column. Peak detection was at 220 nm. Finally Kennedy and McNamara[6] reported a reversed-phase procedure using a mobile phase of 5% aqueous monobasic potassium phosphate at pH 2.5 on a 10-μm μBondapak Phenyl column. Peak detection was at 215 nm.

Unfortunately, all these procedures either produce only a minimal resolution (R_s) between pilocarpine and isopilocarpine or require a lengthy elution time. We report here a reversed-phase HPLC separation for pilocarpine–isopilocarpine which produces a resolution (R_s) of 1.38 in an elution time of 6.9 min. A 5-μm C_{18} radial compression cartridge was used with a mobile phase of 8.7% 2-aminopropane, 14.6% methanol, 22.0% 2 M phosphoric acid, and 54.7% 0.15 M aqueous sodium sulfate at a flow-rate of 3.2 ml/min. Because of the complexity of the mobile phase and the numerous factors which could potentially effect the separation (i.e., pH, ionic strength, polarity, column modification, etc.), a Simplex optimization program was utilized to find a statisfactory mobile phase composition and flow-rate.

EXPERIMENTAL

Materials

Isopilocarpine hydrochloride was purchased from Inland Alkaloid (Tipton, IN, U.S.A.). Analysis of this material by normal-phase HPLC[11] showed it to be a 42:58 mixture of pilocarpine hydrochloride and isopilocarpine hydrochloride. A 2.5 mg/ml solution of this sample was used without further purification as a convenient mixture to demonstrate separation of the two alkaloids. Pure USP-grade pilocarpine hydrochloride (Quimitra S.A., Merck) was used to establish the elution order. 2-Aminopropane was purchased from Aldrich (Milwaukee, WI., U.S.A.) and methanol was HPLC grade from Baker (Phillipsburg, NJ, U.S.A.). All other chemicals were reagent grade.

Chromatography

Separations were performed on a Waters Assoc. (Milford, MA, U.S.A.) radial compression module, Model RCM-100, using a 10 cm × 8 mm 5-μm Radial-Pak C_{18} cartridge. A Waters liquid chromatograph was equipped with a M6000A pump, a U6K syringe injector, and a Model 450 variable-wavelength UV detector. A Sargent (Dallas, TX, U.S.A.) Model SR strip-chart recorder was used. Injections of 5 μl were made and the detection wavelength was 220 nm.

Simplex algorithm

The Simplex method of optimization was first presented by Spendley et al.[12] and later modified by Nelder and Mead[13]. Our version of the Simplex algorithm provides for quadratic interpolations as suggested by Routh et al.[14], when (a) a successful reflection is followed by an unsuccessful expansion or (b) an unsuccessful

reflection is followed by a successful contraction. If the interpolation point is within a predefined narrow range of the centroid, it is rejected to avoid a collapse of the Simplex.

A general form of the objective function is shown in eqn. 1 and contains both resolution and time penalty components. The separation is considered to be optimized when maximum resolution is obtained in a minimum elution time. The specific objective function minimized is defined by eqn. 2.

$$F_{obj} = F_{sep} + F_{time} \tag{1}$$

where F_{sep} = chromatographic resolution function; F_{time} = time penalty function; and

$$F_{obj} = \sum_{i=1}^{N} 100 \, e^{(1.5 - R_{s_i})} + (T_p - T_L)^3 \tag{2}$$

where N = number of peaks -1; R_{s_i} = resolution of the ith pair of peaks as conventionally defined[15]; T_p = elution time at which the penalty is to begin; and T_L = elution time for the last peak.

The time penalty is set to zero when $T_L < T_p$. It is added to the separation function in order to force selection of variables which tend toward shorter elution times. The free parameters for the optimization were as follows:

$$\alpha_1 = f_1 \tag{3}$$

$$\alpha_2 = \frac{f_2}{1 - f_1} \tag{4}$$

$$\alpha_3 = \frac{f_3}{1 - f_1 - f_2} \tag{5}$$

$$\alpha_4 = \text{flow-rate} \tag{6}$$

where f_1 was the mobile-phase fraction of 2-aminopropane, f_2 was the fraction of methanol, f_3 was the fraction of 2 M phosphoric acid (aqueous), and the fourth component of the mobile phase was 0.15 M sodium sulfate (aqueous) defined by:

$$f_4 = 1 - f_1 - f_2 - f_3 \tag{7}$$

Parameter constraints were defined as follows:

$$0 \leqslant f_1 \leqslant 0.15 \tag{8}$$

$$0 \leqslant f_2 \leqslant 1.00 \tag{9}$$

$$0 \leqslant f_3 \leqslant 0.30 \tag{10}$$

$$0.5 \leqslant \text{flow-rate} \leqslant 5.00 \text{ ml/min} \tag{11}$$

The time penalty was set to begin at times greater than 5 min.

RESULTS AND DISCUSSION

Radially compressed HPLC columns were chosen to produce an assay which required a minimal amount of time per injection. The excellent efficiency and low back-pressures exhibited by radially compressed columns supported this objective. Because free silanol groups are not completely end-capped on Waters Radial-Pak C_{18} columns, a column modifier was added to the mobile phase to eliminate peak broadening. When 2-aminopropane was used as a column modifier, an initial separation of pilocarpine and isopilocarpine was readily obtained using a phosphate buffer–methanol mobile phase.

Because of the many factors effecting the separation, a Simplex optimization program was used to find a satisfactory mobile phase composition and flow-rate. The Simplex algorithm described in the Experimental section has been used previously to optimize a number of HPLC separations[16–18]. The mobile phase consisted of four variable components, each representing key factors which control the separation. The mobile phase components were: (1) 2-aminopropane (to control the degree of column modification); (2) methanol (to vary polarity); (3) 2 M phosphoric acid (to vary pH); (4) 0.15 M sodium sulfate (to vary ionic strength). In addition, the flow-rate was also varied.

The objective function (F_{obj}) minimized in eqn. 2 consisted of two parts, chromatographic resolution (F_{sep}) and the time required for separation (F_{time}). The resolution factor (F_{sep}) was designed to vary exponentially so that no baseline resolution (i.e., $R_s < 1.5$) would be penalized more severely than baseline resolution (i.e., $R_s > 1.5$). Baseline resolutions give values less than 100. A time penalty function [$F_{time} = (T_p - T_L)^3$] was added in an attempt to obtain an acceptable resolution in a realistic time. If the time penalty was not added, the separation could have been obtained solely by increasing the polarity of the mobile phase causing broad peaks

TABLE I

SUMMARY OF EXPERIMENTS PERFORMED DURING OPTIMIZATION

Decimal numbers under each of the mobile phase components indicate volume fractional composition.

Point	2-Aminopropane	Methanol	2 M Phosphoric acid	0.15 M Sodium sulfate	Flow-rate (ml/min)
1	0.050	0.150	0.125	0.675	4.5
2	0.100	0.180	0.150	0.570	1.0
3	0.100	0.100	0.200	0.600	2.5
4	0.093	0.110	0.073	0.724	2.6
5	0.025	0.125	0.250	0.600	1.5
6	0.097	0.120	0.175	0.608	3.3
7	0.107	0.106	0.213	0.573	4.0
8	0.087	0.146	0.220	0.547	3.2

TABLE II

SUMMARY OF RESULTS OF EACH OF THE EXPERIMENTS PERFORMED DURING THE OPTIMIZATION UNDER CONDITIONS LISTED IN TABLE I

R_s, F_{sep}, F_{time} and F_{obj} are defined in the text. $T(1)$ and $W(1)$ are the retention time and width for the isopilocarpine peak, $T(2)$ and $W(2)$ are the retention time and width for the pilocarpine peak.

Point	T(1) (min)	W(1) (min)	T(2) (min)	W(2) (min)	R_s	F_{sep}	F_{time}	F_{obj}	Resulting from
1	7.95	0.65	9.09	0.71	1.68	84	68	152	Initial
2	8.13	0.51	8.64	0.55	0.96	171	48	219	Initial
3	8.09	0.51	8.80	0.59	1.29	123	55	178	Initial
4	3.11	0.37	3.31	0.49	0.47	282	0	282	Initial
5	7.28	1.61	7.46	1.61	0.11	401	15	416	Initial
6	3.78	0.31	4.13	0.33	1.09	150	0	150	Reflection
7	4.76	0.39	5.26	0.41	1.25	128	0	128	Expansion
8	6.14	0.51	6.87	0.55	1.38	113	7	120	Contraction

and undesirably long retention times. Having selected a relatively short target elution time (T_L = 5 min for this attempt), separation of pilocarpine and isopilocarpine was obtained by exploiting the selectivity factors of the mobile phase and not just polarity.

A summary of the mobile phases and flow-rates evaluated is in Table I. A summary of the objective function values is shown in Table II. The first five points are part of the initial Simplex. Points six, seven and eight were generated by the Simplex algorithm by a reflection, expansion, and a contraction, respectively. Since point. No. 8 achieved an acceptable resolution of 1.38 in 6.9 min., the search was terminated (see Fig. 2). This corresponded to a mobile-phase composition of 8.7% 2-aminopropane, 14.6% methanol, 22.0% 2 M phosphoric acid, and 54.7% 0.15 M sodium sulfate at a flow-rate of 3.2 ml/min. The resulting chromatogram is shown in Fig. 2.
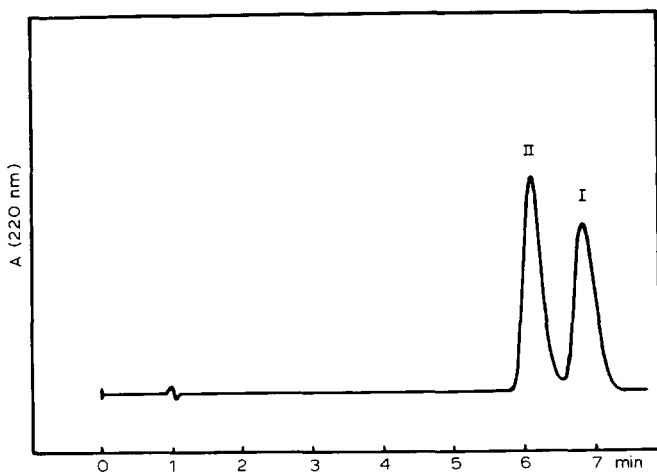

Fig. 2. The separation of pilocarpine (I) from isopilocarpine (II) using a mobile phase consisting of 8.7% 2-aminopropane, 14.6% methanol, 22.0% 2 M phosphoric acid, 54.7% 0.15 M sodium sulfate at a flow-rate of 3.2 ml/min.

TABLE III

SUMMARY OF PUBLISHED SEPARATIONS OF PILOCARPINE AND ISOPILOCARPINE

Method	Column	R_s	Retention time* (min)	Retention time/R_s	Ref.
A	C_{18}	1.10**	15.4 (Pilocarpine)	14.0	7
B	C_{18}	0.95**	11.7 (Pilocarpine)	12.3	9
C	Si 60	2.13	21.9 (Isopilocarpine)	10.3	11
D	Phenyl	0.91**	13.1 (Pilocarpine)	14.3	6
E	Radial-Pak C_{18}	1.38	6.9 (Pilocarpine)	5.0	—

   * The retention time of the longest eluting peak of the pilocarpine–isopilocarpine pair.
   ** R_s values were not reported. They were estimated from measuring the chromatograms given in the references listed.

   Because of the many factors involved, prediction that this mobile-phase composition could produce a satisfactory separation would be difficult even for an experienced chromatographer. The presence of 8.7% 2-aminopropane, which is normally considered as a column modifier, is unusual. The Simplex method of optimization thus appears to be an extremely powerful empirical technique which can produce unique and original mobile phase combinations based only on the demands of the separation and not on preconceived ideas.
   Table III compares this separation of pilocarpine and isopilocarpine with other separations reported in the literature. Clearly, a higher resolution in a shorter time has been achieved. This is illustrated by a comparison of the ratio of retention time to R_s shown in the fifth column of Table III. Using this criterion, method E is clearly the most satisfactory.
   In order to evaluate this HPLC separation as a potential method of analysis for ophthalmic solutions, a commercially available 10% pilocarpine hydrochloride
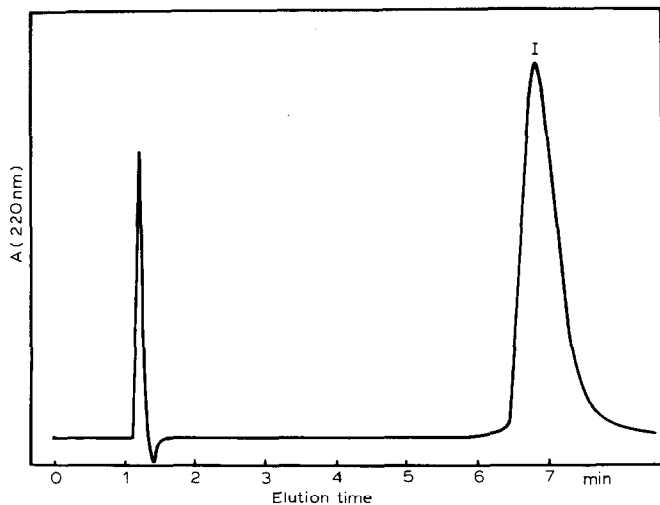

Fig. 3. The elution of pilocarpine (I) from a 10% ophthalmic solution.

solution was diluted to 1.0 mg/ml with water and analyzed using a single-point standard. The chromatogram is shown in Fig. 3 and 102.0% label was obtained. Another degradation product of pilocarpine, pilocarpic acid, was prepared by basic hydrolysis as previously described[6,8]. An injection of this material eluted on the solvent front and did not interfere with either the pilocarpine or isopilocarpine peaks. This is not the situation for other previously reported C_{18} separations[7-10], were pilocarpic acid was found to elute after the pilocarpine and isopilocarpine peaks. This suggests that perhaps a different separation mechanism was involved using this mobile phase and a Radial-Pak C_{18} column.

Because the mobile phase is fairly basic, an extended experiment was devised to check column degradation. The HPLC system was equilibrated and an initial plate count of 6455 plates/m for pilocarpine was calculated. The mobile phase was then pumped through the column for 70 h and another plate count of 6095 plates/m was calculated. This is only a 6% change and it seems that the column should not be greatly degraded with normal day-to-day use. However, the R_s value for pilocarpine and isopilocarpine was noted to vary from column to column, and the mobile phase composition usually needed an adjustment to produce optimal results.

CONCLUSIONS

HPLC separation for pilocarpine and isopilocarpine has been obtained using a Radial-Pak C_{18} column with a mobile phase of 8.7% 2-aminopropane, 14.6% methanol, 22.0% 2 M phosphoric acid, and 54.7% 0.15 M sodium sulfate at 3.2 ml/min. A resolution of 1.38 was achieved in an elution time of 6.9 min. This is a definite improvement over other reported HPLC separations and represents a potential routine method of analysis for pilocarpine ophthalmic solutions.

In addition, it is also apparent that the Simplex algorithm can provide an efficient search strategy in complex chromatographic systems where the effect of several interdependent variables on the separation are difficult to predict. Furthermore, the use of an elution time penalty with solvent flow-rate as a variable resulted in the identification of conditions consistent with both an acceptable resolution and an acceptable separation time.

ACKNOWLEDGEMENTS

REFERENCES

1 J. D. Weber, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 1409.
2 R. K. Hill and S. Barcza, *Tetrahedron*, 22 (1966) 2889.
3 T. A. Anderson and J. B. Cowle, *Brit. J. Ophthalmol.*, 52 (1968) 607.
4 T. Urbanyi, A. Piedmont, E. Willis and G. Manning, *J. Pharm. Sci.*, 65 (1976) 257.
5 S. K. W. Khalil, *J. Pharm. Sci.*, 66 (1977) 1625.
6 J. M. Kennedy and P. E. McNamara, *J. Chromatogr.*, 212 (1981) 331.
7 A. Noordam, K. Waliszewski, C. Olieman, L. Maat and H. C. Beyerman, *J. Chromatogr.*, 153 (1978) 271.

8 A. Noordam, L. Maat and H. C. Beyerman, *J. Pharm. Sci.*, 70 (1981) 96.
9 J. J. O'Donnell, R. P. Sandman and M. V. Drake, *J. Pharm. Sci.*, 69 (1980) 1096.
10 M. V. Drake, J. J. O'Donnell and R. P. Sandman, *J. Pharm. Sci.*, 71 (1982) 358.
11 D. L. Dunn, B. S. Scott and E. D. Dorsey, *J. Pharm. Sci.*, 70 (1981) 446.
12 W. Spendley, S. R. Hext and F. R. Himsworth, *Technometrics*, 4 (1962) 441.
13 J. A. Nelder and R. Mead, *Computer J.*, 7 (1965) 308.
14 M. W. Routh, P. A. Swartz and M. B. Denton, *Anal. Chem.*, 49 (1977) 1422.
15 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience New York, 2nd ed., 1979, p. 34.
16 M. W. Watson and P. W. Carr, *Anal. Chem.*, 51 (1979) 1835.
17 M. L. Rainey and W. C. Purdy, *Anal. Chim. Acta*, 93 (1977) 211.
18 M. R. Detaevernier, L. Dryon and D. L. Massart, *J. Chromatogr.*, 128 (1976) 204.
19 M. A. Nunes and E. Brochmann-Hanssen, *J. Pharm. Sci.*, 63 (1974) 716.