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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PILOCARPINE HYDROCHLORIDE, ISOPILOCARPINE, PILOCARPIC ACID AND ISOPILOCARPIC ACID IN EYE-DROP PREPARATIONS

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SUMMARY

A high-performance liquid chromatographic method is described which allows the simultaneous estimation of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid. The method offers advantages over existing chromatographic procedures in that pilocarpine and the degradation products are eluted within 15 min making the procedure suitable for routine quality control.

Pilocarpine eye-drop preparations available on the Australian market were determined to contain between 0.4 and 1.9% isopilocarpine and between 2.2 and 6.3% total pilocarpic acids*. These findings are comparable to those reported by Weber in a survey of pilocarpine containing products available in the U.S.A.

INTRODUCTION

The alkaloid pilocarpine is used topically as a miotic in the treatment of glaucoma. In aqueous solution degradation can occur either through epimerisation to isopilocarpine or hydrolysis to pilocarpic and isopilocarpic acids[1,2]. Both mechanisms result in a loss of pharmacological activity.

Analytical procedures for pilocarpine based on classical titrimetric or spectrophotometric procedures are described in a number of texts[3–8]. None of these methods is capable of distinguishing the epimers of pilocarpine.

The monographs for pilocarpine salts in the British Pharmacopoeia[6] and the United States Pharmacopoeia (USP)[8] include requirements for specific rotation which may be interpreted as indirectly assigning limits to the total impurity levels. Weber[9] calculated that the range of specific rotation permitted in USP XIX monograph[8] for pilocarpine hydrochloride allows a maximum of either 6% isopilocarpine, or 3% pilocarpic acid. The monographs for pilocarpine preparations, however do not include requirements for specific rotation presumably because excipients may interfere with optical rotation measurements.

* Subsequent reference to pilocarpic acid content shall be understood to mean the total content of pilocarpic and isopilocarpic acids.

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A survey of the literature revealed that nuclear magnetic resonance[2,10] and chromatographic procedures[9,11–14] had been devised for the quantitation of pilocarpine and degradation products.

Urbanyi *et al.*[11] reported the separation of the pilocarpine epimers by ion exchange on Aminex A-7. Weber[9] modified this procedure and estimated the content of pilocarpic acids by cyclizing them to pilocarpine and measuring the resultant increase in alkaloid content of the sample. Weber conceded the inconvenience of the two stage analysis and the possibility that cumulative errors could lead to inaccurate results.

Wahba Khalil[12] joined a μBondapak C_{18} column and a μBondapak CN column in series and used as mobile phase borate buffer (adjusted to pH 9.2)–tetrahydrofuran (7:3). Our investigations to establish the usefulness of this procedure were abandoned after it was determined that the mobile phase had a tendency to dissolve the silica base of the column packing material.

Mitra *et al.*[13] enhanced the detectability of the pilocarpine epimers by forming *p*-nitrobenzyl bromide derivatives which were subsequently separated by ion-pair chromatography on a μBondapak C_{18} column. A sample chromatogram included in the paper showed that the isopilocarpine derivative eluted as an unresolved rider on the tail of the pilocarpine peak which may lead to potential quantitation difficulties. No mention of detection or estimation of the pilocarpic acids was made.

Noordam *et al.*[14] separated pilocarpine and its degradation products on a C_{18} column using a mobile phase of water–methanol (97:3) containing 5% of potassium dihydrogen orthophosphate adjusted to pH 2.5 with orthophosphoric acid. Our attempts to repeat the method gave poor resolution and excessive tailing. The sample chromatogram included in the paper by Noordam *et al.* indicates that these workers experienced similar problems.

In this paper we present a simple method involving minimal sample preparation which would be suitable for routine quality control of pilocarpine eye-drop formulations. Emphasis has been placed on reducing analysis times while maintaining resolution consistent with the needs of quantitation. The procedure described has been used to survey a wide range of pilocarpine eye-drop preparations available in Australia.

EXPERIMENTAL

Materials

Pilocarpine nitrate was obtained from BDH (Poole, Great Britain) and isopilocarpine nitrate from Koch-Light Labs. (Colnbrook, Great Britain). Both alkaloids were used as received. All other reagents and chemicals were either analytical grade or high purity. Water was freshly distilled.

Equipment

The high-performance liquid chromatographic (HPLC) system comprised a Varian 8500 constant-flow pump; an Altex Model 905-42 syringe-loading sample injector fitted with a 20-μl loop; a Waters Assoc. (Milford, MA, U.S.A.) μBondapak phenyl column 30 cm × 3.9 mm; a Varian Aerograph temperature-controlled waterbath; a Perkin-Elmer LC 55 spectrophotometer fitted with a Coleman 55-204 UV accessory and a Hewlett-Packard Model 3380A integrator.

The pH measurements were made with a Radiometer (Copenhagen, Denmark) PHM64 research pH meter which was standardised with Radiometer buffers 51316 (pH 4.01) and s1001 (pH 6.5).

The mobile phase was filtered immediately prior to use with a Millipore filtration assembly XX10047 30 using 0.5-μm filters.

Chromatographic conditions

The column was maintained at 40 $\pm$ 1°C. The mobile phase was a 5% (w/v) aqueous solution of potassium dihydrogen orthophosphate adjusted to pH 2.5 with orthophosphoric acid. Flow-rate was 1 ml/min. The UV detector was set at 215 nm. Integrator settings were attenuation 4, slope sensitivity 0.3 and chart speed 0.5 cm/min.

Samples and standards were chromatographed in the following order, standard 1 followed by 4 samples, consisting of alternated duplicates from 2 batches, then an injection of standard 2 and a group of 4 samples from another 2 batches, followed by a standard 1 and so on.

Before commencing the day's analysis mobile phase was pumped through the column for 1.5 h at a rate of 2 ml/min to establish stable baseline conditions. At the conclusion of each day the column was flushed with 30 ml of water and 30 ml of methanol–water (60:40, v/v).

Preparation of samples and standards

Standards and samples were prepared in 0.2 *M* Na_2SO_4 solution, in order to minimise disturbances of ionic equilibria in the column. Weber[9] had earlier demonstrated the stability of pilocarpine in this medium.

Eye-drop preparations assayed contained pilocarpine as the hydrochloride but the more readily available nitrate salt was chosen as the reference standard. In the preparation of standards 1 and 2 below it is necessary to make allowance for the isopilocarpine content of the pilocarpine nitrate standard.

Each standard contained nominally 1.108 mg/ml of pilocarpine nitrate; in addition standard 1 contained 4.43 μg/ml and standard 2 0.554 μg/ml of isopilocarpine nitrate. These standard solutions (1 and 2) correspond to a nominal content of 1 mg/ml of pilocarpine hydrochloride containing respectively 4% and 0.5% (w/w) of added isopilocarpine hydrochloride. It was anticipated that these levels of isopilocarpine contamination would represent the extremes which may occur in the samples to be surveyed.

Eye-drop preparations were diluted to a nominal concentration of 1 mg/ml of pilocarpine hydrochloride with 0.2 *M* Na_2SO_4. The dilutions were carried out on the day of analysis.

We were unable to locate a source of either pilocarpic or isopilocarpic acid. It was therefore necessary to determine an equivalence factor which related the peak areas of the pilocarpic acids to their concentration. This determination involved the quantitative hydrolysis of a known amount of pilocarpine, following the procedure described below.

About 80 mg of pilocarpine nitrate was weighed accurately and diluted to 200 ml with water. A 25-ml aliquot was refluxed for 1 h with 5 ml of 1 *M* sodium hydroxide. The resultant solution was adjusted to about pH 7 with 0.5 *M* ortho-

phosphoric acid (universal indicator paper), transferred quantitatively to a 50-ml volumetric flask and diluted to volume with water. The hydrolysis procedure was performed in triplicate.

A 25-ml aliquot of the original pilocarpine nitrate solution was diluted to 50 ml with water. Each solution was chromatographed and the mean equivalence factor calculated dividing the pilocarpine peak area by the mean of the pilocarpic acid peaks.

Linearity of standard curves

The relationship between pilocarpine peak areas and concentration was investigated in the concentration range corresponding to a sample assay range of between 70 and 110% of labelled content. A linear regression fit gave a correlation coefficient (r) of 0.99 ($n = 9$).

Solutions of pilocarpine nitrate were spiked with isopilocarpine to investigate the linearity of the relationship between peak height and concentration of isopilocarpine in the concentration range equivalent to 1–5% isopilocarpine contamination. A linear regression fit gave $r = 0.99$ ($n = 15$).

RESULTS AND DISCUSSION

Our investigation of the methods in the literature indicated that the method of Noordam *et al.*[14] appeared to be the most promising for routine quality control of pilocarpine eye-drops. In practice, however, poor resolution due to excessive tailing of the peaks made quantitation difficult. The method was modified as follows.

The ODS column was replaced by a μBondapak phenyl column, which reduced analysis times while improving peak shapes and resolution. Increasing column temperatures led to reduced capacity factors and sharper peaks and a plot of temperature against resolution showed a definite maximum at 40°C. In agreement with the observations of Noordam *et al.*[14] we found that decreasing pH and increasing ionic strength resulted in less peak tailing. However, a compromise was necessary because use of a mobile phase with too low a pH resulted in overlap of the pilocarpic acids, and pilocarpine peaks. A pH of 2.5 was optimum. We adopted the suggestion of Noordam *et al.* that use of a mobile phase consisting of 5% potassium dihydrogen orthophosphate provided a reasonable compromise between peak shape and the life of the column. A discussion of the influence of pH and temperature on chromatographic separations has been given by Horváth *et al.*[15] and Gant *et al.*[16] respectively.

Initially difficulties were experienced with the quantitation of isopilocarpine. It was noted that the response factor for isopilocarpine (peak height per unit concentration) changed slowly but uniformly during the course of a day's analysis. This behaviour appeared to be linked to the age of the mobile phase since results from later experiments conducted with freshly prepared mobile phase showed no drift. Before this link was established the rate of drift was determined by making a series of injections of standard at particular times recorded throughout the day's analysis. The appropriate response factor for each sample could then be interpolated and used to calculate the isopilocarpine content. The analysis results obtained with this drift compensation compared very closely to those obtained when the same samples were re-analysed with freshly prepared mobile phase. Smuckler[17] and Rabel[18] reported

chromatographic problems arising from the gradual accumulation of un-identified UV absorbing impurities in solutions of KH_2PO_4. In order to check the relevance of their findings to our observed drift in response factor, mobile phases were prepared using both highly purified and artificially contaminated KH_2PO_4, prepared according to Smuckler's[17] procedure. No discernable difference was noted in the resultant chromatograms. To date no explanation has been found for the observed drift but the age of the mobile phase appears to be a factor.

The results of the survey are given in Table I. The samples consisted of 29 batches from 4 different manufacturers which are identified by the letters A to D. Weber[9] surveyed 12 batches from 10 manufacturers available on the U.S.A. market. Table II compares the results of our survey to those obtained in Weber's survey and shows the mean pH and mean contents of pilocarpine, isopilocarpine and pilocarpic acid. The range and standard deviation of each set of values are also included.

TABLE I

EXPERIMENTAL RESULTS OF SURVEY

Manufacturer	*Pilocarpine content*		*Isopilocarpine percent of total alkaloids**	*Pilocarpic acid percent of total alkaloids**	*pH*
	Manufacturer label claim (%)	*Percent label claim determined*			
A	0.5	94.4	0.63	2.74	4.46
A	0.5	91.7	0.65	2.38	4.53
B	0.5	96.5	1.23	4.91	4.49
C	0.5	97.6	1.21	5.06	4.49
D	0.5	103.3	1.90	6.26	5.02
A	1.0	96.8	0.51	3.91	4.18
A	1.0	94.5	0.74	3.36	4.24
B	1.0	94.3	0.95	3.79	4.24
C	1.0	93.1	0.96	3.83	4.24
D	1.0	101.1	1.75	5.35	4.81
A	2.0	94.1	0.74	4.01	4.08
A	2.0	95.9	0.67	3.89	4.09
B	2.0	94.0	0.84	3.27	4.19
C	2.0	95.0	0.84	3.13	4.20
D	2.0	103.1	1.15	4.70	4.50
A	3.0	93.0	0.43	4.75	3.98
A	3.0	92.2	0.43	4.61	3.99
B	3.0	96.2	1.37	4.79	3.92
C	3.0	98.5	1.17	3.98	4.36
D	3.0	105.4	1.06	4.98	4.23
A	4.0	93.9	0.63	2.20	3.97
A	4.0	97.4	0.66	4.24	3.96
B	4.0	96.2	1.06	4.16	4.14
C	4.0	95.0	1.04	4.22	4.14
D	4.0	106.2	0.84	3.72	4.25
A	6.0	93.9	0.58	4.42	3.95
A	6.0	95.6	0.76	4.27	4.19
B	6.0	96.3	1.45	4.25	4.40
D	6.0	103.6	1.35	4.23	4.19

* Total content of pilocarpine and isopilocarpine.

TABLE II

MEAN RESULTS OF U.S.A. SURVEY COMPARED TO AUSTRALIAN SURVEY

The first result in each block is the mean and standard deviation, the second is the range of values determined.

	Results	
	U.S.A. survey	*Australian survey*
pH	4.36 ± 0.60 3.55–5.20	4.26 ± 0.26 3.92–5.02
Pilocarpine as % of labelled strength	98.7 ± 3.8 93.7–104	96.9 ± 4.0 91.7–106.2
Isopilocarpine as % of total alkaloids	1.5 ± 0.9 0.4–3.4	1.0 ± 0.4 0.4–1.9
Pilocarpine acids as % of total alkaloids	3.9 ± 2 0.6–6.8	4.1 ± 0.9 2.2–6.3

An investigation of the relationship between isopilocarpine content (as a percentage of total alkaloids) and pH for the data obtained in our survey is shown in Fig. 1. The results of the linear regression analysis, were slope 0.47 (0.54), intercept pH 3.8 (3.5) and r 0.68 (0.84). The results are similar to the results reported by Weber which are given in parentheses. The discrepancy in r values is largely due to the outlying data point circled in Fig. 1. Rejection of this point changes the slope to 0.53 and r to 0.78. A linear regression analysis of content of pilocarpic acids against pH of the samples gave poor correlation ($r = 0.28$) which is in agreement with the data reported by Weber.

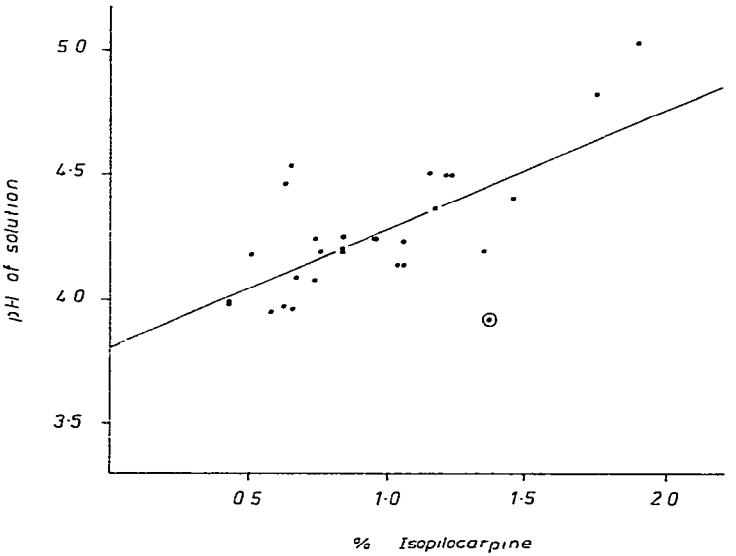

Fig. 1. Correlation of isopilocarpine content and pH of eye-drops solution.

The main difference between the two surveys was the isopilocarpine content of the preparations. Weber found a higher mean content (1.5) and a greater range (0.4–3.4) of individual contents than was found in the Australian formulations, mean (1.0) range (0.4–1.9). This difference is consistent with the following considerations. As demonstrated by Weber and corroborated by our data, isopilocarpine content is often proportional to pH (see Fig. 1). The majority of samples examined by Weber had pH values of either above 5.0 or below 4.0. This contrasts with our narrow pH range of between 4.0 and 4.5 with one sample at pH 5.0. Thus the wider range of isopilocarpine contents found by Weber would appear to be consistent with the wider pH range of the samples examined.

The samples taken for this survey had been manufactured between 4 and 25 months prior to analysis. There does not appear to be a linear relationship between age and content of either degradation product ($r = 0.31$), gradient (m) $= 0.02$ for isopilocarpine and $r = 0.04$, $m = 0.23$ for content of pilocarpic acids. This lack of correlation between age and content of degradation products may suggest good long-term stability for pilocarpine eye-drop preparations. The differences in degradation product concentrations between samples may be accounted for by differences in history of sample preparation and source of pilocarpine. It is intended to establish the stability of the samples analysed in this survey by re-analysing them at a later date.

Of the 41 batches analysed in the two surveys, only 3 samples were found to have isopilocarpine contents in excess of 2% of total alkaloid content. These 3 samples were part of Weber's survey and were found to contain 2.5, 2.6 and 3.4% respectively of isopilocarpine. These same 3 samples also showed atypically high pH

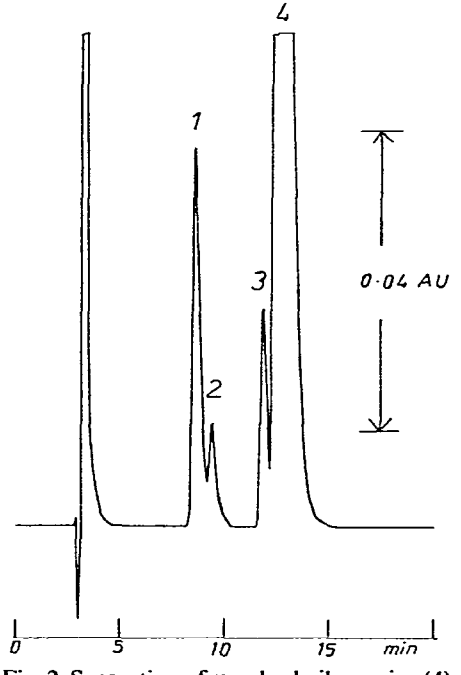

Fig. 2. Separation of standard pilocarpine (4), isopilocarpine (3), isopilocarpic acid (2), and pilocarpic acid (1).

values of 5.20, 5.00 and 5.05 respectively. The normally low isopilocarpine levels found suggest therefore that classical techniques which determine total alkaloid content may give a reasonable indication of pharmacological activity. It should be noted however than Urbanyi *et al.*[11] reported that one preparation of pilocarpine nitrate contained 25.2% isopilocarpine.

CONCLUSION

The HPLC method described allows the simultaneous quantitation of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid in eye-drop preparations. The analysis time, sample preparation and low cost mobile phase make the method suitable for routine quality control and stability trials.

Experience indicates that the mobile phase should be freshly prepared, as ageing results in a drifting isopilocarpine peak height response factor. The cause of this drift is unknown.

As baseline resolution of the pilocarpine epimers was not achieved (Fig. 2) it is recommended that epimer resolution be optimised for each system by manipulation of mobile phase, pH and temperature.

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