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HPLC method for the simultaneous determination of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid*

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Abstract: A high-performance liquid chromatographic method suitable for the determination of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid is described. The column used is a Spherisorb-CN bonded phase at 25°C. An aqueous solution of triethylamine (0.1%, v/v) at pH 2.5 is used as mobile phase. Peak detection is by UV absorption at 220 nm. The elution orders are found to be pilocarpic–isopilocarpic acids with a resolution of 9 and K' of 5 and 6, respectively, isopilocarpine–pilocarpine with a resolution of 1.8 and K' of 13 and 13.5, respectively, the peaks being symmetrical.

Keywords: *Pilocarpine–HPLC; Pilocarpine–isopilocarpine–pilocarpic–isopilocarpic separation; cyano-propyl column; triethylamine eluent modifier.*

Introduction

Pilocarpine (**I**) is a cholinergic alkaloid, with myotic and low intraocular pressure effects, that is widely used in aqueous ophthalmic solutions for the treatment of glaucoma. The epimer isopilocarpine (**II**) and the hydrolysis products isopilocarpic acid (**III**) and pilocarpic acid (**IV**) (Fig. 1) are devoid of pharmacological activity.

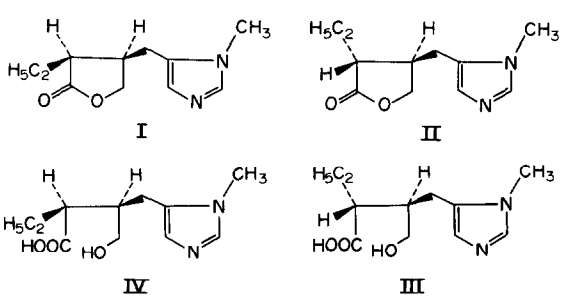

Figure 1
Structure of pilocarpine and its degradation products: **I**, pilocarpine; **II**, isopilocarpine; **III**, isopilocarpic acid; **IV**, pilocarpic acid.

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A specific analytical method for the simultaneous assay of the four products would be useful for the quality control of both raw materials and pharmaceutical preparations. High-performance liquid chromatography (HPLC) is a suitable technique because of its high separation capability and ease of use. The chromatographic conditions should have enough selectivity to resolve the epimeric acids (**III**, **IV**) and bases (**I**, **II**). The pair pilocarpine–isopilocarpine is the most critical because their only structural difference is the α–β position of an ethyl group substituting a cyclic chain; whilst in the pair pilocarpic–isopilocarpic acids the ethyl group substitutes and open chain, having more space mobility (see Fig. 1). So, in the acids pair there is more spatial difference, and in consequence it is easier to resolve them. A reversed-phase technique, with 5% phosphate buffer at pH 2.5 and methanol or acetonitrile mobile phase, has been proposed for the determination of the four products: on a ODS column [1–4] and on a phenyl column [5–7]. An ion-pair reversed–phase method on a ODS column, also has been described [8].

Unfortunately, all these procedures either produce a minimal resolution between pilocarpine and isopilocarpine or require lengthy elution time. The chromatographic separation depends on a hydrophobic interaction between the stationary phase and the solute molecules; which provides slight selectivity for polar solutes with similar molecular structure.

It is known that bonded-silica phases always have a fraction of unbonded and accessible silanols. Products with amino groups suffer hydrogen bonding with these free hydroxyls, reducing selectivity and resulting in the appearance of broad and tailing peaks. The peak shape and in consequence, the selectivity, is improved by adding a silanol blocker agent to the mobile phase. Alkylamines and especially triethylamine (TEA) are claimed to provide good results [9, 10].

In the present work, an easy and fast HPLC method with a Spherisorb-CN stationary phase and a 0.1% TEA at pH 2.5 mobile phase, which resolves **I**, **II**, **III** and **IV**, is developed.

Experimental

Solvents and chemicals

Pilocarpine hydrochloride (USP grade) was supplied by Boehringer Ingelheim (Mannheim, FRG) and isopilocarpine nitrate by Janssen Chimica (Beerse, Belgium). Methanol (HPLC grade), TEA, sodium hydroxide and phosphoric acid (analytical grade) were provided by Scharlau S.A. (Barcelona, Spain). Water was purified by means of a Milli-Q water system. The HPLC column was a Spherisorb-CN (cyano-propyl-bonded phase), 5 μm, 250 × 4.6 mm i.d., from Teknokroma (Barcelona, Spain).

Equipment

A Hewlett–Packard HP 1090 liquid chromatograph with automatic injector, thermostatic oven and diode-array UV detector was used. To print, integrate and compare the obtained chromatograms an HP 79994A ChemStation was used. The statistical calculations were performed by means of Statgraphics software package implemented in an Olivetti M24 personal computer.

Standard solutions

Solutions of pilocarpine hydrochloride and isopilocarpine nitrate were prepared in

purified water. Pilocarpic acid was obtained by hydrolysis of pilocarpine hydrochloride in 0.01 M sodium hydroxide and immediately diluted with 0.04 M phosphoric acid. Isopilocarpine nitrate was treated similarly to obtain isopilocarpic acid.

Chromatographic conditions

The column was kept at 25 ± 0.5°C, the injection volume was 5 μl and the elution was performed at a flow rate of 1 ml min^{-1}. The absorbance was monitored at 220 nm, at a bandwidth of 4 nm and a threshold of 1.0 mAU.

The mobile phase was mainly aqueous and contained triethylamine adjusted to an acid pH. In order to optimize the resolution between pilocarpine and isopilocarpine, the elution of the four compounds was systematically investigated over the pH range 2.5–5.5 (TEA 0.1%); the TEA concentration from 0 to 3%, v/v (pH 2.5) and methanol percentage between 0–5% (TEA 0.1%, pH 2.5).

Results and Discussion

Selectivity

As illustrated in Fig. 2, pilocarpine and isopilocarpine elute faster, and resolution improves as the eluent becomes more acidic. The retention and the resolution of pilocarpic and isopilocarpic acids are not affected by the pH. At the acidic pH the bases (**I** and **II**) are ionized and undergo ion-exchange interaction with the stationary phase, which improves their selectivity and reduces their retention.

As shown in Fig. 3, TEA addition to the mobile phase produces narrow and symmetric peaks, as well as lower retention times. This last effect is critical in the pilocarpine–isopilocarpine separation. 0.1%, v/v, of TEA provides good resolution between the four products in the shortest possible analysis time.

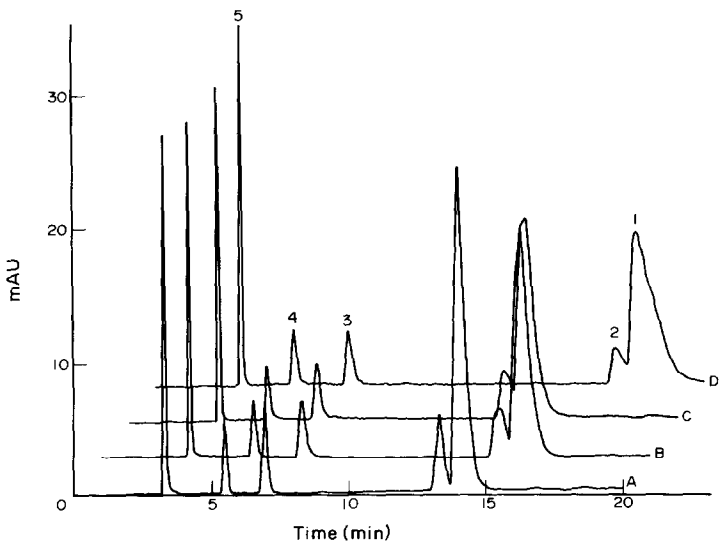

Figure 2
Chromatograms obtained with 0.1% (v/v) TEA in an aqueous mobile phase at different pH values: A, pH 2.5; B, pH 3.5; C, pH 4.5; D, pH 5.5. The peaks shown are: 1, pilocarpine 200 μg ml^{-1}; 2, isopilocarpine 40 μg ml^{-1}; 3, isopilocarpic acid 20 μg ml^{-1}; 4, pilocarpic acid 20 μg ml^{-1}; 5, nitrate ion.

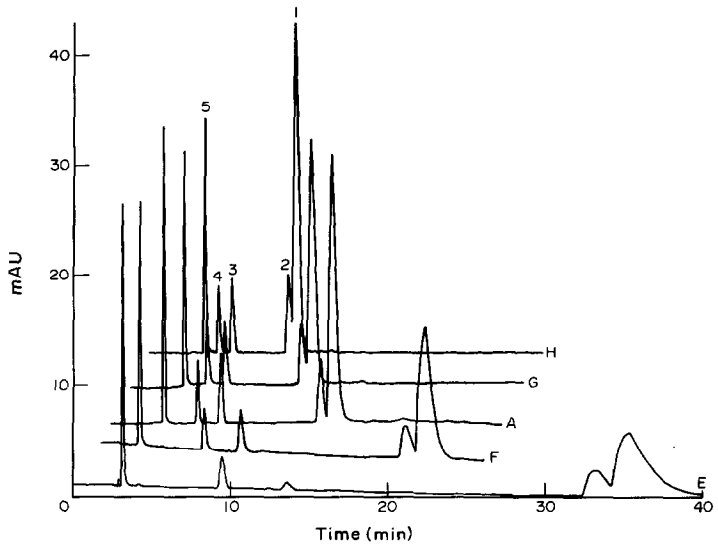

Figure 3
Chromatograms obtained with different concentrations (v/v) of TEA in an aqueous mobile phase, adjusted to pH 2.5: E, 5% phosphate buffer–0% TEA; F, 0.05% TEA; A, 0.1% TEA; G, 0.2% TEA; H, 0.3% TEA. Peaks as in Fig. 2.

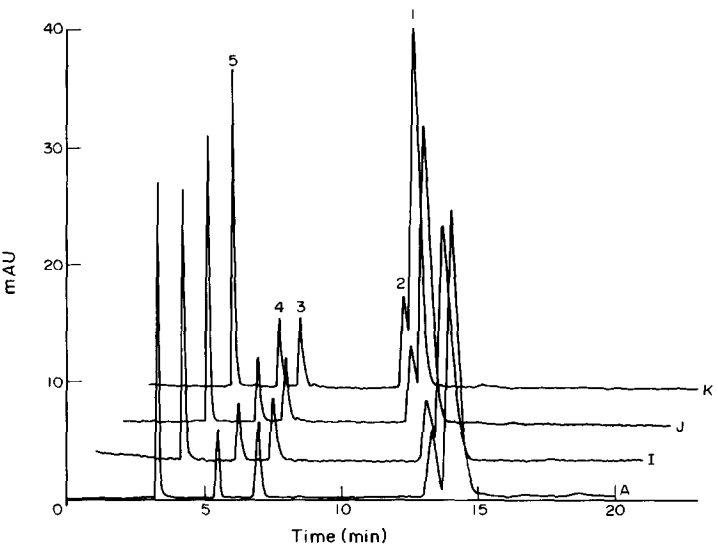

Figure 4
Chromatograms obtained with 0.1% (v/v) TEA–water at pH 2.5 and different methanol percentages mobile phase: A, 0% methanol; I, 1% methanol; J, 3% methanol; K, 5% methanol. Peaks as in Fig. 2.

A negative and linear relation is observed in the retention and resolution of all products when increasing the methanol rate (Fig. 4), the effect being stronger for the pair pilocarpine–isopilocarpine.

The influence of these three factors upon the resolution of pilocarpine-isopilocarpine ($Y1$), which is the critical pair, was quantified by means of a linear regression model with

four independent variables $X1$ = methanol percentage, $X2$ = TEA presence, $X3$ = TEA percentage and $X4$ = pH. As shown in Table 1, the four variables contributed at the significant level of 0.05 to the prediction of the resolution, globally explaining a 84.4% of the model's total variance. $X2$ has a positive effect whilst, $X1$, $X3$ and $X4$ have negative effects.

The mobile phase chosen was 0.1% (v/v) TEA in water, adjusted to pH 2.5 with phosphoric acid. The separation efficacy is summarized in Table 2. Pilocarpine peak has a capacity factor (K') of 13.4, with a correct tailing factor (T 0.05) of 1.15 and a resolution from isopilocarpine (Rs) of 1.8. The achieved selectivity is clearly better than the minimum recommended in the USP XXI [11].

As part of the method validation the stationary-phase stability was investigated in the use of a strongly acidic eluent. The column was exposed exclusively to triethylamine mobile phases, it was washed with water for 30 min after daily use and kept in methanol–water (65:35, v/v). It was found that the column remained practically unaltered for nearly 150 h of exposure to TEA.

Table 1
Linear regression model for the resolution between pilocarpine (**I**) and isopilocarpine (**II**)

Model fitting results for pilocarpine–isopilocarpine resolution ($Y1$)

Independent variable	Coefficient	SE	t-value	Sig. level
Constant	1.782084	0.143792	12.3935	0.0000
$X1$: methanol percentage	−0.164432	0.023239	−7.0756	0.0004
$X2$: amine presence	0.708066	0.144075	4.9146	0.0027
$X3$: amine percentage	−2.130534	0.543306	−3.9214	0.0078
$X4$: pH	−0.152434	0.037494	−4.0655	0.0066

Analysis of variance for the full regression

Source	Sum of squares	d.f.	Mean square	F-ratio	P-value
Model	0.690335	4	0.172584	14.5154	0.0031
Error	0.0713382	6	0.0118897		
Total (corr.)	0.761673	10			

R-SQ. (ADJ.) = 0.8439; SE = 0.109040; MAE = 0.069010; DurbWat= 2.269. (Previously: 0.0000, 0.000000, 0.000000, 0.000).

11 Observations fitted, forecast(s) computed for 0 missing values of dependent variable.

Table 2
Selectivity data of the proposed chromatographic conditions

	K'	W	T 0.05	Rs
Nitrate	2.9	0.10	1.01	
Pilocarpic acid	4.9	0.14	1.06	19.6
Isopilocarpic acid	6.3	0.18	1.08	9.3
Isopilocarpine	12.8	0.30	1.14	26.6
Pilocarpine	13.4	0.40	1.15	1.8

K', capacity factor; W, peak width at the baseline (in min); T 0.05, tailing factor obtained according USP XXI [11]; Rs, resolution.

Linearity and sensitivity

The linearity of response for pilocarpine hydrochloride was verified between 80–800 μg ml^{-1} (six different concentrations and $n = 20$). An equation of a straight line was obtained: peak area = 3.6809 [pilocarpine] − 0.2717, where the correlation coefficient, $r = 0.9999$, the intercept did not significantly differ from zero, and the relative standard deviation for the slope, RSD = 0.010.

Since it was expected that isopilocarpine would be a minor peak, its response was tested with solutions that simultaneously contained 800 μg ml^{-1} of pilocarpine hydrochloride and a range of isopilocarpine nitrate between 4–80 μg ml^{-1} (five different concentrations and $n = 17$), to yield a linearity equation: peak area = 3.4487 [isopilocarpine] + 2.1795, where $r = 0.9997$, the intercept was not significantly different from zero and RSD = 0.018. The response of the more diluted solution was around 16 (peak area), RSD = 5.14%, so empirically determined, the limit of determination of isopilocarpine is therefore better than 0.5% when referred to the pilocarpine content.

The linearity of response of pilocarpic and isopilocarpic acids was not verified because they were obtained by hydrolysis of their respective bases and used without purification.

Precision

The intraday precision was verified for all standard solutions used in the calibration. RSD values between 0.23–1.00% were obtained over the range of 800–80 μg ml^{-1} of pilocarpine hydrochloride and from 0.30 to 5.14% in the range of 80–4 μg ml^{-1} of isopilocarpine nitrate ($n = 3$).

The interday precision ($n = 3$ days) was determined for the 400 μg ml^{-1} of pilocarpine hydrochloride solution and the 20 μg l^{-1} of isopilocarpine nitrate solution, obtaining RSD values of 1.75 and 3.88%, respectively.

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