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Analysis of pilocarpine and its *trans* epimer, isopilocarpine, by capillary electrophoresis

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ABSTRACT

Capillary zone electrophoresis was used for the separation of pilocarpine from its epimer, isopilocarpine, using coated fused-silica capillaries of 20 cm × 25 μm I.D., 8 kV running voltage, migration buffer of 0.1 *M* sodium dihydrogenphosphate pH 8, detection at 217 nm and injection by electromigration. Injections of aqueous, acid and basic solutions were compared. Linearity of the signal for pilocarpine hydrochloride up to 200 μg ml^{-1} in 0.05 *M* hydrochloric acid was obtained, using naphazoline nitrate as internal standard. Optimization of migration buffer pH using coated silica capillaries of 50 cm × 50 μm I.D. showed that at pH 6.9 pilocarpine can be separated from isopilocarpine. Inclusion of β-cyclodextrin in the buffer allows full baseline separation of both epimers. The method was applied to the analysis of a commercial ophthalmic pilocarpine solution.

INTRODUCTION

Pilocarpine, the parasympathomimetic principle isolated from *Pilocarpus jaborandi* (*Rutaceae*), is frequently used in ophthalmic solutions as an antiglaucoma, miotic agent. It is an imidazole-derived alkaloid containing a butyrolactone group, at which both substituents appear in the *cis* configuration. Isopilocarpine, occurring in the plant as well, is the *trans* epimer.

Pilocarpine is more stable at low than at high pH values. The two degradative reactions of pharmaceutical interest are the hydrolysis of the ester linkage of the lactone ring, resulting in pilocarpic acid, and the epimerization about the α-carbon to isopilocarpine, which is further degraded by hydrolysis to isopilocarpic acid [1,2] (Fig. 1). Recent literature data illustrate an increased interest in the area of pilocarpine prodrugs as a means of improving ocular delivery [3–6].

The literature includes reports of several assays for pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid applying HPLC [7–10], analysing commercial pilocarpine preparations in which no significant contamination with the isomer isopilocarpine or the degradation products, pilocarpic acid or isopilocarpic acid, was found [11,12]. In the present study, similar conclusions can be drawn. Preliminary results on the application of capillary zone electrophoresis to the separation of pilocarpine from isopilocar-

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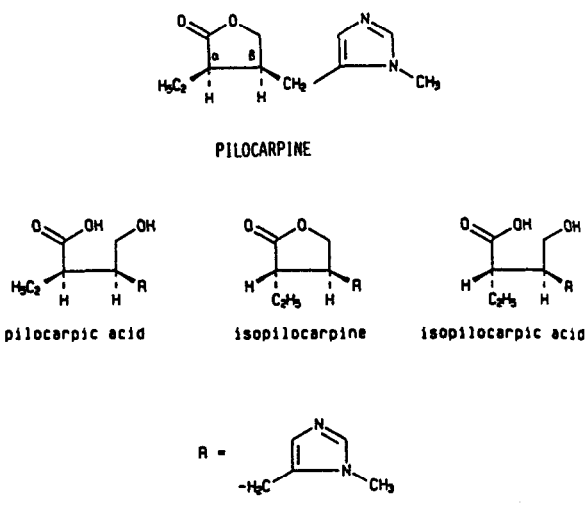

Fig. 1. Structure of pilocarpine, isopilocarpine and their hydrolysis products, pilocarpic acid and isopilocarpic acid.

pine are shown. Inclusion of β-cyclodextrin in the pH 6.9 phosphate eluent buffer using coated columns and UV detection at 217 nm allows full baseline separation of both compounds. The method could be applied to the determination of a commercial ophthalmic solution.

EXPERIMENTAL

Materials and instruments

Pilocarpine hydrochloride and isopilocarpine nitrate were obtained from Aldrich-Chemie (Beerse, Belgium), pilocarpine nitrate from Merck (Darmstadt, Germany) and naphazoline nitrate and β-cyclodextrin from Janssen Chimica (Beerse, Belgium).

Normo-glaukon, a preparation of 2% pilocarpine in the form of 10-ml sterile eyedrops (Dr. Mann Pharma, Berlin, Germany), has the following composition: pilocarpine hydrochloride 20.0 mg, metipranolol hydrochloride 1.118 mg, benzalkonium chloride, disodium EDTA, sodium chloride, dilute hydrochloric acid, water for injections up to 1 ml.

Capillary electrophoresis was performed on a HPE 100 instrument from Bio-Rad, equipped with a variable-wavelength detector. Commercial cartridges (Bio-Rad) containing fused-silica columns were used, with the following specifications: HPE Microsampler cartridge, 20 cm × 25 μm I.D., coated, and Microsampler 100 cartridge, 50 cm × 50 μm I.D., coated. Samples were injected on the anodic side by electromigration, using different loading times and voltages. A constant-voltage mode was applied for the separation of the analytical solutions. For detection, the absorbance was measured at a wavelength (λ) of 217 nm (though any wavelength between 206 and 220 nm may be used), and peak integration was performed by a Shimadzu Chromatopac C-R3A integrator system. Aqueous sodium phosphate buffer solutions were used throughout.

UV absorption spectra were taken on a Perkin-Elmer Lambda 15 double-beam UV spectrophotometer.

Hydrolysis

Hydrolysis of pilocarpine nitrate and of isopilocarpine nitrate was carried out by refluxing 50 mg of each compound (0.184 mmol) for 1 h in about 30 ml of 0.1 *M* sodium hydroxide solution while stirring, followed by cooling of the reaction mixture to room temperature. Next, the solution was treated with 0.5 *M* hydrochloric acid solution to produce a final pH of 7.0, followed by dilution with water up to 50 ml. This solution, containing the hydrolysis products from an original alkaloid concentration of 1 mg ml^{-1} (3.686 m*M*) is used for the UV absorptiometric and capillary electrophoretic experiments. Apart from the hydrolysed solutions of pilocarpine nitrate and of isopilocarpine nitrate, similar hydrolysis was performed on the pilocarpine hydrochloride salt, providing, as expected, completely similar analytical results.

Analysis of a commercial pilocarpine preparation

The ophthalmic solution containing 20.0 mg ml^{-1} pilocarpine hydrochloride solution was diluted 100-fold using 0.05 *M* hydrochloric acid and then mixed with an equal volume of the naphazoline nitrate internal standard solution (50 μg ml^{-1} in 0.05 *M* hydrochloric acid). This sample solution, containing 100 μg ml^{-1} pilocarpine hydrochloride and 25 μg ml^{-1} naphazoline nitrate, was injected onto the column by electromigration at least three times. A standard

solution containing 100 μg ml^{-1} pilocarpine hydrochloride and 25 μg ml^{-1} naphazoline nitrate in 0.05 *M* hydrochloric acid was injected under similar conditions at least three times. By calculating the peak-area ratios of analyte to internal standard in both sample and standard solutions, the pilocarpine content of the eye-drops can be calculated.

RESULTS AND DISCUSSION

As expected, the UV absorption spectra for pilocarpine and isopilocarpine are similar (pilocarpine hydrochloride, $\lambda_{max.} = 214$ nm; pilocarpine nitrate, 206–220 nm plateau; isopilocarpine nitrate, 206–220 nm plateau; all values in water). The spectra from their base-hydrolysed and neutralized solutions are similar to the parent spectra.

Capillary electrophoresis

Coated fused-silica capillaries (see Experimental section) were used throughout so as to decrease electro-endosmosis.

Preliminary experiments were done using short coated columns (20 cm × 25 μm I.D.) at a detection wavelength of 206 nm for aqueous solutions of pilocarpine nitrate, isopilocarpine nitrate, naphazoline nitrate (a suitable internal standard for quantitative work) at concentrations of 50 μg ml^{-1} and equal-volume mixtures of these solutions, applying a loading time of 8 s and a loading voltage of 8 kV (both values experimentally determined to be optimal), and using a 0.1 *M* phosphate buffer pH 8.0. Under these conditions, pilocarpine and isopilocarpine migrate together (about 2.0 min), being separated from the internal standard naphazoline nitrate (about 1.2 min). Reproducibility problems with respect to migration times and peak areas are solved by replacing water with 0.05–0.2 *M* hydrochloric acid as a solvent.

An explanation of the migration and peak-area reproducibility problems depending on the nature of the solution to be injected is as follows. The pilocarpine base has two p*K* values (pK_1 = 7.15; pK_2 = 12.57, both at 20°C). On injection of aqueous solutions, pilocarpine will not be sufficiently protonated to be quantitatively loaded onto the capillary by electromigration. When the pH of the medium is reduced below 7.15, the base becomes diprotonated and the resulting ion will be easily loaded. At pH values between pK_1 and pK_2, pilocarpine is present as the monoprotonated ion, whilst in strong alkaline medium (pH > 12.57) the molecule remains in the non-ionized state. Analyses cannot be carried out starting from basic pilocarpine solutions, which was verified by injecting solutions in sodium hydroxide medium (0.02 *M* and higher), when no peaks could be seen in the electropherograms. Similar problems in quantitatively injecting samples by electromigration may occur when variable amounts of organic solvents (*e.g.* methanol) are present.

The hydrolysed and *ex tempore* acidified solutions of pilocarpine and of isopilocarpine provide an electropherogram showing two non-separated peaks (between 9 and 12 min), without any parent molecule present. When the hydrolysed solutions are acidified down to pH 1.6, in the course of time (days, room temperature) the hydrolysed fraction (non-resolved pilocarpic and isopilocarpic acids) decreases in favour of pilocarpine formation (see *Stability of pilocarpine* section).

Calibration curves

Linear calibration graphs on 20 cm × 25 μm I.D. coated columns were obtained in the 0–200 μg ml^{-1} range ($n = 10$) of pilocarpine nitrate in 0.05 *M* hydrochloric acid ($r = 0.9993$), using naphazoline nitrate as internal standard and applying peak-area ratio calculations. A pilocarpine detection limit of about 1 μg ml^{-1} injected solution ($S/N = 2$) could be reached, though during some analyses this value unexpectedly increased at least five-fold ($\geq$5 μg ml^{-1}), possibly because of a decreased electromigration loading efficiency. Fig. 2 shows a typical electropherogram from pilocarpine in the presence of naphazoline. Under these conditions, isopilocarpine nitrate migrates identically to pilocarpine nitrate, which was confirmed by the addition of isopilocarpine nitrate to a mixture of pilocarpine and naphazoline, the pilocarpine peak remaining qualitatively unaltered. Changing the running buffer pH from 5.0 to 8.0 could

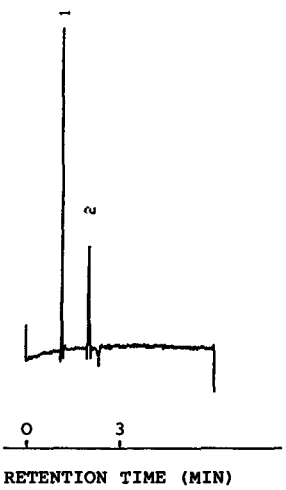

Fig. 2. Electropherogram of a mixture of pilocarpine nitrate and naphazoline nitrate (each at 25 μg ml^{-1} in 0.05 *M* hydrochloric acid). Conditions: 20 cm × 25 μm I.D. coated column; loading by electromigration, 8 s at 8 kV (from + to −); running buffer, 0.1 *M* phosphate buffer pH 8.0; running voltage, 8 kV; detection at 217 nm. Peaks: 1 = naphazoline (1.15 min); 2 = pilocarpine (2.01 min).

not separate the epimers, in spite of the excellent shape and reproducible character of the obtained peaks. The present investigations indicate that the use of longer capillaries may solve this problem.

Separation of pilocarpine and isopilocarpine

Influence of running buffer pH. Mixtures of pilocarpine nitrate and isopilocarpine nitrate were injected onto a 50 cm × 50 μm I.D. coated column applying 0.1 *M* phosphate running buffer solutions with pH values between 6.7 and 7.5, based on the overall identical migration pattern of both molecules over a wider pH range: it was noticed that from pH 5.0 to pH 6.5, both molecules migrate identically (one peak when mixed). From pH 6.6 up to about pH 7.8, separation is observed, whereas at pH 8.0 and 9.0 both compounds again migrate equally. Fig. 3 illustrates the pH effect of the running buffer from pH 6.8 to 7.0. A pH value of 6.9 provides the best electropherogram, though baseline separation is not achieved. Peaks were assigned by repetitive injections of solutions of both compounds, after the addition of each component.

Influence of running voltage. The effect of the running voltage on the separation of pilocarpine nitrate and isopilocarpine nitrate was investigated at the optimum running buffer pH of 6.9. Fig. 4 shows the results on the migration times of both molecules for voltages between 4 and 9 kV. Based on the peak shape, a running voltage of 8 kV was chosen for further experiments. None of the applied voltages could provide a baseline separation.

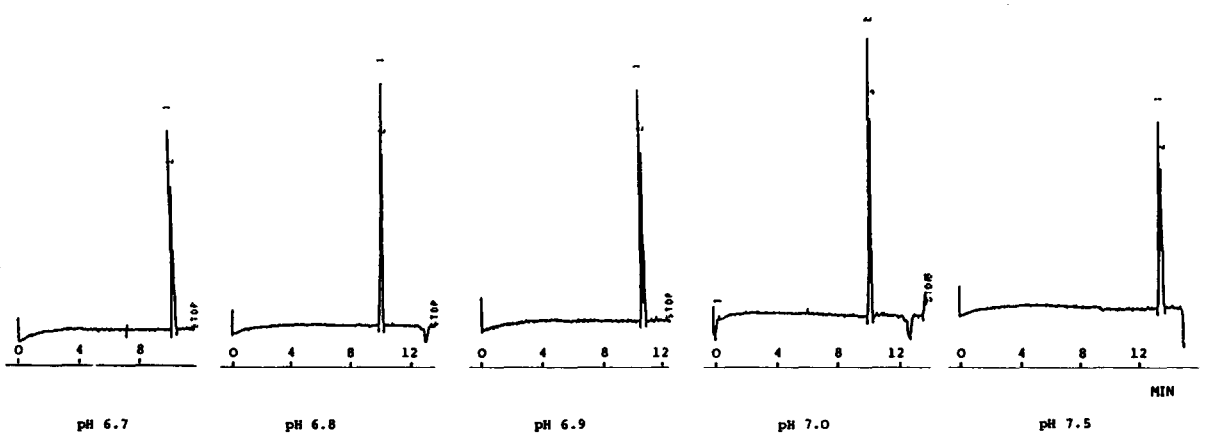

Fig. 3. Electropherogram of a mixture of pilocarpine nitrate and isopilocarpine nitrate (each at 100 μg ml^{-1} in 0.05 *M* hydrochloric acid) at pH 6.7, 6.8, 6.9, 7.0 and 7.5. Conditions: 50 cm × 50 μm I.D. coated column; loading by electromigration, 8 s at 8 kV (from + to −); running buffer, 0.1 *M* phosphate buffer; running voltage, 8 kV; detection at 217 nm. Peaks: 1 = pilocarpine (10.69 min at pH 6.9); 2 = isopilocarpine (10.85 min at pH 6.9).

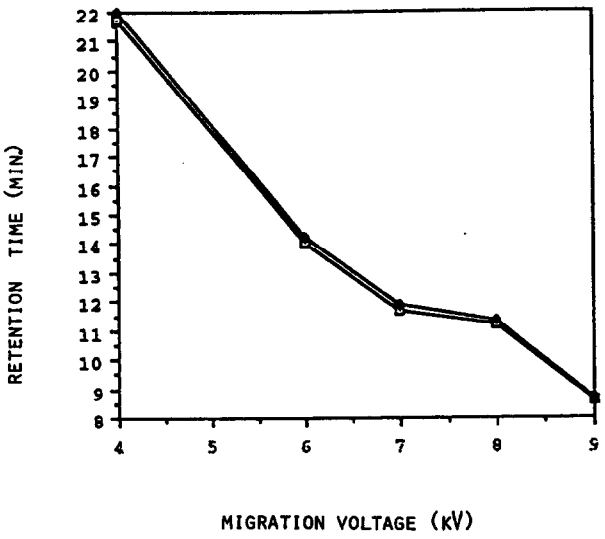

Fig. 4. Influence of migration voltage on the separation of pilocarpine nitrate (□) and isopilocarpine nitrate (◆). Conditions: see Fig. 3 (running buffer pH 6.9).

Influence of β-cyclodextrin. The effect of β-cyclodextrin inclusion in the buffer upon the capillary electrophoretic pilocarpine–isopilocarpine separation was investigated, based on the general use of β-cyclodextrin and similar cyclodextrins as inclusion-complexing and performance-increasing agents in chromatography, spectroscopy and capillary electrophoresis [13–15]. Mixing the pH 6.9 phosphate buffer with an equal volume of 0.02 *M* aqueous β-cyclodextrin under the optimized electrophoretic conditions yielded the electropherogram shown in Fig. 5A, providing baseline separation of the two epimers. Fig. 5B clearly shows that the separation effect is definitely caused by the presence of cyclodextrin in the buffer solution. It is assumed that the increased separation of the two alkaloids, possessing only minor stereochemical differences, is the result of a difference in their interaction with the typical conically shaped β-cyclodextrin molecule, featuring a polar outer surface and a rather non-polar internal cavity.

The influence of the concentration of β-cyclodextrin on the migration difference between both epimers is shown in Fig. 6. Apparently, increasing the β-cyclodextrin concentration of the buffer enhances the difference in the retention time of both migrating compounds. However, at a concentration of 0.03 *M* added β-cyclodextrin onwards (0.015 *M* final concentration), practical problems occur, as cyclodextrin crystallizations in the buffer are noticed. Hence, the capillary flow may be seriously hindered, in addition to the light-scattering effects encountered in the detector, resulting in unstable

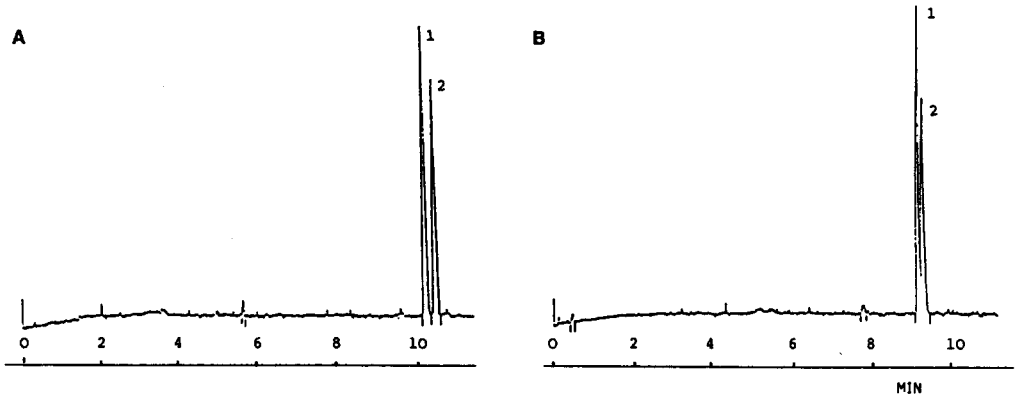

Fig. 5. (A) Electropherogram of a mixture of pilocarpine nitrate and isopilocarpine nitrate (each at 100 μg ml^{-1} in 0.05 *M* hydrochloric acid). Conditions: 50 cm × 50 μm I.D. coated column; loading by electromigration, 8 s at 8 kV (from + to −); running buffer solution, 0.1 *M* phosphate buffer pH 6.9 containing β-cyclodextrin 0.01 *M*; running voltage, 8 kV; detection at 217 nm. Peaks: 1 = pilocarpine; 2 = isopilocarpine. (B) Electropherogram similar to (A), replacing the β-cyclodextrin solutions with pure water.

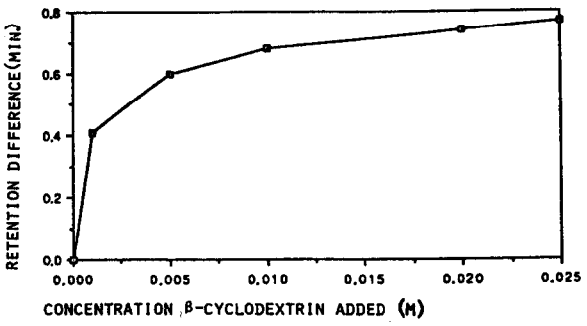

Fig. 6. Influence of β-cyclodextrin concentration on the difference in migration times between pilocarpine nitrate and isopilocarpine nitrate. Conditions as in Fig. 5A with varying β-cyclodextrin concentrations.

baselines. For these reasons, an optimum final β-cyclodextrin concentration of 0.01 *M* was chosen. Peak assignment in the optimized

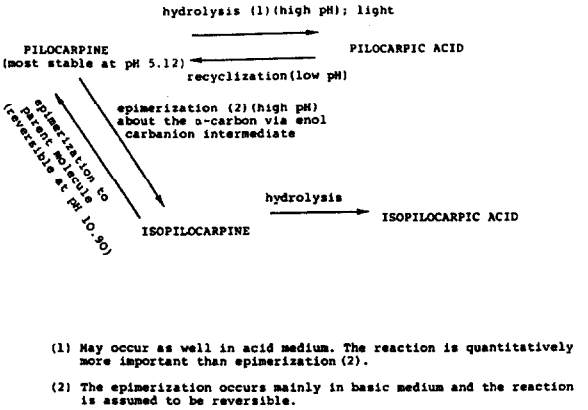

Fig. 7. Chemical stability of pilocarpine and general decomposition reactions [2].

separating system was carried out by injecting mixtures of both alkaloids at different concentrations.

Stability of pilocarpine

A previous report on the chemical stability of pilocarpine [2] states that the hydrolysis of the ester linkage of pilocarpine occurs much faster than the epimerization in alkaline medium. The epimerization reaction of pilocarpine is reversible at pH 10.90. Pilocarpine and isopilocarpine are susceptible to hydrolysis to the same extent at pH 10.90; the hydrolysis reaction of isopilocarpine is probably irreversible in either acid or basic medium. A general scheme on pilocarpine stability is given in Fig. 7.

The fact that pilocarpine is more stable at lower than at higher pH values, with maximum stability at pH 5.12, should be taken into account, *e.g.*, when preparing ophthalmic solutions. In the present study, some useful data on pilocarpine decomposition reactions were obtained. Fig. 8 shows the electropherogram of base-hydrolysed pilocarpine nitrate, adjusted to pH 1.6 and analysed after 1 day and 1 week standing at room temperature. Both electropherograms indicate that pilocarpine nitrate is transformed during the alkaline hydrolysis to isopilocarpine nitrate and hydrolysate. Note that pilocarpic and isopilocarpic acids cannot be separated under the given conditions. When considering peak areas of the different peaks, it could be demonstrated that after 1 week at pH 1.6 the

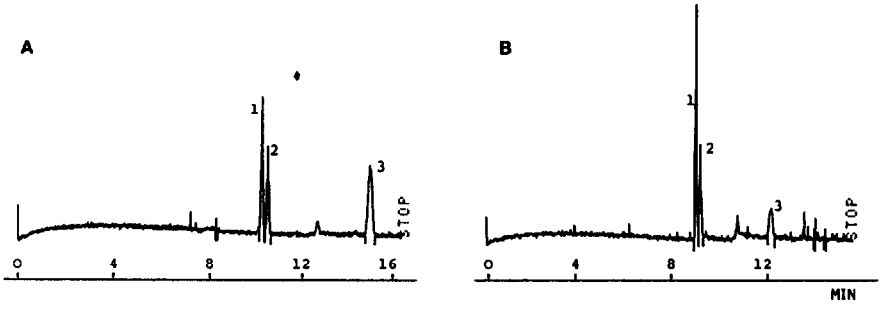

Fig. 8. Electropherogram of base-hydrolysed pilocarpine nitrate (150 μg ml^{-1}), adjusted to pH 1.6 and injected after 1 day (A) and 1 week (B) standing at room temperature. Conditions: see Fig. 5A; detection at 217 nm. Peaks: 1 = pilocarpine; 2 = isopilocarpine; 3 = hydrolysate peak.

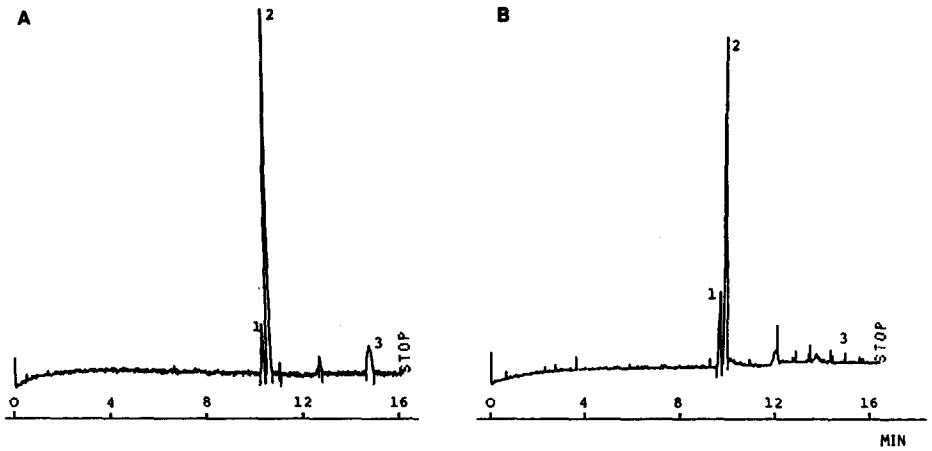

Fig. 9. Electropherogram of base-hydrolysed isopilocarpine nitrate (150 μg ml^{-1}), adjusted to pH 1.6 and injected after 1 day (A) and 1 week (B). Conditions: see Fig. 5A; detection at 217 nm. Peaks: 1 = pilocarpine; 2 = isopilocarpine; 3 = hydrolysate peak (with adjusted integrator attenuation).

(total) acids peak had markedly decreased in favour of a strongly increased pilocarpine nitrate peak. It is assumed that pilocarpic acid is converted into pilocarpine in acid medium. As isopilocarpic acid is not converted to isopilocarpine, the area of the iosopilocarpine nitrate peaks nearly remains unchanged. The same phenomenon could be seen with base-hydrolysed isopilocarpine nitrate, of which the alkaline hydrolysis solution was adjusted to pH 1.6. The obtained electropherograms are illustrated in Fig. 9. Again, three peaks are seen, those of pilocarpine nitrate, isopilocarpine nitrate and the acids. With time, the last peak decreases in favour of pilocarpine nitrate, the isopilocarpine nitrate peak remaining constant. The results of

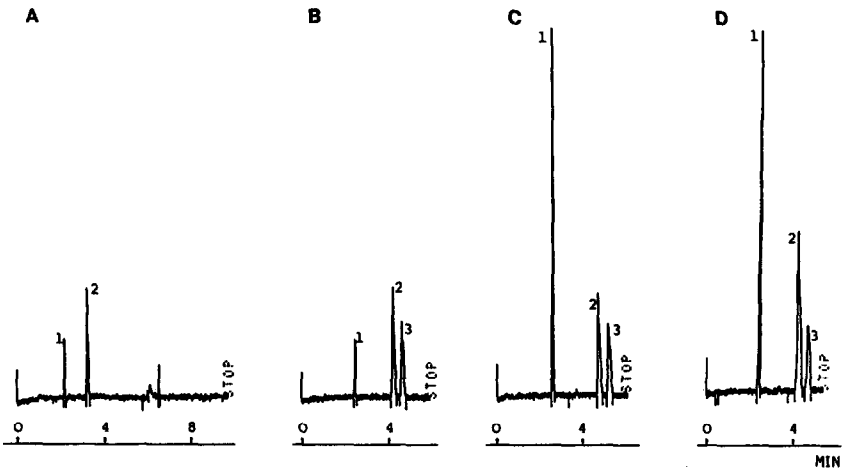

Fig. 10. Electropherograms of solutions of pilocarpine hydrochloride (200 μg ml^{-1}) and naphazoline nitrate (25 μg ml^{-1}) in 0.05 *M* hydrochloric acid (A), to which are subsequently added +4 mg of isopilocarpine nitrate per 20 ml (≈ 200 μg ml^{-1} final concentration) (B), +4 mg of naphazoline nitrate (C), and +4 mg of pilocarpine hydrochloride (D). Peaks: 1 = naphazoline; 2 = pilocarpine; 3 = isopilocarpine. Conditions: 20 cm × 25 μm I.D. coated column; loading by electromigration, 8 s at 8 kV (from + to −); running buffer, 0.1 *M* phosphate buffer pH 6.9 containing 0.01 *M* β-cyclodextrin; running voltage, 8 kV; detection at 217 nm.

the electrophoretic experiments could be confirmed by TLC experiments, a technique that clearly offers poorer resolving capacity than capillary electrophoresis.

Analysis of a commercial pilocarpine preparation

The relative standard deviation of six replicate standard injections was 1.04%. Calibration standard solutions were prepared such that a range of 80–120% of the concentration claimed on the label was covered. A plot of the peak-area ratios of pilocarpine–naphazoline (internal standard) *versus* the amount of pilocarpine in the standard solutions was linear ($r = 0.9995$). The results of the quantitation, calculated for six independently prepared commercial sample solutions, were 101.3% (R.S.D. = 1.43%) with respect to the label claim. It is assumed that the coefficient of variation can be lowered when applying a non-electromigration loading system. Fig. 10 shows the electropherograms, with peak assignment, obtained from standard solutions, indicating the absence of isopilocarpine, which is baseline separated in the electropherogram provided by this system. Fig. 11 shows the electropherogram from the sample solution. As can be seen, the metipranolol peak does not interfere with the peaks of interest. No other pilocarpine decomposition peaks can be identified in the sample solution.

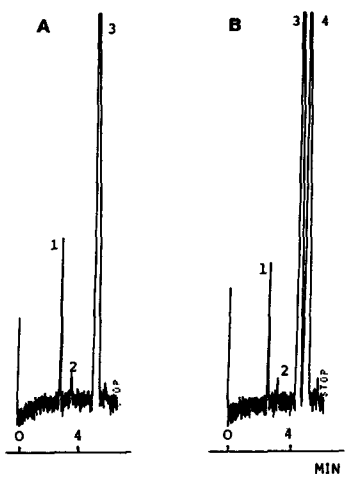

Fig. 11. Electropherogram of a sample solution of the eyedrop preparation (A) and of the same solution after the addition of isopilocarpine nitrate (B). Conditions as for Fig. 8 (with adjusted integrator attenuation). Peaks: 1 = naphazoline; 2 = metipranolol (confirmed by injecting 5 μg ml^{-1} standard solutions); 3 = pilocarpine; 4 = isopilocarpine.

In conclusion, pilocarpine can be separated from its epimer isopilocarpine by applying capillary electrophoresis on coated columns using a 0.1 *M* phosphate buffer pH 6.9 and UV detection; the inclusion of β-cyclodextrin in the buffer allows baseline separations. The method allows quantitative analysis of a commercial ophthalmic preparation using naphazoline as an internal standard. The suggested system opens up the possibility of further analytical research on the chemical stability testing of pilocarpine and related alkaloids.

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