# Analysis of Pilocarpine and Its Degradation Products by Micellar Electrokinetic Capillary Chromatography

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## INTRODUCTION

Pilocarpine is a widely used miotic agent for the treatment of glaucoma. The chemical stability of the drug can be problematic when attempting to prepare solution dose forms with an acceptable shelf life at room temperature. Although a number of liquid chromatographic assays have been developed to characterize the decomposition profile of pilocarpine (to pilocarpic acid, isopilocarpine, and/or isopilocarpic acid; Scheme I), the resolution and separation capacities of the procedures often do not permit quantitation of low concentrations of the degradation products (1–5). For example, peak tailing and poor resolution is often associated with reverse-phase assays (1), while normal-phase assays have been limited to quantifying only the lactones (5). Gas chromatographic methods have been reported, but these require precolumn derivatization of the analyte and generally quantitate only the parent lactones or the carpic acids within a single assay (6,7).

Capillary electrophoresis achieves high separation efficiencies and resolution and, consequently, has found wide application in the analysis of low and high molecular weight species (8–10). Capillary electrophoresis can be performed in different modes with free solution capillary electrophoresis (FSCE) and micellar electrokinetic capillary chromatography (MECC) finding widest application in pharmaceutical analysis. The mechanism of separation in FSCE is based upon differences in solute size and charge (at a particular pH), whereas MECC achieves separation based on the effective partitioning of a solute between an ionic micellar "phase" and the solution phase of the separation buffer. MECC was originally developed for the separation of electrically neutral substances (11,12) which are retained by the slow-moving micellar phase as a function of their effective hydrophobicity. Subsequently, MECC has been extended to the separation of mixtures of both ionic and neutral substances (13–15).

In the present study, MECC was investigated as a means of achieving baseline resolution of pilocarpine and its potential degradation products within a single analysis.

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## MATERIALS AND METHODS

*Chemicals.* Pilocarpine HCl and sodium dodecyl sulfate (SDS; electrophoresis grade) were obtained from Sigma Chemical Company (St. Louis, MO) and isopilocarpine nitrate was obtained from Aldrich Chemical Company (Milwaukee, WI). Sodium deuteroxide and deuterium oxide (both with an isotopic purity of 99.9%) were obtained from Cambridge Isotope Laboratories (Woburn, MA). All other chemicals were of at least analytical reagent grade. Water was obtained from a Milli-Q (Millipore Inc., Milford, MA) water purification system.

*Preparation of Carpic Acid Samples.* Pilocarpic acid and isopilocarpic acid were prepared by hydrolysis of a known quantity (approx 10 mg) of the corresponding lactone with 11% (v/v) 10 *M* NaOD in $D_2O$. The reaction was followed by proton NMR (300 MHz, Bruker) to confirm complete hydrolysis of the lactone to the corresponding carpic acid. From the $^1H$ spectrum, hydrolysis of the lactone was accompanied by an upfield shift and change in the multiplicity of the methylene protons adjacent to the lactone-ring oxygen atom.

*Electrophoresis.* Separation buffers were prepared using acetate (pH 5–5.5), phosphate (pH 6–7.5), or borate (pH 8–9.4) systems by mixing solutions of the acid and conjugate base to give the desired pH and an ionic strength of 0.02 *M*. Final pH measurements were made following the addition of SDS. Samples for analysis were prepared in borate buffer (pH 8.1, $\mu$ = 0.02 *M*) and all solutions (separation buffers and samples) were passed through a 0.22-$\mu$m filter prior to use.

A Beckman (Palo Alto, CA) P/ACE 2100 capillary electrophoresis system and a Beckman 57-cm × 75-$\mu$m-i.d. fused silica capillary column (50 cm to detector) were used for all FSCE and MECC analyses. On-line UV detection was performed at 214 nm and the temperature of the capillary was maintained at 22 ± 0.1°C. Prior to each injection, the column was preconditioned by flushing first with 0.1 *M* NaOH for 2 min and then with separation buffer for 4 min. Samples were introduced onto the column using a 5-sec pressure injection corresponding to approximately 30 nl as determined by the Poiseuille equation. All separations were performed using a constant voltage of 20 kV.

Peak analysis was performed using Beckman System Gold software. The solute electrophoretic mobility ($\mu$), the number of theoretical plates calculated at half-peak height ($N$), and the peak resolution ($R_s$) were determined as described previously (15,16).

## RESULTS AND DISCUSSION

The different ionic character of the lactone compounds and their corresponding carpic acid facilitated separation with FSCE. However, the individual isomeric forms of the lactones or the carpic acids (which are diastereoisomers) remained unresolved. Therefore, MECC was investigated as a means to enhance separation of the diastereoisomers based upon the expected differences in their physicochemical properties.


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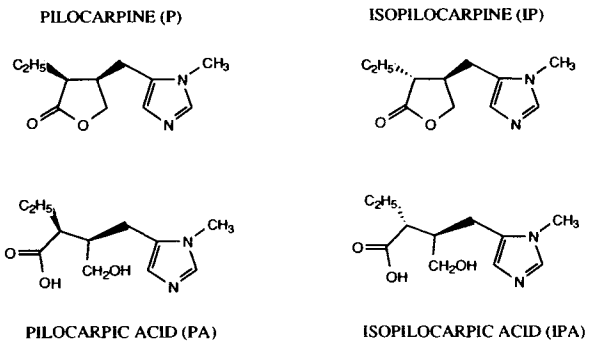


**Scheme I.** Structures of pilocarpine, isopilocarpine, and the respective carpic acids.

*Assay Development.* SDS was chosen as the ionic surfactant for these studies, and the initial experiments were performed at an SDS concentration of 2.5% (w/v) (87 m*M*). The effect of separation buffer pH on the elution characteristics of the four species (and methanol as a marker of the electroosmotic flow) is presented in Fig. 1. As the reported $pK_a$ of the imidazole-ring nitrogen in pilocarpine is approximately 7.2 (17), the data in Fig. 1 cover a range of ionization states for the lactones and carpic acids. It should be noted that the presence of micelles in the separation buffer may alter the apparent $pK_a$ of ionizable solutes, which could affect the interpretation of pH-dependent changes in elution behavior (15).

In the presence of 2.5% (w/v) SDS, the isomeric lactones were well resolved above pH 8.0, with improvements in the separation efficiency, as judged by peak symmetry, occurring above pH 9.0. At pH values less than 8.0, the elution time of the lactones increased, leading to peak broadening and tailing. The longer elution times observed at lower pH's are consistent with the progressive increase in the cationic nature of the solute, leading to increased interaction with the anionic micelle. Additionally, the electrophoretic mobility of SDS micelles is reduced as the pH of the separation buffer is decreased (15), which would also lead to longer elution times for the lactones. The decreased resolution and poorer peak shape observed at these lower pH values may be a manifestation of the secondary and tertiary equilibria which could be involved in the elution characteristics of the pilocarpine cation. Previous investigators have suggested that cationic solutes interact strongly with negatively charged micelles either through an electrostatic interaction with the ionized surface of the micelle or through the formation and subsequent solubilization of ion pairs formed between the cationic solute and the anionic surfactant (15,18). Although the elution times of the isomeric carpic acids were largely independent of pH in the range studied, the resolution improved at low pH values. Loss of baseline resolution of the carpic acids occurred above pH 9.0 when using an SDS concentration of 2.5% (w/v).

The differential and opposing effect of pH on the resolution of the two lactones and carpic acids prevented the use of a single set of conditions which adequately resolved all four compounds using a SDS concentration of 2.5% (w/v). As the lactone peak symmetry improved at high pH's, the effect of SDS concentration on the resolution of the four species was investigated at pH 9.3. Figure 2 displays graphically the change in elution time for each of the four species as the SDS concentration was increased from 0 to 5% (w/w) with a separation buffer pH of 9.3. The effect of SDS concentration on the number of theoretical plates and peak resolution is shown in Table I. Increasing the concentration of SDS in the separation buffer led to improved separation of both the isomeric lactones and the carpic acids. As illustrated in the electropherograms in Fig. 3, increasing the SDS concentration from 2.5 to 5% (w/v) afforded baseline resolution of all four species. Furthermore, the number of theoretical plates calculated for each of the solute species increased with the higher SDS concentrations (Table I).

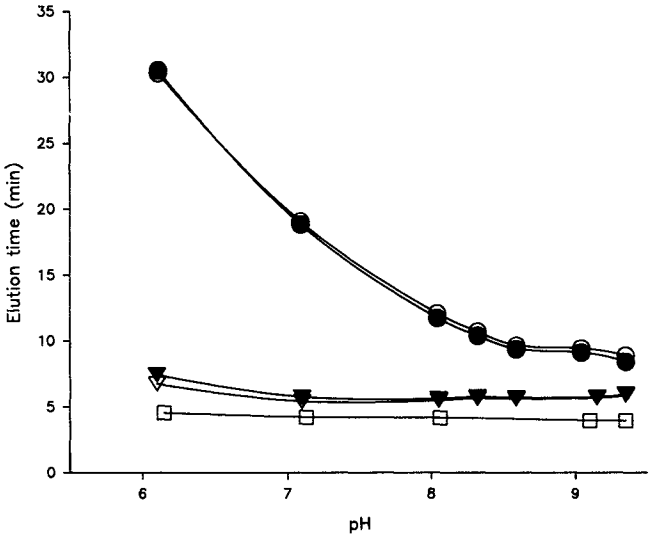


Fig. 1. Relationship between elution time (min) and the pH of the separation buffer for pilocarpine (○), isopilocarpine (●), pilocarpic acid (▽), isopilocarpic acid (▼), and methanol (□). All buffers contained 2.5% (w/v) SDS and the separations were performed at a constant voltage of 20 kV and 22°C.

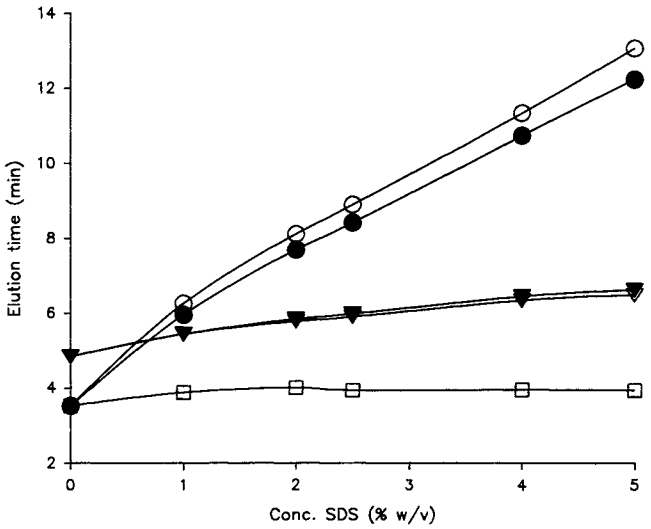


Fig. 2. Effect of SDS concentration on elution time of pilocarpine (○), isopilocarpine (●), isopilocarpic acid (▼), pilocarpic acid (▽), and methanol (□). All separations were performed using pH 9.3 borate buffer ($\mu$ = 0.02 *M*) at a constant voltage of 20 kV and a column temperature of 22°C.

Table I. Effect of SDS Concentration (%, w/v) on the Number of Theoretical Plates and the Resolution of Pilocarpine (P), Isopilocarpine (IP), Pilocarpic Acid (PA), and Isopilocarpic Acid (IPA) (Separation Conditions as Defined for Fig. 2)

| SDS concentration (%) | Number of theoretical plates | | | | Resolution | |
|---|---|---|---|---|---|---|
| | P | IP | PA | IPA | P/IP | PA/IPA |
| 0 | 12,600[a] | | 9400[a] | | NR[b] | NR |
| 1 | 38,000 | 39,000 | —[c] | — | 2.64 | — |
| 2 | 74,000 | 66,000 | 34,000 | 65,000 | 3.60 | 0.55 |
| 4 | 97,000 | 127,000 | 50,000 | 64,000 | 4.56 | 1.03 |
| 5 | 206,000 | 208,000 | 92,000 | 113,000 | 6.96 | 1.63 |

[a] Determined from single-component solutions.
[b] Not resolved.
[c] Inadequate resolution.

From Fig. 2, it is clear that the elution behavior of the lactones was highly dependent upon the SDS concentration, indicating a significant interaction between the lactones and the SDS micelle. In comparison, the elution of the carpic acids displayed little dependency upon the concentration of SDS, although an increase in SDS concentration enhanced their resolution. It is not clear from these data whether the effect of SDS on the resolution of the acids stems from a direct interaction of the solutes with the micelle or from an indirect effect of the SDS on factors affecting the efficiency of the electrophoretic separation (e.g., ionic strength effects, alterations in electrical double layer). From these studies, the optimized conditions consisted of a separation buffer of pH 9.3 and a SDS concentration of 5% (w/v).

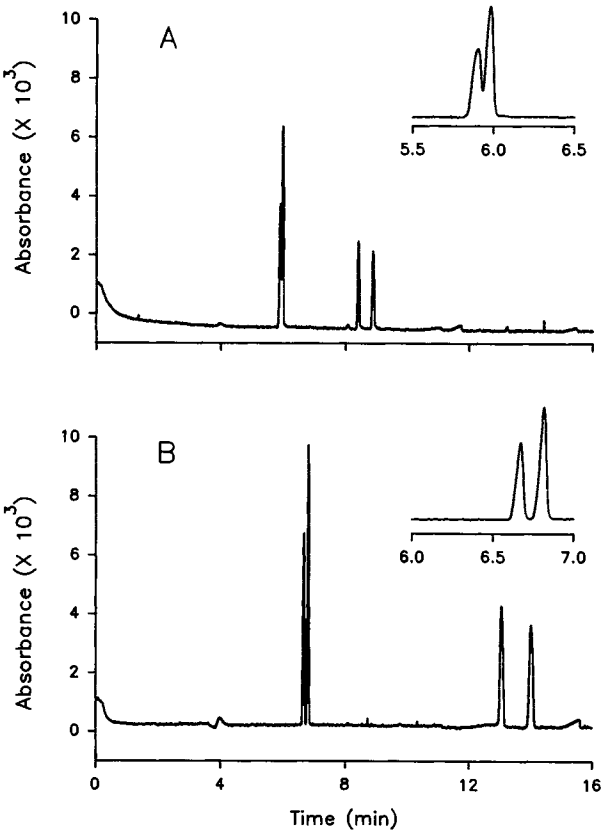


Fig. 3. Electropherograms depicting (in order of elution) the separation of pilocarpic acid, isopilocarpic acid, isopilocarpine, and pilocarpine. Separation performed at 20 kV, pH 9.3 borate buffer ($\mu$ = 0.02 *M*) containing either 2.5% (w/v) SDS (A) or 5% (w/v) SDS (B).

*Validation and Application of the Assay.* The linearity, reproducibility, and limit of detection of the assay were investigated. The relationship between the integrated peak area and the solute concentration was found to be linear in the range 0.007–0.35 m*M* (corresponding to 1.5–75 $\mu$g/ml). For each pair of diastereoisomers, the slopes of the peak area–concentration profiles were not significantly different (slope ± SE: P, 0.316 ± 0.003; IP, 0.315 ± 0.002; PA, 0.458 ± 0.007; IPA, 0.457 ± 0.001). The correlation coefficient describing each of the linear profiles was greater than 0.99, the intercepts were not significantly different from zero, and the limits of detection were approximately 1 $\mu$g/ml for each solute. The within-day reproducibility of the assay was determined for each of the respective solutes and typical data are presented in Tables II and III. The coefficient of variation associated with the elution time measurements was less than 1% for each of the four species. The variability associated with the peak area measurements was typically about 1% for the higher concentrations (approximately 75 $\mu$g/ml), increasing to approximately 5–6% for the lower concentrations.

Examination of the day-to-day variability of the assay has revealed that the coefficient of variation of the peak area data was typically less than 2% for each of the four solutes. The solute elution times, however, displayed a gradual and consistent drift to longer times over a period of weeks following continued use of the same capillary. The elution time drift persisted despite variations in the column rinsing procedure with sodium hydroxide and/or separation buffer. In all instances, the within-day elution time reproducibility was typical of the data presented in Table II.

The observed elution time drifts are consistent with other investigations (19,20) which have implicated a gradual change in the surface characteristics of the silica capillary as the source of the variation. However, when the observed elution times were corrected for changes in the electroosmotic flow by determining the electrophoretic mobility of the particular analyte, day-to-day variations were reduced to negligible levels. From a quantitative standpoint, the incorporation of standards on a daily basis would readily accommodate the gradual change in the elution time of the solutes.

Table II. Reproducibility (Mean ± SD; $n$ = 9) of Elution Time Data and the Corresponding Calculated Electrophoretic Mobilities ($\mu$) of Pilocarpine, Isopilocarpine, Pilocarpic Acid, and Isopilocarpic Acid Using the Separation Conditions Defined for Fig. 3B

| Species | Conc. (m*M*) | Elution time (min) | $\mu$ ($\times 10^4$ cm$^2$/V · sec) |
|---|---|---|---|
| Pilocarpine | 0.169 | 13.77 ± 0.13 | 4.40 ± 0.02 |
| Isopilocarpine | 0.081 | 12.85 ± 0.11 | 4.27 ± 0.02 |
| Pilocarpic acid | 0.032 | 6.68 ± 0.04 | 2.57 ± 0.02 |
| Isopilocarpic acid | 0.025 | 6.82 ± 0.04 | 2.64 ± 0.02 |

Table III. Reproducibility (Mean ± SD; $n$ = 6) of Peak Area Data for Samples Containing Pilocarpine, Isopilocarpine, Pilocarpic Acid, and Isopilocarpic Acid at Two Concentrations Using the Separation Conditions Defined for Fig. 3B

| Species | Conc. (m$M$) | Peak area |
|---|---|---|
| Pilocarpine | 0.2381 | 0.7462 ± 0.0098 |
| | 0.0149 | 0.0430 ± 0.0019 |
| Isopilocarpine | 0.2488 | 0.7935 ± 0.0103 |
| | 0.0156 | 0.0454 ± 0.0035 |
| Pilocarpic acid | 0.1968 | 0.4216 ± 0.0052 |
| | 0.0123 | 0.0266 ± 0.0015 |
| Isopilocarpic acid | 0.3470 | 0.7634 ± 0.0096 |
| | 0.0145 | 0.0361 ± 0.0024 |

An example of the utility of the assay in analyzing commercially available pilocarpine ophthalmic solutions is presented in Figs. 4A and B. Figure 4A is an electropherogram of an "in-date" formulation, with pilocarpine eluting at 15 min. The minor peak at 7 min represents pilocarpic acid corresponding to approximately 3.5% degradation. Figure 4B is an electropherogram of a degraded "out-of-date" formulation and the presence of the pilocarpic acid and isopilocarpine at 7 and 14.3 minutes, respectively, is readily apparent. The presence of pilocarpic acid and isopilocarpine corresponds to approximately 30% degradation as determined by comparison to standard solutions.

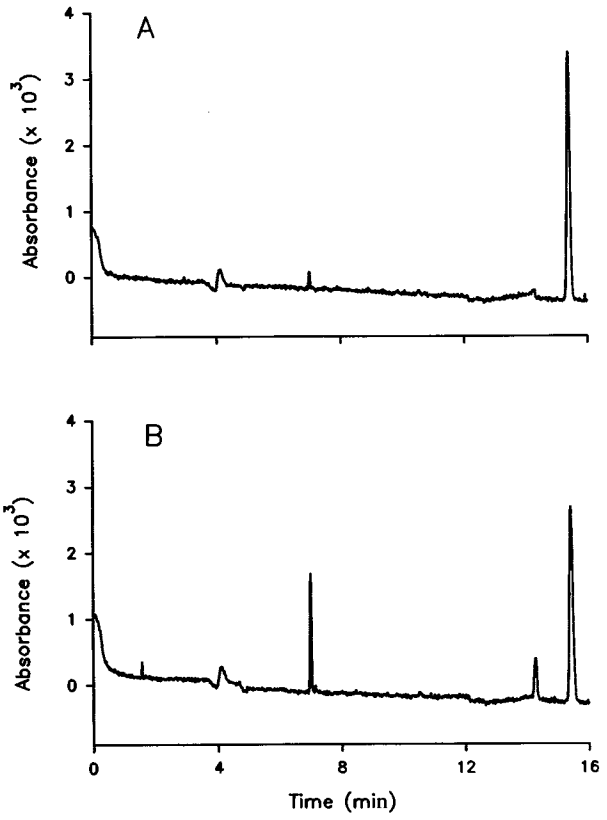


Fig. 4. Electropherograms of different ophthalmic pilocarpine solutions. (A) The major peak is pilocarpine and the minor peak at 7 min represents approximately 3.5% pilocarpic acid. (B) Degraded sample of pilocarpine depicting the presence of pilocarpic acid (7 min) and isopilocarpine (14 · 3 min).

In summary, a MECC assay has been developed for pilocarpine and the three possible degradation products which has the advantages of baseline resolution of all species in a single, rapid analysis. While the mechanistic factors governing the separation of the various pilocarpine species have not been fully elucidated, the assay illustrates the potential utility of MECC for the separation of diastereoisomers which may be difficult to separate by other means.

## ACKNOWLEDGMENT

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