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Separation of pyridinecarboxylic acid isomers and related compounds by capillary zone electrophoresis

Effect of cetyltrimethylammonium bromide on electroosmotic flow and resolution

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ABSTRACT

The effect of the addition of cetyltrimethylammonium bromide (CTAB) to the buffer system in capillary electrophoresis on electroosmotic flow (EOF) is examined. At a CTAB concentration of $2.5 \cdot 10^{-4}$ *M*, EOF is anodal (flow towards the positive detector column end). With bare silica columns, anodal EOF first increases with increasing pH, up to a maximum in the pH range 4–6 depending on CTAB concentration, then decreases as pH is further increased. Optimum resolution of pyridinecarboxylic acid isomers is obtained at pH 2.7 with a 10 m*M* phosphate buffer and 30 m*M* CTAB. Using the same buffer system, optimum resolution for hydroxy-substituted pyridinecarboxylic acid isomers is obtained at pH 7.5. The use of CTAB results in a dramatic improvement in peak shape. Preliminary results, using an excimer laser operated at 248 nm, show that the fluorescence intensity of isonicotinic acid is substantially enhanced with the addition of 0.3% hydrogen peroxide to the phosphate buffer system.

INTRODUCTION

Nicotinic (*m*-pyridinecarboxylic) acid and nicotinamide are water-soluble vitamins that have many important biological functions [1–3], and isonicotinic (*p*-pyridinecarboxylic) acid is a metabolite of isoniazid, which is an anti-tuberculosis drug [4]. Several methods have already been described for the separation and analysis of pyridinecarboxylic (PC) acid derivatives [1–15]. These include colorimetric [5,6], fluorimetric [7,8], gas chromatographic–mass spectrometric [9] and ion-exchange [10] methods and HPLC with various types of detectors [1,3,4,11–15]. The colorimetric and fluorimetric methods have been criticized for excessive analysis time and lack of sensitivity [1], and several of the HPLC methods have been faulted for complicated sample pre-treatment procedures [1,3]. Roberts *et al.* [15] encountered severe problems due to poor peak shapes when analyzing *o*-pyridinecarboxylic (picolinic) acid and related compounds by reversed-phase HPLC, that could only be corrected by addition of the analytes to the mobile phase. Mawatari *et al.* [1,4] reported the fluorimetric determination of 3- and 4-substituted pyridines by HPLC with postcolumn UV irradiation and subsequent fluorescence detection. This interesting approach resulted in signifi-

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cant enhancement of sensitivity; however, post-column sample treatment may result in extra peak broadening and, more importantly, the use of organic solvents in the mobile phase results in decreased fluorescence intensity.

The recent development of capillary zone electrophoresis (CZE) has provided the potential for achieving rapid high-resolution separations of macromolecules as well as small ionic and ionizable compounds [16,17]. Furthermore, the introduction of micellar electrokinetic capillary chromatography (MECC) has allowed the extension of CZE methods for the separation of neutral compounds [18,19]. Because of its strong separation power and its compatibility with aqueous solutions, CZE is particularly suited for the separation of PC acids and related compounds and their analysis in biological fluids. Only three reports on the use of capillary electrophoresis for the analysis of these compounds have been reported; Fujiwara *et al.* [20] and Nishi *et al.* [21] reported the use of MECC for the analysis of water-soluble vitamins including nicotinic acid and Tanaka *et al.* [2] used a tube isotachophoretic method for the separation of nicotinic acid derivatives.

In this study we report the use of capillary electrophoresis with and without micellar additives for the separation of PC acid isomers and related compounds. A number of strategies have been investigated including pH optimization, column coating and control of electroosmosis with the use of cetyltrimethylammonium bromide (CTAB). Preliminary results on the use of an excimer laser at 248 nm for the simultaneous photochemical activation and on-column laser-induced fluorescence (LIF) detection of these compounds will also be presented.

EXPERIMENTAL

A Beckman CZE System 2000 (Model P/ACE) equipped with a UV detector, an autosampler, a liquid-cooled column cartridge and a System Gold software package was used in this study. All experiments were conducted at 25°C. Injections were made using the pressure mode for 2–5 s. The buffers were prepared by dissolving the appropriate amount of reagent in distilled and deionized water and were degassed and filtered through 0.2-μm nylon 66 filters. A Fisher Accumet pH meter 25 (Fisher Scientific, Fair Lawn, NJ, USA) was used to measure pH levels. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were prepared for use by conditioning with NaOH, water and the appropriate buffer. All columns were 57 cm (50 cm, injector–detector) $\times$ 75 μm I.D., unless otherwise specified. Mesityl oxide was used as a neutral marker to monitor the electroosmotic flow. The LIF system was laboratory built as has been described previously [22]. Excitation was provided by a KrF pulsed excimer laser operating at 248 nm (Model GX-500), (Potomac Photonics, Lanham, MD, USA). All chemicals were obtained from Aldrich (Milwaukee, WI, USA).

RESULTS AND DISCUSSION

Effect of CTAB on electroosmotic flow

Cationic surfactants such as CTAB adsorb on the capillary wall surface by dynamic electrostatic interactions between the positively charged tertiary ammonium ion and the negatively charged $Si-O^-$ group [23–25]. A bilayer of CTAB molecules is formed at the capillary wall with a positive charge directed towards the center of the capillary. At a given applied field strength and buffer system, the magnitude and direction of electroosmotic flow (EOF) is controlled solely by the capillary wall surface charge density [26]. The amount of negative charge density on fused-silica surfaces is determined by the degree of ionization of Si–OH groups present on the surface. The ionization constant of the Si–OH group on fused-silica surfaces is not precisely determined, but it is estimated to be about $1 \cdot 10^{-3}$ [26]. As negative charge is built up at the surface with increasing pH, electroosmotic flow increases. This is clearly illustrated in Fig. 1 (curve ■), which shows the effect of pH on EOF with a bare fused-silica capillary in the pH range 3–8. The addition of CTAB to the buffer system, which dynamically adsorbs to the surface, initially slows down the EOF and eventually reverses its direction [23–25,27–29]. The magnitude of change in EOF and the point at which it changes

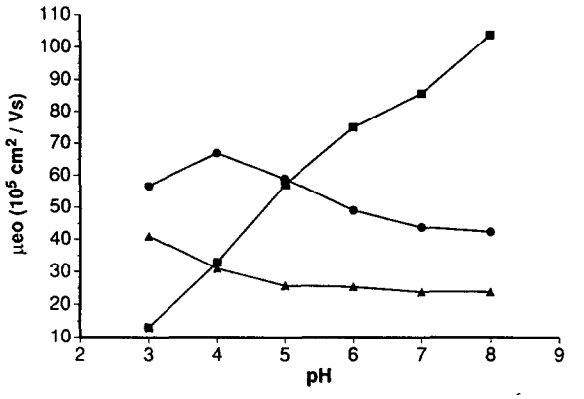

Fig. 1. Electroosmotic mobility (μ_{eo}) *versus* pH. Buffer: 15 m*M* phosphate; neutral marker: mesityl oxide. (■) Column: 50 cm × 75 μm I.D. untreated fused silica; applied voltage: +25 kV; μ_{eo}: positive; (●) Column as for ■; buffer additive $2.5 \cdot 10^{-4}$ *M* CTAB; applied voltage: −25 kV; μ_{eo}: negative; (▲) Column: 50 cm × 50 μm I.D., C_8; buffer additive: $2.5 \cdot 10^{-4}$ *M* CTAB; applied voltage: −25 kV; μ_{eo}: negative.

direction depends on the nature of the capillary surface, pH and the concentration of CTAB in the buffer system [28]. Tsuda [25] and Huang *et al.* [27] both reported reversal of EOF at a surfactant concentration of approximately $3.5 \cdot 10^{-4}$ *M* with bare capillaries, and Pfeffer and Yeung [28] reported a much lower surfactant concentration (*ca.* 1 μ*M* CTAB) for the reversal of flow with a fused-silica column coated with hydrophobic cross-linked polymer PS-264.

In this work we measured the EOF with bare and C_8-coated fused-silica capillaries as a function of pH using buffers modified with CTAB. Fig. 1 (curve ●) shows the dependence of EOF on pH with a bare fused-silica column at a CTAB concentration of $2.5 \cdot 10^{-4}$ *M* and Fig. 1 (curve ▲) shows a corresponding plot with a C_8-coated column. In both situations the direction of EOF is reversed towards the positive electrode. At any given CTAB concentration, EOF with the bare silica column increases as the pH is increased from pH 3, plateaus then decrease with further increase in pH. The pH at which EOF is maximum varies slightly depending on the concentration of CTAB. Assuming that EOF is directly proportional to surface charge density, it could only be concluded that $Si-O^-$ is a more favorable CTAB adsorption site compared to Si–OH. As pH is increased from 3 to 4, the underlying silica surface turns more negative with increased ionization of Si–OH groups and more CTAB is bound to the surface, resulting in higher net positive charge density. As pH is further increased, more Si–OH groups are ionized with no concomitant increase in CTAB binding because of surface saturation. As a result, the net surface charge density turns more and more negative and EOF decreases with increasing pH. The results of the EOF *vs.* pH experiment with the C_8 column (Fig. 1, curve ▲) show that EOF is slower at any given pH compared to the bare silica column. This is presumably because the hydrophobic, C_8-coated surface does not bind the tertiary ammonium ion as strongly as does the negatively charged silica surface. However, even the lowest concentration of CTAB (1 μ*M*) [28] is sufficient for flow reversal because with C_8 bonding most of the surface hydroxy groups are effectively eliminated, especially at low pH, and any slight CTAB binding will develop a positive surface charge density.

Strategies for separating pyridinecarboxylic acid isomers

A number of capillary electrophoresis modes of operation (normal polarity or cathodal, reversed polarity or anodal and MECC) were investigated to arrive at the optimum conditions for the resolution of PC acid isomers. First the normal polarity (cathodal) mode, where the injector end is positive and the EOF is towards the negative electrode, was explored. Fig. 2 shows the effect of pH on the migration relative to mesityl oxide (MO) of *o*-PC (picolinic) acid, *m*-PC (nicotinic) acid and *p*-PC (isonicotinic) acid. The p*K* values of these compounds at 25°C are as follows [30]: picolinic acid, $pK_1 = 1.06$ and $pK_2 = 5.37$; nicotinic acid, $pK_1 = 2.07$ and $pK_2 = 4.73$ and isonicotinic acid, $pK_1 = 1.70$ and $pK_2 = 4.89$. Strictly speaking, these compounds are not zwitterionic even though they possess a positive charge on the pyridine nitrogen at pH < 3, and turn negative at pH > 3 due to the ionization of the carboxylic acid. This dependence of charge on pH is reflected in the trend shown in Fig. 2. The acids migrate faster than MO at pH < 3; the slowest migrating acid being picolinic acid, the

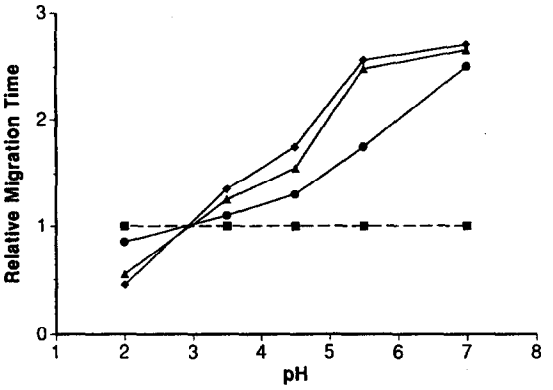

Fig. 2. The effect of pH on the migration order and separation of PC acid isomers. Column: 50 cm × 75 μm I.D. fused silica. Applied voltage: 25 kV; buffer: 25 m*M* phosphate at pH 2.0–4 and pH 5.5–7; 50 m*M* acetate at pH 4.5. ■ = MO; ● = *o*-PC (picolinic) acid; ◆ = *m*-PC (nicotinic) acid; ▲ = *p*-PC (isonicotinic) acid.

isomer that carries the smallest positive charge, at any given pH in this range of pH. As the pH is increased above pH 3 the order of elution is reversed and picolinic acid now carries the smallest negative charge in the pH range and is eluted first. The isomers are best separated at pH ≈ 4.5; however, the peaks exhibit excessive peak asymmetry, the severity of which depends on the pH. Similar problems of poor peak shape that were attributed to a property of the compounds rather than instrumental deficiencies were encountered by Roberts *et al.* [15] during the development of HPLC methods in the analysis of similar compounds.

Fig. 3 is an electropherogram of the PC isomers and nicotinamide under optimum conditions with respect to separation and peak shape. Fig. 4 shows the separation of hydroxy-substituted PC acids, namely, 3-hydroxypicolinic acid (3HPA), 2-hydroxynicotinic acid (2HNA) and 6-hydroxynicotinic acid (6HNA), under two sets of experimental conditions. Although the three isomers could be separated as shown at pH 5.5, peak shape problems are severe and highly dependent on pH. Peak shapes obtained with new columns are reasonable (Figs. 3 and 4); however, peak shape quickly deteriorates with repeated injections. Column reconditioning with NaOH and water did not seem to restore the original conditions. The addition of organic

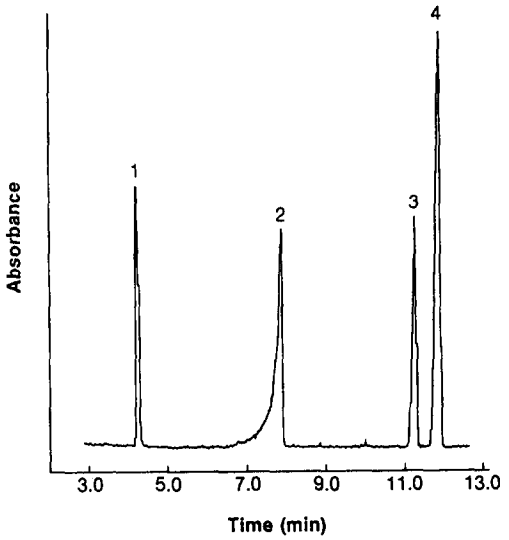

Fig. 3. Separation of PC acid derivatives. Buffer: 50 m*M* acetate, pH 5.5; applied voltage: 25 kV; detection: UV 254 nm; instrument: Beckman Model P/ACE System 2000. Column 50 cm × 75 μm I.D. fused silica. Peaks: 1 = nicotinamide; 2 = picolinic acid; 3 = isonicotinic acid; 4 = nicotinic acid.

solvents, salts and urea to the buffer system was attempted (unpublished results) but did not result in any improvement in peak shape, especially for the most troublesome solutes, namely picolinic acid (Fig. 3) and 3-hydroxypicolinic acid (Fig. 4).

A dramatic improvement in peak shape was obtained by the addition of CTAB to the run-

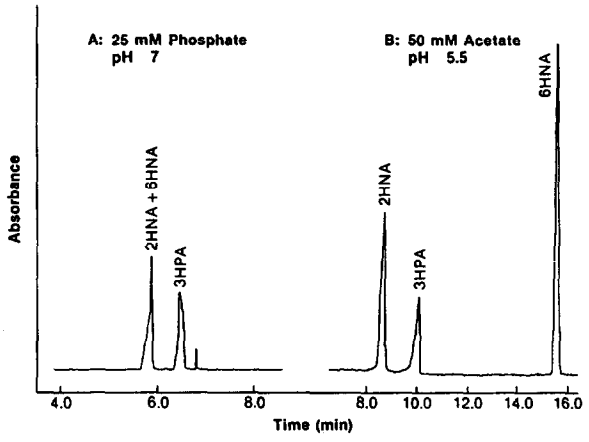

Fig. 4. Separation of hydroxypyridinecarboxylic acid isomers. Applied voltage: 25 kV; detection: UV 280 nm. Other conditions as in Fig. 3.

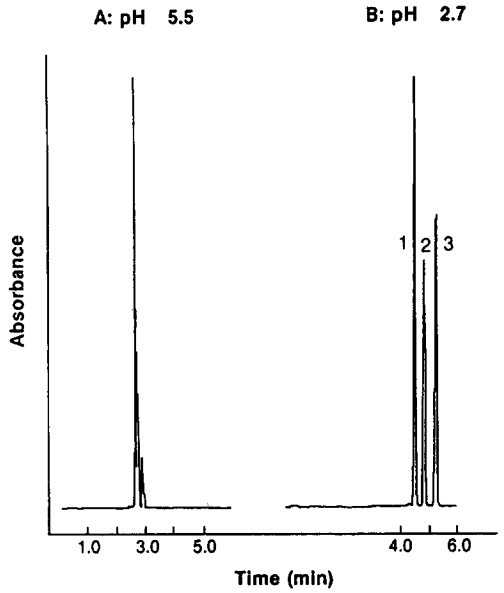

Fig. 5. Separation of PC acid isomers using a CTAB-modified buffer. Buffer: 10 mM phosphate + 30 mM CTAB; applied voltage: 20 kV. Other conditions as in Fig. 3. Peaks: 1 = picolinic acid; 2 = isonicotinic acid; 3 = nicotinic acid.

ning buffer as shown in Figs. 5 and 6. A range of pH and CTAB concentrations was tested to arrive at the best conditions for the separation of these compounds. It was determined that CTAB

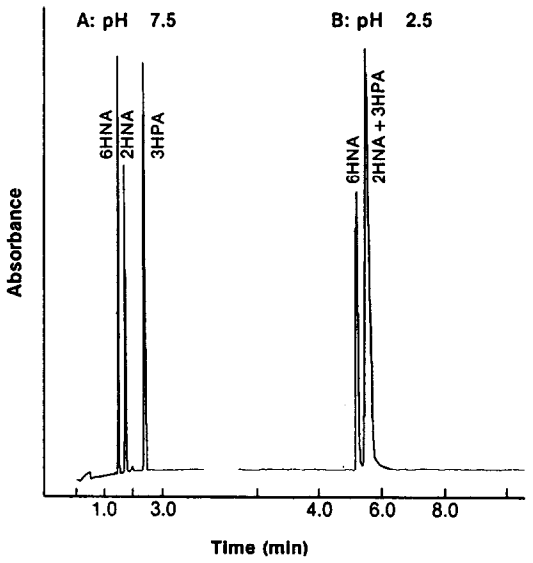

Fig. 6. Separation of hydroxypyridinecarboxylic acid isomers using a CTAB-modified buffer. Experimental conditions as in Fig. 5.

concentrations up to the critical micelle concentration (CMC) of about $1 \cdot 10^{-3}$ M [28] result in shorter analysis time but only slight improvement in peak shape. Even though the use of high CTAB concentrations (up to 30 mM) resulted in high current values (*ca.* 100 μA with 10 mM phosphate buffer, 50 cm × 75 μm I.D. column at 20 kV), it was necessary as it resulted in dramatically improved peak shape at all pH values. MECC is best suited for the separation of neutral compounds, but it has also been shown to improve the separation of some ionic compounds [19]. Analytes of opposite charge to the micelles interact rather strongly with the micelles through ionic forces, while analytes of similar charge interact weakly due to electrostatic repulsion. The separation of PC isomers at pH 2.7 (Fig. 5B), where both the analytes and micelles are positively charged, might be slightly affected by interactions with the micelles, but the order of migration is largely dependent on analyte pK_1 values. On the other hand, comparison of Fig. 4A to Fig. 6A shows that the separation of hydroxypyridinecarboxylic acid isomers in neutral and basic media where the analytes are negatively charged, is greatly influenced by interaction with the micelles. The MECC separation of PC acid isomers also depends on the buffer pH. Fig. 5 shows that complete resolution of the three isomers is obtained at pH 2.7, while higher-pH buffers will result in overlapping peaks. In contrast, Fig. 6 shows that the hydroxy-substituted isomers are better resolved at slightly basic pH values.

The concentration of the individual components of the standard mixtures used to generate Figs. 5 and 6 was about 1–10 μg/ml. The limit of detection obtained under the conditions specified in the Experimental section and figure captions without any attempt at sample stacking was in the range of $1 \cdot 10^{-5}$ to $1 \cdot 10^{-6}$ M. In an attempt to improve the limit of detection, the use of LIF was also investigated. PC acids absorb UV light but their native fluorescence is not particularly strong. Mawatari *et al.* [1,4] previously reported the detection of isonicotinic acid using HPLC coupled with postcolumn photochemical reaction and fluorescence detection. In their study, hydrogen peroxide present in the mobile phase

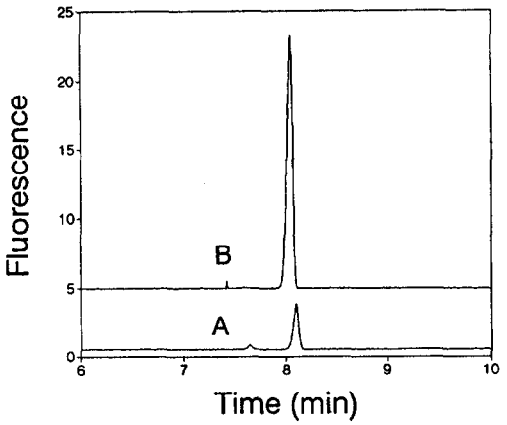

Fig. 7. Effect of H_2O_2 on the fluorescence signal of isonicotinic acid. (A) Buffer: 12 m*M* phosphate, pH 7.7; (B) buffer: 12 m*M* phosphate with 0.3% H_2O_2, pH 7.7; applied voltage: 15 kV; injection: gravity, 10 cm for 15–20 s; isonicotinic acid concentration: 1 μg/ml.

reacted with the analyte to give maximum fluorescence upon irradiation with UV light for about 2.5 min. It was felt that the application of this procedure to capillary electrophoresis, using a UV laser, may have distinct advantages such as the elimination of the postcolumn reactor, instantaneous photochemical reaction, and simultaneous on-column LIF detection. Preliminary results are shown in Fig. 7 which indicates a substantial enhancement in fluorescence signal. The results are encouraging and the subject will be further explored in future communications from this laboratory.

CONCLUSIONS

The CZE separation of pyridinecarboxylic acid isomers presented a challenge because of peak shape problems that seem to be a property of the compounds rather than instrumental deficiencies. The separation and peak shape of these compounds is highly dependent on pH. The addition of CTAB to the buffer provided several advantages including: (1) highly improved peak shape; (2) faster analysis time; (3) better control of EOF and better reproducibility of migration times.

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