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Separation of seven tricyclic antidepressants using capillary electrophoresis

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ABSTRACT

Seven tricyclic antidepressants, protriptyline, desipramine, nortriptyline, nordoxepin, imipramine, amitriptyline and doxepin, were separated using capillary electrophoresis. Because the tricyclic antidepressants were similar in structure and mass, careful manipulation of the electroosmotic flow and the electrophoretic mobilities was required for an optimal separation. In the systematic approach we have developed, the differential electrophoretic mobilities were first maximized by adjusting pH. Next, increasing the buffer concentration improved the separation at the expense of migration times by reducing the electroosmotic flow. Full resolution was achieved by the addition of methanol to the buffer which decreased both the electroosmotic flow and the electrophoretic mobilities of the samples.

INTRODUCTION

Capillary electrophoresis (CE) is a separation technique that has become popular in many areas. Ease of operation and rapid analysis time are two attractive features of CE. Selectivity is based on differences in electrophoretic mobilities between samples. Applications range from the separation of metal cations [1,2] to the resolution of proteins [3–7].

Some of the more elegant work in CE involves the separation of species that are nearly identical, such as isomeric compounds or isotopically substituted species. To effect full resolution, the electroosmotic flow or the electrophoretic mobilities are carefully adjusted. Fujiwara and Honda [8] were able to separate positional isomers of substituted benzoic acids after adding methanol to their buffer to decrease the electroosmotic flow. Grossman *et al.* [9] were able to separate peptides containing seven amino acids that differed in the order of linkage by working at a low pH. Terabe *et al.* [10] were able to separate oxygen-isotopic benzoic acids by carefully adjusting the pH of the buffer to a value close to the pK_a of benzoic acid; hydroxypropyl cellulose was added to the buffer to suppress the electroosmotic flow. Bushey and Jorgenson [11,12] were able to separate dansylated methylamine and dansylated [$^2H_3$]methylamine in a buffer containing micelles by adding 20% methanol to change the capacity factor as well as to reduce the electroosmotic flow.

While there are many ways to improve separations, we are interested in

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providing a systematic approach to separating compounds that are closely related in mass and structure. In the present study we have investigated the effect of buffer pH, buffer concentration and the addition of organic modifiers on the separation of seven tricyclic amines. We are particularly interested in how the electroosmotic flow and the electrophoretic mobilities are affected. We first optimized the differential mobilities by adjusting the pH to a value close to the pK_a values of the seven amines. Increasing the buffer concentration decreased the electroosmotic flow while leaving the electrophoretic mobilities unaltered. Adding methanol to the buffer had the most significant effect on the resolution, decreasing both the electroosmotic flow and the electrophoretic mobilities. The increase in viscosity due to the methanol could not quantitatively account for the changes observed. It appeared that the changes in the dielectric constant affected the degree of dissociation of the protonated samples making them appear more neutral and decreasing the electrophoretic mobility.

EXPERIMENTAL

Instrumentation

The research CE instrument used was similar to those described previously [7,13,14]. A fused-silica capillary column, 1 m × 75 μm I.D. was suspended between two buffer reservoirs. A 30-kV Glassman (Whitehouse Station, NJ, USA) high-voltage power supply was used to apply voltage across the platinum electrodes resting in the buffer reservoirs. Samples were detected on-column using a modified Jasco UV detector set to 210 nm with an 0.05-s time constant. A detector window was fashioned in the column 75 cm from the injection end by burning off a section of the polyimide coating on the capillary column. Injections were done in a hydrodynamic fashion by dipping the injection end of the column into the sample reservoir for 5 s while it rested 3 in. above the detection end reservoir. All separations were run at 30 kV for maximum resolution and minimum migration times. The current did not exceed 20 μA.

The column was etched with 0.1 *M* NaOH for 30 min at the start of each day. Water was used to rinse the column and then the buffer was introduced and allowed to equilibrate with the silica capillary. The column was flushed with buffer after each run.

Chemicals

The buffer used was 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) with a pK_a of 9.6 and was purchased from Sigma (St. Louis, MO, USA). NaOH obtained from J. T. Baker (Phillipsburg, NJ, USA) was used to adjust the buffer pH to values between 9.0 and 10.0. For the studies in which the pH was varied, a 50-m*M* CAPSO buffer with variable concentrations of NaOH was used. For the studies in which the buffer concentrations were varied, both the CAPSO and the NaOH concentrations were changed while keeping the pH constant at 9.55. For the studies where methanol was added, 0.5933 g of CAPSO were dissolved in 50 ml of the appropriate water–methanol mixture and then the pH was adjusted to 9.55 with NaOH.

The samples used in our study were protriptyline, desipramine, nortriptyline, nordoxepin, imipramine, amitriptyline and doxepin obtained from Alltech (Deerfield, IL, USA). Samples were prepared individually in a 50% pH 4.4 phosphate buffer and 50% methanol solution and then mixed together so that the final concentration was

0.14 m*M*. The methanol in the sample solution was used as a neutral marker to monitor the electroosmotic flow with each run.

RESULTS AND DISCUSSION

We have chosen a sample of seven tricyclic amines; the detection and quantitation of tricyclic amines is important because of the wide use of these compounds for the treatment of depression [15]. The structures of the compounds are shown in Fig. 1. Protriptyline (mol.wt. 263.37), desipramine (mol.wt. 266.37), nortriptyline (mol.wt. 263.37) and nordoxepin (ml.wt. 265.37) are all secondary amines that differ from each other only by the substitution of nitrogen or oxygen for a carbon in the ring. Protriptyline and nortriptyline are positional isomers. The pK_a values of these compounds are close to 10.7 [16]. The other three, imipramine (mol.wt. 280.40), amitriptyline (mol.wt. 277.39) and doxepin (mol.wt. 279.37) are tertiary amines that differ from each other only in the substitution of nitrogen or oxygen for a carbon in the ring. The pK_a values of these amines are close to 9.8 [16].

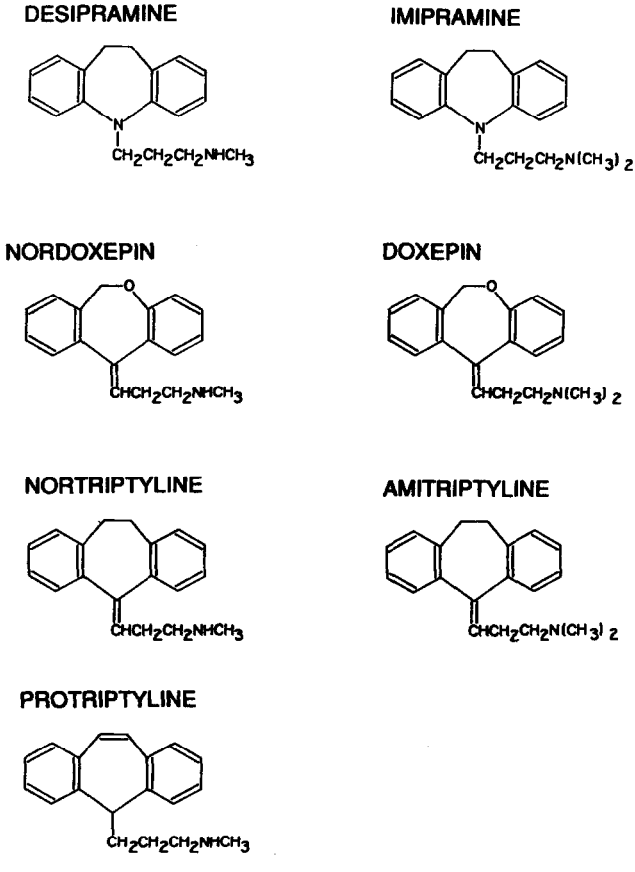

Fig. 1. Structures of the seven tricyclic amines.

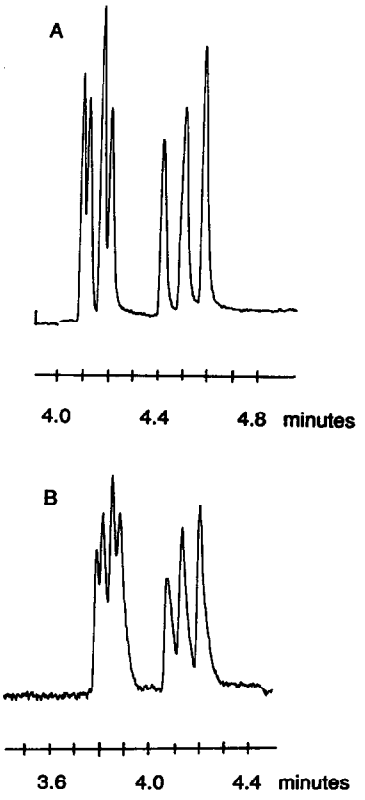

Fig. 2. (A) Separation of the seven tricyclic amines at pH 9.55, 50 m*M* CAPSO–12.5 m*M* NaOH; the order of elution is: protriptyline, desipramine, nortriptyline, nordoxepin, imipramine, amitriptyline and doxepin; (B) separation of the seven tricyclic amines at pH 9.55, 10 m*M* CAPSO–2.7 m*M* NaOH. The order of elution is the same as that for (A); the period of injection has been reduced to 3 s here.

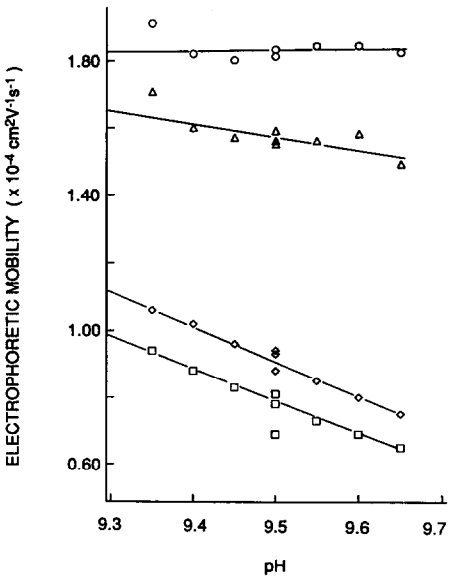

Fig. 3. Electrophoretic mobilities of protriptyline (○), nordoxepin (△), amitriptyline (◇) and doxepin (□) plotted as a function of pH for a 50 m*M* CAPSO buffer. Lines are drawn to emphasize the differences in μ_{ep} between the different compounds.

Effect of pH

At pH 6, all the amines were fully protonated; an electrophoretogram run at this pH resulted in only two peaks, each corresponding to the two different mass groupings. At pH 9.2, the three tertiary amines were baseline resolved, while the four secondary amines eluted as three peaks. At pH 9.4, the secondary amines eluted as four peaks although they were not completely separated. The resolution improved up to pH 9.55 (Fig. 2A); at pH 9.6, the amitriptyline and doxepin peaks began merging together.

Changing the pH from 9.0 to 9.65 had only a negligible effect on the electroosmotic flow (μ_{eo}); however, the change in the elctrophoretic mobilities (μ_{ep}) was pronounced. A plot of the electrophoretic mobilities of protriptyline, nordoxepin, amitriptyline and doxepin *vs.* pH is shown in Fig. 3. With the exception of the electrophoretic mobility of protriptyline (which eluted first), μ_{ep} decreased as the pH was raised.

According to the Stokes–Einstein relationship [17]

$$\mu_{ep} = \frac{q}{6\pi\eta a} \tag{1}$$

where q is the charge of a particular solute (positively charged in this case), a is the hydrodynamic radius of the solute and η is the viscosity of the solution through which the solute moves. If one assumes that the viscosity and the effective radius did not change over the pH region studied, then the observed change in μ_{ep} can be attributed to a change in the charge of the sample. A functional form for the change in the electrophoretic mobility with the degree of protonation of a species is given below [10]

$$\mu_{ep} = f\mu_{ep}(0) \tag{2}$$

where f is the fraction of a compound in its protonated form and $\mu_{ep}(0)$ is the mobility of the solute in its completely protonated form. The largest influence of the pH on the fraction protonated occurs when the pH is close to the pK_a of the solute.

Because the electrophoretic mobilities of all the samples except protriptyline decreased as the pH was raised from 9.0 to 9.6, the differential electrophoretic mobilities, $\Delta\mu_{ep}$, also changed. As the degree of protonation was altered, the differences in electrophoretic mobilities between two compounds became more or less pronounced and the resolution was affected. Resolution, R_s, is directly proportional to the differential electrophoretic mobilities according to the following equation [7,10]

$$R_s = \frac{1}{4}\left(\frac{V}{2D}\right)^{1/2}\left(\frac{l}{L}\right)^{1/2}\frac{\Delta\mu_{ep}}{[\mu_{ep}(\text{ave}) + \mu_{eo}]^{1/2}} \tag{3}$$

where V is the applied voltage, l is the length of the column to the detector window, L is the length of the column, D is the diffusion coefficient, and μ_{ep}(ave) is the average electrophoretic mobility of the two components being separated. For the secondary amines, protriptyline and nordoxepin, $\Delta\mu_{ep}$ increased at higher pH values and an improvement in resolution was observed. For the tertiary amines, amitriptyline and doxepin, $\Delta\mu_{ep}$ became somewhat smaller at pH values >9.5 leading to poorer resolution of these two samples. The change in $\Delta\mu_{ep}$ with pH appeared to provide a window of optimal separation.

According to Terabe *et al.* [10], the optimum pH value for a CE separation lies below the pK_a values of the species being separated. Terabe *et al.* postulated that the optimum pH of a separation of compounds of comparable pK_a values was equal to pK_a − log 2. Assuming that the pK_a of the tertiary amines was close to 9.8, the optimal separation should have been at pH 9.5. Experimentally, pH 9.55 was found to give the best resolution of the tertiary amines which is in fairly good agreement with the theory of Terabe *et al.*

Other studies in the literature on the electrophoretic mobilities measured in a CE system as a function of pH show some dependence of μ_{ep} on pH if the pH is close to the pK_a or pI of the species being separated [18–20]. Overall, our results are consistent with the idea that the separation of similar compounds can be influenced by charge differentiation induced by an adjustment of the pH near the pK_a values of the compounds of interest.

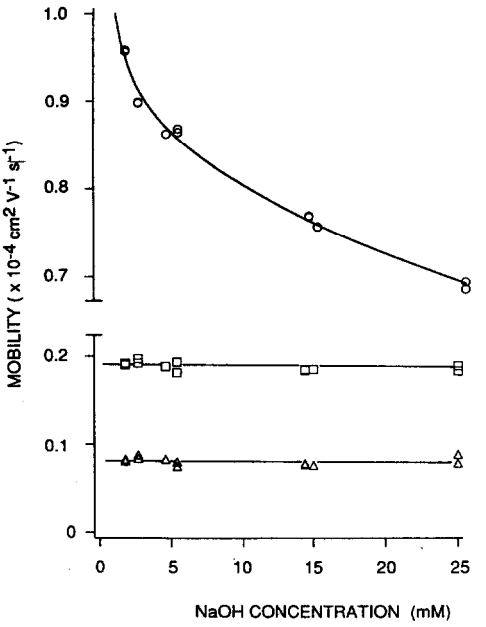

Fig. 4. Electroosmotic flow (□) and electrophoretic mobilities of protriptyline (○) and doxepin (△) as a function of NaOH concentration, at pH 9.55. The solid line drawn through the data points for the electroosmotic mobility are the result of a fit to eqn. 8.

Effect of buffer concentration

The change in the resolution of the seven tricyclic amines with buffer concentration can be seen in Fig. 2. At 10 m*M* CAPSO–2.7 m*M* NaOH, the seven peaks were much less resolved than at 50 m*M* CAPSO–14 m*M* NaOH; the pH is equivalent in both systems. The electroosmotic flow decreased as the buffer concentration increased; a plot of μ_{eo} *vs.* NaOH concentration is shown in Fig. 4. Also shown in Fig. 4 are plots of the electrophoretic mobilities of protriptyline and doxepin as a function of buffer concentration. Unlike the electroosmotic flow, the electrophoretic mobilities were invariant over the concentration range studied. Since the differential mobilities were unchanged, the observed improvement in resolution with increasing buffer concentration must be due to the reduction in the electroosmotic flow (eqn. 3).

The decrease in the zeta potential (which is proportional to μ_{eo}) with increasing buffer concentration is well known in colloid chemistry [21] and it has also been observed in capillary electrophoresis [19,22–27] although an exact relationship between μ_{eo} and buffer concentration had not been formulated. Tsuda *et al.* [22] observed a decrease in the electroosmotic flow with an increase in phosphate buffer concentration and linked it to a decrease in the double-layer thickness which is inversely proportional to the square root of the buffer concentration. However, we were unable to fit their equation to our data.

We have recently derived a relationship between the electroosmotic mobility and

the concentration of monovalent cations (*conc*) in the buffer [28]. We started with the following expression for the electroosmotic mobility

$$\mu_{eo} = \frac{Qx}{\eta} \tag{4}$$

where Q is the charge per unit area at the interface between the capillary wall and the buffer and x is the thickness of the counter-ion layer. We have postulated that the counter-ion layer consists of two regimes: one being a compact layer, the thickness of which is fixed; the other a diffuse layer in which the ions are randomly oriented and which can be described by a Gouy–Chapman model. In such a case, the thickness of the compact layer is defined as d_0 and is not concentration dependent; while the thickness of the diffuse layer is equal to $1/K'(\text{conc})^{1/2}$, where K' is a constant with a value of $3.2 \cdot 10^9\ \text{m}^{-1}\ M^{-1/2}$ (for a monovalent, dilute aqueous buffer at 25°C). The thickness of the counter-ion layer can then be written as:

$$x = d_0 + \frac{1}{K'\sqrt{(conc)}} \tag{5}$$

The charge per unit area at the interface, Q, is also dependent on the buffer concentration. As the buffer concentration is increased, more cations are adsorbed onto the silica surface, leading to a decrease in the charge at the interface. The equilibrium constant K_{wall}, for this process can be written as:

$$K_{\text{wall}} = \frac{[\text{SiO}^-\text{M}^+]}{[\text{M}^+][\text{SiO}^-]} \tag{6}$$

where SiO^- is simply Q, M^+ is a monovalent cation, and SiO^-M^+ is a silanol group with an adsorbed cation. Q can then be written in the following form [28]

$$Q = \frac{Q_0}{1 + [K_{\text{wall}}(conc)]} \tag{7}$$

where Q_0 is the sum of $[\text{SiO}^-]$ and $[\text{SiO}^-\text{M}^+]$ and corresponds to the concentration of ionized groups on the capillary surface. Substituting the above expressions for Q and x into eqn. 4, one obtains an equation for the electroosmotic mobility in terms of the buffer concentration

$$\mu_{eo} = \left(\frac{Q_0}{\eta}\right)\frac{d_0 + \dfrac{1}{K'\sqrt{(conc)}}}{1 + [K_{\text{wall}}(conc)]} \tag{8}$$

Our data was fit to the above equation and the resulting curve was plotted in Fig. 4. Values of the above parameters were found to be: $Q_0 = 1.3 \cdot 10^{16}$ charged sites/m^2; $d_0 = 3.8 \cdot 10^{-8}$ m; and $K_{\text{wall}} = 0.0093\ \text{m}M^{-1}$. In our model, the decrease in μ_{eo} with

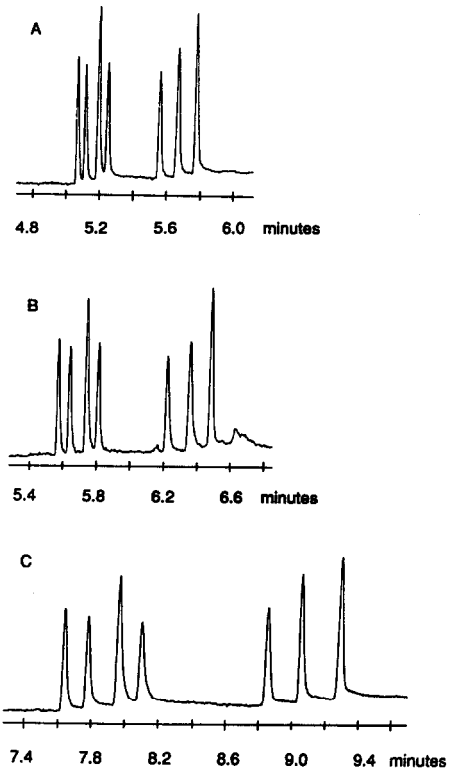

Fig. 5. Separation of seven tricyclic amines as a function of methanol concentration. (A) 4.0% (w/w) methanol; (B) 7.9% (w/w) methanol; (C) 15.8% (w/w) methanol. The order of elution is the same as that for Fig. 2A. Buffer pH 9.55; buffer concentration given in Table I.

increasing buffer concentration is linked to both a decrease in counter-ion layer thickness as well as an increase in the coverage of sites on the silica surface which reduces the charge per unit area at the interface between the capillary wall and the buffer.

Addition of methanol to the buffer

The addition of methanol to the buffer greatly improved the resolution of the seven tricyclic amines as seen in Fig. 5. At 4.0% methanol, only nortriptyline and nordoxepin were not baseline resolved; at 7.9% all peaks were baseline resolved. The resolution was further improved at 15.8% and on up to 23.7% (not shown). There was no change in the order of elution.

One factor in the improved resolution (eqn. 3) was the decrease in the electroosmotic flow (Table I). The decrease in μ_{eo} with the addition of methanol has been noted by others [8,12,27] and attributed to changes in the zeta potential, as well as changes in the viscosity and the dielectric constant of the buffer. Viscosity alone was not responsible for the reduction in μ_{eo}; if it were, the product of μ_{eo} and viscosity would be a constant according to eqn. 4. Values of the product are tabulated in Table I and are seen to decrease with increasing methanol concentration. Another factor

TABLE I

ELECTROOSMOTIC FLOW, μ_{eo}, VISCOSITY, η, AND DIELECTRIC CONSTANT, ε, FOR VARIOUS CONCENTRATIONS OF WATER–METHANOL MIXTURES

Methanol (%, w/w)	[NaOH] (mM)	μ_{eo} ($cm^2\ V^{-1}\ s^{-1}$)	η^a (cP)	ε^b	$\mu_{eo}\eta$ ($cP\ cm^2\ V^{-1}\ s^{-1}$)
0.0	12.5	$7.95 \cdot 10^{-4}$	1.00	80	$7.96 \cdot 10^{-4}$
2.0	13.0	$7.23 \cdot 10^{-4}$	1.07	–	$7.72 \cdot 10^{-4}$
4.0	13.8	$6.47 \cdot 10^{-4}$	1.13	–	$7.30 \cdot 10^{-4}$
5.9	15.5	$6.15 \cdot 10^{-4}$	1.19	–	$7.34 \cdot 10^{-4}$
7.9	16.2	$5.66 \cdot 10^{-4}$	1.26	76	$7.14 \cdot 10^{-4}$
15.8	18.0	$4.16 \cdot 10^{-4}$	1.50	–	$6.23 \cdot 10^{-4}$
23.7	19.8	$3.22 \cdot 10^{-4}$	1.69	71	$5.45 \cdot 10^{-4}$

[a] From. ref. 16.
[b] From ref. 30.

contributing to the reduction in the electroosmotic flow includes the increased NaOH concentrations needed to maintain the buffer at pH 9.55 as the percentage of methanol is increased.

In order to explore more fully the factors controlling the decrease in the electroosmotic flow with increasing methanol concentration, we have measured μ_{eo} as a function of NaOH concentration at a fixed percentage of methanol and fitted the results to eqn. 8. Experiments were conducted at pH 9.55 in CAPSO–NaOH buffers of 7.9% methanol; the concentration of NaOH was varied from 4.5 to 45 mM. A good fit of eqn. 8 with data was obtained with the following values: $K_{wall} = 0.011\ mM^{-1}$; $d_0 = 1.33 \cdot 10^{-8}$ m; and $Q_0 = 2.94 \cdot 10^{16}$ charged sites per square meter. Compared to the values derived without methanol in the buffer, it can be seen that the equilibrium constant between the sodium ions and the wall did not change. However, the value of d_0 decreased when methanol was present in the buffer. It is possible that the presence of methanol in the buffer aided in the solvation of the ions in the buffer and reduced the thickness of the compact layer next to the interface. The initial charge at the wall, Q_0, increased when methanol was present in the buffer; the presence of methanol may have minimized the repulsion between SiO^- groups on the capillary surface, thus allowing more of them to exist in a given area than if methanol were not present in the buffer. The reduction of d_0 as well as the increase in the viscosity and cation concentration are primarily responsible for the decrease in μ_{eo}. We have not found any evidence for changes in the equilibrium constant between the cations and the silica surface.

The electrophoretic mobilities also decreased as the buffer became more concentrated in methanol, leading to an improvement in resolution according to eqn. 3. Part of the decrease could be attributeed to an increase in viscosity as the percentage of methanol was increased. However, if viscosity were the only parameter controlling the electrophoretic mobility, then the product of the electrophoretic mobility and the viscosity would have been a constant at different methanol concentrations according to eqn. 1. We have found experimentally that this is not the case as shown in Fig. 6 for four of the tricyclic amines. For protriptyline which eluted first, the plot sloped downward only slightly. For nordoxepin which eluted fourth, the downward slope was more distinct. For imipramine and doxepin which eluted fifth and seventh, respective-

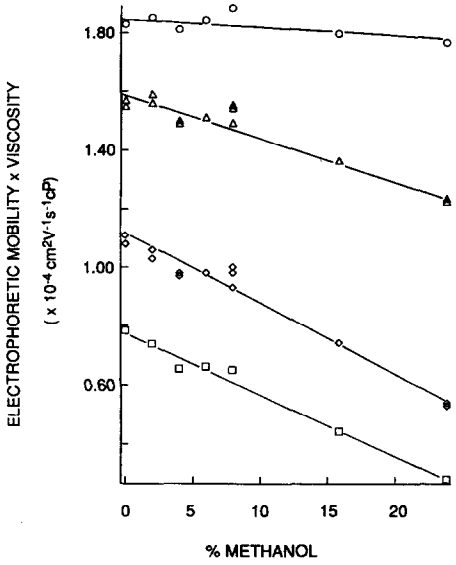

Fig. 6. Plots of the electrophoretic mobility times viscosity as a function of methanol concentration for protriptyline (○), nordoxepin (△), imipramine (◇) and doxepin (□) at pH 9.55. Lines are drawn to emphasize the differences between data sets.

ly, the downward slope was even more pronounced. Clearly, viscosity alone was not responsible for the reduction in the electrophoretic mobilities.

Another factor important in the reduction of the electrophoretic mobilities was the decrease in the degree of protonation as the percentage of methanol was increased. The dielectric constant (Table I) of water–methanol mixtures decreased as the percentage of methanol increased, and the reduction in dielectric constant would favor the more neutral side of the equilibrium between the protonated amines and their neutral bases.

$$RNH^+ + OH^- \rightleftharpoons RN: + H_2O \quad (9)$$

The equilibrium constant would then increase such that the fraction of the solute in the protonated form would decrease. Such a decrease in the concentration of the protonated amine would lead to the observed decrease in electrophoretic mobility according to eqn. 2.

Detection limits

Detection limits were obtained for all seven antidepressants. The detection limits are: 1.1 μg/ml for protriptyline; 1.0 μg/ml for desipramine, nortriptyline and nordoxepin; 0.9 μg/ml for imipramine; 0.8 μg/ml for amitriptyline; and 0.4 μg/ml for doxepin. Some of the differences in detection limits can be attributed to differences in the absorption spectra of the compounds. The detection limits in our CE apparatus are poorer than those in high-performance liquid chromatography (HPLC) which are on

the order of 2–10 ng/ml [29]. The difference in performance is in large part due to differences in the optical path. The inside diameter of a CW column is 75 μm as opposed to 4.6 mm for an HPLC column. Improvements in detection limits for a CE analysis of antidepressants may be achieved by further modifying the detectors specifically for narrow-bore capillary tubes.

CONCLUSIONS

We have shown that it is possible to separate a mixture of seven tricyclic amines that did not differ greatly in structure or molecular weight. In the systematic approach we have developed, the differential mobilities were maximized by adjusting the pH to a value slightly below the pK_a values of the samples being separated. Working at a high buffer concentration was beneficial due to the reduction in the electroosmotic flow. Further improvements in resolution were obtained upon the addition of methanol to the buffer which lead to a reduction in both the electroosmotic flow and the electrophoretic mobilities. The reduction in the electrophoretic mobilities appeared to arise from changes in the sample pK_a values in the methanol–water matrix.

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