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Electroosmotically driven electrochromatography of anions having similar electrophoretic mobilities by ion pairing

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ABSTRACT

Anions of similar electrophoretic mobility are separated using an electrochromatographic technique which involves partitioning the anions, with the aid of the ion-pairing agent, tetrabutylammonium (TBA) cation, onto the surface of a reversed-phase open-tubular capillary liquid chromatographic (OTCLC) column. The anions separate based on differences in retention rather than differences in electrophoretic mobility. Resolution is easily controlled by varying the concentration of TBA in the buffer. Complete separation of anions having similar mobilities is achieved with as little as 600 μM TBA. Mass transfer broadening is inherently smaller for this electroosmotically driven system than that for pressure-driven OTCLC. The efficiency achieved for such a separation in a 40-cm column (N = 140 000) is twice that for a similar chromatographic separation and nearly equal to that for a similar, but poorly resolved, capillary zone electrophoretic separation.

INTRODUCTION

Recently, Everaerts *et al.* [1] discussed the concept of electrophoresis *versus* electrochromatography. They argued that for capillary zone electrophoresis (CZE), a pure electrophoretic separation can only be accomplished in capillaries of 200 μm I.D. or greater. Separations in capillaries of smaller I.D., especially those with I.D. < 50 μm and having electroosmotic flow (EO) as the major mode of solute transport, tend to exhibit behavior that is a hybrid between "purely" electrophoretic and "purely" chromatographic [1]. Nevertheless, in a typical CZE separation of ionic solutes, the separation will be predominantly based on the differential migration of the solutes. Highly efficient separations can be achieved in capillaries without the need for wall adsorption, which is essential for the complementary technique, open-tubular capillary liquid chromatography (OTCLC). In fact, for certain solutes, adsorption on the wall is viewed as disastrous to efficiency and to be avoided if possible [2,3]. However, narrow capillaries (I.D. $<$ 200 μm) are desirable because they increase the surface area-to-volume ratio, which results in efficient heat dissipation.

In the typical CZE separation, the resolution between two solutes will be dependent on their electrophoretic mobility differences and also the EO flow. Increases in resolution are commonly achieved by changing the pH of the buffer, which can alter the degree of charge on the solutes, thereby altering their mobilities. At the same

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time, EO flow can be reduced, eliminated or reversed through buffer additives so that differences in mobility are better exhibited.

Achieving adequate resolution for ionic solutes whose mobilities are nearly identical and whose degrees of charge remain constant over a wide pH range, such as isomeric strong acids or bases, is an exceptionally difficult task. Increasing resolution based on varying pH or EO flow can be ineffective. In these instances, other methods to improve resolution must be adopted. These include the addition of an organic modifier to the buffer [4] and the use of electrokinetic chromatography [5,6].

Our approach to this problem is to take the mixed-mode behavior discussed earlier to an extreme by giving CZE separation a strong chromatographic component. Realizing that isomeric strong acids and bases are easily separated using reversed-phase ion-pair liquid chromatography, we decided to combine the high degree of analyte discrimination associated with the ion-pair technique with the ease and efficiency of a CZE separation. This is accomplished by replacing the CZE column with a reversed-phase OTCLC column of narrow I.D. (*ca.* 10 μm) and by including an ion-pairing agent in the buffer. Because narrow I.D. columns are used, and because the flow profile is flat (electroosmosis) rather than parabolic, the mass transfer contribution to zone broadening is minimized for partitioning solutes [7]. The ion-pairing agent is used to control the affinity of the anions for the stationary phase. We demonstrate the resulting technique, which can be called ion-pair electrochromatography, by separating a series of anions whose mobilities at the working pH are nearly identical.

EXPERIMENTAL

The buffer used throughout was 10 m*M* phosphate. A 0.1% hydroxypropylcellulose (HPC) (Aldrich, Milwaukee, WI, USA) solution was prepared from buffer and the final pH adjusted to 7.0. Ion-pairing solutions were prepared by diluting 40.0% (w/w) tetrabutylammonium hydroxide (TBA) (Aldrich) with the buffer and adjusting the final pH to 7.0. The anions 4-amino-1-naphthalenesulfonic acid (4A1N), 2-amino-1-naphthalenesulfonic acid (2A1N), 5-amino-2-naphthalenesulfonic acid (5A2N), 8-amino-2-naphthalenesulfonic acid (8A2N) (all from Aldrich) and 1-naphthol-4-sulfonic acid (1H4N) (Eastman Kodak, Rochester, NY, USA) were prepared in running buffer prior to their injection.

The fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were 50 cm long and nominally of 10 μm I.D. These capillaries were used as supplied or coated, and the coating later cross-linked, with either 0.9% (w/v) PS-264 (Petrarch Systems, Bristol PA, USA) or 10.0% (w/w) OV-17v (Alltech, Deerfield, IL, USA) as described in ref. 8 or 9, respectively. The polyimide coating was burned off at one tip of the capillary to prevent analytes from possibly adsorbing to it during an injection. It was also burned off at 40 cm from this tip to facilitate on-column detection.

The CZE–electrochromatography system is similar to one described previously [10], except that a stationary phase is used in some instances. The electric field is supplied by either a positive or negative high-voltage supply (0–30 kV) (Glassman High Voltage, Whitehouse Station, NJ, USA; Model NJ30P0400-11 or MJ30N0400-11).

Chromatography was performed by disconnecting the CZE column from the

high-voltage isolation unit and attaching it to a solvent reservoir (Valco Instruments, Houston, TX, USA; Part No. ZU4L) which had a head pressure supplied by a regulated high-pressure helium tank.

Sample introduction for all three types of separations was accomplished by using hydrodynamic injection. The chromatographic solvent velocity and electroosmotic flow velocity were calculated from the baseline disturbance that resulted from the injection of running buffer containing *ca.* 10% of acetonitrile. Detection of the anions in all instances was accomplished by using a laser-based fluorescence detector described previously [7].

All data were either collected on a strip-chart recorder or subjected to analogue to digital conversion (Data Translation, Marlborough, MA, USA; Model DT2827, 5–10 Hz) and later stored on a personal computer (IBM PC-AT).

RESULTS AND DISCUSSION

When an anion in a buffer-filled capillary is subjected to an electric field, E, the anion will migrate with a velocity, v, described by

$$v = v_{ep} + v_{eo} = (\mu_{ep} + \mu_{eo})E \tag{1}$$

where v_{ep}, v_{eo}, μ_{ep} and μ_{eo} represent the electrophoretic velocity component, the electroosmotic velocity component, the electrophoretic mobility of the anion and the coefficient for electroosmotic flow, respectively. When two anions, 1 and 2, are subjected to these conditions, they will separate with a resolution, R, defined by

$$R = \frac{1}{4\sqrt{2}}(\mu_{ep_1} - \mu_{ep_2})\left[\frac{EL}{D(\bar{\mu}_{ep} - \mu_{eo})}\right] \tag{2}$$

where $\bar{\mu}_{ep}$ is their average mobility, D is their diffusion coefficient and L is the length of the column [11].

In a typical CZE separation of anions, μ_{ep} and μ_{eo} are of opposite sign; therefore, by reducing μ_{eo} in eqn. 2, the resolution between the two anions will be increased. In practice, this can be accomplished through the use of certain buffer additives, such as HPC. Fig. 1A represents an attempted separation of two anions using a buffer containing HPC. Although the EO flow has been nearly eliminated, the resolution between the two anions is still close to zero.

If these two anions were subjected to a separation using capillaries similar to those used in OTCLC, two results can be expected. First, owing to the stationary phase shielding the silanol groups, the EO flow will be drastically reduced; therefore, migration of the anions will be based on their mobility alone ($v \approx v_{ep}$). Second, if the anions have enough hydrophobic character, there is the possibility of the solutes partitioning onto the stationary phase. In such a case, an anion will travel with a velocity, v', which is related to v by

$$v' = \frac{v}{(1 + k)} \tag{3}$$

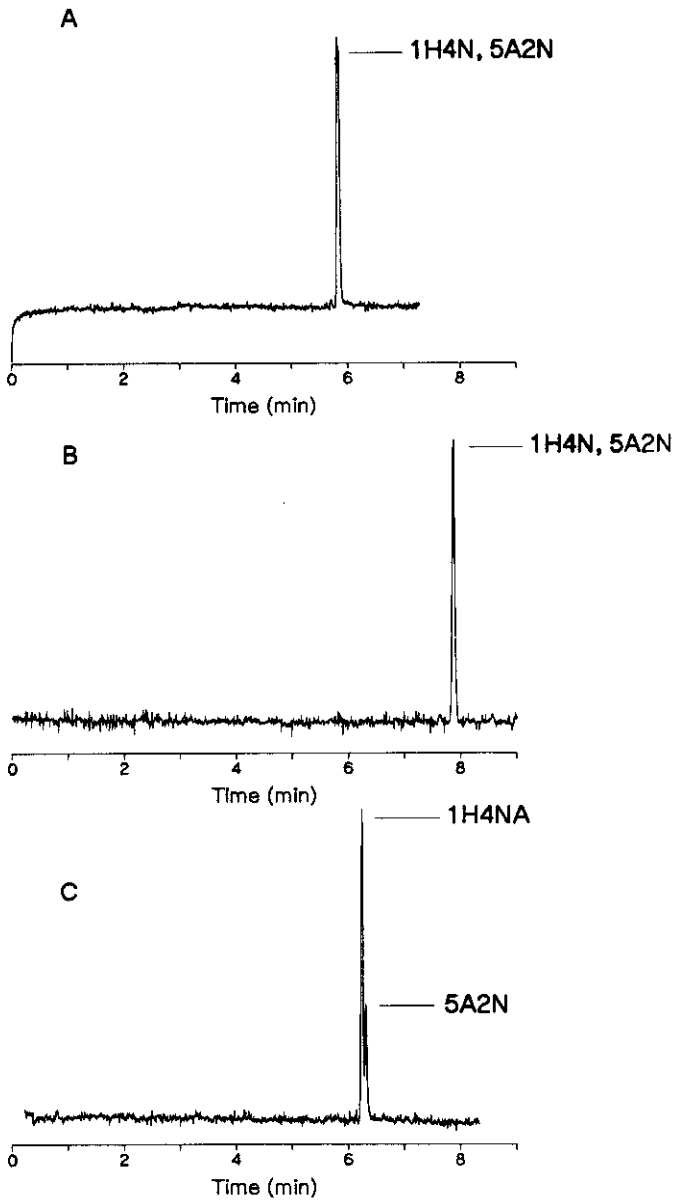

Fig. 1. (A) Elution profile of a CZE separation with reduced EO flow for 5A2N and 1H4N. Buffer conditions: 10 m*M* phosphate, 0.1% HPC, pH 7.0. The separation column is a 50 cm × 10 μm I.D. uncoated capillary having a 40 cm separation distance. Separation voltage, −21 kV. (B) Elution profile for an electrochromatographic separation of the same two anions. Buffer: 10 m*M* phosphate, pH 7.0. The column is a 50 cm × 10 μm I.D. capillary coated with PS-264 with a separating distance of 40 cm. Separation voltage, −21 kV. (C) Electrochromatographic elution profile for the same two anions using a column coated with OV-17v. Buffer, column dimensions and separation voltage are as in (B).

where k is the capacity factor of the anion. In the situation where two anions have similar mobilities but different capacity factors, they will elute with a velocity difference, $\Delta v'$, defined by

$$\Delta v' = \frac{v(k_2 - k_1)}{(1 + k_2)(1 + k_1)} \tag{4}$$

As $\Delta v'$ is proportional to the difference in the capacity coefficients, if a large enough difference exists, complete separation can be expected.

Using this rationale, we attempted to separate the two anions discussed earlier using a column coated with a polymer, PS-264. From Fig. 1B, it can be seen that the electroosmotic flow has been nearly eliminated but, as before, the resolution for the anions is near zero. An attempt to separate the two chromatographically in a pressure-driven system yielded the same results in that the anions were barely retained, if at all, and were inseparable under conditions similar to those in Fig. 1B.

We observe that as the individual capacity factors are small, increasing k for each anion may increase their difference and, hence, $\Delta v'$. The retention of a solute may be increased by modifying the stationary or the mobile phase. In the case of the stationary phase, an OTCLC column can be made more retentive by increasing the film thickness of the stationary phase. Because the column used in Fig. 1B is representative of the most retentive column that we are able to produce repeatedly using the method in ref. 8, we adopted a procedure developed by Dluzneski and Jorgenson [9]. They described a procedure for coating columns of similar I.D. using the polymer OV-17v. After preparing a column from conditions which produced the thickest polymer film in ref. 9, we again attempted to separate the two anions. It can be seen from Fig. 1C that, although incomplete, the beginning of a separation is present.

This approach has several drawbacks. The first is that changing columns to improve a separation is tedious and time consuming. The second, as Fig. 1C demonstrates, is that this technique will fail for many polar, ionic solutes. A similar problem existed in liquid chromatography, but the technique of ion pairing provided a useful solution. Therefore, to increase the retention of these anions through mobile phase modification, the principles of ion-pair chromatography were adopted.

It is well known in ion-pair chromatography that the addition to the eluent of a salt that contains a large organic cation will increase the affinity of an anion for the stationary phase. Although several models exist for reverse-phase ion-pair chromatography [12,13], many experimental results involving a bonded stationary phase have been described by the dynamic ion-exchange model [14–18]. An important requirement for the dynamic ion-exchange model is that the ion-pairing agent, in our case a cation, C^+, be partitioned onto the stationary phase. This can be described through the equilibrium

$$C_m^+ \overset{K_1}{\rightleftharpoons} C_s^+ \tag{5}$$

where the subscripts m and s denote the mobile and the stationary phase, respectively. The anion, A^-, is partitioned onto the column through the equilibrium

$$A_m^- + C_s^+ \overset{K_2}{\rightleftharpoons} (A^- C^+)_s \tag{6}$$

We have observed previously [7] that when a cationic surfactant is incorporated into an electrochromatographic system using a column similar to that in Fig. 1B, the EO flow is in the direction of the anode, and its velocity is dependent on the surfactant concentration. This is due to the surfactant partitioning itself between the mobile and stationary phases, which then modifies the charge on the surface. The EO velocity is directly proportional to this surface charge. Because of the equilibrium that exists, this charge, and hence EO velocity, are dependent on the cation concentration. For the same reason, it is evident from Fig. 2 that the cation, TBA, which is a common ion-pairing, agent, also partitions itself onto such a stationary phase. Based on this we assume that the dynamic ion-exchange model is the dominant retention mechanism. However, as it is unlikely that only one retention mechanism exists, we also take into account the formation of ion pairs.

For the model of ion-pair formation, the anion and the cation form an ion pair through

$$A_m^- + C_m^+ \overset{K_3}{\rightleftharpoons} (A^-C^+)_m \tag{7}$$

and this pair partitions onto the stationary phase through

$$(A^-C^+)_m \overset{K_4}{\rightleftharpoons} (A^-C^+)_s \tag{8}$$

In the absence of ion-pair formation, v' and $\Delta v'$ are described by eqns. 3 and 4, with v now being the sum of v_{ep} and v_{eo}. However, in the presence of ion-pair formation, the ion pair will have zero charge; therefore, it will be transported by EO flow alone. This makes v' and $\Delta v'$ more complicated. In this case, v' becomes

$$v' = \frac{Fv}{1 + k} + \frac{(1 - F)v_{eo}}{1 + k'} \tag{9}$$

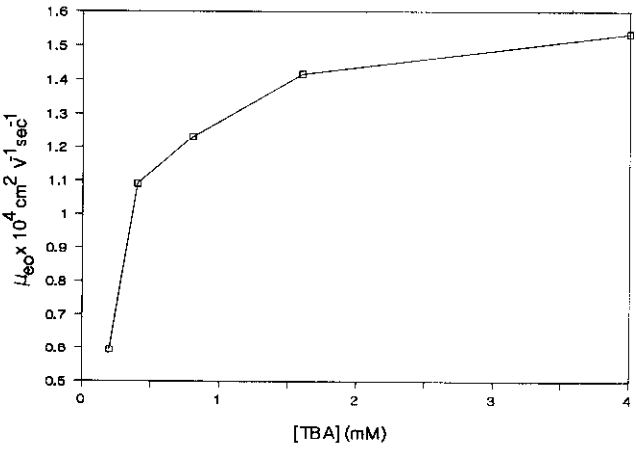

Fig. 2. Dependence of the coefficient for electroosmotic flow (μ_{eo}) on the concentration of TBA in a buffer of 10 m*M* phosphate, pH 7.0. Column and voltage conditions as in Fig. 1B.

and $\Delta v'$ becomes

$$\Delta v' = v\left[\frac{F_1 - F_2 + F_1k_2 - F_2k_1}{(1 + k_2)(1 + k_1)}\right] + v_{eo}\left[\frac{F_2 - F_1 + (1 - F_1)k'_2 - (1 - F_2)k'_1}{(1 + k'_2)(1 + k'_1)}\right] \quad (10)$$

where F is the fraction of free anion, k is the capacity factor for free ion and k' is the capacity factor of the ion pair. F can be determined from

$$F = \frac{[A_m^-]}{[A_m^-] + [A^-C_m^+] + [A^-C_s^+]} = \frac{1}{1 + K_3(1 + K_4)[C_m^+]} \quad (11)$$

We can illustrate the effect of the ion-paring agent on $\Delta v'$ using these equations for the case where the dynamic ion-exchange model is dominant ($K_1K_2 \gg K_3K_4$). At low concentrations of ion-pairing agent, F will be near unity and the second part of eqn. 10 can be neglected. In addition, if $F_1 \approx F_2$, eqn. 10 can be approximated by eqn. 4. With $k \propto K_1K_2[C_m^+]$ [19] and $\Delta v' \propto (k_1 - K_2)$, increasing retention via the ion-pairing agent will increase $\Delta v'$.

To demonstrate this, five anion were subjected to the same separation as in Fig. 1A. From the electropherogram in Fig. 3, it is observed that four of the five anions are unresolved because of similar mobilities. Next, the column was replaced with one identical with it, and equilibrated with a buffer containing 1.25 mM TBA. Separating the same five anions under these conditions resulted in no elution of any anion. To explain this, consider that when large, linear organic cations are incorporated into CZE buffers, the direction and magnitude of the EO flow becomes dependent on the

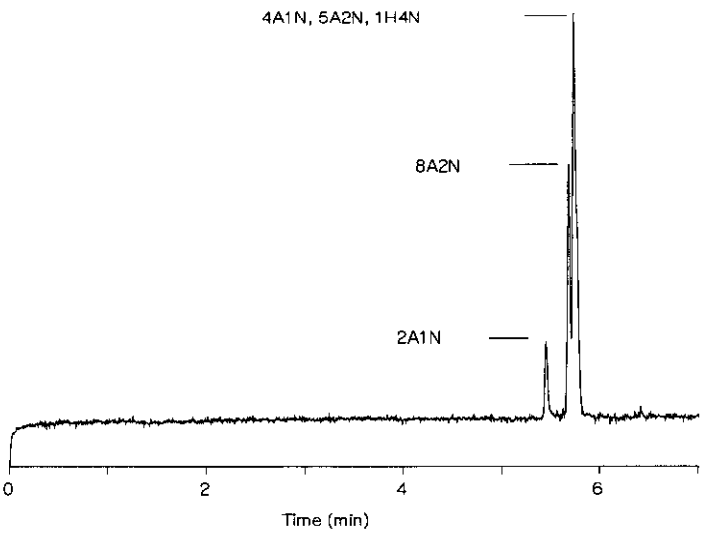

Fig. 3. CZE elution profile for five anions, four of which have similar electrophoretic mobilities. Column, buffer and voltage conditions as in Fig. 1A.

concentration of the cation. This has been discussed previously [20,21], but the effect in those reports is much less than that when a stationary phase is used [7]. In the case above, it was concluded that owing to the bulky nature of TBA, the amount adsorbed on the wall was ineffective at reducing the EO flow, which was later determined to be in the direction of the cathode (away from the detector). Attempting the same separation again, but with the polarity of the electric field reversed, resulted in the electropherogram shown in Fig. 4. Although the four anions with similar mobility are poorly resolved, the fact that 1H4N is separated from 5A2N is of interest. Under similar conditions (5 m*M* phosphate buffer, pH 7.0; +27 kV separating voltage), but with no TBA in the buffer, these two anions do not separate. As dynamic ion echange is unlikely owing to the poor column adsorption of TBA (stationary phase absent), this may be the result of ion-pair formation in the mobile phase.

The discussion above brings up a related point. The cations of salts such as cetyltrimethylammonium bromide and tetradecyltrimethylammonium bromide adsorb on uncoated capillary walls [20,21]. Therefore, CZE separations which have these or similar salts in the buffer may experience an ion-pairing effect. If such a separation is performed in a capillary with I.D. $>>$ 10 μm and if the ion-pairing effect is significant, the bands may exhibit solute zone broadening owing to poor mass transfer.

To switch from CZE to electrochromatography, the CZE column was replaced with an OTCLC column similar to that used in Fig. 1B and equilibrated with a buffer containing 1.25 m*M* TBA. Subjecting the same five anions to a separation under these conditions resulted in the electrochromatogram shown in Fig. 5. Although the TBA concentration in Fig. 5 was 1.25 m*M*, the four anions of similar mobility can be separated completely using TBA even at 600 μM. At that concentration, however, the 2A1N peak is fused with the 1H4N peak. Note that the 2A1N, which was first to elute in Fig. 3, is now second from last. This is due to the amino group being adjacent to the

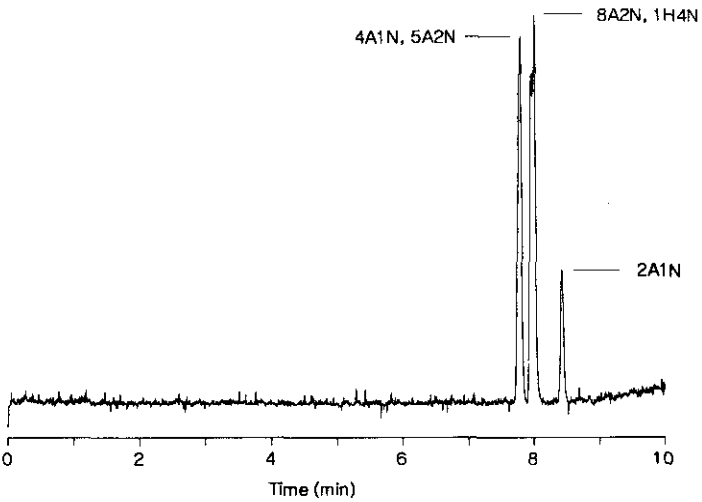

Fig. 4. CZE elution profile for the five anions using a 10 m*M* phosphate buffer, pH 7.0, containing 1.25 m*M* TBA. Column parameters as in Fig. 1A. Separation voltage, +21 kV.

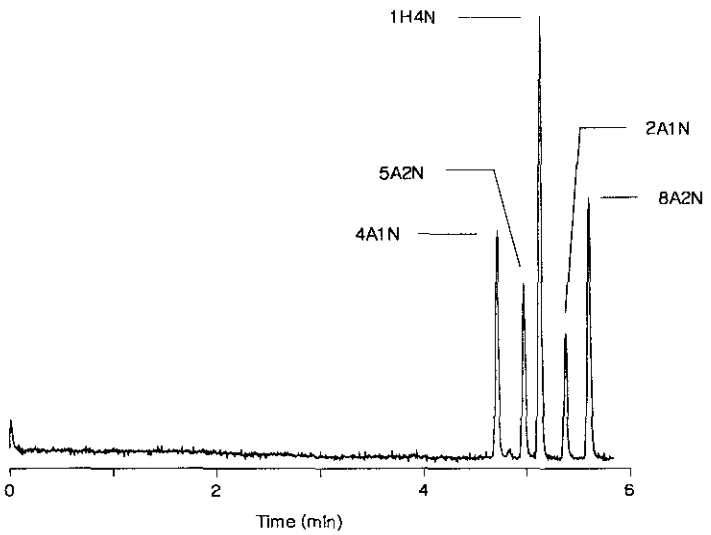

Fig. 5. Electrochromatographic separation of the five anions. Column conditions as in Fig. 1B and buffer conditions as in Fig. 4. Separation voltage, −21 kV.

sulfonic group for 2A1N, which results in a large k value in comparison with its isomers [22]. When TBA is at 7.5 mM, 2A1N becomes the last component to elute. Although not indicated in Fig. 5, all components elute with $v' > v_{eo}$; however, when TBA is at 7.5 mM, only 2A1N elutes with $v' < v_{eo}$. This illustrates the control of retention through $[C_m^+]$.

In order to compare this technique with ion-pair chromatography, the five anions were subjectd to a pressure-driven chromatographic separation using the same column and conditions as in Fig. 5. The result is shown in Fig. 6. For the four anions

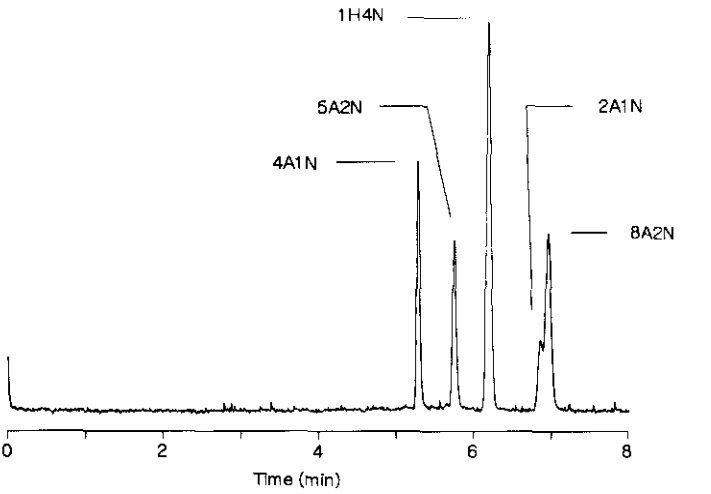

Fig. 6. Pressure-driven chromatographic separation of the five anions using the same buffer and column as in Fig. 5.

of similar mobility, the dominant separation factor present in Fig. 5 is their differences in column affinity, which is also the major separating factor in Fig. 6; hence the similarities in elution order and relative peak positions. As for the anions with different mobilities, the elution order and relative peak position will be a function of both mobility and column affinity, both of which are present only in the electrochromatographic technique. This explains the difference in peak position in Figs. 5 and 6 for the 2A1N peak relative to its neighbors.

The capacity factors for the anions in Fig. 6 are as follows: 4A1N 0.047, 5A2N 0.140, 1H4N 0.228, 2A1N 0.361 and 8A2N 0.380. The fact that these capacity factors are relatively small ($k < 1$) was expected because of the high separation efficiency observed in Fig. 5. The smallest number of theoretical plates, N, in Fig. 5 is for the 4A1N peak, with $N = 140\,000$. Comparing this value with those calculated for Fig. 6 reveals that this N is nearly twice that for the 4A1N peak and three times that for the 1H4N peak (smallest N). This is the result of the flat flow profile of electroosmotic and electrophoretic flow, contributing less to mass transfer broadening than the parabolic flow profile in pressure-driven systems [7,23–25]. Comparing the N value of 145 000 plates for the 2A1N peak in Fig. 5 with that in Fig. 3 or 4 reveals that they are nearly identical. Although no capacity factors were obtained, when TBA is at 7.5 mM 2A1N is eluted with $N = 50\,000$. Therefore, it can be seen that this technique will produce efficient separations only when k is kept small.

The resolution for each of the anion pairs in Fig. 5 was calculated and found to be >1 in all instances. As predicted, resolution can easily be controlled through the concentration of TBA, as shown in Fig. 7. The negative numbers in Fig. 7 results from the fact that the anion, 2A1N, changes elution order with 4A1N as the concentration of TBA is increased, *i.e.*, R actually becomes zero at some point in this range. The leveling off of the curves at high TBA concentration can be attributed to increased mass transfer broadening due to increased retention.

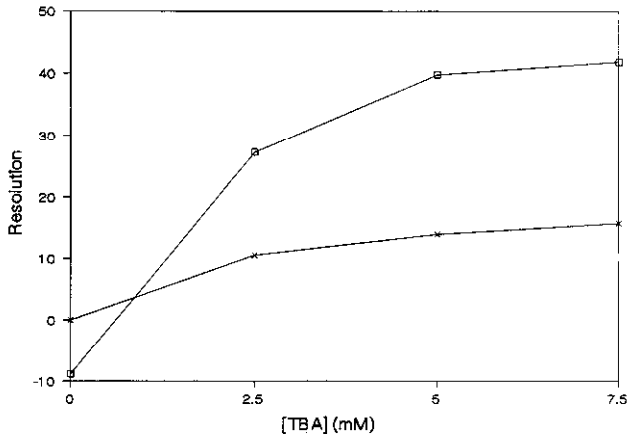

Fig. 7. Dependence of resolution on TBA concentration for ion-pair electrochromatography. Conditions as in Fig. 5. □ = 2A1N and 4A1N; × = 5A2N and 1H4N.

CONCLUSION

We have shown that anions that do not separate based on mobility differences in CZE can be made to separate electrochromatographically. The anions, which need no native affinity for the stationary phase, can be made to partition onto the column via an ion-pairing agent added to the buffer. In so doing, the anions now separate based on affinity differences for the column. Because mass transfer broadening is minimized by using columns of narrow I.D., by keeping the capacity factors small and by the flat flow profile which exists for electroosmotic flow, the efficiency of the resulting separation rivals that of a CZE separation. In addition, as there is a similarity between ion-pair electrochromatography and ion-pair chromatography, foundations for the separation of ionic solutes whose mobilities are similar can be derived from the vast amount of ion-pair chromatography literature. Finally, this technique is expected to be more sensitive than CZE to adjustments of such buffer parameters as pH, ionic strength and percentage of organic modifier, because not only will the electrophoretic component of the separation be affected, but also the chromatographic component.

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