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Packed-capillary electrochromatographic separation of the enantiomers of neutral and anionic compounds using β-cyclodextrin as a chiral selector

Effect of operating parameters and comparison with free-solution capillary electrophoresis

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ABSTRACT

Chiral separation by packed capillary electrochromatography was successfully achieved through the use of 50 μm I.D. fused-silica capillaries packed with a β-cyclodextrin chiral stationary phase. The properties of electroosmosis in these packed capillaries were studied by investigating the influence of buffer composition and pH on the velocity of electroosmotic flow. With the use of triethylammonium acetate (TEAA) as background electrolyte, the direction of electroosmotic flow was reversed compared to the direction of flow observed with a phosphate buffer system. The enantiomers of both neutral solutes (benzoin, hexobarbital) and anionic solutes (dansyl- and dinitrophenyl-amino acids) were resolved; the analysis of anions was facilitated by the reversal of electroosmotic flow with the TEAA buffer system. The effects of methanol concentration and the TEAA buffer concentration on the migration and resolution of the enantiomers were studied. In addition, separations were obtained using β-cyclodextrin as an additive in free-solution capillary electrophoresis using both phosphate and TEAA buffers. Comparison between the packed- and open-tubular systems was made. The same effect of electroosmotic flow reversal with TEAA buffer was observed in open-tubular fused-silica capillaries.

INTRODUCTION

Packed-column electrochromatography was first demonstrated by Pretorius *et al.* [1] in 1974. In this electroseparation method, the column is packed with a HPLC stationary phase and the mobile phase is driven by electroosmotic flow. Analytes are separated by normal chromatographic partitioning between the mobile and stationary phases. Electrochromatography can advantageously be performed in capillary columns, benefiting as with other forms of capillary electrophoresis (CE) from improved heat dissipation. The potential uses and limitations of capillary electrochromatography (cEC) with capillaries filled with ODS packing were discussed by Jorgenson and Lukacs [2] in 1981 and more recently by Yamamoto *et al.* [3] in 1992. One interesting aspect of cEC is that electroosmotic flow has a flat flow profile. Thus there is no contribution to band broadening from the parabolic flow characteristic of pressure-driven systems. Furthermore, as predicted by the theory of electroosmotic flow, the velocity of electroosmotic flow is independent of the geometry and the size of the channels between the packing. This means that electroosmotic flow should proceed at the same rate in a packed capillary

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regardless of packing irregularities down to very small packing particle sizes, until double-layer overlap occurs. The use of very small packing should lead to improved mass-transfer characteristics. In pressure-driven systems the use of very small diameter packing materials is limited by the increase in column back-pressure. Knox and Grant [4] reported results with cEC systems using capillaries packed with 1.5–50 μm diameter silica and ODS-bonded silica gels. Reduced plate heights as low as unity were obtained for unretained solutes and the columns (packed capillaries) driven by electroosmosis showed higher plate efficiencies than when they were driven by pressure. It is expected that selectivities in cEC may be different than those in microcolumn HPLC for ionized analytes since both electrophoresis and partitioning will contribute to the selectivity in cEC.

In our previous work [5], cEC using an α_1-acid glycoprotein (AGP) chiral stationary phase was successfully applied to the direct enantiomeric separation of neutral and cationic chiral compounds. Separation efficiencies were generally somewhat higher than those seen with HPLC separations using analytical-scale AGP columns. However, separation efficiencies were not as high as those typically seen in other CE modes. This could be for a number of reasons, for example a property of the chiral selector itself, of the chiral stationary phase, of the packed capillary arrangement, or overloading. In this study, chiral separation by cEC was investigated using capillaries packed with a β-cyclodextrin chiral stationary phase. The characteristics of this phase when driven by electroosmosis were studied. Comparison is made with electrokinetic separations using β-cyclodextrin as a buffer additive in open-tubular CE.

Cyclodextrins are cyclic oligosaccharide consisting of D-glucose units, all in the classical C_1-chair conformation and linked through α-1,4 bonds. The structure of cyclodextrin is that of a truncated cone with a relatively hydrophobic cavity. The most characteristic property of cyclodextrins is their ability to form inclusion complexes with a wide variety of guest molecules. The stability of inclusion complex is primarily determined by the size, the geometry and the hydrophobicity of the guest molecule. In recent years, cyclodextrins have received much attention in the field of chiral separations [6]. The wide interest in the use of cyclodextrins for chiral discrimination arises from the fact that they can offer a highly stereoselective system for the enantiomeric separation of a wide variety of chiral compounds. In CE, cyclodextrins are the most widely used chiral selector [7]. A wide variety of chiral compounds have been resolved by CE through the use of cyclodextrins as buffer additives [8–10], cyclodextrins combined with sodium dodecyl sulphate micelles [11], cyclodextrins incorporated into a gel matrix [12] and cyclodextrins coated onto the capillary wall [13]. Cyclodextrin bonded stationary phases have become some of the most commonly used chiral stationary phases in both GC [14,15] and HPLC [16,17].

In this paper, we demonstrate the successful enantiomeric separation of benzoin, hexobarbital and a series of amino acid derivatives by cEC using capillaries packed with a β-cyclodextrin bonded stationary phase. The properties of electroosmosis in these packed capillaries is described. The effect of buffer composition and organic modifier concentration on the retention and resolution are discussed in detail. It is shown that reversal of electroosmotic flow can be achieved by the correct choice of buffer system, and that this is most useful in the analysis of anionic solutes which can otherwise be difficult to separate by cEC [5].

EXPERIMENTAL

Apparatus

An Applied Biosystems (Toronto, Canada) Model 270A CE system equipped with a variable-wavelength UV detector was used to perform packed-capillary electrochromatography. The oven temperature was set at 30°C. Samples were electrokinetically introduced into the capillary by applying a voltage of 5 kV (of the appropriate polarity, depending on the direction of electroosmotic flow). The injection time was 1 s which corresponds to approximately a 0.15–0.25 mm long sample zone according to the velocity of electroosmotic flow. On-column de-

tection was carried out by UV absorbance measurements at a wavelength of 200 nm, with a detector rise time of 0.5 s. Free-solution separations in open tubes were performed using an Applied Biosystems model 270A-HT CE system, with hydrodynamic injection for 1 s. The electropherograms were recorded using Model SP4600 integrators (Spectra-Physics, San Jose, CA, USA). Further data manipulation was performed using Spectra-Physics Winner 386 software. A Model 1666 HPLC column slurry packer (Alltech, Deerfield, IL, USA) was used for capillary packing.

Materials

Fused-silica capillary tubes (50 μm I.D. × 365 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). β-Cyclodextrin stationary phase with a 5 μm particle diameter was obtained by emptying an extensively used Cyclobond I HPLC column (Astec, Whippany, NJ, USA). Packing material from near the head of the column was discarded. Benzoin was purchased from Aldrich (Milwaukee, WI, USA). Hexobarbital was from U.S.P.C. (Rockville, MD, USA). 2,4-Dinitrophenyl (DNP)-amino acids, dansyl-amino acids and triethylamine were obtained from Sigma (St. Louis, MO, USA). Disodium hydrogenphosphate and sodium dihydrogenphosphate were obtained from BDH (Toronto, Canada). All organic solvents were from Anachemia (Montreal, Canada).

Procedures

The procedures for the preparation of the packed capillaries are the same as those described in ref. 5, except that the slurry of β-cyclodextrin packing is made in a solution of methanol–10 m*M* phosphate buffer (1:4). Triethylammonium acetate (TEAA) buffer was prepared by neutralizing a 0.10 *M* triethylamine solution with glacial acetic acid. Mobile phase was prepared by mixing methanol with TEAA buffer. The mobile phase was filtered through a 0.45-μm frit (Lida Manufacturing, Kenosha, WI, USA) and degassed in an ultrasonic bath for about 2 min before use. Sample solutions were prepared by dissolving each racemate in methanol to give a concentration of *ca.* 1 mg ml^{-1}.

RESULTS AND DISCUSSION

Properties of electroosmosis in the packed capillary

Electroosmotic flow, the flow of solvent under the effect of an applied potential, is the driving force for the separation in electrochromatography. Therefore, it is clearly of interest to know the behaviour of electroosmosis and to have the ability to control the velocity of electroosmotic flow in the capillaries packed with β-cyclodextrin stationary phase. The velocity of electroosmotic flow, ν, in an open-tubular capillary under the influence of an applied electric field, E, can be described by [1,18]

$$\nu = \frac{\epsilon E \zeta}{\eta}$$

where ϵ is the permittivity of the medium, η is its viscosity and ζ is the ζ potential at the shear plane between the charged surface and the electrolyte solution. The behaviour of electroosmosis in capillaries packed with silica-gel and ODS bonded silica gel was found to be generally similar to that in open-tubular capillaries [2–4,19]. However, the velocity of electroosmotic flow is decreased due to non-alignment of the flow channels in the packed bed with the capillary axis. The velocity of electroosmotic flow in capillaries packed with silica gel and ODS bonded silica gel is about 40–60% of that in open-tubular capillaries [4]. In AGP-packed capillaries, the velocity of electroosmotic flow is approximately 40% of its open-tubular value over a pH range 4.5–7.5 using phosphate buffer with 1-propanol as an organic modifier, but varies from 12 to 40% of its open-tubular value when going from pH 4.5 to 7.5 with 2-propanol as an organic modifier [5]. This behaviour was attributed to an effect of the organic modifier on the immobilized protein; thus in packed-capillary electrochromatography, electroosmosis is controlled by the ζ potential at the surface of the packing particles, and this is not necessarily the same as that at a silica surface. In this study, the behaviour of electroosmosis in the capillaries packed with β-cyclodextrin stationary phase was studied by investigating the effect of the mobile phase composition and pH, and field strength on the electroosmotic flow.

Effect of the type of background electrolyte on the direction of electroosmotic flow. Electroosmotic flow can be measured in packed capillaries by the use of a neutral marker which is unretained on the stationary phase [3]. Because of concerns over finding a truly unretained solute in HPLC with cyclodextrin stationary phases [20], we tested methanol, ethanol and 1-propanol as marker compounds. They were found to all have the same migration time, indicating that they were unretained. Since methanol was present in all samples as a solvent, the methanol peak was used to measure electroosmotic flow. The direction of flow in the β-cyclodextrin packed capillary was found to be determined by the type of background electrolyte used. With sodium phosphate as background electrolyte, the electroosmotic flow is directed towards the cathode (as with ODS or AGP packed capillaries, or with open-tubular fused-silica capillaries), but with TEAA as background electrolyte the flow direction is reversed and the bulk solution is transported towards the anode. This behaviour can be explained by considering the effect of the background electrolyte on the charge at the capillary surface. β-cyclodextrin has a pK_a value of 12.0 [21], thus in the operating pH range (3.5–7.5) of the cyclodextrin stationary phase, β-cyclodextrin is not charged. Therefore, it is expected that under normal aqueous conditions with small binary electrolytes, such as sodium phosphate, both the capillary wall and the unbonded surface of the packing particles will have an excess of anionic charge resulting from ionization of surface silanol functional groups. The resulting direction of electroosmotic flow is toward cathode with sodium phosphate as background electrolyte. As discussed in previous work [5], this direction of electroosmotic flow is advantageous in the cEC separation of cationic analytes. However, anions will migrate against the electroosmotic flow, resulting in an extremely low overall mobility and excessively long migration times. Clearly, this is a severe and general problem in the cEC separation of anionic analytes, and a reversal of the electroosmotic flow direction is desirable for the cEC analysis of anions. The reversal of flow which occurs when using the TEAA buffer system indicates a reversal of the ζ potential at the solid–liquid interfaces within the capillary. This may come about due to adsorption of cations onto the silica surface [22]. The TEAA background electrolyte seems to act in a manner which is in some ways similar to cationic surfactant additives such as cetyltrimethylammonium salts [23] and smaller hydrocarbon chain length homologues [24]. In addition, the efficiency of β-cyclodextrin bonded phase columns can be substantially increased through the use of TEAA buffer. In HPLC separations a TEAA buffer (0.02 *M*, pH 5.0) substituted for water in a methanol–water (40:60) system has produced a four-fold increase in the column efficiency for the separation of phenothiazine derivatives [25].

Effect of pH. The effect of pH on the electroosmotic mobility in β-cyclodextrin packed capillaries was investigated by varying the mobile phase pH from 4.03 to 7.50 with 2 m*M* sodium phosphate and with 15 m*M* TEAA as background electrolytes. It should be noted that the buffer capacity for phosphate is very limited below pH 5, so pH control will be poor for the lower pH data points. For comparison, the dependence of electroosmotic flow mobility on pH in open-tubular capillaries was also studied. Open-tubular capillaries were acid-washed and then allowed to equilibrate with the electrolyte before making electroosmosis measurements. Packed capillaries were allowed to equilibrate with each of the different buffer solutions for 2 h before making mobility measurements. The results are shown in Fig. 1. With sodium phosphate as background electrolyte, the electroosmotic mobility (toward the cathode) in the packed capillary decreases linearly with decreasing pH over the pH range studied. The electroosmotic mobility in an open tubular capillary shows a similar curve, but the velocity is much higher. The difference in mobility is a factor of 3 at pH 7.5, increasing to a factor of 4.5 at pH 4. With underivatized silica packings, electroosmosis in packed systems is expected to be approximately 40–60% of the flow seen in open tubes [4]. The relatively smaller flows seen here are probably due to the surface modification of the silica packing, leading to a reduced surface density of free silanol groups. With TEAA as the back-

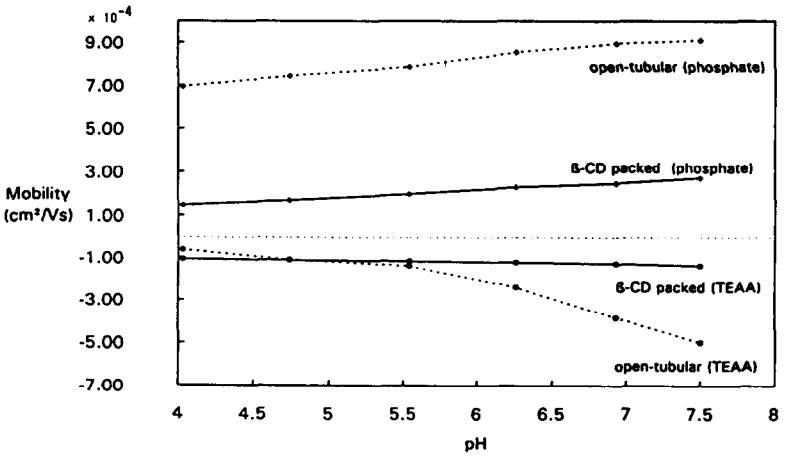


Fig. 1. Dependence of electroosmotic flow mobility on pH. Packed capillary, 42 cm (21 cm to detector) × 50 μm I.D., 17 cm packed with β-cyclodextrin stationary phase; open tubular capillary, 72 cm (50 cm to detector) × 50 μm I.D.; mobile phase: (●) 10 m*M* TEAA, 15% methanol, and (◆) 2 m*M* phosphate, 2% 2-propanol. Solid lines, packed capillary; broken lines, open-tubular capillary.

ground electrolyte, the electroosmotic mobility (toward to the anode) in the packed capillary reduces very slightly in a linear fashion with reducing pH. However, the dependence of electroosmotic flow mobility on pH in the open-tubular capillary shows a significantly different curve. The electroosmotic mobility decreases rapidly with decreasing pH, with values from $5.00 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} at pH 7.50 to $0.62 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} at pH 4.03. In the case of the surfactant additive cetyltrimethylammonium bromide (CTAB), the electroosmotic flow towards the anode in a fused-silica capillary was relatively independent of pH over the pH range 4–11 [26]. This is not surprising, since adsorption of surfactants to the capillary wall is driven by strong adsorption of the hydrophobic surfactant tail. However, association with the capillary surface may come about due to electrostatic as well as hydrophobic effects [22]. The pH dependence of electroosmotic flow with TEAA buffers observed here may be explained by considering that adsorption of the triethylammonium ion (TEA^+) is a result of both hydrophobic effects (probably weaker than for long-chain surfactants) and electrostatic effects. In the open-tubular capillary with TEAA as background electrolyte, the capillary wall is partly covered by TEA^+ ions which are particularly associated with $-SiO^-$ groups on the silica surface. The density of $-SiO^-$ groups on the silica surface increases with increasing pH, resulting in more coverage of TEA^+ on the capillary wall. Electroosmosis is governed by the potential at the shear plane, and so even a one-to-one association of the TEA^+ with the surface SiO^- groups may cause a reversal of the ζ potential, since the cations themselves are closer to the shear plane and thus have a stronger effect on the potential at the shear plane [22]. In the packed capillary, the surface of the packing is bonded with β-cyclodextrin molecules. In TEAA buffer, triethylammonium ions may associate with the bonded cyclodextrin molecules to form an assembly, acting as a reservoir of positive charge which becomes the dominating factor determining the interfacial ζ potential in the packed capillary. pH has little effect, since the cyclodextrin itself is uncharged over this pH range, and the density of free silanol groups should be low.

Effect of methanol concentration. The influence of methanol, added in amounts from 10 to 40% (v/v) to the mobile phase, on the electroosmotic mobility is shown in Fig. 2. The measurements were carried out in 10 m*M* TEAA at a pH of 4.71. It can be seen that the electroosmotic mobility decreases steadily with increasing fraction of methanol in the mobile phase, leading to a reduction in mobility from $-1.50 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} at 10% methanol to $-0.87 \cdot$

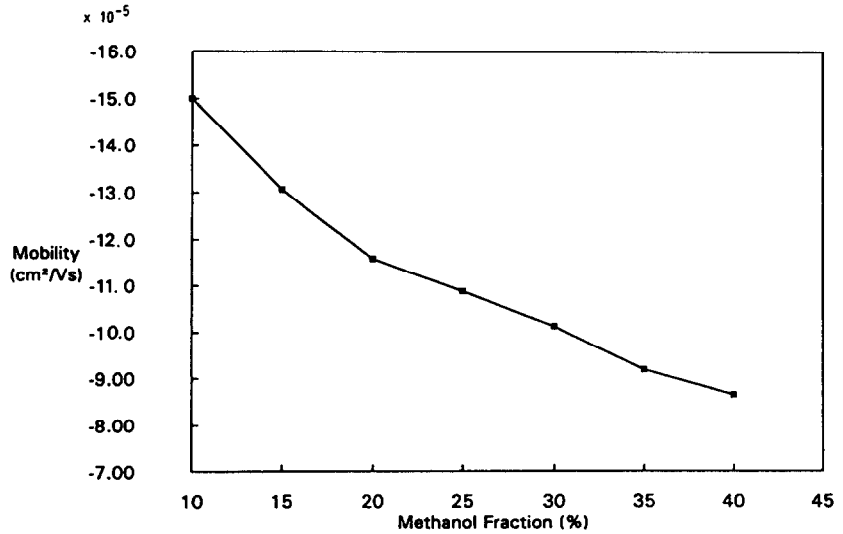


Fig. 2. Dependence of electroosmotic flow mobility on methanol concentration. Packed capillary as in Fig. 1; mobile phase, 10 m*M* TEAA , pH 4.71; applied voltage, 10 kV.

10^{-4} cm^2 V^{-1} s^{-1} in a mobile phase containing 40% (v/v) methanol. These results are similar to those obtained in open-tubular capillaries [27,28] and in an AGP-packed capillary [5]. This has been attributed to a decrease in the ratio of dielectric constant to viscosity for aqueous solutions of organic solvents, with increasing fraction of organic solvent [28]. It can be seen that in changing the percentage of organic modifier in the mobile phase to alter chromatographic resolution the flow-rate through the column will be altered. Higher percentages of organic additive may lead to reduced k' values, but only slight changes in migration time since the effect of reduction in retention is partially compensated by the reduction in electroosmosis.

Effect of TEAA concentration. The variation of electroosmotic flow mobility in a packed capillary as a function of TEAA concentration is shown in Fig. 3. The measurements were carried out at pH 4.71 with mobile phase containing 15% methanol (v/v). The TEAA concentration

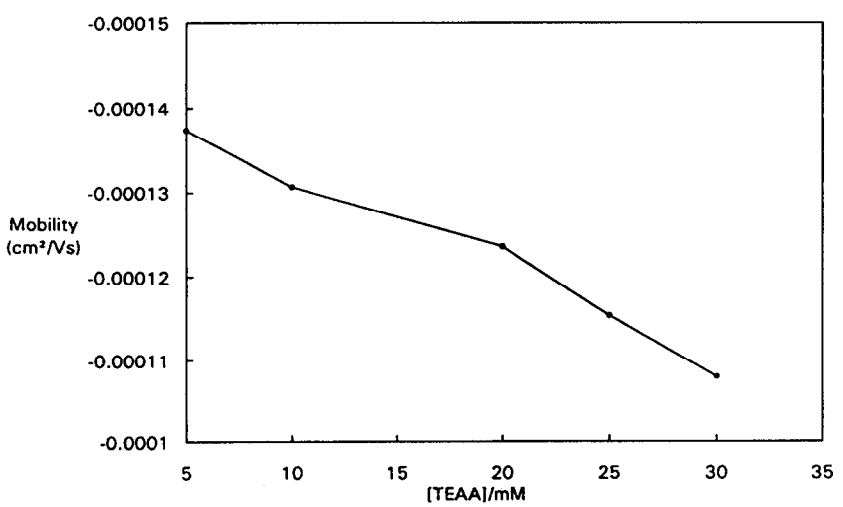


Fig. 3. Dependence of electroosmotic flow mobility on TEAA concentration. Packed capillary as in Fig. 1; mobile phase, 15% methanol, pH 4.71; applied voltage 10 kV.

was changed from 5 to 30 m*M*. As can be seen from Fig. 3, the electroosmotic mobility decreases with increasing buffer concentration, which is the usual behaviour [29]. These results indicate that the absorption of TEAA to the capillary wall and the surface of packing has been saturated even at TEAA concentrations as low as 5 m*M*.

Effect of field strength. The electroosmotic flow velocity should vary in a linear fashion with field strength. However, non-linear deviations have been observed in packed capillaries at high field strengths [3,4]. This is despite the fact that the operating current for cEC is limited by the onset of bubble formation at very low currents (< 10 μA). Using a 2 m*M* phosphate buffer at pH 6.8 with 2% acetonitrile, the equation for the line depicting flow velocity, v (mm s^{-1}), plotted against field strength, E (kV m^{-1}), is $v = 0.026 \cdot E - 0.071$ ($r = 0.997$). Using a 15 m*M* TEAA buffer at pH 4.74 with 15% methanol, the equation of the line for flow velocity plotted against field strength was $v = -0.019 \cdot E + 0.08$ ($r = 0.999$). The maximum current flow was 4 μA with the phosphate buffer and 6 μA with the TEAA buffer. The intercepts reflect a very slight non-linearity at higher field strengths, although this non-linearity is considerably less pronounced than that observed in other packed systems [3].

Chiral separations

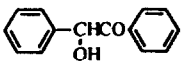
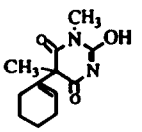
In this study, the enantiomeric separation of benzoin, hexobarbital, dansyl-DL-threonine and eight racemic DNP-amino acids by cEC with a β-cyclodextrin packed capillary was investigated. All these chiral compounds, except hexobarbital, have at least one aromatic ring in the molecule. The cavity of β-cyclodextrin has a internal diameter varying from 6.0 Å at the small side to 7.8 Å at the larger side [30]. It can easily accommodate molecules or parts of molecules having a five-, six- or seven-atom aromatic ring to form inclusion complexes [31]. The presence of aromatic groups in the chiral molecules provides the binding site required for retention. The names, formulae, and mobility and resolution data under the given electrochromatographic conditions are listed in Table I. Under the separation conditions, all the compounds except benzoin and hexobarbital are negatively charged. The calculation of k' and α values is somewhat problematic for charged solutes in cEC [5]. The effective mobility is a useful parameter to measure in free-solution CE, and a mobility parameter calculated in the same way is used here. It should be stressed that although this mobility parameter is calculated in the same way as effective mobilities in free-solution CE, it is not simply related to the electrophoretic mobilities of the individual species in free solution because there is also a retardation of movement due to chromatographic interactions with the stationary phase. In this aspect, the mobility parameter calculated here is rather similar to the pseudo-effective mobility introduced by Ackermans *et al.* [32] in micellar electrokinetic chromatography, and so mobilities calculated for solutes in electrochromatography will be referred to here using the term pseudo-effective mobility. Because of the difficulties of measuring k' and α, only the pseudo-effective mobilities of the first eluted enantiomers, the mobility difference between two enantiomers and the resolution factors are given for charged compounds.

When sodium phosphate was used as background electrolyte, only the enantiomers of two neutral compounds, benzoin and hexobarbital, were separated. The negatively charged compounds, such as dansyl-threonine and DNP-amino acids, migrate against the electroosmotic flow, resulting in extremely low overall mobilities. In order to separate the enantiomers of the anionic chiral compounds, TEAA was used as background electrolyte, to reverse the electroosmotic flow. By changing the polarity of applied potential (detector end as anode), the negatively charged solutes can be electrokinetically injected, and migrate along with the electroosmotic flow in the packed capillary. Enantiomers of the neutral compounds could also be separated using the TEAA buffer. Some electrochromatograms are shown in Fig. 4. For the neutral hexobarbital (Fig. 4A), the plate number for the first eluted peak was *ca.* 9500, corresponding to a height equivalent to a theoretical plate (HETP) of 22 μm. For DNP-DL-methionine (Fig. 4C), $N \approx 15\,700$, and the HETP ≈ 13 μm. For both these compounds the asymmetry factor is be-

TABLE I

CHIRAL SEPARATIONS BY cEC WITH CYCLOBOND I PACKED CAPILLARY

In the structure of solutes, R = 2,4-dinitrophenyl and R_1 = dansyl. The unit of effective mobility (μ_e) is 10^{-6} cm^2 V^{-1} s^{-1}. Packed capillary as in Fig. 1.

Solute	Structure	Mobile phase	μ_e	$\Delta\mu_e$	R_s
Benzoin		4 m*M* Phosphate, 15% MeOH, pH 6.8	−105.8 (k' = 1.58, α = 1.06)	2.5	0.85
		5 m*M* TEAA, 15% MeOH, pH 4.71	−92.4 (k' = 2.16, α = 1.06)	1.7	1.20
Hexobarbital		4 m*M* Phosphate, 5% acetonitrile, pH 6.8	−149.1 (k' = 2.22, α = 1.09)	3.7	1.39
		5 m*M* TEAA, 15% MeOH, pH 4.71	−80.1 (k' = 1.31, α = 1.14)	4.3	1.50
Dansyl-DL-threonine	$CH_3CH(OH)-CHCOOH$ NHR_1	20 m*M* TEAA, 15% MeOH, pH 4.71	−95.6	1.6	1.90
DNP-DL-α-Amino-*n*-butyric acid	$CH_3CH_2\cdot CHCOOH$ NHR	10 m*M* TEAA, 20% MeOH, pH 4.71	−50.7	2.4	0.80
DNP-DL-Norleucine	$CH_3(CH_2)_3\cdot CHCOOH$ NHR	10 m*M* TEAA, 25% MeOH, pH 4.71	−70.9	2.4	1.50
DNP-DL-α-Amino-*n*-caprylic acid	$CH_3(CH_2)_5\cdot CHCOOH$ NHR	15 m*M* TEAA, 15% MeOH, pH 5.54	−101.1	2.2	2.20
DNP-DL-Methionine sulfone	$CH_3SO_2CH_2CH_2\cdot CHCOOH$ NHR	10 m*M* TEAA, 10% MeOH, pH 4.71	−85.9	0.6	0.70
DNP-DL-Methionine	$CH_3SCH_2CH_2\cdot CHCOOH$ NHR	10 m*M* TEAA, 15% MeOH, pH 4.71	−88.0	1.9	1.25
DNP-DL-Ethionine	$CH_3CH_2SCH_2CH_2-CHCOOH$ NHR	10 m*M* TEAA, 15% MeOH, pH 4.71	−104.8	2.2	1.57
DNP-DL-Citrulline	$H_2NCONH(CH_2)_3CHCOOH$ NHR	10 m*M* TEAA, 10% MeOH, pH 4.71	−97.6	1.3	0.70
DNP-DL-Glutamic acid	$HOOCCH_2CH_2CHCOOH$ NHR	10 m*M* TEAA, 10% MeOH, pH 4.71	−110.6	0.9	0.70

tween 3 and 4. Using a phosphate buffer for hexobarbital under the conditions shown in Table I, N was ≈30 000 (HETP ≈ 7 μm). The separation efficiency obtained using this phase may be compared to that achieved using AGP-packed capillaries in a similar experimental setup, where the highest efficiency found for an enantiomerically resolved compound was in the

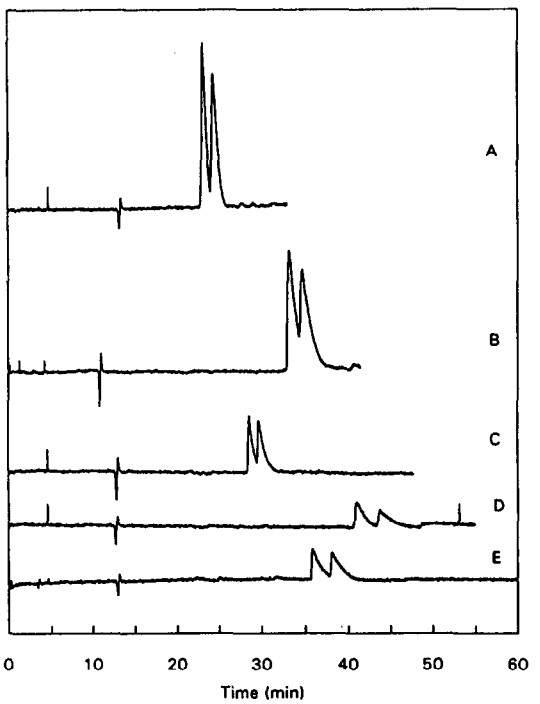


Fig. 4. Electrochromatograms showing the enantiomeric separation of (A) hexobarbital, (B) benzoin, (C) DNP-DL-methionine, (D) DNP-DL-ethionine and (E) DNP-DL-norleucine. Packed capillary as in Fig. 1; mobile phase, 10 m*M* TEAA buffer, pH 4.71, 15% methanol for benzoin, and 25% methanol for all other compounds.

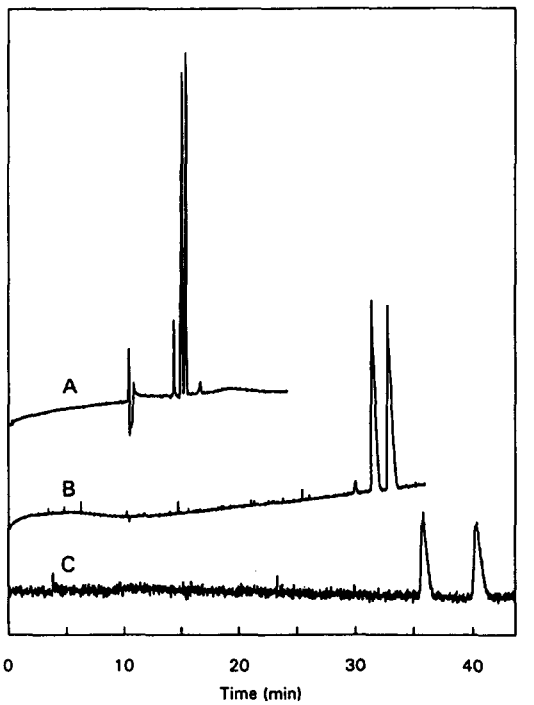


Fig. 5. Enantiomeric separation of DNP-DL-methionine by CE using a 72 cm (50 cm to detector) × 50 μm I.D. open-tubular fused-silica capillary with β-cyclodextrin as a buffer additive. Separation conditions: all separations, 15 m*M* β-cyclodextrin; (A) 100 m*M* sodium phosphate, pH 7.5, applied voltage, +20 kV, sample concentration 500 μg ml^{-1} each enantiomer, (B) 100 m*M* TEAA, pH 4.74, applied voltage, −20 kV, sample concentration 167 μg ml^{-1} each enantiomer, (C) 10 m*M* TEAA, pH 4.74, applied voltage, −30 kV, sample concentration 42 μg ml^{-1} each enantiomer [attenuation reduced by a factor of six times compared to (A) and (B)].

separation of benzoin, with a HETP of 29 μm [5].

By way of comparison with the packed capillaries, the compounds detailed in Table I were also separated using β-cyclodextrin as an additive in free-solution CE. The background electrolyte comprised either TEAA or sodium phosphate solutions with 15 m*M* added cyclodextrin. The polarity of the electric field was set to keep the direction of electroosmotic flow towards the detector. Separations of the enantiomers of DNP-DL-methionine are shown in Fig. 5. Fig. 5A shows the separation using 100 m*M* sodium phosphate buffer at pH 7.5, while Figs. 5B and 4C show the separation using 100 and 10 m*M* TEAA buffers, respectively, at pH 4.74. Separations using the phosphate buffer generally take a shorter time, mainly due to the stronger electroosmotic flow. Asymmetry and broadening of the peaks indicates overloading. The sample concentrations used for each enantiomer were 500 μg ml^{-1} for Fig. 5A, 167 μg ml^{-1} in Fig. 5B and 42 μg ml^{-1} for Fig. 5C. Efficiency is greater in the phosphate buffer; for the first eluted peak of DNP-DL-methionine, $N \approx 75\,000$ (HETP ≈ 7 μm) in 100 m*M* phosphate (Fig. 5A), and ≈ 48 000 (HETP ≈ 10 μm) in 100 m*M* TEAA (Fig. 5B). Efficiency is lower in the 10 m*M* TEAA buffer (Fig. 5C), $N \approx 25\,000$ (HETP ≈ 20

μm), but resolution is increased. With the TEAA buffer there is a degree of peak tailing (asymmetry factor ≈3 for the first eluted enantiomer in Fig. 5C); expansion of the electropherogram in Fig. 5A reveals that with the phosphate buffer the analyte peaks show fronting. Comparison of the separations shown in Fig. 5 may be made with the packed-capillary electropherograms in Fig. 4, particularly Fig. 4C which also shows the separation of DNP-DL-methionine. The main difference in operating conditions between the separations shown in Figs. 4C and 5C is that in the packed column 15% methanol was used as a modifier. The actual efficiency of the shorter packed capillary is somewhat lower than the open-tubular system, but measured in terms of HETP, the packed capillary is slightly superior. The asymmetry is similar in both cases. Resolution is considerably less in the packed capillary, as might be expected because of the added methanol, however elimination of the methanol would lead to unacceptably long migration times.

Perhaps the most striking result of the open-tubular experiments is that using TEAA, all the amino acid derivatives migrate after the electroosmotic flow point, *i.e.* their own mobility is towards the cathode, and thus they must bear a net positive charge. Two possible explanations for this behaviour are that either ion pairing of the amino acid derivatives with triethylamine occurs, or else there is an association of triethylamine with the β-cyclodextrin. In fact, there is evidence to suggest that both of these processes are occurring. By analysing solutes such as DNP-DL-norleucine and DNP-DL-methionine in free solution in open-tubular capillaries using a TEAA buffer (no added cyclodextrin), it was found that these compounds migrated against the electroosmotic flow, *i.e.*, as cations, indicating that ion pairing of these analytes with the buffer ion does occur. Evidence for the binding of triethylamine to β-cyclodextrin comes from observation of the migration behaviour of the neutral solute benzoin in TEAA buffer with and without added β-cyclodextrin. Since both benzoin and β-cyclodextrin are themselves uncharged over the pH range used in these studies, benzoin should be observed to migrate at the same velocity as the electroosmotic flow whether or not β-cyclodextrin is added to the buffer (the separations using packed capillaries reported above have demonstrated that complexation of benzoin with the cyclodextrin does occur). In the TEAA buffer alone, benzoin migrates with the electroosmotic flow, which had a mobility of $-2.15 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} (towards the anode). On addition of 15 m*M* β-cyclodextrin, benzoin was not eluted. However, on reversal of the electric field polarity, a peak for benzoin did appear, with an overall mobility of $1.25 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} (towards the cathode). It seems that a ternary complex must be formed between the solute, cyclodextrin and triethylamine, which bears an overall positive charge. Moreover, the mobility of this complex is quite high. If it is assumed that benzoin is almost completely complexed with the cyclodextrin (not unreasonable, since there was no organic modifier present for these experiments), then the effective mobility of the complex can be estimated as approximately $3.4 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} (towards the cathode).

Regulation of retention and resolution

In HPLC with cyclodextrin bonded phase columns, the retention and selectivity are dependent on inclusion complex formation between cyclodextrin cavity and the solute molecules. The stability of the inclusion complex strongly depends on the nature of the medium in which complexation occurs. In this study, the effect of organic modifier concentration and the concentration of background electrolyte on retention and resolution was investigated.

Effect of organic modifier concentration. As stated previously, inclusion complex formation is usually the critical process in chiral recognition by cyclodextrins. Although inclusion complex formation can take place in pure dipolar organic solvents such as methanol, ethanol and acetonitrile [33], enantioselectivity is usually found only in water and aqueous organic solutions. Aqueous solutions are often used for the mobile phase in liquid chromatographic separations with cyclodextrin bonded phase columns. The common organic modifiers are methanol, ethanol and acetonitrile. In this work, initial studies showed

that a methanol–water system provided much better selectivity than ethanol–water and acetonitrile–water mixtures for the analytes discussed here. Therefore, an aqueous methanol solution was chosen as the mobile phase in all subsequent experiments.

The effect of methanol concentration on the retention and resolution was studied by varying the fraction of methanol in the mobile phase from 10 to 40% (v/v). TEAA concentration was set at 10 m*M* and pH was 4.71. The dependence of pseudo-effective mobility and mobility difference between two enantiomers on the methanol concentration is shown in Fig. 6a and b, respectively. As can be seen, an increase in the methanol concentration resulted in a decrease in the pseudo-effective mobility (and an overall decrease in retention time, despite a reduction in electroosmotic flow) and a decrease in the mobility difference between the enantiomers (a

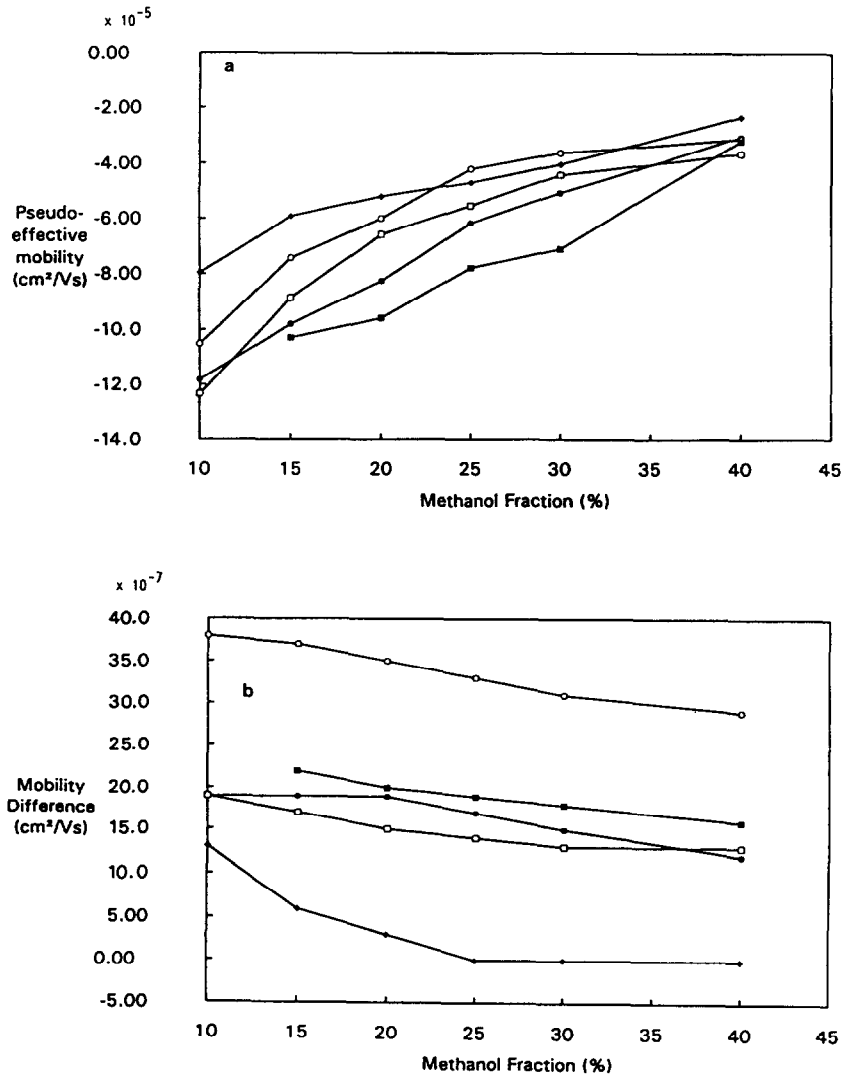


Fig. 6. Effect of methanol concentration on (a) the pseudo-effective mobility (first eluted enantiomer) and (b) the mobility difference between the enantiomers of (□) benzoin, (○) hexobarbital, (■) DNP-ethionine, (◆) DNP-citrulline and (●) DNP-methionine. Packed capillary as in Fig. 1; mobile phase, 10 m*M* TEAA, pH 4.71; applied voltage, 10 kV.

decrease in resolution). This is not surprising since methanol competes with the solute for inclusion in the cyclodextrin cavity. The greater the percentage of methanol in the mobile phase the more easily a solute is displaced from the cyclodextrin cavity, resulting in a shorter retention time and reduced pseudo-effective mobility. In addition, with increasing methanol concentration, the properties of the mobile phase begin to change substantially. The difference in the hydrophobicity between the solvent and β-cyclodextrin cavity becomes smaller, making the inclusion complex formation less favourable. In this study, the enantiomeric resolution of DNP-DL-citrulline, DNP-DL-methionine sulphone and DNP-DL-glutamic acid was only observed at lower methanol concentrations (<15%), while DNP-ethionine and dansyl-threonine were eluted only at higher methanol concentrations (>15%).

Effect of TEAA concentration. The effect of TEAA concentration on the pseudo-effective mobility and mobility difference between the enantiomers was investigated by varying the TEAA concentration from 5 to 30 m*M*. The methanol concentration in the mobile phase was set at 15% (v/v), and the pH was 4.71. Fig. 7a shows the influence of TEAA concentration on the pseudo-effective mobility of benzoin, hexobarbital, DNP-citrulline, DNP-norleucine and DNP-ethionine. It was noted that the overall trend is the same for all of these solutes, a decrease in the magnitude of the pseudo-effective mobility with increasing TEAA concentration. For benzoin and hexobarbital, the change is relatively small. Calculation of k' for these two neutral solutes reveals that there is no significant effect of TEAA concentration on retention. k' values at 5 and 30 m*M* TEAA are 2.17 and 2.12, respectively, for benzoin, and 1.32 at both concentrations of TEAA for hexobarbital. Thus the reduction in the pseudo-effective mobility parameter for these solutes simply reflects a reduction in electroosmotic flow, and there is an overall increase in migration time with increasing TEAA concentration. For the negatively charged DNP-amino acids, the plots show a steeper decrease in pseudo-effective mobility with increasing TEAA concentration, and there is an overall decrease in migration time.

The effect of TEAA concentration on the mobility difference between the enantiomers is shown in Fig. 7b. The slight reduction in mobility difference for the two uncharged solutes is a reflection only of the slight reduction in overall mobility for these compounds. Calculation of the separation factor, α, reveals no change over the TEAA concentration range investigated here ($\alpha = 1.13$, hexobarbital; $\alpha = 1.06$, benzoin). DNP-Norleucine and DNP-ethionine both show an increase in mobility difference with increasing TEAA concentration, and the same is true for DNP-citrulline up to a TEAA concentration of 20 m*M*, after which resolution decreases with no chiral separation seen with 30 m*M* TEAA. The reduction in retention time and increase in stereoselectivity with increasing TEAA over a certain concentration range indicates that the TEAA concentration is an important parameter to consider when optimizing the cEC separation of anionic species using β-cyclodextrin columns.

From the evidence of electroosmotic flow mobility data, it was suggested above that binding of triethylammonium ion to the cyclodextrin occurs, and that this binding is saturated even at 5 m*M* TEAA. The complete lack of effect of TEAA concentration (from 5 to 30 m*M*) on k' and α for the uncharged compounds supports this supposition. If this is the case, then the effect of TEAA concentration on the separation of the anionic solutes should be explained as an effect on the solutes in solution. Measurements of the effective mobility of DNP-ethionine, DNP-norleucine and DNP-citrulline in a phosphate buffer at pH 4.7 indicate that all three compounds are anions at this pH, and so ion-pairing interactions with TEA^+ may be the cause of the observed effects.

Stability and reproducibility of the β-cyclodextrin packed capillaries

The chemical stability of β-cyclodextrin stationary phase is primarily determined by that of silica gel and the bonded β-cyclodextrin. Most aqueous organic solutions can be used if their pH is within the range of 3.5–7.5 [34]. The stability

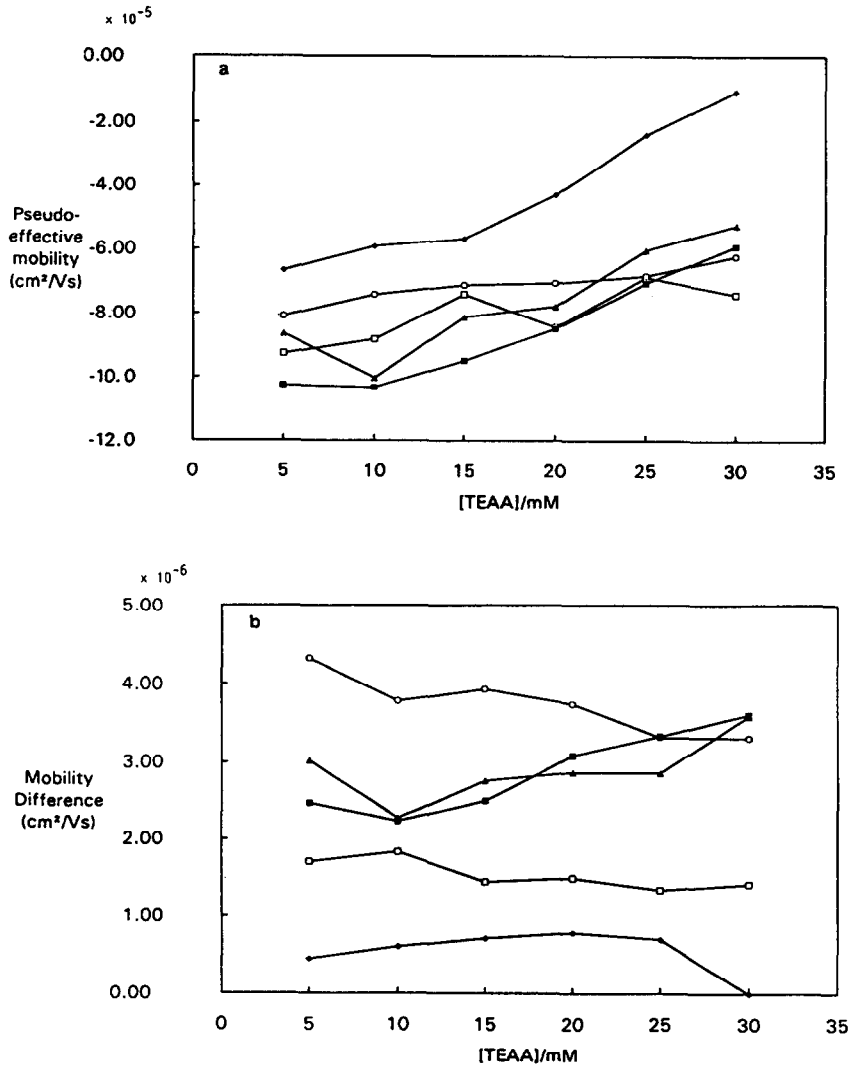


Fig. 7. Effect of TEAA concentration on (a) the pseudo-effective mobility (first eluted enantiomer) and (b) the mobility difference between the enantiomers of (□) benzoin, (○) hexobarbital, (■) DNP-ethionine, (◆) DNP-citrulline and (▲) DNP-norleucine. Packed capillary as in Fig. 1; mobile phase, 15% methanol, pH 4.71; applied voltage, 10 kV.

in aqueous solution at pH > 7.5 is influenced by the hydrolysis of linkage materials. Strongly acidic solutions will cause the hydrolysis of bonded β-cyclodextrin molecules.

As with any packed capillary, air bubbles in the newly packed columns should be washed out by pumping mobile phase through the capillary. Once the capillary is free from air bubbles and well equilibrated with the mobile phase, reasonable reproducibility for retention and resolution can be achieved. The relative standard deviations in the pseudo-effective mobility of benzoin and DNP-norleucine over a period of one week were found to be 2.5 and 3.7%, respectively. The relative standard deviations in the mobility differences were 3.7 and 1.8%, respectively. With TEAA as background electrolyte, the recent history of the capillary is the main factor affecting reproducibility in β-cyclodextrin packed capillaries. Whenever the mobile phase is

changed, the capillary should be equilibrated by electrophoresis of the new mobile phase through the capillary for at least 2 h.

CONCLUSIONS

As in open-tubular capillaries, the direction of electroosmotic flow in β-cyclodextrin packed capillaries is determined by the background electrolyte. With sodium phosphate as background electrolyte, the electroosmotic flow is directed toward the cathode. With TEAA as background electrolyte, the direction of electroosmotic flow is reversed. Thus the choice of background electrolyte will allow for optimized separations of either cationic or anionic species. Of course, neutral compounds may be separated using either buffer system. Judging from the effect of the TEAA buffer on electroosmotic flow in open-tubular fused-silica capillaries, as well as in tubes packed with a β-cyclodextrin stationary phase, this should be a widely applicable method of achieving flow reversal for analysis of anions in cEC. However, the magnitude of electroosmotic flow is likely to vary considerably between different types of stationary phase, being dependent on the degree to which TEAA is adsorbed onto the packing surface. The effect of pH on the electroosmotic mobility in the β-cyclodextrin packed capillaries is less significant than that observed in open-tubular capillaries, probably due to a pH-independent adsorption of TEAA to the uncharged cyclodextrin.

cEC with β-cyclodextrin packed capillaries was successfully applied to the direct enantiomeric separation of both neutral and anionic chiral compounds using methanol–TEAA mobile phases, and of neutral solutes using methanol–phosphate mobile phases. Comparison with free-solution separations using β-cyclodextrin in open-tubular capillaries showed that similar HETP values could be achieved using the TEAA–methanol mobile phase in both open-tube and packed-capillary systems. Free solution measurements revealed that β-cyclodextrin binds TEAA, forming a charged complex. The effect of TEAA concentration and the fraction of methanol in the mobile phase were investigated in packed systems. The effect of increasing fractions of methanol was to reduce retention and enantioselectivity. The effects of TEAA concentration were found to be difficult to interpret for anionic solutes, reflecting the complex set of equilibria which are occurring, involving ion pairing in solution and binding to the stationary phase.

The results reported here suggest that the wide variety of chiral selectors which are used in HPLC may be applied in CE using packed systems. Not all of these may be suitable for use as additives in CE for reasons of solubility, cost or excessive detector response. Separations performed using packing materials with 5 μm particle diameters, such as those shown here with β-cyclodextrin or in our previous results with AGP [5], serve to investigate the effects of the various operating parameters in electrochromatographic systems. The full potential of cEC separation systems will be realized with smaller diameter packings.

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