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Capillary electrochromatography: operating characteristics and enantiomeric separations

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Abstract

Some fundamental aspects of capillary electrochromatography (CEC) (electroosmotic flow, capacity factor, plate height) were studied by carrying out the separation of some neutral compounds with capillaries packed with octadecylsilica particles (ODS). No loss of efficiency up to a linear electroosmotic velocity of 1.3 mm/s was observed for retained analytes (capacity factors varying from 0.7 to 2.5). The extra-column dispersions caused by the frit and the unpacked section separating the frit from the detection window were estimated. Chiral separations of the neutral enantiomers of chlorthalidone by packed capillary electrochromatography was successfully achieved using two approaches: (a) use of the chiral agent, hydroxypropyl-β-cyclodextrin (HPβCD) directly in the mobile phase with an achiral stationary phase (3 μm ODS); (b) use of a chiral stationary phase (5 μm HPβCD-bonded silica particles) with an achiral mobile phase. As with liquid chromatography, the second method achieves higher selectivity and resolution in a shorter analysis time.

Keywords: Capillary electrochromatography; Enantiomer separation

1. Introduction

As first demonstrated by Pretorius et al. [1], capillary electrochromatography (CEC) is an emerging separation technique. It consists in the application of an electrical field between both ends of a capillary (5–500 μm I.D.) that contains a stationary phase. The mobile phase is then driven through the capillary by electroosmosis. The stationary phase may be either bonded to the walls of the capillary (open tubular CEC) [2–11] or packed as small particles (1–10 μm) in the capillary (packed CEC) [1,12–24]. Solutes are separated according to their partitioning between both phases and to their ratio of charge to friction coefficient (electrophoretic mobility). The nearly flat profile of the electroosmotic flow promises higher efficiencies than those obtained with a parabolic pressure-driven flow. In effect, Jorgenson and Lukacs [12] reported reduced plate heights as small as 1.9 for a 170 μm I.D.

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capillary packed with 10-μm octadecylsilica (ODS) particles. Using 3- and 5-μm ODS particles, Knox and Grant [17] confirmed that reduced plate heights were lower in CEC than in LC. Yamamoto et al. [18] observed no loss of column efficiency up to a linear electroosmotic velocity of ca. 3 mm/s. Retention times were reproducible with a relative standard deviation less than 2% [18,19]. Using a pressurized electrochromatographic system that minimizes bubble formation, excellent efficiency (300 000 theoretical plates per meter) was obtained by Smith and Evans [24]. Recently, Yan et al. [20] also demonstrated the high efficiency of packed CEC by separating 16 PAHs. Using a 150 μm I.D. capillary packed with 90% 3-μm ODS and 10% 1-μm bare silica particles, efficiencies up to 400 000 theoretical plates per meter were generated when laser-induced fluorescence detection was performed within the column packing.

Chiral separations of enantiomers have been extensively studied during the last fifteen years. Diverse chromatographic methods including gas (GC) [25], liquid (LC) [26–29] and supercritical fluid chromatography (SFC) [30] are the primary methods for optical isomer separation. Following the first electrokinetic separations of enantiomers by Gassmann and co-workers [31,32], many studies have been carried out to assess the potential of capillary electrokinetic techniques such as zone electrophoresis (CZE), gel electrophoresis, micellar electrokinetic chromatography and electrochromatography [33–37]. These techniques rely on either pre-injection diastereoisomer formation (indirect method) or on formation of diastereoisomers during the separation (direct method). Thus far, most enantiomer separations have been done by the direct method.

Chiral separations by CEC were first demonstrated with open tubular columns [7–11]. Mayer and Schurig achieved the separation of the enantiomers of non-steroidal anti-inflammatory drugs using a 50 μm I.D. open tubular capillary coated with a dimethylpolysiloxane containing chemically bonded permethylated β-cyclodextrin [7,8]. Efficiencies of 30 000 were obtained for a film thickness of 0.2 μm, but a dramatic loss of efficiency was observed when film thickness was increased. With a dual chiral recognition system involving immobilized cyclodextrin (CD) on the walls and CD in the buffer, selectivity was modified as a function of CD concentration. Chiral separations by packed CEC were reported by Li and Lloyd [22,23]. They investigated immobilized α-acid glycoprotein (AGP) [22] and β-cyclodextrin (βCD) [23] as chiral selectors. Using 50 μm I.D. capillaries packed with 5-μm AGP-bonded silica particles, neutral and cationic enantiomers such as benzoin, cyclophosphamide and hexobarbital were baseline-resolved, but efficiencies were low (5000 theoretical plates). Neutral and anionic enantiomers were separated with 5-μm βCD-bonded silica particles [23]. Reversing the electroosmotic flow using triethylamine was necessary to study the anionic solutes. The best efficiency was approximately 30 000 theoretical plates.

Cyclodextrins (CD) have been extensively used as a chiral agent in chromatography and in electrokinetic techniques. They are cyclic oligosaccharides that resemble a truncated cone. The interior of the cavity is relatively hydrophobic while its external surface is hydrophilic. A remarkable property of CDs is their ability to form inclusion complexes. In chromatography, CDs have been used either as an additive in the mobile phase [38–41] or immobilized on the stationary phase [42–45]. In capillary electrophoresis, CDs are in general added directly to the buffer [33–37]. Their immobilization has also been carried out. They can be either coated on the wall [7–11] or bonded to silica particles [23].

In this paper, some basic aspects of CEC (electroosmotic flow, capacity factor, plate height) were studied by carrying out the separation of neutral compounds with ODS-packed capillaries. Two approaches have been investigated to achieve the chiral separation of chlorthalidone, a neutral drug having a diuretic and hypertensive activity, by packed CEC: addition of hydroxypropyl-β-cyclodextrin (HPβCD) directly in the electrolyte while using an achiral stationary phase (ODS), or use of a chiral stationary phase (HPβCD-bonded silica particles). Separation of a basic drug, mianserin, was also studied with the chiral stationary phase. The

influence of organic modifier (acetonitrile) is shown.

2. Experimental

2.1. Apparatus

Separations were performed using a home-built system with an absorbance detector (Isco CV4, Lincoln, NE, USA). Capillaries (50 μm I.D., 363 μm O.D.) were obtained from Polymicro (Phoenix, AZ, USA). The capillary outlet was contained in an interlocked Plexiglas box. The injection end was connected to the ground while the detection end was at high negative voltage. The current through the system was measured by monitoring the voltage drop across a 10-kΩ resistor at the ground side of the capillary. Injections were performed electrokinetically. Data were collected with an Adysis 486 computer (Santa Clara, CA, USA) using Lab Calc data acquisition software and a Chrom-1AT data acquisition board (Galactics, NH, USA). For the packing of the capillaries and for their conditioning, a Brownlee G Model microsyringe pump was used. Frits were made with either a micro-flame or Teledyne wire stripper (Specialized Products, Anaheim, CA, USA).

2.2. Chemicals

Hydroxypropyl-β-cyclodextrin (HPβCD) and 5-μm hydroxypropyl-β-cyclodextrin-bonded silica particles (Cyclobond I 2000 RSP) were supplied as a gift from Astec (Whippany, NJ, USA). Particles of 3-μm octadecylsilica (S3ODS1, 7% endcapped) and 5-μm bare silica were purchased from Phase Separations (Norwalk, CT, USA). Chlorthalidone and mianserin were obtained from Sigma (St. Louis, MO, USA). Disodium hydrogen phosphate, phosphoric acid, methanol, acetonitrile, thiourea (T), benzyl alcohol (BA), benzaldehyde (BD) and benzene (BZ) were purchased from Aldrich (Milwaukee, WI, USA). All solutions were prepared using water from a Millipore filter system (Milli-Q Plus/UV, Milli-RO 6 Plus, Millipore, Bedford, MA, USA). Electrolytes were prepared by mixing in different proportions acetonitrile–disodium hydrogen phosphate adjusted to the desired pH (6.5 or 7.5) with concentrated phosphoric acid. Analytes were dissolved in an electrolyte having an acetonitrile content slightly different from the running electrolyte.

2.3. Preparation of packed capillaries

Capillaries of 50 μm I.D. were packed with either 3-μm ODS or 5-μm HPβCD-bonded silica particles using a procedure similar to the one developed by Yamamoto et al. [18]. An outline of the successive steps is briefly recalled and illustrated in Fig. 1. A thermal wire stripper was used to prepare the middle frit. This device allows us to have a better control of the quality of the frit, which is mainly dependent on the type of particles, the solvent and the diameter of the capillary. The packed part was ca. 25–30 cm long, the window was located 2–4 mm after the outlet on-column frit, and the total length was ca.

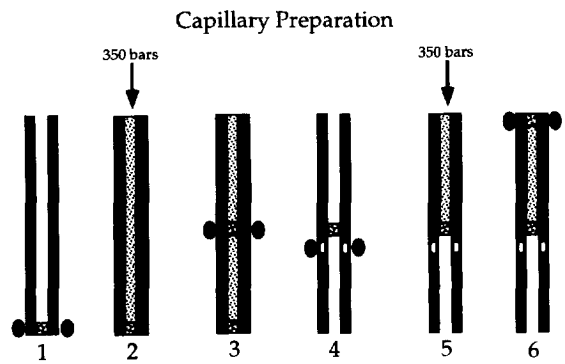

Fig. 1. Packing procedure. (1) Preparation of a frit by sintering 5-μm bare silica particles using a microflame torch. (2) Packing of the capillary by pumping a slurry of 5-μm silica particles (100 mg/ml in methanol) under 350 bars for 3 h. (3) Preparation of a frit at the desired distance from the inlet using a thermal wire stripper. (4) After cutting the initial frit, emptying the capillary by flushing from both ends and preparation of a detection window. (5) Packing of the capillary by pumping the slurry of the particles to be used (100 mg/ml) under 350 bars for 3 h. (6) After replacing the upper layer (ca. 1 mm) of the packing with silica particles, preparation of an inlet frit by gently sintering these particles.

50–60 cm. Once the capillary was packed, it was flushed with the selected mobile phase by pressurizing the capillary inlet at 70 bars for ca. 1 h prior to performing the separations. This procedure conditions the stationary phase and removes any bubbles.

3. Results and discussion

3.1. Preparation of the capillaries

The packing procedure is crucial to the successful development of packed CEC. Our first attempts consisted in connecting a completely packed capillary with an empty one used for the detection through a PTFE sleeve. This approach was less reliable because of the formation of a bubble at this connection. Therefore, we developed a method using an on-column frit that can be generalized to any type of particles. This method was particularly appropriate for HPβCD-bonded silica particles because these particles are difficult to sinter directly. The use of a thermal wire stripper allowed us to prepare narrow and reproducible frits without disrupting the packing. These frits should be sufficiently tight to retain the particles but loose enough to allow fast flow. No attempt was made to optimize the slurry solvent and packing pressure. The conditioning step of the capillary after completion of the packing appeared to be particularly essential for the HPβCD-bonded silica particles. When applying the electrical field at the extremities of a HPβCD-bonded silica particle packed capillary which has been subjected to a short conditioning period (flushing for 1 to 24 h at 70 bars), the current kept increasing and the absorbance signal was erratic, giving ghost peaks. A microscopic observation of the unpacked part of the capillary column showed small zones (approximately 300 μm long) having a different refractive index from that of the buffer. As an example, for one of the capillaries, the current increased from 10 μA to 250 μA over a period of 6 h while applying a voltage of 10 kV and using a CH_3CN–10 m*M* phosphate buffer pH 7.5 (50:50, v/v) electrolyte whereas for an unpacked capillary with similar characteristics, the current was stable at 25 μA. The influence of buffer nature, buffer pH, and temperature were then studied. None of these parameters were shown to be responsible for this behavior. A long conditioning was then attempted: a CH_3CN–1 m*M* phosphate buffer pH 6.5 (50:50, v/v) electrolyte was flushed through the capillary at 210 bars for one week. This represented about 15 ml, i.e. 15 000 times the volume of the capillary. This conditioning resulted in a constant current of ca. 1 μA for a voltage of 15 kV and a stable baseline. It is possible that some molecules were "leaching" from the particles and the particles were not clean enough for electrochromatography. Li and Lloyd [23] used for their experiment some β-cyclodextrin-bonded silica particles from an extensively used Cyclobond I chromatographic column. This fact may explain the successful separations they obtained without further cleaning of the particles.

3.2. Properties of electrochromatography in a packed capillary

The separation of small molecules, thiourea (T), benzyl alcohol (BA), benzaldehyde (BD) and benzene (BZ) was achieved with a capillary packed with 3-μm ODS particles. Fig. 2 shows a typical chromatogram. Theoretical plate numbers per meter were between 70 000 and 175 000 (calculated using the equation $N = 5.54\ (t_R/\omega_{0.5})^2$, where N, t_R, $\omega_{0.5}$ represent the number of theoretical plates, the retention time of the analyte, and the width at half height of the peak, respectively, and normalized per meter of column length).

Electroosmotic flow (EOF)

In packed CEC, electroosmosis is the fundamental process that carries analytes from the injection end toward the detection window. The electroosmotic velocity is defined by:

$$v_{eo} = \mu_{eo} E = \frac{\epsilon \epsilon_0 \zeta}{\eta} E \qquad (1)$$

with

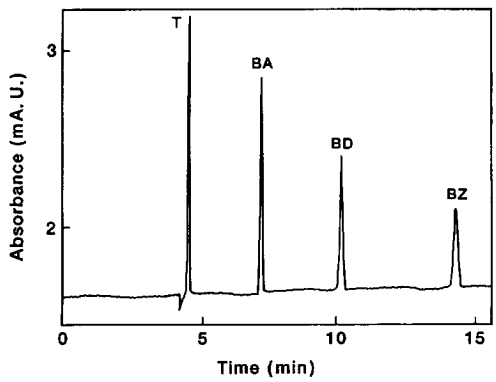

Fig. 2. Separation of thiourea (T), benzyl alcohol (BA), benzaldehyde (BD), and benzene (BZ) by packed capillary electrochromatography. Capillary: 54.6 cm × 50 μm I.D. (28.6 cm to detector), 28.3 cm packed with 3-μm ODS particles. Electrolyte: CH_3CN–phosphate buffer pH 6.5 (50:50, v/v) (5 m*M* Na_2HPO_4, adjusted to pH 6.5 with concentrated H_3PO_4); V = 25 kV (I = 1.8 μA); UV detection at 220 nm. Sample: T, 0.5 m*M*; BA, 1.4 m*M*; BD, 1.4 m*M*; and BZ, 3.75 m*M*. Electrokinetic injection: 5 s at 10 kV.

$$\zeta = \frac{\delta\sigma}{\epsilon\epsilon_0} \quad (2)$$

where μ_{eo} is the electroosmotic mobility, E the electrical field, ϵ_0 the permittivity of vacuum, ϵ the relative permittivity, ζ the zeta potential, η the viscosity, δ the double layer thickness and σ the surface excess charge density. The double layer thickness is inversely proportional to the square root of the molar concentration of the buffer. The flow has a nearly flat profile and is independent of the diameter of the particles d_p provided that $d_p/\delta > 40$ [1,14,17]. Using a buffer with a 0.5 m*M* concentration, the minimum particle diameter is 0.6 μm. Therefore, with 3-μm ODS particles and an acetonitrile–1 m*M* phosphate buffer pH 6.5 (50:50, v/v) electrolyte, the flow is expected to have a plug profile.

The EOF obtained in packed CEC is lower than that obtained in capillary zone electrophoresis (CZE) [17,18,22]. This behavior is caused in part by lack of alignment of the flow channels in the packed bed with the capillary axis and for another part by surface modification of the silica particles, which leads to a decrease of free silanol surface density, i.e., a decrease of the zeta potential. With CH_3CN–(5–20 m*M*) sodium hydrogen phosphate pH 6.5–7.5 (50:50, v/v) electrolytes and a capillary packed with 3-μm ODS particles, the electroosmotic velocity was 40–50% of that in CZE.

Following the work of Yamamoto et al. [18], we intended to use thiourea as a marker for the EOF. Therefore, samples with 0.5 m*M* thiourea were prepared in an electrolyte having an acetonitrile content 10% higher than the running electrolyte. For running electrolytes having a CH_3CN content greater than 40%, the perturbation, created by the difference of acetonitrile between the running and sample electrolyte, appeared before the peak of thiourea whereas the reverse situation was observed when this content was less than 40% (Fig. 3). This observation led us to question the EOF marker and led us to measure the EOF from the perturbation caused by the difference of acetonitrile content between the separation electrolyte and the sample solution.

As shown in Eq. (1), the EOF varies linearly

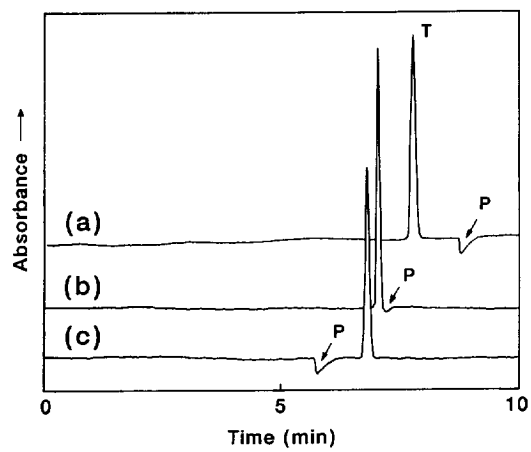

Fig. 3. Influence of acetonitrile percentage on the retention of thiourea (T). Capillary: 53.5 cm × 50 μm I.D. (25.9 cm to detector), 25.7 cm packed with 3-μm ODS particles. Electrolyte: CH_3CN–phosphate buffer pH 6.5 in different proportions (v/v)–(a) 30:70, (b) 40:60, and (c) 50:50 (1 m*M* Na_2HPO_4, adjusted to pH 6.5 with concentrated H_3PO_4); V = 15 kV; UV detection at 220 nm. Sample: T, 0.5 m*M* in an electrolyte CH_3CN–phosphate (v/v)–(a) 40:60, (b) 50:50, and (c) 60:40. Electrokinetic injection: 5 s at 15 kV. P, perturbation caused by the difference of acetonitrile content between the running and sample electrolytes.

with the electrical field as long as Joule heating is negligible. This aspect was shown experimentally by Yamamoto et al. [18] and by Li and Lloyd [22]. In this study, the linearity of EOF versus the electrical field (from 100 to 500 V cm^{-1}) was demonstrated with a 3-μm ODS packed capillary using CH_3CN–5 m*M* phosphate buffer pH 6.5 electrolytes of different proportions. For a 50:50 (v/v) composition, the linear interpolation yielded a correlation coefficient of 0.999. Therefore, Joule heating seemed to be negligible, although no cooling system was used.

The variation of the electroosmotic mobility with the proportion of acetonitrile–1 m*M* phosphate buffer pH 6.5 is shown in Fig. 4. An increase of the electroosmotic mobility was observed when the acetonitrile content was varied from 40% to 80%. This behavior may be explained by the concommitant decrease in viscosity and ionic strength (the higher the acetonitrile content was, the lower the concentration of phosphate was, causing the double layer thickness to increase as well as the zeta potential) resulting from the increased content of acetonitrile. It is to note that Schwer and Kenndler [46] observed the reverse behavior on increasing the acetonitrile content in an empty fused-silica capillary keeping the ionic strength constant. Yamamoto et al. [18] observed a decrease of the electroosmotic mobility with the proportion of acetonitrile–4 m*M* phosphate buffer pH 9.2 in the range of 0 to 60% of acetonitrile with a capillary packed with 1.6-μm ODS particles. This difference of acetonitrile range (which influences the viscosity variations) and the use of thiourea as an electroosmotic flow marker may explain the observed variation difference.

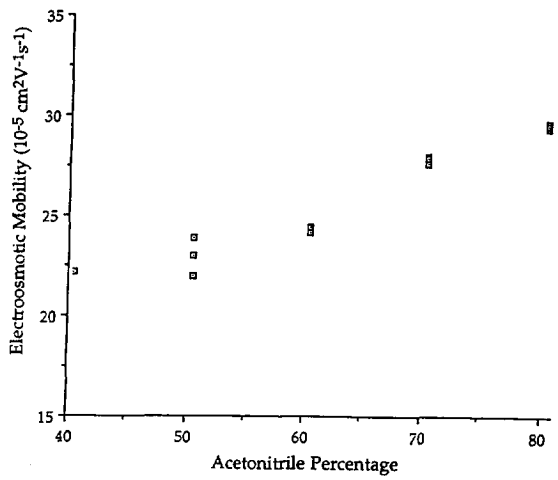

Fig. 4. Variation of the electroosmotic mobility with the proportion of acetonitrile–1 m*M* phosphate buffer pH 6.5. Separation conditions as in Fig. 2, except applied voltage V = 15 kV and acetonitrile content, which varies between 40% and 80%.

Capacity factor

As in conventional LC, the capacity factor (k') can be defined as the ratio of analyte quantity in the stationary phase to analyte quantity in the mobile phase. The velocity v of the analyte is related to its apparent electrophoretic velocity v_{app} in the mobile phase by:

$$v = \frac{1}{1+k'} v_{app} = \frac{1}{1+k'}(v_{eo} + v_{ep}) \quad (3)$$

where v_{ep} is the effective electrophoretic velocity of the analyte.

For neutral solutes, the effective electrophoretic velocity is equal to zero. Hence, Eq. (3) can be written [22]:

$$k' = \frac{t_R - t_{eo}}{t_{eo}} = \frac{\mu_{eo} - \mu}{\mu} \quad (4)$$

with

$$\mu_{eo} = \frac{E}{v_{eo}} = \frac{E t_{eo}}{l}$$

and

$$\mu = \frac{E}{v} = \frac{E t_R}{l}$$

where t_R is the retention time, μ is the apparent electrophoretic mobility of the analyte in the packed capillary, t_{eo} is the electroosmotic time, μ_{eo} is the electroosmotic mobility, and l the distance from the injection point to the detection point.

For charged analytes, Eq. (3) becomes:

$$k' = \frac{t_R - t_{eo}}{t_{eo}} + \frac{t_R}{t_{eff}} = \frac{\mu_{eo} - \mu}{\mu} + \frac{\mu_{eff}}{\mu} \quad (5)$$

where t_{eff} and μ_{eff} are, respectively, the effective retention time and the effective electrophoretic mobility of the analyte in the same analytical conditions (buffer, porosity) as the ones used with the packed capillary but without any partition mechanism between the two phases. Under these conditions, the analyte is characterized by a migration time t' and an apparent mobility μ'. Here, t_{eff} and μ_{eff} are determined by:

$$t_{eff} = \frac{t' t_{eo}}{t' - t_{eo}} \quad (6)$$

and

$$\mu_{eff} = \mu' - \mu_{eo} \quad (7)$$

The values of t' and μ' can be approximated by those obtained in an open tube by CZE using the same buffer conditions, but this approach does not take into account the obstacles arising from the particles in the packed bed. In packed CEC, it is therefore difficult to calculate a capacity factor for charged analytes.

The influence of acetonitrile on capacity factors of benzyl alcohol, benzaldehyde and benzene was studied using CH_3CN–5 m*M* or 1 m*M* phosphate buffer pH 6.5 electrolytes of different proportions. As the acetonitrile content decreases, the expected increase in k' was observed.

Plate height

As in LC, plate height is determined by flow dispersion, axial diffusion, resistance to mass transfer, and extra-column effects (connective tubing, frit, detection window, etc.) [15]. In the range of the reduced velocities experienced ($\nu <$ 3, corresponding to electroosmotic velocities up to 1.3 mm/s) and for the analytes studied representing a k' range of 0.7 to 2.5, no increase of the plate height was observed (Fig. 5). It appeared that the resistance to the mass transfer from one phase toward the other did not make a dominant contribution to the plate height up to a reduced velocity of 3. The best separation regarding the efficiency shown in Fig. 2 was characterized by 52 200 theoretical plates for benzyl alcohol ($k' =$ 0.74), 47 900 for benzaldehyde ($k' = 1.45$) and

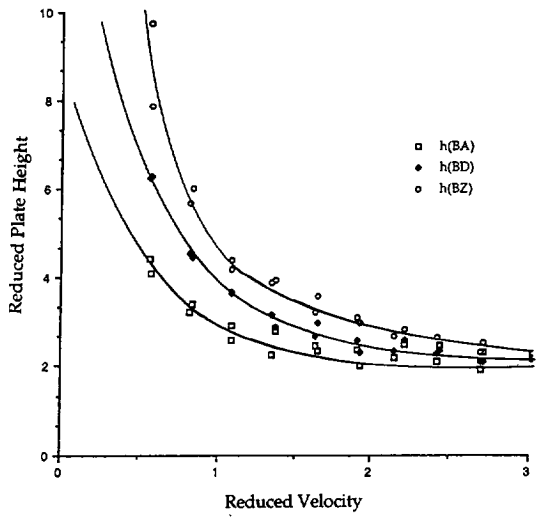

Fig. 5. Reduced plate height versus reduced electroosmotic velocity. Separation conditions as in Fig. 2, except applied voltage, varying between 5 kV and 27.5 kV. A reduced velocity of 3 corresponded to an electroosmotic velocity of 1.3 mm/s and an electrical field of 500 V/cm.

43 000 for benzene ($k' = 2.45$). The benzyl alcohol separation efficiency is equivalent to a reduced plate height of 1.8.

It is interesting to estimate the variances produced by the frit and the connective tubing, and to compare them to the column variance. The minimum volumic variance produced by the packed capillary can be estimated as that of an unretained analyte:

$$(\sigma_V^c)^2 = \frac{(\epsilon_c V_c)^2}{N} \quad (8)$$

where V_c is the volume of the packed part of the capillary and ϵ_c the porosity. Taking $\epsilon_c = 0.75$ and $N = 50\,000$, we obtain a value of 3.5 nL^2 for a 50 μm I.D. capillary packed over a length of 28.3 cm.

The frit is similar to a packed bed without analyte partitioning. If a turbulent regime is assumed to be present in the frit, then the volumic variance is the square of the internal volume. Assuming a frit length of 1 mm and a porosity of 0.5, the frit variance can be estimated to 1 nL^2 in the worst case (turbulent regime), which represents 28% of the column variance.

The variance created by the unpacked section

separating the frit from the detection window should remain less than that produced under laminar flow conditions in an open tube:

$$(\sigma_V^t)^2 = \frac{d_t^4 L_t \pi F}{384 D_m} \quad (9)$$

where d_t and L_t are the diameter and length of the tube, F the flow-rate and D_m the diffusion coefficient of the analyte. Considering here a length of 3 mm, a linear velocity of 1.3 mm/s and a diffusion coefficient of $1.3 \cdot 10^{-9}$ m^2 s^{-1} for an 50:50 (v/v) acetonitrile–phosphate buffer electrolyte, this contribution is estimated to 0.2 nL^2, which represents 6% of the column dispersion.

These extra-column dispersions represent at most together 34% of the column dispersion, which means that the efficiency before the frit should be approximately 70 000 plates, corresponding to 250 000 theoretical plates per meter. For higher efficiency, it appears necessary to reduce these extra-column effects or to perform the detection through the packing material, namely by laser-induced fluorescence [20].

Reproducibility

Reproducibility (run-to-run and day-to-day) of the EOF, retention times and capacity factors are reported in Table 1. Relative standard deviations on retention times were less than 3% with a CH_3CN–5 m*M* phosphate buffer pH 6.5 (50:50, v/v) electrolyte and to 1.1% with a CH_3CN–phosphate (80:20, v/v) electrolyte. It appeared that the reproducibility was better at high percentages of acetonitrile, which can be explained by less occurrence of microbubbles and lower heat generation. Day-to-day reproducibility for retention times was better than 3.7%. The use of a commercial apparatus with thermoregulation is expected to improve these results.

3.3. Chiral separations

Two general approaches exist for the direct liquid chromatographic separation of enantiomers: one involves use of chiral mobile phase additives with an achiral stationary phase; the other makes use of a chiral stationary phase. These two methods were investigated here for packed CEC. Hydroxypropyl-β-cyclodextrin (HPβCD) was selected as the chiral agent either as a mobile phase additive or as a moiety bonded to 5-μm silica. These particles are used in conventional chromatographic columns (Cyclobond I 2000 RSP) and have successfully resolved enantiomers where the chiral center is adjacent to an aromatic ring and/or where hydrogen bonding groups or acceptor sites are within 3–4 carbons of an aromatic group [45,47]. Chlorthalidone (Fig. 6), a drug having a diuretic and hypertensive activity, was chosen as a neutral test analyte. Its enantiomers cannot be separated by CZE using a neutral chiral agent but were previously separated by LC with a Cyclobond I RSP column [45] and a mobile phase of CH_3CN–70 m*M* triethylamine (5:95, v/v) adjusted to pH

Table 1
Reproducibility of retention time and capacity factor in packed CEC for different electrolytes

	CH_3CN (%)	μ_{eo} (10^{-5} $cm^2/V\,s^{-1}$)	R.S.D. (%) (μ_{eo})	R.S.D. (%) BA		R.S.D. (%) BD		R.S.D. (%) BZ	
				t_R	k′	t_R	k′	t_R	k′
A	50	24.8	3.5	2.9	2.2	2.8	2.9	2.8	3.6
	80	30.3	0.8	1.1	2.1	1.1	1.8	1.1	1.7
B	50	24.3	4.1	3.7	2.5	2.7	4.7	2.7	6.3

A = Run-to-run; B = day-to-day. CH_3CN–5 m*M* phosphate buffer pH 6.5 (v/v): A, 50:50 (8 replications) and 80:20 (5 replications); B, 50:50 (4 replications). Experimental conditions as in Fig. 2.

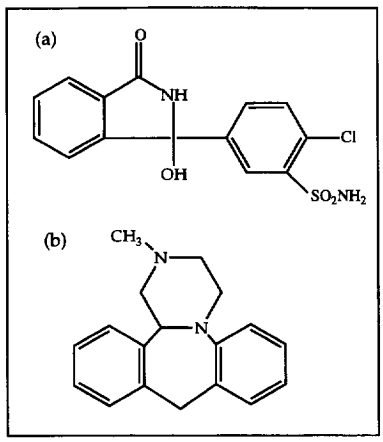

Fig. 6. Optically active analytes studied: (a) chlorthalidone and (b) mianserin.

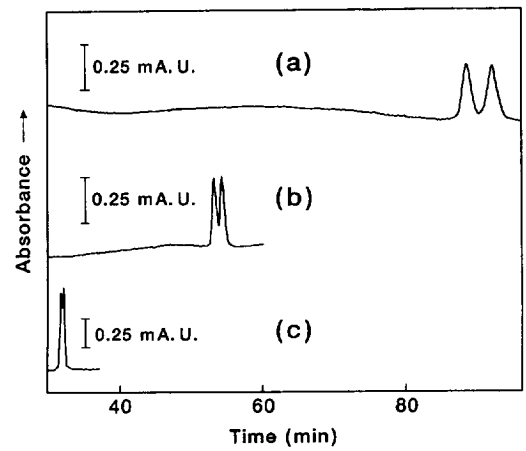

Fig. 7. Influence of acetonitrile percentage on the separation of chlorthalidone enantiomers by packed CEC using HPβCD as a mobile phase additive. Capillary: 52 cm × 50 μm I.D. (26.2 cm to detector), 25.9 cm packed with 3-μm ODS particles. Electrolyte: CH_3CN–phosphate buffer pH 6.5 in different proportions (v/v)–(a) 15:85, (b) 20:80, and (c) 25:75 with 10 m*M* HPβCD (1 m*M* Na_2HPO_4, adjusted to pH 6.5 with concentrated H_3PO_4); V = 15 kV; UV detection at 220 nm. Sample: 0.05 mg/ml chlorthalidone. Electrokinetic injection: 5 s at 15 kV.

4.1 with acetic acid. The enantiomers were baseline-resolved in 9 min with the following characteristics: $\alpha = 1.31$, $k_1 = 1.50$ and $R_s = 2.8$. Mianserin (Fig. 6) was also tested for chiral separation with the HPβCD chiral stationary phase. The enantiomers of this antidepressive cationic drug were baseline-resolved by CZE using 30 m*M* HPβCD in a 50 m*M* phosphate buffer pH 3.3 [48].

Chiral separation with an ODS stationary phase and a mobile phase containing HPβCD

In a totally achiral environment, chlorthalidone was retained by the ODS stationary phase and presented a capacity factor of 4.4 with CH_3CN–1 m*M* phosphate buffer pH 6.5 (20:80, v/v) as the mobile phase. Chlorthalidone enantiomer separation was then studied with 10 m*M* of HPβCD in CH_3CN–phosphate buffer electrolytes of different proportions (v/v): 15:85, 20:80 and 25:75 (Fig. 7). Baseline separation was obtained for 15% acetonitrile. At least three main factors differentiating elution of enantiomers may be responsible for the separation: (a) differences in stability constants of inclusion complexes between the CD and the enantiomers; (b) differences in partition of CD complexes between the two phases; and (c) differences in partition of free enantiomers between the mobile phase and the CD layer that is retained on the surface of the hydrophobic stationary phase. Unlike βCD but like methylated βCD, HPβCD appeared to be retained on the ODS surface. An ODS packed capillary was flushed electrokinetically (15 kV for an hour) with a CH_3CN–1 m*M* phosphate buffer pH 6.5 (25:75, v/v) electrolyte containing 10 m*M* HPβCD (the equilibration volume represents six times the volume of the packed part). With this electrolyte, no separation was obtained for the first runs. But after 15 h of equilibration, the enantiomers were slightly separated as if a chiral stationary phase were dynamically generated. Acetonitrile content is an important parameter affecting both retention times and stability constants of inclusion complexes. As expected, the results showed that upon decreasing the acetonitrile content, retention, selectivity and resolution increased while efficiency decreased (Table 2). To our knowledge, this study is the first report of the use of a chiral mobile phase additive with an achiral

Table 2
Influence of the percentage of acetonitrile on the separation of chlorthalidone enantiomers by packed CEC using HPβCD as a mobile phase additive and an ODS stationary phase

CH_3CN (%)	t_2 (min)	k'_1/k'_2	α	N_1	R_s
25	33.1	1.98/2.02	1.018	n.d.	<0.7
20	54.0	3.42/3.52	1.029	36 000	1.0
15	91.8	6.26/6.55	1.045	22 000	1.4

Experimental conditions as in Fig. 7; n.d. = not determined.

stationary phase for chiral separations in packed CEC.

Chiral separation with a chiral HPβCD-bonded silica stationary phase

The separation of chlorthalidone enantiomers was further studied with a capillary packed with 5-μm HPβCD-bonded silica particles. As emphasized earlier, these particles needed repeated rinsings before being suitable for investigation in CEC.

Baseline separation of chlorthalidone enantiomers was achieved with a CH_3CN–5 m*M* phosphate buffer pH 6.5 (25:75, v/v) electrolyte. The influence of acetonitrile content was studied between 15% and 30% (Fig. 8). Again, the lower this content was, the higher were the selectivity and the resolution, but at the expense of analysis time (Table 3). A 25% acetonitrile content produced a resolution of 1.7 for an analysis time of 35 min, which seemed to be a good compromise. For a series of four runs under these last conditions, the relative standard deviation was 1% for the electroosmotic velocity and 1.7% for the retention time of the first enantiomer. Finally, the analysis time was shorter than that obtained by packed CEC using the chiral agent in the mobile phase, but efficiency was lower.

The chiral separation of the cationic enantiomers of mianserin was also attempted. Slight separation was achieved by chiral packed CEC with a CH_3CN–10 m*M* phosphate buffer pH 7.5 (50:50, v/v) electrolyte (Fig. 9). The peak of the second enantiomer was characterized by a strong tailing owing to the electrostatic interaction with

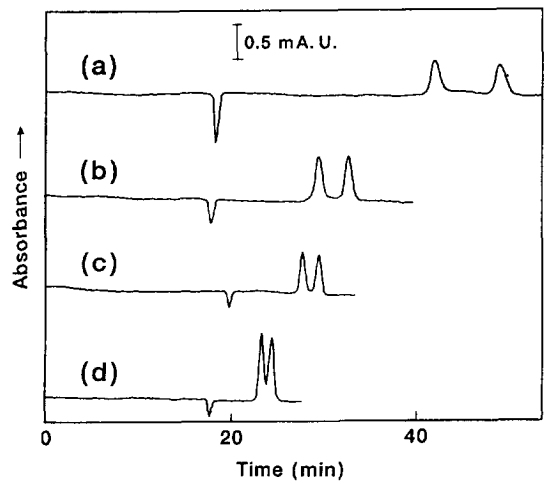

Fig. 8. Influence of acetonitrile percentage on the chiral separation of chlorthalidone enantiomers by packed CEC using a chiral stationary phase. Capillary: 58 cm × 50 μm I.D. (27.2 cm to detector), 27 cm packed with 5-μm HPβCD-bonded silica particles. Electrolyte: CH_3CN–5 m*M* phosphate buffer pH 6.5 in different proportions (v/v)–(a) 15:85, (b) 20:80, (c) 25:75, and (d) 30:70 (5 m*M* Na_2HPO_4, adjusted to pH 6.5 with concentrated H_3PO_4); V = 15 kV; UV detection at 220 nm. Sample: 0.05 mg/ml chlorthalidone in an electrolyte CH_3CN–1 m*M* phosphate pH 6.5 (v/v)–(a) 30:70, (b) 30:70, (c) 35:65 and (d) 40:60. Electrokinetic injection: 5 s at 15 kV.

the negatively charged particles. The influence of acetonitrile (40% to 80%) was also studied. As before, resolution was improved at lower percentages of acetonitrile, but the tailing became more severe. The electrolyte composition for the separation needs to be optimized with respect to pH, organic solvent nature, and introduction of more specific additives (triethylamine, urea).

Table 3
Influence of the percentage of acetonitrile on the separation of chlorthalidone enantiomers by packed CEC using 5 μm HPβCD-bonded silica particles as the chiral stationary phase

CH_3CN (%)	t_2 (min)	k'_1/k'_2	α	N_1/N_2	R_s
30	24.3	0.34/0.40	1.18	8 800/8900	1.0
25	29.5	0.41/0.50	1.23	10 700/11700	1.7
20	32.8	0.68/0.87	1.28	9 900/9400	2.6
15	48.7	1.31/1.69	1.30	10 000/6900	3.4

Experimental conditions as in Fig. 8.

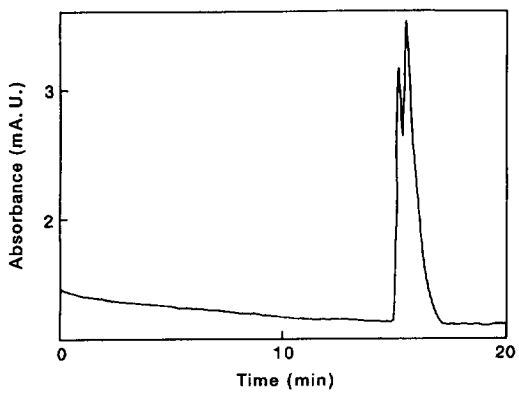

Fig. 9. Chiral separation of mianserin enantiomers by packed CEC using a chiral stationary phase. Capillary: 55 cm × 50 μm I.D. (21.9 cm to detector), 21.6 cm packed with 5-μm HPβCD-bonded silica particles. Electrolyte: CH_3CN–phosphate buffer pH 7.5 (50:50, v/v) (10 m*M* Na_2HPO_4, adjusted to pH 7.5 with concentrated H_3PO_4); V = 10 kV (I = 1.2 μA); UV detection at 200 nm. Sample: 0.1 mg/ml mianserin. Electrokinetic injection: 5 s at 10 kV.

4. Conclusion

The used packing procedure is suitable for capillary columns with 50 to 100 μm I.D. and can be generalized to any kind of rigid particles. This study demonstrated that the conditioning of the particles was an important step in the preparation of capillary columns. It seemed that a higher particle purity is required in CEC than in LC, which is likely caused by the miniaturization of the technique and the use of an electrical field.

Through the separation of neutral molecules, fundamental parameters of CEC were studied. Within the range of reduced electroosmotic velocity usually accessible (up to 3), no loss of efficiency was observed for retained analytes and the resistance to the mass transfer from one phase toward the other did not make a dominant contribution to the plate height. On the contrary, the dispersion from the frit and the connective tubing between the frit and the detection window could represent up to one third of the column dispersion.

This study also confirmed the feasibility of packed CEC for chiral separations. The use of a chiral agent directly in the mobile phase with an achiral stationary phase and that of a chiral stationary phase were both investigated. As in LC, the second approach achieved better resolution and shorter analysis time because of an increased selectivity, but separation efficiency was much lower.

Packed CEC has the capability to separate simultaneously neutral and charged molecules. Preparation of the capillaries (frit, suspension, packing pressure) and optimization of the separations will need to be perfected further for this technique to realize its full potential as a new separation method.

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