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Separation of enantiomers by packed capillary electrochromatography on a cellulose-based stationary phase

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Abstract

Separation of enantiomers was performed by applying packed capillary electrochromatography (CEC). Fused-silica capillaries of different lengths with an inner diameter of 100 μm were packed with a cellulose derivative immobilized onto macroporous silica gel. Parameters such as content of modifier in the mobile phase, concentration and pH of the buffer were varied for a set of test capillaries to determine their influence on enantioselectivity. In packed CEC the highest influence on resolution of the test racemates was found by changing the acetonitrile content, while variation of the buffer concentration mostly affects the electroosmotic velocity. The performance of packed CEC and nano-LC was also compared. Packed CEC showed much better column efficiency and enantioselectivity under similar flow/electroosmotic velocity.

Keywords: Enantiomer separation; Electrochromatography; Chiral stationary phases, electrochromatography; Immobilized cellulose derivatives; Mobile phase composition; Nano-LC

1. Introduction

Since the first application of high voltages over columns packed with macroparticle stationary phases by Pretorius et al. [1], it took about 10 years until capillary electrochromatography (CEC) emerged as one of the most exciting developments in the field of separation technologies. In CEC an electrical field is applied across a capillary that contains a stationary phase, which causes the mobile phase to flow by electroosmosis with a nearly flat flow profile resulting in higher efficiencies compared to those obtained with pressure-driven systems.

For the separation of enantiomers by CEC on chiral stationary phases (CSPs) two approaches, packed or coated capillaries, have been applied. In packed CEC capillaries with inner diameters of 50 to 100 μm are packed with a chiral packing material such as those typically used for high-performance liquid chromatography (HPLC). Various chiral phases have already been evaluated for this purpose, including protein-based stationary phases immobilized on silica particles [2,3], β-cyclodextrin [4], hydroxypropyl-β-cyclodextrin [5], permethyl-β-cyclodextrin [6], Pirkle-type stationary phases immobilized on 3 μm silica [7], quinine-based chiral anion-exchange type stationary phase [8], macrocyclic antibiotic teicoplanin [9], and vancomycin [10]. Recently, enantioseparation using covalently attached poly-*N*-acryloyl-L-phenylalanine ethylester (Chiraspher) and one example of enantiomeric separation on cellulose tris(3,5-dimethylphenylcarbamate) coated on silica gel was also shown [11]. The

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applicability of imprinted polymers as chiral monoliths for CEC has also been reported in several papers [12–15].

In open tubular electrochromatography (OT-EC) the chiral selector is coated or bonded onto the inner surface of a capillary (50 μm I.D. or less) [16–21]. Recently, we demonstrated that neat cellulose derivatives such as 3,5-dimethylphenylcarbamoyl cellulose (DMPCC) and *para*-methylbenzoyl cellulose (PMBC) coated onto silica gel, which exhibit remarkable chiral recognition power in analytical and preparative HPLC [22,23], can also be applied as CSPs in OT-LC and in OT-EC [24]. However, with the elaboration of a process for the immobilization of cellulose and amylose derivatives on silica gel, we were now able to prepare chemically much more stable stationary phases [25]. In the present work, we report on the application of this new immobilized polysaccharide material derived from DMPCC as a CSP for the separation of enantiomers by packed CEC.

2. Experimental

2.1. Chemicals

Thiourea was purchased from Merck (Darmstadt, Germany), benzoin from Fluka (Buchs, Switzerland), *trans*-stilbene oxide from Aldrich (Steinheim Germany), indapamide and lormetazepam from Sigma (St. Louis, MO, USA) and glutethimide was available from Novartis (Basel, Switzerland). Acetonitrile was of HPLC-grade and was purchased from Merck, the citrate buffers were bought from Fluka and the phosphate buffer was obtained from Novartis (Basel, Switzerland). Water was provided by a Millipore Milli-Q system (Volketswil, Switzerland). The test racemates were dissolved in the mobile phase (1 mg/ml).

2.2. Instrumentation

The CEC experiments were carried out with two different instruments, a Beckman P/ACE System 5510 (Palo Alto, CA, USA) and a Prince Technologies CE System (Emmen, The Netherlands). The Beckman instrument was modified to provide about 1.4 bar on the inlet and outlet vial. The Prince CE instrument allows a pressure of up to 10 bar to be applied on both ends of the capillary from an external source. Data were recorded with the P/ACE software version 1.1 (Beckman Instruments) or with the DAX software version 1.1 (Prince Technologies).

The nano-LC experiments were performed on a μ-HPLC pump Evolution 200 (Prolab, Reinach, Switzerland). The detector was a Linear UVIS 200 detector (Applied Biosystems) equipped with a Z-cell.

2.3. Preparation of the packed capillaries

The packing material obtained by immobilization of DMPCC on silica gel was prepared in our laboratory according to a process recently developed in our laboratory [25].

A few capillaries (100 μm) were electrokinetically packed by Unimicro Technologies (Pleasanton, CA, USA) [26]. Capillaries packed in the laboratory (fused-silica capillaries, 100 μm I.D., BGB Analytik, Anwil, Switzerland) were prepared following a procedure elaborated by Bruin and Mayer [27] for silica-based reversed-phase materials, according to the method described by Seifar et al. [28]. As the packing conditions depend on the physico–chemical properties of the packing material, the original procedure had to be adapted to our cellulose-based CSP. All capillaries were packed with a Shandon pump (Shandon Southern Products, UK) at pressure of 500 bar. Two different packing procedure and frit preparation techniques were applied depending on the CE instrument used.

In both cases about 30 mg of the packing material was suspended in 300 μl of acetone and ultrasonicated for about 30 min. The slurry was transferred immediately with a pipette to the slurry reservoir consisting of a steel HPLC column (23 cm×0.5 μm I.D.). The suspension was pumped into the capillary using ethanol at 300 to 500 bar pressure for 1 h. During this step the capillary was placed in a sonication bath. The packing material was retained at the end of the capillary by a HPLC in-line filter. Frits have been prepared in two different ways:

(a) After packing, inlet and outlet frits were made by sintering the CSP on a small portion of the packed capillary using an electric filament heated to

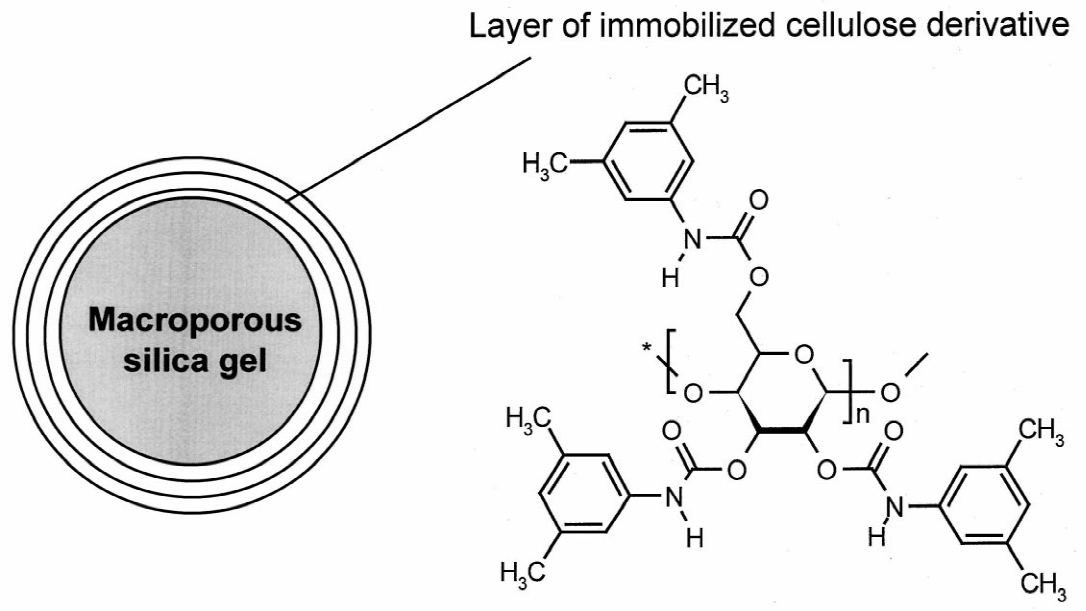

Fig. 1. Structure of the chiral stationary phase.

500°C. First the frit was sintered near the HPLC in-line filter under a pressure of 200 bar by placing a small part (about 1 mm) of the capillary for 60 s on the electric filament. Then the frit near the end of the packing bed was sintered. Afterwards the HPLC in-line filter was removed and the capillary was cut off 0.5 cm after each frit. The capillary was flushed with ethanol at 100 bar to remove the excess of packing material and with buffer at 50 bar for conditioning. The capillaries prepared in this manner were 20 cm and one was 8 cm long. The packed capillary was connected to a 30 cm long transparent fused-silica capillary with a polyethylene tubing. The advantage of using a UV transparent capillary as a transfer line to the outlet buffer vial is that packed columns of different lengths can be installed very easily without burning a new UV window. The distance between the 20 cm long packed section connected to the transparent capillary and the UV cell was 2 cm.

(b) The second packing technique was applied for the preparation of capillaries to be used with the Beckman P/ACE instrument. The cartridge technique of this instrument imposes a minimum capillary length of 27 cm (or plus a multiple of 10 cm). This means that the packed bed has to be maximum 19 cm in length, otherwise the optical window has to be placed in the packed part, what is not recommended due to the decrease in sensitivity. After burning the inlet and outlet frits with the electric filament by heating a small section of the packed column eight times for 6 s at 500°C, the HPLC in-line filter was removed and the capillary was flushed with the mobile phase to remove the packing material contained after the inlet frit. The same operation was conducted to remove the packing material contained in the section before the outlet frit by turning the capillary. The detection window was prepared by scratching or by burning the polyimide layer after the outlet frit. The packed section of the capillary was about 19 cm, the total length of the capillary was 27 cm. Afterwards the capillary was very carefully installed into the cartridge. The Prince CE instrument has the advantage that no cartridges are necessary and it offers the possibility of applying higher pressures.

3. Results and discussion

Enantioselective separations by electrochromatography have been performed on capillaries packed

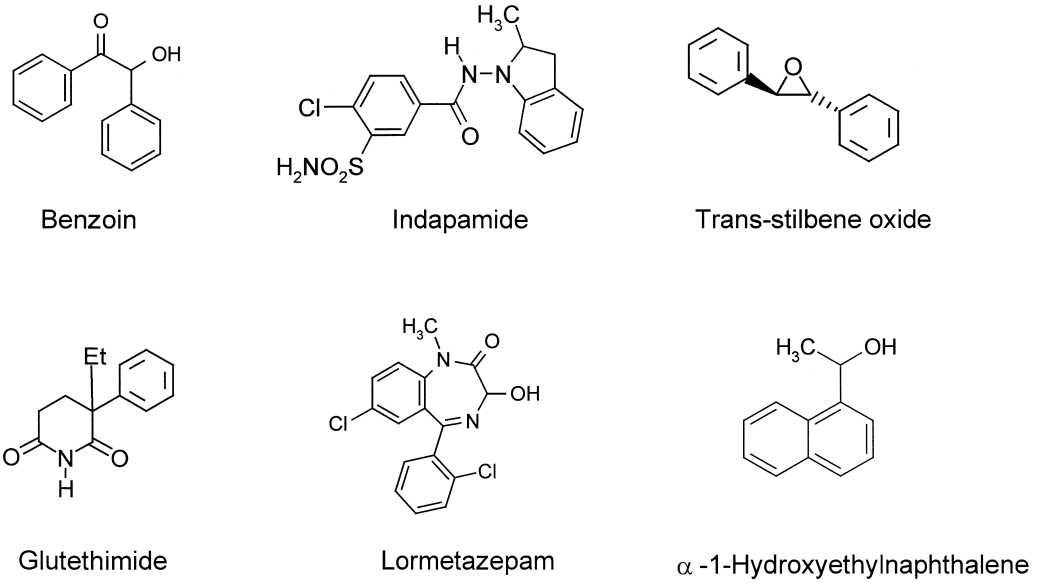

Fig. 2. Structures of the racemic solutes.

with DMPCC immobilized on macroporous silica gel with 5 or 7 μm particle size and 4000 Å pore size. The preparation of the immobilized chiral stationary phase DMPCC was recently described [25] and Fig. 1 shows the structure of the CSP. A series of racemic test compounds (Fig. 2) were injected under various electrochromatographic conditions on the prepared capillaries using mobile phase mixtures containing acetonitrile (ACN) and buffers (citrate or phosphate buffer) in low concentrations.

3.1. Packing of capillaries

Capillaries of 100 μm inner diameter and different lengths ranging between 8 and 20 cm have been packed by the slurry packing method. The frits were

Table 1
Chromatographic results obtained for different racemates under various mobile phase and temperature conditions[a]

Racemate	Mobile phase	Voltage (kV)	T (°C)	Migration time (min)	Theoretical plates, N (enantiomer 1/enantiomer 2)	R_s
Lormetazepam	6.6 m*M* citrate buffer, pH 5–ACN[a] (30:70, v/v)	25	40	36.28/47.28	3173/3059	2.22
α-1-Hydroxyethyl-naphthalene	6.6 m*M* citrate buffer, pH 5–ACN[a] (30:70, v/v)	25	40	26.2/27.92	3281/3655	0.95
Benzoin	0.8 m*M* citrate buffer, pH 6–ACN[a] (40:60, v/v)	15	20	30.3/32.2	5136/5002	1.12
	5 m*M* phosphate buffer, pH 7–ACN[b] (50:50, v/v)	30	20	16.73/19.73	3814/3436	2.31
Indapamide	0.8 m*M* citrate buffer, pH 6–ACN[a] (40:60, v/v)	15	20	15.83/16.78	5322/4318	1.06
	5 m*M* phosphate buffer, pH 7–ACN[b] (50:50, v/v)	20	20	26.86/31.88	4561/3904	2.66
	5 m*M* phosphate buffer, pH 7–ACN[c] (50:50, v/v)	20	20	8.98/9.89	3735/3261	1.35
trans-Stilbene oxide	0.8 m*M* citrate buffer, pH 6–ACN[a] (50:50, v/v)	15	20	52.8/56.25	9353/8649	1.56

[a] Detection: UV at 230 nm. Resolution: $R_s = 1.18(t_2 - t_1)/w_{(0.5)1} + w_{(0.5)2}$. Theoretical plates: $N = 5.54(t/w_{0.5})^2$. Capillary: 27 cm long; packed with DMPCC (7 μm particle size, 4000 Å pore size), packed bed 19 cm; Beckman P/ACE System.
[b] Capillary: 20 cm long packed with DMPCC; Prince Instrument; UV at 230 nm.
[c] Capillary: 8 cm long packed with DMPCC; Prince Instrument; UV at 230 nm.

prepared directly from the CSP by sintering the DMPCC on a small section (about 1 mm) of the capillary with a heated filament as described in Experimental.

3.2. Enantioselective electrochromatography

Table 1 summarizes the chromatographic results (migration time, resolution, R_s, and number of theoretical plates, N) obtained for different racemates under optimized mobile phase and temperature conditions.

Typical electrochromatograms are shown in Fig. 3 for the enantiomeric separation of lormetazepam, *trans*-stilbene oxide, indapamide and benzoin. The separations of lormetazepam and *trans*-stilbene oxide were performed on the Beckman CE instrument using a 27 cm long capillary (packed section 19 cm). For lormetazepam, a plateau was observed between the two enantiomers. This type of plateau is typical for enantiomerization processes and was already observed earlier for the same compound in HPLC [29]. Theoretical plates for the first and second eluting enantiomers were found to be 4561/3904 for indapamide and 3814/3436 for benzoin. Comparing the citrate buffer to the phosphate buffer, higher resolution, R_s for benzoin and indapamide were achieved with the latter one, however the number of

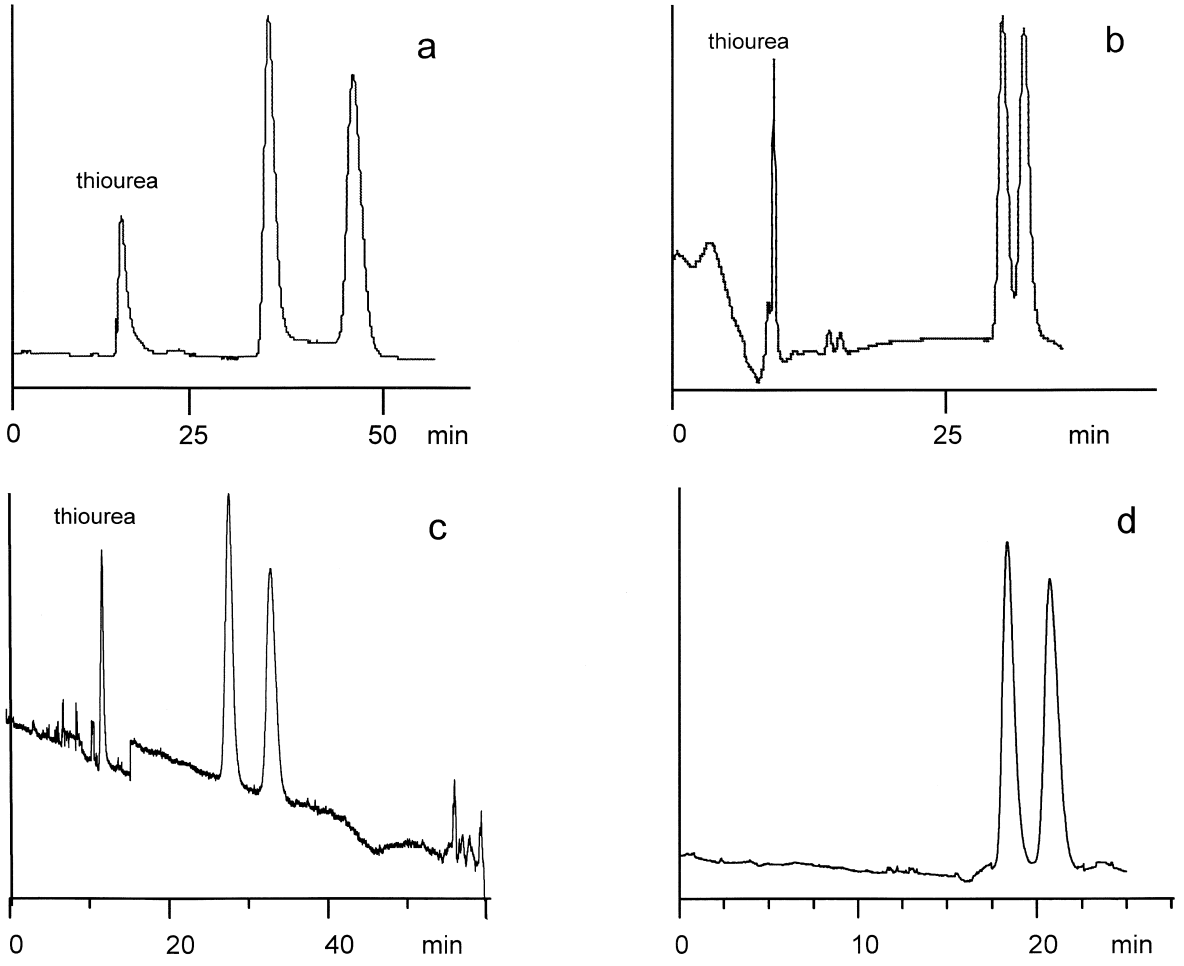

Fig. 3. Electrochromatogram of the enantiomeric separations of (a) lormetazepam, (b) *trans*-stilbene oxide, (c) indapamide, (d) benzoin. For conditions, see Table 1; for indapamide and benzoin, the conditions are those applied with the Prince instrument (20 cm long capillary).

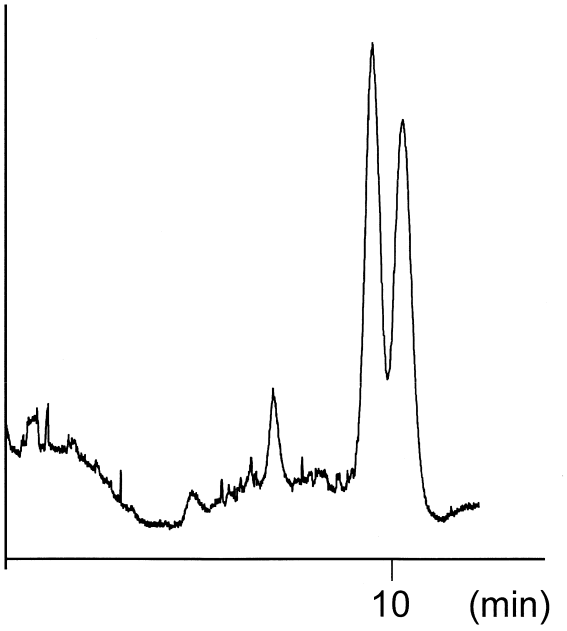

Fig. 4. Electrochromatogram of the enantiomeric separation of racemic indapamide on a short capillary (8 cm). For conditions, see Table 1.

theoretical plates was lower. The possibility to achieve separations with a very short capillary (8 cm), resulting in short migration times, was also demonstrated and is illustrated in Fig. 4 for indapamide.

3.3. Influence of the mobile phase composition

Racemic glutethimide was used to study the influence of the acetonitrile content on selectivity and resolution. The results of these investigations are listed in Table 2. By increasing the amount of acetonitrile in the mobile phase, the migration times of thiourea and of the enantiomers decrease indicating that the electroosmotic flow (EOF) increases. The resolution R_s decreases with increasing content of acetonitrile, whereas the number of theoretical plates per meter is increasing. The separation factors remain almost constant around 1.2. The increase of resolution R_s with lower acetonitrile concentrations can be explained according to the Helmholtz–Smoluchowski equation (Eq. (1)) and to Eq. (2) which give the relationship between mobility and resolution R_s, where ν_{eo} is the electroosmotic velocity, ϵ_R the dielectric constant, ϵ_0 the permittivity of the vacuum, E the electric field, ζ the zeta potential and η the viscosity of the buffer.

$$\nu_{eo} = \frac{\epsilon_R \epsilon_0 E \zeta}{\eta} \quad (1)$$

$$R_s = \frac{\Delta\mu}{\bar{\mu}} \cdot \frac{\sqrt{N}}{4} \quad (2)$$

When decreasing the amount of acetonitrile in the mobile phase its viscosity is increased, resulting in a lower electroosmotic velocity and a lower mobility

Table 2
Influence of the percentage of the acetonitrile content on CEC of racemic glutethimide[a]

	Percentage of acetonitrile		
	70	60	50
Migration time thiourea (min)	17.68	21.22	22.37
Electroosmotic velocity (mm/s)	0.25	0.21	0.20
Migration time enantiomer 1 (min)	27.17	40.37	56.88
Migration time enantiomer 2 (min)	19.17	44.43	65.06
N/m enantiomer 1	19 521	15 872	13 471
N/m enantiomer 2	20 087	15 486	11 489
Selectivity, α	1.21	1.21	1.24
Resolution, R_s	1.26	1.52	1.84

[a] Capillary: Chiral stationary phase DMPCC (5 μm particle size, 4000 Å pore size), 19 cm packed bed, 27 total length (column packed electrokinetically by Unimicro). Thiourea, 4.4 m*M*; glutethimide, 10.35 m*M*. Mobile phase: 10% 20 m*M* sodium citrate solution (pH 5), 20–40% water and 70–50% acetonitrile. Instrument: Beckman P/ACE 5510. Voltage: 25 kV. Injection: 5 kV, 5 s. Temperature: 40°C. Detection: UV at 230 nm. Average of three measurements.

of the analytes. As expressed by the separation factor α, the difference of the mobility ($\Delta\mu$) of the enantiomers of glutethimide remains constant. The average mobility $\overline{\mu}$ of both enantiomers however decreases and causes a gain in resolution although the number of theoretical plates decreases.

3.4. Influence of buffer concentration

The influence of buffer concentration on the separation was examined for different amounts of sodium citrate in water using again glutethimide as a test racemate. The buffer concentration was varied in the range of 2–20 m*M* and 10% in volume of this salt solution were used in all experiments. The results are summarized in Table 3. An increase of the buffer concentration causes a strong decrease of the EOF, resulting in an increase of the migration time for both enantiomers. Compared to the slight variations of the EOF observed by changing the acetonitrile content in the mobile phase, the changes of buffer concentration strongly influenced EOF. Indeed, the zeta potential, ζ, at the electrically double layer at the particle surface depends on the buffer concentration as shown in Eq. (3)

$$\zeta = \sigma\sqrt{\frac{RT}{2\epsilon_R\epsilon_0 cF^2}} \quad (3)$$

where R is the universal gas constant, T is the absolute temperature, ϵ_R is the dielectric constant, ϵ_0 the permittivity of the vacuum, F is the Faraday constant, c is the molar salt concentration and σ the excess charge density.

As the zeta potential is inversely proportional to the square root of the salt concentration, an increase in salt concentration means a decrease of the zeta potential. Since, according to the Helmholtz–Smoluchowski equation (Eq. (1)), the EOF is directly proportional to ζ, a high salt concentration induces a low EOF. Considering Eq. (2), it is expected that at a reduced EOF and at a constant voltage the average mobility of the analytes decreases and resolution R_s increases, while selectivity is practically not influenced.

3.5. Influence of pH

The influence of pH on enantiomeric separation of glutethimide was determined in the pH range of 4–8. As expected, the results (Table 4) show that EOF increases with augmenting pH, but at the same time resolution R_s is decreasing, while the separation factor α remains constant.

In summary, it has been pointed out that different parameters (buffer concentration, percentage of acetonitrile, pH of the buffer) can be modulated to alter resolution R_s and EOF in electrochromatography. The greatest improvements of resolution are achieved by increasing the acetonitrile content, however, higher acetonitrile content has the disadvantage

Table 3
Influence of the buffer concentration on CEC of racemic glutethimide[a]

	Buffer concentration (m*M*)				
	2	5	10	15	20
Migration time thiourea (min)	4.39	8.27	14.09	21.62	17.68
Electroosmotic velocity (mm/s)	1.03	0.544	0.32	0.208	0.25
Migration time enantiomer 1 (min)	6.23	11.61	20.98	32.54	27.17
Migration time enantiomer 2 (min)	6.60	12.29	22.30	34.68	29.12
N/m enantiomer 1	n.c.	12 518	15 258	18 443	19 357
N/m enantiomer 2	n.c.	11 385	16 819	18 046	19 914
Selectivity, α	1.20	1.20	1.19	1.20	1.21
Resolution, R_s	0.88	0.86	1.03	1.10	1.26

[a] Capillary as described in Table 2. Thiourea, 4.4 m*M*; glutethimide, 10.35 m*M*. Mobile phase: 10% sodium citrate solution (pH 5) at different concentrations, 20% water and 70% acetonitrile. Voltage: 25 kV. Instrument: Beckman P/ACE 5510. Injection: 5 kV, 5 s. Temperature: 40°C. Detection: UV at 230 nm. Average of three measurements.

Table 4
Influence of pH on CEC of racemic glutethimide[a]

	pH		
	4	5	8
Migration time thiourea (min)	18.31	14.77	7.33
Electroosmotic velocity (mm/s)	0.25	0.30	0.61
Migration time enantiomer 1 (min)	29.71	21.81	10.52
Migration time enantiomer 2 (min)	32.05	23.08	11.06
Selectivity, α	1.20	1.18	1.17
Resolution, R_s	1.19	1.00	0.90

[a] Capillary as described in Table 2. Thiourea, 4.25 m*M*; glutethimide, 8.75 m*M*. Mobile phase: 10% 4 m*M* sodium citrate solution at different pH, 20% water and 70% acetonitrile. The pH 8 solution was adjusted with 0.1 *M* NaOH. Voltage: 25 kV. Instrument: Beckman P/ACE 5510. Injection: 5 kV, 5 s. Temperature: 40°C. Detection: UV at 230 nm. Average of 3–7 measurements.

to increase migration time (lower EOF). Changing the buffer concentration showed the greatest effect on EOF, but poor resolutions at high EOF.

3.6. Comparison of packed CEC with nano-LC

Indapamide was used as a test racemate to compare the performance of the immobilized DMPCC packing material in the packed CEC and nano-LC modes (Fig. 5). Acetonitrile–phosphate buffer at pH 7 (1:1) was used as a mobile phase in both modes. The nano-LC separations were performed with the μ-HPLC pump Evolution 200, which promotes flow-rates below 1 μl/min under isocratic conditions. The velocity of the mobile phases was adjusted in order to have approximately the same values for the CEC and nano-LC modes. The flow-rate of the μ-HPLC pump was 200 nl/min. Under the applied nano-LC conditions the enantiomers exhibit slightly shorter retention times, but efficiency (1286/1257 theoretical plates for the first and second enantiomer) and resolution (R_s 1.17) were clearly inferior compared to the CEC mode, where theoretical plates of 4561 and 3904 were obtained for the respective enantiomers and a resolution of R_s 2.66 was observed. The

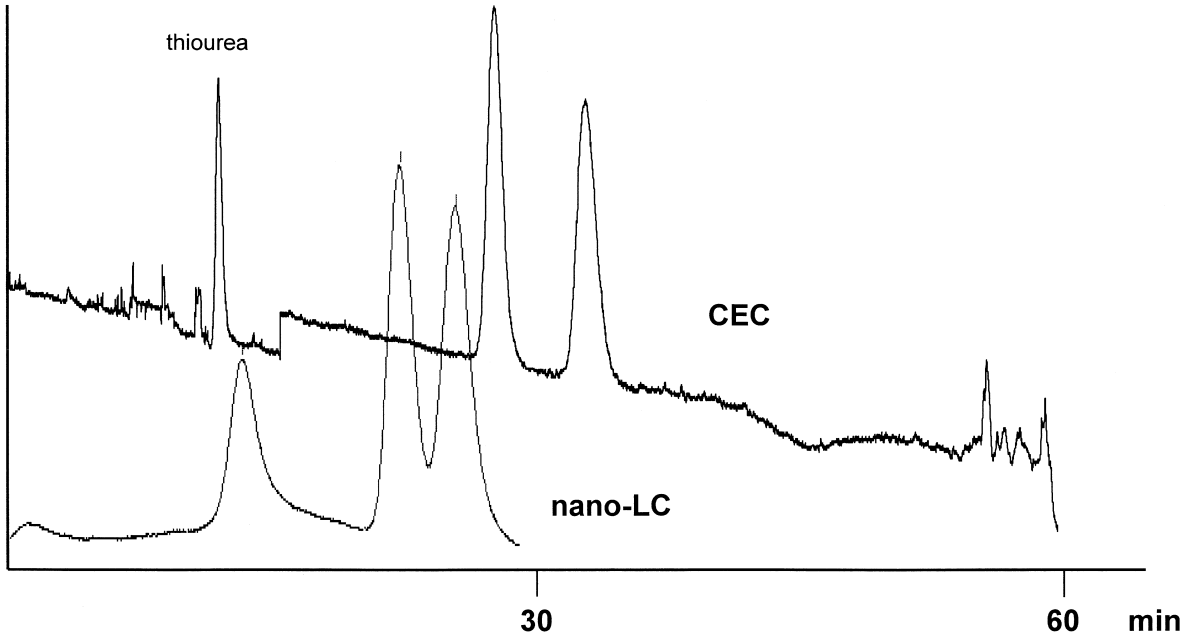

Fig. 5. Enantiomeric separation of racemic indapamide. Capillary: DMPCC immobilized on silica (7 μm, 4000 Å) 20 cm long connected to a UV transparent fused-silica capillary with a polyethylene tubing. Mobile phase: 5 m*M* phosphate buffer, pH 7–acetonitrile (50:50). CEC: Instrument: Prince CE system. Voltage: 20 kV. UV detection at 230 nm. Nano-LC: Instrument: Evolution 200. Flow-rate: 200 nl/min. UV detection at 230 nm.

chiral separation factor α is practically identical with a value of 1.35 in nano-LC and 1.33 in CEC.

4. Conclusion

DMPCC immobilized on macroporous silica has been successfully applied as a chiral stationary phase to the separation of enantiomers by electrochromatography in packed capillaries. Capillaries of 100 μm inner diameter and different lengths could be packed using the slurry packing technique and frits were prepared by sintering the CSP directly. Changes in acetonitrile content showed the highest influence on the resolution of the test racemate glutethimide, while the buffer concentration had the strongest influence on the EOF. The chiral separation factor α remained nearly constant through the different tests. In order to compare both chromatographic modes, one column was operated in CEC and nano-LC modes. Separation efficiency and resolution were higher in the CEC mode when similar mobile phase velocities are applied. The utilization of smaller particle size (3 μm or less) and the combination of pressure and EOF should allow the performance of the technique to be appreciably increased, making electrochromatography a useful alternative for the separation of enantiomeric mixtures.

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