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Use of short-end injection capillary packed with a glycopeptide antibiotic stationary phase in electrochromatography and capillary liquid chromatography for the enantiomeric separation of hydroxy acids

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Abstract

A new chiral stationary phase (CSP) was prepared by reacting MDL 63,246 (Hepta-Tyr), a glycopeptide antibiotic belonging to the teicoplanin family, with 5-μm diol-silica particles. The CSP mixed with 5-μm amino silica particles (3:1) was packed into 75-μm fused-silica capillaries for only 6.6 cm and used for electrochromatographic experiments analyzing several hydroxy acid enantiomers. A reversed electroosmotic flow carried both analytes and mobile phase towards the anode in a short time (1–3 min), being baseline resolved all the studied analytes. In order to achieve the fastest enantiomeric resolution of the studied hydroxy acids, the effect of several experimental parameters such as mobile phase composition (organic modifier type and concentration, pH of the buffer and ionic strength), capillary temperature and applied voltage on enantioresolution factor, retention time, enantioselectivity were evaluated. The packed capillary column allowed the separation of mandelic acid enantiomers in less than 72 s with resolution factor R_s=2.18 applying a voltage of 30 kV and eluting with a mobile phase composed by 50 m*M* ammonium acetate (pH 6)–water–acetonitrile (1:4:5, v/v). The CSP was also tested in the capillary liquid chromatography mode resolving all the studied enantiomers applying 12 bar pressure to the mobile phase [50 m*M* ammonium acetate (pH 6)–water–methanol–acetonitrile, 1:4:2:3, v/v)], however, relatively long analysis times were observed (12–20 min).

Keywords: Injection methods; Chiral stationary phases, electrochromatography; Chiral stationary phases, LC; Electrochromatography; Glycopeptide; Peptides; Antibiotics; Hydroxy acids; Teicoplanin; Mandelic acid

1. Introduction

A wide number of compounds including those of environmental, agrochemical, pharmaceutical interest, because they contain in their chemical structure one or more asymmetric center, exist as one or more couples of enantiomers. Very often the two enantiomers may exhibit different pharmacological or biochemical properties [1] and therefore in the case of the introduction in the market of a new drug, the

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pharmacological effect and the study of the metabolism have to be carefully studied.

In the last few years analytical methods able to resolve chiral compounds were studied focusing attention on the development of new chiral stationary phases (CSPs) and/or chiral selectors achieving good enantioresolution in short time and good efficiency.

Analytical methods so far used for the enantiomeric separations include gas chromatography (GC), high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC) and recently capillary electrophoresis (CE) [2–8]

CE is a powerful electromigration technique exhibiting high efficiency and high resolution in short analysis time towards a wide number of enantiomeric compounds belonging to different classes such as organic and inorganic ions/neutral molecules, peptides, proteins, herbicides, drugs, etc. More recently capillary electrochromatography (CEC) was successfully applied to the enantiomeric resolution of a wide number of compounds utilizing the advantages of both CE and HPLC (high efficiency and selectivity, respectively) [8,9].

In CEC a relatively strong electroosmotic flow (EOF) with a flat flow profile carries to the detector both enantiomers and mobile phase, while the stereoselectivity and the enantioresolution can be achieved because of interactions with the chiral selector. The former is present in the system, e.g., bonded to the capillary wall (open-CEC) or to the packed stationary phase (p-CEC) or to a polymeric material or added to the mobile phase [8,10].

Several chiral selectors were successfully employed in CEC and among them glycopeptide antibiotics (GAs) resulted to be powerful enantiorecognition agents towards a wide number of compounds, e.g., amino acid derivatives, herbicides, drugs, etc.

Vancomycin and teicoplanin, belonging to this class of chiral selectors, exhibited very high enantioresolution capability mainly towards basic compounds. Here the studies were carried out employing either packed or monolithic columns containing separately the two GAs [11–19]. In a recent work [20], with the aim to find a CSP useful for the separation of acidic enantiomeric compounds, we studied a new silica-based CSP employing a Hepta-Tyr antibiotic (MDL 63,246) (a modified teicoplanin) as the chiral selector. The same GAs was studied by us using capillary zone electrophoresis (CZE) for the enantiomeric resolution of acidic compounds such as herbicides, drugs and hydroxy acids [21,22].

Very often it is desirable to perform analyses as fast as possible, especially when a large number of samples have to be handled, therefore appropriate method optimization must be carefully studied. Examples of fast chiral resolutions were already reported in both CZE and CEC [23–25].

In this study part of the short-end injection (8.4 cm) was packed with the CSP containing MDL 63,246 mixed with amino propyl silica (3:1) and used for CEC experiments in order to perform enantiomeric resolution of selected hydroxy acids as fast as possible.

In order to optimize the enantiomeric separation of the selected racemic compounds we studied the effect of the mobile phase composition modifying the content and the type of organic solvent as well as the pH of the buffer.

2. Experimental

2.1. Instrumentation

Electrochromatographic experiments were carried out using an Agilent 3D CE instrument (Waldbronn, Germany) equipped with a UV-diode array detector operated at 195 nm (unless otherwise stated) and a thermostated capillary cartridge applying different voltages in the range 5–30 kV. Injection was done at the short end of the capillary (cathodic polarity) applying 12 bar, 0.2 min. During the experiments both ends of the capillary were pressurized at 8 bar in order to avoid bubble formation.

The fused-silica capillaries, 75-μm I.D.×375-μm O.D., used in this work were purchased from Composite Metal Services (Hallow, UK). Capillary packing was done by using a LC series 10 HPLC pump (Perkin-Elmer, Palo Alto, CA, USA).

2.2. Materials and methods

DL-*m*-Hydroxymandelic acid (m-OH-MA), and DL-3-hydroxy-4-methoxymandelic acid (3-OH-4-MeO-MA) were purchased from Sigma (St. Louis, MO,

USA); D-(−)- and L-(+)-mandelic acids (MA) were from Carlo Erba (Milan, Italy) while DL-*p*-hydroxymandelic acid (p-OH-MA) was from Ega-Chemie (Steinheim, Germany). DL-2-Phenyllactic acid (2-PhL) and 4-chloro-DL-mandelic acid (4-Cl-MA) were purchased from Fluka (Buchs, Switzerland). Methanol (MeOH) and acetonitrile (ACN), of pure reagent grade were obtained from BDH (Poole, UK). Ammonium acetate, sodium cyanoborohydride, sodium periodate, LiChrospher diol silica phase 5-μm particle diameter (pore size 100 Å) were purchased from Merck (Darmstadt, Germany). Kromasil amino silica 5 μm was a gift of Aka Chemicals (Bohus, Sweden), MDL 63,246 glycopeptide antibiotic was synthesized at Lepetit Research Center (Gerenzano, Italy) [26] and purified by Righetti's group with a preparative isoelectric focusing method (see Ref. [27]).

Aqueous mobile phases were prepared by adding the appropriate volume of organic modifier to the buffer solutions at a controlled pH 6.

One mg/ml analyte stock solutions were prepared in methanol and stored at 4 °C; the solutions were daily diluted with the mobile phase at the desired concentrations and injected for the CEC analysis.

2.3. Synthesis of the chiral stationary phase and packing capillary procedure

The Hepta-Tyr chiral stationary phase (MDL-CSP) was prepared in our laboratory according to a previously published method for the synthesis of vancomycin CSP [16].

(A) Four hundred mg of 5 μm LiChrospher DIOL silica particles were added to a 30 ml mixture of water–methanol (4:1, v/v) containing 60 m*M* of $NaIO_4$ and sonicated for 60 min in order to oxidize the diol to aldehyde groups. The mixture was centrifuged at 4000 rpm for 5 min and the solution eliminated; the solid material was washed for three times with 20 ml of water.

(B) One hundred and sixty mg of Hepta-Tyr antibiotic (MDL 63,246) were dissolved in a mixture of 50 m*M* NaH_2PO_4, pH 7.04, titrated with NaOH containing 10 m*M* of $NaCNBH_3$ (24 ml)–acetonitrile (6 ml) in order to have a 3 m*M* solution of MDL. The solution was added to the oxidized diol silica, sonicated for 60 min, centrifuged and washed with water.

The recovered modified silica CSP was treated with 30 ml of 50 m*M* phosphate buffer at pH 3.1 containing 10 m*M* of $NaCNBH_3$ sonicated for 60 min, washed with water as in point (B). The modified silica particles were washed three times with 20 ml each of methanol and the solvent evaporated at room temperature under vacuum.

The following steps were used in order to prepare the CSP packed capillaries: (i) the capillary was connected to a mechanical temporary frit and packed, using an LC pump, with a slurry of LiChrospher diol–silica (2:1) in 10 m*M* NaCl solution; the frit was prepared with a heating wire (about 350 °C, 10 s). The capillary was cut close to the frit, connected to the pump and flushed with water in order to eliminate the excess of silica phase, the capillary was then connected to the pump with the opposite side for next step: (ii) a slurry of 40 mg MDL–amino silica (3:1, w/w) in 1.5 ml water–acetonitrile (1:1, v/v) mixture was prepared, sonicated and used for packing the capillary (about 2000 p.s.i., 6.6 cm; 1 p.s.i.=6894.76 Pa). (iii) The capillary was then packed with diol–silica (2:1, w/w) for 4 cm and the end frit prepared close to the chiral stationary phase (0.4 cm). The part of the capillary where the polyimide was removed was covered with a layer of epoxy resin. The total length of the capillary was 34.4 cm; second frit at 7.0 cm; effective length at 8.4 cm.

Fig. 1 shows the scheme of the separation capillary used for CEC experiments where the effective length was the short end of the tube.

3. Results and discussion

3.1. Test of the capillary packed at the short-end injection

The use of CEC for the separation of chiral compounds can offer great advantages over other established CE modes, e.g., free zone electrophoresis (CZE). In fact in CEC the same column packed with the CSP can be used several times, strongly reducing the amount of expensive chiral selector. On the contrary in CZE, besides only a few milligrams of

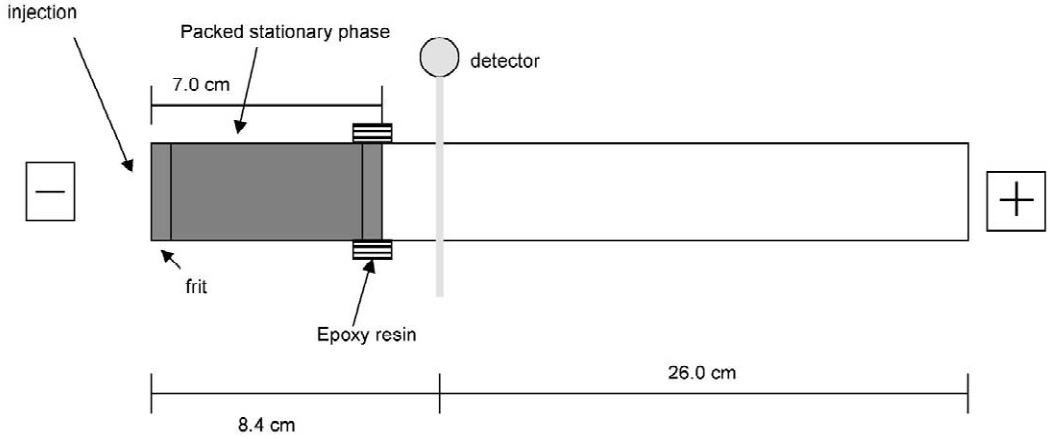

Fig. 1. Scheme of the packed capillary used in this study.

chiral selectors (CSs) being employed, it is necessary to change the BGE after each run. Furthermore the CSs usually not being transparent at the UV wavelengths used for detection, are responsible for the low sensitivity. In this case the partial filling method combined with the counter-current process was proposed analyzing a wide number of chiral compounds [21,22,28,29].

GAs such as vancomycin, teicoplanin and teicoplanin derivatives, resulted to be excellent chiral selectors and successfully tested for the enantiomeric resolution of a wide number of compounds, including amino acid derivatives and drugs in both CZE and CEC.

When analyzing a large number of enantiomeric samples it is desirable to perform the work in a short time and therefore several approaches can be considered. Among them it is noteworthy to mention (i) an increase of the applied electric field (higher voltages or shorter capillary) and (ii) the use of a short effective length of the capillary. In both cases we have some limitations due to the fixed maximum voltage that can be applied to the minimum length that the capillary cartridge can allow and to the fixed effective length (8.4 cm).

In this study the capillary was packed at the short-end injection with slurry composed by MDL 63,246 antibiotic and amino silica. The GA and amino silica present in the packed column, possessing amino groups were positively charged at the operating experimental conditions, pH$<$7. Therefore a strong EOF was expected due to the positive charge of the stationary phase.

The packed capillary at the short-end injection was tested analyzing racemic mandelic acid; the mobile phase was a mixture of ammonium acetate, pH 6, in 50% (v/v) acetonitrile applying a voltage in the range 5–30 kV. The injection was done from the outlet side of the capillary and analytes were moving as anions through the short end (8.4 cm) to the detector carried by a strong electroosmotic flow and the self electrophoretic mobility of the analyte. The experimental set-up resulted to be quite stable, but some instability can be expected due to the fact that two different EOF are involved in the separation process: the first (towards the anode) due to the CSP (positively charged) and the second (towards the cathode) originating from the capillary wall (negatively charged). The measured EOF was in the range 5.8–0.35 min (2.5–30 kV), clearly showing that the influence of the capillary wall was negligible; this was also shown by Dittmann and Rozing using two different reversed-phase columns [30] where the RP18 silica particles were packed into two different capillaries (coated and uncoated), obtaining the same EOF.

The plot of reduced plate heights versus linear velocity of the EOF (see Fig. 2) showed an increase of efficiency by decreasing the velocity of the EOF. However 20 kV was the selected voltage for further

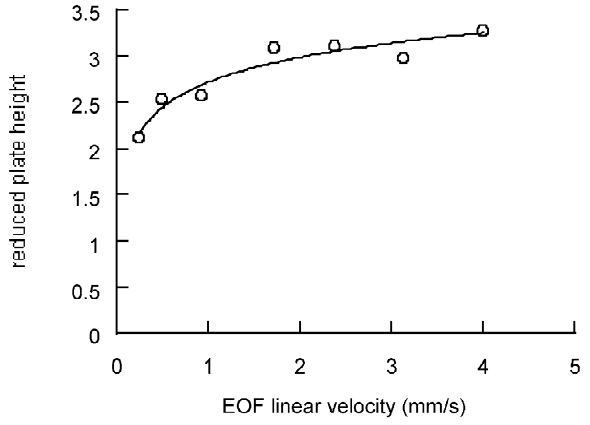

Fig. 2. Plot of reduced plate heights versus linear velocity of electroosmotic flow. Sample L-(+)-mandelic acid. Capillary, 34.4 cm (effective length 8.4 cm, stationary phase, 6.6 cm, frit at 7.0 cm)×75 μm I.D. Mobile phase 50 m*M* ammonium acetate (pH 6)–water–ACN (1:4:5, v/v). Applied voltage, 2.5–30 kV (0.9–9.9 μA); injection at short-end side (cathode) 12 bar, 0.2 min of 0.2 mg/ml of racemic MA followed by a mobile phase plug at 12 bar, 0.15 min. The capillary was pressurized at both ends at 8 bar. Capillary temperature, 20 °C.

experiments achieving good efficiencies and satisfactory analysis time.

The repeatability was calculated by analyzing the racemic mandelic acid mixture ($n=7$), eluting with the mobile phase employed in the above-described experiments and measuring the RSD% of the retention time of the two enantiomers (t_{R1}, t_{R2}), resolution factor (R_s) and enantioselectivity (α) (RSD=1.2, 1.3, 0.7 and 0.7%, respectively). These results were comparable with those previously achieved using a capillary packed with the same stationary phase but with a longer effective length (26 cm) [20].

A sample mixture containing L-(+)- and D-(−)-mandelic acid (3:1, v/v) was analyzed in order to verify the affinity of the two enantiomers towards the chiral selector and we found that the D-(−) enantiomer was the most retained compound.

3.2. Effect of organic modifier on enantioresolution of hydroxy acid compounds

The effect of acetonitrile concentration on enantioseparation of selected hydroxy acid enantiomers, namely mandelic acid, *m*- and *p*-hydroxymandelic acid, 3-hydroxy-4-methoxymandelic acid, 4-chloromandelic acid and 2-phenyllactic acid was studied by CEC using the mobile phase containing ammonium acetate at pH 6 and 30–60% of ACN. The content of the organic modifier was limited at the studied range because current instability and too long analysis time were observed at concentrations higher than 60% and lower than 30%, respectively. All studied compounds were baseline resolved at any concentration of ACN except 2-PhL which showed $R_s=0.8$–1.0. The recorded enantioresolution factors resulted 10–50% lower than those previously observed [20] employing the same stationary phase with a longer effective length (26 cm, with chiral length of 24 cm).

The enantioresolution did not change remarkably by increasing the MeCN concentration while higher efficiencies were observed at 50 and 60%. Therefore we studied the effect of MeOH (0–50%) added to the mobile phase containing ammonium acetate at pH 6 and acetonitrile (50–0%) on enantioresolution.

The trend of R_s versus organic modifier concentration ratio was quite similar to our previous results achieved using the longer effective capillary length, R_s increased by increasing the MeOH concentration with maximum value at 50% of MeOH. However the lowest efficiencies were observed at this MeOH concentration. This is documented in Fig. 3 where mobile phases with different concentration ratio of MeOH–ACN were employed for the enantioresolution of 2-PhL.

Fig. 4a,b shows the effect of organic modifier concentration ratio on efficiency of the first eluting enantiomeric hydroxy acids. As can be observed, the efficiency increased by increasing the MeOH concentration (decreasing ACN%) up to 20% MeOH and than decreased for MA, m-OH-MA, 2-PhL, 4-Cl-MA and 3-OH-4-MeO-MA. In the case of p-OH-MA a similar trend was observed but with a maximum efficiency at 30% MeOH while for 3,4-di-OH-MA the addition of MeOH caused a reduction of efficiency. Twenty or 30% MeOH added to the acetonitrile–water–buffer system seems to offer the highest efficiency with good resolution and reasonably short analysis time. This is shown in Fig. 5a–d where the enantiomeric separation of m-OH-MA, p-OH-MA, 3-OH-4-MeO-MA, 4-Cl-MA, 2-PhL are reported.

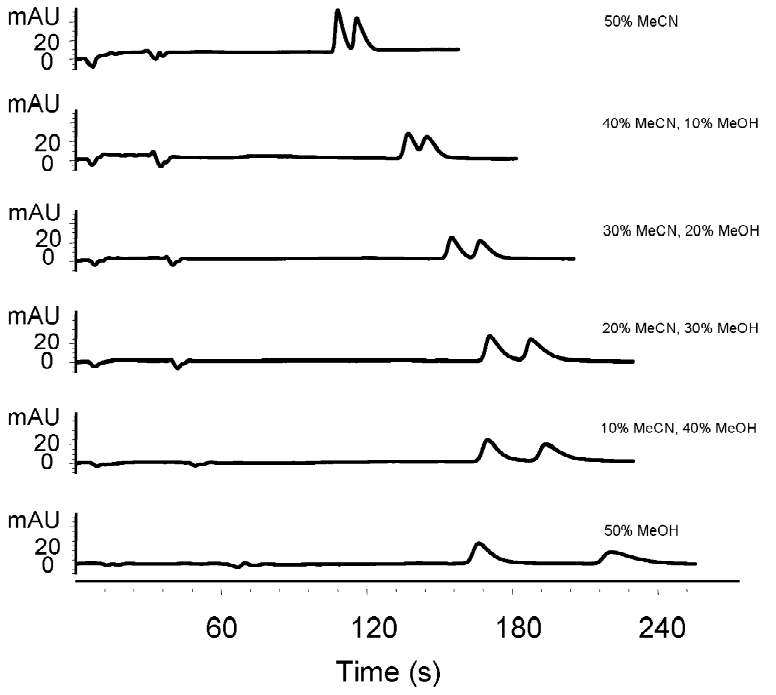

Fig. 3. Effect of organic modifier concentration ratio on enantiomeric separation of 2-phenyllactic acid. Applied voltage, 20 kV; sample concentration, 0.2 mg/ml; mobile phase, 50 m*M* ammonium acetate (pH 6)–water–organic modifier mixture (1:4:5, v/v); sample concentration, 0.2 mg/ml except 3,4-di-OH-MA that was 0.4 mg/ml. MeCN stands for ACN. For other experimental conditions see Fig. 2.

3.3. Use of short-end injection packed capillary in nano-HPLC

The capillary packed at the short-end injection was also evaluated for nano-HPLC enantiomeric separation of selected studied hydroxy acid derivatives running the experiments with the same instrument used for CEC studies. The mobile phase selected was that containing 50 m*M* ammonium acetate (pH 6)–water–MeOH–ACN (1:4:2:3, v/v) which allowed to achieve baseline resolution of all selected hydroxy acid enantiomers by CEC. All studied compounds were baseline resolved by nano-HPLC applying a relatively low pressure: 12 bar (the maximum permitted by the commercial instrumentation). Besides, higher enantioresolution was achieved in nano-HPLC than in CEC, retention times were higher due to the relatively low pressure applied. The higher resolution observed in nano-HPLC can be probably explained by the longer time spent by analytes in contact with the CSP. Efficiencies measured in nano-HPLC analysis were generally lower than those achieved using the same mobile phase in CEC, e.g., N m^{-1} mandelic acid: CEC=83 250; nano-HPLC=43 357.

Table 1 shows the values of retention time, retention factor, enantioselectivity and resolution factor of the studied hydroxy acid enantiomers separated by nano-HPLC. The repeatability was studied analyzing a racemic mixture of *m*-hydroxymandelic acid recording satisfactory results. The RSDs were: 1.4, 1.5 and 1.5% for void volume and retention times of the first and second eluting enantiomer, respectively; 0.4, 1 and 0.1% for retention factors, enantioresolution and enantioselectivity, respectively. From the above-discussed results we can remark that the CSP based on Hepta-Tyr antibiotic can also be used in nano-HPLC; however, an applied pressure higher than 12 bar is necessary in order to reduce the analysis time to the level achieved in CEC.

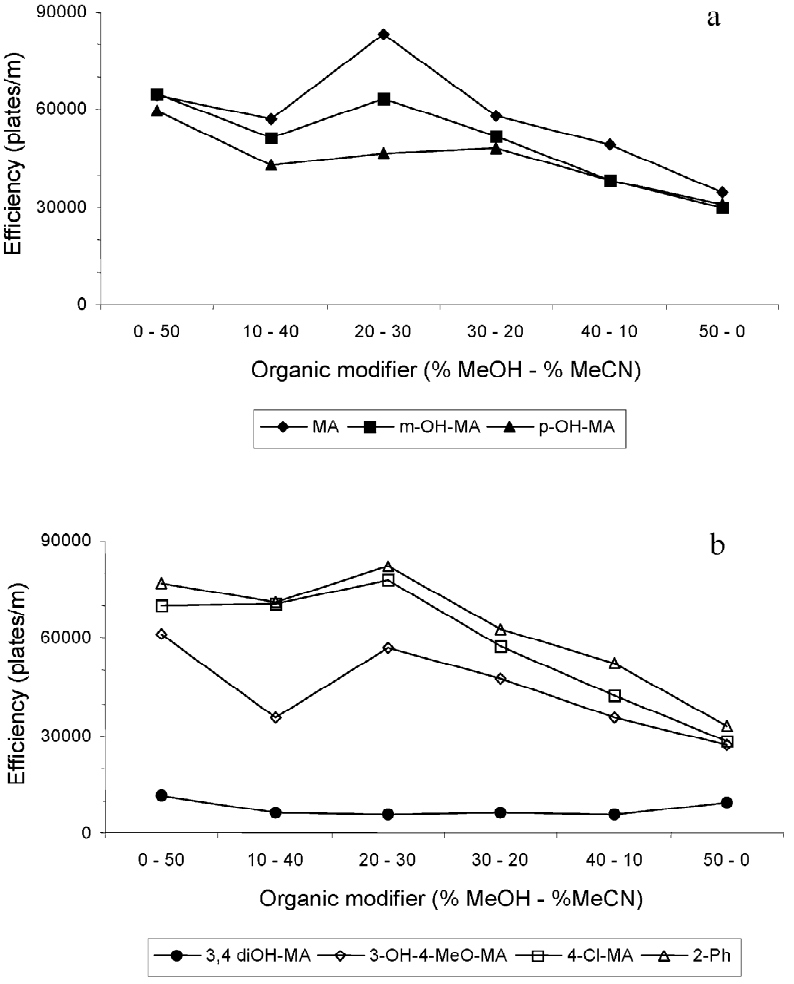

Fig. 4. Effect of organic modifier concentration ratio on efficiency of the first eluting enantiomeric hydroxy acids. (a) MA, m-OH-MA and p-OH-MA; (b) 3,4-di-OH-MA, 3-H-4-MeO-MA, 4-Cl-MA and 2-PhL. MeCN stands for ACN.

4. Conclusions

Hepta-Tyr antibiotic CSP packed in the short-end injection of the capillary was successfully used for the separation of hydroxy acid enantiomeric compounds by CEC. A mobile phase containing ammonium acetate, pH 6, and ACN or ACN–MeOH, allowed the enantioresolution of all studied compounds. Retention time, resolution and efficiency were strongly influenced by the mobile phase composition. In fact the addition of MeOH was a predominant factor for achieving higher enantioresolution factor; however, decreased efficiencies were observed at MeOH concentrations higher than 20–30%. The shortest analysis time was obtained using 50% of ACN, which was also a compromise for good efficiency and enantioresolution. A further increase of applied voltage (30 kV was the maximum allowed by the instrumentation) allowed the enantiomeric resolution of mandelic acid in less than 72 s as is illustrated in Fig. 6.

The CSP was also tested in the nano-HPLC mode achieving interesting results concerning the repeatability data (RSD 1–1.5% for retention time).

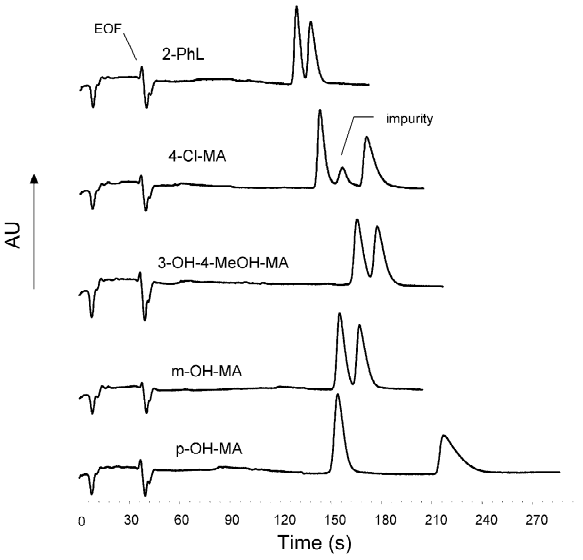

Fig. 5. Electrochromatographic enantiomeric separation of 2-PhL, 4-Cl-MA, 3-OH-4-MeO-MA, m-OH-MA and p-OH-MA. Mobile phase, 50 m*M* ammonium acetate (pH 6)–water–ACN–MeOH (1:4.30:20, v/v). For other experimental conditions see Fig. 2.

Further study is being carried out in our laboratory in order to use pressures higher than 12 bar to find experimental parameters useful for decreasing analysis time at the level observed in CEC. It is not clear whether the Hepta-Tyr antibiotic is bonded or adsorbed on the stationary phase, therefore additional investigations (e.g., NMR) are needed in order to clear this important point.

Table 1
Retention time (t_{R1}), retention factor (k), enantioresolution (R_s) and enantioselectivity (α) achieved using capillary liquid chromatography (CLC) for the enantiomeric separation of hydroxy acids

Compounds	t_{R1}	k_1	k_2	R_s	α
(1) MA	13.2	3.1	4.6	4.1	1.5
(2) m-OH-MA	13.1	2.9	3.5	1.9	1.2
(3) p-OH-MA	12.5	2.9	6.7	2.3	2.3
(4) 3,4-di-OH-MA	14.3	3.2	4.6	2.0	1.4
(5) 3-OH-4-MeO-MA	12.8	2.9	3.5	1.7	1.2
(6) 4-Cl-MA	15.0	3.7	5.4	4.1	1.5
(7) 2-PhL	13.4	3.2	3.7	1.7	1.2

The capillary was the same used for CEC experiments; mobile phase 50 m*M* ammonium acetate (pH 6)–water–methanol–acetonitrile (1:4:2:3, v/v); applied pressure, 12 bar; capillary temperature 20 °C.

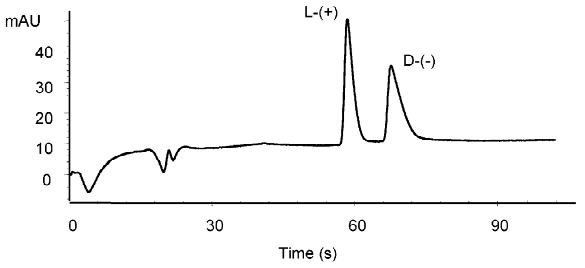

Fig. 6. Fast enantiomeric separation of racemic mandelic acid by CEC. Applied voltage, 30 kV; injection at the short-end of the capillary; mobile phase 50 m*M* ammonium acetate (pH 6)–water–ACN (1:4:5, v/v). For other experimental conditions see Fig. 2.

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