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High-efficiency chiral separations of *N*-derivatized amino acids by packed-capillary electrochromatography with a quinine-based chiral anion-exchange type stationary phase[1]

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Abstract

An anion-exchange type chiral stationary phase (CSP) was evaluated regarding applicability for the separation of enantiomers of chiral amino acids in packed-capillary electrochromatography (CEC). Thus, 5-μm porous silica particles were modified with a basic *tert*.-butyl carbamoyl quinine chiral selector, and this modified chiral sorbent was packed into fused-silica capillaries of 75 and 100 μm I.D., respectively, with a packed bed of 25 cm. When an electric field is applied across the capillary the electroosmotic flow generated by this new packing material may be reversed compared to bare silica, depending on the buffer or mobile phase pH. At pH values below ca. 6.3 the net charge of the chirally modified silica surface is positive and thus the electroosmotic flow is directed towards the anode. Accordingly, electrophoretic migration of the anionic analytes and electroosmotic flow have same directions. This new chiral anion-exchange CEC separation technique was used to separate the enantiomers of *N*-derivatized α-amino acids, e.g. *N*-(9-fluorenylmethoxycarbonyl) α-amino acids and *N*-(3,5-dinitrobenzyloxycarbonyl) α-amino acids. Enantioselectivity values were as high as in HPLC and efficiency was typically by a factor of 2 to 3 higher than in HPLC regarding theoretical plate numbers per meter. The influence of mobile phase parameters (pH, organic modifier, buffer concentration) on electroosmotic flow behaviour as well as on effective retention and separation of the analytes has been investigated. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Electrochromatography; Chiral stationary phases, LC; Amino acids

1. Introduction

Packed-capillary electrochromatography (CEC) is a rapidly developing micro separation technique which combines chromatographic and electrophoretic principles [1–3]. The separation column is a fused-silica capillary packed with the stationary phase, mostly modified silica, and the mobile phase is electroosmotically driven through the capillary column by applying high electric fields. The electroosmotic flow (EOF) is generated due to the electrical double layer which exists at the solid–liquid interface. The flow profile is advantageously plug-like and no column back pressure is produced, thus allowing the use of particles with very small diameter (<5 μm); this results in higher peak efficien-

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cies than in HPLC. More details about terminology, physicochemical background, and advantages over HPLC are discussed elsewhere [4–6,30,31]. Recently, also a review on the current status of CEC and its advances in technology and applications has been presented [7].

Important technical advances since the early beginnings of CEC include pressurization of the separation capillary to avoid bubble formation and gradient elution techniques [8–10]. Also a wide variety of different packing materials specifically developed or modified for CEC applications (ODS, SCX, mixed mode ODS and SCX) are already commercially available [7], and CEC will even more gain in significance, when the versatility of packing materials with tailor-made selectivity, which is characteristic for HPLC, will be transferred to CEC column technology and extended with tailor-made surface chemistry to control the EOF.

However, to date most studies were performed with neutral model compounds, exhibiting ideal behaviour in CEC. Neutral species are transported through the column by the EOF and separation is based purely on their chromatographic properties. For ionized species, however, the transport through the chromatographic bed is based on the electroosmotic flow and is accompanied by electrophoretic migration either with or against the EOF; in addition, chromatographic partition between stationary and mobile phases accounts to effective mobility or retention. At present, relatively little attention has been attributed to the separation of charged species, i.e. basic and acidic analytes. However, the majority of practically relevant analytes are ionizable. Therefore, current interest is in extending CEC technology to analytically relevant compounds (including ionized species), analytical problems of current interest and real life samples. This includes purity determination of pharmaceuticals [11–14], enantioseparations of chiral drugs and chiral building blocks [15–19,32], and analysis of biologically interesting species [10,20]. Recently, basic drugs have been separated upon a strong cation exchanger (SCX) with exceptionally high plate numbers [13]; it seems that focussing effects not yet clearly understood are responsible for the high efficiency of this ion-exchange type separation system.

On the contrary, particularly for acidic analytes, problems arise from the counter-electroosmotic migration of anions in conventional silica-based CEC columns which have a cathodic flow due to their negatively charged surface. Li and Lloyd [16] solved this problem by dynamically modifying the surface charge of a β-cyclodextrin chiral stationary phase with the use of triethylammonium acetate as background electrolyte and low modifier percentage to reverse the cathodic flow of silica to the anode (anodic flow). More conveniently and more stable, the electroosmotic flow direction can be reversed by employing anion-exchange type stationary phases as packing materials. However, such a packed capillary anion-exchange type CEC separation method was not yet reported in the literature. On the other hand, an anion-exchange approach was recently applied in open tubular electrochromatography by Sinibaldi et al. [21]. They used a capillary coated with ergot alkaloid-derived poly-terguride for the separation of the enantiomers of chiral acidic compounds.

In this paper, we present first results of CEC enantioseparations of anionic *N*-derivatized α-amino acids employing a capillary column packed with a weak anion-exchange type chiral stationary phase (WAX type CSP) based on a quinine-derived chiral selector (SO). This CSP was originally developed for HPLC enantioseparation of chiral acidic analytes [22–28], and was used in this study without further optimization for CEC application. The modification of the silica surface with basic quinine carbamate influences considerably the electroosmotic flow characteristics of the stationary phase and may overcome the problem of counter-electroosmotic migration observed for anionic analytes in conventional RP-CEC with ODS stationary phases. However, since both amino and silanol groups are present on the surface of the packing material, magnitude and direction of EOF are dependent on the net charge of the surface at a given pH value. Besides directing the EOF, the quinine carbamate SO provides the chiral moiety for multiple intermolecular interactions with the solute enantiomers, responsible for the high enantioselectivity in the separation process of the given solutes. Factors affecting EOF, effective retention, and enantioselectivity are investigated, and efficiency as well as enantioselectivity of the CEC method are discussed in relation to the corresponding HPLC separations.

2. Experimental

2.1. Materials

The chiral sorbent (see Fig. 1) was prepared according to a standard procedure described elsewhere [29]. Thus, spherical silica with mean particle size of 5 μm (Kromasil® −100 Å from Eka Nobel, Bohus, Sweden) was first modified with 3-mercaptopropyl trimethoxysilane to yield thiopropylsilanized silica. Then, the chiral selector, *tert*.-butyl carbamoyl quinine, was covalently bound onto silica by radical addition of the thiol group to the vinyl group of the quinine selector. Finally, some of the remaining thiol groups were modified with hexyl groups.

The chiral sorbent was either slurry packed into fused-silica capillaries (100 μm I.D.) by a high-pressure packing procedure at Hewlett-Packard (Waldbronn, Germany) or it was electrokinetically packed into fused-silica capillaries (75 μm I.D.) at Unimicro Technologies (Pleasanton, CA, USA). All capillaries had a packed bed of 25 cm and a total length of 33.5 cm.

HPLC experiments were performed by a conventional HPLC technique instead of micro HPLC, since equipment for the latter technique was not available. For the HPLC experiments the same chiral sorbent from the same batch was slurry packed into a stainless steel column of the dimension 150×4.6 mm I.D. by a high-pressure packing procedure.

Racemic *N*-(9-fluorenylmethoxycarbonyl) leucine (FMOC-Leu) and the corresponding (*S*)-enantiomer were purchased from Bachem (Bubendorf, Switzerland); racemic *N*-(3,5-dinitrobenzyloxycarbonyl) leucine (DNZ-Leu) and the (*R*)- and (*S*)-enantiomers thereof were prepared as described [26]. Methanol (MeOH) and acetonitrile (ACN) used for the preparation of the mobile phases were of HPLC grade from J.T. Baker. Ammonia, triethylamine and glacial acetic acid, which were used to prepare the buffer solutions, were of analytical grade. For the preparation of buffers distilled water further purified by a Milli-Q-Plus filtration unit from Millipore (Bedford, MA, USA) was used.

Mobile phases were prepared by mixing organic modifier and acetic acid solution of the given molarity and titrating the mixture with ammonia or triethylamine to the given pH value (apparent pH, pH_a). The mobile phases were filtered through a Nalgene Nylon membrane filter (0.2 μm) (Nalge, New York, NY, USA) and degassed before use by sonication.

2.2. Instrumentation

All CEC experiments were carried out with a Hewlett-Packard HP^{3D} capillary electrophoresis (HP^{3D} CE) system (Hewlett-Packard), which provides the option of applying external pressure of up to 15 bar to the inlet and/or outlet vial. An external pressure of 8 bar has been applied to both inlet and outlet vials during runs throughout this study. Pressurization should prevent formation of gas bubbles in the capillary. Samples (1.0 mg/ml) were injected electrokinetically (−15 kV for 10 s).

3. Results and discussion

3.1. Surface structure of the stationary phase

The WAX type CSP under investigation (see Fig. 1) was originally prepared specifically for HPLC applications. The quinine carbamate SO of this bonded phase offers excellent chiral discrimination capability in HPLC enantioseparations for a broad spectrum of chiral acidic compounds in the anion-exchange mode employing buffered, aqueous–organic mobile phases [22–28]. The specifically charged surface of this modified packing and its operation with buffered mobile phases makes this anion-exchange type separation system also attractive for CEC application. However, to exploit fully the advantages of CEC smaller particle diameters (3 μm or smaller) would be preferred. Nevertheless, this non-optimized 5-μm chiral sorbent was employed for preliminary CEC studies, in order to investigate potential pros and cons regarding applicability and practicality of anion-exchange type stationary phases in CEC.

The chemical heterogeneity of the surface structure of this CSP is a consequence of several synthetic

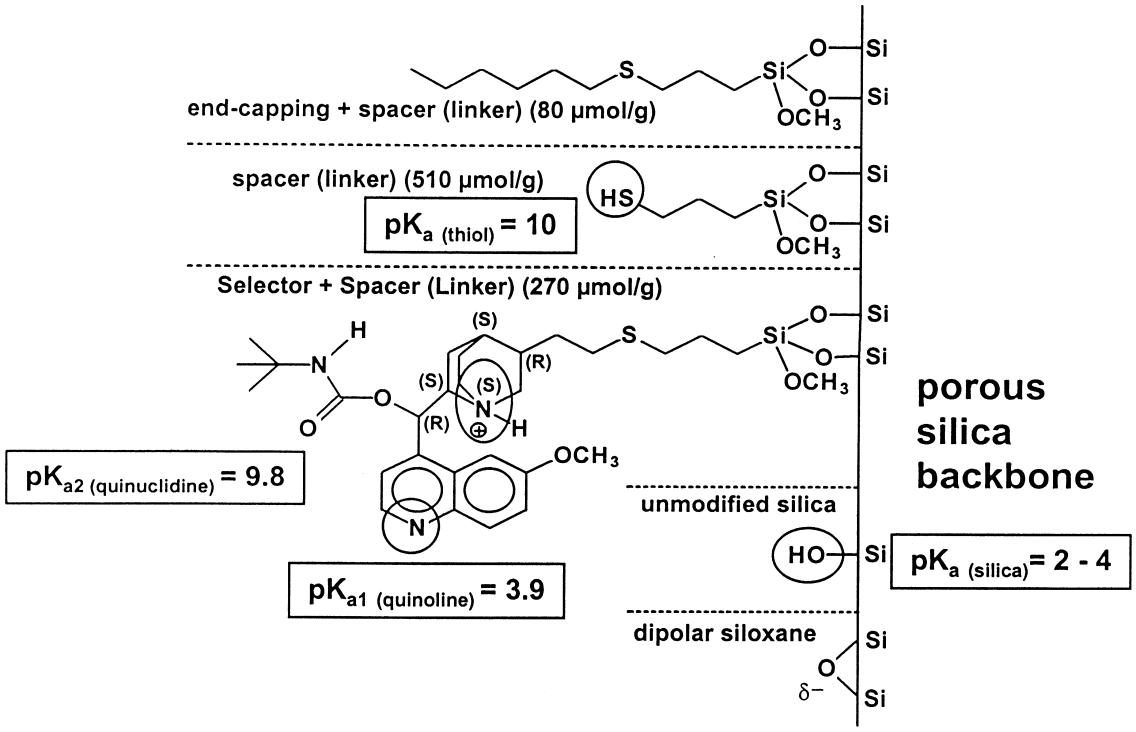

Fig. 1. Chemical heterogeneity of the silica surface modification of the *tert*.-butyl carbamoyl quinine-based weak anion-exchange type CSP.

steps involved in the SO immobilization and modification procedure (see Section 2). In particular, the presence of several positively and/or negatively charged functional groups is of interest with regards to the resulting electrochromatographic behavior. The quinine selector provides two basic amino groups: the tertiary quinuclidine group with a p*K* of about 9.8, fully protonated at pH values below 7.8 and the aromatic quinoline group with a p*K* of about 3.9, predominantly deprotonated at the typical working range between pH 5.5 and 6.0. In this range, thiol groups which have a p*K* of about 10 and which may remain unmodified after completion of the immobilization procedure, i.e. grafting the chiral selector followed by a subsequent end-capping, are non-ionized at working pH. However, since modification of the surface silanol groups is never complete, a lot of silanol groups still remained unmodified after the chemical modification procedure. These acidic silanol groups with p*K* values between 2 and 4 are predominantly deprotonated at the working pH range.

3.2. Characterization of the given chiral anion-exchange type CEC separation system

Generally three processes have to be considered in the CEC separation of chiral anionic solutes upon this WAX type CSP: electroosmotic flow, electrophoretic migration of the charged solutes, and adsorption/desorption processes of analytes to and from the stationary phase similar as in anion-exchange chromatography.

In CEC the electroosmotic flow is basically responsible for the transport of the solutes through the column. Since both amino and silanol groups are present on the surface of the packing material, the mobile phase pH determines the net charge of the surface and hence the magnitude and direction of the EOF. The experimentally obtained electroosmotic

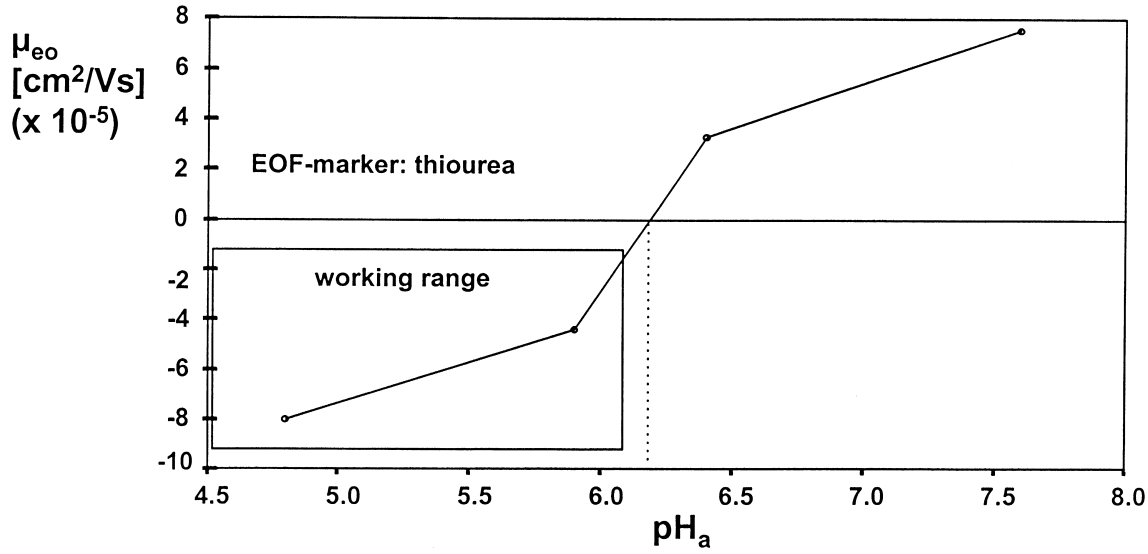

Fig. 2. Influence of mobile phase pH on eluent mobility in CEC employing a capillary column packed with a WAX type CSP derived from quinine. CEC conditions: column dimensions, see Section 2; mobile phase, methanol–10 m*M* acetic acid (80:20) (titrated to the respective apparent pH, pH_a, with triethylamine); *T*, 20°C; detection, UV 254 nm; injection, −15 kV (10 s); electric, −15 kV; pressurization, 8 bar (inlet and outlet).

flow characteristics of the WAX type CSP is depicted in Fig. 2 and will be discussed later in more detail.

Besides, for the given anionic analytes, the electrophoretic mobility also contributes to the transport of the solutes in the mobile phase. In order to avoid counter-electroosmotic migration, an anodic flow is highly desirable.

However, as long as no interaction with a stationary phase is considered, both anionic solute enantiomers move in the mobile phase with same velocities, i.e. by same electrophoretic and electroosmotic velocities.

On the contrary, enantioselectivity is strictly related to the chromatographic process; both enantiomers associate and/or dissociate at different rates with the chiral SO, primarily driven by the ionic interaction between the positively charged quinine selector and the negatively charged solutes (selectands, SAs). Besides, additional intermolecular interactions between the SO and the SA enantiomers as hydrogen bonding, dipole–dipole interactions, π–π interactions, Van der Waals and steric interactions may also get into force: they may support or enable the chromatographic, but stereoselective adsorption behaviour of both SA enantiomers. Accordingly, differences in observed effective retention times occur as a result.

3.3. Factors influencing the EOF

Fig. 2 depicts the experimental results of the influence of the mobile phase pH on eluent mobility. At pH_a values above 6.2 the net charge of the silica-based anion exchanger is negative: therefore, the bulk mobile phase is dragged electroosmotically towards the cathode (cathodic flow). At pH_a values below 6.2 the net charge of the silica surface becomes positive: the electroosmotic flow is therefore directed towards the anode (anodic flow). However, a proportion of the oppositely charged groups of the stationary phase compensates each other and only the excess of charges contribute to the effective EOF: hence, the EOF in the present investigated capillary columns is lower compared to commercial ODS CEC columns. Additionally, this non-uniformly charged surface with its locally different values of ζ

potential might cause turbulent streaming which might have dispersive character resulting in lower peak efficiency terms.

Another crucial factor having influence on the overall and effective EOF is the electrolyte concentration. In order to generate high flow-rates, low buffer concentrations should be used (buffer concentrations of 5–10 m*M* are typical in CEC). In addition, low conductivity buffers would be preferred to avoid high currents and heat generation, and thus bubble formation. Nevertheless, in the present study ammonium or triethylammonium acetate were used as electrolyte, with which better enantioselectivity than with other buffers was expected (this relies on experience from HPLC).

Besides pH and electrolyte concentration, type and percentage of organic modifier can also considerably influence eluent mobility (see Fig. 3). Comparing mobile phases with 80% organic modifier (methanol or acetonitrile) the electroosmotic mobility of the acetonitrile eluent is increased by a factor of about 2.

3.4. Comparison of CEC and HPLC separations

Fig. 4 depicts CEC and HPLC separations of DNZ-leucine enantiomers on the *tert.*-butyl carbamoylated quinine-based anion-exchange type CSP under comparable conditions (exactly the same chiral sorbent was used for preparation of CEC and HPLC columns, and the same mobile phase was run at similar eluent flow-rates).

The CEC separation was achieved with an anodic flow at pH_a of 6.0 and reversed polarity (detector end as anode), with an applied voltage of −15 kV. Under these conditions, the co-electrophoretic elution of the solute anions should be pointed out again; this is indicated by the peak of thiourea in the chromatogram which was used as EOF marker.

Indeed, effective capacity factors and also the enantioselectivity value obtained by the CEC method resemble those of HPLC enantioseparation (see Fig. 4). Thus, α values are at the given applied voltage of −15 kV comparable (2.16 in CEC versus 2.18 in HPLC). In CEC, the chromatographic efficiency of the first eluting (*R*)-enantiomer, which interacts less strongly with the chiral selector, is exceptional; about 122 000 theoretical plates per meter or 30 500 per column (with a 25-cm packed bed) have been obtained at a linear flow velocity of 0.83 mm/s (this corresponds to a reduced plate height of 1.64). Contrary, in the corresponding HPLC run only 57 000 theoretical plates per meter or 8 500 per column were obtained for the (*R*)-enantiomer; this corresponds to a reduced plate height of 3.54. Thus,

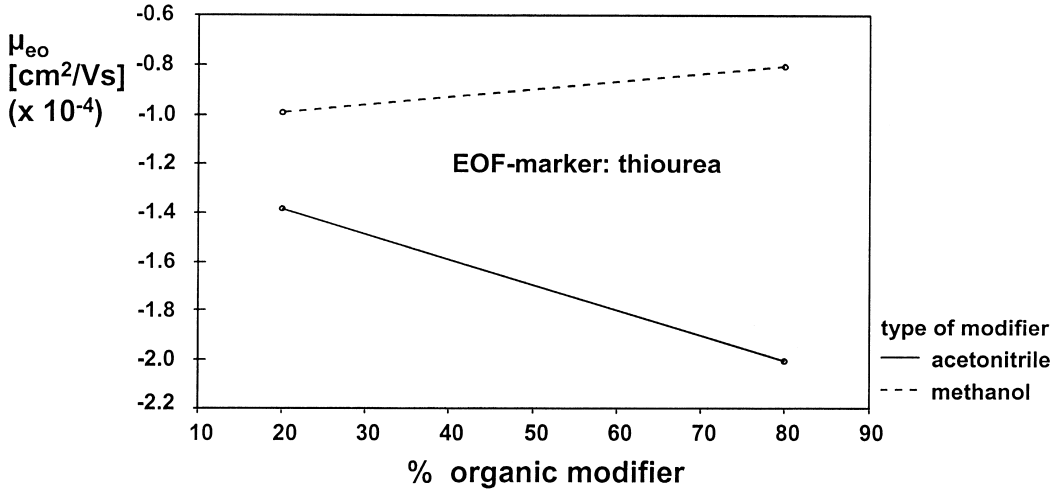

Fig. 3. Influence of organic modifier content on eluent mobility in CEC employing a capillary column packed with a WAX-type CSP derived from quinine. CEC conditions: column dimensions, see Section 2; mobile phase, organic modifier–triethylammonium acetate (total buffer concentration of the mixture, 10 m*M*; pH_a, 6.0); *T*, 20°C; detection, UV 254 nm; injection, −15 kV (10 s); electric, −15 kV; pressurization, 8 bar (inlet and outlet).

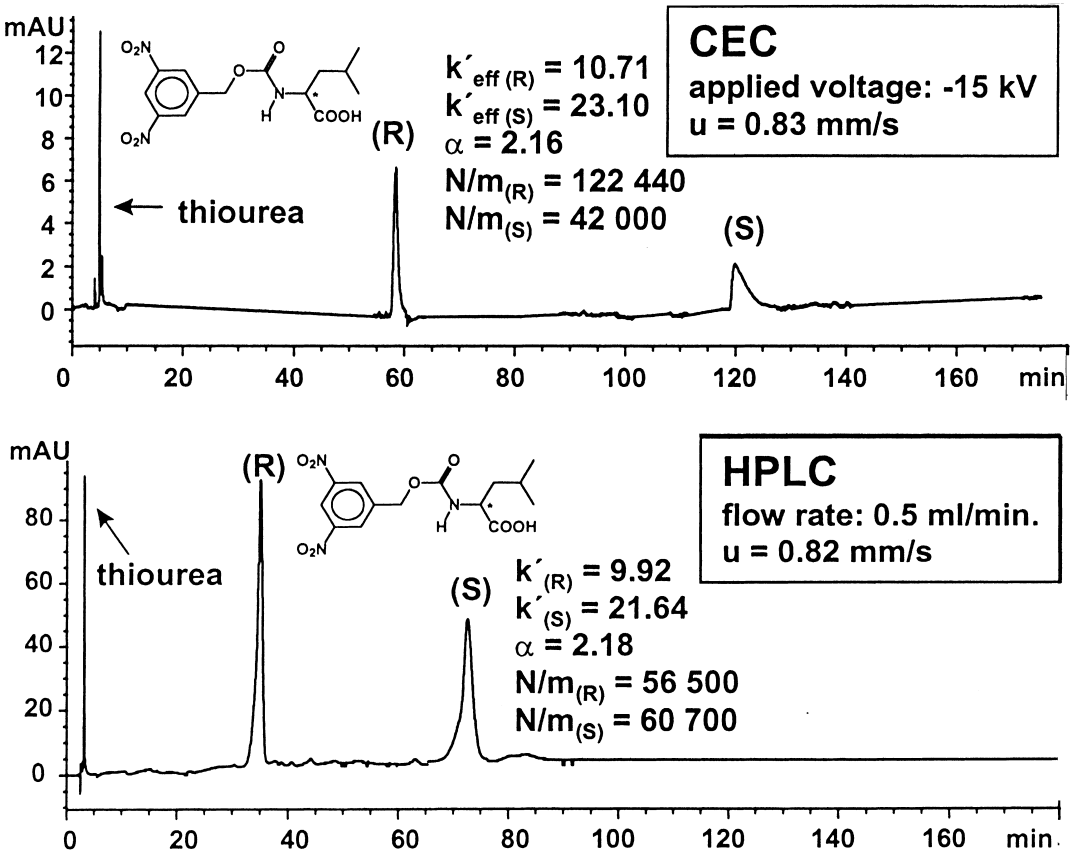

Fig. 4. CEC versus HPLC separations of DNZ-leucine enantiomers on quinine carbamate-based WAX-type CSPs. CEC conditions: column dimensions, see Section 2; mobile phase, acetonitrile–50 m*M* acetic acid (80:20) (mixture titrated to pH_a 6 with triethylamine); *T*, 20°C; detection, UV 254 nm; injection, −15 kV (10s); electric, −15 kV (−1.28 μA); pressurization, 8 bar (inlet and outlet). HPLC conditions: column dimension, see Section 2; mobile phase, as for CEC experiment; flow-rate, 0.5 ml/min; *T*, 20°C; detection, UV 254 nm; EOF marker (CEC) and void volume marker (HPLC), respectively, thiourea.

efficiency of the weakly interacting enantiomer is by a factor of more than 2 higher in the CEC experiment. On the other hand, the dissociation kinetics of the second eluting (*S*)-enantiomer is very slow; theoretical plates of about 42 000 per meter or 10 500 per column are obtained. If considering the theoretical plates per column, which are of more practical relevance, this, however, is still somewhat more than in HPLC (*N* per column for the (*S*)-enantiomer, 9100).

Unfortunately, focussing effects as described by Smith and Evans for basic drugs on strong cation exchangers [13] have not been observed. Although the presented enantioseparation is quite impressive, this CEC method suffers from the extremely long run times. For other analytes this effect may be even more pronounced. Thus in the case of 3,5-dinitrobenzoyl leucine (DNB-leucine) only the (*R*)-enantiomer can be eluted from the *tert*.-butyl carbamoyl quinine-based anion exchanger under the conditions described for DNZ-leucine. The stronger binding (*S*)-enantiomer does not come off the column; it is quasi 'stereoselectively entrapped' on the CSP.

3.5. Effect of pH on the actual capacity of the anion exchanger

In HPLC the actual capacity of the silica-based anion exchanger can easily be adjusted by the mobile phase pH. Using the same CSP in CEC, this is

accompanied by significant problems. When the capacity of the anion exchanger is reduced by increasing the mobile phase pH_a, the EOF will concomitantly be reduced, and above pH_a 6.2 this may even lead to a reversal of the total flow direction. When the pH_a is decreased, also the quinoline nitrogen may become protonated (see Fig. 1) yielding a doubly charged chiral selector moiety and thus a higher positive charge density at the shear of the surface. Consequently, the eluent mobility is slightly increased, but also the actual capacity of the anion exchanger. This, however, results in even stronger binding of the anionic analytes and, simultaneously, enantioselectivity is reduced due to the additional non-stereoselective retention increment based on ionic interaction with the positively charged quinoline nitrogen (see Fig. 5).

3.6. Effect of buffer concentration on the actual capacity of the anion exchanger

In HPLC, the strong ionic interaction between the positively charged anion exchanger and the negatively charged solutes, which is primarily responsible for the strong adsorption and the long retention times, may simply be balanced by the buffer concentration (see Fig. 6). An increase of the buffer concentration causes a decrease in retention times, and fortunately, enantioselectivity remains more or less unaffected. In order to obtain reasonable run times, the concentration of the acetate buffer has to be relatively high (100 m*M* or even higher). In anion-exchange type CEC, the strength of the ionic interaction can principally also be balanced by the buffer concentration. However, by an increase of the buffer concentration the linear flow velocity is simultaneously reduced, i.e. the 'pumping system' is affected significantly. Thus the linear flow-rate decreased from 0.8 mm/s with a 5 m*M* triethylammonium acetate buffer (electric current was −0.6 μA) to 0.7 mm/s with a 10 m*M* buffer (−1.3 μA) and to 0.6 mm/s with a 40 m*M* buffer (−4.2 μA). Much higher total buffer concentrations in the mobile phase than 20 m*M* (depending on the applied electric field strength) are not advisable in the investigated system using 75-μm packed CEC columns due to Joule heating and onset of bubble formation (despite pressurization of both ends of the

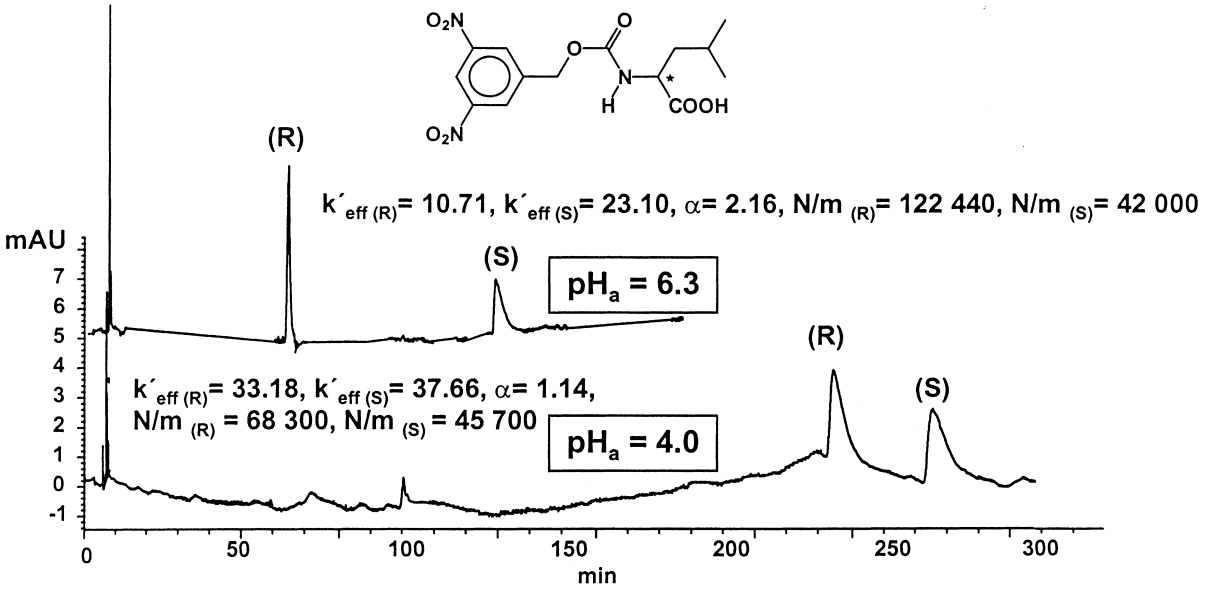

Fig. 5. CEC separations of DNZ-leucine enantiomers employing a capillary column packed with a WAX-type CSP derived from quinine: influence of mobile phase pH on retention, enantioselectivity and efficiency. CEC conditions: column dimensions, see Section 2; mobile phase, acetonitrile–50 m*M* acetic acid (80:20) (mixture titrated to the respective pH_a with triethylamine); *T*, 20°C; detection, UV 254 nm; injection, −15 kV (10s); electric, −15 kV (−1.28 and −1.25 μA, respectively); pressurization, 8 bar (inlet and outlet).

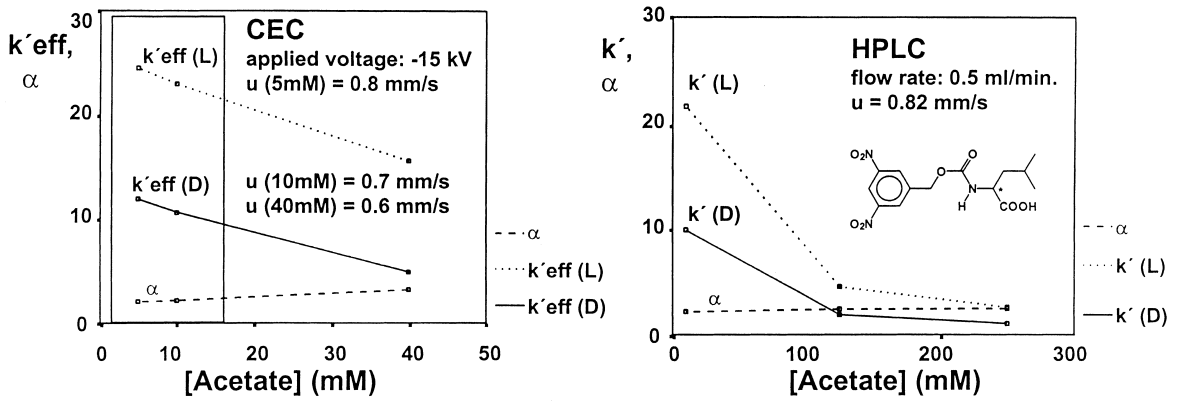

Fig. 6. CEC versus HPLC separations of DNZ-leucine enantiomers on quinine carbamate-based WAX-type CSPs: influence of buffer concentration on retention and enantioselectivity. CEC conditions: column dimensions, see Section 2; mobile phase, acetonitrile–acetic acid (80:20) (mixture titrated to pH_a 6 with triethylamine); *T*, 20°C; detection, UV 254 nm; injection, −15 kV (10 s); electric, −15 kV; pressurization, 8 bar (inlet and outlet). HPLC conditions: column dimensions, see Section 2; mobile phase, as for CEC experiments; flow-rate, 0.5 ml/min; *T*, 20°C; detection, UV 254 nm.

capillary). Hence, the 'useful' experimental range seems limited to a narrow window of buffer concentrations in the low m*M* region.

3.7. Effect of electric field strength

Another option to reduce the long run times would be to apply higher electric field strengths. Usually, EOF and electrophoretic migration increase with increasing electric field strength. Thus, an exceptionally high-efficiency CEC separation of FMOC-leucine enantiomers, which was performed by applying higher voltage (−25 kV), is demonstrated in Fig. 7. More than 15 000 theoretical plates per column for both enantiomers have been obtained compared to about 7000 by the corresponding HPLC method. Additionally, the separation is performed within a few minutes.

Very unfortunately, under these conditions problems with Joule heating and short capillary-column

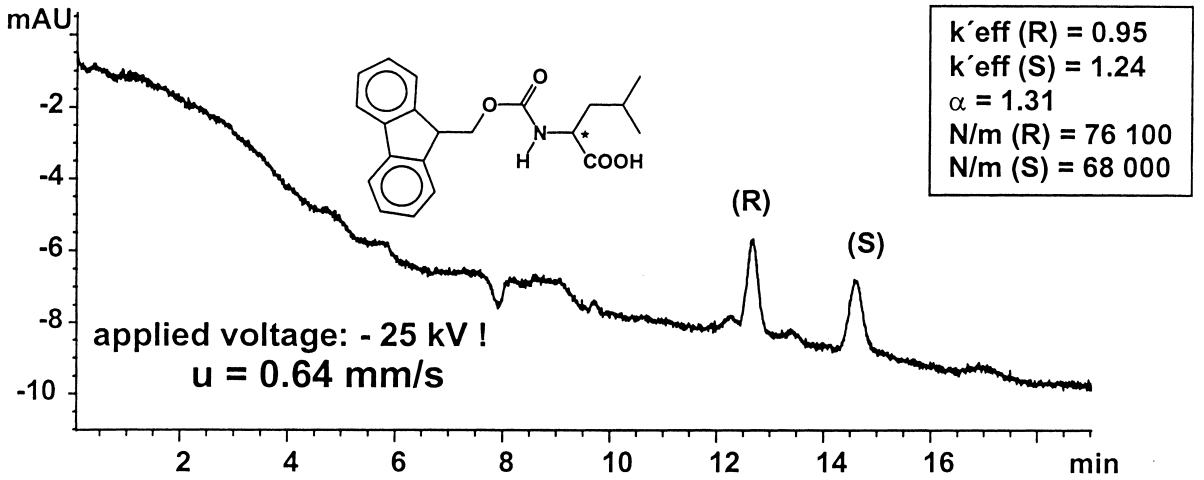

Fig. 7. CEC separation of FMOC-leucine enantiomers employing a capillary column packed with a WAX-type CSP derived from quinine. CEC conditions: column dimensions, see Section 2; mobile phase, methanol–50 m*M* acetic acid (80:20) (mixture titrated to pH_a 6 with ammonia); *T*, 20°C; detection, UV 254 nm; injection, −15 kV (10 s); electric, −25 kV (−2.8 μA); pressurization, 8 bar (inlet and outlet).

life times were more pronounced. Especially the short column life-times, brought about by sparks which were generated at the detection unit, and which destroyed the fused-silica capillaries at the detection window, presently prevents the application of voltages up to −30 kV. However, this technical problem will be solved. Together with improvements in column packing technology and use of small particles rugged and efficient anion-exchange type CEC enantioseparation systems should result.

4. Conclusion

It could be shown that the presented packed-capillary electrochromatographic enantioseparation technique employing WAX-type CSPs based on chiral quinine carbamate selectors principally works. In a certain pH range, co-electrophoretic elution of the anionic analytes is accomplished due to a positive net charge of the surface of the chirally modified silica sorbent. Enantioselectivity may be as high as in HPLC, but typically α values were smaller than in the pure chromatographic technique. For the investigated 5-μm modified silica particles an increase of efficiency by a factor of 2 was commonly observed in CEC compared to the corresponding HPLC method. However, the advantages of CEC with respect to high efficiency have not yet been fully exploited due to the use of only 5-μm particles; 1–2-μm particles will be investigated shortly.

Nevertheless, several obstacles have to be overcome before this technique becomes practically applicable to real-life chiral analysis: In order to get reasonable run times the strong ionic interactions between SO and the SAs have to be balanced; this may be accomplished by the use of higher concentrations of low conductivity buffers. By the use of higher buffer concentrations also the presently moderate run-to-run repeatability (1–5% R.S.D. at stable conditions) should be improved. A further striking disadvantage of the presented CEC technique employing WAX-type stationary phases is also the limited ruggedness (robustness) of the separation system connected with a very small range of usefull experimental conditions. In addition, capillary column hardware (frits, packing stability) is not yet fully optimized so that it can withstand the requirements and working conditions in routine analysis. Besides surface chemistry and particle type of silica backbone to be used for immobilization, a lot of optimization work has to be attributed to inlet and outlet frit making, column packing procedure, and column bed stability in connection with the employed new packing materials. If all the obstacles may be overcome and the column preparation procedures are optimized, chiral CEC with quinine carbamate-based CSPs to be run in ion-exchange and/or non-ion-exchange mode should have considerable potential in the concert of analytical enantioseparation techniques.

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