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High-efficiency enantioseparations of *N*-derivatized amino acids by packed capillary electrochromatography using ODS silica and a quinine-derived chiral selector as ion-pair agent

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Abstract

The enantiomers of *N*-derivatized amino acids, e.g., 3,5-dinitrobenzoyl, 3,5-dinitrobenzyloxycarbonyl, 2,4-dinitrophenyl and 9-fluorenylmethoxycarbonyl amino acids, have been separated by enantioselective ion-pair formation and packed capillary electrochromatography (CEC) using RP18 silica particles. Thus, a CEC-Hypersil ODS-3 μm packed capillary column, 335 mm (effective length 250 mm)×0.1 mm I.D., was used in combination with a quinine carbamate type chiral ion-pair agent (selector, SO) which was added to aqueous and non-aqueous buffered mobile phases, respectively. The negatively charged analyte enantiomers interact with the positively charged chiral SO by multiple intermolecular interactions to form a pair of transient diastereomeric ion-pairs which may differentially adsorb to the ODS-stationary phase. Enantioseparation is achieved due to different observed mobilities of the analyte enantiomers originating from different ion-pair formation rates of the enantiomers and/or differential adsorption of the diastereomeric ion-pairs to the ODS-stationary phase. Countercurrent-like electrophoretic migration of oppositely charged ion-pair agent and solute enantiomers may give rise to enhanced enantioselectivity. At high electrolyte concentrations (>10 m*M*), e.g. if the chiral ion-pair agent is added to the mobile phase that contains, compared to typical RP-CEC conditions (<10 m*M*), relatively high amounts of background electrolyte, electrophoretic transport of the analytes is dominating (negative polarity mode); this method is characterized by high efficiency with theoretical plate numbers up to 170 000 per meter, e.g. for DNP-Val, but with moderate enantioselectivity. In contrast, at low electrolyte concentrations (<10 m*M*), e.g. if the chiral ion-pair agent itself is used as electrolyte without any other background electrolyte, the analytes are driven through the capillary column with the electroosmotic flow (positive polarity mode); this operation mode is characterized by relatively poor efficiency, but high enantioselectivity. The influence of several mobile phase parameters (aqueous versus non-aqueous, selector concentration, type of background electrolyte) on observed mobility, enantioselectivity and efficiency was evaluated.

Keywords: Enantiomer separation; Electrochromatography; Ion-pairing reagents; Chiral selectors; Mobile phase composition; Amino acids; Butylcarbamoylquinine

1. Introduction

Packed-capillary electrochromatography (CEC) is a promising high-efficiency microcolumn separation

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technique in which the mobile phase is electrically driven through a packed capillary column and which combines electrophoretic and chromatographic transport and separation mechanisms [1–7]. While neutral analytes are transported through the packed capillary only by virtue of the electroosmotic flow (EOF), which is generated when an electric field is applied across the capillary column, the transport of charged species depends also on their electrophoretic migration, which may be co-electroosmotic or counter-electroosmotic depending on the charge of the packing material and of the solutes as well as on the presence of certain EOF modifiers in the mobile phase. Consequently, separation of solutes is based on their differential adsorption and/or electrophoretic migration behavior.

Most CEC columns including commercially available ones, i.e. reversed-phase (RP), strong cation exchangers (SCX), and mixed mode RP/SCX CEC columns, are based on silica as chromatographic support. Due to negatively charged residual silanol groups and/or sulfonic acid groups of those packing materials the EOF is directed to the cathode. Accordingly, cations migrate co-electroosmotically and anions, in contrast, counter-electroosmotically. This counter-electroosmotic movement of anions in these CEC columns seems to be the main problem that may lead to inefficient separations and/or long run times.

The majority of current CEC studies deal with separations of pharmaceuticals [4,8–12], biologically interesting compounds [13,14], polycyclic hydrocarbons and other environmentally relevant pollutants [15–17] as well as enantioseparations [18–26] either in packed capillaries or continuous bed capillary columns. However, most of them are concerned with the separation of neutral analytes which exhibit due to absence of strong electrostatic analyte–sorbent interactions more or less ideal behavior. Nevertheless, several studies are also concerned with the separation of basic analytes. Thus, Smith and Evans [11] presented the separation of a series of basic drugs on SCX with efficiencies of $>8\times10^6$ plates per meter. These extremely high theoretical plate numbers are not yet clearly understood and may be due to peak compression and zone focusing effects. Unfortunately, these separations suffer from poor run-to-run reproducability. Recently, a theory for zone migration of charged species in electrochromatography was proposed by Ståhlberg [7] which offers another explanation for the appearance of these extremely sharp peaks. Generally, the mixing of chromatographic and electrophoretic transport mechanisms gives rise to strong non-linear effects, which may cause strong band broadening or, alternatively, a stabilized zone which does not change its form during the migration through the column. Other applications of CEC for the separation of basic drugs include enantioseparations of, e.g. mianserin and chlorthalidon on a hydroxypropyl-β-cyclodextrin chiral stationary phase (CSP) [19], enantioseparations on protein type CSPs [18,27] and enantioseparations of basic drugs with molecular imprint capillary columns [23,24].

On the other hand, only a few attempts to apply CEC to the separation of anionic analytes are published. These include the use of a quinine carbamate based CSP for the separation of the enantiomers of *N*-derivatized amino acids [22] and the use of a cyclodextrin CSP in combination with triethylammonium acetate as background electrolyte (BGE) for the enantioseparation of 2,4-dinitrophenyl (DNP) amino acid derivatives [28]. Both methods enable the reversal of the EOF due to operation as anion exchanger and dynamic anion exchanger, respectively. On the contrary, a set of acidic non-steroidal anti-inflammatory drugs (NSAIDs) were separated by Euerby [29] with a CEC Hypersil mixed mode stationary phase at pH 2.3, at which dissociation of the analytes is suppressed and separation is achieved only by virtue of different chromatographic properties. Similarly, suppressing ionization was also used by Lin et al. for the CEC enantioseparation of Dansyl-amino acid derivatives on molecular imprint capillary columns [30].

Another approach to separate anions is presented in this study by enantioselective ion-pair CEC. This method combines the principles of enantioselective ion-pair capillary zone electrophoresis (CZE) [31,32] and enantioselective ion-pair chromatography which was extensively studied by Pettersson and Schill [33–35]. They also explored quinine as chiral ion-pair agent for the separation of the enantiomers of chiral acids. Recently, a study was presented, in which quinine was employed as chiral additive in non-aqueous CZE [32] and more recently also

carbamoylated quinine selectors were successfully used for enantioseparation of chiral acids in non-aqueous CZE [36].

In this paper, the enantioseparation of various *N*-derivatized amino acids by packed-capillary electrochromatography employing *tert*-butyl carbamoyl quinine (see Fig. 1A) as ion-pair agent and a CEC-Hypersil ODS-3 μm packed-capillary column is reported. The chiral ion-pair agent itself, which has earlier been used as chiral selector grafted to silica [37–39], may be used as electrolyte or it may be added to a background electrolyte. By these two modes either the chromatographic (electroosmotic) or the electrophoretic transport mechanism is favored. The separations are carried out with aqueous–organic mobile phases or non-aqueous mobile phases. Experimental parameters as type of BGE, and concentration of chiral ion-pair agent in the mobile phase are studied. Representative enantioseparations of DNP, 3,5-dinitrobenzoyl (DNB), 3,5-dinitrobenzyloxycarbonyl (DNZ), and 9-fluorenylmethoxycarbonyl (FMOC) amino acids are given.

2. Experimental

2.1. Materials

A CEC-Hypersil ODS-3 μm packed capillary column, purchased from Hewlett-Packard, Waldbronn (Germany), was used as stationary phase. The capillary had a packed bed of 25 cm and a total length of 33.5 cm. The inner diameter of the fused-silica capillary was 100 μm.

The chiral selector, *tert*-butyl carbamoyl quinine (see Fig. 1A), was prepared according to a standard procedure described elsewhere [40]. The pK_a-values of the chiral selector have been calculated with pKalc 3.1, which is a validated computer software from CompuDrug Chemistry (Budapest, Hungary)

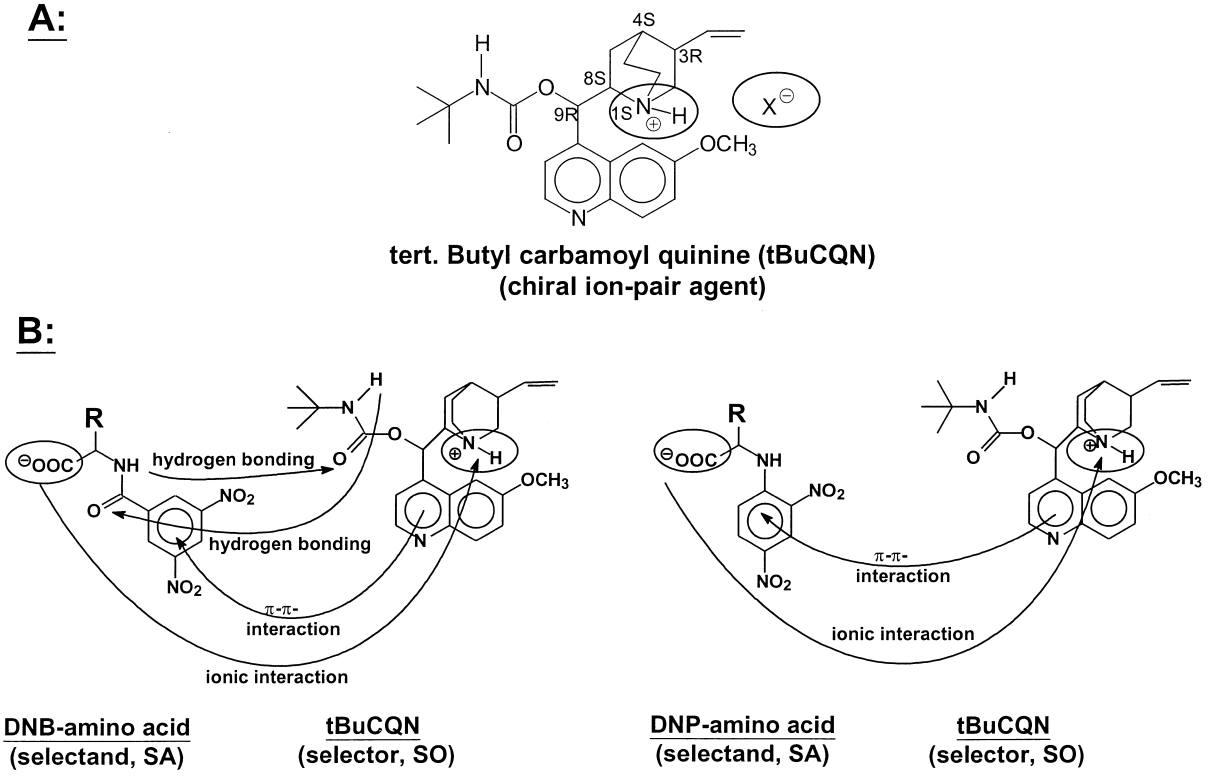

Fig. 1. (A) Structure of *tert*-butyl carbamoyl quinine (tBuCQN) used as chiral ion-pair agent and selector (SO). (B) Binding model for anionic *N*-acyl (e.g. DNB) and *N*-aryl (e.g. DNP) amino acids as selectands (analytes).

and is now commercially available from VCH, Weinheim (Germany). Unfortunately, they could not be experimentally measured due to solubility problems. The calculated pK_a-values are: pK_{a1} (quinuclidine)=9.8 and pK_{a2} (quinoline)=3.9. (For native underivatized quinine, the following experimentally determined pK_a-values are reported in the Merck Index [46]: pK_{a1} (quinuclidine)=9.70 and pK_{a2} (quinoline)=5.07 (measured at 18°C).

(*rac*)- and (*S*)-*N*-(3,5-dinitrobenzoyl) leucine (DNB-Leu) were obtained from Aldrich and (*rac*)- and (*S*)-*N*-(9-fluorenylmethoxycarbonyl) leucine (FMOC-Leu) from Bachem, Bubendorf (Switzerland). The racemic mixture and the (*R*)- and (*S*)-enantiomers of *N*-(3,5-dinitrobenzyloxycarbonyl) leucine (DNZ-Leu) were prepared as described elsewhere [41]. *N*-(2,4-dinitrophenyl) derivatives of leucine, valine and proline (DNP-Leu, DNP-Val, and DNP-Pro) were synthesized by a standard derivatization protocol [42].

Methanol (MeOH) for the preparation of the mobile phases was HPLC grade from J.T. Baker. Ammonia, triethylamine, glacial acetic acid, ammonium acetate and ammonium formate, citric acid, phosphoric acid, formic acid, propionic, butyric, and octanoic acid, which were used to prepare the buffer solutions and mobile phases were of analytical grade, and purchased from different suppliers (Merck, Aldrich, Fluka). For the preparation of buffers and mobile phases, respectively, distilled water that was additionally purified by a Milli-Q-Plus filtration unit from Millipore, Molshem (France) was used.

If indicated, the mobile phase pH refers to the apparent pH (pH_a) which was adjusted by titrating the organic modifier–buffer mixture with the respective acid to the given pH. The mobile phases were filtered through a Nalgene nylon membrane filter (0.2 μm) (Nalge, New York, 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, 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 to 10 bar has been applied to both ends of the capillary throughout this study. Pressurization should prevent formation of gas bubbles in the capillary. The samples (0.5 mg/ml) were injected electrokinetically.

3. Results and discussion

3.1. Basic considerations of retention and selectivity principles of stereoselective ion-pair CEC with quinine carbamate as chiral ion-pair agent

Quinine and derivatives thereof have, although not often used, a long tradition as chiral ion-pair agents (chiral selectors, SOs) in HPLC enantioseparations of chiral acidic analytes (selectands, SAs) [33]. Thus, anionic solute enantiomers and positively charged ion-pair agent form a pair of transient diastereomeric ion-pairs, which may differ in their adsorption behavior to the stationary phase. In this chromatographic experiments retention and separation depend on the concentration of the ion-pair agent in the mobile phase and the rate constants of ion-pair formation as well as on adsorption of the ion-pairs and the individual free solutes to the stationary phase. Many of the theoretical aspects of enantioselective (stereoselective) ion-pairing have been well characterized, particularly by Pettersson [33]. However, practical applications in HPLC remained few, maybe also due to relatively high demands (quantity and quality) on SO products.

On the other side, in enantioselective capillary electrophoresis, where the chiral SO is conceptually added to the buffer, the consumption of high value SOs is not an issue since only small amounts are needed for this micro-scale separation technique. In free solution CZE using chiral ion-pair agents to resolve the enantiomers of acidic analytes, the separation depends on differential ion-pair formation of the two enantiomers with the chiral ion-pair agent and on differences in electrophoretic mobilities between free and complexed solute species. Additionally, the differential overall migration behavior of the both corresponding enantiomers is also a function of the concentration of the ion-pair agent [31,32,43].

Both chromatographic and electrophoretic transport mechanisms are mixed in capillary electrochromatography of ionized species involving ion-pairing as the separation principle. Transport of the several solute species (transient diastereomeric ion-pairs, free SO and free SAs) with the EOF and adsorption to the stationary phase may be termed as chromatographic process. On the other side, in ion-pair processes the both binding partners bear opposite charges. Thus, free chiral SO and free SAs electrophoretically migrate countercurrent-like to the opposite electrodes. This mechanism, self-electrophoretic mobility of the chiral SO in opposite direction to the chiral solutes, was demonstrated to be responsible for enhanced enantioseparation factors of anionic cyclodextrin derivatives in the enantioseparation of basic chiral compounds [44]. Effective rates of the individual transport processes are virtually influenced by secondary chemical equilibria, which are affected by the experimental conditions.

In the presently investigated enantioselective ion-pair CEC method *tert*-butyl carbamoyl quinine (tBuCQN) was employed as chiral selector and ion-pair agent, and a CEC-Hypersil C_{18}-3 μm packed column was used as stationary phase. The chiral SO has a tertiary basic nitrogen functional group (pK_a = 9.8) incorporated in the quinuclidine moiety (see Fig. 1A) that is under working conditions protonated and thus serves as driving force for ion-pairing with dissociated acids, i.e. the anionic SAs. The chiral selector has a second basic site (quinoline) with a pK_a-value of 3.9 that is under experimental conditions as used throughout this study preferentially deprotonated or non-ionized. The positive charge of the SO forces its electromigration towards the cathode. Besides the primary ionic interaction additional intermolecular interaction forces, together with steric factors may be active stereoselectively between the chiral SO and the SA enantiomers; these are the basis for different association constants, resp. ion-pair formation constants (K_{IP}) of both enantiomers. Among them hydrogen bonding between the carbamate group of the chiral SO and complementary functional groups in the SA as well as π–π-interaction between the quinoline and electron-poor aromatic moieties in the SA are the most effective chiral recognition and discrimination forces, especially if they act simultaneously in a co-operative manner and together with Van der Waals type and/or steric binding contributions. Two distinct tentative SO-SA-binding models of quinine carbamate SO and *N*-acyl and *N*-aryl amino acid derivatives, exemplified by DNB- and DNP-amino acid derivatives, respectively, are presented in Fig. 1B. As mobile phase and electrolyte solution, respectively, either aqueous buffered mobile phases with various amounts of organic modifiers or totally non-aqueous mobile phases with organic buffers dissolved in a polar organic solvent (usually methanol) were used.

From a methodological point of view and describing the CEC situation, Fig. 2 depicts a schematic representation of the processes involved in enantioselective ion-pair CEC. The surface of the modified silica particles and also of the capillary contain permanently electrically charged silanol groups, which generate, upon application of an electric field, an electroosmotic flow which is directed towards the cathode (cathodic flow). The movement of the species with the EOF is indicated as electroosmotic mobility (μ_{eo}).

The negatively charged free analytes or selectands, (*S*)- and (*R*)-SAs [in Fig. 2 denoted as (*S*)- and (*R*)-$RCOO^-$] migrate electrophoretically towards the anode (μ_{ep}), and their overall velocity is reduced by the oppositely directed EOF. The positively charged chiral selector (see also Fig. 1A) migrates in its free state electrophoretically towards the cathode and is additionally accelerated by the cathodic EOF. During the binding (ion-pairing) process it forms transient lipophilic ion-pairs with the SA enantiomers which are transported with the EOF and may also adsorb to the ODS-stationary phase. In contrast, considering the presently used mobile phases with high organic modifier content, adsorption of the ionized species, of the SO and of the SAs, to the stationary phase is of minor importance. However, with low organic modifier content enhanced adsorption of the (optionally lipophilized) quinine SO could be exploited to reverse the EOF by generating a more pronounced dynamically coated chiral anion exchanger.

Enantioselectivity is related to differential ion-pair formation ($K_{(R)\text{-IP}} \neq K_{(S)-\text{IP}}$) and/or differential adsorption of the transient diastereomeric ion-pairs to the stationary phase ($K_{\text{Ads. }(R)\text{-IP}} \neq K_{\text{Ads. }(S)\text{-IP}}$) due to differences in their lipophilicity originating from their different overall conformation. From these

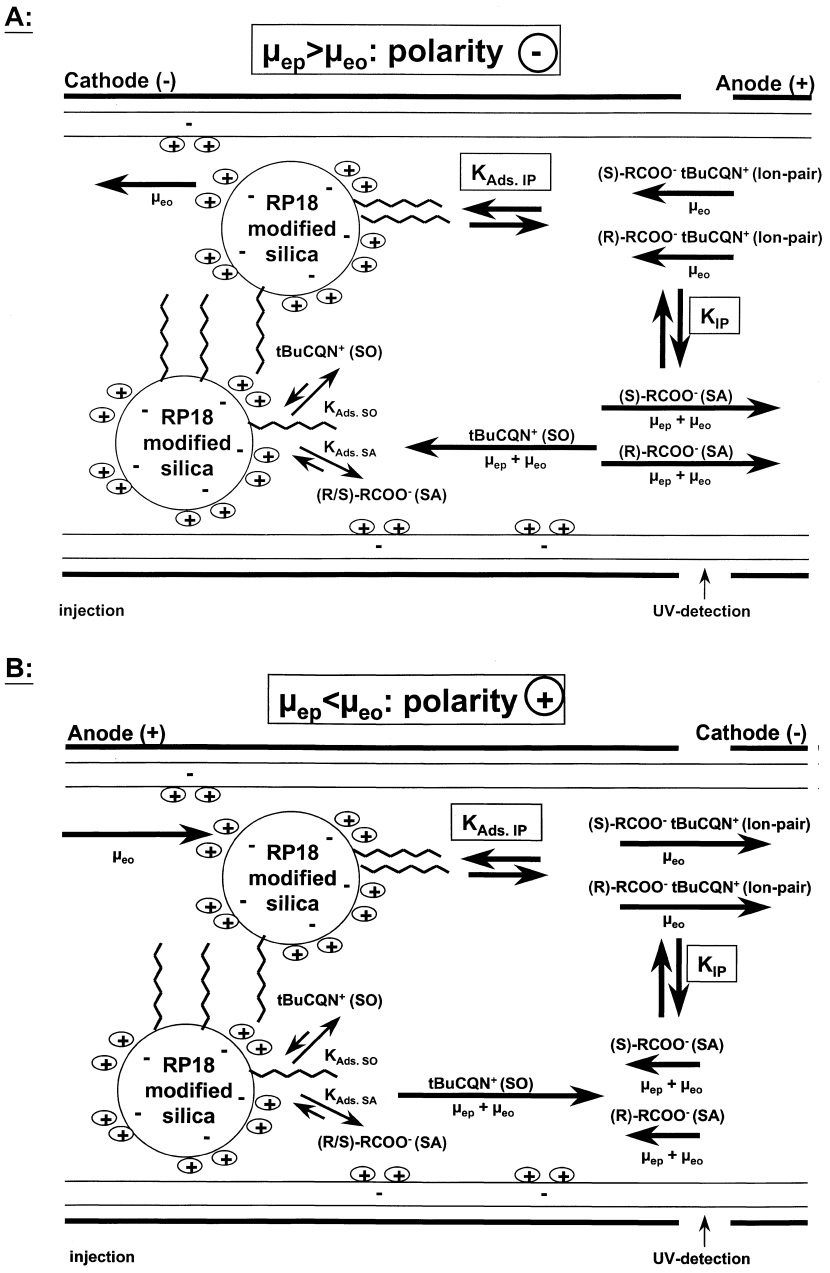

Fig. 2. Schematic representation of the processes involved in enantioselective ion-pair CEC methodologies and relative directions of transport processes. (A) Electrophoretic elution mode: electrophoretically dominated transport (ion-pair agent added to the mobile phase which contains relatively high concentrations of BGE). (B) Chromatographic elution mode: electroosmotically dominated transport (ion-pair agent itself employed as buffer and electrolyte).

differential rate constants differences in observed migration and retention times, respectively, result for both corresponding solute enantiomers. The several interacting equilibria may be influenced and controlled by various mobile phase parameters (pH, buffer type and concentration, temperature) thus

providing enormous flexibility in enantioselectivity and efficiency optimization.

Whereas in CEC of neutral species generally only one axial transport direction exists which is predetermined by the EOF direction, both axial overall transport directions can be realized in CEC of ionized species. Both cases, which are schematically represented by Fig. 2A and B, can be realized under particular experimental conditions. Representative enantioseparations are illustrated in Fig. 3A and B, and the comparisons of the two elution modes clearly demonstrate the advantages and peculiarities of each elution mode.

3.2. Considerations about the electrophoretically dominated separation and elution mode

At high electrolyte concentrations (>10 m*M*), e.g. if the chiral SO is added to the mobile phase which contains substantial amounts of a background electrolyte (BGE) (20 m*M* triethylammonium acetate is a high electrolyte concentration compared to RP-

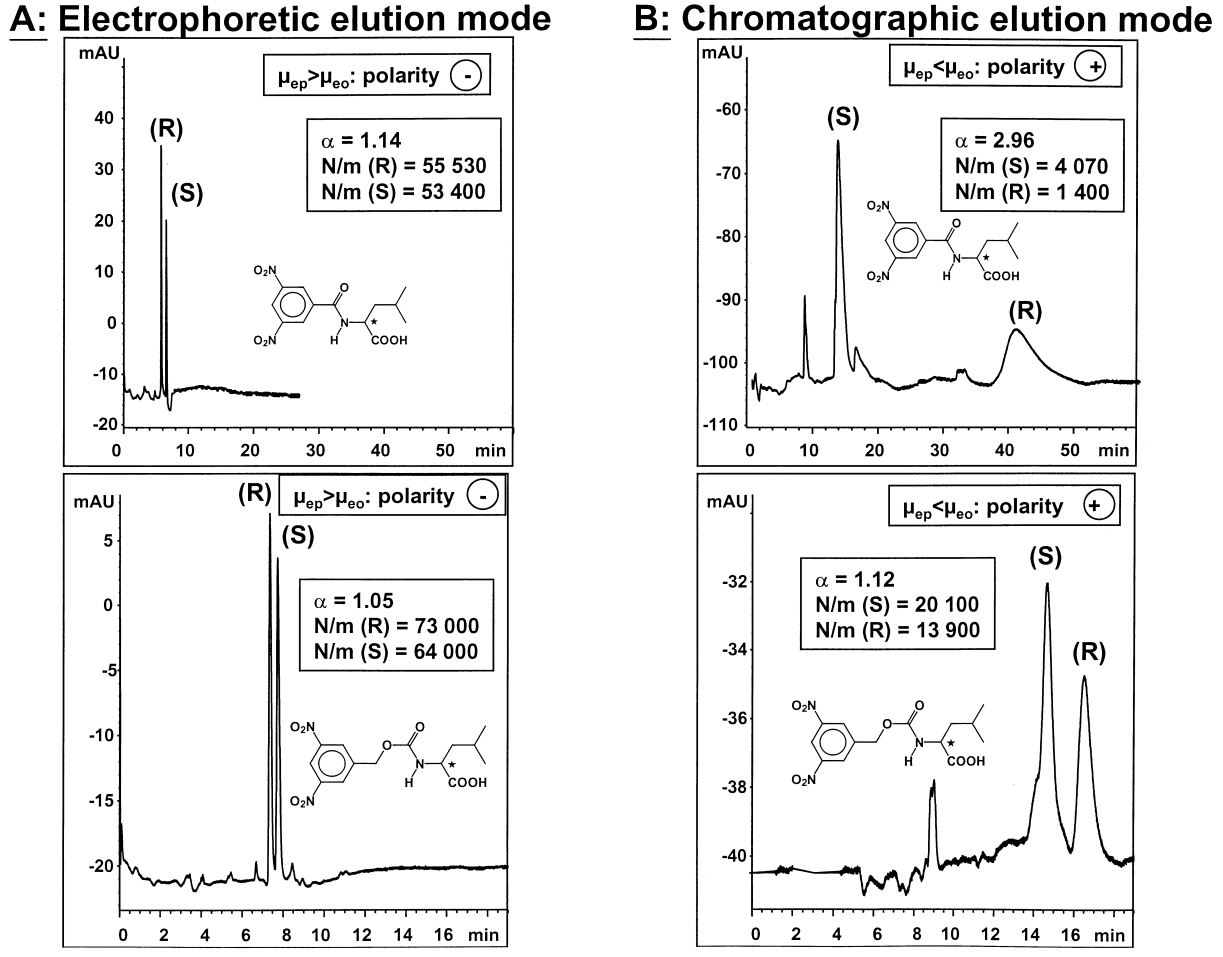

Fig. 3. CEC separation of DNB-Leu and DNZ-Leu enantiomers employing *tert*-butyl carbamoyl quinine as chiral ion-pair agent. (A) Electrophoretically dominated separation (experimental conditions: mobile phase: MeOH+20 m*M* triethylammonium acetate +5 m*M* tBuCQN; 20°C; electric: −25 kV (−22 μA); injection: −10 kV (10 s); pressurization: 8 bar, inlet and outlet). (B) Electroosmotically dominated separation (experimental conditions: mobile phase: MeOH+5 m*M* tBuCQN+5 m*M* HAc; 20°C; electric: +25 kV (+3.3 μA); ; injection: +10 kV (10 s); pressurization: 8 bar, inlet and outlet vial) (for better comparison α is calculated by $\mu_{obs.1}/\mu_{obs.2}$; the peak at ca. 9 min in the electrochromatogram of Fig. 3B is from thiourea that has been added as EOF marker).

CEC where typically buffer concentrations <10 m*M* are used), then the electrophoretic process is the determinant for transport of the SA enantiomers in the capillary column (see Fig. 2A and 3A as well as Tables 1 and 2). This means that the electrophoretic mobility of the anions is higher than its electroosmotic mobility and thus dominates the separation. Accordingly, the SAs are detected when the electric polarity is negative.

The obtained enantioseparations are characterized by high efficiencies, but at the expense of low enantioselectivity. For instance, plate numbers of DNB-Leu and DNZ-Leu enantiomers are ranging between 50 000 and 70 000 per meter (see Fig. 3A). Unfortunately, very small α-values were obtained for DNZ-Leu (1.05) and DNB-Leu (1.14) as well. In contrast, in HPLC with the *tert*-butyl carbamoyl quinine SO immobilized onto silica and a mobile phase as specified in Table 1, we were able to resolve DNB-Leu enantiomers with an α-value of 15.88 and DNZ-Leu enantiomers with $\alpha=2.80$ [37,39], while theoretical plate numbers ranged from 20 000 to 40 000 per meter. In packed capillary anion-exchange CEC with the same SO bound to silica and the resulting chiral stationary phase packed into a capillary column, usually more or less the same enantioselectivity values could be reached as in LC and efficiencies were by a factor of 2 to 3 higher than in LC [22].

3.3. Considerations about the chromatographically (electroosmotically) dominated separation and elution mode

At low electrolyte concentrations, e.g. if the chiral ion-pair agent itself, which a priori has to be a charged compound, serves as electrolyte and if no additional background electrolyte is used, anodic electrophoretic migration of the SA enantiomers is overcompensated by the cathodic eluent mobility which dominates this more chromatography like separation system; in this case, the electric polarity has to be positive (see Fig. 2B and 3B as well as Table 3: DNB, DNZ, FMOC-Leu). However, for DNP-amino acids under the given experimental conditions the electrophoretic process is still dominating the separation (see Table 3). It should be pointed out that the relative directions of the moving species in electrophoretically and electroosmotically dominated CEC separation modes are principally the same, but their total and relative amounts are different.

The electroosmotically dominated ion-pair CEC mode being more similar to ion-pair HPLC is characterized by higher enantioselectivity, but much lower efficiency compared to the electrophoretically dominated mode (compare Fig. 3A and B). Thus, DNB-Leu enantiomers could be resolved with an α-value of 2.96 (versus 1.14 with the electrophoretic

Table 1
Enantioseparations of *N*-derivatized amino acids by aqueous enantioselective ion-pair electrochromatography with a packed RP18 CEC column and *tert*.-butyl carbamoyl quinine as chiral ion-pair agent added to the BGE[a]

Solute	[SO][b] (m*M*)	t_1 (min)	t_2 (min)	$\mu_{obs.1}$ [$\times10^{-4}$] ($cm^2\ V^{-1}\ s^{-1}$)	$\mu_{obs.2}$ [$\times10^{-4}$] ($cm^2\ V^{-1}\ s^{-1}$)	α[c]	N_1	N_2	R_S	m.o.[d]
DNP-Val	5	8.29	8.70	−0.67	−0.64	1.09	25341	21397	1.86	*S*
DNP-Pro	5	8.22	8.69	−0.68	−0.64	1.11	29606	16702	2.04	*S*
DNP-Leu	3	8.04	8.47	−0.69	−0.66	1.10	26337	21787	1.97	*S*
DNZ-Leu	5	11.82	13.47	−0.47	−0.41	1.37	7478	6128	2.67	*R*
DNB-Leu	5	11.48	23.22	−0.49	−0.24	4.21	3919	1627	7.72	*R*
FMOC-Leu	5	19.41	20.97	−0.29	−0.27	1.22	16255	14616	2.40	*R*

[a] BGE: MeOH/0.1 *M* ammonium acetate (80:20); $pH_a=6.0$; $T=20°C$; electric: −25 kV.
[b] t_{EOF} (Thiourea)=22.54 min. (3 m*M* SO) and 22.89 min. (5 m*M* SO); electric: +25 kV.
[c] $\alpha=\mu_{eff\ 1}/\mu_{eff\ 2}$.
[d] m.o.: migration order; configuration of first detected enantiomer.

Table 2
Enantioseparation of *N*-derivatized amino acids by non-aqueous enantioselective ion-pair electrochromatography with a packed RP18 CEC column and *tert*.-butyl carbamoyl quinine as chiral ion-pair agent added to the BGE

SA	BGE[a]	[SO] (m*M*)	kV	t_1 (min)	t_2 (min)	$\mu_{obs.1}$ [$\times 10^{-4}$] ($cm^2\ V^{-1}\ s^{-1}$)	$\mu_{obs.2}$ [$\times 10^{-4}$] ($cm^2\ V^{-1}\ s^{-1}$)	α[b]	N_1	N_2	R_S	m.o.[c]
tBuCQN	A	0	25	17.10		0.33						
Thiourea	A	0	25	17.48		0.32						
	A	5	25	17.82		0.31						
DNB-Leu	A	0	−25	6.11		−0.91			13971			
	A	5	−25	5.76	6.55	−0.97	−0.85	1.14	13881	13350	3.73	*R*
DNZ-Leu	A	0	−25	6.55		−0.85			15641			
	A	5	−25	7.37	7.74	−0.76	−0.72	1.05	18249	15976	1.62	*R*
FMOC-Leu	A	0	−25	8.48		−0.66			14938			
	A	5	−25	7.13	7.36	−0.78	−0.76	1.03	17794	10178	0.89	*R*
tBuCQN	B	0	25	13.13		0.43			17793			
Thiourea	B	0	25	13.17		0.42						
	B	5	25	13.40		0.42						
	C	5	25	18.58		0.30						
DNP-Leu	B	0	−25	5.36		−1.04			8131			
	B	5	−25	4.64		−1.20			6704			
	C	5	−25	6.73	7.03	−0.83	−0.79	1.04	32187	28272	1.84	*S*
DNP-Pro	B	0	−25	5.33		−1.05			7316			
	B	5	−25	4.57		−1.22			5130			
	C	5	−25	8.29	8.74	−0.67	−0.64	1.05	33434	38380	2.51	*S*
DNP-Val	B	0	−25	5.20		−1.07			12753			
	B	5	−25	4.49		−1.24			7347			
	C	5	−25	7.70	8.08	−0.73	−0.69	1.05	37065	41482	2.34	*S*

[a] A: MeOH+20 m*M* triethylammonium acetate; B: MeOH+20 m*M* ammonium acetate; C: MeOH+20 m*M* ammonium acetate+1% acetic acid; T=20°C.
[b] $\alpha=\mu_{eff\ 1}/\mu_{eff\ 2}$.
[c] m.o.: migration order; configuration of first detected enantiomer.

Table 3
Enantioseparation of *N*-derivatized amino acids by non-aqueous enantioselective ion-pair electrochromatography with a RP18 packed capillary column and *tert*.-butyl carbamoyl quinine as chiral ion-pair agent and electrolyte[a]

SA	kV	t_1 (min)	t_2 (min)	$\mu_{obs.1}$ [$\times 10^{-4}$]	$\mu_{obs.2}$ [$\times 10^{-4}$]	$k'_{eff\ 1}$[b]	$k'_{eff\ 2}$[b]	α[c]	N_1	N_2	R_S	e.o.[d]
Thiourea	25	8.50		0.66					1391			
DNB-Leu	25	13.86	41.06	0.40	0.14	0.63	3.83	6.08	1016	348	5.15	*S*
DNZ-Leu	25	14.65	16.48	0.38	0.34	0.72	0.94	1.30	5022	3473	1.88	*S*
FMOC-Leu	25	14.21	14.59	0.39	0.38	0.67	0.72	1.07	6076	3865	0.46	n.d.
DNP-Leu	−25	6.59		−0.85					5546		0.00	
DNP-Pro	−25	6.47		−0.86					3068		0.00	
DNP-Val	−25	6.68		−0.84					5697		0.00	

[a] mob. ph.: MeOH+5 m*M* tBuCQN+5 m*M* acetic acid; T=20°C.
[b] $k'_{eff.}=(t-t_{Thiourea})/t_{Thiourea}$.
[c] $\alpha=k'_{eff\ 2}/k'_{eff\ 1}$.
[d] e.o.: elution order; configuration of first eluted enantiomer, n.d.: not determined.

elution mode). However, efficiencies are even worse than in HPLC. The same trend is observed for DNZ-Leu enantioseparation (compare Fig. 3A and B).

Obviously, the electrophoretically dominated separation mode is preferred for practical applications, as the somewhat lower enantioselectivity is more than compensated by the higher efficiency.

It should be pointed out that in the two distinct elution modes the elution order is reversed (compare Fig 3A and B); this fact is of special interest for the mechanism of the enantioseparation process. However, this reversal is not due to modified binding relations between the chiral SO and the both enantiomers, but due to opposite sign of observed mobilities caused by alterations of the relative magnitudes of EOF and electrophoretic mobility contributions under different experimental conditions.

3.4. Enantiomer migration and elution order, respectively

In contrast to chromatography, where the elution order is directly correlated with the binding affinity of the enantiomers to the chiral SO, in chiral ion-pair CEC, as generally in electrically driven separation systems, the order of migration or elution of two corresponding enantiomers is not only correlated with their binding affinity to the chiral SO. Thus, besides the intrinsic ion-pair rate constant (K_{IP}), that determines which enantiomer has higher affinity to the chiral SO, and differential adsorption constants of the corresponding ion-pairs, also directions and relative magnitude of electrophoretic and electroosmotic mobility contributions of free and complexed SA-species have to be considered. All these factors contribute to the observed mobility, and may be the source for a reversal of the migration, respectively, elution order when experimental conditions are varied and thus relative magnitudes of these contributions change. From a stereochemical point of view the relative affinity of the two corresponding enantiomers to the chiral SO is clearly defined, however, from a separation methodological point of view both enantiomers may migrate or elute in reversed order; this fact, indeed, stresses the necessity of careful evaluation of stereoselective binding mechanisms based on electrophoretic separation methodologies.

Such a phenomenon is observed in the two above presented separation modes. Fig. 4 depicts a scheme which explains the reversal of migration order (elution order) by directions and magnitude of the various processes and rate constants involved.

From LC experiments in which the chiral SO is immobilized onto silica we know that the (*S*)-enantiomer of DNB-Leu, and DNZ-Leu as well, is stronger bound by the chiral SO than the corresponding (*R*)-enantiomers [37,39]; accordingly we may assume that the ion-pair formation constants of the (*S*)-enantiomers of both derivatives are numerically higher than those of the corresponding (*R*)-enantiomers, i.e. $K_{(S)\text{-IP}} > K_{(R)\text{-IP}}$. On the other side, enantioselective ion-pair extraction experiments yielded higher extraction rates for the (*S*)-enantiomers of DNB-Leu and DNZ-Leu [45]; thus, one may conclude that the (*S*)-ion-pair of DNB-Leu and DNZ-Leu, respectively, with tBuCQN as chiral SO is more lipophilic than the corresponding (*R*)-ion-pair. Accordingly, the (*S*)-ion-pair adsorbs stronger to the ODS-stationary phase, i.e. $K_{\text{Ads }(S)\text{-IP}} > K_{\text{Ads }(R)\text{-IP}}$.

Regarding the electrophoretically dominated separation mode (see Fig. 4A), higher ion-pair formation rates for the (*S*)-enantiomer are connected to higher effective migration rates of the (*S*)-IP with the eluent to the inlet end of the capillary ($\mu_{\text{eo (eff) }(S)\text{-IP}} > \mu_{\text{eo (eff) }(R)\text{-IP}}$); thus, achieved retardation is additionally enforced by stronger adsorption of the (*S*)-IP to the stationary phase. Consequently, higher effective electrophoretic migration rates result for the (*R*)-enantiomer ($\mu_{\text{ep (eff) }(R)} > \mu_{\text{ep (eff) }(S)}$), which remains to a higher extent uncomplexed. Overall, higher mobility is observed for the (*R*)-enantiomers which therefore reach the detector end of the capillary first; the (*R*)-enantiomer elutes first (see also Fig. 3A).

Regarding the electroosmotically dominated separation mode (see Fig. 4B), higher ion-pair formation rates of the (*S*)-enantiomer result in higher effective migration rates for the (*S*)-IP with the eluent to the detection end of the capillary ($\mu_{\text{eo (eff) }(S)\text{-IP}} > \mu_{\text{eo (eff) }(R)\text{-IP}}$). Due to lower ion-pair formation rates of the (*R*)-enantiomer and due to lower adsorption rates of the (*R*)-IP, the (*R*)-enantiomer is to a higher extent uncomplexed; accordingly the higher electrophoretic migration rates of the (*R*)-enantiomer to the

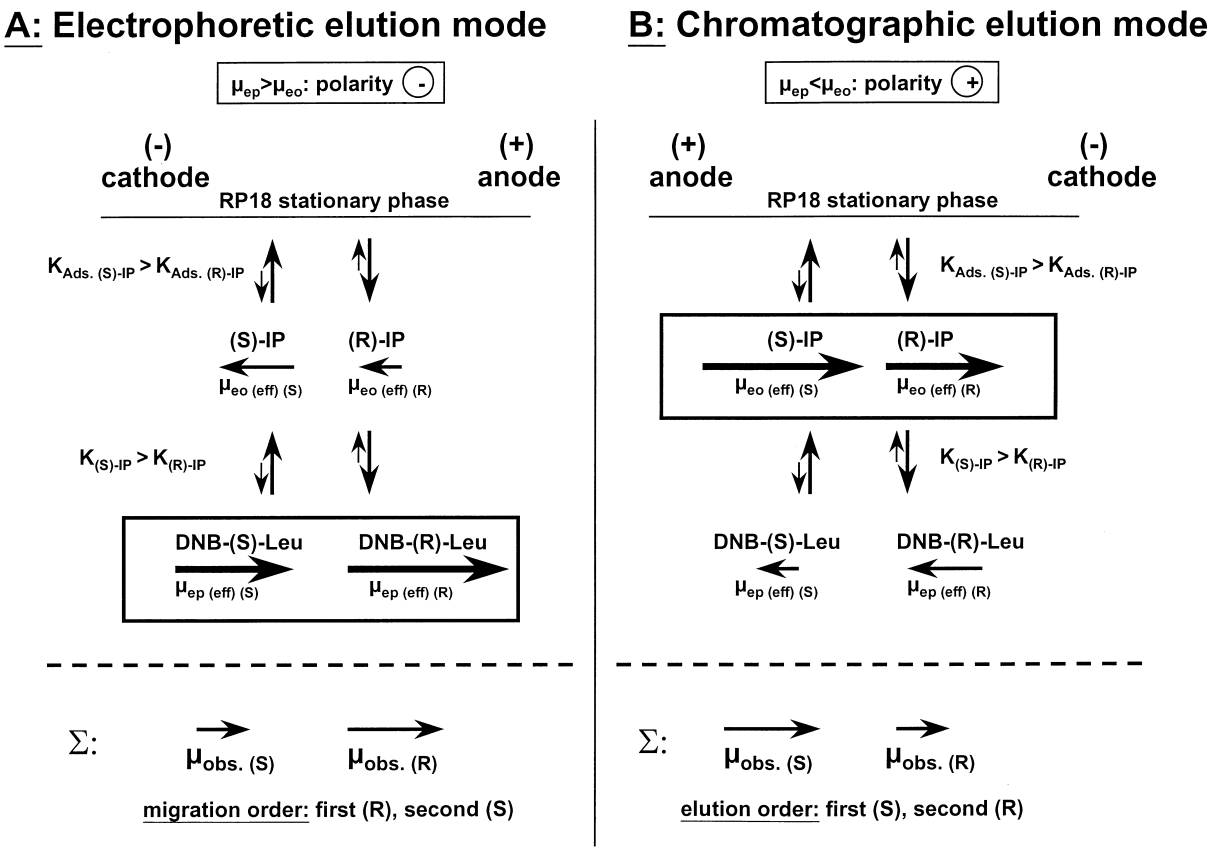

Fig. 4. Schematic representation of the reversal of the migration resp. elution order, exemplified for DNB-Leu as SA. The vectors indicate the relative magnitude of the given transport process with respect to both enantiomers.

injection end of the capillary ($\mu_{ep\ (eff)\ (S)} < \mu_{ep\ (eff)\ (R)}$) result in stronger retardation of the (*R*)-enantiomer. Overall, higher mobility is observed for the (*S*)-enantiomer which is therefore detected before the (*R*)-enantiomer; the (*S*)-enantiomer elutes first (see also Fig. 3B).

3.5. Aqueous versus non-aqueous mobile phases

Commonly, CEC experiments are performed with aqueous, buffered mobile phases and various contents of an organic modifier (preferentially acetonitrile or methanol). However, it is also possible to work in non-aqueous media with organic buffers dissolved in a polar solvent like methanol. The modified mobile phase properties (dielectric constant, viscosity, polarity, acid–base properties, solubilities, etc.) give particular selectivity and efficiency effects. However, the primary intention to use non-aqueous media in the present application was mainly driven by assumption of higher ion-pair rates in less polar media and higher solubility of the lipophilic chiral ion-pair agent (SO) in non-aqueous mobile phases, allowing higher SO concentrations to be used.

The enantioseparation data presented in Table 1 were obtained with an aqueous standard mobile phase which is commonly used in the corresponding anion-exchange type HPLC enantioseparation method [37–39] and addition of the chiral SO to this mobile phase. Various *N*-protected amino acids are well separated into the individual enantiomers with this aqueous mobile phase, usually with better efficiencies (up to 30 000 theoretical plates per column) than in the corresponding anion-exchange type

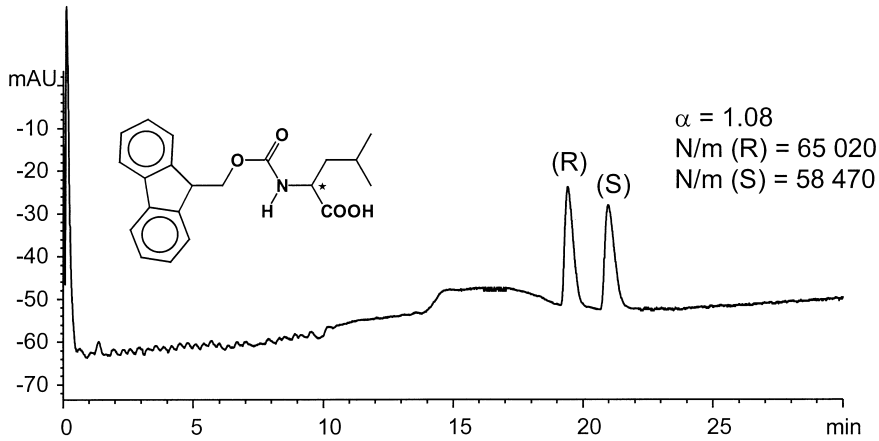

Fig. 5. Enantioseparation of FMOC-Leu by aqueous enantioselective ion-pair CEC with *tert*-butyl carbamoyl quinine as chiral ion-pair agent. Experimental conditions: capillary column: CEC-Hypersil C_{18}-3 μm, 335 mm (250 mm effective length)×0.1 mm I.D.; mobile phase: methanol–0.1 *M* ammonium acetate (80:20) (pH_a=6.0)+5 m*M* *tert*-butyl carbamoyl quinine; 20°C; electric: −25 kV (−28 μA); injection: −5 kV (20 s); pressure: 8 bar (inlet and outlet); detection: UV 254 nm.

HPLC enantioseparation method (see Table 1 and Fig. 5). It has been found that mobile phases used in corresponding HPLC methods are a good choice as starting conditions. However, buffer concentrations should be chosen that do not generate too high electric currents. At this point, it should be mentioned that also the chargeable chiral SO contributes to conductivity and electric current. For example, an electric current of −30 μA was observed under conditions as specified in Table 1 and 10 m*M* SO added to the mobile phase; this was close to the limit of electric currents that can be handled in CEC experiments (100 μm column diameter) without onset of air bubble formation in the capillary column.

In contrast, non-aqueous eluents generate lower currents and as a result lower Joule heat. This, in principle, leads to a flatter profile of the radial temperature gradient and allows the application of higher electric field strengths. Both effects give rise to higher efficiency. Thus, with non-aqueous mobile phases theoretical plate numbers could be increased throughout (see Table 2), and especially DNP-amino acid enantiomers could be resolved with outstanding efficiencies (plate numbers between 130 000 and 170 000 per meter or about 40 000 per column have effectively been calculated) (see Fig. 6).

3.6. Influence of the concentration of the chiral ion-pair agent (SO)

Observed mobilities as well as mobility difference (resp. enantioselectivity), but also efficiency and hence resolution are strongly influenced by the concentration of the chiral ion-pair agent. Increasing amounts of the ion-pair agent added to the BGE yield higher rates of ion-pair formation and therefore adsorption to the stationary phase is favored. Thus, with increasing SO concentrations effective rates of anodic electrophoretic mobility decreased, while simultaneously also the cathodic EOF decreased (under conditions as in Fig. 7 from $\mu_{eo}=+29.8\times 10^{-6}$ at 1 m*M* [tBuCQN] to $+22.9\times10^{-6}$ $cm^2\cdot V\cdot s^{-1}$ at 10 m*M* [tBuCQN]). From these overlaid processes curves of higher order for $\mu_{obs.}$ like the one presented in Fig. 7 resulted.

On the other side, with increasing selector concentrations a consistent increase in mobility difference was observed (see Fig. 7), and it seems that the optimum concentration is not yet reached at 10 m*M* [tBuCQN]. However, the range of useful SO concentrations is limited with respect to solubility, and additionally strong UV absorbance of the quinine derivative is disadvantageous in terms of detection limits (exception: indirect detection of solutes with

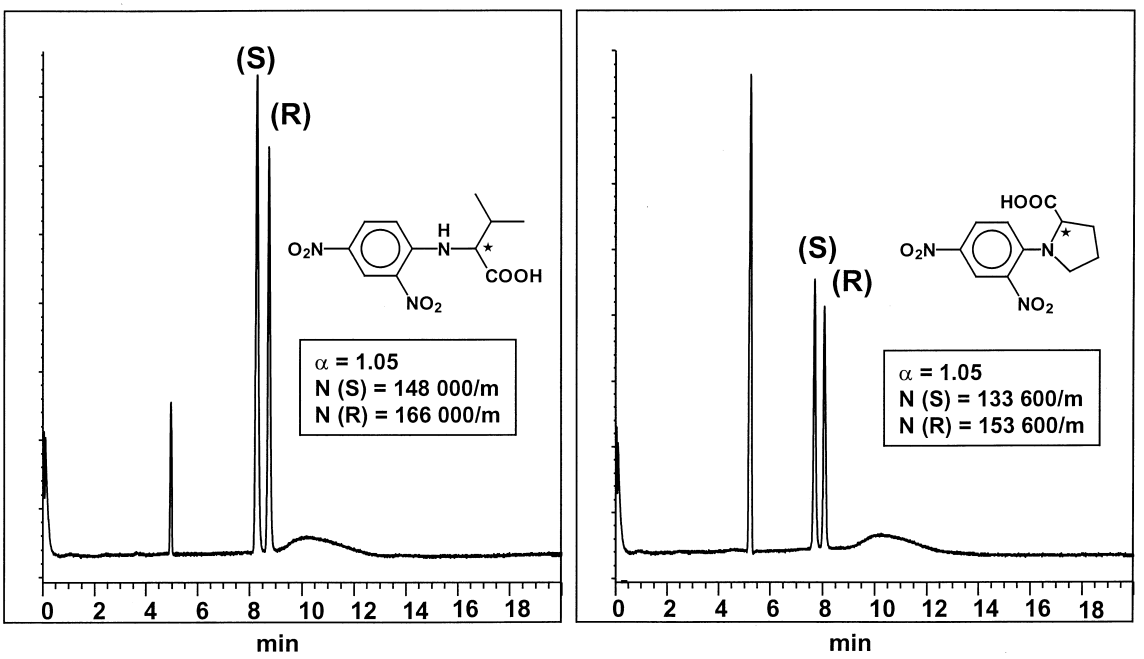

Fig. 6. Enantioseparation of DNP-Val and DNP-Pro by non-aqueous enantioselective ion-pair CEC with *tert*-butyl carbamoyl quinine as chiral ion-pair agent. Experimental conditions: capillary column: CEC-Hypersil C_{18}-3 μm, 335 mm (250 mm effective length)×0.1 mm I.D.; mobile phase: methanol +20 m*M* ammonium acetate +80 m*M* acetic acid +5 m*M* *tert*-butyl carbamoyl quinine; 20°C; electric: −25 kV (−25 μA); injection: −5 kV (20 s); pressure: 10 bar (inlet and outlet); detection: UV 360 nm.

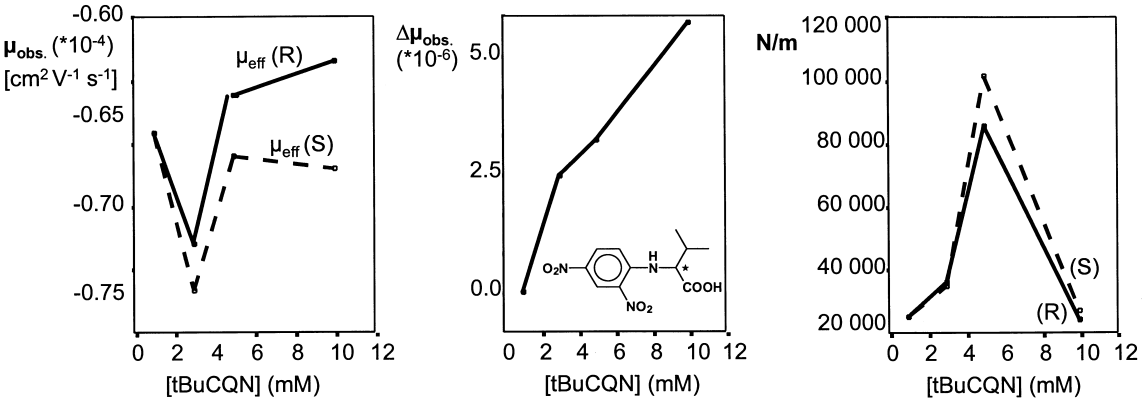

Fig. 7. Influence of SO concentration on observed mobility, mobility difference and efficiency for the enantioseparation of DNP-Val by enantioselective ion-pair CEC with *tert*-butyl carbamoyl quinine as chiral ion-pair agent. Experimental conditions: capillary column: CEC-Hypersil C_{18}-3 μm, 335 mm (250 mm effective length)×0.1 mm I.D.; mobile phase: methanol–0.1 *M* ammonium acetate (80:20) (pH_a=6.0)+*tert*-butyl carbamoyl quinine (tBuCQN); 20°C; electric: −25 kV (between −15 and −30 μA) ; injection: −5 kV (20 s); pressure: 8 bar (inlet and outlet); detection: UV 254 nm; μ_{eo}: 1 m*M*: $+29.8\times10^{-6}$, 3 m*M*: $+25.0\times10^{-6}$, 5 m*M*: $+24.4\times10^{-6}$, 10 m*M*: $+22.9\times10^{-6}$ $cm^2\cdot V\cdot s^{-1}$.

low UV absorption). Moreover, optimal efficiencies have in most cases been received at a SO concentration of about 5 m*M*. For example, up to 100 000 plates per meter (or 25 000 per column) could be achieved in the separation of DNP-Val enantiomers at 5 m*M* SO concentration, whereas plate numbers decreased dramatically at higher concentrations, e.g. 10 m*M*. Optimal resolution was

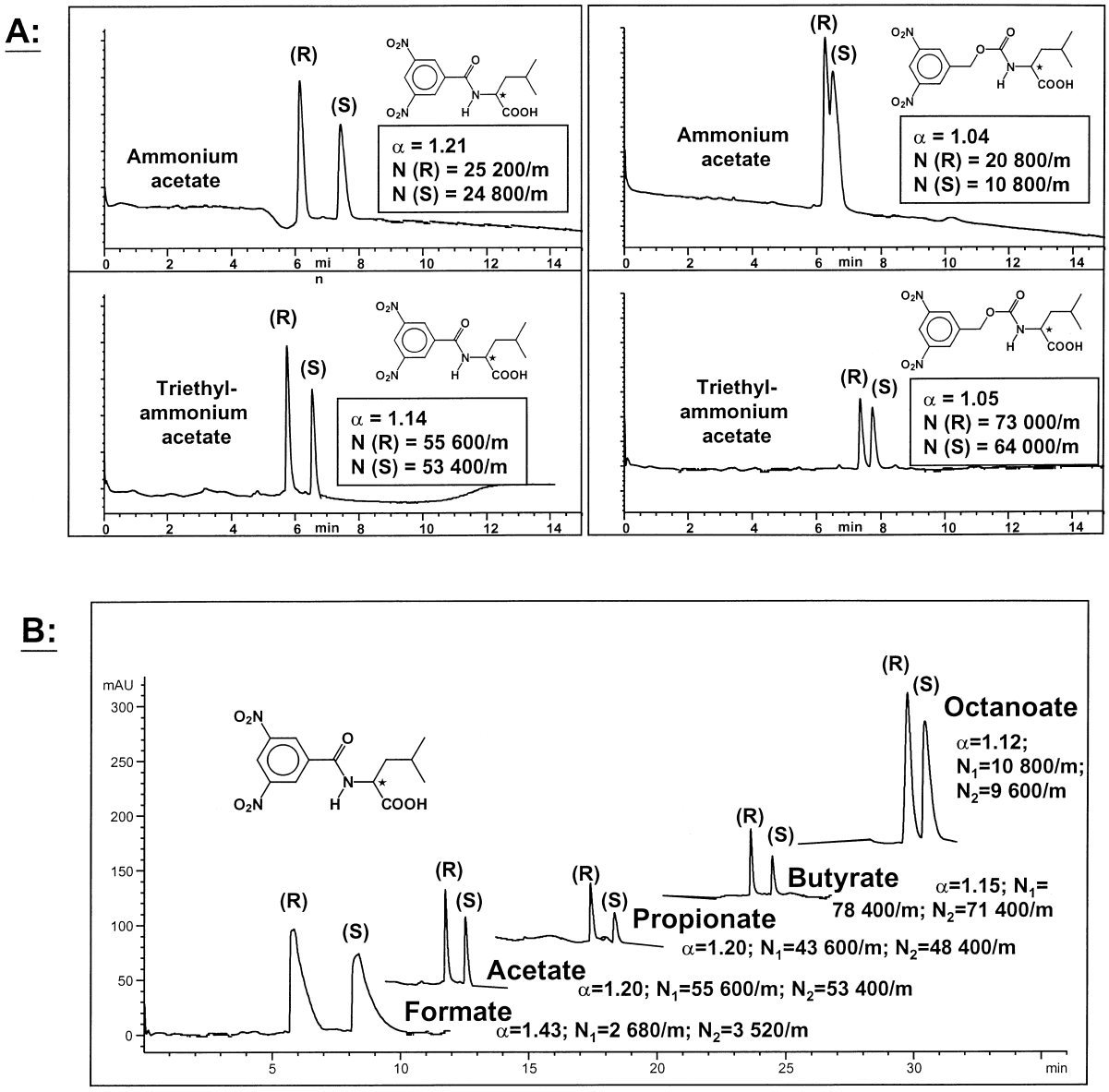

Fig. 8. Influence of type of BGE on enantioseparation in enantioselective ion-pair CEC. (A) Influence of amine component. (B) Influence of acid component (NEt_3 as amine component). Experimental conditions: capillary column: CEC-Hypersil C_{18}-3 μm, 335 mm (250 mm effective length)×0.1 mm I.D.; mobile phase: methanol +20 m*M* buffer +5 m*M* *tert*-butyl carbamoyl quinine; 20°C; electric: −25 kV; injection: −5 kV (20 s); pressure: 10 bar (inlet and outlet); detection: UV 254 nm.

observed for the given solute also at a SO concentration of 5 m*M* (R_S=1.86).

3.7. Influence of the type of BGE on separation and efficiency

Cationic (amine) and anionic (acid) components of the BGE compete with the ion-pair agent and solutes, respectively, for interaction with the oppositely charged species. This competitive effect, by which the strong ionic interaction between SO and SA is balanced, is necessary with respect to effective enantioselective molecular recognition and discrimination, but also with respect to efficiency. If the chiral SO is added to the solvent without any competitive polar constituents, e.g. acetic acid, both enantiomers are more or less equally strong bound by the chiral SO and enantioseparation under such conditions usually fails or at least is very inefficient.

A variety of BGEs have been tested. Among them phosphate and citrate as BGEs, in a non-aqueous system with methanol as solvent and with triethylamine as cation, yielded relatively high differences in observed mobilities, but very poor efficiencies (data not shown).

Better separations could be achieved with single charged organic buffers like acetate, whereby the presence of an amine instead of, e.g. sodium in the mobile phase was generally found to be beneficial for the peak performance. From Fig. 8A it can be seen that with triethylammonium as amine component of the BGE, efficiencies are higher than with the respective ammonium buffer.

The type of buffer anion, which competes with the solutes for association and ion-pair formation with the chiral ion-pair agent, has also a pronounced influence on the separation. The effect of different types of buffer anion on the separation of DNB-Leu enantiomers is depicted in Fig. 8B. Generally, with increasing lipophilicity of the buffer anion enantioselectivity decreases. Within the presented homologues series of single charged buffer anions, formate yielded a remarkable enantioselectivity value (1.43). Unfortunately, low theoretical plate numbers (about 3000/m) prevent the application of formate buffer. The highest efficiencies could be obtained with mediocre lipophilic buffer anions, i.e. acetate, propionate and butyrate. Among them, acetate is the best choice for practical reasons.

4. Conclusion

It has been demonstrated that enantioselective ion-pair CEC is an interesting and highly efficient enantioseparation method to be applied to charged species. The simple set-up as well as the stability and good reproducibility of the method enables to carry out highly efficient CEC separations of anionic solutes which otherwise behave often more problematic in packed enantioselective CEC systems. Furthermore, existing and commercially available RP18 CEC columns may conveniently be employed and the countercurrent-like electrophoretic behavior of solutes and ion-pair agent may give rise to special selectivity effects. Additionally, only small amounts of the chiral selector are consumed by this microscale separation technology. The chiral SO may also be easily back-extracted from the used buffer by two-phase liquid–liquid extraction. Since only small amounts of SO are used and no immobilization procedure of the SO is utilized, this method seems also to be suitable as a screening tool in SO development and optimization. The generally considered disadvantageous strong UV-absorbing properties of the quinoline moiety of the present investigated chiral ion-pair agent may in turn be advantageously exploited for improving detection sensitivity of solutes with low UV-absorbing properties implementing the ‘indirect’ detection methodology. Solubility problems, which may arise when higher SO concentrations have to be used to achieve reasonable enantioseparations, can be overcome by modifying the chiral SO, i.e. introducing polar functional groups via the vinyl double bond of the quinuclidine ring of the SO (see Fig. 1), e.g. by radical addition of 2-hydroxyethanethiol.

Compared to other CEC methods which usually employ electrolyte concentrations below 10 m*M* to guarantee high EOF rates, high electrolyte concentrations (above 10 m*M*) can be beneficial for CEC separation of ionized species in ion-pair CEC in particular with respect to efficiency, stable electric field and current between electrodes and thus with

respect to method robustness. The several experimental parameters to be adjustable provide flexibility in enantioseparation optimization.

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