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Chiral Separation of Amines by Non-Aqueous Capillary Electrophoresis using Low Molecular Weight Selectors

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Abstract

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Three chiral selectors (diketogulonic acid, benzoxycarbonylglycylproline and ketopininc acid) have been introduced for enantioseparation of pharmacologically active amines in non-aqueous capillary electrophoresis. The use of organic solvents, instead of aqueous buffers in the background electrolyte facilitated ion-pair formation between the analytes and the chiral selectors. The enantioresolution was strongly affected by the choice of selector and organic solvent but also depended on the other electrolytes. The most important parameter for the enantioresolution, apart from the choice of chiral selector, was the direction and magnitude of the electro-osmosis. Thus, covalently coated capillaries were used to suppress and to reverse this flow. Furthermore, the alkali metal hydroxide added to the background electrolyte had a great influence on the electro-osmosis. Exchanging LiOH for NaOH, was found to decrease the electro-osmotic flow. Interestingly, the flow was altered from cathodic to anodic, with KOH, RbOH or CsOH added to the ethanolic BGE. The occurrence of a reversed electro-osmosis had a great positive effect on the enantioresolution. An appropriate choice of solvent and electrolytes promoted also fast chiral separations, e.g., the enantiomers of isoprenaline were resolved within one minute.

The capillary electrophoresis systems developed within this work were applied for enantiomeric purity determinations of different pharmaceutical forms of drug products. A detection limit of 0.033 % was achieved for *1S,2R*-ephedrine, the enantiomeric impurity in Efedrin®, when diketogulonic acid was used as the selector.

By using the pre-concentration technique, transient isotachophoresis, the peak efficiency was enhanced for the enantiomers of timolol. This facilitated the introduction of a higher concentration of the sample into the capillary electrophoretic system containing ketopininc acid as the selector, and lowered the detection limit from 2.5 % to 0.2 % for the enantiomeric impurity *R*-timolol compared with injection without transient isotachophoresis.

The volatility of the non-aqueous media in capillary electrophoresis facilitated the hyphenation to mass spectrometry. The partial filling technique ensured that the selector did not contaminate the mass spectrometer, and the separated enantiomers of e.g., pronethalol were detected in the selector-free zone.

Keywords: Chiral Separation, Non-Aqueous Capillary Electrophoresis, Enantioresolution, Electro-osmotic Flow, Pharmaceuticals, Enantiomeric Amines, Low Molecular Weight Selector

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Till Mikael

Papers discussed

This thesis is based on the following papers, which are referred to in the text by the Roman numerals assigned below:

- I Non-aqueous capillary electrophoretic separation of enantiomeric amines with (-)-2,3:4,6-di-*O*-isopropylidene-2-keto-*L*-gulonic acid as chiral counter ion. Y. Carlsson, M. Hedeland, U. Bondesson and C. Pettersson. *J Chromatogr. A* 2001, 922, 303-311
- II Development of a chiral non-aqueous capillary electrophoretic system using the partial filling technique with UV and mass spectrometric detection. H. Lodén, Y. Hedeland, M. Hedeland, U. Bondesson and C. Pettersson, *J. Chromatogr. A* 2003, 986, 143-152
- III Chiral separation of amines with *N*-benzoxycarbonylglycyl-*L*-proline as selector in non-aqueous capillary electrophoresis using methanol and 1,2-dichloroethane in the background electrolyte. Y. Hedeland, M. Hedeland, U. Bondesson and C. Pettersson, *J. Chromatogr. A*, 2003, 984, 261-271
- IV The effect of alkali metal hydroxides on the enantioseparation of amines using di-*O*-isopropylidene-keto-*L*-gulonic acid as the selector in non-aqueous capillary electrophoresis. Y. Hedeland, J. Haglöf, P. Beronius and C. Pettersson, *In manuscript*
- V Determination of the enantiomeric impurity in *1R,2S*-ephedrine and *S*-timolol by chiral ion-pair non-aqueous capillary electrophoresis Y. Hedeland, J. Lehtinen and C. Pettersson, *In manuscript*

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Abbreviations

AP	Aminopropyl
BGE	Background electrolyte
CE	Capillary electrophoresis
CMPA	Chiral mobile phase additive
CS	Chiral selector
CZE	Capillary zone electrophoresis
$\Delta\mu$	Mobility difference between the enantiomers
(-)-DIKGA	(-)-2,3:4,6-di- <i>O</i> -isopropylidene-2-keto- <i>L</i> -gulonic acid
E	Electric field strength
EDL	Electrical double layer
EOF	Electro-osmotic flow
ESI	Electrospray ionisation
ϵ_0	Permittivity (or dielectric constant) of vacuum
ϵ_r	Permittivity (or dielectric constant) of the medium
FASS	Field amplified sample stacking
TITP	Transient isotachopheresis
K_a	Dissociation constant of an acid
K_p	Ion-pair formation constant
KPA	Ketopinic acid
<i>L</i> -ZGP	<i>N</i> -benzoxycarbonylglycyl- <i>L</i> -proline
MS	Mass spectrometry
<i>m/z</i>	Mass-to-charge ratio
μ_{EOF}	Electroosmotic mobility
μ_{ep}	Electrophoretic mobility
μ_{eff}	Effective mobility (i.e. $\mu_{obs} - \mu_{EOF}$)
μ_f	Electrophoretic mobility of free solute
μ_m	Average mobility of the enantiomers
N	Number of theoretical plates
NACE	Non-aqueous capillary electrophoresis
PAC	Polyacrylamide
RMD	Relative mobility difference
R_s	Resolution (refers in this thesis to <i>enantioresolution</i>)
η_{wall}	Viscosity in the EDL
ζ_{ion}	Zeta potential of the ion
ζ_{wall}	Zeta potential across the EDL

Introduction

Today, it is well known that the individual enantiomers of a racemic drug often have different pharmacokinetic and pharmacological properties, as their target structures in the human body are chiral [1]. The first observation of biological differences between enantiomers was made by Louis Pasteur in 1858. By measuring the optical rotation of ammonium tartrate, he discovered that the (*d*)-enantiomer was more rapidly metabolised by the fungus *Penicillium glaucum* than the (*l*)-enantiomer [2, 3]. Fifty years later, Abderhale and Müller [2, 4] reported the first observed pharmacological difference. They found that (*d*)- and (*l*)-epinephrine had different effects on the blood pressure of laboratory animals. Nowadays, there is a broad range of examples where the enantiomers of drugs show differences in their bioavailability, distribution, receptor interaction, metabolic and excretion behaviour, and they should thus be considered to be two different compounds [1]. It has been proven that the use of a single enantiomer may reduce the dose of a drug, simplify the dose-response relationship and minimise the toxicity caused by the therapeutically inactive enantiomer [1]. The Food and Drug Administration (FDA) guidelines for the registration of new drugs state that a chiral impurity should be treated in the same way as any other impurity, and an enantioselective determination should be included in the specification [5]. Fifty percent of the drugs approved by the FDA between 2000 and 2002 were single enantiomers, 6 % racemates and 44 % achiral [6]. That is a significant increase since 1983, when 37 % of the new drugs to be registered world wide were racemates and only 26 % were single enantiomers [6]. Thus the trend in drug discovery is toward single enantiomers. As a result of the pioneering work by Knowles, Noyori and Sharpless, Nobel laureates in 2001, many of the single enantiomeric drugs currently available are produced by asymmetric synthesis [7-9], which results in one of the enantiomers being present in the final product.

The progress toward enantiomerically pure drugs makes selective and rapid analysis of enantiomers an important issue in drug development, especially for chiral purity determinations but also for enantioselective bioanalysis. Only a few techniques can be used for determination without separation of the enantiomers prior to analysis, e.g., polarimetry [10], voltammetry/amperometry with enantioselective sensors [11] and dual circular dichroism with simultaneously ultraviolet detection [12]. The techniques that can be used for chiral separations include high-performance liquid

chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC), supercritical fluid chromatography (SFC), capillary electrophoresis (CEC) and capillary electrophoresis (CE). For general reviews on chiral separations exemplifying the above-mentioned techniques, see the references [13, 14]. The most frequently used analytical technique for enantioseparation today is HPLC [14], but during the last two decades also chiral separation using CE has grown in interest, especially in pharmaceutical analysis [15, 16].

Chiral Capillary Electrophoresis

After the pioneering work by Kolin 1954 [17] and Hjertén 1958 [18] with electrophoresis in glass tubes, Jorgensen and Lukacs published in 1981 a method using glass capillaries with an inner diameter of 75 μm , for zone electrophoresis [19, 20]. Four years later, the first chiral application using capillary electrophoresis was published by Gassman *et al.* [21], who used a copper (II) *L*-histidine complex as the selector for enantiomeric separation of dansyl amino acids. Since then, several different types of chiral selectors have been applied in CE. The most widespread are cyclodextrins [22], but also crown ethers [23], proteins [24], macrocyclic antibiotics [25], and low-molecular weight ion-pair selectors [26, 27] have been utilized. For reviews on chiral separation in CE see the references [13, 28-30].

The high efficiency of CE facilitates the use of the chiral selector for a wide range of analytes, which often makes the selector more general than when the same selector is used as a chiral mobile phase additive (CMPA) in HPLC. The high efficiency also enables more peaks to be resolved in less time, which might shorten the run time. Furthermore, the consumption of the chiral selector is much lower in CE, which makes the analysis more cost-effective and benefits the use of more expensive selectors. CE is also better suited for rapid screening of new chiral selectors, since the long equilibration time that is required at changes between different mobile phases in LC is unnecessary. However, the high detection limit and low loading capacity make CE with UV detection of limited use for the analysis of trace amounts in e.g., biological material without pre-concentration or a more sensitive detector, e.g., mass spectrometer [31].

Chiral Non-Aqueous Capillary Electrophoresis

The use of non-aqueous solvents in capillary electrophoresis was first exploited by Walbroehl *et al.* in 1984 [32]. In 1988, Snopek wrote in a review: "The use of aqueous-organic media and organic media, may extend the appli-

cability and efficiency of HPCZE¹ and/or ITP² methods to a wide range of compounds which have about the same mobilities and pK values and/or are only slightly soluble in water...” [33], but, it was not until the middle of the 1990s that researchers began to explore the use of non-aqueous media in CE. Apart from the advantages listed above, performing CE with non-aqueous solvents allows the use of higher electric field strengths without encountering problems from joule heating, thus faster separations can be performed than with aqueous buffers [34]. However, these advantages (higher separation selectivity, solubility and low electric current) have recently been questioned by Porras and Kenndler in a review [35]. These authors claim that the statements about the advantages of non-aqueous media are often used without convincing facts.

In aqueous solutions, changes in the pH, the selector concentration, the type of buffer and the ionic strength can alter the chiral resolution [36]. In non-aqueous media, also the exchange of one solvent for another or a change in the relative proportions of the solvents used, will affect the enantioresolution. Different organic solvents and solvent mixtures have been used for enantioseparations in CE e.g., methanol (MeOH) [26], acetonitrile (AcN) [27], formamide, *N*-methylformamide and *N,N*-dimethylformamide [37] and binary combinations of ethanol (EtOH)/MeOH [38]. For achiral separations in non-aqueous media also *N,N*-dimethylacetamide, dimethyl sulphoxide (DMSO) [39], buthanol [40], nitromethane [41] have been used. Tetrahydrofurane (THF) has been utilised in mixtures with MeOH [42] or MeOH and AcN [43].

Alteration of the organic solvent composition in the BGE gives rise to changes in several different parameters at the same time. The conditional acid dissociation constant (K_a^*) for the solutes, which is one of the parameters that alter the effective mobility, will be affected [44]. The environment for ion-pair formation (i.e., the dielectric constant, ϵ) will also differ between solvents or solvent mixtures. Tjørnlund and Hansen [39, 45] observed selectivity changes for structurally similar amines when the organic solvent was changed. Recently, Cantu *et al.* [44] determined the K_a^* for these amines in methanol and AcN and correlated their variation in migration order to the change in K_a^* . Furthermore, exchanging the solvent also affects the electroosmosis [46]. Thus, it is difficult to predict the influence of different solvents or solvent mixtures on the chiral separation.

In order to achieve enantioresolution in CE, a difference between the enantiomers in formation of diastereomeric complexes with the selector (or formation of complexes with different mobility) is necessary. The reversible complex formation can be based on: (i) inclusion complexation (ii) ion-pair formation, (iii) a combination of (i) and (ii) or (iv) ligand-exchange mecha-

¹ HPCZE = high performance capillary zone electrophoresis

² ITP = isotachophoresis

nism [30]. Different types of selectors that have been used in non-aqueous solvents are listed in Table 1. Since the work presented in this thesis focuses on ion-pair selectors, all the previously used chiral counter ions (except for the three ones presented in the thesis) are included in Table 1. In addition, combinations of different types of selectors, such as e.g., heptakis (2,3-dimethyl-6-sulphato)- β -CD and camphorsulphonic acid have been used to enhance the chiral separation [47].

Table 1. Chiral selectors used in NACE

Type of interaction	Group of selectors	Example of selector
inclusion complexation	native cyclodextrins and cyclodextrine derivatives	β -cyclodextrin (β -CD) [48]
ion-pair formation	chiral counter-ions	camphorsulfonic acid [27] 3,5-dinitrobenzoyl-leucine [38] quinine [26] quinine derivatives [49], [50]. quinidine [49] cinchonine [49] cinchonidine [49]
inclusion complexation and ion-pair formation	charged cyclodextrins	ammonium β -CD [51] sulphated- γ -CD [52], [53]
	crown ethers with carboxylic acid functions	(+)-18-Crown-6-tetracarboxylic acid [54]
ligand-exchange mechanism		complex of copper (II) and <i>L</i> -proline or <i>L</i> -isoleucine [55]

The first two chiral applications using ion-pair selectors in non-aqueous capillary electrophoresis (NACE) appeared in 1996 in reports by Stalcup and Gahm [26], and Bjørnsdottir *et al.* [27]. These researchers showed that quinine [26] and camphorsulphonate [27], two chiral selectors that had been used previously as CMPAs in HPLC [56, 57], could be used for enantioseparation in NACE. In addition, two applications using neutral [48] and anionic β -cyclodextrins [37] as chiral selectors in NACE were published in the same year. In these and subsequent investigations it was revealed that many of the low molecular weight selectors exhibit low or lack of enantioselectivity [27, 38] or solubility [26, 38] in water based buffers.

A better understanding of the mechanisms underlying the separation systems would facilitate their design. This does not only involve improving the knowledge of the chiral recognition mechanism between analyte and the selector, but also the influence of solvents and the other electrolytes on the separation system.

Aims

The aims of this thesis were:

- to introduce (-)-2,3:4,6-di-*O*-isopropylidene-2-keto-*L*-gulonic acid ((-)-DIKGA), *N*-benzoxycarbonylglycyl-*L*-proline (*L*-ZGP) and *S*-(+)-ketopinic acid ((+)-KPA) as chiral selectors for enantiomeric amines in NACE (**Papers I, III and V**).
- to investigate if the enantioseparation could be improved by manipulation of the electro-osmosis by use of covalently coated capillaries (**Paper I**), non-aqueous solvents with a low dielectric constant (ϵ) (**Papers II, III and V**) or by use of different alkali metal hydroxides in the BGE (**Paper IV**).
- to compare the resolution and migration order of the enantiomers when (-)-DIKGA or (+)-KPA were used as chiral selectors (**Paper V**).
- to design systems suitable for fast chiral separations of amines in CE (**Papers III and IV**).
- to use ion-pair selectors for enantiomeric purity testing (**Paper V**).
- to develop a partial filling system for chiral selectors in non-aqueous media and to demonstrate the use of chiral NACE coupled to tandem mass spectrometric detection (**Paper II**).

Chiral Ion-pair Selectors

The chiral selectors used in this work were (-)-2,3:4,6-di-*O*-isopropylidene-2-keto-*L*-gulonic acid ((-)-DIKGA), in **Papers I, II, IV** and **V**), *N*-benzoxycarbonylglycyl-*L*-proline ((*L*-ZGP), in **Paper III**) and (1*S*)-7,7-dimethyl-2-oxobicyclo[2.2.1]heptane-1-carboxylic acid (*S*-(+)-ketopinonic acid, (+)-KPA), in **Paper V**). Their structures are shown in Figure 1.

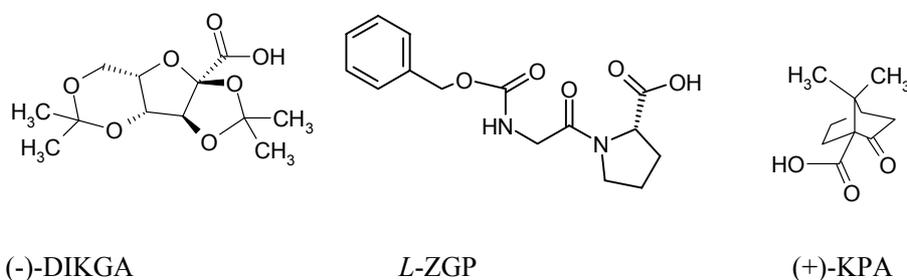


Figure 1. Structures of the chiral selectors

(-)-DIKGA is an *L*-ascorbic acid derivative with a rigid structure and four chiral centers. It has previously been used for purification of chiral amines by crystallisation [58] and as a CMPA in HPLC in polar organic mobile phases such as binary mixtures of MeOH, AcN, 2-PrOH and water [59].

L-ZGP is an *N*-blocked dipeptide that has been successfully used as a CMPA in HPLC for separation of different kinds of enantiomeric amines in mobile phases based on dichloromethane [60] as well as MeOH [61] and mixtures of MeOH and 2-PrOH [62] and MeOH with up to 5% water [63]. An amine had to be added to all solvents except dichloromethane, where enantioseparation was achieved without addition of a base [63].

(+)-KPA is a β -ketonic acid with a rigid structure that is structurally similar to camphorsulphonic acid, except that (+)-KPA contains a carboxylic acid instead of a sulphonic acid near one of its two chiral centres. Camphorsulphonic acid has previously been used by Bjørnsdottir *et al.* [27] in NACE for separation of different pharmacologically active amines, such as e.g., β -adrenoceptor antagonists, local anaesthetics and tricyclic antidepressants. It has also been used by Pettersson and Schill [56] as a CMPA in HPLC.

These three selectors were used for the first time for enantioseparation in CE in the research presented here. The analytes used were different types of pharmacologically active amines, such as e.g., β -adrenoceptor blocking agents, adrenergic agonists and local anaesthetics. The structures of the substances discussed in Figures 3-16 below are presented in Figure 2. Many of these analytes exhibited enantioselectivity with all three selectors, but to a different extent, e.g., pindolol, Figure 3. By using (-)-DIKGA instead of (+)-KPA, the migration order of the enantiomers could be reversed for all substances tested (V).

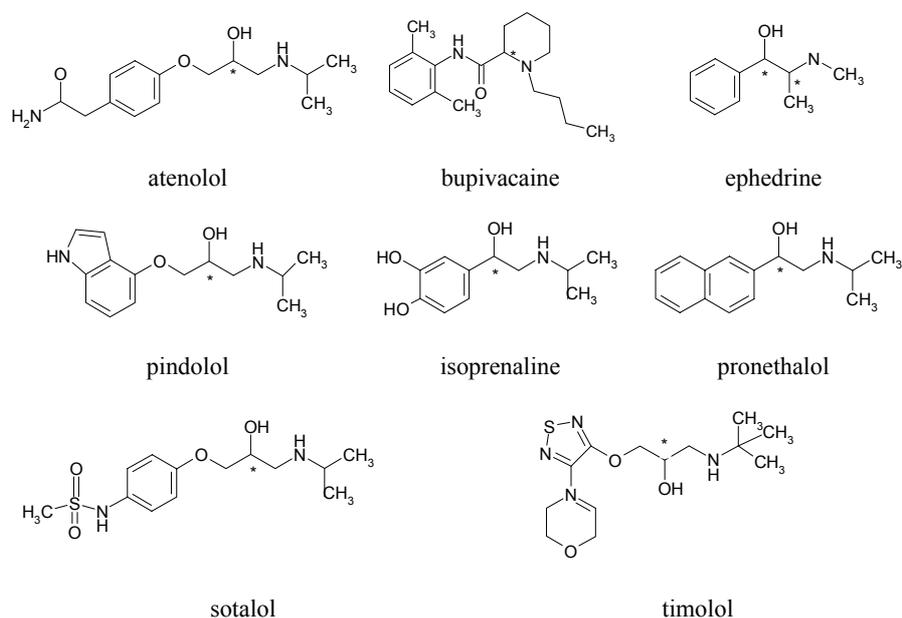


Figure 2. Structures of the chiral solutes

In order to achieve chiral discrimination between the enantiomers, a “three point interaction” is required between at least one of the enantiomers and the selector [64, 65]. These interactions can be of an attractive or a repulsive nature (e.g., a steric interaction).

Beside the chiral selector, a base (e.g., NaOH) needs to be added to obtain chiral selectivity. This indicates that one of the interactions would be ionic attraction between the negatively charged carboxylic groups of the selector and the cationic analytes.

No enantioseparation has been observed with the present selectors in aqueous solution without organic modifiers. The explanation for this is probably that the electrostatic interactions are much weaker in water, which gives a low degree of ion-pair formation [66]. Thus, organic solvents and

mixtures of organic solvents with a lower dielectric constant than water have been used.

If the resolution between the enantiomers is too low ($R_s < 1.5$), the separation can be improved in different ways. According to Equation 1, the resolution will be increased when the difference between the mobilities of the enantiomers ($\Delta\mu$) are increased, the efficiency (N) is increased and when the average of the observed mobilities of the enantiomers (μ_m) is decreased [67]:

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{\Delta\mu}{\mu_m} \quad (\text{Eq. 1})$$

The quotient $\Delta\mu/\mu_m$ is also called the relative mobility difference (RMD), and is sometimes used to describe the enantioseparation. In the research conducted for this thesis, a considerable amount of effort has been put into decreasing μ_m by lowering and reversing the electro-osmotic flow (EOF) (I-V), and also into studying the influence of the selector concentration on $\Delta\mu$ (I, III and IV). In addition, the difference in peak efficiency using different BGEs and coatings has been examined (IV and I).

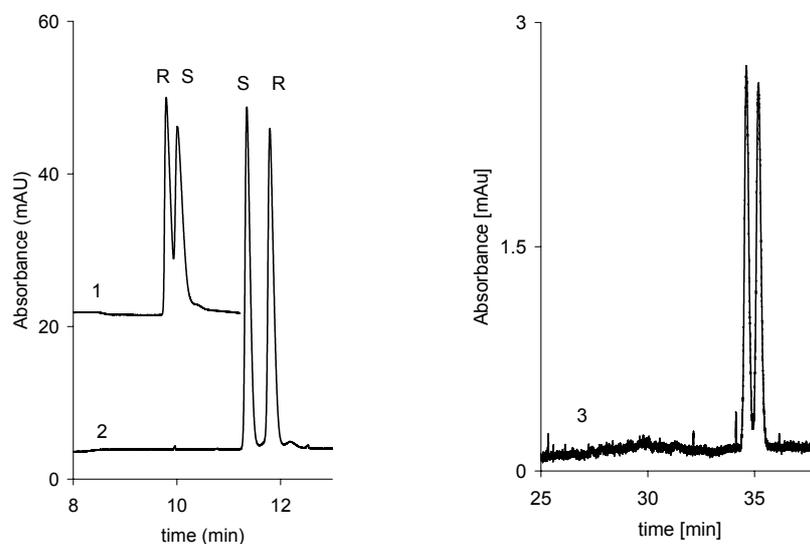


Figure 3. Separation of *rac*-pindolol using different ion-pair selectors (**Papers V** (1, 2) and **III** (3))

L_{det} 23.5 cm (1, 2), L_{det} 60 cm (3), 30 kV. 0.1 mM *rac*-pindolol in MeOH. BGE:

1) 100 mM (+)-KPA, 40 mM KOH in EtOH:MeOH 60:40 (v/v),

2) 100 mM (-)-DIKGA, 40 mM KOH in EtOH:MeOH 60:40 (v/v),

3) 125 mM L-ZGP, 50 mM NH_4Ac in MeOH:dichloroethane 45:55 (v/v).

The migration order in Paper III was not determined because pure enantiomers were not obtained.

Electro-osmosis

The silica surface needs to be at least partially charged in order to give rise to an electro-osmotic flow. The pK_a value for the surface silanol groups has been determined to be 7.1 ± 0.5 [68]. The pK_a value obtained differs from the pK_a for orthosilic acid in water solution, which has been determined to be 9.9 [69]. There are indications that the values for pK_a for the surface silanols are in a range between or near these values and that they are dependent on the chemical environment of each silanol group [69]. In addition, the silica wall becomes positively charged by formation of SiOH_2^+ in water at $\text{pH} < 2$ [69, 70].

The EOF is the flow of uncharged solvent that occurs in an applied electric field. As mentioned above, the silica capillary wall will be negatively charged to some extent, resulting in the formation of an electric double layer (EDL) near the wall. Two regions are distinguished in the EDL, one relatively rigid and stagnant inner layer of attracted counter-ions close to the wall, and one more diffuse outer layer. When an electric field is applied, the excess of counter-ions in the diffuse layer starts to move, and owing to their solvation, the frictional forces make the whole bulk of solvent to move in the same direction. The potential in the boundary between the stagnant and the diffuse layers is called the zeta potential (ζ_{wall}) and is related to the electro-osmotic mobility (μ_{eof}) by [71]:

$$\mu_{eof} = -\frac{\varepsilon_{r,wall} \zeta_{wall}}{\eta_{wall}} \quad (\text{Eq. 2})$$

where $\varepsilon_{r,wall}$ is the relative dielectric constant in the EDL and η_{wall} is the viscosity in the EDL. Despite the fact that the values of $\varepsilon_{r,wall}$ and η_{wall} are different from the values in the BGE [72], the bulk values are often used for comparisons between different solvents. The ζ_{wall} is dependent on the ionic strength and the nature of the ions in the BGE. Since the choice of solvent affects all three terms ($\varepsilon_{r,wall}$, η_{wall} and ζ_{wall}) in Equation 2, the use of different solvents or solvent mixtures can be used to manipulate the EOF. According to Eq.1, a lowered cathodic or a reversed EOF will decrease the μ_m term for cationic analytes, with an increase in enantioresolution as a result (provided that the efficiency and $\Delta\mu$ in the system are preserved).

The influence of capillary coating on EOF

In chiral separations, the mobility difference between the enantiomers is often relatively small (compared to achiral separations), which makes it desirable to suppress or reverse the electro-osmosis in order to enhance the enantioresolution (c.f., Eq. 1). In many of the non-aqueous solvents, e.g., AcN, the EOF is rather high compared to water. The problem with a high EOF in organic solvents has sometimes been addressed in achiral NACE by using dynamic or covalent coatings. Tween 20 [27], hexadimethrine bromide [73], poly(glycidylmethacrylate-co-*N*-vinylpyrrolidone) [74], polyethylene glycol (PEG) [75] and cethylmethylammonium bromide [76] have been used to coat the capillaries dynamically. Covalent coatings with poly(acryloylaminoethoxyethanol) [77], polytetrafluoroethylene [78], polydimethylsilane [75], polyvinyl alcohol [27], PEG [79] and zirconia [80] have also been utilized.

In **Paper I**, covalent coatings were, for the first time, successfully used for chiral separations in non-aqueous solvents. Coatings with polyacrylamide (PAC) and aminopropyl (AP) were investigated. The EOF was lowered in the PAC coated capillaries and reversed in the AP coated capillaries, Figure 4. Note that the absolute values of the reversed EOF in the AP coated capillary are about 50 times higher than in the uncoated capillary.

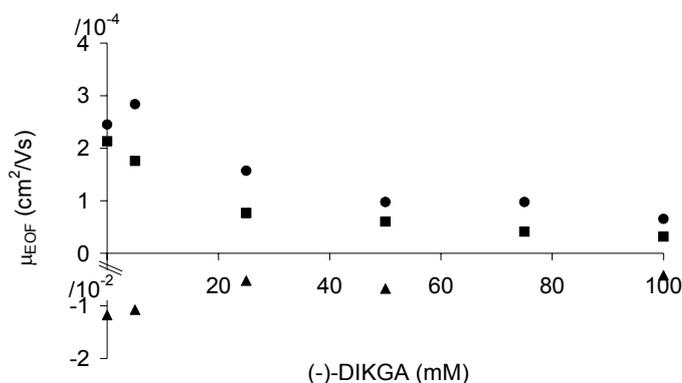


Figure 4. The EOF as a function of the selector concentration. (**Paper I**)

BGE: (-)-DIKGA and NaOH in a molar ratio of 5:2 in MeOH. EOF marker: 0.1 % (v/v) mesityl oxide in MeOH. L_{det} 40 cm, 20 kV. (●) uncoated capillary, (■) PAC-coated, (▲) AP-coated.

The stability of the coatings was good in the solvents tested, and both of them enhanced the resolution for all solutes. Unfortunately, the peak efficiency of the enantiomers was lower than in uncoated capillaries, which to some extent counteracted the enhanced enantioresolution that was achieved

by the lower EOF. The decrease in peak efficiency (N) could not be explained solely by the longer diffusion time, caused by the increased migration time. One possible explanation might be a heterogeneous EOF in the capillary as a result of non-quantitative coverage of the coating. After submission of **Paper I**, a second publication using PAC coated capillaries in NACE was published by Wang *et al.* [81]. Surprisingly, these authors found shortened migration times and higher efficiencies for the cationic analytes (lidocaine and its metabolites) when the neutrally coated PAC capillary was used instead of a negatively charged uncoated capillary in a methanolic BGE [81]. However, no explanation for the shortened migration time was given.

The influence of different organic solvents on EOF

In **Papers II, III, IV** and **V**, different organic solvents and solvent mixtures were used to suppress the electro-osmosis in the chiral separation systems (cf. Eq. 2). The advantage of using mixtures of organic solvents instead of coated capillaries to decrease the EOF is that the properties of the BGE are changed too, enabling the environment for ion-pair formation to be improved subsequently. The physico-chemical properties of the solvents used in this work are listed in Table 2.

Table 2. Properties of the organic solvents used [82]

Solvent	$t_{\text{boil}} (^{\circ}\text{C})^{\text{A}}$	$\eta(\text{kg/ms})^{\text{B}}$	ϵ^{C}	$\epsilon/\eta(\text{kg/ms})^{-1}$	Paper
H ₂ O	100.00	1.137	78.36	69	II
AcN	81.60	0.374	35.94	96	II
MeOH	64.55	0.593	32.66	55	I-V
EtOH	78.29	1.078 ^C	24.55	23 ^D	IV, V
2-PrOH	117.73	3.159	17.51	5.4	II
CH ₂ Cl ₂	39.64	0.449	8.93	20	III
1,2-C ₂ H ₄ Cl ₂	83.48	0.887	10.37	12	III

^A 1 atm

^B 15 °C

^C 25 °C

^D the viscosity was determined at a higher temperature (25 °C) than for the other substances

The magnitude of the electro-osmosis is dependent on the ϵ/η ratio (cf. Eq 2) [34, 83]. Thus, by using a solvent with a lower ϵ/η ratio, the electroosmosis can be decreased, which would benefit the chiral separation (assuming that the enantiomers and the EOF have the same migration direction). In this thesis, only EtOH (IV) and MeOH (I, II) were used without addition of other solvents. Owing to the difficulty in finding comparable ϵ and η values for all the solvent mixtures that have been used, only the prop-

erties of the pure solvents are listed in Table 2. Unfortunately, there is usually non-ideal behavior in solvent mixtures (non-linear trends) that complicates the prediction of their properties [84]. However the trends observed in the EOF when mixing different solvents followed what could be expected from the ϵ/η data in Table 2.

The EOF was increased by addition of AcN and decreased by addition of 2-PrOH to a methanolic BGE containing (-)-DIKGA (**II**). An ethanolic BGE also gave a much lower EOF in comparison to the methanolic one (**IV**, **V**).

In **Paper III**, up to 55 % dichloroethane or dichloromethane was added to the methanolic BGE when *L*-ZGP was used as the selector, Figure 5.

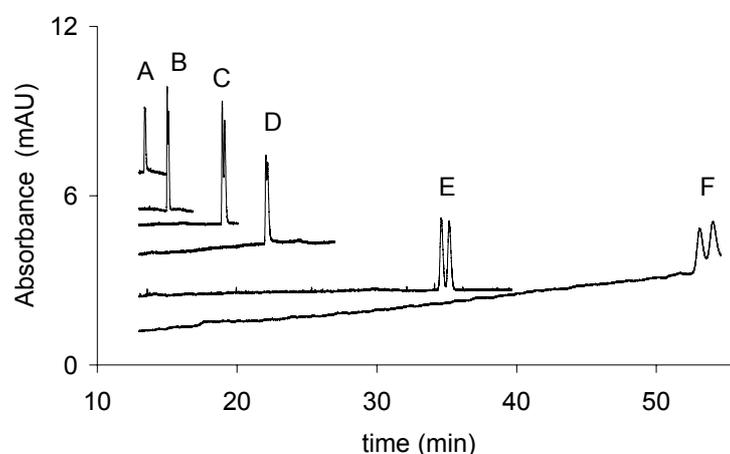


Figure 5. Separation of pindolol in different BGEs. (**Paper III**)

BGE: 125 mM *L*-ZGP and 50 mM NH_4Ac in: (A) MeOH (B) MeOH: 1,2-dichloroethane (80:20 v/v), (C) MeOH:dichloromethane (80:20 v/v), (D) MeOH:2-PrOH (80:20 v/v), (E) MeOH:1,2-dichloroethane (45:55 v/v), (F) MeOH:dichloromethane (45:55 v/v). L_{det} 60 cm, 30kV.

As expected (c.f., Table 2), the EOF was decreased by addition of these solvents. Through the use of 55 % dichloroethane and 45 % methanol (Fig. 5E) instead of pure methanol as BGE solvent (Fig. 5A) the unresolved enantiomers of pindolol could be separated. The addition of 20 % 2-PrOH in methanol also decreased the EOF (Fig. 5D), but the enantioresolution was higher when 20 % 1,2-dichloroethane (Fig. 5B) or 20 % dichloromethane (Fig. 5C) were added. This increase in R_s depends on the higher $\Delta\mu$ in combination with a lower EOF in the latter two solvent mixtures.

Thus, as already discussed above, the observed effect on the enantioresolution might be caused by a decrease in the EOF when a solvent with a lower ϵ/η is added to the BGE (c.f. Eq. 2 and Table 2).

In **Paper V**, ethanol was added to the methanolic BGE when (+)-KPA was used as the selector. The effect of the ethanol concentration on the electro-osmotic flow in a methanolic BGE is presented in Figure 6. Interestingly, the solvent composition influences not only the magnitude, but also the direction of the EOF. The EOF was reversed at ethanol concentrations of above 40 % (v/v). Thus, addition of EtOH to a methanolic BGE containing (+)-KPA and KOH could be used instead of e.g., aminopropyl coated capillaries, to reverse the EOF. A discussion of possible explanations for the reversal in EOF in this separation system is presented in the section below.

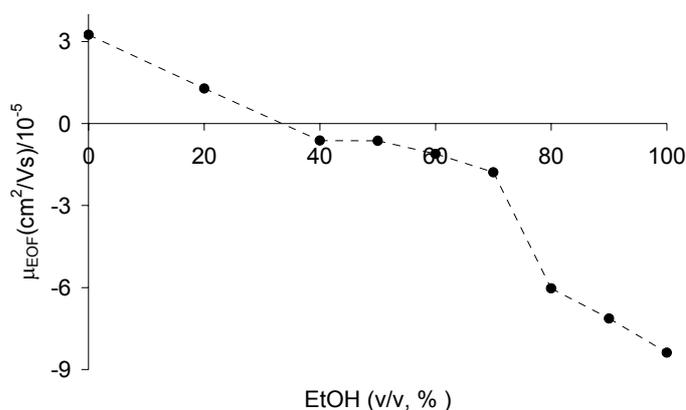


Figure 6. Influence of the EtOH concentration on the electro-osmotic flow (**Paper V**)
*BGE: 100 mM (+)-KPA and 40 mM KOH in MeOH:EtOH (v/v). 30 kV, L_{det} 8.5 cm.
 EOF marker: 0.1 % mesityl oxide in MeOH.*

The influence of different electrolytes on EOF

An example of the importance of making an appropriate choice of electrolytes in the chiral separation system is shown in Figure 7, where the bases LiOH (1) or KOH (2) are added to the chiral selector ((-)-DIKGA) in an ethanolic BGE (**IV**). The same electrophoretic conditions are used for both electrophorograms but no enantioselectivity is observed when LiOH is used, whereas KOH gives baseline resolution between the enantiomers. The observed difference in enantioresolution is primarily a result of variations in the electro-osmosis, Figure 8.

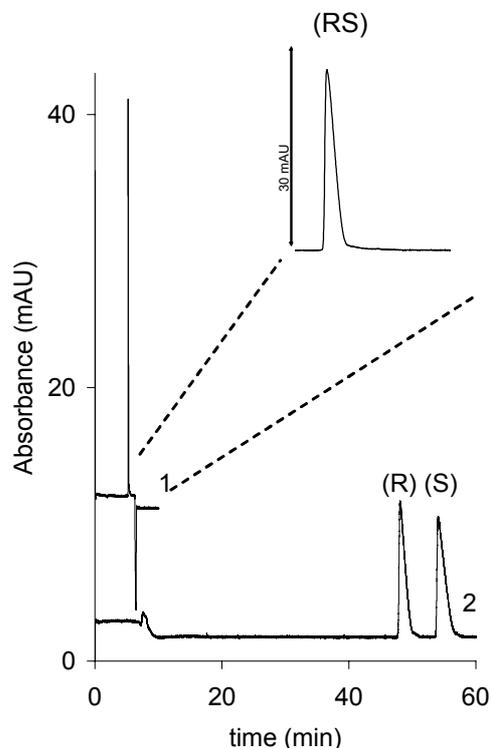


Figure 7. The influence of LiOH and KOH on enantioseparation of atenolol (**Paper IV**)

BGE: 50 mM (-)-DIKGA and 20 mM LiOH (1) or KOH (2) in ethanol. rac-atenolol (0.1 mM) in MeOH. L_{det} 8.5 cm, 30 kV.

In **Paper II**, NH_4Ac was compared with NaOH as an additive to a (-)-DIKGA containing methanolic BGE. The resolution for the solutes was slightly improved by the lowered EOF in the NH_4Ac containing BGE. However, the pH^* is probably lower in the NH_4Ac containing BGE, which decreases the EOF. However, it could not be excluded that the difference in EOF is also dependent on differences in the interaction between the buffer components and the silica wall.

In **Paper IV**, the possibility of using different alkali metal hydroxides to suppress the electro-osmosis in the chiral separation system was demonstrated. The EOF decreased with a decreasing solvated radius of the metal ion and, surprisingly, the EOF was reversed in some of the BGEs, as can be seen in Figure 8. The order of the size of the solvated radius in MeOH and EtOH is $\text{Cs}^+ < \text{Rb}^+ < \text{K}^+ < \text{Na}^+$ [85] i.e., the order is opposite to that of the size of the crystal radius [86]. A reversal of the EOF was observed when the smallest solvated cations, K^+ , Rb^+ and Cs^+ , were used in the ethanolic BGE, but only at higher concentrations in the Rb^+ and Cs^+ based methanolic BGEs.

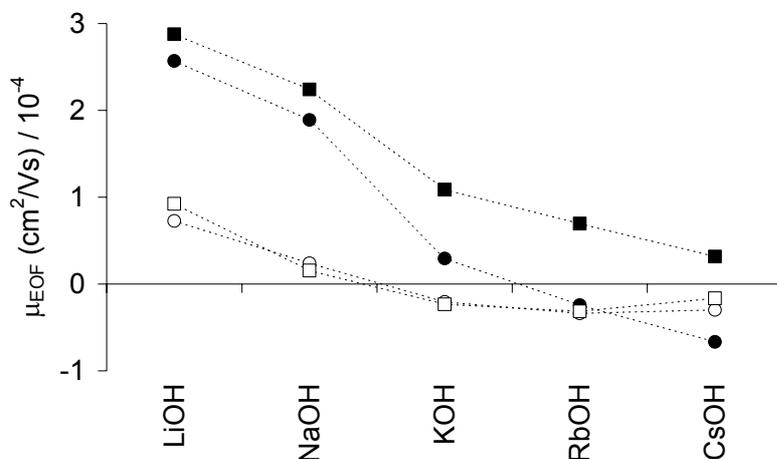


Figure 8. Influence of the alkali metal cation on the electro-osmosis. **(Paper IV)**
BGE: (-)-DIKGA and alkali metal hydroxide in MeOH or EtOH. (■) 25 mM (-)-DIKGA and 10 mM xOH in MeOH, (●) 50 mM (-)-DIKGA and 20 mM xOH in MeOH, (□) 25 mM (-)-DIKGA and 10 mM xOH in EtOH, (○) 50 mM (-)-DIKGA and 20 mM xOH in EtOH. x stand for the alkali metal cations. 30 kV, L_{det} 23.5 cm

A possible explanation to the inversed EOF might be an adsorption of an excess of cations to the silica surface, with a subsequent charge inversion of the EDL. Greberg and Kjellander [87] proposed that a charge inversion may occur in water-based solutions of monovalent electrolytes in a region of the electric double layer when the co-ions are larger than the counter-ions and the surface charge density is low. The difference between the dielectric constants of MeOH ($\epsilon = 32.66$) [82] and EtOH ($\epsilon = 24.55$) [82], makes it reasonable to presume that the surface charge density of silica is higher in the methanolic BGEs than the ethanolic ones. That might explain why only the Rb^+ and Cs^+ systems with high ionic strength gave an anodic EOF in MeOH (Figure 8).

A trend for the EOF of decrease in the order Li^+ , Na^+ , K^+ , Rb^+ , Cs^+ has been found previously when alkali metal acetates were used in MeOH/AcN based BGEs [88] and in water-based buffers [89, 90], but a reversal of the EOF has never been observed. However, a decrease and even a reversal of EOF have been reported in separation systems used for the analysis of inorganic ions [91] and surfactants [92] in methanolic BGEs containing hydrophobic cations like tetraethylammonium (as a chloride and perchlorate salt), imidazole, or 2-naphtalenesulphonic acid [91, 92]. Thus, the present study has not only shown that the solvent can be used to suppress the EOF, but also that the type of electrolytes, e.g., cations in the BGE, can (IV).

Electrophoretic mobility and mobility difference

The electrophoretic mobility is strongly dependent on the choice of solvent, since it influences both the viscosity (η) and the dielectric constant (ε_r) of the medium. According to Henry, the electrophoretic mobility (μ_{ep}) can be expressed by [71]:

$$\mu_{ep} = \frac{2\varepsilon_r \zeta_{ion}}{3\eta} \cdot f_1(\kappa\alpha) \quad (\text{Eq. 3})$$

where ζ_{ion} is the zeta potential of the analyte ion. The function $f(\kappa\alpha)$ is called Henry's function. For small particles in a solvent with a low dielectric constant, $f(\kappa\alpha)$ approaches 1.0, which simplifies Equation 3 (i.e., the Hückel approximation). This approximation is generally used in non-aqueous solvents. Thus, the ε/η ratio for the solvent, which has already been discussed above, is of importance not only for the EOF, but also for the mobility of the analyte.

The model above described includes the influence of the solvent properties, but specific intermolecular interaction between the analyte and other components in the BGE, cannot be derived from the equation. More recently, a model for the electrophoretic mobility in the chiral separation system has been developed by Guttman et al. [22]. In a system with complex formation, the effective mobility is also influenced by the degree of complexation. The relation between the effective mobility of the analyte (μ_{eff}) and the equilibrium concentration of the charged selector ($[CS^-]$) can be expressed by with small modifications to the reference [22]:

$$\mu_{eff} = \frac{\mu_f + \mu_c K [CS^-]}{1 + K [CS^-]} \quad (\text{Eq. 4})$$

where K is the ion-pair formation constant and μ_f is the mobility of the free uncomplexed analyte. In a separation system based on the formation of neutral ion-pairs, the mobility of the complex (μ_c) is zero and Equation 4 can be further simplified to:

$$\mu_{eff} = \frac{\mu_f}{1 + K[CS^-]} \quad (\text{Eq. 5})$$

In order to achieve enantioseparation, it is not only the formation constants for the analyte-selector complexes (K_S , K_R) that need to be different; the electrophoretic mobility of the diastereomeric ion-pair (μ_c) and the free solute (μ_f) must also differ. There are a few examples that can be found for charged cyclodextrins in the literature where the formation constants are equal but the mobilities of the formed diastereomeric complexes are different [93, 94]. However, this is not possible when the ion-pair complex formed is neutral. The mobility difference between the enantiomers ($\Delta\mu$) can be described as a function of the selector concentration according to Wren and Rowe [95]:

$$\Delta\mu = \frac{(\mu_f - \mu_c)\Delta K[CS^-]}{(1 + K_1[CS^-])(1 + K_2[CS^-])} \quad (\text{Eq. 6})$$

K_1 and K_2 are the ion-pair formation constants for the first and second enantiomer respectively, and ΔK is the difference between these ($K_2 - K_1$). As mentioned above, μ_c is zero for a neutral analyte-selector complex. Equation 6 has a maximum, corresponding to the optimal selector concentration at which to achieve $\Delta\mu_{max}$. This is found at [95]:

$$[CS^-]_{opt} = \frac{1}{\sqrt{K_1 K_2}} \quad (\text{Eq. 7})$$

The thermodynamic ion-pair formation constants for (-)-DIKGA and *R*- or *S*-pindolol were determined by precision conductometry to be $1.04 \cdot 10^3$ and $0.995 \cdot 10^3$ respectively (**IV**). The constants were significantly different ($P < 0.01$) from each other and their order in binding strength ($R > S$) was in accordance with the migration order in CE, where *S* was the fastest migrating enantiomer. According to Equation 7, the optimal selector concentration should be at a (-)-DIKGA concentration of 0.98 mM. But, in Figure 9, the $\Delta\mu_{max}$ seems to be at about a total concentration of (-)-DIKGA of 15 mM. The deviation probably depends on the fact that only the charged form of the selector interacts with the enantiomers. Thus, only the fraction of the selector that is present in the charged, uncomplexed form should be used in the calculation of C_{opt} . Unfortunately, it was not possible to determine the ion-pair formation constant for K^+ and (-)-DIKGA with the presently used technique (**IV**). But based on the determined ion-pair constant for CS^+ (-)-DIKGA

($4.71 \cdot 10^3$), Li^+ (-)-DIKGA ($1.08 \cdot 10^3$) and Na^+ (-)-DIKGA ($5.62 \cdot 10^3$) together with Figure 9 and 11, it is reasonable to presume that the ion-pair formation constant of K^+ (-)-DIKGA has a about the same value as Na^+ (-)-DIKGA. Thus, based on an estimated K_a^* value for (-)-DIKGA (10^{-8}) and the thermodynamic ion-pair formation constant for the Na^+ and (-)-DIKGA complex (above) and the Na^+ and hydroxide/ethoxide complex (74.8), the concentration of free charged (-)-DIKGA in a BGE with 15 mM DIKGA and 6 mM NaOH was estimated to be 0.98 mM (IV). This is the same as the value calculated from the determined thermodynamic ion-pair constant, C_{opt} (see above), and indicates that this model [95] is valid for the present system.

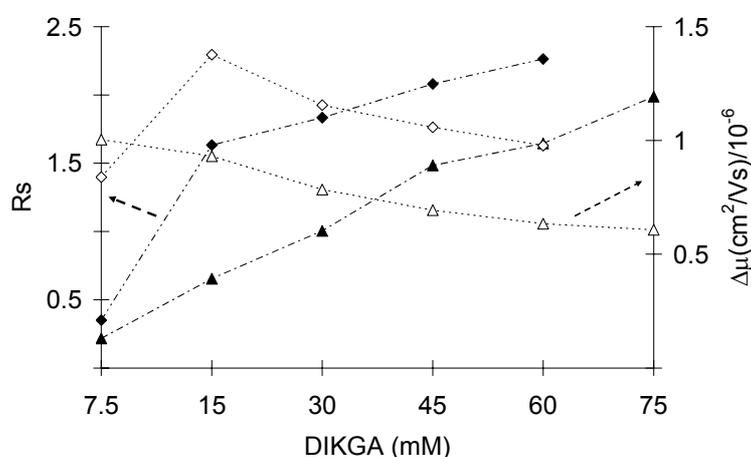


Figure 9. Enantiomeric mobility difference and resolution for different counter-ion concentrations (**Paper IV**).

BGE: (-)-DIKGA and KOH (molar ratio 5:2) in EtOH. L_{det} 23.5 cm. 30 kV.

(\triangle) *rac*-atenolol $\Delta\mu$, (\blacktriangle) *rac*-atenolol R_s , (\diamond) *rac*-pindolol $\Delta\mu$ (\blacklozenge) *rac*-pindolol R_s

The enantiomeric mobility difference at different selector concentrations of (-)-DIKGA has been studied in methanol (I) and ethanol (IV), Figure 9 and 10. However, the theoretical C_{opt} does not always correspond to the maximal enantioresolution, since R_s also depends on the EOF and on the efficiency of the electrophoretic system. (c.f. Eq 1). Also the effective mobility is dependent on the selector concentration (c.f. Eq. 5) and influences the enantioresolution (i.e. μ_m in Eq 1). As shown with atenolol and pindolol in Figure 9, the difference in $\Delta\mu$ in the interval studied in ethanol was moderate, but R_s increased as the selector concentration was increased owing to a decrease in the EOF (IV). This is also illustrated by bupivacaine when *L*-ZGP is used as the chiral selector, Figure 10 (III). The mobility difference (filled symbols) for bupivacaine showed a weak dependence on the selector concentration but

the observed enantioresolution (the Kaiser factor³, unfilled symbols) increased from 0.2 to 0.9 in the concentration interval studied (III). Thus, the lowered EOF, arising from the higher ionic-strength at greater selector concentrations, has a major influence on the enantioresolution.

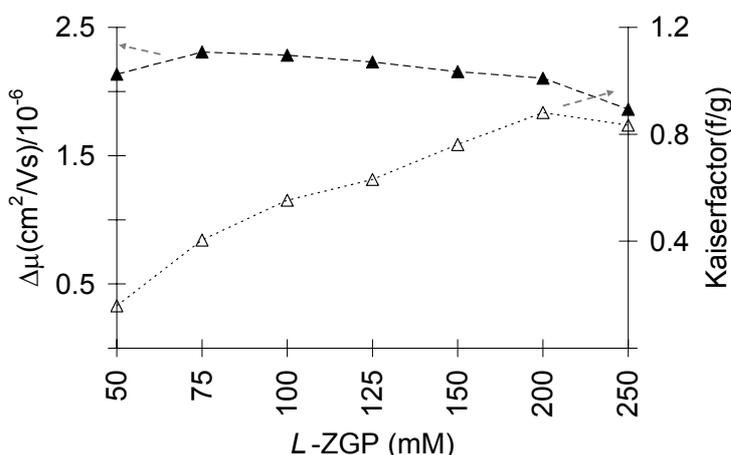


Figure 10. Enantiomeric mobility difference, enantioresolution and selector concentration (**Paper III**).

BGE: L-ZGP and NaOH (molar ratio 5:2) in MeOH. L_{det} 60 cm, 30 kV.

▲ $\Delta\mu$ bupivacaine, △ Kf bupivacaine.

As mentioned above, the ion-pair formation constants of (-)-DIKGA and each one of Li^+ , Na^+ and Cs^+ were determined by precision conductometry (IV). The ion-pair formation constant was lower for the $\text{Li}^+\text{DIKGA}^-$ ion-pair ($K_p = 1.08 \cdot 10^3$) than for the $\text{Na}^+\text{DIKGA}^-$ ($K_p = 5.62 \cdot 10^3$) and $\text{Cs}^+\text{DIKGA}^-$ ($K_p = 4.71 \cdot 10^3$) ion-pair. A higher value of K_p gives a lower equilibrium concentration of free selector due to increased competition. Using NaOH or CsOH in the BGE instead of LiOH increased the $\Delta\mu$, Figure 11. Thus, when LiOH is used, the free selector concentration is higher than C_{opt} , which results in a decrease in $\Delta\mu$.

In **Paper II** $\Delta\mu$ and Rs were studied for pronethalol in methanolic BGEs with the addition of 25-75 % AcN, 5-50 % 2-PrOH or 5-25 % water. The concentration of selector ((-)-DIKGA) and base (NaOH) was kept constant. A steep decrease in $\Delta\mu$ was observed with the addition of water and also the resolution was decreased. For pronethalol, $\Delta\mu$ was increased slightly when AcN was added but it decreased when 2-PrOH was added. However, as far as the resolution was concerned, the relationship was the reverse: 2-PrOH

³ Kaiser factor = f/g . A straight line is drawn between the two peak maxima. g is the distance from the line to the extended baseline and f is the distance from the line to the valley between the peaks. When the Kaiser factor is 1, the peaks are baseline separated.

enhanced the enantioresolution, whereas AcN decreased it. Thus, addition of AcN to the methanolic BGE seems to give more selective ion-pair formation between the selector and pronethalol, but the high EOF decreases the enantioresolution.

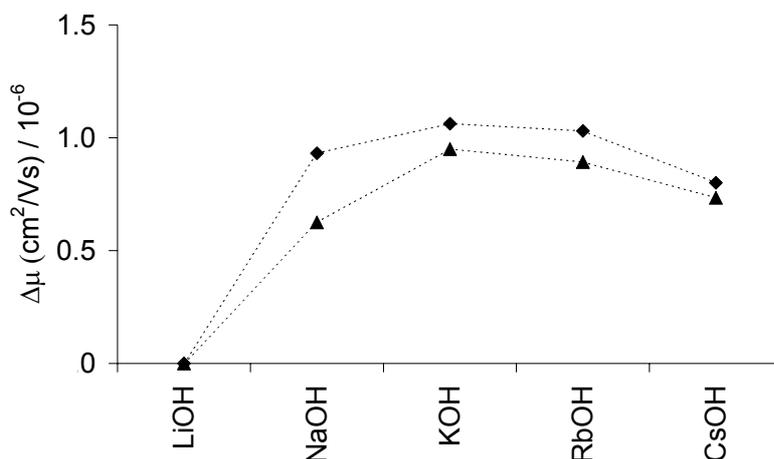


Figure 11. Influence of alkali metal cation on the enantiomeric mobility difference ($\Delta\mu$), (**Paper IV**).

BGE : 50 mM (-)-DIKGA and 20 mM of the alkali metal hydroxide in EtOH. L_{det} 23.5 cm, 30 kV. Sample concentration 0.1 mM in MeOH (▲) atenolol, (◆) pindolol.

In **Paper III**, 50 % of the solutes that possess enantioselectivity with *L*-ZGP in the systems tested had their highest value of $\Delta\mu$ when 20 % dichloromethane or 1,2-dichloroethane were added to the methanolic BGE, and they all showed their highest enantioresolution in these two solvents. Thus, as was discussed above, the low electro-osmosis and high $\Delta\mu$ in these solvent mixtures seem to benefit the resolution. Porras and Kenndler [35] discussed the separation efficiency in different solvents in a recent review in which they concluded that the efficiency is generally lower in organic solvents than in water. This is mainly a result of the lower mobility of the solute and the lower ionic strength in a solvent with a lower dielectric constant. Thus, a careful choice of solvent is of great importance for the resulting enantioresolution. A solvent with a reasonably low dielectric constant should give a low electro-osmosis, however it also facilitates the ion-pair formation with the selector. Thus, the choice of solvent/solvent mixtures and of other electrolytes than the selector influences the $\Delta\mu$. Theoretical models based on the competing equilibria involved, together with determined ion-pair constants, make it possible to determine the optimal selector concentration for a specific separation. However, as discussed above, parameters such as, e.g., the EOF, seem to have a greater influence on the resulting enantioresolution than $\Delta\mu$.

Chiral ion-pair selectors in pharmaceutical analysis

On-line preconcentration

The detection limit in CE can often be improved by the use of a preconcentration technique [96, 97]. Preconcentration can be achieved by manipulating the electrophoretic velocity of the analyte in the injection zone or by partitioning the analyte to a pseudostationary phase. In NACE, preconcentration techniques like field amplified stacking (FASS) [98-100], transient isotachopheresis (tITP) [101], low temperature zones [102], low and high conductivity zones [103] and on-line solid phase extraction [104] have been utilized. In aqueous media, also other electrophoretic techniques [105-107] have been used in addition to chromatographic preconcentration techniques. In the work presented in this thesis, FASS and tITP were evaluated.

In **Paper V** tITP was applied to lower the limit of detection for the *R*-enantiomer of timolol, the enantiomeric impurity in pharmaceutical formulations with *S*-timolol. (+)-KPA was used as the chiral selector. A zone sharpening was observed in the tITP system, as can be observed in Figure 12.

Thus, owing to the injection of a sample with a higher concentration of timolol, the limit of detection decreased from 2.5 % to 0.2 % for the *R*-enantiomer. Furthermore, the migration time decreased from 23 to 10.5 minutes in the tITP system. The decrease in the migration time was due to the cathodic EOF caused by the tailing electrolyte. In the BGE containing the selector, the EOF was anodic. The LOD for this tITP method meets the requirements for determination of *S*-timolol in the European Pharmacopoeia, which is 1 % [108]. An LOD of 0.006 % was previously found for *R*-timolol by using HPLC [109].

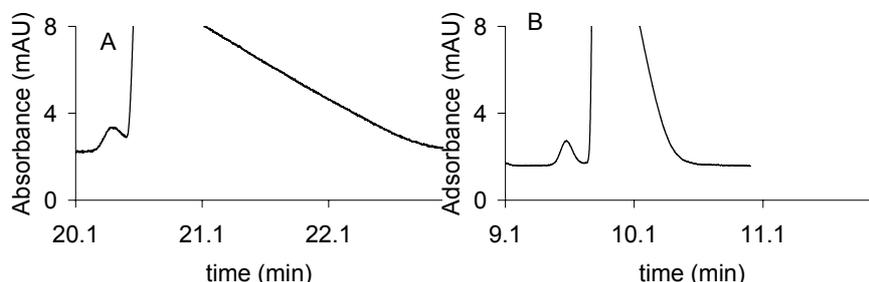


Figure 12. Analysis of *R*-timolol in presence of high amounts of *S*-timolol (**Paper V**).

BGE: 100 mM (+)-KPA 40 mM KOH in MeOH:EtOH (60:40 v/v) PAC coated capillary, L_{det} 23.5 cm, 30 kV, hydrodynamic injection (5 sec 25 mbar), 2mM *S*-timolol with 0.025 % *R*-timolol. (A) Normal injection. (B) tITP system with 100 mM NaAc in MeOH (35mbar 1 sec ,leading electrolyte) and 200 mM triethanolamine as cathodic vial (tailing electrolyte).

Enantiomeric purity determination of *1R*, *2S*-ephedrine

It was possible to inject 25 mM *1R,2S*-ephedrine with a minor impurity *1S,2R*-ephedrine with maintained enantioresolution owing to the high enantioresolution ($R_s = 10.0$), as seen in Figure 13A. The chiral selector (-)-DIKGA was used together with a polyacrylamide coated capillary

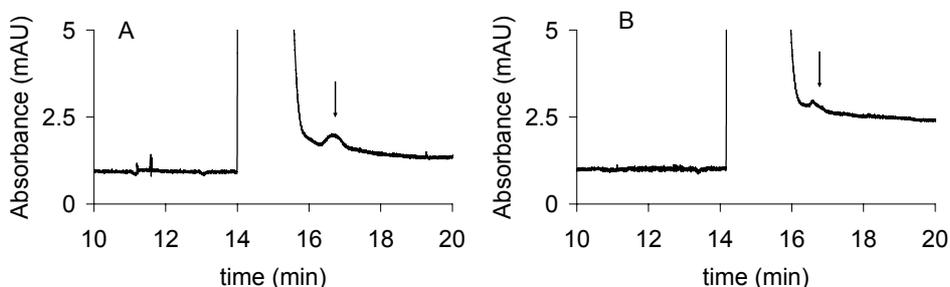


Figure 13. Enantiomeric analysis of *1S*, *2R*-ephedrine (**Paper V**)

BGE: 100 mM (-)-DIKGA and 40 mM KOH in MeOH:EtOH (2:3 v/v), 30 kV, L_{det} 23 cm, 25 mM of *1S,2R*-ephedrine. (A) *1S*, *2R*-ephedrine with 0.071 % (w/w) *1R,2S*-ephedrine, (B) Efedrin® (batch 5A209A),

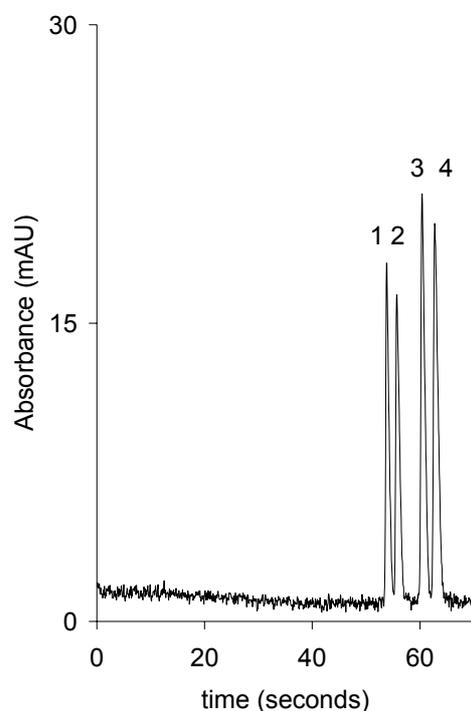
The LOD of the enantiomeric impurity was determined to be 0.033 % ($S/N = 3$), which, to the best of the author's knowledge, is the lowest value obtained for ephedrine by CE. Enantiomeric impurity determinations of ephedrine have been performed by, e.g., NMR [110], HPLC [111], capillary

electrochromatography (CEC) [112], micellar electrokinetic chromatography (MEKC) [113] and CE [110, 114, 115] with LODs in the range of 0.035-0.5 %, with the lowest value being obtained by CEC [112]. The method was subsequently validated according to the ICH guidelines [116, 117] and used for purity analysis of a batch of the injection solution Efedrin[®]. A small peak was observed at the t_{mig} of *1S,2R*-ephedrine (Figure 13B), corresponding to an impurity lower than 0.05 %.

Fast separations

Jansson and Roeraade [118] have derived an expression for the relationship between efficiency per time unit and the solvent properties. They found that in an ideal system, where diffusion alone contributes to the band broadening, a high ε/η ratio of the BGE would promote high efficiency per time unit. However, a high ε/η ratio would also increase the EOF (Eq. 2) and therefore decrease the enantioresolution (c.f. Eq. 1). Of the organic solvents used in this work, AcN has the highest ε/η ratio, followed by MeOH. However, both the efficiency and the efficiency per time unit was decreased for pronethalol as the AcN concentration increased. Furthermore, the efficiency was lower in AcN than in MeOH (**II**). Thus, the relationship between the ε/η ratio and efficiency seems not to be valid for the present systems. This might be due to additional sources of band broadening, such as e.g., electromigration dispersion arising from different ionic-strengths at the same electrolyte concentrations in different solvents [35].

As already discussed above, the low mobility differences for enantiomers that are often observed, make it more difficult to decrease the analysis time through the use of a higher EOF than would be used for achiral separations. However, by a careful adjustment of experimental conditions it has been possible to obtain fast chiral separations. As an example, the enantiomers of isoprenaline were separated within 58 seconds using (-)-DIKGA as chiral selector, Figure 14 (**IV**). When *L*-ZGP was used as the selector, *rac*-mepivacaine was enantio-separated within 72 seconds (**III**). Chiral separation of ormeloxifene using β -cyclodextrins in aqueous CE has been performed in 40 seconds [119] which, to the best of the author's knowledge, is the fastest enantio-separation performed on a conventional CE instrument. The separation speed in chiral separations can be increased further by miniaturizing the system (i.e. decreasing L_{det} and increasing E) whilst using selectors with high selectivity for the analytes of interest, together with a proper choice of solvent and electrolytes.



Figur 14. Separation of *rac*-isoprenaline and *rac*-sotalol (**Paper IV**)

BGE: 100 mM (-)-DIKGA and 40 mM KOH in MeOH. L_{det} 8.5 cm, L_{tot} 32 cm, 30kV. Peak 1 and 2 corresponds to the enantiomers of isoprenaline and peak 3 and 4 to the enantiomers of sotalol.

The use of the Partial Filling in Chiral NACE-MS

The partial filling technique [120] can be used to enhance the detection sensitivity in BGEs with UV absorbing selectors, e.g., CBH I [120, 121] and *tert.*-butylcarbamoyl quinine [122], but it can also be used in conjunction with MS detection to avoid contamination of the interface by non-volatile buffer components, e.g. chiral selectors [123, 124]. With the partial filling technique, only a part of the capillary is filled with the selector solution. This technique requires separation conditions where the enantiomers are separated in the selector plug, and reach the detector before the selector plug. The experimental conditions, such as the plug length and the selector concentration need to be optimised, to increase the enantioresolution. The magnitude of the EOF in the two zones (with and without selector) is of importance too. Zarbl *et al.* [38] used PVA coated capillaries to avoid an early breakthrough of the selector zone into the detection window. They found that

the EOF in the system was lowered, and the repeatability in migration times was comparable to what they had observed previously in uncoated capillaries [122].

The first chiral CE application describing MS detection appeared in 1995 in a publication by Sheppard *et al.* [125]. These authors used complete filling of the capillary with heptakis (2,6-di-*O*-methyl)- β -cyclodextrin in a buffer at low pH, and a sheathless electrospray interface. No ion suppression, which might be caused by the introduction of non-volatile substances into the MS, was observed. Although the flow from the CE is low (in the nL/min range), the introduction of non-volatile substances, e.g., chiral selectors, into the MS would probably require repetitive cleaning of the interface if high sensitivity is to be preserved. Thus, the use of volatile buffers in non-aqueous solvents would also improve the performance of the CE-MS coupling [126].

Although PAC coated capillaries were used in the work conducted for this thesis (II), the EOF was high in the zone without the selector. In order to facilitate an increase in the plug length, acetic acid was added to the zone without the selector, with the effect being apparent in Figure 15. The EOF was lowered, and the selector zone was delayed. Furthermore, the extra band broadening, which was observed to occur in the border between the two zones, decreased when the EOF did not differ too much between the two zones (i.e. N increased c.f. Eq. 1).

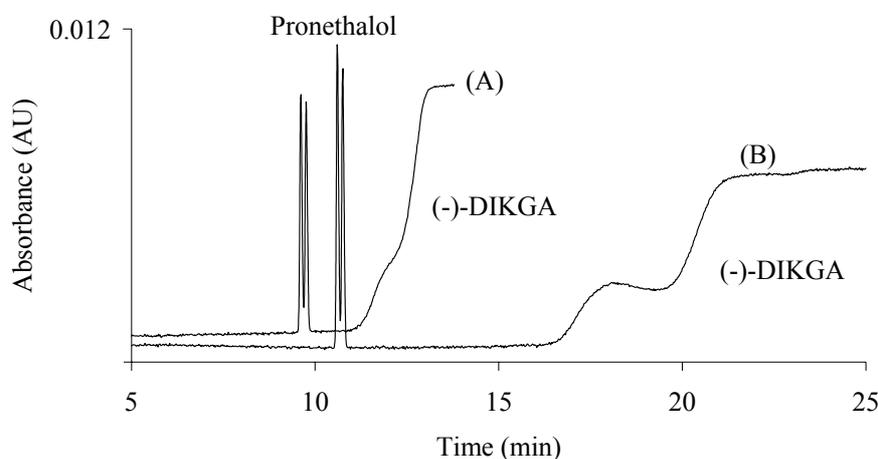
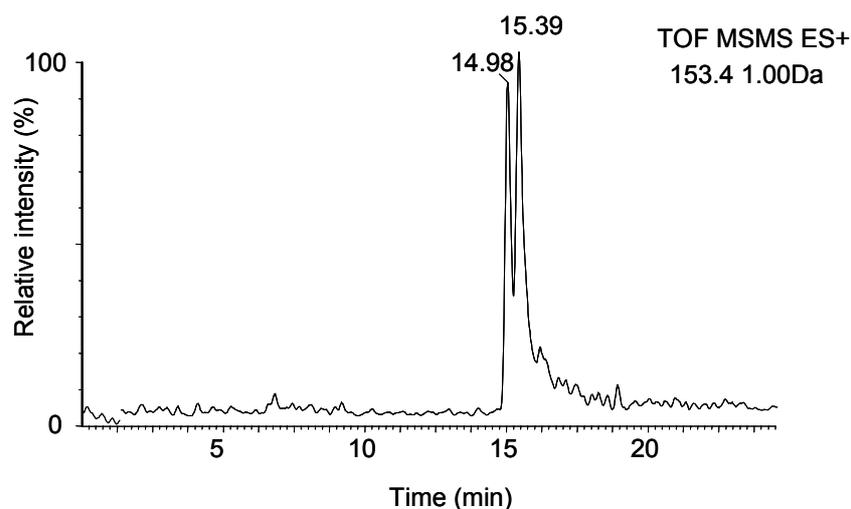


Figure 15. Acetic acid and front migration. (Paper II)

Conditions: BGE with selector : 80 mM (-)-DIKGA and 40 mM NH_4Ac in MeOH.
BGE without selector: (A) 40 mM NH_4Ac or (B) 40 mM NH_4Ac and 30 mM HAC in MeOH. Solute: 0.1 mM pronethalol in MeOH, L_{det} 60 cm, 20 kV, plug length 28 cm.

The MS instrument with which the detection was performed was a Q-TOF model 1 with an orthogonal z-spray sheath liquid electrospray interface. Despite the fact that the apparent applied voltage was 25.5 kV and not 30 kV as was previously the case in UV detection (owing to the cathode being the electrospray ionisation (ESI) interface rather than ground) the migration time was shorter in the CE/MS than the CE/UV system. This is probably due to suction from the sheath liquid interface. To decrease the influence of the suction, a counter pressure of 15 mbar was applied during the runs. Furthermore, the sheath liquid, sheath gas and capillary voltage were switched off during the injection. The lower efficiency observed in the CE/MS system compared to the CE/UV one is probably caused by the same suction from the ESI interface, giving a parabolic flow profile, and by dilution of the sample with the sheath liquid. Through the introduction of a low conductivity zone after the sample (pure MeOH) the efficiency was slightly improved. Further decrease of the EOF was subsequently achieved by adding 25 % 2-PrOH to the methanolic BGEs. A typical CE/MS/MS electrophorogram of *rac*-pronethanol is shown in Figure 16.



Figur 16. MS/MS electrophorogram of pronethalol (**Paper II**).

Conditions: partial filled capillary. BGE zone with selector: 80 mM (-)-DIKGA and 20 mM NH₄Ac in MeOH:2-PrOH (75:25 v/v), BGE zone without selector: 20 mM NH₄Ac and 20 mM HAc in MeOH:2-PrOH (75:25 v/v). 0.01 mM Pronthetalol in MeOH, L_{tot} 58 cm, plug length 32 cm, 30kV, Parent m/z 230, daughter m/z 153.4.

Conclusions

Non-aqueous solvents facilitate the use of ion-pair selectors in chiral CE. In the work presented here, three novel selectors have been introduced. The enantioresolution in CE is always based on stereoselective complexation (“three-point interaction”) between the enantiomers and the selector. In HPLC, on the other hand, a chiral counter-ion may also promote separation by formation of diastereomeric ion-pairs with different distribution properties to the stationary phase.

The enantioresolution obtained for the amines in this work was strongly affected by the choice of other electrolytes (besides the selector), but also by the organic solvent used in the BGE. The most important parameter for regulating the enantioresolution was the direction and magnitude of the electro-osmotic flow. Covalently coated capillaries have been used to suppress the EOF, as have different organic solvents/solvent mixtures and alkali metal hydroxides. Surprisingly, the EOF was anodic when cations such as Rb^+ and Cs^+ were used in a methanolic or ethanolic BGE. The use of organic solvents with a rather low dielectric constant (e.g., EtOH) and a suitable cation that suppresses or reverses the EOF (with or without a coated capillary), increases the enantioresolution and facilitates the use of shorter capillaries, i.e. it promotes a fast chiral separation.

The selectors used were found to give different migration order of the enantiomers. It is often considered to be desirable to be able to choose the migration order of the enantiomers in the electrophorogram. The use of a chiral selector that ensures the desired migration order of the enantiomers, makes it possible to choose e.g., the highest mobility for the minor enantiomer in chiral purity determinations (if enantioresolution is obtained with both selectors). The chiral selectors were successfully applied to purity determinations of enantiomeric amines in pharmaceuticals, with and without the use of an on-line preconcentration technique.

A more sensitive detector such as MS could be used to decrease the limit of detection. The volatility of non-aqueous media would facilitate the CE-MS hyphenation. By using the partial filling technique, the need for extensive cleaning of the interface is avoided which would improve the performance. This technique was adopted for detection of enantiomeric amines in the selector free zone by tandem mass spectrometry.

Future studies

There are still many potential chiral ion-pair selectors that, probably due to their low solubility and selectivity in aqueous solvents, have not been applied. For cationic analytes, only two selectors apart from the three presented in this thesis have been utilized. If a “method development kit” (of the kind available for cyclodextrines) is to be made available for chiral separation with ion-pair selectors in NACE, more selectors need to be discovered.

Since the influence of the solvents and other electrolytes in the BGE have been shown to be of greater importance for the chiral separation than expected, it would also be of interest to study a wider range of different types of electrolytes and solvent compositions, in order to identify suitable conditions for faster chiral separations. Also miniaturisation of the present systems to chip size, would be a feasible way to enhance the separation speed further.

The general knowledge that has been acquired in these NACE systems is also of great value for the understanding of HPLC systems with CMPAs. Further research using the strong combination of NACE and precision conductometry will provide even greater insight into the mechanisms of chiral separation.

Since the number of possible interactions between a low-molecular weight selector and an enantiomer is limited, other techniques for investigation of the stereoselective mechanism would be feasible (e.g., molecular mechanics and NMR)

The further knowledge would also simplify the development of methods using ion-pair selectors for chiral impurity determinations in pharmaceutical formulations and for chiral metabolic profiling in biological samples with MS detection.

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