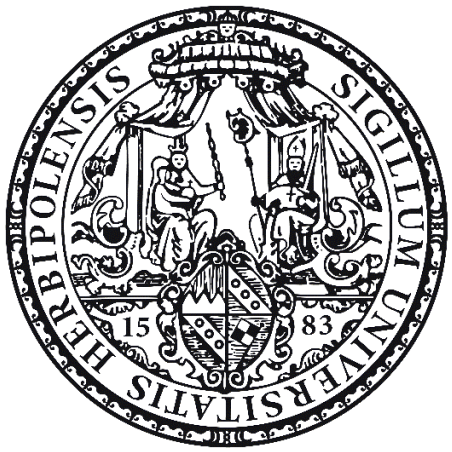
The Use of Ionic Liquids in Capillary Electrophoresis Enantioseparation

DISSERTATION

zur Erlangung des

naturwissenschaftlichen Doktorgrades

der Julius-Maximilians-Universität Würzburg



vorgelegt von

Joachim Wahl

aus Mutlangen

Würzburg 2018

Eingereicht bei der Fakultät für Chemie und Pharmazie am:

……..

Gutachter der schriftlichen Arbeit:

1. Gutachter ……

2. Gutachter ……

Prüfer des öffentlichen Promotionskolloquiums:

1. Prüfer ……

2. Prüfer ……

3. Prüfer ……

Datum des öffentlichen Promotionskolloquiums:

……..

Doktorurkunde ausgehändigt am:

……..

Die vorliegende Arbeit entstand auf Anregung und unter Anleitung von

Frau Prof. Dr. Ulrike Holzgrabe

am Institut für Pharmazie und Lebensmittelchemie der Julius-Maximilians-Universität Würzburg.

Ihr gilt mein besonderer Dank für die freundliche Aufnahme in den Arbeitskreis, das in mich gesetzte Vertrauen und die Unterstützung in jeder Phase der Erstellung dieser Dissertation.

Bedanken möchte ich mich zusätzlich bei Prof. Dr. Dr. Lorenz Meinel, Prof. Dr. Etsuo Yonemochi und Prof. Dr. Tomohiro Endo für die erfolgreiche Kooperation, sowie Takayuki Furuishi für die Zusammenarbeit.

Thorben Link möchte ich für seine Unterstützung danken, ohne die die Erstellung dieser Dissertation nicht möglich gewesen wäre.

Des Weiteren danke ich dem gesamten AK Holzgrabe für die gute Arbeitsatmosphäre, die erfolgreiche Zusammenarbeit und die schöne gemeinsame Zeit inner- und außerhalb des Labors.

Großer Dank gebührt meinen Eltern und meinem Bruder sowie Marleen und Anton, die mich auf meinem Weg zur Dissertation begleitet und moralisch unterstützt haben.

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1 Introduction

Two enantiomers of a chiral drug may have different effects on the human body. Thus, to ensure drug safety the chiral identity and enantiomeric purity of these drugs have to be proven by means of a chiral analytical separation method, such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Compared to other chiral separation techniques, CE has a lot of advantages like low sample volume, high separation efficiency, and low costs. The principles of CE and possible approaches to improve CE enantioseparations are introduced in the following parts.

1.1 Capillary Electrophoresis

In CE the separation of analytes is caused by different migration velocities in an applied electrical field. Usually, CE separations are carried out in a fused silica capillary whose both ends are located in vials containing the background electrolyte (BGE). Since the beginning of CE applications, a lot of method variations were developed and reported in literature. In capillary zone electrophoresis (CZE), separations are performed using conventional buffers, while in capillary gel electrophoresis the capillary is filled with a gel. Further mechanisms for CE separations were introduced by mixing different additives to the BGE. In micellar electrokinetic chromatography (MEKC) and in microemulsion electrokinetic chromatography (MEEKC) micelles and the distribution between two immiscible phases are applied for the formation of pseudo-stationary phases. An additional approach using pseudo-stationary phases in CE is the separation of enantiomers by means of a chiral selector, which is forming diastereomeric complexes with analyte enantiomers. When exclusively organic solvents are used for the separation of analytes in an electrical field, the method is referred to as nonaqueous capillary electrophoresis (NACE).

1.1.1 Apparatus

Figure 1 displays the schematic construction of a CE instrument. The instrument constituted of an adjustable high-voltage power supply: the voltage (-30 kV to +30 kV) is applied on two BGE containing vials by platinum electrodes. The two vials are connected by a thermostated fused silica or coated capillary with a detection window for a diode array detector. Samples can be injected electrokinetically or hydrodynamically and the whole system is operated by a personal computer.

Due to a very small injection volume, the sample injection is a crucial factor for the reproducibility of CE runs. In the course of an electrokinetic injection samples are injected applying

voltage on the sample vial. An advantage, occurring during the application of voltage, is the concentration of sample compounds with high electrophoretic mobilities at the beginning of the capillary. This accumulation of fast migrating ionic compounds improves the limit of detection in CE separations [1]. On the other hand, a great disadvantages of the electrokinetical injection is the discrimination of differently charged analytes, because compounds having lower mobilities are injected in smaller quantities than more mobile species [2]. Furthermore, applying this method the injection of samples with high mobilities towards the EOF is impossible. In case of hydrodynamic injection, the sample solution is either pumped into the capillary or a vacuum is applied at the capillary end.

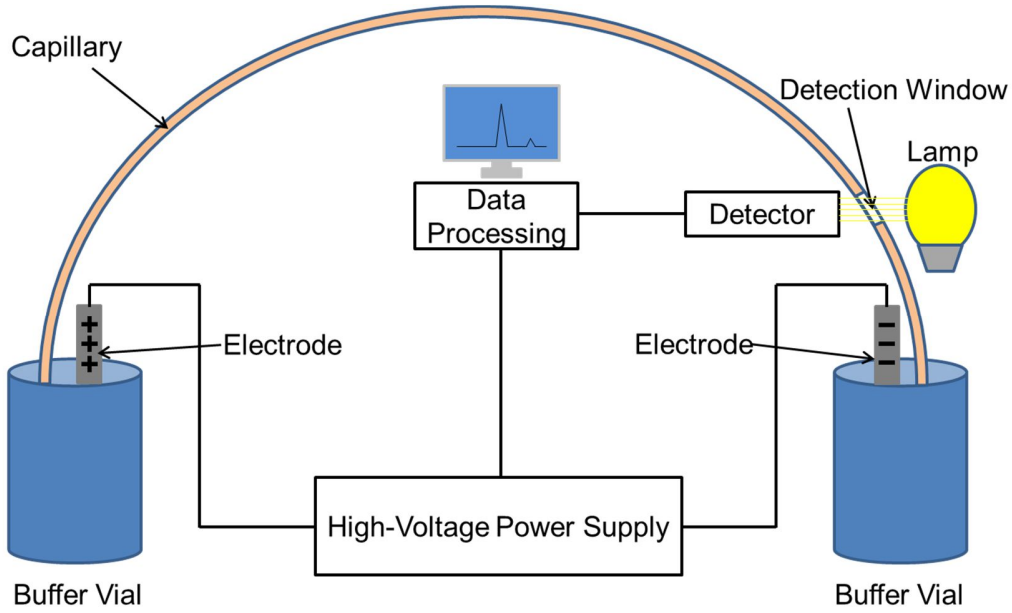


Figure 1 Schematic construction of a CE instrument

In general, CE separations are carried out using polyimide coated fused silica capillaries with a length of 15 to 60 cm and an internal diameter (I. D.) of 50 to 200 µm. The I. D. of a capillary is playing a crucial part in the development of Joule heating during the separation process. Joule heating causes deterioration of separation peaks resulting in peak broadening, and should thus be minimized. Decreasing the capillary I. D. leads to advantages like a lower electrical resistance and an increase of the ratio between capillary surface and capillary volume. A low electrical resistance is beneficial to keep Joule heating low and a large surface of the capillary leads to a fast heat transfer [3]. However, the diminution of the capillary I. D. decreases the path length of the detection cell yielding lower sensitivities in CE [4].

In addition to fused silica capillaries, many capillaries having modified capillary surfaces were reported. Mostly, coatings based on polyacrylamide [5], polyethylene glycol [6], polyvinylpyrrolidone [7], and polyvinyl alcohol [8] are applied for the suppression of the EOF [9].

Usually, UV or diode array detectors (DAD) are applied for the detection in CE. Further methods using a detection inside the capillary are: laser-induced fluorescence detection [10] and refractive index detection [11]. Methods detecting the analytes outside the capillary are electrochemical detection, such as contactless conductivity detection [12, 13] and mass spectrometry (MS) [14].

1.1.2 Theoretical Principles

As mentioned above, separation in CE occurs due to different mobilities of analytes in an applied electrical field. Charged analytes migrate towards the electrode of opposite charge. The velocity of the migration depends on the charge-to-mass ratio of the analytes: higher charged molecules migrate faster than lower charged ones and smaller molecules migrate faster than bigger ones. Figure 2 displays the separation of ions with different charge-to-mass ratios and Eq. (1) summarizes the factors influencing the electrophoretic velocity *u*.

$$u = \frac{z \cdot F \cdot E}{6 \cdot N_A \cdot \pi \cdot r \cdot \eta} \qquad (1)$$

where z is the effective charge and r the Stokes radius of the molecule. F is the Faraday constant, N_A the Avogadro constant, E the applied electrical field and η the dynamic viscosity.

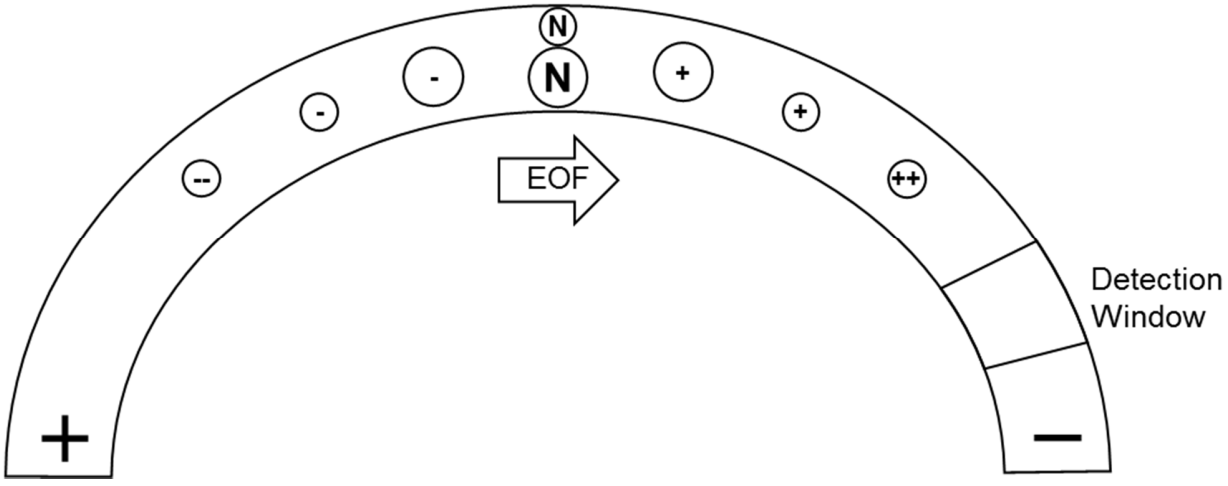


Figure 2 Schematic depiction of the separation of different analyte molecules. Due to the electroosmotic flow, all analytes migrate towards the cathode. Higher charged and smaller molecules possess higher electrophoretic mobilities than lower charged and bigger molecules.

The apparent mobility (μ_{app}) of an analyte is calculated applying the length of the capillary (L), the distance between capillary inlet and detection window (l), the applied voltage (V) and the migration time (t) as follows:

$$\mu_{app} = \frac{l \cdot L}{t \cdot V} \qquad (2)$$

Furthermore, the movements of the ions towards the electrodes are overlaid by the electroosmotic flow (EOF) causing a transport of the whole BGE towards the detection window. The EOF, which doesn't contribute to the separation, is originated by a negatively charged capillary surface caused by a dissociation of silanol groups. The negatively charged silanol groups attract cations from the BGE, which are forming two layers of positively charged ions, a fixed inner (called Helmholtz plane) and a mobile outer layer, which complies with the regulations of the Debye-Hückel theory. By the application of an electrical field the cations of the mobile layer and their solvation shell are pulled towards the cathode entraining the whole BGE. Thus, even negatively charged analyte molecules can be detected at the cathodic side of the capillary.

The electrokinetic potential difference between the stationary inner layer of adsorbed cations and the BGE, the so called zeta-potential, is proportional to the velocity of the EOF and can be decreased by augmenting the ionic strength of the BGE. Additionally, the EOF depends on the amount of deprotonated silanol groups (pK_a of silanol groups on fused silica surfaces: ~ 5 [15, 16]), consequently the pH value of the BGE, and on the properties of the capillary surface. To ensure a homogenous degree of hydroxylation of new fused silica capillaries the capillaries have to be rinsed with sodium hydroxide and hydrochloric acid solution prior to use. Furthermore, to achieve a good run-to-run reproducibility in CE, the capillary has to be conditioned with separation buffer before each consecutive run. The EOF can be calculated from Eq. (2) using an uncharged compound (neutral marker).

In CE, the separation of two analyte peaks is described by the resolution R_S, which is calculated as follows:

$$R_S = 2 \cdot \frac{t_B - t_A}{w_A + w_B} \qquad \text{with } t_A < t_B \qquad (3)$$

where t_A and t_B are the migration times and w_A and w_B the baseline peak widths of the analytes A and B. In the case of overlapping peaks, the determination of w_A and w_B, and consequently the calculation of a R_S value get difficult. Hence, poorly separated peaks are characterized by the electrophoretic selectivity α, which is calculated from the effective electrophoretic mobilities μ_A and μ_B of the analytes A and B.

$$\alpha = \frac{\mu_B}{\mu_A} \qquad (4)$$

1.1.3 Affinity Capillary Electrophoresis

In affinity capillary electrophoresis (ACE), the binding constant between an analyte and a ligand is calculated from the electrophoretic mobility change of the analyte when different concentrations of ligand are added to the BGE [17]. Mostly, binding constants are determined using a constant concentration of analyte whereas the concentration of the ligand is increased. For the determination of binding constants three different linear plotting methods and a nonlinear regression approach can be applied.

For the calculation of binding constants the apparent electrophoretic mobility μ_{app} has to be adjusted by the mobility of the EOF μ_{EOF} resulting in the effective electrophoretic mobility μ_i.

$$\mu_i = \mu_{app} - \mu_{EOF} \qquad (5)$$

Furthermore, 1:1 complexations can be described by the following equation [17]:

$$A + L \leftrightarrows AL$$

$$K[L] = \frac{[AL]}{[A]_f \cdot [L]_f} \qquad (6)$$

where K[L] is the complex formation constant at equilibrium concentration, $[A]_f$ the concentration of free analyte, $[L]_f$ the concentration of free ligand and [AL] the concentration of complex.

The experimentally determined mobility of the sample μ_i is composed of the mobility of the free sample μ_f and the mobility of the complexed sample μ_c weighted by the rate of their existence (X_f and X_c) [18].

$$\mu_i = X_f \cdot \mu_f + X_c \cdot \mu_c \qquad (7)$$

$$X_f = \frac{[A]_f}{[A]_f + [AL]} \qquad (8)$$

$$X_c = \frac{[AL]}{[A]_f + [AL]} \qquad (9)$$

By inserting (8) and (9) into (7) the following equation can be achieved [17]:

$$\mu_i = \frac{[A]_f}{[A]_f + [AL]} \cdot \mu_f + \frac{[AL]}{[A]_f + [AL]} \cdot \mu_c \qquad (10)$$

Combining (10) with (6) results in:

$$K[L] = \frac{\mu_f - \mu_i}{\mu_i - \mu_c} \qquad (11)$$

By rearranging (11) the following equation can be obtained:

$$(\mu_i - \mu_f) = \frac{(\mu_c - \mu_f) \cdot K[L]}{1 + K[L]} \qquad (12)$$

Furthermore, 1:1 molecular complexes can be described by the general form of the binding isotherm [19].

$$y = \frac{ax}{b + cx}, \qquad (13)$$

where y represents an experimental determined value, a, b, and c are constants, and x is the concentration of the free ligand. For graphical analysis (13) can be rearranged into three different forms [19].

x-reciprocal: $$\frac{y}{x} = -\frac{c}{b} \cdot y + \frac{a}{b} \qquad (14)$$

y-reciprocal: $$\frac{x}{y} = \frac{c}{a} \cdot x + \frac{b}{a} \qquad (15)$$

double reciprocal: $$\frac{1}{y} = \frac{b}{a} \cdot \frac{1}{x} + \frac{c}{a} \qquad (16)$$

Comparing Eq. (12) and (13) reveals that both equations have the same mathematical form. For this reason, Eq. (12) can also be rearranged into the forms of (14), (15) and (16).

x-reciprocal: $$\frac{(\mu_i - \mu_f)}{[L]} = -K \cdot (\mu_i - \mu_f) + K \cdot (\mu_c - \mu_f) \qquad (17)$$

y-reciprocal: $$\frac{[L]}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f)} \cdot [L] + \frac{1}{(\mu_c - \mu_f) \cdot K} \qquad (18)$$

double reciprocal: $$\frac{1}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f) \cdot K} \cdot \frac{1}{[L]} + \frac{1}{(\mu_c - \mu_f)} \qquad (19)$$

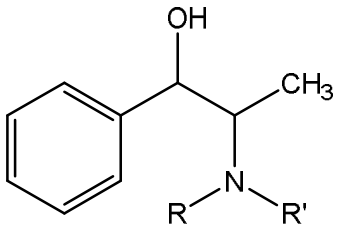
By plotting $\frac{(\mu_i - \mu_f)}{[L]}$ versus $(\mu_i - \mu_f)$ for x-reciprocal, $\frac{[L]}{(\mu_i - \mu_f)}$ versus [L] for y-reciprocal, and $\frac{1}{(\mu_i - \mu_f)}$ versus $\frac{1}{[L]}$ for double reciprocal, the binding constants can be calculated from the slopes and intercepts of the regression lines [17, 19].

Furthermore, nonlinear regression, having benefits like better precision and accuracy [20, 21], can be performed by means of a rearranged form of Eq. (11):

$$\mu_i = \frac{\mu_f + \mu_c \cdot K[L]}{1 + K[L]} \qquad (20)$$

1.2 Phenethylamines

Ephedrine, pseudoephedrine, methylephedrine, and norephedrine are sympathomimetic drugs of the phenethylamine chemical class. Originally, these compounds were found and extracted from various plants of the genus Ephedra [22]. Acting as stimulants of the adrenergic receptor system [23], phenethylamines are used and abused as bronchodilators [24], nasal decongestants [25], to increase blood pressure [26], to lose weight [27], and as stimulants [28]. Ephedrine has been used in “Traditional Chinese Medicine” as anti-asthmatic agent for centuries [29]. In combination with nonsteroidal anti-inflammatory drugs and antihistamines, pseudoephedrine hydrochloride is applied in cold medicines and allergic rhinitis preparations. Nowadays, due to their illicit use for the manufacturing of methamphetamine [30], the distribution of ephedrine, as well as pseudoephedrine, is more and more restricted by public authorities.



	R	R'
(1*R*,2*S*)-, (1*S*,2*R*)-Ephedrine	-H	$-CH_3$
(1*R*,2*R*)-, (1*S*,2*S*)-Pseudoephedrine	-H	$-CH_3$
(1*R*,2*S*)-, (1*S*,2*R*)-N-Methylephedrine	$-CH_3$	$-CH_3$
(1*R*,2*S*)-, (1*S*,2*R*)-Norephedrine	-H	-H

Figure 3 Structures of phenethylamines

As can be seen from Figure 3, ephedrine derivatives consist of a phenyl group, a 2-aminopropyl moiety, and a benzylic alcohol, resulting in two stereogenic centers.

1.3 Chirality

Two chiral chemical molecules appearing as an object and its mirror image are called enantiomers. Enantiomers are characterized by having identical chemical and physical properties. Due to different pharmacokinetic, pharmacodynamic, and toxicological properties [31-33] of drug enantiomers, the interest in the production of enantiomeric pure drugs increases more and more. Enantiopure compounds are prepared by asymmetric synthesis using chiral starting materials or chiral catalyst. A further possibility to obtain single enantiomer drugs is the preparative separation of isomers after preceding conversion into diastereomers. These separations are mostly performed by crystallization or a chromatographic approach in chiral environments [34]. Nevertheless, the performance of a chiral synthesis and/or a preparative chiral purification has to be controlled by an enantioselective analytical technique, such as CE.

In general, enantioseparation in CE is achieved adding a chiral selector to the BGE. Accordingly, the enantioseparation takes place due to different mobilities of the two temporarily formed diastereomeric complexes [35-40] or due to different binding constants between the analyte enantiomers and the chiral selector [41]. Frequently used chiral selectors in CE are chiral surfactants [42], antibiotics [43], polysaccharides [44], crown ethers [45], and cyclodextrins [46, 47].

1.4 Cyclodextrins

As can be seen from Figure 4, cyclodextrins (CDs) consist of α-1,4-glycosidic linked glucose monomers, which form a torus with a larger and a smaller opening. Due to their hydrophilic exterior and their hydrophobic inner cavity, they are able to form inclusion complexes with hydrophobic molecules. CDs are applied in food chemistry [48], in pharmaceutical formulation to enhance the solubility of poorly water soluble compounds [49], and in analytical separation science [50-52], in particular in CE [53, 54]. Due to the formation of diastereomeric complexes by the inclusion of analyte enantiomers into CD cavities, CDs, added to a BGE, are acting as chiral selectors in CE enantioseparations.

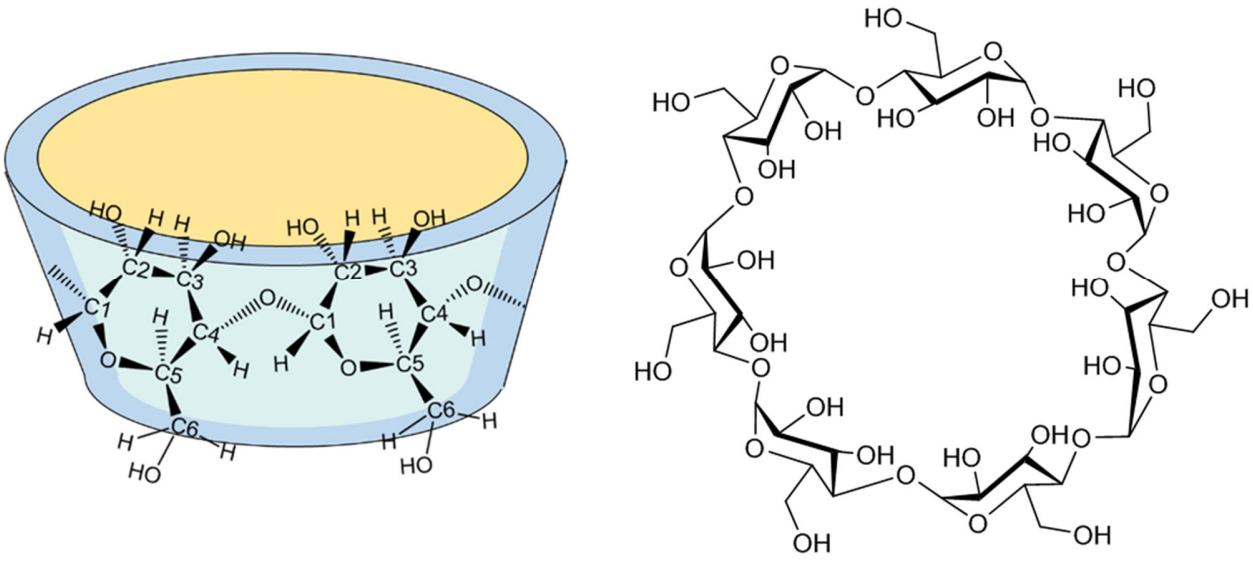
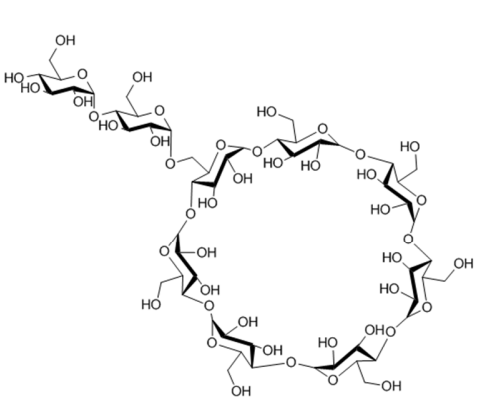


Figure 4 Spatial arrangement and structure of native β-CD

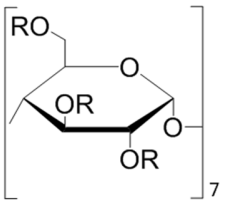
The molecular recognition between uncharged CDs and analytes, forming specific interactions, is driven by hydrophobic forces, van der Waals forces, hydrogen bonding, and steric effects. β-CD, the 7-membered sugar ring molecule (see Figure 4), characterized by an inner diameter of 0.78 nm at the wider rim of the cavity [49], was reported to form 1:1 complexes with the phenyl

moiety of phenethylamines diving into the cavity [55-62]. Furthermore, depending on the pH value, the protonated amino nitrogen of phenethylamines may interact with oxygens at the wider rim of the β-CD by the formation of hydrogen bonds [57, 60].

Different functional moieties, like e. g. 2-hydroxypropyl, methyl, and maltosyl groups were added to the CD rims (see Figure 5). The derivatization of native CDs, changing the solubility, the hydrophobicity, the size of the cavity, and the flexibility of the chiral selector [63-65], may alter the molecular recognition between CDs and guest molecules.



6-O-α-Maltosyl-ß-Cyclodextrin



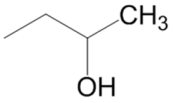
	R
ß-Cyclodextrin	-H
(2-Hydroxypropyl)-ß-cyclodextrin	-H or 
Methyl-ß-cyclodextrin	-H or $-CH_3$

Figure 5 Structures of β-CD derivatives

1.5 Ionic Liquids

Ionic liquids (ILs), also called liquid salts, ionic glasses, or ionic melts, are ionic compounds characterized by a melting point below 100 °C. Due to an effective charge delocalization [66], a high conformational freedom [67], and low lattice forces [68], ILs possess lower melting points than common salts. Cations commonly found in ILs are based on alkyl-substituted imidazolium, alkyl-substituted pyridinium, tetraalkylammonium, and tetraalkylphosphonium, whereas tetrafluoroborate, hexafluorophosphate, bistriflimide, and halides are classical used anions of ILs. By altering the combination of cation and anion the characteristics of an IL can be designed as requested.

Due to their unique properties, like thermal stability [69], miscibility, solvation power, and electrochemical stability [70], ILs are attracting increasing attention in many fields. They are applied in photoelectrochemical solar cells [71], as electrolytes in batteries [72], to transfer and store solar thermal energy [73], for cellulose processing [74], and in fuel cells [75]. Besides the possibility to interact with other compounds by ion-ion and ion-dipol interactions, ILs are able to

form interactions by hydrophobic, π-π, van der Waals, and/or hydrogen bond mechanisms. Thus, and due to their low vapor pressure, ILs are powerful solvents used in organic chemistry [76, 77] and as extracting agents in liquid-liquid and solid-liquid extractions [78]. Furthermore, solubility enhancements of pharmaceutical active organic salts were achieved by the formation of ILs [79, 80].

1.5.1 Ionic Liquids in Analytical Chemistry

In the last years, more and more interest was drawn towards ILs in analytical chemistry. Besides their use for sample preparation by extraction and as solvent for spectroscopy [81], they are applied in electrochemical sensors [82] and electrochemical analysis [83]. In mass spectrometry, ILs are applied as matrices for matrix-assisted laser desorption/ionization [84]. Furthermore, due to their low vapor pressure and their high viscosity, ILs are well suited for the application as stationary phase in gas chromatography columns [85]. In HPLC, ILs, added to the mobile phase, are reported to improve the chromatographic performance by shielding silanol groups [86] and interacting with analyte molecules. Additionally, due to variable chromatographic separation mechanisms (normal-phase, reversed-phase, ion-exchange, hydrophilic, and chiral recognition), IL based stationary phases attract increasing attention in HPLC [87, 88].

1.5.2 Ionic Liquids in Capillary Electrophoresis

In capillary electrophoresis, ILs are used to form a covalent bond capillary surface coating [89] or as BGE additive in CZE [90], MEKC [91], MEEKC [92], and NACE [93]. Due to a dynamical coating of the capillary surface, a reduction or even reversal of the EOF is possible by the addition of ILs to a BGE [94]. Furthermore, the adsorption of IL cations to the silanol groups of the capillary surface leads to a repulsion of positively charged analytes, which makes the application of ILs advantageous for the separation of basic compounds [95, 96], such as phenethylamines. Besides the adsorption of IL cations to the capillary surface, interactions between IL ions and analytes, and between IL ions and other BGE additives are feasible. In CE enantioseparations chiral ILs are applied as sole chiral selector [97, 98] or to achieve a synergistic effect between ILs and commonly used chiral selectors [99, 100].

1.6 Design of Experiments

Statistical methods, like design of experiments (DoE), are used to increase the efficiency of experiments. An extensively planned, conducted, analyzed, and interpreted DoE approach may help saving time and resources, and additionally, may give a lot of information about relationships

between factors and response. Compared with the commonly used approach of holding certain factors constant and altering the levels a single factor, in DoE the levels of different factors are changed simultaneously. By the application of an e. g. three-level full-factorial design investigating two factors, the response of every combination of two factors and three factor levels is investigated [101]. After statistical analysis the factor levels yielding an optimum response are obtained by DoE.

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2 Aim of the Work

In recent years pharmaceutical manufacturers are increasingly interested in the marketing of enantiopure drugs. Furthermore, according to a Food and Drug Administration (FDA) statement, enantiomers of a compound should be treated as different drugs. Additionally, the isomeric composition of a racemic drug and the impurity limits for isomeric compounds in an enantiopure drug have to be specified. Thus, increasing interest is drawn towards enantioselective analytical methods, such as capillary electrophoresis.

The application of ionic liquids as background electrolyte additives in capillary electrophoresis enantioseparations was reported frequently. Nevertheless, until now, the influence of the IL addition to BGEs on the separation of drug enantiomers was not investigated systematically. Conceivable reasons for enhanced chiral separations are interactions between IL ions and analytes and/or chiral selectors, and an influence of ILs on the electroosmotic flow.

In particular, the following influences of tetrabutylammonium based ILs on CE enantioseparations of ephedrine derivatives were to be investigated:

- The influence of ILs on the solubility of cyclodextrins.
- The influence of the adsorption of TBA^+ cations to the silanol groups of the capillary surface on the migration time, on the chiral separation, and on the reproducibility of CE runs.
- The efficacy of different CE rinsing steps on the desorption of TBA^+ cations from the capillary surface.
- The influence of altering the pH value, the buffer concentration, the chiral selector concentration, and the IL concentration on the separation of phenethylamine enantiomers in IL containing BGEs.
- The influence of different anions combined with TBA^+ cations on the enantioseparation.
- The interaction between IL ions and cyclodextrins.

Furthermore, considering the influence of different complex formation constants and different electrophoretic mobilities of temporarily formed analyte CD complexes on the enantioseparation, the complexes between phenethylamine enantiomers and different cyclodextrins were to be investigated by affinity capillary electrophoresis and isothermal titration calorimetry.

3 Results

3.1 Characterization of Complexes Between Phenethylamine Enantiomers and β-Cyclodextrin Derivatives by Capillary Electrophoresis – Determination of Binding Constants and Complex Mobilities

Joachim Wahl, Takayuki Furuishi, Etsuo Yonemochi, Lorenz Meinel, Ulrike Holzgrabe

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Electrophoresis, 38 (2017), 1188-1200

Abstract

To optimize chiral separation conditions and to improve the knowledge of enantioseparation it is important to know the binding constants K between analytes and cyclodextrins and the electrophoretic mobilities of the temporarily formed analyte cyclodextrin complexes. K values for complexes between 8 phenethylamine enantiomers, namely ephedrine, pseudoephedrine, methylephedrine, and norephedrine, and 4 different β-cyclodextrin derivatives were determined by affinity capillary electrophoresis. The binding constants were calculated from the electrophoretic mobility values of the phenethylamine enantiomers at increasing concentrations of cyclodextrins in running buffer. Three different linear plotting methods (x-reciprocal, y-reciprocal, double reciprocal) and nonlinear regression were used for the determination of binding constants with β-cyclodextrin, (2-hydroxypropyl)-β-cyclodextrin, methyl-β-cyclodextrin, and 6-O-α-maltosyl-β-cyclodextrin. The cyclodextrin concentration in a 50 mM phosphate buffer pH 3.0 was varied from 0-12 mM. To investigate the influence of the binding constant values on the enantioseparation the observed electrophoretic selectivities were compared with the obtained K values and the calculated enantiomer cyclodextrin complex mobilities. The different electrophoretic mobilities of the temporarily formed complexes were crucial factors for the migration order and enantioseparation of ephedrine derivatives. To verify the apparent binding constants determined by capillary electrophoresis, a titration process using ephedrine enantiomers and β-cyclodextrin was carried out. Furthermore, the isothermal titration calorimetry measurements gave information about the thermal properties of the complexes.

3.1.1 Introduction

The enantiomers of a chiral drug can have different pharmacodynamic, pharmacokinetic, and toxicological properties [1-3]. Thus, especially new drugs are launched as pure enantiomers. However, the isomeric purity has to be proven by means of chiral chromatography or capillary electrophoresis. In comparison to chiral HPLC methods, capillary electrophoresis (CE) has a lot of advantages including high resolution power, low solvent consumption, low costs, and being easy to perform. Therefore, CE is often used for the chiral separation of drugs and pharmaceutical products.

Phenethylamines, like ephedrine, pseudoephedrine, methylephedrine, and norephedrine are active components extracted from ephedrae herba [4]. They are used as ingredients of e. g. cold medicines and abused as starting material for the synthesis of methamphetamine. Hence, they can be found as impurities in this stimulant [5]. The enantioseparation of these compounds has been studied by different groups [5-11]. Nevertheless, to the best of our knowledge, the determination of binding constants between phenethylamines and cyclodextrins has only been reported for (2,6-di-O-methyl)-β-cyclodextrin complexes [12].

Cyclodextrins (CDs) are frequently used as chiral selectors for enantioseparation. Their application as chiral agent in CE is reviewed in [13]. The toroidal form of CDs, with a hydrophilic exterior whereas the interior acts like a hydrophobic cavity [14], makes them interesting as a host for the solubilization of hydrophobic substances. Adding different functional groups on the CD ring may change the solubility, the size of the cavity, the hydrophobicity, and the flexibility of the chiral selector. The structures of all analytes and CDs used herein are displayed in Figure 1.

The binding constant is used to describe the equilibrium between bound and unbound analyte in a solution containing a ligand. A lot of different techniques have been employed for the determination of binding constants between several analytes and CDs. NMR [15, 16], electroanalytical techniques [17], UV-visible and fluorescence spectrometry [18-20], circular dichroism [20, 21], membrane permeation [22], and thermal analysis [23] were utilized for this purpose.

For the examination of binding constants by CE, methods based on the change in sample mobility at increasing ligand concentrations were used [24-28]. Affinity capillary electrophoresis (ACE) was utilized for the determination of interactions between drugs and CDs and polymers of CDs [29]. Binding constants between enantiomers and CDs were determined for dipeptides [30, 31], vinca alkaloids [32], tadalafil [33], tapentadol [34], nucleoside phosphonates [35], and other analytes as reviewed in [36]. Assuming a 1:1 complexation, the

binding constant is calculated from the change in effective mobility an analyte gets at increasing CD concentrations. The assumption of a 1:1 stoichiometry of uncharged CDs and phenethylamines was corroborated by previous studies using UV spectroscopy [7].

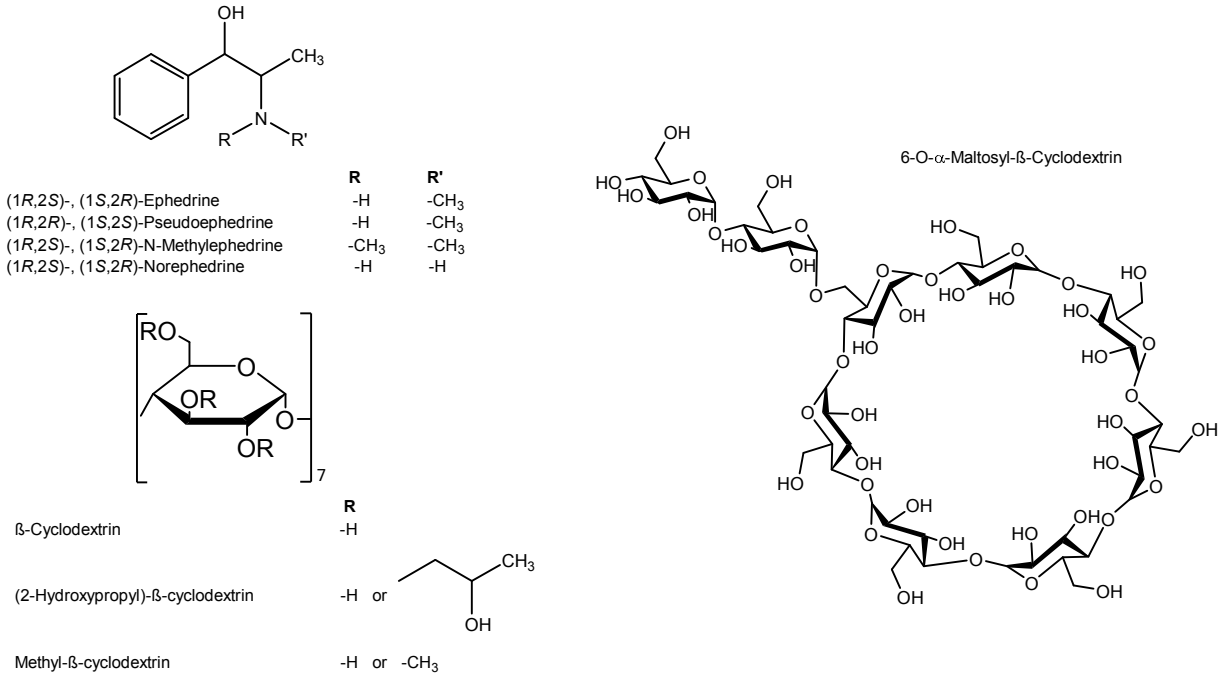

Figure 1 Structures of phenethylamines and cyclodextrins used in this study

The binding constants for 8 phenethylamine enantiomers and 4 β-CD derivatives were determined by ACE using three different linear plotting methods (namely x-reciprocal, y-reciprocal, and double reciprocal). Furthermore, a nonlinear regression method was used for the calculation of the K values. The results obtained by nonlinear regression were compared to those calculated by linear regression. Nonlinear regression was reported to yield more precision and accuracy in determination of binding constants than linearized methods [37, 38]. The linear regression methods may alter the weight of the observed results by changing the space between the measured data points during linearization [38]. If the observed electrophoretic mobility data are not properly weighted the double reciprocal plotting method may mask deviations from linearity. Furthermore, the difference in the relative uncertainties of the x- and y-variables before and after transformation for the linear plotting may yield incorrect results [28]. A problem of the x-reciprocal plot is the use of the dependent variable on both the x- and the y-axes [28]. However, we performed both the linear and the nonlinear regressions and results were compared across these models.

Furthermore, to improve the knowledge about enantioseparation in CE, the correlation between the calculated binding constants and the observed enantioseparation was investigated. Using CDs as chiral selector in CE, there are two basic principles that explain the separation of chiral compounds. The first reason is a difference between the two binding constants of the CD and the analyte enantiomers. Due to different K values the enantiomers stay a different period of time in the CD cavity leading to the separation. The second principle for enantioseparation is a difference in the mobilities of the two temporarily formed diastereomeric complexes. The latter principle was confirmed regarding the migration order of amino acids and peptides under conditions near the pK_a value [30, 39]. Other studies reported that the enantioseparation of phenylalanine derivatives using negatively charged CDs is based on mobility differences of the enantiomer-CD-complexes [40]. A theoretical background on the concept of enantioseparation by differences in complex mobilities is given by different authors [41-43]. The aim of this work is to study the influence of binding constants and of electrophoretic mobilities of analyte-CD-complexes on the migration order and on the enantioseparation of phenethylamines.

It is worth mentioning, that the binding constants determined by ACE and discussed in this publication are apparent binding constants differing from the thermodynamic binding constants. To compare the apparent and the thermodynamic K values of these complexes, isothermal titiration calorimetry (ITC) was carried out, to assess binding constants, reaction enthalpies, and entropies of the cyclodextrin inclusion complexes.

3.1.2 Materials and Methods

3.1.2.1 Reagents and Chemicals

(1*S*,2*R*)-(+)-ephedrine hemihydrate, (1*S*,2*S*)-(+)-pseudoephedrine, (1*R*,2*R*)-(-)-pseudoephedrine, (1*S*,2*R*)-(+)-methylephedrine, (1*R*,2*S*)-(-)-methylephedrine, (1*S*,2*R*)-(+)-norephedrine, (1*R*,2*S*)-(-)-norephedrine, phosphoric acid, dimethyl sulfoxide (DMSO), (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) (average M_r ~ 1460; ~ 0.8 mol 2-hydroxypropyl per sugar unit), and methyl-β-cyclodextrin (Me-β-CD) (M_r ~ 1310; 1.6 - 2.0 mol CH_3 per sugar unit) were purchased from Sigma Aldrich (Schnelldorf, Germany), while (1*R*,2*S*)-(-)-ephedrine hydrochloride was from Caelo (Hilden, Germany). 0.1 mol/L NaOH, 0.1 mol/L HCl, and sodium dihydrogen phosphate monohydrate were acquired from VWR (Darmstadt, Germany), and β-cyclodextrin (β-CD) from Wacker (München, Germany). 6-O-α-Maltosyl-β-cyclodextrin (Mal-β-CD) was a gift from Prof. Dr. Tomohiro Endo (Hoshi University, Tokyo). All chemicals and solvents were of at least analytical grade. Ultra-pure water

was delivered by a “Milli-Q Synthesis” water purification system (Merck Millipore, Schwalbach, Germany).

3.1.2.2 Capillary Electrophoresis

3.1.2.2.1 Apparatus and Methods

The CE separations were performed by means of a Beckman P/ACE System MDQ (Beckman Coulter, Fullerton, USA), equipped with a diode array detector. The uncoated fused-silica capillaries (BGB Analytik, Schloßböckelheim, Germany) employed for the enantioseparation were of 50 µm internal diameter and had an effective length of 50.0 cm (total length of 62.0 cm). A new capillary was conditioned by rinsing for 30 min with 0.1 mol/L NaOH, 2 min with H_2O, 10 min with 0.1 mol/L HCl, and 2 min with H_2O. Before each run the capillary was rinsed for 1 min with H_2O, for 30 min with 0.1 mol/L NaOH, 1 min with H_2O, and 5 min with the running buffer. All rinsing steps were performed with a pressure of 206,843 Pa (≙ 30.0 psi). The samples were injected with a pressure of 3,447 Pa (≙ 0.5 psi) for 5.0 s at the anodic side of the capillary. The capillary was kept at 25 °C and a voltage of +20 kV (current approximately 30 µA) was applied. The samples were detected at 194 nm. All measurements were performed 5 times per CD concentration.

For the determination of the buffer viscosity DMSO was pumped through the capillary with a pressure of 5,516 Pa (≙ 0.8 psi) while the capillary contained background electrolytes with different CD concentrations (0, 2, 4, 6, 8, 10, and 12 mM). The viscosity of the background electrolyte was calculated using the Hagen-Poiseuille law. The velocity of DMSO was calculated from the time needed from the beginning of the capillary to the detection window. Based on this constant velocity the time from the beginning to the end of the capillary was calculated. All viscosity measurements were performed 6 times.

3.1.2.2.2 Preparation of Solutions

A 50 mM phosphate buffer pH 3.0 was prepared by adding a 50 mM H_3PO_4 solution to a 6.9 g/L solution of sodium dihydrogen phosphate monohydrate until the pH was 3.0. Binding constants were determined by dissolving increasing amounts of CD into this phosphate buffer. Solutions with a concentration of 0, 2, 4, 6, 8, 10, and 12 mM CD were prepared in this way. Racemic phenethylamine solutions were prepared by dissolving 5.0 mg of each enantiomer in 10 mL de-ionized water (concentration 0.5 mg/mL). For the determination of the peak order one of the enantiomers was spiked to the racemic solution. In case of an insufficient resolution between two enantiomers the CD concentration was increased, the single enantiomers have been injected

solely, and the peak order was determined from the migration times of at least 3 runs. To determine the strength of the electroosmotic flow 0.25 vol% DMSO (neutral marker) were added to the sample solutions. All solutions were filtered through a 0.2 µm cellulose acetate syringe filter (VWR Darmstadt, Germany) prior to use.

3.1.2.3 Isothermal Titration Calorimetry

3.1.2.3.1 Apparatus and Methods

The parameters of interaction between ephedrine enantiomers and β-CD were determined using a Microcal iTC 200 instrument (GE Healthcare, Buckinghamshire, Great Britain) at 298 K. Each titration was carried out as 20 successive injections of 2 µL of a 14 mM β-CD solution in a 50 mM phosphate buffer pH 3.0 into the reaction calorimetric cell containing 200 µL of a 1 mM ephedrine enantiomer solution in the same buffer. A first injection of 0.4 µL was discarded to eliminate diffusion effects of the material from the syringe to the calorimetric cell. An interval of 150 s was used, which was sufficient to achieve equilibrium and signal return to the baseline, stirring was kept at 750 rpm. The blank values were determined by injecting the 14 mM β-CD solution into phosphate buffer.

3.1.2.4 Theory and Calculation

3.1.2.4.1 Capillary Electrophoresis

Binding constants are calculated from ACE by means of the change in electrophoretic mobility of an enantiomer when a CD is added to the running buffer in increasing concentrations [24]. The K values were determined using a constant concentration of phenethylamine enantiomers whereas the concentration of the CDs was increased. To minimize the error in the calculated binding constants, the fraction of analyte in complexed form has to be between 0.2 and 0.8 [37]. For this reason and considering the solubility of the CDs, the CD concentration was varied from 0 to 12 mM.

The apparent mobility (μ_{app}) of the phenethylamine enantiomers was calculated using the total length of the capillary (L), the distance between capillary inlet and detection window (l), the applied voltage (V), and the migration time (t) as follows:

$$\mu_{app} = \frac{l \cdot L}{t \cdot V} \qquad (1)$$

Since the running buffer's viscosity increased by adding CD, the apparent mobilities were corrected by multiplying with the ratio of this viscosity increase.

$$\mu_{app(corrected)} = \mu_{app(uncorrected)} \cdot \frac{\eta_c}{\eta_0} \quad (2)$$

where η_c is the viscosity of the electrolyte containing CD and η_0 the viscosity without CD. The binding constants were determined using the electrophoretic mobility (μ_i), which is the apparent mobility adjusted by the mobility of the electroosmotic flow (μ_{eof}).

$$\mu_i = \mu_{app} - \mu_{eof} \quad (3)$$

For the determination of binding constants by CE three requirements need to be met. First, the mobilities of the sole analyte and of the complexed analyte have to be different. Second, the formation of the equilibrium between free analyte and analyte-CD-complex has to be fast. And third a 1:1 complexation between the analyte and the CD has to be present. As mentioned before, previous investigations verified 1:1 complexations between phenethylamines and uncharged CDs by means of UV spectroscopy measurements using a method introduced by Job [7]. 1:1 complexes are described by the following equation [24]:

$$K[CD] = \frac{[PCD]}{[P] \cdot [CD]} \quad (4)$$

Transforming the above mentioned equations leads to Eq. (5), which was used for the determination of K and μ_c by nonlinear regression.

$$\mu_i = \frac{\mu_f + \mu_c \cdot K[CD]}{1 + K[CD]} \quad (5)$$

Where μ_i is the electrophoretic mobility of the analyte, which is composed of the mobility of the free analyte μ_f and the mobility of the complexed analyte μ_c. Nonlinear regression was carried out using OriginPro 2016 (OriginLab Corporation, Northampton, MA, USA).

For linear regression Eq. (5) was transformed into three different linear equations. These equations, the plotting methods, and the methods for the determination of the binding constants K and the mobility of the temporarily formed analyte-CD-complex μ_c are shown in Table 1. For further details on the calculation of binding constants using ACE the publications of Rundlett and Armstrong are recommended [28, 44].

Table 1 Linearized plotting methods for the determination of binding constants in ACE [24, 44]

Method	*x-reciprocal*	*y-reciprocal*	*double reciprocal*
Linear equation	$\frac{(\mu_i - \mu_f)}{[CD]} = -K \cdot (\mu_i - \mu_f) + K \cdot (\mu_c - \mu_f)$	$\frac{[CD]}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f)} \cdot [CD] + \frac{1}{(\mu_c - \mu_f) \cdot K}$	$\frac{1}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f) \cdot K} \cdot \frac{1}{[CD]} + \frac{1}{(\mu_c - \mu_f)}$
Plotting method	$\frac{(\mu_i - \mu_f)}{[CD]}$ vs. $(\mu_i - \mu_f)$	$\frac{[CD]}{(\mu_i - \mu_f)}$ vs. $[CD]$	$\frac{1}{(\mu_i - \mu_f)}$ vs. $\frac{1}{[CD]}$
K	$-\text{slope}$	$\frac{\text{slope}}{\text{intercept}}$	$\frac{\text{intercept}}{\text{slope}}$
$\mu_c - \mu_f$	$-\frac{\text{intercept}}{\text{slope}}$	$\frac{1}{\text{slope}}$	$\frac{1}{\text{intercept}}$

3.1.2.4.2 Isothermal Titration Calorimetry

The thermodynamic properties were calculated after subtraction of the blank using the “one binding side” model. We assumed (i) that the complexes are characterized by low Wiseman parameters c value, (ii) a 1:1 stoichiometry, and (iii) that the number n of available sites per CD molecule is one [45].

The raw data was analyzed using Microcal Origin 7.0 for ITC leading to the standard enthalpic variation (ΔH_0), the binding constant (K), the standard Gibbs free energy (ΔG_0), and the standard entropy variation ($T\Delta S_0$).

3.1.3 Results and Discussion

The separation of phenethylamine enantiomers by capillary electrophoresis was investigated using different CD derivatives [7-9, 46, 47]. These studies showed an enhancement of the enantioseparation by using a diacetylated β-CD derivative instead of native β-CD. The best enantioseparation was observed using negatively charged β-CD derivatives, like *heptakis*(2,3-*O*-diacetyl-6-sulfo)β-CD (HDAS) [8, 9, 47]. NMR measurements revealed a penetration of the phenethylamine phenyl ring into the CD cavity while the side chain interacts with the wider rim of the CD [6, 8, 46]. The negatively charged HDAS showed an ion-ion interaction at the narrow CD rim [8, 48]. The results from ACE are in accordance to the previously reported observations made by NMR measurements.

3.1.3.1 Capillary Electrophoresis

ACE was used for further investigations of phenethylamine-CD-complexes. By increasing the concentration of the four CDs a decrease in effective mobility of all 8 phenethylamine enantiomers was observed. Figure 2 displays the electropherograms for the enantioseparation of

pseudoephedrine at increasing concentrations of MaI-β-CD. As can be seen, the migration times and the resolution between the two enantiomers increased by increasing the CD concentration. Furthermore, the electroosmotic flow decreased due to an increase of background electrolyte viscosity.

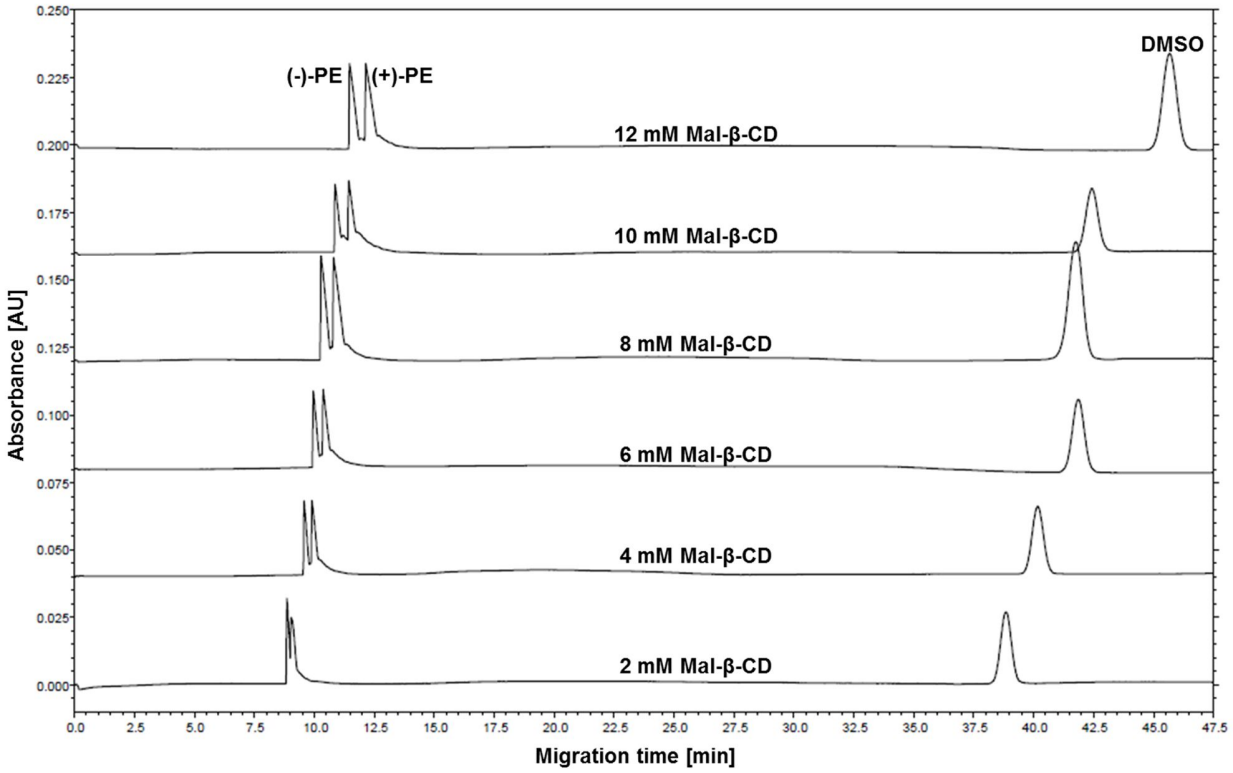

Figure 2 Electropherograms showing the separation of pseudoephedrine (PE) enantiomers at increasing concentrations of MaI-β-CD. Separation conditions: 50 mM phosphate buffer, pH 3.0; voltage: +20 kV; capillary temperature: 25 °C; detection wavelength: 194 nM; fused silica capillary (62.0/50.0 cm, 50 µm I. D.); sample concentration: 0.5 mg/mL; EOF-marker: 0.25 vol% DMSO

Regarding all CDs, the best enantioseparation was always achieved for pseudoephedrine (see Table 2). For ephedrine and methylephedrine only a poor enantioseparation was observed utilizing the four investigated CDs. The separation of norephedrine enantiomers was only possible using Me-β-CD, no separation was achieved with the other three CDs. Nevertheless, the determination of different binding constants for all separated and partial separated enantiomers was feasible. A long rinsing step with a 0.1 M NaOH solution was necessary to achieve a fast and reproducible electroosmotic flow. The rinsing procedure was crucial for the determination of K values because shorter rinsing led to a high variation of the DMSO migration times and therefore to high variations of the calculated electrophoretic mobilities (see Eq. (3)). However, due to peak

broadening, this rinsing procedure led to poor chiral resolution compared to methods optimizing the enantioseparation of ephedrine derivatives [7-9, 59]. Of note, the herein developed methods were optimized for the determination of binding constants and not for the enantioseparation of phenethylamines. Nevertheless, the adsorption of the ephedrine derivatives to the capillary surface might lower the mobility of the analytes and therefore lead to an overestimation of the K values. The performed ITC measurements showed that the K values obtained by ACE are comparable to those determined thermodynamically, indicating no or only a small influence of the capillary surface adsorption on the K value determination.

Table 2 Highest electrophoretic selectivities α, differences in binding constants ΔK, differences in enantiomer-CD-complex mobilities $\Delta\mu_c$, and the from K and $\Delta\mu_c$ backward calculated differences in electrophoretic mobility $\Delta\mu_i$ for complexes between phenethylamines and β-CD derivatives (K, ΔK, $\Delta\mu_c$, and $\Delta\mu_i$ calculated from nonlinear regression)

		β-CD	HP-β-CD	Me-β-CD	MaI-β-CD
Ephedrine	α	1.010	1.006	1.004	1.011
	ΔK [M^{-1}]	1.5	16.7	4.7	2.6
	$\Delta\mu_c$ [$\cdot 10^{-10} m^2V^{-1}s^{-1}$]	3.23	14.27	4.46	7.36
	$\Delta K / \bar{K}$	0.04	0.25	0.05	0.03
	$\Delta\mu_i$ [$\cdot 10^{-10} m^2V^{-1}s^{-1}$]	3.41	13.80	4.38	7.24
Pseudoephedrine	α	1.051	1.062	1.080	1.051
	ΔK [M^{-1}]	33.7	2.5	17.5	28.2
	$\Delta\mu_c$ [$\cdot 10^{-10} m^2V^{-1}s^{-1}$]	14.43	47.30	24.26	51.34
	$\Delta K / \bar{K}$	0.31	0.04	0.29	0.68
	$\Delta\mu_i$ [$\cdot 10^{-10} m^2V^{-1}s^{-1}$]	14.62	46.55	24.37	46.60
Methylephedrine	α	1.011	1.007	1.005	1.012
	ΔK [M^{-1}]	2.2	18.5	22.2	1.0
	$\Delta\mu_c$ [$\cdot 10^{-10} m^2V^{-1}s^{-1}$]	6.76	18.39	4.64	4.93
	$\Delta K / \bar{K}$	0.02	0.16	0.13	0.04
	$\Delta\mu_i$ [$\cdot 10^{-10} m^2V^{-1}s^{-1}$]	6.67	18.14	4.57	4.96
Norephedrine	α	1.000	1.000	1.012	1.000
	ΔK [M^{-1}]	0	0	76.6	0
	$\Delta\mu_c$ [$\cdot 10^{-10} m^2V^{-1}s^{-1}$]	0	0	6.43	0
	$\Delta K / \bar{K}$	0	0	0.28	0
	$\Delta\mu_i$ [$\cdot 10^{-10} m^2V^{-1}s^{-1}$]	0	0	6.37	0

3.1.3.1.1 Determination of Binding Constants K by CE

The binding constants K of 8 phenethylamine enantiomers with 4 different CDs determined here are summarized in Table 3. Values for K have been calculated in cases of baseline or partial enantioseparation. K was determined from three linear graphical plotting methods and from a

nonlinear regression method. Plotting the decreasing electrophoretic mobilities of the phenethylamine enantiomers against the increasing concentration of the CDs led to linear correlations for all three methods. K values were calculated according to the equations given in Table 1.

The coefficients of determination for the binding constants were at least 0.993 (see Table 3). This supports the notion of a 1:1 complexation, because higher order equilibria would lead to a deviation from linearity using the x-reciprocal plotting method [49]. As can be seen from Table 3, the calculated binding constants were very similar for all three linear plotting methods and no deviation from the results obtained by nonlinear regression was observed. Nevertheless, because nonlinear regression is leading to more accurate and precise results, the following findings are discussed considering the values calculated by nonlinear regression.

The calculated binding constants varied over approximately one order of magnitude between 27.4 M^{-1} and 310.5 M^{-1}. Szökő et al. [12] investigated the complex formation between phenethylamines and (2,6-di-O-methyl)-β-cyclodextrin (K values between 43.8 M^{-1} for norephedrine and 77.5 M^{-1} for pseudoephedrine). Furthermore, the influence of the substitution pattern of methylated CDs was investigated using amphetamine derivatives [50]. In both papers the binding constants were in the same order of magnitude (K between 20 M^{-1} and 650 M^{-1}) compared to the results reported here.

Unfortunately, it was not possible to determine a binding constant for the norephedrine-Mal-β-CD-complex, because the analysis of all regression models led to a negative K value, most likely due to a weak association between analyte and CD, leading to a very low fraction of complexed norephedrine [37].

Furthermore, the measurement of (-)-pseudoephedrine at a concentration of 2 mM Mal-β-CD was excluded from the linear and nonlinear regression models. For linear regression models a nonlinear deviation of the plots was observed at this concentration. This can be explained by a saturation of the complexing ligand at low concentrations [51]. To simplify the calculation of binding constants the total concentration of CD added to the buffer is taken for the free equilibrium concentration of CD (see Eq. (4)). This approach is only acceptable when the total CD concentration is much higher than the phenethylamine concentration. Furthermore, using similar concentrations gives a little difference between μ_i and μ_f, leading to a high variation of the term $(\mu_i-\mu_f)$ [52]. This problem was observed for the determination of the binding constant between Mal-β-CD and (-)-pseudoephedrine with a relatively weak interaction (K = 27.4 M^{-1}). Of note, for

Table 3 Apparent binding constants between phenethylamine enantiomers and CDs calculated by linear and nonlinear regression

β-CD

	(+)-Ephedrine		(-)-Ephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	33.7	0.9982	35.7	0.9984
y-reciprocal	33.8	0.9987	35.7	0.9990
double reciprocal	33.0	1.0000	36.3	1.0000
nonlinear	34.2	1.0000	35.7	1.0000

	(+)-Pseudoephedrine		(-)-Pseudoephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	121.9	0.9963	95.9	0.9979
y-reciprocal	124.7	0.9990	94.9	0.9991
double reciprocal	117.9	0.9996	98.1	0.9999
nonlinear	127.2	0.9998	93.5	0.9999

	(+)-Methylephedrine		(-)-Methylephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	100.1	0.9962	90.9	0.9960
y-reciprocal	98.7	0.9989	92.4	0.9988
double reciprocal	103.9	0.9996	87.4	0.9997
nonlinear	96.8	0.9999	94.6	0.9999

	Norephedrine	
	K [M^{-1}]	R^2
x-reciprocal	34.7	0.9972
y-reciprocal	34.7	0.9981
double reciprocal	33.9	1.0000
nonlinear	34.9	0.9999

HP-β-CD

	(+)-Ephedrine		(-)-Ephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	60.5	0.9986	74.3	0.9971
y-reciprocal	60.0	0.9994	74.8	0.9987
double reciprocal	62.1	0.9999	72.7	0.9999
nonlinear	58.9	1.0000	75.7	0.9998

	(+)-Pseudoephedrine		(-)-Pseudoephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	58.6	0.9954	52.9	0.9963
y-reciprocal	58.5	0.9969	53.6	0.9978
double reciprocal	58.5	0.9999	51.7	0.9999
nonlinear	57.9	0.9997	55.4	0.9999

	(+)-Methylephedrine		(-)-Methylephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	124.1	0.9980	105.4	0.9966
y-reciprocal	123.5	0.9992	105.1	0.9986
double reciprocal	123.4	0.9998	106.2	0.9998
nonlinear	123.4	0.9998	104.9	0.9997

	Norephedrine	
	K [M^{-1}]	R^2
x-reciprocal	50.0	0.9956
y-reciprocal	50.7	0.9971
double reciprocal	48.6	0.9999
nonlinear	52.4	0.9998

Table 3 *Continued*

Me-β-CD

	(+)-Ephedrine		(-)-Ephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	89.4	0.9942	101.7	0.9944
y-reciprocal	91.4	0.9975	100.3	0.9976
double reciprocal	87.7	0.9998	102.6	0.9997
nonlinear	93.9	0.9996	98.6	0.9995

	(+)-Pseudoephedrine		(-)-Pseudoephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	73.7	0.9940	48.6	0.9937
y-reciprocal	72.3	0.9973	49.4	0.9966
double reciprocal	77.7	0.9997	46.1	0.9998
nonlinear	69.3	0.9998	51.8	0.9999

	(+)-Methylephedrine		(-)-Methylephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	164.9	0.9981	183.9	0.9974
y-reciprocal	163.5	0.9995	184.5	0.9993
double reciprocal	165.4	0.9998	182.5	0.9997
nonlinear	163.2	0.9998	185.4	0.9997

	(+)-Norephedrine		(-)-Norephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	227.6	0.9969	310.2	0.9987
y-reciprocal	233.4	0.9995	308.7	0.9998
double reciprocal	223.1	0.9994	309.5	0.9997
nonlinear	233.9	0.9997	310.5	0.9999

Mal-β-CD

	(+)-Ephedrine		(-)-Ephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	75.8	0.9935	87.1	0.9933
y-reciprocal	77.6	0.9975	85.7	0.9979
double reciprocal	71.7	0.9996	92.0	0.9994
nonlinear	80.7	0.9998	83.3	0.9998

	(+)-Pseudoephedrine		(-)-Pseudoephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	57.9	0.9954	26.6	0.9949
y-reciprocal	57.3	0.9974	26.8	0.9962
double reciprocal	58.4	0.9999	26.1	0.9999
nonlinear	55.6	0.9998	27.4	0.9999

	(+)-Methylephedrine		(-)-Methylephedrine	
	K [M^{-1}]	R^2	K [M^{-1}]	R^2
x-reciprocal	45.2	0.9957	43.0	0.9959
y-reciprocal	44.8	0.9976	43.4	0.9979
double reciprocal	47.6	0.9999	41.0	0.9999
nonlinear	43.0	0.9999	44.6	1.0000

binding constants higher than 30 M^{-1} (β-CD complexes with ephedrine and norephedrine enantiomers) a deviation from the linear relationship was not observed.

In summary, it can be seen that binding constants for (-)-ephedrine with all four CDs were higher compared to the (+)-enantiomer. The same observation was made for pseudoephedrine where the (+)-enantiomer formed stronger complexes with all CDs. Interestingly, (+)-methylephedrine had higher K values in the β-CD-, HP-β-CD- and Mal-β-CD-complexes whereas for Me-β-CD the binding constant with (-)-methylephedrine was higher.

3.1.3.2 Determination of Binding Constants K by ITC

ITC measurements were carried out at 25 °C to assess interactions between ephedrine enantiomers and β-CD (see Table 4). The binding constants determined by ITC were in good agreement with the results obtained by ACE. Several mechanisms were proposed for the interaction of guests with CDs. Van der Waals force and hydrophobic interaction between the guest molecule and the CD cavity were those among several possible noncovalent interactions (e. g. electrostatic interaction and hydrogen bonding) providing substantial contributions for the complexation of organic guests within cyclodextrins [53]. The enthalpy during inclusion complex formation was exothermic and -5.33 $kJ \cdot mol^{-1}$ for (+)-ephedrine and -16.95 $kJ \cdot mol^{-1}$ for (-)-ephedrine. The negative enthalpy likely reflects guest interaction with β-CD through weak supramolecular forces indicating the formation of host-guest inclusion complexes [54, 55].

Table 4 Thermodynamic parameters for the complexation of ephedrine enantiomers with β-CD determined by ITC

	K [M^{-1}]	ΔH [$kJ \cdot mol^{-1}$]	TΔS [$kJ \cdot mol^{-1}$]	ΔG [$kJ \cdot mol^{-1}$]
(+)-Ephedrine	33.5	-5.33	3.38	-8.70
(-)-Ephedrine	37.4	-16.95	-7.98	-8.98

The results revealed that the complexation of (-)-ephedrine with β-CD is more exothermic compared to the complexation of (+)-ephedrine with β-CD. Negative ΔH and ΔS values have been reported in analogous systems [56, 57]. Upon formation of an inclusion complex between (-)-ephedrine and β-CD, both the β-CD molecules and the (-)-ephedrine molecules lose their degree of freedom of motion and translational entropy. Thus, the (-)-ephedrine-β-CD-complex system in aqueous medium becomes less random, which contributes to the negative entropy change ($\Delta S < 0$) [58]. Due to the stronger van der Waals interaction between (-)-ephedrine and β-CD induced by the good size/shape fit between host and guest [59], the formation of the

(-)-ephedrine complex with β-CD releases more heat than that of (+)-ephedrine. Moreover, the positive value of entropic effects probably results from driving polar water molecules from the cavities of cyclodextrins by (+)-ephedrine molecules present in the solution [60]. The standard Gibbs free energies were negative (ΔG = -8.70 kJ·mol^{-1} for (+)-ephedrine and -8.98 kJ·mol^{-1} for (-)-ephedrine) indicating that both ephedrine-enantiomer-β-CD-interactions in aqueous solution were spontaneous at ambient conditions.

3.1.3.3 Influence of the Binding Constants and the Differences in Electrophoretic Mobility on the Migration Order and the Enantioseparation

In general, there are two principles explaining the separation of enantiomers using chiral discriminators. The first conceptual model is that different binding constants between two enantiomers and a CD are responsible for the enantioseparation. The second principle explains enantioseparation by different mobilities of the temporarily formed complexes between analytes and CD. The background of these two principles was investigated here regarding the migration order and the separation of phenethylamine enantiomers.

The electrophoretic mobilities μ_c of the enantiomer-CD-complexes were calculated by nonlinear and linear regression using the equations displayed in Table 1. With only a few exceptions a good accordance was found for the complex mobilitiy values determined by the different regression methods (see Table 5). The μ_c values calculated by nonlinear regression are used for the following considerations.

3.1.3.3.1 Influence of the Binding Constants and the Differences in Electrophoretic Mobility on the Migration Order

While the calculated electrophoretic complex mobility values μ_c (Table 5) were in accordance with the enantiomer migration order, no correlation between the values of the binding constants and the migration order was found. Theoretically, the enantiomer with the lower K value should have a shorter time in the CD cavity. Furthermore, the larger complexes between CD and ephedrine derivative migrate slower than the uncomplexed analyte. The complexes of pseudoephedrine enantiomers with all CDs, the β-CD-ephedrine-complexes, and the Mal-β-CD-methylephedrine-complexes were in line with this theoretical approach. On the other hand, for the complexes between ephedrine enantiomers and HP-β-CD, Me-β-CD, and Mal-β-CD, for methylephedrine-complexes with β-CD, HP-β-CD, and Me-β-CD, and for the complexes between norephedrine and Me-β-CD, the enantiomer with the higher binding constant migrated faster. These deviations were assigned to differences in the mobility of the CD-analyte-complexes.

Faster migrating enantiomer-CD-complexes with higher binding constants have been reported before e. g. for tadalafil [33].

In summary, the migration order indicated that the mobilities of the temporarily formed enantiomer-CD-complexes were crucial for enantioseparation.

3.1.3.3.2 Influence of the Binding Constants and the Differences in Electrophoretic Mobility on the Enantioseparation

Due to overlapping peaks, a calculation of R_S values for poorly separated peaks was impossible. Hence the electrophoretic selectivity α was calculated as the ratio of the effective mobilities (μ_1, μ_2) of the enantiomers:

$$\alpha = \frac{\mu_1}{\mu_2} \qquad (6)$$

The calculated selectivities for the phenethylamine enantiomers are displayed in Table 2.

As mentioned above, both the difference in interaction strength between phenethylamine enantiomers and a CD and the difference in enantiomer-CD-complex mobilities may impact the chiral resolution of the substances [42, 43]. Considering these theoretical approaches the correlation between the enantioseparation and different calculated parameters was investigated.

With all CDs the best enantioseparation was observed for pseudoephedrine. The value of the calculated binding constants did not correlate with the enantioseparation performance. Looking at the separation of pseudoephedrine, the two enantiomers had the strongest interaction with β-CD compared to the other ephedrine derivatives separated with this CD, whereas the Mal-β-CD-pseudoephedrine-complex was the weakest of the phenethylamine-Mal-β-CD-complexes. Therefore, we conclude that the separation of the enantiomers was independent from the strength of the enantiomer-CD-complex.

The difference between the K values of two enantiomers ΔK seems to be the more predicting factor for the enantioseparation quality than the value of the binding constants. In case of β-CD and Mal-β-CD pseudoephedrine had by far the highest difference (see Table 2). In contrast, the highest difference between the binding constants of two enantiomers was observed for norephedrine and Me-β-CD showing only a poor chiral separation. It is worth mentioning that for these complexes very high K values were calculated. Hence, ΔK was divided by the arithmetic average $\bar{K}$ calculated from the two K values of the corresponding enantiomers. The obtained

Table 5 Electrophoretic mobilities μ_c of enantiomer-CD-complexes and their differences $\Delta\mu_c$

β-CD

	μ_c [$m^2V^{-1}s^{-1}$]		$\Delta\mu_c$ [$m^2V^{-1}s^{-1}$]
	(+)-Ephedrine	(-)-Ephedrine	
x-reciprocal	4.765E-08	4.768E-08	3.477E-11
y-reciprocal	4.760E-08	4.769E-08	9.009E-11
double rec.	4.808E-08	4.737E-08	7.081E-10
nonlinear	1.406E-09	1.083E-09	3.230E-10
	(+)-Pseudoephedrine	(-)-Pseudoephedrine	
x-reciprocal	3.710E-08	3.621E-08	8.900E-10
y-reciprocal	3.694E-08	3.628E-08	6.523E-10
double rec.	3.735E-08	3.604E-08	1.309E-09
nonlinear	1.220E-08	1.364E-08	1.443E-09
	(+)-Methylephedrine	(-)-Methylephedrine	
x-reciprocal	3.414E-08	3.531E-08	1.172E-09
y-reciprocal	3.422E-08	3.515E-08	9.316E-10
double rec.	3.388E-08	3.557E-08	1.689E-09
nonlinear	1.297E-08	1.229E-08	6.762E-10
	Norephedrine		
x-reciprocal	4.840E-08		
y-reciprocal	4.837E-08		
double rec.	4.884E-08		
nonlinear	1.078E-09		

HP-β-CD

	μ_c [$m^2V^{-1}s^{-1}$]		$\Delta\mu_c$ [$m^2V^{-1}s^{-1}$]
	(+)-Ephedrine	(-)-Ephedrine	
x-reciprocal	3.207E-08	3.084E-08	1.231E-09
y-reciprocal	3.210E-08	3.082E-08	1.286E-09
double rec.	3.254E-08	3.095E-08	1.591E-09
nonlinear	1.640E-08	1.782E-08	1.427E-09
	(+)-Pseudoephedrine	(-)-Pseudoephedrine	
x-reciprocal	3.705E-08	3.265E-08	4.402E-09
y-reciprocal	3.706E-08	3.258E-08	4.478E-09
double rec.	3.705E-08	3.279E-08	4.259E-09
nonlinear	1.210E-08	1.683E-08	4.730E-09
	(+)-Methylephedrine	(-)-Methylephedrine	
x-reciprocal	2.918E-08	2.990E-08	7.184E-10
y-reciprocal	2.919E-08	2.990E-08	7.084E-10
double rec.	2.920E-08	2.986E-08	6.691E-10
nonlinear	1.867E-08	1.683E-08	1.839E-09
	Norephedrine		
x-reciprocal	3.073E-08		
y-reciprocal	3.067E-08		
double rec.	3.088E-08		
nonlinear	1.814E-08		

Table 5 *Continued*

Me-β-CD	μ_c [$m^2V^{-1}s^{-1}$]		$\Delta\mu_c$ [$m^2V^{-1}s^{-1}$]
	(+)-Ephedrine	(-)-Ephedrine	
x-reciprocal	3.015E-08	2.940E-08	7.437E-10
y-reciprocal	3.005E-08	2.945E-08	6.003E-10
double rec.	3.024E-08	2.937E-08	8.717E-10
nonlinear	1.627E-08	1.672E-08	4.456E-10
	(+)-Pseudoephedrine	(-)-Pseudoephedrine	
x-reciprocal	3.523E-08	3.371E-08	1.521E-09
y-reciprocal	3.538E-08	3.359E-08	1.792E-09
double rec.	3.481E-08	3.416E-08	6.490E-10
nonlinear	1.133E-08	1.376E-08	2.426E-09
	(+)-Methylephedrine	(-)-Methylephedrine	
x-reciprocal	2.891E-08	2.849E-08	4.223E-10
y-reciprocal	2.893E-08	2.848E-08	4.519E-10
double rec.	2.890E-08	2.851E-08	3.896E-10
nonlinear	1.758E-08	1.805E-08	4.638E-10
	(+)-Norephedrine	(-)-Norephedrine	
x-reciprocal	2.694E-08	2.627E-08	6.723E-10
y-reciprocal	2.690E-08	2.628E-08	6.282E-10
double rec.	2.697E-08	2.627E-08	6.987E-10
nonlinear	1.987E-08	2.051E-08	6.427E-10

Mal-β-CD	μ_c [$m^2V^{-1}s^{-1}$]		$\Delta\mu_c$ [$m^2V^{-1}s^{-1}$]
	(+)-Ephedrine	(-)-Ephedrine	
x-reciprocal	3.272E-08	3.139E-08	1.330E-09
y-reciprocal	3.257E-08	3.148E-08	1.088E-09
double rec.	3.310E-08	3.110E-08	1.996E-09
nonlinear	1.387E-08	1.461E-08	7.361E-10
	(+)-Pseudoephedrine	(-)-Pseudoephedrine	
x-reciprocal	3.993E-08	4.608E-08	6.151E-09
y-reciprocal	4.004E-08	4.594E-08	5.901E-09
double rec.	3.982E-08	4.639E-08	6.577E-09
nonlinear	6.755E-09	1.621E-09	5.134E-09
	(+)-Methylephedrine	(-)-Methylephedrine	
x-reciprocal	3.891E-08	4.044E-08	1.522E-09
y-reciprocal	3.904E-08	4.033E-08	1.287E-09
double rec.	3.830E-08	4.112E-08	2.820E-09
nonlinear	7.028E-09	6.535E-09	4.933E-10

values are listed in Table 2. With the exception of the HP-β-CD-complexes the values calculated this way are in good accordance with the observed enantioseparations. A possible reason for the deviation using HP-β-CD might be the unequal degree and position of substitution of the hydroxypropyl side chains, making it difficult to determine the binding constants for this CD derivative.

Furthermore, the influence of different enantiomer-CD-complex mobilities on the separation of phenethylamine enantiomers was investigated. A very good correlation between the effective electrophoretic selectivities and the differences in complex mobilities was found (Table 2). The $\Delta\mu_c$ values for the pseudoephedrine enantiomers were an order of magnitude higher compared to those of the other phenethylamines. Obviously, a larger difference in complex mobilities was leading to an increase in enantioseparation. Regarding a single CD system, a very good correlation between the observed electrophoretic selectivities and the differences in complex mobilities was found.

To further investigate the influence of binding constants on the enantioseparation, a previously published approach, predicting the selectivity of an enantioseparation, was used [61]. According to this, electrophoretic mobilities μ_i were calculated backward from the determined K and $\Delta\mu_c$ values by means of Eq. (5). The differences $\Delta\mu_i$ between the calculated enantiomer mobilities were determined and are displayed in Table 2. No benefit for the prediction of enantioseparation was found regarding $\Delta\mu_i$ compared to $\Delta\mu_c$.

In summary, a correlation between the electrophoretic selectivity and the mobility of the temporarily formed diastereomeric complexes was found. Although no correlation between electrophoretic selectivity and the calculated binding constants was found, the differences in K values might additionally influence the enantioseparation. It is conceivable that, an overlay of both principles may lead to synergistic effects or work against each other. The possibility of CE to separate enantiomers with identical or similar binding constants to a chiral selector due to different complex mobilities was reported before and a summary of examples can be found in [41]. The different mobilities can be explained by differences in size, shape, solvability, and pK_a-value of the temporarily formed analyte-CD-complexes [39, 62].

3.1.4 Concluding Remarks

The binding constants between four β-CD derivatives and 8 phenethylamine enantiomers were determined using three different linear and a nonlinear regression method. The K values determined by ACE were in good agreement to those obtained thermodynamically. Furthermore,

the electrophoretic mobilities of the temporarily formed enantiomer-CD-complexes were determined by ACE.

We demonstrated the role of differences in enantiomer-CD-complex mobilities for the separation of phenethylamine enantiomers. Furthermore, the migration order of the enantiomers was linked to the mobilities of the temporarily formed analyte-CD-complexes. A strong correlation between the observed electrophoretic selectivities of the phenethylamine enantiomers and the differences in complex mobilities was found, whereas the comparison of the electrophoretic selectivities and the calculated K values showed no direct correlation. Hence, the separation of phenethylamine enantiomers was not driven by complex strength rather than by the different mobilities of the temporarily formed complexes.

Acknowledgement

The authors would like to thank the German Academic Exchange Service (DAAD Project No. 57058983) for the financial support of this research, Prof. Dr. Tomohiro Endo (Hoshi University, Tokyo) for the gift of Mal-β-CD and Cecilia Amstalden for supporting the ITC measurements.

Conflict of interest statement

None of the authors of this paper does have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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3.2 Ionic Liquids in Capillary Electrophoresis

Ulrike Holzgrabe and Joachim Wahl

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Methods in Molecular Biology, 1483 (2016), 131-153

Abstract

Recently, a great interest was drawn towards ionic liquids (ILs) in analytical separation techniques. ILs possess many properties making them excellent additives in capillary electrophoresis (CE) background electrolytes (BGE). The most important property is the charge of the dissolved ions in BGE enabling the cations to interact with deprotonated silanol groups on the capillary surface and thereby modifying the electroosmotic flow (EOF). Ionic and/or proton donor-acceptor interactions between analyte and IL are possible interactions facilitating new kinds of separation mechanisms in CE. Further advantages of ILs are the high conductivity, the environmentally friendliness and the good solubility for organic and inorganic compounds. The most commonly used ILs in capillary electrophoresis are dialkylimidazolium-based ILs, whereas for enantioseparation a lot of innovative chiral cations and anions were investigated.

ILs are reported to be additives to a normal CE background electrolyte or the sole electrolyte in CE, nonaqueous CE (NACE), micellar electrokinetic chromatography (MEKC), and in enantioseparation. An overview of applications and separation mechanisms reported in the literature is given here, in addition to the enantioseparation of pseudoephedrine using tetrabutylammonium chloride (TBAC) as IL additive to an ammonium formate buffer containing β-cyclodextrin (β-CD).

3.2.1 Introduction

Ionic liquids are defined as (semi-)organic salts with a melting point below 100 °C. The ionic bond of a salt is stronger than the van-der-Waals forces in normal solids and liquids. For that reason, salts are usually solid and melt at higher temperatures than other solids. The low melting point of the ILs is explained by a lower degree of symmetry of cation and/or anion, by high conformational freedom and an effective charge delocalization [1, 2]. On the other hand, the high Coulombic forces between the ions in ILs lead to lower vapor pressures compared with molecular liquids of a similar molecular weight [3]. Katritzky et al. assumed in 2002 that there is the possibility to form 10^{18} cation/anion combinations building an ionic liquid [4]. More specifically, a room temperature ionic liquid (RTIL) is a salt with a melting point below ambient temperature. The first RTIL, ethylammonium nitrate (melting point: 14 °C), was described in 1914 by Walden [5]. In most cases, ILs consist of a nitrogen-containing organic cation and an inorganic or less frequently organic anion. The anion is often fluorinated because in this case the negative charge weakens the hydrogen bond to the cation and as a result lowers the melting point [6]. The structural formulas of widely used ILs are shown in Figure 1. Other frequently used anions are halides, hydroxide, sulfate, acetate, and nitrate.

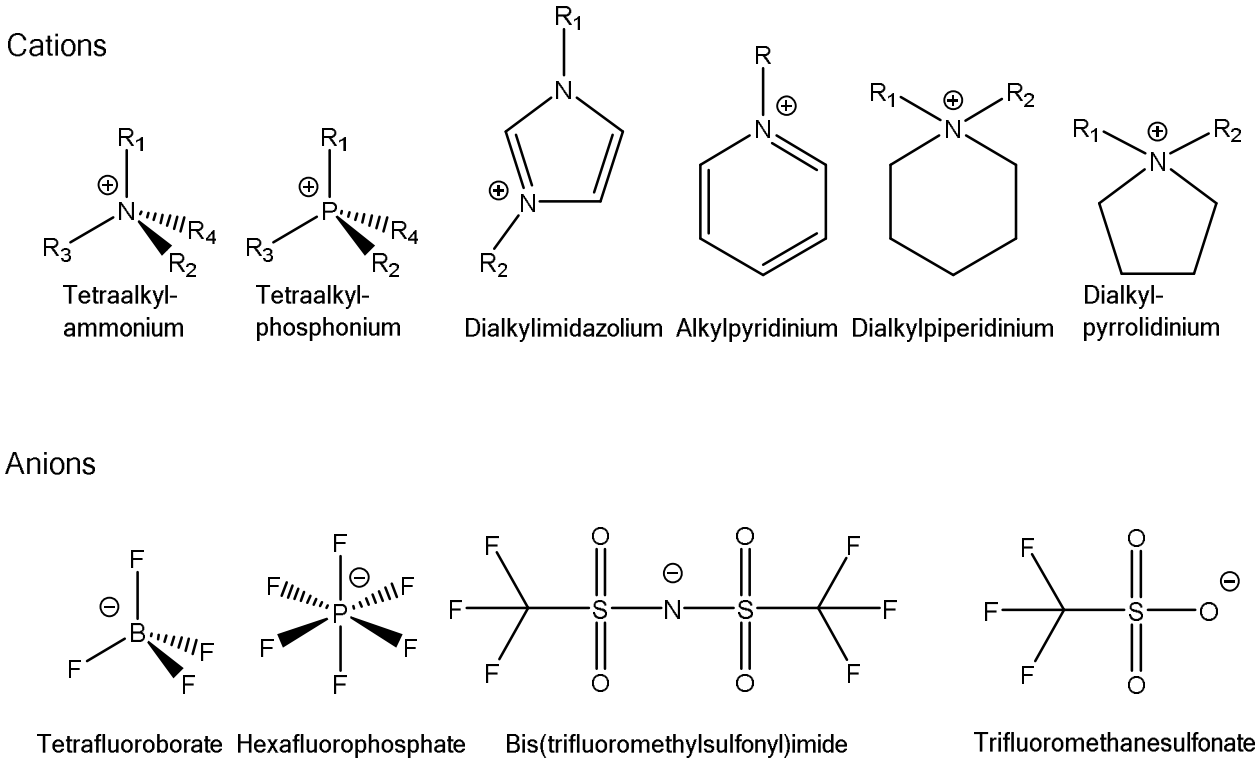

Figure 1 Structures of widely used cations and anions in ionic liquids

In the past few years the interest in ionic liquids increased. One special advantage of ILs is that they can be designed as requested. It is possible to modify the melting point, the viscosity, the

miscibility, and the electrochemical behavior by altering the combination of cations and anions. In most cases, the choice of the anion determines the solubility in water. Water-immiscible ILs often contain PF_6^- or bis(trifluoromethylsulfonyl)imide anions, whereas BF_4^- provides a high water-miscibility. Other advantages are a high thermal stability, a negligible vapor pressure, and therefore an ecological friendliness. Furthermore, they are little or inflammable and can be designed to be protic or aprotic solvents. Some physicochemical properties can be explained by the structure of the cation or the anion. It is also known that a change in cation anion combination can influence the physicochemical properties strongly. For example, the hydrophobicity, density, viscosity, surface tension, and solubility are characteristics depending on the chain length of alkyl substituents of the IL cation. It has to be mentioned that impurities in ILs can affect their physicochemical properties. For example, it was observed that halide impurities can narrow the potential windows because they can be easier oxidized than other IL anions [7].

In the last few years, ILs get an increasing relevance as reaction media and catalyst in organic and inorganic synthesis, for example, in peptide and oligosaccharide synthesis [8, 9]. Considerable advantages are, for example, efficient transfer of microwaves, excellent heat transfer, increased reactivity, and easy product recovery. In biochemical reactions ILs can improve the activity, stability, and enantioselectivity of enzymes. ILs are applied in photoelectrochemical solar cells, as electrolytes in rechargeable lithium-ion batteries, in double-layer capacitors, and in fuel cells. ILs can be used to enhance solubility of poor water-soluble pharmaceutical drugs to improve their bioavailability [10]. Furthermore, ILs can be applied for extraction purpose, for example, in liquid-liquid extraction, liquid-phase microextraction, and solid-phase microextraction.

3.2.2 Ionic Liquids in Chromatography

ILs can also be used in analytical chemistry. In gas chromatography, ILs make it possible to separate complex mixtures of polar and nonpolar compounds [11, 12]. With their thermal stability, wetting ability, high viscosity, and their controllable solvation interactions they are an ideal stationary phase. The thermal stability of ILs depends on many factors, the stability of anions, for example, increases by ascending charge delocalization [12]. For this reason, a lot of anions contain fluorine atoms. Tsunashima et al. reported a higher stability for benzyl-substituted phosphonium ionic liquids in comparison to the analogous ammonium ionic liquids [13]. Even chiral ionic liquid stationary phases are reported in gas chromatography [14]. The first commercially available ionic liquid GC column, based on 1,9-di(3-vinylimidazolium)nonane bis(trifluoromethylsulfonyl)imide, was launched in 2008.

ILs are employed as additives in HPLC mobile phases to suppress interactions between basic analytes and free silanol groups on silica-based reversed phases. Better peak shapes, improved resolution, and shorter retention times can be achieved by addition of ILs to the mobile phase without

having an influence on the pH, which is in contrast to observation made for triethylamine. ILs as additives to the mobile phase can also affect the analyte retention mechanism through interactions with both stationary and/or mobile phase. However, one important disadvantage of ILs used in HPLC is their high viscosity leading to unfavorable high back pressure [15].

Columns with covalent IL coatings, such as butylimidazolium bromide, are reported too [16]. Due to their low vapor pressure, leading to low column bleeding, ILs are employed as column material in mass spectrometry. Thereby, a better identification and quantification of samples is achieved.

RTILs, based on α-cyano-4-hydroxycinamic and sinapic acid anions, are tested as a new class of matrix in MALDI-MS, because they are non-volatile in vacuum, they adsorb laser light, and they dissolve samples even more homogeneous then solid matrices [17]. Because of the higher homogeneity, the results for quantification and determination of molecular weight in MALDI-MS are improved. Even in an ion chromatography-ion association electrospray ionization mass spectrometry (IC/IA-ESI-MS) method for determination of perchlorate ILs showed an advantage [18].

3.2.3 Ionic Liquids in Capillary Electrophoresis

With regard to ILs in capillary electrophoresis, it has to be noticed that they may not be used directly as solvent because their high viscosity and high conductivity is leading to high currents and high Joule heating. In BGE solutions they do not exist as an IL, but as dissolved cations and anions. Therefore, ILs in CE should better be called solutions of ionic liquids. In capillary electrophoresis, ILs could be used as main electrolyte, as electrolyte additive, and for dynamic coating of the capillary surface. ILs have a lot of advantages making them excellent additives in CE background electrolytes, e. g. high solubility, heat stability, good electrical conductivity, and remarkable influence on the EOF.

The application of ILs in capillary electrophoresis had its origin in the 1980s by using surfactants like cetyltrimethylammonium bromide (CTAB) and tetradecyltrimethylammonium bromide (TTAB) to control the electroosmotic flow in CZE [19, 20]. Using these surfactants a change of the EOF direction can be achieved which enables the separation of low molecular weight carboxylic acids. Furthermore, it was shown that a reduction of EOF leads to better separation of analytes of nearly similar mobilities. Based on observations in liquid chromatography, Garner and Yeung, reported an electrochromatography by dynamic ion exchange using CTAB [21]. They performed a CZE with a polyimide coated capillary on which the CTAB adsorbed and formed a double layer. This double layer leads to a direction change of the electroosmotic flow because the stationary phase surface gets charged positively. Beside the coulombic interactions between the negative charged analytes and this positive charged double layer, a small amount of an ion pair between the analytes and CTAB can be formed, which builds hydrophobic interactions with the coated stationary phase [22]. Expectedly, for neutral analytes the reversed electroosmotic mobility in hydrophobic coated capillaries increased by an increasing concentration of CTAB in buffer due to the higher positive

charge on capillary surface. The increase of mobility achieves a maximum when the hydrophobic surface is saturated with CTAB [23].

It is important to note that CTAB and TTAB are no ionic liquids, because their melting points are above 100 °C, but this studies can be considered as a precursor for studying ionic liquids in CE. Furthermore, separation mechanisms and electrophoretic behavior of analytes are comparable between ILs and these surfactants.

3.2.3.1 Ionic Liquid Coated Capillaries

In 2000, the first capillary with a covalent bond IL was reported [24]. The covalent coating with (dialkyl-)imidazolium reversed the EOF. In contrast to bare fused silica, an increase of the buffer pH value can reduce the velocity of the anodic EOF because a higher amount of noncoated silanol groups gets charged negative. The enhanced migration time leads to an increase in resolution of several analytes like carboxylic acids and sildenafil and its metabolite [24, 25]. The positively charged (dialkyl-)imidazolium cations can act as a kind of anion exchanger for buffer and analyte anions and therefore a further separation system is implemented in the capillary electrophoresis system. A covalent coating of silanol groups with IL cations has few advantages like a repulsion of positively charged analytes (e. g. sildenafil, inorganic cations [26], DNA [27]), a stable EOF, coating without deterioration for up to 96 h [27], and the compatibility with MS detection.

3.2.3.2 Ionic Liquids in Capillary Electrophoresis

The first real ILs used for aqueous capillary electrophoresis separation were 1-alkyl-3-methylimidazolium based. In 2001, Yanes et al. separated different phenolic compounds in grape seed extracts using an IL as only background electrolyte [28]. The direction of the EOF was observed to be towards the anode because the imidazolium cations coated the capillary wall silanol groups leading to a positive surface charge. It was found that the polyphenols associate either with the free or the coated imidazolium cations, because uncharged polyphenols migrated after the EOF. A comparison between the change in EOF and the change in polyphenol mobility showed that the interaction with free imidazolium dominates. With increasing IL concentrations the EOF leveled off, the effective electrophoretic mobility ($\mu_{catechin} - \mu_{EOF}$) of catechin increased even above this plateau. The stability of the EOF at high IL concentrations indicates a saturation of the capillary wall with adsorbed IL. These separations were improved for a lot of different compounds in the last few years. Interestingly, not only the migration time gets shorter by increasing IL concentration, even the separation between the peaks gets better. By changing the alkyl group in the cation from ethyl to butyl a better separation could be achieved because longer migration times lead to more interactions with imidazolium cations. Interestingly, an increasing chain length in cathodic detection mode, using same IL concentrations, also causes a decrease in EOF [29-31]. Summarizing it can be seen, that

in both cathodic and anodic detection mode, a longer alkyl chain leads to a decrease in EOF, longer migration times and better separation but also to poorer peak shape. Effects of the alkyl chain length on EOF are shown in Table 1.

When using low IL concentrations in combination with a traditional buffer system, the EOF is directed towards the cathode. An increase in IL concentration leads to a decrease of the EOF velocity. Higher migration times often lead to improved resolutions, due to a higher number of interactions between analyte and the separation system. Further increasing the IL concentration resulted in higher migration time, and occurring of peak tailing decreases the resolution. The EOF can decrease thus far, that a better separation of negative charged analytes can be achieved in reversed mode (shorter migration times, better peak shape) compared to methods without IL addition. To this end inorganic or small organic anions with extremely high electrophoretic mobilities, like nicotinic acid and its isomers, can be separated using ILs [32]. Because of their high electrophoretic mobility, these anions cannot be detected at the cathode in normal mode. In this reversed mode (EOF still cathodic) an increasing IL concentration leads to a decrease in migration time but an increase in resolution and peak shape. Depending on the type of IL a further increase of IL concentration can lead to a change in EOF direction, e. g. for the separation of benzoic acid and chlorophenoxy acid herbicides [33], and anions can be separated in a coelectroosmotic mode at the anodic capillary end [34, 35]. Interestingly, this change in direction is reversed again by raising the pH-value, because the silanol groups get deprotonated [33]. Effects of IL concentration and BGE pH on the EOF are also shown in Table 1 and Figure 2.

Table 1 Effects of IL concentration, pH, and alkyl chain length of the IL cation on the EOF

EOF direction	IL concentration ↑	pH ↑	Alkyl-chain length ↑
Cathodic	EOF ↓ (inversion possible)	EOF ↑	EOF ↓
Anodic	EOF ↑ to plateau	EOF ↓ (inversion possible)	EOF ↓

The separation of inorganic cations can also be improved by addition of ILs to the BGE. Because of their weak UV activity these cations have to be detected in indirect mode using cationic chromophors (protonated form). For that reason, separation and detection of inorganic cations is limited to low pH values. By covalent coating the capillary with an imidazolium-based IL and using this IL as BGE additive a constant mobility of inorganic cations can be achieved in a pH range from 3 to 11 [26].

For the separation of anionic analytes, such as aromatic acids, and basic proteins an additional improvement of separation can be achieved by using a polymeric ionic liquid (poly(1-vinyl-3-butylimidazolium bromide)) for dynamical capillary coating because it reduces the problematic

adsorption of analytes to the capillary wall. The authors hypothesized a higher rate of capillary surface coverage by polymeric ILs compared to IL monomers [36-38].

First studies on interaction mechanisms between IL and analytes were conducted by Yue and Shi [39] who used different 1-alkyl-3-methylimidazolium cations to separate flavonoids. They observed that the H-2 of the imidazolium cation is essential for a hydrogen bonding interaction with the analytes. This observation was proved by a 2-methylated imidazolium cation showing no separation and was confirmed by other studies [40]. When 1-butyl-2-methyl-3-methylimidazolium tetrafluoroborate was added to the BGE no resolution of aryl propionic acids could be achieved. They also reported, that the counterion plays a role in separation. By exchanging counterions (BF_4^-, PF_6^-, Br^-, I^-) differences in migration time, separation, and peak shape can be observed. Interestingly, the resolution increases with a decreasing melting point of the ILs. This phenomenon can be explained by weaker interactions (hydrogen bonds) between IL cation and IL anion leading to a higher amount of free cation that may interact with capillary surface and analyte. The same observation was made in NACE where weaker association between the ions led to a higher current because of an increasing capillary coating [41]. Cabovska et al. investigated the interaction between halophenols and 1-ethyl-3-methylimidazolium cations and found a larger hydrophobic surface of the analyte to elevate the affinity [42].

Using ILs in combination with classical buffers often leads to poor baseline stability. This can be explained by high UV absorption of imidazolium and pyridinium cations. Better results can be achieved by using contactless conductivity detection or electrochemiluminescence (ECL) [43-46]. By addition of ILs into buffer, the ECL intensity increases because of the enhanced conductivity of the BGE, which makes, in comparison to BGE without IL, the electrical resistance of the sample solution passing the detector much higher. Alkylimidazolium based ILs can be used as chromophors in indirect UV detection, for example, in separation of carbohydrates and inorganic cations both lacking a chromophore [30].

An ideal application for ILs in capillary electrophoresis is the separation of basic compounds, because the ILs suppress the adsorption of these analytes to the capillary surface which results in a better separation efficiency and repeatability [47]. This beneficial effect can even be observed in a BGE containing no IL when the capillary was dynamical coated by rinsing with IL prior to the separation [48].

Associations between ILs and analytes can theoretically be a result of hydrogen bonding, hydrophobic bonding, ion-ion, ion-dipole or ion-induced-dipole interactions. The associations between IL cations and analytes seem to be more specific and important for separation in comparison to associations between IL anions and analytes. It goes without saying that the choice

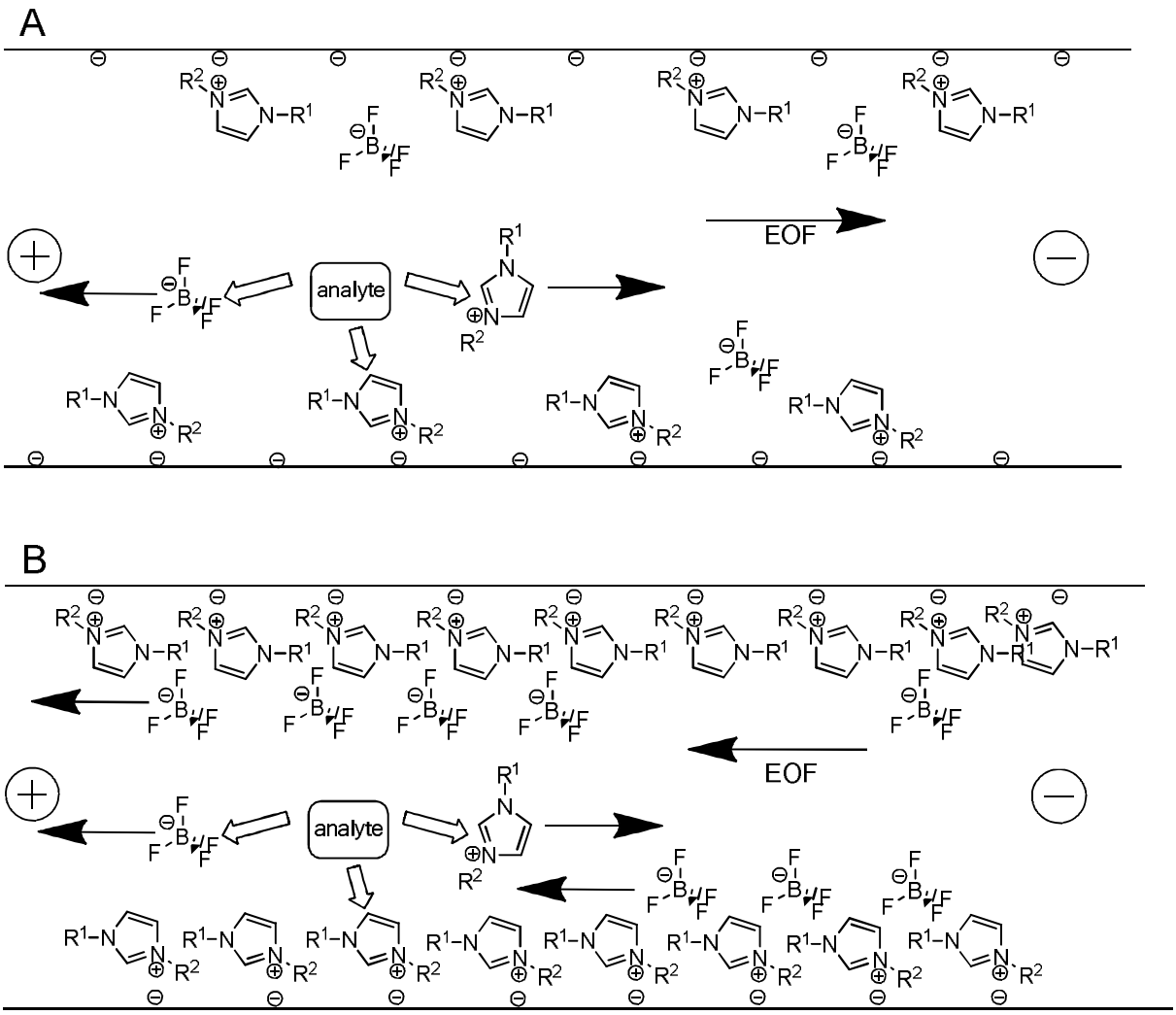

Figure 2 Possible interactions between analyte and IL cations (coated or in BGE) and IL anions. Due to the better capillary surface coating and therefore higher positive surface charge and due to the higher amount of anions migrating to the anode, increasing concentrations of ILs reverse the direction of the EOF.
A: low IL concentration → cathodic EOF; B: high IL concentration → anodic EOF

of the IL cation plays an important role due to its association with the analyte and furthermore to its capillary wall coating effect. Analyte molecules can associate with IL molecules coated on capillary surface and/or IL molecules in bulk solution. Possible interactions of an analyte molecule with the IL and the influence of the IL concentration on the EOF are displayed in Figure 2.

3.2.3.3 Ionic Liquids in Micellar Electrokinetic Chromatography (MEKC) and Microemulsion Electrokinetic Chromatography (MEEKC)

In 2003, the first MEKC method using ILs was reported [49]. By using chiral poly(sodium oleyl-L-leucylvalinate) (poly-SOLV) as surfactant the first enantioseparation of several binaphthyl derivatives in IL modified capillary electrophoresis was reported, too. An increase in cathodic EOF was reported at low concentrations of 1-alkyl-3-methylimidazolium ILs. This can be explained by an

increase of current. However, generally by further increasing the IL concentration in MEKC a decrease in cathodic EOF can be observed as result of capillary coating. No general statement can be made whether ILs are leading to better or worse separation in MEKC. The next step was made by Rizvi and Shamsi who, for the first time, used an amino acid derived cationic IL as chiral selector [50]. They synthesized undecenoxycarbonyl-L-leucinol bromide and undecenoxycarbonyl-L-pyrrolidinol bromide for the application in a MEKC enantioseparation of bromophenylacetate and 2-(2-chlorophenoxy)propanoate anions (structures see Figure 3). The application of these ILs reversed the EOF (cathode to anode) and, by changing the pH value, an electrostatic interaction between IL cations and analyte anions was found. At low pH values the two anionic analytes mentioned earlier are not separated because the protonated acids cannot interact electrostatically with the IL cations. Similarly, the analysis of uncharged aryl-propionic acids in anodic detection mode did not provide any enantioseparation by using this chiral IL as single chiral selector [51]. However, enantioseparation of the acidic analytes was achieved after the addition of a cyclodextrin (CD) in cathodic detection mode. An overview over ILs, additives, and analytes is given in Table 2.

Tian et al. achieved a great improvement in separation and resolution of poorly water-soluble lignans using 1-butyl-3-methylimidazolium tetrafluoroborate and sodium dodecyl sulfate (SDS) [52]. Theoretically, it seems obvious that the imidazolium cations are electrostatically and hydrophobically attracted by the negative charged SDS micelle exterior, which neutralizes the micelle surface. Thereby, the repulsion between the negatively charged hydrophilic “SDS-heads” is reduced leading to a change in charge, shape, and size of micelles and a decrease of the critical micelle concentration. It was reported that lignans could be resolved from other compounds in real samples at low IL and surfactant concentrations.

In 2008, Borissova et al. reported a new long-chain alkylimidazolium IL that acts simultaneously as IL and micelle former [53]. They showed that these alkylimidazolium ILs build micelles like other surfactants and that the CMC decreases with increasing length of hydrophobic tail. Furthermore, they found not only separation of neutral hydrophobic benzene derivatives, due to analyte micelle interaction, but also of phenols, due to electrostatic analyte IL interaction as mentioned earlier.

In 2010, Cao et al. reported a MEEKC method using the hydrophobic IL 1-butyl-3-methylimidazolium hexafluorophosphate instead of oil to form an IL/W microemulsion by addition of SDS [54]. For the separation of phenolic acids they observed a decrease in EOF by addition of the IL, too. The use of IL/W microemulsions introduces some new separation mechanisms compared to O/W microemulsions. There are possible associations between analyte and coated IL and IL in emulsion droplets by hydrogen bonding and Coulombic force.

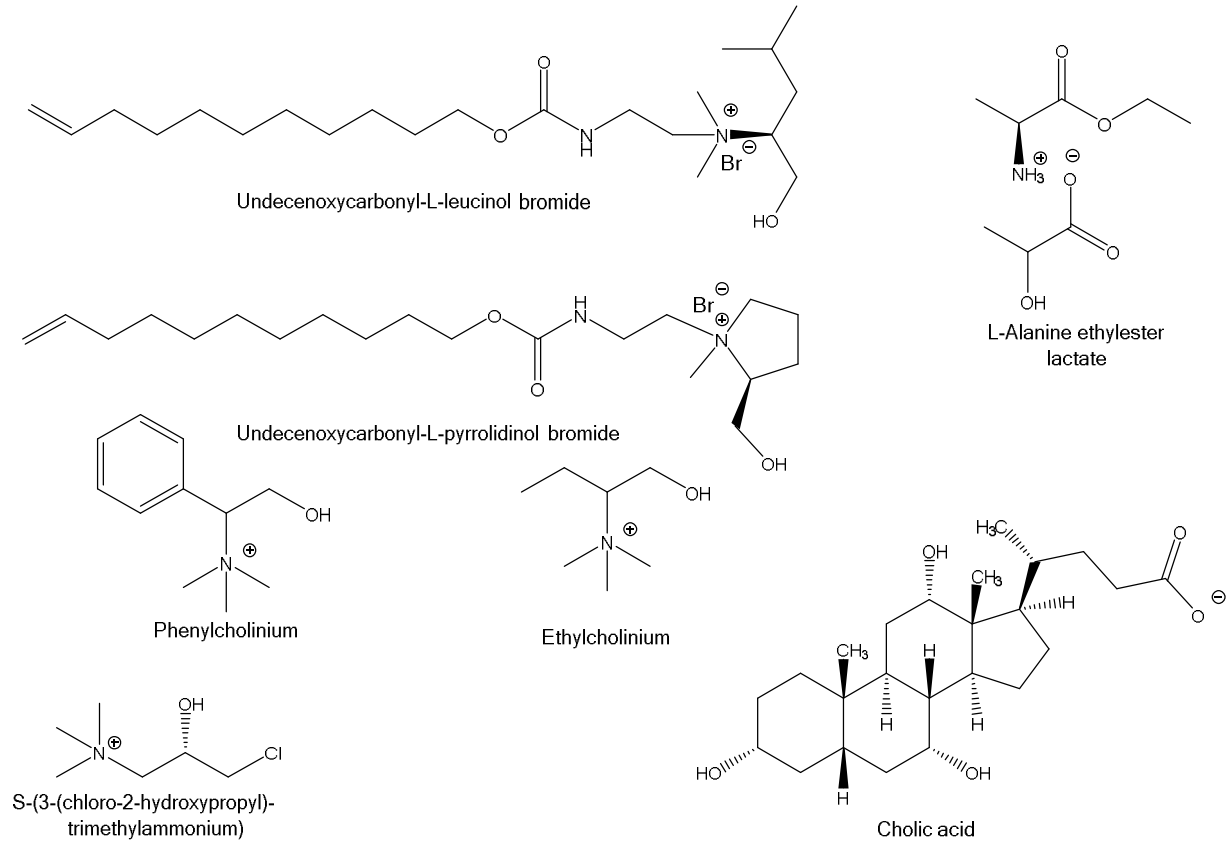

Figure 3 Structures of reported chiral IL cations and anions

3.2.3.4 Ionic Liquids in Capillary Electrophoresis - Enantioseparation

As mentioned before, the first MEKC enantioseparations using ILs as additive in BGE were performed by Mwongela et al. [49, 55]. They investigated the resolution of several enantiomers (shown in Table 2) in a BGE consisting of chiral poly-SOLV micelles and added 1-alkyl-3-methylimidazolium cations to the solute. The enantioselectivity can be either achieved by a chiral selector, like CD, or by the chiral IL itself. Francois et al. investigated the enantioselective power of choline derived ILs (see Figure 3). No enantioseparation of 2-arylpropionic acids was found when these ILs were the sole chiral additives to BGE, but the addition of cyclodextrins revealed a synergistic effect between the two chiral selectors [56]. However, not only synergistic effects between ILs and CDs are possible. Mofaddel et al. reported detrimental interactions between the two additives leading to a decrease in resolution of binaphthyl derivatives enantiomers [57]. Tran and Mejac observed that ibuprofen enantiomers can be separated in a BGE consisting of a chiral IL cation *S*-(3-(chloro-2-hydroxypropyl)trimethylammonium) and chiral cholic acid, but neither with the acid nor with the IL cation solely added to water [58]. Structures of the chiral selectors are shown in Figure 3. This observation shows that there can be cooperative interactions between two chiral selectors, the chiral cation and anion, and the analyte. Salbutamol, cimaterol, and formoterol cannot

be enantioseparated by using β-CD in phosphate buffer only, but by addition of several ammonium, imidazolium and pyridinium based ILs [59].

Taken together, it can be seen that interactions between ILs and CDs in enantioseparation can be synergistic, neutral, or antagonistic. Of note, the synergistic effects in the IL chiral selector system result in optimal separation by much lower concentrations of chiral selectors, like β-cyclodextrin. An antagonistic effect can be explained by the inclusion of the IL ions in the CD cavity in a kind of competitive inhibition. A lot of interactions in enantioseparation using ILs seem to be possible. Beside the inclusion of the enantiomers into the cavity of CDs, the IL or an IL analyte complex can also be included in this cavity. The analytes might associate with IL cations either coated on capillary wall or free in BGE. Furthermore, associations with the negative counterions are possible. Additionally, it seems to be possible that the analyte CD inclusion complex or analyte IL CD inclusion complex can interact with IL ions. For example, Wang et al. measured the binding constants between the three compounds analyte, CD, and IL in MEKC [60]. They found that the enantioseparation was achieved because of the competition between the IL and the analyte for the CD cavity.

A proof of the interactions between IL and CD without the effect of capillary surface coating was performed by using a polyacrylamide coated capillary [61]. An enhancement in chiral resolution by addition of IL to the CD containing BGE could be achieved in this capillary. Due to the interaction between IL cations and the analyte cyclodextrin complex, the migration time was shortened, indicating that IL cations take part in the analyte CD inclusion complex leading to higher electrophoretic mobility. A BGE containing only IL and no CD did not decrease the migration time indicating that there is no interaction between analyte and IL. Furthermore, these observations were manifested by NMR spectroscopy. Possible interactions between these three compounds are shown and listed in Figure 4.

A ligand-exchange enantioseparation of amino acids using an amino acid ionic liquid was reported by Liu et al [62]. Using 1-alkyl-3-methylimidazolium L-proline and copper leads to an adsorption of imidazolium cations on capillary surface. The L-proline anions associated with the imidazolium cations and the copper cations with the L-proline anions. The separation of the amino acid enantiomers was achieved by formation of ternary mixed-metal complexes with different complex stability constants. Similar observations were made by using amino acids as cationic part of ILs in combination with inorganic and organic anions [63]. Mu et al. reported better enantioseparation of amino acids using a L-proline trifluoroacetate IL compared with a BGE containing L-proline and trifluoroacetic acid. Other complexes investigated in ligand-exchange CE are composed of zinc, L-arginine, L-lysine, and different imidazolium cations. An enantioseparation could also be achieved in nonaqueous media. Ma et al. separated rabeprazole and omeprazole enantiomers by using an

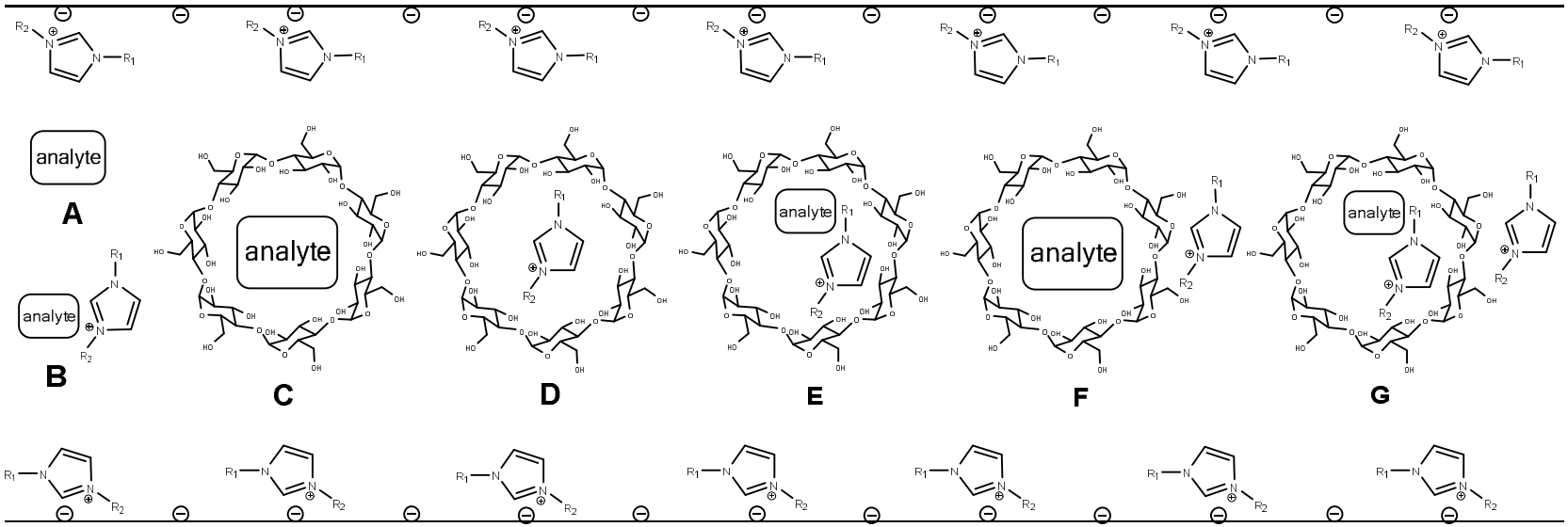

Figure 4 Possible interactions between analyte, IL, and CD.
A: sole analyte
B: analyte IL interaction
C: analyte included in CD
D: IL included in CD
E: analyte and IL included in CD
F: analyte included in CD interacting with IL
G: analyte and IL included in CD interacting with IL.
Furthermore complexes A-G can interact with IL cations coated on capillary surface and with IL anions.

ephedrine based IL ((+)-*N*,*N*-dimethylephedrinium bis(trifluoromethylsulfonyl)imide) as both chiral selector and BGE in acetonitrile methanol mixtures [64]. The first aqueous method with a chiral IL as sole chiral selector was developed by Stavrou et al. in 2013 [65]. They observed the enantioseparation of binaphthyl derivatives by addition of alanine ester based ILs (for details and structure see Table 2 and Figure 3) in a tris-borate buffer. In 2013, the first IL based on a CD was reported: an ammonium-β-CD cation was combined with tetrafluoroborate [66]. A lot of different structures are used as chiral IL ions, for example, amino acids (as cations or as anions), ephedrine based cations, surfactant based, and CD based structures. In the last years, two synergistic system with vancomycin based and glycogen based chiral selectors instead of CDs were reported [67, 68]. It has to be noticed that enantioseparation strongly depends on composition and pH of the BGE. Chiral ILs can be simultaneously used as chiral selectors and electrolyte in CE. Advantages of amino acid based ILs are high biocompatibility, environmental friendliness, stable chirality, weak UV absorption, and low costs.

3.2.3.5 Ionic Liquids in Nonaqueous Capillary Electrophoresis (NACE)

ILs are also applied in nonaqueous capillary electrophoresis. First, they were used as additives to acetonitrile as charge carrier to stabilize the electric field [69-71]. Dialkylimidazolium based ILs are especially suitable, due to their good miscibility with acetonitrile. A separation of 5 hydrophobic dyes (details in Table 2) could be achieved by addition of 1-butyl-3-methylimidazolium ILs to acetonitrile in cathodic detection mode, while no separation can be seen in 100 % acetonitrile [69]. The authors supposed that the analytes get charged in presence of the IL by building heteroconjugates. Two compounds being Brønsted bases (Janus Green, Brilliant Cresyl Blue) interact with the dialkylimidazolium cation and migrate faster than the two Brønsted acids (Thymolphthalein, Phenolphthalein) interacting with the anions. The analyte being neither a proton donor nor an acceptor migrates in middle, with the EOF. The separation mechanism of these compounds and a schematic electropherogram is shown in Figure 5. A similar observation was made when studying the migration order of different phenols, being positional isomers, and carboxylic acids. The migration order depends on the pK_a value, which has an influence on the degree of heteroconjugation [72]. When methanol or water is given to the solute the separation diminishes, because these solvents can act as proton donor and acceptor effecting the breakdown of existing heteroconjugates [70]. Further investigations on separation concept with different anionic counterions confirm the existence of an interaction between Brønsted acids and IL anions by estimation of mobilities [71].

The influence of different anionic counterions on 1-ethyl-3-methylimidazolium cations was tested for flavonoids [41]. By using BF_4^- no anodic mobility of the analytes was observed, whereas

Table 2 Method details to the reported separations in MEKC, MEEKC, enantioseparation, and NACE

Ionic Liquid	BGE Additives	Analyte	Technique	Ref.
1-Alkyl-3-methylimidazolium	SDS, poly-SOLV	Alkyl aryl ketones, Phenols, chiral Binaphthyl derivatives	MEKC	[49]
Undecenoxycarbonyl-L-leucinol bromide, undecenoxycarbonyl-L-pyrrolidinol bromide	-	Enantioseparation of Bromophenylacetic acid, 2-(2-Chlorophenoxy)propanoic acid	MEKC	[50]
Undecenoxycarbonyl-L-leucinol bromide	CD	Aryl-propionic acids	MEKC	[51]
1-Tetradecyl(dodecyl)-3-methylimidazolium	-	Phenols, Benzenes	MEKC	[53]
1-Butyl-3-methylimidazolium hexafluorophosphate	SDS	Phenolic acids	MEEKC	[54]
1-Alkylimidazolium tetrafluoroborate	poly-SOLV	Enantioseparation of Binaphthyl derivatives; Warfarin, Coumachlor, Benzoin derivatives	MEKC	[55]
S-(3-(Chloro-2-hydroxypropyl)trimethylammonium)	Cholic acid	Enantioseparation of Aryl-propionic acids, Atenolol, Propranolol, Warfarin	CE	[58]
Undecenoxycarbonyl-L-leucinol bromide	CD	Aryl-propionic acids	MEKC	[60]
Ammonium based, Pyrrolidinium based, 1-Ethyl-3-methylimidazolium L-lactate	CD	Enantioseparation of Miconazole, Econazole, Ketoconazole, Itraconazole	coated capillary	[61]
L/D-Alanine methyl(ethyl, *tert*-butyl)ester lactate, bis(trifluoromethylsulfonyl)imide	-	Enantioseparation of Binaphthyl derivatives	CE	[65]
6-O-2-Hydroxypropyltrimethylammonium-β-cyclodextrin tetrafluoroborate	-	Different enantiomers	CE	[66]
1-Butyl-3-methylimidazolium hexafluorophosphate, acetate, trifluoroacetate	-	2 Brønsted bases (Janus Green, Brilliant Cresyl Blue), Sudan Black, 2 Brønsted acids (Thymolphthalein, Phenolphthalein)	NACE	[69]
1-Butyl-3-methylimidazolium trifluoroacetate, acetate, hexafluorophosphate, bis(trifluoromethylsulfonyl)imide	-	Phenols, Carboxylic acids	NACE	[70]
1-Butyl-3-methylimidazolium tetrafluoroborate, trifluoroacetate, heptafluorobutanoate	-	(Poly-)phenols	NACE	[71]
1-Butyl-3-methylimidazolium trifluoroacetate, heptafluorobutanoate	-	Phenols	NACE	[72]

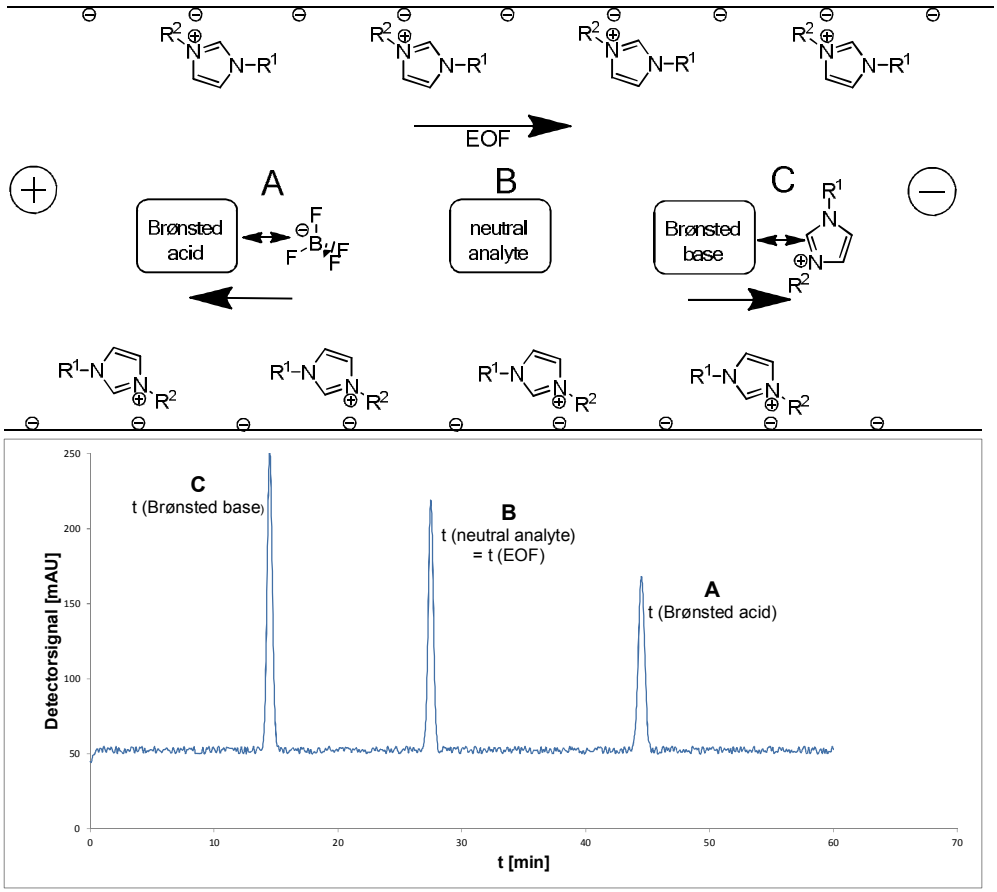

Figure 5 Schematic separation mechanism and schematic electropherogram of three analytes in NACE.
A: Brønsted acids are building a heteroconjugate with IL anions and migrate towards the anode → slower than the EOF.
B: Neutral analytes do not interact with the IL and migrate with the EOF.
C: Brønsted bases are building a heteroconjugate with IL cations and migrate towards the cathode → faster than the EOF

the addition of Cl^- and HSO_4^- led to a mobility toward the anode. This observation verifies a specific interaction between IL anions and the analyte, the heteroconjugation between Cl^- and HSO_4^- on one side and the flavonoids on the other side is much stronger than between BF_4^- and flavonoids. With rising concentration of IL the cathodic EOF is diminished in nonaqueous capillary electrophoresis, too. Like in aqueous CE, a change in EOF direction in nonaqueous CE can be achieved by addition of ILs [64]. Furthermore, Francois et al. demonstrated that there is an affinity of 2-arylpropionic acids to dialkylimidazolium cations in solute as well as to dialkylimidazolium cations coated on capillary surface, indicating a coating of the capillary surface in NACE, too [73]. Interestingly, the separation of 1-alkyl-3-methylimidazolium cations in pure acetonitrile showed in low concentrations (0 - 2 mM) a retention against an EOF marker, which disappears by increasing

concentrations, pointing to the formation of ion pairs at low concentrations. Thus, a good and stable separation could only be achieved at higher concentrations of ILs [43].

Seiman et al. investigated the behavior of the EOF in nonaqueous solvents containing 1-butyl-3-methylimidazolium trifluoroacetate. They observed two different effects [44]: In the first group (acetonitrile, ethanol, propylene carbonate, dimethylformamide) the EOF decreased by increasing IL concentrations, whereas in group two (methanol, nitromethane) upon addition of low concentrations the EOF increased and passed a maximum before it decreased. This can be explained by different conductivities and viscosities between the several solvents used in this investigation.

3.2.4 Conclusions

1. ILs can be applied to coat the capillary surface covalently or dynamically, as single separation medium or binary pseudostationary phase in combination with micelles, CDs, or chiral selectors.
2. The electroosmotic flow can be controlled by addition of different concentrations of ILs to the BGE.
3. The direction of the EOF in CE depends on the concentration and character of the IL. In low concentrations the EOF heads to the cathode. Increase of IL concentration leads to decrease of EOF in cathodic detection mode, because the silanol groups on the capillary surface get shielded. Addition of even higher concentrations of IL to the BGE leads to a change in EOF direction. In anodic detection mode the EOF increases by further raising the IL concentrations (see Table 1).
4. Some observations suggest that the velocity and reversal of the EOF depends on the alkyl chain length of the IL cation. In cathodic detection mode longer alkyl chains effect a decrease and reversal of EOF in lower IL concentrations than shorter alkyl chains. This can be explained by the hydrophobicity of long chain ILs forming a more stable bilayer and thereby the negative charged silanol groups get better shielded. In anodic detection mode an increase in alkyl chain length also causes a decrease in EOF velocity, due to the lower positive charge density (see Table 1).
5. By interaction between silanol groups and IL cations the stationary phase gets more hydrophobic. Therefore, hydrophobic analytes interact more with the stationary phase.
6. A practical problem in working with ILs in capillary electrophoresis is the equilibrium of IL in solution and coated on capillary. So, a few runs can be necessary until the silanol groups

on capillary surface are saturated and measured data gets reproducible [28, 59]. The number of runs is depending on the character of IL.

7. Both IL cation and IL anion play an important role in separation and enantioseparation, whereas interactions between IL cation and analytes seem to be more specific due to the additional adsorption on capillary surface. However, a change in both ions can lead to a change in migration order, peak shape, and migration time [33, 59].
8. ILs are very interesting for the separation of anions with high electrophoretic mobilities. Because of the change in EOF direction anions can be detected in anodic detection mode. The migration time decreases and thereby peak shape and resolution can improve. Furthermore, ILs have an advantage in separation of basic compounds because they suppress the adsorption of these analytes to capillary wall silanol groups.
9. The melting point of an IL allows predictions of the separation with this IL in capillary electrophoresis. A low melting point indicates a weak association between IL cation and IL anion leading to more free ions in solution. Therefore longer migration times, better separation and resolution, and better peak shapes are observed by using ILs with low melting points. The melting point of an IL often depends on the ability of an anion to build a hydrogen bond to its cationic counterion.

3.2.5 Enantioseparation of Pseudoephedrine

Many currently used pharmaceutical drugs are chiral. It is known that the biological activity, the toxicology, and pharmacokinetic parameters of enantiomers can be different. For some drugs it is important to ensure the enantiomeric purity. Therefore, the development of effective methods for enantioseparation is important. Pseudoephedrine is an active compound of ephedrae herba and a popular ingredient in cold medicines. A method using the benefits of ILs to improve the enantioseparation of pseudoephedrine is reported herein. A comparison to other methods separating pseudoephedrine enantiomers developed by our group is also given.

3.2.5.1 Materials

3.2.5.1.1 Apparatus

CE experiments were carried out on a Beckman P/ACE System MDQ instrument (Beckman Coulter, Fullerton, USA) equipped with a DAD detector. The uncoated fused-silica capillaries (BGB Analytik, Schloßböckelheim, Germany) had a total length of 60.2 cm (effective length 50.0 cm) and an internal diameter of 50 µm. The samples were injected hydrodynamically with

0.5 psi for 5 s on the anodic end of the capillary. The separation was carried out at 25 °C and a voltage of +20 kV. The detection wavelength was set to 194 nm.

To check the pH of the buffer solutions a PHM220 pH meter from MeterLab (Villeurbanne, France) was used. For the preparation of homogenous sample solutions a 2510-Branson-Sonicator (Heinemann Ultraschall- und Labortechnik, Schwäbisch Gmünd, Germany) was used.

3.2.5.1.2 Reagents and Chemicals

1. All chemicals used were of analytical grade.

2. Tetrabutylammonium chloride (TBAC), (1*S*,2*S*)-(+)-pseudoephedrine, (1*R*,2*R*)-(-)-pseudoephedrine, ammonium formate, and formic acid were purchased from Sigma Aldrich (Steinheim, Germany), 0.1 M NaOH and 0.1 M HCl from VWR (Darmstadt, Germany), and β-cyclodextrin from Wacker (Munich, Germany).

3.2.5.1.3 Buffers and Samples

1. All buffer and sample solutions were prepared using ultrapure Milli-Q water (Millipore, Milford, MA, USA) and filtered through a 0.2 µm pore-size CA-filter (cellulose acetate) (Carl Roth, Karlsruhe, Germany) prior to use.

2. The running buffer consisted of an aqueous solution of 50 mM ammonium formate, 12 mM β-cyclodextrin, and a step wise raising amount of TBAC.

3. Ammonium formate was dissolved in water and the pH value was adjusted to 3.0 with formic acid and NaOH.

4. β-Cyclodextrin and TBAC were dissolved in this formate buffer using an ultrasonic bath. The pH value was checked and adapted with 0.1 M NaOH and formic acid, if necessary.

5. Pseudoephedrine samples were prepared dissolving 5.0 mg (1*S*,2*S*)-(+)-pseudoephedrine and 5.0 mg (1*R*,2*R*)-(-)-pseudoephedrine in 10.0 ml water. The solution was stored at 8 °C.

3.2.5.1.4 Rinsing Procedure

New capillaries were conditioned at 25 °C by rinsing with 0.1 M NaOH for 20 min, water for 5 min, 0.1 M HCl for 10 min, and again with water for 10 min.

To avoid current breakdown and to achieve repeatable migration times and a stable baseline a steady dynamically capillary coating with TBAC is necessary. To achieve repeatable separations

the capillary was rinsed with water for 5 min, isopropyl alcohol for 10 min, water for 5 min, 0.1 M NaOH for 5 min, water for 5 min, 0.1 M HCl for 5 min, and again with water for 10 min at the beginning of each working day. At the end of each sequence of experiments the capillary was rinsed with water for 5 min, isopropyl alcohol for 20 min, water for 5 min, 0.1 M NaOH for 10 min, water for 5 min, 0.1 M HCl for 5 min, and water for 10 min. Before a new sample was injected the capillary was conditioned by flushing with BGE for 5 min.

All capillary wash cycles were performed at a pressure of 30 psi.

3.2.5.2 Methods

To investigate the influence of the IL concentration on the separation of pseudoephedrine enantiomers the concentration of TBAC in a 50 mM ammonium formate buffer containing 12 mM β-cyclodextrin was step wise raised up to 200 mM. Electropherograms are displayed in Figure 6.

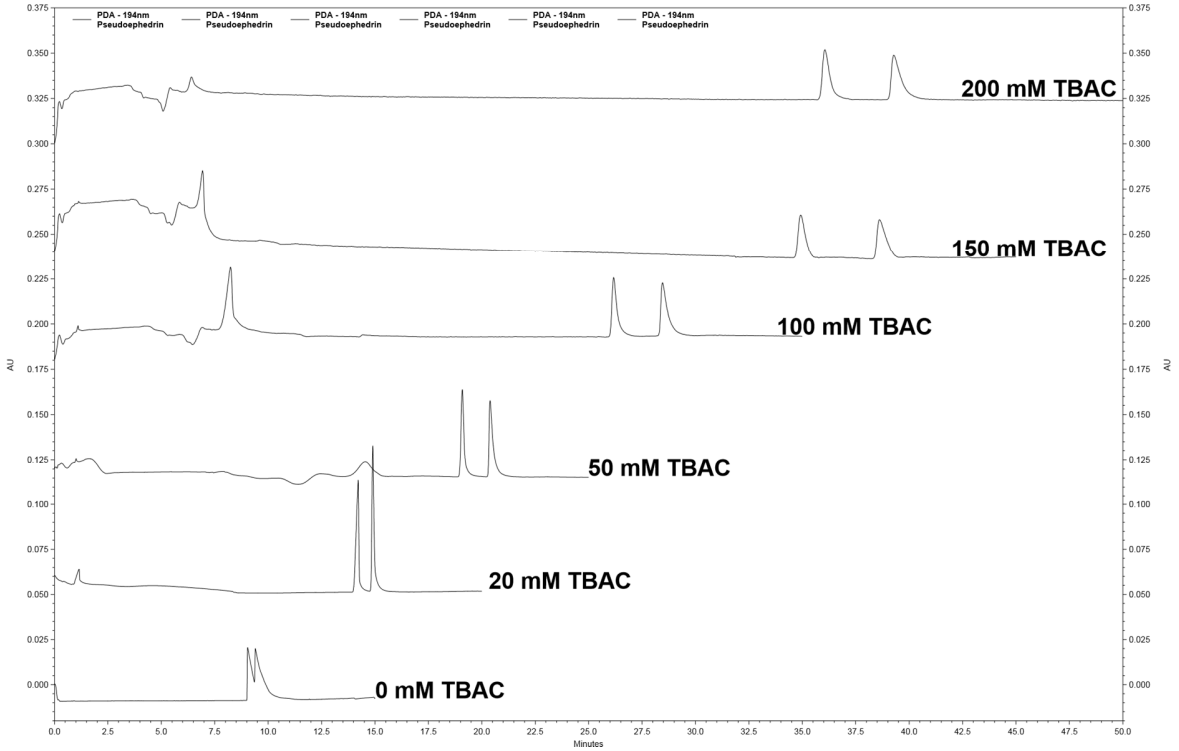

Figure 6 Electropherograms showing the separation of pseudoephedrine enantiomers adding TBAC. Separation conditions: 50 mM ammonium formate buffer, pH 3.0, 12 mM β-cyclodextrin; voltage: +20 kV; temperature: 25 °C; detection wavelength: 194 nm; fused-silica capillary (60.2/50 cm, 50 µm); sample conc.: 0.5 mg/ml

As can be seen in Table 3 and in Figure 7, an enhancement in resolution and a prolongation of migration time, due to capillary coating, was observed. In this concentration range no change

in EOF direction occurred. When the concentration of TBAC exceeds 150 mM no further enhancement of the resolution can be observed. In contrast, due to the increased migration time and peak broadening at a concentration of 200 mM TBAC, a deterioration of the resolution can be seen.

Table 3 Migration time and resolution of pseudoephedrine enantiomers. Separation conditions are the same as in Figure 6.

c(TBAC) [mM]	t_1 [min]	t_2 [min]	R_S
0	9.0	9.4	0.68
20	14.2	14.9	3.27
50	19.1	20.4	4.60
100	26.2	28.5	4.66
150	34.9	38.6	5.01
200	36.1	39.3	4.44

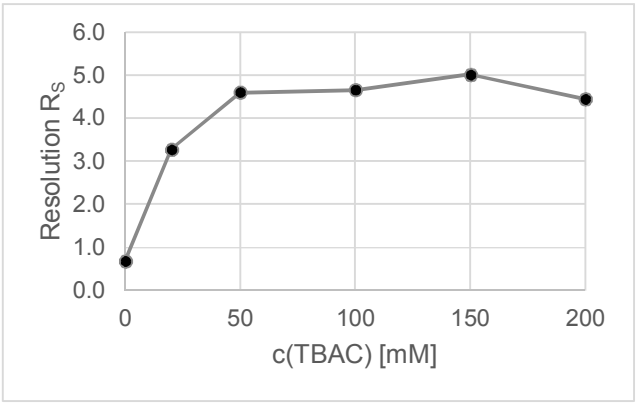

Figure 7 Plot of the chiral resolution of pseudoephedrine. Separation conditions are the same as in Figure 6.

Summarizing, it can be seen that the addition of TBAC to a β-cyclodextrin containing BGE has a synergistic effect on the enantioseparation of pseudoephedrine. By addition of 150 mM TBAC and 12 mM β-CD to the formate buffer, a higher resolution of pseudoephedrine enantiomers can be achieved, compared to a CE method (phosphate buffer, 12 mM β-CD, pH 3) developed by our group (Rs = 3.1) [74]. Our group also reported a MEEKC method using 4.0 % sulfated β-CD, 0.5 % ethyl acetate, 1.0 % SDS, 4.0 % 1-butanol, 2.8 % propan-2-ol in 20 mM phosphate buffer pH 2.5 for the enantioseparation of pseudoephedrine [75]. The IL modified CE method yields to resolutions comparable to those achieved with this method (Rs = 5.7).

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3.3 Separation of Phenethylamine Enantiomers Using Tetrabutylammonium Chloride as Ionic Liquid Background Electrolyte Additive

Joachim Wahl and Ulrike Holzgrabe

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Journal of Research Analytica, 3 (2017), 73-80

Abstract

A capillary electrophoresis method using tetrabutylammonium chloride (TBAC) as ionic liquid buffer additive and different β-cyclodextrin derivatives as chiral discriminators was developed. To find the optimal conditions for the enantioseparation of phenethylamines, the parameters TBAC concentration, pH value, buffer concentration, and cyclodextrin concentration were varied. Best chiral separations have been observed using a 75 mmol/L phosphate buffer pH 2.5 containing 125 mmol/L TBAC. The enantiomers of ephedrine, pseudoephedrine, and methylephedrine were separated employing β-cyclodextrin, (2-hydroxypropyl)-β-cyclodextrin, and 6-O-α-maltosyl-β-cyclodextrin, while the separation of norephedrine enantiomers was only feasible using methyl-β-cyclodextrin. A possible explanation for the improved chiral separation using TBAC as buffer additive, compared to buffers containing no TBAC, is an increased β-cyclodextrin solubility. The solubility of β-cyclodextrin in phosphate buffer and in phosphate buffer containing TBAC was determined by HPLC-CAD. Since the adsorption of tetrabutylammonium cations is of paramount importance and has to be overcome, the influence of different rinsing procedures was investigated. The best reproducibility of separation runs was found for a rinsing step applying voltage and 1 M sodium hydroxide solution.

3.3.1 Introduction

In the last years, ionic liquids (IL) become more and more important in analytical chemistry. They are applied e.g. in liquid chromatography [1, 2], gas chromatography [3], mass spectrometry [4], and capillary electrophoresis [5]. ILs are (semi-)organic salts with a melting point below 100 °C. By changing the combination of cation and anion an IL can be designed as requested. Regarding capillary electrophoresis (CE) separations, solubility, miscibility, viscosity, and electrochemical behavior are some among many characteristics that can be modified by altering the combination of cation and anion. In CE ILs can be used as main electrolyte, electrolyte additive, and for the formation of a dynamical capillary surface coating.

Enantiomers of racemic drugs can have different biological properties, like pharmacodynamics [6], pharmacokinetic [7], and toxicology [8]. The enantiomers of pseudoephedrine are applied in cold medicines. Furthermore, phenethylamines can be found as impurities in methamphetamine stimulants [9]. Enantiomers of ephedrine, pseudoephedrine, methylephedrine, and norephedrine have been separated by CE using different cyclodextrin derivatives [9-12]. Furthermore, the chiral recognition mechanism between ephedrine derivatives and cyclodextrins (CDs) was elucidated by NMR [11-14] and affinity capillary electrophoresis [15]. The derivatization of native CDs can change e. g. their solubility, flexibility, and cavity size; hence, CD derivatives are frequently used for racemic separations in CE [16, 17].

A problem, occurring during CE separation runs, is the adsorption of especially positively charged basic compounds, like ephedrine derivatives at acidic pH values, to the silanol groups of the capillary surface, which leads to peak broadening and poor reproducibility. The adsorption of these analytes can be prevented by dynamical coating of the capillary surface using cationic buffer additives [18], like the IL tetrabutylammonium chloride (TBAC). Due to a change of the zeta potential at the capillary surface, these additives affect the electroosmotic flow (EOF) [19]. Before starting a new run, an entire desorption of cations from the capillary surface has to be ensured to achieve reproducible CE separations. Rinsing procedures using NaOH solution and organic solvents were reported to be beneficial for the desorption of tetramethylammonium cations [20]. Organic solvents cause desorption of organic compounds [21], while a NaOH solution refreshes the capillary surface by breaking up adsorbed layers of ions [22]. Herein, the influence of different rinsing steps on the reproducibility of CE runs, applying an IL buffer additive, was investigated.

The aim of this work was to develop and to optimize a method for the enantioseparation of several phenethylamine derivatives using tetrabutylammonium chloride (melting point 52-54 °C [23]) as IL buffer additive. Furthermore, different β-CD derivatives have been

investigated to improve the enantioseparation. To the best of our knowledge, the separation of phenethylamine enantiomers using Mal-β-CD was not reported before. In addition, the influence of the complexation between tetrabutylammonium cations and β-CD [24] on the solubility of β-CD was investigated by HPLC-CAD.

3.3.2 Materials and Methods

3.3.2.1 Reagents and Chemicals

Tetrabutylammonium chloride (TBAC), (1*S*,2*R*)-(+)-ephedrine hemihydrate, (1*S*,2*S*)-(+)-pseudoephedrine, (1*R*,2*R*)-(-)-pseudoephedrine, (1*S*,2*R*)-(+)-methylephedrine, (1*R*,2*S*)-(-)-methylephedrine, (1*S*,2*R*)-(+)-norephedrine, (1*R*,2*S*)-(-)-norephedrine, phosphoric acid, formic acid, dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), propan-2-ol, ethanol, acetonitrile, (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) (average M_r ~ 1380; ~ 0.6 mol 2-hydroxypropyl per sugar unit), (average M_r ~ 1460; ~ 0.8 mol 2-hydroxypropyl per sugar unit), (average M_r ~ 1540; ~ 1.0 mol 2-hydroxypropyl per sugar unit), and methyl-β-cyclodextrin (Me-β-CD) (average M_r ~ 1310; ~ 1.6-2.0 mol CH_3 per sugar unit) were acquired from Sigma Aldrich (Steinheim, Germany), while (1*R*,2*S*)-(-)-ephedrine hydrochloride was from Caelo (Hilden, Germany). 0.1 M NaOH, 0.1 M HCl, and sodium dihydrogen phosphate monohydrate were purchased from VWR (Darmstadt, Germany), and β-cyclodextrin from Wacker (Munich, Germany). 6-O-α-Maltosyl-β-cyclodextrin (Mal-β-CD) was a gift from Prof. Tomohiro Endo (Hoshi University, Tokyo, Japan). All chemicals and solvents were of at least analytical grade.

3.3.2.2 HPLC – CAD

3.3.2.2.1 Apparatus

HPLC-CAD measurements were carried out using an Agilent 1100 modular chromatographic system consisting of vacuum degasser, binary pump, auto sampler, column compartment, and diode array detector (Agilent, Waldbronn, Germany). The HPLC system was linked to a Corona CAD detector (Thermo Fisher, Courtaboeuf, France) by a PEEK capillary (0.25 mm I. D.) and a stainless steel inlet-frit (0.22 µm). The nitrogen needed for the detector was produced by an ESA Nitrogen Generator (Thermo Fisher, Courtaboeuf, France) and the inlet pressure to the detector was kept constant 241317 Pa (≙ 35.0 psi).

3.3.2.2.2 Preparation of Solutions

The solubility of β-CD was determined by constructing calibration curves from 7 mmol/L to 42 mmol/L β-CD in a 75 mmol/L phosphate buffer pH 2.5 containing 125 mmol/L TBAC. Saturated solutions of β-CD were prepared by weighing 0.5 g β-CD into 2.0 mL (i) 75 mmol/L phosphate buffer pH 2.5 and (ii) 75 mmol/L phosphate buffer pH 2.5 containing 125 mmol/L TBAC. The solutions were mixed for 24 h at 40 °C and 1000 rpm. Samples in buffer were injected directly while the TBAC/phosphate buffer samples were diluted before injection.

3.3.2.2.3 Methods

A RP18 column Eurosphere II (250 x 3 mm, particle size: 3 µm, pore size: 100 Å, C18P, Knauer, Berlin, Germany) was utilized as stationary phase. Separations were carried out isocratically using a mobile phase composed of 0.1 % (V/V) formic acid in water:acetonitril (95:5, V/V). The injection volume was 5 µL.

3.3.2.3 Capillary Electrophoresis

3.3.2.3.1 Apparatus

The CE separations were performed by means of a Beckman P/ACE MDQ system (Beckman Coulter, Fullerton, USA) equipped with a diode array detector. Uncoated fused-silica capillaries (BGB Analytik, Schloßböckelheim, Germany) with an effective length of 50.0 cm (total length 60.2 cm) and an internal diameter of 50 µm were utilized for CE measurements.

3.3.2.3.2 Preparation of Solutions

Phosphate buffers (50, 75, and 100 mmol/L) were prepared by mixing solutions of sodium dihydrogen phosphate monohydrate with phosphoric acid of the appropriate concentration until the required pH value (2.0, 2.5, and 3.0) was achieved. The investigated background electrolytes were prepared by dissolving different amounts of TBAC in these phosphate buffers resulting in TBAC concentrations from 0 to 200 mmol/L. Subsequently, the respective CD was dissolved in the phosphate buffer containing TBAC. Racemic solutions of phenethylamines were prepared by dissolving 5.0 mg of each enantiomer in 10 mL water (concentration 0.5 mg/mL). All buffer and sample solutions were prepared using Milli-Q water (Millipore, Milford, MA, USA) and were filtered through a 0.2 µm cellulose acetate syringe filter (VWR, Darmstadt, Germany) prior to use. Samples and buffers were stored at 8 °C.

3.3.2.3.3 Methods

A new capillary was preconditioned by the following rinsing steps: 5 min H_2O, 30 min 0.1 M NaOH, 2 min H_2O, 15 min 0.1 M HCl, 10 min H_2O. Due to a strong adsorption of tetrabutylammonium cations to the silanol groups of the capillary surface, different rinsing steps were investigated. Before starting a new separation run, the state of the capillary was checked by monitoring the strength of the EOF. Hence, the migration time of a 0.25 % (V/V) DMSO solution in water was measured at 20 kV using a 50 mmol/L phosphate buffer pH 7.4 as background electrolyte. Before each run, the capillary was rinsed for 2 min with water and for 5 min with background electrolyte. A pressure of 206843 Pa (≙ 30.0 psi) was used for all rinsing steps. The samples were injected with a pressure of 3447 Pa (≙ 5.0 psi) for 5.0 s at the anodic side of the capillary. Separations were carried out applying a voltage of 25 kV and the capillary temperature was kept at 25 °C. The samples were detected at 200 nm.

3.3.3 Results and Discussion

3.3.3.1 β-CD Solubility by HPLC-CAD

Compared to phosphate buffer, an improved solubility of β-CD in buffers containing TBAC was observed. Furthermore, the interaction between β-CD and tetrabutylammonium cations was shown by NMR spectroscopy [24]. Hence, the solubility of β-CD in (i) 75 mmol/L phosphate buffer pH 2.5 and (ii) 75 mmol/L phosphate buffer pH 2.5 containing 125 mmol/L TBAC was determined by HPLC-CAD. The calibration curve in TBAC/phosphate buffer gave a good coefficient of determination (R^2 = 0.9975). The solubility of β-CD was found to be 32.0 mmol/L in phosphate buffer and 43.4 mmol/L in TBAC/phosphate buffer. Thus, upon addition of TBAC to the phosphate buffer the β-CD solubility is improved. A similar observation was reported for the solubility of cellulose and was explained by the amphiphilic character of tetrabutylammonium cations [24]. Herein, the enhanced solubility of native β-CD in background electrolytes containing TBAC was utilized to further improve the chiral separation of phenethylamines in CE.

3.3.3.2 Capillary Electrophoresis

3.3.3.2.1 Rinsing Procedure

To ensure a total desorption of tetrabutylammonium cations from the capillary surface different rinsing procedures were tested (see Table 1). Among them were short, long, acidic, basic, organic, and rinsing steps using electric voltage. The effect of the rinsing methods was investigated

regarding the enantioseparation of pseudoephedrine using a 75 mmol/L phosphate buffer pH 2.5 containing 12 mmol/L β-CD and 100 mmol/L TBAC. The reproducibility of CE runs was estimated by regarding the variation of the migration times during a series of 15 runs. For every investigation of a rinsing procedure a new capillary was used and, to avoid possible electrolysis effects by previous runs, every separation run was performed using new buffer vials. To check the capillary condition an EOF test run was performed before each separation run.

Table 1 Investigated rinsing procedures

Short - water	Organic	Acidic	Basic - pressure	Basic - voltage
Preconditioning	Preconditioning	Preconditioning	Preconditioning	Preconditioning
EOF test run	EOF test run	EOF test run	EOF test run	EOF test run
EOF test run	1 min H_2O	1 min H_2O	1 min H_2O	1 min H_2O
EOF test run	12 min 2 % (m/V) SDS/H_2O	12 min 0.1 M HCl	12 min 0.1 M NaOH	2 min 1 M NaOH
run 1	EOF test run	EOF test run	EOF test run	10 min 1 M NaOH, 30 kV
run 2	1 min H_2O	1 min H_2O	1 min H_2O	EOF test run
…	12 min Propan-2-ol	12 min 0.1 M HCl	12 min 0.1 M NaOH	1 min H_2O
	EOF test run	EOF test run	EOF test run	2 min 1 M NaOH
	run 1	run 1	run 1	10 min 1 M NaOH, 30 kV
	EOF test run	EOF test run	EOF test run	EOF test run
	1 min H_2O	1 min H_2O	1 min H_2O	run 1
	12 min 2 % (m/V) SDS/H_2O	12 min 0.1 M HCl	12 min 0.1 M NaOH	EOF test run
	EOF test run	EOF test run	EOF test run	1 min H_2O
	1 min H_2O	1 min H_2O	1 min H_2O	2 min 1 M NaOH
	12 min Propan-2-ol	12 min 0.1 M HCl	12 min 0.1 M NaOH	10 min 1 M NaOH, 30 kV
	EOF test run	EOF test run	EOF test run	EOF test run
	run 2	run 2	run 2	1 min H_2O
	…	…	…	2 min 1 M NaOH
				10 min 1 M NaOH, 30 kV
				EOF test run
				run 2
				…

Figure 1 displays the migration times of pseudoephedrine enantiomers and DMSO for the first 15 runs. The best reproducibility was achieved using basic rinsing procedures. The use of the short rinsing step with water and background electrolyte (Figure 1 A) led to increasing migration times for the EOF test run, while the migration times of the pseudoephedrine enantiomers decreased until run 8. The organic rinsing method (Figure 1 B), using propan-2-ol and SDS, showed increasing migration times for the EOF test run. Furthermore, reproducible migration times for pseudoephedrine enantiomers were not achieved until run 12. Due to a higher amount of protonated silanol groups, the acidic rinsing step (Figure 1 C) led to longer migration times (EOF test run > 7.5 min) and constant pseudoephedrine migration times were achieved after run 8.

The two basic rinsing procedures, using pressure (Figure 1 D) or voltage (Figure 1 E), showed the best reproducibility of migration times. Furthermore, due to the deprotonation of silanol groups, shorter migration times for pseudoephedrine enantiomers were observed. An additional advantage of the basic rinsing methods was a fast equilibration of the capillary leading to

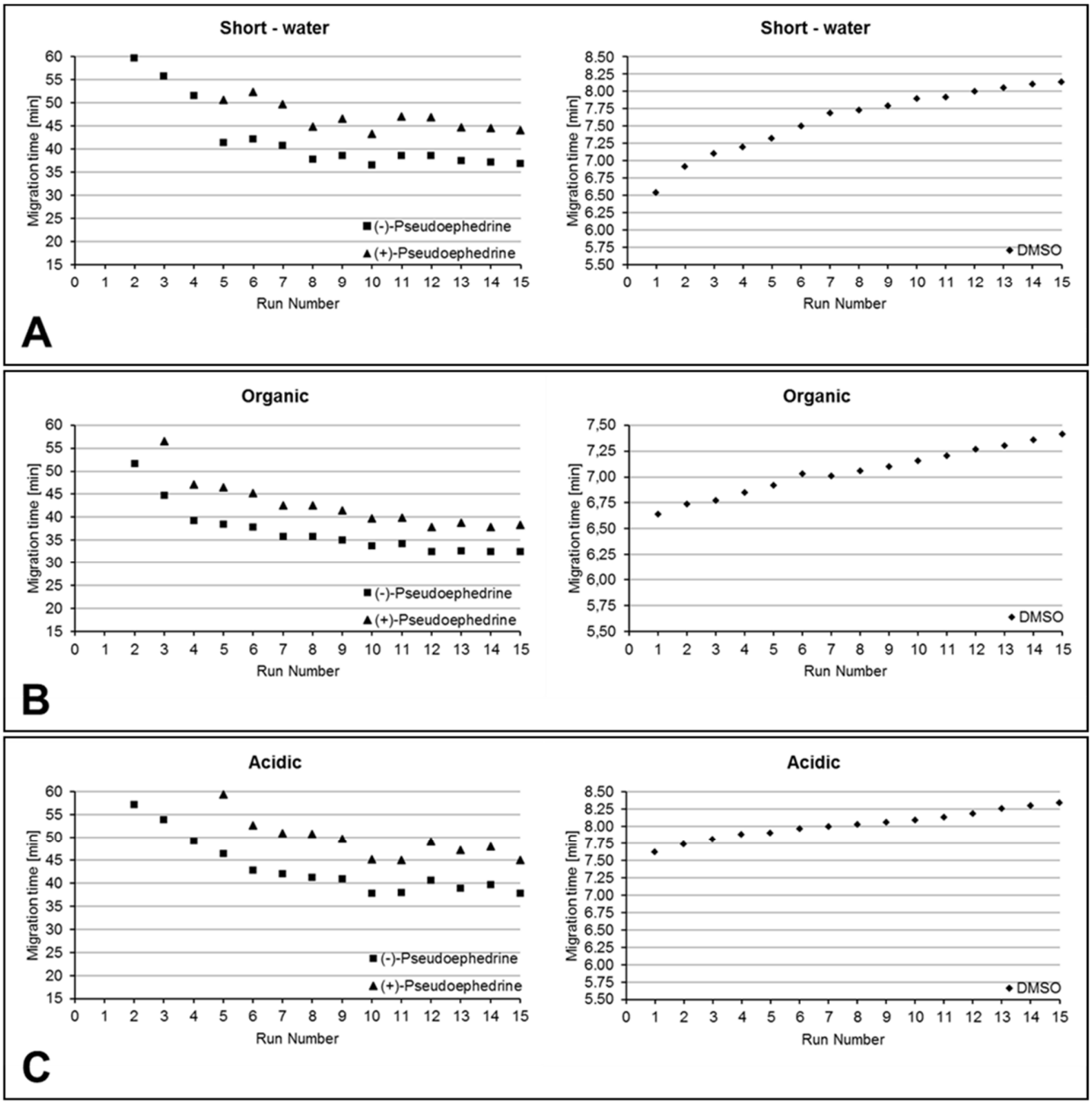


Figure 1 Investigation of the reproducibility of pseudoephedrine enantiomer (left) and DMSO (right) migration times using different rinsing procedures (see Table 1). A: short rinsing procedure using water; B: organic rinsing procedure; C: acidic rinsing procedure; D: basic/pressure rinsing procedure; E: basic/voltage rinsing procedure; F: comparison basic/pressure and basic/voltage rinsing procedure. Scales of the DMSO migration times applying rinsing procedures A and C were changed due to migration times longer than 7.5 min.

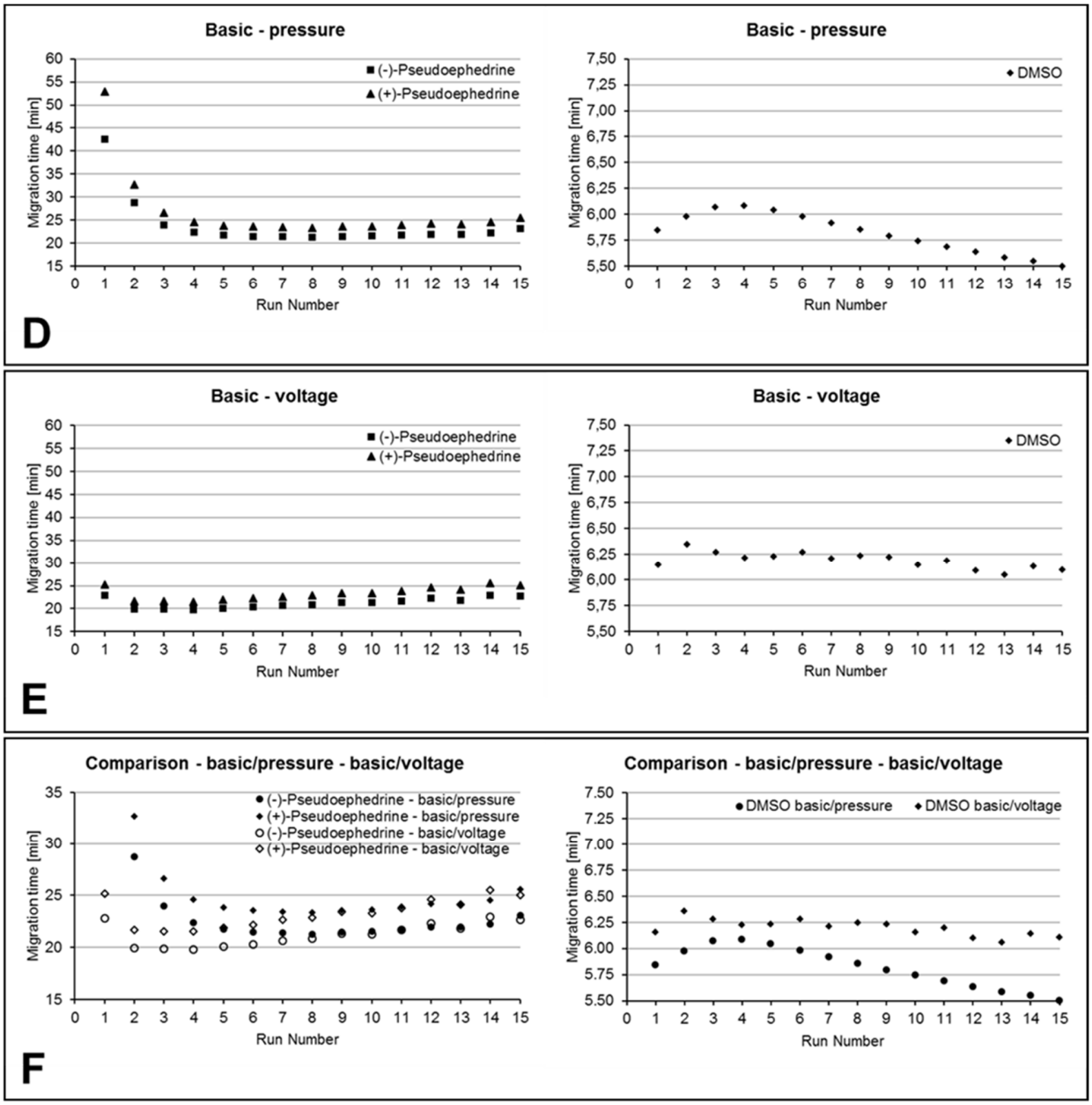


Figure 1 *Continued*

reproducible migration times after two (voltage) and after five (pressure) runs. Furthermore, the relative standard deviations for the migration times, excluding run 1 for equilibration, were lower for the basic/voltage rinsing method compared to the basic/pressure method. Regarding the basic/pressure rinsing procedure the relative standard deviations of the migration times were 8.6 % for (-)-pseudoephedrine and 9.8 % for (+)-pseudoephedrine, respectively, while the basic/voltage rinsing procedure gave relative standard deviations of 5.0 % for (-)-pseudoephedrine or 5.7 % for (+)-pseudoephedrine. Additionally, the migration times of DMSO

in the EOF test runs showed a higher constancy using the basic/voltage rinsing procedure. Figure 1 F displays a comparison between the results of the two basic rinsing methods. Considering these results, the method developments reported hereinafter were made using the basic/voltage rinsing method.

3.3.3.2.2 Method Development – β-CD

Compared to a method using an ammonium formate buffer [5], a better chiral resolution was observed employing phosphate buffer. The influence of TBAC on the enantioseparation was investigated by adding increasing amounts of the IL to a 50 mmol/L phosphate buffer pH 3.0. As can be seen from Figure 2, increasing concentrations of TBAC led to increasing migration times of phenethylamine analytes. A possible explanation is a decrease of the EOF due to an adsorption of the tetrabutylammonium cations to the silanol groups of the capillary surface. Furthermore, an increase of the chiral resolution was observed while the TBAC concentration was increased up to 125 mmol/L. Due to peak broadening, a further increase of the TBAC concentration led to a decrease of the chiral resolution. Using the background electrolyte containing 125 mmol/L TBAC, an enantioseparation of ephedrine, pseudoephedrine, and methylephedrine was feasible, while no separation of norephedrine enantiomers was achieved.

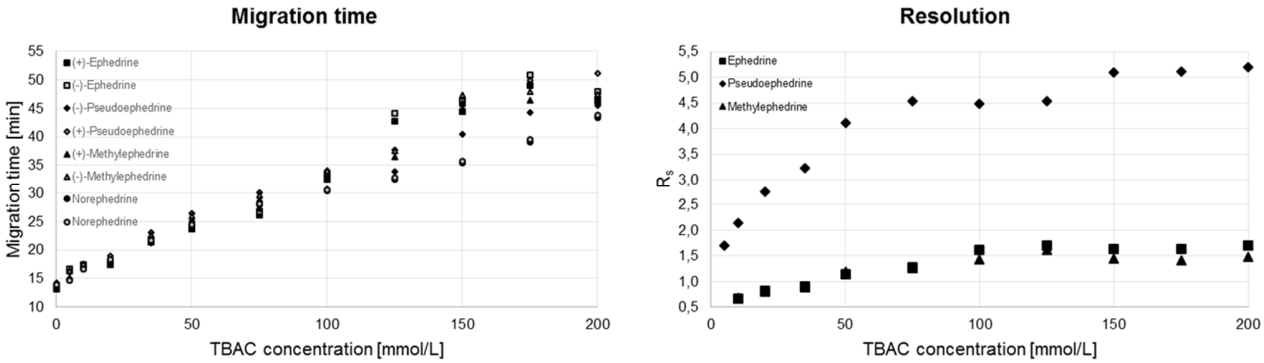
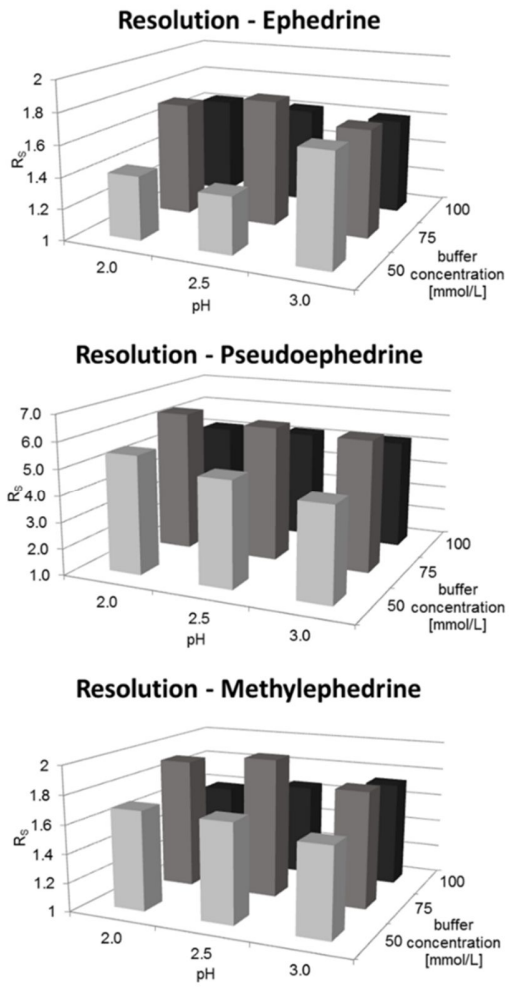


Figure 2 Migration times and resolutions of the enantioseparation of phenethylamines at increasing concentrations of TBAC (separation conditions: 50 mmol/L phosphate buffer pH 3.0, 12 mmol/L β-CD)

Furthermore, the influence of the buffer concentration and the pH value on the chiral resolution were investigated. The results, employing buffer concentrations from 50 to 100 mmol/L and pH values from 2.0 to 3.0, are displayed in Figure 3. The best results have been observed for a 75 mmol/L phosphate buffer pH 2.5. Using this optimized buffer, containing 125 mmol/L TBAC, a baseline separation was achieved for enantiomers of ephedrine, pseudoephedrine and

methylephedrine. Only the enantioseparation of norephedrine was not feasible. Hence, the concentration of β-CD was increased to improve the chiral separation of phenethylamines. Due to the observed solubility improvement of β-CD in phosphate buffer containing TBAC, it was possible to increase the β-CD concentration above the highest soluble concentration in phosphate buffer.



A further enhancement of the chiral resolution was observed while the β-CD concentration was increased to 35 mmol/L. The R_S values were 3.3 for ephedrine enantiomers, 11.0 for pseudoephedrine enantiomers, 2.7 for methylephedrine enantiomers, and 1.0 for norephedrine enantiomers. Unfortunately, due to an increase of the viscosity and due to a higher amount of complexed analyte, it was impossible to separated pseudoephedrine enantiomers within 60 min. Concentrations higher than 35 mmol/L β-CD led to unacceptable long migration times (t > 60 min) and the occurring peak broadening yielded in a decrease of the chiral resolution.

Figure 3 Optimization of buffer concentration and pH value (separation conditions: 12 mmol/L β-CD, 125 mmol/L TBAC)

3.3.3.2.3 Method Development - β-CD Derivatives

Additionally, different β-CD derivatives were tested as chiral discriminators for the enantioseparation of phenethylamines in presence of TBAC. First, the influence of the degree of substitution of different HP-β-CD derivatives on the enantioseparation of phenethylamines was investigated. As can be seen from Figure 4, the best enantioseparation of ephedrine and methylephedrine was achieved using less substituted HP-β-CD derivatives. For pseudoephedrine enantiomers the best separation was achieved using the MW~1460 derivative (R_S = 16.0). Nevertheless, the enantiomers of pseudoephedrine also showed a very good separation using the

MW~1380 derivative of HP-β-CD (R_S = 12.4). Hence, the MW~1380 derivative was used for further investigations.

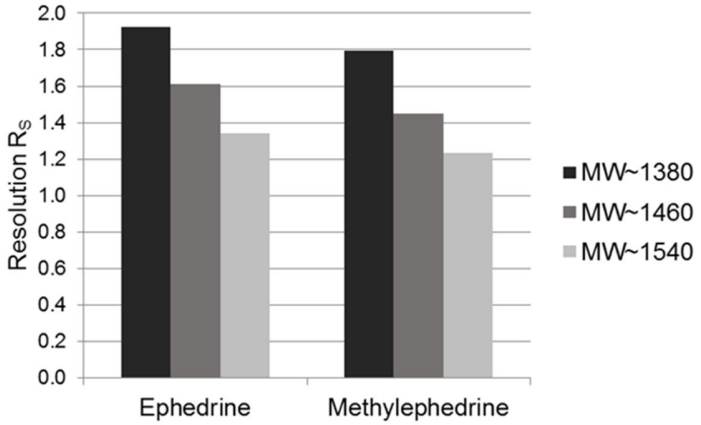


Figure 4 Resolution of phenethylamine enantiomers using HP-β-CD derivatives of different degrees of substitution (separation conditions: 30 mmol/L HP-β-CD, 75 mmol/L phosphate buffer pH 2.5, 125 mmol/L TBAC)

Subsequently, the concentrations of different β-CD derivatives have been optimized and compared to the results achieved using 35 mmol/L native β-CD. The concentrations of all CDs were increased until a maximum resolution was achieved. The best enantioseparations of ephedrine derivatives in a phosphate buffer containing TBAC were obtained using a concentration of 35 mmol/L β-CD, 50 mmol/L HP-β-CD, 60 mmol/L Me-β-CD, and 30 mmol/L Mal-β-CD. The observed chiral resolutions are summarized in Table 2 and electropherograms using optimum separation conditions are displayed in Figure 5. The best results, applying all CDs, have been observed for pseudoephedrine enantiomers. In contrast, the enantioseparation of norephedrine was only feasible using Me-β-CD. On the other hand the separation of ephedrine enantiomers

Table 2 Resolution between phenethylamine enantiomers at optimum CD concentrations (separation conditions: 75 mmol/L phosphate buffer pH 2.5, 125 mmol/L TBAC)

	35 mmol/L ß-CD	50 mmol/L HP-ß-CD	60 mmol/L Me-ß-CD	30 mmol/L Mal-ß-CD
Ephedrine	3.3	2.7	1.3	2.2
Pseudoephedrine	11.0	17.5	19.4	7.9
Methylephedrine	2.7	2.6	1.8	2.0
Norephedrine	1.0	< 1.0	3.4	1.0

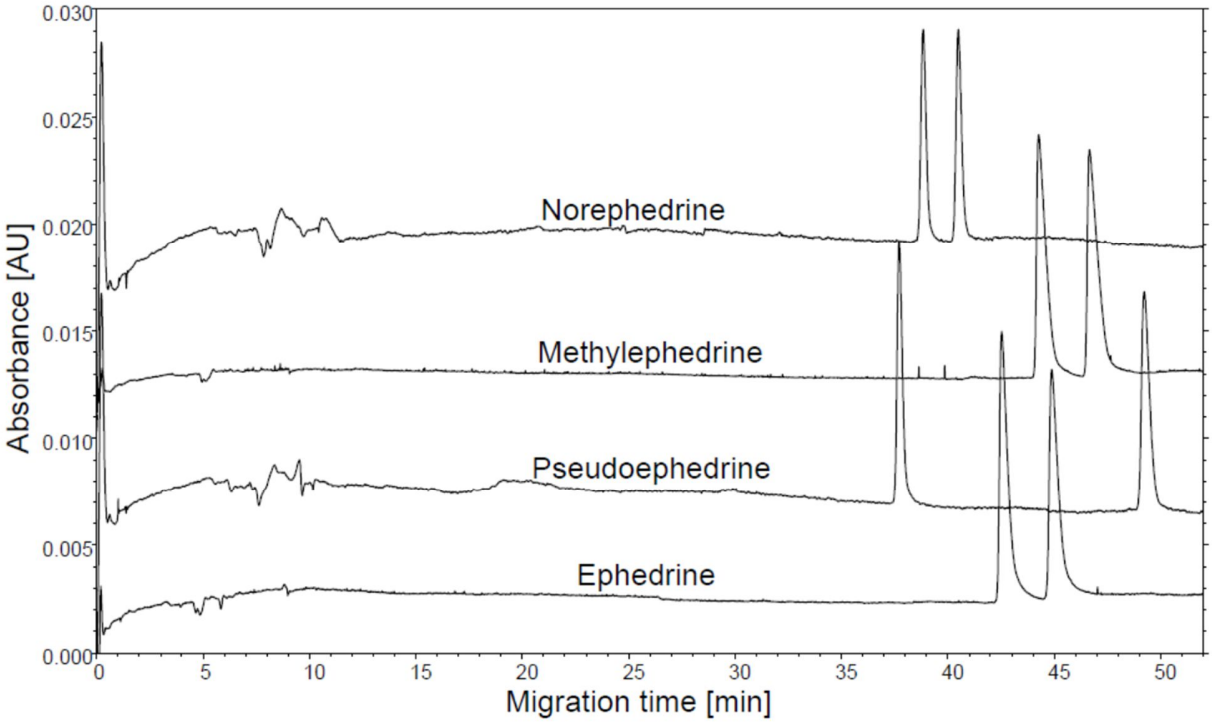


Figure 5 Electropherograms displaying the enantioseparation of ephedrine, pseudo-ephedrine, methylephedrine, and norephedrine at optimum separation conditions (75 mmol/L phosphate buffer pH 2.5, 125 mmol/L TBAC, voltage 25 kV, capillary temperature 25 °C, detection wavelength 200 nm, fused silica capillary (60.2 cm/ 50 cm, 50 µm I.D.), sample concentration 0.5 mg/mL). The displayed separations of ephedrine and methylephedrine were performed using 35 mmol/L β-CD, while pseudoephedrine and norephedrine were separated using 60 mmol/L Me-β-CD.

was impossible in Me-β-CD runs, while these enantiomers were separated applying all other CDs. For methylephedrine all CDs showed at least baseline separation. As can be seen from Figure 5 long migration times (55 min) were observed at optimum separation conditions making it difficult to use the developed methods for daily routine applications.

3.3.3.2.4 Comparison of Chiral Phenethylamine Separations

With the drawbacks of long migration times and a long rinsing procedure, the use of TBAC as buffer additive gave good chiral separations of phenethylamines. To the best of our knowledge, the resolutions between phenethylamine enantiomers observed herein were, with the exception of norephedrine, superior to those published in literature for uncharged CD derivatives. Better chiral separations of ephedrine [25-27] and methylephedrine [28] were only reported using expensive negatively charged CD derivatives. Regarding the enantioseparation of pseudoephedrine, the methods described herein showed better results than all published separations, even compared to methods applying negatively charged CDs [26, 27]. Only for

norephedrine a better enantioseparation was achieved applying the uncharged CD derivative dimethyl-β-CD [29].

3.3.4 Concluding Remarks

The reproducibility of CE separations using cationic buffer additives, like TBAC, is a crucial factor. Thus, the influence of different rinsing procedures on the reproducibility was investigated. Rinsing steps applying voltage and using NaOH solution showed the best results and, additionally, shortened the migrations times.

The influence of a previously reported complexation between tetrabutylammonium cations and β-CD [24] on the solubility of β-CD was investigated by HPLC-CAD. Compared to phosphate buffer, an improved solubility was found. The better solubility of the CDs was utilized to apply higher concentrations of β-CD. Using TBAC as buffer additive, the higher concentrations of dissolved CDs led to enhanced chiral separations of phenethylamines.

Furthermore, the enantioseparation of phenethylamines was investigated using different β-CD derivatives and TBAC as buffer additive. The best separations were achieved using a 75 mmol/L phosphate buffer pH 2.5 containing 125 mmol/L TBAC. With the exception of norephedrine, good chiral separations were achieved using 35 mmol/L β-CD, 50 mmol/L HP-β-CD, and 30 mmol/L Mal-β-CD. A separation of norephedrine was only achieved applying Me-β-CD, while at the optimum concentration of 60 mmol/L Me-β-CD the separation of ephedrine enantiomers was impossible.

A further possible explanation for the increasing chiral resolution by adding TBAC to the background electrolyte, besides the improved solubility of CDs, is the adsorption of tetrabutylammonium cations to the silanol groups of the capillary surface. The resulting decrease of the EOF, due to this adsorption, leads to a longer period of time for the separation of temporarily formed diastereomeric complexes between enantiomers and CDs.

Acknowledgement

The authors would like to thank Prof. Dr. Tomohiro Endo for the gift of Mal-β-CD and Christiane Theiss for supporting HPLC-CAD measurements.

Conflict of interest statement

None of the authors of this paper does have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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3.4 Capillary Electrophoresis Separation of Phenethylamine Enantiomers Using Amino Acid Based Ionic Liquids

Joachim Wahl and Ulrike Holzgrabe

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Journal of Pharmaceutical and Biomedical Analysis, 148 (2018), 245-250

Abstract

In recent years increasing interest was drawn towards ionic liquids in analytical separation science, such as capillary electrophoresis. Ionic liquids combining tetrabutylammonium cations with chiral amino acid based anions were prepared and investigated as capillary electrophoresis background electrolyte additives for the enantioseparation of ephedrine, pseudoephedrine, and methylephedrine isomers. For the optimization of buffer pH and ionic liquid concentration a design of experiments approach was performed. The best results for the separation of all enantiomers were achieved using 125 mmol/L tetrabutylammonium L-argininate in a 75 mmol/L phosphate buffer pH 1.5 containing 30 mmol/L β-cyclodextrin.

3.4.1 Introduction

In the past years ionic liquids (ILs) are increasingly used in analytical separations, especially in capillary electrophoresis (CE). ILs, characterized by a melting point below 100 °C, can establish the running buffer of CE separations. Their properties can easily be adopted for separation optimization by altering the combination of cation and anion. Moreover, chiral ILs can facilitate the enantioseparation of e. g. drugs. The use of ILs in CE is summarized in [1-4].

Advantages like stable chirality, often weak UV absorption, low costs, and environmental friendliness [4] make amino acids excellent counterions for the formation of ILs. Furthermore, amino acids are suitable to be used as both, cation and anion. Amino acids as cationic part of ILs are frequently used as *tert*-butyl esters [5-10]. On the other hand, ILs having amino acids as anionic part are mostly employed for ligand exchange CE [11-13]. Additionally, amino acid based ILs (AAILs), composed of ammonium based cations and amino acid anions, have been reported as additives to β-cyclodextrin (β-CD) containing background electrolytes (BGE). Tetramethylammonium cations were used in combination with anions of L-arginine, L-hydroxyproline, and L-isoleucine [14], while tetrabutylammonium (TBA^+) cations were only reported as L-aspartate salts [15, 16].

The phenethylamines ephedrine, pseudoephedrine, and methylephedrine are pharmacodynamically active components extracted from ephedrae herba [17]. The enantioseparation of these analytes by means of CE was reported frequently [18-24]. Furthermore, the mechanism of chiral interaction between phenethylamine enantiomers with β-CD and their derivatives was investigated by NMR [18, 20, 21, 25] and affinity capillary electrophoresis [26]. Additionally, the separation of phenethylamine enantiomers using the IL tetrabutylammonium chloride (TBAC) as background electrolyte additive was described before [27].

The aim of this study was to investigate the influence of different amino acid based anions in combination with TBA^+ cations on the enantioseparation of phenethylamines. The use of AAILs was thought to be beneficial on the enantioseparation of phenethylamines. Due to the addition of a further chiral compound, a synergistic effect with the conventional used β-CD seemed to be conceivable. For the determination of the best separation conditions (AAIL concentration and pH value) a design of experiments (DoE) approach was used. Furthermore, the β-CD concentration was optimized and the results were compared to a method using achiral TBAC as buffer additive.

3.4.2 Materials and Methods

3.4.2.1 Reagents and Chemicals

Tetrabutylammonium hydroxide solution (~ 40 % in water) (TBAOH), tetrabutylammonium chloride (TBAC), (1*S*,2*R*)-(+)-ephedrine hemihydrate, (1*S*,2*S*)-(+)-pseudoephedrine, (1*R*,2*R*)-(-)-pseudoephedrine, (1*S*,2*R*)-(+)-methylephedrine, (1*R*,2*S*)-(-)-methylephedrine, phosphoric acid, dimethyl sulfoxide (DMSO), benzoic acid, maleic acid (standard for qNMR), L-valine (Val), L-leucine (Leu), L-serine (Ser), L-methionine (Met), L-aspartic acid (Asp), L-glutamic acid (Glu), L-proline (Pro), L-lysine (Lys), and L-arginine (Arg) were purchased from Sigma Aldrich (Steinheim, Germany), while (1*R*,2*S*)-(-)-ephedrine hydrochloride was from Caelo (Hilden, Germany). Glycine (Gly), L-alanine (Ala), L-isoleucine (Ile), L-threonine (Thr), L-cysteine (Cys), L-asparagine (Asn), and L-glutamine (Gln) were acquired from Fluka (Buchs, Switzerland). Deuterium oxide was purchased from Deutero (Kastellaun, Germany). 0.1 M NaOH, 0.1 M HCl, sodium hydroxide, and sodium dihydrogen phosphate monohydrate were acquired from VWR (Darmstadt, Germany), and β-cyclodextrin (β-CD) was from Wacker (Munich, Germany). All solvents and chemicals were at least of analytical grade.

3.4.2.2 Apparatus

3.4.2.2.1 NMR, Melting Point

NMR experiments were carried out by means of a Bruker Avance® (Karlsruhe, Germany; ^{1}H 400.132 MHz). Spectra processing was done using Bruker TopSpin v3.2 software. All ^{1}H experiments were performed with 256 scans, the relaxation delay between two scans was set to 10 s. The flip angle was set to 90° and the sample spinning frequency was 20 Hz. Furthermore, the temperature was kept constant at 300 K.

Melting points were determined using a “melting point meter MPM-H2” (Marienfeld-Superior, Lauda-Königshofen, Germany).

3.4.2.2.2 Capillary Electrophoresis

Capillary electrophoresis runs were performed by means of a Beckman P/ACE MDQ system (Beckman Coulter, Fullerton, USA) equipped with a diode array detector. Uncoated fused silica capillaries, purchased from BGB Analytik (Schloßböckelheim, Germany), with an effective length of 50.0 cm (total length 60.2 cm) and an internal diameter of 50 µm were used for CE separations.

Capillary preconditioning was performed by rinsing 5 min with H_2O, 30 min with 0.1 M NaOH, 2 min with H_2O, 15 min with 0.1 M HCl, and 10 min with H_2O. Before each run the capillary was preconditioned by rinsing with H_2O (2 min) and background electrolyte (5 min).

To ensure a total desorption of TBA^+ cations from the silanol groups, a long rinsing procedure according to [27] was used. Furthermore, the success of the desorption was controlled by means of an electroosmotic flow (EOF) test run (migration time of a 0.25 % (V/V) DMSO solution in water, separated in a 50 mmol/L phosphate buffer pH 7.4, voltage 20 kV).

Between each run the following rinsing procedure was carried out: EOF test run, 1 min H_2O, 2 min 1.0 M NaOH, 10 min 1.0 M NaOH applying 30 kV, EOF test run, 1 min H_2O, 2 min 1.0 M NaOH, 10 min 1.0 M NaOH applying 30 kV, EOF test run. Each rinsing step was carried out applying a pressure of 30.0 psi. Furthermore, samples were injected at the anodic side of the capillary using 5.0 psi for 5.0 s. The separations were performed applying 25 kV (current approximately 95 µA using a 75 mmol/L phosphate buffer pH 1.5 containing 125 mmol/L AAIL and 30 mmol/L β-CD) and the capillary temperature was kept constant at 25 °C. The analytes were detected at a wavelength of 200 and 215 nm. All separation runs were performed in duplicate.

DoE experiment calculations were performed using STATISTICA (Version 12, StatSoft, Hamburg, Germany).

3.4.2.3 Preparation of Amino Acid Based Ionic Liquids

For the preparation of the AAILs the exact concentration of the TBAOH solution was determined by potentiometric titration with a 0.02 mol/L benzoic acid solution. The amino acids were diluted in a volume of TBAOH solution yielding to an equimolar ratio of cation and anion. Subsequently, the solutions were lyophilized to remove water. The contents and the composition of the AAILs were checked by qNMR according to [28].

Contents of at least 95 % AAIL demonstrated the successful remove of water by lyophilisation. With the exception of TBA-L-Arg (melting point: 227.5 °C) all AAILs were liquid at room temperature.

3.4.2.4 Preparation of Solutions

Racemic sample solutions were prepared by dissolving 5.0 mg of each enantiomer in 10 mL water (concentration 0.5 mg/mL). A 75 mmol/L phosphate buffer was prepared by adding 75 mmol/L phosphoric acid solution to a 10.35 g/L solution of sodium dihydrogen phosphate monohydrate until the appropriate pH value was achieved. Subsequently, different amounts of β-CD and of the AAILs were added to the buffer. Samples and buffers were filtered through a

0.2 µm cellulose acetate syringe filter (VWR, Darmstadt, Germany) and were stored at 8 °C. Ultra-pure water, delivered by a “Milli-Q Synthesis” water purification system (Merck Millipore, Schwalbach, Germany), was used for the preparation of all sample and buffer solutions.

3.4.3 Results and Discussion

Previous capillary zone electrophoresis [29, 30] and microemulsion electrokinetic chromatography [31] studies were performed using various β-CD derivatives for the separation of phenethylamine enantiomers. The best results were obtained employing the negatively charged *heptakis*(2,3-di-*O*-acetyl)β-CD [29]. Furthermore, separations of ephedrine, pseudoephedrine, and methylephedrine enantiomers using native β-CD were reported adding the IL TBAC to the BGE [27]. In order to investigate the influence of the anionic part of TBA^+ ILs on the separation, various amino acid based TBA^+ salts were prepared and applied as BGE additive in this study.

3.4.3.1 Optimization of AAIL Concentration, pH and β-Cyclodextrin Concentration

A 75 mmol/L phosphate buffer was reported to be beneficial for the enantioseparation of phenethylamines using TBA^+ cations as BGE additive [27]. Herein, combinations of TBA^+ cations with different amino acid based anions were employed. DoE experiments were carried out for the determination of best separation conditions.

Using the chiral resolution of phenethylamine analytes as response, the influence of AAIL concentration and buffer pH on the enantioseparation was investigated by means of a three-level full-factorial design [32]. In preliminary experiments, carried out using a 75 mmol/L phosphate buffer containing 15 mmol/L β-CD, AAIL concentrations higher than 150 mmol/L and pH values lower than 1.2 showed unfavorable long migration times ($t > 90$ min). Furthermore, at AAIL concentration lower than 100 mmol/L and pH values higher than 2.2 a decrease of resolution between phenethylamine enantiomers was observed. Thus, the concentrations of the AAILs were varied from 100 to 150 mmol/L and the pH values of the phosphate buffer were increased from 1.2 to 2.2, while the concentration of β-CD was kept constant at 15 mmol/L.

Regarding all AAILs, excepting those based on amino acids containing basic side chains, the calculated optimum separation conditions were very similar. An AAIL concentration of 125 mmol/L and a pH value of 1.5 were found to be favorable for enantioseparations in a 75 mmol/L phosphate buffer containing 15 mmol/L β-CD. The results displayed in Figure 1 for TBA-Gly, TBA-L-Ala, TBA-L-Thr, and TBA-L-Asn were comparable to those observed for TBA-L-Val, TBA-L-Leu, TBA-L-Ile, TBA-L-Ser, and TBA-L-Gln.

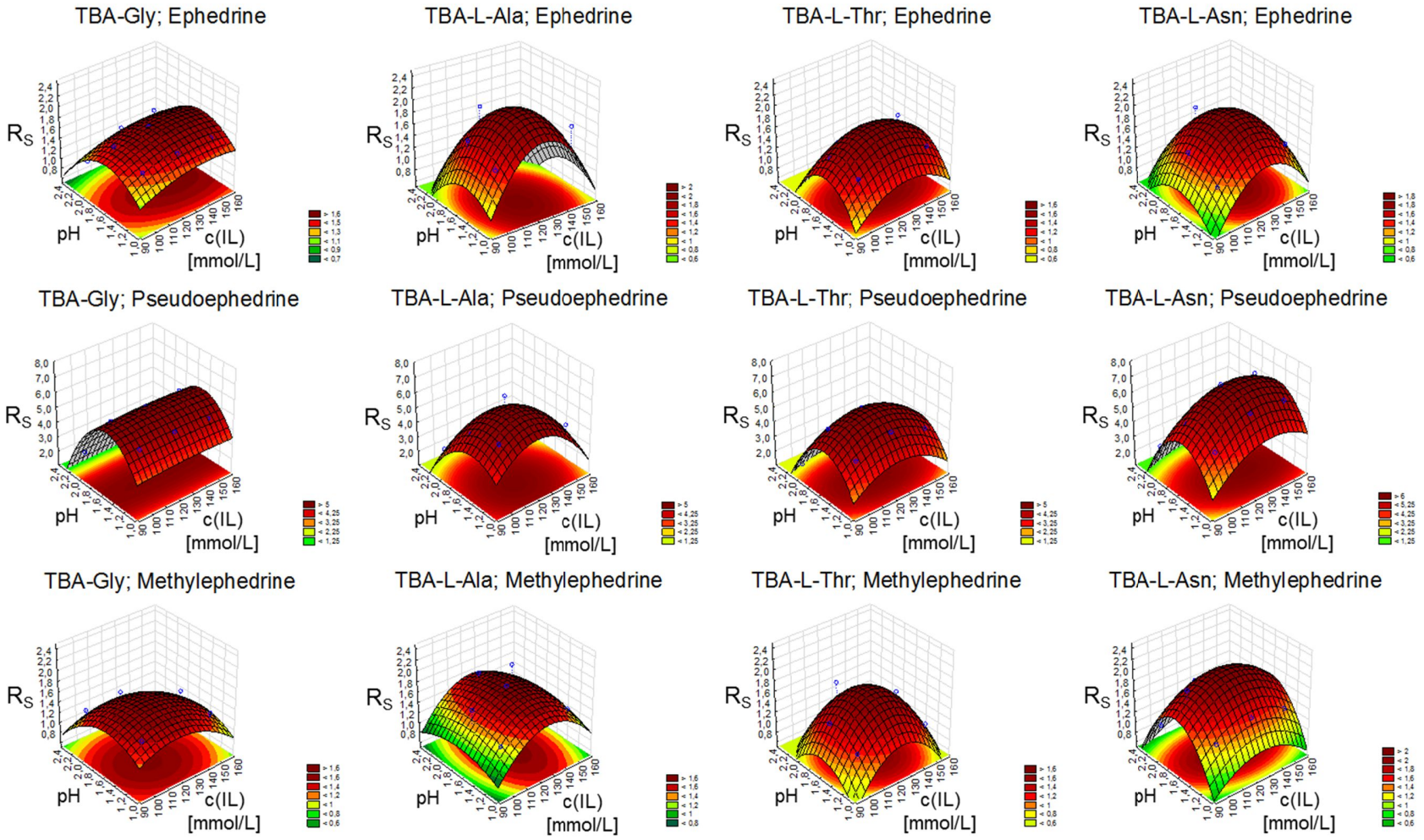

Figure 1 Design of experiments results for the optimization of background electrolyte pH value and AAIL concentration using different AAILs in a 75 mmol/L phosphate buffer containing 15 mmol/L β-CD

Due to a poor solubility at pH 1.7 (maximum solubility: 100 mmol/L), the DoE approach mentioned above was not feasible using AAILs containing an amino acid anion with an acidic side chain, like TBA-L-Asp and TBA-L-Glu. Furthermore, no UV signals were detected adding the sulfhydryl group containing amino acids TBA-L-Cys and TBA-L-Met to the BGE.

TBA-L-Pro and TBA^+ salts from basic amino acids (TBA-L-Lys, TBA-L-Arg) showed best separation results at pH values lower than 1.2 and AAIL concentrations higher than 150 mmol/L.

An increase of the β-CD solubility by the addition of TBA^+ cations to a phosphate buffer was reported before [27]. Thus, subsequently, a further improvement of the enantioseparation of ephedrine, pseudoephedrine, and methylephedrine was achieved by increasing the concentration of β-CD to 30 mmol/L. Of note, increasing the concentration of β-CD led to a shift of the best separation conditions for TBA-L-Pro, TBA-L-Lys, and TBA-L-Arg to a AAIL concentration of 125 mmol/L and a pH of 1.5 (see Table 1). Furthermore, the increase of the β-CD concentration yielded an improved solubility of TBA-L-Glu, while no enhancement of the TBA-L-Asp solubility was observed. Thus, the comparison of chiral resolutions using different TBA^+ salts combined with anions based on amino acids was performed using a buffer containing 30 mmol/L β-CD and 125 mmol/L AAIL at pH 1.5.

Table 1 Chiral resolutions R_S of phenethylamine enantiomers using different concentrations of TBA-L-Arg, β-CD, and different pH values

	15 mmol/L β-CD		30 mmol/L β-CD	
	125 mM TBA-L-Arg, pH 1.7	150 mM TBA-L-Arg, pH 1.2	125 mM TBA-L-Arg, pH 1.5	150 mM TBA-L-Arg, pH 1.2
Ephedrine	1.9	2.4	3.6	3.1
Pseudoephedrine	4.1	7.8	12.2	10.7
Methylephedrine	1.7	2.2	3.3	3.2

3.4.3.2 Comparison of Different Tetrabutylammonium Amino Acid Salts

Table 2 and Figure 2 display the R_S values for phenethylamine enantiomer separations using 30 mmol/L β-CD and 125 mmol/L AAIL in a 75 mmol/L phosphate buffer pH 1.5. The best enantioseparations were achieved adding TBA^+ salts from amino acids containing basic (TBA-L-Arg, TBA-L-Lys) and carboxamide (TBA-L-Gln, TBA-L-Asn) functional groups to the BGE.

The influence of AAILs on the enantioseparation of phenethylamines was investigated by comparing chiral resolutions achieved by adding different combinations of β-CD, TBAC,

Table 2 Chiral resolutions R_S between phenethylamine enantiomers using 125 mmol/L AAIL (Separation conditions: 30 mmol/L β-CD, 75 mmol/L phosphate buffer pH 1.5)

	Chiral Resolution R_S		
	Ephedrine	Pseudoephedrine	Methylephedrine
TBA-Gly	2.2	8.2	2.1
TBA-L-Ala	2.9	9.4	2.3
TBA-L-Val	2.4	7.2	2.2
TBA-L-Leu	2.3	7.7	2.1
TBA-L-Ile	2.3	7.5	2.5
TBA-L-Ser	2.2	7.0	2.1
TBA-L-Thr	2.6	9.2	2.4
TBA-L-Glu	2.2	8.0	1.9
TBA-L-Pro	2.7	8.5	2.8
TBA-L-Asn	2.6	8.5	2.5
TBA-L-Gln	2.8	10.0	2.4
TBA-L-Lys	3.2	9.6	3.2
TBA-L-Arg	3.6	12.2	3.3

TBA-L-Arg, and L-Arg to the BGE (Table 3). Adding solely β-CD to a 75 mmol/L phosphate buffer pH 1.5 gave R_S values of < 1.0 for ephedrine, 1.9 for pseudoephedrine and < 1.0 for methylephedrine. The chiral separation was slightly enhanced by using L-Arg as additive to the β-CD containing BGE. The increased R_S values (< 1.0 for ephedrine, 2.5 for pseudoephedrine, 1.4 for methylephedrine) indicate a synergistic effect between β-CD and L-Arg on the enantioseparations. A possible explanation for this synergy is an influence of L-Arg on the inclusion of analytes into the β-CD cavity. A change of complex stabilities between β-CD and omeprazole [33], and between hydroxypropyl-β-CD and nateglinide [34] by the addition of Arg to the binary systems was reported before. A further explanation for the enhanced enantioseparation might be the adsorption of positively charged L-Arg cations to the capillary surface repelling

Table 3 Comparison of chiral resolutions R_S between phenethylamine enantiomers using different combinations of β-CD, TBAC, TBA-L-Arg, and L-Arg in a 75 mmol/L phosphate buffer pH 1.5

	Chiral Resolution R_S		
	Ephedrine	Pseudoephedrine	Methylephedrine
30 mmol/L β-CD	< 1.0	1.9	< 1.0
125 mmol/L TBAC, 30 mmol/L β-CD	2.6	9.3	2.6
125 mmol/L TBA-L-Arg, 30 mmol/L β-CD	3.6	12.2	3.3
125 mmol/L TBA-L-Arg	no separation	no separation	no separation
125 mmol/L L-Arg, 30 mmol/L β-CD	< 1.0	2.5	1.4
125 mmol/L L-Arg	no separation	no separation	no separation

cationic ephedrine derivatives. Due to a lesser extent of phenethylamine adsorption to the capillary surface, the analyte peaks get narrower and the chiral resolution increases.

As can be seen from Table 3, an enormous increase of R_S values was observed by the addition of TBA^+ salts to the BGE. A possible reason for the achieved enhancement is a competition between the TBA^+ cations and the positively charged analyte enantiomers for the inclusion into the β-CD cavity. Both, the interaction between β-CD and TBA^+ cations [35] and between β-CD and phenethylamine enantiomers [18, 20, 21, 25] were reported before. A further explanation for the increase of the chiral resolution by the addition of TBA^+ based ILs to the BGE is the adsorption of TBA^+ cations to the silanol groups at the capillary surface. Even at low pH values (pH 1.2 and 1.5) a prolongation of migration times was observed by increasing the AAIL concentration, which indicates a decrease or reversal of the EOF, due to the adsorption of TBA^+ cations to the capillary surface. The increasing migration times result in a longer period of time for the separation of temporarily formed diastereomeric complexes between analyte enantiomers and β-CD. On the other hand, one drawback, occurring upon the application of TBA^+ based salts in CE, is the prolongation of migration times. As can be seen from Figure 3, run times of 70 min were necessary to achieve the best enantioseparations of ephedrine, pseudoephedrine, and methylephedrine.

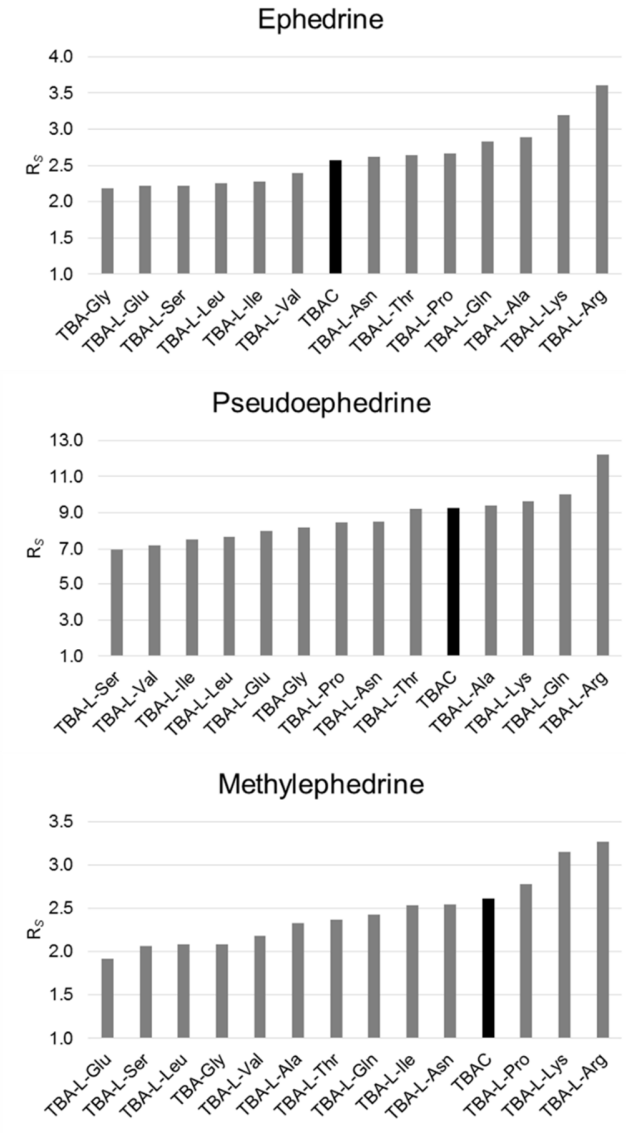

Figure 2 Chiral resolutions between phenethylamine analytes using different AAIL as background electrolyte additive, results for the achiral IL TBAC highlighted (Separation conditions: 125 mmol/L AAIL, 30 mmol/L β-CD, 75 mmol/L phosphate buffer pH 1.5)

For the investigation of the influence of different IL anions on the enantioseparation, runs using achiral TBAC as BGE additive were carried out and compared with the results obtained by the application of chiral AAILs. The chiral resolutions using TBAC and TBA-L-Arg as buffer additive are

displayed in Figure 2 and Table 3. As can be seen from Table 3, the addition of TBA-L-Arg yielded an enhancement of the chiral resolution compared to runs using TBAC only. This observation also indicates the synergistic effect between β-CD and L-Arg on the separation of phenethylamine enantiomers. Nevertheless, this synergy was only observed when using TBA^+ combined with amino acid anions containing basic functional groups (L-Arg and L-Lys), while most other investigated AAILs showed chiral resolutions inferior to those achieved by the addition of TBAC to the BGE (Figure 2).

Furthermore, no enantioseparation of ephedrine derivatives was achieved adding L-Arg and TBA-L-Arg to a BGE containing no β-CD (see Table 3), indicating that the chiral separation of ephedrine derivatives is not caused by the formation of diastereomeric complexes between L-Arg and phenethylamine enantiomers. For the enantioseparation the participation of β-CD in the complexation seems to be a conditio sine qua non.

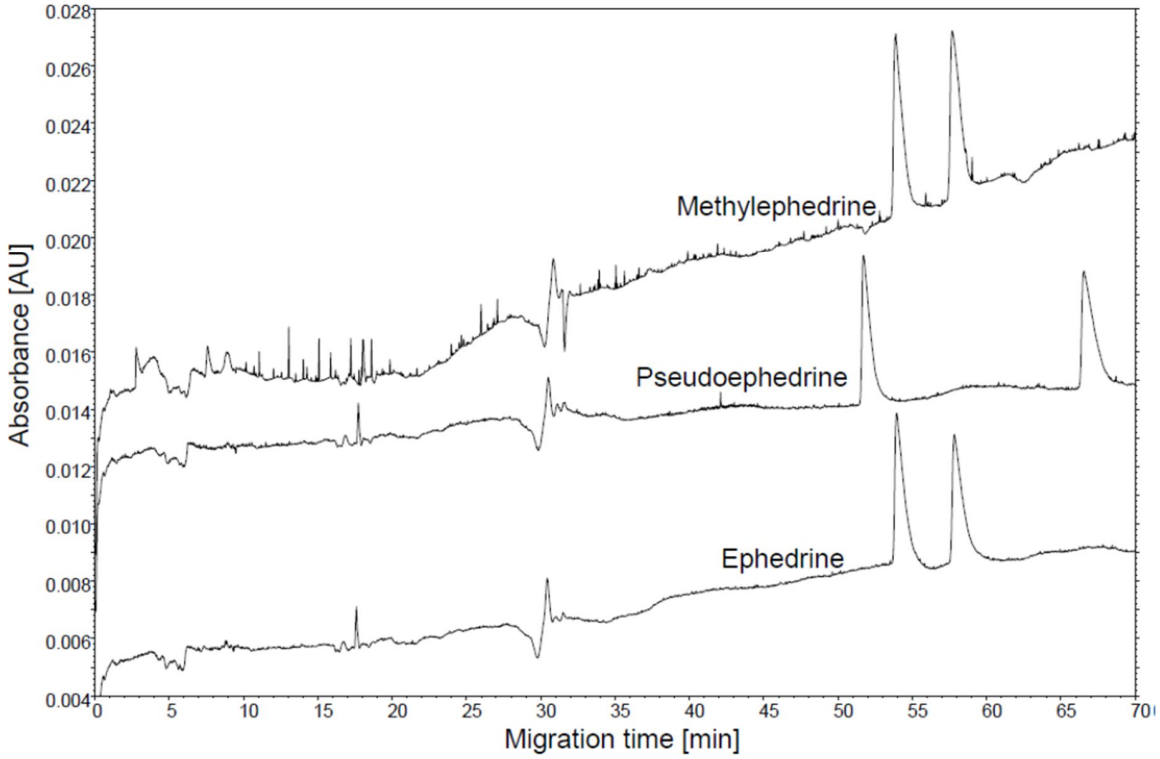

Figure 3 Electropherogramms displaying the enantioseparation of ephedrine, pseudoephedrine and methylephedrine using TBA-L-Arg (Separation conditions: 125 mmol/L TBA-L-Arg, 30 mmol/L β-CD, 75 mmol/L phosphate buffer pH 1.5)

Regarding all applied AAILs, the best results for the enantioseparation of all phenethylamines were achieved using TBA-L-Arg (separations applying β-CD and TBA-L-Arg as BGE additive are displayed in Figure 3). R_S values for enantioseparations were 3.6 for ephedrine, 12.2 for

pseudoephedrine, and 3.3 for methylephedrine. To the best of our knowledge, no better enantioseparations of phenethylamine enantiomers were reported in literature using native β-CD as chiral selector [27]. Better R_S values for ephedrine [29, 31], pseudoephedrine [27, 29, 36] and methylephedrine [29, 31] have only been published applying more expensive CD derivatives.

Nevertheless, the improved chiral separations, reported herein, were achieved at the cost of long migration times. Applying basic AAILs (TBA-L-Arg and TBA-L-Lys) yielded migration times of 70 min, while adding 125 mmol/L of all other reported AAILs to a 75 mmol/L phosphate buffer pH 1.5 containing 30 mmol/L β-CD gave migration times of about 50 min for the slowest migrating phenethylamine enantiomer.

3.4.4 Concluding Remarks

The influence of the anionic part of ILs on the enantioseparation of phenethylamines was investigated by using different amino acid based anions in combination with TBA^+ cations as BGE additive in CE. AAILs with different proteinogenic L-amino acids were prepared and tested as additives to phosphate buffers containing β-CD as chiral selector.

A DoE approach was performed to identify the best separation conditions, like pH value and AAIL concentration. Best separation results were observed by adding AAILs with basic (TBA-L-Arg, TBA-L-Lys) and carboxamide (TBA-L-Gln, TBA-L-Asn) functional groups to a 75 mmol/L phosphate buffer pH 1.5 containing 30 mmol/L β-CD.

A synergy between L-Arg and β-CD, the adsorption of TBA^+ cations to the capillary surface, and a competition between TBA^+ cations and analyte enantiomers for the inclusion into the β-CD cavity were found to be responsible for the enhancement of the phenethylamine enantioseparations by the addition of TBA-L-Arg to the BGE.

Acknowledgement

The authors would like to thank Alexander Becht and Patrick Suppinger for supporting qNMR measurements.

Conflict of Interest Statement

None of the authors of this paper does have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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4 Final Discussion

4.1 Tetrabutylammonium Based Ionic Liquids in Phenethylamine Enantioseparation

The aim of this work was to investigate the influence of ILs, used as BGE additives, on the enantioseparation of phenethylamine enantiomers in CE. ILs based on TBA^+ cations combined with different anions were added to buffers containing different β-CD derivatives as chiral selector. CE methods separating the enantiomers of ephedrine, pseudoephedrine, methylephedrine, and norephedrine were developed and the influences of buffer concentration, pH value, CD concentration, and IL concentration on the chiral resolutions were investigated.

Compared to enantioseparations performed in an ammonium formate buffer the use of sodium phosphate buffers showed advantages like better reproducibility and better chiral separation of phenethylamines. A phosphate buffer concentration of 75 mmol/L was found to be beneficial for the separation of ephedrine derivative enantiomers in buffers containing TBA based ILs. Furthermore, best enantioseparations were achieved at acidic pH values (pH < 3.0). At these low pH values an increasing amount of unionized silanol groups results in a decrease of the EOF and consequently a prolongation of migration times. Due to the increased migration times, the period of time for the enantioseparation of diastereomeric analyte CD complexes increases and results in an enhancement of the chiral separation. A further advantage of a decreased amount of negatively charged silanol groups at acidic pH values is the reduction of the adsorption of positively charged phenethylamine molecules to the capillary surface. Due to enhanced peak shapes and reduced peak widths, the decline of interactions between analyte cations and capillary surface leads to an increase of chiral resolution values.

Subsequently, the concentration of TBA based ILs was optimized. A concentration of 125 mmol/L IL added to BGEs containing different β-CD derivatives was found to yield best enantioseparations of phenethylamine analytes. When the IL concentration was increased from 0 to 125 mmol/L a continuous enhancement of the enantioseparation and prolongation of migration times were observed. Due to peak broadening, increasing the concentration of TBA^+ salts higher than 125 mmol/L resulted in a decrease of chiral resolution. Three different effects, explaining the improved chiral separation of phenethylamines using TBA based ILs as BGE additive, were found. First, an improved β-CD solubility in buffers containing TBA^+ salts was observed, which enables the use of much higher β-CD concentrations for chiral separation. Secondly, a complex formation between CDs and TBA^+ cations (proven for β-CD [1]) was found

to have a positive influence on the separation of phenethylamine enantiomers. A competition between positively charged analyte enantiomers and TBA^+ cations for the inclusion into CD cavities seems to play an important part in the enhancement of the chiral resolution. The third reason, explaining increasing R_S values by the addition of TBA^+ based ILs to the BGE, is the adsorption of TBA^+ cations to the negatively charged silanol groups at the capillary surface. Similar to effects observed by a decrease of the pH value, reported above, the adsorption of TBA^+ cations to the capillary surface prolongs the period of time for the separation of diastereomeric CD analyte inclusion complexes and reduces peak broadening, due to the adsorption of phenethylamine cations to negatively charged silanol groups.

Two drawbacks occurring during the application of ILs as BGE additives in CE are also caused by the adsorption of TBA^+ cations to the capillary surface. Due to a reduction or even a reversal of the EOF at higher IL concentrations, the run times for the enantioseparation of ephedrine derivatives were prolonged up to 50-70 minutes. Secondly, the addition of ILs to the BGE results in a bad reproducibility of CE runs. An approach trying to create a constant dynamical capillary surface coating with TBAC gave strong fluctuations of the migration times. Thus, the aim of a second approach was the development of a rinsing procedure ensuring the total desorption of IL cations from the capillary surface before the beginning of a new separation run. A rinsing procedure applying an electrical voltage of 30 kV on a capillary containing a 1.0 mol/L NaOH solution was found to give reproducible separation runs with the advantage of shortened run times.

Furthermore, the influence of the anionic part of TBA based ILs on the enantioseparation of phenethylamines was investigated applying proteinogenic amino acids as IL anions. The idea behind the use of amino acid based ILs was the addition of a further chiral selector to the BGE, which was though to result in an enhancement of the phenethylamine enantioseparations. Compared to separations performed with achiral TBAC, only the application of the basic amino acid anions argininate and lysinate yielded improved chiral separations. In particular, the use of argininate as TBA^+ counterion gave very good enantioseparations, which might be explained by an effect of arginine on the formation of β-CD analyte complexes [2, 3].

Additionally, the influence of the application of different β-CD derivatives (HP-β-CD, Me-β-CD, Mal-β-CD) on the chiral separation of ephedrine derivatives in buffers containing TBA based ILs was investigated. A baseline separation of ephedrine, pseudoephedrine, and methylephedrine enantiomers was achieved using native β-CD as chiral selector. Nevertheless, the enantioseparation of norephedrine was impossible using underivatized β-CD. The same results (baseline separation of ephedrine, pseudoephedrine, methylephedrine; no separation of norephedrine) were achieved applying the β-CD derivatives HP-β-CD and Mal-β-CD. A baseline separation of norephedrine enantiomers in a TBA^+ containing phosphate buffer was only feasible

using Me-β-CD as chiral selector. One the other hand, the methylated β-CD derivative failed to separate the enantiomers of ephedrine. Thus, the enantioseparation of all four investigated phenethylamines in one run applying a single chiral selector was impossible.

A comparison between the best chiral resolution values for the enantioseparation of ephedrine derivatives reported in literature and those achieved applying the optimized separation methods using TBA based ILs as buffer additives is given in Table 1. As can be seen, with the drawback of a prolongation of migration times, very good chiral resolution values between phenethylamine enantiomers were achieved by the addition of TBA based ILs to phosphate buffers.

Comparing the results of the separation of ephedrine enantiomers achieved applying TBA based ILs as BGE additives with separations reported in literature, better chiral resolutions have only been reported using expensive negatively charged CD derivatives, such as HDAS [8], CM-β-CD [4], and sulfated γ-CDs [6]. The application of ILs in combination with native β-CD yielded results even superior to those reported using sulfated β-CD as chiral selector in a phosphate buffer pH 3.2 [5].

Regarding the enantioseparation of pseudoephedrine, the use of TBAC as BGE additive gave the highest ever reported chiral resolution values. Results superior to those published for negatively charged sulfated β-CD derivatives were achieved applying native β-CD or Me-β-CD as chiral selector [5, 6, 8, 10]. Interestingly, Quang and Khaledi reported a method using different β-CD derivatives in a tetramethylammonium phosphate buffer, which gave excellent separations of pseudoephedrine enantiomers in shorter run times [11].

By the addition of TBAC and TBA-L-Arg to BGEs containing native β-CD separations of methylephedrine enantiomers comparable to those reported for the application of sulfated β-CD [5] and DM-β-CD [9] were achieved. Better chiral resolutions were only reported using HDAS or applying a MEEKC method with sulfated β-CD as chiral selector [10].

Interestingly, a baseline separation of norephedrine enantiomers seems to be impossible employing native β-CD and a great number of uncharged β-CD derivatives. Only methods using methylated uncharged β-CD derivatives (Me-β-CD or DM-β-CD) [9, 11] and negatively charged CDs [5, 6, 10] were reported to yield chiral separation of norephedrine. Furthermore, as can be seen from Table 1, all separations applying methylated β-CD derivatives were performed in buffers containing sterically demanding cations (tetramethylammonium, Tris, tetrabutylammonium) at low pH values. A reduction of the EOF by the protonation of silanol groups and the adsorption of buffer cations to the capillary surface seems to be crucial for the enantioseparation of norephedrine. Regarding the separation of norephedrine enantiomers, the

Table 1 Summary of phenethylamine enantioseparations reported in literature

Separation conditions	Chiral selector	R_S	Migration time	Reference
Ephedrine				
75 mmol/L Phosphate pH 2.5; 125 mmol/L TBAC	35 mmol/L β-CD	3.3	50 min	Chapter 3.2
75 mmol/L Phosphate pH 1.5; 125 mmol/L TBA-L-Arg	30 mmol/L β-CD	3.6	65 min	Chapter 3.3
100 mmol/L Phosphate/Triethanolamine pH 3.0	15 mmol/L CM-β-CD	6.3	20 min	[4]
10 mmol/L Phosphate pH 3.2	2 mmol/L sulfated β-CD	2.2	15 min	[5]
90 % 13.4 mmol/L Phosphate, pH 8.5; 10 % Methanol	10 mmol/L sulfated γ-CD	10.8	not reported	[6]
100 mmol/L Phosphate pH 9.0	15 mmol/L β-CD	1.4	not reported	[7]
50 mmol/L Phosphate pH 3.0	12 mmol/L HDAS	16.9	not reported	[8]
75 mmol/L Tris pH 2.5	40 mmol/L DM-β-CD	2.9	25 min	[9]
0.5 % Ethyl acetate, 1.0 % SDS, 4.0 % 1-Butanol, 3.0 % Propan-2-ol, 91.5 % 20 mmol/L Phosphate pH 2.5	4 % sulfated β-CD	4.0	20 min	[10]
Pseudoephedrine				
75 mmol/L Phosphate buffer pH 2.5; 125 mmol/L TBAC	60 mmol/L Me-β-CD	19.4	50 min	Chapter 3.2
75 mmol/L Phosphate buffer pH 1.5; 125 mmol/L TBA-L-Arg	30 mmol/L β-CD	12.2	70 min	Chapter 3.3
50 mmol/L Tetramethylammonium phosphate pH 2.5	20 mmol/L β-CD 20 mmol/L DM-β-CD 20 mmol/L HP-β-CD	5.7 5.9 8.7	20 min 15 min 15 min	[11]
10 mmol/L Phosphate pH 3.2	2 mmol/L sulfated β-CD	6.4	15 min	[5]
90 % 13.4 mmol/L Phosphate, pH 8.5; 10 % Methanol	10 mmol/L sulfobutyl-ether-β-CD	12.6	10 min	[6]
50 mmol/L Phosphate pH 3.0	12 mmol/L HDAS	12.6	not reported	[8]
0.5 % Ethyl acetate, 1.0 % SDS, 4.0 % 1-Butanol, 3.0 % Propan-2-ol, 91.5 % 20 mmol/L Phosphate pH 2.5	4 % sulfated β-CD	5.7	20 min	[10]
Methylephedrine				
75 mmol/L Phosphate buffer pH 2.5; 125 mmol/L TBAC	35 mmol/L β-CD	2.7	50 min	Chapter 3.2
75 mmol/L Phosphate buffer pH 1.5; 125 mmol/L TBA-L-Arg	30 mmol/L β-CD	3.3	65 min	Chapter 3.3
10 mmol/L Phosphate pH 3.2	2 mmol/L sulfated β-CD	2.2	not reported	[5]
75 mmol/L Tris pH 2.5	40 mmol/L DM-β-CD	2.9	25 min	[9]
50 mmol/L Phosphate pH 3.0	3 mmol/L HDAS	12.4	20 min	[10]
0.5 % Ethyl acetate, 1.0 % SDS, 4.0 % 1-Butanol, 3.0 % Propan-2-ol, 91.5 % 20 mmol/L Phosphate pH 2.5	4 % sulfated β-CD	6.0	20 min	[10]
Norephedrine				
75 mmol/L Phosphate buffer pH 2.5; 125 mmol/L TBAC	60 mmol/L Me-β-CD	3.4	45 min	Chapter 3.2
50 mmol/L Tetramethylammonium phosphate pH 2.5	20 mmol/L DM-β-CD	1.9	15 min	[11]
10 mmol/L Phosphate pH 3.2	2 mmol/L sulfated β-CD	3.4	15 min	[5]
90 % 13.4 mmol/L Phosphate, pH 8.5; 10 % Methanol	10 mmol/L sulfated γ-CD	23.0	10 min	[6]
75 mmol/L Tris pH 2.5	40 mmol/L DM-β-CD	4.0	20 min	[9]
50 mmol/L Phosphate pH 3.0	3 mmol/L HDAS	8.2	20 min	[10]
0.5 % Ethyl acetate, 1.0 % SDS, 4.0 % 1-Butanol, 3.0 % Propan-2-ol, 91.5 % 20 mmol/L Phosphate pH 2.5	4 % sulfated β-CD	5.0	25 min	[10]

CM-β-CD: Carboxymethyl-β-cyclodextrin
DM-β-CD: Dimethyl-β-cyclodextrin
HDAS: *heptakis*(2,3-*O*-diacetyl-6-sulfo)β-CD

influence of the EOF reduction seems to be more important than for the enantioseparation of all other phenethylamines, because shortest migration times have always been observed for norephedrine. The short migration times result in a shorter period of time for the separation of temporarily formed diastereomeric complexes between norephedrine enantiomers and CDs. Thus, especially for norephedrine, the reduction of the EOF by the adsorption of IL cations to the capillary surface results in a significant improvement of the enantioseparation.

4.2 Investigation of Complexes between Cyclodextrins and Phenethylamine Enantiomers

The binding constants and the electrophoretic mobilities of complexes between phenethylamines and different β-CD derivatives were determined by affinity capillary electrophoresis. Furthermore, the influence of these two values on the enantioseparation of ephedrine derivatives was investigated. Comparisons between the achieved chiral resolution values and the calculated binding constants or complex mobilities revealed a strong correlation between the enantioseparation and the difference of the electrophoretic mobilities of diastereomeric complexes. While complexes between phenethylamine enantiomers and CDs with higher mobility differences yielded higher chiral resolution values, no correlation between complex strengths or complex strength differences and chiral resolution was found. Nevertheless, neither the determination of complex formation strengths nor the calculation of complex mobility differences gave indications useful for the prediction whether a CD derivative is able to enantioseparate phenethylamines or not.

Furthermore, the correctness of binding constants determined by CE was validated by ITC. The K values of ephedrine enantiomers were comparable to those calculated by ACE, which proved the applicability of CE for the characterization of complexes between phenethylamine enantiomers and CDs.

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5 Summary

Two chiral chemical molecules being mirror images of each other, also referred to as enantiomers, may have different pharmacokinetic, pharmacodynamic, and toxicological effects. Thus, pharmaceutical manufacturers and authorities are increasingly interested in the approval of enantiopure drugs. However, the isomeric purity and the limits for isomeric impurities have to be specified applying enantioselective analytical methods, such as capillary electrophoresis.

The separation of enantiomers in capillary electrophoresis may be improved by the addition of ionic liquids to the background electrolyte. The aim of this work was to investigate the influence of different separation conditions on the enantioseparation of phenethylamines in background electrolytes containing ionic liquids based on tetrabutylammonium cations.

Best chiral separations were achieved at acidic pH values using phosphate buffers containing 125 mmol/L tetrabutylammonium based salts. Different reasons explaining enhanced enantioseparations in buffers containing ionic liquids were found. First, due to an improvement of the cyclodextrin solubility, the addition of ionic liquids to the background electrolyte enables the use of higher concentrations of these chiral selector. Furthermore, the adsorption of tetrabutylammonium cations to the negatively charged capillary surface results in a reduction of the electroosmotic flow. Hence, the resulting prolongation of migration times leads to a longer period of time for the separation of temporarily formed diastereomeric analyte cyclodextrin complexes, which yields improved enantioseparation. Additionally, due to a decrease of the adsorption of positively charged phenethylamine analyte molecules to capillary surface silanol groups, the adsorption of ionic liquid cations inhibits peak broadening. A further reason explaining an enhanced enantioseparation by the addition of ionic liquids to the background electrolyte is a competition between tetrabutylammonium cations and analyte enantiomers for the inclusion into cyclodextrin cavities.

Furthermore, the influence of different chiral counterions, combined with tetrabutylammonium cations, on the enantioseparation of phenethylamines was investigated. Solely anions based on the basic proteinogenic amino acids L-lysine and L-arginine yielded chiral separation results superior to those achieved using achiral tetrabutylammonium chloride as background electrolyte additive. Especially the application of tetrabutylammonium L-argininate gave very good enantioseparations of all investigated ephedrine derivatives, which might be explained by the ability of L-arginine to affect the formation of complexes between analytes and cyclodextrins.

Besides the investigation of the influence of ionic liquids on the enantioseparation, complexes between phenethylamine enantiomers and β-cyclodextrin derivatives were characterized by affinity capillary electrophoresis. The binding constants between analyte enantiomers and cyclodextrins and the electrophoretic mobilities of the temporarily formed complexes were determined and compared to the observed chiral resolution values. While neither the calculated binding constants nor their differences correlated with the quality of the enantioseparation, a strong correlation between the differences of the electrophoretic mobilities of the complexes and the chiral resolution values was found.

6 Zusammenfassung

Chemische Moleküle, die sich zueinander wie Bild und Spiegelbild verhalten, so genannte Enantiomere, können im menschlichen Organismus unterschiedliche pharmakodynamische und toxikologische Wirkungen zeigen. Aus diesem Grund legen pharmazeutische Unternehmen und Arzneimittelbehörden vermehrten Wert auf die Zulassung enantiomerenreiner Arzneistoffe. Da sowohl die Reinheit eines Enantiomers als auch der Gehalt an isomeren Verunreinigungen spezifiziert werden müssen, besteht ein zunehmender Bedarf an analytischen Methoden zur Enantiomerentrennung, wie zum Beispiel der Kapillarelektrophorese.

Das Ziel dieser Arbeit war die Verbesserung der kapillarelektrophoretischen Enantiomerentrennung von Ephedrin-Derivaten unter Zuhilfenahme von auf Tetrabutylammonium basierenden Ionische Flüssigkeiten. Der Einfluss diverser Parameter auf die Trennung von Phenethylamin-Enantiomeren in Puffern, die Ionische Flüssigkeiten enthalten, wurde systematisch untersucht.

Dabei konnten die besten Trennergebnisse unter stark sauren Bedingungen in Phosphatpuffern, die 125 mmol/L Tetrabutylammonium-Salze enthielten, erreicht werden. Verschiedene Faktoren, die zu einer Verbesserung der Enantiomerentrennung führten, konnten festgestellt werden. Erstens wurde eine Verbesserung der Cyclodextrin-Löslichkeit durch die Zugabe von Ionischen Flüssigkeiten zum Trennpuffer festgestellt. Dies ermöglicht eine Verwendung höherer Konzentrationen dieser chiralen Selektoren. Des Weiteren führt eine Anlagerung von Tetrabutylammonium-Kationen an die negativ geladene Oberfläche der Kapillare zu einer Reduktion des elektroosmotischen Flusses. Daraus resultiert einerseits eine Verlängerung der Migrationszeiten, die bewirkt, dass eine längere Zeit zur Trennung der temporär gebildeten diastereomeren Cyclodextrin-Einlagerungskomplexe zur Verfügung steht. Andererseits wird durch die Adsorption von Tetrabutylammonium-Kationen an die Kapillarwand die Anlagerung von positiv geladenen Phenethylamin-Analyten an die Silanoloberfläche verhindert. Dies führt durch eine Reduktion der Peakbreite zu einer Verbesserung der Trennergebnisse. Als dritter Grund für verbesserte Trennungen nach Zugabe von Ionischer Flüssigkeit zum Trennpuffer kann ein kompetitiver Mechanismus zwischen Analyt-Enantiomeren und Tetrabutylammonium-Kationen um den Einschluss in Cyclodextrine aufgeführt werden.

Zusätzlich wurde der Einfluss verschiedener chiraler Gegenionen, die mit Tetrabutylammonium-Kationen kombiniert wurden, auf die Trennung von Phenethylamin-Enantiomeren untersucht. Dabei konnte ausschließlich unter Verwendung von Anionen der basischen proteinogenen Aminosäuren L-Lysin und L-Arginin eine Verbesserung der Trennung

beobachtet werden. Vor allem die Verwendung von L-Arginin, für welches eine Beeinflussung der Komplexbildung zwischen Analyten und Cyclodextrin vermutet wird, ergab eine starke Verbesserung der Trennung aller Ephedrin-Derivate.

Neben der Untersuchung des Einflusses von Ionischen Flüssigkeiten auf die kapillarelektrophoretische Trennung wurde auch die Komplexbildung zwischen Phenethylamin-Enantiomeren und verschiedenen β-Cyclodextrin-Derivaten mittels Affinitätskapillarelektrophorese untersucht. Die Bindungskonstanten zwischen Analyt-Enantiomeren und Cyclodextrinen und die elektrophoretische Mobilität der gebildeten Komplexe wurden bestimmt und mit den dabei beobachteten chiralen Trennungen verglichen. Dabei konnte eine starke Korrelation zwischen den Unterschieden in den elektrophoretischen Mobilitäten der Komplexe und der Güte der Enantiomerentrennung festgestellt werden, während kein Zusammenhang zwischen den Bindungskonstanten, beziehungsweise deren Differenzen, und der chiralen Auflösung zwischen Phenethylamin-Enantiomeren zu beobachten war.

7 Appendix

7.1 List of Publications and Documentation of Authorship

1. **Characterization of Complexes between Phenethylamine Enantiomers and β-Cyclodextrin Derivatives by Capillary Electrophoresis – Determination of Binding Constants and Complex Mobilities**
   Wahl J., Furuishi T., Yonemochi E., Meinel L., Holzgrabe U.
   Electrophoresis, Volume 38 (2017), Pages 1188-1200
2. **Ionic Liquids in Capillary Electrophoresis**
   Holzgrabe U., Wahl J.
   Methods in Molecular Biology, Volume 1483 (2016), Pages 131-153
3. **Separation of Phenethylamine Enantiomers Using Tetrabutylammonium Chloride as Ionic Liquid Background Electrolyte Additive**
   Wahl J., Holzgrabe U.
   Journal of Research Analytica, Volume 3 (2017), Pages 73-80
4. **Capillary Electrophoresis Separation of Phenethylamine Enantiomers Using Amino Acid Based Ionic Liquids**
   Wahl J., Holzgrabe U.
   Journal of Pharmaceutical and Biomedical Analysis, Volume 148 (2018), Pages 245-250

This section contains a list of the individual contribution for each author to the publications reprinted in this thesis.

P1 **Wahl J**, **Furuishi T**, **Yonemochi E**, **Meinel L**, **Holzgrabe U**, Characterization of Complexes between Phenethylamine Enantiomers and β-Cyclodextrin Derivatives by Capillary Electrophoresis – Determination of Binding Constants and Complex Mobilities Electrophoresis, 38 (2017) 1188-1200

Author	1	2	3	4	5
Study design	x				x
Experimental work – Capillary electrophoresis	x				
Experimental work – Isothermal titration calorimetry		x			
Data analysis and interpretation	x	x	x	x	x
Manuscript planning	x				x
Manuscript writing	x				
Correction of manuscript	x	x	x	x	x
Supervision of Joachim Wahl					x

P2 **Holzgrabe U**, **Wahl J**, Ionic Liquids in Capillary Electrophoresis, Methods Mol. Biol., 1483 (2016) 131-153

Author	1	2
Study design	x	x
Experimental work		x
Data analysis and interpretation	x	x
Manuscript planning	x	x
Manuscript writing		x
Correction of manuscript	x	x
Supervision of Joachim Wahl	x	

P3 **Wahl J**, **Holzgrabe U**, Separation of Phenethylamine Enantiomers Using Tetrabutylammonium Chloride as Ionic Liquid Background Electrolyte Additive, J. Res. Anal., 3 (2017) 73-80

Author	1	2
Study design	x	x
Experimental work	x	
Data analysis and interpretation	x	x
Manuscript planning	x	x
Manuscript writing	x	
Correction of manuscript	x	x
Supervision of Joachim Wahl		x

P4 **Wahl J**, **Holzgrabe U**, Capillary Electrophoresis Separation of Phenethylamine Enantiomers Using Amino Acid Based Ionic Liquids, J. Pharm. Biomed. Anal., 148 (2018) 245-250		
Author	1	2
Study design	x	x
Experimental work	x	
Data analysis and interpretation	x	x
Manuscript planning	x	x
Manuscript writing	x	
Correction of manuscript	x	x
Supervision of Joachim Wahl		x

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Die Beiträge der Mitautoren an den Publikationen sind in den vorausgehenden Tabellen aufgeführt.

Prof. Dr. Ulrike Holzgrabe

Ort, Datum

Unterschrift

Joachim Wahl

Ort, Datum

Unterschrift

7.2 Conference Contributions

Wahl J., Holzgrabe U.

Chiral separation of phenethylamines by capillary electrophoresis using tetrabutylammonium chloride as background electrolyte additive

DPhG Jahrestagung 2016, München

7.3 Abbreviations

AAIL	amino acid based ionic liquid
ACE	affinity capillary electrophoresis
β-CD	β-cyclodextrin
BGE	background electrolyte
CAD	charged aerosol detector
CD	cyclodextrin
CE	capillary electrophoresis
CIL	chiral ionic liquid
CM-β-CD	carboxymethyl-β-cyclodextrin
CTAB	cetyltrimethylammonium bromide
CZE	capillary zone electrophoresis
DAD	diode array detector
DM-β-CD	dimethyl-β-cyclodextrin
DMSO	dimethyl sulfoxide
DoE	design of experiments
ECL	electrochemiluminescence
EOF	electroosmotic flow
FDA	food and drug administration
γ-CD	γ-cyclodextrin
Gly	glycine
HDAS	*heptakis*(2,3-*O*-diacetyl-6-sulfo)β-CD
HP-β-CD	(2-hydroxypropyl)-β-cyclodextrin
HPLC	high performance liquid chromatography
IC/IA-ESI-MS	ion chromatography-ion association electrospray ionization mass spectrometry
I. D.	internal diameter
IL	ionic liquid
ITC	isothermal titration calorimetry
L-Ala	L-alanine
L-Arg	L-arginine
L-Asn	L-asparagine
L-Asp	L-aspartic acid
L-Cys	L-cysteine
L-Gln	L-glutamine
L-Glu	L-glutamic acid

L-Ile	L-isoleucine
L-Leu	L-leucine
L-Lys	L-lysine
L-Met	L-methionine
L-Pro	L-proline
L-Ser	L-serine
L-Thr	L-threonine
L-Val	L-valine
Mal-β-CD	6-O-α-maltosyl-β-cyclodextrin
MALDI-MS	matrix-assisted laser desorption ionization mass spectrometry
Me-β-CD	methyl-β-cyclodextrin
MEEKC	microemulsion electrokinetic chromatography
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry
NACE	nonaqueous capillary electrophoresis
NMR	nuclear magnetic resonance
poly-SOLV	poly(sodium oleyl-L-leucylvalinate)
RTIL	room temperature ionic liquid
SDS	sodium dodecyl sulfate
TBA^+	tetrabutylammonium
TBAC	tetrabutylammonium chloride
TBAOH	tetrabutylammonium hydroxide
TTAB	tetradecyltrimethylammonium bromide