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Review

Fundamental aspects of chiral electromigration techniques and application in pharmaceutical and biomedical analysis

Gerhard K.E. Scriba*

Friedrich Schiller University Jena, Department of Pharmaceutical Chemistry, Philosophenweg 14, 07743 Jena, Germany

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ABSTRACT

Capillary electromigration techniques are often considered ideal methods for the analysis of chiral compounds due to the high resolution power and flexibility of the technique. Therefore, especially capillary electrophoresis using a chiral selector in the background electrolyte, also termed electrokinetic chromatography, has found widespread acceptance in analytical enantioseparations of drug compounds in pharmaceuticals and biological media. Moreover, mechanistic studies on analyte complexation by the chiral selectors have continuously been conducted in an effort to rationalize enantioseparation phenomena. These studies combined capillary electrophoresis with spectroscopic techniques such as nuclear magnetic resonance and/or molecular modeling. The present review focuses on recent examples of mechanistic aspects of capillary electromigration enantioseparations and summarizes recent applications of chiral pharmaceutical and biomedical analysis published between January 2009 and August 2010.

Contents

1. Introduction

The importance of the stereochemistry of pharmaceutical drugs is well recognized as stereoisomers often differ in their pharmacological, toxicological and/or pharmacokinetic profile. This has also affected the requirements for chiral compounds by regulatory authorities worldwide. For example, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) require the development of a single enantiomer of a drug candidate if the enantiomers differ qualitatively or quantitatively in their pharmacological action or toxicological profile. As a consequence, 12 drugs of the top 20 products according to their sale in 2009 are single enantiomer drugs, while 4 drugs are achiral compounds, 3 products are marketed as racemates and one product is a combination of a chiral and a racemic drug [1]. In fact, the top 3 products are single enantiomer drugs. For drug development and quality control the determination of the stereoisomeric composition and purity of a compound requires sensitive and accurate analytical methods in order to determine the stereoisomer ratios in synthetic products, pharmaceutical formulations and biological samples.

Among the separation techniques, high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are most often applied in stereoisomer analysis. Specific advantages of CE are the high separation efficiency, rapid method development and consumption of only small amounts of chemicals and drugs. The most common modes of chiral CE are electrokinetic chromatography (EKC) in the presence of a chiral selector, micellar electrokinetic chromatography (MEKC) and more recently microemulsion

* Fax: +49 3641 949830.
E-mail address: gerhard.scriba@uni-jena.de

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electrokinetic chromatography (MEEKC). Moreover, capillary electrochromatography (CEC) has been investigated combining the high efficiency of electromigration techniques with the high selectivity of stationary phases.

Within the many analytes and applications of pharmaceutical, chemical, environmental and natural products that have been described for chiral electromigration techniques the present review will focus on pharmaceutical and biomedical applications. With a few exceptions of research which appeared to be of specific interest, only publications dating between January 2009 and August 2010 have been considered. Previously published literature has been summarized in recent reviews on the stereoisomer analysis of pharmaceuticals [2–4] and drug analysis in biological media [5,6], general pharmaceutical analysis by CE [7–10] as well as in general reviews on chiral electromigration techniques [11–15]. Furthermore, the reader is referred to recently published monographs on chiral CE [16] and CE in pharmaceutical analysis [17].

2. Fundamental aspects

Efforts have continued to evaluate the structure of analyte–selector complexes in an attempt to understand the complexation behavior and its effect on analyte separation in EKC. Spectroscopic techniques such as nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography as well as molecular modeling have been employed. Furthermore, mathematical models were developed to quantitatively describe the migration behavior of analytes.

2.1. Spectroscopic methods in analyte–selector complexation

A comprehensive review on chiral recognition mechanism studies primarily by spectroscopic and modeling techniques has been published by Lämmerhofer [18]. Uccello-Barretta et al. reviewed the use of NMR for the rationalization of enantiorecognition processes [19]. The application of chemoinformatic techniques to the exploration of enantioselective molecular recognition mechanisms has been summarized in [20]. Furthermore, a web-based program on CD-ligand complexation data has been described [21]. Although these publications focus on enantiorecognition processes in chromatography conclusions may certainly be drawn from these for EKC as well. Most publications combining CE enantioseparations and NMR spectroscopy investigated the interaction between the analytes and CDs. However, an increasing number of studies dealt with other classes of chiral selectors as well as the interaction between analytes and chiral micelles.

Servais et al. have performed a number of NMR and CE studies in an attempt to rationalize the affinity pattern of CDs towards the enantiomers of propranolol in aqueous [22] and non-aqueous background electrolytes [23]. The migration order of the propranolol enantiomers was investigated in the presence of various CDs in aqueous buffers, pH 3.0 [22]. Using β-CD or the neutral derivative 2,3,6-trimethyl-β-CD (TM-β-CD) as chiral selector the S-enantiomer migrated in front of the R-enantiomer while in the presence of sulfated single isomer CDs, i.e. heptakis(6-O-sulfo)-β-CD (HS-β-CD), heptakis(2,3-O-diacetyl-6-O-sulfo)-β-CD (HDAS-β-CD) and heptakis(2,3-O-dimethyl-6-O-sulfo)-β-CD (HDMS-β-CD), the migration order was reversed so that the R-enantiomer migrated in front of the S-enantiomer. The migration order is stated as obtained in the normal polarity mode (detection at the cathode) despite the fact that reversed polarity was applied in some cases. Subsequent NMR measurements including 1D rotating frame nuclear Overhauser effect spectroscopy (1D ROESY) experiments revealed that the naphthyl moiety of propranolol enters the cavity of β-CD from the wider rim containing the secondary hydroxyl groups while the naphthyl residue enters the cavity of HS-β-CD from the narrower rim bearing the primary hydroxyl groups which are derivatized with the sulfate groups. These structural differences of the transient diastereomeric propranolol–CD complexes explain the differences in the enantiomer migration order observed in CE experiments. Comparing the migration behavior of the propranolol enantiomers in aqueous and non-aqueous background electrolytes opposite enantiomer migration order was noted in the presence of HDMS-β-CD when the aqueous buffer was switched to a non-aqueous background electrolyte [23]. In contrast, the migration order remained the same using HDAS-β-CD independent of the nature of the background electrolyte. As above, the migration order is stated as obtained in the normal polarity mode (detection at the cathode). The structures of the complexes between the propranolol enantiomers and both CD derivatives were investigated by 1D ROESY experiments in aqueous and non-aqueous electrolyte solutions. In an aqueous solution, an inclusion complex between HDAS-β-CD and the analytes was apparently not formed as indicated by the lack of significant nuclear Overhauser effects (NOE) between propranolol protons and CD protons located inside the cavity in the 1D ROESY spectra. In contrast, strong interactions between the protons of the side chain of the propranolol molecule and the H3 and H5 protons inside the cavity of HDAS-β-CD were observed as were interactions between protons of the naphthyl moiety and the H2 and H4 protons of the CD located outside of the cavity. This suggests a structure of the complex with the side chain of propranolol entering the CD cavity from the wider, secondary rim of the CD as depicted in Fig. 1A for (R)-propranolol. Despite the fact that different complexes were formed, an identical migration order of the propranolol enantiomers in both types of background electrolyte was observed using HDAS-β-CD as chiral selector. In the case of the complexes between the propranolol enantiomers and HDMS-β-CD in aqueous and non-aqueous solutions the opposite situation was observed in the 1D ROESY experiments. Thus, an inclusion complex was formed in the aqueous background electrolyte, while no analyte inclusion appeared to occur in non-aqueous solutions. Moreover, a completely different complex was formed between the propranolol enantiomers and HDMS-β-CD compared to the propranolol–HDAS-β-CD complex. Thus, as indicated by the NOE the naphthyl moiety of propranolol enters the cavity of HDMS-β-CD from the narrower, primary side as shown in Fig. 1B. The different complexes formed in aqueous and non-aqueous solutions may be the reason for the inversion of the enantiomer migration order observed in the CE experiments in the presence of HDMS-β-CD. Thus, different complexes, i.e. inclusion and non-inclusion complexes, do not necessarily translate into an opposite enantiomer migration order. Moreover, the study demonstrated for the first time that different complexes of an analyte may be formed in aqueous and non-aqueous electrolyte solutions.

Chankvetadze and co-workers have performed a study including NMR and EKC in order to explain the migration behavior of the enantiomers of *cis*-ketoconazole and *cis*-terconazole in the presence of 2-hydroxypropyl-β-CD (HP-β-CD) [24]. Both compounds showed a selector concentration-dependent reversal of the enantiomer migration order in a 100 mM sodium phosphate buffer, pH 3.0, as background electrolyte (Fig. 2). The complexation constants were determined by NMR and CE. Although the absolute values differed depending on the technique, 2S,4R-ketoconazole and (−)-cis-terconazole were complexed stronger which is in agreement with the migration order observed at low HP-β-CD concentrations. However, the stronger complexes also displayed the higher complex mobilities. As a consequence, the enantiomer migration order is reversed at high CD concentrations when the enantiomers essentially exist only in the complexed form and complex mobility becomes the dominant factor responsible for the enantioseparation. The authors concluded that this can be considered as

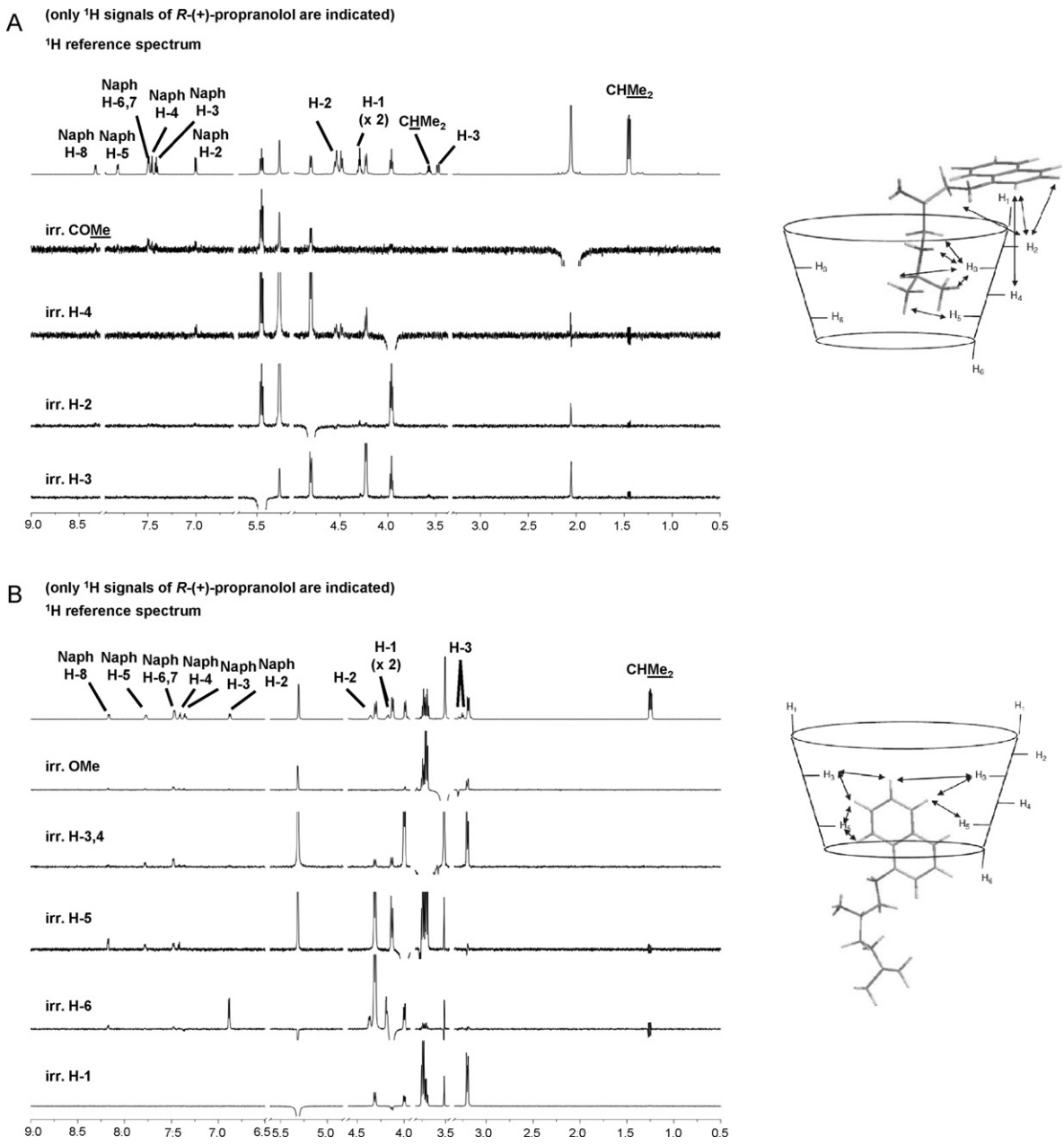

Fig. 1. 1D ROESY spectrum and proposed structure of the R-propranolol–CD complex (A) HDAS-β-CD in methanolic background electrolyte and (B) HDMS-β-CD in aqueous background electrolyte. The spectra show the responses of the propranolol protons upon irradiation of the CD protons.
Adapted from [23] with permission.

experimental proof of the earlier proposed theory of the possibility of EKC separations based solely on differences in the mobilities of the analyte–selector complexes [25,26].

Jamali et al. studied a dual CD system for the enantioseparation of glitazone compounds pioglitazone, rosiglitazone and balaglitazone [27]. Using either 2,6-dimethyl-β-CD (DM-β-CD) or sulfobutylether-β-CD (SBE-β-CD) as chiral selector the separation of the test compounds was observed at pH 2.5 and 9.3, respectively, but a single CD proved to be insufficient to achieve the desired resolution $R_S > 3$. Thus, a dual CD system was optimized by factorial design. Although the optimum concentrations of the CDs were different for each compound a generic separation system consisting of 3 mM DM-β-CD and 12 mM SBE-β-CD in a 50 mM borate buffer, pH 9.7, was identified resulting in $R_S > 5$ for all glitazones. In NMR experiments rosiglitazone was investigated with the individual CDs as well as the CD mixture. SBE-β-CD interacted primarily with the pyridine moiety, while in the case of DM-β-CD the main interaction occurred with the phenyl moiety of the glitazone molecule. The authors concluded from the EKC and NMR experiments that both CDs act as chiral selectors and SBE-β-CD additionally increases the migration time resulting in an optimized enantioseparation. Moreover, the CDs appeared to display opposite chiral recognition towards the enantiomers resulting in the superior enantioseparation compared to the use of a single CD as chiral selector. Further recent studies using NMR spectroscopy for the understanding of analyte complexation by CDs in CE separations include the enantioresolutions of vinca alkaloids by several β-CD and γ-CD derivatives [28], tosyl and dansyl derivatives of pregabalin by β-CD [29] and chiral carboxylic acids by pyrrolidinium-β-CD [30].

A combined CE and NMR study was performed to investigate enantioseparation of catechin by sinorhizobial octasaccharides

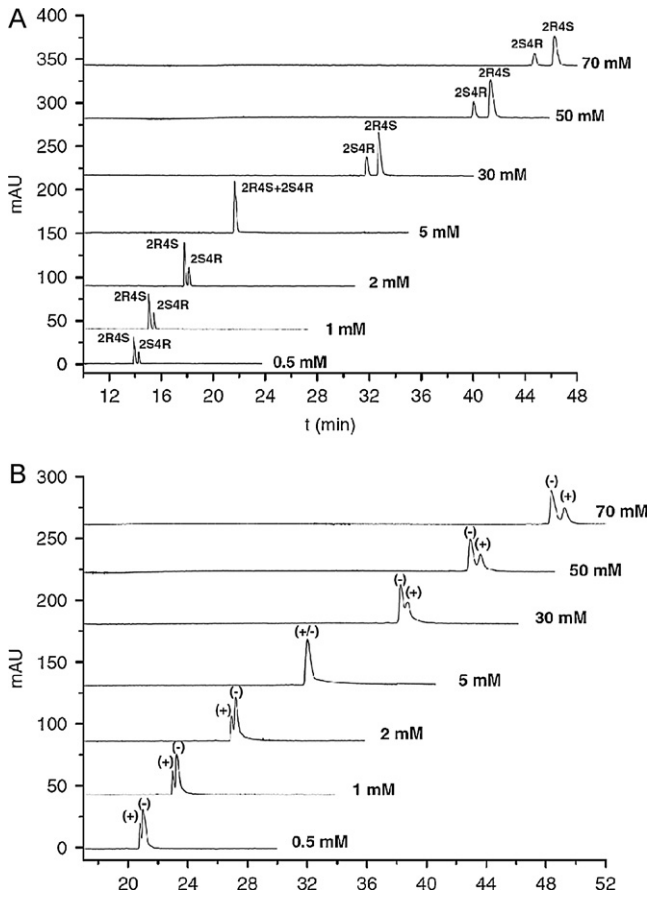

Fig. 2. Effect of the concentration of HP-β-CD on the enantioseparations of *cis*-ketoconazole (A) and *cis*-terconazole. *Experimental conditions*: 64.5/56 cm, 50 μm id fused-silica capillary, 0.1 M sodium phosphate, pH 3.0, 30 kV, 15 °C, 200 nm. Reproduced from [24] with permission.

produced by *Sinorhizobium meliloti* [31]. The linear saccharides differ in the degree of succinylation. While no enantioseparation was observed in the presence of the derivative without a succinyl substituent, derivatives with 1 or 2 succinyl groups effectively separated the enantiomers of catechin. In ^{13}C NMR measurements splitting of signals of catechin was observed in the presence of all studied succinoglycans indicating that chiral recognition occurs with all saccharides. However, as the peak splitting was more pronounced for succinyl substituted derivatives the authors concluded that the succinyl groups could be involved in the recognition or discrimination in the enantioseparation of catechin. ^{1}H and ^{13}C NMR spectroscopy was also employed to explain the chiral recognition ability of amylose towards several basic analytes and ibuprofen [32]. All test compounds were resolved in neutral to acidic background electrolytes containing 1.5% amylose. While the separation of some analytes could be explained by inclusion into the cavities formed by the helical structures of amylose the molecular size of other compounds appeared to be too large for inclusion. Upon increasing the temperature in order to destroy the helical structures of amylose the enantioseparation of the smaller compounds deteriorated while the temperature did not affect the resolution of the larger molecules. NMR measurements indicated signal splitting, i.e. chiral recognition, at low temperatures for the small-sized molecules which disappeared at higher temperatures. Recognition did not change with temperature in case of the larger molecules. A similar effect was observed upon addition of iodine which apparently competed with the smaller molecules for inclusion into the cavities. Thus, chiral recognition of amylose exists even in the absence of helical structures. Consequently, the authors distinguished between a helical-dependent and an ahelical-dependent recognition mechanism.

Besides studies on carbohydrate-derived chiral selectors, the interaction between 1,1′-binaphthyl-2,2′-diylhydrogenphosphate (BNDHP) and chiral micelles has been studied by NMR. The group of Morris investigated molecular micelles containing chiral dipeptide headgroups such as poly(sodium N-undecanoyl-L-leucylleucinate) [33]. Nuclear Overhauser enhancement spectroscopy (NOESY) revealed that the N-terminal amino acid of the dipeptide headgroup was the preferred site of chiral recognition. The H3/H3′ and H4/H4′ protons of BNDHP interacted strongly with the amino acid moiety while the H6/H6′ and H7/H7′ protons primarily interacted with the hydrocarbon side chain. Pulsed field gradient NMR diffusion measurements showed that differences in the free energies of binding of the BNDHP enantiomers correlated with the chiral selectivities observed in MEKC in the presence of the molecular micelles. In ^{31}P NMR experiments of the PNDHP enantiomers in the presence of sodium cholate above the critical micelle concentration a larger upfield shift was observed for the S-enantiomer which correlates with the longer residence times of the S-enantiomer in the micelles found in MEKC [34]. Mobilities of the BNDHP enantiomers in MEKC as a function of the sodium cholate concentration in the background electrolyte were in agreement with the chemical shifts of the ^{31}P NMR signals. In ^{1}H NMR experiments the H5/H5′ and H7/H7′ protons of BNDHP displayed the largest shifts indicating that this part of the molecule interacts primarily with the cholate micelles.

Nagata et al. concluded from a X-ray analysis of single crystals of the chiral crown ether (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid ((+)-18C6H4) with glycine, methylamine, ammonium and H_3O^+ on the conformation of (+)-18C6H4 as selector in chiral separations [35]. In the complex with glycine (+)-18C6H4 exhibited an asymmetric C1-type conformation forming a bowl-like shape with the N–H and C_α–H protons of the amino acid interacting with the oxygen atoms as well as the carboxylate groups of the ring system (Fig. 3A). Complexation of ammonium or methylamine resulted in symmetrical conformations of the crown ether (Fig. 3B). The authors concluded that the asymmetric C1-type conformation is required for chiral separation of racemic amino acids and amines. They suggested a conformational sequence of successive rotations in the macrocyle that results in the formation of the asymmetric structure necessary for enantiorecognition of analytes. Interestingly, an asymmetric complex was also formed in the presence of protons. As EKC separations using (+)-18C6H4 as chiral selector are typically performed in acidic background electrolytes the authors suggested that a pH-dependent promotive effect towards the conformational change of the crown ether exists.

2.2. Chiral CE separations combined with molecular modeling

Zhang et al. used a set of 25 triadimenol analogs for the analysis of the chiral recognition mechanism by carboxymethyl-β-CD (CM-β-CD) [36]. Using standardized conditions of 5 mM of CM-β-CD in 30 mM sodium dihydrogen phosphate, pH 2.2, and a voltage of 20 kV at 20 °C 20 compounds could be resolved. Subsequent molecular docking performed for each compound and CM-β-CD indicated that a hydrophobic interaction and two hydrogen bonds were involved in the interaction between the CD and the triadimenol enantiomers. A mathematical model was constructed correlating the interaction energies of the molecular docking calculations and the resolution found in the EKC enantioseparations. The model could be used to predict the chiral separation of a triadimenol analog. Parametric Method 3 semiempirical molecular orbital calculations have also been used to rationalize the CE enantioseparations of salsolinol, *N*-methyl-salsolinol, and 1-benzyltetrahydroisoquinoline by β-CD [37]. The stabiliza-

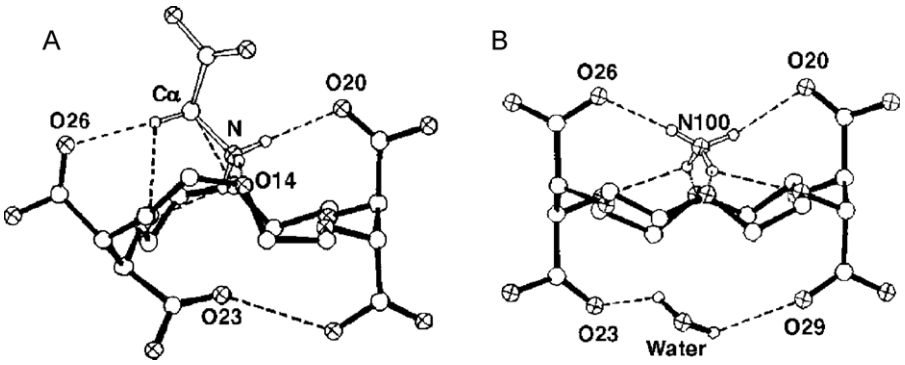

Fig. 3. Molecular conformation and interactions between (+)-18C6H4 and (A) glycine and (B) ammonia. The guest molecule is drawn with open bonds, the dotted lines represent hydrogen bonds.
Adapted from [35] with permission.

tion energies of the inclusion complexes correlated with the enantiomer migration order observed in CE. Molecular modeling studies were also applied to understand the interaction between cathecin enantiomers and mono-succinyl-β-CD [38], between aminoglutethimide and methyl-β-CD [39], and the enantioseparations of modafinil [40] and ofloxacin as well as ornidazole [41] by sulfated β-CD, as well as the separation of the enantiomers of primaquine and quinocide in the presence of α-CD, β-CD or (+)-18C6H4 [42].

Multivariate image analysis-quantitative structure property relationship (MIA-QSPR) modeling has been applied to predict the enantiomer migration order of aromatic amino acids and amino acid esters using (+)-18C6H4 as chiral selector in a Tris-citric acid buffer, pH 4.0 [43]. The MIA descriptors as pixels obtained from images of the structures of the compounds were correlated to the migration time of the compounds reported in the literature by partial least squares analysis. Good correlation between predicted and experimental migration times was achieved with the L-stereoisomers always migrating faster than the respective D-enantiomers. The model could be cross validated by leave-out-samples. The authors concluded that the MIA-QSPR method included physicochemical information related to the spatial arrangement of the molecules.

2.3. Migration models

Two different mechanisms contribute on a macroscopic level to enantioseparations using a chiral selector in CE, a chromatographic and an electrophoretic principle as also discussed in many review papers such as [13,25,26,44,45]. According to the fundamental equation introduced by Wren and Rowe [46], the effective mobility of an analyte, μ^{eff}, interacting with a chiral selector is a function of the mobility of the free analyte, μ_{f}, the (limiting) mobility of the analyte–selector complex, μ^{cplx}, the complexation constant, K (also referred to as binding constant or distribution constant), and the concentration of the chiral selector, c_{CS}, as described by:

$$\mu^{\text{eff}} = \frac{\mu_{\text{f}} + \mu^{\text{cplx}} K c_{\text{cs}}}{1 + K c_{\text{cs}}} \tag{1}$$

An enantioseparation is observed when the effective mobilities of the enantiomers differ, i.e. $\Delta\mu = \mu_1^{\text{eff}} - \mu_2^{\text{eff}}$. The chromatographic enantioselective mechanism (also referred to as the thermodynamic mechanism) is the result of different affinities of the enantiomers towards the chiral selector, i.e. $K_1 \neq K_2$. The electrophoretic enantioselective mechanism is due to differences in the limiting mobilities of the enantiomer–selector complexes, i.e. $\mu_1^{\text{cplx}} \neq \mu_2^{\text{cplx}}$, caused by differences in the hydrodynamic radii of the complexes. Both mechanisms can contribute simultaneously but the chromatographic mechanism is typically the predominant mechanism because the hydrodynamic radii of the two enantiomer–selector complexes do not differ significantly and the effective charges of the two complexes are identical. An exception is the occurrence of a complexation induced pK_{a} shift which can lead to different charge densities of the complexes at a given pH of the background electrolyte [47].

Dubsky et al. have used a theoretical model to explain the often observed superior enantioseparation selectivity of randomly substituted CDs compared to single isomer CDs [48] based on a model derived from an earlier publication on the enantioseparation using multiple chiral selectors [49]. The randomly substituted CD is treated as a mixture of a (large) number of defined chiral selectors with different substitution patterns. In such a system the effective mobility can be described by an equation formally identical to the equation used for a single selector system, i.e.:

$$\mu_i^{\text{eff}} = \frac{\mu_{\text{f}} + \mu_i^{\text{over}} K_i^{\text{over}} c_{\text{cs}}^{\text{tot}}}{1 + K^{\text{over}} c_{\text{cs}}^{\text{tot}}} \tag{2}$$

where μ_{f} is the mobility of the free analyte, $c_{\text{cs}}^{\text{tot}}$ is the total concentration of the mixture of chiral selectors, i.e. the CDs with different substitution patterns. K_i^{over} and μ_i^{over} are the overall (apparent) complexation constants and the overall (apparent) limiting mobilities of the complexes, respectively. K_i^{over} is defined by:

$$K_i^{\text{over}} = \sum_q \chi^q K_i^q \tag{3}$$

where χ^q is the molar fraction of the qth CD substitution isomer in the mixture and K_i^q the individual apparent complexation constants of the analyte with the qth CD isomer. Thus, the overall complexation constant of a multiple isomer CD system is the weighted sum of all individual constants with the mol fractions of the individual CDs as the weights. μ_i^{over} is given by:

$$\mu_i^{\text{over}} = \frac{\sum_q \chi^q K_i^q \mu_i^q}{K_i^{\text{over}}} \tag{4}$$

Eq. (4) illustrates that the overall limiting mobility of an enantiomer interacting with a mixture of CD isomers is no longer an independent physico-chemical (electrophoretic) property but depends on the distribution of the enantiomer between chiral and achiral environments represented by the complexation constants K_i^{over}. Thus, μ_i^{over} becomes an apparent parameter influenced by both, the chromatographic and electrophoretic mechanisms. Consequently, the overall limiting mobilities of two enantiomers, μ_1^{over} and μ_2^{over}, may substantially differ even if resulting from otherwise essentially equal individual mobilities for every qth CD in the

isomer mixture, i.e. $\mu_1^q = \mu_2^q$. Thus, even if enantiomers are separated solely on the chromatographic basis using one or another single isomer CD of the mixture an additional electrophoretic separation mechanism may appear if the two CDs are combined. This is even more likely when a mixture of many individual CD isomers is used as it is the case in randomly substituted CDs. The validity of the theory was demonstrated by comparing the enantioseparation of lorazepam using randomly substituted highly sulfated β-CD (S-β-CD) and the single isomer heptakis(6-O-sulfo)-β-CD (HS-β-CD). Essentially equal limiting mobilities of the enantiomers of μ_i^{over} of $-25.0 \pm 0.8 \times 10^{-9}\,V\,m^2\,s^{-1}$ and $-25.5 \pm 0.8 \times 10^{-9}\,V\,m^2\,s^{-1}$ were found for HS-β-CD while substantially differing mobilities of $-7.9 \pm 0.9 \times 10^{-9}\,V\,m^2\,s^{-1}$ and $-22.8 \pm 0.3 \times 10^{-9}\,V\,m^2\,s^{-1}$ were determined in the case of S-β-CD as a mixture of many CD isomers. The affinity of HS-β-CD towards the enantiomers of lorazepam was about 4-fold higher compared to S-β-CD based on the complexation constants but the chromatographic selectivity expressed as the ratio of the complexation constants was similar for both systems, 1.29 for HS-β-CD and 1.33 in the case of S-β-CD.

Based on the selectivity model developed by Vigh and coworkers [50] for weak bases and neutral chiral selectors a mathematical approach was undertaken in an attempt to rationalize the pH- and selector concentration-dependent reversal of the enantiomer migration order in EKC [51,52]. Although deduced for weak bases and neutral chiral selectors the mathematical approach can be easily transferred to acids or charged selectors by minor modifications of the model. Considering the protonation equilibria of the free base and the analyte–selector complex as well as the complexation equilibria between the protonated and neutral species of the analyte with the selector such as a CD the effective mobility of an analyte, μ_{eff}, can be described by Eq. (5):

$$\mu_{eff} = \frac{\mu_{HB^+} + \mu_{HB^+CD}K_+[CD]}{1 + K_+[CD]} \frac{1}{1 + 10^{pH-(pK_a+\log(1+K_+[CD])/(1+K_n[CD]))}} \quad (5)$$

which expresses μ_{eff} as a function the CD concentration, [CD], the pH of the background electrolyte, the pK_a value of the analyte, the binding constants of the protonated and neutral species, K_+ and K_n, and the mobilities of the protonated free species and the corresponding complex, μ_{HB^+} and μ_{HB^+CD}, respectively.

Considering the separation selectivity, S, described by:

$$S = \frac{\mu_{eff1}}{\mu_{eff2}} \quad (6)$$

an enantioseparation can only be observed for $\mu_{eff1} \neq \mu_{eff2}$. According to Eq. (5) this can be achieved when the enantiomers differ in the binding constants of the protonated form ($K_{+1} \neq K_{+2}$), the neutral form ($K_{n1} \neq K_{n2}$) and/or the mobilities of the two enantiomer–CD complexes ($\mu_{HB^+CD1} \neq \mu_{HB^+CD2}$). Equations describing the effective analyte mobility at fixed CD concentration or pH were described as were equations for the pK_a of the analyte–CD complex as a function of the complexation constants of the charged and electroneutral forms of the analyte. For a systematic classification with regard to the enantiomer migration order the constellation of the operator-dependent parameters, selector concentration and buffer pH, in relation to the pK_a of the analyte were considered. In a 3D plot of the selectivity as a function of [CD] and pH the 4 corner points referred to low/high [CD] and low/high pH. In the mathematical treatment "low" pH was defined with regard to the analyte pK_a as a pH where the analyte is fully protonated while "high" pH as a pH where only the deprotonated state of the analyte exists (Fig. 4). "High" [CD] is treated as an infinite selector concentration and "low" [CD] as an infinitesimal selector concentration. In each corner point the selectivity can be described as a function of the complexation equilibria between the charged and electroneutral species of the base and the selector as well as

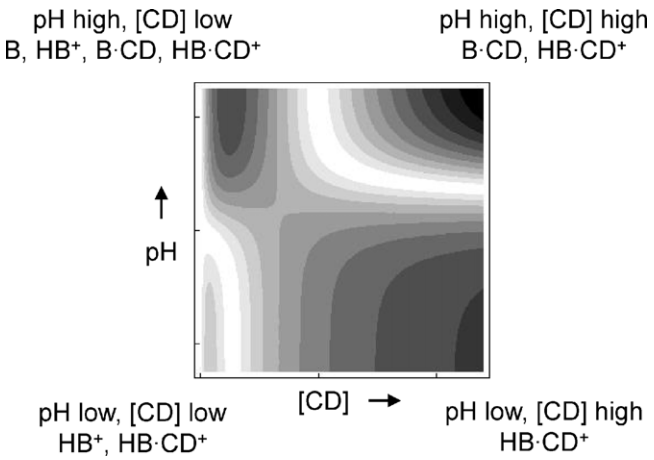

Fig. 4. Schematic representation of a 3D contour plot illustrating the low/high pH and low/high [CD] situation at the respective corner points.

the mobility of the free analyte and the limiting mobility of the analyte–selector complex. Depending on the scenario, only one or several of these factors affected the selectivity. Subsequently, with constant pH or [CD] information of the selectivity (and with it the enantiomer migration order) parallel to the edges of the surface plot can be obtained. Thus, knowing the selectivity in the corner points one can conclude if a selectivity change occurs along the plot upon changing either pH or [CD].

At the corner point low pH and high [CD] essentially only the protonated forms of the analyte enantiomrs exist so that S depends only on the complex mobilities. At the vertex low pH and low [CD] the free and complexed protonated forms coexist. Consequently, complex mobilities and binding constants of the protonated analytes affect S. At high pH and high [CD] (vertex 3) the protonated and neutral forms of the analytes coexist and are essentially complexed so that the selectivity is a function of complex mobilities as well as the binding constants of the protonated and neutral species. Finally, at the vertex high pH, low [CD] S depends on the ratio of the affinity constants of the protonated analyte, K_{+1}/K_{+2}, relative to the ratio of binding constants of the neutral species, K_{n1}/K_{n2}, as well as on the dimensions of the affinity constants K_+ relative to K_n. Thus, depending on the complexation constants and the mobilities of the enantiomer–CD complexes different scenarios may result. With respect to the possibilities of the selectivity at the 4 corner points of the selectivity surface plot 15 cases could be classified mathematically which are summarized in Table 1. Four basic mechanisms were identified leading to the inversion of the enantiomer migration order of a weak base in the presence of a neutral chiral selector, three in a pH-dependent manner and one as a function of the selector concentration. A pH-dependent reversal may be observed due to (1) inversion of the complex mobilities caused by an enantioselective pK_a shift, (2) inversion of the ratio of the mobilities of the analyte-selector complex and the free analyte due to a significant complexation-induced pK_a shift to a higher value and (3) opposite chiral recognition of the protonated and the effectively electroneutral forms of the analyte by the chiral selector.

The applicability of the theoretical model was demonstrated by analysis of the migration behavior of the dipeptides Ala-Phe and Asp-PheOMe using 2,6-dimethyl-β-CD (DM-β-CD) as chiral selector. While Ala-Phe displayed a pH-dependent inversion of the migration order of the enantiomers in the presence of DM-β-CD Asp-PheOMe did not. The pH independent parameters, μ_{HB^+}, μ_{HB^+CD}, K_+ and K_n as well as pK_a and p$K_{a/c}$ were determined in the pH range 1.9–4.8 and DM-β-CD concentrations between 0 and 0.135 mol/L by a three-dimensional, non-linear curve fitting. In the case of Ala-Phe a strong complexation-induced pK_a shift

Table 1
Systematic classification of the separation selectivity with regard to the enantiomer migration order as a function of pH and CD concentration, [CD], derived from the corner points of the 3D contour plot.

Case	Selectivity				EMO reversal			
	Low pH		High pH		pH-dependent		[CD]-dependent	
	High [CD]	Low [CD]	High [CD]	Low [CD]	High [CD]	Low [CD]	Low pH	High pH
I	$S=1$	$S=1$	$S<1$	$S<1$	–	–	–	–
II			$S=1$	$S\leq 1$	–	–	–	–
III				$S>1$	–	+	–	–
IV		$S<1$	$S<1$	$S\leq 1$	–	–	–	–
V				$S>1$	–	+	–	+
VI			$S>1$	$S\leq 1$	–	–	–	+
VII				$S>1$	–	+	–	–
VIII		$S\leq 1$	$S\leq 1$	$S\leq 1$	–	–	–	–
IX				$S>1$	–	+	–	+
X			$S>1$	$S\leq 1$	+	–	–	+
XI	$S<1$			$S>1$	+	+	–	–
XII			$S\leq 1$	$S\leq 1$	–	+	+	–
XIII		$S>1$		$S>1$	–	–	+	+
XIV			$S>1$	$S\leq 1$	+	+	+	+
XV				$S>1$	+	–	+	–

Adapted from [51,52] with permission.

of almost one unit was identified as the reason for the observed pH-dependent reversal of the enantiomer migration order. Asp-PheOMe did not display a significant pK_a shift in the presence of DM-β-CD and, consequently, not reversal of the migration order was found.

The effect of pH on complex formation between β-CD and the enantiomers of the dipeptides Ala-Phe, Ala-Tyr and Asp-PheOMe has been investigated by CE and calorimetry in order to provide a more detailed understanding of the migration behavior of the peptides including the reversal of the enantiomer migration order [53]. The complexation and mobility parameters as well as the pK_a values of Ala-Tyr, Ala-Phe and Asp-PheOMe and the respective peptide-β-CD complexes were determined by CE. The limited aqueous solubility of β-CD prevented the use of extremely high concentrations of the selector even when adding urea as solubilizer to the background electrolyte so that the data were somewhat error prone preventing the unequivocal assignment of the mechanism underlying the pH-dependent reversal of the enantiomer migration order. Most likely the separation behavior of Ala-Phe and Asp-PheOMe was due to inversion of the complex mobility ratio caused by enantioselective complexation-induced pK_a-shifts while in the case of Ala-Tyr the reversal was attributed to either an enantioselective complexation-induced pK_a-shift or opposite chiral recognition of the protonated and effectively electroneutral species by the chiral selector. Complexation constants were also determined by calorimetry. As in CE stronger complex formation between β-CD and the DD-enantiomers of the peptides compared to the LL-stereoisomers was observed and complexation constants of the protonated species exceeded complexation constants of the neutral analytes. The thermodynamic parameters of complexation were affected by both, the configuration of stereoisomers and the pH. Compared to β-CD complexes of the LL-enantiomers, inclusion complexes of the DD-isomers were more entropy stabilized exhibiting less negative enthalpy values.

Mofaddel et al. described a simple model for the enantioseparation of binaphthol and its monoderivatives by CDs [54]. Binaphthol contains two weakly acidic functions so that complexation of three differently ionized binaphthol species and their respective binding constants with the CDs had to be considered. The apparent complexation constants and complex mobilities were determined. Using γ-CD as chiral selector, reversal of the migration order of binaphthol as a function of the CD concentration was observed at pH 10.8. Analysis of the complexation constants and complex mobilities revealed a difference in the limiting apparent mobilities of the binaphthol-γ-CD complexes as well as differences in the strength of the binding of the binaphthol enantiomers. The stronger complexed S-enantiomer also revealed the higher limiting complex mobility. This causes the reversal of the migration order when increasing the concentration of γ-CD at pH 10.8. The authors did not provide a further explanation for this phenomenon. However, due to the fact that pH 10.8 is close to the second dissociation equilibrium of binaphthol it can be concluded that the reversal is due to a complexation induced pK_a shift (either stereoselective or non-stereoselective) based on the mechanistic study described above [51].

3. Capillary electrophoresis in stereoisomer analysis

Within the electromigration techniques CE as well as capillary electrochromatography (CEC) has been applied to drug analysis in pharmaceuticals or biological media. However, in the period of time covered by the present review only application studies by CE have been published. The literature on CEC covered specifically the development and testing of chiral stationary phases but no applications for solving "real" pharmaceutical problems could be found. For recent reviews on chiral stationary CEC phases the reader is referred to [55–58].

CE enantioseparations of drugs can be divided into four major categories: (1) enantioseparations of racemic compounds in studies in order to investigate the separation ability of a new chiral selector or a class of selectors. In these studies, the drugs serve rather as model compounds in contrast to "true" pharmaceutical or biomedical applications. These will not be the focus of this review except in the case of fundamental studies described above. The other categories considered here are (2) separations of (non-)racemic mixtures of enantiomers for assay development and the determination of the enantiomeric purity of compounds, (3) chiral separations of drugs in pharmaceutical preparations and products, and (4) enantioseparations of drugs and metabolites in biological matrices or upon in vitro incubation with metabolizing enzymes. The reader is also referred to earlier general reviews on chiral electromigration techniques [11–15] and chiral drug analysis [2–4] as well as monographs on chiral CE [16] or on pharmaceutical CE analysis in general [17]. Other recent reviews cover specific aspects such as chiral CE-MS [59] or CE for chiral analysis of drugs, metabolites and biomarkers in biological samples [5]. MEEKC has been discussed by Ryan et al. [60]. CE in drug impurity analysis has been summarized in [61].

With regard to the frequency of the application CDs are by far the most "popular" chiral selectors due to their structural diversity and commercial availability. Reviews on CDs in EKC analysis can be found in [45,62–66]. Factors affecting CD-mediated chiral separations have been discussed in a book chapter [67]. Moreover, a summary of glycopeptides and macrocyclic antibiotics as selectors has been published [2].

3.1. Pharmaceutical analysis

Recent examples covering the chiral analysis of drugs in synthetic laboratory samples, bulk drug or pharmaceutical formulations have been compiled in Table 2. All methods employed direct enantioseparation in the presence of a chiral selector except for one example of indirect enantioseparation in case of the analysis of L-tyrosine in an oral solution where derivatization with *R*(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole was used [68]. A number of studies were performed on racemic drugs in their formulations where a chiral assay is not necessarily required, while "true" applications covered either the determination of the enantiomeric purity of chiral compounds in laboratory samples, bulk drug or drug formulations or the simultaneous analysis of enantiomeric impurities as well as related substances.

The separation of clopidogrel from impurities stated by the United States Pharmacopeia, i.e. the R-enantiomer, the carboxylic acid and the positional isomer, was investigated by Fayed et al. [69]. An initial screening of the experimental conditions was performed including buffer type, pH and concentration, cyclodextrin type and concentration as well applied voltage and temperature. The method was further optimized employing a reduced central composite face-centered design comprising buffer pH, buffer concentration, concentration of sulfated β-CD as chiral selector and applied voltage as variables. Resolution between clopidogrel and the first and last migrating enantiomer of the positional isomer, respectively, and total analysis time were selected as responses. However, the optimum conditions predicted by the software resulted in extremely high currents and an unstable baseline. Subsequently, the model was rebuilt including current as a response. As final conditions, a 10 mM phosphate buffer, pH 2.3, containing 5% sulfated β-CD and an applied voltage of −12 kV were selected. Robustness of the method was investigated using a reduced composite face-centered design as well. The method was validated and applied to the analysis of bulk drug. Besides the impurities listed by the United States Pharmacopeia two additional unknown impurities could be detected.

A dual CD system was employed for the impurity profiling of dexamphetamine sulfate [70]. Charged as well as uncharged impurities were considered due to the possible preparation of the drug via several synthetic pathways using chiral (charged) or achiral (uncharged) starting materials. From a screening of CDs using a 50 mM phosphate buffer, pH 2.5, sulfated β-CD and sulfobutylether-β-CD (SBE-β-CD) were identified as chiral selectors for the separation of the enantiomers of amphetamine. In the presence of 20 mg/mL sulfated β-CD providing a resolution of amphetamine of $R_S > 3$ the neutral impurities phenylacetone and phenylacetone oxime could not be detected within 60 min. Broad peaks or peak splitting was observed in the presence of SBE-β-CD below a concentration of 70 mg/mL. At higher concentrations insufficient resolution of the amphetamine enantiomers was still observed. Thus, a combination of both CDs was applied. The final conditions comprised a 50 μm id fused-silica capillary with an effective length of 35 cm, a background electrolyte consisting of 50 mM sodium phosphate buffer, pH 3.0 containing 80 mg/mL SBE-β-CD and 25 mg/mL sulfated β-CD and an applied voltage of −10 kV. During method development it was noted that the substitution degree of the randomly substituted SBE-β-CD could affect the resolution and peak shape of the analytes. However, this could be always fixed by minor adjustments of the CD concentration. The method was validated with a batch of SBE-β-CD with a degree of substitution of 4.9 using 1R,2S-(−)-ephedrine as internal standard. The LOQ of all impurities corresponded to 0.05% based on a concentration of dexamphetamine sulfate of 5 mg/mL. Subsequent analysis of several batches of commercial dexamphetamine sulfate revealed the presence of about 3–4% levoamphetamine as well as phenylacetone and phenylacetone oxime at the LOD level (Fig. 5). In contrast, charged impurities such as norephedrine or norpseudoephedrine could not be detected leading to the conclusion that the investigated samples were synthesized from phenylacetone via the oxime yielding racemic amphetamine which was resolved by fractional crystallization. A subsequently developed CD-modified MEEKC method was able to separate all charged and uncharged impurities as well but proved to be less sensitive than the dual CD system [71]. Interestingly, it was noted in this study that the order of the preparation of the microemulsion affected the resolution between some compounds. Thus, it may be necessary to validate the preparation procedure of the microemulsion as well in order to obtain reproducible MEEKC methods.

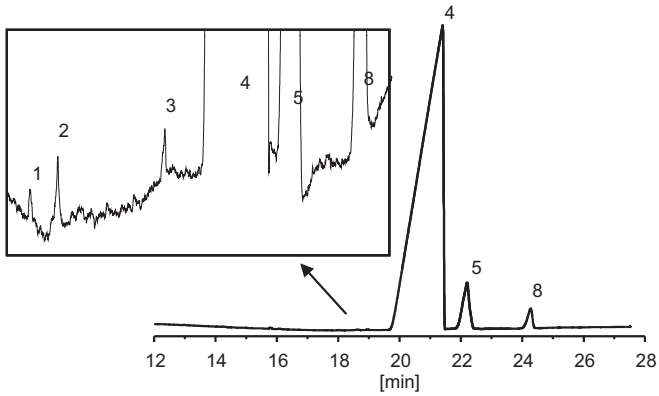

Fig. 5. Electropherogram of a commercial sample of dexamphetamine sulfate analyzed at a concentration of 5 mg/mL. *Experimental conditions*: 40.2/35 cm, 50 μm id fused-silica capillary, 50 mM sodium phosphate buffer, pH 3.0, containing 80 mg/mL SBE-β-CD and 25 mg/mL sulfated β-CD, −10 kV, 20 °C, 200 nm. 1, phenylacetone Z-oxime; 2, phenylacetone E-oxime; 3, phenylacetone; 4, dexamphetamine; 5, levoamphetamine; 6, 1S,2S-(+)-norpseudoephedrine; 7, 1R,2S-(−)-norephedrine; 8, 1R-2S-(−)-ephedrine.
Reproduced from [70] with permission.

Borst and Holzgrabe undertook a systematic study for the establishment of a chiral CD-modified MEEKC method for phenethylamines including ephedrine, norephedrine, N-methylephedrine, epinephrine and further structurally related compounds [72]. The oil-in-water microemulsion consisted of 0.5% ethyl acetate as oil phase, 1.0% SDS as surfactant, 4.0% 1-butanol as cosurfactant, 3.0% 2-propanol as organic modifier, 91.5% 20 mM sodium phosphate buffer, pH 2.5, as aqueous phase, and 4.0% sulfated β-CD was the chiral selector. Except for dipivefrine and 2-(butylamino)-1-phenyl-1-propanol all analytes could be baseline resolved using an applied voltage of −15 kV. The method was validated and the robustness with regard to buffer pH and concentration was assessed. For the simultaneous separation of compounds that are specified as impurities for a certain drug in the European Pharmacopoeia the CD concentration had to be adjusted as shown in Fig. 6 for ephedrine and pseudoephedrine. LODs of the impurities of at least 0.1% were achieved allowing the analysis of the compounds according to the limits of the European Pharmacopoeia.

1S,2R-(+)-ephedrine and 1R,2S-(−)-norephedrine as impurities in pharmaceutical formulations of 1R,2S-(−)-ephedrine have been analyzed using carboxyethyl-β-CD (CE-β-CD) as chiral selector in a

Table 2
Recent examples of drug purity determination by electromigration techniques.

Drug	Chiral selector (concentration)	Background electrolyte	LOD/comment	Ref.
Aliphatic amines	(+)-18C6H4 (5–15 mM) DM-β-CD (5–15 mM)	10 mM citric acid/aspartic acid, pH 2.2	4.2–9.4 μg/mL indirect UV detection, 0.2–0.3 mg/mL CCD, determination of enantiomeric purity	[76]
Aminoglutethimide	M-β-CD (30 mg/mL)	50 mM Tris/phosphoric acid, pH 3.0	2.20–2.13 μg/mL, analysis of racemic drug in formulations	[39]
Amlodipine	HP-β-CD (50 mg/mL)	50 mM glycine/acetate, pH 3.2, 0.2% hydroxyethylcellulose	2.42–3.53 μg/mL, analysis of racemic drug in tablets	[77]
Amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine	HP-β-CD (30 mM)	25 mM sodium acetate/75 mM acetic acid, pH 4.55	0.46–1.8 μg/mL, analysis of illicit tablets	[78]
Baclofen	α-CD (18 mM)	0.1 M sodium borate, pH 9.9, 1% acetonitrile	2–10 μg/mL, analysis of racemic bulk drug and tablets	[79]
Benzimidazole derivatives	Sulfated β-CD (3.5%) HS-β-CD (3.5%) HS-γ-CD (4%)	25 mM triethalamine/phosphoric acid, pH 2.5	0.7–1.0%, analysis of synthetic laboratory samples	[80]
Carnitine, Fmoc derivatized	Succ-γ-CD (10 mM)	0.5 M ammonium formate, pH 2.5	0.002%, MS detection, analysis of infant formulas	[81,82]
Cefotaxime	CM-β-CD (0.5 mM)	75 mM sodium phosphate, pH 7.0	0.4–0.5 μg/mL, analysis of injection formulations	[83]
Clopidogrel	Sulfated β-CD (5%)	10 mM triethylamine/phosphoric acid	0.08–0.33 μg/mL, minor enantiomer and related substances	[69]
Dexamphetamine	HDAS-β-CD (10 mg/mL)	0.1 M sodium phosphate buffer, pH 2.5	0.06%, minor enantiomer and charged related substances	[84]
Dexamphetamine	Sulfated β-CD (25 mg/mL), SBE-β-CD (80 mg/mL)	50 mM sodium phosphate buffer, pH 2.5	0.01–0.02%, minor enantiomer and related substances	[70]
Dexamphetamine	Sulfated β-CD (5.5%)	1.5% SDS, 0.5% ethyl acetate, 3.5% 1-butanol, 2.5% 2-propanol and 92% 50 mM sodium phosphate buffer, pH 3.0	0.05–0.2%, minor enantiomer and related substances	[71]
Econazole	HP-γ-CD (40 mM)	50 mM SDS in 20 mM phosphate buffer, pH 8.0	3.6–4.3 μg/mL, analysis of racemic drug, SPE extraction from cream	[85]
Epinephrine, norepinephrine	CE-β-CD (10 mM)	100 mM Tris/phosphoric acid, pH 5.0	0.8–1.0 μg/mL, stereoisomeric purity of L-epinephrine in formulations	[73]
Growth hormone analogue JMV 1843	HS-γ-CD (5%)	50 mM phosphate buffer, pH 2.5	Poly(diallyldimethylammonium)-coated capillary, analysis of synthetic samples	[86]
Ibuprofen	TM-β-CD (35 mM)	5 mM sodium acetate, 2.63 mM acetic acid, pH 5.0, 1.5 mM *N*-undecenoxy-carbonyl-L-leucinol bromide	LOD not given, analysis of racemic drug in tablet	[87]
Lansoprazole	β-CD (12 mM)	50 mM sodium phosphate, pH 2.2, 5 mM sodium sulfite	0.64–0.72 mg/L, analysis of racemic drug	[88]
Levocetirizine	Sulfated β-CD (5 mg/mL)	25 mmol/L MES/ε-aminocaproic acid, pH 5.3, 0.1% hydroxyethylcellulose	1.7–1.9 μg/mL, analysis of tablets	[89]
Levothyroxine	$CuSO_4$ (0.05 mM) L-proline (0.4 mM)	18 mM borate buffer, pH 9.6, 10% ACN	0.35 μg/mL, analysis of tablets	[74]
Modafinil	Sulfated β-CD (30 mg/mL)	25 mM Tris/phosphoric acid, pH 8.0	1.89–1.07 μg/mL, analysis of racemic drug in tablet	[90]
Ofloxacin and ornidazole	Sulfated β-CD (30 mg/mL)	50 mM Tris/phosphoric acid, pH 1.85	0.46–0.89 μg/mL, racemic drug in injection formulation	[40]
Oxybutinin	HP-β-CD (18 mM)	1.7% n-heptane, 9.2% SDS/n-butanol (1:2), 89.1% 10 mM sodium borate, pH 9.2	3–12 μg/mL, analysis of related substances in tablet	[41]
Phenethylamine drugs	Sulfated β-CD (4–8%)	0.5% ethyl acetate, 1.0% SDS, 4.0% 1-butanol, 3.0% propan-2-ol 91.5% 20 mM phosphate buffer, pH 2.5	0.06–0.1%, minor enantiomer and related substances	[72]
Pheniramine	HP-β-CD (30 mM)	50 mM sodium phosphate, pH 3.0	3.3 μM, analysis of racemic drug in eye drops	[91]
Pregabalin, tosyl or dansyl derivatized	PA-β-CD (3.4 mM)	100 mM sodium phosphate, pH 6.8	0.1%, determination of enantiomeric purity	[29]
Raltitrexed	CM-β-CD (8 mM)	75 mM Tris/phosphoric acid, pH 8.0, 30 mM SDS	0.1 μg/mL, analysis of synthetic laboratory sample	[92]
Sibutramine	M-β-CD (5 mM)	10 mM sodium citrate/20 mM sodium phosphate, pH 4.3	Analysis of racemic drug in formulation	[93]
Tenofovir and related nucleoside phosphonates	β-CD (20 mg/mL)	30–50 mM sodium borate, pH 10–10.5	0.51–1.36 μM, enantiomeric purity of laboratory samples	[94]
DL-Tetrahydropalmitine	BSA (50 μM)	20 mM sodium phosphate, pH 7.4	6–7 ng/mL, analysis of plant extract	[95]

Table 2 (*Continued*)

Drug	Chiral selector (concentration)	Background electrolyte	LOD/comment	Ref.
Tic-hydantoin derivatives	HS-β-CD (10 mM)	50 mM sodium phosphate, pH 2.5, 5% ethanol	0.90–1.31 μM, determination of enantiomeric purity, comparison to HPLC	[96]
Tyrosine	–	15 mM sodium borate, pH 10.5	2.2–2.9 μM, derivatization with DBD-PyNCS, analysis of amino acid formulation	[68]
Zopiclone	β-CD (20 mM)	60.2 mM phosphate buffer, pH 2.0, 1 M urea	2.1–7.2 μg/mL, analysis of racemic drug in tablet	[97]

PA-β-CD, 6-monodeoxy-6-mono-(3-hydroxy)-propylamino-β-CD; CE-β-CD, carboxyethyl-β-CD; CM-β-CD, carboxymethyl-β-CD; M-β-CD, methyl-β-CD; HS-β-CD, highly sulfated β-CD; HS-γ-CD, highly sulfated γ-CD; HP-β-CD, hydroxypropyl-β-CD; HDAS-β-CD, heptakis(2,3-di-O-acetyl-6-O-sulfo)-β-CD; Succ-β-CD, succinyl-β-CD; SBE-β-CD, sulfobutylether-β-CD; HP-γ-CD, hydroxypropyl-γ-CD; PA-β-CD, 6-monodeoxy-6-mono-(3-hydroxy)-propylamino-β-CD; (−)-18C6H4, (−)-(18-crown-6)-2,3,11,12-tetracarboxylic acid; CCD, contactless conductivity detection; DBD-PyNCS, *R*(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole; MES, morpholinoethanesulfonic acid.

0.1 M Tris-phosphate buffer, pH 5.0 [73]. Only 1S,2R-(+)-ephedrine could be detected in the ampoules while both impurities were found in the injection. Moreover, when applying the method to the quantitative determination of 1R,2S-(−)-ephedrine significant differences between the labeled content and the determined quantities were noted.

A chiral ligand-exchange method using the Cu(II)/L-proline complex as chiral selector for the enantioseparation of levothyroxine has been developed and validated [74]. The background electrolyte was optimized regarding the concentration and composition of the chiral selector, the organic modifier and the pH. The final conditions employed a 15 mM borate buffer, pH 9.6 containing 0.05 mM copper(II)sulfate and 0.4 mM L-proline as well as 10% acetonitrile. Using UV detection the method proved to be suitable for the analysis of commercial levothyroxine tablets (Fig. 7). The drug contained two minor impurities one of which could be ascribed to dextrothyroxine. In addition, hyphenation to ICP-MS was performed with reduction of the borate concentration of the background electrolyte to 10 mM. Using L-triiodothyronine as internal standard satisfactory reproducibility of the assay was achieved although the resolution of the thyronine enantiomers was lower compared to the CE-UV method.

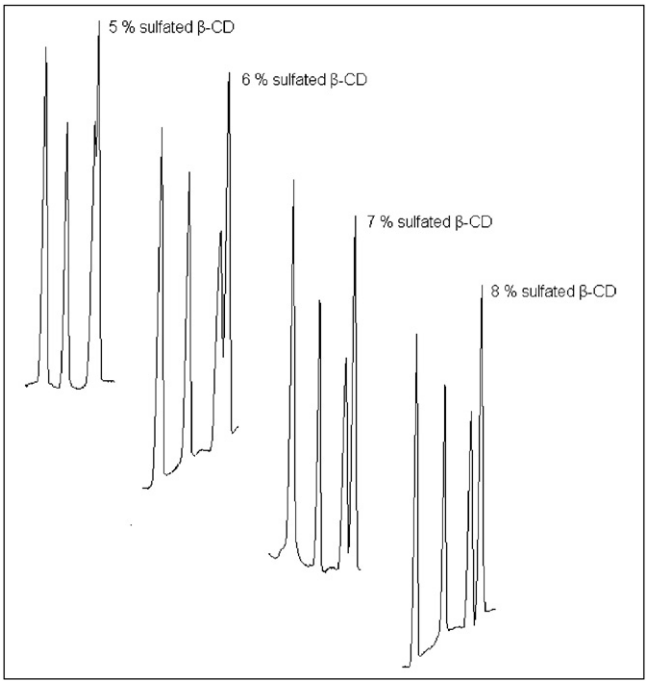

Fig. 6. Dependence of the enantioseparations of ephedrine and pseudoephedrine on the concentration of sulfated β-CD in CD-modified MEEKC. Experimental conditions: 50.2/40 cm, 50 μm id fused-silica capillary; 0.5% ethyl acetate, 1.0% SDS, 4.0% 1-butanol, 3.0% 2-propanol, 91.5% 20 mM sodium phosphate buffer, pH 2.5; −15 kV, 20 °C, 200 nm.
Reproduced from [72] with permission.

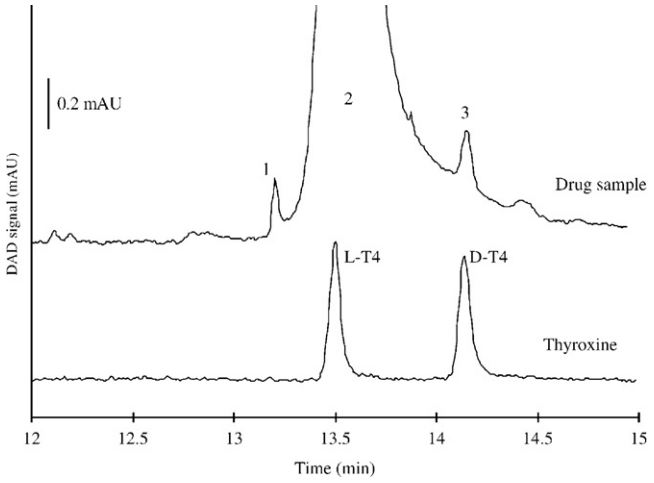

Fig. 7. Electropherogram of (top) a commercial levothyroxine tablet and (bottom) a standard solution of levothyroxine (L-T4) and the enantiomer (D-T4). *Experimental conditions*: 15 mM sodium borate buffer, pH 9.6, containing 10% acetonitrile, 0.05 mM copper(II)sulfate and 0.4 mM L-proline, 15 kV, 25 °C, 226 nm.
Reproduced from [74] with permission.

Although direct application to pharmaceutical analysis was not the topic, Kojtari and Foley investigated the effect of the purity of the chiral detergent N-dodecoxycarbonylvaline (DDCV) as microemulsion component in chiral MEEKC analysis of the ephedrine enantiomers [75]. Either R- or S-DDCV or non-racemic mixtures of the detergent were used. As expected, the enantioresolution of ephedrine decreased with the decreasing enantiomeric excess of the chiral surfactant emphasizing the importance of pure reagents for reproducible analyses.

3.2. Biomedical analysis

Recent examples of bioanalytical studies are summarized in Table 3. These include the analysis of plasma or urine for the determination of the stereoselective metabolism and pharmacokinetics of a drug and the investigation of the in vitro metabolism by liver microsomes or fungi as model organisms.

The stereoselective metabolism of ibuprofen in healthy volunteers with CYP2C8 and CYP2C9 polymorphism has been studied [98]. The enantiomers of ibuprofen and the 2-hydroxy and 2-carboxy metabolites were separated using a 0.2 M triethanolamine/phosphoric acid buffer, pH 5, containing 50 mM 2,3,6-trimethyl-β-CD. In accordance with the unidirectional inversion of R-ibuprofen to S-ibuprofen, plasma levels of S-ibuprofen

Table 3
Recent examples of chiral drug bioanalysis by electromigration techniques.

Analyte	Matrix	Chiral selector (concentration)	Background electrolyte	Extraction	Application/comment	Ref.
Amphetamine, p-chloroamphetamine	Urine	(+)-18C6H4 (0.8 mM)	50 mM *bis*-Tris/citric acid, pH 4.0	SDME	Spiked urine	[102]
Anisodamine	Plasma	HP–CD (20 mg/mL), CM-β-CD (5 mg/mL)	0.11 M Tris/phosphoric acid, pH 4.0	SPE	Pharmacokinetics in rabbits	[103]
Dioxopromethazine	Urine	β-CD (16.5 mM)	25 mM Tris/phosphoric acid, 40 mM boric acid, pH 2.5	LLE	Spiked urine	[104]
Fenbendazole	–	HDAS-β-CD (10 mM), HDMS-β-CD (10 mM)	10 mM ammonium formate, 0.5 M trifluoroacetic acid in methanol	Protein precipitation by MeOH	Stereoselective metabolism by rat liver microsomes	[100]
Hydroxychloroquine	–	Sulfated β-CD (1%), HP-β-CD (30 mM)	0.1 M Tris, pH 9.0	LLE	Stereoselective metabolism by rat and mouse liver microsomes	[105]
Ibuprofen	Plasma, urine	TM-β-CD (50 mM)	20 mM tri-ethanolamine/phosphoric acid, pH 5.0	SPE	Stereoselective metabolism in humans with CYP450 polymorphism	[98]
Ketamine	–	Sulfated β-CD (10 mg/mL)	50 mM Tris/phosphoric acid, pH 2.5	LLE	Stereoselective metabolism by human, equine and canine liver microsomes	[99]
Methamphetamine and related drugs	Urine	β-CD (12 mM), DM-β-CD (6 mM)	125 mM Tris/sodium dihydrogen phosphate, pH 6.15	LLE	PVA-coated capillary, analysis of drug addict samples	[106]
Midodrine, desglymidodrine	–	TM-β-CD (30 mM)	70 mM sodium acetate, pH 5.0	LLE	Stereoselective metabolism by endophytic fungi	[101]
Mirtazapine	Urine	CM-β-CD (0.55%)	50 mM sodium phosphate, pH 2.5	SPME	Stereoselective metabolism after administration of racemic drug	[107]
Propranolol, 4-hydroxypropranolol	–	CM-β-CD (4%)	25 mM triethylamine/phosphoric acid, pH 9.0	LLE	Stereoselective metabolism by endophytic fungi	[108]

LLE, liquid–liquid extraction; SPE, solid phase extraction; SDME, single drop microextraction; SPME, solid phase microextraction; HDMS, heptakis(2,3-di-O-methyl-6-O-sulfo)-β-CD; TM-β-CD, 2,3,6-trimethyl-β-CD; DM-β-CD, 2,6-dimethyl-β-CD; (+)-18C6H4, (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid; for abbreviations of selectors see Table 2.

were always higher during the elimination process. Differences in the pharmacokinetic data such as elimination half-life and clearance depending on genotype were observed. Impaired metabolism of the ibuprofen enantiomers was associated with the presence of the CYP2C9*3 allele and the CYP2C8*1/*3 and CYP2C9*1/*3 genotype. However, pharmacokinetic profiles of ibuprofen and the metabolites also differed for subjects with no detected mutations resulting in similar profiles as found for carriers of mutated alleles.

The stereoselective in vitro metabolism of ketamine by human, equine and canine liver microsomes has been compared by Thromann and co-workers [99]. The enantiomers of ketamine and the major metabolite norketamine as well as hydroxylated norketamine metabolites and 5,6-dehydronorketamine could be resolved simultaneously in a 45/34 cm, 50 μm id fused-silica capillary using a 50 mM Tris/phosphoric acid buffer, pH 2.5, containing 10 mg/mL sulfated β-CD as background electrolyte. As the metabolites were not available except for norketamine, the assignment of the metabolite stereoisomers was achieved by separate incubation of the individual enantiomers of ketamine as well as racemic drug with equine liver microsomes. Ketamine was metabolized stereoselectively by the microsomes of all three species (Fig. 8). Moreover, the metabolites were formed to a different extent by each species. N-demethylation of the parent drug to norketamine was modeled according to the Michaelis–Menten kinetics revealing a reduced conversion of S-ketamine in the racemic drug by human microsomes compared to the administration of the pure S-enantiomer. In contrast, no difference was observed for the other species when administering racemic drug or the single enantiomer. The authors concluded that the reduced conversion can be related to a reduced hepatic clearance of the drug which could be of clinical relevance.

An application of non-aqueous CE (NACE) in biomedical analysis has been published by Rousseau et al. [100]. The in vitro metabolism of the achiral drug fenbendazole to the chiral sulfone metabolites as well as the sufoxide was studied. The compounds were separated using a dual CD system of HDMS-β-CD and HDAS-β-CD in an electrolyte solution of ammonium formate in methanol acidified with trifluoroacetic acid. The simultaneous use of both CDs proved to be essential for complete enantioresolution of the sulfone enantiomers. Initial incubations of fenbendazole with rat liver microsomes indicated a non-stereoselective sulfoxidation. In principle, the method can also be applied to CE-MS because the background electrolyte is volatile and the CDs migrate in the opposite direction to the MS.

The group of Bonato has studied the metabolism of racemic drugs such as propranolol [108] or midodrine [101] by endophytic fungi in order to evaluate these microorganisms as sources for the production of pure metabolites for pharmacological and toxicological studies. In both cases, stereoselective formation of the respective metabolites of the drugs by the fungi was observed.

3.3. Further applications

CE has also evolved as a quick and accurate technique for the determination of interactions between molecules including protein–drug, protein–DNA, peptide–carbohydrate, peptide–peptide, DNA–dye, carbohydrate–drug, antigen–antibody, and other biological interactions. The most recent review on the determination of binding constants by affinity CE (ACE) can be found in [109]. As demonstrated by Chu et al. [110], partial-filling ACE can be employed to study the stereospecific binding of the S-configured rotigotine and its R-enantiomer to bovine and human serum albumin. The method used a 50 mM phosphate buffer, pH

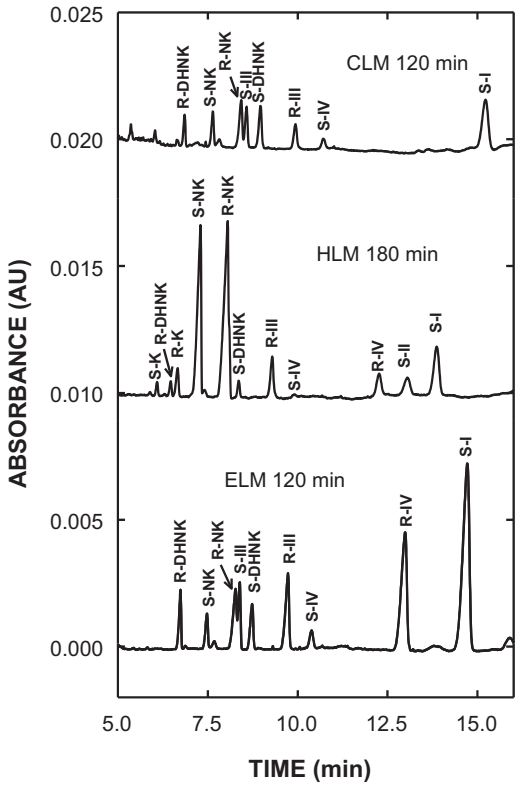

Fig. 8. Comparison of metabolite formation between incubations of 50 μM racemic ketamine with canine liver microsomes (CLM), human liver microsomes (HLM) and equine liver microsomes (ELM). Experimental conditions: 45/34 cm, 50 μm id fused-silica capillary, 50 mM Tris/phosphoric acid, pH 2.5, −20 kV, 20 °C, 200 nm. K, ketamine; NK, norketamine; HNK, hydroxynorketamine; I–IV, different HNK metabolites. R- and S- denote the configuration of the respective analyte. Reproduced from [99] with permission.

7.4, as background electrolyte at 37 °C. The albumin concentration was varied by increasing the length of the injected plug. For both albumins significantly weaker binding of rotigotine than the R-enantiomer was found with a general higher affinity to human serum albumin. Displacement experiments with warfarin and ketoprofen indicated multiple binding sites for rotigotine and the enantiomer. The stereoselective protein binding of amlodipine using chiral CE as analytical method has also been studied [111].

In recent years, CE has been increasingly used in enzyme assays covering all aspects of enzyme-related analysis including the evaluation of the enzymatic activity, enzyme kinetics, identification of enzyme substrates, inhibitors and activators, or the investigation of enzyme-mediated metabolic pathways [112,113]. Chiral ligand-exchange based CE assays have been reported for the determination of the enzyme kinetic constant of amino acid oxidase [114,115]. Furthermore, a stereospecific CE assay for the determination of the activity of 4-hydroxyproline-2-epimerase which is expressed in various pathogenic bacteria has been developed as a stereospecific platform for inhibitor screening [116]. The substrate and potential inhibitors were incubated with the epimerase followed by derivatization with FMOC chloride. Subsequent analyte separation was achieved in a 100 mM HEPES buffer, pH 8, containing 30 mM SDS. The D/L-ratio of proline was determined using 140 mM HEPES, pH 8, containing 30 mM taurodeoxycholate and 30 mM β-CD as background electrolyte. The Michaelis–Menten kinetics were assessed. The enzyme was inhibited by the known competitive inhibitor pyrrole-2-carboxylic acid demonstrating the feasibility of the assay for the identification of inhibitors in compound screening.

4. Conclusions and outlook

Work published in recent years has clearly documented the potential of electromigration techniques for enantioseparations. This is specifically true for chiral EKC employing a chiral selector as pseudostationary phase in a background electrolyte due to the high flexibility and enantioselectivity. Efforts have continued to rationalize chiral separation mechanisms in EKC. New areas such as inhibitor screening have also been reported recently. In contrast, CEC does not yet seem to be valid enough for implementation in routine analysis. Further methodological studies have to be performed until true applications will be presented. The general trend of further miniaturization based on chip technology [117] will also lead to their use in pharmaceutical analysis where reports on drug analysis are still scarce. If such a format can be realized for routine applications they will have a promising future potential for analytical enantioseparations.

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