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Review

Recent advances in capillary electrophoretic migration techniques for pharmaceutical analysis (2013–2015)

This review updates and follows-up a previous review by highlighting recent advancements regarding capillary electromigration methodologies and applications in pharmaceutical analysis. General approaches such as quality by design as well as sample injection methods and detection sensitivity are discussed. The separation and analysis of drug-related substances, chiral CE, and chiral CE-MS in addition to the determination of physicochemical constants are addressed. The advantages of applying affinity capillary electrophoresis in studying receptor–ligand interactions are highlighted. Finally, current aspects related to the analysis of biopharmaceuticals are reviewed. The present review covers the literature between January 2013 and December 2015.

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1 Introduction

CE is an important technique for analyzing many pharmaceutical and biopharmaceutical substances [1–9]. Consequently, protocols for method validation and instrument qualification have been developed and CE methods have been applied during all stages of drug discovery as well as quality control of the

finished products [9–14]. The advantage of CE is its inherent flexibility as well as the various available modes including CZE, capillary EKC, MEKC, MEEKC, cIEF, and CGE among others.

In pharmaceutical analysis, CE has been applied to the determination of the main component as well as the purity of drugs with regard to related substances and stereoisomeric impurities, of drug counter-ions, of physicochemical properties such as log P and pK_a [1, 4–6, 9]. Drug-macromolecule binding properties were studied by ACE [15]. For the analysis of biopharmaceuticals the so called CE-SDS, which refers to CGE replacing the classical SDS-PAGE, and cIEF have been used for protein characterization [1, 2, 9, 16–18]. CE-MS has become a routine method for small molecule analysis [19–21] and an integral part of glycan analysis of biopharmaceuticals [22–27] and natural glycoproteins [27–29].

This review focuses on recent advances and applications of capillary electromigration techniques for the analysis of pharmaceutical substances covering the literature published between January 2013 and January 2016. The analysis of small

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Abbreviations: AQbD, analytical quality by design; CDTA, cyclohexate-1,2-diaminetetraacetic acid; CM- α -CD, carboxymethyl- α -CD; CM- β -CD, carboxymethyl- β -CD; ee, enantiomeric excess; [¹⁸F]FAC, 1-(2'-deoxy-2'-[¹⁸F]fluoro- β -D-arabinofuranosyl)cytosine; [¹⁸F]FLT, 3'-deoxy-3'-[¹⁸F]fluorothymidine; HP- β -CD, hydroxypropyl- β -CD; IS, internal standard; M- β -CD, methyl- β -CD; MODS, method operable design space; PET, positron emission tomography; Phen, 1,10-phenanthroline; QbD, quality by design; TM- β -CD, 2,3,6-trimethyl- β -CD; USP, United States Pharmacopeia; VGCE, velocity gap mode of CE

Colour Online: See the article online to view Fig. 4 in colour.

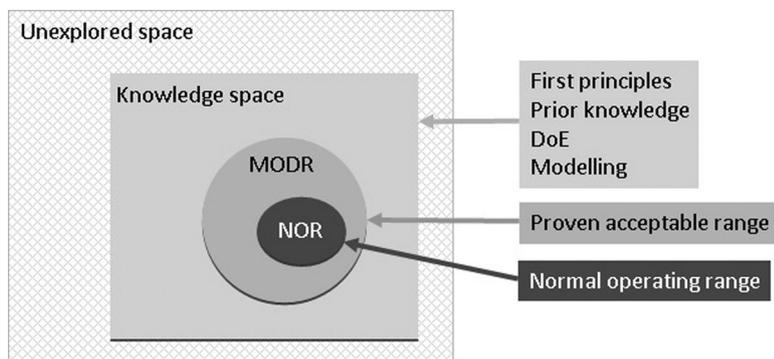


Figure 1. The normal operating range of a method is well within the proven acceptable range, the method operable design region (MODR). Knowledge about these ranges comes from firm knowledge and understanding the method and technique through first principles, prior knowledge of the analytes, designs of experiments (DoE), and modeling. Setting the limits is based on risk management and part of a control strategy.

molecules and large biomolecules will be discussed. The analysis of drugs in biological media or the use of pharmaceutical drugs for the demonstration of the feasibility of a certain separation technique will not be considered. The determination of drugs in biological fluids such as plasma or urine has been summarized [30–32]. CE has also been used for the characterization of herbal drugs including preparations of traditional Chinese medicine or the detection of adulterants in such preparations. These analyses have been summarized, for example, in [33–37] and are not discussed in the present review either. For earlier compilations of the use of capillary electromigration methods in drug analysis see, for example [1–14, 22–27].

2 General considerations of CE methods in pharmaceutical analysis

2.1 Quality by design

The core of the quality by design (QbD) way of thinking is in a crucial sentence in the ICH Q8 Guideline: “quality cannot be tested into products, i.e. quality should be built in by design”. The same attitude is applied in analytical QbD (AQbD). Compliance to requirements should be considered during the method development process and not just tested afterward. The requirements on the method include sufficient selectivity, precision, accuracy, and robustness for the intended purpose. These requirements are set and documented before method development and validation and provide the framework for development activities. Awareness of the requirements leads to conscious choices during development in order to fulfill all these requirements.

However, AQbD encompasses more than a fit-for-purpose methodology. Firm knowledge of method performance and limitations results in a risk management and quality control strategy. This includes marking the normal operating range of a method but also knowing the method operable design space (MODS) as shown in Fig. 1.

Although QbD and AQbD are hot topics in industry, there are very little AQbD publications. Most publications are in the form of lectures and posters at industrial scientific meeting series like “CE in the Pharmaceutical and Biotech Industry”

and “Analytical Technologies Europe” [38]. The research papers that appeared since the previous review are all by the same group [39–45]. These papers give excellent explanations on the DoE procedures used. Unfortunately, the purposes of the methods in these papers were generally not quantitatively described but subjectively phrased as e.g., “baseline separate and accurately quantitate” [41]. There were no preset, specific numbers given on e.g. precision and accuracy requirements and there were no explanations on what “accurately quantitate” actually meant. Neither did the authors explain what conscious decisions they took in order to *design* the quality of the method based on separation mechanisms, good working practices, and/or prior knowledge. The focus was very much on the separation aspects of the methods. The strength of this group is the extension of the normal operating range of the method into the design space.

Although maybe not always of interest for a scientific paper, it is important to realize that AQbD is more than the establishment of an MODS, which in turn is more than applying a multivariate DoE. We hope that more industrial papers describing the application of the full AQbD process will appear in the near future.

2.2 Method injection and detection sensitivity

Sensitivity in CE remains the focus of many publications. Only in *Electrophoresis*, the keywords “capillary electrophoresis stacking” gave 58 hits for 2015. Review papers on sample stacking and injection techniques in CE appear regularly [46–53]. Indeed, one of the most frequently cited concerns in CE is that the concentration limits of detection are inferior to what can be achieved with other liquid separation techniques [54]. Most reviews discuss the various techniques from the mechanistic point of view. Breadmore and Sanger [48] considered in-capillary concentration from a practical point of view and ask the question: I have XYZ in my sample, how do I improve my sensitivity? Although there are many detailed procedures described in literature as reviewed in [46–53], one can generally divide in-capillary sample concentration techniques in three categories, (i) stacking, (ii) sweeping, and (iii) transient-isotachopheresis. In-capillary sample concentration generally speaking means that the analyte ions move in a different velocity in the sample zone

compared to their velocity in the BGE. In stacking the analyte migrates faster in the sample zone and in sweeping they migrate slower in the sample zone compared to the electrolyte. When the analytes reach the electrolyte boundary they slow down resp. are swept together, increasing in concentration.

Another approach to work on the detectability of analytes is of course derivatization. Wuethrich and Quirino wrote a comprehensive review that can be used as a kind of guide on “how to get started” with derivatization for separation and detection in CE [55]. The section about method development and separation efficiency of the former review [1] has been already covered in this section.

3 Separation and analysis of small molecules

In the case of small molecule drugs, capillary electromigration techniques have been applied to all aspects of drug analysis including the determination of the content of the drug as well as the analysis of drug-related impurities, organic and inorganic counter ions, the stereochemical purity, or the determination of physicochemical parameters such as pK_a values, lipophilicity ($\log p$ values) or drug protein binding as summarized earlier [1–9]. Moreover, reviews with a focus on the determination of impurities and counter ions in pharmaceuticals by electromigration techniques have been published [56, 57]. The use of nonaqueous CE for the analysis of pharmaceuticals has been addressed [58]. The publications that have appeared within the period of time covered by the present review employed capillary electromigration techniques including CZE, EKC, MEKC, or MEEKC. CEC as a hybrid technique combining the high resolution of CE with the selectivity of stationary phases has still not matured to a reproducible technique and has hardly been applied to pharmaceutical analysis.

3.1 Analysis of related substances

ICH guideline Q3A(R2), entitled “Impurities in New Drug Substances”, differentiates between organic impurities, inorganic impurities, and residual solvents [59]. The organic impurities, also termed related substances in pharmacopeias, are drug- and process-related comprising starting materials, by-products, intermediates, degradation products as well as reagents. For a drug with a maximal daily dose of 2 g (which applies to most modern drugs), the following thresholds are typically applied: a reporting threshold of 0.05%, an identification threshold of 0.1%, and a qualification threshold of 0.15% where the toxicity of the impurity must be determined [59]. Genotoxic impurities are usually in the ppm range and their determination by CE is challenging due to the low concentration sensitivity of the technique. Nonetheless, CE hyphenated to MS has been applied for the determination of potentially genotoxic alkylating agents upon derivatization [60]. Thus, potentially toxic bromomethylphenyl compounds

were derivatized with either 4-dimethylaminopyridine or butyl 1-pyridinyl-4-yl)piperidine-4-carboxylate to yield quaternary pyridinium derivatives that were separated in a BGE comprising 100 mM Tris adjusted to pH 2.5 with phosphoric acid and 20% acetonitrile. Due to the low EOF at pH 2.5, the phosphate ions did not migrate toward the cathode so that sensitive MS detection could be accomplished despite the fact that a nonvolatile BGE was used. Electrokinetic injection further increased method sensitivity. The composition of the sample matrix, injection voltage and time as well as buffer concentration were optimized by experimental design, resulting in LOD values in the sub ppm range between 0.1 and 0.4 mg/kg. Compared with an HPLC-MS method, the optimized CE-MS assay proved to be about tenfold more sensitive for analyzing the alkylating compounds in model drugs.

Hydrazine and alkylamine impurities were quantified using indirect photometric detection at 350 nm [61]. The separation was carried out in a 50- μ m id fused silica capillary with an extended light path (bubble cell) at an applied voltage of 30 kV. The optimized BGE comprised aqueous 5 mM 4-aminopyridine adjusted to pH 5.5 by the addition of phosphoric acid. In addition, the sampling rate of the detector was investigated and a rate of 20 Hz provided the highest sensitivity and lowest background noise. The LOD values ranged between 0.1 and 0.3 μ g/mL which translated into the low ppm range with regard to the studied drug substances. Consequently, the validated assay was applied to the determination of trimethylamine in emtricitabine as well as hydrazine in cisplatin, carboplatin, and oxaliplatin. The hydrazine content was below 2 ppm in the case of the platinum drugs, whereas 35–275 ppm were found in the emtricitabine batches investigated.

Recent publications on the analysis of related substances in pharmaceutical drugs have been compiled in Table 1 [39, 40, 42, 43, 45, 62–72]. Most separations were carried out in the CZE mode, while four methods applied MEKC [42, 65, 69, 70] and one MEEKC conditions [40]. LODs were in the low microgram per milliliter to sub microgram per milliliter or 0.1% range allowing the sensitive detection of the respective impurities. Furthermore, AQbD approaches have been applied in order to develop robust and precise methods for drug purity control [39, 40, 42, 43, 45]. The topic has also been reviewed [73, 74]. Furthermore, the simultaneous analysis of multiple drugs and their impurities in a single run may be achieved by electromigration methods. This has been demonstrated for an MEEKC method for fluoroquinolone antibiotics although not exact detection limits for the impurities were reported in the study [75].

While most publications dealt with organic impurities, two studies investigated inorganic analytes. Gotti and coworkers reported a method for the determination of free iron(II) in an iron-sucrose product used for the treatment of anemia [68]. The injection is a colloidal solution of ferric hydroxide in complex with sucrose containing 20 mg/mL elemental iron. According to the United States Pharmacopeia (USP) the limit of iron(II) is 0.4% corresponding to about

Table 1. Examples of drug purity analysis by CE

Drug	Sample	BGE	LOD/comment	Ref.
Almotriptan	Tablet	86.72% sodium borate, pH 9.4, 0.91% <i>n</i> -heptane, 12.37% SDS/ <i>n</i> -butanol (1:2, v/v)	0.8–2.0 µg/mL, analysis of related substances	[43]
Amitriptyline	Tablet	10 mM sodium borate, pH 9.1, 4.0% SDS, 7.12% <i>n</i> -butanol, 8.0% acetonitrile, 2.93% urea	0.7–2.0 µg/mL, analysis of related substances	[42]
Cetirizine	Bulk drug, tablet	75 mM sodium phosphate, pH 2.8	10 µg/mL, analysis of synthetic impurities	[62]
Esomeprazole	Coated pellets	100 mM Tris-phosphate, pH 2.5, 20 mM HP-β-CD, 1 mM sodium dithionite	0.6–1.4 µg/mL, simultaneous determination of sulfone impurity and enantiomeric purity	[63]
Fabomotizol (Afobazol)	Synthetic sample, tablet	sodium phosphate, pH 3.9	LOD not determined	[64]
Fexofenadine	Bulk drug, tablet	150 mM sodium phosphate, pH 3.0, 25 mM SDS, 22% acetonitrile	LOQ 50–100 µg/mL, analysis of impurities	[65]
Gemifloxacin	Bulk drug	25 mM sodium phosphate, pH 10	1.0 µg/mL, analysis of synthetic impurity	[66]
Glibenclamide	Tablet	30 mM sodium borate, pH 10.2	0.03–0.05%, analysis of impurities	[45]
Hydrochlorothiazide	Drug substance	20 mM sodium phosphate, pH 8.0	0.03–0.04 µg/mL, Fourier transform convolution of data, determination of hydrolysis kinetics	[67]
Iron sucrose complex	Injection formulation	60 mM sodium borate, pH 9.3	0.04% iron(II), precapillary complexation if iron species with 1,10-phenanthroline and cyclohexane-1,2-diaminetetraacetic acid	[68]
Loratidine	Drug substance, tablet	10 mM sodium borate, pH 9.30, 40 mM SDS, 20 mM HP-β-CD	0.6 µg/mL, analysis of desloratidine	[69]
Metformin	Tablet	30 mM Britton-Britton-Robinson buffer, pH 4.35, 15 mM CM-β-CD	0.03–0.05%, analysis of impurities	[39]
Nelfinavir	Tablet	25 mM sodium borate, pH 9.24, 9 mM SDS, 10% methanol	8.03–18.7 µg/mL, analysis of impurities	[70]
Quetiapine	Drug substance	80 mM sodium phosphate, pH 4.0	0.4 µg/mL impurities	[71]
Zoledronate	Bulk drug	7.5 mM phthalic acid/Tris, pH 3.5	1.8–2.8 µg/mL, analysis of phosphite and phosphate, indirect detection	[72]
Zolmitriptan	Tablet	138 mM sodium phosphate, pH 2.74	0.6–2.0 µg/mL, analysis of related substances	[40]

β-CD, β-cyclodextrin; SDS, sodium dodecyl sulfate.

20% of the total iron. Consequently, the authors developed a method involving mineralization of elemental iron to iron (III) and subsequent determination of iron(III) and iron(II). It is based on the pre-capillary complexation of iron(II) by 1,10-phenanthroline (Phen) and of iron(III) by cyclohexane-1,2-diaminetetraacetic acid (CDTA). Thus, mineralization of iron was accomplished by treatment with 6 M HCl to convert the elemental iron into iron(III) without formation of iron(II). Upon complexation of the iron species by the subsequent addition of Phen and CDTA, separation of the resulting complexes was carried out in a 50-µm-id fused-silica capillary with an extended light path (bubble cell) at an applied voltage of 25 kV at 25°C using a 60 mM sodium borate buffer, pH 9.30, as BGE. Under these conditions the positively charged iron(II)-Phen complex migrated before the EOF, while the negatively charged iron(III)-CDTA complex migrated after the EOF (Fig. 2). Analysis time was about 10 min. Using suprofen as internal standard the method was validated for both iron species according to the ICH guidelines allowing the detection of 1.6 µM iron(II) which corresponds to 0.04% of the total iron. Analyses of iron sucrose injections revealed

an iron(II) content of about 5 % of the total iron content which is in accordance with the USP requirements.

Alvarez and colleagues developed a method for the analysis of phosphate and phosphite ions in the bisphosphonate drug zoledronate [72]. The separation of the analytes was accomplished in a fused-silica capillary employing 7.5 mM phthalic acid adjusted to pH 3.50 by the addition of Tris as BGE. Indirect detection at 205 nm allowed the detection of 1.8 and 2.8 µg/mL of phosphite and phosphate, respectively. The method was validated and applied to monitor the inorganic impurities in zoledronate batches.

In the case of prasugrel hydrochloride, the simultaneous analysis of the counter ion chloride and acetate as an impurity from the synthesis using indirect photometric detection was accomplished in a BGE comprising 3.5 mM 1,2,4,5-benzenetetracarboxylic acid, 20 mM diethylamine, and 0.5 mM myristyltrimethylammonium bromide, pH 8.1 [76]. 1,2,4,5-Benzenetetracarboxylic acid was selected from several aromatic carboxylic acids based on baseline noise and detection sensitivity. The method was validated for both anions and an LOD of 3.8 µg/mL was found for acetate.

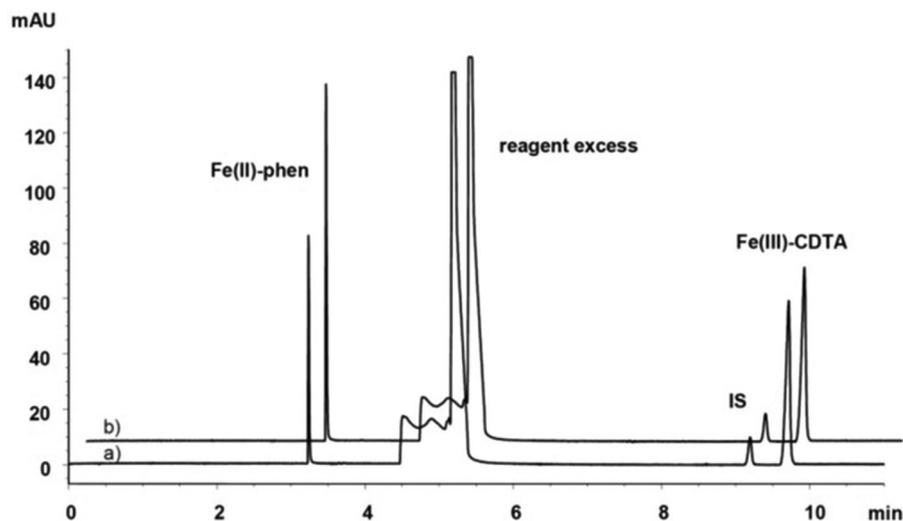


Figure 2. Electropherograms of samples containing iron(II) and iron(III) complexed with Phen and CDTA, respectively. Experimental conditions: 64.5/56 cm, 50 μm id fused-silica capillary, 60 mM sodium borate, pH 9.3, 25 kV, 25°C, 265 nm. Sample (a) contained about 5 % iron(II), sample (b) about 10 % iron(II); IS, internal standard (suprofen). (Reproduced with permission from Wiley-VCH from [68].)

Analysis of two drug batches revealed chloride concentrations of 8.3 and 8.5%, respectively, while the concentrations of the impurity acetate were 0.04 and 0.06%. CE was also applied to the simultaneous determination of diclofenac as well as its common counter ions sodium, potassium, and diethylammonium using capacitively coupled contactless conductivity detection by Cunha and coworkers [77]. Separation of the analytes was achieved in a fused-silica capillary with a BGE comprising 10 mM Tris and 10 mM 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid, pH 8.2. The positively charged counter ions migrated before the EOF, while diclofenac migrated as anion after the EOF. Using salicylic acid as internal standard, the method was validated and applied to the analysis of commercial samples. Good agreement between the CE data and concentrations obtained by HPLC for the analysis of diclofenac and flame photometry for the analysis of sodium and potassium were observed.

A group of pharmaceutical compounds that is not often characterized are positron emission tomography (PET) tracers such as 1-(2'-deoxy-2'-[^{18}F]fluoro- β -D-arabinofuranosyl)cytosine ([^{18}F]FAC) or 3'-deoxy-3'-[^{18}F]fluorothymidine ([^{18}F]FLT) due to the fact that they are synthesized on-site and used immediately. Nonetheless, analytical characterization is required for safety reasons. Cheung and colleagues compared CE and HPLC for the analysis of the above mentioned PET tracers [78]. In the case of [^{18}F]FLT the known impurities and side products could be separated by MEKC in a 21/31.5 cm, 75- μm id fused-silica capillary using a 30 mM sodium phosphate buffer, pH 7.0, containing 100 mM SDS. The system also allowed the separation of the α -anomer of [^{18}F]FAC. Compared to an HPLC method, slightly higher LOD and LOQ values were observed for the impurities when using CE, but analysis time was shorter in CE as compared to HPLC (5 min versus 25 min for the analysis of [^{18}F]FLT and about 3 min versus 5 min for the separation of the [^{18}F]FAC anomers). Moreover, the MEKC method allowed the detection of the toxic crown ether Kryptofix 2.2.2 which is

used as a phase-transfer catalyst in the synthesis of the PET tracers.

3.2 Stereoisomer analysis

CE is an effective technique for stereoisomer analysis due to the high separation efficiency. This is also reflected in the number of recent reviews published on CE enantioseparations [79–84]. The scope of chiral CE-MS [85] as well as the application of nonaqueous CE in chiral analysis [86] have been addressed. Furthermore, reviews have been published on new developments of chiral selectors in general [87, 88] as well as on specific selectors including cyclodextrins (CDs) [89–91], antibiotics [92–94] ligand-exchange selectors [95, 96], and ionic liquids [97]. Finally, modeling of dual-selector systems [98] as well as chiral recognition mechanisms [99] have been summarized. Enantioseparations have been used to demonstrate the superiority of the application of a constant current compared to the application of a constant voltage for method transfer [100].

Pharmaceutical drugs are often used as test racemates in order to evaluate new chiral selectors or for mechanistic studies. This aspect will not be covered in the present review but can be found in some of the reviews mentioned above. Several successful applications of CE for stereoisomeric purity testing have been reported and discussed [1, 56, 79]. Table 2 summarizes recent examples for the determination of the stereoisomeric purity of drug substances [44, 63, 101–122]. Although not always calculated, LODs of 0.1 % or below were typically obtained. As in the past, CDs have been by far the most often applied chiral selectors in CE. This is due to the commercial availability of a wide variety of derivatives. Two studies employing different types of chiral selectors were published, i.e., the enantioseparation of amlodipine in the presence of maltodextrin [104] and the separation of the stereoisomers of palonosetron by sodium cholate [113, 114]. The latter studies are also the only example of a chiral separation within

Table 2. Examples of the determination of the stereochemical purity of drugs by electrophoretic migration techniques

Drug	Chiral selector (concentration)	BGE	Sample	LOD/comment	Ref.
Agomelatine analogs	HS- γ -CD (5 %, w/v), MM- β -CD (10 mM)	25 mM sodium phosphate, pH 2.5	Synthetic samples	0.20–0.25 % minor enantiomer	[101]
Alogliptin	SBE- β -CD (5 mM)	25 mM sodium acetate, pH 4.75	Synthetic sample	2 μ g/mL S-enantiomer	[102]
S-Amlodipine	CM- β -CD (4 mM)	40 mM sodium phosphate, pH 3.5	Bulk drug	1 μ g/mL R-enantiomer	[103]
Amlodipine	Maltodextrin (10%, w/v)	100 mM sodium phosphate, pH 4.0	Tablet	0.52 μ g/mL, analysis of racemic drug	[104]
S-Duloxetine	HP- β -CD (0.5%, w/v)	100 mM sodium phosphate, pH 3.0	Capsule	0.2% R-duloxetine, UV-detection	[105]
	HP- β -CD (0.5%, w/v)	150 mM ammonium formate, pH 3.0	Capsule	0,02% R-duloxetine, MS-detection, partial filling technique	[105]
Esomeparazole	HP- β -CD (20 mM)	100 mM Tris-phosphate, pH 2.5, 1 mM sodium dithionite	Coated pellets	0.06% R-enantiomer, simultaneous analysis of sulfone impurity	[63]
R,S-Glycopyrrolate	Sulfated β -CD (2.0%)	30 mM sodium phosphate, pH 7.0	Bulk drug	0.1% of minor stereoisomers	[106]
Lercanidipine	TM- β -CD (10 mM)	200 mM sodium acetate, pH 4.0	Tablet	0.6–0.8 μ g/mL minor enantiomer, analysis of racemic compound	[107]
Levornidazole	Sulfated α -CD (2% w/v)	20 mM Tris-phosphoric acid, pH 2.1	Bulk drug, injection	0.006% enantiomeric impurity, short end injection	[108]
Levosulpiride	Sulfated β -CD (10 mM), M- β -CD (34 mM)	5 mM Britton-Robinson buffer, pH 3.45	Injection	1.2 μ g/mL R-sulpiride	[44]
Magnesium L-aspartate	HP- β -CD (18 mM)	50 mM sodium phosphate, pH 7.0, 18% (v/v) DMSO	Bulk drug, tablet, granulate	LOQ 0.03% D-Asp, derivatization with OPA and NAC, LIF detection, comparison to HPLC	[109]
Nucleotide analog	β -CD (20 mg/mL)	30 mM sodium borate, pH 10.0	Synthetic sample	Not determined, analysis of ee of product	[110]
Nucleoside phosphonate derivatives	β -CD (20 mg/mL) QA- β -CD (10 mg/mL or 60 mg/mL)	50 mM sodium borate, pH 10.31 or 9.85 40 mM sodium phosphate, pH 2.2	Synthetic samples	3.2–11.6 μ M enantiomer	[111]

(Continued)

Table 2. Continued

Drug	Chiral selector (concentration)	BGE	Sample	LOD/comment	Ref.
Omeprazole	M- β -CD (20 mM)	50 mM sodium phosphate, pH 2.5	Tablet	0.9 μ g/mL minor enantiomer, analysis of enantiomer and racemic drug	[112]
Palonosetron	Sodium cholate (30 mM)	30 mM sodium borate, pH 9.20, 1 mM SDS	Drug substance	0.1 μ g/mL minor stereoisomers	[113]
	Sodium cholate (30 mM)	30 mM sodium borate, pH 9.2, 12% methanol		0.3 μ g/mL minor stereoisomers	[114]
S-Pantoprazole	HP- β -CD (20 mg/mL), L-His (15 mM), Cu(II) acetate (10 mM)	5 mM sodium phosphate, pH 5.0	Bulk drug	0.04% <i>R</i> -enantiomer	[115]
Pantoprazole	SBE- β -CD (5 mM)	50 mM sodium phosphate, pH 7.0	Tablet	1.1 μ g/mL minor enantiomer, analysis of racemic drug	[112]
Tapentadol	Sulfated α -CD (12 mM)	50 mM Tris-acetic acid, pH 4.75	Synthetic sample	not determined, 0.1% <i>S,S</i> -enantiomer detected	[116]
	Sulfated α -CD (1.0 %, w/v)	100 mM sodium borate, pH 9.5	Bulk drug	3.0 μ g/mL minor stereoisomers	[117]
Tolterodine, methoxytolterodine	P- γ -CD (3.0 %, w/v)	70 mM Tris-phosphoric acid, pH 2.5	Tablet	0.33–0.52 μ g/mL, determination of 0.2 % <i>S</i> -enantiomer in <i>R</i> -tolterodine	[118]
Valsartan	β -CD (18 mM)	30 mM sodium acetate, pH 4.5	Bulk drug	2.5 μ g/mL <i>R</i> -enantiomer	[119]
	A- β -CD (10 mM)	25 mM sodium phosphate, pH 8.0	Tablet	0.01% <i>R</i> -enantiomer	[120]
Volinanserin (MDL 100,907), synthetic intermediate	CM- γ -CD (15 mM)	50 mM sodium phosphate, pH 3.0	Synthetic sample	2.5 μ g/mL <i>S</i> -enantiomer	[121]
S-Zopiclone	β -CD (10 mM), [EMIM][L-lactate] (20 mM)	30 mM Tris-phosphoric acid, pH 2.5	Tablet	0.03% <i>R</i> -enantiomer	[122]

A- β -CD, acetyl- β -CD; CM- β -CD, carboxymethyl- β -CD; CM- β -CD, carboxymethyl- γ -CD; HP- β -CD, hydroxypropyl- β -CD; HS- γ -CD, highly sulfated γ -CD; M- β -CD, methyl- β -CD; MM- β -CD, 6-monodeoxy-6-amino- β -CD; P- γ -CD, phosphate γ -CD; QA- β -CD, quaternary ammonium- β -CD; SBE- β -CD, sulfobutylether- β -CD; TM- β -CD, 2,3,6-trimethyl- β -CD; [EMIM][L-lactate], 1-ethyl-3-methylimidazolium-L-lactate; ee, enantiomeric excess.

the reviewed period of time using MEKC for the analysis of the stereochemical purity of a drug. Palonosetron contains two chiral centers so that four stereoisomers (two pairs of enantiomers) result. The pharmacologically active drug is the 3*aS*,2*S* stereoisomer. Using a 30 mM sodium borate, pH 9.20, as BGE in a 50/60 cm, 50 μ m id fused silica capillary, the respective enantiomeric pairs could be separated but the 3*aR*,2*R* and 3*aS*,2*R* diastereomers comigrated. Upon addition of 1 mM SDS to the cholate-containing BGE, the separation of all four stereoisomers of the drug could be achieved. The method was subsequently validated with LODs of the minor

enantiomers of 0.1 μ g/mL [113]. Subsequently, a solvent-modified method was developed also using sodium cholate as chiral micelle-forming agent [114]. The separation of all four stereoisomers was achieved by addition of methanol to a 30 mM sodium borate buffer, pH 9.20. Although the method was somewhat less sensitive as judged from the LODs of the minor isomers of 0.3 μ g/mL as compared to the previous study [113], the solvent-modified assay also allowed the precise quantitation of minor stereoisomers [114].

Another compound with two stereocenters is glycopyrrolate, with the *R,S*-stereoisomer used as an anticholinergic

drug. Zhu and colleagues developed a method for the separation of the four stereoisomers of the compound [106]. Upon screening several neutral and charged CD derivatives in a fused-silica capillary using 30 mM sodium phosphate buffer, pH 3.0, as BGE, sulfated β -CD was selected based on the fact that baseline separation of the four stereoisomers was achieved with this CD under reversed polarity of the applied voltage. Changing the buffer pH to 7.0 and switching to “normal” polarity of the applied voltage resulted in a reversal of the migration order of the stereoisomers with the *R,S*-enantiomer migrating last. The method was subsequently optimized with regard to CD concentration as well as buffer type and concentration and validated according to ICH guidelines. The LOD was 0.3 $\mu\text{g/mL}$ for the stereoisomeric impurities and the LOQ was 1 $\mu\text{g/mL}$ corresponding to a concentration of 0.05 % with regard to the concentration of 2 mg/mL of the drug. Only the *S,S*-isomer could be detected in synthetic samples of *R,S*-glycopyrrolate at concentration levels below 0.1 %.

Tapentadol, 3-[(1*R,2R*)-3-(dimethylamino)-1-ethyl-2-methylpropyl]phenol, is a new μ -opioid receptor agonist. Fejös and colleagues studied the physicochemical constants and separation of the enantiomers of the drug [116]. Initially, the protonation constants of the drug were determined by UV spectroscopy as well as potentiometric and $^1\text{H-NMR}$ titration. For example, the macroscopic constants $\text{p}K_{\text{a}1} = 10.59 \pm 0.01$ and $\text{p}K_{\text{a}2} = 9.44 \pm 0.01$ were obtained by potentiometric titration, indicating that the drug exists predominantly as protonated species at physiological pH. Subsequently, the separation of the drug and its *S,S*-enantiomer by native α -CD and β -CD as well as various neutral and charged derivatives of α -CD and β -CD was investigated in a fused-silica capillary using a 50 mM Tris-acetic acid buffer, pH 4.75 as BGE. Out of the 21 CDs tested, 6 α -CD derivatives and 9 β -CD derivatives were able to separate the enantiomers under the experimental screening conditions. The enantiomer migration order depended on the substitution type and in the case of carboxymethyl CDs also on the size of the cavity. In this case, the *R,R*-enantiomer migrated first in the presence of carboxymethyl- α -CD (CM- α -CD) while it migrated second when carboxymethyl- β -CD (CM- β -CD) was used as chiral selector. Complex formation constants of the enantiomers with α -CDs were about 1 order of magnitude lower than the complexation constants determined for the β -CD derivatives, with the weaker bound enantiomer migrating first in all cases. Differences in the mobilities of the diastereomeric complexes were also observed in some cases leading to high resolution (R_s) values. For example, in the presence of 12 mM sulfated α -CD, $R_s = 16.2$ was found. With a 50 mM sodium borate buffer, pH 9.0, as BGE, similar enantioseparations and peak shape were observed for the CDs as compared to the pH 4.75 BGE. Although no assay validation was performed the authors demonstrated that 0.1 % of the *S,S*-enantiomer could be detected as an impurity in tapentadol using 12 mM sulfated α -CD in 50 mM Tris-acetic acid, pH 4.75. In a subsequent study, the separation of all four stereoisomers of tapentadol was achieved [117]. Native α -, β -, and γ -CD as well as neutral and

charged derivatives were initially screened at pH 2.5 (50 mM sodium phosphate buffer) and pH 9.5 (50 mM sodium borate buffer). In the presence of neither native CDs nor neutral CD derivatives satisfactory resolution of all stereoisomers was observed. The separation could be achieved by a combination of hydroxypropyl- β -CD (HP- β -CD) and HP- γ -CD but separation efficiency was not considered to allow the determination of the minor stereoisomeric impurities in the presence of a large excess of the major enantiomer. Consequently, charged CDs were evaluated at pH 2.5 and pH 9.5. While at least partial resolution of the pairs of enantiomers (i.e. *S,S/R,R* and *S,R/R,S*) was observed under all experimental conditions, separations were improved at pH 9.5 as compared to pH 2.5 especially in the presence of carboxymethyl CDs. Representative electropherograms at pH 9.5 are shown in Fig. 3A. An interesting aspect was the reversal of the migration order within the enantiomeric pairs depending on the cavity size between CM- α -CD and CM- β -CD at pH 9.5. Thus, the order was *R,R* > *R,S* > *S,R* > *S,S* at pH 2.5 and *S,S* > *S,R* > *R,S* > *R,R* at pH 9.5. The latter is favorable considering the fact that small amount of the minor stereoisomers in the presence of a large excess of the *R,R*-configured drug. Therefore, the method comprising a 100 mM sodium borate, pH 9.5, as BGE and a concentration of 1.0 % sulfated α -CD as chiral selector was validated. An electropherogram of tapentadol spiked with 0.15 % of the stereoisomeric impurities is shown in Fig. 3B. In a batch of the drug only the *R,S*-impurity could be detected. The four stereoisomers of ketoconazole could be separated by CD-mediated MEKC using 20-mM 2,3,6-trimethyl- β -CD (TM- β -CD) in a 10 mM sodium phosphate buffer, pH 2.5, containing 5 mM SDS and 1.0 % (v/v) methanol [123]. Although it was found that two stereoisomers were present only at low concentrations, neither were the stereoisomers assigned to the respective peaks nor was the method applied to the analysis of the drug.

Dual selector systems were also applied to the determination of the stereoisomeric purity of drugs. Often, systems comprising a charged CD and an uncharged CD are used but combinations of two neutral CDs have also been reported as summarized, for example, in [98, 124, 125]. Orlandini and coworkers combined sulfated β -CD and methyl- β -CD (M- β -CD) for the analysis of the enantiomeric purity of *S*-configured levosulpiride [44]. In this study, an AQBd approach was taken defining the accurate simultaneous determination of the main component and the chiral impurity in a short analysis time as the analytical target profile of the method. In scouting experiments, the separation of the sulpiride enantiomers could be achieved using sulfated β -CD in a Britton-Robinson buffer, pH 3.0. However, the separation efficiency was not high enough to allow the precise determination of the minor *R*-enantiomer in the presence of a large excess of levosulpiride. From the neutral CDs tested, M- β -CD proved to be a suitable combination. Method optimization was performed via experimental design strategies and Monte-Carlo simulations were applied for defining the design space of the method. Analyzing commercial injection

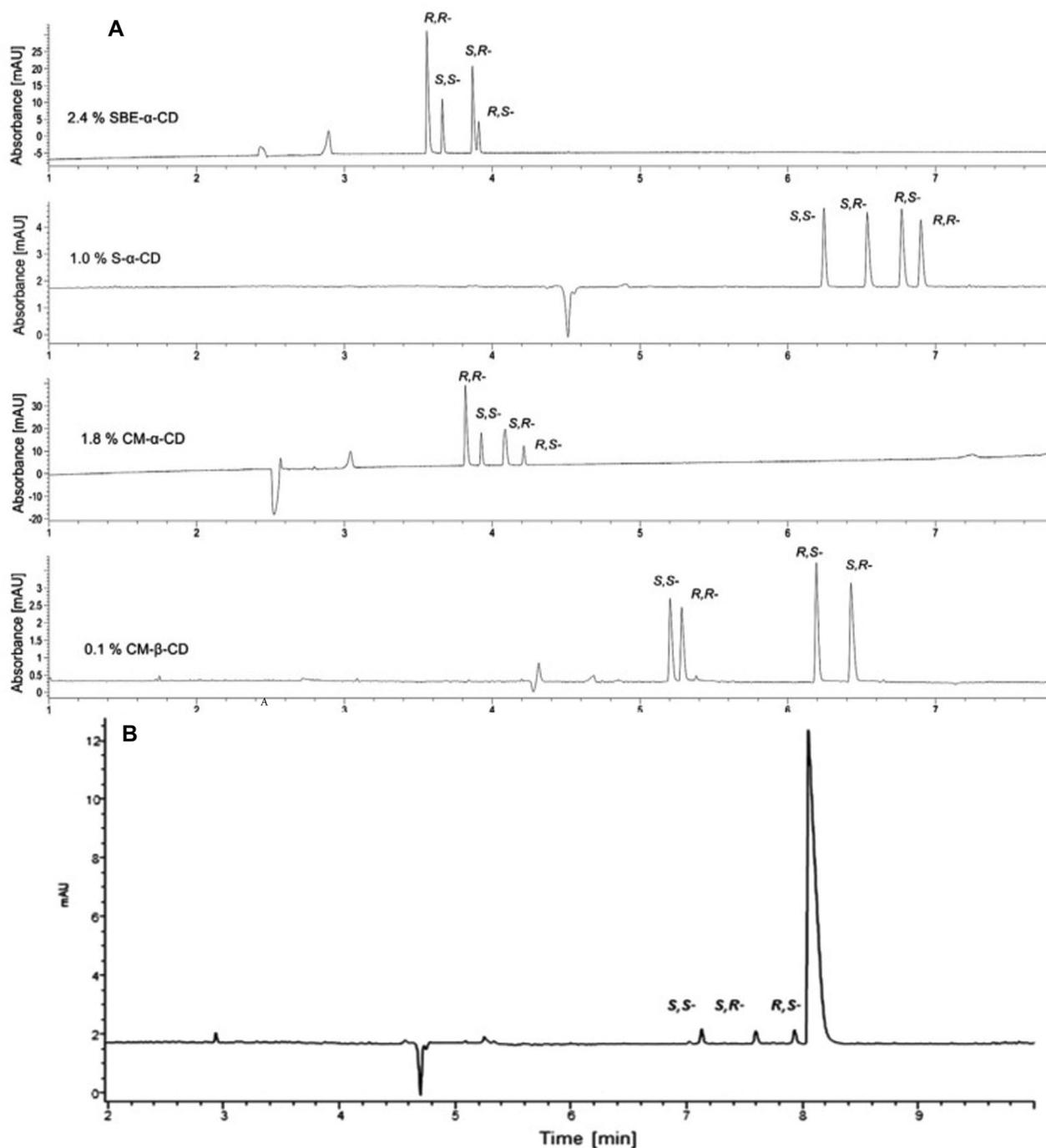


Figure 3. (A) Electropherograms of the separation of the tapentadol stereoisomers in 50 mM sodium borate buffer, pH 9.5 in the presence of negatively charged CD derivatives. (B) Electropherogram of tapentadol spiked with 0.15% of the stereoisomeric impurities using 100 mM sodium borate BGE, pH 9.5, containing 1.0% (w/w) sulfated α -CD. Other experimental conditions: 56.0/64.5 cm, 50 mm id fused silica capillary, 25 kV, 15°C. (Reproduced with permission from Elsevier from [117].)

solutions, concentrations of 100.7 ± 1.1 % for levosulpiride and 0.27 ± 0.02 % for *R*-sulpiride were found. A dual CD system composed of HP- β -CD and mono-6-deoxy-6-piperidine- β -CD in 20 mM sodium phosphate BGE, pH 3.0, was developed for the simultaneous enantioseparation of meptazinol and two chiral synthetic intermediates but not applied to the

determination of the chiral purity of the compound [126]. Lipka and colleagues achieved the enantioseparation of several agomelatine analogs using a combination of the negatively charged highly sulfated γ -CD and the positively charged 6-monodeoxy-6-monoamino- β -CD in a 25 mM sodium phosphate buffer, pH 2.5 [101]. The validated assay allowed the

detection of 0.20–0.25 % of the minor enantiomer based on a concentration of 0.125 mM of the major enantiomer of the respective analog.

Different types of chiral selectors have also been combined. For example, the enantioseparation of several basic drugs by β -CD in a sodium phosphate BGE, pH 2.5, was enhanced upon addition of the chiral ionic liquid 1-ethyl-3-methylimidazolium-*l*-lactate [EMIM][*l*-lactate] [122]. Following method optimization with regard to [EMIM][*l*-lactate] concentration as well as buffer pH and concentration, the method was validated for the enantiomers of the sedative drug zopiclone. The LOD of the *R*-enantiomer in *S*-zopiclone was 0.03 %. The method was applied to the analysis of tablets and a content of the *R*-enantiomer below 0.1 % was noted. Guan and colleagues combined chiral ligand exchange CE using the copper(II)-*l*-His complex with HP- β -CD for the separation of proton pump inhibitors [115]. While enantioresolution with R_S values of about 1.5–2 were observed using the ligand exchange complex, no enantioseparation was observed in the presence of HP- β -CD alone in sodium phosphate BGE, pH 5.0. The combination greatly enhanced the separation efficiency with R_S values between 3 and 6. The system was optimized and eventually validated and applied to analyze the enantiomeric impurity of *S*-pantoprazole. Synthetic batches contained 0.18–0.19% or the *R*-enantiomer.

The velocity gap mode of CE (VGCE) developed earlier by Zhang et al. [127] has been applied to CE enantioseparations by the same group [128]. VGCE is based on differences of the velocities of analytes in two consecutive electric fields. There is no change in the separation mechanism. The experimental set-up includes two capillaries that are joined by an interface allowing the application of a different electric field in each capillary (see [127, 128] for details of the set-up). In the initial step identical fields are applied over both capillaries. Once the first analyte reaches the second capillary, the voltage in the first capillary is switched off so that the velocity of the analyte remaining in the first capillary is zero while the analyte in the second capillary continues to migrate toward the detector. After a given time the electric field is applied to the first capillary again in order to move the remaining analyte to the detector as well. This way, a separation can be tailored to achieve a desired peak resolution by selecting the appropriate experimental conditions. The general feasibility of the approach for chiral CE separations has been demonstrated using the enantioseparations of terbutaline and chlorpheniramine in the presence of a low concentration of β -CD and of promethazine by γ -CD in acidic phosphate buffers [128]. Subsequently, VGCE was applied to the determination of the enantiomeric excess (ee) [129]. As well known, the determination of a low amount of a minor enantiomer in the presence of a large excess of the major enantiomer may be an analytical challenge, especially in the case of low resolution. The peaks of the major and minor enantiomers may overlap in part or even completely due to the size of the peak of the major enantiomer, due to dispersion phenomena or due to peak tailing. During the separation, the minor enantiomer focuses at the edge of the sample zone but may not be detected in a "reg-

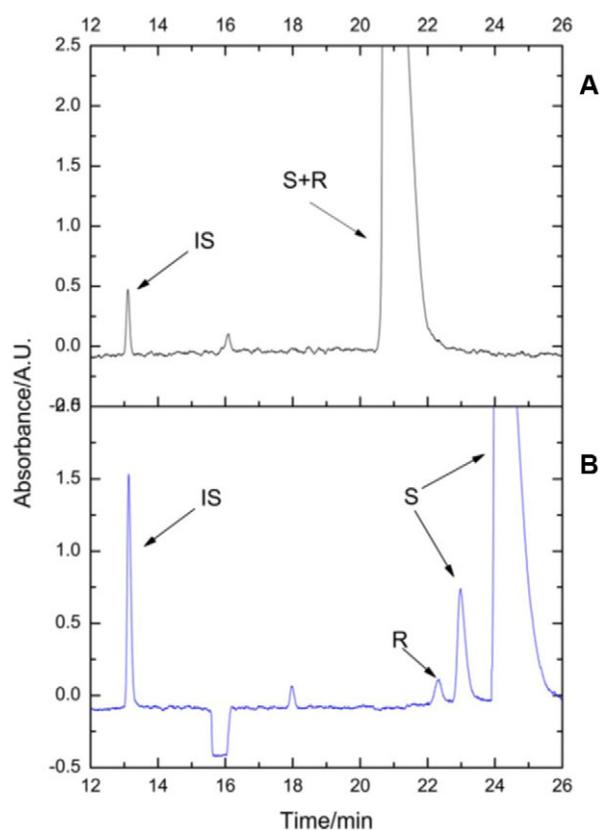


Figure 4. Comparison of conventional CE (A) and VGCE (B) for the determination of ee of *S*-amlopidine. Experimental conditions: 30 mM Tris-phosphoric acid, pH 3.0, 3.0 % (w/v) α -CD, effective separation length 18 cm, electric field $E = 200$ V/cm. The sample ratio *S*-amlopidine/*R*-amlopidine was 100:1. (Reproduced with permission from Elsevier from [129].)

ular" CE experiment. Applying VGCE, it is possible to "cut" the sample zone into two parts, i.e. the edge part containing the minor enantiomer as well as a small amount of the major enantiomer and the main part containing the majority of the major enantiomer. As both parts eventually migrate to the detector, the enantiomers in the edge part can be easily baseline separated because the concentration difference is now much smaller and less dispersion occurs. By selecting the appropriate conditions, the VGCE approach may be applied to the case when the minor enantiomer migrates first as well as to situations when the minor stereoisomer migrates second. The first scenario is illustrated for the determination of the ee of *S*-amlopidine in Fig. 4. The analysis was performed in a fused silica capillary with an effective length of 18 cm using phosphate buffer as BGE in the presence of 3.0 % (w/v) α -CD as chiral selector. The minor *R*-enantiomer cannot be detected by conventional CE under the applied experimental conditions (Fig. 4A). Applying VGCE, i.e. switching off the electric field in the first capillary once the front edge of the peak has reached the second capillary, the front zone continued to migrate to the detector and baseline separation of both enantiomers was achieved due to the low overall analyte concentration. After switching the separation voltage back on

in the first part of the capillary, the remaining major part of *S*-amlodipine migrated toward the detector. Consequently, three peaks were detected (Fig. 4B) and could be used for the calculation of the ee. Analyzing a commercial tablet, an ee of $99.4 \pm 0.2\%$ was determined for *S*-amlodipine. The authors also demonstrated the feasibility of the VGCE concept for the analysis of the ee in the case of the minor enantiomer migrating second using *S*-ofloxacin as an example [129].

CE may also be useful to determine the enantiomeric ratio of a drug and, simultaneously, the resolution agent, i.e. the diastereomeric salt composition, when a chiral drug is obtained via fractionated crystallization. Varga et al. demonstrated the feasibility of the approach using *cis*-permethrinic acid and ibuprofen as model compounds which were resolved via crystallization with *R*-1-phenylethalamine [130]. The analytical enantioseparation of the compounds was achieved in a fused-silica capillary using a 50 mM Britton-Robinson buffer with a pH between 7.0 and 7.2 depending on the analyte using 10 to 12.5 mM TM- β -CD as chiral selector. Under these conditions the positively charged resolving agent *R*-1-phenylethalamine migrated before the EOF while the acidic drug enantiomers migrated after the EOF. Although not fully validated, the method was applied to the analysis of several batches of the diastereomeric salts demonstrating the principal usefulness of the approach for the intended purpose.

The only paper on the application of CEC for the determination of the enantiomeric purity of a pharmaceutical drug within the period of time covered by the present review compared the enantioseparation of amlodipine by CEC and nano-LC using cellulose tris(4-chloro-3-methylphenylcarbamate) as chiral selector [131]. Packed 100 μm id capillary columns were used in combination with a mobile phases composed of acetonitrile/water (90:10, v/v) and containing 15 mM ammonium borate, pH 10.0, in the case of nano LC and 5 mM ammonium borate, pH 9.0, for CEC analysis. The simultaneous separation of amlodipine and 2 chiral impurities could only be achieved under CEC conditions. The CEC method was subsequently validated and applied to the analysis of the racemic drug in a commercial tablet formulation. None of the chiral impurities were found but an additional impurity at a concentration of about 0.45% was detected in the tablets. Aspects of chiral CEC have been summarized [132–134].

3.3 Determination of physicochemical constants

Physicochemical parameters are frequently used as predictors of ADME (absorption, distribution, metabolism, and excretion) properties of drugs. One important physicochemical parameter that affects the pharmaceutical potential of a compound is its acid–base equilibrium, defined by its acidity constant (or $\text{p}K_{\text{a}}$ on a logarithmic scale). There are several methods for the determination of acid dissociation constants. In all of them, a physical property of drug is measured as a function of the pH of a solution. Traditionally, potentiometry [135] and UV-VIS absorption spectrometry [136] have been the most useful techniques for the determination of equilibrium con-

stants. Main drawbacks of potentiometric techniques include the requirements to use pure drugs in aqueous buffers [137]. In UV-VIS spectrophotometry, a drug must contain an UV-active chromophore close enough to the site of the acid–base function in the molecule and it is assumed that its impurities do not absorb in the UV-VIS range, since the spectra of impurities can overlap with those corresponding to the solutes of interest [138].

An alternative to above-mentioned techniques are HPLC and CE. The advantages of separation techniques for the determination of $\text{p}K_{\text{a}}$ values are numerous including the handling of lower sample concentrations, the studied samples do not need to be pure and sample consumption is minimal. One of the most important disadvantages of the LC methods is that the pH of the mobile phase and, therefore, the range of $\text{p}K_{\text{a}}$ values that can be determined are limited by the stability of the column packing. CE permits $\text{p}K_{\text{a}}$ determination in aqueous solutions without difficulties whereas this is not the case for LC, where the retention can also be influenced by the composition of mobile phase [139]. The classic CZE method for $\text{p}K_{\text{a}}$ determination involves measuring the mobility of the substance of interest at several pH values which are set by the preparation of suitable buffers at constant ionic strength in different pH ranges [140, 141]. However, the classic CZE method is quite slow and not very useful for high-throughput screening. Therefore, in drug discovery there is a major need for fast $\text{p}K_{\text{a}}$ determination of a large number of compounds [142–145].

Recently published papers about applications of CE methods to $\text{p}K_{\text{a}}$ determination bring some methodological innovation (e.g., temperature and ionic strength corrections of measured effective mobilities, determination of very low or very high $\text{p}K_{\text{a}}$ values, pressure assisted accelerated measurement of EOF and effective mobilities, and determination of $\text{p}K_{\text{a}}$ in nonaqueous solvents) [146–152]. The determination of the $\text{p}K_{\text{a}}$ of 22 frequently used pharmaceuticals using CE was presented [153]. The data were fitted with to a mathematical model using nonlinear regression analysis to obtain the $\text{p}K_{\text{a}}$ values. Another faster method to determine a $\text{p}K_{\text{a}}$ by CE based on the use of internal standards (IS) was recently developed [143]. The IS-CE method is based on the use of an IS with a similar $\text{p}K_{\text{a}}$ value to that of the test compound (TC). Therefore, if they are injected together, the differences in the mobility values of the compounds can be directly related to differences in their acidity. Just two electropherograms at two different pH values are needed to calculate an acidity constant in less than 3 min of electrophoresis. Furthermore, pH buffer instability during electrophoretic runs is not a problem in the IS-CE method. Furthermore, CE with capacitively coupled contactless conductivity detection (CE-C4D) was successfully applied to the investigation of the $\text{p}K_{\text{a}}$ values of peroxycarboxylic acids [154] and active ingredients [155].

CE is also applicable for the determination of drug lipophilicity ($\log P$ values). $\log P$ values of drugs were estimated by MEKC [156, 157]. Furthermore, liposome electrokinetic chromatography (LEKC) employing liposomes as pseudostationary phase was used for the characterization

of liposomal drug formulations as well as drug lipophilicity and drug–liposome interactions 158–160. These electrokinetic techniques were increasingly employed to predict drug permeability through biological membranes such as skin or the CNS distribution of drugs [1]. Furthermore, cerasome LEKC provided a good basis for the calculation of an Abraham linear free energy relationship for neutral and charged solutes [161]. The EKC system was substantially different compared to systems based on, for example, the traditional octanol-water partition coefficient and provided a basis for the prediction of skin penetration. In other studies, MEEKC provided a better measure for predicting the distribution of drugs into the central nervous system as compared to the octanol-water partition coefficient or calculated lipophilicity parameters [162, 163].

ACE is a particularly promising approach for the examination of ligand–receptor interactions and the estimation of binding constants in free solution [2, 15, 164–166]. Binding constant estimation by ACE is usually achieved by following the change in the electrophoretic mobility of an injected analyte ligand when the interacting molecule is added to the running buffer. Different equations were established for calculating binding constants based on the change in electrophoretic mobility of injected analytes upon complexation [167, 168]. Thus, ACE was successfully applied to study interactions including protein–drug, protein–metal ion, protein–DNA, peptide–carbohydrate, peptide–peptide, DNA–dye, carbohydrate–drug, and antigen–antibody interactions [169]. As separations in ACE can be performed under physiological buffer conditions, ligand and receptors remain in their native state and hence their molecular function is maintained, too. Particular considerations of method validation for ligand binding assays were discussed [22].

3.4 Analysis of organic and inorganic counter ions

A counter-ion is an ion that accompanies the charged drug species forming a salt. The counter-ion of the drug plays an important role in promoting the physicochemical properties such as solubility, stability or bioavailability of a drug, thus, modifying its pharmaceutical properties [170]. Furthermore, the determination of the counter-ion is mandatory to confirm the formation of the correct salt in order to establish the correct molecular mass of the drug and the drug to counter-ion stoichiometry which should also be reproducible from batch-to-batch [171]. A counter-ion in a drug amounts normally to about 2–30% w/w of the drug substance, thus requiring precise and accurate analytical methods for quantification [172]. CE has been used for drug counter-ion analysis for quite some time [173–175]. CE has the advantage of offering the possibility of indirect UV detection, where a chromophore is usually added to the BGE, thus, offering low but in most cases sufficient sensitivity. This mode of detection is possible using commercial common CE equipment and the UV detectors can be used without any modifications. During the past decade many original and review papers as well as mono-

graphs have published on counter ion or impurity determination in drugs [76, 174, 175].

A more sensitive CE approach is the use of conductivity detection, the well-established version is referred to as capacitive coupled contactless conductivity detector (C⁴D). Recent developments in the application of CE-C⁴D for (bio)pharmaceuticals was reviewed [176]. A number of robust, highly sensitive, wide linear range CE-C⁴D methods were validated for the determination of drug counter-ions for manufacturing quality control. Moreover, CE showed high suitability for the simultaneous determination of a drug compound and its counter-ion in a single run. This can easily be achieved in case of cations and slow migrating anions by simply increasing the EOF to force both anions and cations to migrate in the same direction to reach the detector [177, 178].

A more efficient approach for simultaneous analysis of drug compound and its counter ion by CE is referred to as “dual-opposite end injection” [174] which involves the simultaneous injection of the sample at the same time from both anodic and cathodic ends of the capillary. Cations migrating to the cathode and anions migrating to the anode will pass a detector in the center of the capillary. The application of dual-opposite end injection for simultaneous determination of drug and its counter-ion applying either UV or C⁴D detection was reported [179–183]. Particularly the use of dual-opposite end injection and contactless conductivity detection (DOE-CE-C⁴D) is currently an attractive CE approach to easily quantify a drug and its counter-ion in the same run [174].

4 Determination of (bio)pharmaceuticals

CE is one of the established techniques for pharmaceutical quality control and main compound assays. A clear advantage is the usually straightforward sample preparation [184]. Strategies for method development, validation, and aspects of good instrument qualification are meanwhile clearly defined [10, 185–187]. An interesting recent work showed that inter-instrumental method transfer is certainly possible for the GXP environment as well [188]. However, since the thermostating systems vary from one instrument type to another, it sometimes does not suffice to just use the same temperature settings. In some cases the temperature needs to be carefully adjusted during the method transfer, until precisely the same conductivity in both instruments is reached as the target parameter.

Drop-out rates during a sample sequence are still an issue for the analysis of valuable routine samples from the quality control of biologicals [189]. The frequency of these drop-outs, their definition, and a collection of possible reasons have been discussed on a workshop on CE Pharm 2015 dedicated to this subject. Information about this workshop and a summary of the related discussion is published on the conference webpage (<http://www.casss.org/?CE1500>).

Pros and cons of CE compared to LC have already been discussed earlier in [1], hence only further interesting applications and novel approaches will be described here. One of the advantages of CE for bioanalyses has been recently emphasized. It is long-known common knowledge, that injection volumes in CE are in the nanoliter range and extremely small sample amounts can therefore be sufficient. However, the commercially available sample vials typically require sample volumes of at least approximately 50 μL . Using a simple approach by adjusting the level of the sample liquid by another heavier inert solvent, this required volume has been reduced to at least 5 μL and possibly less [190]. Apart from chiral separations (see Section 3.2), CE applications in the pharmaceutical industry can be often found in the analysis of biologicals [191].

CE has substituted traditional slab gel electrophoresis for the analysis of biopharmaceuticals [191, 192]. Nowadays CIEF is widely used for the analysis of protein charge variants [193, 194] and even for antibody drug conjugates [195]. Imaged CIEF (iCIEF) is feasible for the development of biopharmaceutical products as well [195, 196]. It has been shown that iCIEF nicely completes information obtained by LC-MS, providing a good overall picture of the charge variants of IgG antibodies and their conjugates [195]. An earlier interlaboratory study showed excellent between-labs precision for $p\text{I}$ s ($\text{RSD}\% < 0.8\%$) and acceptable reproducibility for peak areas ($\text{RSD}\% < 11\%$) [197]. Parameters to optimize CIEF separations include the ampholyte type and possible ampholyte mixtures as well as the focusing voltage [198].

In addition, CZE is also quite suitable to analyze charge heterogeneity of mAbs, and sometimes even superior to CIEF. A method employing 6-aminocaproic acid (EACA) and hydroxypropylmethyl cellulose (HPMC) has been successfully implemented [199]. Moritz *et al.* [189] achieved excellent validation parameters with a similar approach which, in addition, applied triethylenetetramine. For example, a repeatability of peak areas of approximately 1% RSD has been obtained. The transferability of this robust method has been demonstrated in an intercompany study with 11 participating labs, and another similar method has proven useful for a stability indicating assay of the mAb Rituximab [200]. As described earlier [1, 191], CZE has been used for stability indicating methods, e.g. for recombinant human interleukin-11 [201] and oxytocin [202], as well as for the dose-determination of a dengue fever vaccine candidate [203].

Size variations are now routinely elucidated using CE employing sodium dodecyl sulfate as reagent (“CE-SDS”), similar to SDS-PAGE but superior in terms of validation properties [200, 204–207]. The related general concepts have been already described, e.g. in [1] and the literature cited therein. Today’s methods use very similar approaches. They are typically based on ready-to-use kits with excellent separation efficiency provided by the various instrument manufacturers. The related information can be best found using search engines, e.g. with the key words “capillary electrophoresis supplies” and optionally the names of the various supplying companies. These kits are essentially used as delivered, but

they can also be modified, e.g., by simple dilution [208]. Cianciulli *et al.* [206] demonstrated the use of pressure-assisted injection for CE-SDS as a favourable alternative to the frequently suggested electrokinetic injection. Pressure injection gave better precision and was less sample matrix sensitive. Blotting techniques have been developed for capillary systems as well and have been applied with higher precision compared to traditional gel-based approaches [209, 210]. Therefore, such kits are offering a generic platform for separation methods for monoclonal antibodies. However, sample preparation and sensitivity optimization have to be optimized for each individual case. Differences in sample preparation properties have been observed for different subtypes of IgG [1, 204]. Antibodies of the subtype IgG4 have an increased risk for disulfide scrambling, which can be reduced by optimizing the alkylating agent type and concentration in the sample buffer [211].

Today it is more and more understood how proteins can be stacked and focused, in order to increase sensitivity. The well-described focusing strategies available, point toward a straightforward concept which employs a post-injection of a small plug of a terminating electrolyte, in order to obtain as much isotachophoretic effects as needed [212, 213]. It has to be noted that overdoing is possible here. Highly focused proteins may cause to additional adsorption or even result in precipitation of proteins in the capillary.

Micro-CE-SDS on a chip showed comparable results as the capillary format [211, 214]. Chip separations are very fast and can be accomplished within a few seconds, thus, allowing high-throughput analyses, for example, in process control. However, the precision is still a worse than as compared to “classical” capillary separations. Moreover, the related kits need to be individually assessed for their robustness during long-term analysis and for matrix effects.

CE-MS is a powerful tool for metabolic profiling with many recently published metabolomic studies especially for highly polar metabolites. Applications and novel interfacing techniques have been discussed by R. Ramautar *et al.* [215]. The authors suggest further applications and evaluations for sheathless porous tip and flow-through microvial interfaces to ensure the robustness and reproducibility as well as the development of a migration-time correction strategy for reliable comparative profiling studies. In their review, P. W. Lindenburg *et al.* [216] pointed out a higher sensitivity of the above two new interfacing techniques compared to conventional sheath-liquid ones but agreed with R. Ramautar *et al.* [215] regarding the need for further evaluation of these techniques for routine clinical omics research.

The performance of CE-MS for proteomic, metabolomic, and genomic applications with different mass analyzers has been also reviewed by V. R. Robledo *et al.* where different recent applications are discussed [217]. Studies showed that CE-MS/MS demonstrate significant complementarity to LC-MS/MS in natural peptide identification for possible biomarkers determination [218]. Several urinary proteome-based studies for the discovery of biomarkers of renal diseases have been carried out using CE coupled to MS as reviewed by J. P. Schanstra *et al.* [219].

5 Conclusion

As can be concluded from the examples discussed above, the applications of capillary electromigration techniques for (bio)pharmaceutical analyses are continuously growing for the analysis of small pharmaceuticals as well as large biomolecules. Successful applications related to various aspects of small molecules such as for analysis of drug-related impurities, stereochemical purity, counter ion determination, and calculation of physicochemical parameters have been reported. Furthermore, ACE has been increasingly applied to study different types of analyte interactions including the determination of binding constants.

The ability of CE to resolve different related substances including charged and uncharged species results from the high separation power. Thus, different CE techniques allowed the determination of drug impurities with acceptable detection levels at 0.1% or below. With respect to stereoisomer separations, CE may be considered as the method of choice not only due to its separation efficiency but also to its flexibility in choosing the appropriate chiral selector and reduced cost of selector compared to HPLC. As in the case of other related substances, enantiomeric impurities can be determined at a level of 0.1%. Several chiral based drugs were enantioseparated with sufficiently high resolution using CE techniques, particularly MEKC and MEEKC. The fact, that chiral HPLC methods still dominate industrial applications may be due to the fact that most analytical scientists are trained in HPLC rather than CE.

CE with its different modes is nowadays a well-established qualitative and quantitative analytical technique not only for small molecule pharmaceuticals but also for large biopharmaceuticals. Challenges regarding low sensitivity, precision, or sample drop-outs, especially when analyzing biopharmaceuticals can be significantly improved applying certain method development strategies, validation aspects, and instrument qualification. In the authors' opinion, the major challenges for further implementation of CE in the pharmaceutical industry at the present time are knowledge transfer and an increased need for CE-MS.

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