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Comparison of chiral electrophoretic separation methods for phenethylamines and application on impurity analysis

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ABSTRACT

A chiral microemulsion electrokinetic chromatography method has been developed for the separation of the enantiomers of the phenethylamines ephedrine, *N*-methylephedrine, norephedrine, pseudoephedrine, adrenaline (epinephrine), 2-amino-1-phenylethanol, diethylnorephedrine, and 2-(dibutylamino)-1-phenyl-1-propanol, respectively. The separations were achieved using an oil-in-water microemulsion consisting of the oil-component ethyl acetate, the surfactant sodium dodecylsulfate, the cosurfactant 1-butanol, the organic modifier propan-2-ol and 20 mM phosphate buffer pH 2.5 as aqueous phase. For enantioseparation sulfated β-cyclodextrin was added. The method was compared to an already described CZE method, which made use of *heptakis*(2,3-di-*O*-diacetyl-6-*O*-sulfo)-β-cyclodextrin (HDAS) as chiral selector. Additionally, the developed method was successfully applied to the related substances analysis of noradrenaline, adrenaline, dipivefrine, ephedrine and pseudoephedrine monographed in the European Pharmacopoeia 6.

1. Introduction

Phenethylamines interfere with the peripheral nervous system and are able to induce the release of noradrenalin (norepinephrine), so that they act as oral vasoconstrictors and bronchodilators [1–3]. For example ephedrine, methylephedrine, norephedrine and pseudoephedrine are some of the active components of ephedrae herba [4,5]. Often they are used as ingredients of cold medicines and anorectics because of their aforementioned sympathomimetic qualities. Analysis of ephedra alkaloids is of interest for food, e.g. tea, forensic and pharmaceutical applications [6]. Most of drugs are chiral. Since the biological activity, toxicology and pharmacokinetics of the enantiomers of a chiral drug can be different, it is important to ensure the enantiomeric purity by means of a chiral separation method [1,2,7–10] (Fig. 1).

Based on the principles of electrophoresis, the capillary zone electrophoresis was designed to separate analytes in a small capillary due to their size-to-charge ratio.

Beside the development of many other related techniques, in 1991 [11] the microemulsion electrokinetic chromatography (MEEKC), using mainly oil-in-water (O/W) microemulsions (ME), was invented. Hence, separation of uncharged and hydrophobic substances was made available, because the oil droplets of the ME act as pseudostationary phase and solubilize many hydrophobic analytes. In MEEKC, the chromatographic separation is based on the partition of the analytes between the oil droplets and the buffer solution. For stabilization of the oil droplets surfactants like SDS, and cosurfactants, often short chain alcohols (e.g. 1-butanol), are used. The micelles have a negative charge on the surface because the hydrophilic sulfate head group of the surfactant remains in the aqueous buffer solution, whereas the hydrocarbon tails position in the oil core [12–16].

Application of CDs in CE is a useful technique for resolution of enantiomers and for determination of the enantiomeric excess [17,18]. However, for separation of enantiomers, chiral modifiers such as the cyclodextrins and their derivatives were added to the running buffer [9,10,19–24]. By derivatisation of a native CD many variations are given for enantioseparation of a wide range of chiral compounds. For enantioseparation both hydrophobic and ionic interactions are as necessary as sterically effects and hydrogen bonds [9,25].

In the case the enantiomeric purity of a drug has to be evaluated by means of MEEKC, a chiral selector has to be added to the ME. To form a chiral BGE, chiral oils like (*S*)-(+)-2-octanol [26–28], chiral surfactants like dodecoxycarbonylvaline [29–31] or chiral cosurfactants like (*S*)-2-hexanol [30,32] can be applied. As already mentioned, in CE the mostly used chiral selectors are cyclodextrins, which work in different CE-methods. Good results occurred,

Abbreviations: HDAS, *heptakis*(2,3-di-*O*-diacetyl-6-*O*-sulfo)-β-cyclodextrin; ME, microemulsion; MEEKC, microemulsion electrokinetic chromatography; O/W, oil-in-water; sulf. β-CD, sulfated β-cyclodextrin.

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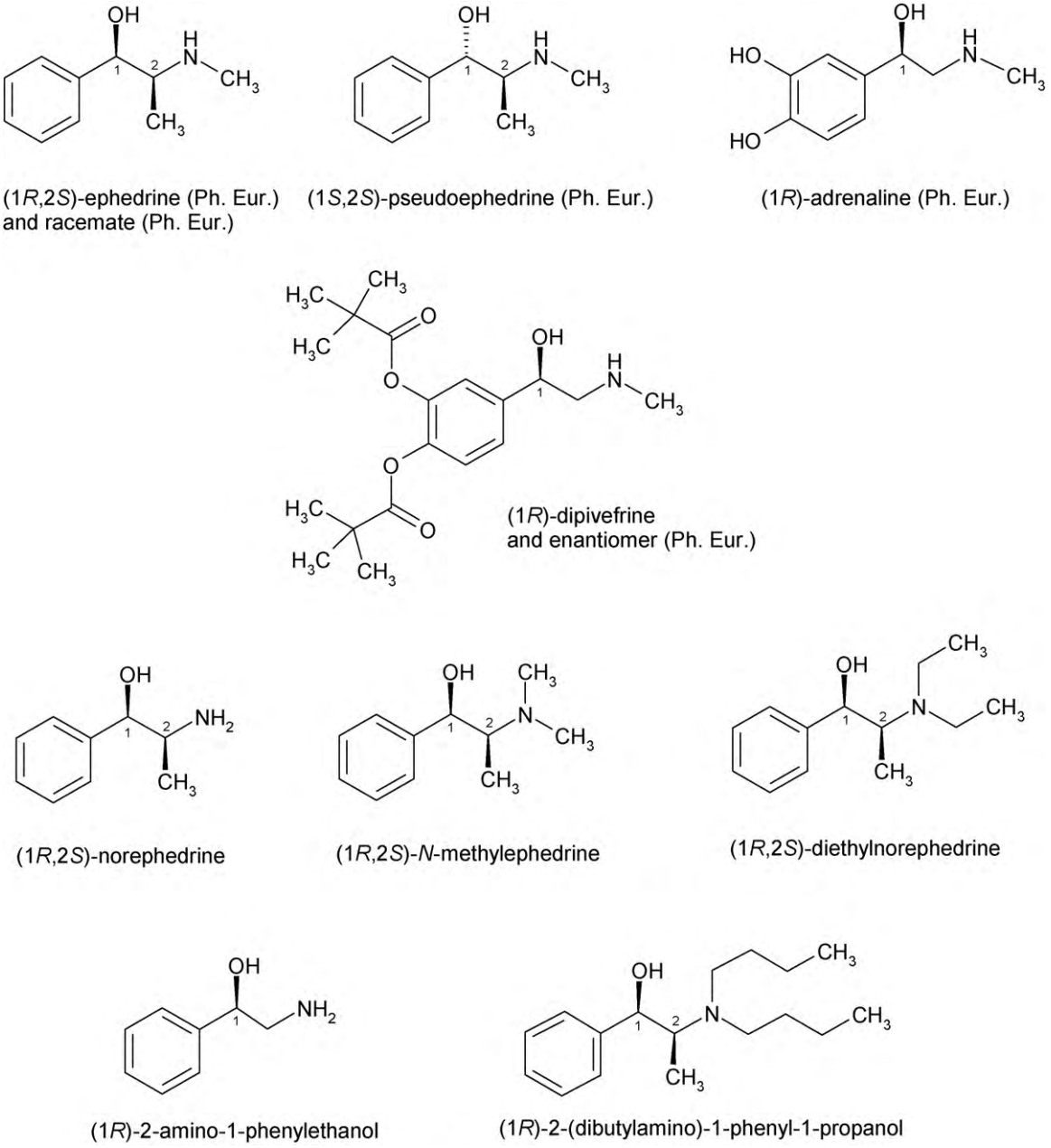

Fig. 1. Structural formulae of the chiral phenethylamines.

e.g. with a dimethyl-β-CD-modified CZE for enantioseparation of 1-aminoindan [9], with a CD-modified MEKC for the determination of isochromene derivatives using hyrdroxypropyl-β-CD [33] and with a CD-modified MEEKC, e.g. for phenylalanine analogues [34] and tropa alkaloids [35].

The purpose of this work was the development and validation of a chiral MEEKC method for the enantioseparation of the aforementioned phenethylamines in clinical use and the comparison of the MEEKC results with results obtained with CD-modified CZE, using *heptakis*(2,3-di-*O*-diacetyl-6-*O*-sulfo)-β-CD [36,37] and dimethyl-β-CD [38], respectively, as chiral selector. Additionally, it was checked, whether CD-modified MEEKC method is appropriate for impurity-profiling of the chiral phenethylamines, as it is typically performed in international pharmacopoeias.

2. Materials and methods

2.1. Instrumentation

All MEEKC separations were performed on a Beckman Coulter P/ACE System MDQ (Fullerton, CA, USA), equipped with an UV-detector measuring at 200 nm. The uncoated fused silica capillaries purchased from BGB Analytik (Schloßböckelheim, Germany) had an internal diameter of 50 μm, an effective length of 40 cm and a total length of 50.2 cm.

The pH of the buffer systems was determined by means of a PHM 220 Lab pH meter (Radiometer Copenhagen, Lyon, France). For the preparation of the ME a 2510-Branson-Sonicator (Heinemann, Ultraschall- und Labortechnik, Schwäbisch Gmünd, Germany) was used.

2.2. Chemicals and materials

The analytes (1*R*,2*S*)-ephedrine and (1*S*,2*R*)-ephedrine hemihydrate, (1*R*,2*S*)-*N*-methylephedrine, (1*R*,2*S*)- and (1*S*,2*R*)-norephedrine, (1*S*,2*S*)- and (1*R*,2*R*)-pseudoephedrine, (1*R*,2*S*)- and (1*S*,2*R*)-2-(dibutylamino)-1-phenyl-1-propanol, (1*R*)-noradrenaline hydrogen-L-tartrate monohydrate, (1*R*)-adrenaline, (1*RS*)-adrenaline hydrochloride and the reagents 1-butanol and propan-2-ol were purchased by Sigma–Aldrich Chemie (Deisenhofen, Germany), (1*S*,2*R*)-*N*-methylephedrine, (1*R*,2*S*)-diethylnorephedrine, (1*S*,2*R*)-diethylnorephedrine hydrochloride, (1*R*)- and (1*S*)-2-amino-1-phenylethanol from Fluka (Fluka Chemie AG, Buchs, Switzerland), as well as the sulfated β-cyclodextrin sodium salt (sulf. β-CD), SDS, orthophosphoric acid (85%) and NaH_2PO_4 (p.a.). Methanol and ethyl acetate was purchased from Fisher Scientific (Loughborough, UK), hydrochloric acid 0.1 M from Grüssing (Fillsum, Germany), native β-cyclodextrin from Wacker (Wacker-Chemie, Burghausen, Germany) and isomerically pure *heptakis*(2,3-di-*O*-diacetyl-6-*O*-sulfo)-β-cyclodextrin from Regis (ict Handels, Bad Homburg, Germany). Racemic dipivefrine hydrochloride was kindly provided by Allergan Pharmaceuticals (Westport, Ireland).

Before running the measurements, all solutions were filtered: aqueous solutions through a 0.22 μm pore-size cellulose mixed ester (cellulose acetate and nitrate) membrane filter and organic solutions through a 0.22 μm pore-size polyvinylidenfluoride membrane filter (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

2.3. Sample preparation

All analyte solutions were prepared in 0.1 M hydrochloric acid. 2 mg/ml stock solutions of (1*R*,2*S*)- and (1*S*,2*R*)-ephedrine, (1*R*,2*S*)- and (1*S*,2*R*)-methylephedrine, (1*R*,2*S*)- and (1*S*,2*R*)-norephedrine, (1*S*,2*S*)- and (1*R*,2*R*)-pseudoephedrine, (1*R*,2*S*)- and (1*S*,2*R*)-2-(dibutylamino)-1-phenyl-1-propanol, (1*R*)-adrenaline, (1*R*,2*S*)-diethylnorephedrine, (1*R*)- and (1*S*)-2-amino-1-phenylethanol were prepared each. Solutions of racemic adrenaline hydrochloride, racemic dipivefrine hydrochloride, (1*R*,2*S*)-diethylnorephedrine and (1*S*,2*R*)-diethylnorephedrine hydrochloride were prepared with 2 mg/ml of each drug calculated as the base. Before each measurement, the stock solutions were diluted with 0.1 M HCl to give 0.2 mg/ml samples.

2.4. Preparation of the microemulsion

250 ml of a buffer stock solution was prepared by dissolving 20 and 50 mM, respectively, of NaH_2PO_4 in about 220 ml ultra pure Milli-Q water (Millipore, Milford, MA, USA). Then, the pH was adjusted to the desired value with concentrated phosphoric acid (85%). Afterwards the solution was diluted to 250.0 ml and the pH value was rechecked and adjusted, if necessary.

For preparation of the standard O/W microemulsion solution ethyl acetate, 1-butanol, SDS and 20 or 50 mM NaH_2PO_4 buffer either of pH 2.5 or of pH 3.0 were mixed. The solutions were sonicated for 20 min to aid dissolution and to form an optically transparent ME. For the CD additives, defined concentrations of the respective CD were added to the ME and sonicated for another 5 min (power 120/240 W and frequency 35 kHz). If the propan-2-ol was used as organic modifier, it was added after the cosurfactant 1-butanol. This additional compound leads to a reduction of the amount of phosphate buffer.

2.5. Methods and conditioning

The samples were injected at the cathodic end of the capillary by a pressure of 3.4 kPa for 4.0 s. Separations were performed at 20 °C using a respective constant voltage in the reversed polarity mode.

New capillaries were conditioned at 25 °C rinsing with 0.1 M NaOH for 10 min, with water for 5 min, with 0.1 M H_3PO_4 for 10 min, and water for 5 min. Before performing the experiments, the capillaries were conditioned at 20 °C rinsing with 0.1 M NaOH for 5 min, with water for 3 min, and the BGE for 6 min. Between each run, the capillaries were rinsed at 20 °C with 0.1 M NaOH for 3 min, with MeOH for 3 min, with 0.1 M H_3PO_4 for 2 min and water for 2 min and conditioned with BGE solution for 5 min. At the end of each working day, the capillaries were rinsed at 30 °C with 0.1 M NaOH for 10 min, water for 5 min, and methanol for 10 min. Capillary wash cycles were performed at a pressure of 207 kPa.

2.6. MEEKC methods

For the CD-modified MEEKC separations of the racemic ephedrine derivatives the ME consisted of 0.5% ethyl acetate, 1.0% SDS, 4.0% 1-butanol, 3.0% of the organic modifier propan-2-ol and 91.5% 20 mM phosphate buffer, previously adjusted to pH 2.5 with 85% phosphoric acid. The BGE was prepared by dissolving 4.0% sulf. β-CD in the ME. Using a fused silica capillary, the CE instrument was set at 20 °C and −15 kV.

A CZE method developed by Wedig and Holzgrabe et al. [36,37] was applied: Baseline separation of racemic ephedrine and the racemic derivatives norephedrine, *N*-methylephedrine and pseudoephedrine was achieved with 50 mM phosphate buffer adjusted to pH 3.0, containing 3 mM HDAS (∼0.7%; w/w), in 20 min. The runs were performed with +20 kV in normal polarity mode at 20 °C in a fused silica capillary (60/50 cm, 50 μm).

3. Results and discussion

For the enantioseparation of the four phenethylamines ephedrine, norephedrine, *N*-methylephedrine and pseudoephedrine a general method was developed, which was based on the parameters of the CZE method [36,37]. Thus, the 50 mM phosphate buffer adjusted to pH 3.0 was used to form a ME with 0.4% ethyl acetate as oil droplet phase, 1.4% SDS as surfactant, 3.6% 1-butanol as cosurfactant and 2.8% 2-propanol as organic modifier. By adding 1.4% (12 mM) native β-CD no separation of any racemic ephedrine derivative was observed. With 4% HDAS, which worked very well in the CZE method, a resolution of approximately 0.5 was achieved for the racemates of ephedrine, *N*-methylephedrine and norephedrine only, but no separation for pseudoephedrine enantiomers.

Hence, the ME was modified: first, the portion of the oil compound was doubled; second, the ionic strength of the phosphate buffer was reduced to 20 mM, and third, the SDS amount was set to 1.0%. By applying these conditions, the resolution of ephedrine could be increased to 1.1, but no improvement was achieved for the other phenethylamines, and additionally the baseline was noisy. Further modifications were performed with regard to the ME, e.g. the increase of the CD concentration in 0.5% steps resulted in resolutions of 1.1 for ephedrine and 1.5 for *N*-methylephedrine. For norephedrine a baseline separation (*Rs*: 2.3) could be achieved by decreasing the pH of the 20 mM phosphate buffer to 2.5, which did not work for pseudoephedrine (*Rs*: 0.8) (see Fig. 2a).

In order to obtain better separations and peak forms, for all ephedrine derivatives the HDAS was replaced with sulf. β-CD. Adding 4% sulf. β-CD to the ME with pH 2.5 an enantioseparation of all four compounds could be achieved (see Fig. 2b). Additionally, baseline and peak form were satisfying.

Spiking the racemic samples of the ephedrine derivatives with the corresponding (+)-enantiomer revealed in each case the first peak to be caused by the (+)-enantiomer, i.e. (*1S,2R*)-

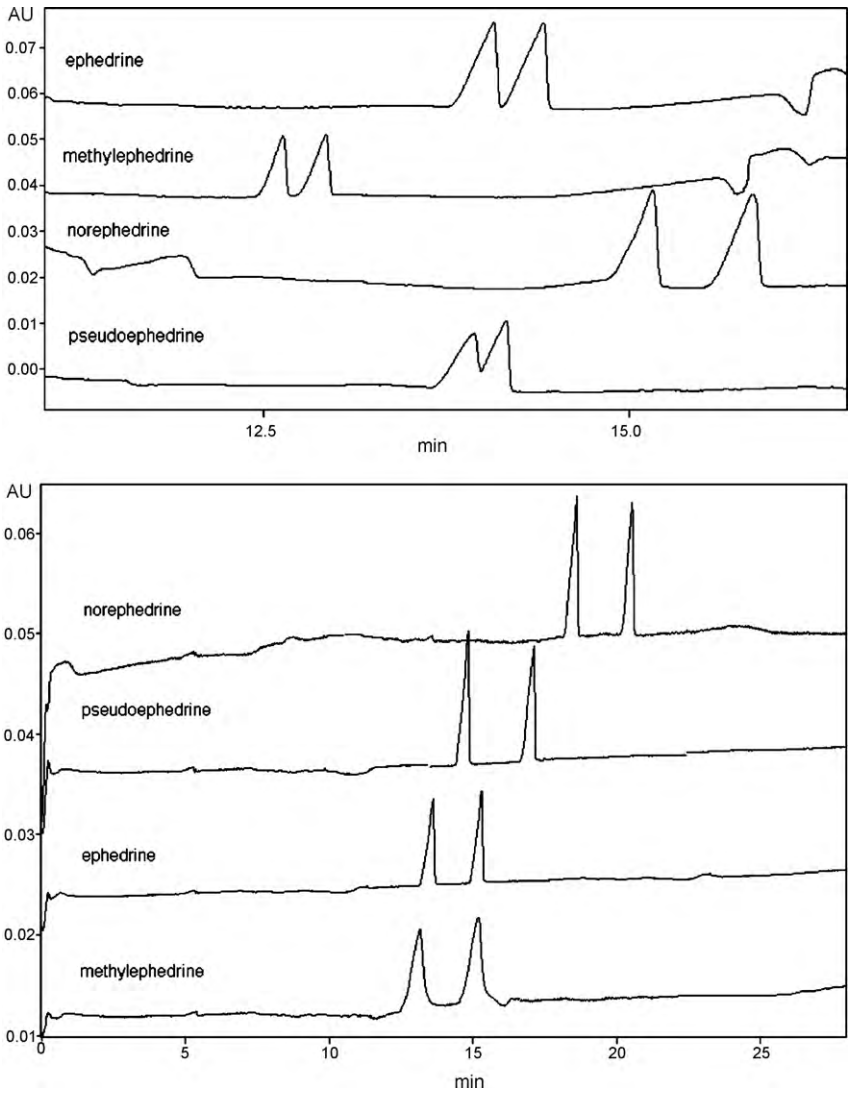

Fig. 2. (a) Chiral CD-modified MEEKC of racemic ephedrine derivatives under individually optimized conditions: separation of racemic ephedrine and racemic methylephedrine using 4.5% HDAS. Separation conditions: ME of 0.8% ethyl acetate, 1.0% SDS, 3.2% 1-butanol, 2.8% propan-2-ol, 92.2% 20 mM phosphate buffer pH 3.0, voltage: −13 kV, temperature: 20 °C. Separation of racemic norephedrine and racemic pseudoephedrine using 5.0% HDAS. Separation conditions: ME of 0.8% ethyl acetate, 1.0% SDS, 3.2% 1-butanol, 2.8% propan-2-ol, 92.2% 20 mM phosphate buffer pH 2.5, voltage: -17 kV, temperature: 20 °C; (b) Chiral CD-modified MEEKC of racemic ephedrine, racemic methylephedrine, racemic norephedrine and racemic pseudoephedrine. Separation conditions: 4.0% (w/w) sulfated β-CD in ME of 0.5% (w/w) ethyl acetate, 1.0% (w/w) SDS, 4.0% (w/w) 1-butanol, 3.0% (w/w) propan-2-ol and 91.5% (w/w) 20 mM phosphate buffer, pH 2.5; voltage: −15 kV, temperature: 20 °C.

ephedrine, (*1S,2R*)-methylephedrine, (*1S,2R*)-norephedrine and (*1S,2S*)-pseudoephedrine.

The resolutions obtained can be explained by the substitution pattern. The highest resolution was revealed for the tertiary amine *N*-methylephedrine, followed by pseudoephedrine with one methyl group attached to the nitrogen, and the primary amine norephedrine at the end. The number of the hydrophobic methyl groups seems to have an influence on the stability of the complexes of the phenethylamines and the chiral selector, sulf. β-CD, in the ME system. By increasing the hydrophobicity, the analyte favours inclusion into the CD and the residence time increases, so that a better enantioseparation occurs. At the same time the exposure time of the substance in the oil droplet of the ME increases. Thereby the migration time is reduced, as the oil droplet is negatively charged which increases the migration rate by reversed polarity.

However, the lowest resolution was achieved for ephedrine. Even though the chemical structure is similar to pseudoephedrine, it acts different in the chiral medium. Pseudoephedrine presents the diastereomeric form of ephedrine by having a *S,S* or *R,R* configuration. These sterical effects may cause the difference in the stability of the complex with the sulf. β-CD and might be responsible for the better resolution of the pseudoephedrine enantiomers than the ephedrine enantiomers.

A study performed by Deeb et al. revealed a resolution between 3 and 4 to be necessary for the quantification of an enantiomeric impurity of 0.1% [39]. Hence, the resolution values found with the MEEKC method are sufficient to obtain a suitable LOD.

For comparison reasons, the four racemates were tried to separate by CZE using sulf. β-CD in concentration of 0.7% and 4% and using the phosphate buffer with 50 mM and pH 3.0 (cf. to Wedig et al. [37]) and with 20 mM and pH 2.5 (cf. to MEEKC method). In contrast to the HDAS-modified CZE method a negative voltage was needed to detect the analytes. The favour of inclusion seems to be greater in the sulf. β-CD than to HDAS, so that the substances migrate to the anode.

However, no complete separation was observed applying the four mentioned buffer systems. Six peaks instead of eight appeared. When using the 20 mM phosphate buffer pH 2.5 containing 0.7% sulf. β-CD, eight peaks could be identified; however, two pairs of peaks are only partly separated (data not shown).

Table 1
Summarized results of the optimized β-sulf. CD modified MEEKC method and the simultaneous HDAS modified CZE determination.

Racemic substance	Migration time of 1st peak [min] MEEKC	*Rs* (MEEKC)	I migration time of 1st peak [min] CZE	*Rs* (CZE)
Dipivefrine	10.4	0		
2-(Dibutylamino)-1-phenyl-1-propanol	11.0	1.1		
Adrenaline	13.0	3.5		
N-methylephedrine	13.2	6.0	14.5	12.4
Ephedrine	13.7	4.0	14.1	11.2
Pseudoephedrine	14.4	5.7	13.9	3.8
2-Amino-1-phenylethanol	15.6	3.4		
Diethylnorephedrine	17.8	2.4		
Norephedrine	18.4	5.0	13.0	8.2

Subsequently, the optimized MEEKC method was transferred to the other phenalkylamines, i.e. diethylnorephedrine, 2-(dibutylamino)-1-phenyl-1-propanol, adrenaline, dipivefrine and 2-amino-1-phenylethanol. Table 1 summarizes the migration times and resolutions of all pairs of enantiomers.

As can be seen from Table 1 baseline separations were achieved for the racemates of adrenaline, 2-amino-1-phenylamine and diethylnorephedrine. Adrenaline and 2-amino-1-phenylamine show similar resolution values which are worse in comparison to the ephedrine derivatives. This might be due to their high polarity which disfavours the CD-inclusion complex. However, diethylnorephedrine is more bulky and more hydrophobic due to its ethyl side chains, and the racemate was less resolved. For racemic 2-(dibutylamino)-1-phenyl-1-propanol a low resolution of $Rs = 1.1$ occurred only, and no enantiomeric separation was observed for dipivefrine racemate. Optimization of this method by varying the pH value, the concentration of the ME compounds or the CDs, showed no improvement of the resolution. The increasing hydrophobicity of these molecules, which was useful for a good separation of *N*-methylephedrine, impinges on these substances. These compounds seem to be too large to fit into a β-CD cavity (Fig. 3).

By means of the CD-modified CZE method [36,37] the four ephedrine derivatives were baseline separated with a high resolution (see Table 1). The ephedrine derivatives were separated with an increasing order of resolution: methylephedrine > ephedrine > norephedrine > pseudoephedrine. Interestingly, the migration times of CZE and MEEKC are antidromic, which holds also true for the resolution with exception of *N*-methylephedrine.

Migration order	CZE: N < P < E < M
	MEEKC: M < E < P < N
Resolution ranking	CZE: M > E > N > P
	MEEKC: M > P > N > E

For example, norephedrine has the shortest migration time using CZE, but the longest migration time using MEEKC and vice versa methylephedrine. This finding can be ascribed to the reversed polarity, as the more hydrophobic substances have a greater exposure to the negatively charged oil droplet of the ME, and therefore the migration to the anodic end of the capillary is faster.

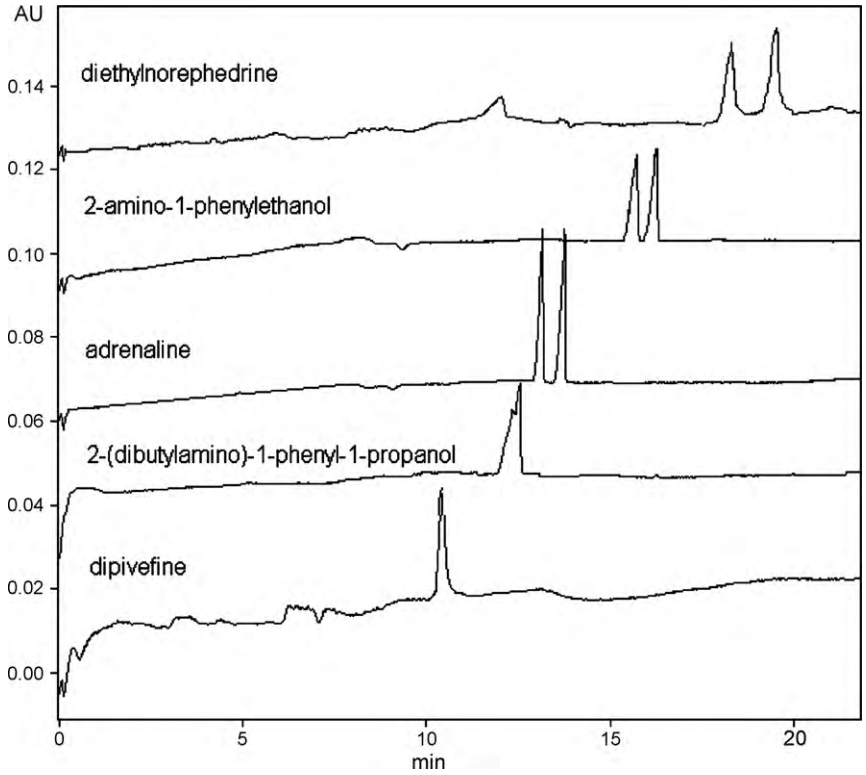

Fig. 3. Chiral CD-modified MEEKC of racemic dipivefrine, racemic 2-(dibutylamino)-1-phenyl-1-propanol, racemic adrenaline, racemic 2-amino-1-phenylethanol and racemic diethylnorephedrine. Separation conditions: 4.0% (w/w) sulfated β-CD in ME of 0.5% (w/w) ethyl acetate, 1.0% (w/w) SDS, 4.0% (w/w) 1-butanol, 3.0% (w/w) propan-2-ol and 91.5% (w/w) 20 mM phosphate buffer, pH 2.5; voltage: −15 kV, temperature: 20 °C.

Interestingly, separation of all ephedrines was achieved by using the neutral CD derivative dimethyl-β-CD as chiral selector in CZE; however, pseudoephedrine was used as enantiomer, not as racemate [38]. Using tris buffer with a pH value of 2.5, a similar migration order to the HDAS-modified CZE method appeared: N > E > M > P, resolution values are not indicated. Using this method, three racemic ephedrine derivatives and (1*S*,2*S*)-pseudoephedrine could be separated in 22 min.

Taken together, the HDAS-modified CZE method as well as the sulf. β-CD-modified MEEKC method is able to separate the racemic ephedrine derivatives in less than 20 min. Both methods show the highest resolution values for methylephedrine. In contrast to the CD-modified MEEKC method the CZE is able to separate all racemates in one run. In spite of MEEKC method optimization only seven single peaks appeared in the electropherogram of all ephedrine derivatives, because the peaks of (1*R*,2*S*)-ephedrine and (1*R*,2*R*)-pseudoephedrine overlap. However, the separation of the racemates of all ephedrine derivatives is not a real life analytical problem.

In view of the increased effort of the four single runs, the MEEKC method has the great advantage of being much cheaper by using the low-priced sulf. β-CD instead of 20-fold more expensive HDAS. Additionally, the MEEKC-method can be applied to other ephedrine derivatives, like diethylnorephedrine, 2-(dibutylamino)-1-phenyl-1-propanol, 2-amino-1-phenylethanol and adrenaline.

4. Validation

The final sulf. β-CD-modified MEEKC method was validated for the separation of two substances: ephedrine and pseudoephedrine.

4.1. Precision

For testing the precision of the method, a racemic ephedrine and a racemic pseudoephedrine solution was prepared in 0.1 M HCl to give 0.2 mg/ml samples (see Section 2.3), respectively. Each solution was injected five times, respectively, using identical ME for enantioseparation and keeping the conditions like voltage and temperature constant. To proof the precision of the developed method, the migration time and the ratio of the percentaged peak areas of both enantiomers was compared by checking the relative standard deviation (RSD) in per cent.

The migration time of pseudoephedrine amounts to 14.4 and 15.2 min with a RSD of 2.8 and 3.2%, respectively, of ephedrine 13.7 and 14.6 min with a RSD of 4.2 and 4.6%, respectively. The ratio of the percentaged peak areas varies with a RSD of 2.2% and 5.4% for pseudoephedrine and ephedrine, respectively. Due to the small RSD values the method can be considered to be precise with regard to migration time and peak areas.

4.2. Robustness—variation of pH values

For analyzing the robustness of the method, the racemic ephedrine and pseudoephedrine solutions (see Section 4.1) were used, again.

The BGE of the final MEEKC method consists of a phosphate buffer of pH 2.5 (see Section 2.6). The robustness of the method was tested by varying the pH values in a range of 2.0–3.0 in 0.25 steps. For this purpose the migration time and the ratio of the corrected peak areas were monitored. For (1*R*,2*S*)- and (1*S*,2*R*)-ephedrine there was no significant difference in the range of 2.0–2.75. Only at pH 3.0 the migration time of both peaks was extended by more than 3 min, for (1*S*,2*S*)- and (1*R*,2*R*)-pseudoephedrine lengthening of the migration time started already at a pH value of 2.75. The resolution differs only in a small range (4.0 ± 0.3 for ephedrine, 5.7 ± 0.3 for pseudoephedrine) between pH 2.0 and 3.0. Interestingly, the resolution values are increased using pH values below 2.5 and smaller values with pH 2.75 and 3.0 for both substances. The ratio of the corrected peak area for both substances remained similar with a RSD of 5.7 and 6.0%, respectively.

4.3. Robustness—variation of buffer concentration

The BGE of the final MEEKC method consists of a 20 mM phosphate buffer (see Section 2.6). For checking the robustness, the concentration of the salt compound NaH_2PO_4 was varied in a range of 10–50 mM in 10 mM steps. Increasing the concentration caused shorter migration times, as the EOF is decreased by the greater ionic strength of the BGE and the anionic micelles are able to migrate faster with the negative voltage. Also the resolution of both substances is changing with varied buffer concentrations. Conspicuously, the greatest resolution is obtained by optimized ME with a 20 mM phosphate buffer. Increasing as well as decreasing the buffer concentration impinges the resolution values, and even differences of the resolution values of 1.5 for ephedrine, and 1.8 for pseudoephedrine, are achieved. The ratio of the corrected peak areas of both substances differs in a very small range, from 1.01 to 1.09, while the used content of the enantiomers were the same.

4.4. Linearity of the content of the impurity

Both substances, pseudoephedrine and ephedrine, considered for validation, are monographed as pure enantiomers in the European Pharmacopoeia (Ph. Eur. 6), i.e. (1*R*,2*S*)-ephedrine and (1*S*,2*S*)-pseudoephedrine. In each case the respective enantiomer is examined as an impurity. Hence, the linearity of the content of the impurity was investigated in the range of 0.1–1.0% of the main compound. On the basis of the corrected peak areas of the impurity peak, a regression line was determined. The figure of merit of this validation parameter is shown by the stability index of 0.9973 and 0.9954 for pseudoephedrine and ephedrine, respectively, indicating that the respective enantiomer can be precisely determined in presence of the main compound.

4.5. Limit of detection

The LOD was investigated according to Ph. Eur. 6, i.e. a signal-to-noise ratio of 3:1. The absolute LOD of each ephedrine derivative was found to be 1.9 and 1.6 μg/ml for pseudoephedrine and ephedrine, i.e. 0.067% and 0.05% of the main compound, respectively.

5. Impurity profiling in comparison to the methods of European Pharmacopoeia

5.1. Impurity profile

The Ph. Eur. 6 contains monographs of ephedrine, pseudoephedrine, adrenaline and dipivefrine: i.e. (1*S*,2*S*)-pseudoephedrine hydrochloride, (1*R*)-adrenaline and (1*R*)-adrenaline tartrate, ephedrine as well as (1*R*,2*S*)-ephedrine anhydrous, (1*R*,2*S*)-ephedrine hydrochloride, (1*R*,2*S*)-ephedrine hemihydrate and racemic ephedrine hydrochloride and dipivefrine hydrochloride as a racemate.

Additionally, ephedrine is listed as an impurity of (1*S*,2*S*)-pseudoephedrine hydrochloride and pseudoephedrine of (1*R*,2*S*)-ephedrine hydrochloride, adrenaline is an impurity of (1*R*)-noradrenaline hydrochloride and (1*R*)-noradrenaline tartrate and noradrenaline of (1*R*)-adrenaline and (1*R*)-adrenaline tartrate.

Furthermore, racemic adrenaline is an impurity of dipivefrine hydrochloride.

5.2. Related substances: separation by means of CD-modified MEEKC

The sulf. β-CD-modified MEEKC enantioseparation method developed here was applied to the separation of the following substances of the Ph. Eur. 6 and its corresponding impurity: ephedrine and pseudoephedrine, adrenaline and noradrenaline, dipivefrine and adrenaline. For this purpose each of the substances and (1*R*)-noradrenaline were dissolved in 0.1 M HCl to give solutions of about 0.2 mg/ml for each enantiomer (see Section 2.3), so all analytes are of the same concentration.

First, the aforementioned MEEKC method was tested for the separation of racemic ephedrine and racemic pseudoephedrine. Using the conditions of the method, only three peaks appeared in the electropherogram, i.e. (1*S*,2*R*)-ephedrine, (1*S*,2*S*)-pseudoephedrine, and (1*R*,2*S*)-ephedrine and (1*R*,2*R*)-pseudoephedrine as one peak. Since the later two substances overlap, the method had to be optimized. By varying the concentration of the sulf. β-CD to 5%, a resolution of the third peak, (1*R*,2*S*)-ephedrine and (1*R*,2*R*)-pseudoephedrine, appeared. Finally a baseline separation of all four peaks was achieved by increasing the concentration of the CD to 8% (see Fig. 4).

Second, the method was also applied to racemic adrenaline and (1*R*)-noradrenaline and accordingly racemic adrenaline and dipivefrine (see Fig. 6). Using 4% sulf. β-CD racemic adrenaline and (1*R*)-noradrenaline could be properly separated (see Fig. 5). Using the same conditions racemic adrenaline and dipivefrine gave three peaks, i.e. racemic dipivefrine could not be separated, as expected (see Fig. 6).

In the next step, the methods were checked according to the limits of the content of the impurity given in Ph. Eur. 6: 1.0% ephedrine in (1*S*,2*S*)-pseudoephedrine, 0.5% pseudoephedrine in (1*R*,2*S*)-ephedrine, 0.2% noradrenaline in (1*R*)-adrenaline and 0.1% racemic adrenaline in racemic dipivefrine. In all cases, a separation of the impurities from the main compound was achieved. The LODs of the impurities were determined (S/N = 3:1) and reported in Table 2. As can be seen from the *R*s and LOD values, the MEEKC methods are able to limit the impurities as good as the methods given in the Ph. Eur. 6.

Finally, one batch of each substance was investigated with the appropriate MEEKC method. Analyzing the electropherograms of each substance ensured that the present batches do not contain the expected impurity or below the disregard limit.

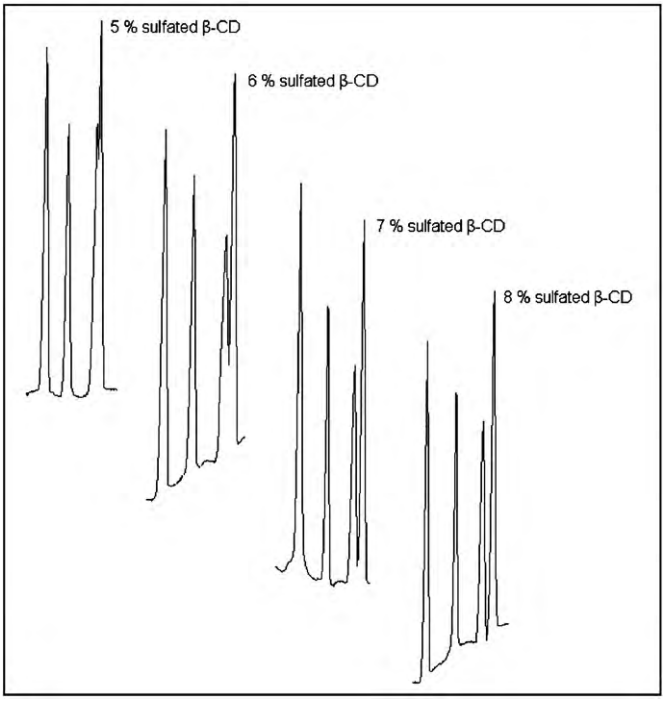

Fig. 4. Development of the chiral CD-modified MEEKC of racemic ephedrine and racemic pseudoephedrine. Separation conditions: 5.0, 6.0, 7.0 and 8.0% (w/w) sulfated β-CD in ME of 0.5% (w/w) ethyl acetate, 1.0% (w/w) SDS, 4.0% (w/w) 1-butanol, 3.0% (w/w) propan-2-ol and 91.5% (w/w) 20 mM phosphate buffer, pH 2.5; voltage: −15 kV, temperature: 20 °C.

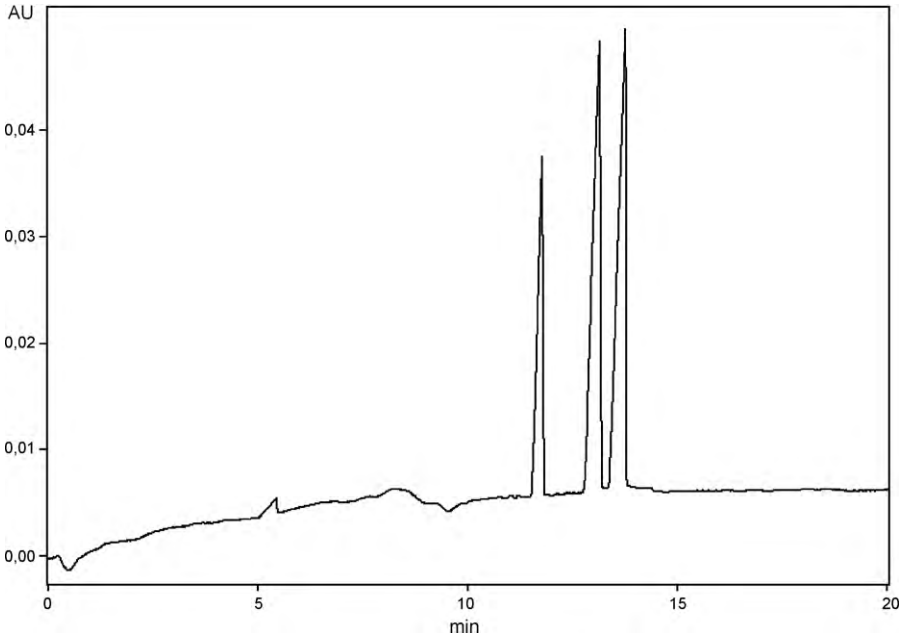

Fig. 5. Chiral CD-modified MEEKC of noradrenaline and racemic adrenaline. Separation conditions: 4.0% (w/w) sulfated β-CD in ME of 0.5% (w/w) ethyl acetate, 1.0% (w/w) SDS, 4.0% (w/w) 1-butanol, 3.0% (w/w) propan-2-ol and 91.5% (w/w) 20 mM phosphate buffer, pH 2.5; voltage: −15 kV, temperature: 20 °C.

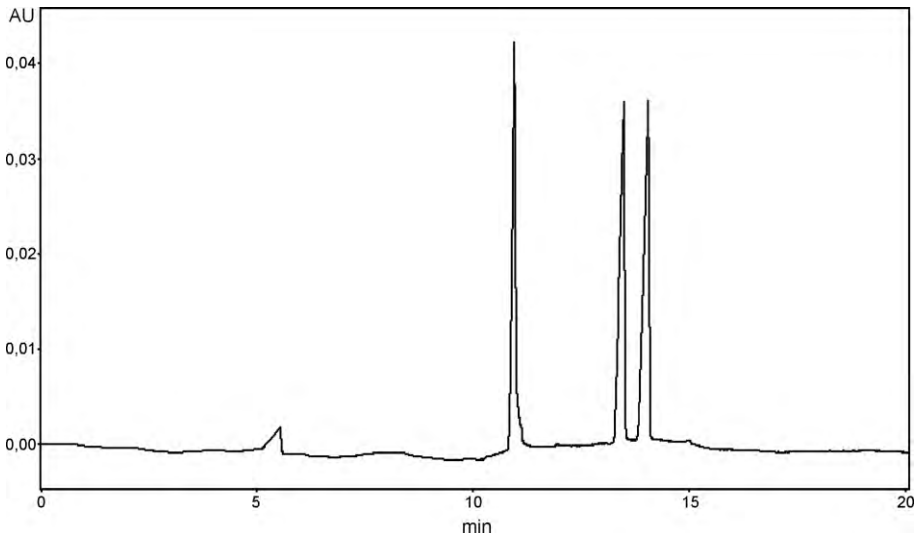

Fig. 6. Chiral CD-modified MEEKC of racemic adrenaline and racemic dipivefrine. Separation conditions: 4.0% (w/w) sulfated β-CD in ME of 0.5% (w/w) ethyl acetate, 1.0% (w/w) SDS, 4.0% (w/w) 1-butanol, 3.0% (w/w) propan-2-ol and 91.5% (w/w) 20 mM phosphate buffer, pH 2.5; voltage: −15 kV, temperature: 20 °C.

Table 2
Summarized results for the determination of the impurities proportional to the main compound given in the Ph. Eur. 6.

Substances	LOD of impurity	Peak order	*Rs* peak1/peak2 peak2/peak3
Ephedrine in (1*S*,2*S*)-pseudoephedrine	0.1%	1. (1*S*,2*R*)-ephedrine 2. (1*S*,2*S*)-pseudoeph. 3. (1*R*,2*S*)-ephedrine	4.4 4.0
Pseudoephedrine in (1*R*,2*S*)-ephedrine	0.1%	1. (1*S*,2*S*)-pseudoeph. 2. (1*R*,2*S*)-ephedrine 3. (1*R*,2*R*)-pseudoeph.	4.0 1.6
Noradrenaline in (1*R*)-adrenaline	0.06%	1. (1*R*)-noradrenaline 2. (1*S*)-adrenaline 3. (1*R*)-adrenaline	5.4 3.5
Rac. adrenaline in rac. dipivefrine	0.067%	1. rac. dipivefrine 2. (1*S*)-adrenaline 3. (1*R*)-adrenaline	12.3 3.5

6. Concluding remarks

The comparison of the two applied CE methods, HDAS-modified CZE and sulf. β-CD-modified MEEKC, revealed different advantages and disadvantages for each of them. Whereas with the HDAS-modified CZE method all four racemic ephedrine alkaloids could be separated in one run, four individual short runs were needed by means of MEEKC. However, the MEEKC method is much cheaper by using sulf. β-CD in MEEKC and provides sufficient resolution for the quantification of the minor enantiomer in presence of the major one. However, the limit of detection might be a problem. Even though Table 2 indicates that the LODs are rather high due to a high level of noise in MEEKC, the values are sufficient for pharmacopoeial purposes.

Taken together, the developed MEEKC method is versatile as it can be used as impurity analysis of the Ph. Eur. 6. The impurities could be separated, and the limits for the impurities, given in the Ph. Eur. 6, could be achieved. Hence, CD-modified MEEKC methods were found to be a powerful tool for chiral and impurity analysis, being as potent as CD-modified CZE.

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