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Do we know the mechanism of chiral recognition between cyclodextrins and analytes?

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday.

Abstract

The resolution of chiral phenethylamine analogue compounds were studied in presence of single-isomer neutral and negatively charged cyclodextrins (CDs) by means of capillary electrophoresis (CE) and NMR spectroscopy. Whereas the native β-CD and *heptakis*(2-*N*,*N*-dimethylcarbamoyl)β-cyclodextrin were not able to separate the racemates of four ephedrine derivatives studied, *heptakis*(2,3-*O*-diacetyl)β-cyclodextrin and especially *heptakis*(2,3-*O*-diacetyl-6-sulfo)β-cyclodextrin could resolve all four pairs of enantiomers in one run. UV and NMR spectroscopic measurements revealed various kinds of complexes of phenethylamines with the CDs. Either defined inclusion complexes or manifold complexes which are mostly characterized by an attachment of the analyte to the outside of the CD cavity were found. No correlation between the kind of complexation and the resolution observed by means of CE could be found. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Chiral recognition; Cyclodextrins; Ephedrine derivatives; Capillary electrophoresis; Nuclear magnetic resonance

1. Introduction

Since the chiral nature of the living systems has implications on biological active compounds interacting with them, the stereoisomers of drugs can differ in both the pharmacodynamic and the pharmacokinetic action [1]. Since the beginning of the ninetieth of the last centuries regulatory authorities encourage pharmaceutical companies to develop single isomers of chiral drugs only [2]. Even though most chiral drugs on the market are still racemates, the situation is changing. Due to the chiral switch of 'old' and the introduction of new enantiomerically pure drugs, among the top-selling 500 drugs more than 50% were single enantiomers in 2000 [3]. Thus, methods are required which are able to evaluate stereospecifically both enantiomers of a drug either in quality control of enantiomerically pure drugs, e.g. the determination of the enantiomeric excess, or in pharmacokinetic studies in order to study the fate of each enantiomer of a chiral drug in the human

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body with respect to pharmacokinetics, toxicology and eventually to interconversion of the enantiomers [4].

The most often applied method in chiral analysis is the high performance liquid chromatography (HPLC) using chiral stationary phases [5,6]. According to Blomberg and Wan [7], HPLC has some drawbacks, such as the lack of general applicability, in which a number of columns are required to cover a reasonably wide application range, the relatively short lifetime and the high costs of chiral columns. Beside NMR spectroscopic applications [8], the capillary electrophoresis (CE) becomes increasingly important, because of the high separation efficiency and analysis speed as well as reduced operating costs. In addition, it is now possible to evaluate chiral impurities at a concentration level below 0.1% with a high reproducibility [9]. Thus, the introduction of CE methods in the US and the European Pharmacopoeia (EP and USP, respectively) has been recently discussed by regulatory authorities [10–13].

Especially, the cyclodextrin (CD)-modified CE, characterized by an orthogonal separation mechanism to LC, is the state-of-the-art in chiral analysis [14] in both quality control [15] and analysis of biological samples [16–22]. Beside the native CDs, α-, β- and γ-CD consisting of a 6-, 7- or 8-membered torus of glucose unit [23], a number of derivatized CDs, either neutral, positively or negatively charged, are commercially available, e.g. hydroxypropyl-β-CD [24], (2,3-di-*O*-methyl)- and (2,3,6-tri-*O*-methyl)β-cyclodextrin [25], sulfobutyl-β-CD [26–28], carboxymethyl-β-CD [29], various sulfated β-CD and some single isomer CDs, such as *heptakis*(2,3-di-*O*-acetyl-6-sulfo)β-CD, *octakis*(2,3-di-*O*-acetyl-6-sulfo)γ-CD and *heptakis*(6-sulfo)β-CD, were developed by Vigh and coworkers [30–34]. However, even though countless applications were described to date (for review see [14]), it is still difficult to predict which kind of CD will be appropriate for the resolution of given analyte. On the one hand the intramolecular modeling approach utilizing the analysis of quantitative structure-enantioselectivity relationships within a structurally related series of compounds can help to predict the optimum CD for resolution [35]. On the other hand, the knowledge of the mechanism of chiral recognition between selectors, in this case the CD, and analyte may also help to find rules for the prediction. Therefore, several groups studied the structures of the diastereomeric complexes by means of IR and NMR spectroscopy, circular dichroism and X-ray analysis [36–43].

The focus of our interest is to elucidate the structure of the diastereomeric complexes formed between the enantiomers of phenethylamines in clinical practice, e.g. ephedrine derivatives, and β-CD and single isomeric acetylated and carbamoylated β-CD derivatives such as the recently synthesized the *heptakis*(2,3-*O*-diacetyl)β-CD (Diac) and *heptakis*(6-*O*-acetyl)β-CD (6-Ac) [44,45] as well as the *heptakis*(2-*N*,*N*-dimethylcarbamoyl)β-CD (HDMC) [46] and the negatively charged *heptakis*(2,3-*O*-diacetyl-6-sulfo)β-CD (HDAS). The resolution power of each CD was simultaneously checked for all compounds by means of CE under almost the same conditions in order to be able to compare the resolution values. NMR spectroscopy, and in particular the complexation induced chemical shifts (CICS) of both ligand and CD in addition to Job plots and ROESY experiments, mass and UV spectroscopy were employed to study the chiral recognition power of each CD. In order to check whether the knowledge of the complex structure can help to predict the most effective CD, data of various studies will be compared.

2. Experimental

2.1. Chemicals

The enantiomers of the ephedrine derivatives were purchased from Fluka (Buchs, Switzerland), β-CD was a gift from the Consortium für Elektrochemische Industrie (München, Germany), Diac was synthesized according to Branch et al. [44], HDMC according to Christians and Holzgrabe [46]. Analytical grade KH_2PO_4 and *ortho*-phosphoric acid was obtained from Merck (Darmstadt, Germany). 50 mM Phosphate buffer pH 3.0 was prepared by mixing appropriate concentra-

tions of H_3PO_4 and KH_2PO_4 solution. The CDs were dissolved in buffer; the samples subjected to the CE were dissolved in de-ionized water (conc. $\approx$ 1 mg/ml). All solutions were filtered through a 0.45 μm syringe (Schleicher und Schüll, Dassel, FRG).

2.2. Capillary electrophoresis

All experiments were performed on a Beckman MDQ system (Beckman Instruments, Fullerton, CA) using a fused-silica capillary with a total length of 60 cm, a detection length of 50 cm, and an internal diameter of 50 μm. Samples were loaded by 5 s of pressure injection (0.5-1.0 p.s.i.) and separated at 25 °C in the cationic injection mode, using a constant voltage of 20 kV. The drug solution had a concentration of 50 μg/ml and was detected using a diode array detector at 194 nm. The capillary was conditioned for 20 min with 0.1 M NaOH, and 10 min with water. Additionally, the capillary was washed for 2 min with 0.1 M NaOH, 1 min with water, and 2 min with the running buffer before each run.

2.3. UV-measurements

The UV spectra were recorded on a Hewlett Packard 8452 A Diode Array Spectrophotometer (Böblingen, Germany). The determination of the stoichiometry of the complexes was done by mixing stock solutions of the enantiomers and the CDs with a molar concentration of 0.1 mM in the ratios of 0.2:0.8, 0.33:0.66, 0.4:0.6, 0.5:0.5, 0.6:0.4, 0.66:0.33 and 0.8:0.2. Comparison of these spectra with solutions of the same concentration of the ephedrine compounds without CDs gave the required changes in absorbance.

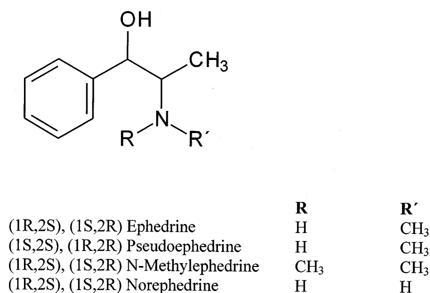
2.4. NMR

All experiments were performed on a Bruker Avance 400 FT NMR spectrometer operating at 400.132 MHz and equipped with a Kayak XA_b computer using the XWIN-NMR program package Version 3.0. The sample temperature was 26.9 °C 32 scans with a frequency range of 4006.4 Hz were collected into 32 050 data points, giving a digital resolution of 0.13 Hz/point. An appropriate Gaussian function was applied before Fourier transformation to enhance the spectral resolution. Solutions having molar ratios of 1:1 (ligand:HDMC) were prepared in deuterated 50 mM phosphate solution (composed of 50 mM KH_2PO_4 in deuterated water, equivalent to pH 4.5 and K_2HPO_4 in deuterated water, equivalent to pH 9.0) in order to measure the induced chemical shifts of the CD and phenethylamine signals. All chemical shifts were referenced to the H^2HO signal at 4.650 ppm.

3. Results and discussion

3.1. Capillary electrophoresis studies

In previous studies, the resolution power of the β-CD, Diac and HDAS in CE was already checked for the pairs of enantiomers of the individual ephedrine derivatives [44,45,47]. As far as the neutral CDs are concerned standard resolution conditions could be worked out for all phenethylamine analogues: pH 3.0, 50 mM phosphate buffer and 12 mM of either CD [45,47,48]. Whereas the native β-CD was not able to separate the racemates of ephedrine (E), pseudoephedrine (P), methylephedrine (M) and norephedrine (N) (Scheme 1), the diacetylated CD, Diac, and the corresponding negatively charged HDAS could baseline separate all enantiomers. In the case of HDAS, only very small amounts are necessary for resolution, i.e. 3 mM HDAS (Fig. 1) when applying pH 3 (Fig. 2).



	R	R′
(1R,2S), (1S,2R) Ephedrine	H	CH_3
(1S,2S), (1R,2R) Pseudoephedrine	H	CH_3
(1R,2S), (1S,2R) N-Methylephedrine	CH_3	CH_3
(1R,2S), (1S,2R) Norephedrine	H	H

Scheme 1. Structural formula of the ephedrine compounds.

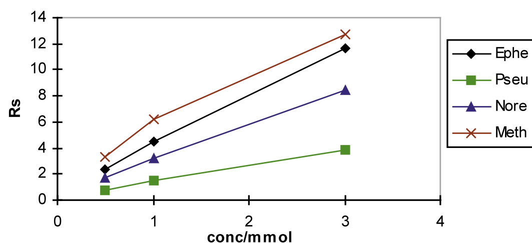


Fig. 1. Resolution of the various ephedrine compounds using different concentrations of HDAS (pH 3).

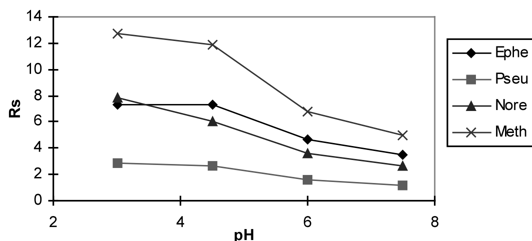


Fig. 2. Resolution of the various ephedrine compounds using 3 mM HDAS with different pH values.

In analogy to the casette dosing experiments in pharmacokinetic studies (for review see [49,50]) the separation power of the CDs was checked in presence of the racemates of all four ephedrine compounds using standard conditions in the case of the neutral β-CD and Diac as well as 18 mM selector in the case of HDMC and 3 mM in the case of HDAS. The electropherograms obtained are displayed in Fig. 3. The assignment of each peak was performed by spiking samples with either compound and enantiomer.

HDMC, which was already successfully applied to CD-modified CE of verapamil, fluoxetine and other phenalkylamines [46], was neither able to separate all compounds nor their enantiomers. Interestingly, the migration time and order was almost not effected by the addition of HDMC (compare Fig. 3a and e) indicating that no complexation has taken place under the applied conditions. β-CD gave three well-separated peaks for the enantiomers of ephedrine, nor- and methylephedrine. Nevertheless, the peaks of the enantiomers were heavily overlapping and the enantiomers of norephedrine were not resolved. The situation could be improved when applying Diac; eight peaks could be identified but were not baseline separated. Only HDAS is able to resolve all isomers of all compounds: eight peaks could be observed.

The spiking experiments also revealed a migration order of major (75%) before minor (25%) enantiomer to be advantageous in terms of resolution. This is especially true with β-CD and Diac (Table 1).

Interestingly, the migration order of the ephedrine compounds was found to be different with either CD: N–M–E–P with β-CD (M and E very close together), N–E–M–P with Diac and N–P–E–M with HDAS. Whereas the migration order of each pair of enantiomers was the same with Diac and HDAS, it was found to be different with β-CD (for assignment see Fig. 3). These results may indicate different modes of complexation for each CD.

Comparing the migration times of each CD revealed Diac to be the ‘fastest’ selector followed by β-CD. Since the neutral CDs are a sort of stationary phase in the capillary at pH 3, the migration times provides information about the complexation strength. The stronger the complexation between CD and analyte is, the longer stays the analyte with the CD and the higher is the migration time. The overall migration time without any CD was observed to be 9–9.5 min, addition of HDMC increased the migration time to 11–12 min, Diac to 11–13 min and β-CD to 13–14.5 min. Thus, β-CD forms stronger complexes with the ephedrine compounds than Diac and Diac stronger complexes than HDMC.

As expected the time to separate the isomers of all ephedrine derivatives was the longest with HDAS. This can be explained by the fact that HDAS carries the positively charged analytes, heading for the cathode at pH 3, in direction of the anode upon complexation. This effect increases the separation way along the capillary and, therefore, improves the resolution, which was found to be true with HDAS. However, the formation of diastereoselective complexes is still a prerequisite for a separation in CE.

3.2. UV measurements

The UV measurements performed to construct Job plots for all ephedrine compounds (Fig. 4) mirrow the complexation behavior observed when using CE [47]. In each case, the curve obtained with β-CD showed a clear maximum at 0.5 with high ΔA values indicating a 1:1 stoichiometry of strong complexes. The curve obtained with Diac also shows a maximum at 0.5, however, the ΔA values are less high again indicating a 1:1 stoichiometry but a lower complex stability. The difference in stability of complexes formed with β-CD and Diac was also derived from the CE measurements (see above) and is in accordance with the complexation constants determined by means of NMR spectroscopy [51].

The comparison of the Job plots obtained with HDAS on the one hand and β-CD and Diac on the other hand clearly revealed the curves of

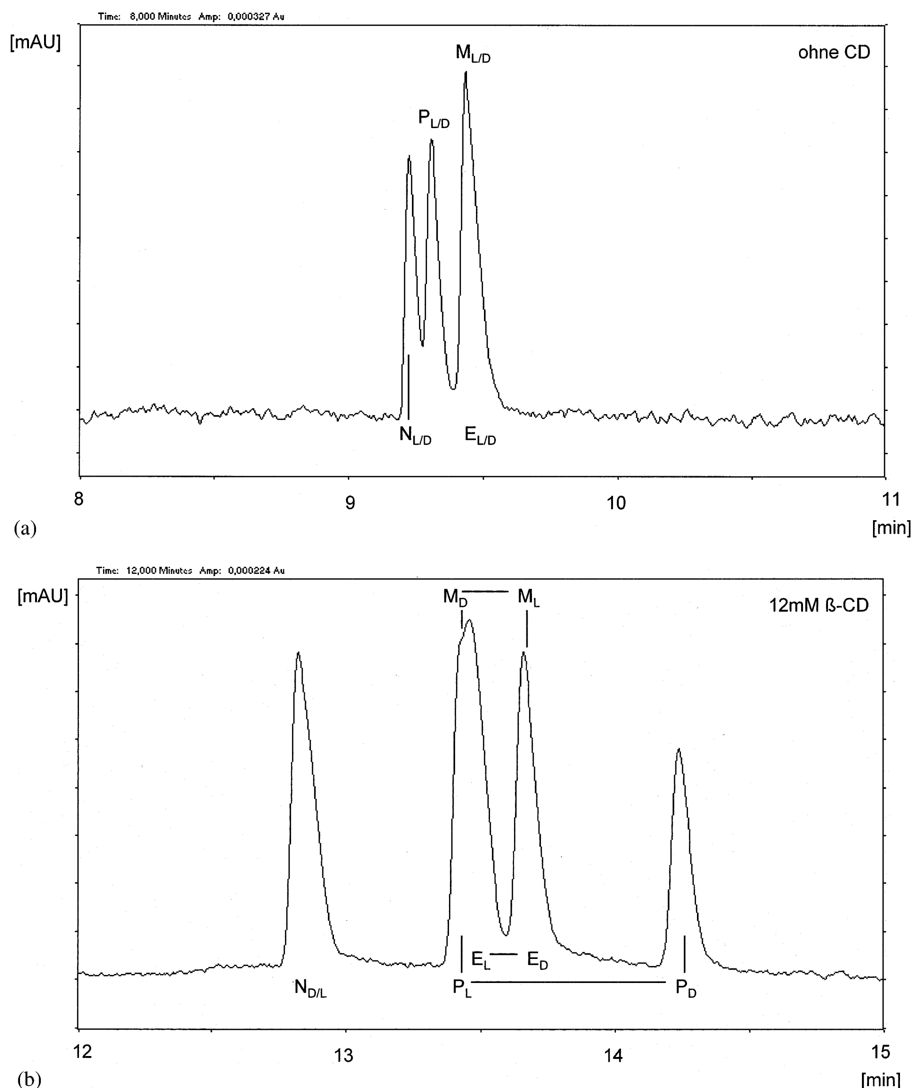


Fig. 3. Electropherograms of racemates (D and L, respectively) of ephedrine (E), pseudoephedrine (P), methylephedrine (M) and norephedrine (N) in (a) absence of CD, (b) in presence of 12 mM β-CD, (c) 12 mM Diac, (d) 3 mM HDAS and (e) 18 mM HDMC using 50 mM phosphate buffer at pH 3 and 20 kV, fused silica capillary of 60 cm (effective length 50 cm) × 50 μm I.D.

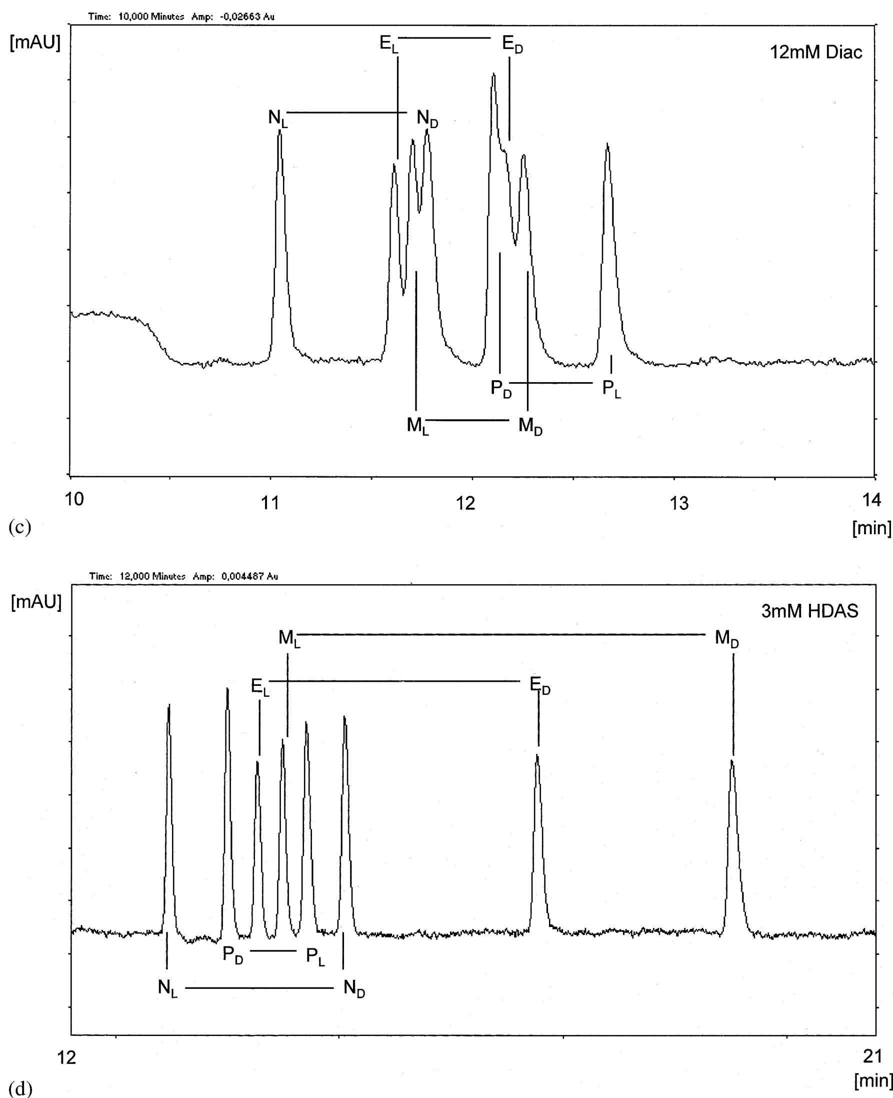


Fig. 3. (*Continued*)

HDAS to be much flatter indicating a lower stability of the HDAS complexes. In addition, the Job plots are rather curved which makes a derivation of the stoichiometry difficult.

3.3. NMR measurements

In previous NMR studies, CICS of the CDs and the phenethylamines as well as ROESY experiments were employed to work out the way of complexation [44,45,47,51,52]. With regard to the neutral CDs, β-CD and Diac, inclusion complexes were found with the aromatic moiety of the phenethylamines diving into the CD cavity and the protonated amino nitrogen (pH 4.5) forming a hydrogen bond to the oxygens of the wider rim of the torus. Interestingly, the coupling constants between the hydrogens did not change upon complexation with the neutral CDs. Thus, the chiral recognition is likely to be caused by a different orientation of the guests in the CD cavity rather than a different conformation. Similar complex

structures were found for brom- and chlorpheniramine, dimethindene or verapamil [37–40]. However, fencamfamine, whose phenethylamine moiety is a part of the rigid norbornane ring system, shows a rather different complexation behavior with Diac and γ-CD, which are both able to baseline separate the four isomers [53]. Job plot, constructed by means of ^{1}H NMR spectra with each isomer separately, were rather curved and did not show any maximum. Since especially the hydrogens located at the outside of the CD cavity were mostly influenced upon complexation, it was concluded that the isomers of the rigid fencamfamine interact mainly with the outside of the cavity rather than forming inclusion complexes. Whereas linear phenethylamines built uniform, defined complexes, manifold complexes were found with fencamfamine (Scheme 2).

The situation becomes even more complicated when considering the negatively charged HDAS.

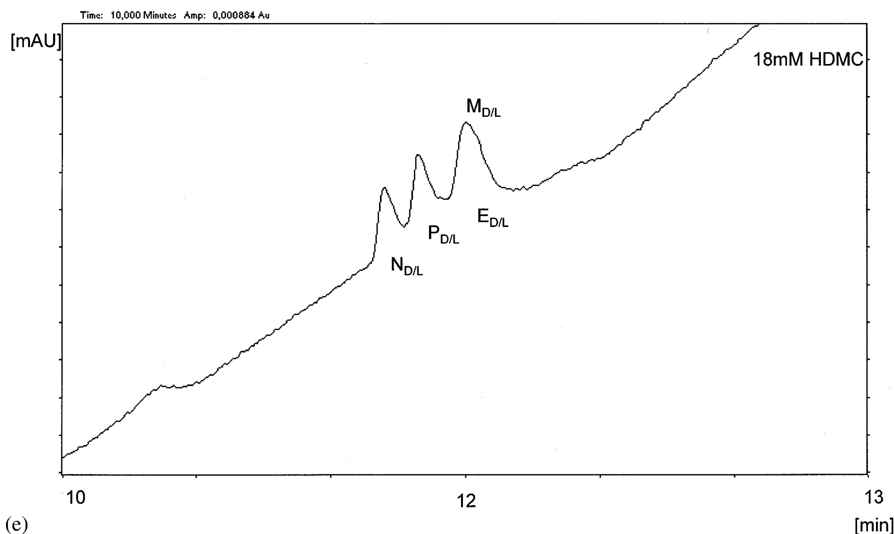


Fig. 3. (*Continued*)

Table 1
Migration times (mt) and resolutions (Rs) at different ratios of the enantiomers in presence of 12 mM β-CD and Diac, respectively, at pH 3 in 50 mM phosphate buffer; the experiments were performed in triplicate

Analyte ratio	Cyclodextrin	mt-L (min)	mt-D (min)	Rs
E 3L+1D	β-CD	13.7	13.6	0.6
	Diac	11.9	12.4	3.7
E 1L+3D	β-CD	13.8	13.6	1.3
	Diac	12.1	12.5	3.0
P 3L+1D	β-CD	13.5	14.4	4.1
	Diac	12.9	12.4	2.6
P 1L+3D	β-CD	13.6	14.2	3.0
	Diac	12.8	12.2	3.4
M 3L+1D	β-CD	13.6	13.5	0.6
	Diac	11.9	12.5	3.7
M 1L+3D	β-CD	14.0	13.7	1.4
	Diac	12.0	12.4	3.3

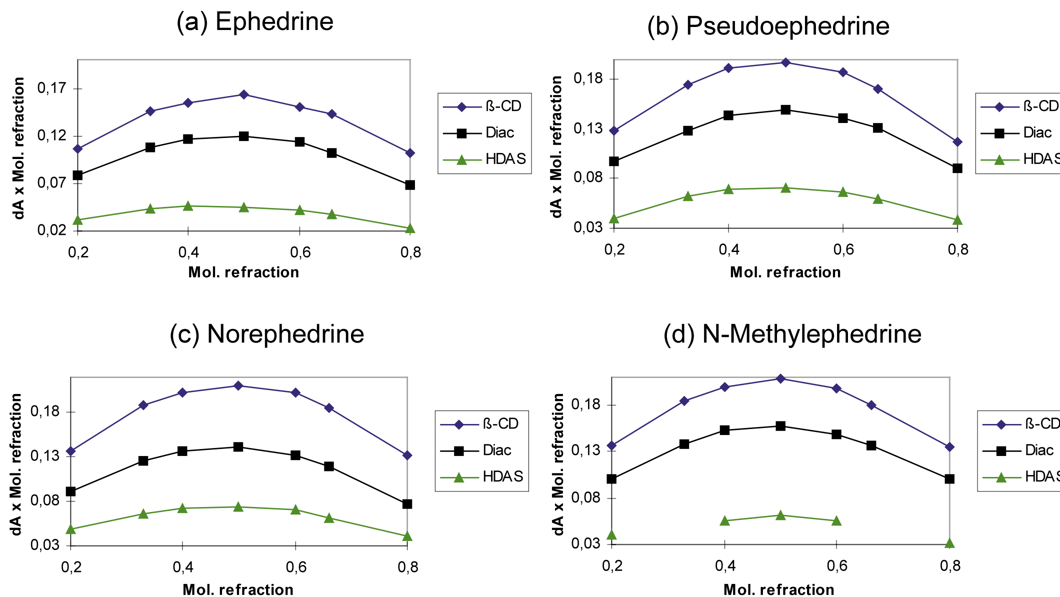
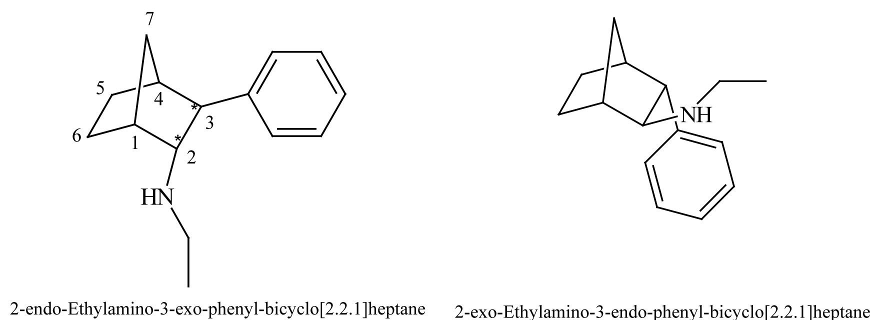


Fig. 4. The Job plot measured by means of UV spectroscopy indicating the interaction between β-CD, Diac and HDAS with (a) ephedrine, (b) pseudoephedrine, (c) norephedrine and (d) methylephedrine; y-axis = ΔA values, x-axis = mol. refraction.



Scheme 2. Structural formula of the isomers of fencamfamine.

In addition to the influence of acetylation on the wider rim ruling the form of the torus of HDAS and Diac, the influence of the negative charges sitting on the opposite narrow rim have to be considered. An ion–ion-attraction between these negative charges of the CD and the positive charge of the analyte (at pH 4.5) is likely to govern the complexation [52]. Both, inclusion complexes with the aromatic moiety sitting inside the cavity and the ethylamine chain pointing out of the narrow (!) rim as well as attachments of the analyte to the outside of the HDAS cavity are likely to occur. Thus, manifold complexes of varying stoichiometries were found with HDAS.

The complexation behavior of HDMC remains to be elucidated. However, using CE no separation of the enantiomers of the ephedrine compounds was observed which does not necessarily mean that no complexation takes place. Thus, NMR spectra of 1:1 mixtures of HDMC and analyte were measured at an acidic and a basic pH value, 4.5 and 9.0, respectively. Applying pH 4.5, the ^{1}H NMR did not show any change of the racemates of the ephedrine compounds on addi-

tion of equimolar amounts of HDMC; not even the pattern of the signals belonging to the aromatic hydrogens changed (data not shown). This finding, which mirrors the CE results, indicate that no complexes were formed at this pH.

Interestingly, NMR spectra of the racemate of pseudoephedrine, which were recorded in presence of HDMC at the basic pH, showed a clear signal splitting of the benzylic hydrogen and the CH-CH_3 group. The signals of the N-CH_3 group and the benzene ring were broadened. In addition, the corresponding signals of the methylephedrine racemate also showed signs of splitting and broadening. These effects indicate a complexation of the analytes by HDMC under basic conditions which cannot applied to CE.

Taking these results obtained from measurements at basic and acidic pH together, it might be concluded that the protonation of the phenethylamino nitrogen at pH 3.0 (CE) or 4.5 (NMR) prevents the complexation whereas the neutral phenethylamine (at pH 9.0) is able to interact with HDMC.

4. Conclusion

Taking the comparison of CE and NMR measurements together it can be stated that the formation of an inclusion complex between a given analyte and either CD is not a prerequisite for the separation of a racemic analyte in CE: even when no defined complex could be detected by means of NMR spectroscopic experiments, a separation of a racemate is possible using CE. This is not only true for negatively charged CD but also for neutral CDs. However, the NMR spectroscopy is a valuable tool to get structural information of the selector-selectand complex, which can help to understand the underlying mechanism of chiral cyclodextrin-modified CE.

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