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Affinity capillary electrophoresis on microchips

Alexandra R. Stettler, Maria A. Schwarz*

Department of Chemistry, University of Basel, Spitalstr. 51, 4056 Basel, Switzerland

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Dedicated to Katharina Helene Schwarz on the occasion of her birth

Abstract

The present study shows that the application of the method of affinity capillary electrophoresis (ACE) to investigate interactions between ligands and their substrates can be realized on microchips. With ACE it is possible to characterize non-covalent molecular interactions (complexation and partition equilibria). Binding constants (K_B) provide a measured value of the affinity of a ligand molecule to a substrate, which is basic information for the understanding of hormones, drugs and their targets, e.g. receptors in the human body. A microchip electrophoresis instrument equipped with a UV-detector and a home-built chip-station with electrochemical detection were used. ACE could be achieved with model solutions of neurotransmitters using sulfated β-cyclodextrin (sCD) as substrate in a background buffer. This paper describes the advantages of microchip-ACE (MC-ACE) to traditional affinity capillary electrophoresis on a capillary. The results show that MC-ACE has great potential as a tool for fast scanning of interactions and to calculate binding constants of ligands with their substrates.

Keywords: ACE; Affinity capillary electrophoresis; Binding constant; Capillary electrophoresis; Chip; Cyclodextrin; Epinephrine; Microchip; Neurotransmitter

1. Introduction

Since the introduction of the miniaturized total analysis system (μTAS [1]) principle, microfabricated devices for increasingly complex analyses have revolutionalized the area of chemical and biological analysis. Particular attention has been paid to micromachined capillary electrophoresis. Miniaturized analysis systems have brought various new schemes of processing analytical operations including the pre-treatment, enrichment, separation, post-treatment and detection of a wide range of sample molecules [2]. This development has mainly been caused by the advantages of miniaturization, the variety of geometry, the layout of microseparation channels and therefore reduced reagent consumption, rapid analysis time, opportunities for parallel screening and ease of automation. Until now electrophoretic processes on micromachined channels have been applied mainly in separation tasks. The separations of proteins [3], DNA [4], biogenetic molecules [5] and enantiomers [6] are only a few applications. A new research field would be the transformation of ACE onto a chip for characterizing of chemical equilibria.

Many techniques have been developed to determine apparent equilibrium constants for molecular association. All experimental techniques are based on titrating changes in physicochemical properties of molecule depending on the presence of substrate. Changes in size and charge (conductivity [7]) might result, in measurable differences in spectroscopic properties (spectrofluorimetry [7–12]), thermo-chemistry (calorimetry [13–15]), surface tension [16], molecular weight (nuclear magnetic resonance (NMR) [17–19]) and electrophoretic migration (ACE) [20–23].

ACE may be classified into three modes: (a) non-equilibrium electrophoresis of equilibrated sample mixture, no additives are in the background electrolyte; (b) dynamic equilibrium affinity electrophoresis, the substrate is dissolved in the running buffer (ACE mode [24,25]); and (c) affinity based CE or affinity capillary electrochromatography, the substrate is immobilized ([26,27]). All types of procedure involve measurements of the migration times (t_m) of solute

* Corresponding author. Tel.: +41 61 2671002; fax: +41 61 2671005.
E-mail address: Maria.Schwarz@unibas.ch (M.A. Schwarz).

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in the presence of complexation agents. Intensity and shape of the peak are not required [28]. Binding constants of many non-covalently bound biomolecules are determined by ACE and compared with the values obtained by other methods. All results indicate that ACE is acceptable for calculations of binding constants [29]. Examples include the action of enzymes upon substrates and the binding of hormones with their receptors [30]. ACE techniques are excellently suited for investigating protein–protein interactions [31,32], in particular because they can be performed in free solutions, generally under non-denaturating conditions and they require only minimal amounts of sample. By adding increasing concentrations of the ligand into the background buffer a shift in the ionic mobility of the substrate can be observed. This shift allows the calculation of the binding constant.

The main focus in this study is on cyclodextrin–drug interactions, because in the literature there are a lot of characterizations on capillary [33]. One kind of neurotransmitter, the natural human sympathomimetics, was chosen as a drug model because epinephrine (EN) and norepinephrine (NEN) have receptors omnipresent in the whole body. Here, we describe the simulation of a simple 1:1 complexation between ligand and "receptor". Additionally, the sample structures had to be measurable with UV- and amperometric detection for comparison of two different microanalytical systems. Beside the affinity studies of these compounds the results obtained can be used for the development of separation methods that is especially important for simultaneous measurements of similar analytes [34]. In addition to the interest in binding reactions and stoichiometry under physiological conditions, interactions under non-physiological conditions have also been taken into account. These data provide more detailed information about the physicochemical parameters influencing the binding interaction and a better insight into the binding mechanism. The formation of inclusion complexes is influenced by the hydrophobic interaction in the cavity and hydrogen bonds/electrostatic interactions between the CDs substituents groups (e.g. carboxymethyl, hydroxypropyl, methyl groups) on the rim of the CD and substituents of the drug.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical grade. (±) Epinephrine (4-[1-hydroxy-2(methylamino)ethyl]-1,2-benzenediol) HCI, (±) norepinephrine (α-(aminomethyl)-3,4-dihydroxybenzyl alcohol) hydrogentartrate, DL-metanephrine (4-hydroxy-3-methoxy-α-(methylaminomethyl)benzene-methanol) HCI and 3,4-dihydroxy-DL-phenylalanine (DL-DOPA) were obtained from Sigma (Buchs, Switzerland). Sulfated β-cyclodextrin was obtained from Aldrich (Buchs, Switzerland). Tris(hydroxymethyl)aminomethane (TRIS), 2-morpholinoethane-sulfonic acid (MES) monohydrate, NaOH and citric acid monohydrate were obtained from Fluka (Buchs, Switzerland). Boric acid and dimethyl sulfoxide (DMSO) were obtained from Acros (Geel, Belgium). *o*-Phosphoric acid was obtained from Merck (Dietikon, Switzerland). The buffers were prepared by mixing a 10 mM solution of TRIS with acid to get to the corresponding pH-value (for pH 6 citric acid, pH 7 boric acid, pH 8 phosphoric acid and pH 9 boric acid) and a 10 mM solution of MES with NaOH to get to pH 6 (for the amperometric measurement). Sample compounds (2 mM for UV-chip, 0.2 mM for UV-CE and 0.5 mM for amperometry-chip) and the internal standard (DMSO for UV and DOPA for amperometry) were dissolved in the buffer solution. CD was dissolved in the buffer solution to yield stock solutions of 10 mg/mL. These were diluted stepwise by the buffer solution to 0.01 mg/mL.

2.2. Apparatus

2.2.1. Microchip with UV detection

On-chip experiments were performed on the commercial Shimadzu MCE-2010 (Kyoto, Japan). The D_2-lamp based instrument possesses a diode array detector located along the separation channel. All microchips for UV detection were purchased from Shimadzu. The 35 mm × 12.5 mm × 2 mm quartz chip has a simple cross injector design. The separation channel is 25 mm long, 20 μm deep and 50 μm wide. Access holes are at the end of each channel. These reservoirs are labelled sample inlet (#1), sample waste (#2), buffer inlet (#3) and buffer waste (#4). All channels are uncoated.

2.2.2. Microchip with amperometric detection

The glass microchip for amperometric detection was purchased from Alberta Microelectronic Corp. (AMC, model MC-BF4-TT100; Edmonton, Canada). The effective length of the separation channel is 8 cm with a semicircular cross-section of 50 μm width and 20 μm depth. The joint has the shape of a double T. The modified chip was mounted on an inverse microscope from Leica (model DM IL) equipped with a XYZ micro-manipulator as a working electrode holder. A laboratory-built amperometric detector was connected to the working electrode. Everything was placed inside a faraday cage, except the high voltage power supplies. A Teflon-coated gold wire (bare wire diameter: 75 μm, Teflon coating: 18 μm; available from Advent Research Materials Ltd. England), attached to a holder for the XYZ micro manipulator, is used as working electrode.

2.2.3. Capillary with UV detection

For the measurements on capillary, a Crystal ATI Unicam Model 310 from PrinCE Technology (Emmen, Netherlands) was used as the CE system. The capillary tubing from BGB Analytik AG (Adliswil, Switzerland) was of uncoated fused silica with an internal diameter of 75 μm, a length from inlet to detector of 65 cm and a length from detector to outlet of 14 cm. The UV detector was a Spectra 100 from thermo separation products (Egelsbach, Germany). Data were collected

and analyzed with a μDAQ AD-modifier from Eagle Technology (Cape Town, South Africa). The conditions used in CE were the following: voltage, +25 kV.

2.3. Methods

All measurements were detected with UV light and electrochemically and were reproduced at least two times. The whole procedures were performed at room temperature (25 °C). Calculations and nonlinear fittings were carried out with a computer program called Origin 6.0 from Microcal Software Inc. (Northampton, USA).

2.3.1. Microchip with UV detection

Flushing the channels was done manually by means of a 2.5 mL syringe with special flushing tip (Shimadzu). Before and after every use, the chip was washed with 0.1 M NaOH at least for 30 s and rinsed afterwards with double deionised water. The channels were dried using the vacuum from a water-pump. Before measuring, the channels were flushed with buffer solution. The four access holes were cleaned with the vacuum (without emptying the channels). Finally, the reservoirs #2–#4 were filled with buffer and #1 with sample solution. This was also done manually using syringes. After setting up the chip into the instrument, the chip was washed again with high voltage. For 60 s, voltages of 0.80 kV, 0.80 kV, 1.50 kV and 0.00 kV, respectively, were applied. Then the measurements were made as pinched injections. This was achieved by a two-step high-voltage program. In a first step (injection mode), a sample plug was performed into the intersection. The applied voltages at the four reservoirs were 0.60 kV, 0.00 kV, 0.32 kV and 0.39 kV. After 10 s, the voltage was switched for a previously chosen length of time (e.g. 15 s) to the separation mode (second step) with 0.80 kV, 0.80 kV, 1.00 kV and 0.00 kV, respectively.

On microchip the detection method was with UV light: at a wavelength of 210 nm. The detection took place over the whole separation channel.

2.3.2. Microchip with amperometric detection

For preconditioning of separation channel a 1 M NaOH-solution (1 min, daily) and the appropriate running buffer (3 min, before every run) was used for flushing the channel. Fresh sample and buffer solutions were prepared daily.

Injection- and separation parameters were controlled and data acquisition was performed with a home-written program in "LabVIEW", Version 6.0.2, a graphical programming language and a controller card (Data Acquisition Devices, PCI-MIO-16XE-50) with a built-in analogue-to-digital converter both from National Instruments (Austin, USA). Two high voltage power supplies model CZE1000R (Spellman HV Electronics Corp., Europe, Haarlem, The Netherlands) were used for switching of the voltages for injection and separation.

Amperometric measurements were performed with a home built detector on the end of the separation channel [35]. The applied potential could be controlled via a voltage divider connected to the non inverting input of an amplifier having the working electrode in its inverting branch. Measuring of the redox current of the working electrode was performed with a precision instrumentation at a 1 MΩ resistor. After passing an amplification stage, the signal was fed to the input of the A/D converter.

2.3.3. Capillary with UV detection

Conditioning was done daily before each use of the capillary: 1 min, 0.1 M NaOH, 300 mbar and 3 min, water, 250 mbar. Before each change of buffer, the capillary was washed: 3 min, water, 250 mbar. The loading method consisted of three programs: Washing for 3 min, 250 mbar with buffer, injection for 0.1 min, 30 mbar with the sample and separation for 20 min, 25 kV with buffer. Preparing buffer and sample vide supra, additionally, these samples had to be diluted by the factor 10.

The detection method on the capillary was with UV light: at a wavelength of 210 nm. In comparison to microchip (MC), it was a normal on-capillary method with detection on the cathode-site.

3. Theory

To achieve qualitatively meaningful results, the following preliminary conditions of ACE must be complied with:

- no interactions between the capillary surface and the molecules under observation;
- low conductivity of the molecules (buffer as well as analyte);
- the background buffer has to be UV-permeable and electrochemically inactive;
- there has to be a difference between the ionic mobility of the ligand and the substrate;
- either the ligand or substrate has to be detectable (by UV or amperometry);
- at least one binding partner has to be charged.

CDs are chiral cyclic oligosaccharides with a shape similar to a truncated cone and containing a hydrophobic cavity. The interactions between the model-drug and CD-molecules are caused by weak physicochemical forces such as hydrophobic (unspecific), electrostatic effects (specifically dipole–dipole or dipole–induced dipole) and steric effects; for the formation of inclusion complexes hydrophobic stereospecific interactions predominate in the cavity of CD-molecule. Using CDs with ionic substituents on the rim, hydrogen bonds and electrostatic interactions are responsible for improving the strength of binding affinity to opposite charged model-drugs.

An indirect indication for the presence of interactions between complexing agent and interacting substance is given by molecular and dynamic parameters of the CD-molecules and the model-substance (ionic mobility, diffusion coefficient,

hydrodynamic radius, apparent molecular mass), which are altered by the aggregation of substances in a characteristic manner.

A more precise characterisation is possible by examining the alteration of the electrophoretic properties of the solubilized drugs than by studying the complex itself or the complete system. With ACE the complexation behaviour of substances can be studied in a straightforward way simply by observing the effect of the composition and concentration of the complexing agent on the ionic mobility. For the calculation of the binding constants according to Eq. (1), [33] the measured ionic mobility of the drug (μ), the mobility of the drug in absence of CD-molecules (μ_S, constant) and the concentration of the free CD-molecules [L] have to be known. In the case of ACE, the free concentration of the ligand is equal to the total concentration of CD because the sample stays a small zone. By non-linear curve fitting assuming a 1:1 interaction, the binding constant (K_B) and the mobility of the drug–CD-complex μ_{SL} (both are parameters) to the experimentally obtained values (measured ionic mobility of drug on the *y*-axis to concentration gradient [L] on the *x*-axis) can be calculated.

$$\frac{\mu - \mu_S}{\mu_{SL} - \mu_S} = \frac{K_B[L]}{1 + K_B[L]} \quad (1)$$

The parameter $\mu = f([L])$ stands for the real electrophoretic mobility of the ions with a contribution from the electroosmotic flow ($\mu = \mu_{measured} - \mu_{EOF}$). The method to calculate μ from the electropherograms depends on the detection method. With the end-channel and on capillary detection methods, the *x*-axis represents the passing time at a certain separation length. With UV detection over the whole channel on MC, the length of the separation channel forms the abscissa at a certain time (see Fig. 1).

$$\mu = \frac{l_{eff} l_{tot}}{U_{Sep}} \left(\frac{1}{t_{Sample}} - \frac{1}{t_{EOF}} \right) \quad (2)$$

$$\mu = \frac{s_{Sample} - s_{EOF}}{E_{Sep} t_{Sep}} \quad (3)$$

In Eq. (2) which is used for on-capillary and end-channel detections, l_{tot} (cm) is the total length of the capillary, l_{eff} is the length from inlet end of the capillary to the detector; t_{Sample} and t_{EOF} are the measured migration times of the sample and the DMSO peak. In Eq. (3) which is especially designed for detection over the whole separation channel, E_{Sep} is the electric field (V/cm), t_{Sep} is the time of the separation and s_{Sample} and s_{EOF} (cm) are the measured migration lengths of the analyte. The shift of μ results from increasing concentrations of the ligand (L) and therefore the position of equilibrium. sCD was added to the background buffer in concentrations from 0 to 3.96 mM.

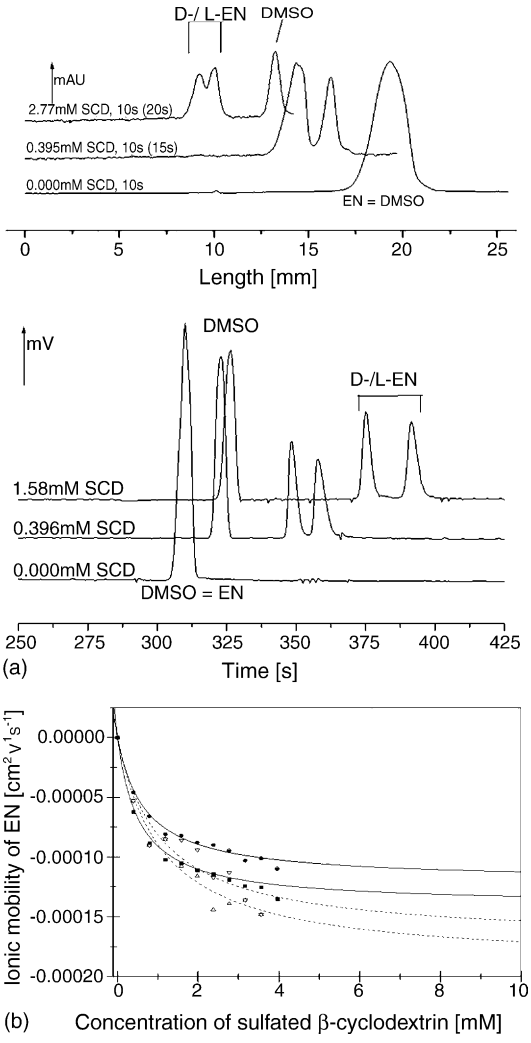

Fig. 1. (a) Electropherograms measured on microchip and capillary. Buffer: 10 mM TRIS at 210 nm containing sulfated cyclodextrin (sCD); internal standard: DMSO; up microchip: 2 mM epinephrine (EN) at pH 7.37. The separation times differed from 10 s up to 20 s (converted to 10 s) at 280 V/cm using an uncoated chip 50 μm × 20 μm i.d. Down capillary: 0.2 mM EN at pH 7.33. Injection time in each run was 6 s at 25 kV using a 65 cm (79 cm in total) 75 μm i.d. open, uncoated quartz capillary. (b) Affinity capillary electrophoresis: comparison between capillary and microchip. The electropherograms of (a) converted into affinity curves with increasing sCD concentrations vs. ionic mobility of EN. The thick lines with the filled symbols is the result of the capillary, the thin lines with the hollow symbols the result of MC.

4. Results and discussion

The strategy for the present work was to verify that MC-ACE would be a useful tool and even advantageous over other techniques. We approached our studies from different angles.

4.1. Comparison between capillary and chip

One electrophoretic study of complex formation between neurotransmitters and CDs on capillary has been reported at a pH of 3.2 [36]. Our work focussed on the idea that ACE can

be transferred from capillary to microchip. We directly compared ACE using uncoated fused-silica capillaries with MC-ACE using quartz microchips, where the separation channel is on average between a 10th and a 20th shorter. Fig. 1a represents a comparison between electropherograms measured with capillary and MC. It shows electropherograms with different concentrations of sCD in the background buffer measured on chip (top) and capillary (bottom). Both diagrams illustrate a shift in migration time of solute depending on the concentration of sCD. Without sCD in the running buffer, at pH of 7 EN migrates with the EOF. With increasing concentrations of the anionic sCD, the EN peak is getting slower. Furthermore, the EOF is decelerated due to the increasing ionic strength. Towards a complete binding, EN migrates with a mobility near of the sCD molecules. Since the enantiomers interact with different intensity, a chiral separation can be observed at a concentration of 0.395 mM sCD on capillary. In consequence of the short migration length (MC/CE: 1/26) at 1.58 mM sCD (not shown) the D- and L-form can be distinguished on chip, however EN could not be separated completely. With further increasing of CD concentration the UV-signal decreased significantly so that an optimum for a baseline separation is not measurable. The experimental data consider [EN] and [EN^+]. In the mathematical derivation of Eq. (1) both are truncated. The calculated K_B-value contains the fraction of [EN] and [EN^+] regarding their interactions.

Due to the noticeable difference in length of the separation ways (65 cm versus 2.5 cm) the migration times on the chip are substantially shorter. The separation time on the chip has to be fixed at a period defined beforehand (e.g. 10 s). The electrophoretical migration can be compared on capillary and MC (accessible by means of the formula $v = s/t$); it is identical for the same sample on both the capillary and chip. The differences between MC and capillary are obvious: the fast separation time and the use of less buffer (about 0.1–0.5 mL for the series of measurements) obtained with the MC competes with the narrow peaks and the more precise separation obtained using capillary. The large peak width on the chip results from both the higher injection volume relative to the channel length and the principle to record the electropherogram (see Section 4.2).

In Fig. 1b, the ionic mobility is set in relation to different concentrations of sCD in the CE-buffer. With both methods (MC and capillary), the ionic mobility behaves similarly with increasing concentrations of sCD in the background electrolyte.

By shifting the complex-formation equilibrium to the side of the complex ($S + L \rightleftharpoons SL$), EN migrates towards longer detection times because sCD is anionic in the pH range used. In addition to the higher scattering of μ-values measured on chip, the resulting functions are analogical and therefore provide similar K_B-values; these are summarized in Table 1. For calculating K_B of the functions, μ_S was applied as a constant value (see Section 4.3).

In a next step, different separation conditions and detection methods were investigated to validate MC-ACE. A sensitive

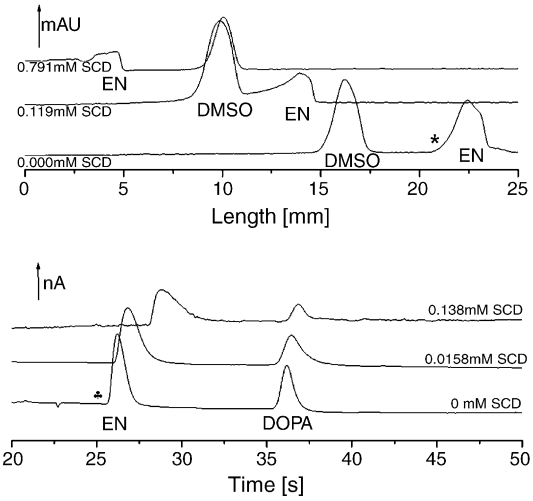

Fig. 2. Electropherograms detected amperometric and with UV. Up amperometric: 0.5 mM EN with DOPA as internal standard at pH 6.00. Buffer was 10 mM MES containing sCD. Injection time was 3 s at 1 kV; separation voltage was 3 kV using an uncoated glass chip 50 μm i.d. and a Au-electrode. Down UV: 2 mM EN with DMSO as internal standard at pH 6.10 at 210 nm. Buffer was 10 mM TRIS containing sCD. The separation time in each run was 10 s at 280 V/cm using an uncoated chip 50 μm × 20 μm i.d.

method of differentiation is required for the application of ACE on the very short distance over which separation occurs.

4.2. Detection methods and EOF-markers

Migration times on MC are typical within a range of 10–20 s (at a 2.5 cm separation way) and 20–40 s (at 8 cm); this requires a particularly high resolution of the detectors and low injection volumes. Especially with MC-UV, the short separation way and the detection limit of UV make it difficult to guarantee these conditions; this leads to wide peaks in relation to the distance over which separation occurs. In order to implement ACE studies on MC, internal standards are necessary. The best method is to use a neutral molecule (migration with the EOF) to get internal standards and EOF-markers. It is important that the molecule does neither interact with CDs nor with the surface of the channel. With an internal standard, the calculated μ will be in relation to the EOF. The UV-active neutral molecule DMSO was chosen as an internal standard for our MC-UV measurements. As DMSO is not electrochemically active, DOPA was used as a reference material. A comparison between UV- and amperometric detection is illustrated in Fig. 2. In both cases – without sCD in the background buffer – EN migrates faster than the EOF due to the positively charged amine group. With increasing of the CD concentration a shift of EN is observed whereas peak broadening is influenced. Above a concentration of about 0.2 mM sCD (not shown) EN migrates as a result of advanced complexation as an anion. The amperometric measurements are more precise with narrower peaks. As above note, the reason for this observation can be explained with the unfavourable ratio of length of injection plug and the migration length

Table 1
Overview of different equilibria at various conditions

Comparison of	Sample	Ligand	Binding constant, K_B (L mol^{-1})	Mobility of complex, μ_{SL} (cm^2 V^{-1} s^{-1})	Detection	Reference	pH	Method	R^2 (%)
Chip vs. capillary	EN	sCD	891[a]	−0.00019	UV	DMSO	7	Chip	96.991
			919	−0.00017					91.614
	EN	sCD	1870[a]	−0.00015	UV	DMSO	7	Capillary	98.675
			1460	−0.00012					98.939
	NEN	sCD	1030	−0.00019	UV	DMSO	7	Chip	95.258
	NEN	sCD	1560	−0.00018	UV	DMSO	7	Capillary	97.053
	MN	sCD	2250[a]	−0.00022	UV	DMSO	7	Chip	99.845
			2560	−0.00018					99.752
	MN	sCD	4100[a]	−0.00020	UV	DMSO	7	Capillary	99.97
			3690	−0.00018					99.974
	EN	sCD	4200[a]	−0.00054	UV	DMSO	6	Chip	93.121
			3880	−0.00047					95.239
	EN	sCD	5420[a]	−0.00060	UV	DMSO	6	Capillary	99.787
			3830	−0.00062					99.802
Detections	EN	sCD	891[a]	−0.00019	UV	DMSO	7	Chip	96.991
			919	−0.00017					91.614
	EN	sCD	671[a]	−0.00027	Amp.	DMSO	7	Chip	99.366
			823	−0.00025					98.741
pH-values	EN	sCD	4200[a]	−0.00054	UV	DMSO	6	Chip	93.121
			3880	−0.00047					95.239
	EN	sCD	891[a]	−0.00019	UV	DMSO	7	Chip	96.991
			919	−0.00017					91.614
	EN	sCD	1870[a]	−0.00056	UV	DMSO	8	Chip	90.694
			1830	−0.00052					91.836
	EN	sCD	2710[a]	−0.00031	UV	DMSO	9	Chip	93.448
			8360	−0.00018					96.117
Neurotransmitter	EN	sCD	891[a]	−0.00019	UV	DMSO	7	Chip	96.991
			919	−0.00017					91.614
	NEN	sCD	1030	−0.00019	UV	DMSO	7	Chip	95.258
	MN	sCD	2250[a]	−0.00022	UV	DMSO	7	Chip	99.752
			2560	−0.00018					99.845

In case of chiral separation there are two results, the first belongs to the analyte with lower interactions to sCD.
[a] Chiral separation of the molecule.

(MC-UV: 50 μm (simple cross)/2.5 cm, MC-Amp: 100 μm (twin tee)/8 cm). Another fact leads to the apparent lower separation efficiency at MC-UV. With recording the detection signal depending on the migration way the peak width results from the sample plug (50 μm) and dispersion of the zone (e.g. in Fig. 2 above, *, the peak width is about 2.5 mm). The concentration profile along the separation channel is a snap-shot at a defined time. With recording the detection signal at one point on the channel in dependence on the time the peak width is given by the width of the sample zone including dispersion and the velocity with that the analyte migrates. In the example given above the following time-interval (peak width (t) on the base) for detection can be calculated: 2.5 mm peak width, velocity of EN: 2.98 mm/s would lead to a time-interval of 0.83 s. This result is clearly narrower than measured in the amperometric experiment (2.4 s, see in Fig. 2 below, ♣) and demonstrates the more unfavourable format of record of MC-UV. The difference in the number of theoretical plates (N_{Amp} = 4110 versus N_{MC} = 151), the standard deviation and the relative standard deviation of migration times ($\sigma_{MC\text{-}Amp}$ = 0.0516 s and R.S.D.$_{MC\text{-}Amp}$ = 0.0949%, $\sigma_{MC\text{-}UV}$ = 0.344 mm and R.S.D.$_{MC\text{-}UV}$ = 8.11%, both calculations were made with the electropherograms of Fig. 2) emphasise this. At the pH-values in the study, DOPA is deprotonated and does not interact with the CDs. In one separate series on MC, the metabolite metanephrine (MN) was measured with DOPA and DMSO. The peak of DOPA stayed constantly at the mobility of 1.95×10^{-4} cm^2 V^{-1} s^{-1} compared with DMSO and was independent of the increasing sCD concentration. As a consequence DOPA can be used as constant bench mark in ACE with CDs. After transferring the results from DOPA standard to DMSO standard, the amperometric approach could be compared with MC-UV measurements. At higher concentrations of sCD (>0.158 mM), it was impossible to measure electrochemically. The background noise increased in both cases, which influenced the peak form and the sensitivity of the measurement.

4.3. pH-values

As we had assumed, the interactions between S and L depend on the pH-value. The part of electrostatic interactions can be estimated by studies outside the physiological pH-range, as ionic interactions are taking place besides the

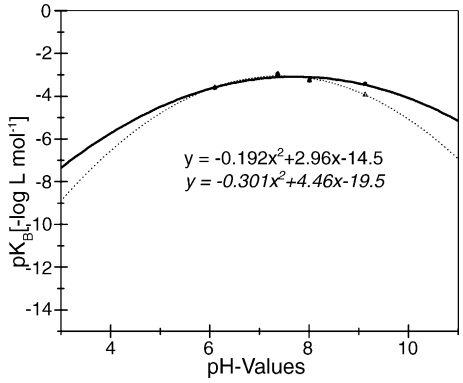

Fig. 3. The dependence of pK_B on different pH-values. Epinephrine at pH 6.10, 7.37, 8.01 and 9.13 the same procedure vide supra. The fat line with the filled symbols is L-epinephrine; the thin line with the hollow symbols represent D-epinephrine.

hydrophobic force. The charge on the drug substance as well as the charge of the CD molecules has an effect on the interactions and thereby on the binding constant. In the case of EN, it was possible to observe how the pH-value (measured in the range between 6 and 9) influences K_B (see Fig. 3). The calculated binding constants describe the sum of all equilibrium constants and result from the dissociation constant of both the EN and sCD molecule as well as from the binding constant of single complexes. A mathematical equation that includes six equilibria (two for the protonated and deprotonated form) would be too complicate for the description of the pH dependence, so that a simple quadratic equation was used to explain the tendencies in the used pH range. In strong acidic and basic buffers (outside the measured range), the pK_BS would diminish tremendously. At pH 7 the pK_B is at a maximum. This means, at the same pH-value as in vivo (pH 7.3–7.4), EN has the lowest affinity to sCD.

With an increase of K_B, the chiral separation of the enantiomers is more obvious. Higher electrostatic interactions between ligand and substrate are responsible for the better separation of the D- and L-forms. At low pH-values, EN is getting protonated and sCD partially deprotonated; at basic pH-values, EN is almost completely presented as the neutral species and sCD as anionic species molecules. Additionally the starting point (μ_S) and μ_L are influenced by them, the migration of the complex formed (μ_{SL}). MC and capillary were compared with results in a similar range.

4.4. Neurotransmitters

The two natural human sympathomimetics norepinephrine (NEN) and epinephrine (EN) and one of their metabolites (metanephrine, MN) were chosen as drug models. Due to their similar structures, the migration times are nearly identical at pH 7. However, after calculating their K_BS, they were easily distinguishable because of their different interactions with sCD.

MN has the largest affinity to sCD followed by NEN and EN. These measurements were repeated on a capillary (for results see Table 1). The difference between the three neurotransmitters is reasonable in terms of the structures of the molecules. MN has the highest K_B (2250, 2560 L mol^{-1}) with a methoxy group on the benzene ring. The increase of lipophilicity on one end of the molecule leads to a higher interaction with the hydrophobic cavity of sCD. The methyl group on the hydrophilic end does not seem to have such a effect. NEN (without methoxy and methyl groups) is sterically more compatible with the hydrophilic sulfated part of sCD than EN, but the two phenol groups decrease the K_B-value (1030 L mol^{-1}) in comparison to MN. EN has the lowest binding constants (891, 919 L mol^{-1}).

The optimal concentration of CDs in chiral separations depends on both the aggregation properties (selectivity) and the stability of the host-guest complex (sensitivity) formed. Theoretical aspects describing the dependence of resolution on chiral selector concentrations are clearly shown by Wren and Rowe [37]. Chiral separations are mainly carried out at acidic pH, avoiding the detrimental effect of the electroosmotic flow on the resolution of cationic analytes and taking into account that most drug compounds contain an amine functionality.

Fig. 4 illustrates the shift of μ_{MN}, μ_{MEN} and μ_{EN} in dependence on sCD. Additionally, the fitted curves calculated with the Eq. (1) are graphed. The starting point without sCD corresponds to the mobility of the free solved sample μ_S. At this pH-value, the mobility of MN starts in the positive range (Fig. 4), whereas the mobilities of NEN and EN start at zero. Both the mobility of the complex μ_{SL} and K_B were calculated with nonlinear fitting using these two variables. The enantiomers of MN and EN could be separated; D- and L-forms have different affinities to sCD. In theory the μ_{SL}s of the enantiomers are identical. However, since this value

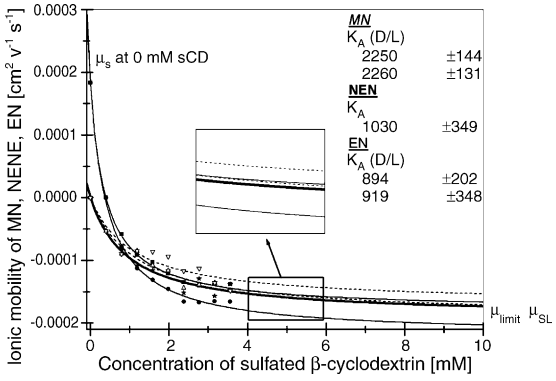

Fig. 4. Affinity capillary electrophoresis of metanephrine (MN), norepinephrine (NEN) and epinephrine (EN): ligand concentration versus ionic mobility of the sample. Buffer: 10 mM TRIS (pH 7.37) at 210 nm containing sCD; internal standard: DMSO; chip: 50 μm × 20 μm i.d., uncoated; voltage: 289 V/cm. Dashed lines with hollow symbols: 2 mM MN. The separation time was 10 s up to 15 s. Thick line with star symbol: 2 mM NEN. The separation time was 10 s. Normal lines with filled symbols: 2 mM EN. The separation time was 10 s up to 20 s.

was set variable in the calculation, the D- and L-form of the complex show slightly different values (see Table 1), this is independent of the K_B-value that mirrors the real facts. In comparison to other calculations of K_B, the mobility of the ligand μ_L can be unknown, but it has to be more negative than μ_{SL}.

4.5. Overview

The efficiency of MC-ACE was tested using different approaches to confirm that miniaturization is a viable alternative to ACE on a capillary. An overview of the results is presented in Table 1. For the resulting chirality, there are two data lines for each sample, only NEN did not separate into its two enantiomers. In each row the measured sample, the ligand in the buffer, the detection method, the reference peak, the pH-value, the binding constant and the mobility of the complex are mentioned. To verify the accuracy of the mathematically fitted data (K_B and μ_{SL}), the coefficient of determination (R^2) in percent is given in the last column. The data achieved from the amperometric measurements are transposed from DOPA to DMSO to make them comparable to UV-detection.

In principle, any ionic or neutral complexing agent as well as model-drugs could be used instead of the neurotransmitters and the sCD.

5. Conclusion

ACE has proved to be an adequate method for studying molecular interactions. We can forsee that there could be important applications in pharmaceutical research and medical diagnostics. The high separation efficiency of CZE in general makes it possible to transfer affinity studies from capillary to miniaturized systems. By choosing a suitable detector, selective measurements are possible whereas impurities in the sample produce no falsification of the results. The limiting factor caused by the small sample volumes is the sensitivity obtained using optical detection techniques. Below 1 mM, the detection limit is reached. On the other hand, traditional binding assays like enzyme linked immuno sorbent assay (ELISA) are tedious, and significant amounts of expensive reagents are consumed.

The introduction of the μTAS concept provides new opportunities for studying binding interactions [21]. MC-ACE is advantageous as a low-cost, rapid and simple screening method, which provides quantitative results on various interactions with minimum reagent consumption and short analysis time (8–60 s). A disadvantage is that MC-ACE provides less precise data compared to capillary. Even this problem, though, can be minimized with an internal standard. The new method described here is a useful tool to obtain an estimation of the strength of interactions between the ligand and its substrate. For the future, more applications of ACE-based chips for high-throughput screening and combinatorial chemistry are to be expected.

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