Applications of Capillary Electrophoresis with Contactless Conductivity Detection

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auf Antrag von

Prof. Dr. Peter C. Hauser

und

Prof. Dr. Milena Koudelka

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Prof. Dr. Hans-Peter Hauri

Dekan

Der Wille öffnet die Tören zum Erfolg. (Stossen wir die Tören auf! Machen wir uns an die Arbeit.).

------Louis Pasteur

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To my daughter

Zi-Yuan Lu

Summary

This thesis focuses on the application of capillary electrophoresis with capacitively coupled contactless conductivity detection (CE-C^4D) in the enantiomeric separation of non-UV active pharmaceutical chemicals and fast detection of non-UV active species in clinical samples.

First CE-C^4D was successfully applied to the direct enantiomeric separation of five basic drugs and nine amino acids. Derivatization of the compounds or the addition of a visualization agent as for indirect optical detection schemes was not needed. Non-charged chiral selectors were employed, hydroxypropylated cyclodextrin (CD) for the more lipophilic basic drugs and 18-crown-6-tetracarboxylic acid ($18C6H_4$) for the amino acids. Concurrent chiral separation of UV and non-UV active amino acids has been realized.

Secondly, CE-C^4D was used for the determination of different organic amines. Direct enantiomeric separation of 1-phenylethylamine and 1-cyclohexylethylamine was obtained with unprecedented high resolution by using a combination of dimethyl-beta-cyclodextrin and the chiral crown ether $18C6H_4$ as selectors. Then the enantiomeric separation of various amines was investigated, the use of contactless conductivity detection for the determination of different species in capillary electrophoresis was successfully demonstrated. The species tested included short chained aliphatic primary, secondary and tertiary amines, branched alipahtic amines, diamines, hydroxyl-substituted amines as well as species incorporating aromatic and non-aromatic cyclic moieties. The determination of the enantiomers of these amines which otherwise can only be achieved with difficulty was possible by using a combination of dimethyl-beta-CD and the chiral crown ether (+)-(18-crown-6)-2, 3, 11, 12-tetracarboxylic acid ($18C_6H_4$) as selectors.

Thirdly the CE-C^4D has also successfully been explored in the separation of diastereomers and enantiomers of various dipeptides, tripeptides and tetrapeptides composed of non-UV active amino acids. These peptides are essential compounds in biological systems as well as important chiral building blocks in pharmaceutical drugs.

At last, the determination of gamma-hydroxybutyrate (GHB) with CE-C^4D, which is a recently introduced party drug and difficult to be analyzed by on-site screening, was developed. The procedure can be used for the detection of GHB in urine and serum samples.

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1. Introduction

1.1 Capillary Electrophoresis and Capacitively Coupled Contactless Conductivity Detection (CE-C^4D)

1.1.1 Introduction of Capillary Electrophoresis

1.1.1.1 History

Electrophoresis is defined as the migration of charged species under the influence of an externally applied electric field. Differences in mobility of the analytes are due to their average charge, size, shape, and on the properties of the electrolyte solution used. Reuss was the first person to carry out separations based on this principle in 1809. During the study of the migration of colloidal clay-particles, he investigated that the liquid adjacent to the negatively charged surface of the wall migrated towards the negative electrode under influence of an externally applied electric field[1]. The theoretical aspects of this electrokinetic phenomenon that Reuss called electro-osmosis were proposed by Kohlrausch in 1897[2]. In their effort to separate diphtheria toxin, globulin, and toxin/antitoxin solutions several researchers carried out electrophoretic separations in so called 'U'-shaped tubes in the late 1800's and early 1900's.

Tiselius greatly contributed to the advancement of the analytical aspects of electrophoresis[3, 4]. During his study on the development of free moving boundary electrophoresis, he successfully separated complex protein mixtures based on differences in electrophoretic mobilities. For this reason, Tiselius was awarded the Nobel prize for chemistry in 1948. At that time, the poor resolution observed due to peak broadening caused by Joule heating and in a minor way caused by molecular diffusion was a big challenge concerning electrophoresis. Starch gel, paper, agarose, cellulose acetate or

polyacrylamide gel were used as stabilizers to prevent this peak broadening[5]. Although the above-mentioned stabilizers minimized the problem of convection, they led to other peak broadening phenomena, like eddy diffusion and undesired interactions between analytes and stabilizer. Later Tiselius and Hjertén developed polyacrylamide gel electrophoresis in 3-mm i.d. rotating capillaries and applied this technique in the separation of ribosomes and viruses[6-8]. Hjertén summarized the technique in 1967, in which stabilization was achieved by continuous rotation of the tube about its longitudinal axis and the peak broadening could be avoided[9].

1.1.1.2 Development

On the basis of Giddings' deduction that a very high efficiency must be possible when longitudinal diffusion is the only cause of peak broadening[10], Everaerts *et al.* in 1970 using 200 μm i.d. Teflon® tubes[11] and Virtanen in 1974 using thin glass tubes[12] did the experiments and confirmed this point. They found the stabilizing "wall effect" by using the thin tubes as a separation column in which the small diameter of the column counteracts the convective flow, leading to an increase in efficiency. The increase in efficiency is proportional to the decrease in diameter of the tube due to the increasing surface-to-volume ratio. In 1981 Jorgenson and Lukacs developed an electrophoresis system using open glass capillaries of 75 μm i.d. with an on-column fluorescence detector[13-15]. They applied the system to the separation of the fluorescent dansylated amino acids and the fluorescamine derivatized amino acids in human urine within 25 minutes, which provided the predicted efficiencies (>400000 plates) and illustrated the high effeciency of electrophoresis in open tubes of small diameter.

The possible advantages of performing zone electrophoresis in open tubes of small diameter were summarized:

1. Efficient heat transfer within the electrophoresis medium is achieved, leading to minimal temperature gradients.
2. Disadvantageous effects of remaining temperature gradients are minimized by solute diffusion back and forth across the tube diameter.
3. The medium is stabilized against convective flow by the wall effect.

After these initial experiments and after the introduction of the first commercially available instruments in 1988, the potential of capillary electrophoresis as a high performance separation technique in analytical (bio)technology, bioanalysis and pharmaceutical analysis was soon acknowledged in some early papers[16-22]. It proved to be a powerful and useful method of analysis, especially when only small amounts of sample are available.

1.1.2 Principle of Capillary Electrophoresis

Modern capillary electrophoresis consists of a high-voltage power supply, two buffer reservoirs, a capillary and a detector (see Figure 1). This basic set-up can be elaborated upon with enhanced features such as autosamplers, multiple injection devices, sample/capillary temperature control, programmable power supply, multiple detectors, fraction collection and computer interfacing.

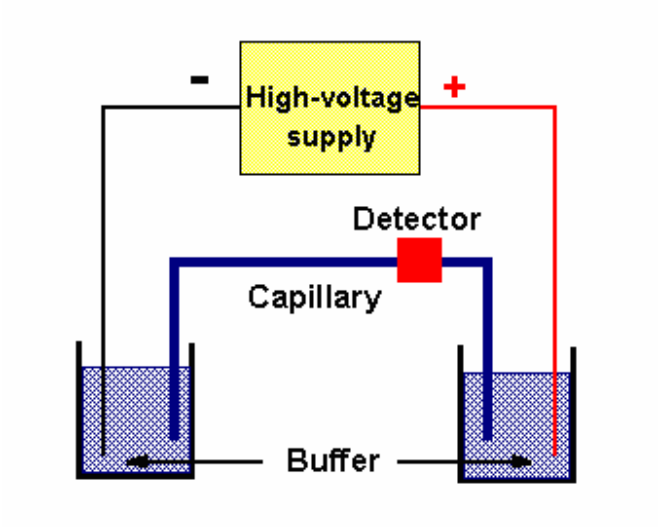


Figure 1. Structure of capillary electrophoresis

1.1.2.1 Electrophoretic Mobility

The transport of a charged particle in an electric field is very similar to that found for the sedimentation of a particle in a centrifugal field. A molecule with charge q in an electric field E (V cm^{-1}) experiences an electronic force F_E:

$$F_E = qE \qquad (1)$$

Just as in sedimentation, the molecule quickly reaches a velocity, v, and receives a frictional force F_F given by:

$$F_F = -6\pi\eta rv \qquad (2)$$

At a steady state during electrophoresis, the two forces are equal but opposite, thus

$$qE = 6\pi\eta rv \qquad (3)$$

The electrophoretic mobility of the molecule, μ, is the velocity per unit field.

$$\mu = v/E \qquad (cm^2/V\text{-}sec) \qquad (4)$$

Combination of equations (3) and (4) yields

$$\mu = q / (6\pi\eta r) \qquad (5)$$

From equation (5), it can be seen that species with small size and higher charge numbers will have high mobilities, while species with large size and lower charge numbers will have low mobilities. Except the above factors, mobility is affected by temperature and electroosmotic flow.

1.1.2.2 Electro-Osmotic Flow (EOF)

When a current is applied to the capillaries there is a bulk flow of movement through the system. This is known as electroosmotic flow (**EOF**) and is a result of the surface charge on the capillary wall (Figure 2).

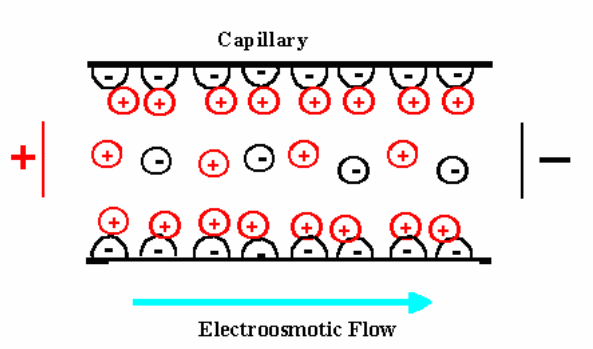


Figure 2. Electroosmotic flow

When an aqueous buffer is placed inside a fused silica capillary, its inner surface acquires an excess of negative charges. This is due to the ionisation of the silanol groups

(SiOH) that can exist in anionic form (SiO^-) and or the absorption of ions from the buffer onto the capillary. EOF becomes significant above pH 4. The negatively charged silanoate groups attract counter ions from the buffer which form an inner layer of tightly held cations, also termed the fixed layer, at the capillary wall. However these cations are not of sufficient density to neutralise all negative charges, so a second outer layer of cations forms which makes up the diffuse layer. The fixed and diffuse layers make up the diffuse double layer of cations. When an electric field is applied, the outer layer of cations is pulled toward the negatively charged cathode. Since these cations are solvated, they drag the bulk buffer solution with them, thus causing electroosmotic flow. EOF drags all analytes irrespective of charge in one direction. Consequently cations and anions may be simultaneously analysed in one run, which otherwise is not possible in the absence of EOF. EOF makes it possible to analysis analytes with different charge mass ratios within reasonable analysis times.

Compared to pumped or laminar flow in HPLC, electroosmotic flow in CE has a flat profile. The advantage of the flat flow profile is that all solutes experience the same velocity component caused by electroosmotic flow regardless of their cross-sectional position in the capillary, and they elute as narrow bands giving narrow peaks of high efficiency.

1.1.3 Modes of Capillary Electrophoresis

The origins of the different modes of separation may be attributed to the fact that capillary electrophoresis has developed from a combination of many electrophoresis and chromatographic techniques. In general terms, it can be considered as the electrophoretic

separation of a number of substances inside of a narrow tube. The distinct capillary electroseparation methods include:

Capillary Zone Electrophoresis (CZE)

Capillary Isotachophoresis (CITP)

Capillary Isoelectric Focusing (CIEF)

Capillary Gel Electrophoresis (CGE)

Capillary zone electrophoresis (**CZE**) is the most commonly used technique in CE. Many compounds can be separated rapidly and easily. The separation in CZE is based on the differences in the electrophoretic mobilities resulting in different velocities of migration of ionic species in the electrophoretic buffer contained in the capillary. Both anions and cations can be separated in the same run. Cations are attracted towards the cathode and their speed is augmented by the electroosmotic flow. Anions, although electrophoretically attracted towards the anode, are swept towards the cathode with the bulk flow of the electrophoretic medium.

Capillary Isotachophoresis **(CITP)** is performed in a discontinuous buffer system. Sample components condense between leading and terminating constituents, producing a steady-state migrating configuration composed of conservative sample zones. The isotachopherogram obtained contains a series of steps, with each step representing an analyte zone. The quantitation in CITP is mainly based on the measured zone length which is proportional to the amount of analyte present.

Capillary Isoelectric Focusing (CIEF) is based on the isoelectric points (pH values) of the substances to be separated. The most common type of sample that utilizes this analytical method is proteins. Under the influence of an applied electric field,

charged proteins migrate through the medium with pH gradient until they reside in a region of the pH where they become electrically neutral and therefore stop migrating. Consequently, zones are focused until a steady state condition is reached. After focusing, the zones can be migrated (mobilized) from the capillary by a pressurized flow. Sharp peaks are obtained with good resolution, and a large peak capacity is observed mainly because the whole tube is simultaneously used for focusing.

Capillary gel electrophoresis (CGE) is based on differences in solute size as analytes migrate through the pores of the gel-filled column. Gels are potentially useful for electrophoretic separations mainly because they permit separation based on 'molecular sieving'. They serve as anti-convective media, minimize solute diffusion, which contributes to zone broadening, prevent solute adsorption to the capillary walls and they help to eliminate electroosmosis.

1. 1.4 Detection in Capillary Electrophoresis

Most detectors in capillary elctrophoresis have been adapted from HPLC. Good detector should supply a stable baseline and be responsive to all type of compounds, rugged and not too expensive. Also they must be versatile, provide high sensitivity and low noise level. They may be situated on-column, end-column or post-column. Two types of detectors have been developed for capillary electrophoresis: optical detection and eletrochemical detection.

1.1.4.1 Optical Detection Techniques

Optical detection was successfully implemented in commercial state-of-the-art CE

instruments, which include UV absorbance and fluorescence detectors. On-column detection is often used, since the light source can be directly focused on to the capillary whilst the electronic transduction of the signal remains galvanically separated from the DC influence of the high-voltage.

1.1.4.1.1 UV/Vis

Due to its relatively universal nature; any molecules possessing a chromophore can be detected by UV/Vis; and because of its availability from HPLC work, the UV/Vis absorbance detector is the most commonly used detector in capillary electrophoresis. The advantages of the UV/Vis detector include: non-destructive to the analytes, insensitivity to temperature and gradient changes, low cost, and simplicity. The detector necessitates the presence of an optical window, which is created by removing a small section of the polyimide coating, which renders the capillary fragile and vulnerable to breakage. It should be noticed that not all species of interest possess chromophores, such as most amino acids, sugars and inorganic ions. This problem could be solved by detection in the indirect mode where a chromophore is added into the background electrolyte. This however yields lower sensitivity. Furthermore there are some restrictions in the running buffers due to the optical properties of the buffers themselves.

1.1.4.1.2 Fluorescence Detection

A fluorescent detector is used for fluorescent molecules which absorb light at one wavelength and then re-emit it instantaneously at a longer wavelength. Two types of fluorescence detectors have been developed according to the light sources used: lamp-

based and laser induced fluorescence detectors (LIF). In the former, light sources such as deuterium, tungsten or xenon lamps are used for excitation, whilst lasers are used as excitation sources in the latter. Laser induced fluorescence gives rise to even higher sensitivities due to the high intensity of its incident light and the ability to accurately focus light to the small diameter capillaries. Detection limits in the 10^{-12} M range have been reported. Lasers available include argon ion, helium-cadmium and helium-neon. The LIF detector is expensive and generally limited by the range of excitation wavelengths offered by the laser, there are also possibilities of photo-degradation of the analytes caused by the high light intensity. The detector is also less versatile because many solutes of interest do not exhibit native fluorescence.

1.1.4.2 Electrochemical Detection Methods

Electrochemical detection methods in capillary electrophoresis have been reviewed in recent articles. As a universal method, conductometric, amperometric, and potentiometric modes of detection have been successfully coupled to capillary electrophoresis. The positioning of the electrodes, interferences of high electric field, and the materials employed in the fabrication and modification of the electrodes are the main topics concerned. The advantages of the use of electrochemical detection with capillary electrophoresis, regarding to the sensitivity and selectivity, is exemplified with a large number of applications[23-25]. Especially, the use of electrochemical detection systems in microchip technology is addressed[26-29].

1.1.4.2.1 Potentiometric Detection

Potentiometric detection can be a powerful alternative in capillary electrophoresis. In this detection mode, a potential developing on an ion-selective electrode or membrane in contact with an analyte ion is measured. Potentiometric detectors can easily be miniaturised without loss of sensitivity because their response is quasi-independent on flow rate. Since potentiometric electrodes respond only to ions with a charge of the correct sign, they are more selective. Miniaturised forms of liquid membrane ion-selective electrodes, which are routinely applied in physiological studies, have been used as detectors in CE. They were used for the detection of both cations and anions[30]. In recent works potentiometric detection was carried out with coated-wire liquid membrane electrodes. The detection properties of these electrodes were comparable to those of the micropipette electrodes but they were easier to handle and had a longer lifetime. Reviews on potentiometric detection for capillary electrophoresis are available[31-33].

The disadvantages of potentiometric detection include the complication of sensor preparation, and handling, fragile micromanipulations and limited lifetime.

1.1.4.2.2 Amperometric Detection

Amperometric detection is an important method of detection for CE because it has attractive features including high sensitivity, good selectivity, and low cost. CE with amperometric detection has been established as a powerful analytical technique, especially for the analysis of biological microenvironments such as single cells.

Amperometric detection is based on the application of a fixed potential at an electrode. Electroactive compounds gain (reduction) or lose (oxidation) electrons to the electrode

and the resulting current can be directly correlated with analyte concentration. Amperometric detection requires three electrodes; a working electrode, reference electrode and an auxiliary electrode, which controls the potential difference between the working and reference electrode. A potential is applied across a supporting electrolyte between the working and reference electrode effecting solute oxidation or reduction. Amperometric detection may be carried out in the oxidative or the reductive mode. In the oxidative mode, a negative potential is applied by the auxiliary electrode. This results in a positive potential difference between the working and reference electrodes. As a result, electrons are transferred to the working electrode. In the reductive mode, the opposite occurs. Reviews on amperometric detection for capillary electrophoresis are available[31, 34-36].

1.1.4.2.3 Conductivity Detection

Compared to potentiometric and amperometric detection, conductivity detection does not rely on electrochemical reactions on the surface of the electrode but measures an electrical signal (conductance) between electrodes contacting the solution. In this detection mode, analytes have to be charged in solution for the determination. A conductivity detector cell comprises two inert electrodes across which a high frequency AC signal is applied. Alternating current is used during the detection in order to avoid electrolysis reactions on the surfaces of the electrodes. The signal arises from the difference in conductance between the analyte and the background electrolyte. The higher the conductivity differences between the analyte-molecules and background co-ion, the larger the detector response. Reviews on conductivity detection for capillary

electrophoresis can be found in the literature[32, 37-40]. The following section will emphasis on the contactless conductivity detection.

1.1.5 Contactless Conductivity Detection

Conductivity detection may be carried out in the contact or contactless mode. In contact conductivity detection, the electrodes are in galvanic contact with the electrolyte solution. The fouling problem of the electrode is a big issue in the contact conductivity detection. For this reason, capacitively coupled contactless conductivity detection has been developed. In 1998 two research groups, the group of Zemann and the group of Fracassi da Silva and do Lago reported on a new design suitable for standard capillaries which was based on two tubular electrodes for capacitive coupling placed side by side on the capillary in an axial arrangement[41, 42]. Electrodes were either cut from syringe needles or painted directly onto the capillary with silver varnish. These two pioneering publications were subsequently followed by a number of reports dealing with the CE-C^4D in order to facilitate its handling, to allow easy incorporation into available instruments, to achieve higher sensitivity, and to expand the application[37, 43].

Basic Principle of Contactless Conductivity Detection: The basic cell configuration for axial contactless conductivity detection is shown in the Figure 3.

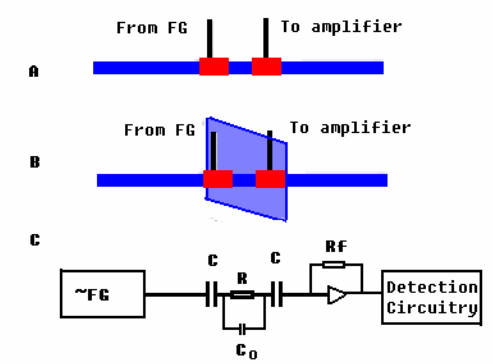


Figure 3. Schematic of the cell design for contactless conductivity detection: A) without shielding; B) with shelding; C) equivalent circuitry. FG = functional generator, C = cell capacitance, R = cell resistance, C_0 = stray capacity, Rf = feedback resistor on the operational amplifier.

The detection cell is formed by two tubular electrodes of about 2 – 30 mm length with a gap of 1 – 5 mm (Figure 3A). A grounded Faraday shield made of a thin copper foil is usually used to avoid direct capacitive coupling between the two electrodes as illustrated in Figure 3B. As shown in Figure 3C, the two electrodes form capacitors (C) with the electrolyte solution inside the capillary, which are connected by a resistor (R) formed by the solution in the gap between the two electrodes. C_0 represents a stray capacitance, which originates from direct coupling between the electrodes when a grounded shield is not used. Under the excitation with an ac-voltage it leads to an ac-current flowing through the cell, which can be picked up at the second electrode and transformed back to an ac-voltage using an operational amplifier in the appropriate configuration. Thus conductivity changes of the solution in the capillary between the two electrodes can be monitored.

1.1.6 Contactless Conductivity Detection on Microchip

Microchip electrophoresis, i.e electrophoresis performed on microfabricated devices, represents the platform for a new generation of miniaturized analyzers where all operations, sample cleanup, preconcentration, mixing, derivatization, separation, and detection, are fully integrated and automated in the so called “Lab on a chip”. Due to their fast analysis times and less sample consumption, they are considered to become the most powerful tools of analytical chemistry in the 21st century with a broad application in life sciences, biotechnology, and drug development, particularly in genomic, proteomic, and metabolomic research[44]. A general microchip used for electrophoretic separations is simply shown in Figure 4.

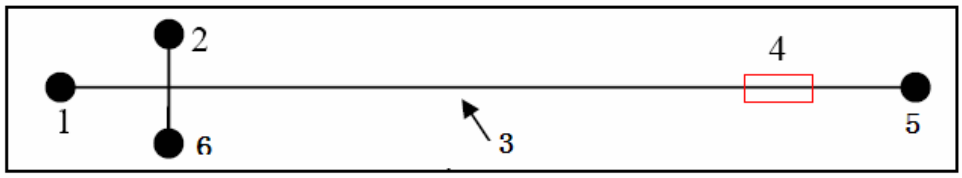


Figure 4.Scheme of a microchip, 1: electrolyte inlet, 2: sample inlet, 3: separation channel, 4: detector, 5: electrolyte outlet, 6: sample outlet

The device with micrometer dimensions is mainly prepared by photolithographic and wet etching techniques. Glass, silicon, and more recently polymers are often used as substrates. From inlet 1, background electrolytes are injected into the chip by pressure-driven or electrokinetic’s flow. From Inlet 2, sample is introduced. Then the sample are passed through and separated in the separation channel 3, and then the interested species

are detected by a sensitive detector 4. The waste of sample and electrolyte are collected from outlet 5 and 6, respectively.

Because of its high sensitivity, fluorescence detection has often been used in microchip electrophoresis, but considerable effort has also been spent on the development of alternative detection techniques, and amperometric and conductometric detection have been regarded to be most attractive alternatives[32, 45, 46]. Different techniques for the conductivity detection have been developed, such as a four electrode conductivity detector[47] and two electrode conductivity detector[48]. Contactless conductivity detectors are also applied to microchip electrophoresis[49, 50].

1.1.7 Application of Contactless Conductivity Detection in CE and Microchip CE

Contactless conductivity detection in capillary electrophoresis has been growing steadily and has been widely applied in the analysis of inorganic ions, organic ions, and bio-molecules in recent years.

Inorganic Ions: The determination of small, charged species, including cations and anions, has been largely favored by CE-C^4D technique from its basic principle[51-53]. The detection limits achieved for the determination of alkali and alkaline earth cations and ammonium ions with C^4D are generally one to two orders of magnitude better than those for the indirect absorption method, which has to be employed for these ions with the standard UV-detectors. Due to their good separation efficiency, low specific conductivity and higher ionic strength, a series of electrolyte solutions containing organic acid / base (e.g., citric , lactic or acetic acids and histidine) have been developed for the CE-C^4D system[54, 55]. The concurrent determination of alkali and alkaline earth metals with transition metals has been achieved by adding α-hydroxy-isobutyric acid (HIBA) in

the buffers[56, 57]. The determination of anions was also realized by suppressing EOF with electrolyte solutions of low pH-value or reversing the direction of the EOF by adding an EOF modifier (e.g., hexadimethrine bromide (HDB) or cetyltrimethylammonium bromide (CTAB)[58-60].

The determination of both cations and anions in a single sample by capillary electrophoresis has also been achieved with CE-C^4D. The technique is based on the dual opposite ends injection of analytes and the center-positioned detector on the capillary. This was first reported by Kubáň et al., they successfully applied the technique to the analysis of rain and surface water samples and proved the possibility to determine simultaneously a total of 21 cations and anions in one run[61, 62]. The simultaneous separation of anions and cations with dual opposite-end injection was also demonstrated in PEEK capillaries and has been automated by using a flow-injection-approach[63, 64]. Recently a new portable CE-C^4D system was developed and optimized for the sensitive field measurements of ionic compounds in environmental samples[65].

Organic Ions: Organic bases and acids can be determined after protonation or deprotonation with CE-C^4D. Depending on the nature of the background electrolyte, indirect and direct conductivity detection was applied. Alkylammonium cations, alkylsulfonic anions, and fatty acids were determined in CE with indirect contactless conductivity detector[66-68]. Partial separation of 9 small haloacetic acids was reported in phosphate, citrate and borate electrolytes after the EOF was reversed with several modifiers[69].

Contactless conductivity detection has proven to be a suitable method for underivatized amino acids, which are impossible to be directly detected using

commercially available UV or fluorescence detectors because of their poor UV absorption. Underivatized amino acids in beer and yeast samples, even amino acid profiles obtained from hydrolysed collagen, egg white and milk casein samples, were determined with CE-C^4D system[70, 71]. Determination of free amino acids and related compounds in amniotic fluid have been developed with CE-C^4D, 20 proteinogenic amino acids and 12 other biogenic compounds such as ethanolamine, choline, gamma-aminobutyric acid etc. have been identified[72]. The determination of mono and di-saccharides as well as fructose, glucose, galactose and sucrose in soft drinks and sugarcane spirit were also presented in recent research[73].

Biomolecules: Not only small building blocks for biomolecules such as amino acids, glucose, fatty acids can be determined with contactless conductivity detector, but also it is possible to be used in the analysis of the larger biomolecules. For example, Baltussen et al. presented the determination of 9 peptides. The determination of human immunoglobulin M (IgM), immunoglobulin G (IgG) (an antihuman IgM) and the complex formed in the immunoreaction between these two species was investigated by Abad-Villar et al. The determination of the antibiotic tobramycin in human serum, which is high hydrophilic and non UV-active, was demonstrated with CE-C^4D.

Contactless Conductivity Detection in Microchip Electrophoresis: A range of applications of microchip electrophoresis with C^4D has been reported. Wang and co-workers[48, 74] and Tanyanyiwa et al.[75, 76] demonstrated the determination of inorganic cations in glass and poly(methylmethacrylate) (PMMA) devices. When PMMA channels are used, the EOF is significantly reduced and the separation of anionic compounds is possible in common electrolyte solutions even without addition of EOF

modifiers[48, 74]. Organic species are also separated on microchip with contactless conductivity detection, for example several amino acids were separated in PMMA and glass microchips[76]. Organophosphorus pesticides and their degradation were also detected by using chip techniques[77]. Even large biomolecules and their bio-transformation were monitored on chip with contactless conductivity detector[78]. A wide application of CE-C^4D in microchip was recently reviewed[38].

1.2 Enantiomeric Separation

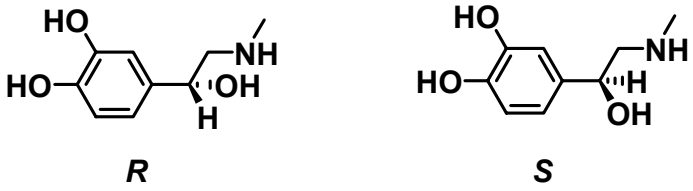
1.2.1 Importance of Enantiomeric Separation

Chirality has become vitally important in the pharmaceutical, chemical, and agricultural industries. The differences which make compounds chiral can produce critically different pharmacological effects in biological systems since the majority of bioorganic molecules are chiral. Living organisms, for example, are composed of chiral biomolecules such as amino acids, sugars, proteins and nucleic acids. In nature these biomolecules exist in only one of the two possible enantiomeric forms, e.g., amino acids in the L-form and sugars in the D-form. Because of chirality, living organisms show different biological responses to one of a pair of enantiomers in drugs, pesticides, etc.

Louis Pasteur was the first person who realized the importance of enantiomeric separations. During his study on the stereochemical differences between the *dextro* and the *levo* form of ammonium tartrate in 1858, he noticed that the *dextro*-isomer was more readily degraded by a mold than the *levo*-isomer. In 1908 Abderhalde and Müller for the first time observed the pharmacological differences in pressor effects between the two enantiomers of 4-(hydroxy-2-(methylamino)ethyl)benzene-1,2-diol. The *R* configuration

is a hormone and secreted from the adrenal medulla with a common name adrenaline, while the S configuration is not from metabolism, see Scheme 1[79].

Scheme 1



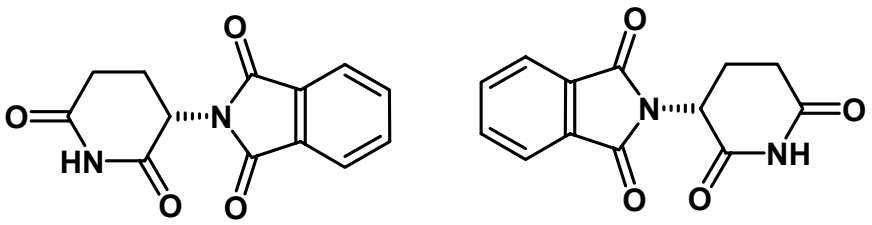
Adrenaline

The body being amazingly enantiomeric selective, will interact with each racemic drug differently and metabolize each enantiomer by a separate pathway to produce different pharmacological activity. Thus, one isomer may produce the desired therapeutic activities, while the other may be inactive or, in the worst case, produce unwanted effects. Consider the tragic case of the racemic drug of n-phthalyl-glutamic acid imide that was marketed in the 1960's as the sedative Thalidomide. Its therapeutic acitivity resided exclusively in the R-(+)-enantiomer. It was discovered only after several hundred births of malformed infants that the S-(+)-enantiomer was teratogenic (Scheme 2).

Nowadays, the high demand for pure chiral compounds in the field of pharmaceutical drug research and production, life sciences, food chemistry, agriculture chemistry has greatly stimulated the fast and accurate enantiomeric analysis. In the drug market, the US Food and Drug Administration (FDA), as well as regulatory authorities in Europe, China and Japan have provided guidelines indicating that preferably only the active enantiomer of a chiral drug should be brought[80-82]. In addition, a rigorous

justification is required for market approval of a racemate of chiral drugs. Presently, a majority of commercially available drugs are both synthetic and chiral. However, a large number of chiral drugs are still marketed as racemic mixtures. Nevertheless, to avoid the possible undesirable effects of a chiral drug, it is imperative that only the pure, therapeutically active form be prepared and marketed.

Scheme 2



(S)-thalidomide, Teratogenic **(R)-thalidomide, Sedative**

Chiral compounds are also utilized for asymmetric synthesis, i.e., for the preparation of pure optically active compounds, which is an important tool to get large quantity of chiral chemicals[83]. They are also used in studies for determining reaction mechanisms, as well as reaction pathways.

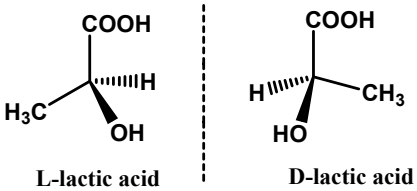
As a result, the development of stereoselective separation techniques and analytical assays to evaluate the enantiomeric purity of chiral compounds has greatly increased.

1.2.2 Some Terms Related to Enantiomeric Separation

Chirality and Chiral Molecules

Chirality refers to the geometric property of a rigid object being nonsuperposable on its mirror image, like left and right hands**.** The compound with this property is named as **chiral molecule**, such as lactic acid (Scheme 3). L-Lactic acid and D-lactic acid are mirror images to each other, but nonsuperimposable on each other.

Scheme 3



Chiral molecules possess either: an asymmetrically substituted atom or an overall chiral shape. An asymmetric substituted atom refers to the atom containing four different groups, it is also called chiral atom. Chiral molecules containing asymmetrically substituted carbon atoms are the most frequently encountered.

Enantiomers, Enantiomeric Pure, and Racemate

The two forms of a chiral object are called enantiomers, which are mirror images of each other and nonsuperimposable. They are also called **mirror image stereoisomers** or **optical isomers** (since enantiomers have the same physical and chemical properties but different properties to plane-polarized light). It should be noticed that they are different compounds.

A collection containing only one enantiomeric form of a chiral molecule is called **enantiopure,** enantiomerically pure, or optically pure.

A collection containing equal amounts of the two enantiomeric forms of a chiral molecule is called a racemic mixture or **racemate**.

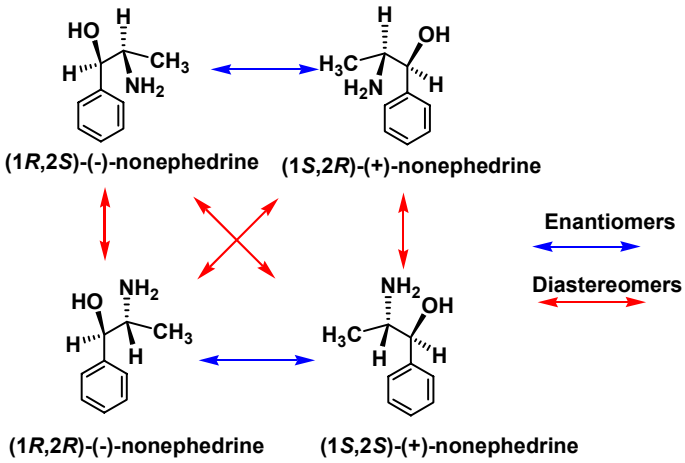
Enantiomeric Excess (Ee) and Optical Purity

If a mixture contains two enantiomers at different amount, enantiopurity is usually reported in terms of "**enantiomeric excess**" (e.e.).

$$\%ee = \frac{\text{major - minor}}{\text{major + minor}} \times 100$$

Sometimes the term **optical purity** is used, which is defined as the ratio of the observed optical rotation of a mixture of enantiomers to the optical rotation of one pure enantiomer. It can be seen that the value of ee is equal to optical purity.

Scheme 4



Diastereomer

If a molecule contains two different chiral carbon atoms, there exist four stereoisomers. Two pairs of them are enantiomers, which are mirror images. The

relationships between other stereoisomers, i.e. non-enantiomeric isomers are called diastereomers (Scheme 4). Diastereomers are characterised by differences in physical properties, and by differences in chemical behaviour towards achiral as well as chiral reagents.

Nomenclatures of Chiral Molecules

Optical Rotation d(+)/l(-) Nomenclature: Enantiomers can rotate the plane of polarization of plane-polarized light, **Dextrorotatory (+)** enantiomers give a positive optical rotation; **Levorotatory (-)** enantiomers give a negative optical rotation. Although "dl" shows very important physical properties of the molecule, they unfortunately do not give any information about spatial arrangement of the chiral center. Presently **(+)** and **(-)** symbols are preferred.

Fischer Projections DL Nomenclature: This was invented by Fischer in 1891. It works by having horizontal bonds in front of the plane and vertical bonds behind the plane. D and L symbols are based on the comparison of the substituents of the chiral center of the compound with that of (+)-glyceraldehyde. However, the D and L convention can be confused with dl terminology. They are still used for sugars, but generally discouraged.

Cahn-Ingold-Prelog R/S Nomenclature: This is the system most frequently used for designating absolute configurations of chiral compounds. Here the priority of the ligands to the asymmetric center is based on the atomic number controlled, and the group with lower priority is positioned far from the observation point. The priority of the other groups is examined. If it decreases clockwise, the R configuration is assigned, otherwise S configuration is given (Figure 5).

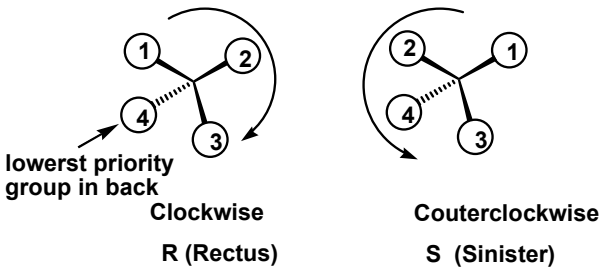


Figure 5 Cahn-Ingold-Prelog R/S Nomenclature

Enantiomeric Separation

Enantiomeric separation, also known as enantiomeric resolution or enantiomeric separation, is the process for the separation of racemic compounds into their enantiomers. It can be achieved by using a chiral stationary phase or a chiral mobile phase.

Resolution (Rs) and Separation Factor (α)

As for chromatographic separation, resolution (Rs) and separation factor (α) are also used in the enantiomeric separation process.

The separation factor α is calculated by:

$$\alpha = \frac{t_2}{t_1}$$

where t_1 is the migration time of the first eluting enantiomer and t_2 the migration time of the second enantiomer (also called as antipode).

The resolution is calculated by:

$$Rs = \frac{2\,(t_2 - t_1)}{(w_1 + w_2)}$$

where w_1 and w_2 are the peak widths of both enantiomers.

1.2.3 Principle of Enantiomeric Separation:

Enantiomers have identical physical properties, and consequently cannot be directly separated by conventional methods such as distillation, crystallization, sizing, or chromatography on conventional stationary phases. Physical separation of the enantiomers comprising a racemic mixture requires the use of some external enantiopure or enantioenriched material or device.

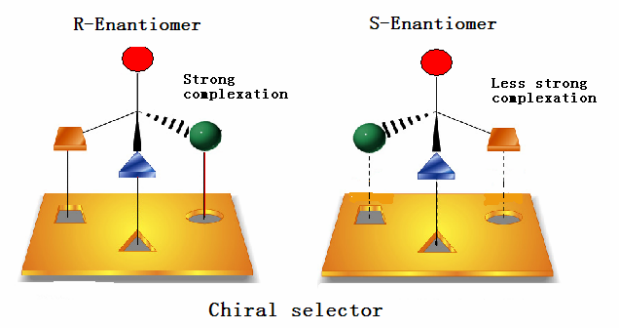


Figure 6. Transient diastereomeric complexes dynamically formed during electrophoresis

No matter whether the enantiomeric separation is achieved by using chiral stationary phase (in GC, HPLC) or using chiral mobile phase (in CE), the key point is the preferential interaction with the chiral selector of one enantiomer over the other. It results in the formation of the transient diastereomers (Figure 6), which show different mobility. The different effective migration velocity results from two independent stereoselective phenomena, first from differences in the strength of interactions between the enantiomers and selector (different complexation constant), and second, from electrophoretic mobility differences of the diastereomeric complexes owing to their different shapes and / or pKa values.

1.2.4 Methods for Enantiomeric Separation

Enantiomeric separation represents a field of vast still growing importance in chemical, pharmaceutical, clinical, agrochemical, and environmental research and development. Since the late 1960s, instrumental techniques for analytical as well as preparative–scale enantiomeric separations have become more and more advanced. For analytical purposes, GC, HPLC, supercritical fluid chromatography (SFC), thin layer chromatography (TLC), and recently capillary electrophoresis (CE) have been used for the separation of chiral compounds.

GC and HPLC were firstly developed for the enantiomeric seperation. Enantiomeric GC covered almost adequately volatile and thermo-stable analytes. It is not as common as liquid chropmatography, but nevertheless there are some very effective optically active stationary phases that have been used in GC for the separation of enantiomers. Several review articles reported on the enantiomeric separation with GC[84-86].

Enantiomeric HPLC has proven to be one of the best methods for the direct separation and analysis of enantiomers[87-89]. It is more versatile than enantiomeric GC because it can separate a wide variety of nonvolatile compounds. It provides fast and accurate methods for enantiomeric separation, and allows on-line detection and quantitation of both mass and optical rotation of enantiomers if appropriate detection devices are used. Current enantiomeric HPLC methods are either direct, which utilizes chiral stationary phases (CSPs) and chiral additives in the mobile phase, or indirect, which involves derivatization of samples. Direct enantiomeric separations using CSPs are more widely used and are more predictable, in mechanistic terms, than those using chiral

additives in the mobile phase.

Since the 1990s capillary electrophoresis has been considered as one of the most powerful analytical tools for enantiomeric separations. Especially enantiomeric CZE developed with a fascinating speed and became a mature and versatile technique within a few years. In recognition of this, the journal *Electrophoresis* has published two special issues on enantioseparations in 1999 and 2007.

Some major advantages of enantiomeric separations in CE in comparison with HPLC are the low consumption of the chiral selector (reduced costs) and the high plate numbers due to a reduced peak broadening as a consequence of the absence of eddy diffusion and mass transfer between two phases (the A- and C-term of the Van Deemter equation, respectively). Also the selectivity that is defined as the extent to which the analyte under study can be assessed in a complex mixture without interference from the other components in the mixture, obtained by a difference in mobility between the solutes under the influence of an external electrical field, is high in CE. The latter expression was extensively discussed as one of the key parameters in electrokinetic separation sciences. Because of the combination of the high plate numbers and a high selectivity, baseline separations with CE can be achieved at lower concentration levels of the chiral selector.

1.2.5 Indirect and Direct Enantiomeric Separation in CE

Enantiomeric separation by electrophoresis techniques needs the formation of diastereomers. To this end, two inherently different concepts for the separation of corresponding enantiomers have been used in CE. The diastereomers can be formed by a chemical reaction between analytes and optically pure chiral derivatization reagent before

the electrophoresis separation. Or they can be transient diastereomeric complexes formed during electrophoresis between the enantiomeric analytes and a chiral selector in the mobile phase. The former is called indirect enantiomeric separation, and the later is called direct enantiomeric separation.

In the indirect enantiomeric separation, the derivatized chiral analytes can subsequently be separated with a non-enantioselective CE system as a pair of diastereomers. Due to several reasons the indirect approach is not very popular: the chemical derivatisation of the enantiomers not only requires additional work but is also prone to errors which may be significant especially in the case of quantitative analysis.

The far more often employed concept for the electrochromatographic separation of enantiomers is based upon the direct enantiomeric separation for which chiral media are necessary. As a result of specific interactions between the chiral selector and the enantiomeric mixture differing in their strength and/or nature of the individual enantiomers of the analytes a discrimination of the stereoisomers can be achieved. Noncovalent binding forces typically involved in the stereochemical recognition are mainly attractive or repulsive electrostatic (ionic), van der Waals, π-π or dipolar interactions and hydrogen bondings besides steric phenomena.

The direct separation of enantiomers by CE is easier to perform than the indirect separation. The direct method is less time consuming, a wide number of chiral selectors (see below) are commercially available, and small amounts of chiral selectors can be used.

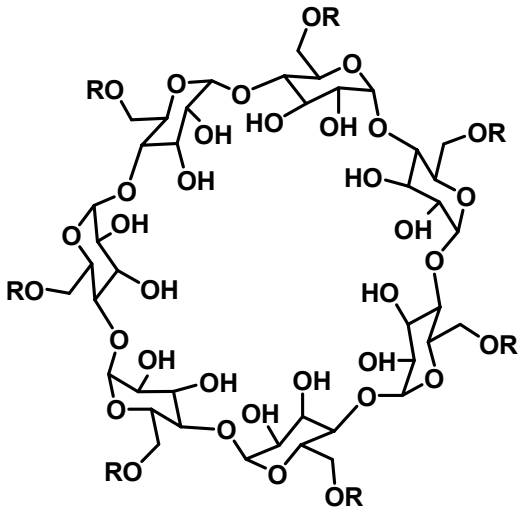
1.2.6 Chiral Selectors and Mechanisms for Chiral Recognition

As in the LC with chiral stationary phases, general types of chiral selectors used in CE include: cyclodextrins (CDs), chiral crown ethers, macrocyclic antibiotics, chiral metal complexes, proteins etc. Depending on the types of the chiral selectors used and analytes to be separated, different mechanisms of chiral separation have been proposed.

1.2.6.1 Cyclodextrins and Their Derivatives:

Cyclodextrins and their derivatives are the most popular and widely used chiral selectores for many analytical chemists[90-95]. Cyclodextrins are cyclic oligosaccharides containing from six to twelve D(+) glucopyranose units bonded through alpha-(1,4) linkages. Three sizes are commercially available alpha, beta & gamma corresponding to 6, 7 and 8 glucopyranose units respectively (for β-CD see Scheme 5). The cyclodextrin molecule forms a truncated conical cavity the diameter of which depends on the number of glucopyranose units. The size of the cavities and the type of molecules that may be accommodated is listed in the following table.

Scheme 5



The cyclodextrin molecule has secondary 2- and 3- hydroxyl groups lining the mouth of the cavity and primary 6-hydroxyl groups at the rear of the molecule. This means that the cavity itself is a relatively hydrophobic region of the molecule and permits inclusion of hydrophobic portions of solute molecules. Interaction of any polar regions of a solute molecule with the surface hydroxyls combined with the hydrophobic interactions in the cavity provides the 3-point interaction required for enantiomeric recognition.

CD	No. of units	Size Å	Molecules included	Chiral centers
alpha	6	4.5-6.0	5-6 membered aromatic	30
beta	7	6.0-8.0	Biphenyl or naphthalene	35
gama	8	8.0-10.0	Substituted pyrenes and steroids	40

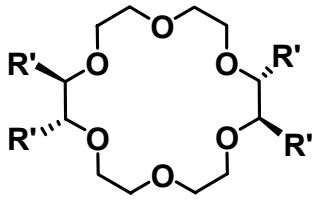
The resolution of enantiomers is obtained through a inclusion-complexation mechanism[96]. A portion of the molecule must enter the hydrophobic cavity and a hydrogen bonding region of the molecule must interact with the mouth of the cavity. The beta form has been found to have the widest application. The selectivity of a cyclodextrin is dependent on the size of the analyte. Alpha-cyclodextrin will include single phenyl groups or napthyl groups end-on. Beta-cyclodextrin will accept Napthyl groups and heavily substituted phenyl groups. Gamma-cyclodextrin is useful for bulky steroid-type molecules. Separations will be greater if the hydrogen bonding groups in the analyte are brought into close proximity to the surface hydroxyls. This is illustrated by the separation of D,L-phenylalanine analogs on beta-cyclodextrin. Meta-substituted analogs tilt the molecule thereby enhancing hydrogen bonding in the mouth of the cavity.

More recently a new range of modified cyclodextrins have been developed which expand the range of compounds which can be resolved. The derivatives are formed by bonding various groups onto the surface hydroxyls of the cyclodextrin cavity. This extends the area available for chiral interactions[91, 97].

1.2.6.2 Chiral Crown Ether

Another class of compounds used for enantiomeric resolution by the inclusion-complexation mechanism is represented by crown ethers[98-101]. These macromolecules are able to form inclusion complexes with guest compounds through weak bonds with the etheroatoms of the crown. 18-Crown-6-ether tetracarboxylic acid ($18C_6H_4$) is the most often used one (Scheme 6). Here the inclusion complexation is different from that of CDs, the hydrophilic part of the analyte is included, while with CDs it is the hydrophobic part.

Scheme 6



R' = COOH, $18C6H_4$

Two different enantiomeric resolution mechanisms have been proposed by Kuhn in which the four carboxylic groups of the crown are involved in the enantiomeric recognition process, either forming electro-static interaction or causing a steric barrier

effect with the included molecule[102]. The presence of a primary amino group in the analyte is fundamental for inclusion-complexation. Non-polar and branched substituents on the asymmetric center as well as the distance of the amino group from the chiral center have strong influence on the enantiomeric resolution.

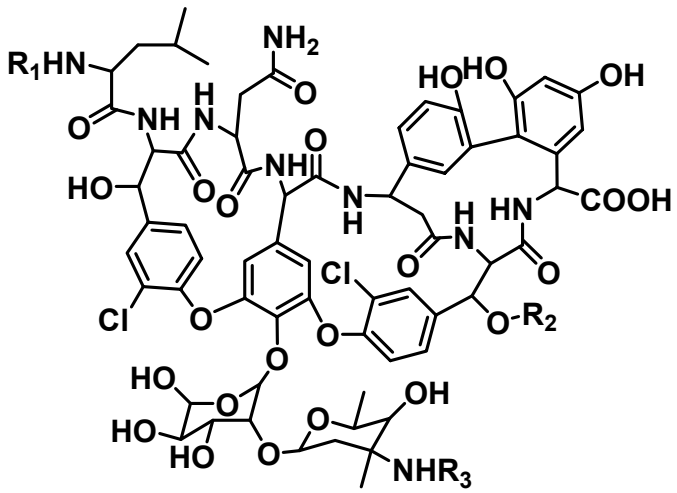
1.2.6.3 Macrocyclic Antibiotics

Recently macrocyclic antibiotics have been used as a new class of chiral selectors. Macrocyclic antibiotic selectors are glycopeptides or cyclic peptides, which supply in many cases highly enentioselective properties. The most popular selectors of this group include Vancomycin, Restocetin A, Ticoplanin, and Rifamycins etc[103-106]. Rifamycin has been more successful in the CE field where it has been used as a mobile phase additive. The glycopeptides Vancomycin and Ticoplanin have a cup like region and a sugar "flap". The enantiomeric resolution mechanism with these selectors is based on the pi-pi interactions, hydrogen bonding, inclusion complexation, ionic interactions and peptide binding. Glycopeptides have considerable UV-absorption in the commonly chosen detection wavelength range, thus the so-called partial filling technique combined with a countermigration mode was developed to overcome this problem.

Vancomycin (Scheme 7) has been received particular attention, since it is well soluble in aqueous buffers, commercially available at a reasonable price, and it is highly selective to anionic enantiomers. It has a molecular weight of 1449, 18 chiral centres and three fused rings. It has a basket like structure with a single flexible sugar flap that can enclose a molecule sitting in the basket. A carboxylic acid and a secondary amine group

sit on the rim of the basket and can take part in ionic interactions. Vancomycin also separates amines, amide neutrals and esters.

Scheme 7



$R_1 = CH_3$, $R_2 = R_3 = H$

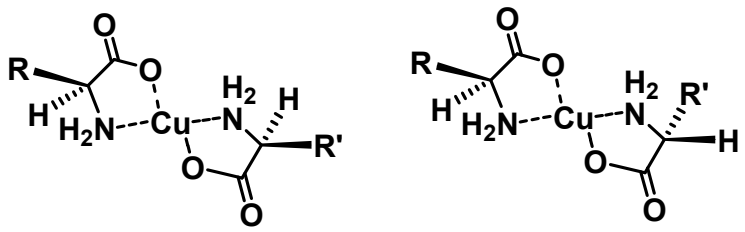
Ticoplanin has three sugar groups and four fused rings with a molecular weight of 1885 and 20 chiral centres. Ticoplanin is very useful in the enantiomeric separation of all amino acids. The mechanism of enantiomeric recognition involves the ionic interaction from both the acid group at one end of the peptide cup/cleft and a basic group at the other end. The sugar groups are arranged in three flaps that can fold over to enclose a molecule in the peptide cup.

1.2.6.4 Ligand-Exchange Type Selectors

Metal complexes with a chiral ligand, normally a chiral amino-acid-copper complex, can be used for the enantiomeric separation of amino acids[107, 108]. The separation is

based on the ligand exchange mechanism by forming diastereomeric copper complexes. The analytes normally contain at least two coordinating groups which are able to form coordination interaction with the metal ion. It should be noticed that the pH of the BGE plays a key role for the successful enantiomeric separation, as the complex stability is very much pH dependent. For example, copper (II) complex with amino acid containing R group which act as chiral selector can form two transient diastereomers (Scheme 8) with two enantiomers of amino acids to be separated, they exhibit different mobility and could be separated during the electrophoresis. The separation of D- and L-phenyllactic acid was achieved when Cu(II)-proline complexes were used as the chiral selector[109]. Phenyllactic acid forms a tertiary complex with Cu(II), causing a reduction of the effective mobility of both enantiomers; the two complexes posses different stability constants and thus are separated by the end of the electrophoretic run.

Scheme 8



1.2.6.5 Proteins

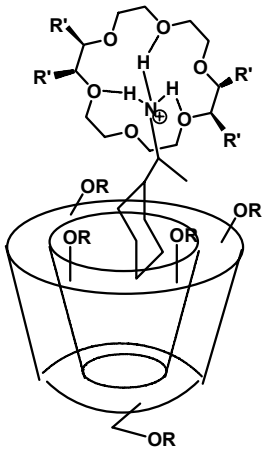
Proteins are natural biopolymers with helical conformation able to interact selectively with a wide number of compounds of small size, such as pharmaceuticals. Several types of proteins have been used as chiral selectors in CE[110-113]. The

enantiomeric separation is based on stereoselective interactions between the protein and the two analytes, with the formation of two labile diastereoisomeric complexes during the electrophoretic run. Usually the separations are not as efficient as achieved by means of low-molecular-weight selectors. Partial-filling technique has to be used.

1.2.6.6 Dual Chiral Selectors

Mixed selectors can be used to obtain the good selectivity of an enantiomeric separation. This approach consists of a mixture of either chiral/chiral or chiral/achiral compounds. A mixture of CDs are usually applied to separate several related analytes and enantiomers[114-116]. Especially dual CD systems consisting of a charged and a neutral CD proved to be effective in chiral discrimination compared with the use of either charged or neutral CD alone. Verleysen and Sandra successfully separated the enantiomers of a tryptophan derivative and a small peptide by using the combination of cyclodextrins with the chiral crown ether[117]. Lin et al. separated the isomers of fluor phenylalanine[118] and Kuhn et al. used it to resolve the enantiomers of adrenaline[102]. In this separation both inclusion- and outer-sphere complexation are involved in the separation mechanism, a sandwich structure of the transient diastereomer can be used to explain the chiral recognition during the separation process (Scheme 9).

Scheme 9



1.2.7 Optimization of the Enantiomeric Separation

To achieve good enantiomeric separation, the following aspects have to be considered: resolution, analysis time, and robustness of the system. It has been shown that the enantiomeric resolution is strongly influenced by several parameters, including structure of analyte, chiral selector type and concentration, composition of background electrolyte (BGE) (ionic strength, ion type and concentration, pH, organic solvent), polymeric additives in the BGE, applied voltage, and capillary temperature[90, 119-126].

Structure of Analyte

Depending on the structures of the analytes, different chiral selector and separation conditions are to be designed. Here a simple discussion from the viewpoint of acidic and basic analytes is given, more details will be discussed in the following section combined with the type of chiral selectors.

For basic samples, an acidic pH of the BGE should be selected and the separation can be performed either in a coated or uncoated capillary. Normally a pH one unit lower than the pKa of the enantiomers allows the migration of analytes as cation. To achieve good

resolution, the electro-osmotic flow should be controlled so that the time spent by the analytes in contact with the chiral selector is sufficient. When the enantiomeric separation of negatively charged compounds must be performed, selecting a pH for the BGE in the range 4.5-8 will charge (negatively) the two enantiomers and produces a sufficient electro-osmotic flow for the movement of the analytes toward the cathode.

Selection of the Chiral Selector and Its concentration

The features and the potential as well as the applications of various chiral selectors have been widely discussed and reviewed (see also part 2.6).

Due to commercial availability, low cost, and wide variety, native cyclodextrins and their derivatives have been widely used for the enantiomeric separation in CE. According to their size, α-cyclodextrin or its derivatives are often selected for analytes without containing aromatic rings or containing only one aromatic ring with substituent on the para position. β-Cyclodextrins are often used for compounds possessing two aromatic rings. For analytes with more than two aromatic groups γ-cyclodextrin could be the appreciative chiral selector. The wide number of modified cyclodextrins should be considered for further optimization if native ones do not work well.

For the compounds containing primary amines in the chemical structures, 18-crown-6 ether is the favorite chiral selector for the separation. This is also applicable to the chiral separation of some pepetides.

For the enantiomeric separation of amino acids or hydroxycarboxylic acids, cyclodextrins could be used, however a ligand exchange electrophoretic system can also be an alternative method.

To achieve better resolution, the concentration of selector should also be optimized.

The effect of the chiral selector concentration on the enantiomeric separation are complicated, some points are summarized here. For neutral and charged selectors, there is a maximum in the selectivity vs. selector concentration curve. In this case, increasing concentration of selector is better for the resolution of analytes at the lower concentration range and reaches the highest resolution value at certain point, increasing the concentration of selector will lower the resolution. For negatively charged selectors, they often have strong binding constants. The curve of the corresponding selectivity and selector concentration does not show a maximum but a discontinuity at a certain critical selector concentration. It proved that the optimum selector concentration is pH dependent in the region near to the pKa values of weakly acidic or basic groups present either in the analytes or the selector. Furthermore the addition of organic solvent also affects the optimum selector concentration.

Composition of the Background Electrolyte

Enantiomeric resolution can be strongly influenced by the composition of the BGE. Decreasing the ionic strength of the BGE generally causes a reduction of migration time and resolution, peak tailing has also been observed. A BGE at a concentration 100 times higher than that of the sample is recommended. The peak shape can be controlled by selecting a co-ion with similar electrophoretic mobility to that of analyte. For UV detection, either the analytes contain UV active groups or the BGE must contain a UV active component for non-UV active analytes such as inorganic ions. Inorganic buffers such as phosphate can be used for the UV measurement. For electrochemical detection, especially for conductivity detection, organic acids are often used as BGE while inorganic buffer is not applicable due to high conductivity.

Another component often used in BGE is an EOF modifier. EOF moves towards the cathode, whereas anions migrate towards the anode. Thus for the analysis of negatively charged analytes, the EOF must be reduced or better reversed towards the anode to shorten the analysis time. Amines such as diethylamine are often used for reducing EOF, and single or mixtures of quaternary ammonium salts are used to reverse the EOF, for example cetyl trimethylammonium bromide (CTAB).

The pH value of BGE is a main parameter affecting selectivity, efficiency and resolution, its influence has been discussed by several authors and a theoretical model has also been described. The pH of the BGE not only influences the electro-osmotic flow but can also affect the charge of the analytes and selectors. For selectors containing weakly acidic or basic groups, the pH of BGE can evidently influence the binding strength and complex mobility. For chiral analytes containing weakly acidic or basic groups, varying the pH can produce different speciation, thus resulting in different intrinsic selectivity, which could be significantly reduced or be inverted. An inversion of the migration order of enantiomers can be realized through this strategy. Certainly the analysis time and resolution are greatly effected via the effective net-charge and the effective mobility of the complexes by the pH of the BGE. The pH value also strongly influences the EOF, especially for non-coated fused silica capillaries. It is often noticed that only a small pH window can be optimized.

Organic Additives

The addition of an organic solvent to the BGE can improve the selectivity of the enantiomeric separation by a differential influence on the binding constants of the two enantiomers. At the same time, the affinity of the analytes for the organic additive must

be considered. Thus organic solvents such as ethanol, methanol, acetonitrile are often used. Organic solvents can enhance or reduce the effective selectivity of a separation depending on the concentration of chiral selector. Above the optimum concentration of selector, addition of organic solvent normally enhances the selectivity value. Higher concentration of organic solvents can also shift the pKa values of analytes and selectors.

Capillary Temperature

Efficient temperature control is recommended to prevent loss in resolution. Temperature increase causes a decrease in buffer viscosity, and thus a decrease in migration time. Furthermore a change in the temperature can strongly affect the stability of the complex formed between analytes and selector. For good repeatability, the temperature of studies should be maintained at 20-25 °C.

1.3 Research Objectives

Several interrelated objectives were pursued for this dissertation. The first objective was to develop a systematic method for optimized separation of racemic chemicals which are non-UV absorbing or not so sensitive for direct UV detection. These chemicals include amino acids, alkyl amines and peptides. They are very important building blocks for biomolecules, pharmaceutical products, and widely used in asymmetric catalysis. Normally they were chirally separated by indirect methods, which are time consuming and have lower sensitivity. Direct enantiomeric separation of them will be important for quality control and process chemistry monitoring. The study will focus on the optimization of the method, which includes selection of chiral selectors, buffer composition, pH control, effects of additive, and concentration dependence. The aims of

the study were to get good resolution, short separation time, and sometimes also concurrent separations.

The second objective of this study was to investigate the possible enantiomeric recognition mechanism. It is well known that chiral selectors play a key role in the enantiomeric separation. Different analytes need different chiral selectors, to achive good resolution, sometimes dual chiral selectors have to be used. From the optimization of chiral selectors for different analytes, it is hoped to get some experience or conclusion for further enantiomeric separation studies.

Thirdly, enantiomeric separations of non-UV active analytes on chip with C^4D were to be explored. The main advantage of electrophoresis chips, planar devices based on embedded channels for separation, is the fast analysis times which can be achieved due to their short separation lengths and efficient injection regime. The fast separation of enantiomers detected amperometrically on such devices had been demonstrated, but detection with contactless conductivity technique on electrophoresis chips for enantiomeric separations had not yet been demonstrated.

The fourth goal of this research was to explore the application of CE-C^4D for clinical samples such as the street drug - gamma-hydroxybutyric acid (GHB). GHB is a recently introduced party drug, which is presently mainly analyzed by gas chromatography after derivatization. UV-detection is not suitable and therefore conductometric detection is an attractive proposition. On-site screening of street samples should be possible with a portable CE-C^4D instrument. The aim is to obtain best sensitivity and selectivity and apply the method to the determination in urine or blood samples.

2. Results and Discussion

Parts of this thesis have been reported in various journals of analytical chemistry, thus the results and discussion chapter is composed of 5 publications and one manuscript.

In Part 2.1, chiral separations of basic drugs and non-UV active amino acids were studied. Enantiomeric separation of basic drugs with contacless conductivity detection has been successfully achieved after extensive optimization of the separation conditions including type and concentration of chiral selectors, type and the pH of background electrolyte. Hydroxypropyl-beta-cyclodextrin was chosen as chiral selector, 100 mM lactic acid with 5 mM L-histidine at pH 2.75 was used as background electrolyte. At the conditions employed, a good separation of all samples could be achieved. Enantiomeric excess calibration for the separation of pseudoephedrine has been investigated. The detection limits for the two enantiomers of pseudoephedrine were determined as 3.0×10^{-7} M and 3.8×10^{-7} M for the lS,2S-(+)- and lR,2R-(-)-pseudoephedrine, respectively. Calibration curves were linear up to at least 1.0×10^{-4} M, they are over more than two orders of magnitude, using peak areas for quantitation. On the basis of the above results, enantiomeric separation of amino acids was investigated. The successful enantiomeric separation of nine amino acids with contactless conductivity detection has been realized for the following condition: chiral crown ether $18C6H_4$ was adopted as chiral selector, 25 mM citric acid or 10 mM citrate/Tris at pH 2.1 were used as background electrolyte. The detection limits ranged between 2.5 and 20 μM for the species examined. The concurrent enantiomeric separation of a mixture of six amino acids was achieved.

In Part 2.2, 1-phenylethylamine and 1-cyclohexylethylamine were chosen as model substances to study the mechanism of enantiomeric separation of small amines which are very important in chiral synthesis. After many efforts, direct enantiomeric separation of both amines was obtained with unprecedented high resolution by using a combination of dimethyl-β-cyclodextrin and the chiral crown ether $18C6H_4$ as selectors. The enantiomeric separation mechanism has been attributed to the cooperative interaction of the tertiary system among the two chiral selectors and amines.

Before we go to the enantiomeric separation of various amines, the use of contactless conductivity detection for the determination of non-chiral amines in capillary electrophoresis was successfully demonstrated in Part 2.3. Aliphatic non-UV-absorbing species could be determined along absorbing compounds by measuring the conductivity of their protonated forms. The species tested included short chained aliphatic primary, secondary and tertiary amines, branched aliphatic amines, diamines, hydroxy amines as well as species incorporating aromatic and non-aromatic cyclic moieties. Highest sensitivity was obtained with background electrolyte solutions containing solely acetic acid. A concentration of 0.5 M at a pH-value of 2.5 was used. Detection limits were in the order of 1 μM. Inadequate separation of *cis*- and *trans*-1, 2-diaminocyclohexane could be resolved by adding 18-crown-6 as modifier to the electrolyte solution.

After optimization of the determination condition for various amines, the enantiomeric separations of the chiral species among them were achieved by using a combination of the chiral crown ether (+)-(18-crown-6)-2, 3, 11, 12-tetracarboxylic acid ($18C_6H_4$) and dimethyl-beta-CD as selectors in CE and contactless conductivity measurement for detection in Part 2.4. A BGE solution consisting of acetic acid was employed. Compared

to the method using a single chiral selector, the binary selector approach is much better. The resolutions range from 1.2 to 6.0, most of them are above 1.5. The detection limits were found to be about 1.0 μM and the determination is possible up to at least 1.0 mM. The determination of enantiomeric ratios of up to 99.5:0.5 was also found feasible. The baseline separation of the enantiomers of *trans*-cyclohexane-1, 2-diamine is possible in less than 2 min on a chip, as opposed to the 8 min required on the standard capillary.

On the basis of the successful direct enantiomeric separation of basic dugs, amino acids, various amines, the research work was further extended to the optimization of the enantiomeric and diasteromeric seaprations of various peptides and oligo-peptides in Part 2.5. The chiral separation of these small bio-molecules are becoming increasingly important in pharmaceutical industry and fine chemical industries as well as organo catalysis research, however, many of them are poorly UV-active because of non-UV active amino acid units. With the advantage of CE-C^4D, chiral separation of peptides has been optimized through background electrolyte composition, the pH of the BGE, kinds of chiral selector, organic additives and operation conditions. Separation of the stereoisomers was achieved in typically 10–15 min by using either dimethyl-beta-CD, (+)-(18-crown-6)-2, 3, 11, 12-tetracarboxylic acid ($18C_6H_4$), a combination of the two substances, or of histidine, as buffer additives. Calibration curves were determined for isomers of Gly-Asp and H-Pro-Asp-NH_2, in the range of 0.05–0.5 mM and 0.1–1 mM, respectively, and were found to be linear. LODs were determined to be in the order of 1 μM. The determination of isomeric impurities down to about 1% was found possible. Species showing good separation could also be successfully determined on an electrophoretic lab-on-chip device, with analysis times of a few minutes.

In Part 2.6, the procedure for the fast determination gamma-hydroxybutyric acid with CE-C^4D has been developed. Gamma-hydroxybutyric acid (GHB) is a recently introduced party drug, which is presently mainly analyzed by gas chromatography after derivatization. UV-detection is not suitable and therefore conductometric detection is an attractive proposition. Analysis of GHB with CE-C^4D has been successfully developed through optimization of separation condition and calibration with standards. Furthermore, urine and serum samples from the clinical laboratory of the University Hospital in Basel have been measured. It proves that the analysis of GHB with CE-C^4D is possible and accurate, and the method is simple and efficient. The analytical data of GHB in various samples with CE-C^4D are comparable to those of the standard method are comparable.

2. 1 Enantiomeric separation of basic drugs and amino acids

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Separation of enantiomers in capillary electrophoresis with contactless conductivity detection

Xiao Yang Gong, Pavel Kubáň, Jatisai Tanyanyiwa, Peter C. Hauser*

Department of Chemistry, University of Basel, Spitalstrasse 51, 4004 Basel, Switzerland

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Abstract

Contactless conductivity detection is successfully demonstrated for the enantiomeric separation of basic drugs and amino acids in capillary electrophoresis (CE). Derivatization of the compounds or the addition of a visualization agent as for indirect optical detection schemes were not needed. Non-charged chiral selectors were employed, hydroxypropylated cyclodextrin (CD) for the more lipophilic basic drugs and 18-crown-6-tetracarboxylic acid ($18C6H_4$) for the amino acids. Acidic buffer solutions based on lactic or citric acid were used. The detection limits were determined as 0.3 μM for pseudoephedrine as an example of a basic drug and were in the range from 2.5 to 20 μM for the amino acids.

Keywords: Enantiomeric separations; Basic drugs; Amino acids; Capillary electrophoresis; Contactless conductivity detection

1. Introduction

Most pharmaceutical compounds have chiral centres and their effects are due to interactions with chiral biological compounds. Different enantiomers of the Pharmaceuticals therefore usually have different pharmacological properties in terms of activity, toxicity, transport mechanism and metabolic route. For this reason drugs are administered in enantiomeric pure form. The determination of the enantiomeric purity of intermediates used for, and products of, enantioselective syntheses is therefore an important analytical task. This is often carried out with separation methods such as HPLC or GC (see for example, the following review [1]). However, the approach is challenging as the fundamental chromatographic separation mechanisms are not adequate for distinction between enantiomers because their physical and chemical properties are too similar. Therefore, special chiral reagents have to be bonded onto the chromatographic column.

The suitability of capillary electrophoresis (CE) for enantiomeric separation was first demonstrated by Gassmann et al. [2], and this approach has become an important technique. Besides the general advantages of CE, high separation efficiency and short analysis times, the method is less costly than chromatography as the preparation of special chiral columns is not necessary. In CE small amounts of reagents are dissolved in the background electrolyte solution, which interact with the analytes, thus modify their electrophoretic mobilities and act as enantiomeric selectors. A range of selectors has been used, such as cyclodextrins (CDs), a chiral crown ether, macrocyclic antibiotics, polysaccarides and chiral surfactants. The use of CDs, introduced by Terabe [3], is the most common approach in CE to obtain enantiomeric separations and a range of modified CDs have been used for this purpose. A number of recent reviews are available (see for example, [4–8]).

In CE, UV or fluorescence detectors are most often used and commercially available. Therefore, in the enantiomeric separation of non-UV absorbing compounds, such as alkylamines and most amino acids, these are often measured after derivatization [9–12], or by indirect methods in which a chromophore is displaced by the analyte leading to peaks due to a reduction in the background absorbance [13,14]. However, derivatization adds an additional step to the analytical process and may also affect the interaction of the analytes with

* Corresponding author. Tel.: +41 612671003; fax: +41 61 2671013.
E-mail address: peter.hauser@unibas.ch (P.C. Hauser).

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the chiral selector. Indirect methods generally show relatively poor sensitivity.

Electrochemical detection techniques are a good alternative, in particular the recently developed capactively coupled contactless conductivity detector (C^4D) has a number of advantages. The technique is universal in capillary electrophoresis in that all ionic compounds can be detected without derivatization or indirect approaches. Except for the electrodes, no physical parts are needed, all the other components consist of inexpensive electronic circuitry. The contactless approach features also unprecedented ease of the cell arrangement and inherent prevention of electrode fouling. Handling of the separation capillary is facile as it is not necessary to remove the polymeric cladding to create a detection window. For these reasons, contactless conductivity detection has been adopted by a number of research groups and the successful application of this detector for organic molecules of different classes, incuding basic drugs [15] and amino acids [15–18] has been reported. Recent reviews on contactless conductivity detection are available [19,20]. However, to our knowledge, the use of C^4D in chiral separations has not been previously explored, and a study of this application is presented here.

2. Experimental

2.1. Instrumentation

Separations were carried out on an instrument, which was built in-house and is based on a high voltage power supply with interchangeable polarity (CZE 2000R) from Start Spellman (Pulborough, UK). The contactless conductivity detector consists of two tubular electrodes of 4 mm length separated by a gap of 1 mm and a Faradaic shield. Cell excitation was carried out with a sine-wave with a frequency of 100 kHz and a peak-to-peak amplitude of 450 V. The resulting current signal was amplified, rectified and low pass filtered with a circuitry described elsewhere [21,22] before passing to a MacLab/4e data acquisition system (AD Instruments, Castle Hill, Australia) for recording of the electropherograms. All electropherograms were inverted for presentation of the peaks in the normal orientation. Detection limits are reported as the concentrations giving peak heights corresponding to three times the baseline noise.

2.2. Reagents and methods

All chemicals were of analytical reagent grade and were obtained from Fluka (Buchs, Switzerland) with the exception of hydroxypropyl-β-cyclodextrin (HP-β-CD) which was purchased from Acros (Geel, Belgium) and sulphated β-cyclodextrin (HS-β-CD), which was purchased from Aldrich (Buchs, Switzerland). All solutions were degassed by ultrasonication and filtered through 0.2 μm nylon filters before use. Fused-silica capillaries of 10 μm i.d. and 375 μm o.d. were used for performing the electrophoretic separations. These were purchased from Polymicro Technologies (Phoenix, AZ, USA) and were preconditioned with a 0.1 M sodium hydroxide solution before flushing with water followed by flushing with the running buffer. All capillaries had a total length of 48 and 43 cm effective length. Sample injection was carried out electrokinetically at 5.0 kV for 7 s, the separation voltage was 15 kV unless stated otherwise. Standard solutions were diluted with background electrolyte solution to ensure injection under non-stacking conditions.

3. Results and discussion

3.1. Basic drugs

In order to explore the feasibility of using contactless conductivity detection in enantiomeric separations first experiments were conducted with a relatively well-established method, the separation of basic drugs with cyclodextrins as chiral selector. Frequently, sulfated cyclodextrins are used for this purpose (see for example, [23,24]) and therefore the use of such an enantiomeric selector was first investigated. It was however found, that it was not possible to obtain a stable baseline when including sulfated β-CD into an electrolyte solution consisting of 20 mM lactic acid. This was ascribed to the fact that at the high concentration of 1% at which the modifier has to be used, this highly charged substance leads to a marked increase of conductivity of the running buffer (from 692 to 3260 $\mu S\,cm^{-1}$ for HS-β-CD), which is not compatible with conductivity detection. Lower concentrations of the sulfated cyclodextrins on the other hand are not adequate for achieving enantiomeric separations. It appears that the use of cyclodextrins must thus be limited to non-charged derivatives when employing conductivity detection.

Further trials were therefore carried out with the neutral hydroxypropyl-β-cyclodextrin, the use of which had been described previously for the purpose of the enantiomeric separation of basic drugs employing optical detection (see for example, [25,26]), and this was indeed successful. The separation of the enantiomers of adrenaline as an example for the use of HP-β-CD with C^4D is shown in Fig. 1. The four electropherograms were obtained with different concentrations of the enantiomeric selector. As evident, the concentration in the range from 10 to 40 mM has a strong effect on the separation, which is expected from the previous results reported in the literature (see for example, [26]). Interesting is also the effect of the concentration on the sensitivity, which is in contrast to results obtained with optical detection. Peak heights are smaller for the higher concentrations of the cyclodextrin. Presumably this is caused by the partial complexation of the analyte by the selector, which lowers the sensitivity to conductometric detection. A similar effect was observed previously for amperometric detection as well [27]. Besides the concentration of the chiral selector, the overall buffer composition also strongly influences the separation. In Fig. 2, the

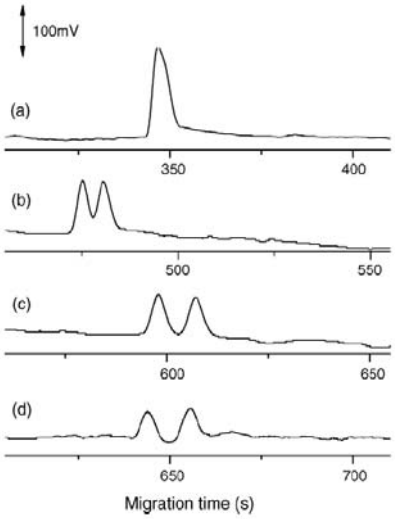

Fig. 1. Influence of the concentration of HP-β-CD on the resolution of D- and L-adrenaline (100 μM): (a) 10 mM; (b) 20 mM; (c) 30 mM; and (d) 40 mM HP-β-CD in 100 mM lactic acid, 5 mM L-histidine at pH 2.75. Capillary: fused silica (48/43 cm × 10 μm i.d.), separation voltage: 15 kV, and injection: 7 s/5 kV.

effect of adding histidine to the lactic acid buffer is illustrated. The addition of this compound leads to an increase of the pH-value from 2.40 to 2.75. However, the effect cannot be completely reconciled with a rise in the pH-value alone as an alteration of the concentration of lactic acid in the absence

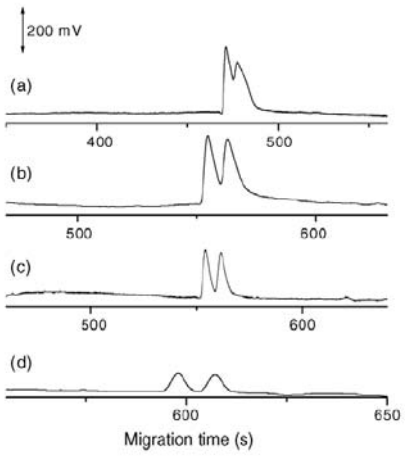

Fig. 2. Influence of the pH-value and the concentration of histidine on the resolution of D- and L-adrenaline (100 μM) in buffers of 100 mM lactic acid and various concentration of L-His containing 30 mM HP-β-CD: (a) pH 2.4; (b) 1 mM His, pH 2.5; (c) 2.5 mM His, pH 2.6; and (d) 5 mM His, pH 2.75. Other condition as for Fig. 1.

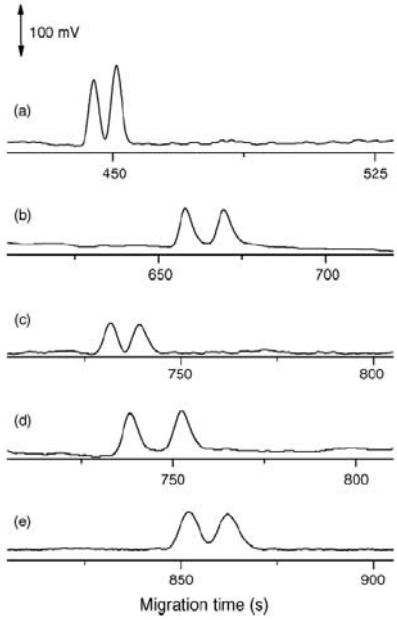

Fig. 3. Enantiomeric separation of: (a) doxylamine using 40 mM HP-β-CD in 100 mM lactic acid and 5 mM L-His at pH 2.75; (b) ephedrine; (c) noradrenaline; (d) isoproterenol using 30 mM HP-β-CD in 100 mM lactic acid with 5 mM L-His at pH 2.75; and (e) propranolol using 10 mM HP-β-CD in 60 mM lactic acid with 7.5 mM L-His at pH 3.06. All at 100 μM. Other conditions as for Fig. 1.

of histidine had a much less pronounced effect. Again, an effect on the peak height is noted.

The optimized buffer system (lactic acid, histidine, and HP-β-CD) was then applied to the separation of several further basic drugs. In Fig. 3, the enantiometric separation of doxylamine, ephedrine, isoproterenol, noradrenaline, and propanolol is shown. At the conditions employed, a good separation of all samples could be achieved. The resolution values, R, were determined as 1.34, 0.95, 1.21, 1.36, 0.96, 0.94 for adrenaline, doxylamine, ephedrine, isoproterenol, noradrenaline, and propanolol, respectively.

In enantiomeric separations it is often important to accurately measure the enantiomeric excess (ee). The separation of pseudoephedrine, as a further example, at enantiomeric ratios of 99:1 and 1:99 is illustrated in Fig. 4. The resolution for the separation of lS,2S-(+)- and lR,2R-(−)-pseudoephedrine was determined as $R = 1.86$. The quantitative results for pseudoephedrine are reported in Table 1 and are showing a good accuracy. The detection limits for the two enantiomers of pseudoephedrine were determined as 3.0×10^{-7} M and 3.8×10^{-7} M for the lS,2S-(+)- and lR,2R-(−)-pseudoephedrine, respectively. Calibration curves were linear up to at least 1.0×10^{-4} M, that is over more than two orders of magnitude, using peak areas for quantitation. The

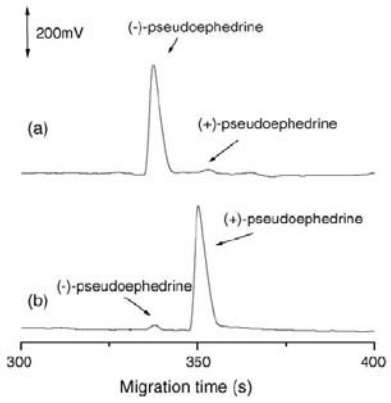

Fig. 4. Enantiometric separation of lS,2S-(+)- and lR,2R-(−)-pseudoephedrine at ratios of 1:99 and 99:1. Total concentration: 100 μM. Buffer: 20 mM lactic acid and 20 mM HP-β-CD at pH 2.45. Other conditions as for Fig. 1.

R-values of the regression analysis were 0.9989 and 0.9999 for the + and − forms, respectively.

3.2. Amino acids

Although enantiomers of amino acids have been separated with cyclodextrins, the reported results appear to be limited to species bearing a phenol group or to species which had been labelled for UV- or fluorescence detection [8]. Our attempts to carry out the separation of underivatized amino acids with HP-β-CD were not successful, presumably due to unsufficient lipophilicity of the analytes. For this reason the chiral crown ether (+)-(18-crown-6)-2,3,ll,12-tetracarboxylic acid ($I8C6H_4$) was adopted as chiral selector. This use of this compound had previously been reported for the successful separation of some amino acids [28–31]. These applications had, however, been limited to UV-absorbing amino acids, or necessitated analyte derivatization or undirect detection with a chromophore added to the buffer.

The successful enantiomeric separation of six underivatized amino acids using $I8C6H_4$ and contactless conductivity detection is illustrated in Fig. 5. Note that aspartic acid is one of the species. It was reported previously that this compound could not be separated using $I8C6H_4$ and the failure was ascribed to electronic repulsion between the crown ether

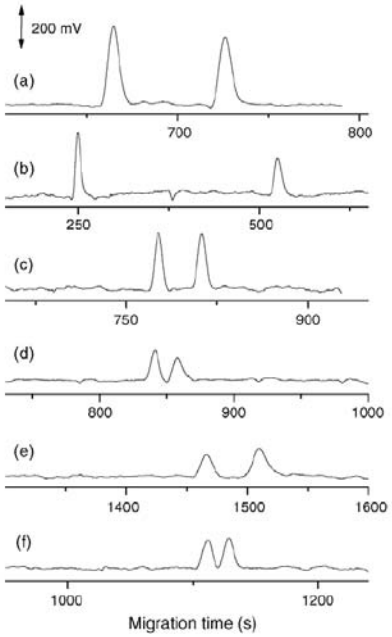

Fig. 5. Enantiomeric separation of amino acids at 250 μM using 10 mM $I8C6H_4$ in 25 mM citric acid at pH 2.1: (a) arginine; (b) serine; (c) threonine; (d) methionine; (e) tryptophan; and (f) aspartic acid. Other conditions as for Fig. 1 except for serine (separation at 25 kV).

and the side chain of the amino acid [30]. Enantiomeric separation was achieved only after one of the carboxylic acids of the amino acid was blocked by derivatization to form an ester. However, our result indicates that the previous explanation is not sufficient. A possible interaction between the acidic part of aspartic acid and benzyl trimethyl ammonium chloride, which had been included in the buffer for indirect UV-detection, might have to be taken into account.

The method is not restricted to the species shown in Fig. 5. A total of nine amino acids, including examples from all classes of these species, were investigated. The following *R*-values were obtained: arginine 3.1, valine 1.2, serine 7.7, phenylalanine 1.5, tyrosine 1.7, aspartic acid 1.1, threonine 2.3, methionine 1.1 and tryptophan 1.9. Note the particularly strong separation of the enantiomers of serine. The reason for this is not known. The separation was therefore successful for

Table 1
Determination of the enantiomeric ratio for lS,2S-(+)-and lR,2R-(−)-pseudoephedrine

Ratio 1R,2R to 1S,2S	1R,2Rin 1S,2S		Bias (%)	Ratio 1S,2S to 1R,2R	1S,2S in 1R,2R		Bias (%)
	Added	Found			Added	Found	
1: 99	0.0101	0.019	+88	1: 99	0.0101	0.018	+80
10: 90	0.1111	0.119	+6.8	15: 85	0.1765	0.182	+2.8
25: 75	0.3	0.318	+6.0	25: 75	0.300	0.320	+6.6
50: 50	1	1.05	+5.0	50: 50	1.00	0.948	−5.2

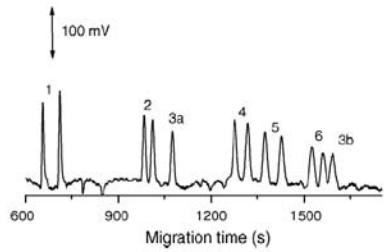

Fig. 6. Enantiomeric separation of a mixture of amino acids (2 mM) using 10 mM $I8C6H_4$ in 10 mM citrate/Tris at pH 2.2: (1) arginine; (2) valine; (3a and 3b) serine; (4) phenylalanine; (5) tyrosine; (6) aspartic acid. Other condition as for Fig. 1.

all species studied, and it is expected that the approach can be extended to all amino acids. The detection limits ranged between 2.5 and 20 μM for the species examined. The concurrent enantiomeric separation of a mixture of six amino acids is illustrated in Fig. 6. Good resolution was achieved in all cases.

4. Conclusions

Contactless conductivity detection was found suitable for enantiomeric separations in capillary electrophoresis and to our knowledge this is the first report on this application. The method is particular useful for non-UV-absorbing compounds as derivatization is not needed and any possible complications due to dyes added for indirect optical detection can be avoided. The enantiomeric separation of the non-absorbing amino acids demonstrated is thus unprecedented in its simplicity. Absorbing and non-absorbing species may also be determined in the same run, due to the universality of the conductometric detection method.

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2. 2 Enantiomeric separation of 1-phenylethylamine and 1-cyclohexylethylamine

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Short communication

Enantiomeric separation of 1-phenylethylamine and 1-cyclohexylethylamine in capillary electrophoresis with contactless conductivity detection

Xiao Yang Gong, Peter C. Hauser*

Department of Chemistry, University of Basel, Spitalstrasse 51, 4004 Basel, Switzerland

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Abstract

Contactless conductivity detection was employed for the detection of the enantiomers of 1-phenylethylamine and 1-cyclohexylethylamine which were separated in capillary electrophoresis with unprecedented high resolutions R_s of 2.3 and 3.3, respectively, by using a combination of dimethyl-β-cyclodextrin and the chiral crown ether $18C6H_4$ as chiral selectors in a citric acid buffer of pH 2.4. The conductivity measurement enabled the direct detection, i.e. without having to derivatize or resort to indirect methods, of all species including the non-UV-absorbing enantiomers of cyclohexylamine. Detection limits of 0.5 μM were achieved and the determination of enantiomeric ratios of up to 99:1 was found possible.

Keywords: Enantiomeric separations; 1-Phenylethylamine; 1-Cyclohexylethylamine; Capillary electrophoresis; Contactless conductivity detection; Cyclodextrin; $18C6H_4$

1. Introduction

Analytical enantiomeric separations have become more important in recent times as there is a continuing trend towards the use of enantiomerically pure compounds [1]. Quantitative determinations are often carried out by HPLC, which however requires the use of specially designed columns with immobilized chiral selectors. Furthermore the sample throughput is commonly not adequate [2]. Capillary electrophoresis (CE) is an attractive alternative method. Enantiomeric separation is achieved by simply dissolving a small amount of a chiral selector in the buffer solution, and the analysis times are significantly reduced. This has therefore become an important field of application of capillary electrophoresis (for recent reviews see [3–7]). The most commonly employed chiral selectors are derivatives of cyclodextrins (CDs) and the chiral crown ether 18-crown-6-tetracarboxylic acid ($18C6H_4$).

The development of a method for the enantiomeric separation of the optical isomers of 1-phenylethylamine (α-methylbenzylamine) and 1-cyclohexylethylamine is reported herein. These compounds have been widely used as intermediates for industrial asymmetric synthesis. Their enantiomeric separation is difficult due to the shortness of the substituents on the chiral centres and to our knowledge, only a few reports on CE-methods for the phenylethylamines have appeared [8–12]. Only Mori et al. [10] and Armstrong et al. [11] achieved a useful resolution. The enantiomeric separation of the 1-cyclohexylethylamines was studied solely by Reetz et al. [12], using fluorescence detection, as the compounds are not accessible by the UV-detection employed by the other workers. However, Reetz et al. needed to derivatize the analytes.

The limitations of optical detection can be overcome with the relatively new method of axial capacitively coupled contactless conductivity detection (C^4D). This C^4D detector was introduced in 1998 and consists of a pair of short tubular electrodes which encompass the capillary [13,14]. The conductivity is probed by applying a sine voltage to the first electrode and picking up the resulting cell current at the second electrode. The chief advantages of this detector are its universality to all ionic species, its robustness and its low cost. It has been thoroughly characterized and its operation is straightforward [15–19]. The main requirement for a sensitive and stable output is the use of buffers of low conductivity. Many applications to inorganic as well as organic cations and anions have been reported (for recent reviews see [20,21]). It has also been shown recently by our group that this mode of detection is useful in the electrophoretic

* Corresponding author. Tel.: +41 61 2671003; fax: +41 61 2671013.
E-mail address: peter.hauser@unibas.ch (P.C. Hauser).

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separation of enantiomers [22]. Non-UV-absorbing amino acids could be analysed directly with good sensitivity without having to resort to chemical derivatization for fluorescence detection.

2. Experimental

2.1. Instrumentation

The capillary electrophoresis instrument was purpose-built around a commercial high voltage power supply module (CZE 2000R, Start Spellman, Pulborough, UK). The detector is based on two electrodes of 4 mm length, consisting of steel tubing with an internal diameter of about 400 μm, and a detection gap of 1 mm. A sine wave voltage of 100 kHz and an amplitude of 400 V_{pp} (peak-to-peak) was used for cell excitation. The cell current was converted to a voltage, which was then rectified, low-pass filtered and digitized with a MacLab/4e data acquisition system (AD Instruments, Castle Hill, Australia). More details can be found elsewhere [23,24]. All electropherograms were inverted for presentation of the peaks in the normal orientation.

2.2. Reagents and methods

All chemicals were of analytical reagent grade and were obtained from Fluka (Buchs, Switzerland) with the exception of hydroxypropyl-β-cyclodextrin (HP-β-cyclodextrin) and dimethyl-β-cyclodextrin (DM-β-CD), which were purchased from Acros (Geel, Belgium). All solutions were degassed by ultrasonication and filtered through 0.2 μm nylon filters before use. The fused-silica capillary of 10 μm I.D. and 375 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) and total and effective lengths of 48 and 43 cm was preconditioned with a 0.1 M sodium hydroxide solution before rinsing with water followed by flushing with the running buffer. Sample injection was carried out electrokinetically at 5.0 kV for 7 s, the separation voltage was 15 kV unless stated otherwise. Standard solutions were obtained by diluting stock solutions (containing 20 mM of the amine in water) with the background electrolyte solution to ensure injection under non-stacking conditions.

3. Results and discussion

3.1. Cyclodextrins

Reetz et al. [12] reported at least partial separation for both compounds with different β- and γ-cyclodextrins at pH 9.1. Our attempts with seven different CDs, i.e. native α-, β-, and γ-cyclodextrins, HP-α-, β-, and γ-cyclodextrins, and DM-β-cyclodextrin, with different buffer solutions between pH-values of 2.4 and 9.1 were futile for both compounds. The reason for this discrepancy must be the fact that Reetz et al. had derivatized the analytes with fluorescein in order to render them accessible to their detection technique and it can be assumed that interaction of the fluorescein moiety with the cavity of the cyclodextrin was taking place.

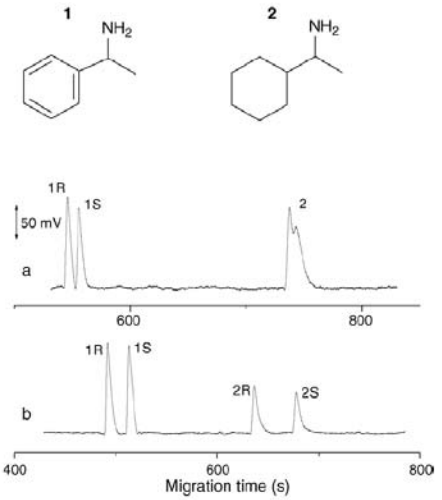

Fig. 1. Electropherograms for a mixture of (*R/S*)-1-phenylethylamine (1) and (*R/S*)-1-cyclohexylethylamine (2) for different conditions: (a) 25 mM citric acid with 10 mM DM-β-CD and 5 mM 18-crown-6; (b) in 25 mM citric acid with 5 mM DM-β-CD and 5 mM 18C6H_4 (pH 2.4).

3.2. Cyclodextrins with 18-crown-6

The combination of a cyclodextrin and the non-chiral crown ether 18-crown-6 has reportedly led to successful enantiomeric separations where cyclodextrins on their own have not been adequate, possibly through the formation of ternary, sandwich-type complexes [9,11,25,26]. As illustrated in Fig. 1a, we were able to reproduce the results reported by Armstrong et al. [11] with DM-β-CD, and an R_s value of 0.8 was achieved with HP-β-CD and 18-crown-6 in our case. The conductivity detection employed by us also allowed the examination of 1-cyclohexylethylamine but none of the combinations of 18-crown-6 with either of the cyclodextrins HP-α-CD, HP-β-CD, HP-γ-CD and DM-β-CD was found to lead to a good resolution of this non-aromatic compound.

3.3. Chiral crown ether (18C6H_4)

The chiral crown ether (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18C6H_4) was found not to be adequate for the enantiomeric separation of either of the two species. Kuhn et al. [8] also unsuccessfully attempted the same approach for the phenylethylamine enantiomers, but note that Mori et al. [10] succeeded in separating the isomers of 1-phenylethylamine in an organic, rather than aqueous, solvent employing the chiral crown ether.

3.4. Cyclodextrins with 18C6H_4

The combination of cyclodextrins with the chiral crown ether is not common but has been reported before for the enantiomeric

Table 1
Determination of enantiomeric ratios for (*R*/*S*)-1-cyclohexylethylamine and (*R*/*S*)-1-phenylethylamine using a background electrolyte solution of 25 mM citric acid containing 5 mM DM-β-CD and 5 mM 18C6H$_4$

Ratio *R*-to-*S*	Expected	Found[a]	Bias (%)	Ratio *S*-to-*R*	Expected	Found[a]	Bias (%)
1-Cyclohexylethylamine							
1:99	0.010	0.018	+80	1:99	0.010	0.019	+85
25:75	0.333	0.35	+4	25:75	0.333	0.34	+2
50:50	1.000	1.02	+2	50:50	1.000	0.98	−2
1-Phenylethylamine							
1:99	0.010	0.018	+82	1:99	0.010	0.019	+90
25:75	0.333	0.35	+6	25:75	0.333	0.32	−5
50:50	1.000	1.02	+1	50:50	1.000	0.96	−2

[a] Ratio of peak areas.

separation of a few compounds [27–29]. Our investigation of using the chiral crown ether together with DM-β-CD showed that this combination can indeed be used for the enantiomeric separation of both, the optical isomers of 1-phenylethylamine and of 1-cyclohexylamine. The results are illustrated in Fig. 1b. As evident, very good baseline resolution is obtained for both compounds. The corresponding R_s values are 2.3 and 3.3 for 1-phenylethylamine and 1-cyclohexylethylamine, respectively. In case of 1-phenylethylamine the separation is clearly better than what can be obtained with the non-chiral crown ether in combination with DM-β-CD as reported above. The application of this combination was then tested for quantification of the enantiomers. Calibration curves were acquired from 2 μM to 0.2 mM and a linear response was found for at least this range. As shown in Fig. 2 for the non-aromatic compound, it is possible to detect the enantiomers in ratios of 99:1, allowing the determination of large enantiomeric excess (ee) values. The results for the quantitative determination of different ratios for the enantiomers of both compounds are given in Table 1. The detection limits (3× signal-to-noise ratio) were determined as 0.5 μM for all four species. Due to the lack of published data, a comparison of this value with those obtained by other capillary electrophoresis methods is not possible, but it is generally comparable to what is achieved with direct UV-absorption detection for aromatic compounds.

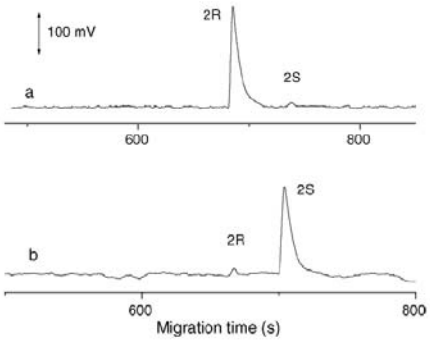

Fig. 2. Electropherograms for 0.2 mM (*R*/*S*)-1-cyclohexylethylamine (2) in 25 mM citric acid with 5 mM DM-β-CD and 5 mM 18C6H$_4$ (pH 2.4). (a) R 99%, S 1%; (b) R 1%, S 99%.

4. Conclusions

The use of a combination of two chiral selectors, the chiral crown ether and DM-β-cyclodextrin, led to unprecedented good enantiomeric separations of 1-phenyethylamine and 1-cyclohexylethylamine. Conductivity detection allowed the measurement of the non-aromatic cyclohexylamine without having to resort to optical methods based on dye displacement or derivatization. This is, to our knowledge, the first report on the separation of the underivatized enantiomers of this compound in capillary electrophoresis. The combination of the efficient separation approach and the impedance method for detection is expected to also lead to the facile enantiomeric analysis of other small, non-UV-absorbing amines, which to date has been carried out only with difficulty.

Acknowledgements

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2. 3 Determination of different classes of amines with CE-C^4D

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Xiao Yang Gong
Peter C. Hauser

Department of Chemistry,
University of Basel,
Basel, Switzerland

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Research Article

Determination of different classes of amines with capillary zone electrophoresis and contactless conductivity detection

The use of contactless conductivity detection for the determination of different organic amines in CE was successfully demonstrated. Aliphatic nonUV-absorbing species could be determined along absorbing compounds by measuring the conductivity of their protonated forms. The species tested included short-chained aliphatic primary, secondary and tertiary amines, branched aliphatic amines, diamines, hydroxyl-substituted amines as well as species incorporating aromatic and nonaromatic cyclic moieties. Highest sensitivity was obtained with BGE solutions containing solely acetic acid. A concentration of 0.5 M at a pH value of 2.5 was used. Detection limits were in the order of 1 μM. Complete separation of *cis*- and *trans*-1,2-diaminocyclohexane could be achieved by adding 18-crown-6 as modifier to the electrolyte solution.

Keywords: Amines / Capillary electrophoresis / Contactless conductivity detection / Dimethylamine / Methylamine DOI 10.1002/elps.200500423

1 Introduction

CZE has certain advantages compared to the longer established method of HPLC, such as reduced sample and reagent volumes and lower cost of the separation columns. Currently, most commonly detection for CE is carried out by UV-absorption and fluorescence measurements featured by commercial instruments. As electrophoresis is based on ionic separations, the universal detection method for ions, conductivity measurement, is an attractive alternative. Indeed, for ion chromatography, conductimetric detection is the norm. However, for CE conductometric detection has long seen relatively little attention. The reason for this was the difficulty to construct small cells which match the internal diameters of separation capillaries (typically 50 μm) and the potential interference by the electric field applied for separation. A contactless conductivity detector, which overcame these limitations, had been available for ITP for some time but was not suitable for the narrower capillaries usually required for zone electrophoresis [1]. The introduction of a new contactless conductivity detector for CZE by Zemann *et al.* [2] and Fracassi da Silva and do Lago [3] in 1998 changed this situation. This detector is based on two tubular electrodes placed on the capillary along its axis for capacitively coupling an excitation signal into the capillary and read-out of the conductivity-dependent cell current. The fundamental characteristics of this arrangement have since been thoroughly studied [4–9] and are straightforward. Recent reviews are available [10–12].

Initial studies on applications of this new type of detector have focussed on inorganic species as these can otherwise only be detected by indirect optical methods and their small size leads to high detection sensitivity. However, it became apparent in recent years that the detector is also well suited for organic ions of different classes, such as carboxylic acids [13, 14], sulfonic acids [15], quaternary amines [3, 15, 16], amino acids [17–19], catecholamines [20], basic drugs [21] and polysaccharides, which were ionized at high pH values [22]. The detection limits are typically about 1 μM. This is comparable with the common UV-absorption, but not quite as good as the commercially available LIF detection, which allows detection limits which are one to two orders of magnitude lower. However, for most analytes fluorescence detection requires derivatization which is not possible for all species. The main strength of conductometric detection is its universality, which enables the concurrent determination of ionizable organic species even if they are not accessible by the optical methods.

Correspondence: Professor Peter C. Hauser, Department of Chemistry, University of Basel, Spitalstrasse 51, CH-4004 Basel, Switzerland
E-mail: peter.hauser@unibas.ch
Fax: +41-61-2671013

Different classes of organic amines are of relevance in many areas. Biogenic amines such as catecholamines are important in neuroscience. Small amines such as dimethylamine, trimethylamine, 1,5-diaminopentane and histamine need to be determined in food such as fish and wine. Other amines are important precursors and catalysts in organic synthesis. Many of these species do not possess aromatic moieties and therefore are only poor UV-absorbers. In CE such compounds have therefore been detected by indirect UV-absorption [23], after derivatization [24] or *via* chemiluminescence [25]. Contactless conductivity detection is an attractive and simpler alternative. Previous reports on the use of this detection methods for amines have been limited to amino acids [17–19], a few catecholamines [20], and several basic drugs [21]. A detailed investigation of the detection of further classes of amines is reported herein.

2 Materials and methods

2.1 Instrumentation

Separations were performed with a purpose-made instrument featuring a 30-kV-high-voltage power supply with dual polarity (CZE 2000R) from Start Spellman (Pulborough, UK). The contactless conductivity detector is based on two tubular electrodes of 4 mm length which are separated by 1 mm and a Faradaic shield. Excitation is achieved with a sine-wave of a peak-to-peak amplitude of 450 V and a frequency of 100 kHz. The cell current was amplified, rectified and low pass filtered with a circuitry described elsewhere [15, 26] and passed on to a MacLab/4e data acquisition system (AD Instruments, Castle Hill, Australia), which was used to record the electropherograms. The traces for Figs. 3, 5, and 7–9 have been inverted to show the peaks in the usual positive-going orientation. The reported detection limits are the concentrations giving peak heights corresponding to three times the baseline noise.

2.2 Reagents and methods

All chemicals were of analytical reagent grade. 3-Amino-1-propanol, 2-amino-2-methyl-1-propanol, (1*S*,2*R*)-2-amino-1,2-diphenylethanol, (1*R*,2*S*)-(+)-*cis*-1-amino-2-indanol, *R*-(−)-2-amino-3-methyl-1-butanol, L-prolinol, butylamine, histamine dihydrochloride, *trans*-1,2-diaminocyclohexane, *cis*-1,2-diaminocyclohexane, 3-cyclohexyl-D-alanine hydrate, dopamine, ammonium, methylamine, dimethylamine, trimethylamine, acetic acid, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-Tris), 2-(*N*-morpholino)-ethanesulfonic acid (MES), L-histidine (His), L-arginine and 18-crown-6 were obtained from Fluka (Buchs, Switzerland). 1,3-Dimethylbutylamine, 2-heptylamine and 2-octylamine were obtained from Acros (Geel, Belgium) and ethyl-3-aminobutyrate, 1,2-dimethylpropylamine, 1-cyclohexyl-ethylamine, 1-benzyl-ethylenediamine and 2-adamantanamine hydrochloride were purchased from Aldrich (Buchs, Switzerland). 2-Aminononane and (*S*)-1-amino-2-propanol were purchased from Lancaster (Frankfurt am Main, Germany). All solutions were degassed by ultrasonication and filtered through 0.2 μm nylon filters before use. Fused-silica capillaries of 25 μm ID and 375 μm OD were used for the separations. These were purchased from Polymicro Technologies (Phoenix, AZ, USA) and were preconditioned with a 0.1 M sodium hydroxide solution before flushing with water followed by flushing with the running buffer. All capillaries had a total length of 60 and 55 cm effective length. Sample injection was carried out manually with the help of a stopwatch by applying 5.0 kV to the sample vial for 7 s; the separation voltage was 15 kV unless stated otherwise. Standard solutions were diluted with BGE solution to ensure injection under nonstacking conditions.

3 Results and discussion

First experiments were conducted with simple aliphatic amines. As the pK_a values of these compounds are all well over 9, the species are present in the protonated cationic form over a wide pH range and correspondingly a range of buffers may be employed for their separation. For conductivity detection buffers of low conductivity must be chosen to minimize Joule heating as the temperature coefficient of the measured parameter is high. A very high conductivity results in baseline instability. In our experience the requirement in this regard is more stringent than for CE in general and for this reason capillaries with a relatively narrow internal diameter of 25 μm were employed. On the other hand, for capillaries with even smaller diameters a deterioration in the S/N has to be expected [27]. The electropherograms for a mixture of methylamine, ethylamine, *n*-butylamine and 2-heptylamine obtained in five different buffers of pH 8.3, 6.0, 4.0, 3.0 and 2.5 are given in Fig. 1. Clearly, the compounds are well separated following the order of increasing size, and the determination is possible in all buffers as expected. However, some striking differences are apparent. For the first three electropherograms the peaks are all positive-going and the peak heights are decreasing with size of the ion, whereas for the last two electropherograms the peaks are negative-going, taller but all similar in size. Comparable patterns are obtained for the separation of a mixture of ammonium, methylamine, dimethylamine and trimethylamine in the same buffer solutions as shown in Fig. 2.

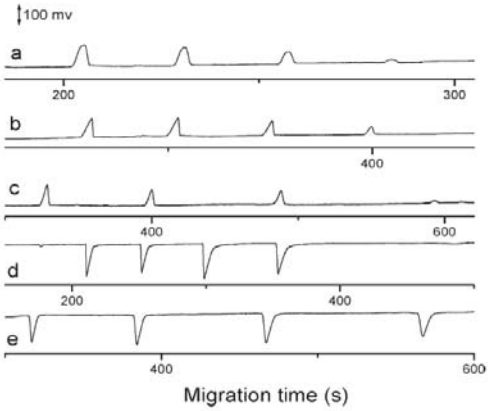

Figure 1. Electropherograms of a mixture of methylamine, ethylamine, *n*-butylamine and *n*-heptylamine (in the respective order) at 100 μM in different BGE: (a) 15 mM arginine and 10 mM acetic acid, pH 8.3; (b) 10 mM MES/His, pH 6.0; (c) 50 mM acetic acid and 10 mM Bis-Tris, pH 4.0; (d) 25 mM acetic acid, pH 3.0; (e) 0.5 M acetic acid, pH 2.5.

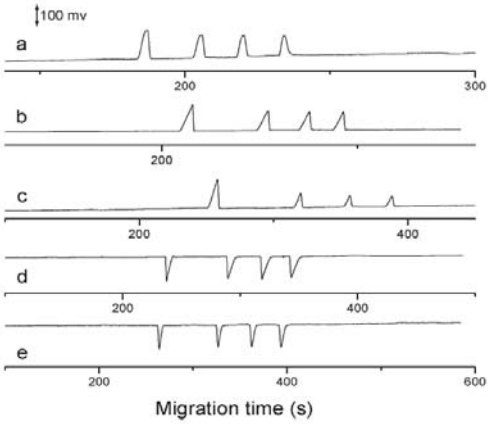

Figure 2. Electropherograms of a mixture of ammonium, methylamine, dimethylamine and trimethylamine (in the respective order) at 100 μM in different BGE solutions: (a) 15 mM arginine and 10 mM acetic acid, pH 8.3; (b) 10 mM MES/His, pH 6.0; (c) 50 mM acetic acid and 10 mM Bis-Tris, pH 4.0; (d) 25 mM acetic acid, pH 3.0 and (e) 0.5 M acetic acid, pH 2.5.

This behavior can be rationalized by examining the response mechanism in conductivity detection. The presence of buffer ions leads to a baseline signal which is always present but its magnitude is modified by the presence of bands of analyte ions causing the peaks. The analyte zone is composed of cations in the present case. In a simplified view (which does not consider components of the BGE solution or the sample that are not completely ionized, nor electromigrative peak dispersion) analyte cations displace buffer cations to maintain a balance of overall charge neutrality with the buffer anions. As buffer cations and analyte cations possess different limiting equivalent conductivities, this leads to the measured conductivity changes. As the first three buffer solutions contain relatively large buffer cations, which therefore have relatively low limiting equivalent conductivities, positive-going peaks are obtained for the small analytes. As the difference diminishes with increasing size of the analyte ions, the peaks become smaller. For the last two BGE solutions, the cations are protons which have very high limiting equivalent conductivities. Displacement of the protons by the analyte cations therefore leads to tall negative-going peaks with minimal variation in peak height. For a more detailed discussion of the response mechanism and quantitative predictions see [10, 28].

The acidic buffers are thus preferable for the determination of the amines because of the high and uniform sensitivity. Intrinsically the direction of the peak is of no consequence and there is no disadvantage in the fact that the peaks are negative-going in these solutions. Also note that the terms direct and indirect detections are not meaningful in this context. Other BGE solutions may be used if the amines are to be determined concurrently with other compounds which require different pH values, albeit at reduced sensitivity.

Extension to amines with branched hydrocarbon chains is possible as evidenced by the electropherogram of Fig. 3, which includes several such species with differing chain lengths determined in an acetic acid-based electrolyte

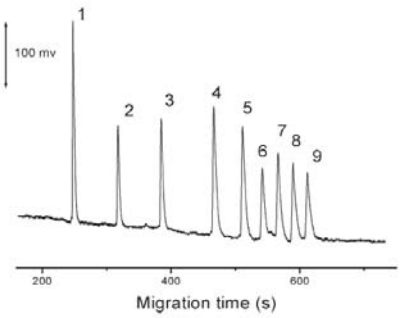

Figure 3. Electropherogram of a mixture of 100 μM of each (1) ethylenediamine, (2) methylamine, (3) ethylamine, (4) *n*-butylamine, (5) 1,2-dimethylpropylamine, (6) 1,3-dimethylbutyamine, (7) 2-heptylamine, (8) 2-octylamine and (9) 2-nonanylamine in 0.5 M acetic acid, pH 2.5.

solution. Note that the traces have been inverted for this and the subsequent electropherograms to follow the usual convention of positive-going peaks. Please refer to Fig. 4 for the structures of the compounds. Note that also ethylenediamine is included in the standard mixture illustrating the possibility of determining this industrially important diamine species by this means. In Fig. 5, a separation of a number of amines with aromatic moieties is shown. The structures are also given in Fig. 4. The interest in their determination lies in their biological activity: although some play a natural role in human physiology (specifically in cell metabolism and growth), high concentrations can have deleterious effects on health. Histamine, for example, is important in food analysis (*e.g.* in fish and wine), nicotinamide is an important metabolite and dopamine is a catecholamine representative here also for other neurotransmitters with similar structure.

The method can also be extended to small aliphatic amino alcohols as shown in Fig. 6. The structures of the compounds are given in Fig. 7. Small organic species

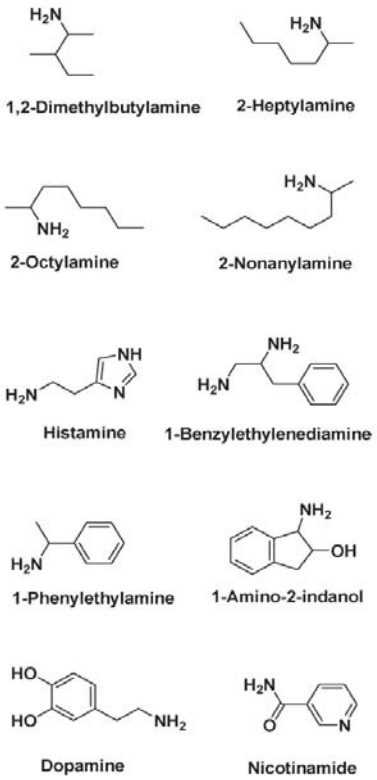

Figure 4. Structures of some of the species determined for Figs. 3 and 5.

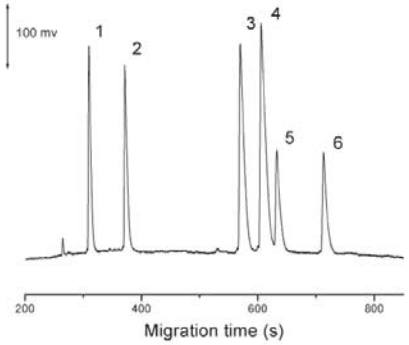

Figure 5. Electropherogram of a mixture of 100 μM each of (1) histamine, (2) 1-benzylethylenediamine, (3) 1-phenyl-ethylamine, (4) 1-amino-2-indanol, (5) dopamine and (6) nicotinamide in 0.5 M acetic acid, pH 2.5.

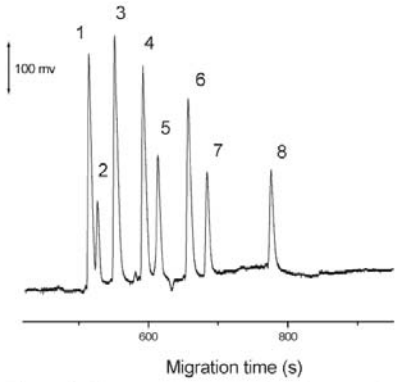

Figure 6. Electropherogram of a mixture of 100 μM each of (1) 3-amino-1-propanol, (2) 1-amino-2-propanol, (3) prolinol, (4) 2-amino-2-methyl-1-propanol, (5) 2-amino-3-methyl-1-butanol, (6) 1-amino-2-indanol, (7) dopamine and (8) nicotinamide in 0.5 M acetic acid, pH 2.5.

such as these amino alcohols as well as diamines are widely used in the pharmaceutical industry as building blocks in synthesis and have, in recent years, also gained importance as catalysts [29]. In Fig. 8a, the separation of some more complex amines, which include nonaromatic ring moieties in their structures, is shown. The structures of the molecules can also be seen in Fig. 7. It is evident that complete separation is not possible for the last two peaks corresponding to 2-adamantanamine and 1-cyclohexyl-ethylamine are not completely resolved. However, this can be improved by addition of the crown ether 18-crown-6 as evidenced by the electropherogram of Fig. 8b. 18-Crown-6 is frequently used in the separation of small inorganic cations by CE due to its ability to

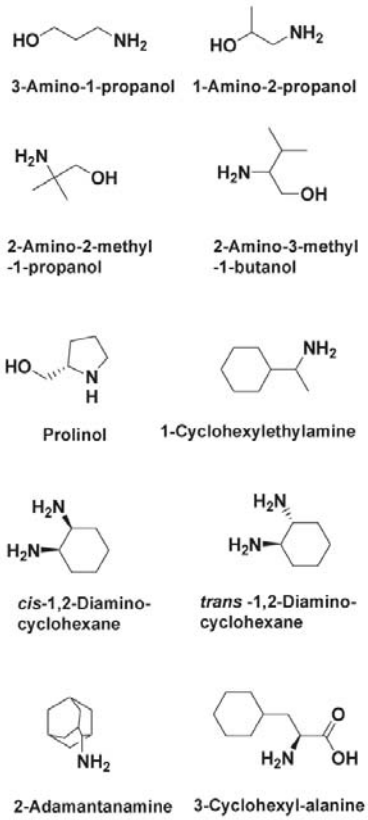

Figure 7. Structures of some of the species determined for Figs. 6, 8 and 9.

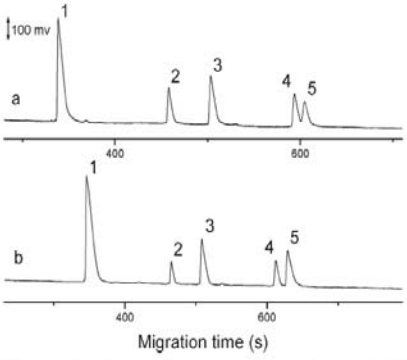

Figure 8. Electropherogram of a mixture of 100 μM each of (1) *cis*-1,2-diaminocyclohexane, (2) 3-cyclohexyl-D-alanine-hydrate, (3) prolinol, (4) 2-adamantanamine, (5) 1-cyclohexylethylamine in (a) 0.5 M acetic acid, pH 2.5 and (b) 0.5 M acetic acid with 50 mM 18-crown-6, pH 2.5.

modify their electrophoretic mobility by complexation. Chiou and Shih [30] have demonstrated the use of this additive to improve the separation of some amines as well. This approach can also be used to separate *cis*- and *trans*-1,2-diaminocyclohexane, compounds which are important in stereoselective synthesis. As can be seen from Fig. 9a, a mixture of the two species cannot be resolved at all without additive. The concentration of crown ether of 50 mM employed for Fig. 8 is not adequate, but an increase to 200 mM leads to an adequate separation of the two isomers as shown in Fig. 9b.

Essential calibration data for a range of compounds representing different classes of amines is given in Table 1. The detection limits were found to be all around 1–2 μM and the determination is possible up to at least 1 mM. A precision in peak area of typically 5% was achieved. Please note that this is limited by the manual injection method employed in our case and better precision can be expected for a fully automated system.

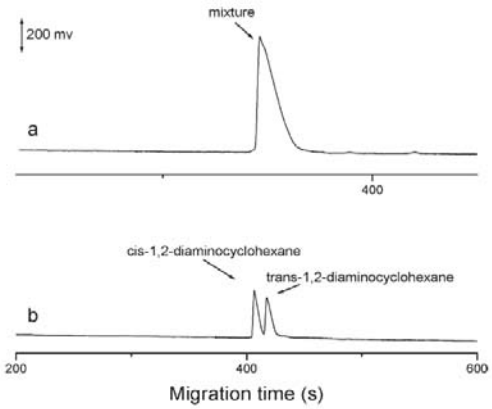

Figure 9. Electropherograms of a mixture of 100 μM each of *cis*-1,2-diaminocyclohexane and *trans*-1,2-diaminocyclohexane in (a) 0.5 M acetic acid and (b) 0.5 M acetic acid with 200 mM 18-crown-6, pH 2.5.

Table 1. Calibration data for the determination of amines

	r	RSD,%	LOD, μM
Ammonium	0.989	5.5	2.1
Histamine	0.998	5.4	2.4
Ethylamine	0.996	5.3	2.1
3-Amino-1-propanol	0.999	3.8	1.1
L-Prolinol	0.9999	7.9	2.7

r: correlation coefficient for calibration curves from 0.1 to 1 mM; RSD: relative standard deviation for the peak areas of injections of 100 μM, $n = 5$; LOD: limit of detection, concentrations corresponding to peak heights 3× the baseline noise.

4 Concluding remarks

Contactless conductivity detection was found suitable for a wide range of amines. The use of a buffer that contains protons as the only background cation yielded best sensitivity. The method is versatile and allows the concurrent determination of UV-absorbing as well as nonabsorbing species. The detection limits are generally better then those achieved with indirect UV detection [23] and comparable to direct UV detection where this is possible. The method was also found compatible with the use of the crown ether 18-crown-6, a useful additive to improve selectivity of the separation.

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2. 4 Enantiomeric separation of underivatized amines

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Xiao Yang Gong
Peter C. Hauser

Department of Chemistry,
University of Basel,
Basel, Switzerland

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Research Article

Enantiomeric separation of underivatized small amines in conventional and on-chip capillary electrophoresis with contactless conductivity detection

The determination of the enantiomers of small non-UV-absorbing amines which otherwise can only be achieved with difficulty was possible by using a combination of the chiral crown ether (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid ($18C_6H_4$) and dimethyl-β-CD as selectors in CE and contactless conductivity measurement for detection. Alkylamines without any other functional group, amino alcohols, species with ether or ester groups and with a cyclic moiety were investigated. The detection limits were found to be about 1.0 μM and the determination is possible up to at least 1.0 mM. The determination of enantiomeric ratios of up to 99.5:0.5 was also found feasible.

Keywords: Amines / Capillary electrophoresis / Contactless conductivity detection / Enantiomeric separation
DOI 10.1002/elps.200600258

1 Introduction

Small chiral aliphatic amines constitute important building blocks and intermediates in the synthesis of pharmaceuticals, plant protecting agents, and fragrances and there has also been a trend toward the increased use of such amines as enantiomeric catalysts instead of the more traditional metallic compounds [1]. The determination of the enantiomeric purity of these compounds is therefore becoming increasingly important. These analyses are often carried out by HPLC, using special separation columns with immobilized chiral selectors. These usually have to be developed for the task at hand and therefore are expensive. Also, the analysis times with HPLC are long and thus the sample throughput is often not adequate for high-throughput screening of the enantiomeric excess (ee) in asymmetric catalysis [2].

Due to its high versatility, high efficiency, short analysis time, and low cost, CE represents an attractive alternative to HPLC and also to GC for the separation of enantiomers. Chiral selectors can be dissolved in the relatively low volumes of separation buffer required and the preparation of chiral columns is thus not necessary. CDs, a chiral crown ether, macrocyclic antibiotics, proteins, and chiral surfactants have been used as reagents to achieve the separation of enantiomers in CE (see for example these recent reviews [3–7]).

In the analysis of aliphatic amines by separation methods, detection is a challenge as these compounds usually do not have strong UV-absorbance nor fluorescence and thus direct quantification with the established detection methods is not possible. In HPLC, chemical derivatization to enable optical detection is most often employed or alternatively MS detection may be used. Relatively few publications have appeared on the determination of this class of compounds by CE. Detection had to be carried out either by derivatization for absorbance (*e.g.*, using salicylaldehyde-5-sulfonate [8]) or fluorescence (*e.g.*, using FITC [9]), or indirectly *via* displacement of a dye of the same charge which is added to the BGE solution (*e.g.*, using imidazole [10] or copper ions [11]).

Contactless conductivity detection has been demonstrated as an alternative detection method for enantiomeric separations in CE which is suitable for the direct determination of UV-absorbing and nonabsorbing species [12]. This approach eliminates the need for the chemical derivatization of compounds and the resulting complications, and also allows better detection limits

Correspondence: Professor Peter C. Hauser, Department of Chemistry, University of Basel, Spitalstrasse 51, CH-4004, Basel Switzerland
E-mail: peter.hauser@unibas.ch
Fax: +41-61-2671013

Abbreviation: DM-β-CD, dimethyl-β-CD

than indirect UV-absorption methods. The applicability of this type of detector for the direct sensitive quantification of a range of small aliphatic amines in nonenantiomeric separations has also been shown [13]. Furthermore, the efficient separation and conductometric detection of the enantiomers of the non-UV-absorbing 1-cyclohexylethylamine by using a combination of two chiral separators, namely dimethyl-β-CD (DM-β-CD) and the chiral crown ether (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid ($18C_6H_4$), was very recently also demonstrated in preliminary work published as a short communication [14]. The contactless conductivity detector employed was based on a design independently introduced in 1998 by Zemann *et al.* [15] and Fracassi da Silva and do Lago [16], which features two tubular external electrodes arranged axially along the capillary. The advantages of this detector for CE are its universality for charged species, its high sensitivity, as well as simplicity and inertness to electrode fouling. Recent reviews on its applications are available [17, 18], as are detailed studies on its working principles [19–25].

In this publication, we report on the direct enantiomeric separation of various aliphatic and saturated cyclic amines and related hydroxylamines, amino ester and amino ethers and also demonstrate a fast enantiomeric separation in a microfabricated lab-on-chip device.

2 Materials and methods

2.1 Instrumentation

Separations on conventional capillaries were carried out with an instrument constructed in-house using a 30 kV-high-voltage power supply with dual polarity (CZE 2000R) from Start Spellman (Pulborough, UK). Fused-silica capillaries of 25 μm id and 375 μm od, which were purchased from Polymicro Technologies (Phoenix, AZ, USA), were used. The detector consisted of two tubular electrodes of 4 mm length which are separated by a gap of 1 mm and a Faradaic shield. A sinusoidal voltage with a peak-to-peak amplitude of 400 V and a frequency of 200 kHz was used for cell excitation. The detector current was amplified, rectified, and low pass filtered [26, 27] and the signal acquired with a MacLab/4e system (AD Instruments, Castle Hill, Australia). The electrophoresis microchips were obtained from the Microfluidic Chip Shop (Jena, Germany), consisted of polymethylmethacrylate (PMMA) and had dimensions of 90 mm × 16 mm. The separation channel was 85 mm in length (75 mm from the injection cross). The depth and width of this channel was 50 μm. The chip was placed on a holder made from PMMA which was fitted with a pair of antiparallel orientated electrodes of 1 mm width, 1.4 cm length, and with a detection gap of 0.5 mm. A vertical Faraday shield was placed between the excitation and pick up electrodes in order to minimize direct capacitive coupling. The excitation frequency was set to 600 kHz and the amplitude to 20 Vpp (peak to peak). The chip experiments were conducted using two high-voltage power supplies (CZE 1000R; Start Spellman), which were controlled with a multifunctional I/O card (PCI-MIO-16 XE-50; National Instrument, Austin, TX, USA) and a LabVIEW program (National Instrument). The data were also acquired and analyzed with a Maclab/4e system.

2.2 Reagents and methods

All chemicals were of analytical reagent grade. 4-Methylpentan-2-amine, 3-methylbutan-2-amine, 1-methoxypropan-2-amine, heptan-2-amine, octan-2-amine, nonan-2-amine, and dimethyl-β-CD were purchased from Acros (Geel, Belgium). 5-Methylheptan-2-amine, 6-methylheptan-2-amine, hexan-2-amine, 2-aminobutan-1-ol, prolinol, 2-aminopropan-1-ol, 1-aminopropan-2-ol, ethyl 3-aminobutanoate, alanine ethyl ester hydrochloride, and alanine methyl ester hydrochloride, and (THF-2-yl)methanamine were purchased from Lancaster (Eastgate, England). Histidine, 2-cyclohexylalanine hydrate, (±)-*trans*-cyclohexane-1,2-diamine, (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid and acetic acid were purchased from Fluka (Buchs, Switzerland). All solutions were degassed by ultrasonication and filtered through 0.2 μm nylon filters before use. The fused-silica capillaries were preconditioned with a 0.1 M sodium hydroxide solution before flushing with water followed by flushing with the running buffer. All capillaries had a total length of 60 cm and 55 cm effective length. Sample injection was carried out electrokinetically at 5.0 kV for 7 s, the separation voltage was 15 kV unless stated otherwise. Standard solutions were diluted with BGE solution to ensure injection under nonstacking conditions. The chip was rinsed with water (5 min) and conditioned with running buffer for 10 min before the first measurement. Electrokinetic injection was conducted at 1 kV for 1 s, and the separation was achieved at 2 kV. Microchips were flushed with running buffer between runs to ensure reproducibility and maintain a stable baseline. All experiments were performed at a room temperature of 22 ± 1°C. The reported detection limits are the concentrations giving peak heights corresponding to three times the baseline noise.

3 Results and discussion

The enantiomeric separation of a range of aliphatic amines was tested and the structures of all substances are given in Table 1. The selection made includes a varia-

Table 1. Enantiomeric resolution of amines (at 100 μM) using an electrolyte solution of 0.5 M acetic acid containing 5 mM DM-β-CD and 5 mM $18C_6H_4$, a fused-silica capillary of 25 μm id and 48 cm total and 43 cm effective lengths, and a separation voltage of 15 kV

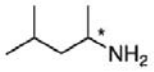
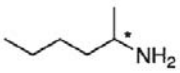
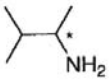
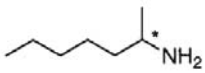
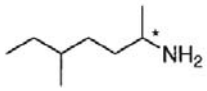
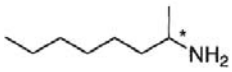
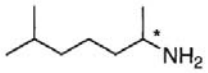
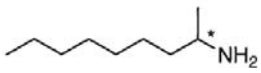
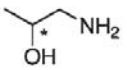
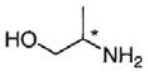
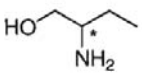
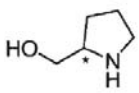
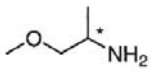
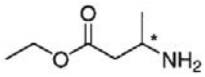
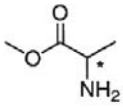
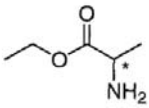
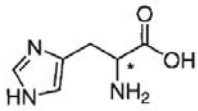
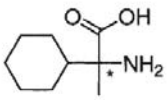
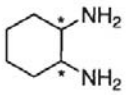
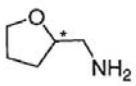
No.	Compound	Structure	Migration time, *t* (min)	Separation factor, α	Resolution, R_s
1	4-Methylpentan-2-amine		8.3	1.008	1.4
2	Hexan-2-amine		9.8	–	–
3	3-Methylbutan-2-amine		10.6	1.018	1.6
4	Heptan-2-amine		12.3	1.017	1.5
5	5-Methylheptan-2-amine		13.3	1.021	1.9
6	Octan-2-amine		15.3	1.016	1.5
7	6-Methylheptan-2-amine		15.8	1.028	2.6
8	Nonan-2-amine		16.8	1.013	1.5
9	1-Aminopropan-2-ol		7.2	1.037	2.8
10	2-Aminopropan-1-ol		9.5	1.026	1.3
11	2-Aminobutan-1-ol		6.7	1.032	1.5
12	Prolinol		5.5	–	–

Table 1. Continued

No.	Compound	Structure	Migration time, *t* (min)	Separation factor, α	Resolution, R_s
13	1-Methoxypropan-2-amine		7.9	1.013	1.2
14	Ethyl 3-aminobutanoate		6.9	1.028	1.7
15	Alanine methyl ester		7.3	–	–
16	Alanine ethyl ester		8.9	–	–
17	Histidine		5.3	1.152	5.1
18	2-Cyclohexylalanine		14.2	1.007	3.9
19	*trans*-Cyclohexane-1,2-diamine		8.7	1.090	6.0
20	(THF-2-yl)-methanamine		9.2	–	–

tion in size, different degrees of branching of aliphatic chains, cyclic compounds, and species with additional functional groups. The choice was partly dictated by the commercial availability of compounds. A classification with increasing complexity can arbitrarily be made as follows: (i) alkylamines without any other functional group; (ii) alkylamines with a hydroxyl group (*i.e.*, amino alcohols); (iii) alkylamines with ether or ester groups, and (iv) alkylamines with a cyclic moiety. On the basis of the excellent enantioresolution obtained in preliminary work for 1-cyclohexylethylamine [14] with the combination of dimethyl-β-CD and the chiral crown ether (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (subsequently abbreviated as $18C_6H_4$), this binary selector approach was adopted again. A BGE solution consisting of acetic acid was employed. The low pH-value of this solution (pH 2.45) assures protonation of the analytes rendering them in cationic form, and good sensitivity is obtained with this buffer which does not contain cations other than protons. Acetic acid was used rather than citric acid as reported previously [14], due to to the somewhat better baseline stability obtained.

3.1 Separation of alkylamines without a further functional group

First the effect of the chiral selectors was tested individually. The addition of 5 mM of DM-β-CD, which interacts with the lipophilic end of the analytes, to the acetic acid BGE solution was not effective as not even partial separation of any of compounds 1–8 could be observed. This is in agreement with the observation that these selectors on their own work best with aromatic analytes [5]. The use of the chiral crown ether $18C_6H_4$, which interacts with the amine group of the molecules, on its own did also not lead to a separation of the enantiomers of the alkylamines. On the other hand, as in our preliminary work on 1-cyclohexylethylamine [14], the combination of the two selectors at 5 mM each was found to be highly effective for chiral recognition for each racemic amine, except for hexan-2-amine. The separation factors, α, and resolution values, R_s, are given in Table 1. As shown in Fig. 1, it is even possible to carry out the enantiomeric separation at baseline resolution of all seven amines concurrently. It is presumed that most likely the formation of weak sandwich complexes, in which the amine functionality of the analyte interacts with the crown ether and the lipophilic end of the molecule with the cavity of the CD, is responsible for the selective modification of the electrophoretic mobility and hence the separation of the enantiomers [28]. The relatively short unbranched hydrocarbon chain on hexan-2-amine must not provide sufficient steric hindrance for selectivity in the interactions of the two enantiomers with the CD cavity. Despite the interaction with the chiral selectors, the relative peak heights and the retention times of the species separated for Fig. 1 can be correlated with the size of the molecules, the smallest ones giving highest sensitivity and having highest electrophoretic mobility.

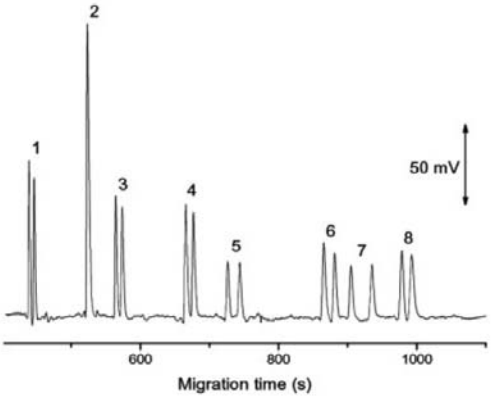


Figure 1. Electropherogram illustrating the concurrent separation of the enantiomers of aliphatic amines (compounds 1–8 in Table 1) at 100 μM in a BGE solution of 0.5 M acetic acid with 5 mM of DM-β-CD and 5 mM of (+)-$18C_6H_4$, pH 2.45. Capillary: fused silica (48/43 cm × 25 μm id), separation voltage: 15 kV.

3.2 Separation of alkylamines with hydroxyl group

Four amino alcohols, compounds 9–12 in Table 1, were examined. For the noncyclic compounds, 1-aminopropan-2-ol, 2-aminopropan-1-ol, and 2-aminobutan-1-ol, partial separation could be achieved with $18C_6H_4$ alone. However, only for 1-aminopropan-2-ol an acceptable resolution, with an R_s value of 1.8, could be achieved. The combination of DM-β-CD and $18C_6H_4$ led again to a further improved resolution for all three compounds. The separation parameters are listed in Table 1. As an example, the electropherogram of each enantiomer of 1-aminopropan-2-ol and 2-aminopropan-1-ol, which are structural isomers, is shown in Fig. 2. The fact that a separation of the enantiomers of prolinol could not be achieved can be attributed either to it being a secondary amine, which results in weak interaction with $18C_6H_4$ [29] or to steric hindrance.

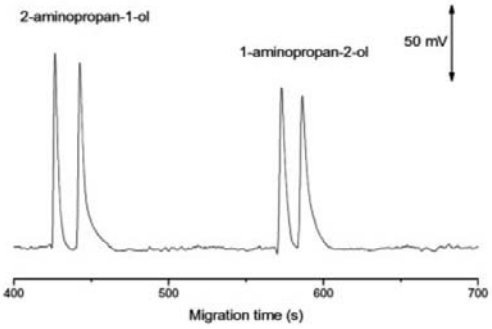


Figure 2. Electropherogram of the concurrent separation of the enantiomers of 2-aminopropan-1-ol and 1-aminopropan-2-ol at 100 μM. Conditions as for Fig. 1.

3.3 Separation of alkylamines with ether or ester groups

One compound which contains an ether functionality besides the amine group, and three esters were also examined, see compounds 13–16 in Table 1. Again, the use of the chiral crown ether on its own was insufficient. Partial resolution only was achieved for 1-methoxypropan-2-amine (R_s = 0.9) and ethyl 3-aminobutanoate (R_s = 1.1) with this separator. When using the combination of DM-β-CD and $18C_6H_4$, the separation for these two

compounds was improved with R_s values of 1.2 and 1.7, respectively. Baseline separation of the enantiomers of both compounds concurrently is illustrated by the electropherogram of Fig. 3. On the other hand, for the methyl and ethyl esters of alanine not even partial separation could be achieved for the chiral crown ether on its own nor the combination of the two chiral selectors.

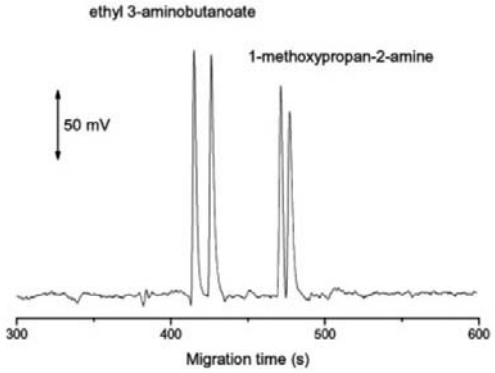


Figure 3. Electropherogram of the concurrent separation of the enantiomers of ethyl 3-aminobutanoate and 1-methoxypropan-2-amine at 100 μM. Conditions as for Fig. 1.

3.4 Separation of alkylamines with a cyclic moiety

Four alkylamines which incorporate aliphatic or aromatic cyclic groups, namely histidine, 2-cyclohexylalanine, *trans*-cyclohexane-1,2-diamine, and (THF-2-yl)methanamine (compounds 17–20 in Table 1), were also investigated. With the chiral crown ether alone, it was found that enantiomeric separation was not achieved for histidine and (THF-2-yl)methanamine, partial separation for 2-cyclohexylalanine (R_s = 1.0), and good baseline separation for *trans*-1,2-diaminocyclohexane (R_s = 2.3). When employing the combination of the two selectors excellent baseline separation (with R_s values of between about 4 and 6) of the enantiomers of three of the compounds, namely histidine, 2-cyclohexylalanine, and *trans*-1,2-diaminocyclohexane, was observed. The concurrent separation of the enantiomers of 2-cyclohexylalanine and of *trans*-cyclohexane-1,2-diamine is shown in Fig. 4. The lack of enantiomeric separation for (THF-2-yl)methanamine is thought to be resulting from the fact that the chiral center is not directly connected to the amine group, which is consistent with the findings of Hilton and Armstrong [30] on the enantioselectivity of the chiral crown ether in LC for small peptides.

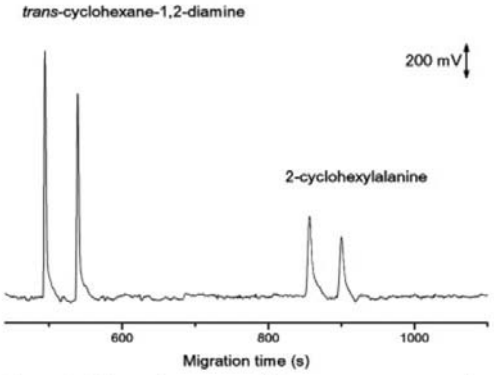


Figure 4. Electropherogram of the concurrent separation of the enantiomers of *trans*-cyclohexane-1,2-diamine and 2-cyclohexylalanine at 100 μM. Conditions as for Fig. 1.

3.5 Quantification

Calibration curves were acquired for the enantiomers of *trans*-cyclohexane-1,2-diamine taken as a representative examples for five concentrations in the range from 0.05 to 1.0 mM. Linear regression analysis gave a slope of 535 ± 24 (r = 0.9971) for the *S*-enantiomer and a slope of 531 ± 22 (r = 0.9974) for the *R*-enantiomer. Slight nonlinearities are evident for the low and the high end of the calibration curve. The detection limits (by comparing peak height with baseline noise) for the compounds were determined as 1.0 and 1.2 μM (S/N = 3). The determination of the ee is possible to a ratio of about 0.5–99.5 as illustrated by the electropherograms of Fig. 5 and the data of Table 2. The experimentally determined enantiomeric ratios given in the table were calculated directly by

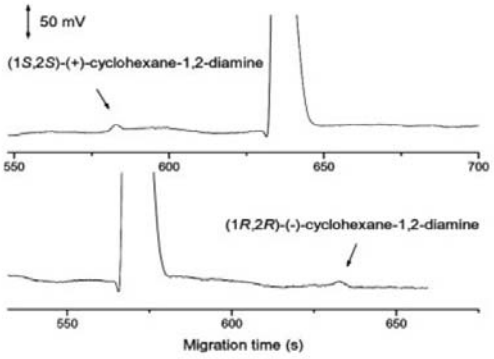


Figure 5. Electropherogram of the separation of (1*S*,2*S*)-(+)- and (1*R*,2*R*)-(−)-*trans*-cyclohexane-1,2-diamine at ratios of 0.5:99.5 and 99.5:0.5. Total concentrations: 200 μM. Conditions as for Fig. 1.

Table 2. Determination of enantiomeric ratios by comparing the peak areas for the isomers of *trans*-cyclohexane-1,2-diamine obtained by using an electrolyte solution of 0.5 M acetic acid containing 5 mM DM-β-CD and 5 mM $18C_6H_4$, a fused-silica capillary of 25 μm id and 48 cm total and 43 cm effective lengths, and a separation voltage of 15 kV

Ratio *R*-to-*S*	Expected	Found (RSD)	Bias (%)	Ratio *S*-to-*R*	Expected	Found (RSD)	Bias (%)
0.5:99.5	0.0050	0.0080 (4.0%)	+60	0.5:99.5	0.0050	0.0080 (4.8%)	+60
1.0:99.0	0.010	0.012 (3.0%)	+20	1.0:99.0	0.010	0.014 (3.1%)	+40
2.0:98.0	0.020	0.023 (2.6%)	+15	2.0:98.0	0.020	0.022 (2.8%)	+10
5.0:95.0	0.053	0.051 (1.6%)	−3.8	5.0:95.0	0.053	0.049 (1.5%)	−7.5

The total concentration of the isomers was 200 μM. $n = 4$.

ratioing the peak areas. The relatively large biases obtained for the extreme ends are due to the non-linearities of the calibration curves mentioned above. As the precision of the measurements are good (see the table), these biases can in practice be compensated for by calibration.

3.6 Electrophoresis chip

The main general advantage of electrophoresis chips, planar devices based on embedded channels for separation, is the fast analysis times which can be achieved due to their short separation lengths and efficient injection regime. The fast separation of enantiomers detected amperometrically on such devices has been demonstrated [31, 32]. Contactless conductivity detection with external electrodes has also been implemented for electrophoresis chips [33–36] but detection with this technique for enantiomeric separations has not yet been demonstrated. As is illustrated in Fig. 6, the baseline separation of the enantiomers of *trans*-cyclohexane-1,2-diamine is possible in less than 2 min on a chip, as opposed to the 8 min required on the standard capillary. Note, however, that this is only possible due to the outstanding resolution value achieved in this case (compare Table 1) with the combination of the chiral separators. The approach was not successful for other compounds for which lower R_s-values are reported in Table 1. For example, the peaks for the enantiomers of 5-methylpentan-2-amine (R_s = 1.9 on conventional capillary) were still severely overlapped. The separation efficiency is limited by the short length of the separation channel.

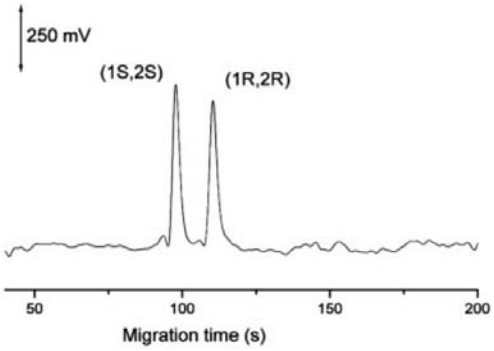


Figure 6. Electropherogram of the fast separation of (1*S*,2*S*)-(+)- and (1*R*,2*R*)-(−)-*trans*-cyclohexane-1,2-diamine at 200 μM on an electrophoresis chip with an effective separation length of 75 mm. BGE solution: 0.5 M acetic acid with 5 mM of DM-β-CD and 5 mM of (+)-$18C_6H_4$, pH 2.45. Electrokinetic injection at 1 kV for 1 s; separation voltage: 2 kV.

4 Concluding remarks

The enantiomeric separation of a range of small amines was found possible by employing a combination of the chiral crown ether $18C_6H_4$ and the CD DM-β-CD. Both chiral selectors are neutral and therefore do not interfere with the conductometric detection method which works very well for the non-UV-absorbing analytes. The general approach should be useful also for other chiral amines and optimization for different compounds should be possible by using CDs with different cavity size and different, neutral, functionalization.

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2. 5 Separating stereoisomers of di-, tri-, and tetra-peptides

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Xiao Yang Gong
Dominik Dobrunz
Michael Kümin
Markus Wiesner
Jefferson D. Revell
Helma Wennemers
Peter C. Hauser

Department of Chemistry, University of Basel, Basel, Switzerland

Original Paper

Separating stereoisomers of di-, tri-, and tetrapeptides using capillary electrophoresis with contactless conductivity detection

The separation and detection of small oligopeptides in CE with contactless conductivity detection were demonstrated. A strongly acidic separation buffer (0.5 M acetic acid) was employed in order to render the species cationic. Separation of the stereoisomers was achieved in typically 10–15 min by using either dimethyl-β-CD (DM-β-CD), (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid ($18C_6H_4$), a combination of the two substances, or of histidine, as buffer additives. Calibration curves were determined for isomers of Gly-Asp and H-Pro-Asp-NH_2, in the range of 0.05–0.5 mM and 0.1–1 mM, respectively, and were found to be linear. LODs were determined to be in the order of 1.0 μM. The determination of isomeric impurities down to about 1% was found possible. Species showing good separation could also be successfully determined on an electrophoretic lab-on-chip device, with analysis times of a few minutes.

Keywords: Capillary electrophoresis / Contactless conductivity detection / Peptides / Separation of stereoisomers

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1 Introduction

As an important class of biomolecules, peptides play a key role in nature serving for example as neurotransmitters, immunomodulators, enzyme substrates and inhibitors, coenzymes, drugs, toxins, and antibiotics. In recent years, many small peptides such as di- and tripeptides and peptidomimetics have received much attention in efforts to understand the molecular basis of natural processes and for the discovery of novel drug targets in the pharmaceutical industry [1–6]. The synthesis and production of biologically active small peptides, especially through solid phase methods, is becoming more widely used. The analysis of these peptides requires not only the determination of by-products from the synthesis, but also of their stereoisomers. The determination of the stereoisomeric, and in particular the enantiomeric, purity is an important part of their analysis. Various methods have been developed for the enantiomeric analysis of peptides such as GC [7, 8], HPLC [9–11], TLC [12, 13], and CE [14–17]. Due to its high resolution power, low consumption of analytes, chemicals and solvents, as well as the high flexibility with regard to using and changing chiral selectors, CE has proven to be a premier technique for the enantiomeric analysis of peptides. Kašička and Prusik [18] early on reviewed the application of CE to the analysis of peptides. Tran *et al.* [19] first demonstrated the separation of the enantiomers of small peptides with CE utilizing an indirect method which was based on the derivatization of the enantiomers with a stereochemically pure agent to form diastereomers. Direct separation, using a chiral crown ether as the enantiomeric selector, has been demonstrated by Kuhn *et al.* [20, 21] and Sandra and coworkers [23, 24]. Wan and Blomberg demonstrated the enantiomeric separation of many di- and tripeptides by using vancomycin and CDs as selectors, and also used 1-(9-fluorenyl)ethyl chloroformate (FLEC) and 9-fluorenylmethyl chloroformate (FMOC) as derivatization reagents [16, 25–27]. In recent years, Scriba and coworkers [28–31] developed the chiral separation of di- and tripeptides by using various CDs as selectors and studied the migration of the enantiomers at different pH values in detail. Comprehensive reviews on the enantiomeric separation of peptides in CE are available [2, 32–37].

Correspondence: Professor Peter C. Hauser, Department of Chemistry, University of Basel, Spitalstrasse 51, 4004 Basel, Switzerland
E-mail: peter.hauser@unibas.ch
Fax: +41-61-2671013

Abbreviations: C^4D, capacitively coupled contactless conductivity detection; **FMOC,** 9-fluorenylmethyl chloroformate; **PVA,** polyvinylalcohol

Quantification of peptides in CE has usually been carried out using the standard built-in UV-absorption detector. The peptide bond shows an absorbance band in the low UV range. However, most of the work reported in the literature has been carried out with peptides made up of amino acids containing aromatic groups [21, 34]. Peptides completely composed of aliphatic amino acid have often been modified with chromophoric reagents such as FMOC to improve the detection sensitivity [37].

Electrochemical detection techniques are a good alternative to optical detection, in particular capacitively coupled contactless conductivity detection (C^4D) has a number of advantages. All ionic compounds can be detected with the latter method without derivatization or other indirect approaches. Contactless conductivity detection has been adopted by a number of research groups and the successful application of this detection method for organic molecules of different classes, including amino acids and basic drugs has been reported [38–45]. Recent reviews on the applications of contactless conductivity detection are available [46–50]. It has also been demonstrated that CE-C^4D can be applied in enantiomeric separations. Methods for the enantiomeric separation of basic drugs, underivatized amino acids, and various aliphatic amines with a CE-C^4D system have been reported [51–53]. As continuation of our work on enantiomeric separations in CE with C^4D, we report here the separation of stereoisomers of underivatized peptides which are composed of amino acids without aromatic moiety.

2 Materials and methods

2.1 Instrumentation

Separations in conventional capillaries were carried out on an instrument which was built in-house and is based on a high voltage power supply with interchangeable polarity (CZE 2000R) from Start Spellman (Pulborough, UK). The contactless conductivity detector consists of two tubular electrodes of 4 mm length separated by a gap of 1 mm and a Faradaic shield. Cell excitation was carried out with a sine wave with a frequency of 300 kHz and a peak-to-peak amplitude of 400 V. The resulting current signal was amplified, rectified, and low pass filtered with a circuitry described elsewhere before passing to a MacLab/4e data acquisition system (AD Instruments, Castle Hill, Australia) for recording of the electropherograms [54, 55]. Electrophoretic microchips were purchased from the Microfluidic Chip Shop (Jena, Germany). This was placed on a purpose built holder which was carrying two planar electrodes of 1 mm width and separated by a gap of 1 mm (etched in printed circuit board material) located underneath the separation channel. Injection and separation were performed by applying voltages generated with two units from Spellman (CZE 1000R) to the short and long channels of the manifold in the form of an elongated cross. The sequence was controlled by a LabVIEW program (National Instruments, Austin, TX, USA) running on a standard PC *via* a multifunctional I/O-card (PCI-MIO-16XE-50, National Instruments). Detection was carried out by employing the same circuitry as used for standard capillaries by applying a sine wave with 25 V peak-to-peak amplitude and a frequency of 600 kHz. Further details on the chip instrumentation can be found in ref. [56].

2.2 Reagents and methods

DL-Ala–DL-Ala, DL-Ala–DL-Leu, DL-Leu–DL-Leu, Gly–DL-Ala, Gly–DL-Ala, DL-Ala–Gly, and Gly–DL-Asp were obtained from Sigma (Buchs, Switzerland); DL-Ala–Gly–Gly and DL-Ala–DL-Leu–Gly from Bachem (Bubendorf, Switzerland). The following isomers of oligo-peptides were prepared by solid phase synthesis following the standard FMOC/t-Bu protocol for peptide synthesis [57]: H–L-Pro–L-Pro–L-Asp–NH_2, H–L-Pro–L-Pro–L-Asp–O–Me, H–L-Pro–L-Pro–L-Asp–OH, (4*S*)Azp–L-Pro–L-Asp–NH_2 ((4*S*)Azp = (2*S*,4*S*)-azidoproline), (4*R*)Azp–L-Pro–L-Asp–NH_2 ((4*R*)Azp = (2*S*,4*R*)-azidoproline), H–L-Pro–L-Asp–NH_2, H–L-Pro–D-Asp–NH_2, H–L-Pro–Aib-L-Asp–NH_2, H–L-Pro–Aib-D-Asp–NH_2, H–L-Pro–L-Glu–NH_2, H–L-Pro–D-Glu–NH_2, (4*S*)Azp–(4*S*)Azp–L-Asp–NH_2, (4*R*)Azp–(4*R*)Azp–L-Asp–NH_2, H–L-Pro–L-Pro–L-Asp–D-Pro–NH_2, H–L-Pro–L-Pro–L-Asp–L-Pro–NH_2, H–L-Pro–L-Pro–D-Asp–D-Pro–NH_2, H–L-Pro–D-Pro–L-Asp–L-Pro–NH_2, H–D-Pro–L-Pro–L-Asp–L-Pro–NH_2, H–L-Pro–L-Pro–D-Asp–L-Pro–NH_2, H–L-Pro–D-Pro–L-Asp–D-Pro–NH_2, H–L-Pro–D-Pro–D-Asp–L-Pro–NH_2. (+)-(18-Crown-6)-2,3,11,12-tetracarboxylic acid ($18C_6H_4$), L-His, D-His, DL-His, acetic acid, and polyvinylalcohol (PVA) were purchased from Fluka (Buchs, Switzerland) and 2-hydroxypropyl-β-CD (HP-β-CD, degree of substitution 2–4) and dimethyl-β–CD (DM-β–CD, heptakis(2,6-di-*O*-methyl)-beta-CD) from Acros (Geel, Belgium).

All solutions were degassed by ultrasonication and filtered through 0.2 μm nylon filters before use. Untreated fused-silica capillaries of 25 μm id and 375 μm od were used for the conventional electrophoretic separations unless stated otherwise. These were purchased from Polymicro Technologies (Phoenix, AZ, USA) and were preconditioned with a 0.1 M sodium hydroxide solution before flushing with water followed by flushing with the separation buffer. All capillaries had a total length of 50 and 45 cm effective length. The coated capillary used for some of the experiments as indicated was prepared according to the following procedure: (i) the capillary was rinsed with 0.1 M NaOH for 10 min, followed by 1 M HCl for 10 min, water for 10 min, and air for 10 min; (ii) the PVA solution (10% PVA in water) was passed through the capillary for 5 min, followed by air for 5 min; (iii) the

capillary was then emptied and dried in an oven at 120°C overnight. Sample injection was carried out electrokinetically at 5.0 kV for 7 s, the separation voltage was 15 kV unless stated otherwise. Standard solutions were diluted with separation buffer to ensure injection under nonstacking conditions. The reported LODs are the concentrations giving peak heights corresponding to three times the baseline noise. The electrophoretic microchip was conditioned by flushing with the buffer solution.

3 Results and discussion

3.1 Separation of a mixture of three peptides

In order to explore the feasibility of using contactless conductivity detection in peptide separations, a first experiment was conducted with three tripeptides which are very similar in structure. The three compounds possess different moieties on one of the terminal amino acids, namely amide, ester, and acid residues: H–L-Pro–L-Pro–L-Asp–NH_2, H–L-Pro–L-Pro–L-Asp–O–Me, H–L-Pro–L-Pro–L-Asp–OH. As illustrated in Fig. 1, it was found that the three peptides could be separated and detected in a separation buffer consisting of 0.5 M acetic acid at pH 2.5. It was not necessary to add any auxiliary reagent to achieve separation. At this pH value the free amine groups at the *N*-termini of the peptides are fully protonated, the species are therefore positively charged and separated as cations. The separation order is consistent with the expected mobility of the three peptides. The last one in the sequence is the species with the free carboxylic acid, which is fractionally deprotonated at the pH value employed and thus the positive charge on the amine group is partially neutralized. The choice of dilute acetic acid as separation buffer was dictated by the need for low background conductivity. A consequence is a certain degree of mismatch of mobilities between buffer and analyte ions leading to peaks which therefore tend to show the skewing typical for CE and evident for the last peak of Fig. 1.

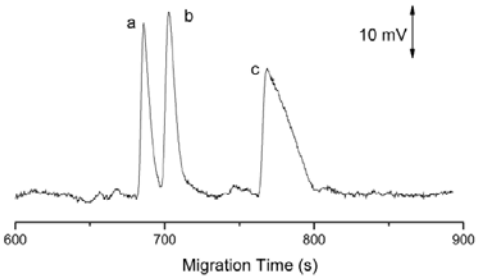


Figure 1. Electropherogram of the separation of (a) H–L-Pro–L-Pro–L-Asp–NH_2, (b) H–L-Pro–L-Pro–L-Asp–OMe, (c) H–L-Pro–L-Pro–L-Asp–OH at 0.1 mM in a separation buffer consisting of 0.5 M acetic acid. Capillary: fused-silica (50/45 cm × 25 μm id), separation voltage: 15 kV.

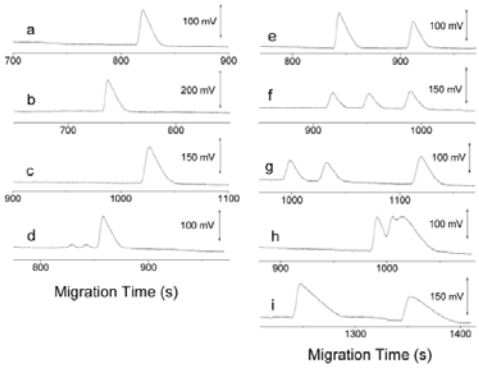


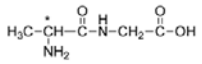
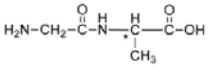
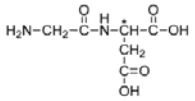
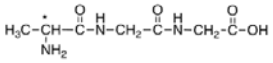
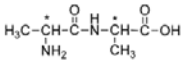
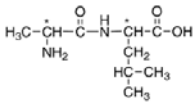
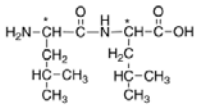
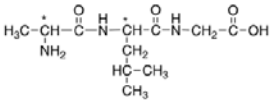
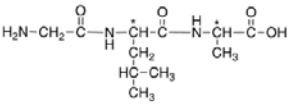
Figure 2. Electropherograms of the separation of peptides at 0.5 mM in a separation buffer consisting of 0.5 M acetic acid and containing 10 mM DM-β-CD. Other conditions as for Fig. 1. (a) DL-Ala–Gly, (b) Gly–DL-Ala, (c) Gly–DL-Asp, (d) DL-Ala–Gly–Gly, (e) DL-Ala–DL-Ala, (f) DL-Ala–DL-Leu, (g) DL-Leu–DL-Leu, (h) DL-Ala–DL-Leu–Gly, (i) Gly–DLLeu–DL-Ala (designations correspond to Table 1).

3.2 Separation of peptide enantiomers with a single chiral selector

The use of three types of chiral selectors individually, DM-β-CD, HP-β-CD, and 18C_6H_4, for the separation of peptide enantiomers was first investigated. Mixtures of the enantiomers of the following nine di- and tripeptides with one or two chiral centers were tested: (a) DL-Ala–Gly, (b) Gly–DL-Ala, (c) Gly–DL-Asp, (d) DL-Ala–Gly–Gly, (e) DL-Ala–DL-Ala, (f) DL-Ala–DL-Leu, (g) DL-Leu–DL-Leu, (h) DL-Ala–DL-Leu–Gly, (i) Gly–DL-Leu–DL-Ala. The structures of the compounds are given in Table 1.

The addition of 5 mM DM-β-CD to the separation buffer (0.5 M acetic acid) resulted in the separation of one of the two pairs of enantiomers for the dipeptides DL-Ala–DL-Leu and DL-Leu–DL-Leu in each case. The identity of the isomers could not be established as the pure compounds were not available. Enantiomeric separation for the other peptides could not be observed. As shown in Fig. 2, an increase in the DM-β-CD concentration to 10 mM led to a slightly higher separation of the two pairs which could be separated at the lower concentration and a partial separation of one of the pairs of DL-Ala–DL-Leu–Gly. There was no effect on the separation of the other enantiomers. A further increase in the concentration of the chiral selector to 20 mM did not lead to an improvement. Similar effects of the concentration of the chiral selector on the resolution of enantiomers in CE have been reported in the literature [21, 26, 58].

Table 1. Migration times (t) and resolution (R_s) for the separation of the stereoisomers of peptides using a separation buffer consisting of 0.5 M acetic acid containing 5 mM DM-β-CD and 5 mM $18C_6H_4$

Mixture of isomers	General structure	t_1 (min)	R_s
[a] DL-Ala – Gly		8.71	3.09
[b] Gly – DL-Ala		26.5	2.56
[c] Gly – DL-Asp		7.92	2.76
[d] DL-Ala – Gly – Gly		9.75	3.54
[e] DL-Ala – DL-Ala		16.49 (1) 16.79 (2) 18.18 (3) 19.13 (4)	1.73 (1, 2) 8.19 (2, 3) 5.11 (3, 4)
[f] DL-Ala – DL-Leu		13.01 (1) 13.39 (2) 14.42 (3) 15.99 (4)	4.04 (1, 2) 10.05 (2, 3) 13.85 (3, 4)
[g] DL-Leu – DL-Leu		12.37 (1) 12.98 (2) 13.14 (3) 14.64 (4)	7.74 (1, 2) 2.11 (2, 3) 17.72 (3, 4)
[h] DL-Ala – DL-Leu – Gly		10.10 (1) 10.28 (2) 11.01 (3) 11.26 (4)	2.73 (1, 2) 11.79 (2, 3) 3.79 (3, 4)
[i] GlyDL-Leu – DL-Ala		15.35 (1) 16.54 (2) 17.30 (3) 20.24 (4)	2.97 (1, 2) 1.87 (2, 3) 6.28 (3, 4)

It was found that HP-β-CD is a poorer chiral selector for the separation of these peptides than DM-β-CD. The addition of HP-β-CD (at 5 and 10 mM) to the separation buffer did not lead to any enantiomeric separation. As shown in Fig. 3, even the separation of the diasteromers (DL-Ala – DL-Leu, Gly – DL-Leu – DL-Ala, and DL-Ala – DL-Leu – Gly) was generally not as good as for DM-β-CD. Thus further optimization with this chiral selector was not attempted.

Thereafter, the use of the chiral crown ether $18C_6H_4$ as selector for the separation of these peptides was investigated. At 5 mM $18C_6H_4$ and 0.5 M acetic acid (pH 2.5), the peaks for the enantiomers of the two of the four peptides with a single chiral center were baseline separated (DL-Ala – Gly – Gly and Gly – DL-Asp) while for the other two partial separation was obtained (DL-Leu – Gly, Gly – DL-Ala). For the compounds with two chiral centers different degrees of partial separation of the four isomers were observed. Increasing the concentration of chiral crown ether from 5 to 10 mM further improved the resolutions for most of the peptides studied, but the resolution became poor for the peptides Gly – DL-Ala, and Gly – DL-Leu – DL-Ala. On the other hand, as can be seen from Fig. 4, the baseline at this concentration of this partially charged additive was not very stable, presumably due to

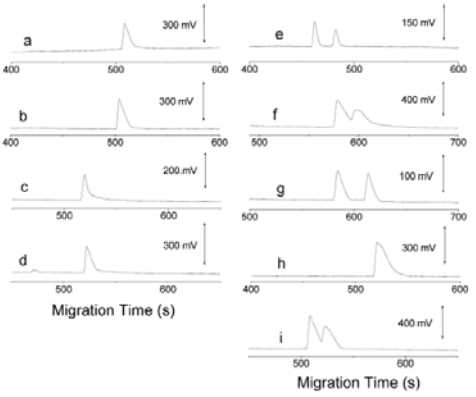


Figure 3. Electropherograms of the separation of peptides at 0.5 mM in a separation buffer consisting of 0.5 M acetic acid and containing 10 mM HP-β-CD. Other conditions as for Fig. 1 and designations according to Fig. 2 and Table 1.

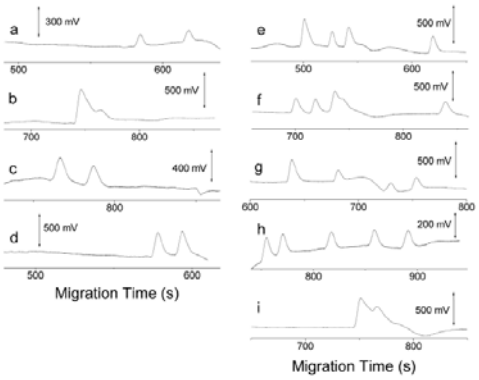


Figure 4. Electropherogram of the separation of peptides at 0.5 mM in a separation buffer consisting of 0.5 M acetic acid and containing 10 mM crown ether $18C_6H_4$. Other conditions as for Fig. 1 and designations according to Fig. 2 and Table 1.

the increase in Joule heating caused by the higher background conductivity. The degree of deprotonation of the chiral selector has an effect on the binding ability with peptides and it is therefore worthwhile to investigate the effect of the pH value of the buffer on separation. On the other hand, a low pH value is necessary to maintain the protonation of the peptides. Therefore, the range in which the concentration of free acid in the separation buffer may be varied is limited. When the pH value was changed from 2.5 to 2.1 for the two concentrations of crown ether (5 and 10 mM), the resolution for the separation of DL-Leu–DL-Leu, and DL-Ala–DL-Leu–Gly became a little better, but the resolution for the peptides DL-Ala–

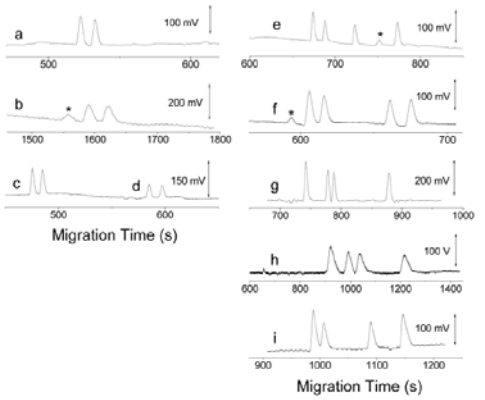


Figure 5. Electropherogram of the separation of peptides at 0.2 mM in a separation buffer consisting of 0.5 M acetic acid and containing 5 mM DM-β-CD and 5 mM $18C_6H_4$. Other conditions as for Fig. 1 except for compound (d) (PVA-coated capillary, 50/45 cm × 75 μm id) and designations according to Fig. 2 and Table 1. The asterisks denote impurities.

DL-Ala, DL-Ala–DL-Leu, Gly–DL-Ala, Gly–DL-Leu–DL-Ala became poor.

From the experiments reported above, it is clear that the chiral crown ether $18C_6H_4$ is a better chiral selector for the separation of the peptide enantiomers than DM-β-CD and HP-β-CD, but adequate resolution of the enantiomers cannot be achieved for all peptides.

3.3 Separation of peptide enantiomers with two chiral selectors

The use of a combination of two chiral selectors to achieve improved enantiomeric separations had been reported by a number of researchers [21, 24, 59–63]. The combination of chiral crown ether and CD had also been used successfully for the analysis of the enantiomers of underivatized small amines using CE with contactless conductivity detection [51, 52] and was therefore now also tested for the separation of small peptides. The chiral crown ether interacts with the amine groups through hydrogen bonds, while the CD interacts with the lipophilic end of the peptides.

First, the enantiomeric separation of di- and tripeptides containing one chiral center was investigated. A separation buffer of 0.5 M acetic acid containing 5 mM DM-β-CD and 5 mM $18C_6H_4$ was used. The separations of Gly–DL-Asp and DL-Ala–Gly–Gly (concurrently), DL-Ala–Gly, and Gly–DL-Ala are shown in Figs. 5(a–d). The four pairs of enantiomers could be separated successfully. However, for the separation of Gly–DL-Ala, a PVA-coated capillary had to be used. This modification suppresses the EOF

and hence leads to longer residence times and therefore improved separations. It had been demonstrated that the distance of the interactive amine and the stereogenic center influences the chiral recognition, the closer, the better [20]. Although the chiral center, the α-carbon, is adjacent to the amine in DL-Ala–Gly and DL-Ala–Gly–Gly, four bonds separate the chiral center from the amine in Gly–DL-Asp and Gly–DL-Ala. The di- and tripeptides containing two chiral centers were also tested. The separations of DL-Ala–DL-Leu, DL-Ala–DL-Leu–Gly, DL-Leu–DL-Leu, Gly–DL-Leu–DL-Ala, and DL-Ala–DL-Ala are also shown in Figs. 5(e–i). The four isomers of all five compounds could be separated well.

In comparison to the separation with just the chiral crown ether alone (Fig. 4), it is found that generally a better baseline separation is achieved for the combination of both selectors. For those two species which could not be separated properly, (b) Gly–DL-Ala and (i) Gly–DL-Leu–DL-Ala, now also baseline separation was obtained. Note also that the electropherograms for (b) Gly–DL-Ala, (e) DL-Ala–DL-Ala, and (f) DL-Ala–DL-Leu, reveal impurities which were not clearly resolved with just the chiral crown ether (compare Fig. 4).

The analytical data for all nine compounds obtained for the combination of the selectors is summarized in Table 1. Note that the peptide samples were obtained as racemic mixtures, the pure compounds were not available, thus the elution orders of the isomers could not be assigned.

3.4 Separation of diastereomers of catalytically active oligopeptides

In recent years, peptides have become popular as organocatalysts (for a recent review see [64]). For example, H–Pro–Pro–Asp–NH_2 is a highly active and selective catalyst for asymmetric aldol reactions. A study by Wennemers coworkers [57, 65, 66] further demonstrated that peptidic catalysts offer distinct advantages over the other small organocatalysts such as facile tunability of the selectivity by simple modifications of the primary and thereby secondary structure of peptides. For chiral catalysts, stereoisomeric purity is very important in order to obtain good enantioselectivity during the catalysis. Thus, methods for the analysis of these stereoisomers are very valuable. On the other hand, the presence of several chiral centers leads to a multitude of stereoisomers and the simultaneous analysis of all possible forms is a tedious if not an impossible task.

The separation of a number of stereoisomeric pairs of peptides (mainly of H–Pro–Pro–Asp–Pro–NH_2) was investigated. First tests were carried out with the diastereomer pair of L-Pro–L-Asp and L-Pro–D-Asp. It was found that the pair could not be separated in pure acetic acid separation buffer (at 2.3 M) nor on addition of 18C_6H_4,

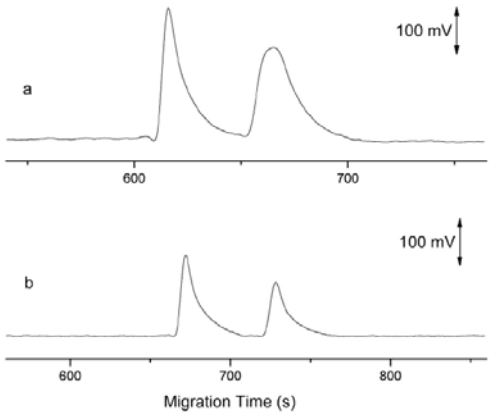


Figure 6. Electropherogram of the separation of (a) H–L-Pro–L-Pro–D-Asp–D-Pro–NH_2 and H–L-Pro–D-Pro–L-Asp–L-Pro–NH_2, and (b) H–L-Pro–D-Pro–L-Asp–D-Pro–NH_2 and H–L-Pro–D-Pro–D-Asp–L-Pro–NH_2 at 0.5 mM in a separation buffer consisting of 0.5 M acetic acid and containing 5 mM L-histidine. Other conditions as for Fig. 1.

DM-β-CD or the combination of these two separators. Partial separation could however be achieved by use of L-His as an additive (5 mM). This approach had previously been found to be useful in optimizing the separation of organic amines [67].

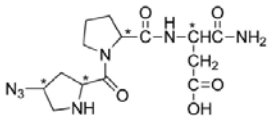
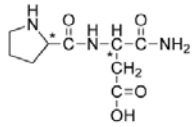
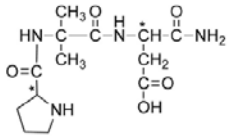
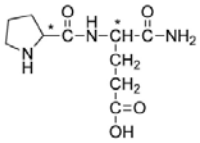
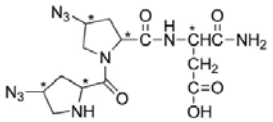
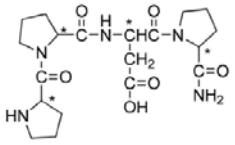
An optimization was carried out by changing the pH value of the separation buffer by setting the concentration of the acetic acid to 2.3 M (pH 2.1), 1.5 M (pH 2.2), 1.0 M (pH 2.3), and 0.5 M (pH 2.5) while keeping the concentration of L-His at 5 mM. Best separation was obtained for a concentration of 0.5 M acetic acid. Subsequently the concentration of L-His was varied between 2.5, 5, and 7.5 mM while keeping the acetic acid concentration at 0.5 M. Best resolution was obtained with the original concentration of L-His at 5 mM.

As examples, the separation of H–L-Pro–L-Pro–D-Asp–D-Pro–NH_2 and H–L-Pro–D-Pro–L-Asp–L-Pro–NH_2 and the separation of H–L-Pro–D-Pro–L-Asp–D-Pro–NH_2 and H–L-Pro–D-Pro–D-Asp-L-Pro–NH_2 are shown in Figs. 6(a) and (b), respectively. The numerical data on the resolution of these compounds as well as of a number of several other pairs tested are given in Table 2. For many of these, baseline separation was possible, while others could only be separated partially or not at all using the working conditions optimized as detailed above.

3.5 Quantification

Calibration curves were first acquired for the enantiomers of Gly–DL-Asp in the concentration range of 0.05–0.5 mM. The regression analysis for the peak areas of two

Table 2. Migration times (t) and resolution (R_s) for the separation of the stereoisomers of proline containing peptides using a separation buffer consisting of 0.5 M acetic acid containing 5 mM histidine

Mixture of isomers	General structure	t_1 (min)	R_s
H–(4*S*)Azp–L-Pro–L-Asp–NH_2H–(4*R*) Azp–L-Pro–L-Asp–NH_2		10.11	1.55
H–L-Pro–L-Asp–NH_2H–L-Pro–D-Asp–NH_2		7.96	3.44
H–L-Pro–Aib–L-Asp–NH_2H–L-Pro–Aib–D-Asp–NH_2		14.04	1.01
H–L-Pro–L-Glu–NH_2H–L-Pro–D-Glu–NH_2		8.05	no separation
H–(4*S*)Azp–(4*S*)Azp–L-Asp–NH_2H–(4*R*) Azp–(4*R*)Azp–L-Asp–NH_2		9.65	1.32
H–L-Pro–L-Pro–L-Asp–L-Pro–NH_2H–L-Pro–L-Pro–L-Asp–D-Pro–NH_2		10.34	2.48
H-L-Pro-L-Pro-D-Asp-D-Pro–NH_2H–L-Pro–D-Pro–L-Asp–L-Pro–NH_2		10.28	3.66
H–D-Pro–L-Pro–L-Asp–L-Pro–NH_2H–L-Pro–L-Pro–D-Asp–L-Pro–NH_2		13.37	3.45
H-L-Pro-D-Pro-L-Asp-D-Pro-NH_2H–L-Pro–D-Pro–D-Asp–L-Pro–NH_2		11.20	5.85

enantiomers gave straight lines through zero with slopes of $(1.06 \pm 0.03) \times 10^5$ ($r = 0.9982$) for the first enantiomer, and $(1.06 \pm 0.02) \times 10^5$ ($r = 0.9991$) for the second enantiomer. Calibration curves were also acquired for the diastereomer L-Pro–DL-Asp–NH_2 using concentrations in the range of 0.1–1.0 mM. The regression analysis for the two diastereomers gave straight lines through zero with slopes of $(3.56 \pm 0.05) \times 10^4$ ($r = 0.9994$) for L-Pro–L-Asp–NH_2, and of $(4.88 \pm 0.09) \times 10^4$ ($r = 0.9997$) for L-Pro–D-Asp–NH_2. The LODs for the compounds were determined as 1.0 and 1.2 μM (S/N = 3). As illustrated in Fig. 7, the determination of the enantiomeric excess (ee) is possible. It was found that 1% of L-Ala–Gly in a mixture with 99% D-Ala–Gly could be detected with a bias of −10%, for the analysis of 1% D-Ala–Gly in a mixture with 99% L-Ala–Gly the bias was about −40%.

3.6 Separation of peptide enantiomers on an electrophoresis chip

The main general advantage of electrophoresis chips, planar devices based on embedded channels for separation, is the fast analysis times which can be achieved due to their short separation lengths and efficient injection

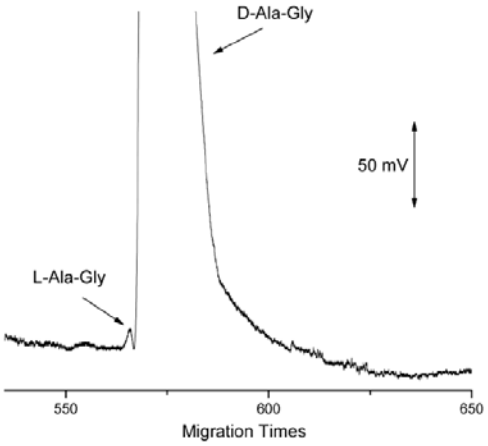


Figure 7. Electropherogram of the separation of L-Ala–Gly and D-Ala–Gly at a ratio of 1:99 (total concentration: 1 mM) in a separation buffer consisting of 0.5 M acetic acid containing 5 mM DM-β-CD and 5 mM $18C_6H_4$. Other conditions as for Fig. 1.

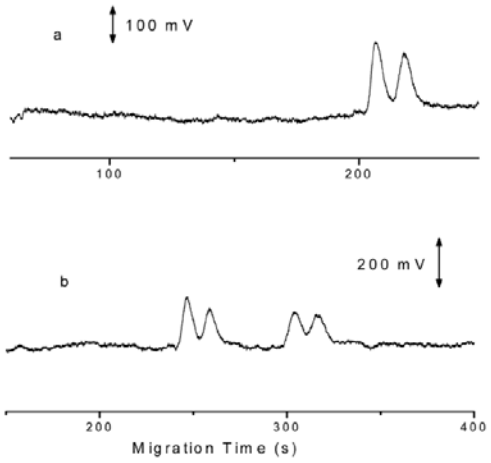


Figure 8. Electropherogram of the separation of (a) DL-Ala–Gly and (b) DL-Ala–DL-Leu–Gly at 0.2 mM on an electrophoresis chip with an effective separation length of 75 mm using a separation buffer consisting of 0.5 M acetic acid and containing 5 mM DM-β-CD and 5 mM $18C_6H_4$. Electrokinetic injection at 1 kV for 1 s; separation voltage: 2 kV.

regime. Contactless conductivity detection with external electrodes has also been implemented for electrophoresis chips [54, 68], and detection with this technique for enantiomeric separations has been demonstrated [52]. As illustrated in Fig. 8, close to baseline separation of the enantiomers was possible for the peptides DL-Ala–Gly and DL-Ala–DL-Leu–Gly. The separation times for the two peptides are 3.4 and 4.1 min, respectively, on a chip, as opposed to 8.7 and 10.1 min required on the standard capillary.

4 Concluding remarks

The determination of aliphatic di-, tri-, and tetrapeptides was found to be possible in CE using contactless conductivity detection with good LODs. Enantiomeric separations of species which lack aromatic moiety are generally more difficult, as the interaction with CDs is weak, nevertheless a variety of separations was achieved using different additives. Analyte derivatization was thus not necessary, neither for the purpose of detection sensitivity, nor for aiding the separation. The results show that one particular buffer composition is not suitable for all separation problems and individual optimization is required for each task at hand. It is hoped that the method in general will prove useful for challenging separation and detection needs in peptide chemistry.

This work was supported by project grants from the Swiss National Science Foundation (grants 200020-113335/1 and 200020-109511) and Bachem. X. Y. G. gratefully acknowledges a Marie Heim-Vögtlin subsidy (PMCD2-114322/1) from the Swiss National Science Foundation and H. W. is grateful to Bachem for an endowed professorship.

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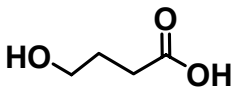
2. 6 Determination of gamma-hydroxybutyric acid (GHB) using capillary electrophoresis with contactless conductivity detection

(A manuscript in preparation)

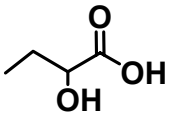
Introduction

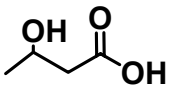
Gamma-hydroxybutyric acid (GHB) is made up of a hydroxyl group, three straight chain carbons and a carboxylic group (Scheme 1). Although its chemical structure is very simple, GHB acts as an neurotransmitter/neuromodulator via binding to the gamma-aminobutyric acid (abbreviated as GABA, an inhibiting neurotransimitter) receptor[1] and has been used as an intravenous anaesthetic agent[2] and to treat alcohol dependence[3] and sleep disorders[4]. The compound also stimulates secretion of growth hormone and has therefore been used by body builders[5]. Since 1990s, GHB is abused for recreational purpose because its effects on euphoria, sedation and disinhibition, it has also been used as a "date rape drug"[6]. For these reasons, GHB is placed in the Schedules of Controlled Substances world-wide.

Scheme I



γ-hydroxylbutyric acid (GHB)





α-hydroxylbutyric acid (AHB) **β-hydroxylbutyric acid (BHB)**

In recent years, the number of seizures of GHB is steadily increasing and GHB abuse has become a serious social problem in recent years. To develop a fast and efficient method

for the analysis of GHB is becoming important for screening purposes in clinical and forensic toxicology. The difficulties for the analysis of GHB include: i) GHB is also an endogenous substance, to recognize the presence of exogenous GHB, a cut-off level should be established[7]; ii) the small polar nature of the molecule and the lack of an UV chromophore complicate the chromatographic and spectrophotometric analysis of GHB ; iii) its short half-life of GHB in plasma (0.2-1h) and urine (3-6 hours)[8].

For analysis of GHB in urine, rapid colorimetric screening tests capable of recognizing GHB concentration larger than 100 mg/ml were reported[9, 10]. Gas chromatography (GC) with FID detection and Gas-chromatography-mass spectroscopy (GC-MS) has been mainly applied to the determination of GHB in biological fluids[11, 12]. However, because its nature of small polar molecule/small anion and its thermal instability, GHB is not directly suitable to GC. Consequently a conversion of GHB to gamma-butyrolacton (GBL) in strong acid before injection is applied. Silylation of hydroxyl and carboxyl groups has been used as well[13]. Recently capillary electrophoresis has been developed for GHB determination. The CE method was based on micellar electrokinetic capillary chromatography (MECC) with indirect detection[14, 15]. Capillary zone electrophoresis with indirect detection was reported for the quantitative determination of GHB in urine[7, 16]. The determination of GHB using dynamically coated capillaries with a running buffer of 50 mM phosphate + 3%SDS has also been investigated[17].

The aim of this work was to develop a rapid analytical method based on capillary electrophoresis with capacitively coupled contactless conductivity detection (CE-C^4D). The determination of GHB in clinical urine and serum samples was studied.

Materials and methods

Instrumentation

CE was conducted on a purpose-built instrument and is based on a high voltage power supply with interchangeable polarity (CZE 2000R) from Start Spellman (Pulborough, UK). Fused-silica capillaries (25 μm id and 375 μm od) were products of Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillary was 70 cm and the effective length was 65 cm. A function generator (Model GFG 8019G, Goodwill Instrument, Taiwan) was employed to provide a sinusoidal excitation signal. The excitation frequency was set to 300 kHz and the amplitude was boosted to 400 Vpp (peak to peak) using a purpose-made amplifier. This signal was fed to the excitation electrode of 4 mm length and an internal diameter of approximately 400 μm. This electrode was separated by a gap of 1 mm from a second identical electrode which served to pick up the cell current. An operational amplifier (OPA655, Texas Instrument, Dallas, TX, USA) fitted with a feedback resistor of 1.0 MΩ was utilized to convert the cell current to an AC voltage. This raw output voltage was then rectified, offset, amplified, and low pass filtered before passing to the data acquisition system. More details on the C^4D used have been presented previously[18, 19]. Data were acquired and analyzed with a Maclab/4e system (AD Instrument, Hastings, UK).

Reagents and samples

All chemicals were either of analytical grade or reagent grade. Sodium-gamma-hydroxylbutyric acid (sodium GHB) was obtained from Dr. Scholer (University-hospital Basel), beta-hydroxybutyric acid (BHB), alpha-hydroxybutyric acid (AHB) were

purchased from Sigma (St. Louis, MO, USA). 18-Crown-6, histidine, (S)-2-nonylamine, 2-(N-morpholino)ethanesulfonic acid (MES), 2-(N-morpholino) propanesulfonic acid (MOPS), maleic acid, cetyltrimethylammonium bromide (CTAB) were obtained from Fluka (Buchs, Switzerland). Arginine, hydroxypropyl-ß-cyclodextrin (HP-beta-cyclodextrin) and vancomycin were bought from Acros (Geel, Belgium). The human urine and blood serum samples were obtained from the Clinical Chemistry Laboratory of the University Hospital Basel. All samples were kept in a refrigerator until the experiments and were diluted 1:4 with the buffer solution prio to analyses.

Stock solutions of sodium GHB, BHB, AHB, arginine, MES, Mops, CTAB were prepared separately and stored in a refrigerator. All solutions were degassed in an ultrasonic bath and filtered through 0.2 μm nylon filters (BGB Analytik AG, Boeckten, Switzerland) before analyses.

Separation procedure

Capillaries were rinsed with 0.1 M NaOH (10 min), followed by water (10 min), and running buffer (15 min), respectively, before commencing experiments every morning or when peak distortion and poor peak shapes were observed. Standard solutions and samples were dissolved in the running buffer to ensure injection under non-stacking conditions and were introduced electrokinetically by applying 5 kV for 7 s. The reported detection limits are the concentrations giving peak heights corresponding to three times the baseline noise. Experiments were carried out at constant laboratory temperature of 25 ± 1 °C. The pH-value of all running buffers was measured using a pH-meter (model 744, Metrohm, Herisau, Switzerland).

All samples from subjects were analyzed after 4 fold dilution. The results were analyzed with calibration curve obtained from the above experiments. Reference measurements were performed at the Clinical Chemistry laboratory using standard methods based on gas chromatography.

Results and Discussion

Optimization of background electrolyte (BGE) composition

The pK_a of GHB is 4.72, thus it is best to use BGE with a pH > 5.70 to achieve full deprotonation of GHB for the detection as anion. As the approach chosen requires a slow EOF toward the anode, a small amount of CTAB (30 μM) was added to the BGE.

A BGE based on an organic acid and organic base has commonly used for the separation of small ions in CE-C^4D due to their high ionic strength, low conductivity and suitable migration rate of the co-ions and counter ions. For this reason, a different composition of BGE with pH values in the range from 5.5 - 7.0 were employed: 20 mM MES/His, pH 6.00; 25 mM MOPS / 15 mM arginine, pH 7.00; and 15 mM Arginine / 13 mM maleic acid pH 5.5. Both peak position and magnititude varied in different BGEs, it was found that a BGE based on arginine and maleic acid afforded good sensitivity.

Optimization of the pH of the background electrolyte

On the basis of the above results, the pH of BGE was further optimized by varying the ratio between arginine and maleic acid. The following compositions of arginine and maleic acid were tested in the presence of 30 μM CTAB: 15 mM Arginine/13 mM maleic acid, pH 5.7; 20 mM Arginine / 13 mM maleic acid, pH 6.14; 20 mM arigine / 10 mM maleic acid, pH 7.35; 22 mM arginine / 10 mM maleic acid, pH 8.00; 25 mM arginine /

10 mM maleic acid, pH 8.30. It was found that the combination of 20 mM arginine and 10 mM maleic acid at pH 7.35 gave the best results, and then was used for all further studies.

Internal standard selection

Alpha-hydroxybutyric acid (AHB) was employed as a suitable internal standard (I.S.) because it exhibits a similar structure and property as GHB, the trace amount of AHB present in biological fluids can be ignored.

Separation of GHB and BHB in standard and urine sample

Beta-hydroxybutyric acid (BHB) is commonly present in urine samples and difficult to be separated from GHB. To overcome this problem, different additives to the BGE were tested. Crown ether 18C6 with concentrations from 10 mM to 50 mM were tested, no good result was obtained. Addition of HP-β-CD also gave no good separation. (S)-2-Nonylamine was thought to form different salts with GHB and BHB and expected to help the separation of GHB and BHB, but it proved not successful. At last it was found that vancomycine could help to resolve two substances, and 5.0 mM vancomycine resulted in the baseline separation of GHB and BHB. The separation of a standard sample of sodium GHB and BHB at BGE of 20 mM arginine, 10 mM maleic acid, and 30 μM CTAB (pH 7.35) without and with vancomycin are shown in Figure 1.

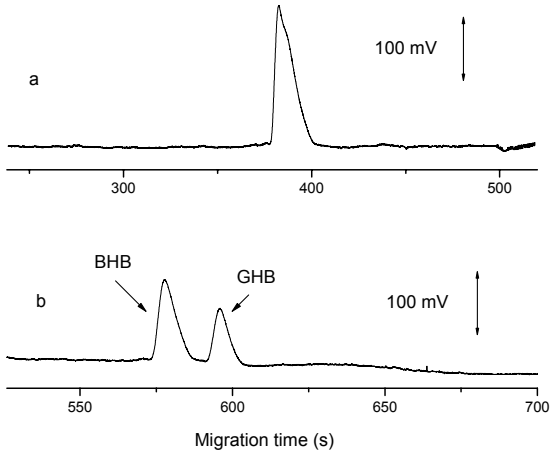


Figure 1. Electropherograms of sodium GHB and BHB. Buffer: pH 7.3. a) 20 mM of Arginine, 10 mM of Maleic acid, and 30 uM of CTAB; b) 20 mM of Arginine, 10 mM of Maleic acid, 30 uM of CTAB, and 5 mM Vancomycin; concentrations, 0.05 mg/ml. Separation condition: injection, 5 kV, 7s; separation voltage, 20 kV; capillary, length 48/43 cm, id 25 um.

Typical electropherograms of a positive urine sample and urine sample spiked with 0.03 mg/ml of GHB in BGE of 20 mM arginine, 10 mM maleic acid, and 30 μM CTAB at pH 7.35 are shown in Figure 2.

The effect of vancomycine on the separation of GHB and BHB in urine sample has also been checked, the resolution was not so ideal.

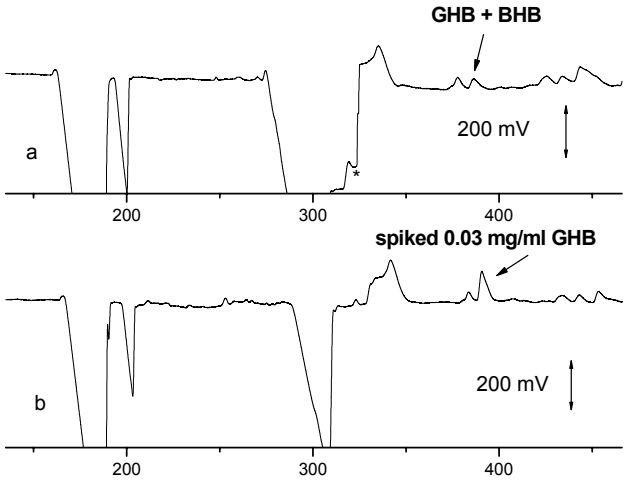


Figure 2. Electropherograms of positive urine samples with 4 fold dilution a) and with 4 fold dilution and spiked with 0.03 mg/ml of sodium GHB b). Buffer: pH 7.3, 20 mM of Arginine, 10 mM of Maleic acid, and 30 uM of CTAB. Separation condition: the same as figure 1.* System peak and unknown peak.

Effect of the length of capillary

The separation of GHB in urine sample was optimized by changing the length of capillary. With addition of vancomycin in the background electrolyte, GHB peaks are still overlapped due to the presence of the matrix in the urine sample. In this case, a longer capillary was tried to be used for the separation. It turned out that the separation of urine sample with 70 cm length of capillary is much better when compared with those of using a 48 cm length of capillary (see Figure 3).

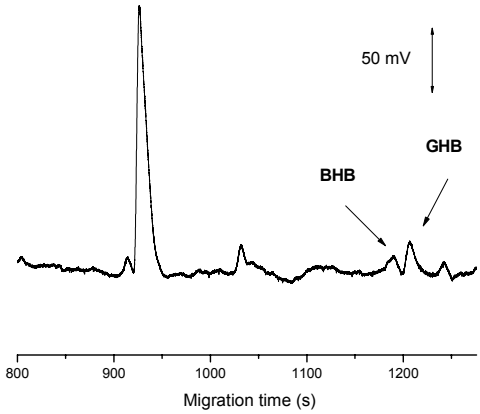


Figure 3. Electropherograms of urine positive sample of 4 fold dilution. Buffer: pH 7.3, 20 mM of Arginine, 10 mM of Maleic acid, 30 uM of CTAB, and 5 mM of Vancomycin. Separation condition: capillary length, 70/65 cm; other conditions the same as Figure 1.

Calibration curve

With the background electrolyte composition: 20 mM arginine, 10 mM maleic acid, 30 μM CTAB, 5.0 mM vancomycin, pH 7.35, a calibration curve was obtained by varying the concentration of sodium GHB. The response was calculated as the correlation of the area ratio of GHB/I.S. (AHB) vs. GHB concentration (Table 1). The studied concentration ranged from 5.0 to 150 μg/ml. Good linearity was obtained, the correlation is described by the following equation: $y = 0.022x + 0.05$ ($R^2 = 0.999$).

Detection of GHB in urine

Different urine samples obtained from the hospital stated in the experimental part were determined with the above-developed procedure. Quantitative results were calculated from the calibration curve and are summarized in Table 1.

Table 1 Analytical data of the determination of GHB in Urine samples in 20 mM arginine, 10 mM maleic acid, 0.03 mM CTAB, 5.0 mM vancomycin, pH 7.35

Samples	Concentrations of GHB (μg/ml)	
	CE-C^4D method	Reference method
Urine-1	< 10	<10
Urine-2	< 10	<10
Urine-3	32.27	24.5
Urine-4	15.96	16
Urine-5	33.16	47
Urine-6	98.34	98
Urine-7	248.51	203
Urine-8	< 10	<10
Urine-9	< 10	<10
Urine-10	< 10	<10
Urine-11	< 10	<10
Urine-12	< 10	<10
Urine-13	< 10	<10
Urine-14	< 10	<10
Urine-15	< 10	<10
Urine-16	< 10	<10
Urine-17	< 10	<10
Urine-18	< 10	<10

All samples were analyzed with internal addition method. It was found that 5 samples among 17 samples displayed intoxication cases, the others were below the endogenous concentration. It can be seen that most values determined with CE-C^4D methods generally correlate very well with those obtained by reference methods.

Analytical sensitivity

Analytical sensitivity (as limit of detection) was calculated as the lowest GHB concentration in water giving a signal-to-noise ratio >3. Under the optimized conditions, the limit of detection for the urine sample is about 0.6 μg/ml. This sensitivity is sufficient for identification of acute intoxication, the endogenous GHB concentration is below 10 μg/ml.

Detection of GHB in serum

With the above method, six serum samples were also analyzed. It turned out that three samples (Numbered as S-4 to S-6) displayed high intoxication, the other three samples (number as S-1 to S-3) were below toxic limits. (See Table 2).

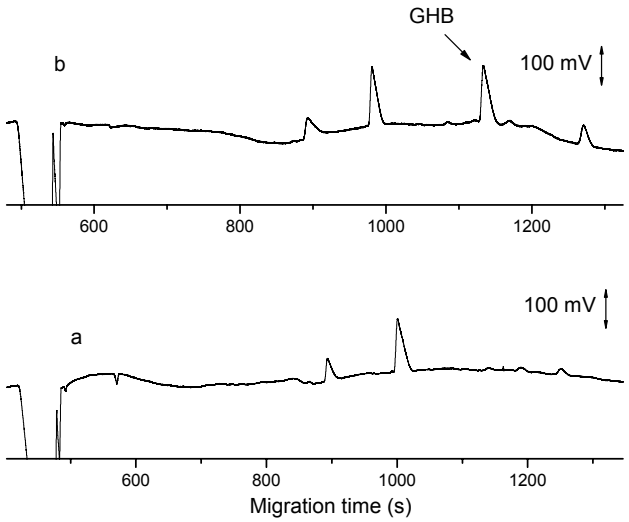


Figure 4. Electropherograms of a negative serum sample of 4 fold dilution a) and a positive serum sample of 4 fold dilution b). Buffer: pH 7.3, 20 mM of Arginine, 10 mM of Maleic acid, 30 uM of CTAB, and 5 mM of Vancomycin. Separation condition: capillary length, 70/65 cm; other conditions the same as Figure 1.

As an example, the electropherogram for samples S-3 and S-5 are shown in Figure 4. The assays for GHB in serum were also compared with the results obtained from the reference method. It can be seen that most results are comparable between the CE-C^4D method and the reference method.

Table 2 Analytical data of the determination of GHB in serum samples in 20 mM arginine, 10 mM maleic acid, 0.03 mM CTAB, 5.0 mM vancomycine, pH 7.35

Samples	Concentrations of GHB (μg/ml)	
	CE-C^4D method	Reference method
Serum-1	---	---
Serum-2	---	---
Serum-3	---	---
Serum-4	201.14	230
Serum-5	128.21	132
Serum-6	256.51	172

Summary

In this part of the work, the determination of gamma-hydroxybutyrate (GHB) with CE-C^4D, which is a recently introduced party drug and difficult to be analyzed by on-site screening, was developed. The composition of background electrolyte, the pH of background electrolyte, additives, and the length of capillary have been optimized to get good resolution. The procedure has been used to the detection of GHB in urine and serum sample.

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4 Contributions and Collaborations

In 5 papers published I am the first author as I carried out the majority of the experiments and prepared the manuscripts for publication. Various co-authors contributed to the thesis to different extents.

Jatisai Langholz-Tanyanyiwa, co-author of the first paper, introduced me to practical capillary electrophoresis and helped to the optimization of the enantiomeric separation of basic drugs.

Dominik Dobrunz, the second author of paper 5, worked on his diploma project under my supervision. He optimized the separation condition for the dipeptides. Michael Kuemin, Markus Wiesner, and Jefferson D. Revell supplied the oligomer peptides to be separated. Helma Wennemers is their supervisor.

Dr. Scholer, co-author in the manuscript to be published, from the Clinical Chemistry Laboratory of the University Hospital Basel, supplied sodium gamma-hydroxybutyric acid (GHB) and urine and serum samples. He contributed to the standard method determination of GHB in clinical samples and the project progress.

5. List of Publications and Posters

Publications

1. **Xiao Yang Gong**, Pavel Kubáň, Jatisai Tanyanyiwa, and Peter C. Hauser. Separation of enantiomers in capillary electrophoresis with contactless conductivity detection, *J. Chromatogr. A*., 2005, 1082, 230-234.
2. **Xiao Yang Gong** and Peter C. Hauser. Enantiomeric separation of 1-phenylethylamine and 1-cyclohexylethylamine in capillary electrophoresis with contactless conductivity detection. *J. Chromatogr. A*., 2005, 1094, 196-199.
3. **Xiao Yang Gong** and Peter C. Hauser. Determination of Different Classes of Amines with Capillary Zone Electrophoresis and Contactless Conductivity Detection, *Electrophoresis*, 2006, 27, 468-473.
4. **Xiao Yang Gong** and Peter C. Hauser. Chiral separation of various aliphatic amines with capillary zone electrophoresis and contactless conductivity detection, *Electrophoresis,* 2006, 27, 4375-4382.
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