# Development of Capillary Electrophoresis with Capacitively Coupled Contactless Conductivity Detection for Clinical Analysis and Related Applications

## Inauguraldissertation

zur
Erlangung der Würde eines Doctors der Philosophie
vorgelegt der
Philosophisch-Naturissenschaftlichen Fakultät
der Universität Basel

von
WORAPAN PORMSILA
aus
Thailand

Basel 2010

Genehmigt von Philosophisch-Naturissenschaftlichen Fakultät
auf Antrag von

Prof. Dr. Peter C. Hauser
und
Prof. Dr. Stephan Krähenbühl

Basel, den 16 November 2010

Prof. Dr. Martin Spiess
Dekan

# ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to many contributors for their support during my Ph.D. period since May 2007.

Firstly, I would like to thank my supervisor; Prof. Peter. C. Hauser for giving me the great opportunity to join in his research group. I sincerely appreciate his supervision and providing the CE-$C^4$D equipment throughout my research. I am truly grateful to the co-authors; Prof. Stephan Krähenbühl and Rejáne Morand for their various suggestions and assistances. My sincere gratitude also goes to the members of Division of Clinical Pharmacology & Toxicology, University Hospital of Basel for providing clinical samples throughout my research.

Secondly, I would like to thank all members in Hauser's group and contributors; Dr. Xiao Yang Gong, Dr. Aiping Schuchert-Shi, Dr. Huong Thi Anh Nguyen, Thanh Duc Mai, Benjamin Bomastyk, Marko Stojkovic, Milica Popovic. Dr. Krisnin Chansanroj, Dr. Xin Xiao Rong and Dr. Natasa Cmiljanovic for their discussions are acknowledged. Special thanks to Dr. Hong Heng See for his assistances. Thanks to Mrs. Beatice Erisman who regulated all of my official documents and to Mr. Markus Hauri who ordered the chemicals and endless helps throughout my study. I also would like to extend my recognition to all Thai friends in Switzerland who share the greatest times with me.

Thirdly, I owe to my family and boyfriend for their loves and encouragement during my stay overseas. Without their strong support, it would be difficult for me to progress my study. I would like to acknowledge Assoc.Prof.Atchana Wongchaisuwat for inspiring me to study a doctoral degree in Basel.

Finally, I would like to express my sincere gratitude to the Thai Government Scholarship through the Rajamangala University of Technology Krungthep (RMUTK) for the financial support and to the officers in RMUTK for their proceeding.

Basel, November 2010

# TABLE OF CONTENTS

**Summary** **6**

**1. Introduction** **7**

1.1. Capillary Electrophoresis (CE) 7
- 1.1.1 Historical background and development of capillary electrophoresis 7
- 1.1.2 Basic principles of capillary electrophoresis 8
- 1.1.3 Modes of capillary electrophoresis 12
- 1.1.4 Detection in capillary electrophoresis 13

1.2. Capillary electrophoresis with capacitively coupled contactless conductivity detection (CE-$C^4D$) 15
- 1.2.1 Basic principles of conductivity detection 15
- 1.2.2 Capacitively coupled contactless conductivity detection 16

1.3. Applications of capillary electrophoresis 19

1.4. Aims of this thesis 21

**2. Results and discussion** **23**

2.1. Capillary electrophoresis with contactless conductivity detection for uric acid determination in biological fluids 23
*(Anal.Chim.Acta, 2009, 224-228)*

2.2. Quantification of plasma lactate concentrations using capillary electrophoresis with contactless conductivity detection 25
*(accepted for publication in Electrophoresis)*

2.3. Capillary electrophoresis with contactless conductivity detection for the determination of carnitine and acylcarnitines in clinical samples 27
*(Submitted article to Journal of Chromatography B)*

2.4. Determination of the enantiomers of α-hydroxy- and α-amino acids in capillary electrophoresis with contactless conductivity detection 31
*(Electrophoresis, 2010, 2044-2048)*

2.5. Determination of carnitine in food and food supplements by capillary electrophoresis with contactless conductivity detection 32
*(Electrophoresis, 2010, 2186-2191)*

**3. References** **102**

**4. Curriculum Vitae** **106**

**5. List of publications and presentations** **108**

# SUMMARY

This thesis is focused on the development of capillary electrophoresis with capacitively coupled contactless conductivity detection (CE-$C^4$D) for quantification of small organic ions that are poorly UV absorbing molecules in clinical analysis and related applications.

CE-$C^4$D is suitable for clinical analysis that usually involves small sample volumes in the nanolitre range. Due to the high separation efficiency obtained in CE measuring the analyte ions in the presence of complex matrices is possible. The determination of uric acid was firstly examined and to our knowledge CE-$C^4$D had not yet been explored for the routine compound analysis. We successfully determined uric acid in plasma and urine samples in its anionic form using MES/His buffer. The detection limit was acceptable and the results obtained were well correlated with the standard enzymatic method. We further investigated the determination of non-UV absorbing molecules, namely lactate, carnitine and acylcarnitines. CE-$C^4$D proved to be suitable for the analysis of these compounds with little sample preparation and without any derivatization steps. The developed method was much simpler compared to the enzymatic as well as chromatographic methods. Plasma lactate, which is an important parameter to evaluate the anaerobic metabolism and the exercise intensity in terms of the lactate threshold, was quantified. We successfully performed the quantification of plasma lactate in its anionic form using MES/His buffer in CE-$C^4$D. The plasma lactate concentrations at different exercise conditions and the lactate threshold curve were achieved under optimum CE conditions. The results obtained by CE-$C^4$D corresponded to the results observed by the enzymatic method with acceptable correlation. We also demonstrated the determination of carnitine and its derivatives (acylcarnitines) in various clinical samples. Free carnitine, short- and medium-chain acylcarnitines, consisting of acetyl-, propionyl-, isovaleryl-, hexanoyl-, octanoyl-, and valproyl-carnitines in plasma and urine samples were successfully quantified. The similarity of their chemical structures posed a high challenge in this study and required comprehensive optimization of the running buffer. An acetic acid buffer at pH 2.6 in the presence of hydroxypropyl-β-cyclodextrin (HP-β-CD) as a modifier was found to be the optimum. The isomers of valproyl- and octanoyl-carnitines could additionally be distinguished under this optimum condition. The LODs of carnitine and acylcarnitines were generally acceptable. The results obtained by CE-$C^4$D in various clinical samples were compared to standard enzymatic- and LC/MS-methods. The results were satisfactory.

In food analysis, the determination of lactic acid and carnitine in various food samples were also achieved using CE-$C^4$D with simple sample preparation steps. The determination of D- and L-lactic acid in fresh and spoiled milk and yogurt samples were successfully obtained in the presence of Tris/Maleic acid buffer using vancomycin as a chiral selector. The LODs were generally satisfactory. In addition, the determination of carnitine in food samples was investigated in the presence of acetic acid buffer at pH 2.6. The validated method was further utilized in food samples analysis. The carnitine contents in various samples consisting of meat, dairy foods and fruit products and food supplements were successfully quantified with good method reproducibility, and the results obtained were acceptable.

# 1. INTRODUCTION

## 1.1 Introduction to Capillary Electrophoresis (CE)

### 1.1.1 Historical background and development of electrophoresis

Electrophoresis is a separation technique for charged species employing the influence of an electric field. Electrophoresis was introduced by Arne Tiselius in 1937. Tiselius studied the moving boundary of protein mixtures using a U-shape tube under the gradient of applied voltage. He found that the migration of moving boundary of protein was in the same direction at a rate determined by their charge and mobility under an electric field. Hence, he called this feature of migration as "moving-boundary electrophoresis" [1, 2]. However, the moving boundary electrophoresis by Tiselius was unsuitable for high electric potentials, required large sample volumes and it was inappropriate for the separation of similar structure compounds. In addition, the separation efficiency was limited by diffusion and convection effects caused by "Joule heating". This heating is principally determining the resolution efficiency in electrophoresis. In 1955, Smithies invented electrophoresis in a starch gel to separate the serum proteins [3]. He used a starch gel as an anti-convective supporting medium in order to prevent the heating diffusion and convection effects when a high potential was applied. The use of the anti-convection medium could significantly improve the resolution efficiency. Thus, proteins with closely similar mobility could be successfully separated in starch gel. This separation mode has been called "zone electrophoresis". Consequently, the use of anti-convection media such as starch gel, filter paper, agarose, cellulose acetate or polyacrylamide gel has been widely employed for the separation of various biological macromolecules [4, 5]. However, disadvantages such as long analysis times, low efficiency, difficulties in detection and automation were still observed. Hence, electrophoresis in a narrow-bore tube was developed.

Electrophoresis in a narrow-bore tube has been known as capillary electrophoresis (CE). CE was initially described by Hjertén in 1967. He invented a fully automated version of capillary free zone electrophoresis apparatus by using a 3 mm internal diameter (id) rotated millimeter-bore quartz-glass capillary. This capillary was coated with methylcellulose in order to minimize the convection effect and heating diffusion [6]. In 1974, Virtanen followed Hjertén's work by using a small glass capillary tube of approximately 200 μm id in order to increase separation efficiency [7]. A different capillary consisting of a teflon capillary tube of 200 μm id was also reported by Mikkers, *et.al* [8]. In early 1980s, Jorgenson and Lukacs advanced the CE technique by using 75 μm id of fused silica capillary to separate charged compounds and amino acids with on-column fluorescent detection [9, 10]. The use of a narrow bore capillary has presented better separation efficiency for zone electrophoresis. This is mainly due to the increasing of surface to capillary volume ratio that can significantly reduce the heating and convection effects. Moreover, only a small amount of solution is required to perform the entire separation.

### 1.1.2 Basic principles of capillary electrophoresis

Capillary electrophoresis (CE) is the separation of charged species in a narrow capillary under the electrical field. The migration of charge species depends on their velocity that relies on their charge, size and shape. The CE system is generally comprised of a narrow-bore capillary with diameter between 25 to 100 µm, high voltage power supply with operating voltage at 5 to 30 kV, buffer reservoirs, detector and data acquisition system, see the schematic in Figure 1. A fused silica capillary is commonly used in CE. Each end of a narrow bore fused silica capillary is placed in the reservoirs filled with an electrolyte buffer solution (or sometimes called running buffer). Electrodes usually made from platinum are dipped into the electrolyte solution and connected to a high voltage supply. A sample is introduced by applying a difference of height, pressure or voltage between each end of capillary for a specific period. A detector is set on-column for detecting the signal of the species. An output signal is then transferred to be recorded by a data acquisition system.

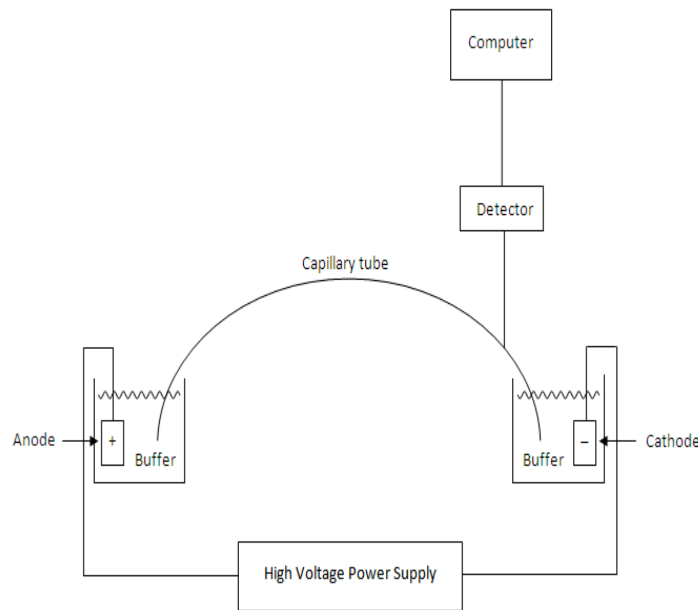



Figure 1. Schematic of a capillary electrophoresis system

A separation of ions in CE is based on their velocity in an electric field. An ion velocity ($v$) can be given by the equation below

$$v = \mu_e E \qquad (1)$$

where $v$ = ion velocity in cm $s^{-1}$
$\mu_e$ = electrophoretic mobility in $cm^2\ s^{-1}\ V^{-1}$
$E$ = electric field strength in V $cm^{-1}$

An electric field ($E$) is a function of the applied voltage and the capillary length. The electrophoretic mobility ($\mu_e$) of ion is a constant which is characteristic of that ion and it is determined by the electric force ($F_E$) and the frictional force ($F_F$) by the equation below

$$\mu_e = \frac{\text{Electric force}(F_E)}{\text{Frictional force}(F_F)} \tag{2}$$

where the electric force ($F_E$) is the force of velocity of ion under medium which is a function of the electric field and ion charge, and it can be given by

$$F_E = qE \tag{3}$$

and the frictional force ($F_F$) is the force of viscosity of ion under medium which can be expressed by Stokes' Law,

$$F_F = 6\pi\eta rv \tag{4}$$

where
$q$ = ion charge
$\eta$ = solution viscosity
$r$ = ion radius
$v$ = ion velocity

Those two forces are competing for an ion of radius $r$ during electrophoresis and they are equal at a steady state in electrophoresis, but opposite direction. It can be given as shown below

$$qE = -6\pi\eta rv \tag{5}$$

The electrophoretic mobility ($\mu_e$) can be obtained by solving of equations (1) and (5) which yields:

$$\mu_e = \frac{q}{6\rho\eta r} \tag{6}$$

From equation (6), it is demonstrated that ions with smaller size and higher charge possess higher mobility than ions with larger size and smaller charge. Thus, ions with higher size and smaller charge will tend to migrate slower towards the detector. However, the mobility of ions not only depends on charge and ion size, but is also affected by the electroosmotic flow (EOF) as discussed below.

Electroosmotic flow (EOF) is an important movement mechanism in CE. It is a flow to drive all species towards the detector. EOF reflects to the bulk flow of liquid inside a fused silica capillary caused by a surface charge on the interior capillary wall. A fused silica capillary used in CE has numerous silanol groups (SiOH) on the interior surface. When an aqueous buffer is placed inside a capillary, the silanol groups are partially ionized to silanol anionic form ($SiO^-$). This feature is presented in Figure 2. The silanol anionic forms will pair with counter cations from the buffer in order to maintain a charge balance. This ionic attraction leads to the formation of a layer which is known as "the fixed layer". However, the cationic forms are not able to neutralize all the negative charges. Thus, a second layer of cationic ions is presented forming "the diffuse layer". The double layers of cationic ions cause the EOF flow and this flow will drag all species according to their mobility through the capillary towards the detector. Under the electric field applied, the diffuse layer is migrated towards the negative electrode (cathode). Hence, cationic species migrate fastest toward the cathode. The EOF and neutral ions migrate together and anionic species migrate slowest to detector.

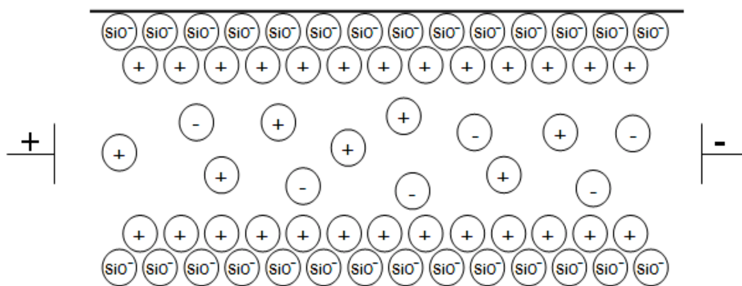



Figure 2. Interior capillary surface to form EOF

The magnitude of the EOF is controlled by the isoelectric point (pI) of the silanol groups and electrolyte pH value. Thus, the EOF becomes significantly strong when electrolyte flowing through the capillary has a pH value above 4. At high pH, a rapid EOF can elute the solute before the separation is occurred. In contrast, low pH leads to protonate and therefore, the EOF is lower at low pH.

The magnitude of the EOF can be defined as a proportion of potential difference across the layer. This differential potential is known as the zeta potential (ζ). The function of the magnitude of the EOF and the zeta potential can be expressed by the equations below

$$v_{EOF} = \frac{\varepsilon\zeta}{\eta} E \quad (7)$$

or $$\mu_{EOF} = \frac{\varepsilon\zeta}{\eta} \quad (8)$$

where
$v_{EOF}$ = velocity of the buffer
$\mu_{EOF}$ = EOF mobility
$\zeta$ = zeta potential
$\varepsilon$ = dielectric constant of the buffer

The EOF mobility can be obtained experimentally from the migration time of the neutral markers such as DMSO and acetone. It is a function of the capillary effective length which is the length from inlet to detector, as well as applied electric field according to the equation below

$$\mu_{EOF} = \frac{l_{eff}L}{Vt} \quad (9)$$

where
$l_{eff}$ = effective length of capillary
$L$ = total length of capillary
$V$ = applied voltage
$t$ = migration time of EOF marker

The feature of the EOF is a flat and uniform profile throughout the capillary as opposed as the laminar flow presented in HPLC, see for the comparative features in Figure 3. The homogeneous flow supports the dispersion of an analyte zone in the same velocity and it approaches a narrow peak in CE.

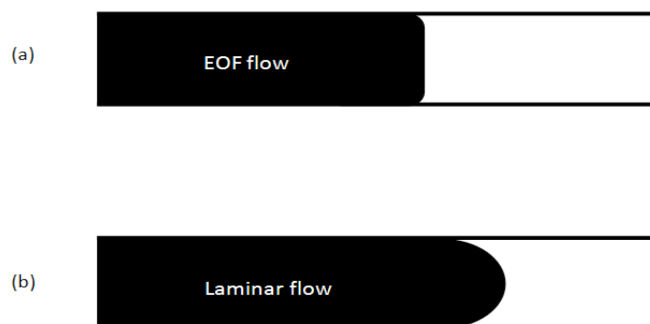



Figure 3. The flow profiles in CE (a) compared to HPLC (b)

The optimization of the EOF can be obtained in many ways in order to improve separation and resolution efficiency, for example:

1) Varying the applied potential

Increasing of the applied potential can improve the separation and resolution efficiency and increase the EOF. However, the joule heating may be problematic extremely when high potentials are applied.

2) Varying the buffer pH

The buffer pH value can significantly change the EOF magnitude and importantly affect to dissociation constant of analyte species in conductivity detection method. At high pH value, the silanol groups are fully ionized and a strong zeta potential is generated that leads to the increase of the EOF. In contrast, the EOF is lower at low pH.

3) Modifying the ionic strength of the buffer concentration

Increasing of the ionic strength not only decreases the zeta potential by compressing of double layer, it also generates more heating. Moreover, higher concentration of running buffer has a higher viscosity that leads to lower EOF. Usually in CE, buffer concentrations are employed in the range from 10 to 100 mmol/L.

4) Increasing temperature

The temperature can change the viscosity of buffer by 2-3% per ºC due to a decrease in the buffer viscosity. This results in higher EOF.

5) Adding the buffer modifier

Modifiers can create an effect on the double layer and decrease the EOF. Buffer modifiers, for example adding organic solvents (methanol or acetonitrile) as well as adding polymer such as polyethylene glycol (PEG) can be used to reduce the EOF, or the addition of quaternary alkyl ammonium compounds (CTAB or TTAB) can be utilized to reverse the EOF while the addition of surfactant such as polysorbate 20 (commercially known as TWEEN 20) can modify the EOF and also minimize sample adsorption on the interior wall.

### 1.1.3 Modes of capillary electrophoresis

The operations of CE have generally been classified as follows:

1) Capillary zone electrophoresis (CZE)

CZE is the most widely used mode due to its simplicity and versatility of operation. It is performed in a homogeneous background electrolyte and in a constant field strength applied along the capillary. The separations of both cationic and anionic species are governed by the EOF and all species migrate according to their individual mobility, based on charge to size ratio.

2) Capillary isoelectric focusing (CIEF)

CIEF is a separation according to the basis of isolectric point (pI) of analyte under a pH gradient in buffer. Anayte is migrated to a point at which a net charge is zero according to its pI. This mode is useful for protein characterization according to its pI as well as for determining a pI value of an unknown protein. It is also used as the first step in multi-dimentional separations of protein mixtures, see for example by Dickerson e*t.al* [11] that they used CIEF and CZE to create a 2-dimention separation method for proteins.

3) Capillary isotachophoresis (CITP)

CITP is a separation under a discontinuous electrical field to create a boundary of analyte between two zones consisting of leading and terminating zones. Analyte is focused between these two zones according to its mobility. This mode is commonly used as a sample pre-concentration or sample purification step. Purification of Nucleic acids, for example, it can be carried out to the purification and quantifification from nanoliter volume of whole blood [12].

4) Micellar electrokinetic capillary chromatography (MECC)

MECC is a separation mode, which is based on differential partitioning between micelles (pseudo stationary phase) and running buffer (mobile phase). Sodium dodecyl sulfate (SDS) is normally used for the micelles. This technique can separate charged and neutral species in the same run. The different migrations of ions are from the variations of ion characteristics across micelles. The method is usually utilized for peptides separation.

5) Capillary gel electrophoresis (CGE)

CGE is an adaptation technique of traditional gel electrophoresis by using polymers to create a molecular sieve. This mode is based on size difference of ions that leads to size-exclusion separation. Thus, similar charge-to-mass ratio compounds can be separated using CGE. The applications method is commonly employed for weight analysis of proteins and sizing of DNA fragment.

6) Capillary electrophoresis chromatography (CEC)

CEC is a hybrid of the two separation methods CZE and HPLC by using an electric field to propel the mobile phase through a packed bed. The use of a small-diameter packed bed can minimize backpressure and can achieve a higher efficiency in this mode. CEC mostly appears in on-line pre-concentration prior to separation and detection [13].

### 1.1.4 Detection for capillary electrophoresis

Most of the detection methods for CE have been adapted from chromatography and the most common ones are described:

1) UV/Vis detection is widely used in CE due to its high sensitivity, low cost and simplicity. The detection is responsive to UV-absorbing molecules. Creatinine, for example a UV absorbing molecule, can be directly detected in urine by UV detection method at 214 nm [14]. For non-UV-absorbing molecules such as inorganic ions, amino acids and sugars are necessary to perform with indirect modes. Indirect detection is usually carried out by adding an intermediate absorbing molecule into the running buffer such as the use of 3,5-dinitrobenzoic acid (DNTB) as a background absorbance for indirect UV absorbance detection at 254 nm in order to determine organic acids in wine, see for example [15]. However, the drawbacks of indirect detection method are low sensitivity, high detection limit and poor linearity range.

2) Fluorescent detection is a highly sensitive detection method. Detection limits can be obtained in the range of $10^{-7}$–$10^{-6}$ mol/L. However, it has not been widely used as most compounds do not fluoresce. Thus, derivatization procedures are usually needed to label compounds with a fluorophore group prior to measurement. The determination of low molecular mass aldehydes in drinking water, for example, can be achieved at sub-

microgram/liter lever (0.15-0.35 μmol/L) after labeling with fluorescein 5-thio-semicarbazide (FTSC) [16]. However, the detection is limited in linear range. Several recent review articles on fluorescent detection method for bioanalytical application, pharmaceutical and environmental samples are available, see for example [17-19].

3) Mass spectrometric detection is highly sensitive which provides a desirable detection limit. The measurement generally provides important information on analyte structure. However, it is an expensive detection method and unavailable for many laboratories. The use of CE coupling with MS detection has been explored for many applications, see for example [20, 21]. Desiderio, *et.al* recent reviewed CE-MS method for the quantification and characterization of proteins and peptides in clinical samples [22].

4) Potentiometric detection is a simple electrochemical method. The detector consists of two electrodes namely working and reference electrodes. The working electrode normally uses a crystalline-, liquid- or glass- membrane that is specific and permeable only to ion of interest. Thus, the working electrode is also termed ion-selective electrode (ISE). The potential change between electrodes is measured according to the Nernst's equation. The applications of potentiometric detection have been described [23, 24]. Bakker, *et.al* reviewed the achievement of potentiometry electrodes coupled with analytical techniques including CE for diverse applications such as bioanalysis [25]. Kubáň, *et.al* reviewed CE-electrochemical detection methods in which the principle and application of potentiometry detection has been reported [26].

5) Amperometric detection is a method that measures the current change during oxidation or reduction of ions of interest. It generally requires three electrodes namely working-, reference- and auxiliary-electrodes. A constant potential is applied between working and reference electrodes and the current resulting from electron transfer is measured between working and auxiliary electrodes. The recent review on amperometric detection method for CE, especially for microchip CE, has been summarized by Wang [27], and a portable microchip CE with amperometric detection has been invented by Villa, *et.al* for separation of neurotransmitters [28]. The electrode maintenance is important for amperometric detection as well as potentiometric detection in order to achieve the desirable reproducibility.

6) Conductivity detection is the most general electrochemical detection method in CE. All charged species can be determined. Conductivity requires two platinum electrodes to measure the current that passes through the buffer when a potential is applied. The current measured rely on the conductivity according to Ohm's law. The applications of conductivity detection are primarily for poor- or non-UV absorbing molecules determinations such as inorganic cations [29, 30] and metal ions [31, 32]. The principle of electrochemical detection methods including conductivity detection and its application for inorganic species has been reviewed by Kappes, *et.al* [33]. The conductivity detection method can be utilized in many fields of application such as in pharmaceutical and clinical analysis [34, 35]. The conductivity detection can be performed with and without a galvanic contact of electrolyte solution as will be discussed below. However, the detection has limited by choices of buffer due to a response to any ions in background solution. High background conductivity may affects the detection and sensitivity of analyte species. Thus, the selection of background with low conductivity and high ionic strength is suitable for this detection method.

## 1.2 Capillary electrophoresis with capacitively coupled contactless conductivity detection (CE-$C^4D$)

### 1.2.1 Basic principles of conductivity detection

The conductivity detection is a universal and inexpensive detection method that can be combined with various separation modes in CE. Unlike potentiometric or amperometric detection method, conductivity detection does not rely on an electrochemical reaction on the working electrode surface but it measures the electrical signal (conductance) between electrodes.

When the voltage is applied to the electrodes, the current from the ion movement passes through the buffer that is based on Ohm's Law as given by the equation below

$$V = i R \tag{10}$$

where $V$ = applied voltage
$i$ = current measured
$R$ = resistance between two electrodes.

The resistance ($R$) is directly proportional to the distance between electrodes ($l$) and inversely proportional to the surface area ($A$) of electrodes and it follows that

$$R = \frac{\rho l}{A} \tag{11}$$

where $\rho$ is the resistivity of an aqueous solution which is a constant value at a fixed concentration and a particular temperature. The reciprocal of the resistance of electrolyte is called the conductance ($G$), given by

$$G = \frac{1}{R} = \frac{\kappa A}{l} \tag{12}$$

The conductivity ($\kappa$) is an intrinsic property of solution that contains all chemical properties of ions which is expressed by

$$\kappa = \frac{1}{\rho} = \frac{l}{RA} \tag{13}$$

The conductivity can be given by the following equation

$$\kappa = F \sum_i Z_i u_i C_i \qquad (14)$$

where $C_i$ is the concentration of ion, $u_i$ is the mobility of ion correlating to its charge ($Z_i$), solvated radius ($R_i$), the elementary charge constant ($e$) and the viscosity of the solvent ($\eta$), it follows that

$$u_i = \frac{Z_i e}{6\pi\eta R_i} \qquad (15)$$

Thus, conductivity can be written in term of the mobilities of all ions

$$\kappa = F \sum_i Z_i u_i C_i = F \sum_i Z_i \left( \frac{Z_i e}{6\pi\eta R_i} \right) C_i \qquad (16)$$

where $F$ = the Faraday constant (96,485 C/mol)
$C_i$ = the concentration of ion "$i$" in mol/cm$^3$.

Considering equation (16), the conductivity relies on the ion charge ($Z_i$), mobility of the ions present ($u_i$) and concentration of ions ($C_i$) in the solution.

### 1.2.2 Capacitively coupled contactless conductivity detection (C$^4$D)

Conductivity detection in CE can be performed either through contact or in a contactless mode and there is no fundamental difference between conventional contacted conductivity and capacitively coupled contactless conductivity detection [26]. Working with the contactless method requires high frequency at several hundred kHz for excitation while in contact mode an operating frequency around 1 kHz is used. The advantages of contactless conductivity mode are, avoidance of corrosion of electrode, prevention of electrode fouling, simple construction of the detection cell and then its possibility to be miniaturized. Electrodes placing around the capillary leads to an easy setup, an exact alignment of electrodes matching of various diameters of capillary used as opposed as the difficulty of alignment in the contact conductivity detection method in which it is problematic to position the electrodes matching those of small internal diameter of capillary. Due to the easy of construction, a contactless conductivity detector can also be combined with a commercial CE-instrument. Commercial versions of C$^4$D detection have recently become available in the market that can be coupled with analytical techniques such as IC, HPLC or FIA (www.edaq.com and www.istech.at).

Contactless conductivity detection was introduced in 1980 by Gaš [36]. It was described as a new detection method for capillary isotachophoresis (CITP). A high frequency was applied to a four electrodes system that was placed externally around a 800 μm outer diameter of narrow bore tube. The mobilities of anionic species in aqueous solution were tested. The electrodes in this configuration did not contact the electrolyte and therefore any undesirably interfering and fouling effect were prevented. In 1998, two independent research groups; Zemann, *et.al* [37] and Fracassi da Silva and do Lagodo [38] developed the contactless conductivity detection with two axial tubular electrodes which are made of a conducting silver vanish. Electrodes were painted around the outer polyimide coating of the fused-silica capillary (< 400 μm outer diameter) with 2 mm gap in between. In 1999, Mayrhofer, *et.al* demonstrated the contactless conductivity detection with a detection gap between electrodes at 1 mm using high frequency input between 40 to 100 kHz for excitation [39] and a different contactless conductivity detector arrangement had also been reported by Kaniansky, *et.al* [40]. In 2001, Tuma, *et.al* constructed a contactless conductivity detection cell by using semi-tubular electrodes instead of tubular electrode [41]. The electrodes were made of a strip of aluminum foil using an operating ac signal with frequency as high as 200 kHz and amplitude of ± 10V and an improved version of contactless conductivity detector was reported in 2002 by the same research group [42]. Using the ac signal at 12 V and the frequency up to 460 kHz has been employed for the improved version in order to study the effect of the gap between electrodes (0.2-1.0 cm in between) on the behavior of the conductivity cell. In 2002, Tanyanyiwa, *et.al* described a different improved design of $C^4D$ which could carry out the detection at higher amplitude with input voltage up to 250 $V_{p\text{-}p}$ and successfully increased the detection sensitivity [43]. An excitation voltage up to 500 $V_{p\text{-}p}$ was also achieved for microchip CE [44].

The configuration of the contactless conductivity detection method in principle required two tubular electrodes which are normally made of a conductive silver varnish or short metallic tubes. Electrodes namely actuator and pick-up electrodes are removed from the solution but they are placed around a narrow bore capillary with a millimeter gap in between. Between two electrodes, a grounded Faraday shield which is made of a thin copper foil is usually used to avoid direct capacitive coupling, see the schematic configuration of $C^4D$ in Figure 4. At electrode surface, a double layer is established by a coupling between charges in the electrodes and an opposite charge in the electrolyte solution inside the capillary. This double layer leads to a capacitive behavior on each electrode. Between the two plates of a capacitor, the resistor behavior is formed by the resistance of solution. Thus, a capacitor-resistor-capacitor arrangement is presented and the equivalent electronic circuitry in a $C^4D$-cell is shown in Figure 5. An ac signal with sufficient frequency is applied for excitation in order to suppress the influence of any reduction/oxidation occurring at the electrodes, minimizing the influence of reactance of the liquid and prevent a current limitation. An ac signal, mostly employing a frequency between 20 to 900 kHz and an amplitude about 20 to 24 V is applied at the first electrode, namely actuator electrode [45]. When the ionic species run through the detection gap, a voltage drop on the resistance is noticed and the difference of conductivity between the electrolyte and anlayte ions is observed. The relationship between voltage drop and current resulted can be calculated according to Ohm's law, yielding the resistance or the

conductance of the electrolyte solution. The conductance ($G$) is dependent on surface area of electrode ($A$), distance between electrodes ($l$), concentration of ion ($C_i$) and mobility ($u_i$) as given in the equations in the section 1.2.1. The output signal is picked up at the second electrode which is connected to a current-to-voltage convertor. This output is further amplified, rectified, and reported to a read out system.

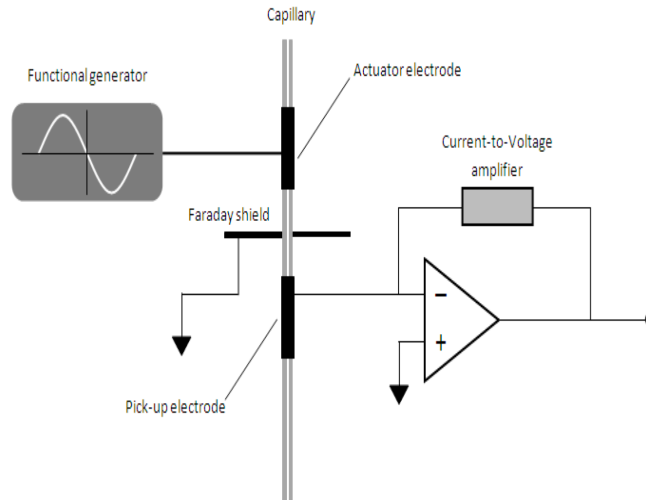



Figure 4. The configuration of CE-$C^4D$

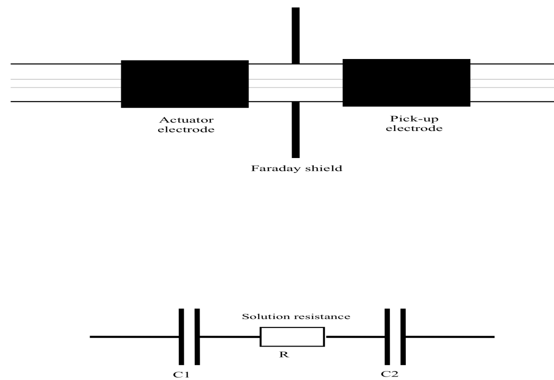



Figure 5. Schematic drawing of $C^4D$

## 1.3 Applications of capillary electrophoresis

There are several reasons why CE has been utilized in many fields. CE gives fast results and provides high resolution and separation efficiency due to the use of high voltages for separation. Compared to the enzymatic or chromatographic methods, CE has advantages such as short analysis time, low reagent and sample consumption, high selectivity, low effects from matrices in samples, and simple sample preparation. Several modes of CE provide various applications for cationic or anionic forms of inorganic and organic compounds, or simultaneous analysis. Besides, CE is highly profitable for the separation of chiral compounds. The separation only requires the addition of a specific chiral selector into the background electrolyte. Unlike in HPLC, a special and expensive chiral column is not needed for separation. The applications of CE have been recently reviewed for diverse applications such as inorganic species analysis [46], enantioseparation [47], and forensic science [48]. Moreover, CE can be coupled with several detection methods such as UV, MS and conductivity detection. Nicholas, *et.al* recently summarized capillary electrophoresis consisting of fundamental background, modes of separation and detection and variety fields of applications [49]. However, detection on commercial CE instruments is generally performed by photometric (UV) detection. Thus, for non-UV-absorbing compounds, indirect detection must be employed. However, indirect methods generally result in small linearity ranges. Electrochemical detection is hence an alternative detection method for a wide variety of compounds especially for non-UV-absorbing species measurements. Particularly, conductivity detection is a universal detection method and is easy to fabricate at low cost compared to other electrochemical detection methods.

The applications of CE with $C^4D$ had been previously reviewed by Zemann, *et.al* in 2001 which included the discussion on the theoretical and instrumental background of conductivity detection [50]. The detection in the early stage was applied to the analysis of non UV-absorbing species such as inorganic ions. Direct detection can be carried out by $C^4D$ which provides high separation efficiency. The analysis of inorganic species in complex sample matrices have been successfully reported, see for example [30, 40]. Alkali and alkaline earth metal determinations had also been achieved with CE-$C^4D$ with detection limits lower than 1 μmol/L. The determination of heavy metal ions has also been possible. CE-$C^4D$ had been employed for metal ion analysis [51, 52]. Moreover, organic molecule determinations are also achievable for species that can be rendered in charged forms depending on the pH value of the buffer. The pH value is an important parameter in order to carry out deprotonation or protonation of organic molecules in dependence of their dissociation constants. For example, the separation of amines in their protonated form can be carried out using an acidic buffer [53, 54], while the separation of carboxylic acids generally requires pH values higher than 5 to achieve deprotonation of the carboxylic group, see example [55]. Hence, CE-$C^4D$ provides many advantages for both inorganic and organic compounds. Several review articles for CE-$C^4D$ in various applications have been published emphasizing environmental, pharmaceutical and forensic fields [13, 48, 56-58].

In clinical analysis, CE has many attractive attributes. The particular features of biological samples consist of small sample volumes and complex matrices. Thus, an effective and selective technique is required for these samples. CE is a suitable assay for clinical samples analysis whereby the method only requires sample amounts that are less than 10 nL and a sample preparation step is not needed. CE in general is suitable for clinical samples such as blood, urine, saliva and tissues and the determination of inorganic, organic and macro molecules is possible in the same run. CE for clinical analysis has primarily been utilized for small inorganic ions and later been facilitated for organic acids which are biomarkers for clinical diagnosis, see for example [59-62]. Besides, CE had been used to separate amino acids in human body fluids, such as human plasma and amniotic fluid for the diagnosis of amino acid metabolic disorders [63, 64]. The determination of heparin, an anticoagulation agent in blood plasma had also been successfully examined by Tuma, *et.al* [65] and the same research group recently reported the fast monitoring of 28 biogenic amino acids in body fluid using a short a capillary of 25 μm id with an effective length of 18 cm [66]. CE-$C^4D$ has been used in the analysis of larger bio-molecules such as hemoglobin [67], serum protein [68], human immunoglobulin M (IgM) and immunoglobin G (IgG) [69]. The determination of nerve agents had also been accomplished [70]. Recently, the monitoring of enzyme reactions was also successfully conducted using CE-$C^4D$ [71, 72]. An indirect measurement has also been employed for this monitoring. Urea in human blood serum, which is a non-ionic substance, was converted to ammonia by urease and detected by $C^4D$ [73]. A comprehensive review article for the determination of enzymatic activity by CE has been written by Glatz [74].

In pharmaceutical analysis, CE can also be utilized for the measurement of drugs and their metabolites. Several pharmaceutical drug determinations have been achieved using CE-$C^4D$, such as ethambutol [75], atenolol and amiloride [76] as well as methotrexate and its metabolites [77]. Chiral analysis in the field of pharmaceutical drug development is also important. The presence of different enantiomeric structures in the pharmaceutical products generally represents different medical effects. The specific enantiomers usually demonstrate different activity, toxicity, transport mechanisms and metabolic routes. CE-$C^4D$ for chiral selection has been explored. The enantiomeric operation in CE can be carried out by adding a specific chiral selector additive into the buffer. Cyclodextrin or its derivatives (CDs) have been largely used to achieve the enantiomeric separation of many basic drugs [78, 79]. A chiral crown ether, namely 18-crown-6-tetracarboxylic acid ($18C_6H_4$) have been utilized for the enantiomeric separation of drugs, basic amino acids or amine compounds [80]. Hence, CE has been considered as a powerful method and multifunctional assay method for clinical purposes, as reviewed by Sniehotta, *et.al* [81].

The applications of CE are not only limited to clinical and pharmaceutical analysis, but it is also useful for food analysis. Food is usually based on complex mixtures that consist of both inorganic and organic species in various products. Food composition is important for investigation to guarantee the quality and to ensure consumption safety. Analytical methods for food must be well-suited in terms of efficiency and resolution. The most frequently determined compounds in food analysis are inorganic ions that can be

measured at relatively low concentrations in various samples by CE. The determination of the small organic ions was achieved in conventional CE [82] as well as in microchip CE [83]. Organic acid determinations in food have also been successfully examined by CE-$C^4$D. The determination of organic acids is known to be important for monitoring quality during food storage. The presence of organic compounds in food can also be used to indicate the stability of food products and to prevent an antibacterial and anti oxidant activities [84]. Moreover, organic acids play an important role in creating taste and aroma. That is the reason why they are normally present in various foods and beverages. Organic acids such as α-hydroxy acids (AHAs) are widely used in many cosmetic products such as exfoliants, moisturizers and emollients to correct skin disorders, increase skin hydration, induce the removal of the outer layers of the skin, and improve some of the visible effects of ageing by reducing lines and wrinkles as well as stimulating skin cell removal [85]. Benzoic acid and sorbic acid can be used as antifungal preservatives against molds and yeasts to ensure long-term microbial stability [86]. Besides, various macromolecules in food such as carbohydrates [87], proteins and amino acids [88-90], fatty acids [91] have been comprehensively determined. The analysis of contaminants has also been achieved by CE-$C^4$D such as metals [92], vitamins [93], pesticides [94] and microbial contamination [95]. The applications of CE-$C^4$D in food analysis can be found in the review article by Kubáň, *et.al* [57] and in the recent review articles by Vallejo-Cordoba and González-Córdova [96] and Herrero *et.al [97].*

## 1.4 Aims of this thesis

The aim of this thesis was to develop CE-$C^4$D techniques for the determination of small organic metabolites, namely uric acid, lactic acid, carnitine and acylcarnitines which are not sensitive for UV detection. The CE conditions were comprehensively optimized which included buffer selection, pH control and additive effect. The optimum conditions were systematically validated (LOD, linearity, repeatability, reproducibility and recovery) and applied in quantifying the ions of interest in clinical and food samples.

Firstly, uric acid was chosen as our target compound. Determination of uric acid in plasma and urine is important to estimate the risk of gout and kidney failure. Many methods for the determination of uric acid with different detection limits have been reported. However, to our knowledge CE-$C^4$D has not yet been utilized to analyze this compound. Hence, we selected uric acid as an exemplary analyte to explore our scope of CE-$C^4$D for routine clinical analysis. We studied the composition of buffer and its pH in order to achieve an effective determination. The validated method was utilized for direct determination of uric acid in human plasma and urine samples and the developed method was compared to the standard enzyme assay.

Secondly, the concentration of lactate was quantified in various clinical samples. The use of conductivity detection is well suited for lactate due to its poor responses to UV detection. Lactate is related to glycolysis during energy production which results from the anaerobic metabolism. The determination is important to estimate exercise performance which is known by the term of "lactate threshold". The quantification of plasma lactate under different exercise conditions was investigated and the lactate threshold curve was obtained. CE conditions were optimized and a method validation was carried out. The quantitative results of plasma lactate were compared to the standard enzyme assay.

Thirdly, the determination of carnitine, as well as of short- and medium-chain acylcarnitines was developed. Carnitine acts as the transporter of long chain fatty acids to mitochondria and generates different acylcarnitines. To our knowledge, the use of CE-$C^4D$ for the determination of free carnitine and acylcarnitines has not yet been reported. The similarity of their structures created a big challenge for high efficiency separation. Hence, we optimized and developed the method for direct quantitative determination of carnitine and six acylcarnitines in various clinical samples. CE conditions were studied and the results were compared with enzymatic and chromatographic methods.

Fourthly, we further worked on food analysis in measuring the enantiomers of D- and L-lactic acid. The CE-conditions were adoped from the work on gamma-hydroxybutyric acid (GHB) reported by Gong, *et.al* [98]. The presence of vancomycin as an additive was successfully utilized to separate enantiomerically and quantified the hydroxyl acid compounds in clinical samples. It was thus of interest to further explore the use of vancomycin for the determination of $\alpha$-hydroxyl acid mainly lactic acid in food samples. The optimization and method validation for D- and L-Lactic acid were investigated. The quantifications of D- and L-lactic acid in dairy products are reported.

Finally, using the optimized CE-conditions from the previous work, an acetic acid buffer was used for the determination of carnitine and acylcarnitines in clinical samples to investigate the carnitine concentrations in various food and food supplements. Only a few reports had focused on the carnitine determination in food. Hence, we decided to employ the developed method for carnitine determination in food analyses. The method was validated and applied for carnitine determination in food and food supplements.

# 2. RESULTS AND DISCUSSION

The results and discussion chapter consists of five research articles. These consist of three publications reprinted (one publication in Analytica Chimica Acta and two publications in Electrophoresis), one preprint of a paper recently accepted for publication in Electrophoresis and one manuscript that has been submitted to the Journal of Chromatography B. The first three articles, which are related to the clinical applications, are presented in the sections 2.1-2.3. The other two articles (sections 2.4-2.5) are based on the work in food analysis.

## 2.1 Capillary electrophoresis with contactless conductivity detection for uric acid determination in biological fluids

Uric acid is a heterocyclic compound with the chemical structure shown in Figure 6. It is a weak organic acid with dissociation constant of 5.4 $pK_{a1}$ and 10.3 $pK_{a2}$. In mammals, uric acid is a final metabolite of the purine metabolism, see the schematic of the purine metabolism in Figure 7.

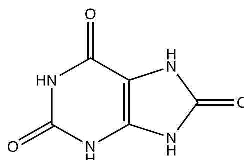



Figure 6. Chemical structure of uric acid

In the normal serum, the pH value is slightly alkaline (pH 7.35-7.45). Thus, almost all the uric acid is presented as a monovalent anion and is usually found in serum in the concentration range from 100 to 400 μmol/L. It can go lower or higher than the normal range depending on the metabolism or uptake of purine. A high level of uric acid in blood is called hyperuricemia which represents a risk factor for gout. Furthermore, the decrease of renal excretion of urate generally is associated with cardiovascular disease and metabolic disorders. To keep the balance of plasma urate, 70% of urate is eliminated by the kidney which performs the main regulatory function and 30% of urate is excreted via the intestines. Urinary excretion of urate is presented in the range from 2 to 4 mmol/24h and results in a renal clearance of 6 to 11 mL/min. A disorder of the excretion that leads to the crystallization of urate in kidney is commonly known as uric acid kidney stone.

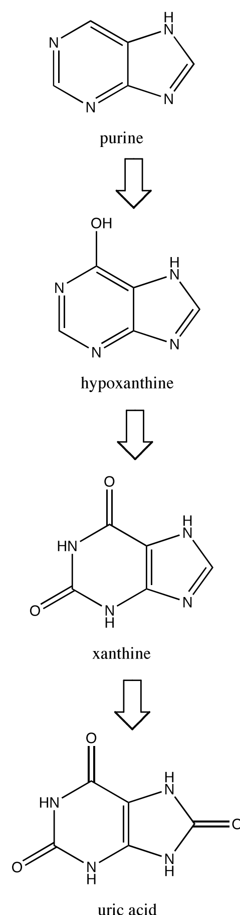



Figure 7. Purine metabolism

We investigated the determination of uric acid by CE-$C^4D$ and it was noticed that extensive optimization of the buffer was needed to achieve its quantification in clinical samples with complex matrices. Uric acid could be measured in the anionic form when the electrolyte pH was higher than its $pK_a$ of 5.7. The arginine/maleic acid buffer system consisting of 20 mmol/L L-arginine and 10 mmol/L maleic acid together with 50 μmol/L of CTAB at pH 7.0 was first investigated to analyze standard uric acid in aqueous solution. However, its response to clinical samples was unsatisfactory. An unstable baseline was observed and this may be due to heating or the adsorption of interferants inside the capillary. An alternate buffer of MES/His was later investigated. We found that the optimal buffer condition of 10 mmol/L of MES/His and 0.1 mmol/L CTAB at pH 6.0 could be used to achieve the accurate quantification of uric acid in biological samples. By performing method characterization, the accuracy of the developed method was checked by analyzing a certified bovine serum and eventually recovery up to 92% was obtained. The detection limit was at 3.3 μmol/L and the repeatability as well as reproducibility were acceptable. The results agreed well with the standard enzymatic method and the paired *t-test* value showed no significant difference between the two methods at the 95% confidence level.

## 2.2 Quantification of plasma lactate concentration using capillary electrophoresis with contactless conductivity detection

Lactic acid, 2-hydroxypropanoic acid, has the chemical formula $C_3H_6O_3$. It is commonly known as milk acid. Lactic acid was first found in 1780 by the Swedish chemist Carl Wilhelm. Lactic acid has two isomeric forms; L- and D-lactic acid, see the difference isomeric structures in Figure 8.

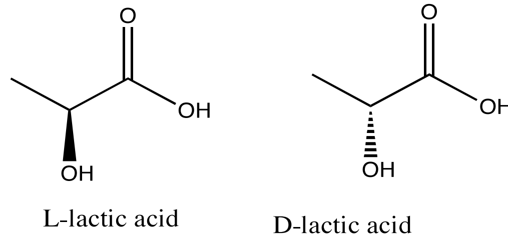
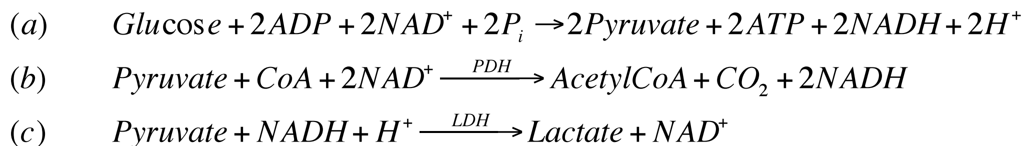



Figure 8. The enantiomeric structures of L- and D-lactic acid

Lactate is a metabolite compound present in several metabolic processes. In the carbohydrate pathway, carbohydrate are first broken down to glucose and then converted to pyruvate. This process is called glycolysis as shown in equation (a). Pyruvate can be further oxidized to acetylCoA in aerobic conditions through the aerobic metabolism. Pyruvate can be also oxidized to lactate in the presence of the lactate dehydrogenase enzyme (LDH) in anaerobic conditions. Both aerobic and anaerobic metabolisms are using NADH and $H^+$ as co-factors, see the reactions given below



$$(a) \quad Glucose + 2ADP + 2NAD^{+} + 2P_i \rightarrow 2Pyruvate + 2ATP + 2NADH + 2H^{+}$$

$$(b) \quad Pyruvate + CoA + 2NAD^{+} \xrightarrow{PDH} AcetylCoA + CO_2 + 2NADH$$

$$(c) \quad Pyruvate + NADH + H^{+} \xrightarrow{LDH} Lactate + NAD^{+}$$

| where | (a) | = | glycolysis |
|---|---|---|---|
| | (b) | = | aerobic metabolism |
| | (c) | = | anaerobic metabolism |

In the aerobic metabolism, pyruvate is shuttled into mitochondria and oxidized into acetylCoA entering the citric acid cycle. Thus, the production of lactic acid is still small. In contrast, the mitochondria becomes unable to continue ATP production at the sufficient rate for any muscle cell activity due to the absence of oxygen during exercise. Consequently, glycolysis is stimulated and increases the metabolism rate in order to produce extra energy. This eventual process leads to a higher pyruvate production. A high amount of pyruvate will be converted into lactate rather than turning into citric acid. However, L-lactic acid is the only lactic acid form which is metabolized in mammalian metabolism while D-lactic acid is not a human metabolite. D-lactic acid can be formed

by some micro-organisms only or produced by the bacterial flora of the gut in pathological conditions [99]. Thus, high amounts of L-lactate produced accumulate in the organs where it is produced. Lactate will be released to the blood stream and transported to the liver and kidneys in order to convert it to glucose in order to balance its normal value. Blood lactate is usually found in the range from 1 to 2 mmol/L and it might also able to reach values as high as 20 mmo/L after prolonged exercise. High levels of blood lactate can lead to its accumulation in muscle and reduces blood pH. Several physical signs, such as rapid breathing, vomiting or muscle fatigue is presented when lactic acid accumulation has occurred.

Blood lactate level in human can be utilized for evaluating an exercise performance for physical training. This monitoring is known as lactate threshold study. Lactate threshold (LT) means the exercise intensity that is associated with a substantial increase in blood lactate during an incremental exercise test [100]. The relationship between blood lactate concentration and exercise period is normally acquired as an exponential increase, see in Figure 9.

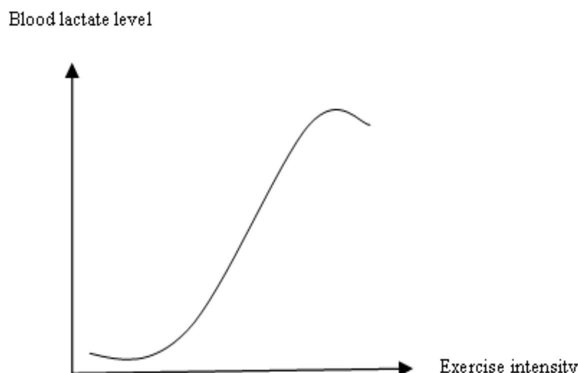



Figure 9. The model of lactate threshold curve

A lactate threshold curve generally represents the ratios between the rate of production and removal of blood lactate during exercise. At the beginning of exercise, the rate of lactate production is still stable. During exercise, the ATP is more demanded. As consequence, the lactate production is higher than the removal process until it reaches the maximum point and goes down to the normal value in rest condition. The LT value is normally set in plasma lactate as 2 mmol/L [101]. Hence, the exercise performance that reaches this concentration level will be utilized to investigate the exercise period and exercise capacity.

We reported the quantification of plasma lactate in various clinical samples. Lactate was characterized in its anionic form by using the buffer at a pH higher than its $pK_a$ at 3.86. MES/His buffers at different concentrations in the range from 5 to 50 mmol/L were investigated. Additions of CTAB at different concentration were also tested in order to reverse the EOF. The buffer at 10 mmol/L MES/His and 70 µmol/L CTAB at pH 6.0 was found to be the optimum. Calibration was carried out in a concentration range from 10 to 1000 µmol/L and good linearity with a correlation coefficient of 0.9994 was

obtained. The LOD of 3.3 μmol/L was achieved. Certified reference standard solutions and various plasma samples were quantified. The lactate threshold curve was noticed as exponential increasing trend. The quantitative results were found comparable with the standard enzymatic method and the correlation between two methods was satisfactory.

## 2.3 Capillary electrophoresis with contactless conductivity detection for the determination of carnitine and acylcarnitines in clinical samples

L-carnitine ((R)-3-carboxy-2-hydroxypropyl) trimethylammonium) was first isolated from muscle tissue and functions as a growth factor for mealworm. It is commonly known as vitamin Bt. Carnitine generally exists in two enantiomeric forms, L- and D-carnitine, see the different structures in Figure 10. However, only L-carnitine is biologically active while D-carnitine is considered to be a toxic influence on biochemical processes.

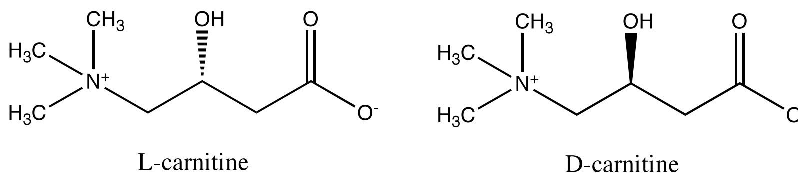



Figure 10. Chemical structures of L- and D-carnitine

In human body, carnitine is synthesized from the amino acid lysine and methionine in the liver and kidney, see the metabolism pathway in Figure 11. Carnitine plays an important role in fatty acid metabolism. It acts as a carrier to shuttle the long chain acyl-fatty acids into mitochondria for β-oxidation and energy production. The inner mitochondria membrane is otherwise impermeable for fatty acids. Hence, fatty acids must be reacted with ATP to form the fatty acyl adenylates, see the process in Figure 12. The fatty acyl adenylates are activated to acylCoA in the outer mitochondria membrane and conjugated to carnitine using carnitine palmitoyltransferase (CPI) enzyme generating with different chain lengths of acylcarnitines. Acylcarnitines are shuttled across the inner mitochondria membrane using enzyme translocase and are converted to acylCoA by carnitine palmitoyltransferase (CPT II) in the inner mitochondria. Free carnitine is then released and returned to the cytosol while the free fatty acid is entering into β-oxidation.

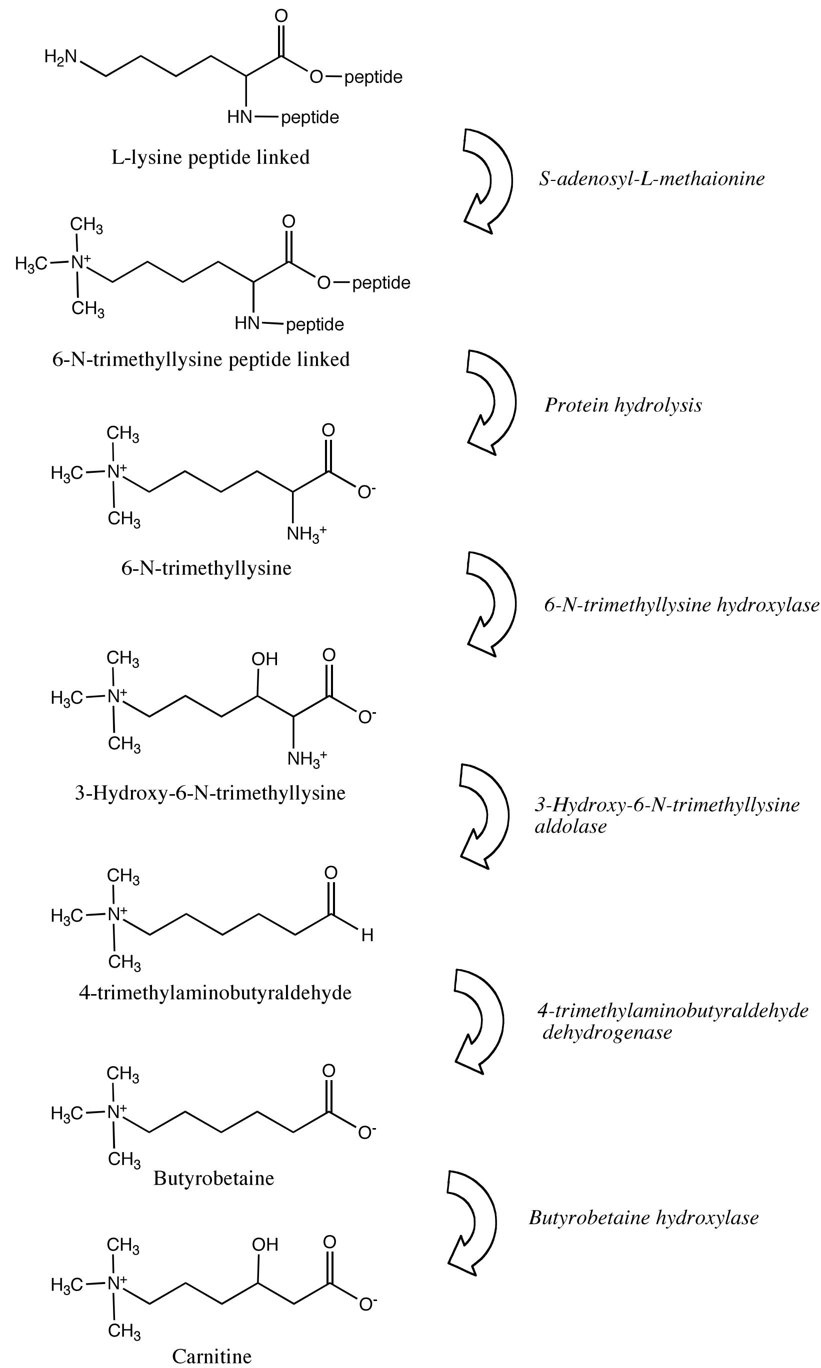



Figure 11. Pathway of carnitine biosynthesis

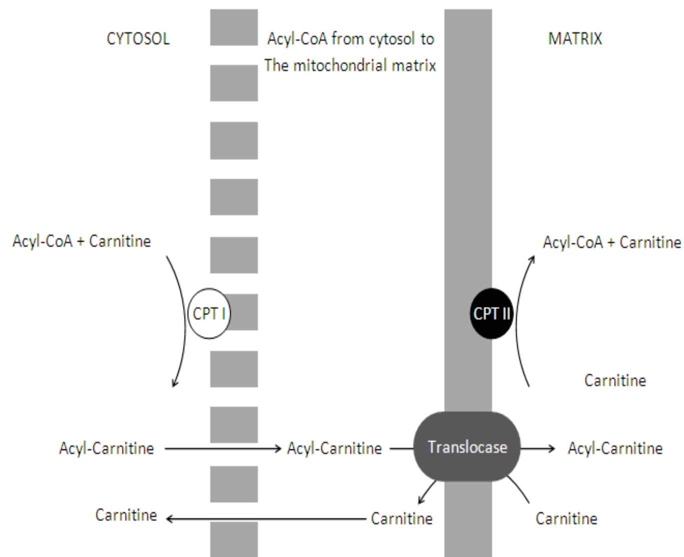
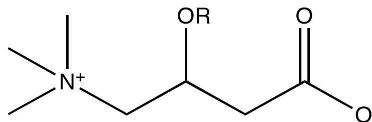



Figure 12. The process of transportation of fatty acids by carnitine

The different short and medium chains of aclycarnitines are listed as follows:




| Compounds | R = |
|---|---|
| Carnitine | H |
| Acetylcarntitine | $COCH_3$ |
| Propionylcarnitine | $COCH_2CH_3$ |
| Isovalerylcarnitine | $COC(CH_3)$ |
| Hexanoylcarnitine | $CO(CH_2)_4CH_3$ |
| Octanoylcarnitine | $CO(CH_2)_6CH_3$ |
| Valproylcarnitine | $CO(CH_2)_6CH_3$ |
| Myristoylcarnitine | $CO(CH_2)_{12}CH_3$ |
| Palmitoycarnitine | $CO(CH_2)_{14}CH_3$ |
| Stearoylcarnitine | $CO(CH_2)_{16}CH_3$ |

Carnitine not only functions as fatty acid shuttle but it is also believed to be important to regulate the acetylCoA/free CoA ratio of the mitochondria. It maintains sufficient cellular levels of free coenzyme A (CoA) and acts as a scavenger of unwanted acyl groups. High amounts of carnitine and acylcarnitines are usually discovered in various tissues. In particular, tissues such as cardiac and muscle tissues require extremely high energy uptake. Free carnitine and acylcarnitines namely acetylcarnitine, are mostly found in plasma and tissue of healthy persons. Reported plasma carnitine in healthy adults is usually in the range from 38 to 44 μmol/L and acetylcarnitine was found in the range from 6 to 7 μmol/L [102]. In order to regulate plasma carnitine in the human body, the kidneys play a vital role to perform a regulatory function via urine excretion. The excretion of carnitine is reported in the range from 81 to 290 nmol/ml and acetylcarnitine is removed in the range from 22 to 100 nmol/ml. If plasma carnitine decreases below reference value, fatty acid metabolism disorder will occur that might lead to primary carnitine deficiency. Consequently, it will cause the loss of energy and an accumulation of free fatty acid in various organs. Secondary carnitine deficiency can be associated with other metabolic disorders such as amino acid disorder and chronic renal disease.

In this section, the determination of carnitine and acylcarnitines consisting of short-, medium- and long chain-acylcarnitines by CE-$C^4D$ is reported. Carnitine is a poor UV-absorbing molecule, but it can be readily detected by conductivity detection. The separation of the structure similar carnitine and acylcarnitines needed to be carefully investigated and optimized. We successfully achieved the determination of carnitine and acylcarnitines in their cationic forms using an acetic buffer system. Acetic acid without additives was found to be inadequate for clinical sample analysis because of an unstable migration time of compounds. This may be due to the adsorption of protein from the sample onto the capillary wall. Besides, acetic acid buffer was not able to separate the two carnitine isomeric structures, namely valproyl- and hexanoyl-carnitine. In addition, the long chain acylcarnitines consisting of myristoyl-, palmitoyl- and stearoyl-carnitine were found to be requiring extremely long migration times using the same buffer. Hence, we decided to focus on short- and medium-chain acylcarnitines in our scope and the target analytes consisted of free carnitine, acetyl-, propionyl-, isovaleryl-, hexanoyl-, valproyl- and octanoyl-carnitine. In order to achieve desirable reproducibility, Tween 20, a non-ionic surfactant, was added to the buffer with the optimal content as 0.05%. To distinguish the isomeric compounds of valproyl- and hexanoyl-carnitine, buffer modifiers consisting of 18-crown-6, heptafluorobutyric acid, cyclodextrin and its derivatives were investigated. We also found that an acetic acid buffer in the presence of hydroxypropyl-β-cyclodextrin (HP-β-CD) was optimal to separate those two carnitine isomers. The developed method was utilized to determine plasma samples from patients in a study to investigate the effect of oral carnitine supplementation on exercise performance. Further to access the quantity of valproylcarnitine, the plasma and urine samples from the patients who orally consumed valproic acid were analyzed. The results were found comparable with the enzymatic- and HPLC-MS method. The correlation between the methods was acceptable.

## 2.4 Determination of the enantiomers of α-hydroxy- and α-amino acid in capillary electrophoresis with contactless conductivity detection

Hydroxy acids namely lactic acid are not only important in the clinical field, but are also widely utilized in many applications. The use of lactic acid can be easily be found in the cosmetic field due. L-lactic acid is one of the α-hydroxy acids (AHAs). AHAs are believed to keep the skin healthy. In addition, lactate in chinese medicine is also the main water soluble compound in herbal drugs for the treatment of coronary heat disease, cerebrovascular disease, bone loss, hepatitis, hepatocirrhosis and chronic renal failure [103]. Besides, lactic acid is considered to be the most common acidic constituent of fermented milk products. That is the reason why it is also called milk acid. Lactic acid is responsible for the sour taste and used to improve microbiological stability and safety in foodstuffs. L-lactic acid is the non-toxic form while the presence of D-lactic acid in the manufacturing may indicate a microbial contamination. The presence of D-lactic acid in water has a different sour taste from L-lactic acid [104].

The determination of D- and L-lactic acids in food samples by CE-$C^4D$ is demonstrated. The CE conditions such as type of buffer, buffer pH and chiral selector selection were adopted from the original work reported by Gong, *et.al in which t*hey reported the determination of γ-hydroxyl-acid, namely gamma-hydroxybutyric acid (GHB) in clinical samples [98]. The use of vancomycin as a chiral selector can be separated enantiomerically of α-, β- and γ-hydroxylbutyric acids. Using the Arginine/maleic acid buffer at pH 7.35 and vancomycin as a chiral selector, the determination of γ-hydroxylbutyric acid (GHB) in urine and serum samples has been reported [98]. Gong later developed the Tris/Maleic acid buffer to separate the enantiomers of α-hydroxy and α-amino acids namely, namely lactic acid, 2-hydroxybutyric acid, 2-hydroxycaproic acid, 2-hydroxyoctanoic acid and 2-hydroxydecanoic acid, aspartic acid and glutamic acid. The best buffer at pH 7.35 consisting of 10 mM of Tris, 4.4 mM of maleic acid, 0.03 mM of CTAB and 5.0 mM of vancomycin was obtained. Under these conditions, the enantiomeric separations for all compounds were considered adequate with $R_s$-values ≥1.5. A detection limit of 2-hydroxybutyric acid was established as 0.3 μmol/L while the enantiomeric separations of D- and L-lactic acid in milk and yogurt samples were carried out. However, the validation of the method for D- and L-lactic acid quantification has not yet been reported.

As a further development of this method, we utilized the CE conditions for the enantiomeric separation and quantification of α-hydroxy-acid, mainly lactic acid. We investigated and extended herein the validation of the method (linearity, LOD, LOQ, intra-day and inter-day variabilities of migration and peak area) in order to quantify D- and L-lactic acids in dairy food samples. We also achieved the separation of D- and L-lactic acid in Tris/Maleic acid using vancomycin as the modifier. We performed method characterizations for linearity at the concentration range of 10 to 500 μmol/L and the correlation coefficient values up to 0.9990 were obtained. The LODs were calculated as 2.8 μmol/L for L-lactic acid and 2.4 μmol/L for D-lactic acid. The intra- and inter-day variabilities of the migration time and peak area were less than 10%. The validated method was applied for determination of L- and D-lactic acid in fresh and spoilt milk and

yogurt samples (2 and 10 days spoiling). The results showed that the present of L-lactic acid could be easily detected while D-lactic acid was also found after spoiling.

## 2.5 Determination of carnitine in food and food supplements by capillary electrophoresis with contactless conductivity detection

As discussed previously, L-carnitine in the body is biosynthesized from the amino acids lysine and methionine. However, the synthesis of carnitine in human body is generally of low amount. The majority of the carnitine in human body originates from food intake. High amounts of L-carnitine are particularly found in red meat, fish and dairy products while smaller amounts are usually present in fruits and vegetables, except for avocardo and some fermented soy products where a high content of carnitine is found [105]. The normal need for daily L-carnitine intake for an adult is between 0.3 and 1.9 mg per kg body weight [106]. The partial diet of carnitine in food will lead to a diminished of L-carnitine synthesis and muscle fatigue [107]. Due to its function in the fatty acid metabolism, it is also widely utilized in weight management programs as it is believed able to increase a fatty acid metabolism. That is the reason why carnitine supplements are often found in markets nowadays. Persons such as athletes, vegetarians and pregnant women may consider an oral intake of a carnitine supplement.

The determination of carnitine in food and food supplements was developed using the same CE conditions as for the clinical analysis. The optimized buffer consisted of 500 mmol/L acetic acid and 0.05% Tween 20. Linearity was obtained with correlation coefficients up to 0.9996 in a concentration range of 5 to 500 μmol/L. The LOD was determined as 2.6 μmol/L and the intra- and inter-day variabilities were acceptable with RSD < 10%. The accuracy of the method was checked by taking the contents declared on the product labels. The method was utilized for the determination of carnitine in several foodstuffs, namely fruit juices, milk, yogurt, cheese and meat.

## 1$^{st}$ Project

# CAPILLARY ELECTROPHORESIS WITH CONTACTLESS CONDUCTIVITY DETECTION FOR URIC ACID DETERMINATION IN BIOLOGICAL FLUIDS

**Analytica Chimica Acta 2009, Volume 636, Issue 2, pages: 224-228.**

Analytica Chimica Acta 636 (2009) 224–228

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

# Capillary electrophoresis with contactless conductivity detection for uric acid determination in biological fluids

Worapan Pormsila [a,b], Stephan Krähenbühl [c], Peter C. Hauser [a,*]

[a] Department of Chemistry, University of Basel, Spitalstrasse 51, 4056 Basel, Switzerland
[b] Department of Chemistry, Rajamangala University of Technology Krungthep, 2 Nanglingi, Sathorn, 10120 Bangkok, Thailand
[c] Division of Clinical Pharmacology & Toxicology, University Hospital Basel, Hebelstrasse 2, 4031 Basel, Switzerland

ARTICLE INFO

*Article history:*
Received 7 October 2008
Received in revised form 5 February 2009
Accepted 5 February 2009
Available online 14 February 2009

*Keywords:*
Uric acid
Plasma
Urine
Capillary electrophoresis
Contactless conductivity detection

ABSTRACT

The suitability of capillary electrophoresis (CE) with capacitively coupled contactless conductivity detection ($C^4D$) for the direct determination of uric acid in human plasma and urine was investigated. It was found that a careful optimization of the buffer composition and pH was necessary to achieve selective determination in the complex sample matrices. An electrolyte solution consisting of 10 mM 2-morpholinoethanesulfonic acid (MES), 10 mM histidine and 0.1 mM hexadecyltrimethylammonium bromide (CTAB), pH 6.0, was finally found suitable for use as running buffer for both sample matrices. The limit of detection (3 S/N) was determined as 3.3 μM. The linearity of the response was tested for the range between 10 and 500 μM and a correlation coefficient of 0.9996 was obtained. Intra- and inter-day variabilities were <10%. Quantitative analysis of urine and plasma samples showed a good correlation with the routine enzymatic method currently used at the University Hospital of Basel.

© 2009 Elsevier B.V. All rights reserved.

## 1. Introduction

Capacitively coupled contactless conductivity detection ($C^4D$) can be considered a universal detection technique for capillary electrophoresis (CE) which shows good sensitivity for all ionic species. Since relatively large external tubular electrodes are used for the contactless approach, alignment of capillary and cell is uncritical and electrode fouling is not possible. An introduction to the method can be found in Ref. [1] and for the fundamental principles the reader may consult Refs. [2,3]. The combination of CE with $C^4D$ is an attractive proposal as both steps, separation and detection, are achieved directly by electronic means only and the instruments are thus very simple and inexpensive. Methods based on UV-absorption are usually considerably more complicated since light sources, monochromators, radiation detectors and other optical components are required. Consequently, inexpensive commercial versions of the contactless conductivity detector, which can also be fitted to existing CE instruments, have been produced [4,5].

A recent summary of reported applications is available [6]. Because of the large number of samples to be processed, the inexpensive CE-$C^4D$ method may prove to be useful for clinical assays, if only for cost savings in the health sector. Capillary electrophoresis in general is an attractive alternative for the analysis of clinical samples. The method has a high separation efficiency, allowing the determination of species in complex matrices with minimum sample pre-treatment and with a short analysis time. The required volumes are generally small, representing a large advantage in many clinical situations with limited amounts of material. The investigation of clinical applications of CE-$C^4D$ has indeed been reported, including the determination of the major inorganic ions in blood and urine samples [7,8]. The determination of the non-UV absorbing drugs fosfomycin [9], tobramycin [10] or valproic acid [11] has also been reported. In a recent publication the determination of the party and rape drug GHB (γ-hydroxybutyrate) was reported [12]. These previous CE-$C^4D$ projects have indeed demonstrated the potential of the new method for clinical analysis in biological fluids but, with the exception of creatinine [7], have not touched the small organic ions routinely determined in clinical blood and urine samples. One such analyte regularly quantified in clinical laboratories is uric acid. As the application of CE-$C^4D$ for this task has not been studied yet, uric acid was chosen as an exemplary analyte to further explore the scope of CE-$C^4D$ in clinical analysis.

Uric acid, the end product of the human purine metabolism, is mainly excreted by the kidneys. High serum or plasma concentrations represent a strong risk factor for the development of gout [13]. Determination of the serum or plasma uric acid concentration is therefore important for estimating the risk for developing gouty arthritis and also for monitoring patients treated with drugs lowering serum urate levels [14]. Since the determination of urate

* Corresponding author. Tel.: +41 61 267 10 03; fax: +41 61 267 10 13.
*E-mail address:* Peter.Hauser@unibas.ch (P.C. Hauser).

0003-2670/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2009.02.012

elimination and renal urate clearance can be helpful in determining the exact reason for hyperuricemia [15], urate determination in urine is also important. In humans, the normal serum or plasma concentrations of uric acid range from about 100 to 400 μM [16]. Above serum concentrations of 400 μM, the risk for developing gouty arthritis rises sharply [16]. Urinary excretion of urate is in the range of 2–4 mmol/24 h, resulting in a renal clearance of 6–11 mL $min^{-1}$ [15].

Many different methods for the determination of uric acid in biological samples have been reported. For example, mass spectrometry [17], HPLC [18–20], colorimetry [21], as well as enzymatic [22,23], amperometric and voltammetric methods [24,25] have been described. The determination of uric acid in human clinical samples with capillary electrophoresis using UV-detection has been reported by Grune et al. [26] with a detection limit of 0.5 μM by using direct absorbance measurement at 260 nm, by Cheng et al. [27] (254 nm, detection limit not given), by Wang and Liao [28] (210 nm, detection limit 0.87 ppm) and by Zinellu et al. [29] (292 nm, 0.5 mg $L^{-1}$). The use of amperometric and voltammetric methods in CE has also been described [30–35]. The detection limits achieved by amperometric detection vary considerably, as values between $3 \times 10^{-8}$ and $2 \times 10^{-6}$ M have been reported.

## 2. Experimental

### 2.1. Reagent and solutions

All chemicals were of analytical reagent grade. Maleic acid, hexadecyltrimethylammonium bromide (CTAB), L-histidine (His) and L-arginine were obtained from Fluka (Buchs, Switzerland). 2-Morpholinoethanesulfonic acid monohydrate (MES) was obtained from Merck (Darmstadt, Germany). The uric aid (Ultra minimum 99%) was from Sigma (Buchs, Switzerland). The separation buffers were prepared freshly every day. Standard solutions were also prepared daily by diluting a stock solution of 10 mM of uric acid in de-ionized water with the separation buffer. The pH-values of the buffers were determined with a pH-meter (model 744, Metrohm, Herisau, Switzerland). A certified bovine serum sample (product Nr. DE14-801F) was obtained from Lonza (Verviers, Belgium).

### 2.2. Instrumentation and conditioning

The capillary electrophoresis instrument was purpose-built around a commercial high voltage power supply module (CZE 2000R, Spellman, Pulborough, UK). The detector was also constructed in-house and 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 300 kHz and an amplitude of 300 $V_{pp}$ (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). Further details can be found in these references [2,3,36–38]. A fused-silica capillary of 50 μm I.D. and 375 μm O.D (Polymicro Technologies, Phoenix, AZ, USA) and total and effective lengths of 60 and 52 cm, respectively, was employed. The new capillary was conditioned by flushing with a 0.1-M hydrochloric acid (HCl) solution (5 min), followed by de-ionized water (5 min) and then with 0.1 M sodium hydroxide (NaOH) solution (10 min) before rinsing with the running buffer (15 min). Each day before starting experiments, the capillary was rinsed with the sodium hydroxide solution (2 min) and running buffer (5 min). After each analysis run the capillary was rinsed for 2 min with the running buffer to maintain the reproducibility of the analysis. Injection of standards and samples was carried out hydrostatically by siphoning at 10 cm height difference for 20 s. The separation was carried out with a negative applied voltage of 25 kV.

### 2.3. Sample preparation

Plasma and urine samples were obtained from the clinical chemistry laboratory of the University Hospital Basel, Switzerland. All samples were kept at −20 °C in a freezer until the experiments. The plasma samples were centrifuged for 20 min at 2000 × *g* for complete separation from the coagulant and the supernatant was used for analysis after dilution 1:5 (v/v) with running buffer. Urine samples were not centrifuged but also diluted 1:5 (v/v) with running buffer before CE-analysis.

## 3. Results and discussion

### 3.1. CE conditions

For contactless conductivity detection in capillary electrophoresis it is necessary to use a background electrolyte solution of low conductivity in order to obtain good sensitivity and to minimize the electrophoretic current. The electrophoresis current influences the detection stability due to the effects of Joule heating. A buffer consisting of 15 mM L-arginine and 13 mM maleic acid had previously been successfully used for the determination of inorganic cations and anions in body fluids [8], and was therefore considered also for the determination of uric acid. Uric acid is a weak organic acid which is characterized by a dissociation constant, $pK_a$, of 5.7. The buffer used in the separation should thus have a pH-value which is higher then 5.7 in order to render a substantial fraction of uric acid in its anionic form, which is a prerequisite for its electrophoretic separation and detection by conductivity measurement. The original arginine/maleic acid buffer had a pH-value of 5.5 which was considered too low for the determination of the urate anion. Thus, the composition of the buffer was altered to 20 mM L-arginine and 10 mM maleic acid, yielding a pH value of 7.0. A first test indicated that it is indeed possible to detect uric acid using this buffer. However, the analysis time of approximately 10 min was fairly long. This is due to the pronounced electroosmotic flow (EOF) towards the cathode, the injection end, which is present at this pH-value. Therefore an EOF-modifier was included in the buffer in order to reverse the EOF and obtain a flow towards the anode. This concurrent electrokinetic phenomenon then does not delay the electrophoretic migration of the anions but speeds up their transport to the detector. The addition of 10, 50 and 100 μM CTAB (hexadecyltrimethylammonium bromide) was tested in seeking an optimal concentration. The lowest concentration (10 μM) was not sufficient to reverse the EOF. With the highest concentration (100 μM), the anodic EOF was found to be so strong that the residence time of urate and other anions was too short so that the peak for uric acid was found to overlap with the large peak of chloride. Thus, the intermediate concentration of 50 μM was adopted, resulting in a migration time of about 200 s for a uric acid standard.

The possibility of determining uric acid in urine with this buffer was tested and the result is shown in Fig. 1. The identity of uric acid was demonstrated by spiking of the urine sample with uric acid. However, a partial overlap occurred with a peak following that of uric acid, rendering proper quantification impossible. The experiment was repeated with a human plasma sample in the same buffer and the uric acid peak was also identified by spiking. A peak overlap did not occur in this matrix, but a general baseline instability was encountered. This may be partly due to Joule heating, but cannot be completely explained by this effect as much more stable baselines were observed when injecting aqueous standards, and so must to some extent have been caused by the sample matrix. Due to the uncertainty of the baseline, proper peak quantification is not pos-

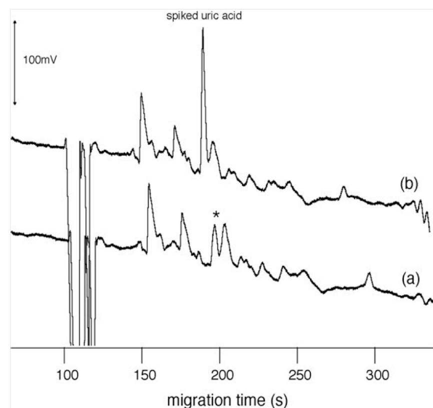



**Fig. 1.** Electropherograms of a urine sample (dilution 1:5) without (a) and with addition of 1 mM uric acid (b). CE conditions: 20 mM L-arginine, 10 mM maleic acid and 0.05 mM CTAB pH 7.0, siphoning injection at 10 cm height difference for 20 s and 25 kV separation voltage. Uric acid is denoted with an asterisk.

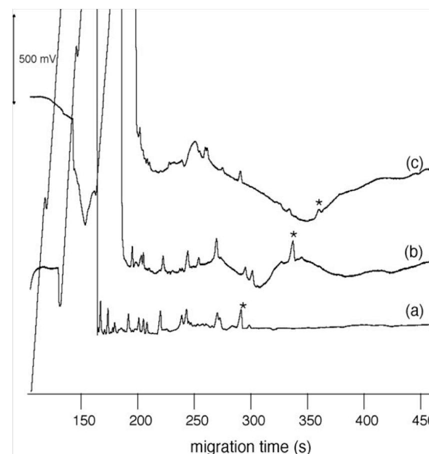



**Fig. 2.** Electropherograms of a urine sample (dilution 1:5) in 10 mM MES-HIS and 0.1 mM CTAB at different pH-values: (a) pH 6.0, (b) pH 6.5, (c) pH 7.0. CE conditions: siphoning injection at 10 cm height difference for 20 s and 25 kV separation voltage. Uric acid is denoted with an asterisk.

sible, and thus the buffer was not suitable for the determination of uric acid in the clinical samples even though it had been used successfully for the inorganic ions.

To overcome the overlap and baseline problems, the use of an alternative buffer consisting of equimolar amounts of 2-morpholinoethanesulfonic acid monohydrate (MES) and L-histidine (His) was investigated. Due to the zwitterionic nature of the buffer substances, this electrolyte solution has a very low conductivity and is therefore well suited for conductivity detection. The buffer has been used frequently for the determination of inorganic cations and anions, but had been found not to be suitable for the determination of these species in serum and urine [8]. The native pH-value of a 10-mM equimolar MES/His buffer is 6.0. As uric acid is not completely deprotonated at pH 6, small amounts of a NaOH solution was added to the buffer to raise its pH to approximately 6.5 and 7. The electropherograms obtained for this buffer solution at the 3 pH values are shown in Figs. 2 and 3, for urine and plasma samples respectively. Note that the buffers also contained CTAB at a concentration of 100 μM. Clearly, uric acid can be determined in this buffer without problems from peak overlaps. The peak heights for the three pH-values do not follow the pattern expected from deprotonation of the analyte alone, which must be due to the alteration of the buffer composition. For the buffer with pH 6.0 the most stable baseline was obtained. This correlates well with the electrophoretic currents of 3, 7 and 15 μA determined for the three buffers at pH 6.0, 6.5 and 7, respectively. For the arginine/maleic acid buffer used before, an electrophoretic current of 15 μA had been obtained. But note, that the pronounced feature present at about 250 s in the electropherogram for plasma recorded in the buffer at pH 7 (electropherogram (c) in Fig. 3) corresponds to a similar feature for plasma observed in the arginine/maleic acid buffer, which must thus be caused by a pH-dependent species. The effect of the concentration of the buffer without pH adjustment was also investigated. Concentrations of 5, 10 and 20 mM of the buffer led to peak areas of 124, 211 and 358 mV s at migration times of 150, 230 and 410 s for an injection of 200 μM uric acid standard. The peak for the latter condition was found to be considerably broadened so that selectivity in the clinical samples might not have been assured. The value of 10 mM was thus adopted as a good compromise condition. Uric acid is not completely deprotonated at the pH 6 of the buffer, but the peak area of 211 mV s obtained was still considerably larger than the 62 mV s determined for the same concentration of uric acid in the arginine/maleic acid buffer.

### 3.2. Quantification

A calibration curve was acquired and the linearity was studied by testing standard solutions of uric acid at 10, 20, 50, 100, 250 and 500 μM. The curve of peak area vs. concentration was found to be linear for this range and is described by the following regression equation: peak area [V s] = 0.015 [V s $\mu M^{-1}$] · concentration [μM] + 0.0023 [V s]. The correlation coefficient, $r$, is 0.9996. The concentrations used covered well the uric acid concentrations expected

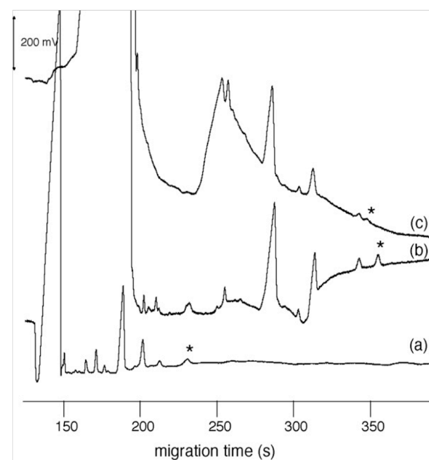



**Fig. 3.** Electropherograms of a plasma sample (dilution 1:5) in 10 mM MES-HIS and 0.1 mM CTAB at different pH-values: (a) pH 6.0, (b) pH 6.5, (c) pH 7.0. CE conditions: siphoning injection at 10 cm height difference for 20 s and 25 kV separation voltage. Uric acid is denoted with an asterisk.

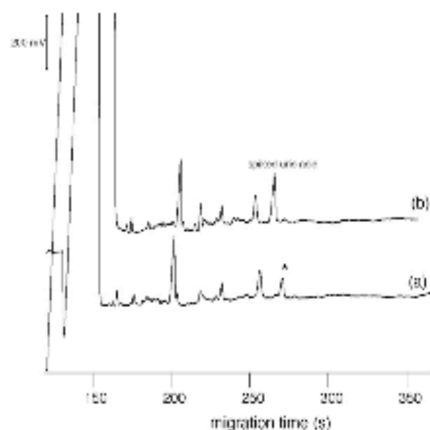



**Fig. 4.** Electropherograms of a urine sample (dilution 1:5) without (a) and with addition of 1 mM uric acid (b). CE conditions: 10 mM MES-HIS and 0.1 mM CTAB, pH 6.0, siphoning injection at 10 cm height difference for 20 s and 25 kV separation voltage. Uric acid is denoted with an asterisk.

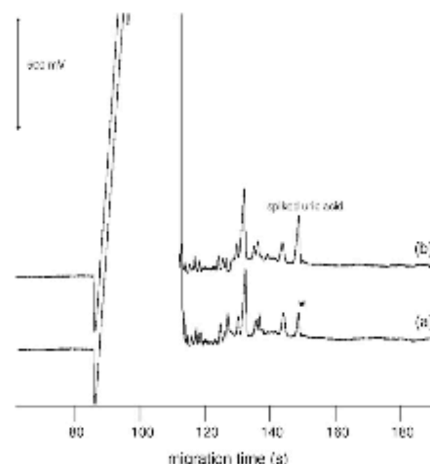



**Fig. 5.** Electropherograms of a plasma sample (dilution 1:5) without (a) and with addition of 200 μM uric acid (b). CE conditions: 10 mM MES-HIS and 0.1 mM CTAB, pH 6.0, siphoning injection at 10 cm height difference for 20 s and 25 kV separation voltage. Uric acid is denoted with an asterisk.

in the clinical samples after dilution. The limit of detection (LOD) ($3 \times$ signal-to-noise) was determined as 3.3 μM. A 200-μM uric acid standard was used to investigate the intra-day and inter-day (4 days) variability. The intra-day variability of for the peak area was found to be 4.8% (R.S.D.) ($n = 10$), whereas for the inter-day study this value was 9.0%.

### 3.3. *Analysis of samples*

The CE-C$^4$D method was validated by analysing a certified bovine serum sample with a level of 17 mg L$^{-1}$. This corresponds to 101 μM. A concentration of $93.1 \pm 8.2$ μM (standard deviation, $n = 3$) was obtained with the new method, hence the recovery was 92%. Uric acid was then quantified by CE-C$^4$D in a batch of 10 urine and 15 plasma samples. The electropherograms for one example from each group are shown in Figs. 4 and 5. There was a certain variation in the migration times between the runs for each figure, and the runs containing plasma were generally slower than those containing urine. This was true also for the electropherograms of the urine and plasma samples in Figs. 2a and 3a, which had been acquired with the same buffer solution. For aqueous samples, consistently shorter migration times with a high reproducibility were obtained (variability of 2%, $n = 10$). It is assumed that the reason for this difference in behaviour between aqueous and clinical samples is an interaction of species present in the clinical samples with the surface of the capillary. In particular proteins are known to show a high degree of affinity to the surface of fused silica capillaries. This most probably leads to a disturbance of the dynamic coating with CTAB and hence to an instability in the electroosmotic flow. The more pronounced effect of the plasma samples on the EOF (as evidenced by a higher residence times for uric acid) is most probably due to the high protein content in these samples, which is not the case in urine or water. In practice, this effect was not a problem for quantification, however; the uric acid peaks could always be identified by spiking. Note also the differences in overall appearance between the electropherograms of Figs. 2a and 4a as well as Figs. 3a and 5a, for urine and plasma samples respectively, which were obtained under the same conditions, but for different samples. These illustrate the variations to be expected, but also indicate the possibility of other clinical applications of the method.

The results for the 10 urine and the 15 plasma samples are given in Tables 1 and 2, respectively, along with the concentrations determined using the standard enzymatic method used at the University Hospital Basel. The correlation coefficients, $r$, for the two pairs of data were determined as 0.974 and 0.953 for urine (Table 1) and plasma (Table 2), respectively. The data sets for the two types of samples were further evaluated by applying the paired $t$-test [39], and $t$-values of 0.042 and 3.18 were calculated for urine and plasma, respectively. This indicates that at the 95% confidence level the values for urine were in very good agreement, but not the plasma values. This is not surprising, as an inspection of the results of Table 2 reveals some gross outliers. The reason for this is not clear, but it cannot be assumed that any deviation is necessarily due to the CE-C$^4$D method. The enzymatic method used routinely is based on single measurements of the samples and thus these results cannot be taken as certified reference values. Some results may also have been affected by the storage of the samples prior to CE-C$^4$D analysis.

**Table 1**
Quantitative results for uric acid in urine samples.

| Urine sample | Concentration of uric acid determined by CE-C$^4$D (μM)[a] | Concentration of uric acid determined with the standard method (μM)[b] |
|---|---|---|
| 1 | $938 \pm 7$ | 925 |
| 2 | $1638 \pm 12$ | 1500 |
| 3 | $526 \pm 7$ | 404 |
| 4 | $637 \pm 12$ | 659 |
| 5 | $840 \pm 8$ | 855 |
| 6 | $1550 \pm 19$ | 1498 |
| 7 | $1275 \pm 13$ | 1164 |
| 8 | $375 \pm 11$ | 481 |
| 9 | $1140 \pm 72$ | 1101 |
| 10 | $600 \pm 19$ | 518 |

[a] Errors are standard deviations ($n = 5$).
[b] Enzymatic method ($n = 1$).

**Table 2**
Quantitative results for uric acid in plasma samples.

| Plasma Sample | Concentration of uric acid determined by CE-C$^4$D (μM)[a] | Concentration of uric acid determined with the standard method (μM)[b] |
|---|---|---|
| 1 | 410 ± 25 | 391 |
| 2 | 207 ± 22 | 193 |
| 3 | 335 ± 31 | 302 |
| 4 | 252 ± 14 | 213 |
| 5 | 242 ± 20 | 103 |
| 6 | 158 ± 11 | 163 |
| 7 | 275 ± 19 | 247 |
| 8 | 1135 ± 49 | 959 |
| 9 | 275 ± 9 | 200 |
| 10 | 760 ± 26 | 518 |
| 11 | 44 ± 7 | 48 |
| 12 | 130 ± 18 | 112 |
| 13 | 336 ± 11 | 345 |
| 14 | 608 ± 13 | 497 |
| 15 | 633 ± 21 | 596 |

[a] Errors are standard deviations ($n$ = 5).
[b] Enzymatic method ($n$ = 1).

## 4. Conclusions

The suitability of CE-C$^4$D as an alternative method for the determination of uric acid in urine and plasma samples has been demonstrated. The limit of detection of 3 μM (≈0.5 mg L$^{-1}$) obtained with this simple and inexpensive technique is adequate for clinical analysis and comparable to the values obtained for CE with the more complex and more expensive UV-detection. In comparison to the other electrochemical detection technique which has repeatedly been reported for this application, namely amperometry, C$^4$D has the advantage of greater simplicity and robustness. Amperometry is problematic in routine use as the precise alignment of the narrow capillary and electrode is difficult and electrode fouling tends to affect the long-term stability. Commercial CE-detectors based on this technique are not available.

An important finding of the work reported herein is that the nature of the background electrolyte solution has an extraordinarily strong effect when employing the universal conductivity detection for clinical samples. It was found that the exact composition and pH-value of the buffer has a very pronounced influence on the selectivity of the analysis, and its optimization was far more challenging then for previous applications of CE-C$^4$D investigated in our laboratory. This is of course due to the complex nature of the clinical samples. On the other hand, the peak richness of the electropherograms obtained for the plasma and urine samples suggests that the method has the potential for successful detection of many other analytes, which may possibly be determined simultaneously in a single analytical run.

## Acknowledgements

The authors thank Dr. André Scholer and Ms. Sandra Grauwiler of the Clinical Chemistry Laboratory, University Hospital Basel, Switzerland, for providing the plasma and urine samples and the reference values. Partial funding for this work was provided by the Thai Government through a grant to the Rajamangala University of Technology Krungthep, Bangkok, Thailand.

## References

[1] P. Kubáň, P.C. Hauser, Electrophoresis 30 (2009) 176.
[2] P. Kubáň, P.C. Hauser, Electrophoresis 25 (2004) 3387.
[3] P. Kubáň, P.C. Hauser, Electrophoresis 25 (2004) 3398.
[4] TraceDec: www.istech.at.
[5] eDAQ: www.edaq.com.
[6] P. Kubáň, P.C. Hauser, Anal. Chim. Acta 607 (2008) 15.
[7] P. Tůma, E. Samcová, F. Duška, J. Sep. Sci. 31 (2008) 2260.
[8] Q.J. Wan, P. Kubáň, J. Tanyanyiwa, A. Rainelli, P.C. Hauser, Anal. Chim. Acta 525 (2004) 11.
[9] M. Petsch, B.X. Mayer-Helm, R. Sauermann, C. Joukhadar, E. Kenndler, Electrophoresis 25 (2004) 2292.
[10] W.S. Law, P. Kubáň, L.L. Yuan, J.H. Zhao, S.F.Y. Li, P.C. Hauser, Electrophoresis 27 (2006) 1932.
[11] G.K. Belin, S. Krähenbühl, P.C. Hauser, J. Chromatogr. B 847 (2007) 205.
[12] X.Y. Gong, P. Kubáň, A. Scholer, P.C. Hauser, J. Chromatogr. A 1213 (2008) 17.
[13] E.W. Campion, R.J. Glynn, L.O. DeLabry, Am. J. Med. 82 (1987) 421.
[14] M.P. Keith, W.R. Gilliland, Am. J. Med. 120 (2007) 221.
[15] B.T. Emmerson, Lancet 337 (1991) 1461.
[16] V.L. Star, M.C. Hochberg, Drugs 45 (1993) 212.
[17] L. Siekmann, J. Clin. Chem. Clin. Biochem. 23 (1985) 129.
[18] J. Perello, P. Sanchis, F. Grases, J. Chromatogr. B 824 (2005) 175.
[19] V.F. Samanidou, A.S. Metaxa, I.N. Papadoyannis, J. Liq. Chromatogr. 25 (2002) 43.
[20] Y.D. Yang, Biomed. Chromatogr. 12 (1998) 47.
[21] P. Miller, V. Oberholzer, Clin. Chem. 36 (1990) 668.
[22] H.E. Lane, J.L. Dwyer, C.J. Lai, Clin. Chem. 21 (1975) 1015.
[23] K.H. Zimelka, E. Munz, R. Boguslawski, J. Clin. Chem. Clin. Biochem. 17 (1979) 201.
[24] S.A. Abakshina, L.S. Anisimova, B.F. Nazarov, Y.A. Belikhmaer, J. Anal. Chem. 49 (1994) 886.
[25] Y. Wei, M.G. Li, B. Fang, Chin. J. Chem. 25 (2007) 1622.
[26] T. Grune, G.A. Ross, H. Schmidt, W. Siems, D. Perrett, J. Chromatogr. 636 (1993) 105.
[27] M.-L. Cheng, T.-Z. Liu, F.-J. Lu, D.T.-Y. Chiu, Clin. Biochem. 32 (1999) 473.
[28] S.-P. Wang, C.-S. Liao, J. Chromatogr. A 1051 (2004) 213.
[29] A. Zinellu, C. Carru, S. Sotgia, L. Deiana, Anal. Biochem. 330 (2004) 298.
[30] X. Yao, Y.T. Wang, G. Chen, Biomed. Chromatogr. 21 (2007) 520.
[31] Q.C. Chu, M. Lin, C.H. Geng, J.N. Ye, Chromatographia 65 (2007) 179.
[32] Y.Q. Guan, T. Wu, J.N. Ye, J. Chromatogr. B 821 (2005) 229.
[33] Y.Q. Guan, Q.C. Chu, J.N. Ye, Anal. Bioanal. Chem. 380 (2004) 913.
[34] J.L. Boughton, B.W. Robinson, T.G. Strein, Electrophoresis 23 (2002) 3705.
[35] D.K. Xu, L. Hua, Z.M. Li, H.Y. Chen, J. Chromatogr. B 694 (1997) 461.
[36] L. Zhang, S.S. Khaloo, P. Kubáň, P.C. Hauser, Meas. Sci. Technol. 17 (2006) 3317.
[37] J. Tanyanyiwa, P.C. Hauser, Electrophoresis 23 (2002) 3781.
[38] J. Tanyanyiwa, B. Galliker, M.A. Schwarz, P.C. Hauser, Analyst 127 (2002) 214.
[39] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, Ellis Horwood, Chichester, 1993.

## $2^{nd}$ Project

# QUANTIFICATION OF PLASMA LACTATE CONCENTRATION USING CAPILLARY ELECTROPHORESIS WITH CONTACTLESS CONDUCTIVITY DETECTION

**Accepted article for publication in Electrophoresis**

# Quantification of plasma lactate concentrations using capillary electrophoresis with contactless conductivity detection

**Worapan Pormsila[1,2], Réjane Morand[3], Stephan Krähenbühl[3], Peter C. Hauser[1]**

*[1]Department of Chemistry, University of Basel, Basel, Switzerland*

*[2]Department of Chemistry, Rajamangala University of Technology Krungthep, Bangkok, Thailand.*

*[3]Division of Clinical Pharmacology & Toxicology, University Hospital Basel and Department of Biomedicine, University of Basel, Basel, Switzerland*

Correspondence: Professor Peter C. Hauser, Department of Chemistry, University of Basel, Spitalstrasse 51, 4056 Basel, Switzerland

E-mail address: Peter.Hauser@unibas.ch

Fax: ++41 61 267 10 13

**Abstract**

The quantification of plasma lactate and evaluation of the lactate threshold by capillary electrophoresis with capacitively coupled contactless conductivity is demonstrated. The only sample preparation needed was deproteinization with a acetonitrile/methanol mixture. A solution of 10 mmol/L 2-morpholinoethanesulfonic acid monohydrate (MES), 10 mmol/L DL-histidine (His), 70 µmol/L hexadecyltrimethlammonium bromide (CTAB), pH 6.0 was found suitable as running buffer. Linearity was achieved for the concentration range of 10-1000 µmol/L with a correlation coefficient of 0.9994. The limit of detection (3 S/N) was determined as 3.2 µmol/L. Intra- and inter-day variability were less than 7% RSD. The suitability of the method could be demonstrated by analyzing various clinical samples, where the results correlated satisfactorily with those of an established enzymatic method.

**Keywords:**

Lactate, capillary electrophoresis, contactless conductivity detection, plasma lactate, lactate threshold

**Abbreviations:**

CE-$C^4$D, capillary electrophoresis with capacitively coupled contactless conductivity detection; His, DL-histidine; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide.

## 1 Introduction

Glucose molecules are broken down and converted to pyruvate via the glycolysis pathway. Pyruvate can be transported into mitochondria and be degraded by the citric acid cycle, where it is metabolized to $CO_2$ and $H_2O$. When the glycolytic activity exceeds the activity of the citric acid cycle (e.g. exercise above the lactate threshold) or when oxygen is lacking, pyruvate can be reduced to lactate by the enzyme lactate dehydrogenase (LDH). Lactate accumulates in the organ where it is produced. After release into blood, lactate is transported to the liver and kidneys, which can convert it to glucose via gluconeogenesis. At rest, the concentration of blood lactate is normally 1-2 mmol/L in healthy persons, but can rise to over 20 mmol/L during intense exertion [1].

The measurement of plasma lactate concentrations can be useful in athletes during training, mainly for the determination of the lactate threshold. The lactate threshold corresponds to the exercise intensity (workload) associated with a substantial increase in blood lactate during an incremental exercise test [2]. In addition, the plasma lactate concentration is a very useful clinical parameter, mainly in patients with intoxications and/or with a compromised circulation.

The routine methods for lactate determination in clinical samples are based on the enzymatic conversion to pyruvate in presence of LDH. The NADH formed as byproduct is measured optically for indirect quantification. The pyruvate produced has to be removed by an auxiliary enzymatic reaction in order to drive the main process to completion [3]. Relatively large sample volumes are required when using photometric absorbance measurements. For this reason fluorimetric methods have been developed (see for example [4], but these require careful attention to potential interferences due to possible quenching and inner filter effects

[5]. Amperometric sensors based on the enzymatic conversion are also available (see for example [6]). The use of chromatographic separation methods has also been reported. Gas-chromatography is possible after derivatization to render lactate volatile [7], but also determination by HPLC requires derivatization to allow UV- or fluorescence detection as lactate is a poor UV-absorber [8-11].

Capillary electrophoresis (CE) is an attractive alternative method for clinical analysis, which is of particular interest when only small sample volumes are available. Several reports on the determination of lactic acid in clinical samples by this method have indeed appeared (see references [12-16]), but indirect detection methods (via displacement of charged dye molecules) had to be employed. Such approaches are not necessary when using conductivity detection. Due to the difficulty of constructing such detection cells, this method had however not been widely used until the development of contactless conductivity cells (often termed $C^4D$ for 'capacitively coupled contactless conductivity detection'), which are much easier to build and use than conventional units. $C^4D$ in the arrangement currently employed was introduced to capillary electrophoresis independently by Zemann and coworkers [17] and da Silva and do Lago [18] in 1998. For further details see for example [19-26]. Contactless conductivity detectors which can be fitted to standard CE-instruments have recently become available (www.edaq.com, www.istech.at). The method has indeed been useful for the determination of poorly UV-absorbing analytes in clinical samples. The determination of the major inorganic blood electrolytes in plasma and urine samples was, for example, demonstrated by Wan *et al.* [27], Tůma *et al.* [28] and Kubáň and Hauser [29]. Tůma and coworkers demonstrated also the determination of native organic ions in clinical samples, namely free amino acids [30, 31], 1-methylhistidine and 3-methylhistidine [32] and creatinine [28]. Pormsila *et al.* described the quantification of uric acid [33]. Furthermore, the

determination of non-UV absorbing organic pharmaceuticals, such as fosfomycin [34], tobramycin [35], valproic acid [36] and gamma-hydroxybutyric acid [37] is also possible by CE-$C^4D$. While the determination of lactate in food samples by this method has been described [38]Law, 2007 #30][39], as well as the separation of the enantiomers [38, 40] and its use as internal standard in the determination of formaldehyde [41], the application of CE-$C^4D$ for the determination of this species in clinical samples has to our knowledge not yet been reported.

## 2 Material and methods

### 2.1 Reagents

All chemicals were of analytical reagent grade. Hexadecyltrimethylammonium bromide (CTAB), DL-histidine (His) and L-lactic acid sodium salt were obtained from Fluka (Buchs, Switzerland). 2-Morpholinoethanesulfonic acid monohydrate (MES) was obtained from Merck (Darmstadt, Germany). Methanol (Fluka) and acetonitrile (Fisher Scientific, Wohlen, Switzerland) were used for deproteinization. The de-ionized water used throughout this work had a resistivity of 18 MΩ·cm and was obtained from a Millipore Milli-Q Plus 185 purifier (Labtec Services, Wohlen, Switzerland). The running buffer consisting of 10 mmol/L MES-His and 70 µmol/L CTAB, pH 6.0 was prepared fresh daily. An aqueous standard solution was also prepared daily by diluting a stock solution of 10 mmol/L of lactate in de-ionized water with the running buffer, and all prepared solutions were degassed in an ultrasonic bath. The pH-value of the buffer was determined with a pH-meter (model 744, Metrohm, Herisau, Switzerland). The certified serum reference material analyzed was obtained from Bio-Rad (Reinach, Switzerland) and consisted of the products Nr. 694, 695 and 696 ("Liquid Assayed Multiqual", Level 1, 2 and 3 respectively). For the enzymatic fluorimetric assay used as

reference method, the plasma samples were deproteinized and centrifuged and the quantification carried out as detailed by Olson [4].

**2.2 Instrumentation**

The capillary electrophoresis instrument was purpose-built around a commercial high voltage power supply module capable of delivering up to 30 kV (CZE 2000R, Spellman, Pulborough, UK) and otherwise similar to the design described in [42]. The detector was also built in-house. It 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 [43]. A sine wave voltage of 320 kHz and an amplitude of 340 Vpp (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 an e-corder data acquisition system (eDAQ, Denistone East, NSW, Australia). A fused-silica capillary of 50 μm I.D. and 375 μm O.D (Polymicro Technologies, Phoenix, AZ, USA) and total and effective lengths of 60 and 52 cm respectively was employed. The new capillary was conditioned by flushing with a 0.1 mol/L sodium hydroxide (NaOH) solution (5 min), followed by de-ionized water (5 min), with 0.1 mol/L hydrochloric acid (HCl) solution (5 min), and then with de-ionized water (5 min) before rinsing with the running buffer (20 min). Each day before starting experiments the capillary was rinsed with 0.1 mol/L NaOH solution (2 min), de-ionized water (2 min) and running buffer (10 min) to prevent peak distortions. After each sample analysis run the capillary was rinsed for 5 min with the running buffer to maintain reproducibility. Injection of standards and samples was carried out hydrostatically by siphoning at 10 cm height difference for 15 sec. The separation was carried out with a negative applied voltage of 20 kV at the injection end.

### 2.3 Sample preparation

Heparinized plasma samples were obtained from the Clinical Pharmacology and Toxicology Laboratory of the University Hospital Basel, Switzerland. All plasma samples were kept at -20°C in a freezer until the experiments. For the precipitation of the protein content, different ratios of acetonitrile to methanol were investigated and a ratio of 3:1 (v/v) was adopted. To 50 µL of the samples 50 µL of the solvent mixture was added. The mixture was centrifuged for 5 min at 3000×g, and the supernatants were collected. If necessary, the samples were diluted with running buffer to a desired concentration within the linear range of the calibration curve before injection into the CE-instrument.

## 3 Results and discussion

### 3.1 Optimization of the CE conditions

Lactic acid is a weak organic acid, which is characterized by the dissociation constant, $pK_a$, of 3.86. The separation buffer used should thus have a pH-value higher than 3.86 in order to render a substantial fraction of lactate in its anionic form. A buffer consisting of 2-morpholinoethansulfonic acid monohydrate (MES) and DL-histidine (His) was considered as electrolyte for the separation. The low background conductivity of these zwitterionic substances makes this buffer suitable for conductivity detection. An equimolar amount of MES-His is commonly used in the concentration range between 5 to 50 mmol/L for CE with a pH value of 6.0 and it has been successfully employed for the determination of organic species in serum and urine with CE-$C^4D$ [33, 35, 36]. To find the optimal concentration of an equimolar MES-His buffer, concentrations of 5, 10, 20, 40 and 50 mmol/L were investigated. Since lactic acid is present in its anionic form at this pH-value, the addition of an electroosmotic flow (EOF) modifier is necessary. CTAB (hexadecyltrimethylammonium

bromide) was thus added to the running buffer to reverse the direction of the EOF and obtain migration towards the anode. Additions of 10, 30, 50, 70, 100 and 150 μmol/L of CTAB were tested. An equimolar amount of 10 mmol/L of MES-His, 70 μmol/L of CTAB, at pH 6.0, was found to be suitable with the application of a negative voltage of 20 kV for separation and injection by siphoning for 15 sec at 10 cm elevation. The electrophoretic current was found to be as low as 4 μA, the EOF peak was observed after 11 min., and the migration time for lactate was about 4 min. In addition, non-equimolar ratios of MES-His were investigated by varying the content of MES to decrease the pH value. It was found that the sensitivity for lactate could not be improved by this variation, but longer migration times and broader peaks were the result. Thus, an equimolar amount of MES-His (10 mmol/L) at pH 6.0 was finally adapted as the best condition for further studies.

### 3.2 Analytical characteristics of the method

The electropherogram of a plasma sample is shown in trace (a) of Fig. 1. The identity of the peak for lactate was verified by spiking with a lactate standard (electropherogram b). Note that pyruvate, a precursor of lactate, could also be identified, and the method is in principle also suitable for the concurrent determination of both species. However, no attempt was made to quantify pyruvate in samples, due to the low stability of the compound [44]. A calibration was carried out with aqueous standard solutions of 10, 20, 30, 50, 100, 300, 600 and 1000 μmol/L (8 points). The curve of peak area vs. concentration was found to be linear for this range, which covers the concentrations expected in the diluted samples. The following regression equation was obtained: $y = 0.0022\,x + 0.0420$ (y in mV·sec, and x in μmol/L). The corresponding correlation coefficient, $r$, was determined as 0.9994 (n=3). The limit of detection (LOD, 3 × signal to noise) was 3.2 μmol/L (n = 10). The intraday reproducibility for a standard of 200 μmol/L was determined as 3.4% (relative standard

deviation for n=10) for peak area and 2.3% for migration time. The respective inter-day values were found to be 6.1% and 4.3% (5 days). The method was then further tested by analyzing certified serum reference material at three different concentration levels within the clinically relevant range. The results are summarized in Table 1. It can be seen that the CE-$C^4D$-method gave satisfactory results, as two of the three concentrations obtained were within the certified range, while the third concentration was slightly above the range given.

**3.3 Analysis of clinical samples**

The method was tested for its usefulness in clinical studies by analyzing the plasma samples from 10 volunteers between 25 and 40 years of age who were performing a workout on an exercise bicycle for 60 minutes. Blood samples were taken before and after the physical activity. The test was repeated 12 weeks later, so that a total of 4 samples were obtained from each person. The quantitative data for the 10 subjects is given in Table 2. Clearly, as expected, a significant increase of lactate levels was caused by the physical exertion, the lactate levels were found to increase from typically 1-2 mmol/L to levels as high as 15 mmol/L after the exercise. During the exercise above the lactate threshold, skeletal muscles generate more pyruvate than can be metabolized by the citric acid cycle. Pyruvate is reduced to lactate, which accumulates in skeletal muscle from where it is transported via the bloodstream to the liver, thus increased levels of lactate are found in the plasma samples. Also given in the table are the results obtained by using the established enzymatic method. Some deviations can be expected, in particular considering that only a single quantification was carried out with the enzymatic approach, but the agreement is generally satisfactory. The correlation coefficient for the two sets of data was calculated as $r = 0.9486$.

In a further test, the method was applied to the ergometric determination of the lactate threshold, the exercise intensity at which the lactate level starts to rise, of a 56-years old healthy male. Starting at 220 W, the workload was increased every 2 minutes by 20 W until exhaustion. Blood samples were collected every 2 minutes during the bicycle run, and every 5 minutes during recovery. As shown in Figure 2, the subject was exhausted after 18 minutes, corresponding to a maximal workload of 380 W, and the exercise then stopped. Interestingly, the peak lactate concentration was determined only during the first 5 minutes of recovery; afterwards, the plasma lactate concentration gradually decreased. The lactate threshold was estimated as described previously (workload at a plasma lactate concentration of 2 mM) [1] as 260 W. In addition to capillary electrophoresis, the lactate concentrations were also determined using the enzymatic method [4]. The corresponding values are given in Table 3. This time 4 measurements for each sample were carried out with both methods. The correlation between the two methods is satisfactory ($r = 0.9815$), but it is evident that the precision of the data of the capillary electrophoresis method was much better than that obtained by the enzymatic method. Any deviation is thus not necessarily due to the CE-method.

**4 Concluding remarks**

Capillary electrophoresis with contactless conductivity detection (CE-$C^4D$) was found to be suitable for the determination of plasma lactate. The method was successfully applied for the determination of the lactate threshold, sometimes also called anaerobic threshold, of a volunteer. Generally good accuracy and precision were achieved, and only small sample volumes are required. The technique may therefore be considered an alternative to the

commonly used enzymatic approach, which has the advantage of potentially allowing the concurrent determination of additional species.

**Acknowledgements**

The authors thank the members of the ergometry team of the Division of Cardiology of the University Hospital Basel, Switzerland. Partial funding for this work was provided by the Thai Government through a grant to the Rajamangala University of Technology Krungthep, Bangkok, Thailand and the Swiss National Science Foundation through grant No. 200020-126384/1.

**5 References**


[1] Friolet, R., Hoppeler, H., Krähenbühl, S., *J. Clin. Invest.* 1994, *94*, 1490-1495.

[2] Svedahl, K., MacIntosh, B. R., *Can. J. Appl. Physiol.-Rev. Can. Physiol. Appl.* 2003, *28*, 299-323.

[3] Alderman, J. A., Cross, R. E., *Clin. Chem.* 1977, *23*, 1917-1920.

[4] Olson, C., *Clin. Chim. Acta* 1971, *33*, 293-300.

[5] de Marcos, S., Galban, J., Castillo, J. R., *Anal. Sci.* 1995, *11*, 233-238.

[6] de Keijzer, M. H., Brandts, R. W., Brans, P. G. W., *Clin. Biochem.* 1999, *32*, 109-112.

[7] Paik, M. J., Cho, E. Y., Kim, H., Kim, K. R., Choi, S., Ahn, Y. H., Lee, G., *Biomed. Chromatogr.* 2008, *22*, 450-453.

[8] Koike, K., Urata, Y., Hiraoka, N., *J. Clin. Biochem. Nutr.* 1990, *9*, 151-161.

[9] Bhaskar, V. V., Gurupadayya, B. M., Manohara, Y. N., Reddy, D. V. P., *Asian J. Chem.* 2007, *19*, 5049-5056.

[10] Hasegawa, H., Fukushima, T., Lee, J. A., Tsukamoto, K., Moriya, K., Ono, Y., Imai, K., *Anal. Bioanal. Chem.* 2003, *377*, 886-891.

[11] Bleiberg, B., Steinberg, J. J., Katz, S. D., Wexler, J., Lejemtel, T., *J. Chromatogr.-Biomed. Appl.* 1991, *568*, 301-308.

[12] Markuszewski, M. J., Szczykowska, M., Siluk, D., Kaliszan, R., *J. Pharm. Biomed. Anal.* 2005, *39*, 636-642.

[13] Dolník, V., Dolníková, J., *J. Chromatogr. A* 1995, *716*, 269-277.

[14] Oefner, P. J., *Electrophoresis* 1995, *16*, 46-56.

[15] Hiraoka, A., Akai, J., Tominaga, I., Hattori, M., Sasaki, H., Arato, T., *J. Chromatogr. A* 1994, *680*, 243-246.

[16] Xue, Q. F., Yeung, E. S., *J. Chromatogr. A* 1994, *661*, 287-295.

[17] Zemann, A. J., Schnell, E., Volgger, D., Bonn, G. K., *Anal. Chem.* 1998, *70*, 563-567.

[18] da Silva, J. A. F., do Lago, C. L., *Anal. Chem.* 1998, *70*, 4339-4343.

[19] Kubáň, P., Hauser, P. C., *Electrophoresis* 2009, *30*, 176-188.

[20] Kubáň, P., Hauser, P. C., *Anal. Chim. Acta* 2008, *607*, 15-29.

[21] Pumera, M., *Talanta* 2007, *74*, 358-364.

[22] Matysik, F. M., *Microchim. Acta.* 2007, *160*, 1-14.

[23] Brito-Neto, J. G. A., da Silva, J. A. F., Blanes, L., do Lago, C. L., *Electroanalysis* 2005, *17*, 1207-1214.

[24] Brito-Neto, J. G. A., da Silva, J. A. F., Blanes, L., do Lago, C. L., *Electroanalysis* 2005, *17*, 1198-1206.

[25] Kubáň, P., Hauser, P. C., *Electrophoresis* 2004, *25*, 3398-3405.

[26] Kubáň, P., Hauser, P. C., *Electrophoresis* 2004, *25*, 3387-3397.

[27] Wan, Q. J., Kubáň, P., Tanyanyiwa, J., Rainelli, A., Hauser, P. C., *Anal. Chim. Acta* 2004, *525*, 11-16.

[28] Tůma, P., Samcova, E., Duska, F., *J. Sep. Sci.* 2008, *31*, 2260-2264.

[29] Kubáň, P., Hauser, P. C., *Lab Chip* 2008, *8*, 1829-1836.

[30] Tůma, P., Samcova, E., Andelova, K., *J. Chromatogr. B* 2006, *839*, 12-18.

[31] Tůma, P., Samcova, E., *Chem. Listy* 2007, *101*, 200-207.

[32] Tůma, P., Samcova, E., Opekar, F., Jurka, V., Stulik, K., *Electrophoresis* 2007, *28*, 2174-2180.

[33] Pormsila, W., Krähenbühl, S., Hauser, P. C., *Anal. Chim. Acta* 2009, *636*, 224-228.

[34] Petsch, M., Mayer-Helm, B. X., Sauermann, R., Joukhadar, C., Kenndler, E., *Electrophoresis* 2004, *25*, 2292-2298.

[35] Law, W. S., Kubáň, P., Yuan, L. L., Zhao, J. H., Li, S. F. Y., Hauser, P. C., *Electrophoresis* 2006, *27*, 1932-1938.

[36] Belin, G. K., Krähenbühl, S., Hauser, P. C., *J. Chromatogr. B* 2007, *847*, 205-209.

[37] Gong, X. Y., Kubáň, P., Scholer, A., Hauser, P. C., *J. Chromatogr. A* 2008, *1213*, 100-104.

[38] Pormsila, W., Gong, X. Y., Hauser, P. C., *Electrophoresis* 2010, *31*, 2044-2048.

[39] Masár, M., Poliaková, K., Danková, M., Kaniansky, D., Stanislawski, B., *J. Sep. Sci.* 2005, *28*, 905-914.

[40] Maier, V., Petr, J., Knob, R., Horakova, J., Sevcik, J., *Electrophoresis* 2007, *28*, 1815-1822.

[41] Rocha, F. R., Coelho, L. H. G., Lopes, M. L. A., Carvalho, L. R. F., da Silva, J. A. F., do Lago, C. L., Gutz, I. G. R., *Talanta* 2008, *76*, 271-275.

[42] Kubáň, P., Nguyen, H. T. A., Macka, M., Haddad, P. R., Hauser, P. C., *Electroanalysis* 2007, *19*, 2059-2065.

[43] Zhang, L., Khaloo, S. S., Kubáň, P., Hauser, P. C., *Meas. Sci. Technol.* 2006, *17*, 3317-3322.

[44] Chuang, C. K., Wang, T. J., Yeung, C. Y., Hsieh, W. S., Lin, D. S., Ho, S. C., Lin, S. P., *Clin. Biochem.* 2006, *39*, 74-77.

**Table 1.**

Quantification results for lactate in certified serum reference material.

| No | Certified lactate concentration in reference material (mmol/L) | | Concentration determined with CE-C$^4$D (mmol/L)[1] | Concentration determined with the enzymatic method (mmol/L)[2] |
|---|---|---|---|---|
| | mean | range | | |
| 1 | 1.37 | 1.08-1.65 | 1.7±0.2 | 1.9 |
| 2 | 3.33 | 2.63-4.03 | 3.6±0.4 | 3.3 |
| 3 | 5.25 | 4.15-6.35 | 6.0±0.2 | 5.5 |

[1]mean±standard deviations (n = 3)

[2]Quantification by fluorescence measurement according to reference [4] (n = 1).

**Table 2.**
Quantitative results for plasma lactate. Subjects (1 to 10) were tested at rest (samples a, c) and after ergometry for 1 h at 65% of the maximal workload (samples b, d) determined earlier. As the determinations were repeated after 12 weeks, 4 samples were obtained from each participant.

| Candidate | Sample | Concentration determined by CE-$C^4D$ (mmol/L)[1] | Concentration determined with the enzymatic method (mmol/L)[2] |
|---|---|---|---|
| 1 | 1a | 1.08±0.07 | 1.30 |
| | 1b | 4.26±0.04 | 3.51 |
| | 1c | 2.14±0.13 | 2.01 |
| | 1d | 6.67±0.24 | 4.62 |
| 2 | 2a | 1.95±0.02 | 1.51 |
| | 2b | 6.35±0.22 | 4.93 |
| | 2c | 1.63±0.06 | 1.32 |
| | 2d | 4.90±0.16 | 3.87 |
| 3 | 3a | 2.24±0.06 | 1.33 |
| | 3b | 12.56±0.98 | 10.80 |
| | 3c | 2.48±0.10 | 2.19 |
| | 3d | 15.32±0.30 | 15.72 |
| 4 | 4a | 1.90±0.03 | 1.39 |
| | 4b | 5.94±0.11 | 5.08 |
| | 4c | 2.40±0.08 | 1.44 |
| | 4d | 4.56±0.09 | 4.36 |
| 5 | 5a | 1.07±0.02 | 0.73 |
| | 5b | 3.77±0.08 | 5.45 |
| | 5c | 2.24±0.04 | 1.70 |
| | 5d | 13.31±0.05 | 11.00 |
| 6 | 6a | 0.89±0.02 | 0.71 |
| | 6b | 6.66±0.05 | 6.89 |
| | 6c | 0.93±0.08 | 0.84 |
| | 6d | 5.70±0.36 | 6.59 |
| 7 | 7a | 0.90±0.04 | 0.77 |
| | 7b | 4.70±0.09 | 4.44 |
| | 7c | 1.01±0.09 | 0.79 |
| | 7d | 3.44±0.12 | 2.34 |
| 8 | 8a | 1.03±0.07 | 0.72 |
| | 8b | 7.06±0.38 | 6.00 |
| | 8c | 0.79±0.01 | 0.46 |
| | 8d | 2.91±0.09 | 2.63 |
| 9 | 9a | 1.71±0.09 | 1.17 |
| | 9b | 3.77±0.07 | 3.46 |
| | 9c | 2.99±0.10 | 3.96 |
| | 9d | 2.84±0.23 | 3.69 |
| 10 | 10a | 0.98±0.02 | 0.86 |
| | 10b | 2.96±0.08 | 3.59 |
| | 10c | 1.10±0.06 | 0.86 |
| | 10d | 2.79±0.23 | 2.99 |

[1]mean±standard deviations (n = 3), [2] n = 1.

**Table 3.**

Quantitative results for exhaustive exercise testing and determination of the lactate threshold in one subject.

| Sample | Exercise time (min) | Concentration determined with CE-$C^4D$ (mmol/L)[1] | Concentration determined with the enzymatic method (mmol/L)[1] |
|---|---|---|---|
| 1 | 0 | 1.31±0.13 | 1.19±1.04 |
| 2 | 2 | 1.40±0.02 | 1.45±0.77 |
| 3 | 4 | 1.67±0.31 | 1.54±0.29 |
| 4 | 6 | 2.04±0.09 | 2.35±0.52 |
| 5 | 8 | 2.80±0.15 | 3.49±0.74 |
| 6 | 10 | 3.87±0.06 | 2.83±0.39 |
| 7 | 12 | 4.94±0.11 | 4.70±0.56 |
| 8 | 14 | 5.37±0.16 | 6.30±1.05 |
| 9 | 16 | 8.56±0.22 | 10.51±1.84 |
| 10 | 18-20 | 11.61±0.21 | 13.15±0.31 |
| 11 | 25 | 12.30±0.24 | 13.54±0.69 |
| 12 | 30 | 8.63±0.33 | 8.25±0.87 |
| 13 | 35 | 7.89±0.09 | 7.64±1.58 |

[1]mean±standard deviations (n = 4, in both cases)

**Figure Captions**

Figure 1. Electropherogram of plasma. a) sample diluted 5-fold, b) spiking 100 μmol/L pyruvate and 200 μmol/L lactate. CE conditions: 10 mmol/L of MES-His, 70 μmol/L CTAB, siphoning injection at 10 cm height difference for 15 s and -20 kV separation voltage.

Figure 2. Plasma lactate concentrations (circles) determined during ergometry at rising workloads (bars) until exhaustion. The workload started at 220 W and was increased by 20 W every 2 minutes. Exhaustion was achieved after 18 minutes at 380 W.

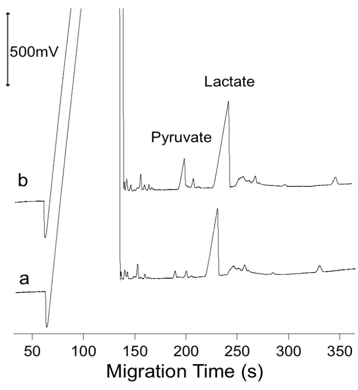



Fig. 1

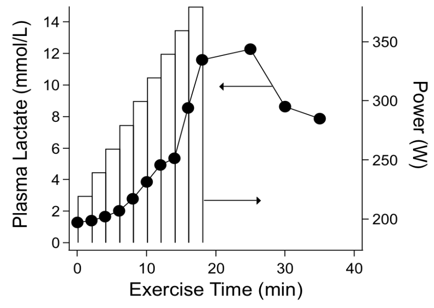



Fig. 2

## 3rd Project

# CAPILLARY ELECTROPHORESIS WITH CONTACTLESS CONDUCTIVITY DETECTION FOR THE DETERMINATION OF CARNITINE AND ACYLCARNITINES IN CLINICAL SAMPLES

**Submitted article to Journal of Chromatography B**

*Manuscript
Click here to view linked References



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

# Capillary electrophoresis with contactless conductivity detection for the determination of carnitine and acylcarnitines in clinical samples

Worapan Pormsila[a,b], Réjane Morand[c], Stephan Krähenbühl[c], Peter C. Hauser[a]*

[a]Department of Chemistry, University of Basel, Spitalstrasse 51, 4056 Basel, Switzerland.

[b]Department of Chemistry, Rajamangala University of Technology Krungthep, 2 Nanglingi, Sathorn, 10120 Bangkok, Thailand

[c]Division of Clinical Pharmacology & Toxicology, University Hospital Basel and Department of Biomedicine, University of Basel, Hebelstrasse 2, 4031 Basel, Switzerland

*Corresponding author. Tel: ++41 61 267 10 03; Fax: ++41 61 267 10 13

E-mail address: Peter.Hauser@unibas.ch (P.C. Hauser)

## ABSTRACT

A capillary electrophoresis method with contactless conductivity detection was developed for the quantification of carnitine and six acylcarnitines in plasma and urine samples. The running buffer arrived at consisted of 500 mmol/L acetic acid, 1.0 mmol/L hydroxypropyl-β-cyclodextrin and 0.05% Tween at a pH of 2.6. Under these conditions, the isomeric valproyl- and octanoyl-carnitines could be distinguished. The linearity was in the range from 5.0 to 200.0 μmol/L with correlation coefficients between 0.9992-0.9997. The limits of detection were between 1.0 and 3.2 μmol/L. Intra- and inter-day precisions as %RSD were below 10%. The method allows for direct determination without derivatization or extraction processes. The method was applied for the quantification of carnitine and acetylcarnitine in plasma pre- and post-exercise, and to measure valproylcarnitine in plasma and urine of patients undergoing valproate therapy.



Keywords:

Capillary electrophoresis; Conductivity detection; Carnitine; Acylcarnitines; Clinical samples

## 1. Introduction

Carnitine is an endogenous molecule, which is present as L-carnitine in most mammalian tissues [1]. Mammals ingest the largest portion of the carnitine body needs by the diet [2]. In particular meat, and, to a lesser extent, also dairy products are rich in carnitine, whereas plants contain almost no carnitine [1-3]. Carnitine can also be biosynthesized by mammals from the amino acids lysine and methionine primarily in skeletal muscle, liver and kidney [1,2,4]. In omnivores, however, biosynthesis is less important than dietary intake.

Carnitine plays an important role for mitochondrial β-oxidation of long-chain fatty acids by enabling the transport of long-chain fatty acids across the inner mitochondrial membrane for energy production [1,2,5]. Carnitine can be esterified at the hydroxy group by acyltransferases to generate acylcarnitines with different chain lengths (see Figure 1). This esterification is not only essential for the transport of long-chain fatty acids into the mitochondrial matrix [6], but also for the role of carnitine in buffering the cellular pool of free coenzyme A [7].

Since acylcarnitines are intermediates of many metabolic pathways and can be transported from cells into plasma and excreted in the urine [1,2,8], the determination of carnitine and acylcarnitines in plasma and/or urine is helpful in the diagnosis of metabolic diseases such as organic acidurias and disturbances in amino acid metabolism. Analytical techniques used for the quantification of carnitine and acylcarnitines in biological samples include enzymatic, chromatographic and mass-spectrometric methods. Since carnitine exists in biochemical samples usually only in its L-enantiomeric form, most methods published for this purpose are not

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

enantioselective. The enzymatic methods are based on carnitine acetyltransferase (CAT) and the cofactor acetyl-coenzyme A (acetyl-CoA), leading to the generation of acetylcarnitine and free CoA [9]. Using this method, with the exception of acetylcarnitine, specific acylcarnitines cannot be determined. For the determination of specific acylcarnitines, HPLC methods have first been developed, but carnitine and acylcarnitines have to be derivatized before detection by UV or fluorescene. For instance, 4'-bromophenacyl trifluoromethanesulfonate has often been used as a pre-column derivative reagent for UV-absorption detection [10-12], while 1-aminoanthracene and 1-ethyl-3-(3-di-methylaminopropyl) [13] have been used for fluorescence detection. Mass spectrometric detection following separation of acylcarnitines by HPLC has also been reported, but these methods still require sample work-up, and, for some methods, analyte derivatization [14-19].

Capillary electrophoresis (CE) is an alternative separation method for the carnitines, which usually does not require extensive sample work-up and has the advantage of only requiring small amounts of sample. Mass spectrometric detection can also be used following separation with CE and in this case, derivatization is not required [20,21]. By employing UV detection, carnitine and acylcarnitines can be measured as derivatives of 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde [22], 4'-bromophenacyl trifluoromethanesulfonate [23], or 9-anthryldiazomethane [24], or they can be determined indirectly using a buffer containing quinine sulfate or copper sulfate [25]. An attractive alternative detection method for CE is capacitively coupled conductivity detection ($C^4D$), which is universally applicable for ions, but particularly attractive for non-UV absorbing species. Details on this relatively new method can be found in recent review articles [26-30]. A number of reports on the use of CE-$C^4D$

for the determination of small organic species of clinical interest have been published, for instance the determination of tobramycin [31], and the quantification of valproic acid [32] and uric acid [33]. Recently, Pormsila *et al.* [3] reported the determination of carnitine in food and food supplements. As a further development of this method towards clinical application, we report herein the determination of carnitine as well as of short- and medium-chain acylcarnitines in human plasma and urine samples.

## 2. Materials and methods

### 2.1. Instrumentation

The capillary electrophoresis instrument was purpose-built and utilized a commercial high voltage power supply module (CZE 2000R, Spellman, Pulborough, UK). The detector was 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 320 kHz and amplitude of 330 Vpp (peak-to-peak) was used for cell excitation. The cell current was converted to a voltage, which was then rectified, low pass filtered and finally acquired with an e-corder system (eDAQ, Denistone East, NSW, Australia). A fused-silica capillary of 50 µm I.D. and 375 µm O.D. (Polymicro Technologies, Phoenix, AZ, USA) and total and effective lengths of 40 and 32 cm, respectively, was employed. A new capillary was conditioned by flushing with 0.1 mmol/L sodium hydroxide for 5 min, followed by deionized water for 5 min and then by 0.1 mmol/L hydrochloric acid for 5 min before rinsing with the running buffer for 15 min. Each day before starting experiments, the capillary was rinsed with the sodium hydroxide solution for 2 min, deionized water for 2 min and running buffer for 10 min. Between runs, the capillary was reconditioned with the running buffer for

2 min to maintain the reproducibility of the analysis. Injection of standards and samples was carried out hydrostatically by siphoning at 10 cm height difference for 15 sec. The separation was carried out with a positive voltage of 15 kV applied at the injection end.

### 2.2. Reagents and standards

All chemicals were of analytical reagent grade. L-Carnitine hydrochloride (MW 197.7 g/mol), DL-acetylcarnitine hydrochloride (MW 239.7 g/mol), DL-hexanoylcarnitine hydrochloride (MW 295.8 g/mol) and Tween®20 (Polyethylene glycol [20] sorbitan monolaurate) were obtained from Sigma-Aldrich (Buchs, Switzerland). Acetic acid, 18-crown-6, β-cyclodextrin (β-CD), hydroxypropyl-α-cyclodextrin (HP-α-CD), (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) and (2-hydroxypropyl)-γ-cyclodextrin (HP-γ-CD) were purchased from Fluka (Buchs, Switzerland). L-Propionylcarnitine hydrochloride (MW 253.7 g/mol), L-isovalerylcarnitine hydrochloride (MW 281.8 g/mol), L-octanoylcarnitine hydrochloride (MW 323.9 g/mol) and L-valproylcarnitine hydrochloride (MW 323.9 g/mol) were gifts from the VU Medical Center, Metabolic Laboratory (Amsterdam, Netherlands). The biological samples were obtained at the Division of Clinical Pharmacology & Toxicology, University Hospital of Basel. Methanol (Fluka) and acetonitrile (Fischer Scientific, Wohlen, Switzerland) of HPLC grade were used for deproteination. The deionized water used throughout this work had a resistivity of 18 MΩ·cm and was obtained from a Millipore Milli-Q Plus 185 purifier (Labtec Service AG, Wohlen, Switzerland). The running buffer (500 mmol/L acetic acid, 1.0 mmol/L [2-hydroxypropyl]-β-cyclodextrin (HP-β-CD) and 0.05% Tween 20) was adopted at pH 2.6, prepared freshly every day and was degassed before use. An aqueous stock

solution of 200 μmol/L of carnitine and 100 μmol/L acylcarnitines was prepared and diluted in the running buffer. The pH-value of the buffer was determined with a pH-meter (model 744, Metrohm, Herisau, Switzerland).

### 2.3. Samples

The biological samples were obtained from the Division of Clinical Pharmacology & Toxicology, University Hospital of Basel and were kept frozen at -20°C until the experiments. The studies where the samples originated from had been accepted by the Ethics Committee of the two Cantons of Basel. The plasma samples were deproteinized to prevent the adsorption of proteins on the capillary wall, which aids in maintaining the reproducibility. This was achieved by adding 50 μL of a acetronitrile/methanol mixture (3:1 v/v) to 50 μL of plasma sample. The urine samples were analysed without the deproteinization procedure. Both types of samples, plasma and urine, were centrifuged for 5 min at 3000 × g for complete separation of solids, and the supernatant was diluted 1:4 with running buffer before direct injection into the CE system. The enzymatic determinations were carried out using the method described by Brass and Hoppel [9] while the LC/MS methods employed are described in two publications by Vernez *et al.* for urine [16] and plasma [17].

## 3. Results and discussion

### 3.1. CE conditions

The determination of free carnitine ($C_0$) and the six short chain acylcarnitines acetylcarnitine ($C_2$), propionylcarnitine ($C_3$), isovalerylcarnitine ($C_5$),

hexanoylcarnitine ($C_6$), valproylcarnitine ($C_{8V}$) and octanoylcarnitine ($C_{8O}$), see the structures in Figure 1, was investigated. Acetylcarnitine is the most abundant acylcarnitine in plasma and in tissues of healthy persons [34,35]. It is formed from acetyl-CoA and carnitine by the action of carnitine acetyl transferase and has an important buffer function for the coenzyme A pool [7,34]. Propionylcarnitine, isovalerylcarnitine, hexanoylcarnitine and octanoylcarnitine are almost absent in plasma and urine of healthy persons [35], but can accumulate in patients with organic acidurias [36-38] Valproylcarnitine does not occur naturally, but can be detected in patients treated with the antiepileptic valproic acid [39].

In order to avoid the zwitterionic form and to allow separation and detection by $C^4D$ as a cation, a pH-value below the $pK_a$ of carnitine at 3.8 is required. A solution of acetic acid at 500 mmol/L (pH 2.6), which had been used successfully in previous work for the determination of carnitine in food and food supplements [3], was thus adopted as the running buffer. The solution included 0.05% Tween 20 (a nonionic detergent) in order to prevent adsorption of analytes on the capillary wall. A separation voltage of +15 kV (40 cm capillary length) and injection by siphoning for 15 seconds at 10 cm elevation was employed. Using this system, the separation of carnitine and acylcarnitines could be achieved within 7 minutes. The migration times of carnitine and its esters followed the expected sequence according to the size of the molecules. However, as shown in electropherogram (a) of Fig. 2, a serious peak overlap between the isomeric valproylcarnitine ($C_{8V}$) and octanoylcarnitine ($C_{8O}$) was observed. Hence, the inclusion of buffer additives was studied intensively in order to achieve separation of these isomers.

### 3.2. Effect of buffer additives

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

The first attempt to overcome the overlap between valproyl- ($C_{8V}$) and octanoyl-carnitine ($C_{8O}$) was made by including heptafluorobutyric acid (at 5.0 mmol/L) in the separation buffer. This substance had been employed successfully as an ion-pairing reagent by Vernez et al. [16,17] in the chromatographic separation of the acylcarnitines. However, this additive was found not to be suitable as it did not lead to an improved separation (see trace (b) of Fig. 2) and caused a strongly fluctuating baseline due to Joule heating due to a significantly increased conductivity of the buffer.

The addition of complexing agents used in the separation of cationic enantiomers and other isomers was then investigated. 18-crown-6 had been found useful to achieve the separation of *cis*- and *trans*-1,2-diaminocyclohexane in CE-$C^4$D [40] and was thus considered, but, as can be seen from trace (c) of Fig. 2, was of no benefit in this case. The use of neutral cyclodextrins was studied next. This class of toroidal compounds has been employed successfully for the separation of the enantiomers of organic amines in CE-$C^4$D [41-44]. These compounds are effective by forming weak complexes with the analytes in which the hydrophilic rim interacts with the charged parts of the analytes, while the relatively hydrophobic cavity interacts with the lipophilic ends. As evidenced by electropherogram (d) of Fig. 2, hydroxypropyl-β-cyclodextrin (HP-β-CD) had indeed a strong effect on the retention time of the acylcarnitines with longer chain lengths, and in particular the isomeric species valproylcarnitine and octanoylcarnitine could be well separated. Subsequently the effect of several other cyclodextrins was also investigated, and, as seen in Fig. 3, underivatized β-cyclodextrin (β-CD), hydroxypropyl-α-cyclodextrin (HP-α-CD) as

well as 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD) were found almost equally well suitable. Hydroxypropyl-β-cyclodextrin (HP-β-CD) was finally chosen and investigated in a concentration range between 0.5 and 5 mmol/L in order to optimize analysis time and analyte separation. As shown in Fig. 4, a level of 1.0 mmol/L allowed baseline separation of the 7 compounds in 7 min.

### 3.3. Quantification

Calibration curves for carnitine and its esters were acquired in a concentration range of 5.0 to 200.0 µmol/L. Good linearity was obtained with correlation coefficients between 0.9992-0.9997. The limits of detection (3 signal to noise) were determined to be between 1.0 and 3.2 µmol/L, with a clear trend to higher values for the larger carnitine derivatives. The detailed data is given in Table 1. The intraday- and interday- repeatability was also determined and quantification could be reproduced to within a few percent. See Table 2 for the detailed data.

### 3.4. Analysis of biological samples

The first set of samples consisted of plasma samples which had been collected in a clinical study investigating the effect of oral carnitine supplementation on physical performance (S. Krähenbühl, unpublished data). During a period of 12 weeks, 10 volunteers ingested 2.0 g/day of L-carnitine. Four sets of samples were obtained, the first two at the beginning of the study, and the second set at its end. At each of the collection times, the volunteers were carrying out an ergometric test on an exercise bicycle of 60 minutes duration at 65% of their maximal performance, which had been determined previously [34]. Venous blood samples were obtained before and immediately after the end of the physical activity. Of interest were the pre- and post-

exercise levels of free carnitine ($C_0$) and of acetylcarnitine ($C_2$). An example for the plasma electropherograms obtained for samples taken before and after the exercise are shown in Fig. 5. The quantitative data for the 10 subjects is given in Table 3. Several trends are discernable. Firstly, the free carnitine levels are generally higher at the end of the study, which can be explained by the ingestion of the L-carnitine supplement [45]. Then, the levels of free carnitine decrease during the 60 minutes of ergometric exercise, while the acetylcarnitine levels significantly increase. Since also the plasma lactate levels increased with exercise (data not shown), the exercise level was above the lactate threshold. The pyruvate produced by glycolysis in skeletal muscle therefore exceeded the activity of the Krebs cycle, leading to cellular accumulation of acetyl-CoA, which can be converted to acetylcarnitine [34]. Acetylcarnitine can be transported out of the cells and enter plasma, where it can be detected. For comparison, values for carnitine determined using the radioenzymatic method [9] and for acetylcarnitine using LC/MS [17] are also given in the table. As shown in Fig. 6, a correlation of the values obtained by the different methods showed an acceptable result.

The second set of the plasma and urine samples were collected from 8 patients who were orally treated with valproic acid at 500-2000 mg/day (S. Krähenbühl, unpublished data). Valproic acid is an anticonvulsant and mood-stabilizing agent widely used in the treatment of epilepsy, bipolar disorders and prophylaxis of migraine headaches [46-48]. Not only free carnitine ($C_0$) and acetylcarnitine ($C_2$), but also valproylcarnitine ($C_{8V}$) would be expected in these samples. Valproate can be activated to the corresponding CoA-derivative, which can be converted to valproylcarnitine, transported into plasma and excreted in the urine [39]. An

electropherogram for a urine sample containing valproylcarnitine is shown in Fig. 7. The quantitative results for the three species for all plasma and urine samples are given in Table 4, along with the results obtained by LC/MS. As seen, carnitine could be successfully quantified in all samples. Acetylcarnitine could be measured in all but two of the urine samples, in which the levels were below the detection limits for the CE method. Valproylcarnitine was present at low levels, and the quantification by CE was only possible for three of the 8 urines sampled.

## 4. Conclusion

Under optimal conditions, carnitine and six short chain acylcarnitines, especially the isomeric compounds octanoyl- and valproyl-carnitine could be separated by inclusion of a cyclodextrin additive. Separation of carnitine and acylcarnitines was also possible in clinical samples. If acylcarnitine species not included here need to be determined, this should be possible by using the same approach. The agreement with the currently used more complex methods such as LC/MS was good, but the detection limits of the CE method were not quite adequate for the quantification of the derivatives present at low concentrations, as for instance valproylcarnitine in the plasma samples. It is expected that a preconcentration method, such as solid-phase extraction, would enable an extension of the CE-$C^4D$-method to such tasks as well.

## Acknowledgements

The authors would like to thank Dr. Herman J. ten Brink, of the VU Medical Center, Metabolic Laboratory (Amsterdam, Netherlands) for providing the acylcarnitines

standards. Partial funding was supported by the Thai Government Scholarship through the Rajamangala University of Technology Krungthep, Bangkok, Thailand and the Swiss National Science Foundation through grant No. 200020-126384/1.

## References


1
2
3 [1] J. Bremer, Physiol Rev 63 (1983) 1420.
4
5 [2] C.J. Rebouche, D.J. Paulson, Annu Rev Nutr 6 (1986) 41.
6
7 [3] W. Pormsila, S. Krähenbühl, P.C. Hauser, Electrophoresis
8 31 (2010) 2186.
9
10
11 [4] F.M. Vaz, R.J. Wanders, Biochem J 361 (2002) 417.
12
13 [5] I.B. Fritz, Acta Physiol Scand 34 (1955) 367.
14
15 [6] J. Kerner, C. Hoppel, Biochim Biophys Acta 1486 (2000) 1.
16
17 [7] E.P. Brass, C.L. Hoppel, Biochem J 190 (1980) 495.
18
19
20 [8] R.A. Chalmers, C.R. Roe, T.E. Stacey, C.L. Hoppel, Pediatr Res 18 (1984)
21 1325.
22
23 [9] E.P. Brass, C.L. Hoppel, J Biol Chem 253 (1978) 2688.
24
25
26 [10] P.E. Minkler, S.T. Ingalls, L.S. Kormos, D.E. Weir, C.L. Hoppel, J Chromatogr
27 336 (1984) 271.
28
29 [11] P.E. Minkler, C.L. Hoppel, Clin Chim Acta 212 (1992) 55.
30
31 [12] P.E. Minkler, E.P. Brass, W.R. Hiatt, S.T. Ingalls, C.L. Hoppel, Anal Biochem
32 231 (1995) 315.
33
34
35 [13] Q.R. Cao, S. Ren, M.J. Park, Y.J. Choi, B.J. Lee, Arch Pharmacol Res 30
36 (2007) 1041.
37
38 [14] P.E. Minkler, S.T. Ingalls, C.L. Hoppel, Anal Chem 77 (2005) 1448.
39
40
41 [15] C.L. Hoppel, P.E. Minkler, Molecul Gen Metabol 98 (2009) 547.
42
43 [16] L. Vernez, G. Hopfgartner, M. Wenk, S. Krähenbühl, J Chromatogr A 984
44 (2003) 203.
45
46
47 [17] L. Vernez, M. Wenk, S. Krähenbühl, Rapid Commun Mass Spectrom 18 (2004)
48 1233.
49
50 [18] A.K. Ghoshal, T.D. Guo, N. Soukhova, S.J. Soldin, Clin Chim Acta 358 (2005)
51 104.
52
53
54 [19] F. Hirche, M. Fischer, J. Keller, K. Eder, J Chromatogr B 877 (2009) 2158.
55
56 [20] K. Heinig, J. Henion, J Chromatogr B 735 (1999) 171.
57
58 [21] C. Desiderio, A. De Rossi, R. Inzitari, A. Mancinelli, D.V. Rossetti, M.
59 Castagnola, I. Messana, Anal Bioanal Chem 390 (2008) 1637.
60
61
62
63
64
65

[22] O. Boulat, D.G. McLaren, E.A. Arriaga, D.D.Y. Chen, J Chromatogr B 754 (2001) 217.

[23] L. Vernez, W. Thormann, S. Krähenbühl, J Chromatogr A 895 (2000) 309.

[24] S. Kiessig, C. Vogt, J Chromatogr A 781 (1997) 475.

[25] C. Mardones, N. Vizioli, C. Carducci, A. Rios, M. Valcarcel, Anal Chim Acta 382 (1999) 23.

[26] P. Kubáň, P.C. Hauser, Electrophoresis 30 (2009) 176.

[27] M. Trojanowicz, Anal Chim Acta 653 (2009) 36.

[28] P. Kubáň, P.C. Hauser, Anal Chim Acta 607 (2008) 15.

[29] F.M. Matysik, Microchim Acta 160 (2008) 1.

[30] M. Pumera, Talanta 74 (2007) 358.

[31] W.S. Law, P. Kubáň, L.L. Yuan, J.H. Zhao, S.F.Y. Li, P.C. Hauser, Electrophoresis 27 (2006) 1932.

[32] G.K. Belin, S. Krähenbühl, P.C. Hauser, J Chromatogr B 847 (2007) 205.

[33] W. Pormsila, S. Krähenbühl, P.C. Hauser, Anal Chim Acta 636 (2009) 224.

[34] R. Friolet, H. Hoppeler, S. Krähenbühl, J Clin Invest 94 (1994) 1490.

[35] L. Vernez, M. Dickenmann, J. Steiger, M. Wenk, S. Krähenbühl, Nephrol Dial Transplant 21 (2006) 450.

[36] F. Deodato, S. Boenzi, F.M. Santorelli, C. Dionisi-Vici, Am J Med Genet C Semin Med Genet 142C (2006) 104.

[37] E.H. Touma, C. Charpentier, Arch Dis Child 67 (1992) 142.

[38] H. Ogier de Baulny, J.M. Saudubray, Semin Neonatol 7 (2002) 65.

[39] H. Muro, T. Tatsuhara, T. Sugimoto, M. Woo, N. Nishida, K. Murakami, Y. Yamaguchi, J Chromatogr B 663 (1995) 83.

[40] X.Y. Gong, P.C. Hauser, Electrophoresis 27 (2006) 468.

[41] M. Lecoeur-Lorin, R. Delepee, P. Morin, Electrophoresis 30 (2009) 487.

[42] X.Y. Gong, D. Dobrunz, M. Kümin, M. Wiesner, J.D. Revell, H. Wennemers, P.C. Hauser, J Sep Sci 31 (2008) 565.

[43] X.Y. Gong, P.C. Hauser, Electrophoresis 27 (2006) 4375.

[44] X.Y. Gong, P. Kubáň, J. Tanyanyiwa, P.C. Hauser, J Chromatogr A 1082 (2005) 230.

Table 1

Linearity and LOD for carnitine and six acylcarnitines in aqueous solutions (n = 3)

| Compounds | y = mx+c | | Correlation coefficient, r | LOD (μmol/L) |
|---|---|---|---|---|
| | m (V·s·μM$^{-1}$) | c (V·s) | | |
| Carnitine ($C_0$) | 0.0058 | 0.0097 | 0.9996 | 1.0 |
| Acetylcarnitine ($C_2$) | 0.0034 | -0.0012 | 0.9993 | 1.6 |
| Propionylcarnitine ($C_3$) | 0.0033 | 0.0007 | 0.9992 | 2.3 |
| Isovalerylcarnitine ($C_5$) | 0.0040 | -0.0024 | 0.9994 | 2.0 |
| Hexanoylcarnitine ($C_6$) | 0.0041 | -0.0028 | 0.9997 | 2.2 |
| Valproylcarnitine ($C_{8V}$) | 0.0049 | 0.0044 | 0.9994 | 2.0 |
| Octanoylcarnitine ($C_{8O}$) | 0.0041 | 0.0085 | 0.9992 | 3.2 |

Table 2

Intraday- and interday-variability data for carnitine and six acylcarnitines.

| Compounds | Intra-day variability (%RSD) | | Inter-day variability (%RSD) | |
|---|---|---|---|---|
| | Peak area | Migration time | Peak area | Migration time |
| Carnitine ($C_0$) | 1.1-4.5 | 6.5-9.5 | 2.6 | 8.0 |
| Acetylcarnitine ($C_2$) | 1.3-2.3 | 4.3-9.4 | 1.8 | 6.9 |
| Propionylcarnitine ($C_3$) | 0.6-1.4 | 4.8-9.0 | 1.0 | 6.9 |
| Isovalerylcarnitine ($C_5$) | 1.4-2.3 | 5.2-9.7 | 1.9 | 7.5 |
| Hexanoylcarnitine ($C_6$) | 1.6-2.5 | 5.4-9.3 | 2.1 | 7.4 |
| Valproylcarnitine ($C_{8V}$) | 1.7-2.6 | 5.3-7.6 | 2.2 | 7.4 |
| Octanoylcarnitine ($C_{8O}$) | 1.2-2.1 | 5.6-9.2 | 1.7 | 6.5 |

10 determinations for intra-day variability

10 determinations and 3 consecution days for inter-day variability

Table 3

Quantitative results for plasma carnitine ($C_0$) and acetylcarnitine ($C_2$). Subjects (1 to 10) were tested at rest (samples A, C) and after ergometry for 1 h at 65% of the maximal workload (samples B, D). The second set of the samples (C, D) was obtained after oral treatment with L-carnitine (2 g per day) for 12 weeks.

| Candidate | | $C_0$ (µmol/L) | | $C_2$ (µmol/L) | |
|---|---|---|---|---|---|
| | | CE[1] | Enzymatic[2] | CE[1] | HPLC[2] |
| 1 | 1A | 44.1±3.2 | 41.2 | <LOD | 4.7 |
| | 1B | 28.0±4.3 | 34.6 | 14.2±2.6 | 14.8 |
| | 1C | 33.8±1.3 | 52.1 | 7.2±2.9 | 9.8 |
| | 1D | 56.5±3.3 | 49.8 | 18.4±3.2 | 19.0 |
| 2 | 2A | 42.3±3.6 | 42.1 | 10.3±3.6 | 6.9 |
| | 2B | 44.2±2.3 | 35.4 | 23.1±4.3 | 19.2 |
| | 2C | 43.6±5.4 | 51.6 | 7.2±4.0 | 11.0 |
| | 2D | 55.5±1.0 | 51.4 | 33.4±3.9 | 21.9 |
| 3 | 3A | 28.1±1.0 | 36.8 | <LOD | 4.6 |
| | 3B | 40.0±1.1 | 33.5 | 9.5±3.2 | 8.6 |
| | 3C | 50.3±3.1 | 45.2 | 7.5±2.2 | 5.8 |
| | 3D | 43.1±1.3 | 42.1 | 10.0±1.2 | 11.9 |
| 4 | 4A | 55.5±2.3 | 49.3 | <LOD | 5.0 |
| | 4B | 50.8±3.5 | 54.1 | 14.8±2.1 | 15.1 |
| | 4C | 50.3±5.4 | 65.2 | 9.0±2.2 | 6.2 |
| | 4D | 69.7±3.5 | 64.1 | 15.3±2.4 | 18.0 |
| 5 | 5A | 45.2±4.3 | 39.5 | <LOD | 3.4 |
| | 5B | 42.6±2.9 | 31.2 | 11.3±1.4 | 8.0 |
| | 5C | 75.7±4.3 | 70.6 | 7.9±2.7 | 7.4 |
| | 5D | 80.3±2.0 | 74.8 | 28.3±4.0 | 15.2 |
| 6 | 6A | 26.9±3.5 | 29.6 | <LOD | 4.8 |
| | 6B | 23.1±2.2 | 27.4 | 7.7±2.2 | 7.5 |
| | 6C | 42.1±1.9 | 45.6 | <LOD | 6.3 |
| | 6D | 41.1±2.1 | 44.9 | 11.2±3.1 | 9.3 |
| 7 | 7A | 32.2±2.0 | 37.3 | 9.8±1.0 | 8.6 |
| | 7B | 31.4±0.9 | 33.6 | 15.1±2.3 | 16.8 |
| | 7C | 44.9±4.5 | 49.6 | 6.3±2.0 | 7.7 |
| | 7D | 35.1±2.2 | 43.2 | 17.5±3.4 | 10.2 |
| 8 | 8A | 34.4±1.6 | 37.0 | 6.3±1.6 | 7.4 |
| | 8B | 34.0±1.1 | 33.4 | 13.4±2.5 | 11.6 |
| | 8C | 44.4±1.6 | 42.7 | 10.0±2.9 | 13.4 |
| | 8D | 41.6±4.0 | 41.2 | 14.0±3.6 | 16.3 |
| 9 | 9A | 66.4±5.4 | 44.2 | 9.2±2.0 | 8.8 |
| | 9B | 50.2±2.5 | 40.2 | 18.1±1.0 | 15.8 |
| | 9C | 77.1±5.3 | 70.5 | 10.9±2.3 | 9.6 |
| | 9D | 78.8±2.3 | 70.6 | 28.4±3.0 | 25.1 |
| 10 | 10A | 43.6±1.0 | 42.8 | <LOD | 5.2 |
| | 10B | 40.1±5.6 | 39.2 | 15.7±1.9 | 13.6 |
| | 10C | 58.6±4.4 | 64.2 | 9.7±2.5 | 8.0 |
| | 10D | 68.2±3.2 | 60.0 | 21.5±3.1 | 23.6 |

[1]mean±standard deviations (n = 3), [2]n = 1,

Table 4

Quantitative results for carnitine ($C_0$), acetylcarnitine ($C_2$) and valproylcarnitine ($C_{8V}$) in plama andurine samples of patients treated with valproic acid

| Samples | Concentration by CE (μmol/L)[1] | | | Concentration by LC/MS (μmol/L)[2] | | |
|---|---|---|---|---|---|---|
| | $C_0$ | $C_2$ | $C_{8V}$ | $C_0$ | $C_2$ | $C_{8V}$ |
| Plasma | | | | | | |
| 1 | 45.6 | 5.3 | <LOD | 39.7 | 5.8 | ND |
| 2 | 43.1 | 3.6 | <LOD | 42.8 | 6.3 | ND |
| 3 | 47.5 | 11.2 | <LOD | 36.7 | 6.7 | 0.02 |
| 4 | 26.5 | 2.7 | <LOD | 31.6 | 3.9 | 0.05 |
| 5 | 28.6 | 3.0 | <LOD | 31.2 | 5.2 | 0.06 |
| 6 | 36.8 | 3.6 | <LOD | 38.7 | 4.7 | 0.09 |
| 7 | 31.2 | 4.5 | <LOD | 28.6 | 5.2 | ND |
| 8 | 24.5 | 3.9 | <LOD | 27.1 | 5.3 | 0.02 |
| Urine | | | | | | |
| 1 | 11.5 | 4.2 | <LOD | 14.0 | 5.2 | ND |
| 2 | 67.5 | 11.1 | <LOD | 50.4 | 10.1 | ND |
| 3 | 7.3 | <LOD | <LOD | 2.7 | 1.1 | 0.82 |
| 4 | 11.1 | <LOD | <LOD | 2.9 | 0.8 | 1.64 |
| 5 | 13.3 | 7.8 | 2.2 | 12.3 | 9.6 | 2.91 |
| 6 | 68.5 | 32.4 | 5.4 | 76.8 | 29.0 | 5.24 |
| 7 | 11.1 | 12.7 | <LOD | 15.0 | 12.7 | ND |
| 8 | 16.6 | 3.6 | 2.7 | 9.1 | 2.0 | 2.8 |

[1]mean (n=3), [2] n=1 and ND = not detected

Figure Captions

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Fig. 1. The stuctures of carnitine ($C_0$) and the acylcarnitines acetylcarnitine ($C_2$), propionylcarnitine ($C_3$), isovalerylcarnitine ($C_5$), hexanoylcarnitine ($C_6$), valproylcarnitine ($C_{8V}$) and octanoylcarnitine ($C_{8O}$).

Fig. 2. Electropherogram of standard solutions; 100 µmol/L for carnitine and and 50 µmol/L for acylcarnitines. 1) $C_0$, 2) $C_2$, 3) $C_3$, 4) $C_5$, 5) $C_6$, 6) $C_{8V}$ and 7) $C_{8O}$. CE conditions; A 500 mmol/L acetic acid, 0.05% Tween 20 at pH 2.6, +15 kV for separation voltage and 15 sec injection time (a) without modifier (b) with 5.0 µmol/L heptafluorobutyric acid, (c) with 5.0 mmol/L 18-crown-6 and (d) with 5.0 mmol/L hydroxypropyl-β-cyclodextrin (HP-β-CD).

Fig. 3. Electropherogram of standard solutions; 100 µmol/L for $C_0$ and 50 µmol/L $C_2$, $C_3$, $C_5$, $C_6$, $C_{8V}$ and $C_{8O}$ with different modifiers as 1.0 mmol/L, (a) β-CD, (b) HP-α-CD, and (c) HP-β-CD, CE conditions as for Fig. 2.

Fig. 4. Electropherogram of standard solutions; 100 µmol/L for $C_0$ and 50 µmol/L $C_2$, $C_3$, $C_5$, $C_6$, $C_{8V}$ and $C_{8O}$ with different concentrations of HP-β-CD, (a) 0.5 mmol/L, (b) 1.0 mmol/L, (c) 3.0 mmol/L and (d) 5.0 mmol/L. CE conditions as for Fig. 2.

Fig. 5. Electropherograms of plasma diluted 4-fold. a) before exercise and b) after exercise. CE conditions: A 500 mmol/L acetic acid, 1.0 mmol/L HP-β-CD and 0.5% Tween at pH 2.6, +15 kV for separation voltage and 15 sec injection time.

Fig. 6. Regression analysis of the carnitine and acetylcarnitine concentrations displayed in Table 3. The 10 subjects were tested at two occasions at rest and after ergometry for 1 h at 65% of the maximal workload as described in Table 3. The regression equations are: $y = 1.02 x – 0.277$ ($r^2 = 0.738$) for carnitine and $y = 1.09 x + 0.006$ ($r^2 = 0.736$) for acetylcarnitine.

Fig. 7. Electropherograms of urine diluted 4-fold. a) sample without spiking and b) spiked with $C_0$, $C_2$, $C_{8V}$ standards. CE as for Fig. 5.

**Figure**
**Click here to download Figure: Fig 1.pdf**

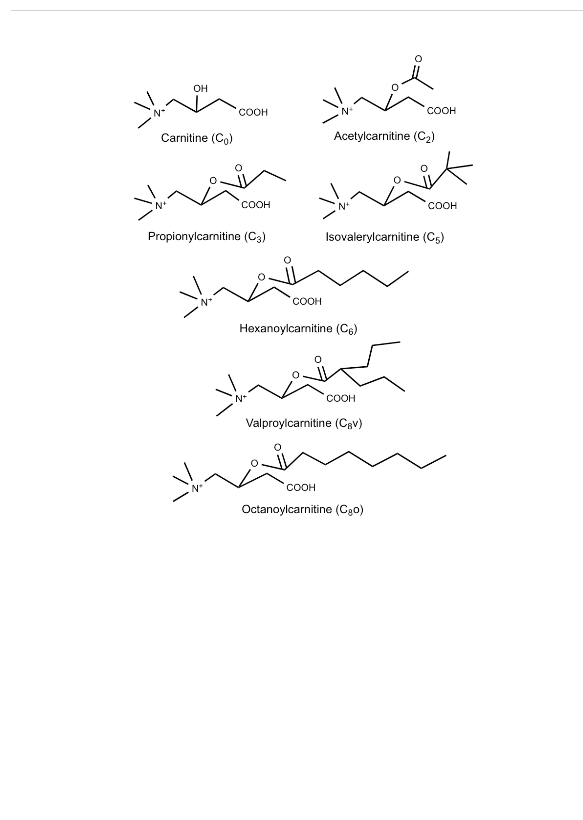


Figure
Click here to download Figure: Fig 2.pdf

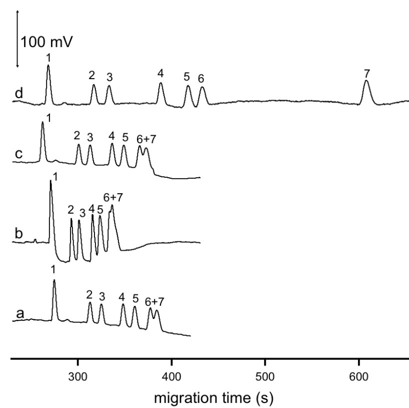



Fig. 2

**Figure**
**Click here to download Figure: Fig 3.pdf**

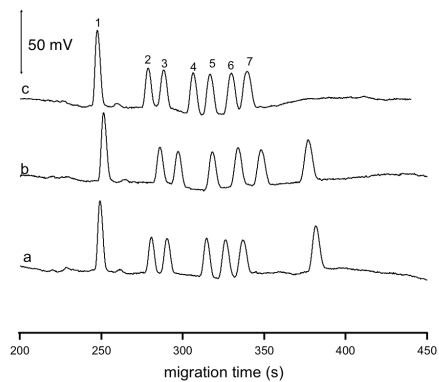



Fig. 3

Figure
Click here to download Figure: Fig 4.pdf

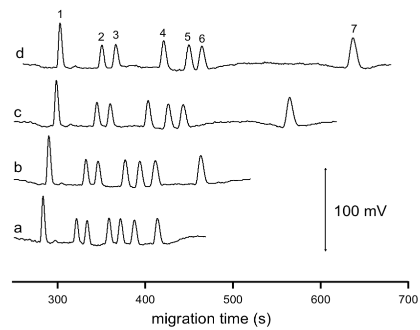



Fig. 4

**Figure**
Click here to download Figure: Fig 5.pdf

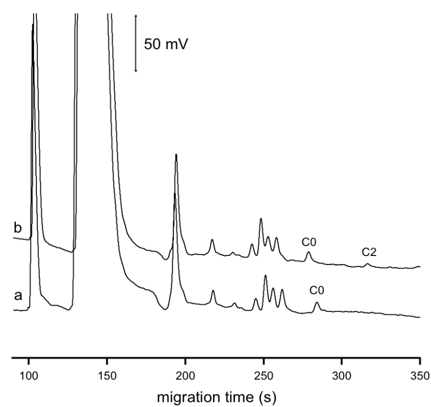
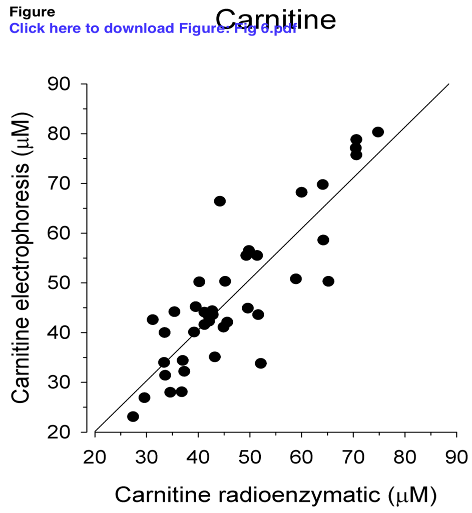



Fig. 5

Figure
Click here to download Figure: Fig 6.pdf



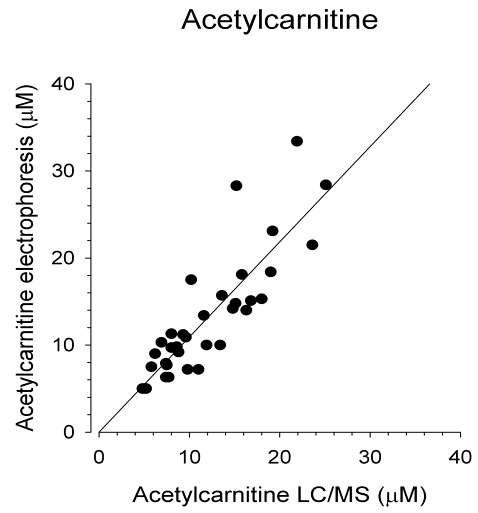



Figure
Click here to download Figure: Fig 7.pdf

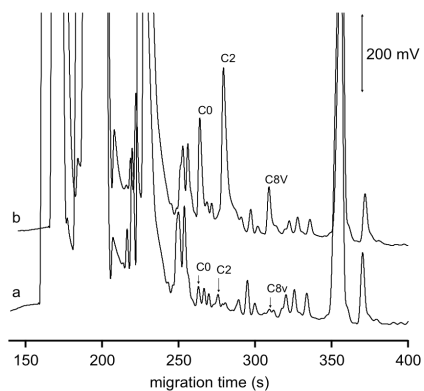



Fig. 7

## $4^{th}$ Project

# DETERMINATION OF THE ENANTIOMERS OF α-HYDROXY- AND α-AMINO ACIDS IN CAPILLARY ELECTROPHORESIS WITH CONTACTLESS CONDUCTIVITY DETECTION

**Electrophoresis 2010, Volume 31, Issue 12, Pages: 2044-2048**

**Worapan Pormsila**[1,2]
**Xiao Yang Gong**[1]
**Peter C. Hauser**[1]

[1]Department of Chemistry, University of Basel, Basel, Switzerland
[2]Department of Chemistry, Rajamangala University of Technology Krungthep, Bangkok, Thailand

Received October 14, 2009
Revised March 3, 2010
Accepted March 19, 2010

Research Article

# Determination of the enantiomers of α-hydroxy- and α-amino acids in capillary electrophoresis with contactless conductivity detection

The enantiomers of the anions of five α-hydroxy acids, namely lactic acid, α-hydroxybutyric acid, 2-hydroxycaproic acid, 2-hydroxyoctanoic acid and 2-hydroxydecanoic acid, as well as the two α-amino acids aspartic acid and glutamic acid, were baseline separated and detected by CE with contactless conductivity detection. Vancomycin was employed as chiral selector and could be used with conductivity detection without having to resort to a partial filling protocol as needed when this reagent is used with UV absorbance measurements. The procedure was successfully applied to the determination of the lactic acid enantiomers in samples of milk and yogurt. Linearity was achieved in the concentration range of 10–500 μmol/L with good correlation coefficients (0.9993 and 0.9990 for L- and D-lactic acid, respectively). The LODs (3 $S/N$) for L- and D-lactic acid were determined as 2.8 and 2.4 μmol/L, respectively.

**Keywords:**
Amino acids / CE / Contactless conductivity detection / Enantiomeric separation / Hydroxycarboxylic acids DOI 10.1002/elps.201000127

## 1 Introduction

The high demand for enantiomerically pure chiral compounds, in particular in the field of pharmaceutical drug research and production, has greatly stimulated the development of methods for fast and accurate enantiomeric analysis. Such determinations play an important role in monitoring the asymmetric synthesis of chiral compounds, in enantiomeric purity control and in pharmacological studies. CE has proven to be an effective technique for this task. This is due to the fact that the chiral reagents, which are needed to achieve the separation of enantiomers in separation techniques, can be dissolved in the relatively small volumes of buffer and do not have to be immobilized on a stationary phase. This allows a much higher flexibility in choosing and optimizing chiral selectors than in chromatography. In fact, the separation of enantiomers is one of the most important fields of application of CE. Summaries of the current developments of enantiomeric separations in CE can be found in recent reviews [1–3].

**Correspondence:** Professor Peter C. Hauser, Department of Chemistry, University of Basel, Spitalstrasse 51, 4056 Basel, Switzerland
**E-mail:** Peter.Hauser@unibas.ch
**Fax:** +41-61-267-1013

**Abbreviations: AHA,** α-hydroxy acid; **C$^4$D,** capacitively coupled contactless conductivity detection

However, the enantiomeric analysis of species that are not UV-absorbing has been relatively little explored. Standard instruments are fitted with photometric detectors; therefore, analyte derivatization or the use of indirect detection methods is necessary for non-UV-active compounds. Capacitively coupled contactless conductivity detection (C$^4$D) is an alternative for quantification, which, in contrast, allows the direct determination of all charged species. The method has recently become available commercially and the detectors can be fitted to existing instrumentation. A few reports on the use of C$^4$D for the analysis of enantiomeric mixtures have indeed appeared. Gong *et al.* [4–7] have demonstrated the use of C$^4$D for the determination of the enantiomers of different small and non-UV-active amines, which were separated and detected in their protonated, cationic form. This has been expanded on by Lecoeur-Lorin *et al.* [8], who used the method for the determination of the enantiomeric purity of a range of amines and demonstrated clearly that improved sensitivity is possible compared to indirect UV detection.

Maier *et al.* [9] have extended the use of CE-C$^4$D to the determination of the enantiomers of the anionic lactate. The macrocylic antibiotic vancomycin was used as the chiral selector, which was employed by applying a special protocol in which the capillary was only partially filled with a plug of the reagent solution. This procedure had been developed for optical detection and prevents the reagent from entering the detector cell in order to avoid its detrimental high background absorbance. Vancomycin contains 18 stereogenic centres, multiple hydrogen bonding groups (hydroxyl,

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

amine and amide), aromatic moieties and hydrophobic clefts; it can provide simultaneous interactions needed for chiral recognition [10]. Thus, it is an effective chiral selector for enantioseparation by HPLC and CE [11, 12]. It was recently also found that vancomycin is useful as an additive in CE-C$^4$D to achieve the separation of the anions of different positional isomers of hydroxybutyric acid, namely α-, β- and γ-hydroxybutyrate [13].

The successful separation of the enantiomers of different α-substituted aliphatic carboxylic acids by using vancomycin as selector is reported here. The separation of the optical isomers of α-hydroxy acids (AHAs) is described, with particular focus on lactic acid. The optimized CE method does not require a partial filling technique and is applied to the determination of the enantiomers of the lactate in samples of yogurt and milk. Chiral carboxylic acids such as lactic acid exist in various fruit and milk products and are key metabolites of virtually all metabolic pathways. They are also used in cosmetic products. For quality control, as well as detecting the adulterative addition of the synthetic racemic acids, it is important to determine the enantiomeric purity of the products. In particular, the presence of D-lactate in dairy products may indicate a microbial contamination [14]. Kodama *et al.* demonstrated the separation of the enantiomers of lactic acid in food with CE, but had to use a stacking procedure due to the low sensitivity of direct UV detection at 200 nm [15]. Also detailed herein is the separation of the optical isomers of aspartic and glutamic acid. According to Bednar *et al.*, the determination of the ratio of the isomers of aspartic acid is used for dating of teeth and archeological artefacts [16]. These authors have shown that the separation of the isomers is possible with the aid of vancomycin, but had to use the partial capillary filling protocol to avoid a detrimental background absorbance signal on the detector, and had to employ indirect absorbance detection [16].

## 2 Materials and methods

### 2.1 Instrumentation

The separations were carried out on a purpose-built instrument, which is based on a high voltage power supply with interchangeable polarity (CZE 2000R) from Spellman (Pulborough, UK). Fused-silica capillaries (25 μm id and 365 μm od) were products of Polymicro Technologies (Phoenix, AZ, USA). A function generator (Model GFG 8019G, Goodwill Instruments, Taiwan) was employed to provide a sinusoidal excitation signal for detection. The excitation frequency was set to 300 kHz and the amplitude was boosted to 400 $V_{pp}$ (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$^4$D used have been presented previously [17–19]. Data were acquired and analysed using a Maclab/4e system (AD Instruments, Hastings, UK).

### 2.2 Reagents and samples

All chemicals were either of analytical grade or reagent grade. D- and L-2-hydroxybutyric acid, D- and L-2-hydroxyoctanoic acid, D- and L-lactic acid, D- and L-aspartic acid, D- and L-glutamic acid, maleic acid and CTAB were obtained from Fluka (Buchs, Switzerland). Tris, D- and L-2-hydroxycaproic acid and D- and L-2-hydroxdecanoic acid were purchased from Sigma (Steinheim, Germany). Vancomycin was bought from Acros (Geel, Belgium). Water used throughout this experiment had a resistivity ≥18.2 MΩ cm and was obtained from a NANO-Pure water purification system (Barnstead, IA, USA). Stock solutions of all analytes and 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 analysis.

### 2.3 Separation procedure

Capillaries were rinsed with 0.1 M NaOH (10 min), followed by deionized water (10 min), and running buffer (15 min), before commencing experiments at the beginning of a working day or when peak distortions 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. The pH value of all running buffers was measured with a pH-meter (model 744, Metrohm, Herisau, Switzerland). Milk and yogurt samples were purchased from a local supermarket. 0.2 mL of milk (or 0.2 g of yogurt) was sampled and 0.6 mL of ACN was added, and the mixture was then centrifuged at 3000 rpm for 5 min. The supernatant solutions were then transferred to clean tubes and the solvent was allowed to evaporate at room temperature. The dried samples were reconstituted in 2 mL of the separation buffer and filtered prior to analysis.

## 3 Results and discussion

The structures of the chiral α-substituted carboxilic acids investigated are given in Fig. 1. Of primary interest were the hydroxyl substituted aliphatic acids with different chain lengths, starting with lactic acid. Also included where the two α-amino acids: aspartic acid and glutamic acid. The separation of the enantiomers of aspartic acid in CE-C$^4$D

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

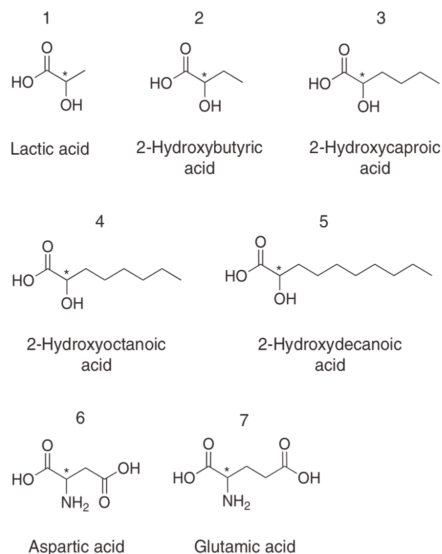



**Figure 1.** Structures of the compounds.

had previously been reported at low pH *via* the protonated cationic form and a combination of a cyclodextrin and a chiral crown ether as separator [4]. It was of interest, therefore, to see if the separation would also be possible for the anionic form of the compound using vancomycin as chiral selector (Figs. 2–4).

### 3.1 Optimization of the separation buffer

The experience gained in previous work on the determination of γ-hydroxybutyrate [13], in which vancomycin was employed to achieve its separation from positional isomers, indicated that the use of a partial filling method (as used by Maier *et al.* [9] for lactic acid) would not be necessary. Indeed, preliminary tests confirmed that the simple inclusion of vancomycin in the separation buffer used in conventional zone electrophoresis would be adequate and thus the complication of a sequential partial filling protocol could be avoided. Optimization therefore concerned the pH value of the buffer and the concentration of the chiral selector.

The pH value of the BGE is an important parameter to consider as it not only governs the charge and migration behaviour of the analytes but also of the chiral selector. The pH value has to be sufficiently high to render the analytes in the anionic deprotonated form, but at pH values significantly higher than 7.2 vancomycin would be present predominantly in anionic form [10], which is deemed not suitable for interaction with the anionic analytes. The influence of the buffer pH on enantioselectivity was therefore investigated in a range of pH from 4.5 to 7.35 using different buffer solutions consisting of appropriate mixtures of maleic acid with Tris, with the inclusion of CTAB for reversal of the electroosmotic flow. Best results in terms of separation were obtained for the buffer at pH 7.35 consisting of 10 mM of Tris, 4.4 mM of maleic acid, 0.03 mM of CTAB, pH 7.35.

Also investigated was the effect of the concentration of vancomycin, which was varied in the Tris/maleate buffer of pH 7.35 between concentrations of 1.5 and 5.0 mM. An almost linear increase of resolution with the concentration of vancomycin was obtained for all compounds. Concentrations higher than 5 mM were not considered as this was expected to lead to excessive background conductivity and thus to an instable baseline due to Joule heating. However, as seen in Table 1, the separation was successful for all compounds as $R_s$ values $\geq 1.5$ are considered adequate.

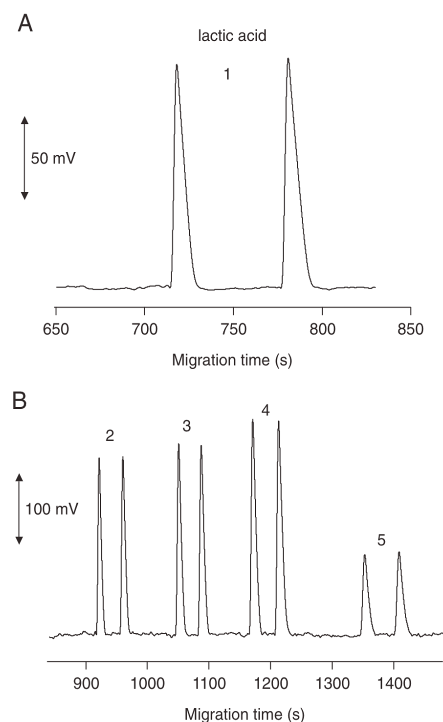



**Figure 2.** Electropherograms illustrating the separation of the enantiomers of the α-hydroxy acids with 5 mM vancomycin in the BGE. (A) DL-lactic acid. (B) DL-2-hydroxybutyric acid, DL-2-hydroxycaproic acid, DL-2-hydroxyoctanoic acid and DL-2-hydroxydecanoic acid. Concentration: 0.3 mM. Buffer: 10 mM of Tris, 4.4 mM of maleic acid, 0.03 mM of CTAB, pH 7.35. Separation conditions: injection, −5 kV, 7 s; separation voltage, −15 kV; capillary length, 60 cm (55 cm to detector); capillary id, 25 μm.

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

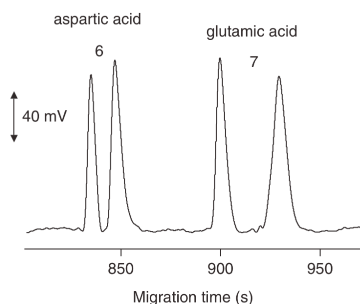



**Figure 3.** Electropherogram illustrating the separation of the enantiomers of DL-aspartic acid and DL-glutamic acid. Conditions as in Fig. 2.

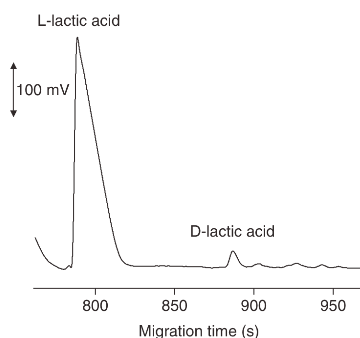



**Figure 4.** Electropherogram for a sample of spoiled yogurt, extracted and diluted tenfold. Conditions as in Fig. 2.

The migration times of the anions of the AHAs correlate with the length of the alkyl chain, as expected. The $R_s$ values for the AHAs generally also show a trend related to size, with the separation being less pronounced for the larger species. It is interesting to note, though, that the α-hydroxydecanoic acid deviates from this pattern as the resolution is relatively high. The reason is not clear, but may be related to association of analyte molecules due to interaction of the lipophilic parts. The best resolution, with an $R_s$ value of 3.8, was obtained for lactic acid, which compares well with the value of 2.6 reported by Maier *et al.* for the partial filling technique [9].

### 3.2 Quantification

Calibration curves were acquired by using the optimized buffer as described above for standard solutions of L- and D-lactic acid at concentrations of 10, 25, 50, 100, 250 and 500 μmol/L. The curves of peak area (mVs) *versus* concentration (μmol/L) were found to be linear for this range (regression equations: $y = 0.0019x-0.0052$ for L-lactic acid and $y = 0.0023x-0.0055$ for D-lactic acid); the correlation coefficients, $r$, were determined as 0.9993 and 0.9990 for the L- and D-forms, respectively. The LOD (3 × signal-to-noise) were determined as 2.8 μmol/L for L-lactic and 2.4 μmol/L for D-lactic acid. The intra-day variabilities (TEN consecutive determinations) were found to be 3% (RSD) for migration time and 6% for peak area, whereas for an inter-day study (3 days) of migration time and the peak area these values were found to be 5 and 10%, respectively.

### 3.3 Determination of lactic acid in milk and yogurt

As an example to illustrate a potential application, the method was then applied to the quantification of L- and D-lactic acid in fresh and spoilt samples of milk and yoghurt. Pasteurized milk and yoghurt samples (plain and flavored) were opened, analysed immediately and then left at room temperature for several days. The analysis was repeated after 2 and 10 days. The results are given in Table 2. As expected, in fresh milk neither L- nor D-lactic acid could be detected, while the fresh yoghurt sample contained L-lactic acid at different levels. The presence of the latter in yogurt is of course due to the normal fermentation process in the production of this dairy food by addition of lactic acid bacteria. As the products were left to spoil, L-lactate was found to increase in the yogurts, while the milk samples on going sour also started to show L-lactate. In one of the yogurt samples, D-lactate was found to be present when analysing 10 days after opening the product, most likely due to a contamination with a corresponding strain of undesirable bacteria.

**Table 1.** Analytical data for the separation of the enantiomers using a BGE composed of 10 mM Tris, 4.4 mM maleic acid, 0.03 mM CTAB and 5 mM vancomycin (pH 7.35)

| Compounds | Migration time ($t_1$) (min) | Separation factor (α) | Resolution ($R_s$) |
|---|---|---|---|
| DL-Lactic acid | 11.9 | 1.09 | 3.8 |
| DL-2-Hydroxybutyric acid | 15.3 | 1.04 | 2.8 |
| DL-2-Hydroxycaproic acid | 17.5 | 1.04 | 2.3 |
| DL-2-Hydroxyoctanoic acid | 19.5 | 1.04 | 2.2 |
| DL-2-Hydroxydecanoic acid | 22.5 | 1.04 | 2.6 |
| DL-Aspartic acid | 13.9 | 1.01 | 1.5 |
| DL-Glutamic acid | 15.0 | 1.03 | 2.1 |

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

**Table 2.** Quantification of L- and D-lactic acid in milk (mg/100 mL sample) and yogurt (mg/100 mg sample)

| Sample | Fresh | | After 2 days | | After 10 days | |
|---|---|---|---|---|---|---|
| | L-lactate | D-lactate | L-lactate | D-lactate | L-lactate | D-lactate |
| Plain pasteurized milk | nd | nd | 610 | nd | 1450 | nd |
| Chocolate flavored milk | nd | nd | nd | nd | 160 | nd |
| Caramel flavored milk | nd | nd | nd | nd | 75 | nd |
| Plain yogurt | 1420 | nd | 1910 | nd | 3310 | 130 |
| Strawberry flavored yogurt | 810 | nd | 1310 | nd | 1640 | nd |
| Orange flavored yogurt | 250 | nd | 1210 | nd | 1400 | nd |

nd = not detectable; the values given are the averages of three determinations.

## 4 Concluding remarks

The direct separation and detection of a selection of non-UV-absorbing α-hydroxy- and amino-substituted alkyl carboxylate enantiomers could be readily achieved using vancomycin as chiral selector. Contactless conductivity detection was found to allow the incorporation of this reagent in the BGE without having to use the relatively complicated partial filling technique, which is needed for UV detection in order to avoid saturation of the detector. It is likely that the method will also work with other compounds of similar constitution and it is thought that the approach would also be useful for UV-absorbing species, considering the complications caused by the reagent when employing UV detection. The procedure was successfully applied to the determination of the lactic acid enantiomers in samples of yogurt and milk. It is hoped that this facile technique will prove useful for food research and may find application in the food industry.

*This work was partly supported by a project grant from the Swiss National Science Foundation (No. 200020-113335/1). Partial funding for this work (W. Pormsila) was provided by the Thai Government through a grant to the Rajamangala University of Technology Krungthep, Bangkok, Thailand. X. Y. Gong gratefully acknowledges a Marie Heim-Voegtlin scholarship (PMCD2-114322/1) from the Swiss National Science Foundation.*

*The authors have declared no conflict of interest.*

## 5 References

[1] Preinerstorfer, B., Lammerhofer, M., Lindner, W., *Electrophoresis* 2009, *30*, 100–132.

[2] Sanchez-Hernandez, L., Crego, A. L., Marina, M. L., Garcia-Ruiz, C., *Electrophoresis* 2008, *29*, 237–251.

[3] Gübitz, G., Schmid, M. G., *J. Chromatogr. A* 2008, *1204*, 140–156.

[4] Gong, X. Y., Kubáň, P., Tanyanyiwa, J., Hauser, P. C., *J. Chromatogr. A* 2005, *1082*, 230–234.

[5] Gong, X. Y., Hauser, P. C., *J. Chromatogr. A* 2005, *1094*, 196–199.

[6] Gong, X. Y., Hauser, P. C., *Electrophoresis* 2006, *27*, 4375–4382.

[7] Gong, X. Y., Dobrunz, D., Kümin, M., Wiesner, M., Revell, J. D., Wennemers, H., Hauser, P. C., *J. Sep. Sci.* 2008, *31*, 565–573.

[8] Lecoeur-Lorin, M., Delepee, R., Morin, P., *Electrophoresis* 2009, *30*, 487–498.

[9] Maier, V., Petr, J., Knob, R., Horáková, J., Ševèik, J., *Electrophoresis* 2007, *28*, 1815–1822.

[10] Hui, F., Caude, M., *Analusis* 1999, *27*, 131–137.

[11] Armstrong, D. W., Nair, U. B., *Electrophoresis* 1997, *18*, 2331–2342.

[12] Gübitz, G., Schmid, M. G., *Mol. Biotechnol.* 2006, *32*, 159–180.

[13] Gong, X. Y., Kubàň, P., Scholer, A., Hauser, P. C., *J. Chromatogr. A* 2008, *1213*, 100–104.

[14] Garvie, E. I., *Microbiol. Rev.* 1980, *44*, 106–139.

[15] Kodama, S., Yamamoto, A., Matsunaga, A., Soga, T., Minoura, K., *J. Chromatogr. A* 2000, *875*, 371–377.

[16] Bednar, P., Aturki, Z., Stransky, Z., Fanali, S., *Electrophoresis* 2001, *22*, 2129–2135.

[17] Tanyanyiwa, J., Galliker, B., Schwarz, M. A., Hauser, P. C., *Analyst* 2002, *127*, 214–218.

[18] Tanyanyiwa, J., Hauser, P. C., *Electrophoresis* 2002, *23*, 3781–3786.

[19] Zhang, L., Khaloo, S. S., Kubáň, P., Hauser, P. C., *Meas. Sci. Technol.* 2006, *17*, 3317–3322.

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

## 5th Project

# DETERMINATION OF CARNITINE IN FOOD AND FOOD SUPPLEMENTS BY CAPILLARY ELECTROPHORESIS WITH CONTACTLESS CONDUCTIVITY DETECTION

**Electrophoresis 2010, Volume 31, Issue 13,**

**Special issue: Food and Beverage, Analysis, Pages: 2186-2191**

*Electrophoresis* 2010, *31*, 2186–2191

**Worapan Pormsila**[1,2]
**Stephan Krähenbühl**[3]
**Peter C. Hauser**[1]

[1]Department of Chemistry, University of Basel, Basel, Switzerland
[2]Department of Chemistry, Rajamangala University of Technology Krungthep, Bangkok, Thailand
[3]Division of Clinical Pharmacology & Toxicology, University Hospital Basel, Basel, Switzerland

Received November 16, 2009
Revised February 1, 2010
Accepted February 3, 2010

Research Article

# Determination of carnitine in food and food supplements by capillary electrophoresis with contactless conductivity detection

A CE method with capacitively coupled contactless conductivity detection was developed and tested for the quantification of carnitine in different types of foodstuffs, namely fruit juices, milk, yogurt, cheese, red meat and chicken meat. Sample preparation was minimal as chemical or enzymatic conversion of the analyte is not necessary with the non-UV-absorbing compound when conductivity detection is employed. A 500 mmol/L acetic acid solution at pH 2.6 with 0.05% Tween-20, was used as the optimized running buffer. The analysis time was approximately 4 min. Linearity was achieved for the concentration range of 5–500 μmol/L with a correlation coefficient of 0.9996. The LOD (3 signal/noise) was determined as 2.6 μmol/L. Intra- and inter-day variabilities were less than 10% for both migration time and peak area, indicating a good precision of the method.

**Keywords:**
Carnitine / CE / Conductivity detection / Food / Food supplements
DOI 10.1002/elps.200900692

## 1 Introduction

L-Carnitine ((R)-(3-carboxy-2-hydroxypropyl)trimethylammonium), the structure is given in Fig. 1, plays an important role in the metabolism of fatty acids, acting as a carrier to transport the long-chain acyl compounds into the mitochondria for β-oxidation and energy production [1]. It reaches therefore high concentrations in organs or tissues where fatty acids are important substrates for energy production, such as skeletal muscle, the heart or in sperm. L-carnitine is produced to a small extent in the human body from the amino acids lysine and methionine but it is mainly introduced by food intake. High amounts of L-carnitine are found in meat, especially in red meat, and dairy products (*e.g.* milk or yogurt), but much smaller amounts are present in fruits and vegetables. An adult body contains around 25 g of L-carnitine, and the normal daily need for L-carnitine is between 0.3 and 1.9 mg/kg body weight [2]. Due to its important role in energy metabolism, carnitine deficiency is a serious problem, leading to hypoglycemia, hyperammonemia and hypoketosis during starvation [3]. Besides primary carnitine deficiency, which is caused by mutations in the gene coding for the renal carnitine carrier OCTN2 (organic cation/carnitine transporter No. 2) [4], there are secondary carnitine deficiency states, which are primarily caused by renal excretion of acylcarnitines [5, 6], interactions of drugs or toxins with OCTN2 [7, 8], or due to the removal of carnitine by hemodialysis [9]. While treatment with carnitine is life saving for patients with primary carnitine deficiency and can be associated with symptomatic relief in those with secondary deficiency, it is so far not clear whether other individuals could also profit from intake of supplemental carnitine. Groups of persons who may profit from supplemental carnitine include pregnant women [10], vegetarians [11] or athletes in the training phase for minimizing the recovery period after heavy exercise [12]. Individuals belonging to such groups often ingest food rich in carnitine and/or pharmaceutical preparations containing carnitine. Therefore, the quantification of carnitine needs to be carried out not only in body fluids and tissues but also in different foodstuffs and pharmaceutical preparations.

Most reported methods for the quantification of carnitine concern its determination in clinical samples, such as plasma or urine, for diagnostic purposes (see, for example, [13–15]). In 2005, Möder *et al.* thoroughly reviewed the current methods for the quantification of carnitine in different matrices [16]. Most currently used methods are either based on enzymatic conversion, involving the formation of a radioactive or UV-absorbing compound as an auxiliary step to enable quantification, or on chromatographic separation. As carnitine is only very weakly UV absorbing, derivatization is also necessary for sensitive determination by HPLC [17]. However, such methods are

**Correspondence:** Professor Peter C. Hauser, Department of Chemistry, University of Basel, Spitalstrasse 51, 4056 Basel, Switzerland
**E-mail:** Peter.Hauser@unibas.ch
**Fax:** +41-61-267-10-13

**Abbreviation:** **C$^4$D,** capacitively coupled contactless conductivity detection

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

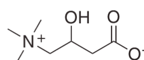



**Figure 1.** The structure of carnitine.

time consuming and special care has to be taken to assure the completeness of the derivatization step. Alternatively, mass spectrometry is employed [14, 15], but this is expensive, and the volatilization/ionization step may also present difficulties.

Only a few reports have focused on the determination of carnitine in foodstuffs and food supplements. Demarquoy *et al.* [2] and Knüttel-Gustavson and Harmeyer [18] determined the content of free carnitine in an extensive range of raw and processed foods commonly consumed in Western countries by using radio-enzymatic assays. Using these methods, they could determine the levels of carnitine in different meats, dairy products, fruits and vegetables. Woollard and Indyk reported the determination of carnitine in milk and infant formula using an enzymatic method coupled to the generation of a UV-absorbing dye [19, 20]. For food supplements, an ion-pair chromatographic method with indirect conductometric quantification was developed, which does not require the derivatization of carnitine prior to its detection [21].

Due to the charged nature of carnitine, CE is an attractive alternative method for its determination, as already reported for biological fluids [13, 22, 23] and food supplements [24]. UV-absorbance detection was used in these studies, either following derivatization [13, 23] or by employing the relatively insensitive indirect approach using a charged dye molecule in the separation buffer [22, 24].

For CE, the relatively new method of capacitively coupled contactless conductivity detection ($C^4D$), which has recently become available commercially (see www.istech.at or www.edaq.au), allows the facile and sensitive quantification of any charged species (for recent reviews, see [25, 26]). Previous work in our research group demonstrated the potential of CE-$C^4D$ for food and beverage analysis [27–29]. A report by Tůma *et al.* on the determination of amino acids in amniotic fluid indicated that carnitine is indeed detectable by CE-$C^4D$ [30]. In the current report, a method for the direct determination of carnitine in food and food supplements is presented, eliminating the need for derivatization or indirect approaches.

## 2 Materials and methods

### 2.1 Reagents

All chemicals were of analytical reagent grade. L-carnitine hydrochloride (~98%) and Tween-20 (70% in water) were obtained from Sigma-Aldrich (Buchs, Switzerland). Acetic acid was purchased from Fluka (Buchs, Switzerland). The deionized water used throughout this work had a resistivity of 18 MΩ cm and was obtained from a NANO-Pure water purification system (Barnstead, IA, USA). The background electrolyte (500 mmol/L acetic acid and 0.05% Tween-20 at pH 2.6) was prepared freshly every day. Standard solutions were also prepared daily by diluting a stock solution of 10 mmol/L of L-carnitine in deionized water with the background electrolyte. The pH value of the background electrolyte was determined using a pH-meter (model 744, Metrohm, Herisau, Switzerland). The 2.0-mL test tubes to contain the sample were obtained from Vaudaux-Eppendorf (Schönenbuch, Switzerland).

### 2.2 Instrumentation

The CE instrument was purpose-built, is similar to a previously reported design [31], but based on a high voltage power supply module capable of delivering up to 30 kV (CZE 2000R, Spellman, Pulborough, UK). The detector, also built in-house, is based on two electrodes of 4 mm length each, consisting of steel tubing with an internal diameter of about 400 μm and a detection gap of 1 mm. A sine wave voltage of 320 kHz and an amplitude of 280 Vpp (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 in [32]. Note that the peaks are due to a reduction in conductivity, but that the electropherograms are presented in the conventional way, *i.e.* with positive going peaks. A fused-silica capillary of 50 μm id and 365 μm od (Polymicro Technologies, Phoenix, AZ, USA) and total and effective lengths of 40 and 32 cm, respectively, was employed. The new capillary was conditioned by flushing with a 0.1 mol/L HCl solution (5 min), followed by deionized water (5 min) and then with 0.1 mol/L NaOH solution (10 min) before rinsing with the background electrolyte (15 min). Each day before starting experiments, the capillary was rinsed with the sodium hydroxide solution (2 min), deionized water (2 min) and background electrolyte (10 min). After each analysis run, the capillary was rinsed for 30 s with the sodium hydroxide solution and for 2 min with the background electrolyte to maintain the reproducibility of the analysis. Injection of standards and samples was carried out hydrodynamically by siphoning at 10-cm height difference for 15 s. The separation was performed with a positive voltage of 20 kV applied at the injection end.

### 2.3 Sample preparation

The food supplements analyzed were commercial non-prescription formulations obtained from a local pharmacy. Product A was an effervescent tablet for ingestion in liquid form, which besides auxiliary substances also contained magnesium as a second active ingredient. Product B

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

consisted of a non-effervescent tablet. Food samples were purchased from local supermarkets. All were measured as soon as possible after sampling and kept in a refrigerator at 4°C. The semi-solid and solid samples were minced before weighing. 100 mg each of the solid samples or 100 μL of the liquid samples were placed into vials and then diluted with 100 μL of the acetic acid background electrolyte. The solutions were stirred for 5 min and then centrifuged for 30 min at 3000 × *g* for complete separation. The supernatant was used for analysis. The clear solutions were injected directly into the CE system, or when necessary diluted with the background electrolyte to yield a final concentration in the linear range of the calibration curve. The tablets were ground to a powder before weighing and then prepared for CE analysis as described above for the food samples.

## 3 Results and discussion

### 3.1 Optimization the CE conditions

Carnitine (see Fig. 1) is a zwitterionic compound containing a positively charged quaternary amine group and a carboxylic acid group with a p$K_a$ of 3.8. In CE, carnitine therefore needs to be determined as a cation at low pH value where the acid group is protonated. A strongly acidic background electrolyte with a high ionic strength but low conductivity background was required for conductivity detection. Three acidic background electrolyte solutions, namely citric acid, lactic acid and acetic acid, which had previously been used successfully for CE, were selected for preliminary testing. While these solutions of acids cannot be considered ideal buffers, the lack of any cations, other than protons, is beneficial for detection sensitivity. Background electrolyte solutions with approximate pH values of 3 were prepared consisting of 10 mmol/L citric acid (pH 2.8), 20 mmol/L lactic acid (pH 2.9) and 100 mmol/L acetic acid (pH 2.8). As evidenced in Fig. 2, carnitine can indeed be detected in any of the three solutions, but the stability of the background signal was adequate only for the acetic acid electrolyte. This effect clearly is related to Joule heating as the electrophoretic currents were measured as 8, 12 and 22 μA for acetic, lactic and citric acid, respectively. Note that conductivity detection is more susceptible to Joule heating than other modes of detection due to the temperature coefficient of ionic conductivity. For this reason, the separation voltage was limited to 20 kV. A further optimization was carried out by varying the concentration of acetic acid in the range from 50 to 1000 mmol/L (corresponding to pH values between 3.2 and 2.4); best peak heights were obtained at 500 mmol/L acetic acid at pH 2.6. Another important issue is, of course, selectivity. In particular, in food samples and supplements, it can be expected that the major inorganic cations are present. As demonstrated by the electropherogram of Fig. 3, under these conditions, carnitine is well separated from $K^+$, $Ca^{2+}$, $Na^+$, $Mg^{2+}$ and $Li^+$.

To examine the feasibility of analyzing food samples, a sample of milk was investigated in preliminary tests. One hundred microliter of a raw milk sample were weighed and then diluted (1:2) with background electrolyte (500 mmol/L acetic acid), centrifuged for 30 min and then injected directly into the CE system. While carnitine could be detected without peak overlap in this matrix, a significant variation in migration time was observed (>10% RSD, $n = 3$). It was thought that the adsorption of proteins from the sample on the capillary wall, leading to unstable electroosmotic flow, is responsible for this effect. The possibility of using Tween-20, a non-ionic surfactant, to prevent the wall interaction of the larger species was therefore investigated. This approach had been found useful in earlier work with conductivity detection [33, 34]. Different concentrations

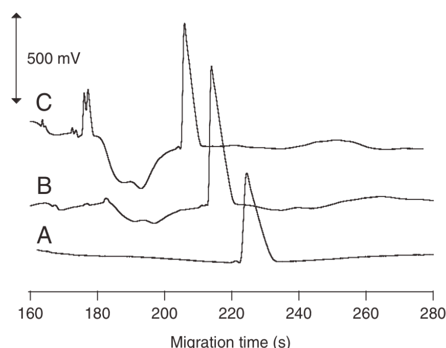



**Figure 2.** Electropherograms of 0.5 mmol/L L-carnitine standard solution in (A) 100 mmol/L acetic acid, (B) 10 mmol/L citric acid and (C) 20 mmol/L lactic acid. CE conditions: siphoning injection at 10 cm height difference for 20 s and +25 kV separation voltage.

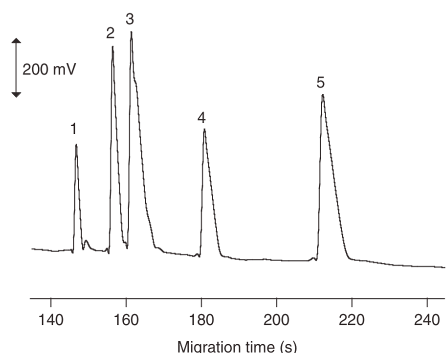



**Figure 3.** Electropherogram for 0.3 mmol/L L-carnitine and selected inorganic cations at 300 μmol/L separated in 500 mmol/L acetic acid at pH 2.6. Other conditions are as for Fig. 2. (1) $K^+$, (2) $Ca^{2+}$, (3) $Na^+$ and $Mg^{2+}$, (4) $Li^+$, (5) carnitine.

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

of Tween-20 were tested. It was found that with the inclusion of 0.05% of Tween-20 an acceptable stability of migration time of 2.2% RSD ($n = 3$) could be obtained.

### 3.2 Analytical characteristics of the method

A calibration curve was acquired and the linearity was studied by testing standard solutions of L-carnitine at 5, 10, 25, 50, 100, 250 and 500 μmol/L. The curve of peak area (Vs) *versus* concentration (μmol/L) was found to be linear for this range and gave the following regression equation: $y = 0.0034x–0.0151$ (correlation coefficient, $r = 0.9996$). The concentrations used covered well the carnitine concentrations expected in the food samples after dilution. The LOD (concentration giving a peak height corresponding to three times the amplitude of the baseline noise) was determined as 2.6 μmol/L and the LOQ (concentration giving a peak height corresponding to ten times the amplitude of the baseline noise) as 7.9 μmol/L. An L-carnitine standard of 100 μmol/L was used to investigate the intra-day and inter-day (5 days) repeatability. The intra-day variabilities for the migration time and peak area were found to be 1.4 and 7.2% (RSD, $n = 10$), respectively, and for the inter-day study (5 days) these values were 1.9% RSD for migration time and 8.4% RSD for peak area.

### 3.3 Analysis of food samples and food supplements

Two dietary products were tested; product A comprising of both magnesium and carnitine as active ingredients, and product B containing only carnitine. Both preparations also contained a number of additional auxiliary substances, in particular product A, this being a tablet to produce an effervescent beverage. The electropherogram obtained in the analysis of product A is given in Fig. 4. In addition to carnitine, there is a large peak originating from the second active ingredient, magnesium. Although the $Mg^{2+}$-peak is overlapping with a peak from $Na^{+}$, it is feasible that, after optimization of the conditions, both active constituents ($Mg^{2+}$ and carnitine), which otherwise require quantification by different means, may be determined concurrently when using CE-C$^4$D. The results of the successful quantification of carnitine in both products are given in Table 1. The recoveries, calculated by taking the contents declared on the labels as reference value, are acceptable.

Carnitine was then identified and quantified in several foodstuffs. An attempt to determine carnitine in apple juice is illustrated in Fig. 5. Two electropherograms are shown: one for apple juiced spiked with carnitine, the other for the unspiked apple juice. In the unspiked sample, there clearly is no discoverable peak at the position where carnitine is expected, indicating that the carnitine content is below the

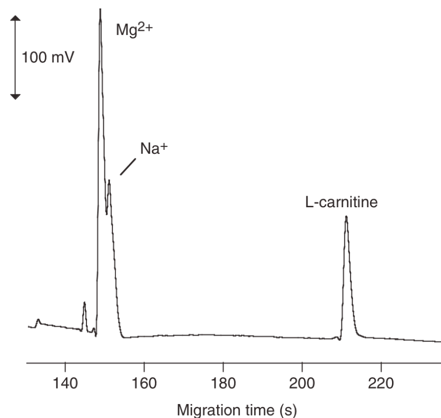



**Figure 4.** Electropherogram for L-carnitine food supplement A containing $Mg^{2+}$ as second active ingredient. CE conditions: 500 mmol/L acetic acid background electrolyte, 0.05 % Tween-20, pH 2.6, siphoning injection at 10 cm height difference for 15 s and +20 kV separation voltage.

**Table 1.** Quantitative results for carnitine in food supplements

| Food supplement | Determined amount of carnitine by CE-C$^4$D (mg/tablet)[a] | Declared amount of L-carnitine (mg/tablet) | Recovery of declared content (%) |
|---|---|---|---|
| A | 468±28 | 500 | 93.5±5.7 |
| B | 611±20 | 600 | 102.4±3.4 |

a) Mean±standard deviations ($n = 3$).

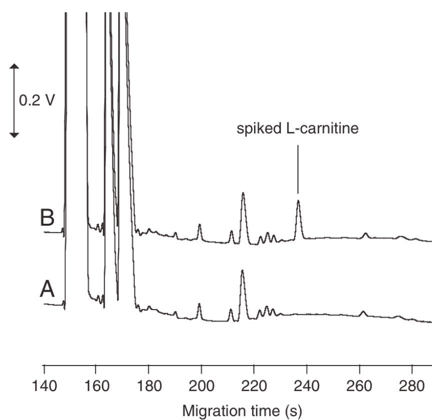



**Figure 5.** Electropherogram of apple juice (dilution 1:2) without (A) and with addition of 50 μmol/L L-carnitine (B). CE conditions are as for Fig. 4.

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

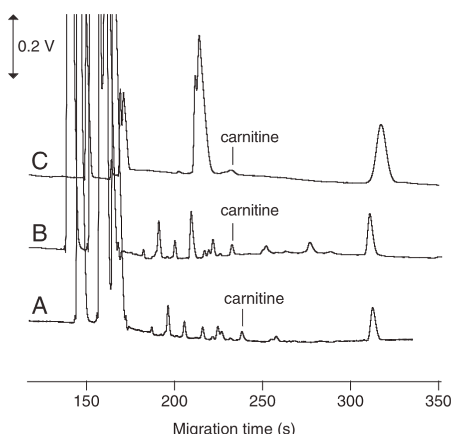



**Figure 6.** Electropherograms of (A) choco milk (dilution 1:2), (B) mixed fruit yogurt (dilution 1:2) and (C) chicken meat extract (dilution 1:50). CE conditions are as for Fig. 4.

**Table 2.** Quantitative results for carnitine in various foodstuffs

| Food samples | Carnitine (μmol/100 g or 100 mL sample)[a] |
|---|---|
| *Fruit products* | |
| Apple juice | <LOD |
| Orange juice | <LOD |
| *Dairy products* | |
| Milk, 3.5% fat | 62.8±6.8 |
| Cream, 25% fat | 21.5±4.5 |
| Chocolate milk A | 86.7±7.6 |
| Chocolate milk B | 64.5±5.7 |
| Mocca milk | 50.0±5.0 |
| Banana/kiwi yogurt | 26.0±1.3 |
| Apricot yogurt | 35.6±2.9 |
| Acerola/orange/acai yogurt | 40.0±1.0 |
| Peach yogurt | 46.3±4.8 |
| Strawberry yogurt | 39.1±4.6 |
| Mixed fruits yogurt | 62.1±3.0 |
| Cheese A | 70.1±3.8 |
| Cheese B | 34.5±4.9 |
| Cheese C | <LOD |
| *Meat* | |
| Beef | 1270±40 |
| Pork | 520±30 |
| Chicken | 290±30 |

a) Mean±standard deviations ($n = 3$).

LOD for the apple juice. The recovery for 100 μmol/100 mL L-carnitine added into apple juice was determined as 106%. The analysis of orange juice also revealed an absence of a detectable level of carnitine.

The analysis of a range of dairy products was successful. Electropherograms (A) and (B) of Fig. 6, for example, illustrate the determination of carnitine in samples of chocolate-flavored milk and of a fruit yogurt, respectively. The values obtained for a number of samples are given in Table 2, along with the precision of the determinations. The recoveries for 100 μmol/100 mL of L-carnitine spiked into the chocolate-flavored milk and mixed fruit yoghurt were determined as 113 and 115%, respectively. Also analyzed were samples of beef, pork and chicken meat. Electropherogram (C) of Fig. 6 was obtained for the latter. The amounts of carnitine in these samples where much higher than for the products derived from milk, with beef showing the highest level. These findings, and the general values for the food samples investigated, compare well with results compiled by Steiber et al. [35].

## 4 Concluding remarks

The determination of carnitine in various foodstuffs and food supplements was carried out successfully with CE-C$^4$D, generally with baseline resolution. An enzymatic conversion or color-forming derivatization reaction is not needed with this new approach for quantification of carnitine. Furthermore, it was found that deproteinization of samples is not required when using a neutral surface active compound in the separation buffer. The main advantages compared to existing methods are the simple sample preparation and the low cost for the equipment and for running the analyses. Simultaneous determinations of other constituents are also possible in this approach. It is therefore hoped that this facile method will find wider use for quantification of carnitine in food samples. Note that the procedure does not allow a differentiation between the L- and the D-enantiomers, but as the latter does not occur naturally this distinction is not always needed.

*The authors thank the partial funding for this work by the Thai Government through a grant to the Rajamangala University of Technology Krungthep, Bangkok, Thailand. Partial funding was also provided by the Swiss National Science Foundation through Research Grant No. 200020-105176/1.*

*The authors have declared no conflict of interest.*

## 5 References

[1] Bremer, J., *Physiol. Rev.* 1983, *63*, 1420–1480.

[2] Demarquoy, J., Georges, W., Rigault, C., Royer, M. C., Clairet, A., Soty, M., Lekounoungou, S., Le Borgne, F., *Food Chem.* 2004, *86*, 137–142.

[3] Treem, W. R., Stanley, C. A., Finegold, D. N., Hale, D. E., Coates, P. M., *N. Engl. J. Med.* 1988, *319*, 1331–1336.

[4] Nezu, J., Tamai, I., Oku, A., Ohashi, R., Yabuuchi, H., Hashimoto, N., Nikaido, H., Sai, Y., Koizumi, A., Shoji, Y., Takada, G., Matsuishi, T., Yoshino, M., Kato, H.,

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

Ohura, T., Tsujimoto, G., Hayakawa, J., Shimane, M., Tsuji, A., *Nat. Genet.* 1999, *21*, 91–94.

[5] Brass, E. P., Mayer, M. D., Mulford, D. J., Stickler, T. K., Hoppel, C. L., *Clin. Pharmacol. Ther.* 2003, *73*, 338–347.

[6] Todesco, L., Bodmer, M., Vonwil, K., Häussinger, D., Krähenbühl, S., *Chem. Biol. Interact.* 2009, *180*, 472–477.

[7] Todesco, L., Bur, D., Brooks, H., Torok, M., Landmann, L., Stieger, B., Krähenbühl, S., *Cell Mol. Life Sci.* 2008, *65*, 1596–1608.

[8] Spaniol, M., Brooks, H., Auer, L., Zimmermann, A., Solioz, M., Stieger, B., Krähenbühl, S., *Eur. J. Biochem.* 2001, *268*, 1876–1887.

[9] Vernez, L., Dickenmann, M., Steiger, J., Wenk, M., Krähenbühl, S., *Nephrol. Dial. Transplant.* 2006, *21*, 450–458.

[10] Keller, U., van der Wal, C., Seliger, G., Scheler, C., Ropke, F., Eder, K., *Eur. J. Clin. Nutr.* 2009, *63*, 1098–1105.

[11] Lombard, K. A., Olson, A. L., Nelson, S. E., Rebouche, C. J., *Am. J. Clin. Nutr.* 1989, *50*, 301–306.

[12] Volek, J. S., Kraemer, W. J., Rubin, M. R., Gomez, A. L., Ratamess, N. A., Gaynor, P., *Am. J. Physiol. Endocrinol. Metab.* 2002, *282*, E474–482.

[13] Vernez, L., Thormann, W., Krähenbühl,S., *J. Chromatogr. A* 2000, *895*, 309–316.

[14] Vernez, L., Hopfgartner, G., Wenk, M., Krähenbühl, S., *J. Chromatogr. A* 2003, *984*, 203–213.

[15] Vernez, L., Wenk, M., Krähenbühl, S., *Rapid Commun. Mass Spectrom.* 2004, *18*, 1233–1238.

[16] Möder, M., Kiessling, A., Löster, H., *Mon. Chem.* 2005, *136*, 1279–1291.

[17] Minkler, P. E., Hoppel, C. L., *J. Chromatogr.* 1993, *613*, 203–221.

[18] Knüttel-Gustavsen, S., Harmeyer, J., *Food Chem.* 2007, *105*, 793–804.

[19] Woollard, D. C., Indyk, H. E., Woollard, G. A., *Food Chem.* 1997, *59*, 325–332.

[20] Indyk, H. E., Woollard, D. C., *J. AOAC Int.* 1995, *78*, 69–74.

[21] Kakou, A., Megoulas, N. C., Koupparis, M. A., *J. Chromatogr. A* 2005, *1069*, 209–215.

[22] Mardones, C., Vizioli, N., Carducci, C., Rios, A., Valcárcel, M., *Anal. Chim. Acta* 1999, *382*, 23–31.

[23] Kiessig, S., Vogt, C., *J. Chromatogr. A* 1997, 475–479.

[24] Prokorátová, V., Kvasnička, F., Ševčík, R., Voldrich, M., *J. Chromatogr. A* 2005, *1081*, 60–64.

[25] Kubáň, P., Hauser, P. C., *Anal. Chim. Acta* 2008, *607*, 15–29.

[26] Kubáň, P., Hauser, P. C., *Electrophoresis* 2009, *30*, 176–188.

[27] Tanyanyiwa, J., Hauser, P. C., *Anal. Chem.* 2002, *74*, 6378–6382.

[28] Law, W. S., Kubáň, P., Zha, J. H., Li, S. F. Y., Hauser, P. C., *Electrophoresis* 2005, *26*, 4648–4655.

[29] Kubáň, P., Hauser, P. C., *Electrophoresis* 2005, *26*, 3169–3178.

[30] Tůma, P., Samcová, E., Andělová, K., *J. Chromatogr. B* 2005, *839*, 12–18.

[31] Kubáň, P., Nguyen, H. T. A., Macka, M., Haddad, P. R., Hauser, P. C., *Electroanalysis* 2007, *19*, 2059–2065.

[32] Zhang, L., Khaloo, S. S., Kubáň, P., Hauser, P. C., *Meas. Sci. Technol.* 2006, *17*, 3317–3322.

[33] Abad-Villar, E. M., Tanyanyiwa, J., Fernandez-Abedul, M. T., Costa-Garcia, A., Hauser, P. C., *Anal. Chem.* 2004, *76*, 1282–1288.

[34] Abad-Villar, E. M., Kubáň, P., Hauser, P. C., *Electrophoresis* 2005, *26*, 3609–3614.

[35] Steiber, A., Kerner, J., Hoppel, C. L., *Mol. Aspects Med.* 2004, *25*, 455–473.

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

# 3. REFERENCES


[1] Tiselius, A., *Biochem. J.* 1937, *31*, 313-317.
[2] Tiselius, A., *Biochem. J.* 1937, *31*, 1464-1477.
[3] Smithies, O., *Biochem. J.* 1955, *61*, 629-641.
[4] Smithies, O., *Adv.Protein Chem.* 1959, *14*, 65-113.
[5] Hjertén, S., *Biochimica Et Biophysica Acta* 1959, *32*, 531-534.
[6] Hjertén, S., *Chromatographic Reviews* 1967, *9*, 122-219.
[7] Virtanen, R., *Acta Polytech. Scand.-Chem. Technol. Ser.* 1974, 1-67.
[8] Mikkers, F. E. P., Everaerts, F. M., Verheggen, T., *Journal of Chromatography* 1979, *169*, 11-20.
[9] Jorgenson, J. W., Lukacs, K. D., *Anal. Chem.* 1981, *53*, 1298-1302.
[10] Jorgenson, J. W., Lukacs, K. D., *Clin. Chem.* 1981, *27*, 1551-1553.
[11] Dickerson, J. A., Ramsay, L. M., Dada, O. O., Cermak, N., Dovichi, N. J., *Electrophoresis* 2010, *31*, 2650-2654.
[12] Persat, A., Marshall, L. A., Santiago, J. G., *Anal. Chem.* 2009, *81*, 9507-9511.
[13] Kašička, V., *Electrophoresis* 2010, *31*, 122-146.
[14] Liotta, E., Gottardo, R., Bonizzato, L., Pascali, J. P*., et al.*, *Clin. Chim. Acta* 2009, *409*, 52-55.
[15] Peres, R. G., Moraes, E. P., Micke, G. A., Tonin, F. G*., et al.*, *Food Control* 2009, *20*, 548-552.
[16] Baños, C. E., Silva, M., *Electrophoresis* 2010, *31*, 2028-2036.
[17] Kim, H. S., Wainer, I. W., *J. Pharm. Biomed. Anal.* 2010, *52*, 372-376.
[18] Qin, J. H., Fung, Y. S., Zhu, D. R., Lin, B. C., *J. Chromatogr. A* 2004, *1027*, 223-229.
[19] Tseng, H. M., Li, Y., Barrett, D. A., *Bioanalysis* 2010, *2*, 1641-1653.
[20] Ravelo-Pérez, L. M., Asensio-Ramos, M., Hernandez-Borges, J., Rodriguez-, M. A., *Electrophoresis* 2009, *30*, 1624-1646.
[21] Servais, A. C., Crommen, J., Fillet, M., *Electrophoresis* 2006, *27*, 2616-2629.
[22] Desiderio, C., Rossetti, D. V., Iavarone, F., Messana, I., Castagnola, M., *J. Pharm. Biomed. Anal.* 2010, *53*, 1161-1169.
[23] DeBacker, B. L., Nagels, L. J., *Anal. Chem.* 1996, *68*, 4441-4445.
[24] van Staden, R. I. S., Mashile, T. R., *Sens. Actuator B-Chem.* 2006, *120*, 295-297.
[25] Bakker, E., Pretsch, E., *Trac-Trends Anal. Chem.* 2008, *27*, 612-618.
[26] Kubáň, P., Hauser, P. C., *Electrophoresis* 2009, *30*, 3305-3314.
[27] Wang, J., *Electroanalysis* 2005, *17*, 1133-1140.
[28] Fernández-la-Villa, A., Pozo-Ayuso, D. F., Castaño-Álvarez, M., *Electrophoresis* 2010, *31*, 2641-2649.
[29] Nussbaumer, S., Fleury-Souverain, S., Bouchoud, L., Rudaz, S*., et al.*, *J. Pharm. Biomed. Anal.* 2010, *53*, 130-136.
[30] Masár, M., Sydes, D., Luc, M., Kaniansky, D., Kuss, H. M., *J. Chromatogr. A* 2009, *1216*, 6252-6255.
[31] Nguyen, H. T. A., Kubáň, P., Pham, V. H., Hauser, P. C., *Electrophoresis* 2007, *28*, 3500-3506.
[32] Tan, F., Yang, B. C., Guan, Y. F., *Anal. Sci.* 2005, *21*, 955-958.

[33] Kappes, T., Hauser, P. C., *J. Chromatogr. A* 1999, *834*, 89-101.
[34] Tůma, P., Samcová, E., Duska, F., *J. Sep. Sci.* 2008, *31*, 2260-2264.
[35] Petsch, M., Mayer-Helm, B. X., Sauermann, R., Joukhadar, C., Kenndler, E., *Electrophoresis* 2004, *25*, 2292-2298.
[36] Gaš, B., Demjaněnko, M., Vacík, J., *Journal of Chromatography* 1980, *192*, 253-257.
[37] Zemann, A. J., Schnell, E., Volgger, D., Bonn, G. K., *Anal. Chem.* 1998, *70*, 563-567.
[38] da Silva, J. A. F., do Lago, C. L., *Anal. Chem.* 1998, *70*, 4339-4343.
[39] Mayrhofer, K., Zemann, A. J., Schnell, E., Bonn, G. K., *Anal. Chem.* 1999, *71*, 3828-3833.
[40] Kaniansky, D., Zelenská, V., Masár, M., Iványi, F., Gazdíková, Š., *J. Chromatogr. A* 1999, *844*, 349-359.
[41] Tůma, P., Opekar, F., Jelínek, I., *Electroanalysis* 2001, *13*, 989-992.
[42] Tůma, P., Opekar, F., Štulik, K., *Electrophoresis* 2002, *23*, 3718-3724.
[43] Tanyanyiwa, J., Galliker, B., Schwarz, M. A., Hauser, P. C., *Analyst* 2002, *127*, 214-218.
[44] Tanyanyiwa, J., Hauser, P. C., *Anal. Chem.* 2002, *74*, 6378-6382.
[45] Zemann, A. J., *Electrophoresis* 2003, *24*, 2125-2137.
[46] Timerbaev, A. R., *J. Sep. Sci.* 2008, *31*, 2012-2021.
[47] Prokhorova, A. F., Shapovalova, E. N., Shpigun, O. A., *J. Pharm. Biomed. Anal.* 2010, *53*, 1170-1179.
[48] Tagliaro, F., Pascali, J., Fanigliulo, A., Bortolotti, F., *Electrophoresis* 2010, *31*, 251-259.
[49] Nicholas W. Frost, M. J., and Michael T. Bowser, *Anal.Chem.* 2010, *82*, 4682-4698.
[50] Zemann, A. J., *Trac-Trends Anal. Chem.* 2001, *20*, 346-354.
[51] Fritz, J. S., *J. Chromatogr. A* 2000, *884*, 261-275.
[52] Timerbaev, A. R., Shpigun, O. A., *Electrophoresis* 2000, *21*, 4179-4191.
[53] Gong, X. Y., Hauser, P. C., *J. Chromatogr. A* 2005, *1094*, 196-199.
[54] Gong, X. Y., Kubáň, P., Tanyanyiwa, J., Hauser, P. C., *J. Chromatogr. A* 2005, *1082*, 230-234.
[55] Caslavska, J., Thormann, W., *Electrophoresis* 2006, *27*, 4618-4630.
[56] Sánchez-Hernández, L., García-Ruiz, C., Marina, M. L., Crego, A. L., *Electrophoresis* 2010, *31*, 28-43.
[57] Kubáň, P., Hauser, P. C., *Electrophoresis* 2009, *30*, 176-188.
[58] Dabek-Zlotorzynska, E., Celo, V., Yassine, M. M., *Electrophoresis* 2008, *29*, 310-323.
[59] Hiraoka, A., Akai, J., Tominaga, I., Hattori, M., *et al.*, *J. Chromatogr. A* 1994, *680*, 243-246.
[60] Petucci, C. J., Kantes, H. L., Strein, T. G., Veening, H., *J. Chromatogr. B-Biomed. Appl.* 1995, *668*, 241-251.
[61] Dolnik, V., Dolnikova, J., *J. Chromatogr. A* 1995, *716*, 269-277.
[62] García, A., Barbas, C., Aguilar, R., Castro, M., *Clin. Chem.* 1998, *44*, 1905-1911.
[63] Tůma, P., Samcová, E., Andelová, K., *J. Chromatogr. B* 2006, *839*, 12-18.
[64] Samcová, E., Tůma, P., *Electroanalysis* 2006, *18*, 152-157.

[65] Tůma, P., Samcová, E., Opekar, F., Štulik, K., *Collect. Czech. Chem. Commun.* 2008, *73*, 187-200.
[66] Tůma, P., Málková, K., Samcová, E., Štulik, K., *J. Sep. Sci.* 2010, *33*, 2394-2401.
[67] Jenkins, M., Ratnaike, S., *Clin. Chem. Lab. Med.* 2003, *41*, 747-754.
[68] Oda, R. P., Clark, R., Katzmann, J. A., Landers, J. P., *Electrophoresis* 1997, *18*, 1715-1723.
[69] Abad-Villar, E. M., Tanyanyiwa, J., Fernández-Abedul, M. T., Costa-García, A., Hauser, P. C., *Anal. Chem.* 2004, *76*, 1282-1288.
[70] Xu, L., Hauser, P. C., Lee, H. K., *J. Chromatogr. A* 2009, *1216*, 5911-5916.
[71] Schuchert-Shi, A., Hauser, P. C., *Chirality* 2010, *22*, 331-335.
[72] Schuchert-Shi, A., Hauser, P. C., *Electrophoresis* 2009, *30*, 3442-3448.
[73] Schuchert-Shi, A., Hauser, P. C., *Anal. Biochem.* 2008, *376*, 262-267.
[74] Glatz, Z., *J. Chromatogr. B* 2006, *841*, 23-37.
[75] da Silva, J. A. F., de Castro, N. V., de Jesus, D. P., Faria, A. F*., et al.*, *Electrophoresis* 2010, *31*, 570-574.
[76] Al Azzam, K. M., Saad, B., Aboul-Enein, H. Y., *Biomed. Chromatogr.* 2010, *24*, 948-953.
[77] Cheng, H. L., Liao, Y. M., Chiou, S. S., Wu, S. M., *Electrophoresis* 2008, *29*, 3665-3673.
[78] Nielen, M. W. F., *Anal. Chem.* 1993, *65*, 885-893.
[79] Aumatell, A., Guttmán, A., *J. Chromatogr. A* 1995, *717*, 229-234.
[80] Kuhn, R., *Electrophoresis* 1999, *20*, 2605-2613.
[81] Sniehotta, M., Schiffer, E., Zürbig, P., Novak, J., Mischak, H., *Electrophoresis* 2007, *28*, 1407-1417.
[82] Law, W. S., Zha, J. H., Hauser, P. C., Li, S. F. Y., *J. Sep. Sci.* 2007, *30*, 3247-3254.
[83] Lee, C. Y., Chen, C. M., Chang, G. L., Lin, C. H., Fu, L. M., *Electrophoresis* 2006, *27*, 5043-5050.
[84] Mato, I., Suárez-Luque, S., Huidobro, J. F., *Food Chem.* 2007, *102*, 104-112.
[85] Dutra, E. A., Santoro, M., Micke, G. A., Tavares, M. F. M., Kedor-Hackmann, E. R. M., *J. Pharm. Biomed. Anal.* 2006, *40*, 242-248.
[86] Tang, Y. J., Wu, M. J., *Food Chem.* 2007, *103*, 243-248.
[87] Zidkova, J., Chmelik, J., *Chem. Listy* 2000, *94*, 1093-1103.
[88] Xu, Y. H., Tang, Y. J., Wu, M. J., *Chin. J. Anal. Chem.* 2005, *33*, 1794-1798.
[89] Zhai, C., Sheng, J., Qiang, W., Lei, J. P., Ju, H. X., *Talanta* 2010, *82*, 67-71.
[90] Peng, Y. Y., Pallandre, A., Tran, N. T., Taverna, M., *Electrophoresis* 2008, *29*, 157-178.
[91] Heinig, K., Hissner, F., Martin, S., Vogt, C., *Am. Lab.* 1998, *30*, 24-29.
[92] Sze, K. L., Yeung, W. S. B., Fung, Y. S., *Electrophoresis* 2007, *28*, 4156-4163.
[93] Law, W. S., Kubáň, P., Zha, J. H., Li, S. F. Y., Hauser, P. C., *Electrophoresis* 2005, *26*, 4648-4655.
[94] Blasco, C., Picó, Y., Andreu, V., *Electrophoresis* 2009, *30*, 1698-1707.
[95] García-Canas, V., Cifuentes, A., *Electrophoresis* 2007, *28*, 4013-4030.
[96] Vallejo-Cordoba, B., González-Córdova, A. F., *Electrophoresis* 2010, *31*, 2154-2164.

[97] Herrero, M., Garcia-Cañas, V., Simo, C., Cifuentes, A., *Electrophoresis* 2010, *31*, 205-228.
[98] Gong, X. Y., Kubáň, P., Scholer, A., Hauser, P. C., *J. Chromatogr. A* 2008, *1213*, 100-104.
[99] Barbas, C., Saavedra, L., *J. Sep. Sci.* 2002, *25*, 1190-1196.
[100] Svedahl, K., MacIntosh, B. R., *Can. J. Appl. Physiol.-Rev. Can. Physiol. Appl.* 2003, *28*, 299-323.
[101] Friolet, R., Hoppeler, H., Krähenbühl, S., *J. Clin. Invest.* 1994, *94*, 1490-1495.
[102] Reuter, S. E., Evans, A. M., Chace, D. H., Fornasini, G., *Ann. Clin. Biochem.* 2008, *45*, 585-592.
[103] Liu, X. M., Zhang, J. S., Chen, X. G., *J. Chromatogr. B* 2007, *852*, 325-332.
[104] Simó, C., Barbas, C., Cifuentes, A., *Electrophoresis* 2003, *24*, 2431-2441.
[105] Prokorátová, V., Kvasnička, F., Ševčík, R., Voldřich, M., *J. Chromatogr. A* 2005, *1081*, 60-64.
[106] Demarquoy, J., Georges, W., Rigault, C., Royer, M. C., *et al.*, *Food Chem.* 2004, *86*, 137-142.
[107] Held, U. S., *Chimia* 2007, *61*, 8-10.

# 4. CURRICULUM VITAE

**Personal Information**


| | |
|---|---|
| Family name | Pormsila |
| First name | Worapan |
| Date of Birth | 5 July 1976 |
| Nationality | Thai |
| Gender | Female |
| Current address | Herbergsgasse 7, Basel 4051, Switzerland |
| Office address | The Rajamangala University of Technology Krungthep,<br>Fackulty of Science and Technology, Department of Chemistry<br>2 Nanglinji road, Thungmahamek subdistrict, Sathorn district,<br>Bangkok 10120, Thailand |
| Email | worapan.pormsila@unibas.ch |


**Education**

| | |
|---|---|
| 2007-present | Ph.D. candidate, Department of Chemistry, University of Basel, Basel, Switzerland<br>Doctoral thesis: Development of capillary electrophoresis with capicitively coupled contactless conductivity detection for clinical analysis and related applications.<br>Supervisor: Prof. Peter C. Hauser |
| 1996-2000 | M.S. (Chemistry), Kasetsart University, Bangkok, Thailand<br>Master thesis: Determination of lead and cadmium in various water samples by adsorptive stripping voltametry at iridium-based mercury electrode. |
| 1993-1996 | B.Sc. (Chemistry), Naresuan University, Phitsanulok, Thailand<br>Bachelor thesis: Synthesis of the copolymer for polyurethane. |

**Work Experience**

| | |
|---|---|
| 2000-2002 | Lecturer at the Pibulsongkram Rajabhat University, Facultry of Science and Technology, Department of Chemistry, Phitsanulok, Thailand |
| 2002-2005 | Scientist at the Ministry of Science, Technology and Environment, Department of Science Service, Chemistry Program, Water Testing Section, Bangkok, Thailand |
| 2005-present | Lecturer at the Rajamangala University of Technology Krungthep, Facultry of Science and Technology, Department of Chemistry, Bangkok, Thailand |

**Trainings**

| | |
|---|---|
| 2002 | Quality documents and related documents for ISO/IEC17025, Department of Science Service, Bangkok, Thailand *(Dec, 2002)* |
| 2003 | Glassware Calibration, Department of Science Service, Bangkok, Thailand *(Jan, 2003)*<br>ISO/IEC Guide 58 and ISO/IEC 17011, Department of Science Service, Bangkok, Thailand *(March, 2003)*<br>Requirements for ISO/IEC Guide 58 and ISO/IEC 17011, Department of Science Service, Bangkok, Thailand *(Sep, 2003)*<br>Uncertainty of Measurement, Department of Science Service, Bangkok, Thailand *(Nov, 2003)*<br>AAS technique for research, Department of Science Service, Bangkok,Thailand *(Dec, 2003)* |
| 2004 | pH meter Calibration, Department of Science Service, Bangkok, Thailand *(Jan, 2004)*<br>Statistic for research, Department of Science Service, Bangkok, Thailand *(Feb, 2004)*<br>Chemical Abstract, Department of Science Service, Bangkok, Thailand *(May, 2004)* |

# 5 LIST OF PUBLICATIONS AND PRESENTATIONS

## 5.1 Publications

5.1.1 Worapan Pormsila, Stephan Krähenbühl and Peter C. Hauser. Capillary electrophoresis with contactless conductivity detection for uric acid determination in biological fluids, *Anal.Chim.Acta, 636 (2009) 224-228.*

5.1.2 Worapan Pormsila, Xiao Yang Gong and Peter C. Hauser. Determination of the enantiomers of α-hydroxy- and α-amino acids in capillary electrophoresis with contactless conductivity detection, *Electrophoresis, 31 (2010) 2044-2048*.

5.1.3 Worapan Pormsila, Stephan Krähenbühl and Peter C. Hauser. Determination of carnitine in food and food supplements by capillary electrophoresis with contactless conductivity detection, *Electrophoresis, 31 (2010) 2186-2191*.

5.1.4 Worapan Pormsila, Rejáne Morand, Stephan Krähenbühl and Peter C. Hauser. Quantification of plasma lactate concentration using capillary electrophoresis with contactless conductivity detection, *accepted for publication in Electrophoresis.*

5.1.5 Worapan Pormsila, Rejáne Morand, Stephan Krähenbühl and Peter C. Hauser. Capillary electrophoresis with contactless conductivity detection for the determination of carnitine and acylcarnitines in clinical samples, *submitted to Journal of Chromatography B.*

## 5.2 Conferences with Posters

5.2.1 Worapan Pormsila and Atchana Wongchaisuwat, Determination of Lead and Cadmium in Various Sample Waters by Adsorptive Stripping Voltammetry at Iridium-based Mercury Electrode, The 2nd Asian International Conference on Ecotoxicology and Environment Safety (SECOTOX), 2004, Songkhla, Thailand.

5.2.2 Worapan Pormsila, Oi Jin Wan, Gamze Belin, Wai Siang Law and Peter C. Hauser, Application of Capillary Electrophoresis with Contactless Conductivity Detection in Clinical Analysis. The Fall Meeting of the Swiss Chemical Society (SCS Fall Meeting), 2008, University of Zurich (Irechel), Switzerland.

5.2.3 Worapan Pormsila and Peter C. Hauser, Capillary Electrophoresis with Contactless Conductivity Detection in Clinical Analysis. The Analytical Chemistry Symposium, 2009, ETH Zurich, Switzerland.