Review

Beatriz Baena[1]
Alejandro Cifuentes[2]
Coral Barbas[1]

[1]Sección Química Analítica, Fac. CC. Experimentales y de la Salud, Universidad San Pablo-CEU, Madrid, Spain
[2]Inst. Fermentaciones Industriales (CSIC), Madrid, Spain

Analysis of carboxylic acids in biological fluids by capillary electrophoresis

This review article addresses the different capillary electrophoretic methods that are being used for the study of both short-chain organic acids (including anionic catecholamine metabolites) and fatty acids in biological samples. This work intends to provide an updated overview (including works published until November 2004) on the recent methodological developments and applications of such procedures together with their main advantages and drawbacks. Moreover, the usefulness of CE analysis of organic acids to study and/or monitor different diseases such as diabetes, new-borns diseases or metabolism disorders is examined. The use of microchip devices and CE-MS couplings for organic acid analysis is also discussed.

Keywords: Acidurias / Bioanalysis / Capillary electrophoresis / Electromigration / Fatty acids / Indirect detection / Metabolism diseases / Micellar eletrokinetic chromatography / Organic acids / Review

DOI 10.1002/elps.200410329

Contents

1 Introduction 2622
2 Short-chain organic acids 2623
2.1 Metabolic significance 2623
2.2 Analysis of short-chain organic acids 2623
3 Anionic catecholamine metabolites 2631
4 Fatty acids 2631
4.1 Metabolic significance 2631
4.2 Sample treatment 2631
4.3 Fatty acids analysis 2631
4.4 Miscellaneous 2634
5 Concluding remarks and future outlook 2634
6 References 2634

1 Introduction

Carboxylic acids are a biochemically heterogeneous group of compounds containing a carboxylic group. Other functional groups (-keto, -hydroxyl) can also be present; some nitrogen-containing compounds are included such as pyroglutamate, but amino acids are excluded. There is misleading information with the terms commonly employed for the different carboxylic acids. Organic acid is the name generally employed for short-chain organic acids. They are water-soluble compounds which are intermediate metabolites of all major groups of organic cellular components: amino acids, lipids, carbohydrates, nucleic acids, and steroids [1]. Fatty acids are carboxylic acids found in nature, free or bonded to triglycerides, phospholipids, *etc.* Their chain length ranges from 4 to 30 carbons, with 12 to 24 carbons being the most common. The chain is typically linear, and usually contains an even number of carbons. Their polarity is lower, and decreases with increasing carbon number.

From a chemical point of view, most of the carboxylic acids present the same drawback for their determination, *i.e.*, the lack of a characteristic chromophore, because the carboxylic group presents a weak absorption around 200 nm (except the compounds containing an aromatic moiety).

Organic acids comprise key metabolites of virtually all metabolic pathways as well as exogenous compounds. Comprehensive quantitative analysis of organic acids in body fluids has therefore the potential of yielding information on the physiological and pathophysiological status of different metabolic pathways, as well as their interrelationships. For that reason, organic acids can play an important role as biomarkers for monitoring different diseases such as organic acidurias, metabolic diseases, diabetes, central nervous system diseases, *etc*.

Correspondence: Dr. Coral Barbas, Sección Química Analítica, Fac. CC. Experimentales y de la Salud, Universidad San Pablo-CEU, Urbanización Montepríncipe, E-28668 Boadilla del Monte, Madrid, Spain
E-mail: cbarbas@ceu.es
Fax: +34-91-3724711

Abbreviations: DAD, diode array detection; **DHAP**, dihydroxyacetonephosphate; **FFA**, free fatty acid; **LCFA**, long-chain fatty acid; **PEP**, phosphoenolpyruvate; **PUFA**, polyunsaturated fatty acid

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

CE is a powerful analytical tool for diagnostic applications suitable to detect important changes in the metabolic profiles of body fluids. CE is easy to include in routine analytical systems and it can offer a quick and simple alternative or complement other techniques such as HPLC or GC, mainly for a first screening of the pathologies included in the study and for monitoring the therapy once the patient has been diagnosed. One of the main drawbacks of CE is related to the detection systems. Since the sample volume employed is very small (nanoliters) and the pathlength is short, the LODs in CE-UV are not the best feature of the technique. Nevertheless, this is not a critical point when an increase has to be detected.

Although the use of CE for the analysis of organic acids has been reviewed either for general purposes [2] or for detecting acidurias [3], the present work will focus on the determination of carboxylic acids in different biological fluids by capillary electromigration methods, including the most recent contributions that have been published on this topic.

2 Short-chain organic acids

2.1 Metabolic significance

Organic acidurias are a heterogeneous group of inborn errors of metabolism. They are biochemically characterized by the accumulation of organic acids, *i.e.*, metabolites that are not present under physiological conditions, produced by the activation of alternative pathways in response to the loss of function of a specific gene product (enzyme), or by the accumulation of pathological amounts of normal metabolites. These disorders share a common natural history, which is the occurrence of either acute life-threatening illness in early infancy or unexplained developmental delay with intercurrent episodes of metabolic decompensation in later childhood [4]. Central nervous system diseases, neuroblastoma, nephrolithiasis and other pathologies are also related to an increase in organic acids in body fluids. Table 1 includes a general overview of organic acids, the biological fluid assayed, the electrophoretic conditions and the relevance of the compounds analyzed in the biological context.

The best fluid to analyze for organic acids is urine, because: (i) organic acids are concentrated in the urine so that most of them are present in the urine in a much higher concentration than in the blood; (ii) the virtual lack of protein facilitates the analysis of the sample; and (iii) a specimen consisting of a simple voiding is easy to obtain and is adequate for analysis [5]. In rare instances, and/or for follow-up or prenatal diagnostic purposes, quantitation of specific metabolites in other physiological fluid specimens is appropriate.

2.2 Analysis of short-chain organic acids

Although out of the scope of the present work, it is interesting to indicate that GC-MS has been used routinely as a screening method for the analysis of patient urine for the diagnosis of metabolic disorders [6, 7]. A recent publication of Kuhara [8] establishes a practical (yet comprehensive) diagnostic procedure of inborn errors of metabolism (IEM). This procedure involves the use of urine or eluates from urine on filter paper, stable isotope dilution, and GC-MS. This procedure not only offers reliable and quantitative evidence for diagnosing, understanding, and monitoring the diseases, but also provides evidence for the diagnosis of new kinds of IEM. In spite of its unquestionable sensitivity, selectivity, and identification capability, significant drawbacks of the GC-MS technique are the long time required for sample preparation and analysis, and the need for trained personnel. These limitations have impeded the use of GC-MS for general screening purposes and, moreover, its use is often unnecessary for the monitoring the therapy adhesion/progression. Early and accurate diagnosis of metabolic diseases is very important in determining a patient's prognosis. Many of these disorders are now treatable by simple dietary measures, yet when undiagnosed and untreated, they result in death or irreversible mental retardation.

As mentioned above, CE can be an interesting alternative to solve these analytical problems. In this sense, an interesting work was recently published describing the prediction of the electrophoretic mobilities of some carboxylic acids from theoretically derived descriptors based on quantitative structure-properties relationships [9]. This could help with the easiest development of new separation methods for these compounds as well as with the understanding of migration orders of the compounds. In the same line, another work was focused on the way that several electrolyte components could affect the EOF and the capillary electrophoretic migration of aliphatic or aromatic (hydroxyl)carboxylic acids. The effects exerted by the EOF modifier, hexadecyltrimethylammonium bromide, the addition of metal salt to the electrolyte and the chromophore used for indirect detection were investigated. Retention of the organic acids was demonstrated and its magnitude was shown to depend on the amount of cationic surfactant adsorbed onto the capillary wall. Physiologically relevant acids such, as glycolic and glyceric acids, are included in the study [10].

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Short-chain organic acids, CE conditions, biological sample and relevance

Organic acids (see also Relevance)	Conditions	Detection	Type of sample	Relevance	Ref.
Uric (1), hippuric (2) and other acids	Normal polarity, uncoated capillary, borate, pH 9.0	UV diode-array	Uremic and normal serum	1,2) Renal disease	[60]
Pyruvic (1), citric (2), malic (3), acetoacetic (4) and lactic (5) acids	Reversed polarity Capillary coated with linear PAA ε-aminocaproic and 2-hydroxyphenylacetic acid at pH 3.8	UV 220 nm Indirect detection	Serum	1,4,5) Carbohydrate metabolism marker 2,3) Central energy pathway marker	[61]
Methylmalonic acid	Reversed polarity Capillary coated with linear PAA Tris-acetate pH 6.4 Hydroxypropylmethyl-cellulose and dimethyl-formamide	Fluorescence detection LIF He-Cd	Serum	Methylation pathway marker	[62]
Short-chain organic acids	Normal polarity Uncoated capillary a) Borate pH 10.0, SDS and urea (MEKC) b) Tris and benzoate	a) Fluorescent detection LIF argon 488 nm b) Indirect detection at 220 nm	Serum	Metabolic disorders	[45]
Lactic (1), acetoacetic (2) and β-hydroxybutyric (3) acids	Reversed polarity Uncoated capillary 3,5-dinitrobenzoate 5 mM, 0.1 mM CTAB, pH 3.5	UV Indirect detection	Serum	1,2,3) Carbohydrate metabolism marker	[12]
Lactic acid	Normal polarity Uncoated capillary 1 mM NADH in 30 mM HEPES buffer, pH 7.0	UV 266 nm Direct detection	Serum	Carbohydrate meta-bolism marker	[14]
Lactic (1), acetoacetic (2) β-hydrxoybutyric (3), uric (4) and pyruvic (5) acids	Normal polarity PAA-Coated capillary a) phosphate 0.150 M with $CaCl_2$ 0.3 mM, pH 6.2 b) phosphate 200 mM acetate 10 mM, pH 4.0	UV 200 nm Direct detection	Plasma	1,2,3,5) Carbohydrate metabolism marker 4) Antioxidant defense	[13]
Methylmalonic (1) and short-chain dicarboxylic acids (2) (n = 6)	Normal polarity Uncoated capillary Phosphate-acetate, 50% organic modifier, pH 4.8	Fluorescence LIF He-Cd	Urine and serum	1) Methylation pathway marker 2) Metabolic disorders	[63]
Short-chain organic acids (n = 14)	Normal polarity Uncoated capillary Carbonate and phthalate at pH 7	UV 230 nm Indirect detection	Serum and urine	Metabolic disorders	[64]
Oxalic (1), formic (2), methyl-malonic (3), fumaric (4), succinic (5) and 2-keto-glutaric (6). (n = 12)	Reversed polarity Uncoated capillary Tetraborate and commercial EOF modifier pH 10.0	UV 185 nm Direct detection	Urine	1) Nephrolithiasis 2) Metabolic disorder 3) Methylation pathway marker 4,5,6) Central energy pathway marker	[65]

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Continued

Organic acids (see also Relevance)	Conditions	Detection	Type of sample	Relevance	Ref.
Methylmalonic (1), citric (2), 2-ketoglutaric (3) and succinic (4) acids	Reversed polarity Uncoated capillary Phthalate, phosphate, CTAB and 30% v/v ACN	UV 210 nm Indirect detection	Urine	1) Methylation pathway marker 2) Central energy pathway marker 3,4) Central energy pathway marker	[66]
Methylmalonic (1), glutaric (2), *N*-acetylaspartic (3), aminoadipic, propionic acids (n = 10)	Reversed polarity Uncoated capillary Sodium sulfate, calcium chloride and commercial EOF modifier	UV 185 nm Direct detection	Urine	1) Methylation pathway marker 2) Fatty acid oxidation 3) Metabolic disorder	[67]
Oxalic (1), malonic, maleic, succinic (2), pyruvic (3), lactic (4), 3-hydroxybutyric (5) and hippuric (5) acids	Reversed polarity Uncoated capillary Tetraborate and commercial EOF modifier (TTAB) and calcium salt pH 10.0	UV 196 nm Direct detection	Urine	1) Nephrolitiasis 2) Central energy pathway marker 3,4,5) Carbohydrate metabolism marker 5) Renal disease	[68]
Orotic acid	Normal polarity Capillary coated with polyvinyl alcohol Phosphate, pH 3.0	Direct detection UV Diode-array	Urine	Detoxication indicators	[69]
Orotic acid	Reversed polarity Uncoated capillary Phosphate, pH 2.23	Direct detection UV Diode-array	Urine	Detoxication indicators	[70]
Mevalonic (1), glutaric, glyceric (2) and methylmalonic (3) acids	Normal polarity Uncoated capillary Borate, pH 8.5	Direct detection UV Diode-array	Urine	1,2) Metabolic disorder 2) Methylation cofactor markers	[71]
Short- and medium-chain organic acids (n = 9)	Reversed polarity Capillary coated with linear PAA Phosphate pH 6.0 + 10% v/v methanol	UV 200 nm Direct detection	Urine	Metabolic disorders	[72]
Short- and medium-chain organic acids (n = 27)	Reversed polarity Capillary coated with linear PAA a) Phosphate pH 6.0 + 10% v/v methanol b) Phosphate and acetate pH 4.0	UV 200 nm Direct detection	Urine	Metabolic disorders	[73]
Orotic acid	Normal polarity Uncoated capillary a) Glutamate and spermine pH 5.2 b) Phosphate and glycine pH 2.15	a) UV 254 nm b) UV 280 nm	a) Urine from healthy people b) Urine from patients	Detoxication indicators	[74]
Homogentisic (1), pyroglutamic (2) acids and others compounds	Normal polarity Uncoated capillary Ammonium bicarbonate pH 8.5	CE-MS-MS ESI interface IT MS	Urine	1) Alkaptonuria 2) Detoxication indicator	[75]

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Continued

Organic acids (see also Relevance)	Conditions	Detection	Type of sample	Relevance	Ref.
Propionic, benzoic (1), homogentisic (2), homovanillic (3), vanillylmandelic (4), glyceric (5), orotic (6) and more acids	Normal polarity Uncoated capillary Ammonium acetate pH 8.5	CE-MS/MS ESI interface Triple quadrupole MS	Urine	1) Intestinal dysbiosis marker 2) Alkaptonuria 3,4) Neurotransmitter metabolism marker 5) Metabolic disorder 6) Detoxication indicator	[76]
Oxalic (1) and citric (2) acids	Reversed polarity Uncoated capillary Chromate and TTAB, pH 8.1	UV 254 nm Indirect detection	Urine	1) Nephrolithiasis 2) Central energy pathway marker	[77]
Oxalic (1), ascorbic and uric (2) acids	Reversed polarity Uncoated capillary Phosphate and CTAB, pH 5.7	Amperometric detection 0.8 V *versus* Ag/AgCl Cobalt phthalocyanine-modified carbon electrode	Urine	1) Nephrolithiasis 2) Renal disease	[78]
Oxalic (1), citric (2), glyoxylic and glyceric (3) acids	Reversed polarity Capillary coated with linear PAA a) phosphate pH 6.0 + 10% v/v methanol b) phosphate and acetate pH 4.0	UV 200 nm Direct detection	Urine	1) Nephrolithiasis 2) Central energy pathway marker 3) Metabolic disorder	[79]
Vanillylmandelic (1) and homovanillic (2) acids	Normal polarity Uncoated capillary Acetate buffer, pH 4.10	UV 214 nm Direct detection	Urine	1,2) Neurotransmitter metabolism	[80]
Vanillylmandelic (1), homovanillic (2), 5-hydroxyindoleacetic (3) acids and others urinary indole derivatives	Normal polarity Uncoated capillary MEKC Phosphate-tetraborate buffer with SDS, pH 9.2	a) Absorption at 220 nm Direct detection b) Fluorescence at 340 nm	Urine	1,2,3) Neurotransmitter metabolism	[81]
Creatinine (1), vanillylmandelic (2), homovanillic (3) and uric (4) acids	Normal polarity Uncoated capillary Phosphate buffer with SDS, pH 7.0	UV 245 nm Direct detection	Urine	1) Marker of renal function 2,3) Neurotransmitter metabolism 4) Renal disease	[82]
Vanillylmandelic (1) and homovanillic (2) acids (only detection)	Normal polarity Uncoated capillary Borate, pH 8.5	Direct detection UV Diode-array	Urine	1,2) Neurotransmitter metabolism	[71]
Biogenic amines and vanillylmandelic (1), homovanillic (2) and 5-hydroxyindoleacetic (3) acids	Normal polarity Uncoated capillary Ammonium acetate buffer pH 4.0	UV 220 nm Direct detection	Urine	1,2,3) Neurotransmitter metabolism	[83]

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Continued

Organic acids (see also Relevance)	Conditions	Detection	Type of sample	Relevance	Ref.
Vanillylmandelic (1), homovanillic (2) and 5-hydroxyindoleacetic (3) acids	Reversed polarity Capillary coated with linear PAA Phosphate-acetate buffer pH 4.4 with 10% v/v methanol or pH 4.3 with 5% v/v methanol	UV 192 nm Direct detection	Urine	1,2,3) Neurotransmitter metabolism	[84]
Vanillylmandelic (1), homovanillic (2) and more organic acids	Normal polarity Uncoated capillary Ammonium acetate buffer, pH 8.5	CE-MS/MS ESI interface Triple quadrupole MS	Urine	1,2) Neurotransmitter metabolism	[76]
Vanillylmandelic (1) and homovanillic (2) acid	Normal polarity Uncoated capillary Phosphate buffer, pH 5.2	Amperometric detection 1.1 V *versus* Hg/Hg_2Cl_2 Carbon fiber microdisk bundle electrode	Urine	1,2) Neurotransmitter metabolism	[85]
Orotic acid	Uncoated capillary Phosphate 10 mM, glycine, αCD 30 mM, βCD 10 mM, PVP 1 (% w/v), DMDAPS 100 mM, MHEC 0.2 (% w/v) pH 2.8	CZE in tandem-coupled columns with DAD detection 200–350 nm	Urine	Detoxication indicators	[15]
Creatine, creatinine, *p*-aminohyppuric and uric acids	Phosphate buffer 20 mM pH 9.5 containing enzymes	CE chip-based detection system	Urine	1,2,3,4) Renal markers	[19]
Γ-Hydroxybutiric	Uncoated capillary, normal polarity 5 mM phosphate and 15 mM sodium barbital, pH 12	UV 214 nm Indirect detection	Urine and serum	Central nervous system depressant	[18]
D- (1) and L-Lactic (2) acid	Reversed polarity Capillary coated with linear PAA Phosphate pH 6.0 and 2-hydroxypropyl-β-CD	UV 200 nm Direct detection	Urine, CSF, amniotic fluid	1) Dysbiosis markers 2) Carbohydrate metabolism	[86]
Oxalic acid	Reversed polarity Uncoated capillary Chromate, TTAB, EDTA, pH 8	UV 254 nm Indirect detection	Amniotic fluid and plasma	Nephrolithiasis	[87]
Formic (1), succinic (2), acetic, lactic (3) and propionic (4) acids	Reversed polarity Uncoated capillary Phthalate and commercial EOF modifier (OFM Anion BT) pH 5.6	UV 254 nm Indirect detection	Standards and human saliva	1) Metabolic disorder 2) Central energy pathway marker 3) Carbohydrate metabolism marker 4) Propionic acidemia	[88]
Acetic, lactic (1), citric (2), tartaric, malic (3) and succinic (4) acids. Not with fumaric and orotic acids	Reversed polarity Uncoated capillary Phthalate and commercial EOF modifier	UV 254 nm Indirect detection	Foods and urine	1) Carbohydrate metabolism marker 2,3,4) Central energy pathway marker	[89]

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Continued

Organic acids (see also Relevance)	Conditions	Detection	Type of sample	Relevance	Ref.
Lactate (1) and pyruvate (2)	Reversed polarity Uncoated capillary Tetraborate and TTAB, pH 9.2	UV 185 nm Direct detection	CSF	1,2) Carbohydrate metabolism marker	[90]
Fumaric, citric (1), succinic (2), pyruvic (3), acetic and lactic (4) acids	Reversed polarity 20 mM MES/His buffer to which 0.2 mM of TTAB was added to reverse the EOF, pH 5.8	On-chip contactless four-electrode conductivity detection for CE devices	Standards	1,2) Central energy pathway marker 3,4) Carbohydrate metabolism marker	[11]
Glyoxylate, glycolate, pyruvate, lactate, fumarate, succinate, malate, 2-oxoglutarate, PEP, DHAP, glycerol3P and others (n = 20)	Cationic polymer-coated capillary 50 mM ammonium acetate solution, pH 9.0. Reverse polarity	DAD detector CE-ESI-MS	Cell cultures	Metabolites of glycolisis and the TCA cycle	[20]

CSF, cerebrospinal fluid; DAD, diode-array detector; DHAP, dihydroxyacetonephosphate; DMDAPS, 3-(*N*,*N*-dimethyldodecylammonio)-propanesulfonate; MHEC, methylhydroxyethylcellulose; PAA, polyacrylamide; PEP, phosphoenolpyrovate; TTAB, tetradecyltrimethylammonium bromide

Laugere *et al.* carried out an innovative study in which a CE microdevice with an integrated on-chip contactless four-electrode conductivity detector was presented [11]. A 6 cm long, 70 μm wide, and 20 μm deep channel was etched in a glass substrate that was bonded to a second glass substrate in order to form a sealed channel. Four contactless electrodes (metal electrodes covered by 30 nm silicon carbide) were deposited and patterned on the second glass substrate for on-chip conductivity detection. The four-electrode configuration allows for sensitive detection for varying carrier-electrolyte background conductivity without the need for adjustment of the measurement frequency. Reproducible electrophoretic separations of three inorganic cations (K^+, Na^+, Li^+) and six organic acids (citric, succinic, pyruvic, acetic, and lactic acids) was presented.

Related to real sample analysis, Jager and Tavares [12] have developed a method for lactate, acetoacetate and β-hydroxybutyrate in blood serum samples of diabetic patients using CZE with indirect detection and reversed polarity. The running buffer contained 5 mM 3,5-dinitrobenzoate, 0.1 mM CTAB at pH 3.5. The LODs obtained under these conditions were 0.039 mM for acetoacetate and 0.046 mM for β-hydroxybutyrate. Lactate could be clearly measured, but only traces of acetoacetate and β-hydroxybutyrate could be found in the control sample.

In a recent paper, a CZE method was optimized to analyze low-molecular-mass organic acids to monitor diabetes in rat plasma [13]. Separation was carried out applying reversed voltage by using a polyacrylamide-coated capillary. Two electrophoretic buffers were employed: 0.150 M H_3PO_4 with 0.3 mM $CaCl_2$ at pH 6.20 for acetoacetic, β-hydroxybutyric, lactic and uric acids (see Fig. 1), and 200 mM phosphate with 10 mM acetate at pH 4.0 for pyruvic acid, both with direct detection at 200 nm. LOD values were 0.002 mM for acetoacetic acid, 0.006 mM for lactic acid, 0.004 mM for β-hydroxybutyric acid, 0.0001 mM for uric acid and 0.002 mM for pyruvic acids. The method was successfully applied to analyze these organic acids in control and diabetic animals. Although the LOD values greatly depend on calculation method used, these results show that indirect detection is not necessarily the best option for the analysis of organic acids. The main advantages of CE for the analysis of these organic acids were the avoidance of the use of different enzymatic assays (saving time and money), and the small amount of sample consumed (a very important issue when a large number of determinations has to be done with experimental animals of small size).

A novel use of catalyzed reactions to increase sensitivity in CE analysis of lactate was recently described [14]. Two reactions, catalyzed by lactate oxidase (LO) and peroxidase, are initiated by a single injection of the enzymes and the substrate 2,2'-azino-bis(3-ethylene-thiazoline-6-sulfonic acid) (ABTS) into the capillary previously filled with the sample (lactate or lactate-oxalate mixture) and the run buffer containing NADH. The oxidized ABTS product upon reaction with NADH is converted into

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

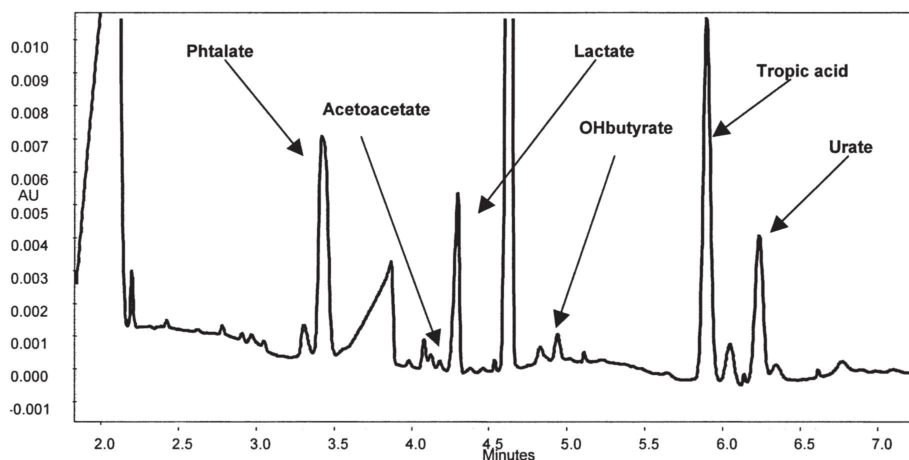

Figure 1. Rat plasma sample analyzed by CE using a buffer composed of 150 mM sodium phosphate, 0.3 mM $CaCl_2$ at pH 6.2. Redrawn from [13].

NAD(+), which is separated and detected in less than 2 min at 266 nm with a sample throughput of 7 min (including wash steps between samples). Linearity was established within the range from 0.0025 to 1 mM with r^2 = 0.998. Recoveries of lactate from five spiked serum samples averaged 101%. Application of this method to the determination of oxalate as an inhibitor of LO was also demonstrated.

Analytical capability of CZE using tandem-coupled columns for the spectral identification and determination of orotic acid (OA) in urine by diode-array detection (DAD) was investigated [15]. A very significant "in-column" cleanup of OA from the urine matrix was reached in the separation stage of the tandem by combining a low pH (2.8) with complexing reagents (namely, α- and β-CDs, poly(vinylpyrrolidone) and 3-(*N*,*N*-dimethyldodecylammonio)-propanesulfonate). Due to this, its DAD spectral data could be acquired in the detection stage of the tandem with almost no disturbances by matrix comigrating compounds. The LODs obtained under such working conditions for a 200-nL sample load of OA and 320 μm ID capillary tubes were 3.5 μM at 218 nm, and 0.4 μM at 280 nm. Using chemometric procedures (target transformation factor analysis, fixed-size moving window-evolving factor analysis, orthogonal projection approach and fixed-size moving window-target transformation factor analysis) to process the acquired spectral data, the presence of OA in the loaded urine matrix could be confirmed with confidence at concentrations higher than 10 μM. Interestingly, LOD values for HPLC methods are close to CE values according to a review from Salerno *et al.* [16], moreover, most of the ranges of urinary orotic acid levels reported in patients with abnormal pyrimidine metabolism are higher than the value (*i.e.*, 10 μM) reported above [16].

γ-Hydroxybutyric acid (GHB) is a central nervous system depressant and hypnotic which, in recent times, has shown increasing abuse either as recreational drug (due to its euphoric effects and ability to reduce inhibitions) or as doping agent (enhancer of muscle growth). Analogues of GHB, namely γ-butyrolactone and 1,4-butanediol, share its biological activity and are rapidly converted *in vivo* into GHB. At present, GHB and analogues are placed in the Schedules of Controlled Substances. Two analytical methods based on CZE for the direct determination of GHB in human urine and serum at potentially toxic concentrations have been developed [17, 18]. Baldacci *et al.* have developed an assay for urinary GHB that is based upon liquid-liquid extraction and CZE with indirect UV absorption detection [17]. The BGE is composed of 4 mM nicotinic acid (compound for indirect detection), 3 mM spermine (reversal of electroosmosis) and histidine (added to reach pH 6.2). Using a 50 μm ID capillary with 40 cm of effective length, 1-octanesulfonic acid as internal standard, solute detection at 214 nm and diluted urine with a conductivity of 2.4 mS/cm, GHB concentrations higher or equal to 2 μg/mL can be detected. LOD and LOQ were determined to be dependent on urine concentration and varied between 2 and 24 and 5 and 60 μg/mL, respectively. In the method by Bortolotti *et al.* [18] a capillary with 40 cm of detection length and 75 μm ID was employed, together with a CE buffer composed of 5.0 mM Na_2HPO_4, 15 mM sodium barbital adjusted to pH 12 with 1.0 M NaOH; the running voltage was 25 kV, the separation temperature 23°C, and detection was carried out using indirect UV detection at 214 nm together with hydrodynamic injection at 0.5 psi for 5 s. α-Hydroxyisobutyric acid was used as internal standard (IS). This method can be applied to both urine and plasma samples. Sample pretreatment was limited to 1:8 dilution with NaOH. Under these conditions, the sensitivity was ap-

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

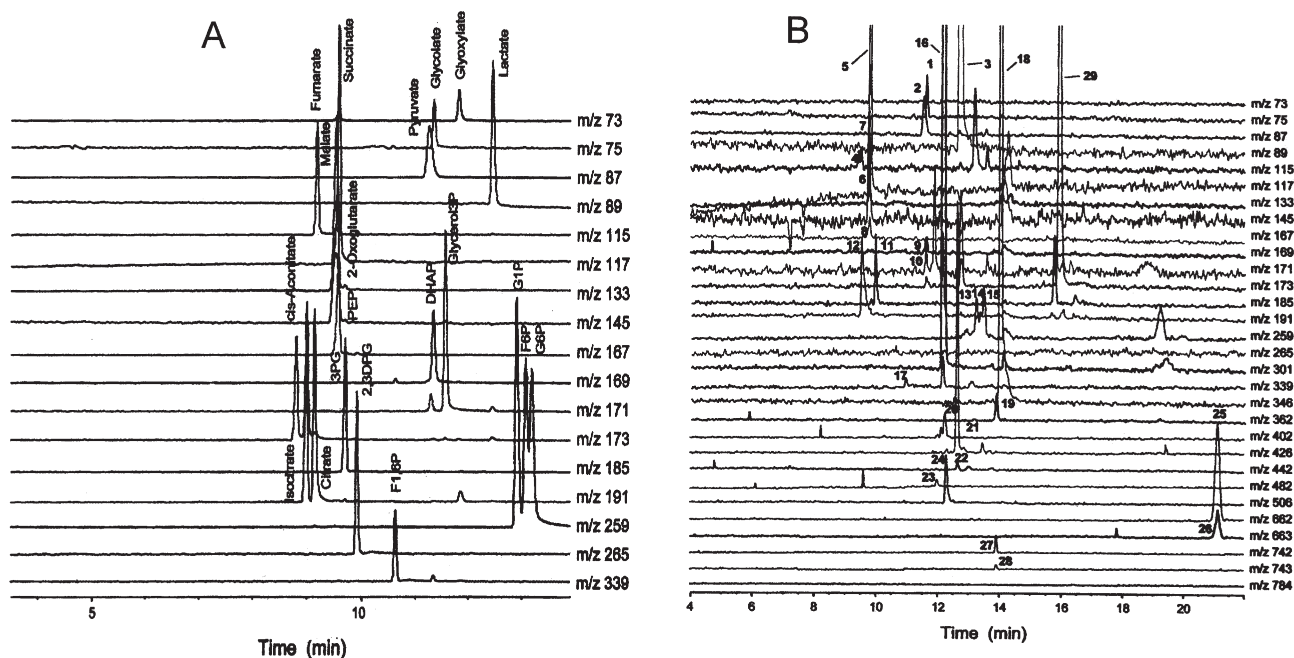

Figure 2. (A) CE-ESI-MS selective ion electropherograms for a standard mixture of metabolites of glycolysis and the TCA cycle. Conditions: capillary SMILE 50 μm ID × 100 cm; electrolyte, 50 mM ammonium acetate at pH 9.0; applied potential, −30 kV; temperature, 20°C; sheath liquid, 10 μL/min 5 mM ammonium acetate in 50% v/v methanol-water. (B) Selected ion electropherograms for metabolic intermediates of *Bacillus subtilis* JH642. Solutes: 1. glycolate; 2. pyruvate; 3. lactate; 4. fumarate; 5. succinate; 6. malate; 7. 2-oxoglutarate; 8. PEP; 9. DHAP; 10. glycerol3P; 11. 3PG; 12. citrate; 13. G1P; 14. F6P; 15. G6P; 16. PIPES(is); 17. F1,6P; 18. AMP; 19. GMP; 20. CDP; 21. ADP; 22. GDP; 23. CTP; 24. ATP; 25. NAD; 26. NADH; 27. NADP; 28. NADPH; 29. unknown. Redrawn from [20].

proximately 3.0 μM (S/N ratio = 3). No interferences were found in either the most common "drugs of abuse" or from endogenous compounds.

Uric acid has been measured in a novel CE chip-based detection system for simultaneous measurements of the renal markers creatine, creatinine, *p*-aminohippuric acid, and uric acid [19]. Fluid control is used for mixing the sample with the enzymes creatininase (CA), creatinase (CI), and sarcosine oxidase (SOx) and for separating the neutral hydrogen peroxide end product from the anionic *p*-aminohippuric and urate species. The peroxide product, together with *p*-aminohippuric and uric acids, are detected electrochemically at a downstream gold-coated thick-film amperometric detector. The four renal markers are readily measured within 5 min, and creatinine/creatine in less than 2 min. Applicability to urine samples is demonstrated. Such a multianalyte microchip detection device would allow renal function testing to be performed more rapidly, easily, and economically in the point-of-care setting.

An innovative work in this area has been the simultaneous determination of anionic metabolites based on CE coupled to ESI-MS [20]. To prevent current drop by the system, EOF reversal by using a cationic polymer-coated capillary was indispensable. A mixture containing 32 standards including carboxylic acids, phosphorylated carboxylic acids, phosphorylated saccharides, nucleotides, and nicotinamide and flavin adenine coenzymes of glycolysis and the tricarboxylic acid cycle pathways were separated by CE and selectively detected by a quadrupole mass spectrometer with a sheath-flow ESI interface. Key to the analysis was, as indicated above, EOF reversal using a cationic polymer-coated capillary together with a volatile electrolyte consisting of 50 mM ammonium acetate at pH 9.0. The RSDs of this method were better than 0.4% for migration times, and between 0.9 and 5.4% for peak areas. The concentration detection limits for these metabolites were between 0.3 and 6.7 μM/L with pressure injection of 50 mbar for 30 s (30 nL); *i.e.*, mass detection limits ranged from 9 to 200 fmol, at an S/N ratio of 3. This method was applied to the comprehensive analysis of metabolic intermediates extracted from *Bacillus subtilis*, and 27 anionic metabolites could be directly detected and quantified, among them: glyoxylate, glycolate, pyruvate, lactate, fumarate, succinate, malate, 2-oxoglutarate, cis-aconitic and citric acids have been determined. An example of this CE-MS analysis is shown in Fig. 2.

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

3 Anionic catecholamine metabolites

These compounds, although containing a carboxylic group and being small molecules, are not commonly considered organic acids. These metabolites are particularly useful for detecting pheochromocytomas, Parkinson's disease and stress. A micellar electrokinetic capillary chromatographic method for determining homovanillic (HVA), vanillic mandelic (VMA), 5-hydroxyindolacetic (5-HIAA) acids and other anionic catecholamine metabolites from patient urine samples has recently been established [21]. The optimum electrolyte solution (at pH 10.6) was made of tetraborate and SDS in water. Furthermore, studies were focused to optimize SPE steps to concentrate patient urine samples for identification of catecholamines with UV detection. LODs and LOQs were near 0.05 and 0.1 μg/mL, respectively, except for dopamine. The concentrations of the catecholamine metabolites in the studied patient urines varied from 0.186 to 76.4 μg/mL.

4 Fatty acids

4.1 Metabolic significance

Long-chain fatty acids are important constituents of the diet and they contribute to a multitude of cellular pathways and functions. Long-chain fatty acids (LCFAs) are taken up by cells and used for many biological functions. In addition to their important role in energy generation and storage, LCFAs contribute to the synthesis of phospholipids, molecules necessary for the structure, integrity, and function of plasma membranes, and are precursors for prostaglandins, which have numerous functions. Fatty acid acylation of proteins regulates their function and recruitment to plasma membranes. Fatty acids modulate the function of numerous enzymes and regulate the expression of multiple genes, notably those involved in fatty acid metabolism. Fatty acids also play an important role in nerve system function and synaptic transmission. As a result of their many actions, fatty acids play a role in many chronic diseases, such as arteriosclerosis and cancer, and in the body's response to infection and inflammation [22].

Free fatty acids differ by their chain length and branching, degree of unsaturation, and position and configuration of their double bonds. During the last few years, there has been an increasing interest in the properties of polyunsaturated fatty acids (PUFAs), especially in w-3 PUFAs because they seem to be bioactive compounds that play a critical role in human health in relation to cardiovascular disease, cancer, diabetes mellitus and neuropsychiatric disorders [23]. Plasma-free fatty acids (FFAs) are indicator substances in lipid, glucose, and hormone metabolism and are useful in the diagnosis of various diseases, such as diabetes mellitus, acute pancreatitis, angina pectoris and thyroidism [24–26]. On the other hand, individual fatty acids seem to be related to diabetes incidence, 16:0 in plasma cholesterol ester and phospholipid (PL), 16:1n-7 and 20:3n-6 in cholesterol ester and 18:0 in PL showed independent positive associations with diabetes incidence [27]. Fatty acids also seem to be affected for some dietary treatments in specific disease as phenylketonuria (PKU) [28].

4.2 Sample treatment

Fatty acids occur in limited amounts in the free form. Bound fatty acids are ionic or covalently linked to alcohol, glycol, glycerine, phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidyl inositol, monogalactosylglycerol, digalactosylglycerol, galactosylglucosylglycerol, aminosaccharides, proteins or other molecules. It is out of the scope of this review to consider the technique of lipid extraction that has been described by Christie [29] and Kates *et al.* [30], among other authors. But, it is necessary to comment that in the methods described above, only the free form can be determined, and therefore, a hydrolysis would be necessary to obtain the total amount of this type of compounds if desired.

4.3 Fatty acid analysis

In most cases, the analysis of FFAs is accomplished either by GC or HPLC [31–33]. For the particular determination of FFAs in plasma and serum, GC or GC-MS [34, 35] and HPLC with fluorescent detection [36–42] for electrochemical detection [43] have been used. These techniques frequently involve a precolumn derivatization step to enhance the volatility in GC and detectability in HPLC and it carries with it the risk of PUFA modifications and problems with quantification due to the loss of target compounds and incomplete reactions.

An alternative method to analyze fatty acids is the employment of CE. Table 2 summarizes the methods developed up to now for standards and biological samples. Unlike short-chain organic acids, separation is usually accomplished with normal polarity (injection in the positive end and detection in the negative end) with electrophoretic mobility of the analytes opposite to EOF.

Likewise, for short-chain organic acids, fatty acids are difficult to detect by direct UV detection due to the lack of a strongly absorbing chromophore. In contrast, PUFAs exhibit a significant absorption between 200 and 250 nm,

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 2. Different conditions for the CE analysis of saturated and unsaturated fatty acids

Compounds	Conditions	Detector	Ref.
C12–C20 saturated unsaturated	Fused-silica capillary 20 kV normal polarity 10 mM *p*-hydroxybenzoate, 5 mM Tris, 40 mM Brij 35 50%	Indirect UV	[44]
C18:1, C18:2, α and γ C18:3 and hydroperoxides	Fused-silica capillary normal polarity (526 V/cm) 70% sodium phosphate (50 mM, pH 4.85), 30% methanol, 1.1 g/L Brij 35	UV DAD 195 nm for fatty acids and 234 nm for hydroperoxides	[54]
C14–C19 (AF-labeled fatty acids)	Fused-silica capillary 30 kV normal polarity 25 mM borate buffer 30% ACN	LIF (488–520 nm)	[51]
C10–C20 saturated and unsaturated	Fused-silica capillary 25 kV normal polarity 5 mM buffer phosphate, pH 7.0 containing 4 mM SDBS, 4 mM β-CD and 35% ACN	DAD 224 nm (206 nm for PUFAs)	[52]
C4–C24 saturated C4–C18:2	Fused-silica capillary 30 kV normal polarity 40°C 5 mM diethylbarbiturate buffer, pH 10.5 containing 0.5 M z1-methyl and 70% ethyleneglycolmonomethyl ether	Indirect UV 240 nm	[46]
C14–C20	Fused-silica capillary 30 kV normal polarity 25°C 10 mM Tris – 5 mM *p*-anisic (pH 8.1) containing 10 mM Brij 35 in methanol-water (50:50 v/v)	Indirect UV 254 nm	[47]
C14–C18	Fused-silica capillary 30 kV normal polarity 27°C 10 mM Tris – 5 mM *p*-anisate (pH 8.1) and 1 mM dimethyl-β-CD in methanol-water (50:50 v/v)	Indirect UV 260 nm	[49]
C12–C31 saturated C14–C22 unsaturated	Fused-silica capillary 20 kV normal polarity 40°C 40 mM Tris, NMF-dioxane (3:2) and 2.5 mM AMP 0.5 mM Brij For unsaturated: 40 mM Tris, NMF-dioxane (5:4:1) and 2.5 mM AMP 20°C	UV indirect 259 nm	[55]
C14–C26 saturated	PTFE-coated capillary 20 kV normal polarity 2.5 mM antraquinone- 2-carboxylic acid 40 mM Tris in *N*-methylformamide-dioxane (3:1 v/v)	Indirect UV 264 nm	[56]
C5–C18	Fused-silica capillary 28 kV normal polarity, 50°C, 80 mM Tris, 10 mM benzoic acid, pH 8.0	Indirect UV	[45]
C8–C11	Fused-silica capillary 27 kV normal polarity 100 mM borate, pH 10 20 mM SDS, 4 M urea	LIF	
C8, C9, C10 in serum	Serum	LIF	
C12–C18 saturated and unsaturated	Fused-silica capillary 20 kV normal polarity 15 mM phosphate buffer, pH 7.0 containing 4 mM SDBS, and 45% ACN, 10 mM Brij 35 and 2% 1-octanol	Indirect UV 224 nm	[53]

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 2. Continued

Compounds	Conditions	Detector	Ref.
C8–C18 saturated	Fused-silica capillary 30 kV normal polarity, 30°C, 20 mM Tris – 10 mM *p*-anisate (pH 8.2) containing 1 mM trimethyl-β-CD in methanol-water (50:50 v/v)	Indirect UV 270 nm	[48]

AMP, adenosine monophosphate; NMF, *N*-methylformamide; SDBS, sodium dodecyl benzene sulfonate

owing to the presence of double bonds. Nevertheless, for both saturated and unsaturated FFAs, indirect UV detection using a UV absorbing co-ion seems to provide superior performance in terms of sensitivity compared to direct UV detection. Thus, for optimized indirect A detection of FFA, the chromophore should preferably bear a negative charge and have a high molar absorptivity for high sensitivity, while its effective mobility must be close to that of the analytes to reduce electromigration dispersion. Different chromogenic species, such as dihydroxybenzoate [44], benzoate [45], diethylbarbiturate [46], chromate, naphtalenesulfonate and *p*-anisate [47], have been tested. Among these compounds, *p*-anisate seems to give the most satisfactory baseline stability, peak symmetry and sensitivity [48].

5-Bromomethylfluorescein derivatization together with LIF has also been employed as a detection technique, but only for medium-chain (C8 to C11) fatty acids. Nevertheless, this application has the interest of being the only work published so far where a pathological serum sample has been measured after 3000-fold dilution in water. It is also interesting to mention that the methyl esters of these fatty acids could not be measured by GC-MS, because of coelution with other peaks, and therefore they could not be identified by this latter procedure [45].

As the chain length of FFAs increases, several analytical difficulties arise owing to the lower analyte solubility, micelle formation in the separation electrolyte, and decreasing separation selectivity between the consecutive homologues [48]. This problem can be partially overcome through the addition of organic solvents to the carrier electrolyte [49]. CE separations of fatty acids up to C18 have been achieved using up to 60% ACN [44, 50–53] or up to 60% methanol [48], [47, 49] adding CD [52] and/or Brij 35 [44] to increase solubility and selectivity [47, 48, 54]. The use of CD and its derivatives has shown to be necessary as electrolyte additives to improve resolution of very similar solute pairs [49]. Moreover, in our experience, the presence of Brij has proved to be fundamental in the BGE, when the concentration or number of FFA increases to avoid micelle formation. On the other hand, the use of nonaqueous media is an interesting alternative for electrophoretic separation of LCFAs. However, few organic solvents have been investigated as electrophoretic media [55]. The use of nonaqueous electrolyte for the CZE separation of very-long-chain FFAs has been demonstrated by Drange *et al.* [56]. In this work, the authors achieved the separations of C14 to C26 FFAs but their method was not optimized for the resolution of unsaturated FFAs [56]. Haddadian *et al.* optimized a partially aqueous CZE system for the separation of a complicated mixture of unsaturated (C14 to C22) FFAs isomers, which is shown in Fig. 3 [55].

It is noteworthy that some organic solvents used in nonaqueous CE such as formamide and its N-substitutes possess UV A. Although they might cause problems in indirect UV detection, this can be solved by using *e.g.,* indirect fluorescence detection together with a BGE containing a fluorophore with both maximum excitation and emission wavelengths far away from the absorption range

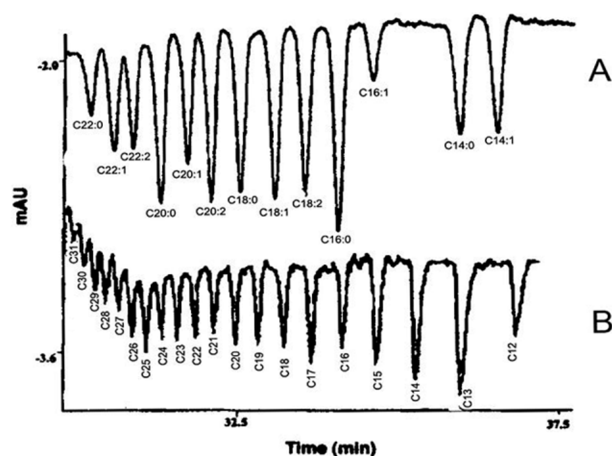

Figure 3. (A) Electropherogram of saturated and unsaturated FFAs (C14–C22). Background electrolyte, 40 mM Tris, NMF-dioxane-water (5:4:1), and 2.5 mM AMP. Applied voltage, +20 kV; 259 nm; capillary temperature, 20°C. (B) Electropherogram of saturated FFAs (C12–C31). Background electrolyte, 0.5% w/v Brij, 40 mM Tris, NMF-dioxane (3:2), and 2.5 mM AMP. Applied voltage, 20 kV; 259 nm; capillary temperature, 40°C. Redrawn from [55].

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

of the organic solvent. Wang *et al.* developed a method for separation of saturated C6 to C24 fatty acids using nonaqueous CZE with indirect fluorescence detection [57]. The results showed that such a wide range of medium- and long-chain free fatty acids could be separated between any two consecutive homologues in one run and be detected at a level of about 0.01 to 0.02 mM in highly basic methanol/ACN media containing fluorescein as a coion of BGE for indirect fluorescence detection.

4.4 Miscellaneous

Sialic acid is the general name for neuraminic acid derivatives, *N*-acetylneuraminic acid (NANA) being one of the most common and representative substances among these derivatives. In a recent work, introduction of a sialoglycan (*N*-acetylneuraminyllactose) or a sialoglycoprotein (bovine serum fetuin) sample in a CE capillary containing a running buffer at pH 5.0 with *N*-acetylneuraminidase followed by application of separation voltage resulted in the release of NANA which could be estimated by CE with UV detection [58].

Hyaluronic acid (hyaluronan) is a ubiquitous extracellular matrix component presents at high concentrations in skin, joints and cornea. In the skin, it is synthesized primarily by dermal fibroblasts and by epidermal keratinocytes. Hyaluronic acid usually exists as a high-molecular-mass macromolecule (600 000 to 1 000 000) and non-sulfated glycosaminoglycan composed of a disaccharide unit of [bond]3GlcNAc β 1[bond]4GlcA β 1[bond]. Hyaluronic acid has widely been used not only for osteoarthritis and ophthalmology but also for skin care cosmetics. A recent review includes the development in the analysis of hyaluronic acid having various molecular sizes using electrophoretic and chromatographic techniques [59].

5 Concluding remarks and future outlook

In this work, we have revised the most recent developments for the analysis of short-chain organic acids, anionic catecholamine metabolites and fatty acids using capillary electromigration procedures. Although it is clear that the use of CE still shows some important drawbacks for the analysis of these small solutes (mostly due to its low sensitivity), it is also clear that CE can provide interesting advantages compared to other more classical, laborious and expensive procedures based on the use of chromatographic techniques. Thus, in many cases, CE with UV detection would be the best option for monitoring previously diagnosed metabolic diseases, when the structural identification of unknown diagnostic metabolites is not necessary. It permits the development of fully automatic methods, with minimum or no sample pretreatment, small sample volumes, high separation efficiency and low cost. On the other hand, when the patient is acutely ill, the procedures utilized must be capable of providing not only accurate but also rapid results and CE can comply with this requirement. Moreover, the good analytical capabilities of CE-MS have still to be fully explored and realized in carboxylic acids analysis in real samples. Finally, a new methodology, such as CE in a microchip, is also getting into organic acids analysis and can provide new possibilities in the not to distant future derived from the high throughput and easy portability of these microdevices.

Financial support from the CICYT projects (AGL2002-04621-C02-01 and AGL2002-04621-C02-02, Ministerio de Ciencia y Tecnología) is acknowledged.

Received November 16, 2004

6 References

[1] Lehotay, D. C., *Biomed. Chromatogr.* 1991, *5*, 113–121.

[2] Galli, V., Garcia, A., Saavedra, L., Barbas, C., *Electrophoresis* 2003, *24*, 1951–1981.

[3] Garcia, A., Barbas, C., *Clin. Chem. Lab. Med.* 2003, *41*, 755–761.

[4] Chalmers, R. A., Mistry, J., Penketh, R., McFadyen, I. R., *J. Inherit. Metab. Dis.* 1989, *12 Suppl 2*, 283–285.

[5] Tuchman, M., Ulstrom, R. A., *Adv. Pediatr.* 1985, *32*, 469–506.

[6] Tanaka, K., Hine, D. G., West-Dull, A., Lynn, T. B., *Clin. Chem. (Winston-Salem, N. C.)* 1980, *26*, 1839–1846.

[7] Tanaka, K., Hine, D. G., *J. Chromatogr.* 1982, *239*, 301–322.

[8] Kuhara, T., *J. Chromatogr. B* 2002, *781*, 497–517.

[9] Fatemi, M. H., *J. Chromatogr. A* 2004, *1038*, 231–237.

[10] Martin-Jouet, M., Hagege, A., Leroy, M. J., *J. Chromatogr. A* 2002, *946*, 255–263.

[11] Laugere, F., Guijt, R. M., Bastemeijer, J., van der Steen, G., Berthold, A., Baltussen, E., Sarro, P., van Dedem, G. W., Vellekoop, M., Bosschet, A., *Anal.Chem.* 2003, *75*, 306–312.

[12] Jager, A. V., Tavares, M. F., *Electrophoresis* 2003, *24*, 1208–1214.

[13] Baena, B., García-Martínez, D., Barbas, C., *J. Chromatogr. A* 2004, *1051*, 199–205.

[14] Qi, L., Danielson, N. D., *Electrophoresis* 2003, *24*, 2070–2075.

[15] Dankova, M., Strasik, S., Kaniansky, D., *J. Chromatogr. A* 2003, *990*, 121–132.

[16] Salerno, C., Crifo, C., *J. Chromatogr. B* 2002, *781*, 57–71.

[17] Baldacci, A., Theurillat, R., Caslavska, J., Pardubska, H., Brenneisen, R., Thormann, W., *J. Chromatogr. A* 2003, *990*, 99–110.

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[18] Bortolotti, F., De Paoli, G., Gottardo, R., Trattene, M.,Tagliaro, F., *J. Chromatogr. B* 2004, *800*, 239–244.

[19] Wang, J., Chatrathi, MP., *Anal. Chem.* 2003, *75*, 525–529.

[20] Soga, T., Ueno, Y., Naraoka, H., Ohashi, Y., Tomita, M., Nishioka, T., *Anal. Chem.* 2002, *74*, 2233–2239.

[21] Sirén, H., Mielonen, M., Herlevi, M., *J. Chromatogr. A* 2004, *1032*, 289–297.

[22] Hajri, T., Abumrad, N. A., *Annu. Rev. Nutr.* 2002, *22*, 383–415.

[23] Duo, L., *Chin. Med. J.* 2003, *116*, 453–458.

[24] Jensen, T., *World. Rev. Nutr. Diet.* 1994, *76*, 15–20.

[25] Harlan, W. R., Laszol, J., Bogdonoff, M. D., Estes, E. H., *J. Clin. Endocrinol.* 1963, *23*, 33–40.

[26] Shimomura, Y., Sugiyama, S., Takamura, T., Kondo, T., Ozawa, T., *J. Chromatogr.* 1986, *383*, 9–17.

[27] Wang, L., Folsom, A. R., Eckfeldt, J. H., *Nutr. Metab. Cardiovasc. Dis.* 2003, *13*, 256–266.

[28] Poge, A. P., Baumann, K., Muller, E., Leichsenring, M., Schmidt, H., Bremer, H. J., *J. Inherit. Metab. Dis.* 1998, *21*, 373–381.

[29] Christie, W. W., *Lipid. Analysis*, Pergamon Press, Oxford 1973.

[30] Kates, M. R., Burdon, H., van Knippenberg, P. H., *Techniques of Lipidology: Isolation, Analysis and Identification of Lipids*, Elsevier, Amsterdam 1986, Vol. 3, part 2.

[31] Ackman, R. G., *Anal. Chim. Acta* 2002, *465*, 175–192.

[32] Lima, E. S., Abdalla, D. S. P., *Anal. Chim. Acta* 2002, *465*, 81–91.

[33] Brondz, I., *Anal. Chim. Acta* 2002, *465*, 1–37.

[34] Costa, C. G., Dorland, L., Holwerda, U., de Almeida, I. T., Poll-The, B. T., Jakobs, C., Duran, M., *Clin. Chem.* 1998, *44*, 463–71.

[35] Magni, F., Piatti, P. M., Monti, L. D., Lecchi, P., Pontiroli, A. E., Pozza, G., Galli Kienle, M., *J. Chromatogr. B* 1994, *657*, 1–7.

[36] Mehta, A., Oeser, A. M., Carlson, M. G., *J. Chromatogr. B* 1998, *719*, 9–23.

[37] Inoue, H., Ikeno, M., Ishii, Y., Tsuruta, Y., *J. Chromatogr. A* 1998, *816*, 137–143.

[38] Nakashima, K., Taguchi, Y., Kuroda, N., Akiyama, S., Duan, G., *J. Chromatogr.* 1993, *619*, 1–8.

[39] Toyo'oka, T., Takahashi, M., Suzuki, A., Ishii, Y., *Biomed. Chromatogr.* 1995, *9*, 162–170.

[40] Asakura, K., Ohrui, H., Meguro, H., *Analyst* 1993, *118*, 765–768.

[41] Yasaka, Y., Tanaka, M., Shono, T., *J. Chromatogr.* 1990, *508*, 133–140.

[42] Yamaguchi, M., Matsunaga, R., Hara, S., Nakamura, M., Ohkura, Y., *J. Chromatogr.* 1986, *375*, 27–35.

[43] Kotani, A., Fuse, T., Kusu, F., *Anal. Biochem.* 2000, *284*, 65–69.

[44] Heinig, K., Hissner, F., Martin, S., Vogt, C., *Am. Lab.* 1998, *30*, 24–29.

[45] Zuriguel, V., Causse, E., Bounery, J. D., Nouadje, G., Simeon, N., Nertz, M., Salvayre, R., Couderc, F., *J. Chromatogr. A* 1997, *781*, 233–238.

[46] Buchberger, W., Winna, K., *Mikrochim. Acta* 1996, *122*, 45–52.

[47] Collet, J., Gareil, P., *J. Chromatogr. A* 1997, *792*, 165–177.

[48] Roldan-Assad, R., Gareil, P., *J. Chromatogr. A* 1995, *708*, 339–350.

[49] Collet, J., Gareil, P., *J. Capil. Electrophor.* 1996, *3*, 77–82.

[50] Gutnikov, G., Beck, W., Engelhardt, H., *J. Microcol. Sep.* 1994, *6*, 565–572.

[51] Brando, T., Pardin, C., Prandi, J., Puzo, G., *J. Chromatogr. A* 2002, *973*, 203–210.

[52] de Oliveira, M. A., Micke, G. A., Bruns, R. E.,Tavares, M. F., *J. Chromatogr. A* 2001, *924*, 533–539.

[53] de Oliveira, M. A., Solis, V. E., Gioielli, L. A., Polakiewicz, B.,Tavares, M. F., *Electrophoresis* 2003, *24*, 1641–1647.

[54] Schmitz, O., Gäb, S., *J. Chromatogr. A* 1997, *767*, 249–253.

[55] Haddadian, F., Shamsi, S. A., Warner, I. M., *J. Chromatogr. Sci.* 1999, *37*, 103–107.

[56] Drange, E., Lundanes, E., *J. Chromatogr. A* 1997, *771*, 301–309.

[57] Wang, T., Wei, H., Li, S., *Electrophoresis* 1998, *19*, 2187–2192.

[58] Taga, A., Sugimura, M., Suzuki, S., Honda, S., *J. Chromatogr. A* 2002, *954*, 259–266.

[59] Kakehi, K., Kinoshita, M., Yasueda, S., *J. Chromatogr. B* 2003, *797*, 347–355.

[60] Petucci, C. J., Kantes, H. L., Strein, T. G., Veening, H., *J. Chromatogr. B* 1995, *668*, 241–251.

[61] Dolnik, V., Dolnikova, J., *J. Chromatogr. A* 1995, *716*, 269–277.

[62] Schneede, J., Ueland, P. M., *Anal. Chem.* 1995, *67*, 812–819.

[63] Schneede, J., Mortensen, J. H., Kvalheim, G., Ueland, P. M., *J. Chromatogr. A* 1994, *669*, 185–193.

[64] Chen, H., Xu, Y., Lente, F. V., *J. Chromatogr. B* 1996, *679*, 49–59.

[65] Shirao, M., Furuta, R., Suzuki, S., Nakazawa, H., Fujita, S., Maruyama, T., *J. Chromatogr. A* 1994, *680*, 247–251.

[66] Marsh, D. B., Nuttall, K. L., *J. Capil. Electrophor.* 1995, *2*, 63–67.

[67] Jariego, C. M., Hernanz, A., *Clin. Chem.* 1996, *42*, 477–478.

[68] Willetts, M., Clarkson, P., Cooke, M., *Chromatographia* 1996, *23*, 671–674.

[69] Franke, D. R., Nuttall, K. L., *J. Capil. Electrophor.* 1996, *3*, 309–312.

[70] Sevcik, J., Adam, T., Sazel, V., *Clin. Chim. Acta* 1997, *259*, 73–81.

[71] Jellum, E., Dollekamp, H., Brunsvig, A., Gislefoss, R., *J. Chromatogr. B* 1997, *689*, 155–164.

[72] Barbas, C., Adeva, N., Aguilar, R., Rosillo, M., Rubio, T., Castro, M., *Clin. Chem.* 1998, *44*, 1340–1342.

[73] Garcia, A., Barbas, C., Aguilar, R., Castro, M., *Clin. Chem.* 1998, *44*, 1905–1911.

[74] Procházková, A., Kriváneková, L., Bocek, P., *J. Chromatogr. A* 1999, *838*, 213–221.

[75] He, T., Quinn, D., Fu, E., Wang, Y. K., *J. Chromatogr. B* 1999, *727*, 43–52.

[76] Elgstoen, K. B., Zhao, J. Y., Anacleto, J. F., Jellum, E., *J. Chromatogr. A* 2001, *914*, 265–275.

[77] Holmes, E., Bonner, F. W., Nicholson, J. K., *Arch. Toxicol.* 1995, *70*, 89–95.

[78] Fu, C., Wang, L., Fang, Y., *Talanta* 1999, *50*, 953–958.

[79] García, A., Muros, M., Barbas, C., *J. Chromatogr. B* 2001, *755*, 287–295.

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[80] Issaq, H. J., Delviks, K., Janini, G. M., Muschik, G. M., *J. Liq. Chromatogr.* 1992, *15*, 3193–3201.

[81] Caslavska, J., Gassmann, E., Thormann, W., *J. Chromatogr. A* 1995, *709*, 147–156.

[82] Shirao, M. K., Suzuki, S., Kobayashi, J., Hiroyuki, N., Mochizuki, E., *J. Chromatogr. B* 1997, *693*, 463–467.

[83] Siren, H., Karjalainen, U., *J. Chromatogr. A* 1999, *853*, 527–533.

[84] Garcia, A., Heinanen, M., Jimenez, L. M., Barbas, C., *J. Chromatogr. A* 2000, *871*, 341–350.

[85] Li, X., Jin, W., Weng, Q., *Anal. Chim. Acta* 2002, *461*, 123–130.

[86] Saavedra, L., Barbas, C., *J. Chromatogr. B* 2002, *766*, 235–242.

[87] Nelson, B. C., Rockwell, G. F., Campfiel, T., O'Grady, P., Hernandez, R. M., Wise, S. A., *Anal. Chim. Acta* 2000, *410*, 1–10.

[88] Romano, J., Jandik, P., Jones, W. R., Jackson, P. E., *J. Chromatogr.* 1991, *546*, 411–421.

[89] Kenney, B. F., *J. Chromatogr.* 1991, *546*, 423–430.

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

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim