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Determination of carnitine and acylcarnitines in biological samples by capillary electrophoresis–mass spectrometry

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Abstract

Free carnitine and acylcarnitines (carnitine esters) play an important role in the metabolism of fatty acids. Metabolic disorders can be detected by abnormal levels of these compounds in biological fluids. Capillary electrophoresis–mass spectrometry has the advantage of combining an efficient separation technique with highly selective detection. Therefore, we have developed a method for the determination of carnitine and several of its esters implementing electrospray capillary electrophoresis–mass spectrometry in the positive ion selected reaction monitoring mode. A sheath-flow interface with a mixture of 2-propanol or methanol, water and acetic acid as sheath liquid and nitrogen as nebulizing gas was used. The zwitterionic analytes migrated as cations in the applied electric field using ammonium acetate–acetic acid or formic acid electrolytes. Separations were performed in aqueous, mixed organic–aqueous and non-aqueous media. The influence of the electrolyte composition on the separation efficiency was investigated. The electrospray conditions have been optimized regarding ion current stability and sensitivity. Ammonium acetate (10 mmol/l)–0.8% formic acid in water or 6.4% formic acid in acetonitrile–water (1:1) were used as running buffers for the determination of carnitine and acylcarnitines in human biological samples. Methanol extracts of dried blood spots were analyzed as well as urine and plasma following sample preparation via solid-phase or liquid–liquid extraction. Recoveries approaching 100% were achieved depending on the analytes and sample preparation procedures employed. Endogenous carnitine and acetylcarnitine were determined at concentrations between 2.7 and 108 nmol/ml in normal human urine and plasma. Other acylcarnitines were detected at levels of below the limit of detection to 12 nmol/ml. Good precision (0.8 to 14%) and accuracy (85 to 111%) were obtained; the achieved limits of quantitation (0.1 to 1 nmol/ml) are sufficient to characterize carnitine and acylcarnitine levels occurring as markers for metabolic disorders.

Keywords: Mass spectrometry; Carnitine; Acylcarnitines

1. Introduction

Free carnitine (3-hydroxy-4-*N*,*N*,*N*-trimethylammonium butyrate) and acylcarnitines (carnitine esters) play an important role in the metabolism of fatty acids [1,2]. Carnitine is introduced into the body during the intake of food and is also synthesized in small amounts by the organism. About 98% of the entire carnitine content is found in skeletal and cardiac muscles [3]. Carnitine works as a carrier molecule for the transport of long-chain fatty acids across the mitochondrial membrane,

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which then undergo β-oxidation for energy production. Carnitine and its acylmetabolites, usually the acetyl form, are present in blood and normally excreted in urine [4], with only the L-enantiomers occurring. In normal human plasma about 30–50 nmol/ml carnitine, 2–5 nmol/ml acetylcarnitine and only 0–1 nmol/ml propionylcarnitine were detected [5]. In normal human urine 81–290 nmol/ml carnitine, 22–100 nmol/ml acetylcarnitine and 0–5 nmol/ml other acylcarnitines (propionyl-, isovaleryl-, isobutyryl-, octanoyl-, decanoylcarnitine) can be found [6–9]. Many genetic and acquired disorders affect the carnitine concentration in biological fluids. Carnitine deficiencies which develop with certain diseases (e.g., renal tubular disease) or as a result of hemodialysis and total parenteral nutrition (secondary deficiencies) lead to serious damage to the organism, including failure of cardiac function [10]. Therefore, the determination of carnitine levels in those patients is important. Many genetic disorders, such as medium chain acyl-CoA dehydrogenase deficiency (MCAD), methylmalonic aciduria, propionic acidemia and isovaleric acidemia, are characterized by abnormal production of carnitine and its acyl metabolites [5,6,9–12]. The excretion of free carnitine is usually decreased whereas the excretion of atypical acylcarnitines, such as propionyl-, *n*-butyryl-, isovaleryl- or octanoylcarnitine, is increased. The lack of acylcarnitines with chain length $>C_{10}$ is an additional evidence for MCAD [11]. Thus carnitine and acylcarnitines can be used as biomarkers for genetic inborn errors during the routine neonatal screening of blood. The determination of carnitine and acylcarnitines after carnitine administration is necessary to monitor therapeutic levels and control the course of the treatment.

Although the total carnitine concentration can be used to detect a deficiency, the differentiation between homologous and even isomeric acylcarnitines is essential to determine the nature of the metabolic disorder (e.g., *n*-butyrylcarnitine may be present with MCAD, isobutyrylcarnitine occurs in normal urine [12]). Sophisticated analytical methods, such as tandem mass spectrometry (MS–MS), gas chromatography (GC), GC–MS, liquid chromatography (LC), LC–MS and capillary electrophoresis (CE), are necessary to meet the requirements for selective and sensitive analysis. The application of these techniques for the determination of carnitine and acylcarnitines has been reviewed elsewhere [10,13,14]. Fast atom bombardment (FAB) MS–MS using flow-injection analysis (FIA) is very selective, sensitive and fast, and has been routinely used in neonatal screening of blood spots [15–18]. Also electrospray ionization (ESI) MS–MS has been applied recently for the detection of genetic inborn errors [19–21]. Quantitation by those techniques requires a stable labeled internal standard for each analyte. Limits of quantitation (LOQs) are typically 0.1 nmol/ml. The distinction between isomeric forms of acylcarnitines was only possible by high collision energy fragmentation FIA–FAB-MS–MS with the use of sophisticated, expensive instruments [22]. Thus separation techniques are still of importance as alternative methods for acylcarnitine determination and differentiation. LC is capable of separation and identification of carnitine and acylcarnitines in complex mixtures [5–8,12,14,23–27]. Adequate sensitivity was obtained via UV and fluorescence detection after derivatization [6–8] or by using the radioisotope exchange method [5]. The developed LC methods are able to distinguish between homologous and isomeric acylcarnitines, but require long separation times (up to 90 min for a mixture of C_1 to C_{18} acylcarnitines [8]) for sufficient peak resolution and separation from matrix interferences. Thermospray and FAB LC–MS analyses have been reported [16,24]. ESI-LC–MS has been applied only for the determination of carnitine, acetyl- and propionylcarnitine [25–27]. Liquid chromatographic techniques give LOQ values between 0.2 and 5 nmol/ml. Sample preparation methods include simple protein precipitation for plasma [27] or methanol extraction of dried blood, plasma and urine [17] as well as solid-phase extraction (SPE) procedures using silica gel [6,12] or cation exchanger material [8,9] for urine and plasma.

CE, a well-suited separation method for charged compounds, is another possible technique for the separation of carnitine and acylcarnitines in biological matrices. Advantages over HPLC are the possibility of fast method development, robustness and inexpensive replacement of capillaries, low sample and solvent consumption, higher resolution capability, feasibility for fast separations and simultaneous determination of polar and nonpolar compounds. The ability to resolve homologues and isomers as well as enantiomers has been shown

previously by CE with UV and fluorescence detection [28–32]. The separation of carnitine enantiomers was achieved either after reaction with fluorenylethylchloroformate (FLEC) to form diastereomeric derivatives [28,29] or in electrolyte solutions containing cyclodextrins [29,30]. For acylcarnitine determination an electrolyte consisting of phosphoric acid–5% sodium dodecyl sulfate solution–methanol (4:8:88) was used [31,32]. Derivatization was performed by reaction with fluorenylmethylchloroformate (FMOC) or 9-anthryldiazomethane (ADAM). Laser-induced fluorescence detection provided excellent sensitivity (estimated LOD 0.01 nmol/ml). The analysis of spiked plasma samples suffered from interferences with matrix components that overlap with the analyte peaks. Long run times (>45 min for a mixture of acylcarnitines to chain length C_{16}) were required for a good separation of the analytes from these interfering compounds.

CE–MS determination of carnitine and its esters has not been described previously, although the technique has progressed substantially in recent years, providing validated bioanalytical methods [33–35]. One advantage of CE–MS is that derivatization of the carnitines is not required. Furthermore, less influence of matrix components occurs, shorter run times are possible (complete peak resolution is not necessary because extracted ion current profiles may be studied to distinguish each component), and CE–MS techniques provide the unique possibility to identify the sample constituents. Therefore, the aim of our work was to develop CE–MS methods to determine carnitine and acylcarnitines in biological samples. The influence of the buffer composition on the separation of standard mixtures was investigated. The electrospray conditions were optimized to provide adequate sensitivity and good ion current stability. Sample preparation strategies for urine and plasma samples were investigated and quantitation experiments were carried out.

2. Experimental

2.1. Materials

The analytes carnitine, acetylcarnitine and palmitoylcarnitine were purchased from Sigma (St. Louis, MO, USA). The other acylcarnitines (C_3–C_{18}) were obtained from Stichting Klin. Genetica (Amsterdam, The Netherlands). The deuterated internal standards carnitine-d_3 and acetylcarnitine-d_3 were obtained from Cambridge Isotope Labs., Andover, MA, USA. A mixture of deuterated internal standards (304 μmol/l carnitine-d_9, 76 μmol/l acetylcarnitine-d_3, each 15.2 μmol/l propionyl-, butyryl-, octanoyl- and myristoylcarnitine-d_3 and 30.4 μmol/l palmitoylcarnitine-d_3) was supplied by Neo Gen Screening (Pittsburgh, PA, USA). Ammonium acetate was obtained from Sigma, acetic acid from Fisher Scientific (Fair Lawn, NJ, USA) and formic acid from GFS Chemicals (Columbus, OH, USA). Ethyl acetate, *n*-butanol, chloroform, methanol, acetonitrile and 2-propanol (HPLC grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA). All solutions were prepared with a laboratory-generated deionized water (Barnstead Nanopure II filtration system, Boston, MA, USA). Silica Gel 3 ml SPE cartridges were obtained from J.T. Baker.

2.2. Capillary electrophoresis

A Crystal CE model 310 (ATI Unicam, Madison, WI, USA) was used for the electrophoretic separations. Uncoated fused-silica capillaries of 80 cm×75 μm I.D.×360 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). The CE electrolyte solutions were ammonium acetate (10–30 mmol/l) with acetic or formic acid in aqueous and organic (methanol, acetonitrile) solution. The separation voltage was 30 kV. A pressure between 0 and 50 mbar was applied at the injection (inlet) end during the electrophoretic separation. Injection was performed hydrodynamically or electrokinetically. The specific separation conditions for each application are given in Results.

2.3. Mass spectrometry

The PE Sciex API 365 tandem mass spectrometer (Concord, Ontario, Canada) was coupled to the CE apparatus via a laboratory-built coaxial sheath-flow interface, placed into the commercial ion spray interface housing. A 2 MΩ resistor was used to decouple the electrospray voltage from the CE voltage. Pneumatically assisted electrospray (ion

spray) with 2-propanol–water or methanol–water containing 0.1% acetic acid as sheath liquid at 4–10 μl/min and nitrogen as nebulizer gas (set at position 1–4 on the API 365) was used for all CE–MS experiments. The sheath liquid was delivered by a Harvard Apparatus syringe pump (Natick, MA, USA). The electrospray voltage, sheath liquid and nebulizing gas were turned on 0.2 min after the start of the CE separation. This was necessary to prevent vacuum formation and liquid siphoning from the capillary outlet, leading to a ''break down'' of the separation current. Data acquisition was started 1 min after sample injection. The nitrogen curtain gas was maintained at 10, the collision-induced dissociation (CID) gas at 3 and the ionspray voltage at 5100 V. All experiments were carried out in the positive ion mode. Full-scan, selected ion monitoring (SIM) or selected reaction monitoring (SRM) modes were used. The Q1 and Q3 quadrupoles were operated at unit mass resolution (0.7 Da at half-height). The dwell time for monitoring selected ions or transitions was set at 150 ms. Prior to CE–MS experiments the monitored ions, transitions and fragmentation energies were optimized by infusion of standard solutions in methanol–water (50:50), 1% acetic acid at 10 μl/min.

2.4. Sample preparation

Stock solutions of the standards were prepared in methanol at levels of 1.61 mg/ml C_0, 2.03 mg/ml C_2, 1.2 mg/ml C_3, 1.4 mg/ml C_4, 1.3 mg/ml C_5, 1.5 mg/ml C_6, 1.6 mg/ml C_8, 1.4 mg/ml C_{10}, 0.8 mg/ml C_{12}, 1.3 mg/ml C_{14}, 0.34 mg/ml C_{16} and 1.35 mg/ml C_{18}. Internal standard stock solutions were prepared in methanol at 1 mg/ml carnitine-d_3 and acetylcarnitine-d_3. The stock solutions were diluted with water–methanol (1:1) to obtain the spiking solutions.

2.4.1. Blood

Methanolic extracts of blood spots (approx. 50 μl) on filter paper were provided by Neo Gen Screening. The dried extracts (control sample and patient sample with elevated propionylcarnitine) were reconstituted in 50 μl methanol.

2.4.2. Urine

A 2-ml volume of human urine (obtained from healthy female and male volunteers) was evaporated to dryness under nitrogen at 60°C, reconstituted with 1 ml methanol and centrifuged 2 min in a Shelton Scientific VSMC-13 micro centrifuge (Shelton, CT, USA) at 3000 *g*. The supernatant was applied to the Silica Gel SPE column (preconditioned with 1 ml methanol) for further clean-up. A vacuum manifold Vac Elut (Analytichem, Harbor City, CA, USA) and pump (KNF Neuberger, Princeton, NJ, USA) were used for the SPE procedures. After rinsing the SPE column with 2 ml methanol, the analytes were eluted with 2 ml methanol–water–acetic acid (50:45:5). The eluate was evaporated to dryness under dry nitrogen at 50°C using a Reacti-Vap blow-down manifold and Reacti-Therm heater (Pierce, Rockford, IL, USA) and reconstituted in 200 μl of a solution containing methanol–water–formic acid (50:48.5:1.5).

Liquid–liquid extraction of human urine was performed by adding 500 μl ethyl acetate–acetonitrile (9:1) to 500 μl spiked or blank urine sample, followed by mixing (rotation on a Fisher roto-rack hematological mixer) for 5 min and centrifugation at 3000 *g*. The organic layer was removed and evaporated under dry nitrogen at 40°C. The dried residue was reconstituted in 200 μl methanol–formic acid (98.5:1.5).

2.4.3. Plasma

For protein precipitation 1.2 ml acetonitrile–methanol (3:1) was added to 300 μl spiked or blank human control plasma (Lampire Biological Labs., Pipersville, PA, USA), mixed manually, and centrifuged 5 min at 3000 *g*. The supernatant was applied to a silica gel SPE cartridge (preconditioned with 2 ml methanol). The cartridge was rinsed with 2 ml methanol before eluting the analytes with 2 ml methanol–water–acetic acid (50:45:5). The evaporated extract was reconstituted in 100 μl methanol–water (1:1) containing 1.5% formic acid.

Liquid–liquid extraction of human plasma was performed by adding 500 μl ethyl acetate to 500 μl spiked or blank sample, mixing 5 min (rotation on hematological mixer) and centrifuging 2 min at 3000 *g*. The organic layer was removed and evaporated under dry nitrogen at 40°C. The dried residue was

reconstituted in 100 μl of 3% formic acid in methanol.

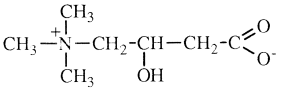
3. Results and discussion

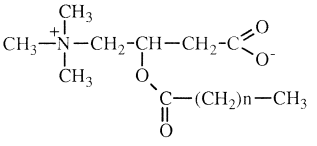
3.1. Method development

Carnitine and acylcarnitines are zwitterionic compounds because they contain both a tertiary ammonium group and a carboxyl group (see Table 1 for structures). Therefore, a low pH (2–3) is required to convert all analytes into positively charged compounds. Furthermore, a decrease of the dissociation of the silanol groups on the capillary wall under acidic conditions can prevent adsorption of the cationic analytes and also reduce (or prevent) the electroosmotic flow (EOF), leading to better peak resolution of homologues and isomers with similar migration behavior. MS-''friendly'' ammonium acetate–acetic- or formic acid electrolytes were used, because nonvolatile buffers and additives lead to salt buildup on the sprayer, clogging of narrow apertures and decreased electrospray sensitivity. Separations of aqueous standard mixtures of carnitine and acylcarnitines were possible in completely aqueous or nonaqueous as well as mixed organic–water electrolytes. The analytes reach the capillary end as a function of their charge/frictional drag ratios. With positive voltage polarity carnitine migrated as the fastest ion followed by the acylcarnitines in sequence of their increasing chain length.

With mass spectrometric detection complete electrophoretic peak resolution is not required because selected ion current profiles can be used for quantitation, although good resolution means also possibly good separation from chemical interferences. The full-scan mass spectral acquisition mode provides simultaneous detection of all compounds that exit the capillary end in the form of positive ions under the applied electrospray conditions. Selected ion monitoring (SIM) of the $[M+H]^+$ ions or selected reaction monitoring (SRM) for selected precursor–product ion transitions provide higher selectivity and sensitivity (see Table 1 for molecular masses and monitored transitions). Fig. 1 shows the product ion spectra for carnitine and selected acylcarnitines. The acylcarnitines form the same product ions at m/z 85 as the base peak in their CID mass spectra with a proposed structure $^+CH_2$-CH=CH-COOH (Fig. 1B–D). The second most abundant product ion peaks contain the alkyl chain [proposed structure: $^+CH_2$-CH(OCO-$(CH_2)_n$-CH_3)-CH_2COOH] and therefore, differ for the homologous acylcarnitines (compare

Table 1
Structures, molecular masses, peak numbers and MS–MS transitions of carnitine and acylcarnitines

Carnitine 

Acylcarnitines 

Compound	*n* (see structure)	Peak number[a]	Abbreviation	M_r (g/mol)	Transition (m/z)
Carnitine	–	1	C_0	161	162.2→103.1
Acetylcarnitine	0	2	C_2	203	204.2→85.1
Propionylcarnitine	1	3	C_3	217	218.2→85.1
Butyrylcarnitine	2	4	C_4	231	232.3→85.1
Valeroylcarnitine	3	5	C_5	245	246.3→85.1
Hexanoylcarnitine	4	6	C_6	259	260.3→85.1
Octanoylcarnitine	6	7	C_8	287	288.3→85.1
Decanoylcarnitine	8	8	C_{10}	315	316.3→85.1
Dodecanoylcarnitine	10	9	C_{12}	343	344.3→85.1
Tetradecanoylcarnitine	12	10	C_{14}	371	372.3→85.1
Palmitoylcarnitine	14	11	C_{16}	399	400.3→85.1
Stearoylcarnitine	16	12	C_{18}	427	428.3→85.1

[a] Refers to Figs. 2, 4, 6.

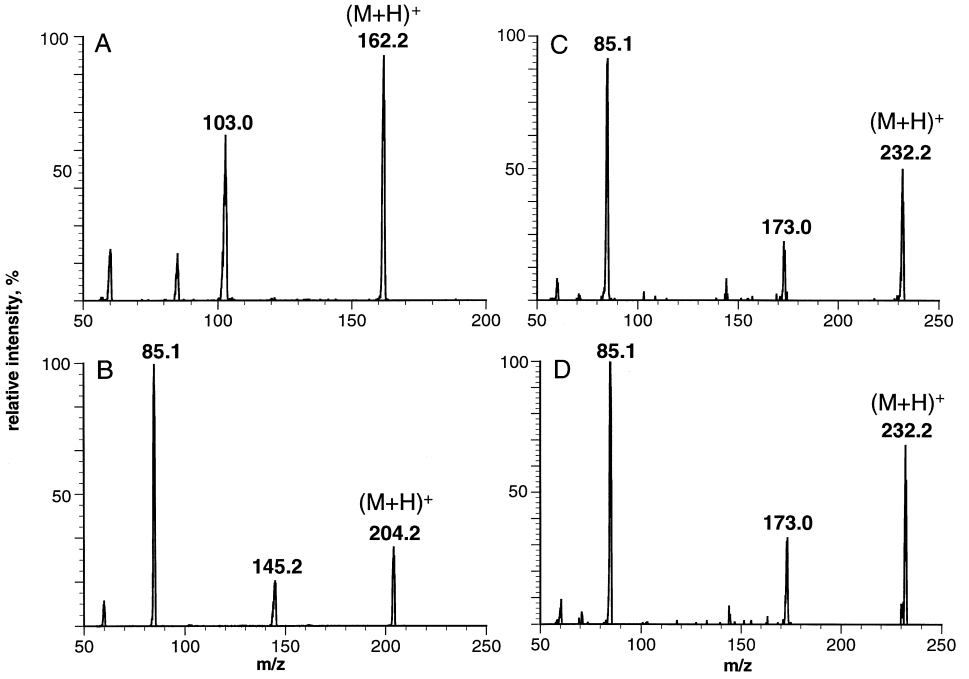


Fig. 1. Product ion spectra for carnitine and selected acylcarnitines. (A) Carnitine, (B) acetylcarnitine, (C) *n*-butyrylcarnitine, (D) isobutyrylcarnitine; obtained by infusion of 1 μg/ml standard solutions in methanol–water (1:1) (1% acetic acid) at 10 μl/min.

m/*z* 145.2 in Fig. 1B with *m*/*z* 173 in Fig. 1C). Isomeric forms show no differences in the product ion spectra (see Fig. 1C and 1D for the product ion spectra of *n*- and isobutyrylcarnitine). Differentiation between these isomeric compounds benefits from the analytical potential of the CE separation. Carnitine displays lower sensitivity in electrospray MS than the acylcarnitines presumably due to the surface tension effect during droplet formation that causes better ionization of more hydrophobic compounds [36].

Prior to sample analysis, the CE separation and ion spray mass spectrometric conditions were optimized to obtain component resolution while maintaining a reasonable separation time as well as adequate sensitivity. The SIM mode was used to monitor the $[M+H]^+$ ions to obtain the CE–MS results shown in Fig. 2 and Table 2. Using an aqueous 10 mmol/l NH_4OAc–3.2% acetic acid buffer and an analyte mixture of C_2 to C_6 acylcarnitines the influence of the ion spray conditions was investigated. Sheath liquid composition, flow-rate and nebulizing gas flow-rate were optimized.

In the next experiments systematic studies of varying electrolyte concentration, acid content and organic solvent content were performed. Representative results are shown in Table 2. Separations of a synthetic standard mixture of carnitine and 11 selected acylcarnitines in different electrolyte solvents are presented in Fig. 2. The electrolytes consisted of 10 mmol/l ammonium acetate containing acetic or formic acid in 100% water (Fig. 2A), water–acetonitrile (50:50) (Fig. 2B), water–methanol (50:50) (Fig. 2C) and methanol–acetonitrile (50:50) (Fig. 2D).

The 100% aqueous electrolytes are well suited for the determination of short to medium chain acylcarnitines and carnitine itself, but less useful for the longer chain homologues. Therefore, organic solvents were added to the electrolyte because they can improve separation of the homologues and peak shape for these analytes. In these experiments we found the addition of methanol or acetonitrile essential for the determination of the C_{16} and C_{18} homologues, but this did not result in better separation efficiency for the short-chain compounds. The organic solvents help to solubilize the more hydro-

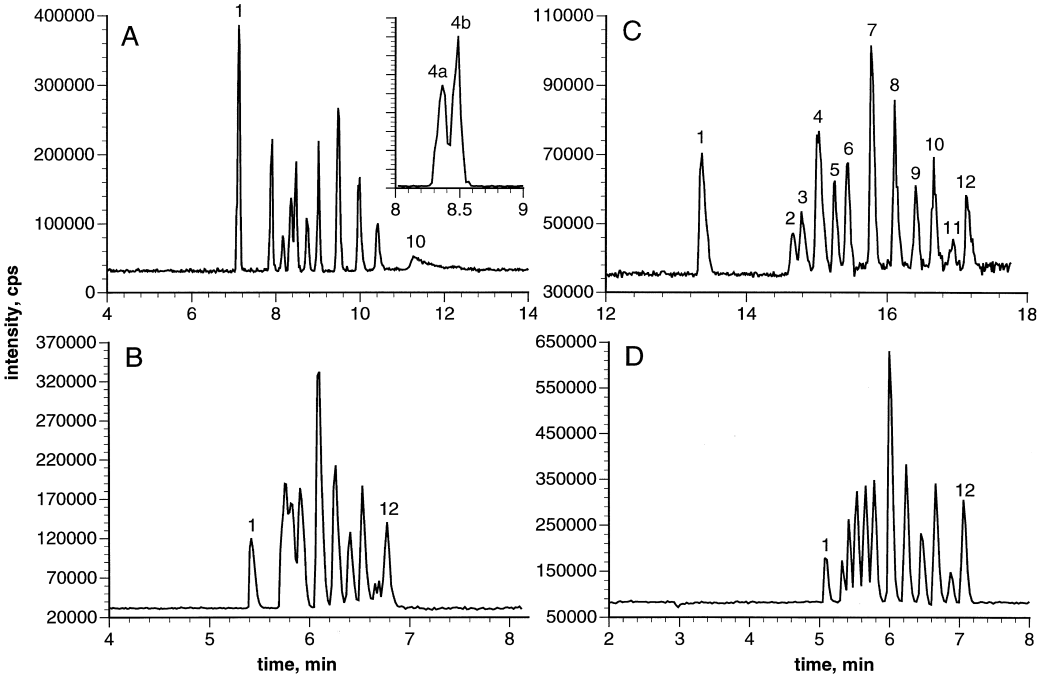


Fig. 2. Separation of synthetic carnitine/acylcarnitine standard mixtures in different electrolytes by SIM CE–MS. (A) Aqueous buffer containing 0.8% formic acid, (B) buffer containing 3.2% acetic acid and 50% acetonitrile, (C) buffer containing 3.2% acetic acid and 50% methanol, (D) nonaqueous buffer (6.4% formic acid in methanol–acetonitrile, 1:1); buffer: 10 mmol/l ammonium acetate; voltage: 30 kV; injection: 0.1 min 50 mbar standard mixture 1.5 μg/ml; for peak assignment see Table 1. (a) Isobutyrylcarnitine, (b) *n*-butyrylcarnitine.

phobic compounds, prevent the formation of micelles and also lead to a different selectivity due to changes in dissociation of both analytes and silanol groups of the capillary surface [37–40]. Fig. 2B shows the separation of carnitine and 11 acylcarnitines in ammonium acetate–acetic acid containing 50% acetonitrile, while the data shown in Fig. 2C used the same electrolyte containing 50% methanol. With methanol longer migration times and broader peaks, but better peak resolution were observed than using acetonitrile as organic additive due to the higher viscosity and lower dielectric constant of methanol. The addition of acetonitrile led to increased sensitivity, while in methanol–water electrolytes the lowest signal intensities were observed. The dissociation of carnitine and acylcarnitines is influenced by the electrolyte composition (water content, pH), and affects the migration behavior of these zwitterionic analytes. With the use of organic solvents the dissociation is reduced compared to aqueous conditions leading to different electrophoretic properties. The acid content in the buffer exerts a different influence in organic media which is also due to changes in dissociation. In general, more acid is required in highly organic electrolytes than in aqueous media to obtain sharp peaks with good resolution. The influence upon resolution and migration times of acetonitrile and methanol content in the electrolyte is shown in Table 2. When the methanol content was increased to 50% the peak resolution and migrations times were also increased. Between 50 and 75% methanol no significant additional changes in these parameters were observed, but at 100% methanol migration times and peak resolution were reduced. This is likely due to different migration behavior of the analytes under these nonaqueous conditions. The results obtained using acetonitrile containing buffers were similar to the results achieved with methanol, although the decrease in migration times and resolution occurred at 50 and 75%, respectively. An increase in acetic or formic acid concentration (investigated applying 50% methanol in the running

Table 2
Influence of the electrophoretic conditions on separation of acylcarnitines[a]

Electrolyte composition			Peak resolution	Migration time (min)
100% Water[b]	% Acetic acid	1.2	0.67	7.4
		2.4	0.98	7.8
		3.6	1.2	8
		4.8	1.42	8.8
	% Formic acid	1.2	1.88	7.1
Water–organic solvent with 3.6% acetic acid[c]	% Acetonitrile	25	1.04	8.25
		50	1.1	6.8
		75	0.71	6.85
		100	0.45	6.05
	% Methanol	25	1.29	11.6
		50	1.57	14.6
		75	1.54	15
		100	0.79	11.1
Water–50% methanol[b]				
	% Acetic acid	3.2	1.12	14.7
		6.4	1.68	15.7
		9.6	1.48	16.3
	% Formic acid	3.2	1.83	15
100% Methanol[c]				
	% Acetic acid	3.2	0.77	11.2
		6.4	0.98	10.4
		9.6	1.25	10.8
	% Formic acid	3.2	1.53	8.8
		6.4	2.15	11.6
		9.6	1.7	15
50% Methanol, 50% acetonitrile[c]	% Acetic acid	3.2	0.4	5.8
		6.4	1.2	6.2
	% Formic acid	6.4	1.5	6.1

[a] Electrolyte: 10 mmol/l ammonium acetate containing acids and organic solvents as described in the Table above; voltage: 30 kV; injection: 0.1 min 50 mbar standard mixture 1.5 μg/ml; sheath liquid: 2-propanol–water (1:1) with 0.3% acetic acid at 6 μl/min; nebulizer gas flow-rate: 1 l/min; SIM.
[b] Results derived from average peak resolution of C_0–C_4 analytes and migration time of C_2.
[c] Results from average peak resolution of C_5–C_{18} analytes and migration time of C_5.

buffer) led to longer migration times and better peak resolution (see Table 2). However, broader peaks and reduced resolution resulted with a content of 9.6% acetic acid. Formic acid as a stronger acid improved the peak resolution to a greater extent than an equal percent of acetic acid.

Nonaqueous electrolyte systems for CE [37,38,40] have the advantage of improved evaporation of the electrospray droplets, good solubility of hydrophobic compounds and often better selectivity compared to aqueous electrolytes, as has been previously described for CE–MS [41,42]. The disadvantages are the high volatility which can lead to unwanted evaporation, bubble formation in the capillary due to Joule heating, and subsequent loss of the electrophoretic current. In 100% methanol all but one of the standards in the reference mixture were separated and better sensitivity was obtained than in aqueous/organic buffers (data not shown). When the electrolyte consists of 100% organic solvent a higher acid content compared to aqueous media is necessary to achieve adequate peak resolution. Table 2 shows the influence of acetic and formic acid content on the separation of C_5 to C_{18} acylcarnitines in a 100% methanolic electrolyte. The increase in the acetic acid concentration led to better peak resolution and

shorter migration times for lower acid contents (to 6.4%), but resulted in longer migration times for higher acid concentrations. Formic acid was especially well suited for obtaining sharp peaks and good resolution at contents up to 6.4%. However, a higher formic acid content (9.6%) led to broader peaks, reduced resolution and longer migration times. The use of acetonitrile–methanol (50:50) reduced the peak resolution, but faster migration was obtained. Balancing the acetonitrile–methanol ratio, peak resolution and migration time adjustments are possible. Increased acetic or formic acid content in the methanol–acetonitrile electrolyte also improved the electrophoretic peak resolution (see Table 2). Fig. 2D shows the separation of carnitine and acylcarnitines in 10 mmol/l NH_4OAc–6.4% formic acid in methanol–acetonitrile (1:1). These electrophoretic conditions provided good peak resolution of all 12 analytes in a reasonable migration time.

In summary, selectivity adjustments were obtained by variation of type and content of organic solvent and organic acid in the separation electrolyte. The use of a pure aqueous buffer was advantageous for isomeric separation, while for the simultaneous determination of all homologues (but no isomeric separation) a nonaqueous electrolyte was well-suited, maintaining good signal intensity in both conditions.

3.2. Analysis of biological samples

The goal of our further experiments was to demonstrate that CE–MS techniques can be used to determine the targeted carnitines in human blood, urine and plasma. The composition of the sample matrix can significantly influence the separation efficiency and sensitivity of the method. Introducing a sample zone with high ionic strength may result in decreased sensitivity and reduced efficiency. The separation conditions optimized for synthetic standards often need to be modified to overcome these problems. Careful sample preparation with selective enrichment of the targeted analytes is advantageous. Electrokinetic injection can be used to selectively introduce the cationic analytes while discriminating against neutral and anionic sample components. Low ionic strength matrices support the application of electrokinetic injection (field amplified sample injection occurs). If the sample matrix has a higher ionic strength than the buffer, the migration velocity of the sample components is reduced leading to the injection of a relatively low quantity of analyte ions. Analytes with lower mobility are discriminated. Furthermore, calibration curves are often non-linear if internal standards are not available for each compound, especially with large conductivity differences between the standards with lowest and highest concentration. Therefore, hydrodynamic injection was preferred in this work for quantitation studies.

3.2.1. Blood spot extracts

As a first example for a real world application, blood spot extracts normally used in routine screening of genetic disorders by FIA electrospray tandem MS [19] were analyzed by SRM CE–MS. Both electrolyte and injection conditions were optimized for maximum sensitivity. The determinations were performed using a nonaqueous separation medium (methanol–acetonitrile, 1:1) because of the good sensitivity provided by these electrolyte conditions. Electrokinetic injection was applied to introduce the cationic components selectively, and to concentrate the analytes by field amplified sample injection. Fig. 3A–D shows a comparison of a control sample with a patient sample containing an elevated level of propionylcarnitine. The acetylcarnitine concentration in the patient sample (Fig. 3B) seems to be unchanged compared to a normal control blood (Fig. 3A), whereas the propionylcarnitine peak is clearly elevated in the patient (Fig. 3D) compared to the normal sample (Fig. 3C). Although only low analyte concentrations were present in the obtained blood spot samples, and the small sample volume did not allow preconcentration, reproducible results with a relative standard deviation (RSD) of 8.2% for propionylcarnitine peak area were achieved. However, CE–MS may not provide adequate sensitivity for samples containing lower analyte contents prepared for FIA analysis.

To explore the potential of CE–MS for the analysis of biological samples we further investigated the determination of carnitine and acylcarnitines in human urine and plasma using the benefits of analyte enrichment via sample preparation techniques.

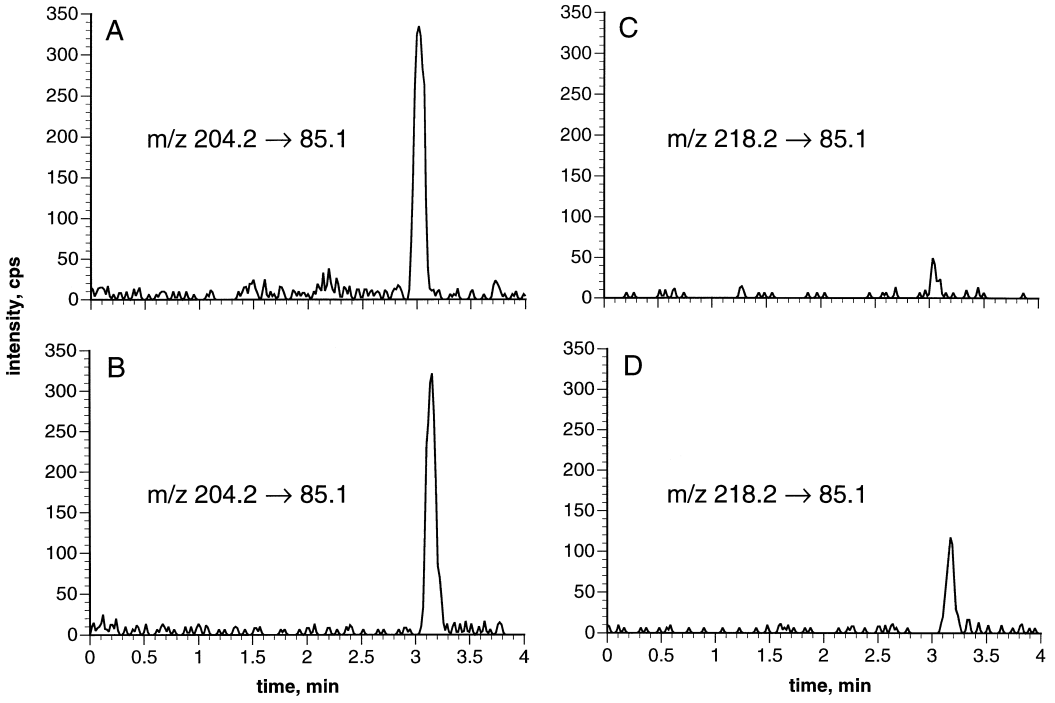


Fig. 3. Determination of acylcarnitines in human blood spot extracts using SRM CE–MS. (A) Selected ion current profile from acetylcarnitine control, (B) selected ion current profile from acetylcarnitine patient, (C) selected ion current profile from propionylcarnitine control, (D) selected ion current profile from propionylcarnitine patient; buffer: 10 mmol/l ammonium acetate, 6.4% formic acid in methanol–acetonitrile (1:1); voltage: 30 kV; injection: 2 min, 10 kV.

3.2.2. Urine and plasma

3.2.2.1. Method development. Endogenous carnitine and acetylcarnitine can be detected by CE–MS in normal human urine and plasma by direct injection of untreated sample because the concentrations of these compounds are relatively high (unpublished results). For the determination of other acylcarnitines enrichment and selective sample clean-up was necessary. Furthermore, crude biological samples contain a large variety of possible interfering compounds. Direct injection of large numbers of samples can have a negative influence on separation efficiency and detection sensitivity, although CE capillaries are relatively robust and can accommodate ''dirty'' samples better than LC columns. The analysis of plasma and urine for carnitine and acylcarnitines required a sample preparation strategy which would concentrate and isolate the polar carnitine as well as the hydrophobic long-chain acylcarnitines from the biological matrix. Due to the large differences in polarity of the analytes it is difficult to develop a technique that can enable comparable recoveries of each targeted compound. Simple protein precipitation of plasma is not well-suited if very low concentrations have to be determined because sample dilution occurs. Furthermore, there are still many matrix constituents in the sample which can exert a negative influence on the determination of the targeted compounds.

Further clean-up of plasma and urine was performed by solid-phase extraction on silica gel [6] leading to a selective enrichment of the desired analytes and removal of matrix constituents. Injecting the biological sample extracts into nonaqueous or partly organic CE electrolytes caused a negative effect on the separation efficiency. The peak resolution and sensitivity deteriorated considerably, and

the peaks became very broad and noisy, making quantitation very difficult. A modification of the separation conditions (e.g., increased ammonium acetate concentration) and sample preparation steps (e.g., filtration and centrifugation) could not improve the results significantly. In contrast, aqueous buffers which contained no organic solvents could accommodate biological matrices derived from different sample preparation steps while maintaining good separation efficiency and sensitivity. Therefore, our method for determining the short- and medium-chain compounds (to C_{14}) applied isolation by SPE on silica gel cartridges and separation in an aqueous 10 mmol/l ammonium acetate–0.8% formic acid electrolyte. Under these conditions the C_{16} and C_{18} acylcarnitine homologues could not be detected. Fortunately for our study, the short and medium chain analytes are of primary importance to characterize metabolic disorders (e.g., propionyl-, isovaleryl- or octanoylcarnitine [5–10]).

However, long-chain acylcarnitines are also present in normal urine and plasma at low concentrations and can be used as an additional source of information for detection of abnormal metabolism [11]. Liquid–liquid extraction of human urine was investigated for these compounds using different solvents. It was found that ethyl acetate containing 10% acetonitrile showed the best extraction efficiency and sample clean-up for urine. No negative influences on the analyte peak shape were obtained injecting even large sample volumes into a nonaqueous electrolyte after ethyl acetate extraction, leading to the conclusion that these samples are relatively clean. For the hydrophobic long-chain compounds (C_{12} to C_{18}) recoveries up to 95% could be achieved, while the more polar analytes ($<C_{10}$) remained to a high extent in the aqueous phase and gave poor recoveries. The use of the less polar chloroform solvent resulted in reduced recoveries of all analytes. Butanol extracts gave the same results as the SPE procedure: noisy peaks in organic buffers. This may suggest that some polar matrix components cause this effect. Ethyl acetate was also well-suited to extract long-chain acylcarnitines from plasma without a prior protein precipitation step. The final method for determining the longer chain analytes ($>C_8$) included liquid–liquid extraction (ethyl acetate–acetonitrile for urine or only ethyl acetate for plasma) and separation via CE in a nonaqueous electrolyte (10 mmol/l ammonium acetate–6.4% formic acid in methanol–acetonitrile, 1:1).

3.2.2.2. Analysis of urine. Normal human urine extract prepared by SPE and analyzed by SRM CE–MS using the aqueous buffer (Fig. 4A) showed high concentrations of carnitine and acetylcarnitine as well as lower concentrations of several other acylcarnitines with hydrocarbon chain lengths up to C_{10}. The endogenous acylcarnitines marked with an asterisk are present as other isomers than the *n*-form (with the exception of acetyl- and propionylcarnitine, which do not possess different isomers), because additional peaks were obtained in the electropherogram of a spiked sample shown in Fig. 4B. When isobutyrylcarnitine was co-injected with the unspiked urine, the peak area of the endogenous peak increased, and no additional peak was observed. To confirm the peak identity, product ion scans of a spiked urine sample over the range of m/z 50 to 250 were performed for the precursor ions at m/z 232.2 (butyrylcarnitine) and m/z 246.2 (valerylcarnitine). The compounds present in the control urine samples are likely to be closely related structural isomers of the *n*-acylcarnitines, because they form not only the same base peak product ion ($m/z=85.1$, monitored in SRM) but also the same second most abundant ion ($m/z=173$ for butyryl-, 186.8 for valerylcarnitine). A separation method is necessary to distinguish between these analytes when applying ESI-MS.

Small quantities of endogenous long-chain acylcarnitines extracted from urine using liquid–liquid extraction could be detected only by injecting 2 min at 10 kV for on-line analyte enrichment and applying 10 mbar pressure during the separation in the nonaqueous electrolyte (results not shown). A breakdown of the electrophoretic current occurred randomly. The peak areas were not reproducible under these conditions (noisy peaks and concentrations near LOD). Using hydrodynamic injection conditions no compounds were detected in the blank sample (Fig. 4C). The contents of long-chain compounds are normally low and could also not be quantitated by other authors [8,12]. An electropherogram of spiked urine extract showed that the long-chain acylcarnitines $>C_8$ were recovered from the biological sample (Fig. 4D).

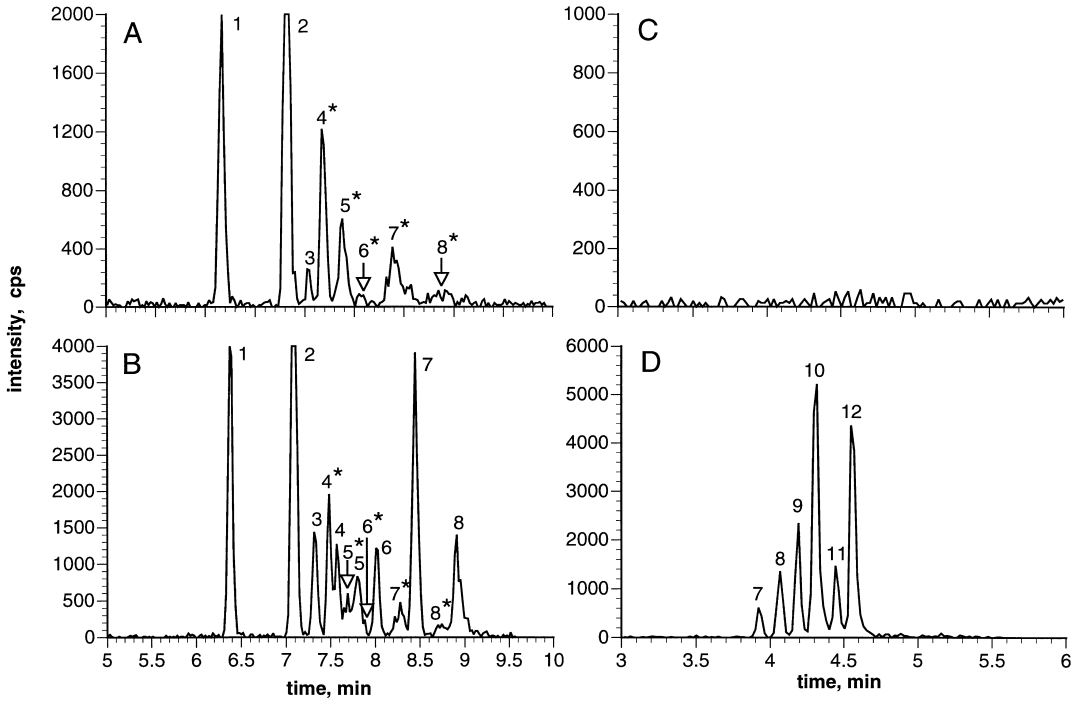


Fig. 4. Determination of carnitine and acylcarnitines in human urine extracts by SRM CE–MS. (A) Endogenous carnitine and short-chain acylcarnitines, (B) carnitine and C_2–C_{10} acylcarnitine spike at 100 ng/ml–1 μg/ml, (C) blank (endogenous long-chain compounds expected), (D) C_8–C_{18} acylcarnitine spike at 400 ng/ml. Conditions: (A) and (B) buffer: 10 mmol/l ammonium acetate, 0.8% formic acid in water; sample preparation: SPE; (C) and (D) buffer: 10 mmol/l ammonium acetate, 6.4% formic acid in methanol–acetonitrile (1:1); sample preparation: liquid–liquid extraction; voltage: 30 kV; injection: 0.1 min 50 mbar; acylcarnitines different from *n*-isomers are marked with *; see Table 1 for monitored transitions and peak assignments.

To quantitatively measure carnitine and acylcarnitines, calibration was carried out using six concentrations spiked into human urine in the range of 0.04 to 20 μg/ml, depending on the compound. Table 3 summarizes the quantitation results. Deuterated (d_3) acetylcarnitine (1 μg/ml) was added as internal standard for the determination of all analytes. For the long-chain compounds acetylcarnitine-d_3 was added after the extraction to correct for CE–MS variation, exclusive of sample preparation variation. The reproducibility of six replicate injections of normal urine extract for carnitine to C_{12} acylcarnitine (SPE, aqueous electrolyte) and of a medium concentration for the C_8 to C_{18} analytes (determined by liquid–liquid extraction–nonaqueous separation) was determined to be between 5 and 13%. The limits of detection at signal/noise=3 were calculated using $LOD=3h/s$ (h=average height of noise peak, s=slope of calibration function determined with peak heights). The LOD values are between 28–50 ng/ml (acylcarnitines) and 200 ng/ml (carnitine), which correspond to 0.1 to 1 nmol/ml and are comparable to those values reported for LC–MS [27]. Carnitine and acetylcarnitine are usually present in high quantities in human urine, so it is not essential to achieve a low LOD for these compounds. The limits of quantitation were determined as the concentration equal to a signal-to-noise ratio of 10, with the criterion of precision <20% for six replicate analyses. For carnitine and acylcarnitine the LOQs were determined using the deuterated compounds carnitine-d_3 and acetylcarnitine-d_6 as model analytes, resulting in values of 400 and 40 ng/ml, respectively. The other targeted compounds showed LOQ values between 40 and 80 ng/ml.

The applicability of full-scan CE–MS was tested for the detection of carnitine and C_2 to C_{10} acylcarnitines in urine. Separation was carried out in the

Table 3
Quantitation results for carnitine and acylcarnitines in human urine using SRM CE–MS

Compound	Concentration range (μg/ml)	R^2	Recovery (%)	RSD (%)		LOD (ng/ml)
				Area	Time	
Carnitine	1.8–30	0.989	87.5	7.0	0.08	200
Acetylcarnitine	0.60–12	0.992	96.5	6.6	0.18	30
Propionylcarnitine	0.144–4.8	0.990	85.0	6.6	0.18	27.6
Butyrylcarnitine	0.168–5.6	0.989	85.2	9.7	0.22	28.7
Valeroylcarnitine	0.156–5.2	0.989	70.4	10.2	0.14	35.1
Hexanoylcarnitine	0.18–6	0.988	78.6	10.1	0.42	44.3
Octanoylcarnitine	0.192–6.4	0.991/0.990[a]	82.9/3[a]	9.4/19.7[a]	0.15/0.8[a]	34.4/640[a]
Decanoylcarnitine	0.168–5.6	0.996/0.987[a]	83.1/14[a]	6.5/9.3[a]	0.42/0.7[a]	28/196[a]
Dodecanoylcarnitine	0.096–3.2	0.996/0.990[a]	81.2/49.0[a]	5.3/5.1[a]	0.66/0.8[a]	49.2/59[a]
Tetradecanoylcarnitine	0.156–5.2	0.999/0.998[a]	80.5/85.5[a]	11.1/7.6[a]	0.68/0.9[a]	49.9/36[a]
Palmitoylcarnitine	0.041–1.36	0.997[a]	93.8[a]	7.1[a]	1.2[a]	29[a]
Stearoylcarnitine	0.162–5.4	0.992[a]	95.3[a]	12.8[a]	1.3[a]	38[a]

[a] Sample preparation by liquid–liquid extraction, all others by SPE.

aqueous electrolyte after SPE of the control urine sample. Fig. 5 (top portion) shows the total ion electropherogram obtained by scanning from m/z 150 to 320. Although the limits of detection are relatively high in the full-scan mode, several electrophoretic peaks appear in the migration time window expected for carnitine to C_{10} acylcarnitine. To identify the targeted compounds among these peaks, extracted ion current profiles were obtained, allowing for the detection of carnitine and acylcarnitine peaks in the selected ion traces. This urine sample possessed a comparatively high level of these target analytes. In Fig. 5A and B representative extracted ion profiles at m/z 232.2 and 246.2 (butyryl- and valerylcarnitine, respectively) are shown. The corresponding background-subtracted CE–MS mass spectra at 7.86 and 8.1 min, are presented in Fig. 5C and D, respectively. The extracted ion electropherogram m/z 246.2 for valerylcarnitine (Fig. 5B) also shows another peak 2 resulting from an unidentified sample component. In the absence of MS–MS, a separation technique such as LC or CE is essential to avoid interference by those isobaric matrix constituents with the target compounds. Full-scan CE–MS experiments could be applied to higher analyte concentrations (the LODs were determined to be in the lower nmol/ml range). An advantage of the full-scan mode is the possibility to detect and identify unknown sample constituents; from the on-line full-scan mass spectra precursor ions can be selected for a subsequent product ion scan. SIM CE–MS may be an alternative to the SRM mode if one is limited to a single quadrupole mass spectrometer. The achieved sensitivity is comparable to SRM, while the electrophoretic separation provides the necessary selectivity (data not shown).

The SRM CE–MS method for short- to medium-chain compounds was used to compare the urinary acylcarnitine composition of different individuals. Carnitine concentrations were determined to be between 20.7 and 104.2 nmol/ml. Table 4 shows the C_2 to C_{10} acylcarnitine content in human urine normalized to 100% as well as the concentration range for these analytes. The concentrations differ significantly between the samples due to normal inter-individual variability. For isobutyrylcarnitine, for example, values between 0.45 and 17.15 nmol/ml were obtained. Therefore, the carnitine and acylcarnitine concentrations by themselves are not representative for characterization of metabolic disorders although during diseases the levels may be significantly elevated (e.g., at propionic aciduria up to 90 nmol/ml propionylcarnitine has been detected [8]). Nevertheless, the ratios of the various acylcarnitines compared to physiological compounds, such as acetylcarnitine, can give valuable information about possible diseases. The advantage of urine as matrix for acylcarnitines determination is that the samples can be obtained noninvasively and are available in larger volumes than blood.

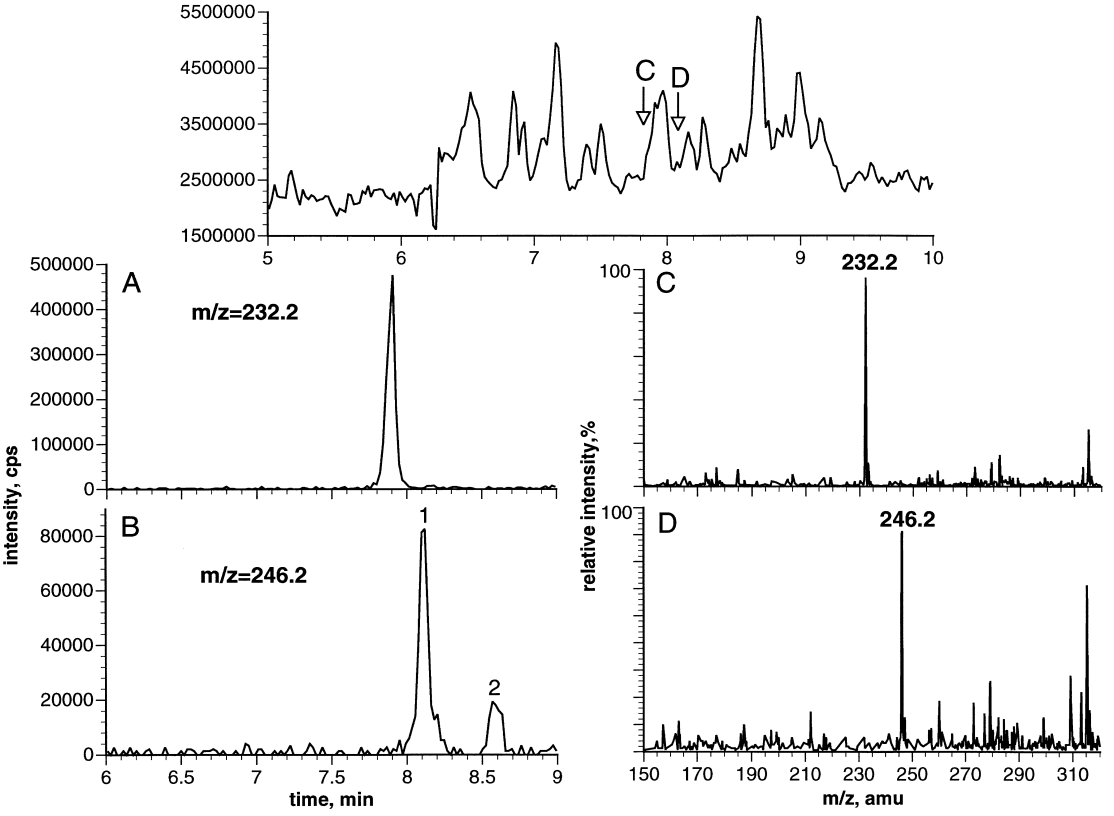


Fig. 5. Determination of carnitine and acylcarnitines in human urine extract using full-scan CE–MS mode. Total ion chromatogram (top figure), (A) extracted ion current profile for butyrylcarnitine, (B) extracted ion current profile for valerylcarnitine, (C) on-line mass spectrum at 7.86 min (background subtracted), (D) on-line mass spectrum at 8.1 min (background subtracted); buffer: 10 mmol/l ammonium acetate, 0.8% formic acid in water; voltage: 30 kV; injection: 0.2 min 50 mbar; sample preparation: SPE; scan range: m/z 150–320.

3.2.2.3. Analysis of plasma. The analysis of human plasma is more suited to determine metabolism-related diseases because in normal plasma only very low levels of other acylcarnitines besides acetylcarnitine are present. The SRM CE–MS determination of the short- to medium-chain analytes in plasma

Table 4
Comparison of urinary acylcarnitine levels of different healthy people (A–E) determined by SRM CE–MS

Compound	Acylcarnitine content (%) normalized to 100					Concentration range[a] (nmol/ml)
	A	B	C	D	E	
Acetylcarnitine	36.7	63.3	32.7	44.1	45.6	2.74–28.02
Propionylcarnitine	<LOD	11.9	9.4	10.5	5.0	0.64–1.88
Butyrylcarnitine	48.3	8.5	33.7	26.0	22.4	0.45–17.15
Valeroylcarnitine	13.1	11.0	10.6	12.2	9.2	0.13–5.01
Hexanoylcarnitine	<LOD	3.6	8.9	3.2	7.9	0.20–1.88
Octanoylcarnitine	1.8	1.7	3.0	2.3	5.9	0.02–0.61
Decanoylcarnitine	<LOD	<LOD	1.7	1.7	4.0	0.10–0.27

[a] Lowest to highest concentration found in the different individuals, compare literature values [6–9].

samples after SPE was performed using the aqueous electrolyte. The unspiked sample (Fig. 6A) showed carnitine and acetylcarnitine peaks as well as low concentrations of C_3, C_4, C_5, C_8 and C_{10} acylcarnitines. The comparison with a spiked plasma containing carnitine and C_2 to C_{12} acylcarnitines is shown in Fig. 6B. The nonaqueous methanol–acetonitrile electrolyte was used for determining the long-chain acylcarnitines. Endogenous compounds (C_{14} to C_{18}) were detected in normal plasma after liquid–liquid extraction at levels near the LOD (see Fig. 6C). The electropherogram of a sample extract obtained from plasma spiked with C_6 to C_{18} acylcarnitines is presented in Fig. 6D.

For quantitation of the analytes calibration using six standard concentrations between 0.02 and 19 μg/ml was carried out, adding 4 μg/ml carnitine-d_3 as the internal standard for carnitine determination and 1.6 μg/ml acetylcarnitine-d_3 as the internal standard for all other compounds. Table 5 summarizes the results. Reproducibilities of six replicate determinations were determined to be 4.5 to 15%. The limits of detection at a signal-to-noise ratio of 3 are relatively low for the short-chain compounds (7 to 30 ng/ml or 0.03 to 0.13 nmol/ml, respectively). The LODs obtained for long-chain homologues are higher than 60 ng/ml (corresponds to 0.16–0.3 nmol/ml) due to the extraction efficiency of about 50%. Carnitine and acetylcarnitine are present at high levels in human control plasma meaning that it is difficult to obtain control plasma free of these analytes. Therefore, the determination of the LOQs for these compounds were not determined in this work. Therefore, carnitine-d_3 and acetylcarnitine-d_6 served as model compounds for this purpose, resulting in LOQ values of 160 ng/ml and 40 ng/ml, respectively. Because other acylcarnitines were also detected in normal plasma, the limits of quantitation for these compounds were set equal to the concentrations in the ''blank'' sample, with precision of

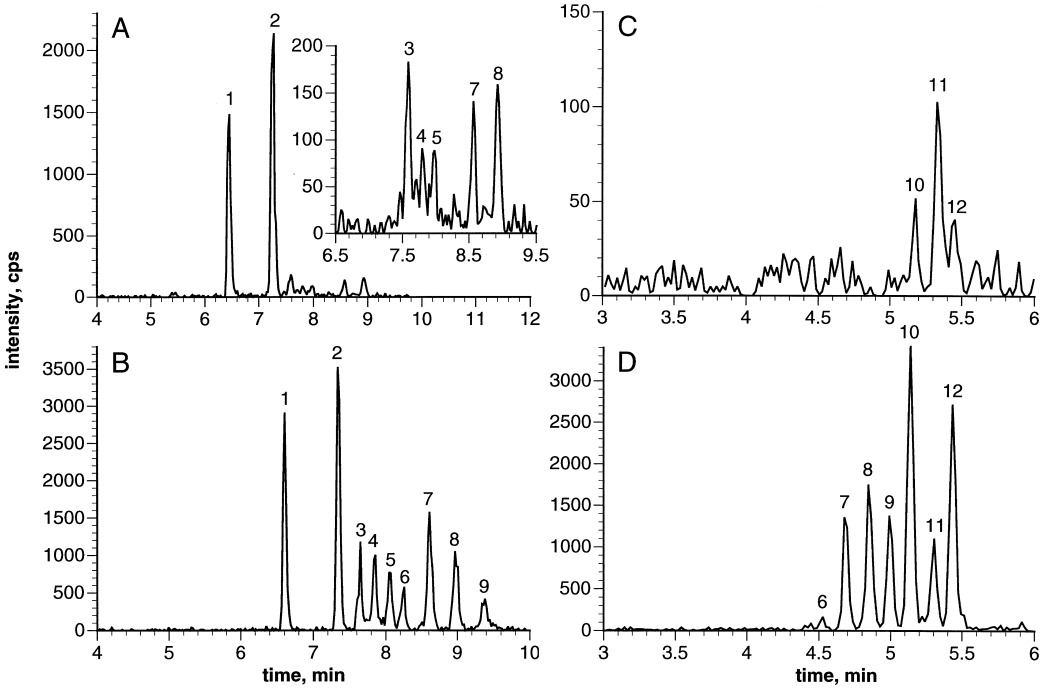


Fig. 6. Determination of carnitine and acylcarnitines in human plasma extracts by SRM CE–MS. (A) Endogenous carnitine and short-chain acylcarnitines, (B) C_3–C_{12} acylcarnitines spike at 160–300 ng/ml, (C) endogenous long-chain acylcarnitines, (D) C_8–C_{18} acylcarnitines spike at 400 ng/ml. Conditions: (A) and (B) buffer: 10 mmol/l ammonium acetate, 0.8% formic acid in water; sample preparation: SPE; (C) and (D) buffer: 10 mmol/l ammonium acetate, 6.4% formic acid in methanol–acetonitrile (1:1); sample preparation: liquid–liquid extraction; voltage: 30 kV; injection: 0.2 min 50 mbar; see Table 1 for monitored transitions and peak assignments.

Table 5
Quantitation results for carnitine and acylcarnitines in human plasma using SRM CE–MS

Compound	Concentration range (μg/ml)	R^2	Recovery (%)	RSD (%)		LOD (ng/ml)
				Peak Area	Time	
Carnitine	0.167–16.7	0.999	75.0	7.4	0.86	222
Acetylcarnitine	0.04–4.0	0.990	76.3	4.8	0.2	26.7
Propionylcarnitine	0.024–4.8	0.991	105.9	10.1	0.18	29.9
Butyrylcarnitine	0.028–5.6	0.995	97.6	4.5	0.16	14.9
Valeroylcarnitine	0.026–5.2	0.995	97.7	10.8	0.18	15.2
Hexanoylcarnitine	0.03–6	0.998	91.4	11.4	0.23	25
Octanoylcarnitine	0.032–6.4/0.32–12.8[a]	0.9957/0.988[a]	91.0/6.6[a]	11.3/8.5[a]	0.31/0.8[a]	6.9/330[a]
Decanoylcarnitine	0.028–5.6/0.28–11.2[a]	0.998/0.995[a]	102.2/16.6[a]	7.9/9.2[a]	0.44/0.9[a]	10.3/114[a]
Dodecanoylcarnitine	0.016–3.2/0.16–6.4[a]	0.999/0.990[a]	81.5/43[a]	7.3/10.4[a]	1.13/1.1[a]	19.3/50[a]
Tetradecanoylcarnitine	0.260–10.4[a]	0.989[a]	44.6[a]	11.2[a]	1.4[a]	33.8[a]
Palmitoylcarnitine	0.068–2.72[a]	0.993[a]	65.8[a]	8.5[a]	1.1[a]	31.5[a]
Stearoylcarnitine	0.27–10.8[a]	0.990[a]	47.2[a]	14.1[a]	1.7[a]	45.9[a]

[a] Sample preparation by liquid–liquid extraction, all others by SPE.

4.5 to 20%. The endogenous carnitine and acylcarnitine concentrations in plasma from different donors showed only minor variances. Average concentrations of 24.0 nmol/ml carnitine, 3.72 nmol/ml acetylcarnitine, 0.29 nmol/ml C_3, 0.19 nmol/ml C_4, 0.15 nmol/ml C_5, 0.02 nmol/ml C_8 and 0.12 nmol/ml C_{10} were determined in control plasma.

Using SIM CE–MS the above mentioned acylcarnitines were also detected, whereas in the full-scan mode only carnitine and acetylcarnitine could be determined in control plasma (data not shown). The analysis of spiked plasma allowed for the determination of the detection limits between 0.05 and 0.85 nmol/ml in the SIM mode and 1.7 to 5.8 nmol/ml in full-scan mode for C_3 to C_{10} acylcarnitines. The higher baseline noise obtained with SIM compared to SRM led to the slightly decreased sensitivity in this mode for the analysis of plasma extracts. The SIM mode could be used for CE–MS analysis if tandem MS is not available. CE–MS in the full-scan mode may be applicable to measure elevated acylcarnitine levels, and could be used for the identification of unknown peaks. The SRM mode is preferred for better selectivity and sensitivity.

To demonstrate the robustness of the developed SRM CE–MS technique for determining short- and medium-chain acylcarnitines in plasma, an abbreviated method validation was carried out. Propionyl-, butyryl-, valeryl-, octanoyl- and decanoylcarnitine were selected as important representatives. As internal standards propionylcarnitine-d_3 (for C_3), butyrylcarnitine-d_3 (for C_4 and C_5) and octanoylcarnitine-d_3 (for C_8 and C_{10}) were added. Calibration using seven concentrations over a range of 200 (0.055 nmol/ml to 24.2 nmol/ml, depending on the compound) was performed in duplicate over two separate days. Table 6 summarizes the results. Quality control samples at low, medium and high levels (six replicates each) showed acceptable intra- and inter-day precision and accuracy. The results show that CE–MS can meet the acceptance criteria for bioanalytical determinations.

4. Conclusions

This report describes the development and application of capillary electrophoretic methods with electrospray mass spectrometric detection for the determination of carnitine and acylcarnitine homologues in human blood, urine and plasma. The selected reaction monitoring mode provides high selectivity and sensitivity, although SIM and full-scan analyses are also possible for the detection of the targeted analytes in biological samples. Performing a CE–MS analysis provides the possibility to separate isomers that are not distinguishable by MS detection without prior separation. Faster run times than in LC could be achieved. We have shown the feasibility for determining carnitine and acylcar-

Table 6
Precision and accuracy for selected acylcarnitines in human plasma

		C_3	C_4	C_5	C_8	C_{10}
Concentration range (μg/ml)		0.024–4.8	0.028–5.6	0.026–5.2	0.016–3.2	0.028–5.6
R^2 day 1		0.996	0.997	0.997	0.999	0.990
R^2 day 2		0.994	0.996	0.995	0.998	0.995
Concentration (μg/ml)						
	Low QC	0.072	0.084	0.078	0.048	0.084
	Medium QC	1.68	1.96	1.82	1.12	1.96
	High QC	3.6	4.2	3.9	2.4	4.2
Intra-day assay						
Low QC	Accuracy (%)	99.4	92.0	101.8	100.4	90.3
	Precision (%)	8.5	4.9	8.2	7.4	3.4
Medium QC	Accuracy (%)	105.4	110.2	106.9	100.2	96.1
	Precision (%)	13.9	3.7	8.7	6.8	7.3
High QC	Accuracy (%)	99.3	103.5	104.8	99.2	85.2
	Precision (%)	4.2	5.2	9.2	4.8	8.4
Inter-day assay						
Low QC	Accuracy (%)	92.3	92.0	91.5	97.1	89.5
	Precision (%)	6.5	0.5	11.8	3.66	8.3
Medium QC	Accuracy (%)	111.3	106.4	107.0	101.5	95.2
	Precision (%)	4.0	8.2	6.0	4.5	2.3
High QC	Accuracy (%)	105.5	99.9	105.3	100.6	94.2
	Precision (%)	0.8	0.9	5.2	3.2	2.9

nitines in human urine and plasma samples with acceptable selectivity, sensitivity, precision and accuracy. The simultaneous determination of all analytes with one sample preparation and separation method was not practical in this work. Nonaqueous CE electrolytes (methanol–acetonitrile as solvent mixture) provided good homologous peak resolution of carnitine and all acylcarnitines inclusive of C_2 to C_{18} in a relatively short separation time, but could only accommodate relatively ''clean'' biological samples prepared by liquid–liquid extraction. Therefore, this approach can be applied if the determination of long-chain homologues is required. A 100% aqueous electrolyte was well-suited for each sample matrix studied and showed good isomeric acylcarnitine separation efficiency. Although the C_{16} and C_{18} homologues could not be detected, this method is applicable to characterize important metabolic disorders, such as propionyl and isovaleryl acidemia and MCAD. Therefore, CE–MS might be used as an alternative for acylcarnitine determinations in clinical samples. With the application of automated sample preparation an increased throughput may be possible. Miniaturization (CE on chip) could be a further step to higher speed and sensitivity.

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