ORIGINAL PAPER

Optimization of a rapid capillary electrophoresis ESI-IT tandem mass spectrometry method for the analysis of short-chain carnitines in human plasma

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Abstract A capillary electrophoresis (CE) method coupled to electrospray ionization ion trap tandem mass spectrometry (ESI-IT-MS/MS) is described for the rapid analysis of carnitine, acetylcarnitine, and propionylcarnitine in human plasma. Optimization of the procedure was achieved by a reduced sample pretreatment and after examining several physicochemical parameters that influence both the CE separation and the MS analytes detection. The analysis of total carnitine in human plasma after hydrolysis of short-chain metabolites is also shown. The analysis of carnitine and metabolites was obtained in less than 10 min using a 200 mM ammonium formate buffer, pH 2.5, with high sensitivity and specificity using the MS detection in product ion scan mode. The method was tested for quantitative recovery using dialyzed human plasma as matrix and showed linearity in the concentrations ranges 20–160, 1–32, and 0.25–8 μM for carnitine, acetylcarnitine, and propionylcarnitine with (squared) correlation coefficients of 0.9984, 0.9995, and 0.9991, respectively. The intraday and intermediate analysis repeatability and accuracy are within 15% of relative standard deviation (RSD) at low, medium, and high concentration and within/or slight exceeding 20% at the lower limit of quantitation (LLOQ). The method is sensitive for determining carnitine and its metabolites in human plasma with high specificity.

Keywords Capillary electrophoresis · Mass spectrometry · Carnitines · Biological fluids · Sample preparation

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Introduction

Carnitine has a pivotal role in energy metabolism through its action on the oxidation of long-chain fatty acids and their transport into the mitochondria. Only a small quantity of the total body carnitine content in humans is endogenously biosynthesized, and the majority of it comes from the diet. Carnitine shuttles acyl groups to its 3-hydroxyl

group to generate short-chain (namely, acetylcarnitine, propionylcarnitine, butyrylcarnitine, isobutyrylcarnitine, valerylcarnitine, and isovalerylcarnitine), medium-chain (octanoylcarnitine), and long-chain (palmitoylcarnitine) acylcarnitines [1]. Figure 1 shows the chemical structures of the short-chain carnitine metabolites. In plasma and urine both carnitine and its esterified forms (acylcarnitines) are present only in their L-enantiomeric form. The plasma levels of endogenous carnitines are greatly affected by the presence of genetic or metabolic disorders [2, 3].

The availability of selective and sensitive analytical methods for rapid profiling of carnitines in body fluids is therefore relevant for diagnostic purposes and therapeutic monitoring. The increasing interest in carnitine and its acetylated and propionylated metabolites for various therapeutic goals stimulated the development of analytical methods for their rapid and reliable determination in human plasma. These include high-performance liquid chromatography (HPLC) with different derivatization procedures, gas chromatography (GC) approaches, MS/MS, and enzymatic and radioenzymatic techniques that have been recently reviewed by Marzo and Curti [1]. In recent years several HPLC methods coupled to MS have been applied to the analysis of carnitines in plasma [4–7] or urine [8]. ESI-MS/MS flow injection approaches have been recently introduced for the determination of carnitines both for screening of metabolic disorders and inborn errors in newborns [9–11] and drug therapy monitoring [12], and some of these methods have been developed in combination with solid-phase microextraction [13]. A biochemical perspective on the use of tandem mass spectrometry for metabolic screening of inherited diseases and clinical testing has been r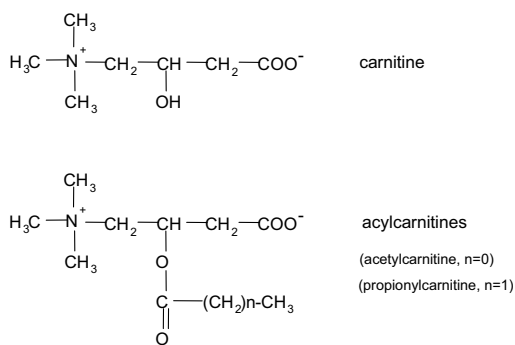ecently reviewed [14]. In our previous paper an ESI-IT-MS/MS flow injection method was optimized and validated for the analysis of short-chain carnitines in human plasma using a very reduced sample pretreatment procedure [15].

CE has been successfully applied to the analysis of carnitines in urine after derivatization [16] and in biological fluids with ESI-MS/MS detection using a triple quadruple instrument [17]. In these methods the biological sample was treated with liquid/liquid or solid-phase extraction procedures before injection. The use of CE coupling with mass spectrometry for the screening of metabolic disorders in newborns, including carnitines analysis, has been recently reviewed [18]. Chip-based CE/MS was also used for quantitation of carnitines in human urine and plasma [19, 20].

Our aim was to investigate the use of CE coupled to ESI-IT-MS detection for developing a fast and sensitive analytical method for quantitating carnitine and its short-chain metabolites in human plasma.

With respect to conventional chromatographic techniques, CE exhibits some important features, particularly advantageous in the field of biomedical analysis. Its high separation efficiency and resolution power provide analysis free from matrix interferences, advantageously allowing one to minimize the sample pretreatment procedure and to strongly reduce the total analysis time. Owing to the injection of only nanoliter sample quantities, CE is particularly suitable for the analysis of biological fluids available in minute volumes (e.g., newborn serum, lachrymal fluids, saliva), allowing several injections per few microliters of samples.

The coupling of CE with ESI-IT-MS strongly increases method sensitivity and specificity owing to the possibility of isolating and fragmenting specific ions and selectively extracting them from possible comigrating analytes and matrix interferences.

Experimental

Chemicals

Methanol, acetonitrile, water, and formic acid (Backer analyzed, 98%) were purchased from Mallinckrodt Baker B.V. (Deventer, Holland). All the organic solvents were for HPLC use and gradient grade. Ammonium hydroxide solution (30%) RPE was from Farmitalia-Carlo Erba (Milan, Italy). Sodium hydroxide pellets, pro analysis, were from Merck (Darmstadt, Germany). L-Carnitine (C), L-acetylcarnitine (AC), and L-propionylcarnitine (PC) reference standards were provided by Sigma-Tau Industrie Farmaceutiche Riunite S.r.L. (Rome, Italy). Deuterated L-carnitine (Cd$_3$) and L-acetylcarnitine (ACd$_3$) were purchased from CDN Isotopes (Quebec, Canada).

compound	molecular mass	ESI MS/MS Transitions (m/z)
Carnitine	161	162 -> 103.1 + 60.5
Carnitine-d$_3$	164	165 -> 103.1 + 63.5
Acetylcarnitine	203	204 -> 85.2 + 145.0
Acetylcarnitine-d$_3$	206	207 -> 85.2 + 148.0
Propionylcarnitine	217	218 -> 85.2 + 159.0

Fig. 1 Molecular structures and MS/MS transition patterns of the carnitine compounds studied

The stock (1 mg mL^{-1}) and diluted standard solutions were prepared in water/methanol (1:1) and stored at −20 °C. Starting from the stock standard solution, different C, AC, and PC working standard solutions were prepared in order to spike the dialyzed plasma sample with a 10% volume aliquot before analysis pretreatment. The addition of the internal standard (IS) mixture (i.e., Cd$_3$ and ACd$_3$) was also performed using a 10% sample volume aliquot.

Apparatus

Capillary zone electrophoresis experiments were performed on an Agilent Technologies capillary electrophoresis automated apparatus (Waldbronn, Germany) equipped with diode array UV detector and external nitrogen source. Fused silica uncoated capillaries of 75-μm I.D., 375-μm O.D. (Composite Metal Services, Hallow, UK), and total length 70 cm were inserted into the CE-MS assembly cartridge and used for CE-MS experiments. Before starting CE experiments, new capillaries were conditioned using the following procedure: (1) water (12 bar×1 min);,(2) 0.1 M sodium hydroxide (10 bar×10 min), (3) 0.1 M sodium hydroxide (10 min, without flushing), (4) water (12 bar× 5 min). Between runs the capillary in use was rinsed with pure acetonitrile (8 bar×0.8 min) followed by water (12 bar×0.5 min) and conditioned with background electrolyte (BGE) (12 bar×1.2 min). Samples were injected by high pressure application at 2 bar×0.01 min followed by buffer injection at 2 bar×0.02 min to prevent sample loss on application of voltage and to improve data repeatability.

The running voltage was 20–30 kV. The ammonium formate BGE was prepared by titrating a 200 mM formic acid solution with ammonium hydroxide to the final pH of 2.5. The BGE was prepared every 3 days and stored at 4 °C in three different aliquots, each for 1 day of use. The temperature of the CE-MS assembly cartridge was set at 25 °C.

The CE apparatus was coupled to the mass spectrometer Esquire 3000 plus (Bruker Daltonics, Bremen, Germany) via a coaxial sheath liquid electrospray ionization (ESI) interface (Agilent Technologies, Waldbronn, Germany). The sheath liquid was delivered by an external syringe pump (Cole Palmer, Vernon Hills, Illinois, USA) at a constant flow rate of 240 μL h^{-1}. Nebulizing and drying gas (nitrogen) were set at 5 psi and 5 L min^{-1}, respectively. Dry gas temperature was 300 °C. The detection of the analytes was performed in product ion scan mode by activating the multiple reaction monitoring (MRM) windows using an isolation width of ± 2.0 and fragmentation amplitude of 1.0 in positive ionization and extended resolution scan. The acquisition of the extracted ion current (EIC) signals was made over the m/z 50–280 mass range using a maximum accumulation time of 200 ms and a target value of 50,000.

Preparation of the spiked samples of dialyzed plasma

A pool of heparinized human plasma was collected, dialyzed (carnitine free), and stored at −80 °C in different aliquots of about 4 mL. Each day ($n=5$) an aliquot of 4 mL was thawed at room temperature, distributed into different aliquots, and spiked with the ×10 concentrated mixtures of carnitines and IS in order to prepare different spiked samples to be used for testing the method precision, accuracy, and linearity. The signals from the EIC produced by the summation of the main fragments for each analytes were used for quantitation using the analyte/IS peak area ratio data. Each calibration curve consisted of a blank sample, a zero sample (i.e., blank sample with only IS added) and six calibration samples in duplicate. Method linearity ($n=6$ calibration points) was tested in the plasma concentration ranges of 5–160, 1–32, and 0.25–8.00 μM for C, AC, and PC, respectively. The first calibration point corresponded to the LLOQ. Intraday and interday precision, recovery, and accuracy were evaluated each day on five replicated injections of four different concentration samples, the LLOQ and a low, medium, and high concentration sample corresponding to points 1, 2, 4, and 5 of the calibration, respectively, for C, AC, and PC.

Plasma sample preparation

A 100-μL aliquot of dialyzed plasma was spiked with carnitine and deuterated carnitine standard mixtures (10-μL volumes each) and vortex mixed for a few seconds. The sample was then deproteinized by adding 600 μL of cold acetonitrile (4 °C) and vortex mixed for 2 min. After holding on ice for 15 min, the plasma samples were centrifuged at 13,400 rpm for 5 min and the supernatant (400 μL) injected.

The blank and zero samples were added of 20 and 10 μL of water, respectively, in order to afford the same homogeneous matrix dilution in the final sample.

For the analysis of total carnitine (C$_T$) the first step of sample pretreatment was modified as following: a 100-μL aliquot of dialyzed plasma was added up to 10-μL volume of internal standard (Cd$_3$) and hydrolyzed by the addition of 100 μL of 0.1 M aqueous KOH and 10 μL of 1 M KOH (100% methanol solution) at room temperature for 10 min. The sample deproteinization step was then performed following the above-described procedure.

Results and discussion

On the basis of the chemical structures reported in Fig. 1 and the physicochemical properties of C, AC, and PC, 100 mM ammonium formate buffer, pH 2.5, compatible

with CE-MS applications, was chosen as BGE for preliminary experiments in full scan MS detection and positive ESI ionization. The CE separation capillary was coupled with the mass spectrometer via a coaxial sheath liquid interface. A mixture of methanol/water (70:30 v/v) containing 0.1% formic acid was used as the sheath liquid at flow rate of 240 μL h^{-1}. Under these conditions the analytes were positively charged and migrated towards the cathode under the applied electric field. In spite of the small molecular differences between AC and PC metabolites, their baseline separation was easily obtained in less than 7 min. In order to improve the separation efficiency and to increase the detection sensitivity, the effect of several physicochemical parameters, influencing both the CE separation and the MS analyte ionization, were investigated.

Method optimization

The increase of BGE concentration from 100 to 200 mM produced longer analysis time and improved the separation between AC and PC compounds (data not shown). The migration time of PC, the last migrating peak, corresponded to 6.8 and 7.5 min in 100 and 200 mM BGE, respectively. Thus, 200 mM BGE was selected for further investigations.

The effect of applied voltage was studied at 20, 25, and 30 kV. Voltages higher than 30 kV are not allowed on the CE instrument used. At both 20 and 30 kV we observed a decrease of the peak efficiency and analyte separation. Values lower than 20 kV were not investigated because at low voltages the peak efficiency is usually strongly decreased. Thus, 25 kV was the optimum running voltage.

The effect of buffer pH was studied at three different values: 2.5, 3.0, and 3.5. Higher pH values were not considered to avoid analyte adsorption on the capillary wall. At pH 3.5 the generated current was too high and unsuitable for CE-MS applications where a maximum current of 50 μA is allowed. At pH 3.0, tested both in 100 and 200 mM BGE, we observed a worse analyte separation than that obtained at pH 2.5, probably due to starting adsorption effects which heavily affected the peak shape.

The optimized CE operating conditions, i.e., 200 mM ammonium formate buffer, pH 2.5, and 25 kV voltage, were applied to investigate the effect of the sheath liquid composition on analytes separation and detection sensitivity. The choice of sheath liquid composition strongly influences CE separation. In fact the sheath liquid provides both the electrical contact with the capillary during CE and the analyte electrospray ionization [21]. Different water/organic mixtures were tested as sheath liquid solutions at a flow rate of 240 μL h^{-1}. The use of acetonitrile as organic modifier produced very low analyte signal compared with methanol (data not shown). The effect of methanol concentration in the sheath liquid was studied in the range

30–70% (v/v). Figure 2 shows the effect of three different methanol percentages in the sheath liquid on the separation of carnitines: 70% (panel A), 50% (panel B), and 30% (panel C) (v/v). The use of 70% methanol produced the strongest peak intensity; however, the baseline noise showed several disturbances. At lower methanol content, namely 50% and 30% (v/v), the signal/noise ratios were increased in spite of the lower peak intensity observed. The 70:30 water/methanol mixture (v/v) was chosen as sheath liquid to investigate the effect of 0.1% (v/v) formic acid on the analyte ionization. Formic acid produced stronger analyte peak intensity, longer migration times, and increased separation of AC and PC (Fig. 1d). In fact, as reported in the literature [21], the use of a sheath liquid which differs from BGE in composition, ionic strength, and/or counterions can affect both the migration time and analyte separation due to formation of ionic boundaries.

The effect of a reduced sheath liquid flow rate (120 μL h^{-1}) on detection sensitivity was also investigated. In spite of the lower dilution of the CE eluate, the analyte signal was not improved, and the electrospray ionization process was relatively unstable.

The method, optimized in full scan detection, was applied in product ion scan mode using the characteristic MS/MS transitions reported in Fig. 1 to increase the specificity of the detection of carnitines.

Figure 3 shows the base peak electropherograms, the MS/MS EIC plots, and the MS/MS product ion spectra of
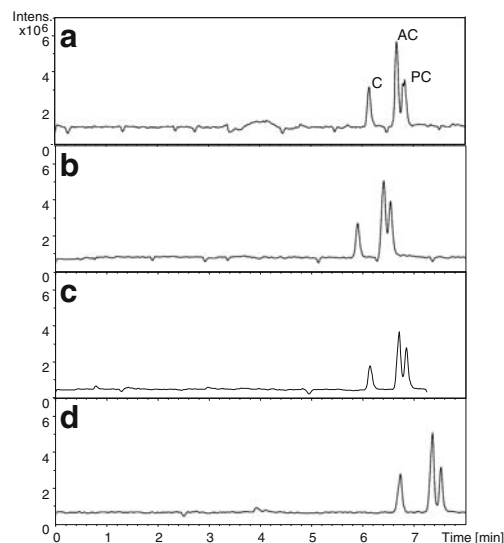

Fig. 2 Effect of sheath liquid composition on separation of carnitines *C*, *AC*, and *PC*: **a** water/methanol (70:30, v/v), **b** water/methanol (50:50, v/v), **c** water/methanol (30:70, v/v), **d** water/methanol (70:30, v/v) 0.1% formic acid. Operating conditions: 200 mM ammonium formate buffer, pH 2.5, running voltage 25 kV, sheath liquid flow rate 240 μL h^{-1}. For other experimental conditions see the text

Fig. 3 Base peak electrophero-gram (*BPC*), MS/MS extracted ion current plots (*EIC*), and product ion spectra (+*MS2*) of a *C*, *AC*, and *PC* standard mixture under the optimum CE/MS separation conditions. Operating conditions: 200 mM ammonium formate buffer, pH 2.5, running voltage 25 kV, sheath liquid water/methanol 70:30 (v/v) 0.1% formic acid at 240 µL h^{-1} flow rate, dry gas temperature 200 °C. For other experimental conditions see the text
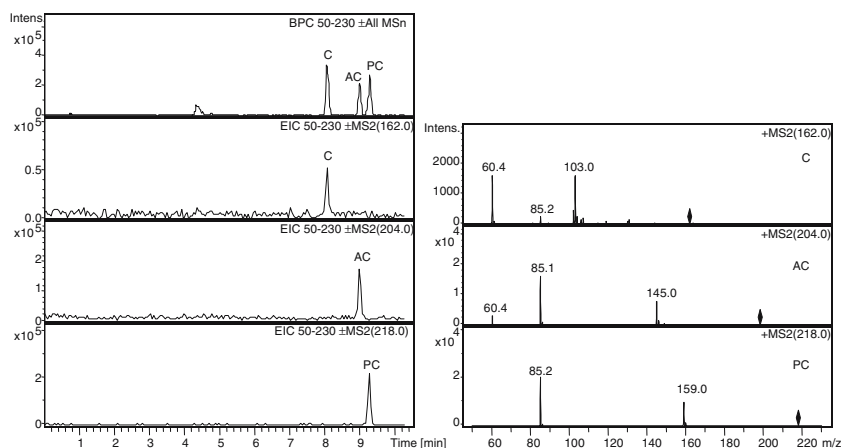
C, AC, and PC standard compounds analyzed in a mixture under the optimum experimental conditions. The analytes were baseline separated in less than 10 min. C showed a weaker ionic current signal than the AC and PC metabolites.

Analysis of human plasma

An important step in the method development was the optimization of a fast and simple pretreatment procedure for the analysis of plasma samples. A small volume of plasma (100 µL) was deproteinized with cold acetonitrile (1:5), centrifuged, and directly injected into the CE/MS apparatus. In spite of the sample dilution produced by the organic solvent, analyte peak intensity did not significantly decrease, probably due to a stacking effect of the acetonitrile. The C, AC, and PC peak intensity values were 9.2, 6.1, and 6.1×10^5 and 8.2, 4.1, and 5.5×10^5 for the sample injected in water and in water/acetonitrile (1:5) solution, respectively. The presence of acetonitrile in the injected sample did not affect analyte separation. The peak efficiency values of C and AC were higher in water/acetonitrile than in water

samples, but comparable for PC: they were 24,622, 41,732, and 44,874 and 134,821, 99,942, and 39,980 for C, AC and PC, in water and water/acetonitrile samples, respectively.

Figure 4 shows the CE-MS/MS analysis of carnitines in a sample pool of human plasma using Cd$_3$ as internal standard. The endogenous C and its short-chain metabolites could be identified with high specificity free of matrix interferences.

The method exhibited a limit of detection (LOD) of 4.5 nM (injected value) for PC, the compound present at the lowest concentration in plasma.

Quantitation

A pool of dialyzed human plasma was spiked with different carnitine concentrations (see the Experimental section) and used for evaluating the intraday and interday precision, accuracy, and linearity of the optimized CE-MS/MS analytical method. The d_3-carnitine (Cd$_3$) was used as the IS. Analytes were detected and quantified by calculating the ratio of analyte/IS peak area, the latter measured in the MS/

Fig. 4 Product ion scan mode extracted MS/MS ion current plots (*EIC*) and MS/MS spectra (+*MS2*) of *C*, *AC*, and *PC* analysis in human plasma. *Cd$_3$*, internal standard. For other experimental conditions see text and Fig. 3
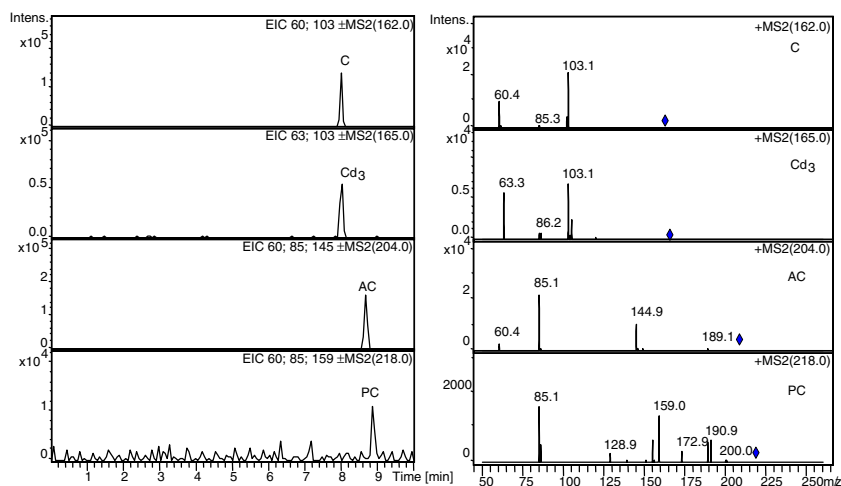
MS EIC plots generated by the sum of the main fragment ions intensities (see Fig. 1 for the considered MS/MS transitions of the analytes).

Under these experimental conditions, the method did not show the required RSD values for data repeatability, i.e., within 20% for LLOQ and 15% for higher concentrations. This was not caused by analyte adsorption on the capillary wall because a very high analyte migration time repeatability (less than 2% RSD) was observed.

Therefore, the effects of different MS acquisition parameters, i.e., the target values and the ion charge control, were investigated. The increase of the target value from 25,000 to 50,000 with a dry gas temperature of 300 °C provided RSD values for repeatability <15% and were used for testing linearity, repeatability, and accuracy of the method. In order to improve the method precision, d_3-acetylcarnitine (ACd$_3$) was used as IS for AC and PC. Cd$_3$ was used as IS only for C. Linearity was investigated in the concentration ranges 5–160, 1–32, and 0.25–8.00 μM for C, AC, and PC, respectively, using six calibration points. The investigated concentration ranges were selected on the basis of the endogenous levels of C, AC, and PC in human plasma reported in the literature [1, 17, 22–24]. The average plasma concentration of total carnitine (C$_T$), C, AC, and PC were 55, 48, 5, and 0.8 μM and 37, 33, 3, and 0.6 μM in males and females, respectively [1, 23]. In 16 men and 45 women without muscular disorders the mean C values were 57.3±12.8 μM (SD) for men and 46.5±12.4 μM (SD) for women [24].

AC and PC showed linearity in the selected concentration ranges with correlation coefficients (R^2) of 0.9995 and 0.9991, respectively. The calibration curve of C showed an R^2 of 0.9779 ($n=6$), that changed to 0.9984 in the restricted concentration range 20–160 μM ($n=4$ calibration points) by excluding the lower concentrations (5 and 10 μM). The poor ionization efficiency of C probably made the quantitative analysis critical at low levels.

For AC and PC the lower calibration point corresponded to the LLOQ, i.e., 1 and 0.25 μM, respectively. The LLOQ showed intraday RSD values for repeatability (on five replicated runs) of 12.8% and 21.6% for AC and PC, respectively, with accuracy values of 96.5% and 124%. Calibration points 2, 4, and 5 of AC and PC, corresponding to low, medium, and high concentration, respectively, were tested for intraday ($n=5$ replicated analysis) and interday ($n=5$ days) repeatability, accuracy, and method recoveries. The RSD values, reported in Table 1, were within the acceptable data range. The method recoveries were calculated on the average values ($n=5$ runs) of two determinations per concentration level and were in the range 88–86% (low), 101–112% (medium), and 96–105% (high concentration) for AC and 105–118% (low), 99–112% (medium), and 96–105% (high concentration) for PC.

The intraday and interday repeatability, the accuracy, and method recovery for C compound were calculated at 40 and 80 μM concentrations. They exhibited acceptable RSD values as shown in Table 1. The method recovery for C ($n=5$ runs, two determinations) showed RSD values in the range 78–88% and 101–102%, for 40 and 80 μM concentrations, respectively.

By increasing the IT target value from 50,000 to 200,000, the linearity of C response was obtained in a

Table 1 Intraday and interday precision and accuracy data

Analyte	Concentration level	Nominal concentration (μM)	Mean values found (μM)	Intra-assay repeatability ($n=5$ runs)		Interassay repeatability ($n=5$ days)		Intra-assay accuracy (%)
				SD	RSD (%)	SD	RSD (%)	
C[a]	m-QCs	40	35.28	0.084	9.10	0.130	14.10	88
	h-QCs	80	82.23	0.100	6.24	0.248	4.83	103
C[b]	LLOQ	5	5.57	0.042	26.33	nd	nd	111
	l-QCs	10	10.76	0.042	14.62	nd	nd	108
	m-QCs	40	40.47	0.091	8.88	nd	nd	101
	h-QCs	80	79.80	0.158	7.95	nd	nd	100
AC	LLOQ	1	0.97	0.024	12.80	0.024	11.47	97
	l-QCs	2	1.76	0.044	13.62	0.033	9.32	88
	m-QCs	8	8.96	0.139	8.65	0.124	8.65	112
	h-QCs	16	16.50	0.214	7.27	0.427	12.98	103
PC	LLOQ	0.25	0.31	0.016	21.60	0.020	21.31	124
	l-QCs	0.50	0.59	0.017	10.95	0.018	12.94	117
	m-QCs	2	2.24	0.046	6.76	0.085	12.48	112
	h-QCs	4	4.18	0.153	11.90	0.246	16.69	104

l low, m medium, h high, nd not determined
[a] IT target value 50,000
[b] IT target value 200,000

wider concentration range, from 5 to 80 μM ($n=5$ calibration points) ($R^2=0.9970$). The relative data of intraday repeatability and accuracy are reported in Table 1 and were calculated on calibration points 1, 2, 4, and 5, corresponding to LLOQ and low, medium, and high concentration, respectively. Under these conditions the C interday repeatability was not evaluated. Although the repeatability of C at the LLOQ (5 μM concentration) exceeded the 20% of RSD it showed an accuracy largely within 20%. Since 5 μM concentration is much lower than the C concentrations reported in human plasma [24], this method could in any case be used for the evaluation of abnormally low carnitine levels in human plasma.

Analysis of total carnitine in human plasma

The plasma C_T is the combination of the endogenous C and short-chain, medium-chain, and long-chain metabolites. The determination of C_T can help in the estimation of the long-chain carnitine levels for the evaluation of clinical states of pathological interest.

The analysis of C_T was performed after the hydrolysis of the carnitine ester metabolites with KOH solution, as reported in detail in the Experimental section. The efficiency of the hydrolysis procedure was tested on the palmitoylcarnitine (PmC) in mixture with C, AC, and PC standard compounds. For the CE analysis of PmC the optimized method needed a slight modification, i.e., the addition of 70% acetonitrile to the 200 mM buffer concentration, to obtain complete solubilization of the compound. The addition of acetonitrile negatively affected the peak shape due to the dilution of the ammonium formate buffer from 200 mM to 60 mM. To restore the analytes separation previously obtained, the buffer concentration was increased up to 150 mM (final concentration in the presence of acetonitrile).

After the hydrolysis of the standard mixture, only C was detectable in the analyzed sample (data not shown). The acetonitrile buffer was used only for testing the efficacy of the hydrolysis process, since the organic solvent is necessary in order to solubilize and analyze the PmC.

The determination of C_T in human plasma was performed using the CE/MS method optimized for the short-chain carnitines (buffer free of acetonitrile). The same human plasma sample previously analyzed for the short-chain carnitines was found positive only for carnitine. The hydrolysis reaction reduced the AC and PC metabolites to levels below the LLOQ of the method. Figure 5 depicts the relative electropherogram showing the peak of the C_T and Cd_3 IS compound and the blank profiles for the AC and PC metabolites.

It is noteworthy to underline that for C_T analysis in plasma, the sample pretreatment procedure was also very fast and simply included the hydrolysis step before the acetonitrile deproteinization.

Conclusions

A CE-MS/MS method was optimized for the rapid analysis of carnitine and its acetylated and propionylated metabolites and total carnitine in human plasma. The method combined the high efficiency of CE with the high selectivity of ESI-IT-MS detection, particularly advantageous for achieving successful and sensitive analysis of non-UV-absorbing analytes in biological matrix. The analysis of carnitines was obtained in less than 10 min using 200 mM ammonium formate, pH 2.5, as BGE. The optimized method was suitable for the quantitation of endogenous levels of carnitines in human plasma. The method showed good linearity, precision, and accuracy for AC and PC in the concentration ranges 1–32 and 0.25–

Fig. 5 Product ion scan mode extracted MS/MS ion current plots (EIC) and MS/MS spectra (+MS2) of the analysis of total C in hydrolyzed human plasma. Cd_3 internal standard. For other experimental conditions see text and Fig. 3
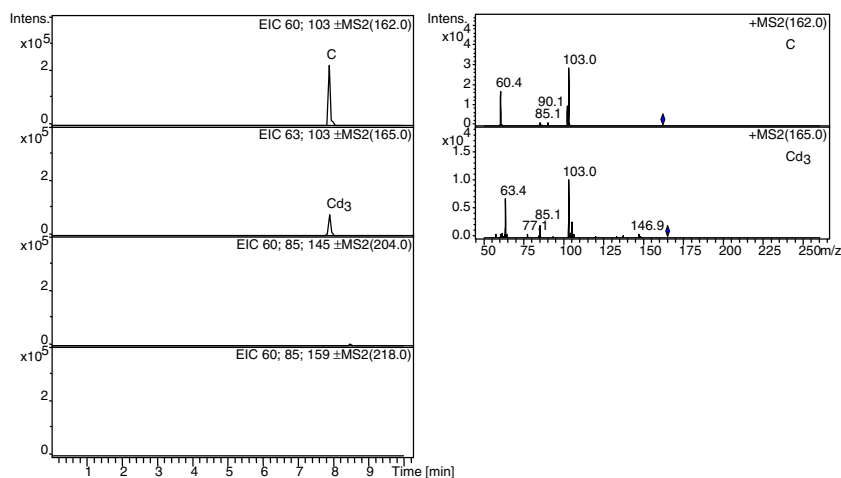
8.00 μM ($n=6$ calibration points), respectively. The quantitative analysis of carnitine showed a linear response in the range 20–160 μM ($n=4$ calibration points). The plasma samples were directly injected into CE after a simple and rapid sample pretreatment procedure. It required only 22 min, including the acetonitrile protein precipitation step (2 min vortex and 15 min holding on ice) and centrifugation (5 min). A set of samples could be simultaneously prepared with rapidity and simplicity. The method showed high sensitivity, allowing the determination of AC and PC at 0.143 and 0.036 μM (injected concentrations) corresponding to LLOQ in plasma of 1 and 0.25 μM, respectively.

The short analysis time, the direct MS detection of the analytes with no labor-intensive derivatization procedures, and the minimal plasma sample pretreatment contributed to the development of a sensitive and selective analytical method to assay carnitines in human plasma with a strong reduction of the total analysis time.

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