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RESEARCH PAPER

Rapid and sensitive HILIC–MS/MS analysis of carnitine and acetylcarnitine in biological fluids

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Abstract Monitoring carnitine and acetylcarnitine levels in biological fluids is a powerful tool for diagnostic studies. Research has recently shown that the analysis of carnitine and related compounds in clinical samples can be accomplished by different analytical approaches. Because of the polar and ionic nature of the analytes and matrix complexity, accurate quantitation is a highly challenging task. Thus, sample processing factors, preparation/cleanup procedures, and chromatographic/ionization/detection parameters were evaluated. On the basis of the results obtained, a rapid, selective, sensitive method based on hydrophilic interaction liquid chromatography–tandem mass spectrometry for the analysis of carnitine and acetylcarnitine in serum and urine samples is proposed. The matrix effect was assessed. The proposed approach was validated, the limits of detection were in the nanomolar range, and carnitine and acetylcarnitine levels were found within the micromolar range in both types of sample.

Keywords Carnitine · Acetylcarnitine · Biological fluids · Hydrophilic interaction liquid chromatography–tandem mass spectrometry

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Introduction

Carnitine is an endogenous metabolite found in most mammals. It is synthesized in tissues such as brain, liver, and kidney from the amino acids lysine and methionine [1, 2]. Carnitine is involved in decreasing the formation of free radicals in various tissues, the maintenance of the cellular concentration of free coenzyme A [3], the elimination of potentially toxic compounds, and transport of long-chain fatty acids [3–8]. During the transfer of long-chain fatty acids from the cytosol to the mitochondrial or peroxisomal matrix for β-oxidation of fatty acids, various acylcarnitines of different length are generated, with acetylcarnitine being the most abundant among these acyl derivatives. Skeletal muscle is the major reservoir of carnitine (approximately 95 %) [9]. However, only a quarter of this reserve comes from endogenous synthesis, and the rest comes from the diet [9]. Carnitine metabolism is closely linked to a variety of metabolic problems, which lead to a redistribution of carnitine and the different acylcarnitines [10–12]. Any increase or decrease in the carnitine and acylcarnitine contents could be mirrored by a simultaneous increase or decrease in their serum and urine levels. Thus, determination of carnitine and acetylcarnitine in biological fluids is a powerful means for the diagnosis and management of these disorders [13–19].

However, slight, but metabolically important, variations can only be detected by cutting-edge technology instruments and analytical methods. Mass spectrometry (MS) is a powerful tool for the analysis of carnitine and acetylcarnitine with electrospray ionization (ESI) as the ionization source, since carnitine and acylcarnitines contain a stably charged quaternary ammonium group and thus exhibit high ionization efficiency [20]. In addition, tandem MS (MS/MS) is often used as a detection technique to improve the detection selectivity and sensitivity of these analytes in complex samples.

The pattern of urinary acylcarnitines has already been determined by nonchromatographic approaches coupled with MS [16, 18]; however, in these methods not enough analytical tools have been used to confirm the identity of the compounds under study. In this sense, for robust identification of analytes at low concentrations in complex matrices, some requirements have been established: retention times and accurate masses or, retention times and at least two specific transitions should be used [21]. Nevertheless, in both works, a single-quadrupole instrument without application of any chromatographic preseparation was used. In addition, although a matrix effect was observed, no significant details about the study of the matrix effect were given.

To deal with the complexity of biological samples, the coupling of MS and chromatographic separations is required in most cases to obtain identification accuracy, high separation capabilities, high sensitivity, and high selectivity [22, 23].

Liquid chromatography (LC) remains the method of choice for the quantitative determination of several compounds in different matrices, including carnitine and acylcarnitines [23, 24]. Ultra-high-pressure LC using smaller particles (less than 2.0 μm) and higher linear velocities is able to achieve high-efficiency separations in faster analysis times compared with conventional high-pressure LC separations [25, 26]. In addition, polar stationary phases such as in hydrophilic interaction LC (HILIC) are better suited to retain diverse polar analytes and offer improved sensitivity over reversed-phase (RP) chromatography [27, 28]. In HILIC, analyte retention is believed to be caused by partitioning of the analyte between a water-enriched layer of stagnant eluent on a hydrophilic stationary phase and an eluent with more hydrophobic properties, with the main components usually being 5–40 % water in acetonitrile. This high acetonitrile content gives HILIC two additional advantages: high sensitivity when coupled with ESI-MS [28] and faster separations compared with standard RP eluents. The elution order in HILIC is more or less the opposite of that seen in RP separations [28, 29]. Thus, HILIC can be used to optimize the retention of very polar compounds with poor retention in RP chromatography [30, 31]. In fact, when carnitine and/or several of its acyl derivatives were studied by RP chromatography, the most polar analytes were eluted almost with the solvent front, showing in general very poor resolution performance [32, 33].

When different chromatographic separation strategies are applied, a sample preparation procedure is often mandatory owing to the complexity and, sometimes, the low analyte concentrations in biological matrices. For this reason, automated off-line procedures are generally preferred because they do not depend on the chromatographic separation, and a great number of samples can be treated rapidly. In the particular case of blood samples, protein precipitation approaches have been mainly used for sample treatment prior to analysis [25].

To the best of our knowledge, a few methods combining polar chromatographic strategies and MS have been reported for the analysis of carnitine and acylcarnitines in different samples [24, 27, 34, 35]. In 1998, Tallarico et al. [34] were pioneers in the use of a polar functionalized column for the separation of carnitine, acetylcarnitine, propionylcarnitine, and their deuterated analogues. The method applied was suitable for the analysis of the compounds mentioned in plasma samples, but no information regarding optimization of the chromatographic approach was given. Later, Hirche et al. [35] proposed a normal-phase separation–MS method for monitoring carnitine, its short acyl esters, and some other metabolic precursors in plasma and pig tissues. Both of these works made significant contributions to the topic under study; however, important analytical aspects related to chromatographic retention/MS performance have been greatly improved, especially in the work presented here. Recently, Miller et al. [27] developed a method based on HILIC and MS/MS to identify fatty acid oxidation, amino acid metabolism, and organic acid disorders. Despite the satisfactory analytical features obtained, only whole blood was analyzed in this study. On the other hand, Sowell et al. [24] applied HILIC and MS to the analysis of only carnitine in plasma samples.

The present study is based on the separation/determination of carnitine and acetylcarnitine in biological fluids such as serum and urine by HILIC coupled with MS/MS. The method incorporated a deproteinization step for serum and a careful assessment of the matrix effects for both types of samples. Both analytes were identified in a single run with optimized ESI and multiple reaction monitoring (MRM) conditions.

Experimental

Chemicals and reagents

L-Carnitine inner salt (98 %) and *O*-acetyl-L-carnitine hydrochloride were purchased from Sigma-Aldrich (St. Louis, USA). Water, methanol, and acetonitrile of Optima® LC–MS grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (98 %) was obtained from Fisher Scientific (Loughborough, UK). Acetic acid (glacial, trace metal grade), high-pressure LC grade ammonium acetate, and certified ammonium formate were obtained from Fisher Chemical (Fisher Scientific, Pittsburgh, PA, USA). Ultrapure water (18 MW cm) was obtained with an EASYpure RF purification system (Barnstead International, Dubuque, IA, USA).

Methanolic standard solutions of carnitine and acetylcarnitine were prepared daily by appropriate dilutions of a 1 mg L^{-1} aqueous stock solution. Quantification was achieved

by preparing spiked serum and urine samples with proper amounts of the analytes. The solutions were maintained at 4 °C.

Mobile phase preparation

The mobile phase for HILIC was prepared by first dissolving a known amount of ammonium acetate or ammonium formate in water (stock solution), and then mixing the salt solution with the desired volume of acetonitrile. The salt concentration in the text refers to the final concentration in the mobile phase. Mobile phases A and B were also prepared by adding 0.1 % (v/v) formic acid to both water and acetonitrile.

Sample collection and preparation

After written informed consent had been obtained, blood (serum) and urine samples were collected from healthy female and male adults, ranging from 40 to 60 years old. The whole procedure was made anonymous, and the study was approved by the Local Ethics Committee. All participants were informed about the purposes and scope of the study, and signed appropriate consent forms. Data assessment was complete for all participants.

Serum samples

Venous blood samples (5 mL) were obtained from the people studied after a 12-h overnight fast. Sterile disposable plastic syringes were used. Serum from blood left to clot was separated by centrifugation and immediately stored at −20 °C. Serum samples free of hemolysis were used. For protein precipitation, a 3:1 solvent mixture of acetonitrile and methanol was prepared. Firstly, the serum was thawed and a 25-μL aliquot was pipetted into 500 μL of the acetonitrile–methanol solvent mixture. Subsequently, the mixture was frozen for 18 h. The mixture was then thawed again and centrifuged for 15 min at 12,000 rpm. The supernatant was retaken and the pellets were discarded; there were two more successive centrifugations. Finally, the resulting supernatant was evaporated with a stream of gaseous nitrogen. Samples were reconstituted with 300 μL of methanol and analyzed immediately.

Urine samples

Urine samples were collected in sterile specimen collection bottles. The urine samples were centrifuged for 10 min at 12,000 rpm. A 50-μL aliquot of the supernatant was diluted with ultrapure water up to 1-mL final volume, filtered through a 0.22-μm syringe filter (Osmonics®), and stored in an amber vial suitable for LC–MS/MS analysis.

Ultra-high-pressure LC instrumentation and conditions

An ACQUITY® ultra-high-performance LC system (Waters, Milford, MA, USA) equipped with autosampler injection and pump systems (Waters, Milford, MA, USA) was used. The autosampler vial tray was maintained at 15 °C. The needle was washed with proper mixtures of acetonitrile and water. The separation was performed by injecting a 10-μL sample onto an ACQUITY UPLC® BEH HILIC analytical column (Waters, Milford, MA, USA) of 2.1-mm internal diameter, 50-mm length, and 1.7-μm particle size. The binary mobile phases consisted of water with 7.5 mM ammonium formate (solvent A) and acetonitrile with 7.5 mM ammonium formate (solvent B) delivered at 0.25 mL min^{-1}. The HILIC gradient was started at an initial composition of 10 % solvent A and 90 % solvent B, followed by a 3-min linear gradient to 30 % solvent A, held for 0.2 min. Return to the initial conditions was accomplished by a 0.2-min gradient to 90 % solvent A, held for 1.8 min. Thus, the total chromatographic run time was 5.0 min. The column was maintained at 30 °C. Under these conditions, no sample contamination or sample-to-sample carryover was observed.

MS instrumentation and MS/MS conditions

MS analyses were performed with a Micromass Technologies Quattro Premier™ XE triple-quadrupole mass spectrometer with a ZSpray™ ESI source (Waters, Milford, MA, USA). The source was operated in the positive ESI mode at 345 °C with nitrogen as the nebulizer, and the source temperature was kept at 150 °C. The capillary voltage was maintained at 3.75 kV and the extractor voltage was set at 5.0 V. Ultrapure nitrogen was used as the desolvation gas at a flow rate of 800 L h^{-1}. Argon was used as the collision gas at a flow rate of 0.18 mL min^{-1}, achieving an analyzer pressure of approximately 3×10^{-5} Torr. After optimization, detection was performed in MRM mode of selected ions at the first quadrupole (Q_1) and third quadrupole (Q_3). To choose the fragmentation patterns of m/z (Q_1)→m/z (Q_3) for the analyte in MRM mode, direct infusions (via a syringe pump) into the MS system of carnitine and acetylcarnitine standard solutions in methanol were performed, and the product ion scan mass spectra were recorded. Quantification of carnitine and acetylcarnitine was done by measuring the area under the specific peak using MassLynx (Waters, Milford, MA, USA).

Evaluation of the matrix effect

One downside of ESI-MS/MS ionization/detection is that the ionization process is susceptible to matrix signal suppression or enhancement [36–38]. The LC–MS response obtained from a standard can differ significantly from that obtained from matrix samples. In the work presented here, after

selecting the proper chromatographic approach for each sample, we assessed the effect of serum and urine matrices by comparing the signals of carnitine and acetylcarnitine in pure solvent (methanol) with the signals in the sample matrices. The calibration curves obtained for carnitine and acylcarnitine in spiked biological fluids and in the pure solvent were created. The percentage of the quotient of the slopes (b) in the spiked and solvent samples was used as an indicator of the extent of the matrix effect, which was calculated as $100-(b_{spiked}/b_{solvent}\times 100)$.

Assay validation

Linearity

The calibration plots were constructed under optimal experimental conditions for each type of sample (reconstituted serum and diluted urine samples). Six points of the calibration curve were determined (ten technical replicates at each concentration). The calibration equations were calculated by the least-squares linear regression method. Thus, linearity was evaluated from values close to the limits of detection up to approximately 1,000 μg L^{-1} for carnitine and 500 μg L^{-1} for acetylcarnitine.

To estimate the trueness, intraday repeatability, and interday reproducibility, spiked serum and urine samples were analyzed. For serum samples, five blank samples were measured and ten replicate measurements at carnitine concentrations of 0, 10, 25, 50, 75, and 100 μg L^{-1} and acetylcarnitine concentrations of 0, 5, 10, 30, 50, and 75 μg L^{-1} were performed.

Statistical method

Analysis of variance was used to determine significant differences among data. Each statistical analysis was done using the software program Statgraphics Centurion XV (version 15.2.06), and normality of the data was tested before applying the analysis of variance approach.

Results and discussion

Sample preparation optimization

Although the technology related to chromatographic separations and MS techniques is advancing, sample cleanup is still one of the most important bottlenecks of the analytical process. Effective sample preparation is essential for achieving good analytical results because matrix-related compounds may be co-extracted and interfere in the analysis.

The selection of the sample cleanup conditions in the analysis of carnitine and acetylcarnitine in biological material is a difficult task because of the nature of both analytes. For this reason, optimization of the protein precipitation step paying special attention to the recovery of the analytes was conducted as detailed in "Serum samples." The accuracy of the proposed method was evaluated by recovery experiments involving spiking diluted sample solutions. Recoveries higher than 95 % reveal sufficient accuracy. As already mentioned, urine samples were diluted and centrifuged only prior to their analysis. The recovery studies demonstrated recoveries above 95 %.

Chromatographic procedure

For polar analytes, HILIC separation has proven to provide excellent efficiency and peak shape [27, 31]. In HILIC, a mobile phase containing a high concentration of organic (less polar) solvent and a low concentration of aqueous (polar) solvent is used. The aqueous portion constitutes a strong solvent and hence the elution of compounds occurs in the order of increasing hydrophilicity [39]. In this assay, a HILIC column was selected for the method development because it was suitable for the separation of polar and ionic compounds such as carnitine and acetylcarnitine, which contain quaternary amine functional groups.

As illustrated by Ruta et al. [40], when compounds are analyzed by HILIC, the buffer is an essential component in the mobile phase since peak shapes are affected if only weak acids are used as mobile phase additives. It is therefore important to use a buffer compatible with the acetonitrile-rich mobile phase. Three different types of mobile phase buffers were compared: formic acid and different concentrations of ammonium acetate and ammonium formate. The addition of formic acid failed to produce satisfactory separation of carnitine and acetylcarnitine. The addition of ammonium acetate allowed the separation of both analytes, but the performance was inferior compared with the use of ammonium formate. The latter succeeded in the separation of the analytes at all concentrations tested (2.5 mM, 5.0 mM, 7.5 mM, and 10.0 mM), but 7.5 mM was selected because the addition of this concentration to the mobile phase resulted in more reproducible retention times with repeated injections. The HILIC column used for this assay showed good peak shape and retention time stability throughout the analysis.

The effect of the mobile phase flow rate on the separation/retention of carnitine and acetylcarnitine was evaluated using van Deemter plots. A 10-μL standard sample injection volume was loaded onto the system at various flow rates, from 0.1 to 0.5 mL min^{-1}, with isocratic separation. A flow rate of 0.25 mL min^{-1} gave the best results in terms of chromatographic conditions and ESI efficiency. Figure 1 depicts the van Deemter plots obtained for both analytes.

In addition, the effect of column temperature on the retention of carnitine and acetylcarnitine was studied. The

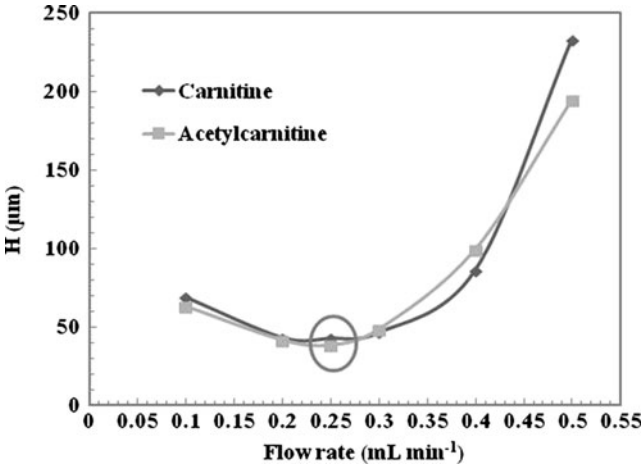

Fig. 1 Van Deemter curves for carnitine and acetylcarnitine. The conditions were as follows: hydrophilic interaction liquid chromatography (HILIC) column; mobile phase of acetonitrile–water in isocratic mode containing 7.5 mM ammonium formate; temperature 30 °C; concentration of carnitine and acetylcarnitine standards 25 μg L^{-1}; injection volume 10 μL

van't Hoff plot for both analytes on the HILIC column in the temperature interval from 15 to 70 °C was evaluated. The elution times of carnitine and acetylcarnitine increased from seconds to minutes when the column temperature was increased. The optimal retention conditions were obtained when the temperature was fixed at 30 °C. This temperature was selected for further experiments.

Under optimal conditions, acetylcarnitine and carnitine were eluted from the column at 2.74 and 2.98 min; respectively, in a total run cycle of 5.0 min (Fig. 2).

Optimization of the ionization/MS conditions

Owing to the properties of carnitine and its derivatives, ESI is currently the most used strategy for their ionization. The ESI method was optimized with respect to dominant conditions such as capillary voltage, source temperature, probe temperature, drying gas flow rate, and drying gas temperature (the optimal parameters were mentioned in "MS instrumentation and MS/MS conditions").

Preliminary experiments were conducted with the purpose of finding the best instrumental conditions that would allow identification of carnitine and acetylcarnitine in serum and urine samples at trace levels. Carnitine and acetylcarnitine standard solutions (1 mg L^{-1}) in methanol were introduced into the MS system at a flow rate of 30 μL min^{-1} via a syringe

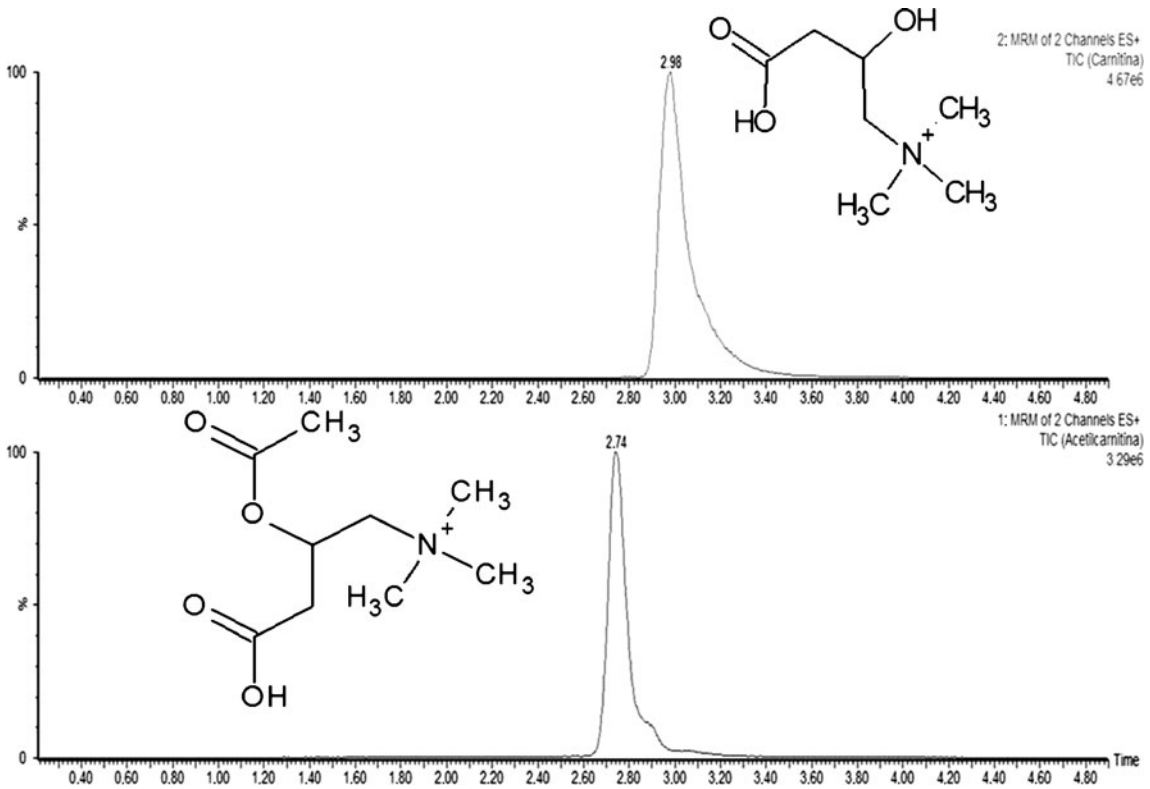

Fig. 2 Representative total ion chromatograms of the HILIC separation of carnitine and acetylcarnitine. The ionization and mass spectrometry (MS) conditions were as follows: electrospray ionization in positive mode associated with MS detection in multiple reaction monitoring mode (experimental parameters were as described in "MS instrumentation and MS/MS conditions"). The chromatographic conditions were as follows: HILIC column; mobile phase of acetonitrile–water in gradient mode containing 7.5 mM ammonium formate; mobile phase flow rate 0.25 mL-min^{-1}; temperature 30 °C; concentration of carnitine and acetylcarnitine standards 25 μg L^{-1}; sample injection volume 10 μL

Table 1 Mass spectrometry conditions for the generation and fragmentation of carnitine and acetylcarnitine

	Dwell time (s)	Cone voltage (V)	Collision voltage (V)
Carnitine transitions (*m*/*z*)			
162.2→103.1[a]			
162.2→85.1	0.08	15	17
162.2→60.3			
Acetylcarnitine transitions (*m*/*z*)			
204.0→145.2[a]			14
204.0→85.1	0.08	18	15
204.0→60.3			15

[a] Transition used for quantification

pump. The positive ion, full scans (mass spectra from *m*/*z* 100 to *m*/*z* 300 were recorded) of carnitine and acetylcarnitine indicated the presence of their pseudomolecular ion $[M+H]^+$ as the predominant specie, with *m*/z values of 162.2 and 204.0, respectively. MRM of the precursor–product ion transitions was optimized. Specific charged fragments for both analytes are listed in Table 1. The optimization of the ion production and fragmentation conditions permitted sensitive and selective detection of the analyte. As a result, the areas under the most sensitive transitions, 162.2→103.1 and 204.0→145.2 for carnitine and acetylcarnitine, respectively, were measured for quantification purposes.

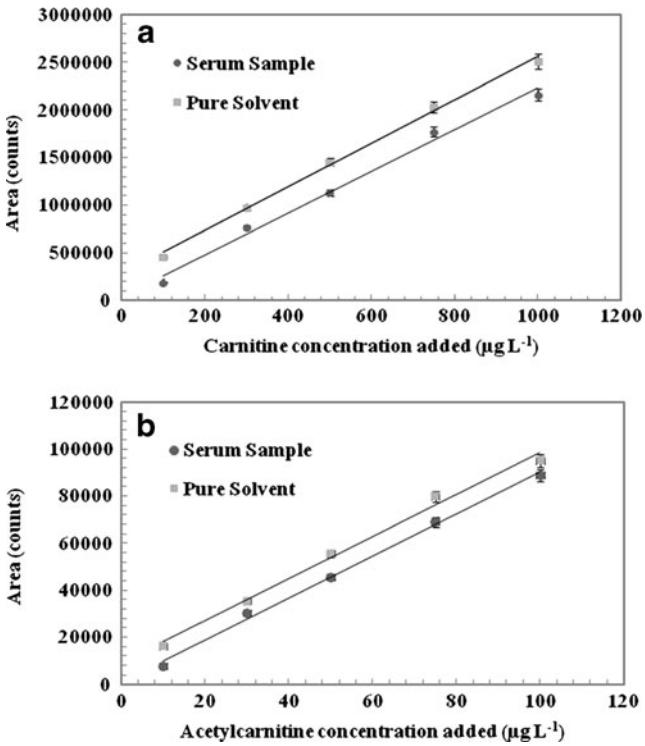

Fig. 3 Calibration plots from spiked matrix (serum) and spiked pure solvent (methanol) samples: **a** carnitine and *b* acetylcarnitine. The chromatographic and ionization/MS conditions were as described in "Ultra-high-pressure LC instrumentation and conditions" and "MS instrumentation and MS/MS conditions"

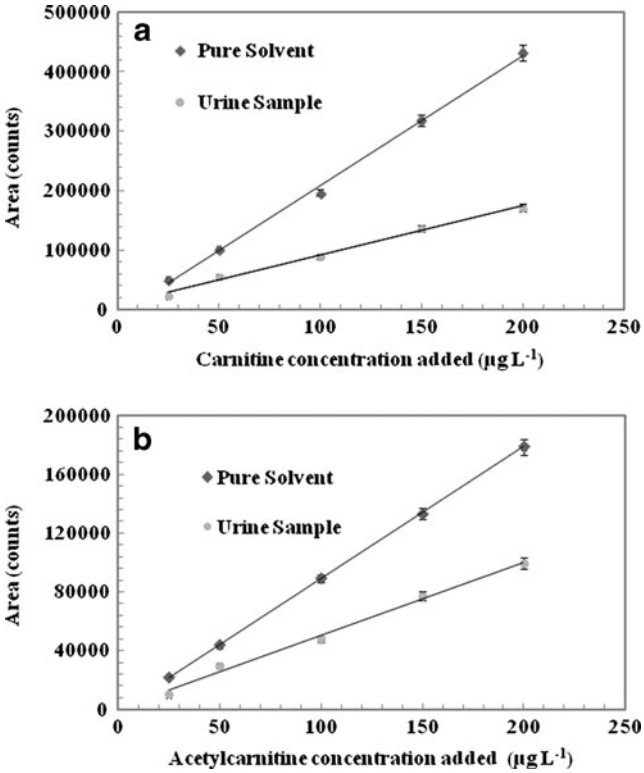

Fig. 4 Calibration plots from spiked matrix (urine) and spiked pure solvent (methanol) samples: **a** carnitine and **b** acetylcarnitine. The chromatographic and ionization/MS conditions were as described in "Ultra-high-pressure LC instrumentation and conditions" and "MS instrumentation and MS/MS conditions"

Evaluation of the matrix effect

The origin and mechanism of matrix effects are not understood fully. As mentioned in "Experimental," after selecting the proper chromatographic approach, we evaluated the effect of the two matrices under study (serum and urine) by comparing the signals of carnitine and acetylcarnitine in pure solvent (methanol) with the signals in the sample matrices. Thus, calibration curves from spiked matrix and spiked pure solvent samples were created. The resulting calibration plots for serum and urine samples are shown in Figs. 3 and 4. In the case of carnitine, the slopes (*b*) obtained in pure solvent, spiked serum, and spiked urine were 2,280.50, 2,190.70, and 833.18; respectively. On the other hand, the slopes obtained for acetylcarnitine in pure solvent, serum, and urine samples were 895.97, 875.56, and 498.89. These values were used as described in "Experimental" as indicators of the extent of the ion suppression or signal enhancement. From our results, no signal enhancement was observed, but the serum and urine matrices diminished the response of the analytes by between 5 and 50 %. As a consequence, carnitine and acetylcarnitine

Table 2 Analytical efficiency for the analysis of carnitine and acetylcarnitine by hydrophilic interaction liquid chromatography–tandem mass spectrometry

LoD Limit of detection, *LoQ* limit of quantification, *RSD* relative standard deviation

Analyte	R^2	LoD (nM)	LoQ (nM)	Repeatability (RSD %)	Reproducibility (RSD %)
Carnitine (serum)	0.9980	1.42±0.04	4.34±0.13	2.99	6.09
Acetylcarnitine(serum)	0.9978	1.51±0.05	4.33±0.12	3.03	6.66
Carnitine (urine)	0.9990	1.05±0.03	3.22±0.09	2.96	6.56
Acetylcarnitine (urine)	0.9923	0.98±0.02	3.15±0.11	3.58	7.39

quantifications were conducted following the standard addition method.

Analytical performance

Certified reference materials for the matrices studied with an informed value for carnitine and acetylcarnitine do not exist. However, it is acceptable to assess the trueness of the measurements through recovery of additions of known amounts of the analyte to a blank matrix. Thus, spiked serum and urine samples were analyzed. Peak integration, regression, and calculation of concentrations were computed using MassLynx.

For the urine samples, the concentrations of the carnitine and acetylcarnitine standards at the respective points on the calibration curves were 0, 25, 50, 100, 200, and 350 μg L^{-1}.

The same experiment was repeated on three other independent occasions with at least a 1-week interval. Repeatability as intraday variability was determined by calculating the relative standard deviation for the replicated measurements. The values obtained were 2.99 % and 3.03 % at a concentration of 10 μg L^{-1} for carnitine and acetylcarnitine, respectively. The overall within-laboratory reproducibility was 6.09 % for carnitine and 6.66 % for acetylcarnitine at the concentrations tested. In summary and if we take into account the matrix complexity, the reported values for the method assessment parameters can be considered highly satisfactory.

The limit of detection and limit of quantification were calculated as the signal equivalent to, respectively, three times and ten times the background chromatography noise under working conditions. The analytical efficiency of the method is shown in Table 2.

Sample analysis

Once the optimal conditions had been established for the different matrices under study, the methods developed were applied to the analysis of real samples. The analysis of serum and urine samples to assess the levels of carnitine and acetylcarnitine was performed. Carnitine concentrations ranged between 30 and 50 μM, and acetylcarnitine levels were between 5 and 10 μM. The values obtained were in good agreement with those reported by other research groups [16, 41].

In the case of urine samples, the carnitine concentration ranged between 81 and 290 μM, whereas the concentration of acetylcarnitine was lower, on the order of 15–70 μM [16]. These results are in good agreement in terms of concentration and biological significance with those reported by Reuter and Evans [3].

Conclusions

We have developed and proposed a sensitive and selective analytical method for the separation and determination of carnitine and acetylcarnitine in biological samples based on the use of HILIC coupled with MS/MS. The advantages of using HILIC for the analysis of these polar compounds in the complex samples under study were demonstrated. Our studies showed that matrix effects should be carefully assessed when biological fluids are involved. Finally, the method developed could be of great interest for monitoring variations of carnitine and acetylcarnitine in metabolic studies.

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