Quantification of Total and Free Carnitine in Human Plasma by Hydrophilic Interaction Liquid Chromatography Tandem Mass Spectrometry

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Abstract

Carnitine is an endogenous quaternary amine whose primary function is to shuttle long chain fatty acids to the mitochondrial matrix, where they subsequently undergo beta oxidation. Accurate quantification of total and free carnitine is essential for the accurate diagnosis of a number of inborn errors of metabolism, including disorders of fatty acid oxidation as well as various organic acidurias. Early methods for carnitine measurement were enzyme based. Recently, liquid chromatography tandem mass spectrometry has become the method of choice for carnitine measurement. Typically, carnitine is derivitized to from a butyl ester, thus improving its ionization and retention characteristics. A potential problem with this approach is that the acidic conditions used to carry out the reaction may hydrolyze other acyl esters, resulting in ex-vivo artifacts. Consequently, we developed a hydrophobic interaction chromatography (HILIC) tandem mass spectrometry method for the quantification of carnitine. The use of HILIC allows for the derivitization step to be circumvented, while still allowing for favorable chromatographic performance. The method was shown to be accurate, precise, and robust.

Introduction

Carnitine (3-carboxy-2-hydroxy-N,N,N-trimethylpropan-1-aminium) is an endogenous quaternary ammonium compound synthesized from lysine and methionine.

While carnitine has a wide range of biological functions, its most important role involves the transport of long chain fatty acids into the mitochondrial matrix (1). Fatty acids first undergo a conjugation reaction forming acyl-CoA. The acyl group is then transferred to carnitine, forming an acylcarnitine. The transport of acylcarnitines from the cytosol of the cell to the matrix side of the inner mitochondrial membrane is mediated by a translocase (Figure 1). After traversing the membrane, the acyl group is transferred from carnitine to Coenzyme A, where it ultimately enters the citric acid cycle as acetyl CoA after undergoing β oxidation. Carnitine is shuttled back across the membrane, where the process is then repeated.

Inborn errors of metabolism are typically caused by genetic errors in genes coding for metabolic enzymes. The affected enzyme operates at a reduced capacity, or loses functionality entirely. Consequently, metabolic intermediates may accumulate to toxic levels and downstream metabolic pathways may be adversely affected (2,3). Disruptions in fatty acid metabolism result in metabolic disorders including very long chain acyl

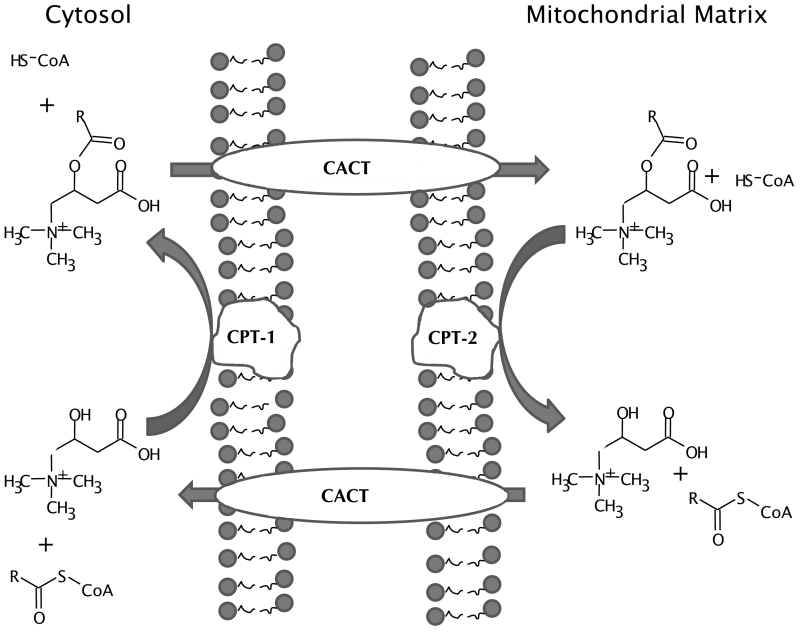

Figure 1. Acyl-carnitine conjugation is catalyzed by CPT-1 (Carnitine Palmitoyle Transferase I) in the cellular cytosol. The conjugate is shuttled across the mitochondrial membrane by CACT (Carnitine Acylcarnitine Translocase) to the mitochondrial matrix. There, the aliphatic ester bond is broken, Acyl-CoA conjugation is catalyzed by CPT-2 (Carnitine Palmitoyle Transferase II), and then carnitine is shuttled back across the membrane by CACT.

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coenzyme A dehydrogenase (VLCAD) deficiency, long chain acyl-coenzyme A dehydrogenase (LCAD) deficiency, medium chain acyl-coenzyme A dehydrogenase (MCAD) deficiency, etc (4–8). These acyl coenzyme A dehydrogenase deficiencies result in accumulation of the corresponding acylcarnitine and may be diagnosed by measuring plasma acylcarnitine levels. However, acylcarnitine concentrations are dependent on the availability of carnitine. Consequently, measurement of carnitine is essential for the diagnosis of several inborn metabolism errors, including disorders of fatty acid oxidation and various organic acidurias. Low carnitine levels can mask diagnostic elevations in plasma acylcarnitines. Therefore, interpretation of plasma acylcarnitines without evaluating the total carnitine pool can lead to misdiagnosis. Because many of the adverse effects of the described disorders may be mitigated through dietary intervention and supplementation, early diagnosis is critical. Thus accurate and robust methods for carnitine quantification are critical.

Given the need to quantify total and free carnitine, various analytical methods have evolved for carnitine measurement. Traditionally, enzymatic assays using radioactive labels were used for this purpose (9,10). Typically, carnitine is enzymatically converted to radio-labeled acyl carnitine, which can then be quantified and correlated to the concentration of carnitine. Total and free carnitine is differentiated by utilizing a base hydrolysis step. While effective, the enzymatic assays are labor intensive and therefore not conducive to high throughput formats. As a result, liquid chromatography tandem mass spectrometry (LC–MS–MS) methods for the quantification of total and free carnitine have been developed (2–8,11–15). The implementation of tandem mass spectrometry based assays for the quantification of carnitine has resulted in significantly increased throughput, specificity, and sensitivity. Consequently, LC–MS–MS is typically the method of choice for carnitine measurement.

We have developed a tandem MS assay for the quantification of total and free carnitine that utilizes hydrophilic interaction liquid chromatography (HILIC) and requires no derivitization of carnitine. HILIC, originally coined by Alpert is unlike reversed-phase chromatography in that polar analytes are strongly retained (16). The stationary phase in HILIC is usually bare silica, though other stationary phases such as mixed diol packings may be used (17–19). HILIC separations are typically mixed mode, in that a combination of hydrophilic interaction, ion exchange, and to a lesser extent, reverse-phase retention, combine to provide for superior retention and selectivity for polar analytes. Consequently, HILIC is ideal for separation and retention of small polar molecules. When coupled with tandem MS for detection, overall assay sensitivity is greatly increased due to improved desolvation in the ionization process. Sensitivity is also improved, when compared to reverse-phase chromatography, because polar molecules exhibit improved retention and, therefore, are less likely to succumb to ion suppression regions, which are usually more prevalent in chromatographic regions that show poor retention. These characteristics make HILIC useful for the quantification of small, polar analytes.

Typically, LC–MS–MS analysis of carnitine has utilized an acid-catalyzed reaction with butanol to form butyl esters. This process is not only time consuming, but the acidic reaction conditions promote the hydrolysis of acylcarnitines, and can result in an overestimation of the carnitine concentration. The carnitine assay described here requires no pre- or post-analytical derivitization. Furthermore, the assay is sensitive, robust, and amenable to high throughput formats as total sample prep time is less than 5 min.

Materials and Methods

Materials

Carnitine, ammonium acetate, formic acid, sodium hydroxide, hydrochloric acid, bovine serum albumin sodium chloride, potassium chloride, dibasic sodium phosphate, and monobasic sodium phosphate were purchased from Sigma (St. Louis, MO). D3-carnitine was purchased from Cambridge Isotopes (Andover, MA). Acetonitrile was purchased from Fisher Scientific (Pittsburg, PA). Heptafluorobutyric acid was purchased from Thermo Scientific (HFBA) (Waltham, MA).

Reagents

Carnitine and D3-carnitine stock solutions (1 mg/mL) were prepared in Milli-Q water. In order to verify stock solution accuracy for carnitine, duplicate stock solutions were prepared from independent weighings. The solutions were combined if their area percent difference was less than five percent (measured by LC–MS–MS). Working internal standard solution was prepared by mixing 121 µL of the internal standard stock solution and 14.879 mL of Milli-Q water. Surrogate matrix solution was prepared by dissolving sodium chloride, potassium chloride, dibasic sodium phosphate, monobasic sodium phosphate, and bovine serum albumin in Milli-Q water such that the final concentrations were 137 mM, 2.7 mM, 4.3 mM, 1.47 mM, and 40 g/L, respectively. Standard calibrator (SC) solutions for carnitine were prepared in surrogate matrix solutions from the carnitine stock solution such that the final concentrations were 200, 100, 50, 20, 10, 5, and 1 µM. Quality control (QC) samples for carnitine were prepared in surrogate matrix solution from the carnitine stock solution such that the final concentrations were 100, 20, and 5 µM.

Samples

Samples were obtained from healthy volunteers or from residual, anonymized clinical samples.

Sample preparation

Fifty microliters of sample, standard, QC, or blank was transferred to a 1.5 mL polypropylene tube. For clinical samples, two aliquots were prepared, one for free carnitine and one for total carnitine. To the total carnitine tube, 50 µL of 1 M KOH was added, the tube was mixed at 65°C for 15 min, then 50 µL of 1 M HCl was added. Fifty microliters of working internal standard solution was then added. Protein precipitation was accomplished through the addition of 100 µL of 0.1% formic acid in acetonitrile. Samples were then briefly vortexed and centrifuged at 13,000 RPM for 5 min. Following centrifugation, 100 µL of the supernatant was transferred to an autosampler vial. Finally, 100 µL of 0.1% formic acid in Milli-Q water was added to each vial.

High-performance liquid chromatography

Reverse phase

All chromatography experiments were performed on a Waters 2795 separation module using a Waters Symmetry C8 column (2.1 × 100 mm, 3 µm). The injection volume was 5 µL. Mobile phase A consisted of 0.1% formic acid and 0.02% HFBA in Milli Q water. Mobile phase B consisted of 0.1% formic acid and 0.02% HFBA in acetonitrile. Analytes were separated using a gradient elution profile where mobile phase B changed from 3% to 8% over 5 min. The column was then flushed using 95% mobile phase B for 1 min, then re-equilibrated for 4 min. A flow rate of 300 µL/min was used for all separations.

HILIC

All chromatography experiments were performed on a Waters 2795 separation module using a Waters Atlantis HILIC Silica column (2.1 × 100 mm, 3 µm). The injection volume was 5 µL. Mobile phase A consisted of 0.1% formic acid and 20 mM ammonium acetate in Milli-Q water. Mobile phase B consisted of 0.1% formic acid and 20 mM ammonium acetate in 90% acetonitrile–10% Milli-Q water. Mobile phase B was used for the needle wash. Analytes were separated using a gradient elution profile where mobile phase B changed from 70% to 40% over 5 min. The column was held at 40% mobile phase B from 5–6 min then re-equilibrated for 4 min at 70% mobile phase B. The flow rate throughout the separation was 300 µL/min.

Mass spectrometry

All experiments were performed on a Micromass Quatro Micro triple quadrupole mass spectrometer, operated in positive ion mode (electrospray ionization). Nitrogen was used for the source gas and argon was used for the collision gas. The capillary voltage was 1 kV, and the cone voltage was 20 V. A source temperature of 150°C and desolvation temperature of 400°C were used for all analysis. For quantification, the instrument was operated in selected reaction monitoring mode (SRM) at unit resolution. SRM transitions were (*m/z* 162 → 102) for carnitine and (*m/z* 165 → 102) for D3-carnitine. SRM transitions were determined and optimized by infusing dilute stock solution via a T junction and monitoring the fragments of *m/z* 162. A 100 ms dwell time was used between transitions. A 20 eV collision energy was used for collision induced dissociation.

Post column infusion ion suppression

A solution of carnitine and carnitine internal standard (100 µM each in acetonitrile) was infused post column via a T junction. A matrix blank was prepared and analyzed (See experimental section for details on sample preparation). The SRM transitions for carnitine and D3-carnitine were continuously monitored to determine suppression regions.

Data analysis

Data analysis was performed using Masslynx/Quanlynx version 4.1. A quadratic fit with 1/x2 weighting was used for quantification. The best fit and weighting were determined through construction of a residuals plot using a sum of squared deviations approach. Accuracy was determined by calculating the percent deviation from fit. Precision was determined by calculating the relative standard deviation for a given QC level.

Results and Discussion

Mass spectrometry

Product ion scans were performed on carnitine in order to select the best transition for selected reaction monitoring. Using a syringe pump a carnitine solution was infused post column, into the mass spectrometer. Following optimization of Q1 parameters, product scans of *m/z* 165 were obtained while ramping the collision energy. Data is shown in Figure 2B. Fragments were tentatively identified as 3-carboxy-2-hydroxyprop-1-ylium (*m/z* 102.4), N,N,N-trimethyl(oxo)methanaminium (*m/z* 101.8), 3-carboxyprop-2-en-1-ylium (*m/z* 84.5), and N,N-dimethylmethanaminium (*m/z* 59.5). Further data supporting these fragment assignments were obtained by repeating the above experiment using a solution of D3-carnitine. As can be seen in Figure 2A, fragments containing the deuterated methyl group are shifted by 3 daltons, suggesting that the structural assignments in the figure are correct. The 3-carboxy-2-hydroxyprop-1-ylium ion at *m/z* 102.4 was chosen for the fragment to be used for SRM due to its favorable intensity and stability.

Chromatography

While reverse-phase chromatography is the most popular chromatographic mode, it is not well suited for analysis of highly polar compounds, due to their poor retention on the column.

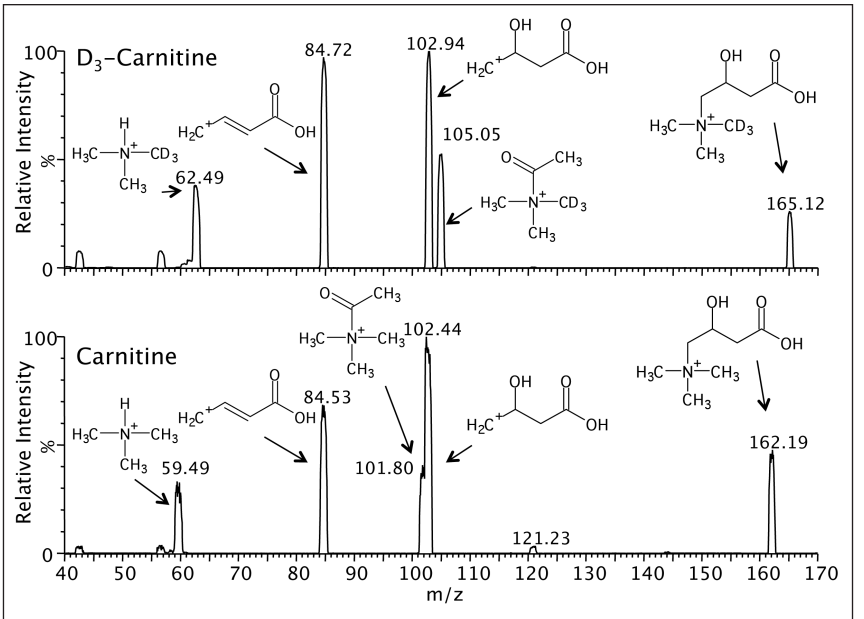

Figure 2. Product ion spectra of carnitine and D3-carnitine. The molecular ions are present at *m/z* 165 and *m/z* 162 Putative ion fragment structures are shown.

Poorly retained compounds often elute in a chromatographic region that is highly susceptible to ion suppression. Consequently, signal response from these compounds is diminished and unstable. Use of ion-paring agents has been employed as a means to increase the retention of highly polar compounds with a fair degree of success. However, this is not a universal solution. While developing and optimizing the chromatographic conditions for the carnitine assay, we initially tried using a traditional reverse approach coupled with the ion pairing agent heptafluorobutyric acid. A neat solution of 10 µM carnitine and 100 µM D3-carnitine in acetonitrile was used for chromatographic method development. Further details of the chromatographic conditions may be found in the Experimental Section. Results are shown in the bottom two panels of Figure 3, which correspond to the SRM transitions for the internal standard (Figure 3C) and analyte (Figure 3D), respectively. Under these conditions, carnitine had a retention time of 2 min. Using an approximated dead volume of 220 µL, the retention factor for carnitine is 1.6, a region that is prone to ion suppression. As can be seen from the figure, carnitine was not detected under these conditions, likely due to poor chromatographic characteristics adversely affecting ionization. Consequently, we used a HILIC column in an effort to improve the retention characteristics of carnitine (Details of the chromatography conditions may be found in the Experimental section). Results are shown in the top two panels of Figure 3. Implementation of the HILIC column resulted in a calculated retention factor of 3. Not only is this chromatographic region ideal with respect to optimizing resolution versus chromatographic time, but it is also removed from the typical suppression region. Comparing the results obtained using reverse phase and HILIC separations, the advantages of HILIC become apparent. In Figure 3D, carnitine is not detected, presumably due to significant ion suppression and poor desolvation due to the high aqueous content of the mobile phase. However, signal-to-noise increased from 1.5 to 150 for carnitine when the HILIC separation was used. This significant improvement can be attributed to improved desolvation and decreased ion suppression. Due to the superior performance observed with the HILIC method we chose to use this LC technique for the assay.

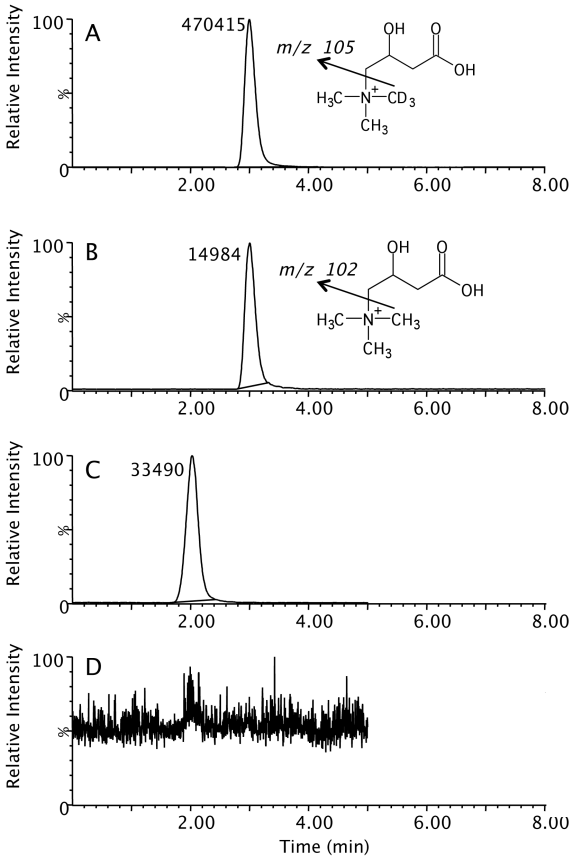

Figure 3. Selected reaction monitoring chromatogram comparing chromatographic performance of the analyte and internal standard when using HILIC conditions at *m/z* 165 → 102 (A) and *m/z* 162 → 102 (B) and using reverse phase conditions *m/z* 165 → 102 (C) and *m/z* 162 → 102 (D).

Despite the improvement in chromatographic performance and signal response that was observed when comparing HILIC and reverse phase separations, it was still necessary to determine if suppression effects were influencing the instrument response with the HILIC separation. In order to accomplish this, a solution of carnitine and carnitine internal standard (100 µM each in acetonitrile) was infused post column via a T junction. A matrix blank was then injected and analyzed (See experimental section for details on sample preparation).

The direct infusion of analyte will result in an elevated baseline of the SRM chromatogram. Any compounds present in the matrix that contribute to ion suppression, and their retention behavior, will be evident as dips in the elevated baseline. The results of the experiment are shown in Figure 4. As can be seen in the figure, a significant suppression region exists prior to the elution of carnitine. Based on these results, no further manipulation of the chromatographic parameters was done.

Method performance and evaluation

Multiple carnitine analysis methods require derivitization and/or sample dry down followed by reconstitution, thereby increasing sample preparation time. We have developed a

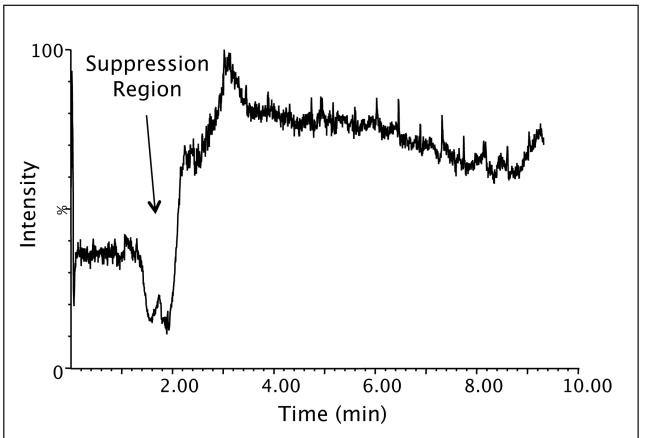

Figure 4. Post column infusion suppression experiments. SRM chromatogram illustrating ion suppression regions. A dilute solution of carnitine and D3-carnitine was infused post column into the mass spectrometer. A matrix blank was prepared and analyzed as described in the experimental section. Analyte suppression regions are shown in the figure.

simple protein precipitation technique that requires no derivitization or dry down steps. Using this sample preparation technique, and the chromatography conditions discussed previously, method performance was evaluated for the analysis of total and free carnitine. Standard calibrators were prepared at 1, 5, 10, 20, 50, 100, and 200 µM. The signal to noise ratio at the lower limit of quantification (1 µM) was approximately 100 (peak to peak). Two standard curves were prepared for each run and placed at the beginning and end of the run. Curve type and curve weighting were determined using a least squares deviation approach. Standard calibrators were deemed suitable if their percent deviation from fit was less than 15%, or 20% at the lower limit of quantification (LLOQ). Method performance was determined by evaluating QC precision and accuracy. QC samples were prepared at low, mid, and high concentrations of carnitine, with $n = 6$ replicates at each concentration. Method suitability was evaluated using the criteria that QC deviation from fit and QC relative standard deviation must be equal to or less than 15%.

Three analytical runs were performed to assess intra and inter day method performance. Data for method performance (inter and intra run) are shown in Table I. As can be seen from the results in Table I, the method appears to be both precise and accurate. Additional parameters tested included long term matrix stability, room temperature matrix stability, and freeze thaw stability (3 freeze thaw cycles). Stability samples were prepared at two concentrations in the same manner as described in the experimental section, with the exception that long term stability samples were stored at –20°C for 90 days, short term stability samples were stored at room temperature for 24 hours, and freeze thaw stability samples were subjected to 3 freeze thaw samples. The stability samples were run with freshly prepared SC and QCs. Carnitine was observed to be stable under all conditions tested (data not shown). Matrix and reagent blanks were analyzed and analyte response was observed to be less than 20% of the analyte response at the LLOQ for all runs (data not shown). Finally, carryover was investigated by injecting a reagent blank after injection of a sample at the upper limit of quantification. Carryover impact was evaluated by comparing analyte signal in this sample to the analyte response in the LLOQ samples. Significant carryover was not observed for the assay.

Table I. The Intra- and Inter-Run Method Performance Statistics*

	Average Conc. (µM)	Standard Deviation	Precision (%CV)	Accuracy
Analytical run 1				
QC-5	4.9	0.5	10.5	2.7
QC-20	20.5	1.5	7.4	–2.5
QC-100	103.3	10.8	10.5	–3.3
Analytical run 2				
QC-5	5.3	0.6	11.9	–5.0
QC-20	19.1	2.3	12.0	4.6
QC-100	96.5	9.2	9.5	3.5
Analytical run 3				
QC-5	5.7	0.4	7.2	–13.3
QC-20	20.8	1.8	8.8	–4.2
QC-100	97.7	11.9	12.23	2.3
Interday results				
QC-5	5.3	0.5	9.9	–5.2
QC-20	20.1	1.9	9.4	–0.7
QC-100	99.2	10.6	10.7	0.8

* Six replicates were used for each QC level (5, 20, and 100 µM). Shown for each QC level is the average concentration, standard deviation, precision (expressed as percent coefficient of variation), and percent accuracy (expressed as percent deviation from fit).

Serum sample analysis

Ideally, when developing LC–MS assays, calibrators and standards should be made up in the same matrix as the analyte. Endogenous compounds can be problematic in that it is sometimes impossible to obtain analyte-free plasma. A potential solution to this dilemma is to use a standard addition approach, where carnitine would be spiked into plasma and the endogenous concentration of carnitine would be calculated and accounted for. However, we did not want to use this approach, as it might be possible for acetylated carnitine to undergo hydrolysis, thereby changing the carnitine concentration in the standard calibrator or quality control sample. Consequently, we used a surrogate plasma solution designed to approximate possible matrix effects. In order to determine if the developed method was suitable for plasma analysis, plasma samples of known total and free carnitine were analyzed. The results are shown in Table II. As can be seen from the table, good agreement exists between the known values of the standards and the values that were determined using the HILIC–MS–MS method, suggesting that the method is indeed suitable for analysis of total and free carnitine in plasma. It should be noted that total carnitine is the sum of free carnitine and carnitine which exists as acylcarnitine.

Table II. A Comparison of Serum Results Obtained with HILIC–MS–MS Method with Known Standards

Sample ID	HILIC–MS–MS Serum Conc. (µM)	Known Serum Conc. (µM)	Percent Difference
Sample 1 Free	31.2	28.4	–9.4
Sample 1 Total	37.6	30.9	–19.6
Sample 2 Free	35.5	31.9	–10.7
Sample 2 Total	44.1	43.3	–1.8
Sample 3 Free	50.1	46.9	–6.6
Sample 3 Total	63.9	62.4	–2.4
Sample 4 Free	42.4	48.8	14.0
Sample 4 Total	57.7	57.6	–0.2
Sample 5 Free	28.4	30.2	6.1
Sample 5 Total	38.5	35.5	–8.1

Conclusion

An LC–MS–MS method for the quantification of total and free carnitine has been developed. Unlike other methods that require derivitization, typically through formation of esters, the present method uses simple protein precipitation. The advantages of this are two-fold. First, sample preparation takes approximately 5 min, making the method amenable to high-throughput formats. Second, the derivitization procedures utilized in other methods are typically acid catalyzed. Acidic conditions result in ester hydrolysis. Consequently, the total and free carnitine values may be artificially altered and thus contribute to the formation of ex-vivo artifacts. As the present assay is not subjected to these conditions, it is potentially more reflective of the true endogenous concentrations. Furthermore, the assay was shown to be accurate and precise, through statistical evaluation of QC performance over the analytical range of the assay. Finally, the advantages of employing HILIC chromatography for the analysis of total and free carnitine were demonstrated.

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