Measurement of Stable Isotopic Enrichment and Concentration of Long Chain Fatty Acyl-carnitines in Tissue by Ion-pairing HPLC / MS

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Running title: Quantitative method for long chain fatty acyl-carnitines

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Abstract:

We have developed a new method for the simultaneous measurements of stable isotopic tracer enrichments and concentrations of individual long chain fatty acyl-carnitines in muscle tissue using ion-pairing high performance liquid chromatography - electrospray ionization quadrupole mass spectrometry in the selected ion monitoring (SIM) mode. Long chain fatty acyl-carnitines were extracted from frozen muscle tissue samples by acetonitrile / methanol. Base-line separation was achieved by a reversed phase HPLC in the presence of the volatile ion-pairing reagent, heptafluorobutyric acid (HFBA). The SIM capability of a single quadrupole mass analyzer allows further separation of the ions of interest from the sample matrixes, providing very clean total and selected ion chromatograms that can be used to calculate the stable isotopic tracer enrichment and concentration of long chain fatty acyl-carnitines in a single analysis. The combination of these two separation techniques greatly simplifies the sample preparation procedure and increases the detection sensitivity. Applying this protocol to biological muscle samples proves it to be a very sensitive, accurate and precise analytical tool.

Keywords:

Stable isotope enrichment, Concentration, Long chain fatty acyl-carnitine, Muscle tissue, Ion-pairing HPLC, Mass spectrometry
Introduction:

Carnitine (L-3-hydroxy-4-aminobutyrobetaine), and its acyl esters (acyl-carnitines) are central in the pathway of the oxidation of fatty acids. Carnitine combines with fatty acyl-coenzyme A (CoA) esters to form acyl-carnitines in order to transport fatty acids from the cytosol into the mitochondria for oxidation\(^1\). Fatty acyl-carnitines are therefore the direct precursors for oxidation of long chain fatty acids. The measurement of their isotopic enrichments is useful for calculating rates of fatty acid oxidation accurately since the measurement of the rate of oxidation of any substrate with a stable isotopic tracer involves dividing the rate of excretion of the labeled CO\(_2\) by the precursor enrichment. Use of the acyl-carnitine as a precursor enrichment, rather than plasma free fatty acids (FFA) would control for any dilution of the plasma enrichment by intracellular unlabelled products. Further, by comparing the measured enrichment of fatty acyl-carnitines with the corresponding plasma FFA enrichment, it is possible to distinguish the relative contributions of plasma FFA and other intracellular sources of fatty acids (i.e., intracellular triglyceride) as precursors for fatty acid oxidation\(^2\).

Simultaneous measurement of the concentration of acyl-carnitines is useful for a variety of applications, including determining points of regulation of fatty acid metabolism\(^3\). When combined with the concentration of fatty acyl-CoA, the measurement of acyl-carnitine concentration can provide an estimation of the activity of the carnitine palmitoyl transferase I (CPT1) enzyme, which is responsible for the binding of the fatty acyl-CoA and carnitine, and thus the initial step in the transport of fatty acids into the mitochondria for oxidation. CPT1 is the rate-limiting step in the oxidation of fatty acids in a number of circumstances, so the estimation of its activity in varied physiologic states is central to understanding the regulation of fatty acid
metabolism. Thus, a method to measure both fatty acyl-carnitine concentration and enrichment would be useful for a variety of reasons. In addition to measuring the total concentration, this method allows for the identity of the FFAs in the acyl-carnitines. Thus, if there is a difference in the profile of the FFAs from varied sources, i.e. phospholipid or plasma free FFA, the profile may be matched to determine the source of the FFA.

There is currently no method described to simultaneously measure stable isotopic enrichment and concentration of individual long chain fatty acyl-carnitines. Previous reports have focused on free and total carnitine screening and quantitative short and medium chain acyl-carnitine profiles on blood, plasma or urine samples using a radio-enzymatic method, HPLC, capillary electrophoresis (CE) and recently ESI/MS/MS in the field of newborn screening. These methods are either time-consuming with limited information concerning individual acyl-carnitines, especially long chain fatty acyl-carnitines, or involve the use of expensive instruments with complicated sample preparation and derivatizations, which usually cause a loss of sensitivity, difficulties in interpreting the spectrum and measuring stable isotopic enrichment due to possible incomplete derivatizations, hydrolysis of acyl-carnitines or limited quantitative dynamic range of the mass analyzer, such as ion trap. The selected ion monitoring (SIM) technique enabled by quadrupole mass analyzers has been widely used in the field of tracer methodology to achieve highly sensitive, accurate and precise measurement of stable isotopic tracer enrichment and concentration. This technique has been extensively applied in the GC/MS analysis, but its application to LC/MS is not as well documented. In this paper, we report the use of SIM analysis combined with ion paring HPLC to simultaneously detect the stable isotopic enrichments and concentrations of six individual long chain fatty acyl-carnitines extracted from frozen tissue samples. We found that this method is simple, sensitive, accurate and precise.
Materials and Methods:

The chemical structures of the long chain fatty acyl-carnitines analyzed and the ions used for SIM monitoring are shown in Table 1.

1. Reagents

Long chain acyl-carnitine standards were purchased from Sigma (St. Louis, MO, USA) as chloride salts and stored at –20°C. Deuterated internal standard d₃-palmitoyl-carnitine was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and was prepared in a stock solution by dissolving in 3:1 Acetonitrile / Methanol to obtain a concentration of 1 ng/µl and stored at –80°C. Uniformly ¹³C labeled palmitoyl-carnitine was purchased from Isotec Inc. (St. Louis, MO, USA) as the chloride salt and stored at –20°C. Heptafluorobutyric acid (HFBA) 99% was purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile and methanol were of HPLC-grade from Fisher (Fair Lawn, NJ, USA). Silica gel (230~400 mesh, 60Å) used for solid phase extraction (SPE) was purchased from Aldrich (Milwaukee, WI, USA).

2. Instrumentation

The HPLC/MS analysis was performed using an Agilent 1100 series liquid chromatograph – 1956B SL single quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with a binary gradient pump, a heated column compartment, an auto-sampler, and was equipped with an electrospray ionization source. The system was controlled by LC/MSD Chem-Station.
software Rev. A.10.02 (Agilent Technologies). The analytical column was a reversed phase Zorbax Eclipse XDB-C8 4.6 × 150 mm, 5µm with corresponding guard cartridge (Agilent Technologies). GC/MS analysis was performed using an Agilent 6890 GC – 5973 MSD system (Agilent Technologies, Palo Alto, CA, USA) with an electron impact ion source, split-splitless injector, and an auto-sampler. A Supelco omega-wax 250 capillary column 30 m × 0.25 mm × 0.25 µm was used.

3. Analytical conditions

HPLC separation was achieved by means of a binary gradient with a volatile ion-pairing reagent that consisted of 0.05% heptafluorobutyric acid in water (A) and acetonitrile (B) at a flow rate of 1 ml/min. The gradient started at 10% B, increased to 80% B over 16 min and stayed at 80% for 9 min. The post-run re-equilibrium time was 5 min for a total run time of 30 min. A faster run could be achieved by increasing B from 10% to 80% over 4 min when running the standard samples or only measuring isotopic enrichments. The column temperature was 30°C during the run and 4°C for the auto-sampler. The sample injection volume was 1~10 µl.

Electrospray mass spectrometry was performed in the positive ion mode. Electrospray ion source parameters were: drying gas flow: 13 L/min; nebulizer pressure: 60 psi; drying gas temperature: 350°C; capillary voltage: 400 V; fragmentor voltage: 200 eV. The detector was turned on from 15 min till 25 min. The ions listed in Table 1 were monitored in the SIM mode. Scan mass range was 200 ~ 600 amu when run in the scan mode. The mass spectrometer was tuned with Agilent electrospray tuning mix solution and checked daily before running any samples. All standard and
tissue samples were analyzed in duplicate by LC/MS and the average values were used for the calculations.

GC/MS conditions were: positive-ion EI mode, injector temperature 250°C; He constant flow 1 ml/min; column temperature started at 100°C for 1 min, and then increased at the rate of 15°C/min to 260°C and kept for 2 min; solvent delay time 9 min. Ions at m/z 270.2 and 286.2 were monitored for unlabelled-palmitate and U-\textsuperscript{13}C\textsubscript{16}-palmitate respectively under SIM mode.

4. Biological sample preparation

Male New Zealand white rabbits (Myrtle’s Rabbitry, Thomson Station, TN), weighing 4-5 kg, were anesthetized with ketamine and xylazine after a 48 hours fast. An incision was made on the neck for placement of catheters in the carotid artery and jugular vein, and for tracheotomy. U-\textsuperscript{13}C\textsubscript{16}- or d\textsubscript{2}-palmitate as potassium salt was bound to albumin and infused via the jugular vein. For the study to analyze the reproducibility of sample analysis, two rabbits were infused with U-\textsuperscript{13}C\textsubscript{16}- palmitate at 0.114 and 0.355 µmol/kg/min respectively, and tissue samples were collected from the adductor muscle of the hindlimb at 5 hours. For biological inquiry on the relationship between plasma FFA and tissue long chain fatty acyl-carnitines, another four rabbits were infused with either U-\textsuperscript{13}C\textsubscript{16}- or d\textsubscript{2}-palmitate (n = 2 each) at 0.2-0.4 µmol/kg/min for 2 hour. Blood samples were taken from the arterial catheter and tissue samples were taken from the adductor muscle in the hindlimb before the tracer infusion and every 30 min during the tracer infusion. Plasma was separated from blood by centrifugation and stored at -20°C. Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C for later analysis. This protocol
complied with NIH guidelines and was approved by the Animal Care and Use Committee of The University of Texas Medical Branch.

Plasma samples were extracted, isolated and derivatized to their methyl esters for measurement of U-\textsuperscript{13}C\textsubscript{16}-palmitate enrichment by GC/MS.\textsuperscript{25} The frozen muscle sample (20-50 mg) was pulverized into a fine powder under liquid nitrogen using a medium size tissue pulverizer (Spectrum Laboratories Inc. Rancho Dominguez, CA, USA). 50 µl freshly made 1 M potassium phosphate monobasic KH\textsubscript{2}PO\textsubscript{4} solution and 20 µl 1 ng/µl internal standard solution were added to the sample. 1 ml freshly made extraction solution of 3:1 Acetonitrile/Methanol (v/v) was then added, and the resulting mixture was vortexed for 2 min and further homogenized with a mechanical grinder for 2 min. After centrifugation at 14,000 g for 20 min at 4°C, the supernatant was transferred to a 1.5ml plastic centrifuge tube and dried under gentle N\textsubscript{2} flow. 100 µl of 3:1 Acn/MeOH was then added and vortexed for 5 min and sonicated for 15 min. The sample was then centrifuged at 14,000 g for 20 min at 4°C, and the clear solution was carefully transferred into analytical vials and kept at -20°C until LC/MS analysis.

The above procedure was compared with additional use of SPE purification. After the above procedure was completed, the extraction supernatant was decanted onto a 0.5 ml silica gel column (made with 300 mg 230-400 mesh, 60Å silica gel in 3:1 Acn / MeOH). The column was washed with 2 ml methanol followed by 1 ml of 1% acetic acid in methanol. Carnitines were eluted with 4 ml 1% acetic acid in methanol. The elute was dried under N\textsubscript{2} and re-dissolved in 100µl 3:1 Acn/MeOH. The mixture was then centrifuged and filtered at 14,000g for 3 hr for LC/MS analysis.
5. Method validation

5.1 Recovery study

The recovery study was carried out by comparing the LC/MS peak areas of a standard sample with and without processing as described above. A mixed standard solution was prepared by dissolving acyl-carnitine standards in 3:1 Acn/MeOH. 100 µl of the standard solution was directly analyzed by LC/MS, while another 100 µl solution was dried under gentle N₂ flow and processed as described above and re-dissolved in 100 µl 3:1 Acn/MeOH for LC/MS analysis. The peak areas for acyl-carnitines [M+0]⁺ ions were integrated and compared. This parallel experiment was carried out three times and the average recovery was calculated.

“In vivo” extraction efficiency has been estimated by comparing the peak areas of internal standard recovered from a spiked muscle sample and from a pure solution, and they were very close. In addition, after the extraction procedure, the remaining tissue was subjected to further extraction with a 2nd portion of extraction solution, and only trace amount of acyl-carnitines were detected, indicating nearly complete of extraction from the muscle sample.

5.2 Isotopic ratio measurement accuracy

To test the accuracy of the isotopic ratio measurement, a series of standardized solutions of U-\(^{13}\)C₁₆-palmitoyl-carnitine and unlabeled palmitoyl-carnitine with the molecular ratio ranging from 0.0002 to 1 were prepared by mixing their stock solutions, followed by dilution to the same concentration. The mixed solutions were dried under N₂ flow, processed as the above described
procedure, and analyzed by LC/MS under SIM mode. The calculated isotopic ratio and measured isotopic ratio were compared and a standard curve was drawn.

5.3 Quantification accuracy

The quantification accuracy was determined by comparing the added theoretical concentration with the measured concentration by using the internal standard method. Standardized solutions were prepared at seven concentration levels at 50, 100, 200, 400, 600, 800 and 1000 pg/µl in 3:1 Acn/MeOH. To 100 µl of such solution, 20 µl of 1 ng/µl D3-palmitoyl-carnitine solution was added as internal standard. The mixed solutions were dried under gentle N2 flow, and processed as described above, re-dissolved in 100 µl 3:1 Acn/MeOH, and analyzed by LC/MS under SIM.

5.4 Sensitivity

Sensitivity was determined with the standard solutions of as low as 0.0002 tracer / tracee ratio of U-13C16-palmitoyl-carnitine / palmitoyl-carnitine, 20 pg/µl of concentration, and with 10 mg tissue samples respectively.

5.5 Reproducibility and reliability studies

Two independent rabbit muscle samples taken after 5 hours U-13C16-palmitate infusion were used to determine the method reproducibility. To test the sample-to-sample reproducibility, these two muscle samples were prepared and analyzed by LC/MS six times from tissue amounts ranging from 15 to 90 mg. The isotopic enrichment and concentrations of individual long chain acyl-
carnitines were calculated and compared. In order to study day-to-day reproducibility, the samples were analyzed on three different days.

Results and Discussion:

1. Tissue extraction

Long chain fatty acyl-carnitines can be readily extracted from frozen tissue by using the plasma extractions conditions with some modifications. Long chain acyl-carnitines are highly polar molecules, and therefore tend to dissolve in polar solvents. 3:1 Acn/MeOH was found to be a good extraction solvent system for tissue samples. We found that tissue samples must be ground into a fine powder at liquid N<sub>2</sub> temperature before extraction. A special tissue pulverizer was very helpful in this step. It enabled the frozen tissue to be pulverized into a fine powder, while also making it possible to collect the sample without cross contamination and to obtain a precise weight of the tissue. Potassium phosphate monobasic KH<sub>2</sub>PO<sub>4</sub> was added to provide an acidic environment in which to stabilize the acyl-carnitines during the extraction. The homogization process is also an essential step to assure the completion of the extraction of the acyl-carnitines.

Solid phase extraction (SPE) has been frequently used to provide a further purification and enrichment of acyl-carnitine species before HPLC, CE or tandem mass spectrometry analysis. However, with the combination of ion-pairing HPLC and the selected ion monitoring separation, the SPE step can be avoided, which could reduce the sample preparation time and improve the recovery and analytical sensitivity. We found that even without the SPE, both the total ion chromatograms (TIC) and selected ion chromatograms (SIC) resulting from the
biological samples were reasonably clean with few impurities. The guard column and electrospray ion source did not require cleaning after analyzing hundreds of samples. Using the simplified sample preparation procedure without SPE, the extraction recoveries for long chain acyl-carnitines were found to be over 75%.

2. **Analytical conditions**

Ion-pairing HPLC is a good option for long chain fatty acyl-carnitines to improve the chromatographic separation and peak shape. Trace amount of acidic ion-pairing reagent in the mobile phase could effectively react with the basic amino groups of acyl-carnitines to form weakly interacted ion-pair complexes during the HPLC separation to prevent them from interacting with the stationary phase silanol active sites, which usually causes the peak broaden and tailing problem. A volatile ion-pairing reagent must be used to avoid ion suppression in electrospray ionization because it evaporates in the ion source and only the acyl-carnitine molecules will be ionized. Heptafluorobutyric acid (HFBA) was chosen over the commonly used ion-pairing reagent - trifluoroacetic acid (TFA) because HFBA has a longer alkyl chain and therefore will be more attracted to the XDB-C8 column stationary phase, resulting in better retention of the analytes and improved chromatographic separation. Using this approach, long chain fatty acyl-carnitines were separated with good resolution and peak shape (Fig. 1).

The scan positive ion electrospray mass spectrum of long chain acyl-carnitines was clean with few gas phase ionization fragmentations when a proper fragmentor voltage was used. Palmitoyl-carnitine only showed a protonated molecular ion \([M+H]^+\) at \(m/z = 400.4\) and its naturally occurring isotopomer peaks with theoretical isotopic ratios. It is important that the mass spectrum
be clean to avoid co-elution with contaminating impurity peaks because the chance that an impurity compound would have the same molecular ion and the same HPLC retention time as the acyl-carnitines is greatly minimized, which improves the analytical sensitivity and resolution. This phenomenon has been well known in the isotopic tracer enrichments measurement by GC/MS.\textsuperscript{25} Chemical ionization (CI) usually has greater sensitivity and precision than electron impact (EI) ionization because CI is a much “softer” ionization technique with less fragmentation. The electrospray ionization method used in this paper is an even “softer” ionization than CI, so excellent analytical data could be obtained under SIM analysis.

3. Method Validation

a. Accuracy

The accuracy of the isotopic enrichment measurement was tested by analyzing a series of standard stock solutions containing U-\textsuperscript{13}C\textsubscript{16}-plamitoyl-carnitine and unlabelled palmitoyl-carnitine in the molecular ratio range from 0.0002 to 1. Ions at m/z 400.4 and 416.4 were chosen to be monitored under SIM mode. The peak areas of those two selected ions were integrated and their relative ratio was calculated. As shown in Fig 2, the measured tracer-tracee ratio (TTR) closely matched the theoretical TTR with the linear correlation $R^2$ of 0.9998, the slope of 1.0317 and the y-intercept of 0.0009. The values were calculated to be 92.8 \textasciitilde 102.7\% of the known values over the entire range of enrichment, indicating a good analytical accuracy of the isotopic enrichment measurement.
In order to achieve reliable quantification of the concentration of long chain acyl-carnitines by using D3-palmitoyl-carnitine as the internal standard, the isotopic ratio of internal standard and unlabeled acyl-carnitines must be measured accurately. A series of standard stock solutions containing D3-plamitoyl-carnitine and unlabelled palmitoyl-carnitine in the molecular ratio range from 0.001 to 1 were therefore analyzed. Ions at m/z 400.4 and 403.4 were chosen to be monitored under SIM mode. The peak areas of those two selected ions were integrated and their relative ratio was calculated. Different from the case with \([M+16]^+ / [M+0]^+\), the measured ratios must be corrected by subtracting the naturally occurring distribution of \([M+3]^+ / [M+0]^+\), which is 0.51%. As shown in Fig 3, the measured isotopic ratio closely matched the theoretical isotopic ratio and there was an excellent linear relationship over the entire range with the linear correlation \(R^2\) of 0.9986, the slope of 1.0185 and the y-intercept of −0.0051. A standard curve is shown in Fig 4. There was a linear relationship between the expected and the measured isotopic ratio \([M+0]^+ / [M+3]^+\) of palmitoyl-carnitine.

The accuracy of the concentration measurement was tested by analyzing a series of standard solutions containing long chain fatty acyl-carnitines at concentration levels from 50 pg/µl to 1000 pg/µl containing known amounts of D3-palmitoyl-carnitine as internal standard. All the standard solutions were processed as described above. Base mass peaks \([M+0]^+\), which were actually the protonated acyl-carnitines \([M+H]^+\) ions, were monitored under SIM mode and their peak areas were integrated and the relative ratios with the peak area of internal standard were calculated. The concentrations of long chain acyl-carnitines were calculated based on the peak area ratios and amounts of internal standard. As shown in Table 2, the measured concentration of palmitoyl-carnitine was nearly identical to the calculated theoretical concentration over seven different concentrations. The accuracy of the palmitoyl-carnitine concentration measurement was between
95~102% with relative standard deviation (RSD) less than 4%. With the same method, the accuracies of the concentration measurements were calculated to be between 91~103% with RSD less than 5% for other long chain acyl-carnitines.

b. Sensitivity

With the above sample preparation and analytical procedure, it was found that tissue samples as small as 10 mg could be reliably quantified, which is convenient for biopsies obtained using a Bergstrom needle in human subjects, which are generally 50 mg or less. It is not recommended to use less than 10 mg tissue sample because the possibility of contamination during sample preparation is increased. The limit of concentration detection was found to be 20 pg/µl. The stable isotopic ratio detection limit was 0.001.

c. Reproducibility of Biological Samples

Two rabbit muscle samples were used to study the biological sample analytical reproducibility for isotopic enrichment and concentration measurements. Using the above sample preparation and analytical conditions, a clean total ion chromatogram was obtained (Fig 1) with base line chromatographic separation of long chain acyl-carnitines. The selected ion chromatograms for each long chain fatty acyl-carnitine were even cleaner than the total ion chromatogram with few impurities, and even with a fast gradient, a clean selected ion chromatogram could be obtained. As shown in Fig 5, [M+0]⁺, [M+1]⁺, [M+3]⁺ and [M+16]⁺ could be clearly detected and integrated for the calculations.
Six replicate muscle samples with weights ranging from 15 to 90 mg were prepared from each rabbit sample. Table 3 shows the isotopic enrichment measurement reproducibility results of these replicate samples over three days. For muscle sample A, the TTR was measured to be $0.01966 \pm 0.00081$ with a RSD of 4.142% over 18 analyses. Sample B had a TTR of $0.05265 \pm 0.00140$ with a RSD of 2.664% over 18 analyses. Both samples showed excellent sample-to-sample and day-to-day reproducibility. As shown in Table 4, six individual long chain fatty acyl-carnitines were quantified by using the internal standard method. Their concentrations show excellent sample-to-sample and day-to-day reproducibility. These results demonstrated that the analytical method was reliable for the measurement of stable isotopic enrichment as well as concentration in a biological matrix.

4. Biological application

A close relationship between the enrichment of plasma FFA and tissue long chain fatty acyl-carnitines was found in the samples from rabbits used in the reproducibility study described above. The more stable isotopic tracer was infused into blood, the higher the tissue long chain fatty acyl-carnitines’ enrichment was. In order to demonstrate this more clearly, a further study was designed and performed, as described above in the experimental section. Over 2 hrs of infusion, blood and tissue samples were collected at 4 different time points, and were analyzed by GC/MS and LC/MS, respectively. The measured tissue palmitoyl-carnitine enrichments were compared with the corresponding plasma palmitate enrichments for assessment of percent contribution of plasma FFA to muscle fatty acid oxidation. As shown in Fig 6, palmitoyl-carnitine enrichments in the muscle were constantly lower than palmitate enrichments in the
plasma, yet the enrichments of both increased in apparent parallel through the duration of the infusion. This finding indicates that plasma FFA is an important source of fatty acid oxidation in the muscle. After 48 hours fast, plasma FFA accounted for 44 ± 3% (means ± SE) of fatty acid oxidation in the muscle [(palmitoyl-carnitine enrichment / plasma palmitate enrichment) × 100%].

Conclusion:

We have found that ion-pairing HPLC / electrospray ionization quadrupole mass spectrometry under SIM mode is a simple, sensitive, accurate and precise analytical tool to simultaneously detect the stable isotopic tracer enrichments and concentrations of long chain fatty acyl-carnitines in small samples of muscle tissue. Biological muscle samples with as low as 0.001 tracer / tracee ratio, or 20 pg/µl of acyl-carnitine concentration, or as little as 10 mg tissue can be reliably analyzed with excellent quantification accuracy and sample-to-sample and day-to-day reproducibility. Applying this method to rabbits revealed that after 48 hours fast, plasma FFA accounts for 44% of fatty acid oxidation in the muscle.

Acknowledgements:

This study was funded by Shriners Hospitals for Children Grant 8490, and NIH Grant P30 AG024832. The authors thank Ms. Yunxia Lin for the assistance in the preparation of rabbit muscle samples and Ms. Deborah Viane for the assistance in the preparation of the manuscript.
Table 1. Chemical structure of long chain fatty acyl-carnitines analyzed, and the SIM ions used for quantification.

![Chemical structure of long chain fatty acyl-carnitines analyzed](attachment:image.png)

<table>
<thead>
<tr>
<th>R¹</th>
<th>R²</th>
<th>Name</th>
<th>Abbreviations</th>
<th>SIM ions (m/z)</th>
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<tr>
<td>H₃</td>
<td>OCO(CH₂)₁₂CH₃</td>
<td>Myristoyl-carnitine</td>
<td>C14:0</td>
<td>372.4</td>
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<tr>
<td>H₃</td>
<td>OCO(CH₂)₁₄CH₃</td>
<td>Palmitoyl-carnitine</td>
<td>C16:0</td>
<td>400.4</td>
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<tr>
<td>D₃</td>
<td>OCO(CH₂)₁₄CH₃</td>
<td>D₃-Palmitoyl-carnitine</td>
<td>D₃-C16:0</td>
<td>403.4</td>
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<tr>
<td>H₃</td>
<td>O¹³CO(CH₂)₁₄¹³CH₃</td>
<td>U-¹³C₁₆-Palmitoyl-carnitine</td>
<td>U-¹³C₁₆-C16:0</td>
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<td>Linolenoyl-carnitine</td>
<td>C18:3</td>
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Table 2. Accuracy and precision of the measurement of the concentration of palmitoyl-carnitine.  

<table>
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<tr>
<th>Theoretical concentration (pg/µl)</th>
<th>Measured concentration (pg/µl)</th>
<th>Accuracy (%)</th>
<th>S. D. (n=3)</th>
<th>Precision (%)</th>
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<tr>
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<td>101.65</td>
<td>22.99</td>
<td>2.26</td>
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</tbody>
</table>

1. Results are from quantification of internal standard.
Table 3. Reproducibility of measurement of U-\(^{13}\)C\(_{16}\)-Palmitoyl-carnitine enrichment.\(^1,2\)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Replicate</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day to Day</th>
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<td>A</td>
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<td>0.01991</td>
<td>0.01954</td>
<td>0.01838</td>
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<td>0.01961±0.00054</td>
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<td>0.01883</td>
<td>0.01944</td>
<td>0.02068</td>
<td>0.01965±0.00094</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.01984</td>
<td>0.02142</td>
<td>0.02085</td>
<td>0.02070±0.00080</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.01909</td>
<td>0.01878</td>
<td>0.01986</td>
<td>0.01924±0.00056</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.01970</td>
<td>0.01996</td>
<td>0.02096</td>
<td>0.02021±0.00067</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0.01940</td>
<td>0.01961</td>
<td>0.01996</td>
<td>0.01966</td>
</tr>
<tr>
<td>S.D.</td>
<td></td>
<td>±0.00047</td>
<td>±0.00097</td>
<td>±0.00088</td>
<td>±0.00081</td>
</tr>
<tr>
<td>R.S.D.</td>
<td></td>
<td>±2.409%</td>
<td>±4.945%</td>
<td>±4.391%</td>
<td>±4.142%</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>0.05274</td>
<td>0.05234</td>
<td>0.05374</td>
<td>0.05294±0.00072</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.05068</td>
<td>0.05386</td>
<td>0.05197</td>
<td>0.05217±0.00160</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.05265</td>
<td>0.05248</td>
<td>0.05374</td>
<td>0.05296±0.00068</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.05481</td>
<td>0.05379</td>
<td>0.05448</td>
<td>0.05436±0.00052</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.05277</td>
<td>0.05278</td>
<td>0.05356</td>
<td>0.05304±0.00045</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.05107</td>
<td>0.05025</td>
<td>0.05006</td>
<td>0.05046±0.00054</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0.05245</td>
<td>0.05258</td>
<td>0.05293</td>
<td>0.05265</td>
</tr>
<tr>
<td>S.D.</td>
<td></td>
<td>±0.00147</td>
<td>±0.00131</td>
<td>±0.00163</td>
<td>±0.00140</td>
</tr>
<tr>
<td>R.S.D.</td>
<td></td>
<td>±2.497%</td>
<td>±2.989%</td>
<td>±2.978%</td>
<td>±2.664%</td>
</tr>
</tbody>
</table>

1. Sample A and B from rabbit muscle were each analyzed six times on three different days.
2. Values are mean ± S.D. (Standard Deviation)
Table 4. Reproducibility of measurement of concentrations of individual long chain fatty acyl-carnitines.1,2

<table>
<thead>
<tr>
<th></th>
<th>C14:0 (ng/mg)</th>
<th>C16:0 (ng/mg)</th>
<th>C18:0 (ng/mg)</th>
<th>C18:1 (ng/mg)</th>
<th>C18:2 (ng/mg)</th>
<th>C18:3 (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Muscle Sample A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>0.18±0.01</td>
<td>0.86±0.04</td>
<td>0.47±0.07</td>
<td>1.18±0.13</td>
<td>0.56±0.03</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.15±0.01</td>
<td>0.86±0.04</td>
<td>0.39±0.02</td>
<td>1.17±0.05</td>
<td>0.51±0.03</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.15±0.01</td>
<td>0.86±0.04</td>
<td>0.37±0.02</td>
<td>1.12±0.05</td>
<td>0.52±0.04</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Day to day (n = 18)</td>
<td>0.16±0.02</td>
<td>0.86±0.04</td>
<td>0.41±0.06</td>
<td>1.16±0.08</td>
<td>0.53±0.04</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td><strong>Muscle Sample B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>0.14±0.01</td>
<td>0.56±0.02</td>
<td>0.38±0.02</td>
<td>0.84±0.06</td>
<td>0.42±0.05</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.14±0.01</td>
<td>0.56±0.02</td>
<td>0.36±0.01</td>
<td>0.82±0.06</td>
<td>0.41±0.05</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.12±0.01</td>
<td>0.55±0.02</td>
<td>0.33±0.01</td>
<td>0.76±0.05</td>
<td>0.38±0.05</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>Day to day (n = 18)</td>
<td>0.13±0.01</td>
<td>0.56±0.02</td>
<td>0.36±0.03</td>
<td>0.81±0.06</td>
<td>0.40±0.05</td>
<td>0.07±0.01</td>
</tr>
</tbody>
</table>

1. Two different samples (A+B) from rabbit muscle were analyzed six times each on three different days.
2. Values are mean ± S.D. (Standard Deviation)
References:


Figure legends

FIG. 1. Total ion chromatogram of a rabbit muscle sample prepared without SPE purification and analyzed using a slow gradient over 25 min.

FIG. 2. Relationship between theoretical and measured tracer / tracee ratio of $^{13}$C$_{16}$-palmitoyl-carnitine to palmitoyl-carnitine. ($y = 1.0317 x - 0.0009$, $R^2 = 0.9998$ for entire test range of 0.0002~1, and $y = 0.9878 x - 0.0002$, $R^2 = 0.9999$ for zoom-in range of 0.0002~0.1)

FIG. 3. Relationship between theoretical and measured isotopic ratio of D$_3$-palmitoyl-carnitine to palmitoyl-carnitine. ($y = 1.0185 x - 0.0051$, $R^2 = 0.9986$ for entire test range of 0.0002~1, and $y = 0.9469 x - 0.0006$, $R^2 = 0.9998$ for zoom-in range of 0.0002~0.1)

FIG. 4. Standard curve of palmitoyl-carnitine concentration. ($y = 0.0049 x + 0.0276$, $R^2 = 0.9996$). Normal tissue concentration is approximately 150~600 pg/µl.

FIG. 5. Selected ion chromatograms of palmitoyl-carnitine for a rabbit muscle sample analyzed under a fast gradient containing long chain acyl-carnitines:

(a) [M+0]$^+$ - unlabeled palmitoyl-carnitine, as tracee;
(b) [M+1]$^+$ - 1-$^{13}$C-palmitoyl-carnitine, as naturally occurring isotopomer;
(c) [M+3]$^+$ - D$_3$-palmitoyl-carnitine, as internal standard;
(d) [M+16]$^+$ - U-$^{13}$C$_{16}$-palmitoyl-carnitine, as tracer.

FIG. 6. Plasma palmitate and muscle palmitoyl-carnitine enrichments. The values are means ± SE from 4 rabbits infused with either d$_2$- or U-$^{13}$C$_{16}$-palmitate (n = 2 each).
FIG. 1

Total Ion Abundance ($\times 10^5$)

- C14:0
- C16:0 + D3-C16:0
- U-13C16-C16:0
- C18:0
- C18:2
- C18:3
- C18:1

Time (min)

0 2 4 6 8 10 12 14 16

0 6 8 10 12 14 16 18

min
FIG. 2

Theoretical tracer / tracee ratio vs. Measured tracer / tracee ratio.
FIG. 3

Theoretical isotopic ratio $[M+3]^+ / [M+0]^+$

Measured isotopic ratio $[M+3]^+ / [M+0]^+$
FIG. 4

Measured isotopic ratio $[M+0]/[M+3]$ (%) vs. Concentration of Palmitoyl-carnitine (pg/µl)
FIG. 5
FIG. 6

- O - Plasma palmitate  ■ - Muscle palmitoyl-carnitine