methods

Measurement of stable isotopic enrichment and concentration of long-chain fatty acyl-carnitines in tissue by HPLC-MS

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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. Baseline separation was achieved by reverse-phase HPLC in the presence of the volatile ion-pairing reagent heptafluorobutyric acid. 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.—** Sun, D., M. G. Cree, X-j. Zhang, E. Bøersheim, and R. R. Wolfe. **Measurement of stable isotopic enrichment and concentration of long-chain fatty acyl-carnitines in tissue by HPLC-MS. *J. Lipid Res.* 2006.** 47: **431–439.**

Supplementary key words muscle tissue • ion-pairing high-performance liquid chromatography • high-performance liquid chromatography-mass spectrometry

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-CoA esters to form acyl-carnitines to transport fatty acids from the cytosol into the mitochondria for oxidation (1). Therefore, fatty acyl-carnitines are direct precursors for the oxidation of long-chain fatty acids. The measurement of their isotopic enrichments is useful for calculating rates of fatty acid oxidation accurately, because 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 FFAs, would control for any dilution of the plasma enrichment by intracellular unlabeled products. Furthermore, 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 FFAs 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 palmitoyltransferase-1 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. Carnitine palmitoyltransferase-1 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 (3). 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 would allow 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 FFAs), the profile may be matched to determine the source of the FFAs.

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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 radioenzymatic method (4), HPLC (5–11), capillary electrophoresis (12, 13), and, recently, ESI/MS/MS in the field of newborn screening (14–24). 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 as a result of possible incomplete derivatizations, hydrolysis of acyl-carnitines, or the limited quantitative dynamic range of the mass analyzer, such as the ion trap (23, 24). 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 (25). This technique has been applied extensively in GC-MS analysis, but its application to LC-MS is not as well documented (26). Here, we report the use of SIM analysis combined with ion-pairing 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**.

Reagents

Long-chain acyl-carnitine standards were purchased from Sigma (St. Louis, MO) as chloride salts and stored at −20°C. Deuterated internal standard [D_3]palmitoyl-carnitine was purchased from Cambridge Isotope Laboratories (Andover, MA) and was prepared in a stock solution by dissolving in 3:1 acetonitrile-methanol (Acn/MeOH) to obtain a concentration of 1 ng/μl and stored at −80°C. Uniformly ^{13}C-labeled palmitoyl-carnitine was purchased from Isotec, Inc. (St. Louis, MO), as the chloride salt and stored at −20°C. Heptafluorobutyric acid (HFBA) 99% was purchased from Aldrich (Milwaukee, WI). Acetonitrile and methanol were of HPLC grade from Fisher (Fair Lawn, NJ). Silica gel (230–400 mesh, 60 Å) used for solid-phase extraction (SPE) was purchased from Aldrich.

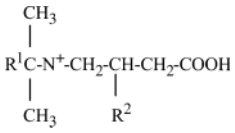
Instrumentation

The HPLC-MS analysis was performed using an Agilent 1100 series liquid chromatograph-1956B SL single quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA) with a binary gradient pump, a heated column compartment, and an autosampler 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 reverse-phase Zorbax Eclipse XDB-C8 4.6 × 150 mm, 5 μm with corresponding guard cartridge (Agilent Technologies). GC-MS analysis was performed using an Agilent 6890 GC-5973 MSD system (Agilent Technologies) with an electron-impact ion source, split-splitless injector, and an autosampler. A Supelco omega-wax 250 capillary column (30 m × 0.25 mm × 0.25 μm) was used.

Analytical conditions

HPLC separation was achieved by means of a binary gradient with a volatile ion-pairing reagent that consisted of 0.05% HFBA 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 postrun reequilibrium 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.

TABLE 1. Chemical structures of long-chain fatty acyl-carnitines analyzed, and the SIM ions used for quantification



R^1	R^2	Name	Abbreviation	SIM Ion
				m/z
H_3	$OCO(CH_2)_{12}CH_3$	Myristoyl-carnitine	C14:0	372.4
H_3	$OCO(CH_2)_{14}CH_3$	Palmitoyl-carnitine	C16:0	400.4
D_3	$OCO(CH_2)_{14}CH_3$	[D_3]palmitoyl-carnitine	D_3-C16:0	403.4
H_3	$O^{13}CO(^{13}CH_2)_{14}{}^{13}CH_3$	[U-$^{13}C_{16}$]palmitoyl-carnitine	U-$^{13}C_{16}$-C16:0	416.4
H_3	$OCO(CH_2)_{16}CH_3$	Stearoyl-carnitine	C18:0	428.4
H_3	$OCO(CH_2)_{14}(CH)_2CH_3$	Oleoyl-carnitine	C18:1	426.4
H_3	$OCO(CH_2)_{12}(CH)_4CH_3$	Linoleoyl-carnitine	C18:2	424.4
H_3	$OCO(CH_2)_{10}(CH)_6CH_3$	Linolenoyl-carnitine	C18:3	422.4

SIM, selected ion monitoring.

The column temperature was 30°C during the run and 4°C for the autosampler. The sample injection volume was 1–10 μl.

Electrospray mass spectrometry was performed in the positive ion mode. Electrospray ion source parameters were as follows: 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 to 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 average values were used for the calculations.

GC-MS conditions were as follows: positive ion electron impact mode; injector temperature, 250°C; He constant flow, 1 ml/min; column temperature started at 100°C for 1 min, 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 unlabeled palmitate and [U-$^{13}C_{16}$]palmitate, respectively, in SIM mode.

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 h fast. An incision was made on the neck for placement of catheters in the carotid artery and jugular vein and for tracheotomy. [U-$^{13}C_{16}$]palmitate or [D_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-$^{13}C_{16}$]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 h. For biological inquiry on the relationship between plasma FFA and tissue long-chain fatty acyl-carnitines, another four rabbits were infused with either [U-$^{13}C_{16}$]palmitate or [D_2]palmitate (n = 2 each) at 0.2–0.4 μmol/kg/min for 2 h. 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 National Institutes of Health 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-$^{13}C_{16}$]palmitate enrichment by GC-MS (25). The frozen muscle sample (20–50 mg) was pulverized into a fine powder under liquid nitrogen using a medium tissue pulverizer (Spectrum Laboratories, Inc., Rancho Dominguez, CA). Fifty microliters of freshly made 1 M potassium phosphate monobasic KH_2PO_4 solution and 20 μl of 1 ng/μl internal standard solution were added to the sample. One milliliter of freshly made extraction solution of 3:1 Acn/MeOH (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 1.5 ml plastic centrifuge tube and dried under gentle N_2 flow. One hundred microliters 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.

This procedure was compared with the additional use of SPE purification. After the procedure described above was completed, the extraction supernatant was decanted onto a 0.5 ml silica gel column (made with 300 mg of 230–400 mesh, 60 Å silica gel in 3:1 Acn/MeOH). The column was washed with 2 ml of methanol followed by 1 ml of 1% acetic acid in methanol. Carnitines were eluted with 4 ml of 1% acetic acid in methanol. The eluate was dried under N_2 and redissolved in 100 μl of 3:1 Acn/MeOH. The mixture was then centrifuged and filtered at 14,000 *g* for 3 h for LC-MS analysis.

Method validation

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. One hundred microliters of the standard solution was directly analyzed by LC-MS, and another 100 μl solution was dried under gentle N_2 flow, processed as described above, and redissolved in 100 μl of 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 second portion of extraction solution, and only trace amounts of acyl-carnitines were detected, indicating nearly complete extraction from the muscle sample.

Isotopic ratio measurement accuracy. To test the accuracy of the isotopic ratio measurement, a series of standardized solutions of [U-$^{13}C_{16}$]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 N_2 flow, processed as described above, and analyzed by LC-MS in the SIM mode. The calculated isotopic ratio and measured isotopic ratio were compared, and a standard curve was drawn.

Quantification accuracy. The quantification accuracy was determined by comparing the added theoretical concentration with the measured concentration using the internal standard method. Standardized solutions were prepared at seven concentration levels: 50, 100, 200, 400, 600, 800, and 1,000 pg/μl in 3:1 Acn/MeOH. To 100 μl of each solution, 20 μl of 1 ng/μl [D_3]palmitoyl-carnitine solution was added as an internal standard. The mixed solutions were dried under gentle N_2 flow and processed as described above, redissolved in 100 μl of 3:1 Acn/MeOH, and analyzed by LC-MS in the SIM mode.

Sensitivity. Sensitivity was determined with the standard solutions of as low as 0.0002 tracer-to-tracee ratio (TTR) of [U-$^{13}C_{16}$] palmitoyl-carnitine to palmitoyl-carnitine, 20 pg/μl concentration, with 10 mg tissue samples.

Reproducibility and reliability studies. Two independent rabbit muscle samples taken after 5 h of [U-$^{13}C_{16}$]palmitate infusion were used to determine method reproducibility. To test the sample-to-sample reproducibility, these two muscle samples were prepared and analyzed by LC-MS six times in tissue amounts ranging from 15 to 90 mg. The isotopic enrichment and concentrations of individual long-chain acyl-carnitines were calculated and compared. To study day-to-day reproducibility, the samples were analyzed on three different days.

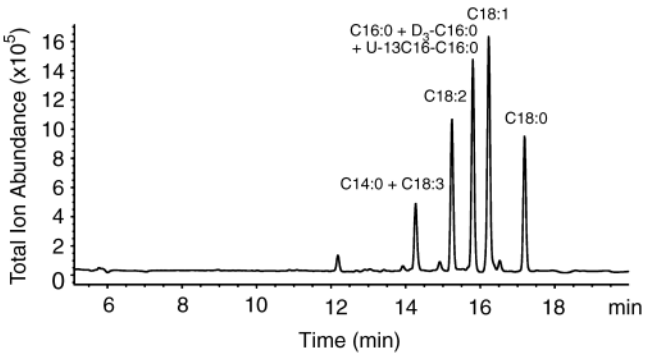


Fig. 1. Total ion chromatogram of a rabbit muscle sample prepared without solid-phase extraction purification and analyzed using a slow gradient over 25 min.

RESULTS AND DISCUSSION

Tissue extraction

Long-chain fatty acyl-carnitines can be readily extracted from frozen tissue using the plasma extraction conditions with some modifications (6). Long-chain acyl-carnitines are highly polar molecules; therefore, they tend to dissolve in polar solvents. Acn/MeOH (3:1) 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_2 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_2PO_4 was added to provide an acidic environment in which to stabilize the acyl-carnitines during the extraction. The homogenization process is also an essential step to ensure the completion of the extraction of the acyl-carnitines.

SPE has been used frequently to provide a further purification and enrichment of acyl-carnitine species before HPLC, capillary electrophoresis, or tandem mass spectrometry analysis (5–7, 23, 24). However, with the combination of ion-pairing HPLC and SIM 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 and selected ion chromatograms 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 >75%.

Analytical conditions

Ion-pairing HPLC is a good option for long-chain fatty acyl-carnitines to improve the chromatographic separation and peak shape. Trace amounts 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. HFBA was chosen over the commonly used ion-pairing reagent trifluoroacetic acid 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**).

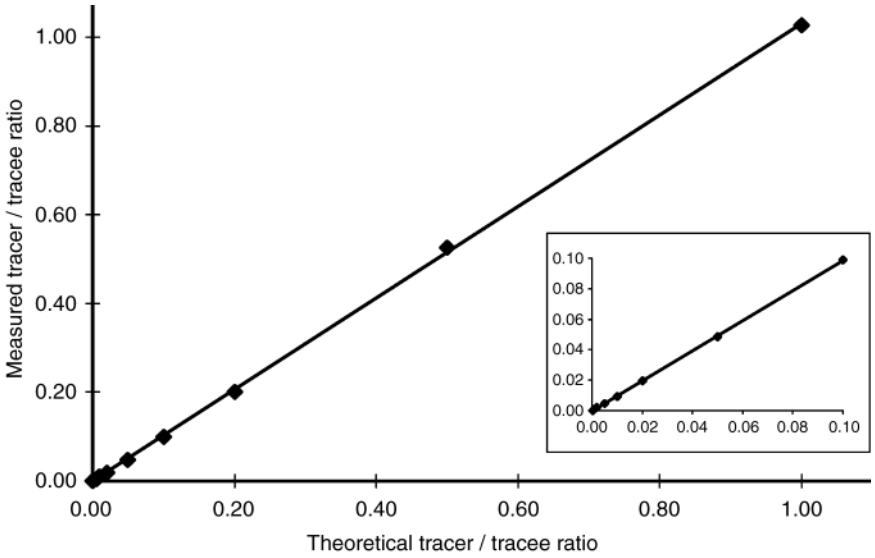


Fig. 2. Relationship between the theoretical and measured tracer-to-tracee ratio of [U-$^{13}C_{16}$]palmitoyl-carnitine to palmitoyl-carnitine (y = 1.0317, x = −0.0009, R^2 = 0.9998 for the entire test range of 0.0002–1; y = 0.9878, x = −0.0002, R^2 = 0.9999 for the zoom-in range of 0.0002–0.1).

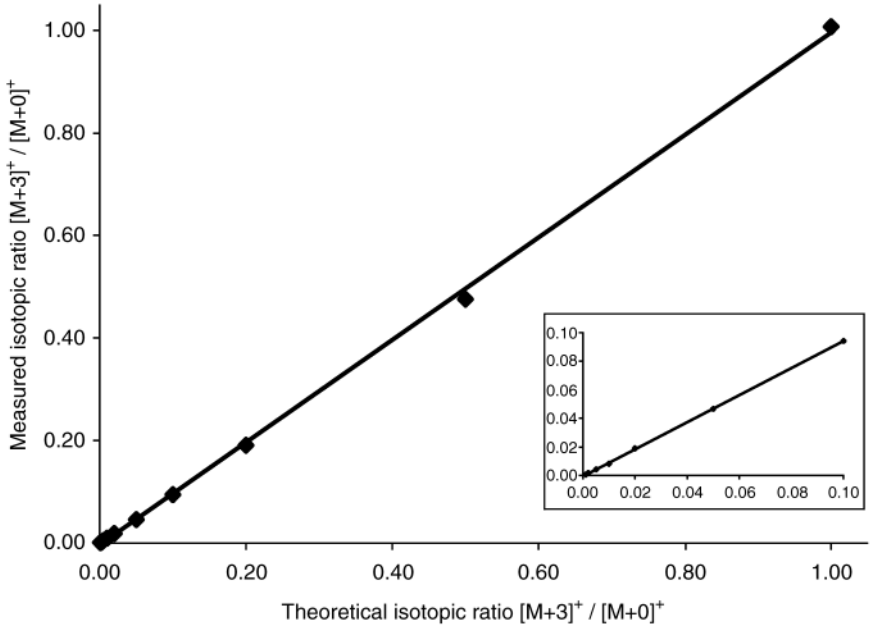


Fig. 3. Relationship between the theoretical and measured isotopic ratio of [D_3]palmitoyl-carnitine to palmitoyl-carnitine (y = 1.0185, x = − 0.0051, R^2 = 0.9986 for the entire test range of 0.0002–1; y = 0.9469, x = − 0.0006, R^2 = 0.9998 for the zoom-in range of 0.0002–0.1).

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 showed only 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 coelution 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 is well known in isotopic tracer enrichments measured by GC-MS (25). Chemical ionization (CI) usually has greater sensitivity and precision than electron impact ionization because CI is a much "softer" ionization technique with less fragmentation. The electrospray ionization method used here is an even softer ionization than CI, so excellent analytical data could be obtained by SIM analysis.

Method validation

Accuracy. The accuracy of the isotopic enrichment measurement was tested by analyzing a series of standard stock solutions containing [U-$^{13}C_{16}$]palmitoyl-carnitine and unlabeled 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 in the 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 TTR closely matched the theoretical TTR, with a linear R^2 of 0.9998, a slope of 1.0317, and a y-intercept of 0.0009. The values were calculated to be 92.8–102.7% of the known values over the entire range of enrichment, indicating a good analytical accuracy of the isotopic enrichment measurement.

To achieve reliable quantification of the concentration of long-chain acyl-carnitines using [D_3]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 [D_3]palmitoyl-carnitine and unlabeled palmitoyl-carnitine in the molecular ratio range from 0.001 to 1 were analyzed. Ions at m/z 400.4 and 403.4 were chosen to be monitored

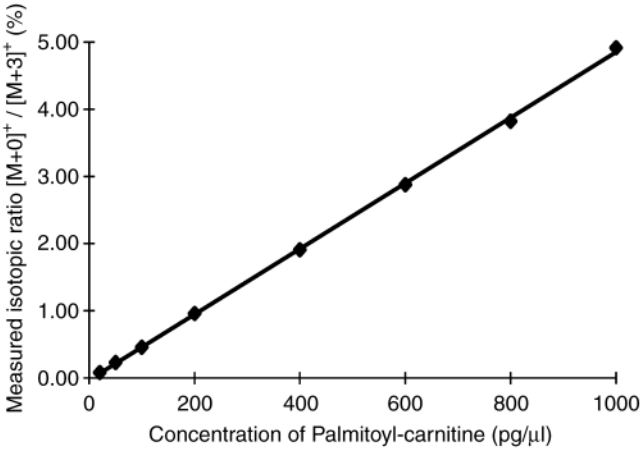


Fig. 4. Standard curve of palmitoyl-carnitine concentration (y = 0.0049, x = +0.0276, R^2 = 0.9996). Normal tissue concentration is ~150–600 pg/µl.

TABLE 2. Accuracy and precision of the measurement of the concentration of palmitoyl-carnitine

Theoretical Concentration	Measured Concentration	Accuracy	SD (n = 3)	Precision
pg/μl	%		%	
50	47.87	95.74	1.07	2.24
100	100.29	100.29	1.94	1.93
200	193.99	96.99	2.02	1.04
400	396.51	99.13	8.46	2.13
600	596.85	99.47	7.94	1.33
800	812.75	101.59	25.80	3.17
1,000	1,016.49	101.65	22.99	2.26

Results are from quantification of the internal standard.

in the 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 a linear R^2 of 0.9986, a slope of 1.0185, and a 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 to 1,000 pg/μl containing known amounts of $[D_3]$palmitoyl-carnitine as internal standard. All of the standard solutions were processed as described above. Base mass peaks $[M + 0]^+$, which were actually the protonated acyl-carnitine $[M + H]^+$ ions, were monitored in the SIM mode, and their peak areas were integrated and the relative ratios with the peak area of the 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% and 102%, with relative standard deviation (RSD) < 4%. With the same method, the accuracies of the concentration measurements were calculated to be between 91% and 103%, with RSD < 5% for other long-chain acyl-carnitines.

Sensitivity. With the sample preparation and analytical procedure described above, 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 a tissue sample of <10 mg 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.

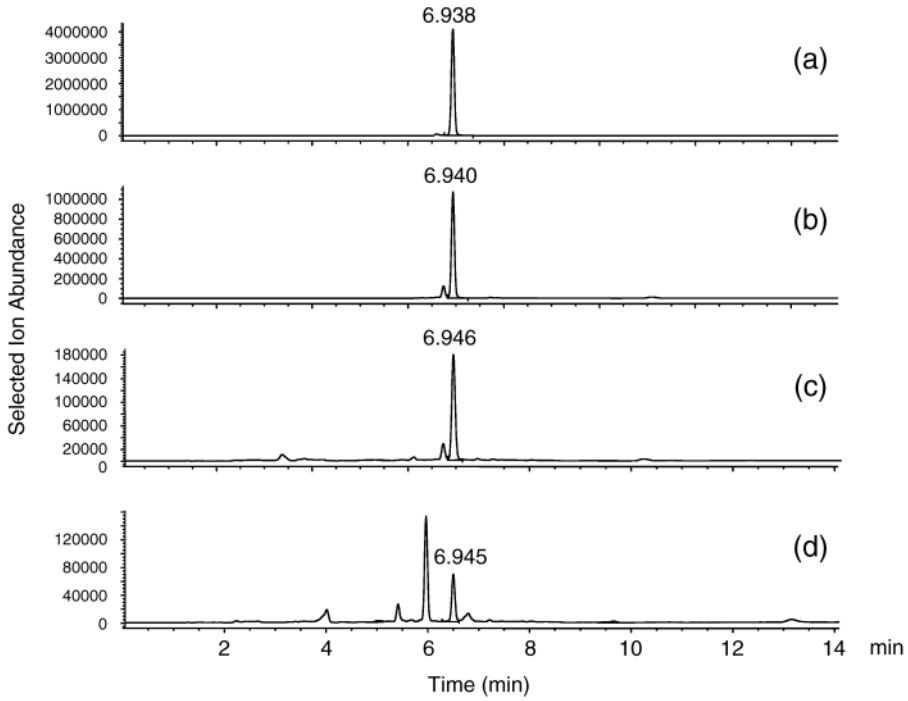


Fig. 5. Selected ion chromatograms of palmitoyl-carnitine for rabbit muscle samples analyzed under a fast gradient containing long-chain acyl-carnitines. A: $[M + 0]^+$, unlabeled palmitoyl-carnitine, as tracee. B: $[M + 1]^+$, [1-^{13}C]palmitoyl-carnitine, as naturally occurring isotopomer. C: $[M + 3]^+$, $[D_3]$palmitoyl-carnitine, as internal standard. D: $[M + 16]^+$, [U-$^{13}C_{16}$]palmitoyl-carnitine, as tracer.

TABLE 3. Reproducibility of the measurement of [U-$^{13}C_{16}$]palmitoyl-carnitine enrichment

Sample	Replicate	Tracer-to-Tracee Ratio			
		Day 1	Day 2	Day 3	Day to Day
A	1	0.01991	0.01954	0.01838	0.01928 ± 0.00080
	2	0.01903	0.01971	0.02010	0.01961 ± 0.00054
	3	0.01883	0.01944	0.02068	0.01965 ± 0.00094
	4	0.01984	0.02142	0.02085	0.02070 ± 0.00080
	5	0.01909	0.01878	0.01986	0.01924 ± 0.00056
	6	0.01970	0.01996	0.02096	0.02021 ± 0.00067
	Mean	0.01940	0.01961	0.01996	0.01966
	SD	±0.00047	±0.00097	±0.00088	±0.00081
	RSD	±2.409%	±4.945%	±4.391%	±4.142%
B	1	0.05274	0.05234	0.05374	0.05294 ± 0.00072
	2	0.05068	0.05386	0.05197	0.05217 ± 0.00160
	3	0.05265	0.05248	0.05374	0.05296 ± 0.00068
	4	0.05481	0.05379	0.05448	0.05436 ± 0.00052
	5	0.05277	0.05278	0.05356	0.05304 ± 0.00045
	6	0.05107	0.05025	0.05006	0.05046 ± 0.00054
	Mean	0.05245	0.05258	0.05293	0.05265
	SD	±0.00147	±0.00131	±0.00163	±0.00140
	RSD	±2.497%	±2.989%	±2.978%	±2.664%

RSD, relative standard deviation. Sample A and B from rabbit muscle were each analyzed six times on three different days. Values are means ± SD.

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 sample preparation and analytical conditions described above, a clean total ion chromatogram was obtained (Fig. 1) with baseline 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 3 days. For muscle sample A, the TTR was measured to be 0.01966 ± 0.00081, with a RSD of 4.142% over 18 analyses. Sample B had a TTR of 0.05265 ± 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 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.

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 here. The more stable isotopic tracer that was infused into blood, the higher the tissue long-chain fatty acyl-carnitine enrichment was. To demonstrate this more clearly, a further study was designed and performed, as described above in Materials and Methods. Over 2 h of infusion, blood and tissue samples were collected at four 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 assess-

TABLE 4. Reproducibility of the measurement of concentrations of individual long-chain fatty acyl-carnitines

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

Two different samples (A and B) from rabbit muscle were analyzed six times each on three different days. Values are means ± SD.

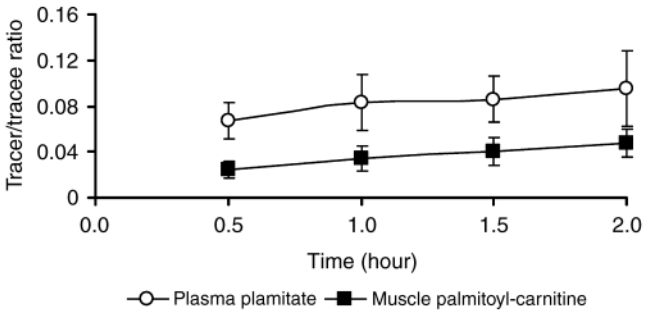


Fig. 6. Plasma palmitate and muscle palmitoyl-carnitine enrichments. The values are means ± SEM from four rabbits infused with either [D_2]palmitate or [U-$^{13}C_{16}$]palmitate (n = 2 for each).

ment of percentage 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 a 48 h fast, plasma FFA accounted for 44 ± 3% (means ± SEM) 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 in the 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 little as 0.001 TTR, or 20 pg/μl acyl-carnitine concentration, or as little as 10 mg of 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 a 48 h fast, plasma FFA accounts for 44% of fatty acid oxidation in the muscle.

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