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Liquid chromatography/mass spectrometry determination of endogenous plasma acetyl and palmitoyl carnitines as potential biomarkers of β-oxidation in mice

Zhi Liu*, Abdul E. Mutlib, Jianyao Wang and Rasmy E. Talaat
Drug Metabolism Department, Drug Safety and Metabolism, Wyeth Research, Collegeville, PA 19426, USA

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A robust bioanalytical method capable of measuring acetyl and palmitoyl carnitines was developed and validated. Application of hydrophilic interaction chromatography (HILIC) enabled retention of these highly polar and difficult to analyze compounds on a silica HPLC column. The chromatography was conducted with a high percentage of an organic component in the mobile phase, allowing high sensitivity for the pre-existing positively charged quaternary ammonium ions by electrospray ionization mass spectrometry. Successful application of the method to reliably quantify naturally occurring acyl carnitines in mouse plasma depended on the use of corresponding deuterated analogues. The specificity of the method, achieved through the use of stable isotope labeled compounds in combination with a mass spectral multiple reaction monitoring technique, permitted a non-invasive assessment of the overall change in the levels of these acyl carnitines in the plasma of intact animals administered peroxisome proliferator activated receptor (PPAR) agents. These acyl carnitines, as carriers of the corresponding long-chain fatty acids for transport into mitochondria, can be employed as potential biomarkers for significant alteration in the β-oxidation process in an intact animal. Copyright © 2008 John Wiley & Sons, Ltd.

The physiological role of acyl carnitines is to facilitate the transfer of long-chain fatty acids from the cytosol to the mitochondrial or peroxisomal matrix for β-oxidation of fatty acids. Under normal conditions, the acetyl coenzyme (CoA) produced from fatty acid oxidation is further processed through the tricarboxylic acid (TCA) cycle to ultimately yield the energy (as ATP) required by the cells. However, in the event of an increased β-oxidation process, the level of acetyl CoA rises to a point where the excess amount cannot be handled by the TCA cycle. Excess acetyl CoA is then shunted off as acetyl carnitine, releasing CoA, which is required for the β-oxidation of fatty acids.[1] Maintaining a continuous reservoir of free CoA in the mitochondria for energy production is an important function of the carnitine system.[2–5]

An increase or decrease in the overall β-oxidation process in the two high-energy demanding tissues, muscle and heart, may be reflected by systemic changes in the carnitine and acyl carnitine levels. Hence a non-invasive technique, such as monitoring plasma levels of acyl carnitines, may be more appropriate to use in assessing the overall β-oxidation status in an animal exposed to a drug or environmental conditions that may stimulate this process. Unfortunately, due to the lack of specific methods to detect individual fatty acyl carnitine levels in biological fluids, this area of research has been largely ignored. It is proposed that analytical measurement of these acyl carnitines can provide very useful information on the overall energy consumption process via the β-oxidation in mitochondria or peroxisomes in the body. We herein propose that measuring plasma acyl carnitines, especially acetyl carnitine and palmitoyl carnitine, by a non-invasive specific liquid chromatography/tandem mass spectrometry (LC/MS/MS) method is a more relevant, practical and robust way of monitoring overall systemic changes in the β-oxidation process. Any increase or decrease in the utilization of fatty acids by the body should be mirrored by a simultaneous increase or decrease in these acyl carnitines in plasma. For example, upon administration of a peroxisome proliferator activated receptor (PPAR) agent, known to increase fatty acid β-oxidation,[6–8] a decrease in the plasma levels of long-chain acyl carnitines such as palmitoyl carnitine is expected.

Several methods have been developed for the quantitation of carnitine and acyl carnitines such as high-performance liquid chromatography/ultraviolet (HPLC/UV),[9] HPLC/fluorescence,[10] flow injection analysis,[11] and tandem mass spectrometry (MS/MS).[12–15] Among these, HPLC-based methods with UV or fluorescence detection need derivatization steps. Fast-atom bombardmemt tandem mass spectrometry (FAB-MS/MS) was used first for the determination of carnitine and acyl carnitines as methyl esters,[12] followed by electrospray ionization tandem mass spectrometry

**Correspondence to*: Z. Liu, Wyeth Research, Bldg S3250, Collegeville, PA 19426, USA.
E-mail: liup2@wyeth.com

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(ESI-MS/MS).[13] A LC/MS/MS method for the online extraction of acyl carnitines in plasma without derivatization that requires only precipitation and centrifugation has been published.[14] A quantitation of the short-, medium- and long-chain acy lcarnitines from dried human blood spots by using 59 amu neutral loss (trimethylamine) LC/MS/MS was also reported.[15]

A rapid LC/MS/MS method, which relied on the use of a hydrophilic interaction chromatography (HILIC) column capable of retaining highly polar compounds, was developed to simultaneously quantify acetyl carnitine (**I**) and palmitoyl carnitine (**II**), as potential biomarkers of the β-oxidation process. Because of the presence of endogenous acyl carnitines, deuterated analogues of these compounds were used for quantitation purposes. The validation of the assay and the application of this method to assess changes in the fatty acyl carnitine levels in response to two PPAR agents, compounds **A** and **B**, with differing potencies will be reported in this paper. It is believed that this is the first publication linking the β-oxidation activity with the level of acyl carnitines.

EXPERIMENTAL

Chemicals and reagents

Stable isotope labeled d_3-acetyl carnitine, d_3-palmitoyl carnitine and d_3-octanoyl carnitine (internal standard) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) and the structures are shown in Fig. 1. Non-labeled acetyl, palmitoyl carnitines and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO, USA). All solvents and reagents were of HPLC or analytical reagent grade and were purchased from EM Sciences (Gibbstown, NJ, USA).

Instruments

LC/MS/MS was performed using an Applied Biosystems Sciex API 4000 tandem triple quadrupole mass spectrometer interfaced via an ESI probe to a liquid chromatography system consisting of one Agilent 1100 solvent delivery unit (Agilent Technologies, Palo Alto, CA, USA) and a CTC high-throughput autosampler (Carrboro, NC, USA). An Atlantis HILIC silica column (50×2.0 mm i.d., 4 μm), supplied by Waters (Milford, MA, USA), coupled with a 0.5 μm in-line filter from Mod-Mac Analytical, Inc. (Chadds Ford, PA, USA), was used at ambient temperature. The protein precipitation was performed using a Tomtec Quadra 96-320 station (Hamden, CT, USA) and an Isolute PPT+ protein precipitation plate, supplied by Biotage AB (Charlottesville, VA, USA).

Calibration curves

A stock solution of the deuterated analogues of **I** and **II** (1 mg/mL) was prepared in acetonitrile (ACN). This solution was further diluted with a mixture of ACN/water (1:1, v/v) to give a series of working standard solutions in order to prepare the plasma standards needed to construct the calibration curves. The internal standard, d_3-octanoyl carnitine (**III**), was also prepared as a stock solution (1 mg/mL) in ACN. An internal standard working solution of 250 ng/mL was prepared by diluting the stock solution of **III** with a 1:1 ACN/water mixture and was used for all analyses. All stock and working standard solutions were stored at 4°C. The concentrations of the standards at the respective points on the calibration curves were 1, 2, 5, 10, 20, 100, 200, 900 and 1000 ng/mL in mouse plasma for the deuterated analogues of **I** and **II**, respectively. The quality control (QC) samples, at concentrations of 3, 80 and 800 ng/mL, in mouse plasma for the deuterated analogues of **I** and **II** were prepared similarly from different stock solutions. Aliquots (0.3 mL) of these QC samples were placed in 2.0 mL polypropylene tubes, stored at −70°C in a freezer, and analyzed daily with study samples.

The calculated concentrations of the QC samples were compared on a day-to-day basis to assess the inter-day assay performance. Peak integration, regression and calculation of concentrations were computed using Analyst™ software (version 1.4.1). The calibration curve was constructed using a weighted ($1/x^2$) linear regression of the standard mouse plasma concentrations to measured peak-area ratios.

Sample preparation

For each batch, a calibration curve, control blank samples and QC samples were used. A measured portion (50 μL) of each mouse plasma sample was placed in an individual well in a 1 mL 96-well collection plate. The following were added in succession: 10 μL of calibrator spiking solution (standard curve only), 10 μL of 50% ACN (excluding standards) and 20 μL of internal standard working solution.

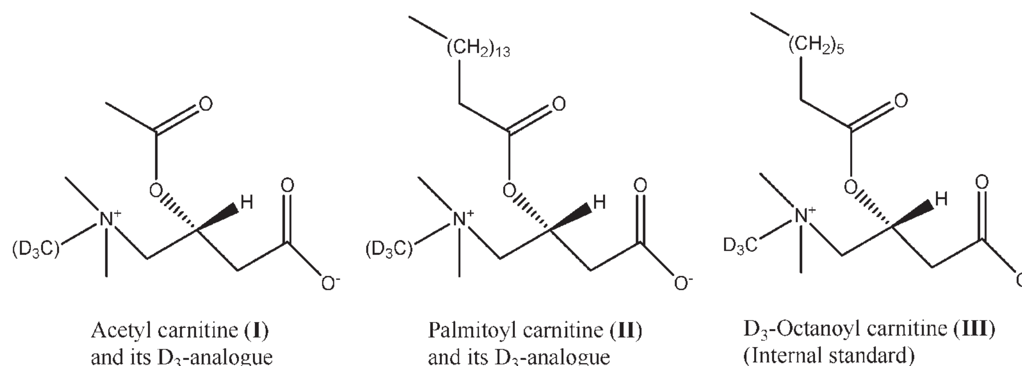

Figure 1. Structures of acetyl, palmitoyl, d_3-acetyl, d_3-palmitoyl and d_3-octanoyl (internal standard) carnitines.

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The 96-well plate was vortexed for 30 s and then transferred to a Tomtec Quadra 96-320 for extraction. Using a protein precipitation filter plate (Isolute® PPT +, supplied by Biotage), the Tomtec dispensed 200 μL of ACN to each well. The mouse plasma samples were then applied to the filter plate that was subsequently allowed to stand for 2 min. No vortex mixing was required. With a 96-well collection plate underneath, vacuum was then applied for 3 min. The filtrates were evaporated to dryness under nitrogen and the residue was reconstituted with 200 μL of ACN/water (9:1, v/v). The plate was vortexed gently and sealed with a cap mat. An aliquot (10 μL) of sample was injected onto an Atlantis HILIC silica column (50 × 2.0 mm i.d., 4 μm particle size).

Chromatography and mass spectrometry conditions

HPLC separation was performed on an Atlantis HILIC silica column. A gradient condition with 5% of ACN in 5 mM ammonium acetate solution as aqueous phase (A) and 95% of ACN in 5 mM ammonium acetate solution as organic phase (B) was used. The initial mobile phase consisting of 90% B was delivered to the mass spectrometer at a flow rate of 0.4 mL/min. At 1 min, the gradient started at 90% B, decreased to 10% B over 1 min, and stayed at 10% B for 1 min, the post-run requilibrium time was 2 min, for a total runtime of 5 min. The retention times for compounds **I**, **II**, and **III** were between 2–3 min.

The analytes were detected using the positive multiple reaction monitoring (MRM) mode of the transitions m/z 204 → 145 for compound **I** (m/z 207 → 145 for its d_3-analogue), m/z 400 → 341 for compound **II** (m/z 403 → 341 for its d_3-analogue), and m/z 291 → 229 for compound **III** (internal standard), respectively. The instrument was operated at a turbo temperature of 500°C, nebulizing gas flow of rate of 50 L/min and dwell time of 100 ms per transition.

Method validation

The quantitation range was 1–1000 ng/mL for both **I** and **II** in mouse plasma. A calibration curve containing both compounds was prepared on three separate days. The curves were fit by the $1/x^2$ weighted linear regression method. Each analytical run included one calibration curve with nine different concentrations, control blank samples, and precision/accuracy QC samples (at low, mid and high concentration levels) in replicates of five. The precision and accuracy of the lower limit of quantitation (LLOQ) were examined by analyzing five replicates of 1 ng/mL concentration samples. Dilution of above quantitation limit (AQL) samples was performed in replicates of five by diluting 16000 ng/mL of AQL samples 20-fold with 4% BSA solution. The recovery of compounds **I** and **II** from mouse plasma was determined by comparing the mean peak areas of **I** and **II** (at three concentrations) spiked in control mouse plasma before extraction (pre-extraction samples) with the mean peak areas of samples spiked after extraction (post-extraction samples) at equivalent concentrations. The effect of ion suppression was determined by spiking the compounds **I** and **II** into control mouse plasma samples after the extraction procedure (post-extraction samples). The samples were assayed and the mean peak area responses at each concentration were compared to the mean peak areas of samples spiked with the compounds **I** and **II** in neat (matrix-free) solutions at equivalent concentrations. Carryover was determined by injecting an extracted control plasma sample immediately following the highest calibrator. The bench-top stability of **I** and **II** in mouse plasma was determined by storing low and high concentration QC samples for 4 h at room temperature and under normal light conditions prior to extraction. The freeze/thaw (F/T) stability of compounds **I** and **II** in mouse plasma was determined by subjecting low and high concentration QC samples to three F/T cycles before processing.

Data management and quantitation

To quantify acetyl and palmitoyl carnitines using the stable isotope labeled surrogate calibrator, it was necessary to process the calibrators and study samples separately during the quantitation. From the Sciex Analyst software quantitation wizard, the calibrators and quality control samples were processed first with the transitions of deuterated analogues of **I** and **II** (e.g. m/z 207 → 145 for the d_3-analogue of acetyl carnitine and m/z 403 → 341 for the d_3-analogue of palmitoyl carnitine). Next, all study samples were processed with the transitions of unlabeled **I** and **II**. The saved electronic result files were then imported into the Watson DM laboratory information management system (LIMS) for the regression and calculation of concentrations. The chromatographic peaks of unlabeled and stable isotope labeled acetyl and palmitoyl carnitines were integrated using Analyst software (version 1.4.1) with a smooth factor of 1. Quantitation was based on linear regression analysis of calibration curves (weighted $1/x^2$) using the calibrator to internal standard peak area ratio vs. nominal concentration utilizing Watson LIMS software (version 7.0.0.01).

Animal studies

Male mice (CD-1) weighing between 15–20 g were used for the studies. Groups of mice (3 animals/group) were dosed orally with dosing vehicle, Methocell/Tween 80 (0.5%:2%), and 20 mg/kg of test compound **A**, a PPAR agent. Studies were conducted for either a single day, whereby animals were sacrificed within 24 h after a single dose, or for 7 days in which animals received a daily dose of test compound or vehicle until sacrificed. Animals dosed with either dosing vehicle or test compound were sacrificed at 0, 1, 4, 8 and 24 h post-dosing on day 1 or day 7 of the study. Blood samples, collected by cardiac puncture at the time of sacrifice, were processed to obtain plasma which was immediately stored frozen at −70°C until analysis.

Plasma samples were also obtained from a 14-day toxicokinetic study in which mice were dosed with a second test compound, **B**, also known to be a potent PPAR agent and capable of inducing changes in the β-oxidation process in mice. Compound **B** was administered orally by gavage to male CD-1 mice (3 animals/group) at dosages of 0.3, 1, and 3 mg/kg/day once daily for 14 consecutive days.

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RESULTS AND DISCUSSION

Method development

Due to the fact that acetyl and palmitoyl carnitines are present endogenously in biological fluids, it is difficult to use plasma as a matrix for the preparation of calibration curves. Even though dialyzed plasma can be used as an alternate matrix, it is usually not easy to prepare. In order to circumvent this problem, deuterated analogues of acetyl and palmitoyl carnitines were used to prepare standard calibration curves. As part of the validation process, it was imperative to demonstrate that the mass spectral response of the stable isotope labeled analogues was identical to the naturally occurring carnitines. The experimental results (Table 1) showed that the peak area ratios of these carnitines and their d_3-analogues to the internal standard (d_3-octanoyl carnitine) were identical. Therefore, it was reliable to quantify the concentrations of naturally occurring acetyl carnitine (**I**) and palmitoyl carnitine (**II**) in plasma using calibration curves prepared using the deuterated analogues. Several investigators have used this approach in the past to quantify endogenous biomarkers.[12,16]

Application of the deuterated analogues of acetyl carnitine (I) and palmitoyl carnitine (II) to quantify endogenous acetyl and palmitoyl carnitine

Both d_3-acetyl carnitine and d_3-palmitoyl carnitine were used to prepare standard curves in the range 1–1000 ng/mL of plasma. The deuterated analogues were used so that the endogenous levels of acetyl carnitine (**I**) and palmitoyl carnitine (**II**) could be measured.

MS/MS optimization

Quantitation was conducted by operating the mass spectrometer in the positive ion mode. The mass spectrometer was programmed to transmit the protonated molecules (precursor) through the first quadrupole (Q1). Following collision-induced dissociation (fragmentation) in the collision cell with nitrogen gas, product ions were transmitted through the third quadrupole (Q3). The positive ion full scans of **I**, **II** and **III** indicated the presence of the molecular ion $[M^+]$ as the predominant ion for each compound, with *m/z* values of 204 (207 for the d_3-analogue), 400 (403 for the d_3-analogue) and 291 for **I**, **II** and **III** (internal standard), respectively. The fragment ions observed at *m/z* 145 for **I** and its d_3-analogue, *m/z* 341 for **II** and its d_3-analogue, and *m/z* 229 for **III** could be attributed to the loss of trimethylamine (C_3H_7N) group from the molecular ion. Multiple reaction monitoring of the precursor → product ion transitions at *m/z* 204 → 145 for compound **I**, *m/z* 400 → 341 for compound **II**, and *m/z* 291 → 229 for compound **III**, respectively, permitted sensitive and selective detection of the analytes and internal standard.

Chromatography

Various bonded phases including ODS (C18), Octyl (C8), phenyl, silica and CN were evaluated in an attempt to achieve a satisfactory peak shape for the analytes of interest. Among them, the Atlantis HILIC silica column (50 × 2.0 mm i.d., 4 μm) provided the best overall chromatographic separation and peak shape. In HILIC, first introduced by Alpert in 1990,[17] a mobile phase which contains a high concentration of organic (non-polar) solvent and a low concentration of aqueous (polar) solvent is used. The aqueous portion constitutes a strong solvent and hence the compound elution takes place in the order of increasing hydrophilicity. The retention mechanism of HILIC is the partitioning of the polar analyte between the water-rich stationary phase and the water-poor mobile phase. On a silica stationary phase, polar bases can also undergo weak exchange with the negatively charged silanols. This combination of partitioning and cation exchange results in retention of the highly polar compounds that circumvents some of the problems associated with reversed-phase chromatography such as low retention or phase collapse (dewetting). In this assay, a HILIC silica column was selected for the method development because it was suitable for the separation of polar and ionic compounds such as acylcarnitines containing quaternary amine functional groups. The reconstituting solvent, consisting of 90% ACN, has a significant effect on the resolution of the analytes and sensitivity. Hence, the analytes were concentrated as a

Table 1. Peak area ratios of acetyl carnitine (**I**) and palmitoyl carnitine (**II**) and their d_3-analogues to the internal standard (d_3-octanoyl carnitine)

	Acetyl carnitine (ng/mL)			d_3-Acetyl carnitine (ng/mL)		
	Low QC 3.00	Mid QC 80.0	High QC 800	Low QC 3.00	Mid QC 80.0	High QC 800
Mean	0.0235	0.577	3.77	0.0245	0.614	4.00
SD	0.00147	0.00782	0.0466	0.000493	0.0137	0.0327
CV (%)	6.3	1.4	1.2	2.0	2.2	0.8
n	5	5	5	5	5	5

	Palmitoyl carnitine (ng/mL)			d_3-Palmitoyl carnitine (ng/mL)		
	Low QC 3.00	Mid QC 80.0	High QC 800	Low QC 3.00	Mid QC 80.0	High QC 800
Mean	0.00424	0.102	0.688	0.00415	0.0985	0.727
SD	0.000188	0.00337	0.0240	0.000482	0.00106	0.0173
CV (%)	4.4	3.3	3.5	11.6	1.1	2.4
n	5	5	5	5	5	5

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narrow band at the beginning of the column to give sharp peaks. The addition of 5 mM ammonium acetate solution instead of water resulted in more reproducible retention times and higher sensitivity on repeated injections. Peak shapes and retention times were optimized at a flow rate of 0.4 mL/min. The HILIC column used for this assay showed good peak shape and retention time stability throughout the analysis. The excellent column stability is attributed to the use of aqueous organic mobile phase, which washes off endogenous compounds, thus avoiding their accumulation on the column.

Sample preparation

A protein precipitation technique was used to extract compounds **I**, **II** and the internal standard (**III**) from plasma samples. However, unlike the traditional centrifugation-based technique, which involves precipitation in vials or collection plates followed by centrifugation and analysis of the supernatants, the protein precipitation followed by filtration (96-well format) in one step was used as a high-throughput, easy to automate alternative. The functionalized bottom frit holds up organic solvents, allowing the precipitating solvent to be dispensed into each well prior to sample addition. This 'solvent first' methodology is optimal for both high efficiency protein precipitation and automation, since the solvent first approach negates the need for vortex mixing. The system has an optimized porosity distribution and acts as a depth filter, retaining the precipitated protein without the potential for well blockage.

Validation of the assay method

Specificity

Six different lots of control mouse plasma were screened and no interfering peaks were observed at the retention times corresponding to compounds **I–III**. Typical chromatograms of an extracted control mouse plasma blank and plasma spiked with compounds **I** and **II** at 800 ng/mL (High QC) are illustrated in Figs. 2 and 3. The retention times of **I**, **II**, and **III** are 2.8, 2.4 and 2.5 min, respectively.

Linearity

A regression analysis of the peak area ratio versus concentration showed linearity over the 1–1000 ng/mL range for mouse plasma. The calibration curves were calculated using weighted linear regression, with weight $= 1/x^2$ in the equation, $y = ax + b$, where y is the peak area ratio of analyte to internal standard, a is the slope of calibration curve, b is constant, and x is the analyte concentration (ng/mL). Coefficients of determination (r^2) of 0.9890 or better were obtained in the validation experiments. The linear regression parameters obtained from three separate calibration curves of d_3-acetyl and d_3-palmitoyl carnitines is summarized in Table 2.

Lower limit of quantitation

The lower limit of quantitation (LLOQ) is defined as the lowest concentration on the calibration graph for which an acceptable accuracy (nominal ±20%) and precision (<20% coefficient of variation (CV)) were obtained. The current assay had an LLOQ of 1 ng/mL in mouse plasma based on

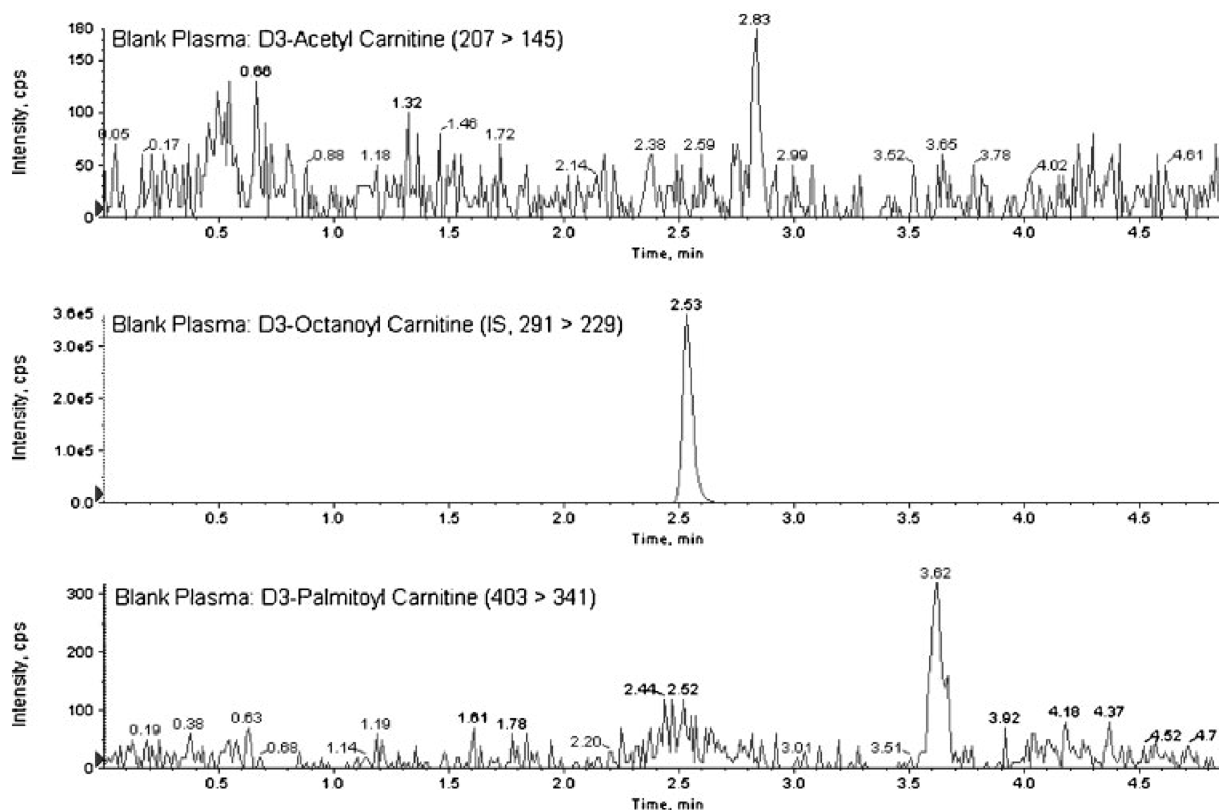

Figure 2. Representative chromatograms of extracted and spiked blank plasma sample.

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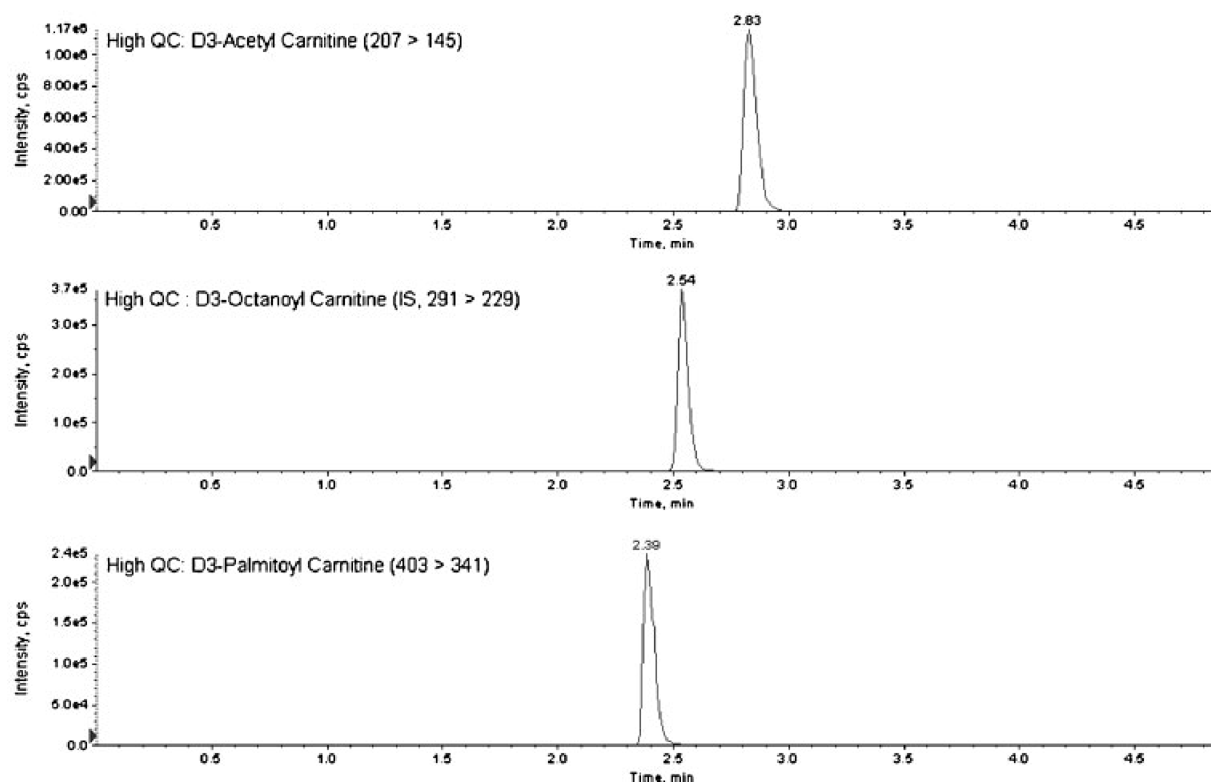

Figure 3. Representative chromatograms of extracted High QC sample.

the analysis of 50 μL of plasma sample and the signal/noise (S/N) ratio is more than 5. For compound **I**, the CV and bias values for replicate (n = 5) samples prepared at 1 ng/mL (LLOQ) were 8.1% and −8.5%, respectively. For compound **II**, the CV and bias values for replicate (n = 5) samples prepared at a concentration of 1 ng/mL (LLOQ) in mouse plasma were 11.2% and –2.5%, respectively.

Precision and accuracy

To test the precision and accuracy of this assay, QC samples at low, mid and high concentrations were prepared and interpolated against the respective calibration curve. The intra- and inter-day precision and accuracy were determined by calculating daily and overall CV and bias values for validation study sample sets that were assayed in three analytical runs. For compound **I**, the intra-day CV and bias values ranged from 1.5% to 9.5% and −17.7% to 0.2%, respectively. The overall inter-day CV and bias values ranged from 6.5% to 7.7% and −14.6% to −5.3%, respectively (Table 3). For compound **II**, the intra-day CV and bias values ranged from 2.3% to 6.5% and −18.2% to −2.7%, respectively. The overall inter-day CV and bias values ranged from 6.1% to 7.6% and −13.6% to −8.8%, respectively (Table 3).

Table 2. Linear regression parameters obtained from the calibration curves of deuterated analogues of acetyl carnitine (**I**) and palmitoyl carnitine (**II**) in mouse plasma

	d_3-Acetyl carnitine (ng/mL)			d_3-Palmitoyl carnitine (ng/mL)		
Batch No.	Slope	Intercept	r^2	Slope	Intercept	r^2
1	0.00114	0.000243	0.9890	0.000802	0.0000459	0.9892
2	0.00114	0.000641	0.9892	0.000911	0.000154	0.9914
3	0.00138	0.0000908	0.9902	0.000877	0.0000448	0.9938
Mean	0.00122	0.000325	0.9895	0.000863	0.0000816	0.9915
SD	0.000139	0.000284	0.000643	0.0000558	0.0000627	0.00230
CV (%)	0.11			0.06		
n	3	3	3	3	3	3

Recovery

The recovery of compounds **I** and **II** from mouse plasma was determined by comparing the mean peak areas of **I** and **II** (at three concentrations) spiked in control mouse plasma before extraction (pre-extraction samples) with the mean peak areas of samples spiked after extraction (post-extraction samples) at equivalent concentrations. The mean recoveries were 87.8% and 85.4% for compounds **I** and **II**, respectively. The recovery of the internal standard (**III**) was 88.7%, determined in the same manner.

Matrix effect and carryover

The matrix effect was determined by spiking the compounds **I** and **II** into control mouse plasma samples after the extraction procedure (post-extraction samples). The samples were assayed and the mean peak area responses at each concentration were compared to the mean peak areas of samples spiked with the compounds **I** and **II** in neat (matrix-free) solutions at equivalent concentrations. There was no significant signal suppression due to matrix effect during the ionization process. The average matrix effects measured were 0.843 and 0.826 for compounds **I** and **II** (at three concentration levels), respectively. Similarly, the matrix effect for the internal standard (**III**) was 0.844. These

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Table 3. Intra- and inter-day precision and accuracy determination of deuterated analogues of acetyl carnitine (**I**) and palmitoyl carnitine (**II**) spiked in mouse plasma

		d_3-Acetyl carnitine (ng/mL)			d_3-Palmitoyl carnitine (ng/mL)		
Batch No.		Low QC 3.00	Mid QC 80.0	High QC 800	Low QC 3.00	Mid QC 80.0	High QC 800
1	Mean	2.74	78.4	760	2.75	77.2	779
	CV (%)	3.0	1.7	3.4	6.5	3.1	3.0
	Bias (%)	−8.7	−1.9	−5.0	−8.3	−3.4	−2.7
2	Mean	2.47	80.2	773	2.45	72.4	673
	CV (%)	9.5	3.9	2.3	5.7	5.1	2.3
	Bias (%)	−17.7	0.2	−3.3	−18.2	−9.5	−15.8
3	Mean	2.60	68.7	682	2.57	69.1	718
	CV (%)	3.2	1.5	4.6	6.5	4.3	3.5
	Bias (%)	−13.4	−14.1	−14.8	−14.4	−13.6	−10.2
	Overall Mean	2.60	75.8	738	2.59	72.9	723
	CV (%)	7.7	7.3	6.5	7.6	6.1	6.8
	Bias (%)	−14.6	−5.3	−7.7	−13.6	−8.8	−9.6

negative values indicate a small ion suppression effect present from the plasma matrix. However, the matrix did not adversely affect the precision, accuracy, sensitivity, or selectivity of the assay as all validation acceptance criteria were met. Carryover was determined by injecting an extracted control plasma sample immediately following the highest calibrator. No such carryover was observed in this assay.

Dilution integrity

Dilution of above limit of quantitation (AQL) samples was accomplished by diluting 16000 ng/mL of AQL samples 20-fold with 4% BSA solution. For compound **I**, the CV and bias values for replicate (n = 5) samples were 2.1% and –7.8%, respectively. For compound **II**, the CV and bias values for replicate (n = 5) samples were 2.5% and 4.7%, respectively.

Stability

All stability experiments were performed with two different concentration levels (3.00 and 800 ng/mL) in five replicates. Compounds **I** and **II** were found to be stable in mouse plasma for at least 4 h at ambient temperature. After 4 h at ambient temperature, the concentration of **I** decreased 7.9% and 7.8% at the low and high concentrations, respectively, from their corresponding nominal concentrations. Similarly, the concentration of **II** decreased 6.2% and 13.7% at the low and high concentrations, respectively, from their corresponding nominal concentrations after 4 h at ambient temperature. The stability of **I** and **II** to repeated freeze/thaw (F/T) cycles was also examined using the spiked mouse plasma samples. After three F/T cycles, the proportion of **I** remaining, relative to the nominal values, was 86.7 and 95.1% for low and high concentrations, respectively. The proportion of **II** remaining, relative to the nominal values, was 98.6 and 90.6% for low and high concentrations, respectively, after three F/T cycles. Compounds **I** and **II** in mouse plasma can, therefore, tolerate at least three F/T cycles without significant degradation.

Application of the validated analytical method

The validated method was successfully used to analyze over 500 plasma samples to assess the relative levels of acetyl and palmitoyl carnitines in plasma of mice dosed with PPAR agents. Measurement of acyl carnitines was conducted to confirm the increase in the β-oxidation process in response to single and multiple administrations of PPAR agents. Analysis of control mouse plasma samples taken at various time points from mice showed some expected variation in the control levels of acetyl and palmitoyl carnitines over a 7-day period (Tables 4 and 5). It appears that the concentration of acetyl carnitine fluctuated slightly during the course of the day and after 7 days of administering dosing vehicle. The average plasma concentration of acetyl carnitine in control plasma on day 1 was found to be 12900 ng/mL, as compared to 11500 ng/mL on day 7. Upon administration of compound **A**, at 20 mg/kg, the level of acetyl carnitine did not change much from the control values on day 1, except at the 24-h time point, where it showed a significant increase ($p < 0.05$) as compared to control values at the same time point. This elevated level of acetyl carnitine was sustained on day 7 after the animals were administered multiple doses of compound **A**, suggesting that the effect of the compound which was discernable at 24 h after the first dose continued with multiple dosing (Table 4). On day 7, the average plasma concentration of acetyl carnitine was 17 500 ng/mL, which was higher than the average control value.

The average plasma concentration of palmitoyl carnitine on day 1 was 138 ng/mL as compared to the day 7 value of 153 ng/mL in vehicle-treated animals (Table 5). On administrating compound **A** at 20 mg/kg, a dramatic drop (approximately 50% of the average control value) in the level of palmitoyl carnitine was observed at 24 h after the first dose. This reduction in the palmitoyl carnitine level was sustained at all time points on day 7 after multiple administration of the compound (Table 5).

Compound **B**, which is a more potent PPAR agent, showed a greater than two-fold increase in the acetyl carnitine levels as compared to control values at all doses studied. Furthermore, the magnitude of change in the acetyl carnitine levels appeared similar at all doses. The effect on plasma acetyl and palmitoyl carnitine concentration was more dramatic with compound **B** than with compound **A** (Tables 4 and 5). Similar to what was observed with compound **A**, a sustained reduction in the palmitoyl

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Table 4. Acetyl carnitine concentrations[a] (ng/mL) in plasma of mice at various time points after single and multiple doses of vehicle and PPAR test compounds

	Vehicle		Compound **A** (20 mg/kg)		Compound **B**		
Time (h)	Day 1[b]	Day 7[b]	Day 1	Day 7	Day 14 (0.3 mg/kg)	Day 14 (1 mg/kg)	Day 14 (3.0 mg/kg)
0	13100	10500	14100	19600*	—	—	—
1	15400	12100	12700	17100*	28300**	24500**	22000[b]**
4	12700	7500	13000	15500	28100*	25100**	24900*
8	10600	13900	11600	17500*	27700**	27800**	31800[b]**
24	12500	13500	20000*	18000	25500**	20900**	18000**
Average	12900	11500	14300	17500	28900	25100	23200

*Significant difference ($p < 0.05$) as compared to the concentrations of acetyl carnitine present in the vehicle-dosed animals at the same time point on the respective day. Two-sample *t*-test assuming equal variance was used to calculate the *p*-values.
**Significant difference ($p < 0.05$) as compared to the concentrations of acetyl carnitine present in the vehicle-dosed animals; however, comparisons were made with the day 7 acetyl carnitine vehicle control concentrations at or nearest the closest time point.
[a] Samples from three animals (n = 3) at each time point were analyzed to obtain the average concentrations.
[b] Average of two samples, as insufficient number of animals to obtain standard deviation values
— Samples not taken at these times.

Table 5. Palmitoyl carnitine concentrations[a] (ng/mL) in plasma of mice at various time points after single and multiple doses of vehicle and PPAR test compounds

	Vehicle		Compound **A** (20 mg/kg)		Compound **B**		
Time (h)	Day 1	Day 7	Day 1	Day 7	Day 14 (0.3 mg/kg)	Day 14 (1 mg/kg)	Day 14 (3 mg/kg)
0	89	118	104	67*	—	—	—
1	130	157	121	58*	122	53**	55**
4	166	113	126	66*	141	62**	62**
8	139	214	122	73*	162	74**	40[b]**
24	167	162	61*	71*	64**	49**	34**
Average	138	153	107	54	121	62	46

*Significant difference ($p < 0.05$) as compared to the concentrations of palmitoyl carnitine present in the vehicle-dosed animals at the same time point on the respective day. Two-sample *t*-test assuming equal variance was used to calculate the *p*-values.
**Significant difference ($p < 0.05$) as compared to the concentrations of palmitoyl carnitine present in the vehicle-dosed animals; however, comparisons were made with the day 7 palmitoyl carnitine vehicle control concentrations at or nearest the closest time point.
[a] Samples from three animals (n = 3) at each time point were analyzed to obtain the average concentrations.
[b] Only one sample at this time point available for analysis.
— Samples not taken at these times.

carnitine level was observed at 1 and 3 mg/kg doses of compound **B** after 14 days of dosing. It is interesting to note that, at 0.3 mg/kg, the level of palmitoyl carnitine remained at the control value through 8 h post-dose and only decreased at 24 h post-dose. Besides day 1 and day 14 sampling, no other samples were obtained.

Hence it appears that the trend observed in the increase and decrease of acetyl and palmitoyl carnitines, respectively, in plasma of mice was reproducible with at least two PPAR agents capable of modulating the β-oxidation process. The data obtained from these studies suggested that, due to an increase in the β-oxidation process, the levels of palmitoyl carnitine had decreased substantially as it was being fed into the β-oxidation machinery of mitochondria and peroxisomes. A corresponding increase in the levels acetyl carnitine was also observed and was particularly pronounced with the more potent PPAR agent, compound **B**.

CONCLUSIONS

A robust bioanalytical method capable of measuring polar acetyl and palmitoyl carnitines was developed and validated. The specificity of the method achieved through multiple reaction monitoring of the analytes allows a non-invasive assessment of the overall change in the β-oxidation process to be made in an intact animal responding to a drug such as a PPAR agent. The LC/MS method can be used to simultaneously monitor the acetyl and palmitoyl carnitines. Further studies are being conducted to fully validate the quantification of these acyl carnitines in plasma of various species.

The effects of PPAR agents are an area of active research. While it is understood that these agents affect the β-oxidation process, as one of their primary pharmacological targets, the consequences of long-term administration of such

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compounds are poorly understood. Having an analytical method such as the one described here provides a researcher with a powerful tool to better understand the interplay between various biochemical processes that may be altered in response to such compounds. This LC/MS method has demonstrated that it could be applied easily in assessing an overall global response by an animal exposed to compounds capable of modulating the β-oxidation as well as perhaps other biochemical processes involved in energy production.

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