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Quantitative Acylcarnitine Determination by UHPLC-MS/MS – Going Beyond Tandem MS Acylcarnitine “Profiles”

Paul E. Minkler^a, Maria S.K. Stoll^a, Stephen T. Ingalls^a, Janos Kerner^a, and Charles L. Hoppel^{a,b,*}

^aCenter for Mitochondrial Diseases, Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, OH, U.S.A. 44106

^bDepartment of Medicine, Case Western Reserve University School of Medicine, Cleveland, OH, U.S.A. 44106

Abstract

Tandem MS “profiling” of acylcarnitines and amino acids was conceived as a first-tier screening method, and its application to expanded newborn screening has been enormously successful. However, unlike amino acid screening (which uses amino acid analysis as its second-tier validation of screening results), acylcarnitine “profiling” also assumed the role of second-tier validation, due to the lack of a generally accepted second-tier acylcarnitine determination method. In this report, we present results from the application of our validated UHPLC-MS/MS second-tier method for the quantification of total carnitine, free carnitine, butyrobetaine, and acylcarnitines to patient samples with known diagnoses: malonic acidemia, short-chain acyl-CoA dehydrogenase deficiency (SCADD) or isobutyryl-CoA dehydrogenase deficiency (IBD), 3-methyl-crotonyl carboxylase deficiency (3-MCC) or β -ketothiolase deficiency (BKT), and methylmalonic acidemia (MMA). We demonstrate the assay’s ability to separate constitutional isomers and diastereomeric acylcarnitines and generate values with a high level of accuracy and precision. These capabilities are unavailable when using tandem MS “profiles”. We also show examples of research interest, where separation of acylcarnitine species and accurate and precise acylcarnitine quantification is necessary.

Keywords

Quantitative acylcarnitine analysis; second-tier analysis; newborn screening follow-up; carnitine analysis; metabolism research

Corresponding author: Charles L. Hoppel: charles.hoppel@case.edu.

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1. Introduction

The widespread implementation of expanded newborn screening programs has been effective and beneficial [1]. These programs became possible because of advances in tandem mass spectrometric (MS) instrumentation and data analysis [2], allowing for “profiling” of amino acids and acylcarnitines using tandem MS [3,4]. Originally, “profiling” of acylcarnitines was conceived of as a qualitative screening method for detection of grossly elevated levels observed in rare, severe metabolic diseases [5]. The use of the jargon “profile” was intended to convey the inherent lack of analytical specificity and lack of quantitative accuracy that first-tier analyses sacrifice in exchange for rapidity, thus conceding the necessity of confirmatory second-tier analysis. The final validation of disease then often relied on enzyme activity measurements, while currently, a patient’s diagnosis is usually established using mutational analysis.

Today, the very different handling of the amino acid “profile” versus the acylcarnitine “profile” is a consequence of the historical development of their respective analytical technologies. Quantitative amino acid analysis has a long history, beginning with ion-exchange chromatography and ninhydrin derivatization in the 1950s [6], and continuing recently with HPLC-MS methods [7]. More importantly today, there are validated, commercially available amino acid analysis kits for selective, accurate, and fully quantitative amino acid analysis [8,9,10]. These kits provide both established chromatographic conditions for separation of isobaric compounds and standard compounds for quantitative accuracy. As a result, when first-tier tandem MS amino acid “profiles” were adopted in expanded newborn screening, the obvious second-tier analysis was already widely accepted. However, this was not the case with acylcarnitines, where qualitative soft ionization MS technologies were the dominant methodology [11,12]. With no generally accepted alternative second-tier methodology, the first-tier “profile” was simply repeated, coupled with semi-quantitative organic acid analysis of the patient’s urine [13]. This approach is and has been the state of the art for decades. However, today we can take advantage of more accurate and precise techniques due to advances in technology.

To move forward with acylcarnitine analysis, there are two features which any universally applicable, comprehensive, second-tier acylcarnitine method must provide:

1. *Discrimination of acylcarnitine constitutional isomers and diastereomers.* Acylcarnitine diagnostic markers are often specific to a particular acylcarnitine isomer, reflecting the metabolic pathways that generated them. However, endogenous acylcarnitine species may be present in multiple isomeric forms, and since their isomer masses are identical, they are indistinguishable by tandem MS “profiling”. (U)HPLC separation of acylcarnitine constitutional isomers and diastereomers has been shown by several groups [14,15,16].
2. *Accurate Quantification.* Acylcarnitine mass spectral responses are variable and compound specific. Therefore, accurate quantification requires standard compounds with accurately known concentrations to calibrate individual acylcarnitine compound responses. Tandem MS

“profile” values are generated using single point calibration with labeled, standardized internal standards. The calculation of these values assumes that the relative responses of each acylcarnitine and its internal standard are proportional and linear throughout the entire detected range. This approach has been shown to be quantitatively inaccurate and imprecise [17,18]. However, protocols which comply with the FDA’s “Guidance for Industry: Analytical Procedures and Methods Validation for Drugs and Biologics” [19,20] can provide excellent accuracy and precision. These procedures use calibrated standards and internal standards to construct multiple-point calibration curves.

Although no commercial kit (analogous to those for amino acid analysis) exists for acylcarnitine analysis, many groups have published second-tier methods that are, to varying degrees, more selective and quantitatively rigorous than tandem MS “profiles” [21,22,23,24]. We developed second-tier methods for the quantification of carnitine beginning 40 years ago, using radioactively labeled acetyl-CoA and measuring the production of radiolabeled acetylcarnitine [25,26]. We next prepared a highly reactive, strongly UV absorbing derivatization reagent, 4'-bromophenacyl trifluoromethanesulfonate [27], and used it to develop HPLC-UV methods for the quantification of carnitine, butyrobetaine [28,29], and acylcarnitines [30,31]. These methods were further enhanced using the intensely fluorescent derivatization reagent 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate, and we quantified carnitine and acylcarnitines in plasma and tissues by HPLC-fluorescence [32,33]. These methods were selective (using HPLC), sensitive (spectrophotometric or fluorescence detection), and truly quantitative (with internal standards and calibration curves). With the advent of atmospheric pressure ionization (API) mass spectrometers, even more selectivity was available. We modified our procedure to use an ion trap mass spectrometer for detection following derivatization of carnitine and acylcarnitines with the MS specific reagent, pentafluorophenacyl trifluoromethanesulfonate [34,35]. Most recently, we incorporated greatly improved fast chromatography using fused-core C8 UHPLC columns, and used triple quadrupole mass spectrometers with multiple-reaction monitoring (MRM) for data collection [36]. Our improved, fast, validated method for quantification of total carnitine, free carnitine, butyrobetaine, and acylcarnitines fulfills both assay requirements for a second-tier acylcarnitine analysis method: 1) chromatographic separation of acylcarnitine constitutional isomers and diastereomers, and 2) accurate quantification using multiple point calibration curves.

For chromatographic separation of acylcarnitines, we developed a two-dimensional orthogonal chromatographic scheme in which cation-exchange is used to trap on-line all carnitine species (short-chain, medium-chain, long-chain, etc.) due to the positively charged trimethylammonium functional group of carnitine. This was followed by separation of carnitine and individual acylcarnitine constitutional isomers and diastereomers using reversed-phase UHPLC. We call this technique “sequential ion-exchange / reversed-phase chromatography” [28].

Few acylcarnitine standards are commercially available. Therefore, we used published methods for synthesis of acylcarnitines [37,38,39,40], and we prepared 66 different

acylcarnitines, along with appropriate internal standards. Purification of synthesized acylcarnitines was accomplished using a two-step chromatographic process, combining cation-exchange solid-phase extraction (to remove unreacted acid) followed by preparative HPLC (to remove carnitine and synthetic impurities). We then standardized stock solutions of carnitine. This was necessary, since we have found errors in making standard solutions by weighing “pure” acylcarnitines because: 1) there is usually some free carnitine contamination in acylcarnitines that needs to be accounted for, and 2) synthesized acylcarnitines usually do not crystallize into readily transferable solids. They often are isolated as wet, viscous, hygroscopic materials that rapidly absorb additional atmospheric moisture. This prevents accurate weighing. Therefore, using standardized carnitine stock solutions [36], we standardized the acylcarnitine stock solutions with our method for free and total carnitine.

Based on literature reports, tandem MS acylcarnitine “profile” analysis has two basic problems: 1) Selectivity–isobaric contaminants and isomeric acylcarnitines have led to false negative [41] and false positive results [42,43,44], and 2) Quantification–Preparation of acylcarnitine butyl ester derivatives partially hydrolyzes some acylcarnitines, giving inaccurate free carnitine values [45]. Lacking rigorous quantitation (standardized calibrants, calibration curves, etc.), most reported “profile” values are pseudo-concentrations [17,18]. With our validated method, both of these problems are eliminated. This allows us to use our procedure for several distinct purposes:

1. *Follow-up to newborn screening* – When confronted with ambiguous findings, we use our methodology to validate the indefinite screening results with a more rigorous technique, and thus resolve false-positive or false-negative screening results. We also use accurate quantification values for carnitine and butyrobetaine to characterize carnitine biosynthesis diseases.
2. *Follow-up to Patient Treatment* – Patients receiving treatment are monitored, and require methodology capable of separating isomeric acylcarnitines with accurate quantification of carnitine, butyrobetaine, and acylcarnitines. From these determinations, appropriate modifications to the patient’s treatment regimen can be formulated.
3. *Metabolism Research* – Acceptable research protocols require analytical methods that are selective, accurate, and precise. Selectivity is accomplished with UHPLC chromatography and mass spectrometry with multiple reaction monitoring (MRM). Accurate and precise values are generated using calibration standards and internal standards to construct multiple-point calibration curves. Our results are accurate (within $\pm 20\%$ of the true value at the lowest concentrations) with excellent precision [36].

This manuscript reports the application of this validated, second-tier method for quantification of total carnitine, free carnitine, butyrobetaine, and acylcarnitines in patient samples with known diagnoses, providing results that are not attainable when using tandem MS “profiles”. We also show examples of research interest, where accurate and precise quantification of acylcarnitines, as surrogates of their respective acyl-CoAs, is necessary.

2. Material and methods

2.1 Instrumentation

The instrumentation consisted of two Agilent UHPLC 1290 Infinity binary pumps, autosampler, and thermostated column compartment with a 6-port valve, and a 6460 QQQ triple quadrupole LC/MS purchased from Agilent Technologies (Santa Clara, CA). The chromatographic separation was accomplished with an SCX trap cartridge (2.1 mm × 5 mm) contained in an in-line holder purchased from Optimize Technologies (Oregon City, OR), connected in series (through the 6-port valve) with an Agilent Poroshell 120 EC-C8 column (3.0 × 100 mm, 2.7 μm). Configuration of the instrument and instrument parameters, construction of calibration curves, and generation of quantification values were as described [36].

2.2 Biological samples

Human plasma and urine from patients with known diagnoses were obtained from the College of American Pathologists (CAP; Northfield, IL). We participate in their Biochemical Genetic Survey program, and we analyzed these samples for proficiency testing purposes. Human cerebral spinal fluid was purchased from Golden West Biologicals, Inc. (Temecula, CA).

We also show results from analysis of perfused rat heart [46]. All procedures with animal materials were approved by the Case Western Reserve University Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines for care and use of animals in research. Six months old Fisher 344 rats were injected with 500 U of heparin (IP), anesthetized with sodium pentobarbital (100 mg/kg body weight), and the hearts were removed and cannulated for perfusion. The perfusion protocol consisted of a 15 min non-recirculating perfusion in the Langendorff mode with Krebs–Henseleit buffer containing 5.5 mM glucose and 0.1 U/L insulin, followed by 60 min of perfusion in the working heart mode with 5.5 mM glucose/0.1 U/L insulin, and with either 1.2 mM palmitic acid or [1,2,3,4-¹³C₄]-palmitic acid complexed to 3% BSA. At the end of perfusion, the heart was freeze-clamped, powdered under liquid nitrogen, and the powdered tissue was stored at –60 °C.

2.3 Sample preparation and analysis

Samples were prepared and analyzed as described [36]. Briefly, to 10 μL of plasma, diluted urine, homogenized tissue, or cerebral spinal fluid (plus internal standards) was added organic solvents to precipitate salts and proteins. The resulting supernatant was then applied to a mixed-mode, reversed-phase/strong cation-exchange solid-phase extraction plate (Oasis MCX, purchased from Waters Corporation, Milford, MA). Carnitine and acylcarnitines were eluted, evaporated, and derivatized with pentafluorophenacyl trifluoromethanesulfonate [34], then injected into the UHPLC-MS/MS system. Carnitine and butyrobetaine were eluted in a 4 min chromatogram; optimized MRM transitions were collected for carnitine, d₃-carnitine internal standard, butyrobetaine, and d₃-butyrobetaine internal standard. Acylcarnitines were eluted in a 14 min chromatogram, and optimized MRM transitions were collected for acylcarnitines and their internal standards (see Fig. 1).

3. Results and Discussion

3.1 Accurate Quantification: Malonylcarnitine in a CAP Plasma Sample from a patient with Malonic Acidemia

To comply with the requirements for our CLIA certification, we participate in the College of American Pathologists' (CAP) proficiency testing for plasma acylcarnitine analysis. In one plasma sample we received, malonylcarnitine was highly elevated, consistent with a diagnosis of "malonic acidemia" (MA [47]). Fig. 2 shows part of the chromatogram from that analysis, highlighting malonylcarnitine (Peak 27) and its internal standard d₃-malonylcarnitine (Peak 28). We quantified malonylcarnitine in this plasma (using a calibration curve constructed with standardized malonylcarnitine and with d₃-malonylcarnitine as the internal standard) at 2.51 $\mu\text{mol/L}$. Normal values are less than 0.05 $\mu\text{mol/L}$. Later, we received our Participant Summary from CAP [48]. There were 46 total laboratories reporting, of which 44 used tandem MS; one lab used GC/MS and we were, presumably, the lab identified as "other". From these participants, 39 (85%) reported the correct diagnosis, with 40 labs observing elevated malonylcarnitine. It is significant that 6 labs missed this large increase in malonylcarnitine. The Participant Summary also included a histogram showing the frequency distribution of the quantitative malonylcarnitine results from 32 participating labs, which we have reproduced in Fig. 2. We have highlighted the bar at 2.5 $\mu\text{mol/L}$, which was our result. CAP does not grade the quantitative value; the acceptable response for this specimen was detection of malonylcarnitine and reporting malonic acidemia as the correct diagnosis. However, our validation studies demonstrated accuracy and precision for malonylcarnitine quantification within $\pm 15\%$ of the correct value when using our method [36]. Therefore, we assert that the malonylcarnitine concentration value for the patient sample is indeed 2.5 $\mu\text{mol/L}$. We and 6 other labs measured the same quantitative value, but 26 other labs show widely variable results (from 1 to 7.5 $\mu\text{mol/L}$). Inconsistent quantitative results using tandem MS "profiles" is not unexpected. Chace et al. [17] reported a wide variation of results from 101 laboratories given identical bloodspots containing 0.58 $\mu\text{mol/L}$ of malonylcarnitine. Using a variety of tandem MS methods, reported values varied from 0.2 to 2.0 $\mu\text{mol/L}$. The initial goal of their study was to determine what bias, if any, from the correct analytical result tandem MS "profile" quantification would yield. Assuming that the results would be precise and that any deviation from accuracy (bias) could be compensated for with correction factors, these authors instead found that "profile" results were neither accurate nor precise. The authors then abandoned the use of correction factors, and concluded that the numerical results from tandem MS analysis were pseudo-quantitative. This same group attempted a similar study with 3-hydroxyisovalerylcarnitine measurements by tandem MS, and they found again that tandem MS "profile" results were inconsistent and not quantitative [18].

In Table 1, we report the absolute quantification of total carnitine, free carnitine, and the sum of all acylcarnitines quantified in this sample. We also calculated the concentration of the acylcarnitine pool by subtracting the free carnitine concentration from the total carnitine concentration (Total - Free). From these values, we then determined the percent of acylcarnitines accounted for by quantification of the individual acylcarnitines. For this sample the value is 93%. We call this calculation a "balance study". Thus, we have

demonstrated agreement (within 7%) between what the total and free carnitine concentrations tell us what the acylcarnitine concentration should be, and what we have actually identified and quantified. If the value for malonylcarnitine were 1.0 $\mu\text{mol/L}$ (the low value on the histogram) or 7.5 $\mu\text{mol/L}$ (the high value on the histogram), the “balance study” results would be 71% or 160%, respectively, and suggest quantitative inaccuracy. Instead, a value for malonylcarnitine of 2.51 $\mu\text{mol/L}$ is consistent with the independently determined concentrations of free carnitine and total carnitine. The full analysis report is provided in the Supplemental Material.

3.2 Discrimination of Constitutional Isomers: Short-chain Acyl-CoA Dehydrogenase Deficiency or Isobutyryl-CoA Dehydrogenase Deficiency in a CAP Plasma Sample

In another plasma sample we received from CAP, we determined that butyryl- but not isobutyrylcarnitine was elevated. The diagnosis we reported was short-chain acyl-CoA dehydrogenase deficiency (SCADD [49]). The expected diagnosis by CAP was either SCADD or isobutyryl-CoA dehydrogenase deficiency (IBD [50]). Because tandem MS “profiles” cannot distinguish between butyrylcarnitine (the marker for SCADD) and isobutyrylcarnitine (the marker for IBD), the diagnosis accepted by CAP was the less definite SCADD/IBD. Fig. 3 shows part of the chromatogram from our analysis, highlighting isobutyrylcarnitine (Peak 10), butyrylcarnitine (Peak 11) and their internal standard d_3 -butyrylcarnitine (Peak 12). We chromatographically resolved isobutyrylcarnitine from butyrylcarnitine, and determined their concentrations to be 0.05 $\mu\text{mol/L}$ and 0.83 $\mu\text{mol/L}$, respectively. Normal values are $<0.2 \mu\text{mol/L}$. Since only butyrylcarnitine was elevated, the correct diagnosis was SCADD and not IBD. Table 1 includes the balance study for this sample (90% of acylcarnitines are accounted for) consistent with highly accurate quantification. The full analysis report is provided in the Supplemental Material. Later, we received our Participant Summary from CAP [51], and were informed that the sample had come from a patient with SCADD. There were 50 participants in the survey; we were the only lab not using tandem MS. Of the participants, 89% correctly identified an elevation in C4 acylcarnitine; however, because we separated butyryl- from isobutyrylcarnitine, we were the only lab that could make the correct diagnosis. The ability to resolve isomeric acylcarnitines is often underappreciated; we contributed to a report in which the inability of tandem MS “profiles” to resolve C8 acylcarnitine isomers resulted in misdiagnoses in newborn screens (see McCandless et al. [52], Supplemental Fig. 1).

3.3 Discrimination of Diastereomers: β -Ketothiolase Deficiency in a CAP Plasma Sample

We analyzed a plasma specimen provided by CAP with the expected diagnosis of either 3-methyl-crotonyl carboxylase deficiency (3-MCC, [53]) or β -ketothiolase deficiency (BKT, [54]). The marker for these diseases is HO-C5 acylcarnitine, with 3-hydroxy-isovalerylcarnitine specific for 3-MCC and 3-hydroxy-2-methyl-butyrylcarnitines specific for BKT. Fig. 4 shows an acylcarnitine chromatogram from the sample with a tiny amount of 3-hydroxy-isovalerylcarnitine (Peak 5; 0.03 $\mu\text{mol/L}$). We identified this peak based on its identical retention time to that of the purified and standardized 3-hydroxy-isovalerylcarnitine, which was used to generate a calibration curve. However, there are 4 additional HO-C5 acyl-L-carnitine isomers (Peaks 5a–d), which by tandem MS “profile” analysis are indistinguishable from 3-hydroxy-isovalerylcarnitine. These are the four

different diastereomers (*RR*-, *SS*-, *SR*-, and *RS*-) of 3-hydroxy-2-methyl-butyryl-L-carnitine, and they are chromatographically separable. Their concentrations, determined using the 3-hydroxy-isovalerylcarnitine calibration curve, were 0.09, 0.10, 0.06 and 0.07 $\mu\text{mol/L}$. The other marker for 3-MCC and BKT is C5:1 acylcarnitine, with 3-methyl-crotonylcarnitine present in 3-MCC, while tigloylcarnitine is present in BKT. The chromatogram in Fig. 4 shows an enormous peak of tigloylcarnitine (Peak 13) and essentially no 3-methyl-crotonylcarnitine (Peak 14). Table 1 includes the balance study for this sample, which accounts for 77% of the acylcarnitines. We included the 3-hydroxy-2-methyl-butyrylcarnitines in the calculation, with the remaining 23% consisting of small contributions from many other acylcarnitines. Because tandem MS acylcarnitine “profiles” cannot distinguish between 3-MCC and BKT, either diagnosis was acceptable to CAP. However, not only does our UHPLC-MS/MS procedure correctly identify an elevation in HO-C5 acylcarnitine, we can definitively identify the increase as 3-hydroxy-2-methyl-butyrylcarnitines and not 3-hydroxy-isovalerylcarnitine. Since we can also identify the elevated C5:1 acylcarnitine as tigloylcarnitine and not 3-methyl-crotonylcarnitine, we can unambiguously and correctly identify the patient’s condition as BKT and not 3-MCC. In our publication describing this procedure [36], we showed a 3-MCC sample in which we correctly identified the HO-C5 acylcarnitine as 3-hydroxy-isovalerylcarnitine and the C5:1 acylcarnitine as 3-methyl-crotonylcarnitine, providing in that case the correct and unambiguous diagnosis of 3-MCC.

3.4 Urine Acylcarnitines for Diagnosis and Treatment: Methylmalonic Acidemia in a CAP Urine Sample

We also participated in CAP’s proficiency testing for urinary organic acid analysis. We analyzed a provided urine sample for urinary organic acids, made a diagnosis of methylmalonic acidemia (MMA), and this was the correct diagnosis. Concurrently, we analyzed this same sample for acylcarnitines. Tandem MS “profiles” of urine acylcarnitines are not commonly performed, and there is no proficiency testing for urine acylcarnitines from CAP. However, our sample preparation scheme is essentially sample matrix independent. We have analyzed a wide variety of specimen types without altering the basic protocol, including plasma, urine, tissue homogenates, cultured cells, blood spots, dietary supplements, and samples from a wide variety of research protocols. Fig. 5 shows the UHPLC-MS/MS chromatograms of the short- and medium-chain acylcarnitines, free carnitine, butyrobetaine, and total carnitine from analysis of the urine provided by CAP with the diagnosis of MMA, showing elevations in propionylcarnitine and methylmalonylcarnitine. Initially, the sample was prepared by first diluting by 1:5 with water, but the resulting chromatograms contained peaks that exceeded some of the calibration curves. The sample was then diluted 1:50 with water and reanalyzed, and this result is shown in Fig. 5A. Peak 6 is enormous, and it represents the excretion of propionylcarnitine (6950 $\mu\text{mol/g}$ creatinine, normal values <2.8). Peak 7 is the internal standard d_3 -propionylcarnitine. The inset (Fig. 5Aa) is an expanded view highlighting methylmalonylcarnitine (Peak 34), along with the internal standard (Peak 33, d_3 -glutaroylcarnitine) used to construct the methylmalonylcarnitine calibration curve (N.B. Because we generate calibration curves from standardized calibrants, it is not necessary for us to use a heavy-labeled internal standard for each compound measured. We empirically

determine the response relationship between each acylcarnitine and its internal standard using a multiple-point calibration curve constructed with standardized calibrants and internal standards). Other peaks in the inset figure are succinylcarnitine (Peak 30), glutaroylcarnitine (Peak 32) and Peak 31, the C8:1 acylcarnitine *cis*-3,4-methylene-heptanoylcarnitine [40]. Peak 31a is another C8:1 acylcarnitine, presumed to be the *trans*- isomer. Fig. 5Ab is from the initial analysis of urine diluted 1:5. Here, the methylmalonylcarnitine peak (Peak 34) is nearly as large as the d₃-glutaroylcarnitine internal standard peak (Peak 33). When comparing the peak shape of methylmalonylcarnitine with d₃-glutaroylcarnitine or glutaroylcarnitine (Peak 32), it is apparent that methylmalonyl-L-carnitine is much wider, less symmetrical, and shows chromatographic peak splitting. This is because methylmalonylcarnitine is actually composed of two diastereomeric forms (*R*-methylmalonyl- and *S*-methylmalonyl-L-carnitine). In this case, these diastereomers are not completely chromatographically resolved. However, we observe that both diastereomers are present in this sample in approximately equal amounts. Fig. 5B shows, in a separate chromatogram, the analysis of free carnitine and butyrobetaine for this sample. Fig. 5C is the total carnitine chromatogram. The urinary excretion of total carnitine and free carnitine is 50 times the normal value. The excretion of butyrobetaine (the biosynthetic precursor of carnitine [55]), at 1944 μmol/g creatinine, is 400 times the normal value. Table 1 shows the “balance study” for this analysis, with 80% of the calculated acylcarnitine pool accounted for by the sum of the quantified individual acylcarnitines. The full analysis report is provided in the Supplemental Material.

With these enormous excretion values, the patient is undoubtedly being treated with carnitine. These results illustrate the utility of urine acylcarnitine analysis for therapeutic monitoring and perhaps carnitine dose adjustment. Ideally, the carnitine treatment dose in MMA should be sufficient to prevent sequestration of free CoASH, which is required for normal metabolism, while limiting the negative consequences of high dose carnitine therapy [56,57]. Therapeutic carnitine treatment facilitates excretion of the accumulated excess acyl groups in the form of acylcarnitines, i.e. propionyl- and methylmalonylcarnitine, and the patient’s dose should be sufficient to promote this. However, the carnitine dose can be too high. The excretion of acetylcarnitine is very high (3243 μmol/g creatinine, normal value < 44.15) and 47% as much as the propionylcarnitine. Because the bioavailability of oral carnitine is only 10–20%, the high butyrobetaine value is consistent with some of the carnitine dose being degraded by colonic bacteria to butyrobetaine [58]. These values suggest that the patient’s carnitine dose may be too high and could be reduced. However, if the acetylcarnitine excretion were to become low despite carnitine treatment, then as a consequence of MMA, propionic acid would accumulate, leading to CoASH sequestration and to carnitine insufficiency. Therefore, the careful monitoring of butyrobetaine and acetylcarnitine excretion, in addition to propionylcarnitine, methylmalonylcarnitine, and free carnitine excretion, can be used to optimize the patient’s therapeutic carnitine dose levels, potentially leading to improved patient care.

In another example of urine analysis, we participated in a metabolomics study in which untargeted metabolomics identified changes in acylcarnitine concentrations [59]. We validated these untargeted findings with our targeted acylcarnitine analysis. Our collaborators then showed that acylcarnitines vary as a function of both kidney cancer status

and cancer grade. The study concluded with the observation that urine contains possible acylcarnitine biomarkers for kidney cancer. These results were obtainable only because of our ability to accurately and reproducibly quantify acylcarnitines in urine.

3.5 Rat Heart Acylcarnitines for Research: Fatty Acid Chain Elongation in Palmitate-Perfused Working Rat Heart

Using acylcarnitine analysis for research protocols requires accuracy, precision, and flexibility in sample type choices and analysis strategies. We conducted studies in fatty acid perfused working rat hearts, looking for the source of the two-carbon units for fatty acid chain elongation [46]. We measured acylcarnitines as a proxy for their respective acyl-CoA derivatives formed from both β -oxidation and chain-elongation of palmitic acid. Powdered rat heart (44.4 mg) was homogenized in 750 μ L of acetonitrile / 2-propanol (3+1) + 250 μ L of 0.1 M KH_2PO_4 (pH 6.7) [60]. The sample was centrifuged (5 min, 16,000 $\times g$), and the supernatant was transferred to a new clean tube. Total carnitine, free carnitine, and butyrobetaine were quantified using 10 μ L of the supernatant, and acylcarnitines were quantified using a second 10 μ L aliquot. Fig. 6 shows UHPLC-MS/MS chromatograms resulting from acylcarnitine, free carnitine, butyrobetaine, and total carnitine analysis of palmitate-perfused rat heart. In Fig. 6, a large amount of palmitoylcarnitine (Peak 74, reflecting palmitoyl-CoA) and myristoylcarnitine (Peak 67, from β -oxidation of palmitoyl-CoA and reflecting myristoyl-CoA) were observed as expected. The surprising result was the large amount of stearoylcarnitine (Peak 78), reflecting chain elongation of palmitoyl-CoA [61]. Table 1 shows the “balance study” for this analysis, with 93% of the calculated acylcarnitine pool accounted for by the sum of the quantified individual acylcarnitines. The full analysis report is provided in the Supplemental Material.

In a separate experiment to further characterize chain elongation, rat heart was perfused with [1,2,3,4- $^{13}\text{C}_4$]-palmitic acid, and a rat heart homogenate was prepared and analyzed [46]. The mass spectrometer acquisition method was modified to collect data for isotopomer analysis, and the chromatogram was extended to observe C20:0 and C22:0 acylcarnitines (arachidyl- and behenoylcarnitine). We found evidence of both chain elongation and β -oxidation in stearoyl-, arachidyl-, and behenoylcarnitine (reflecting their respective acyl-CoAs), and this can be seen in the complex isotope enrichment pattern of arachidylcarnitine (Peak 303) shown in Fig. 7A. Fig. 7A also shows the necessity for the chromatographic separation of the C20:2 (Peak 301) and C20:1 (Peak 302) from C20:0 acylcarnitine, since the mass of the M+0 C20:0 chromatographic peak is identical to and indistinguishable from the M+2 peak of C20:1 and M+4 peak of C20:2. This study would not be possible with tandem MS “profiles” (beyond the problem of analyzing tissue homogenates by that technique) because, for example, the M+0 C20:0 value would be incorrectly quantified due to contributions from M+2 of C20:1 and M+4 of C20:2. Without chromatographic separation of saturated from unsaturated acylcarnitines, the mass spectral results in this study would be uninterpretable due to the multiple overlapping isomeric contributions from the unsaturated isomers of myristoyl-, palmitoyl-, stearoyl-, arachidyl-, and behenoylcarnitine.

Comparison of the unlabeled and labeled analyses was done using the enrichment calculator IsoPat² [62]. With the unlabeled isotope pattern of each acylcarnitine used as a standard, the relative enrichment of its analogous labeled acylcarnitine was then determined (see Fig. 7B). For palmitoylcarnitine, 94% of the enrichment was in the M+4 isotope, as expected following perfusion with [1,2,3,4-¹³C₄]-palmitic acid, conversion to palmitoyl-CoA, and then reflected in palmitoylcarnitine. Myristoylcarnitine was determined to have 94% enrichment in the M+2 isotope, consistent with β -oxidation of M+4 palmitoyl-CoA to M+2 myristoyl-CoA, and then reflected in M+2 myristoylcarnitine. However, stearoyl-, arachidyl-, and behenoylcarnitine were enriched in M+6 as well as M+4 isotopes. The formation of M+4 and M+6 stearoylcarnitine from M+4 palmitate reflects chain elongation of M+4 palmitate using M+0 and M+2 two-carbon chain extender units derived from β -oxidation of M+4 palmitoyl-CoA. This effect is further demonstrated in the isotopomer patterns observed in arachidyl- and behenoylcarnitine (Fig. 7B). Arachidyl- and behenoylcarnitine also showed apparent enrichment in M+5 and M+7 isotopes. We believe this is an artifact resulting from M+4 and M+6 signal spilling into M+5 and M+7, respectively, and correctable with more precise mass designations for MRM isolation and sampling.

3.6 Human Cerebral Spinal Fluid: High Sensitivity Accurate Analysis

We purchased human CSF from a biological products company and analyzed this sample using our standard procedure. Since the concentration values for carnitine and acylcarnitines in CSF were significantly lower as compared to plasma, urine, and tissues, we diluted our calibrants and internal standards 1:10 to accommodate the lower concentrations. Fig. 8 shows UHPLC-MS/MS chromatograms of acylcarnitine, free carnitine, butyrobetaine, and total carnitine analysis of a normal CSF sample. Acetylcarnitine (Peak 3) accounts for 71% of the acylcarnitine pool (see Supplemental Material). Propionylcarnitine (Peak 6) is also quantified. There are no long-chain acylcarnitines in this specimen, but there are quantifiable amounts of isobutyryl-, butyryl-, tigloyl-, 2-methyl-butyryl-, 3-hydroxy-isovaleryl-, succinyl-, methylmalonyl-, and glutaroylcarnitine. The C5 dicarboxylic acylcarnitines are specifically highlighted in Fig. 8D. The peak labeled 41a is isomeric with glutaroyl- and ethylmalonylcarnitine but chromatographs between them; Peak 41a is presumed to be methylsuccinylcarnitine. These three isomeric forms of C5 dicarboxylic acylcarnitine were clearly chromatographically separated, and definitive compound assignments were made based on co-chromatography with standard compounds. Methylsuccinylcarnitine was observed in all the CSF control samples we analyzed. Again, tandem MS “profiles” do not resolve isomeric acylcarnitines and would report all these compounds together as C5 dicarboxylic acylcarnitine.

This CSF sample was quantified using 10 μ L of sample. Analysis was performed without any modification to the sample isolation protocol, but to accommodate the lower concentrations, the calibrant and internal standard concentrations were reduced. The analysis remains highly accurate; Table 1 also shows the “balance study” for this analysis. The entire calculated acylcarnitine pool (102%) is accounted for by the sum of the quantified individual acylcarnitines.

4. Conclusions

We have shown the application of our validated UHPLC-MS/MS second-tier method for quantification of total carnitine, free carnitine, butyrobetaine, and acylcarnitines to CAP plasma testing samples, a CAP urine sample, research rat heart homogenates, and a CSF sample. We have demonstrated quantitative accuracy in diagnosing malonic acidemia (MA), which is problematic with tandem MS “profiles”. Since tandem MS “profiles” cannot distinguish among isomeric acylcarnitine species, elevated C4 acylcarnitine could be diagnosed as either SCADD or IBD. In contrast, with our method, we unambiguously determined the correct diagnosis as SCADD. In a second example, elevated HO-C5 and C5:1 acylcarnitines could be diagnosed as either 3-MCC or BKT as tandem MS “profiles”. However, with our method, we unambiguously determined the correct diagnosis as BKT. In analyzing urinary acylcarnitines in a CAP sample from a patient with MMA, we demonstrated the flexibility of our second-tier system to accurately quantify a urine sample with no modification to our method or sample workup. This provided valuable information for follow-up to treatment with carnitine. Urine is not typically analyzed by tandem MS. For perfused rat hearts, we provided both accurate quantification and isotopomer analysis, demonstrating the robustness of both the sample preparation scheme and the chromatographic and mass spectral analysis capabilities. In a high sensitivity application, we analyzed a CSF sample without modification to our sample preparation protocol. In all of these examples, we show that going beyond tandem MS acylcarnitine “profiles” is both feasible and advantageous using our validated second-tier UHPLC-MS/MS method. The accuracy and precision of the procedure should make it the method of choice for clinical and basic research projects, including treatment protocol studies, acylcarnitine biomarker studies, and metabolite studies using tissue, urine, or other sample matrices. Due to these unique capabilities, numerous collaborations and publications [59,63,64,65,66] have resulted from the application of this validated acylcarnitine analysis method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Applied our UHPLC-MS/MS acylcarnitine method to clinical and research samples
- Absolute quantification of 66 acylcarnitine species in 14 min chromatograms
- Examined plasma malonic acidemia, SCADD vs. IBD, 3-MCC vs. BKT, and urine MMA
- Show labeled fatty acid perfused rat heart isotopomers and CSF research samples
- These results are not possible when using tandem MS acylcarnitine “profiles”

Fig. 1.

UHPLC-MS/MS chromatogram of an acylcarnitine calibration curve medium point.

Overlaid extracted ion chromatograms of 78 transitions monitored for 66 compounds and 12 internal standards. Internal standards are colored red. Peak Identities:

1) *S*-3-hydroxy-butyryl-, 2) *R*-3-hydroxy-butyryl-, 3) acetyl-, 4) d_6 -acetyl-, 5) 3-hydroxy-isovaleryl-, 6) propionyl-, 7) d_3 -propionyl-, 8) *S*-3-hydroxy-hexanoyl-, 9) *R*-3-hydroxy-hexanoyl-, 10) isobutyryl-, 11) butyryl-, 12) d_3 -butyryl-, 13) tigloyl-, 14) 3-methyl-crotonyl-, 15) benzoyl-, 16) pivaloyl- 17) 2-methyl-butyryl-, 18) isovaleryl-, 19) d_9 -isovaleryl-, 20) valeryl-, 21) phenylacetyl-, 22) *S*-3-hydroxy-octanoyl-, 23) *R*-3-hydroxy-octanoyl-, 24) phenylpropionyl-, 25) hexanoyl-, 26) 4-phenyl-butyryl-, 27) malonyl-, 28) d_3 -malonyl-, 29) 4-methyl-hexanoyl-, 30) succinyl-, 31) *cis*-3,4-methylene-heptanoyl-, 32) glutaroyl-, 33) d_3 -glutaroyl-, 34) methylmalonyl-, 35) valproyl-, 36) *S*-3-hydroxy-decanoyl-, 37) *R*-3-hydroxy-decanoyl-, 38) adipoyl-, 39) 5-decynoyl-, 40) 3- methyl-glutaroyl-, 41) ethylmalonyl-, 42) octanoyl-, 43) d_3 -octanoyl-, 44) 2,6-dimethyl-heptanoyl-, 45) suberoyl-, 46) 4-methyl-octanoyl-, 47) *cis*-3,4-methylene-nonanoyl-, 48) *cis*-4-decenoyl-, 49) *S*-3-hydroxy-lauroyl-, 50) *R*-3-hydroxy-lauroyl-, 51) sebacoyl-, 52) decanoyl-, 53) undecanedioyl-, 54) undecanoyl-, 55) *S*-3-hydroxy-myristoyl-, 56) *trans*-2-dodecenoyl-, 57) *R*-3-hydroxy-myristoyl-, 58) *cis,cis*-5,8-tetradecadienoyl-, 59) lauroylcarnitine, 60) myristoleoylcarnitine, 61) d_3 -myristoleoylcarnitine, 62) *cis*-5-tetradecenoylcarnitine, 63) *S*-3-hydroxy-palmitoyl-, 64) *R*-3-hydroxy-palmitoyl-, 65) *trans*-2-tetradecenoyl-, 66) α -linolenoyl-, 67) myristoyl-, 68) γ -linolenoyl-, 69) palmitoleoyl-, 70) linoleoyl-, 71) *S*-3-hydroxy-stearoyl-, 72) *R*-3-hydroxy-stearoyl-, 73) *trans*-2-hexadecenoyl-, 74) palmitoyl-, 75) d_3 -palmitoyl-, 76) oleoyl-, 77) heptadecanoyl-, 78) stearoylcarnitine.

Fig. 2.

UHPLC-MS/MS chromatogram of the acylcarnitine analysis of a plasma specimen provided by CAP with the expected diagnosis of malonic acidemia. Peak identities and colors are the same as in Fig. 1. Malonylcarnitine is Peak 27 and d_3 -malonylcarnitine internal standard is Peak 28. The inset graph is a histogram showing the frequency distribution of quantitative values determined by us and other labs participating in CAP testing. The highlighted bar at 2.5 $\mu\text{mol/L}$ is the value we and six others determined from this analysis. Also present in this chromatogram are *S*-3-hydroxy-octanoyl- (Peak 22), *R*-3-hydroxy-octanoyl- (Peak 23), hexanoyl- (Peak 25), succinyl- (Peak 30) and *cis*-3,4-methylene-heptanoylcarnitine (Peak 31).

Fig. 3.

UHPLC-MS/MS chromatogram of the acylcarnitine analysis of a plasma specimen provided by CAP with the expected diagnosis by tandem MS of either short-chain acyl-CoA dehydrogenase deficiency (SCADD) or isobutyryl-CoA dehydrogenase deficiency (IBD). Peak identities and colors are the same as in Fig. 1. This chromatogram illustrates the resolution between isobutyryl- (Peak 10) and butyrylcarnitine (Peak 11), leading us to the correct diagnosis of SCADD. Peak 12 is d_3 -butyrylcarnitine internal standard.

Fig. 4.

UHPLC-MS/MS chromatogram of the acylcarnitine analysis of a plasma specimen provided by CAP with the diagnosis of either 3-methyl-crotonyl carboxylase deficiency (3-MCC) or β -ketothiolase deficiency (BKT). Peak identities and colors are the same as in Fig. 1. Peaks 5a–d are the four stereoisomeric forms (*RR*-, *SS*-, *SR*-, and *RS*-) of 3-hydroxy-2-methyl-butyrylcarnitine. Tigloylcarnitine (Peak 13) and 3-methyl-crotonylcarnitine (Peak 14) are also chromatographically separated. This chromatogram illustrates the separation of HO-C5 and C5:1 acylcarnitines, leading us to the correct diagnosis of BKT.

Fig. 5. UHPLC-MS/MS chromatograms of the short- and medium-chain acylcarnitines, free carnitine, butyrobetaine, and total carnitine from analysis of a urine specimen provided by CAP with the expected diagnosis of methylmalonic academia (MMA). Peak identities and colors are the same as in Fig. 1. A) Acylcarnitine chromatogram from a 1:50 dilution of the urine. Peak 6 is propionylcarnitine. The inset chromatograms a) accentuate methylmalonylcarnitine and b) 10x more concentrated urine accentuating methylmalonylcarnitine. Peak 34 is methylmalonylcarnitine. B) Free carnitine and butyrobetaine chromatogram. Peak Identities: 101) free carnitine, 102) d₃-carnitine, 103) butyrobetaine, 104) d₃-butyrobetaine. C) Total carnitine chromatogram. Peak Identities: 105) total carnitine, 106) d₃-carnitine.

Fig. 6.

UHPLC-MS/MS chromatograms of acylcarnitines, free carnitine, butyrobetaine, and total carnitine from analysis of a rat heart perfused with palmitic acid. Peak identities and colors are the same as in Fig. 1 and Fig. 5. The chromatogram displays prominent increases in myristoyl- (Peak 67), palmitoyl- (Peak 74), and stearoylcarnitine (Peak 78). Analysis was performed using 10 μ L of a 1 mL homogenate of 44.4 mg of powdered rat heart. A) acylcarnitine chromatogram B) free carnitine and butyrobetaine chromatogram. C) total carnitine chromatogram.

Fig. 7. Perfused Rat Heart with Isotopomer Analysis. Rat heart was perfused for 60 min with [1,2,3,4- $^{13}\text{C}_4$]-palmitic acid in the working heart mode. A) The chromatographic separation and the determination of the mass isotopomer distribution in arachidylcarnitine is shown. Peak Identities: 301) C20:2 acylcarnitine, 302) C20:1 acylcarnitine, 303) C20:0 acylcarnitine (arachidylcarnitine) B) The % isotope enrichment compared to unlabeled myristoyl-, palmitoyl-, stearoyl-, and arachidylcarnitine are presented. The isotopomer patterns of stearoyl-, arachidyl-, and behenoylcarnitine show evidence of both chain elongation and β -oxidation of their analogous acyl-CoAs.

Fig. 8.

UHPLC-MS/MS chromatograms of acylcarnitines, free carnitine, butyrobetaine, and total carnitine from analysis of a human cerebral spinal fluid (CSF) specimen. Analysis was performed using 10 μ L of CSF with calibrants and internal standards diluted 1:10 to accommodate the low concentration of carnitine and acylcarnitines in CSF. Peak identities and colors are the same as in Fig. 1 and Fig. 5. A) acylcarnitine chromatogram B) free carnitine and butyrobetaine chromatogram. C) total carnitine chromatogram. D) Expanded range showing only C5 dicarboxylic acylcarnitines. Peak 32 is glutaroylcarnitine, Peak 41 is ethylmalonylcarnitine, and Peak 41a is presumed to be methylsuccinylcarnitine.

Table 1

Balance Studies: Demonstration of Quantitative Accuracy by Comparing Calculated (Total-Free) and Summed Acylcarnitine Values

	MA CAP plasma ($\mu\text{mol/L}$)	SCADD CAP plasma ($\mu\text{mol/L}$)	BKT CAP plasma ($\mu\text{mol/L}$) [*]	MMA CAP urine ^{**} ($\mu\text{mol/g creatinine}$)	Palmitate Perfused Rat Heart (nmol/g ww)	Cerebral Spinal Fluid ($\mu\text{mol/L}$) ^{***}
Total Carnitine	40.8	47.1	80.7	24311	678.3	1.93
Free Carnitine	33.5	36.0	67.3	11049	284.1	1.41
Total - Free	7.27	11.1	13.4	13262	394.2	0.520
Sum of Acylcarnitines	6.74	9.92	10.3	10664	364.9	0.531
% Acylcarnitines Accounted For	93%	90%	77%	80%	93%	102%

MA = malonic acidemia; SCADD = short-chain acyl-CoA dehydrogenase deficiency; BKT = β -ketothiolase deficiency; MMA = methylmalonic acidemia; CAP = College of American Pathologists

^{*} 3-hydroxy-2-methyl-butyrylcarnitines were included in the calculation

^{**} patient is receiving carnitine therapy

^{***} calibrants and internal standards were diluted 1:10 to accommodate the lower concentrations