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Selective and accurate C5 acylcarnitine quantitation by UHPLC–MS/MS: Distinguishing true isovaleric acidemia from pivalate derived interference

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ABSTRACT

Tandem MS acylcarnitine "profiles" are extremely valuable. Although used appropriately in newborn screening programs to identify patients with possible diseases, their inadequate quantitative accuracy and lack of selectivity is problematic for confirmatory testing. In this report, we show the application of our validated, selective, accurate, precise, and robust UHPLC–MS/MS method for quantitation of acylcarnitines, specifically to C5 acylcarnitines: pivaloyl-, 2-methylbutyryl-, isovaleryl-, and valerylcarnitine. Standardized calibrants were used to generate 13-point, 200-fold concentration range calibration curves. Samples were isolated by solid-phase extraction and derivatized with pentafluorophenacyl trifluoromethanesulfonate. Acylcarnitine pentafluorophenacyl esters were eluted in 14 min chromatograms. Data demonstrating quantitative stability and method robustness over a five year time period are shown and these results validate the method's accuracy and robustness. Urine from patients with isovaleric acidemia (with the disease marker isovalerylcarnitine) and with pivaloylcarnitine present are shown. These results demonstrate the method's ability to distinguish true isovaleric acidemia from pivalate derived interference. Our method for acylcarnitine quantitation is shown to be accurate, precise, and robust for selective quantitation of isovalerylcarnitine, and thus is recommended for confirmatory testing of suspected isovaleric acidemia patients.

1. Introduction

Expanded newborn screening programs require rapid analysis of many patient samples, identifying the very few patients with positive results, followed by validation of the positive results with confirmatory tests [1]. Since elevated amino acids and acylcarnitines are diagnostic markers for many metabolic diseases, screening of dried blood spots for amino acid and acylcarnitine "profiles" is a usual component of expanded newborn screening programs [2]. The original mass spectrometric technique was pioneered by Millington et al. [3,4]. Eventually using a triple quadrupole mass spectrometer, they collected precursor ion scans whose responses were predominantly but not exclusively from characteristic product ion fragments of acylcarnitine molecular cations. The analysis generated a qualitative metabolic "profile" of acylcarnitines [5]. Many groups contributed to produce the mature technique used today [6–8], and for speed and breadth, there is no technology to rival tandem MS. However, correcting false positive results using confirmatory tests are essential for appropriate patient care [9].

For confirmation of positive amino acid "profiles", analysis kits for selective (using chromatography) and accurate (using standardized calibrants) amino acid analyses are widely available [10–12]. However, this is not the case with acylcarnitines. Without commercially produced confirmatory methodologies, the "profile" is repeated as the confirmatory test. Reports of quantitative problems and false positive results from insufficient selectivity from tandem MS "profiles" are longstanding and widespread [13–17], particularly with C5 acylcarnitines [18,19]. Although "profiling" is appropriately used in newborn screening programs to identify patients with possible diseases, the inadequate quantitative accuracy and lack of selectivity in tandem MS "profiles" is problematic for confirmatory testing, where more rigorous methodology is recommended [20,21].

Some groups have developed specialized chromatographic methods for C5 acylcarnitine analysis [22–24]. In contrast, we developed a comprehensive, accurate, precise, selective, and robust second-tier method for the quantitation of 66 different short-, medium, and long-chain acylcarnitines by UHPLC–MS [25,26]. This method is capable of analyzing plasma, bloodspots, urine, tissue homogenates, and other sample materials without modification. As recommended [27],

Abreviations: IVA, isovaleric acidemia; SCADD, short chain acyl-CoA dehydrogenase deficiency; GAI, glutaric acidemia type I; SCX, strong cation exchange; SPE, solid-phase extraction; LLOQ, lower limit of quantitation; ULOQ, upper limit of quantitation; CAP, College of American Pathologists; CIDEM, Center for Inherited Disorders of Energy Metabolism

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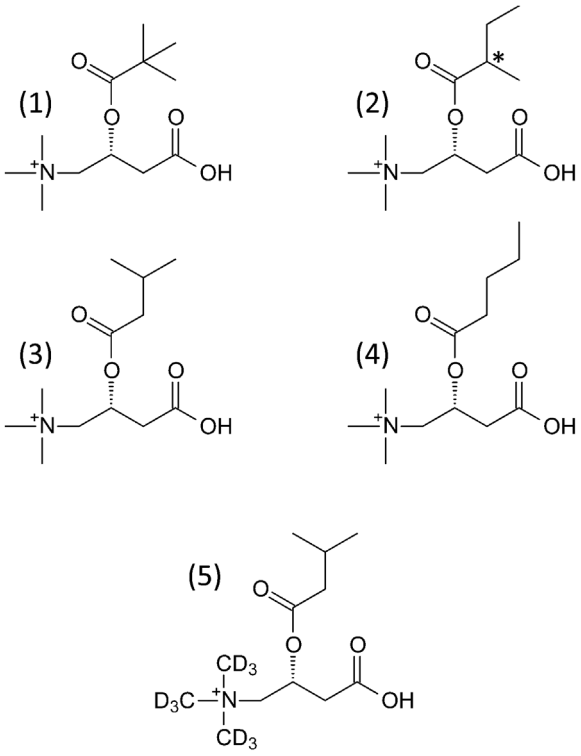

Fig. 1. C5 Acylcarnitines. We synthesized the four constitutional isomers of C5 acylcarnitines, and purchased isovaleryl-d_9-carnitine for use as an internal standard. Their structures are labeled as follows: (1) pivaloylcarnitine, (2) 2-methylbutyrylcarnitine, (3) isovalerylcarnitine, (4) valerylcarnitine, (5) isovaleryl-d_9-carnitine. Synthesis of 2-methylbutyrylcarnitine yielded R- and S- isomers due a chiral center at carbon 2. The chiral center is marked with "*" in this figure.

acylcarnitines are chromatographically separated and quantitated with standardized calibrants using multiple-point calibration curves. We use this method as a confirmatory test for newborn screening results [28], to monitor patient treatment [29], and for metabolism research [30].

There are four constitutional isomers of C5 acylcarnitines (see Fig. 1): (1) pivaloylcarnitine (trimethylacetylcarnitine), (2) 2-methylbutyrylcarnitine, (3) isovalerylcarnitine, and (4) valerylcarnitine. Also shown in Fig. 1 is (5), isovaleryl-d_9-carnitine, which we purchased for use as the C5 acylcarnitine internal standard. Pivalate is used to generate prodrugs to increase oral bioavailability. Metabolically, pivalate is converted to pivaloyl-CoA by acyl-CoA synthetase. However, pivaloyl-CoA cannot be oxidized, and it accumulates. In humans, pivaloylcarnitine is the dominant route of pivalate elimination [31]. In addition, pivalate derivatives are used in the cosmetic industry as emollients [19]. Pivalate can also be generated from neo fatty acids, which come from bacteria in the intestine, or from dairy products and meat [32]. 2-Methylbutyrylcarnitine is a normal endogenous acylcarnitine species, which can accumulate due to a defect in isoleucine catabolism in the very rare disease 2-methylbutyryl-CoA dehydrogenase deficiency [33,34]. There are R- and S- isomers of 2-methylbutyryl-L-carnitine resulting from the chiral center at carbon 2, marked with "*" in Fig. 1. Because L-carnitine is also chiral, the R- and S- isomers of 2-methylbutyryl-L-carnitine are diastereomers and potentially separable using achiral chromatographic stationary phases (see Supplementary Material, pages S01-S02 and S05). Isovalerylcarnitine is the diagnostic marker for isovaleric acidemia (IVA) [35]. Elevated C5 acylcarnitines reported from expanded newborn screening programs, using tandem MS "profiling" of acylcarnitines, are interpreted as suspected IVA. Therefore, it is essential that isovalerylcarnitine is distinguishable from the other three C5 isomers by the confirmatory test. Valerylcarnitine is normally present at very low concentrations, but it may be elevated as a result of odd chain fatty acid degradation.

We synthesized the four C5 acylcarnitines in Fig. 1 as described [36]. For use as analytical standards, we purified small amounts of the raw synthetic products, standardized solutions of purified C5 acylcarnitines, and incorporated them into our calibration solutions [25]. This report describes a selective analysis of C5 acylcarnitines provided by our comprehensive acylcarnitine analysis method. Included are examples of an IVA patient urine and urine containing pivaloylcarnitine provided to us by the College of American Pathologists, which we used in our proficiency testing.

2. Materials and methods

2.1. Chemicals

Labeled internal standards (including isovaleryl-d_9-carnitine) were either purchased from Cambridge Isotope Laboratories (Andover, MA) or synthesized using methyl-d_3-L-carnitine purchased from Cambridge. Acylcarnitine reference standards were synthesized using L-carnitine and established methods [35,37–39]. The synthesized acylcarnitines were purified using solid-phase extraction (SPE) and preparative HPLC, and solutions were standardized using our free and total carnitine assay as described [17,25]. Oasis MCX 96-well SPE plates (mixed-mode cation-exchange, 30 µm, 10 mg) were purchased from Waters Corporation (Milford, MA). Acetonitrile, acetic acid, triethylamine, and tetrahydofuran were HPLC grade from Fisher Scientific (Pittsburgh, PA). Formic acid, diisoproylethylamine, and pyridine were purchased from Sigma-Aldrich (St. Louis, MO). USP 200 proof ethanol was from Decon Labs, Inc (King of Prussia, PA). Gibco bovine albumin fraction V solution (7.5%) in phosphate-buffered saline was purchased from Fisher. Pentafluorophenacyl trifluoromethanesulfonate was prepared as described [25].

2.2. Instrumentation

The instrumentation consisted of two Agilent UHPLC 1290 Infinity binary pumps, autosampler, thermostated column compartment with a six-port valve, an Agilent 1260 DAD UV detector (used to monitor the loading of the SCX trap with detection at 260 nm), and an Agilent 6460 QQQ triple quadrupole electrospray mass spectrometer (Agilent Technologies, Santa Clara, CA). Sequential ion-exchange/reversed-phase chromatographic separations were accomplished with gradients containing acetonitrile, water, triethylamine, and acetic acid. An SCX trap cartridge (EXP Trap Cartridge 2.1 × 5 mm, 3 µm, Optimize Technologies, Oregon City, OR), connected in series through the six-port valve with an Agilent Poroshell 120 EC-C8 column (3.0 × 100 mm, 2.7 µm) was used to perform the two-dimensional chromatographic separations. Full method details and instrument parameters were as described [25].

2.3. Calibration curves and quality controls

Stock solutions contained 66 different standardized acylcarnitines. The calibrants for multiple-point calibration curves (including pivaloyl-, 2-methylbutyryl-, isovaleryl-, and valerylcarnitine) were formulated by accurately dispensing specific volumes of the stock solutions into microcentrifuge tubes, along with internal standards, and then evaporating them to dryness. To dried calibrants and quality control samples was added 10 µL of bovine albumin in phosphate-buffered saline (a sample matrix proxy for both plasma and urine specimens) and these were then treated the same as the samples. For pivaloyl-, 2-methylbutyryl-, isovaleryl-, and valerylcarnitine, the lower limit of quantitation (LLOQ) was the lowest nonzero calibrant (0.05 nmol/mL), and the upper limit of quantitation (ULOQ) was 200-fold greater (10.0 nmol/mL). Three quality control samples were prepared concurrently (QC-Low, QC-Medium, and QC-High) in the same way as the