Molecular Genetics and Metabolism 120 (2017) 363–369

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Correcting false positive medium-chain acyl-CoA dehydrogenase deficiency results from newborn screening; synthesis, purification, and standardization of branched-chain C8 acylcarnitines for use in their selective and accurate absolute quantitation by UHPLC–MS/MS

Paul E. Minkler [a], Maria S.K. Stoll [a], Stephen T. Ingalls [a], Charles L. Hoppel [a,b,*]

[a] Center for Mitochondrial Diseases, Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA
[b] Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

ARTICLE INFO

Article history:
Received 1 December 2016
Received in revised form 18 January 2017
Accepted 6 February 2017
Available online 8 February 2017

Keywords:
Acylcarnitine
Medium-chain acyl-CoA dehydrogenase deficiency (MCADD)
Newborn screening
False positive
Valproate
Valproylcarnitine

ABSTRACT

While selectively quantifying acylcarnitines in thousands of patient samples using UHPLC–MS/MS, we have occasionally observed unidentified branched-chain C8 acylcarnitines. Such observations are not possible using tandem MS methods, which generate pseudo-quantitative acylcarnitine "profiles". Since these "profiles" select for mass alone, they cannot distinguish authentic signal from isobaric and isomeric interferences. For example, some of the samples containing branched-chain C8 acylcarnitines were, in fact, expanded newborn screening false positive "profiles" for medium-chain acyl-CoA dehydrogenase deficiency (MCADD). Using our fast, highly selective, and quantitatively accurate UHPLC–MS/MS acylcarnitine determination method, we corrected the false positive tandem MS results and reported the sample results as normal for octanoylcarnitine (the marker for MCADD). From instances such as these, we decided to further investigate the presence of branched-chain C8 acylcarnitines in patient samples. To accomplish this, we synthesized and chromatographically characterized several branched-chain C8 acylcarnitines (in addition to valproylcarnitine): 2-methylheptanoylcarnitine, 6-methylheptanoylcarnitine, 2,2-dimethylhexanoylcarnitine, 3,3-dimethylhexanoylcarnitine, 3,5-dimethylhexanoylcarnitine, 2-ethylhexanoylcarnitine, and 2,4,4-trimethylpentanoylcarnitine. We then compared their behavior with branched-chain C8 acylcarnitines observed in patient samples and demonstrated our ability to chromographically resolve, and thus distinguish, octanoylcarnitine from branched-chain C8 acylcarnitines, correcting false positive MCADD results from expanded newborn screening.

1. Introduction

The widespread implementation of expanded newborn screening programs using tandem MS "profiling" of amino acids and acyl-L-carnitines from bloodspots has been effective and beneficial [1]. The original technique was pioneered by Millington et al. [2], who dubbed it "tandem mass spectrometry" or tandem MS [3,4]. They used first a magnetic sector instrument, and then a triple quadrupole mass spectrometer to collect precursor ion scans, whose responses were predominantly from acylcarnitine molecular cations. The analysis generated a qualitative metabolic "profile" of acylcarnitines. Many groups contributed to advances to the technique, including derivatization with HCl butanol for sensitivity and selectivity improvements [5], use of electrospray ionization and flow-injection analysis, and software to assist in the interpretations [6]. Transitions for amino acids were added and full blown expanded newborn screening was established [7]. For speed and breath of metabolite coverage of newborn screening of dried bloodspots, there is no technology to rival tandem MS. The technique retained the original jargon of tandem MS generating "profiles".

The use of the term "profile" was intended to convey the inherent lack of analytical specificity and quantitative accuracy that "profiling" sacrifices in exchange for rapidity. The requirement for confirmation of screening results using more rigorous methodologies is implicit. In the case of amino acids, analysis kits for selective (using chromatography) and accurate (using standardized calibrants) amino acid analysis are commercially produced and widely available [8,9,10]. However, this is not the case with acylcarnitines. Without commercially produced confirmatory methodologies, the decade's long practice has been to simply repeat the "profile" and ignore its short-comings. As expected, widely variable quantitative results from acylcarnitine "profiling" of proficiency test samples have been shown [11,12]. Reports of

* Corresponding author at: Center for Mitochondrial Diseases, Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA.
E-mail address: charles.hoppel@case.edu (C.L. Hoppel).

http://dx.doi.org/10.1016/j.ymgme.2017.02.006
1096-7192/

quantitative problems and false positive results from insufficient selectivity when using "profiling" are long-standing and widespread [13,14,15,16,17]. Although "profiling" is appropriately used in expanded newborn screening programs to identify patients with possible diseases, the inadequate quantitative accuracy and lack of selectivity in tandem MS "profiles" is unacceptable for standard clinical uses [18,19].

We developed a comprehensive accurate, precise, selective, and robust second-tier method for the quantitation of 66 different short-, medium-, and long-chain acyl-L-carnitines by UHPLC–MS [20,21]. The procedure is a second-tier method which we use for 1) confirmation of expanded newborn screening results, 2) monitoring patient treatment protocols, and 3) metabolism research. The procedure is fast, accurate, precise, selective, and robust. It has been in use in our laboratory for >6 years, during which we have analyzed and reported the results from >5000 patient samples. This method is sample matrix independent – capable of analyzing plasma, bloodspots, urine, tissue homogenates, etc. without modification. Isomeric acylcarnitines are chromatographically separated and quantitated with standardized calibrants using multiple-point calibration curves, as recommended by the FDA Guidelines [22]. One result of our adherence to these sound analytical principles is that the patient values we generate from our L-carnitine determination today (with UHPLC–MS/MS) are unchanged from those values we generated 10 years ago (using HPLC–Ion trap MS), 20 years ago (using HPLC-fluorescence), 30 years ago (using HPLC-UV), or even 40 years ago (using radio-labeled scintillation counting) [23].

In the course of analyzing patient samples, we have occasionally observed unidentified branched-chain C8 acylcarnitines. Our colleagues reported that some of these samples were, in fact, false positive newborn screening results [14]. The newborn screen indicated elevated C8 acylcarnitine, suggesting medium-chain acyl-CoA dehydrogenase deficiency (MCADD). Using our procedure (which separates C8 acylcarnitine isomers), we found normal octanoylcarnitine. (N.B. octanoylcarnitine is the specific C8 acylcarnitine isomer elevated in MCADD). Instead of octanoylcarnitine, we observed a branched-chain C8 acylcarnitine which was responsible for the vast majority of the C8 acylcarnitine response.

Chromatographically, the branched-chain C8 acylcarnitine was distinct from both of the C8 acylcarnitines that we already separate and quantify: octanoylcarnitine, the marker for MCADD, and valproylcarnitine, which we observe in patients receiving valproate therapy [24]. Recently, Kratz and Albert [25] reported observing a branched-chain C8 acylcarnitine in their patient's plasma. By GC/MS urinary organic acid analysis, they identified 2-ethylhexanoic acid in the urine of these same patients. Then they synthesized 2-ethylhexanoylcarnitine and showed that, by LC–MS/MS, it was indistinguishable from the branched-chain C8 acylcarnitine they had observed in their patient's plasma. This strong evidence for the identity of the branched-chain C8 acylcarnitine was further bolstered when they proposed that the source of 2-ethylhexanoic acid was plasticizers in medical devices, and that their patients were exposed to these in the course of their treatment. We synthesized 2-ethylhexanoylcarnitine for our own use, but when we compared its chromatographic behavior to our observed branched-chain C8 acylcarnitine [14], the retention times were different.

To investigate the presence of branched-chain C8 acylcarnitines in patient's samples, we undertook the synthesis of several additional branched-chain C8 acylcarnitines. Along with octanoylcarnitine and valproylcarnitine, there are 24 other possible isomeric forms of C8 acylcarnitines (see Fig. 1). We prepared seven branched-chain acylcarnitines from acid or acid chlorides that were commercially available. To use as analytical standards, we purified small amounts of the raw synthetic products, standardized solutions of purified acylcarnitines, and incorporated them into calibration solutions. We now report on the UHPLC–MS/MS behavior of these branched-chain C8 acylcarnitines, and their presence in patient samples.

2. Materials and methods

2.1. Chemicals

L-Carnitine HCl, dry acetonitrile, silver perchlorate, 2-ethylhexanoyl chloride, 2-methylheptanoic acid, and thionyl chloride were purchased from Sigma-Aldrich (St. Louis, MO). 6-Methylheptanoic acid and 2,2-dimethylhexanoic acid were purchased from Alfa Aesar (Haverhill, MA). 3,5-Dimethylhexanoic acid and 2,4,4-trimethylpentanoic acid were purchased from Aurum Pharmatech (Howell, NJ). 3,3-Dimethylhexanoic acid was purchased from TripleBond Corp. (Guelph, Ontario, Canada). Oasis MCX mixed-mode reversed-phase/strong cation-exchange solid-phase extraction columns (6 cm^3/500 mg) and plates (96 well, 10 mg) were purchased from Waters Corporation (Milford, MA). Methanol and acetonitrile used for chromatographic separations were HPLC grade from Fisher Scientific (Pittsburgh, PA). Pentafluorophenacyl trifluoromethanesulfonate was prepared as described [26].

2.2. Instrumentation

As described before [20], the instrumentation consisted of two Agilent UHPLC 1290 Infinity binary pumps, autosampler, and thermostated column compartment with a 6-port valve, and an Agilent 6460 QQQ triple quadrupole LC/MS (Agilent Technologies, Santa Clara, CA). Sequential ion-exchange/reversed-phase chromatographic separation was accomplished with an SCX trap cartridge (EXP Trap Cartridge 2.1 × 5 mm, 3 μm, 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). Instrument parameters were as described [20].

2.3. Synthesis, purification, and standardization of branched-chain C8 acylcarnitines

Using the procedure of Brendal and Bressler [27], we combined L-carnitine perchlorate with 2-ethylhexanoyl chloride to prepare 2-ethylhexanoylcarnitine. For other branched-chain C8 acylcarnitines, we replicated our described synthesis of *cis*-3,4-methyleneheptanoylcarnitine [28]. Thus, we combined each of six branched-chain C8 acids (2-methylheptanoic acid, 6-methylheptanoic acid, 2,2-dimethylhexanoic acid, 3,3-dimethylhexanoic acid, 3,5-dimethylhexanoic acid, and 2,4,4-trimethylpentanoic acid) with thionyl chloride to generate acid chlorides, and then prepared the acylcarnitines. Purification of synthesized acylcarnitines was accomplished using a two-step chromatographic process, combining mixed-mode reversed-phase/strong cation-exchange solid-phase extraction (to remove unreacted acid or acid chloride) followed by preparative HPLC (to remove carnitine and synthetic impurities). We then standardized our stock solutions of branched-chain C8 acylcarnitines. This was necessary because: 1) the small amount of free carnitine contamination needed to be accounted for, and 2) synthesized acylcarnitines often do not crystallize into readily transferable solids and are therefore not amendable to accurate weighing. Using standardized L-carnitine stock solutions, we standardized stock solutions of the branched-chain C8 acylcarnitines with our method for free and total carnitine [20]. Detailed descriptions of these procedures are contained in the Supplemental Material S1–S7.

2.4. Calibrants and samples, preparation and analysis

Calibrants and samples were prepared and analyzed as described [20]. Briefly, to 10 μL of plasma, diluted urine, or one 4 mm dried blood spot, (plus internal standards), was added organic solvents to precipitate salts and proteins. Calibrants were prepared by transferring