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Separation and identification of plasma short-chain acylcarnitine isomers by HPLC/MS/MS for the differential diagnosis of fatty acid oxidation defects and organic acidemias

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Abstract

This paper reports a new, high-performance liquid chromatography/tandem mass spectrometry method for the separation and identification of human plasma short-chain acylcarnitine isomers. This simple, rapid procedure involves the use of a single sample previously shown to contain elevated acylcarnitine concentrations by flow injection analysis, and can separate two C4, three C5, two C5:1 and four C5-OH acylcarnitine isomers, thus permitting the differential diagnosis of certain fatty acid oxidation defects and organic acidemias.

Keywords: Acylcarnitine isomers; Tandem mass spectrometry; Fatty acid oxidation defects; Isovaleryl-CoA dehydrogenase; 3-Methylcrotonyl-CoA carboxylase; β-Ketothiolase; 3-Hydroxy-3-methylglutaryl-CoA lyase; Pivaloylcarnitine

1. Introduction

The analysis of plasma acylcarnitines is important in the diagnosis of defects related to branched-chain amino acids metabolism (BCAAM) and fatty acid oxidation [1]. Over the last decade, significant advances in the analysis of acylcarnitines have been made due to the development of techniques such as fast atom bombardment/mass spectrometry (FAB/MS), liquid secondary ion tandem mass spectrometry (LSIMS/MS) [2], high-performance liquid chromatography coupled with ultraviolet diode-array detection (HPLC/UV) [3], matrix-assisted laser desorption ionization/time-of-flight/mass spectrometry (MALDI/TOF/MS) [4,5], and high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) [6]. The analysis of plasma acylcarnitines in the form of their butyl esters by flow injection analysis/electrospray ionization/tandem mass spectrometry (FIA/ESI/MS/MS) is also a fast, accurate method with high sensitivity and reproducibility. In addition it allows the analysis of the large numbers of samples that have to be processed in the screening of newborns [7]. However, this method cannot identify different acylcarnitine isomers. Four-carbon acylcarnitine exists as two isomers: butyrylcarnitine and isobutyrylcarnitine. The plasma concentration of the first is increased in patients with short-chain acyl-CoA dehydrogenase (SCAD) deficiency, and the second increased in those with isobutyryl-CoA dehydrogenase (IBD) deficiency. However, both these genetic defects are associated with an increased C4-carnitine signal, and are indistinguishable by conventional screening methods. There are also three acylcarnitine isomers with five carbons: isovalerylcarnitine – the diagnostic marker of isovaleryl-CoA dehydrogenase (IVD) deficiency, 2-methylbutyrylcarnitine – which is found in greater amounts in patients with 2-methylbutyryl-CoA dehydrogenase (MBD) deficiency, and pivaloylcarnitine (2,2-dimethylpropionylcarnitine), which appears after the administration of pivoxyl-containing medications such as certain antibiotics. Two C5:1 isomers also exist: tiglylcarnitine, the plasma concentration of which increases in β-ketothiolase (β-KT) deficiency, and 3-methylcrotonylcarnitine, which is increased in 3-methylcrotonyl-CoA carboxylase (MCC)

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deficiency. Finally, there are three C5-OH isomers: 3-hydroxyisovalerylcarnitine, which appears in patients with either an MCC defect or 3-hydroxy-3-methylglutaryl-CoA lyase (HMGCL) deficiency, and two diastereoisomers of 2-methyl-3-hydroxybutyrylcarnitine, which appear in 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency. The identification of these isomers, however, is not possible with current techniques [8].

Although gas chromatography [9], tandem mass spectrometry with collisionally activated decomposition of the parent ions produced by FAB [10], and radioisotopic exchange/high-performance liquid chromatography [11] have been used in the past to separate plasma acylcarnitine isomers, these labor intensive methods are incompatible with routine laboratory work. However, an HPLC/MS/MS method has recently been reported that can separate and quantify certain acylcarnitine isomers and dicarboxylic acylcarnitines [12], as well as a chromatographic method for the separation and detection of carnitine and acylcarnitines in the form of their pentafluorophenacyl esters [13].

The aim of this work was to develop an HPLC/MS/MS method for the separation and identification of isomeric short-chain acylcarnitine species in human plasma samples previously analyzed by FIA, thus allowing the differential diagnosis of a number of disorders in patients with pathological acylcarnitine profiles.

2. Materials and methods

2.1. Patients

Following the detection of increased concentrations of short-chain acylcarnitines by FIA in the plasma of eight patients with suspected metabolic disease, the same samples were subjected to acylcarnitine isomer separation by HPLC/MS/MS. Patient A was a male who presented with an episode of fever with diarrhea, microcephaly, a dysmorphic phenotype, and metabolic acidosis with hyperlactacidemia. An elevated level of ethylmalonic acid was detected in his urine at diagnosis. SCAD deficiency was subsequently confirmed by enzyme and genetic analyses [14].

Patient B was a female with persistent diarrhea, malnutrition, failure to thrive, generalized hypotonia, no head control and petechiae on her upper limbs. Urine analysis revealed elevated excretion of ethylmalonic and succinic acids as well as short branched-chain acylglycines. Ethylmalonic encephalopathy (EE) caused by mutations in the ETHE1 gene was subsequently confirmed [14].

Patient C was a female who suffered kidney failure, and psychomotor and growth retardation. Her urine organic acids were normal.

Patient D was a female with bradypsychia and somnolence. Her urine had a 'sweaty feet' odor. Analysis of her urine's organic acids revealed elevated excretion of 3-hydroxyisovaleric acid and isovalerylglycine. An IVD defect was confirmed through the deficient incorporation of [1-^{14}C]-isovalerate by cultured skin fibroblasts.

Patient E was a female who suffered lethargy and episodes of vomiting. Her urine organic acids were normal.

Patient F was a male who suffered diarrhea, episodes of vomiting, and ketoacidosis. Urine organic acid analysis revealed elevated excretion of 2-methyl-3-hydroxybutyric, 2-methylacetoacetic acids and tiglylglycine. A diagnosis of β-KT deficiency was confirmed by measuring the activity of this enzyme in cultured fibroblasts.

Patient G was a male who presented with abdominal pain, muscle fatigue and depression. Urine analysis revealed the elevated excretion of 3-hydroxyisovaleric acid and 3-methylcrotonylglycine. MCC deficiency was confirmed by determining the β-methylcrotonyl-CoA carboxylase activity in cultured lymphocytes.

Patient H was a female who presented with epilepsy, a failure to thrive, and who had suffered an episode of aborted sudden death. Her excretion of organic acids in the urine was normal.

2.2. Chemicals and reagents

The acylcarnitines labeled with stable isotopes (used as internal standards) were purchased from Cambridge Isotope Laboratories Inc. Acetonitrile and hydrogen chloride in 1-butanol were obtained from Fluka, formic acid, methanol and water from MERCK, and ammonium acetate from BDH. L-Carnitine was obtained from Sigma. Acetyl-, propionyl-, butyryl-, isobutyryl-, 2-methylbutyryl- and isovalerylcarnitine were synthesized by Prof. E. Brunet, Department of Organic Chemistry (Universidad Autónoma de Madrid, Spain).

2.3. Sample preparation

Seventy-five microliters of a deuterated standard mixture (7.6 μM d9-C0, 1.9 μM d3-C2, 0.38 μM d3-C3, 0.38 μM d3-C4, 0.38 μM d9-isoC5, 0.38 μM d3-C8, 0.38 μM d9-C14 and 0.76 μM d3-C16) were added to 50 μL of plasma. This mixture was subsequently deproteinized with 500 μL of acetonitrile. It was then centrifuged (15,000 rpm) for 15 min and the supernatant dried under a nitrogen stream and derivatized with 50 μL of hydrogen chloride (3 M) in 1-butanol for 20 min at 65 °C. These samples were dried under a nitrogen stream and redissolved in 200 μL acetonitrile/water (60:40) and 0.1% formic acid.

2.4. Instrumentation and operating parameters

The method made use of a Q TRAP mass spectrometer equipped with a TurboIonSpray source (Applied Biosystems/MDS Sciex).

- FIA method

  Plasma acylcarnitines were quantified using a scanning precursor ion with a relative mass of 85 (*m/z* 215–600) for 5 min. Acetonitrile/water (60:40) and 0.1% formic acid was used as the mobile phase (flow rate: 50 μL/min). Forty microliters of the sample were injected.

Table 1
MRM transitions (Q1/Q3) for short-chain acylcarnitines

Acylcarnitine	Abbreviation	Q1/Q3
Carnitine	C0	218/85
d9-Carnitine	d9-C0	227/85
Acetylcarnitine	C2	260/85
d3-Acetylcarnitine	d3-C2	263/85
Propionylcarnitine	C3	274/85
d3-Propionylcarnitine	d3-C3	277/85
Butyrylcarnitine	C4	288/85
Isobutyrylcarnitine	C4	288/85
d3-Butyrylcarnitine	d3-C4	291/85
Tiglylcarnitine	C5:1	300/85
3-Methylcrotonylcarnitine	C5:1	300/85
Isovalerylcarnitine	C5	302/85
Pivaloylcarnitine	C5	302/85
2-Methylbutyrylcarnitine	C5	302/85
d9-Isovalerylcarnitine	d9-C5	311/85
3-Hydroxyisovalerylcarnitine	C5-OH	318/85
2-Methyl-3-hydroxybutyrylcarnitine	C5-OH	318/85

The working parameters were: curtain gas, 35 psi; GS1, 45 psi; GS2, 40 psi; temperature, off; mode of analysis, positive; ion spray voltage, 5500 V.

- HPLC/MS/MS method

High-performance liquid chromatography was performed using an Agilent 1100 series HPLC with a Symmetry C18 column (100 mm × 2.1 mm, particle size 3.5 μm, Waters). Ammonium acetate (10 mM) in water (eluent A) and ammonium acetate (10 mM) in methanol (eluent B) were used to make the gradient of the mobile phase (flow rate: 200 μL/min). The eluents were linearly changed from 40% B to 60% B for 20 min. They were subsequently changed from 60% to 40% for 5 min and held for 10 min to re-equilibrate the column.

The working parameters were: curtain gas, 30 psi; GS1, 40 psi; GS2, 40 psi: probe temperature 500 °C; dwell time per transition, 100 ms; mode of analysis, positive; ion spray voltage, 5500 V.

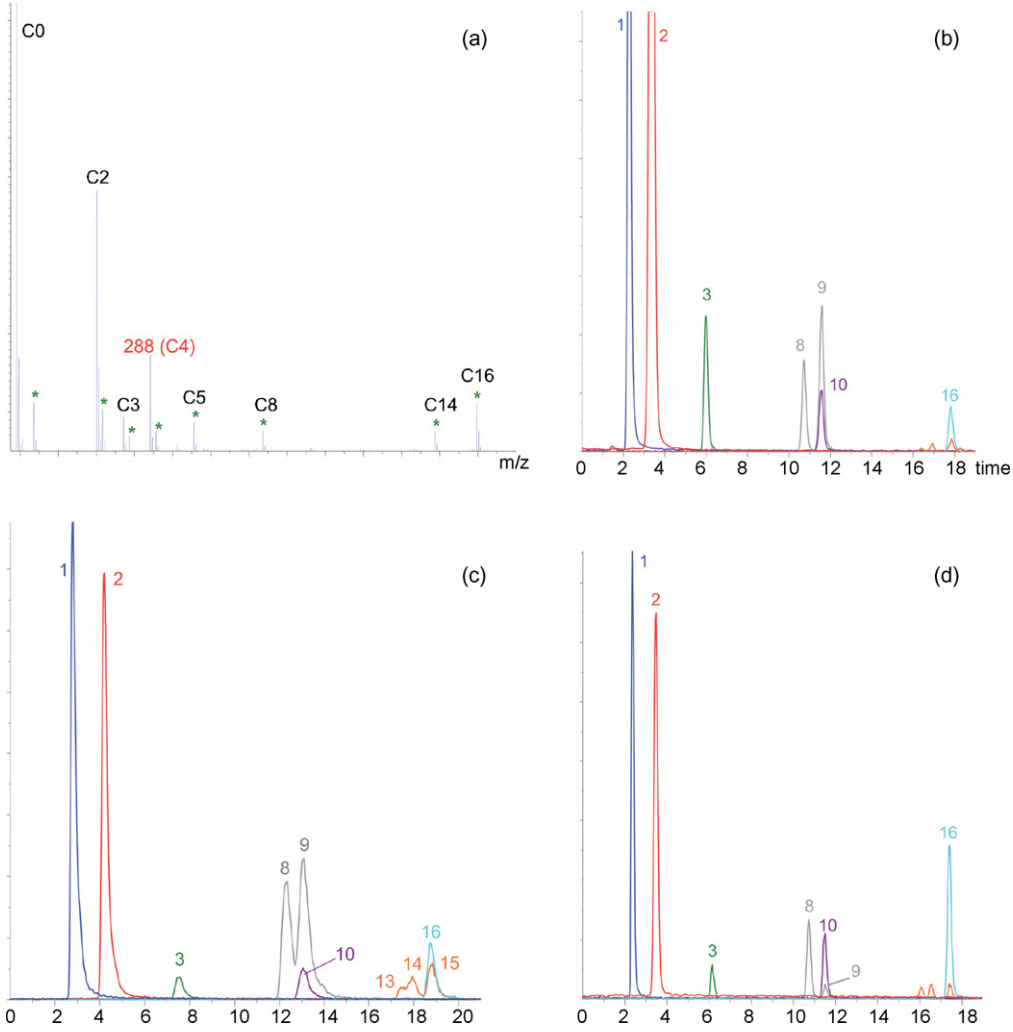
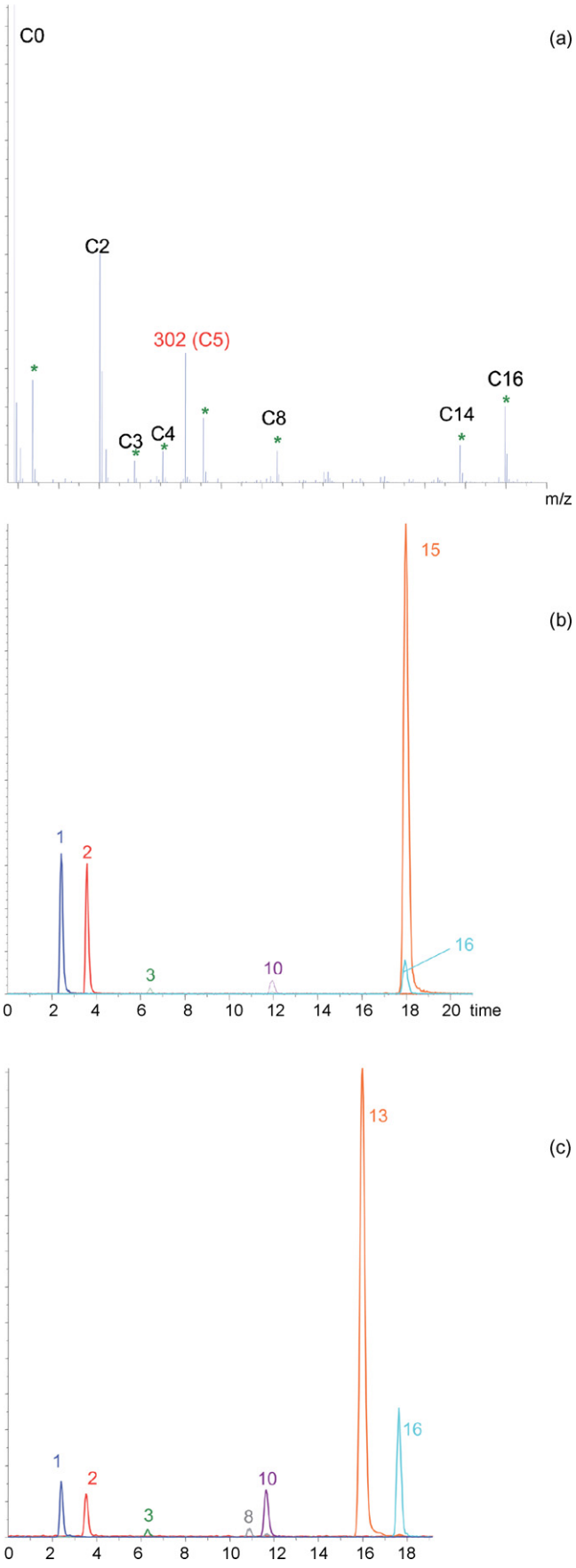


Fig. 1. Acylcarnitine profiles with elevated quantities of C4 carnitine. Profile determined by FIA (a); *deuterated internal standards. Short-chain acylcarnitine isomer separation by HPLC/MS/MS in samples from patients with SCAD deficiency (b), EE (c), and an elevated concentration of isobutyrylcarnitine (d). (1) Carnitine; (2) acetylcarnitine; (3) propionylcarnitine; (8) isobutyrylcarnitine; (9) butyrylcarnitine; (10) d3-butyrylcarnitine; (13) pivaloylcarnitine; (14) 2-methylbutyrylcarnitine; (15) isovalerylcarnitine; and (16) d9-isovalerylcarnitine.



Two microliters of the sample, previously analyzed by FIA, were injected and analyzed using the multiple reaction monitoring (MRM) mode to distinguish the plasma short-chain acylcarnitine isomers (see Table 1 for MRM transitions).

3. Results and discussion

The separation of isomeric short-chain acylcarnitines species in plasma was performed by HPLC/MS/MS using those samples that showed increased concentrations of C4 carnitine (>0.80 μM), C5 carnitine (>0.65 μM), C5-OH carnitine (>0.15 μM) and/or C5:1 carnitine (>0.10 μM) in FIA. The latter technique showed patient A to have increased levels of C4 carnitine (Fig. 1a), like patients B and C; patient D to have elevated levels of C5 carnitine (Fig. 2a), like patient E; patient F to have increased levels of C5-OH and C5:1 carnitines (Fig. 3a), like patient G; and patient H to have increased levels of C4 and C5 carnitines (Fig. 4a).

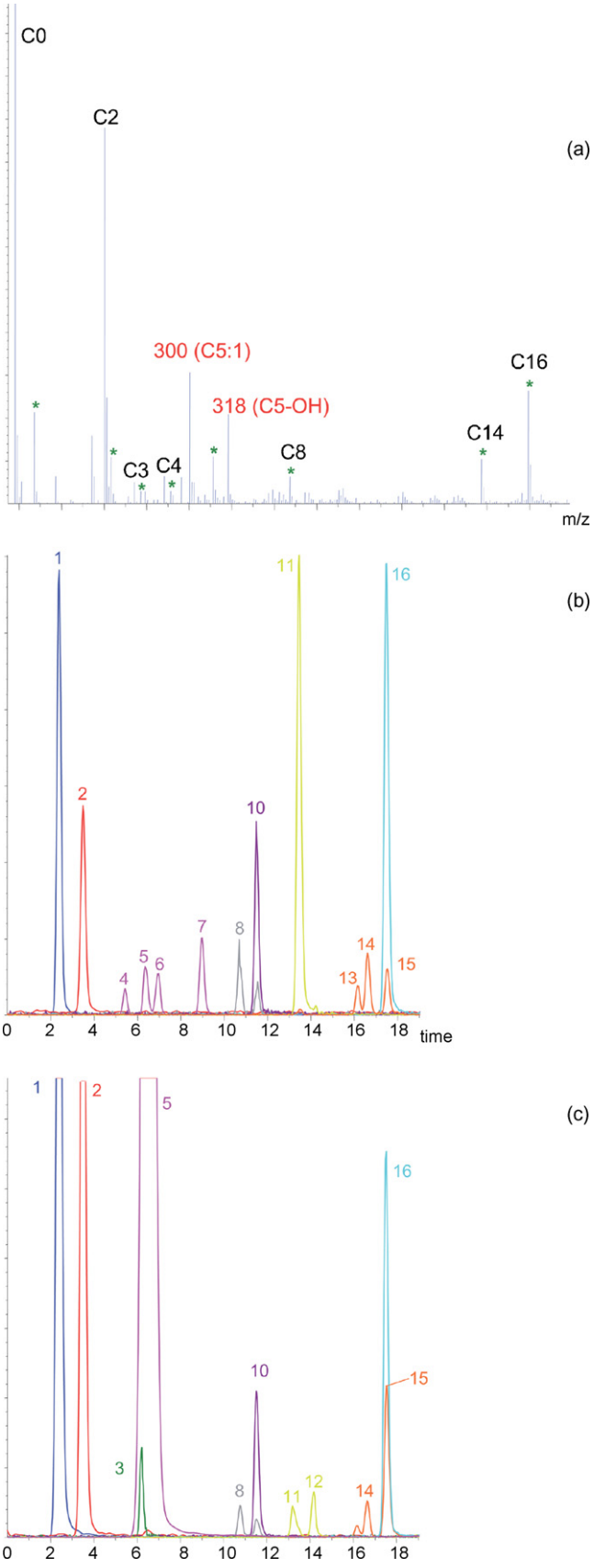
The HPLC/MS/MS identification of butyrylcarnitine (C4), isobutyrylcarnitine (C4), 2-methylbutyrylcarnitine (C5) and isovalerylcarnitine (C5) was confirmed against standards. The peak eluting just before the less branched C5 carnitines (2-methylbutyrylcarnitine and isovalerylcarnitine) in patients with high C5 levels (as determined by FIA) could be pivaloylcarnitine (2,2-dimethylpropionic carnitine) (see Fig. 2c). The evidence for this includes: (a) in patients in whom C5 levels were raised, the 2-methylbutyrylcarnitine and isovalerylcarnitine levels were normal; and (b) complementary analyses showed that these patients did not have isovaleryl-CoA dehydrogenase or 2-methylbutyryl-CoA dehydrogenase defects.

For the identification of the C5:1 and C5-OH isomers, samples from patients with confirmed disease in which these acylcarnitines accumulate (patients F and G) were examined. No interfering compounds were observed after isomer separation by HPLC/MS/MS.

The C5:1 carnitines identified were tiglylcarnitine (2-methyl-2-butenoylcarnitine) and 3-methylcrotonylcarnitine (3-methyl-2-butenoylcarnitine). In the proposed reverse phase chromatography procedure, both of these compounds eluted before the isomers of the saturated C5 carnitine group, and in the same relative order (first the 2-methyl isomer, then the 3-methyl branched isomer).

In patient A, HPLC/MS/MS identified the major C4 isomer present as butyrylcarnitine (the butyrylcarnitine/isobutyrylcarnitine ratio was >1); this is a known feature of SCAD deficiency (Fig. 1b) [14]. In patient B, both C4 carnitine isomers were found, the butyrylcarni-

Fig. 2. Acylcarnitine profiles with elevated amounts of C5 carnitine. Profile determined by FIA (a); *deuterated internal standards. Short-chain acylcarnitine isomer separation by HPLC/MS/MS in samples from a patient with IVD deficiency (b) and form a patient receiving pivalic acid treatment (c). (1) Carnitine; (2) acetylcarnitine; (3) propionylcarnitine; (8) isobutyrylcarnitine; (10) d3-butyrylcarnitine; (13) pivaloylcarnitine; (15) isovalerylcarnitine; and (16) d9-isovalerylcarnitine.



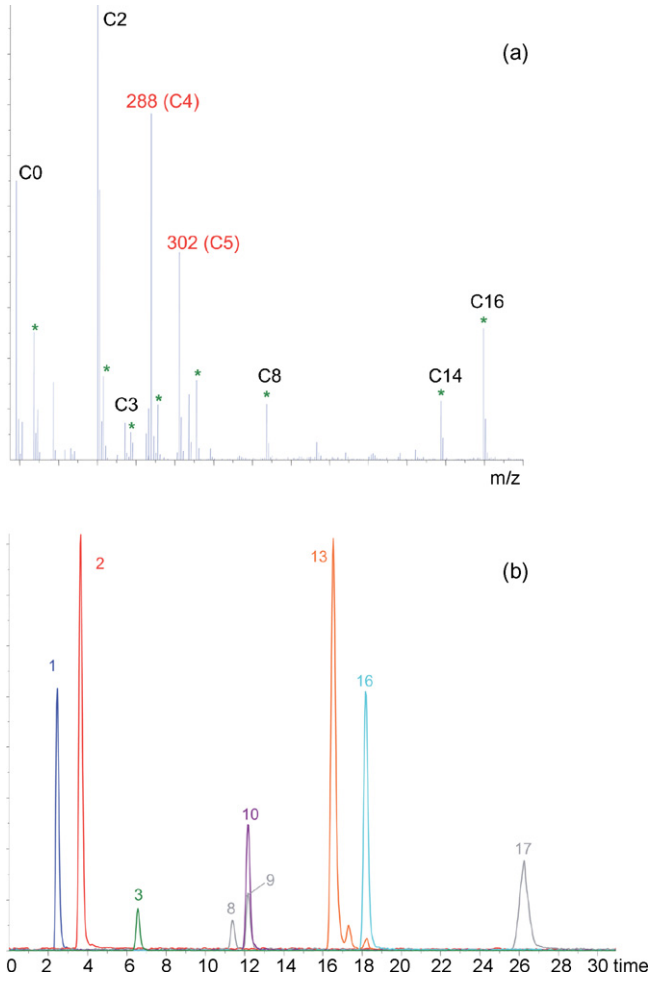


Fig. 4. Acylcarnitine profiles with elevated amounts of C4 and C5-carnitines. Profile determined by FIA (a); *deuterated internal standards. Short-chain acylcarnitine isomer separation by HPLC/MS/MS in a sample from a patient with an interfering compound (*m/z* 288) (b). (1) Carnitine; (2) acetylcarnitine; (3) propionylcarnitine; (8) isobutyrylcarnitine; (9) butyrylcarnitine; (10) d3-butyrylcarnitine; (13) pivaloylcarnitine; (16) d9-isovalerylcarnitine; and (17) interfering compound of *m/z* 288.

tine/isobutyrylcarnitine ratio was ≈1; this is a known feature of EE (Fig. 1c) [14]. In patient C, the majority isomer was isobutyrylcarnitine (Fig. 1d). The absence of urine ethylmalonate and isobutyrylglycine in GC/MS analysis, together with the slight increase in plasma isobutyrylcarnitine, made an IBD defect unlikely in this patient. In patient D, the separation of the C5 carnitine isomers confirmed the major presence of isovalerylcarnitine—a known feature of IVD deficiency

Fig. 3. Acylcarnitine profiles with elevated amounts of C5:1 and C5-OH-carnitines. Profile determined by FIA (a); *deuterated internal standards. Short-chain acylcarnitine isomer separation by HPLC/MS/MS in a sample from a patient with β-KT deficiency (a), and in another from a patient with MCC deficiency (b). (1) Carnitine; (2) acetylcarnitine; (3) propionylcarnitine; (4), (6) and (7) isomers of *m/z* 318; (5) 3-hydroxyisovalerylcarnitine; (8) isobutyrylcarnitine; (10) d3-butyrylcarnitine; (11) tiglylcarnitine; (12) 3-methylcrotonylcarnitine; (13) pivaloylcarnitine; (14) 2-methylbutyrylcarnitine; (15) isovalerylcarnitine; and (16) d9-isovalerylcarnitine.

(Fig. 2b). In patient E, pivaloylcarnitine was identified—a consequence of the medication she took (Fig. 2c). In patient F (who suffered β-KT deficiency), HPLC/MS/MS analysis permitted the separation of several compounds thought to be tiglylcarnitine plus four C5-OH isomers (Fig. 3b) representing 3-hydroxyisovalerylcarnitine (normal levels), two diastereoisomers of 2-methyl-3-hydroxybutyrylcarnitine (elevated levels), and an as yet unidentified isomer. These peaks, were also observed in other patients with β-KT deficiency (not shown). In patient G (who suffered MCC deficiency), two peaks were produced, probably corresponding to 3-hydroxyisovalerylcarnitine and 3-methylcrotonylcarnitine (Fig. 3c).

HPLC/MS/MS facilitated the separation of the plasma isomeric short-chain acylcarnitine isomers with high-chromatographic resolution ($R > 1$). The method offers several advantages: samples prepared for FIA analysis can be re-used for HPLC/MS/MS analysis, the entire process takes less than 60 min to complete, and it allows the initial differential diagnosis of, EE, MCC, β-KT, SCAD and IVD deficiencies. It could also be used for diagnosing other diseases in which the characteristic plasma metabolites are short-chain acylcarnitines, e.g., in IBD, HMGCL, MHBD or MBD defects. The identification of the C5 compound pivaloylcarnitine, which is found in plasma after the use of certain medications, is also possible. Another important use of this method is the potential discrimination of compounds that might interfere with FIA results. For example, patient H showed high *m/z* 288 and 302 signals in FIA (Fig. 4a). However, HPLC/MS/MS analysis detected no elevated levels of either isobutyrylcarnitine or butyrylcarnitine that would correspond to *m/z* 288 (Fig. 4b). When the elution conditions were greatly modified (i.e., eluent B at 100%, held for 15 min), an MRM signal of 288/85 was recorded (see Table 1). The extended chromatographic retention time led us to suppose that the *m/z* 288 signal, as measured by FIA, was in fact an interfering compound. A product ion of *m/z* 288 confirmed this suspicion, showing that its mass spectrum did not correspond to any of the acylcarnitines despite an *m/z* 85 signal being present (the daughter ion of acylcarnitines). This observation underscores the importance of identifying the isomers of short-chain acylcarnitines in order to discard false positives related to fatty acid oxidation or branched-chain amino acid metabolism defects.

This method could facilitate diagnosis in cases where plasma is the sole biological sample available. It could also prove suitable for inclusion in MS/MS neonatal screening programs as a means of eliminating the interference of medications or other compounds before complementary tests are performed. Cut-off values for dried blood samples could then be established.

In conclusion, the proposed method is a simple and rapid diagnostic procedure of potentially great use in the differential diagnosis of patients presenting with pathological acylcarnitine profiles.

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