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Measurement of succinyl-carnitine and methylmalonyl-carnitine on dried blood spot by liquid chromatography-tandem mass spectrometry

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ABSTRACT

Methylmalonic aciduria (MMA) is one of the most frequent organic acidurias, a class of diseases caused by enzymatic defects mainly involved in the catabolism of branched-chain amino acids. Recently, mild MMA and C4-dicarboxylyl-carnitine (C4DC-C) accumulation have been reported in patients carrying mutation in genes encoding the α-subunit (*SUCLG1*) and the β-subunit (*SUCLA2*) of the ADP-forming succinyl-CoA synthetase (SCS). We developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify in dried blood spot the two isobaric compounds of C4DC-C, succinyl-carnitine and methylmalonyl-carnitine, to allow the differential diagnosis between classical MMA and SCS-related defects.

This method, with an easy liquid-phase extraction and derivatization procedure, has been validated to demonstrate the specificity, linearity, recovery, lowest limit of quantification (LLOQ), accuracy and precision for quantitative determination of blood succinyl-carnitine and methylmalonyl-carnitine. The assay was linear over a concentration range of 0.025–10 μmol/L and achieved the LLOQ of 0.025 μmol/L for both metabolites. The average slope, intercept, and coefficient of linear regression (r^2) were respectively: 0.3389 (95% confidence interval 0.2888–0.3889), 0.0113 (95% confidence interval −0.0157 to 0.0384), 0.9995 (95% confidence interval 0.9990–1.0000) for succinyl-carnitine and 0.5699 (95% confidence interval 0.5263–0.6134), 0.0319 (95% confidence interval −0.0038 to 0.0677), 0.9997 (95% confidence interval 0.9995–1.0000) for methylmalonyl-carnitine. Within-day and between-day coefficients of variation (CV) were 1.94% and 3.19% for succinyl-carnitine and 3.21%, and 2.56 for methylmalonyl-carnitine.

This method is accurate and provides a new tool to differentiate patients with classical methylmalonic acidemia from those with SCS-related defects.

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1. Introduction

Methylmalonic acidurias (MMAs) are a group of genetically heterogeneous autosomal recessive disorders of methylmalonate and cobalamin metabolism caused by a complete (mut^0) or partial (mut^-) deficiency of the enzyme methylmalonyl-CoA mutase (EC 5.4.99.2), for the conversion of methylmalonyl-CoA to succinyl-CoA, or by defects in the cobalamin metabolism (CblA, CblB, CblC, CblD, CblF) [1]. Recently a few patients have been described with mild methylmalonic aciduria with deficiencies in the two subunits α and β of the mitochondrial matrix enzyme succinyl-CoA synthetase caused by *SUCLG1* and *SUCLA2* gene mutations. Succinyl-CoA synthetase (SCS), also called succinate ligase, is a Krebs cycle enzyme that not only converts succinyl-CoA to succinate and free Coenzyme A, but also converts ADP to ATP and GDP to GTP. The substrate specificity for ADP and GDP is determined by the β-subunits, whereas the α subunit is shared. The α-subunit is coded by the gene *SUCLG1*, whereas the β-subunit is encoded by *SUCLA2* for the ADP specificity, and by *SUCLG2* for the GDP specificity [2]. Patients with classical MMA or with SCS-related defects *SUCLG1* or *SUCLA2* mutation present hypotonia, muscle weakness,

Abbreviations: MMAs, Methylmalonic acidurias; SCS, succinyl-CoA synthetase; C3-C, propionyl-carnitine; C4DC-C, C4-dicarboxylyl-carnitine; C3-C/C2-C, propionyl-carnitine/acetyl-carnitine ratio; C3-C/C16-C, propionyl-carnitine/palmitoyl-carnitine ratio; MS/MS, tandem mass spectrometry; SC, succinyl-carnitine; MMC, methylmalonyl-carnitine; TDHFA, tridecafluoroheptanoic acid; FOA, formic acid; DP, declustering potential; CXP, collision cell exit potential; CE, collision energy; LOD, limit of detection; LLOQ, lower limit of quantification; S/N, signal-to-noise ratio; SD, standard deviation; MRM, multiple reaction monitoring.

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hypoacusis, Leigh disease, lactic acidosis, polyneuropathy, mild methylmalonic aciduria and mild elevation of C4DC-C [2,3].

The diagnosis of MMAs is based on the presence of characteristic compounds in body fluids detected by organic acids analysis in urine (methylmalonic acid, 3-hydroxy-propionic acid and methylcitric acid) and blood acylcarnitine profiling (propionyl-carnitine (C3-C), C4-dicarboxylyl-carnitine (C4DC-C) and propionyl-carnitine/acetyl-carnitine (C3-C/C2-C) and propionyl-carnitine/palmitoyl-carnitine (C3-C/C16-C) ratios [4,5].

The introduction of tandem mass spectrometry (MS/MS) based on rapid and simultaneous quantification of acylcarnitines and aminoacids on dried blood spot [6], has significantly increased early diagnosis of inborn errors of metabolism [7].

The commonly used method for the determination of acylcarnitines on dried blood spot quantifies the whole C4DC-C which is the sum of two different isobaric compounds: succinyl-carnitine (SC) and methylmalonyl-carnitine (MMC).

In this paper we report a new liquid chromatography-tandem mass spectrometry (LC-MS/MS) method able to identify and quantify SC and MMC. This allows the differential diagnosis between classical MMA and *SUCLA2* or *SUCLG1* defects where MMC or SC was respectively elevated. Our study adds a new method to the diagnosis of MMAs and *SUCLA2* or *SUCLG1* defects, which may also be relevant for a second tier test when C4DC-C is found elevated in routine acylcarnitines analysis.

2. Materials and methods

2.1. Reagents

SC, MMC and labelled internal standards [$^2H_3$]-SC and [$^2H_3$]-MMC, were synthesized by Dr. David Johnson (Department of Chemical Pathology, Women's and Children's Hospital, North Adelaide, Australia). HPLC grade acetonitrile and water were purchased from Romil Ltd. (The Source Convent Drive Waterbeach Cambridge, United Kingdom). Tridecafluoroheptanoic acid (TDHFA) 98/100% and butanolic-HCl were obtained from Sigma-Aldrich (Steinheim, Germany). Formic acid (FOA) 98/100% was obtained from BDH (VWR Ltd, Poole, England).

2.2. Preparation of standard solutions

We prepared [$^2H_3$]-SC and [$^2H_3$]-MMC stock solutions (Stock-1) in water 1 mmol/L in order to investigate molecular fragmentation and also to obtain calibration curves for SC and MMC. This was stored frozen at −20 °C. "Daily standard solution" containing 10 μmol/L [$^2H_3$]-SC and [$^2H_3$]-MMC was prepared by dilution of Stock-1 solution 1:100 (*v*/*v*) with methanol.

2.3. Sample treatment procedure

Whole blood was drawn by heel prick or venipuncture and dried on a filter paper (W303). We punched one disk of 5 mm diameter containing ~10 μL of whole blood from each dried blood spot into a vial and mixed it with 50 μL of "Daily standard solution". Four hundred microliters of methanol was added to precipitate proteins and to extract the metabolites. Each vial was mixed by vortex for 20 min, then centrifuged for 5 min at 10,000 rpm. The supernatant fluid was transferred into a clean vial and dried at 40 °C under nitrogen stream. Eighty microliters of butanolic-HCl was added to derivatize metabolites as butyl-esters and heated at 65 °C for 15 min. Samples were dried again at 40 °C under nitrogen stream and finally they were reconstituted with 200 μL of acetonitrile–water 50:50 (*v*/*v*) containing 0.05% FOA to obtain pH 4 which is the appropriate pH for metabolites ionization.

2.4. Liquid chromatography-mass spectrometry

Chromatography was performed on an Agilent series 1200 pump and autosampler (Agilent Technologies Inc., Wilmington, DE, USA). The column for chromatographic separation was a Zorbax Eclipse XDB-C8 column 5 μm, 4.6 × 150 mm, (Agilent Technologies, Santa Clara, CA). The mobile phase was solution A (water containing 0.5 μmol/L TDHFA) and solution B (acetonitrile containing 0.5 μmol/L TDHFA. Chromatographic separation of metabolites was obtained with gradient elution. Gradient starts from 90% A to 10% in 10 min then 3 min at 10% A. Flow rate was 1.0 mL/min. The column was maintained at room temperature. 5 μL of sample were injected into the column. The total run was 16 min.

Tandem mass spectrometry experiments were carried out on an API3200 triple quadrupole mass spectrometer (Applied Biosystems-Sciex, Toronto, Canada), equipped with a Turbo Ion Spray Source operating in positive ion mode with a needle potential of 5500 V; the source temperature was 550 °C. Declustering Potential (DP), Collision Cell Exit Potential (CXP) and Collision Energy (CE) were optimized by direct infusion at flow rate 10 μL/min of each analyte in the mass spectrometer. The injected solutions were 5.0 μmol/L in water/acetonitrile 50/50 v/v containing 0.05% FOA. The resulting DP was 40 V and optimal CE and CXP were found at 40 and 3 V for SC, and MMC. The following transitions were monitored: m/z 374.3 > 85.1 for SC and MMC and m/z 377.3 > 85.1 for [$^2H_3$]-SC and [$^2H_3$]-MMC.

2.5. Standard curves for quantification

Methanolic standard solutions of 10, 5, 1, 0.5, 0.1, 0.05 0.01 μmol/L for SC and MMC were analyzed with the same procedure of blood spot samples. The acquired data were processed using Analyst® version 1.4.2 software (Applied Biosystems-Sciex), including option for chromatographic and spectral interpretation and for quantitative information generation. Calibration curves were constructed with the Analyst Quantitation program using a linear least-square regression non-weighted.

The limit of detection (LOD) was determined by progressive dilutions of calibrator solutions of each analyte and was considered as the lowest concentration for which the signal-to-noise ratio (S/N) was indicated by the Analyst software to be at least 3. The lower limit of quantification (LLOQ) was determined by preparing calibrator solutions with decreasing concentration of each analyte and was considered as the lowest concentration for which the signal-to-noise ratio (S/N) was indicated by the Analyst software to be at least 10.

2.6. Sample collection of patients and controls

Twenty children's blood spot, for reference values, were obtained from hospitalized neurologically healthy subjects (10 females and 10 males; age: 1 day to 18 year, mean 7 year).

Blood spot samples from patients where C4DC-C was found elevated (>1 μmol/L) using routine MS/MS screening method [6] were reanalyzed with LC-MS/MS method (2 samples of 1 patient affected by methylmalonyl-CoA mutase; 6 samples of 5 patients with mutation of *SUCLA2* and 3 samples of 1 patient with mutation of *SUCLG1*). One of *SUCLA2* blood spot was a neonatal blood spot. All samples were treated as described in sample treatment procedure.

Control's and patient's blood samples were collected after obtaining informed consent. Patient's blood was collected during routine outpatient evaluation following an overnight fast before receiving the first morning dose of medication.

2.7. Statistical analysis

The SPSS version 11.5.1 (SPSS Inc., Chicago, US) was used as statistical software. A preliminary test (Kolmogorov–Smirnov) was performed

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