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Quantification of methylcitrate in dried urine spots by liquid chromatography tandem mass spectrometry for the diagnosis of propionic and methylmalonic acidemias

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ABSTRACT

Accumulation of methylcitrate is a biochemical hallmark of inborn errors of propionate metabolism, a group of disorders that include propionic acidemia, methylmalonic aciduria and cobalamin defects. In clinical laboratories, this analyte is measured without quantification by gas chromatography mass spectrometry as part of urine organic acids. Here we describe a simple, sensitive and specific method to quantify methylcitrate in dried urine spots by liquid chromatography tandem mass spectrometry.

Methylcitrate is extracted and derivatized with 4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole in a single step. A derivatization mixture was added to 3.2 mm disc of dried urine spots, incubated at 65 °C for 45 min and 4 µl of the reaction mixture were analyzed.

Separation was achieved on C18 column with methylcitrate eluting at 3.8 min. Intraday and interday imprecision (n = 17) were ≤20.9%. The method was applied on dried urine spots from established patients and controls. In controls (n = 135), methylcitrate reference interval of 0.4–3.4 mmol/mol creatinine. In patients, methylcitrate ranged between 8.3 and 591 mmol/mol creatinine.

Quantification of methylcitrate provides important diagnostic clues for propionic acidemia, methylmalonic aciduria and cobalamin disorders. The potential utilization of methylcitrate as monitoring biomarker of patients under treatment and whether it correlates with the clinical status has yet to be established.

1. Introduction

A common intermediate in the catabolism of branched chain amino acids and odd chain fatty acids is propionyl CoA [1]. Biotin-requiring propionyl CoA carboxylase catalyzes the carboxylation of this metabolite in the mitochondrial matrix to produce methylmalonyl CoA [2]. Catalyzed by methylmalonyl CoA mutase in the presence of vitamin B12, methylmalonyl CoA is converted to succinyl CoA which is utilized in the Krebs cycle, the final common oxidative pathway of carbohydrates, lipids and proteins [3]. Genetic defects in propionyl CoA carboxylase cause propionic acidemia (PA). Methylmalonic aciduria (MMA) is a genetically heterogeneous and can be caused by methylmalonyl CoA mutase deficiency, methylmalonyl CoA epimerase deficiency or cobalamin (Cbl) defects. PA and MMA result in the accumulation of propionyl CoA metabolites [4–6]. Metabolic acidosis, ketosis and hyperammonemic episodes that may lead to severe sequela including neurological dysfunction or death in infancy are potential complications of these disorders [7–10].

In PA and MMA, the pathognomonic marker MCA is produced from the condensation of propionyl CoA with Krebs cycle intermediate oxaloacetate in a reaction thought to be catalyzed by citrate synthase [3]. Accumulation of MCA and depletion of critical Krebs cycle intermediates may negatively impact the overall mitochondrial energy metabolism. Studies on the pathogenic role of MCA are scarce, however,

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recent reports indicated that this compound is significantly involved in neurotoxicity and brain damage using in vitro model of MMA [1,11,12].

Analysis of MCA is traditionally carried out through urine organic acid analysis profiling by gas chromatography mass spectrometry (GC–MS) or by selected ion monitoring GC–MS [8,13,14]. These methods, however, are tedious and labor intensive. Determination of MCA as part of organic acid profiling is commonly qualitative and sensitivity is compromised by the poor and variable extraction recovery and the presence of multiple isomer peaks. Recently, successful determination of MCA using liquid chromatography tandem mass spectrometry (LC-MS/MS) was reported [15–18]. However, these methods were specifically applied as second tier tests in newborn screening laboratories using dried blood spot samples.

In this paper, we describe the quantitative analysis of MCA by LC-MS/MS in urine, the matrix of choice for hydrophilic low molecular weight organic acids. The method utilizes 4-[2-(N,N-dimethylamino) ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD AE) as derivatization reagent to improve chromatographic and mass spectrometric properties of MCA. The improved sensitivity and specificity allowed for the detection of this biomarker in single 3.2 mm discs excised from dried urine spot (DUS) specimens that contains minute amount of urine (3 µl). The method was validated and applied to MCA quantification in samples from healthy individuals and patients with established diagnosis of PA, MMA and Cbl disorders.

2. Materials and methods

2.1. Chemicals and solvents

LC MS/MS grade water and methanol were provided by Merck (Darmstadt, Germany). MCA and d3-MCA used as internal standard were purchased from Cambridge Isotopes Laboratories (Tewksbury, Massachusetts, USA). DAABD-AE, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), 4-(dimethylamino) pyridine (DMAP) and perfluorooctanoic acid (PFOA) were from Sigma Aldrich (Taufkirchen, Germany). Stock solutions of MCA and d3-MCA were prepared by dissolving specific amounts of each compound in 50% acetonitrile to obtain a concentration of 1 mg/ml. Stock solutions were placed in tightly capped amber glass vials at − 20 °C and were stable for at least 6 months.

2.2. Controls and patient samples

This study was approved by Al Ain Medical District Human Research Ethics Committee. We analyzed random urine from patients (n = 17) with established diagnosis of PA, MMA or Cbl defects. For the determination of the reference range of MCA, urine specimens from individuals of 1 month to 60 years of age received at the metabolic laboratory for organic acids analysis and reported as “not remarkable” were used (n = 135). DUS samples (calibrators, patients, controls) were prepared by applying 70 µl aliquots of urine using an Eppendorf pipette onto Whatman 903 Specimen Collection Paper. Samples were left to dry overnight at room temperature and the resultant DUS samples were stored in sealed plastic bags at 4 °C when they are not in use.

2.3. Sample preparation

Single 3.2 mm DUS discs (calibrators, patients, controls) were excised and placed in a 1.5 ml Eppendorf Snap-Cap microcentrifuge tube. To each tube, 10 µl of 24 µM d3-MCA were added followed by an aliquot of 100 µl of a mixture (2:1:1 v/v/v) of DAABD-AE (2 mM in 90% acetonitrile), DMAP (25 mM in acetonitrile) and EDC (25 mM in water). Tightly capped tubes were vortexed for 30 s, centrifuged for 30 s and incubated at 65 °C. After 45 min, the reaction was stopped by adding 200 µl of water. Four µl of the resultant mixture were injected into the LC-MS/MS system. For liquid urine analysis, the DUS disc was replaced with 5 µl aliquots of urine as sample.

2.4. LC-MS/MS system

For solvent delivery and sample introduction, a Shimadzu Nexera X2 UHPLC consisting of two pumps, cooled autosampler, column oven, degasser and system controller was used (Shimadzu, Kyoto, Japan). An LC-MS 8060 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source was used for detection (Shimadzu). The system was operated in the positive ion mode with capillary voltage of 3.4 kV, cone voltage of 35 V and collision energy of 22 eV. Argon was used as collision gas. The desolvation and ion source temperature were set at 120 °C and 350 °C, respectively. Multiple reaction monitoring (MRM) was used to detect MCA and d3-MCA using mass/charge (m/z) transitions of 499.0 > 151.1 and 502.0 > 151.1, respectively. Separation was achieved on an Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 µm) column (Waters, Milford, USA) using a gradient program. Mobile phase A was 100% water and mobile phase B was 100% methanol both contained 2 mM ammonium acetate and 0.1% formic acid. Using a flow rate of 0.4 ml/min, the following gradient program was employed: 0–2 min 2% B, 2–5.2 min 2–85% B, column was re-equilibrated for 2.8 min with 2% B. The column temperature was maintained at 40 °C.

2.5. Method validation

A standard curve of MCA was prepared by adding specific amounts of MCA stock solution to a known volume of pooled control urine to obtain a final concentration of 500 µM which was further diluted to achieve the following concentrations: 250, 100, 50, 25, 10, 5, 1, and 0.5 µM. Control urine without enrichment was used as a blank to correct for endogenous MCA. Quality control (QC) samples were prepared at 7.5, 75 and 500 µM representing low, intermediate and high concentrations. A trained technician applied these samples onto Whatman 903 Specimen Collection Paper as described previously.

Intraday (n = 17) and interday (n = 17) imprecision were assessed by repeated analysis of QC samples. Coefficient of variation (CV%) was calculated according to the following eq. [CV% = 100 x standard deviation/mean]. Analytical recovery was calculated as follow: [analytical recovery (%) = 100 x (concentration measured – concentration in nonenriched sample)/concentration added].

Stability of MCA was evaluated by repeatedly analyzing DUS specimens containing MCA at 200 µM stored at different temperatures (i.e. -20, 4, 23 and 50 °C) over a period of 4 weeks.

3. Results

3.1. Sample preparation

Sample preparation for analysis was achieved in a single Eppendorf tube in which 100 µl of premixed reagent solution composed of DAABD-AE, DMAP and EDC (2:1:1 v/v/v) was added to a 3.2 mm DUS disc. The premixed reagent preparation was stable for 7 days when stored in tightly capped amber glass vial at 4 °C. Derivatization Reaction conditions were as previously described [15,16].

Using a reversed phase C18 column, adequate chromatographic separation of MCA from other components in the sample was achieved with a gradient mixture of water and methanol containing formic acid (0.1%) ammonium acetate (2 mM) as additives. Target peaks that eluted at 3.8 min were well separated from interfering compounds. Fig. 1 shows extracted mass chromatograms obtained with DUS from a control individual and a patient with Cbl B deficiency. To eliminate late eluting compounds, column washing and reconditioning step was included making the total run time 8.0 min. It is noteworthy that derivatized MCA samples were stable for at least 24 h if stored at 4 °C and