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Quantifying MMA by SLE LC-MS/MS: Unexpected challenges in assay development

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ABSTRACT

Objectives: Analysis of serum/plasma methylmalonic acid (MMA) is important for the diagnosis and management of methylmalonic acidemia in pediatric populations. This work focuses on developing and validating a liquid chromatography tandem mass spectrometry (LC-MS/MS) method to monitor methylmalonic acidemia using a simple method preparation.

Design and methods: MMA and stable isotope labeled d_3-MMA was extracted using supported liquid extraction (SLE). Assay imprecision, bias, linearity, recovery and carryover were determined. The relationship between MMA and propionyl acylcarnitine (C3-acylcarnitine) was also evaluated using historical paired results from 51 unique individuals.

Results: Baseline separation between MMA and succinic acid was completed in 7 min. The assay was linear from 0.1 to 500 μM. The intra-day and inter-day imprecision CV ranged from 4.1 to 13.2% (0.3 to 526 μM) and 5.0 to 15.7% (0.3 to 233 μM), respectively. Recovery ranged from 93 to 125%. The correlation with a national reference laboratory LC-MS/MS assay showed a Deming regression of 1.026 and intercept of −1.335. Carryover was determined to be <0.04%. Patient-specific correlation was observed between MMA and C3-acylcarnitine.

Conclusion: This report describes the first LC-MS/MS method using SLE for MMA extraction. In addition, we illustrate the challenges encountered during this method development that should be assessed and resolved by any laboratory implementing a SLE LC-MS/MS assay designed to quantify analytes across several orders of magnitude.

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1. Introduction

Methylmalonic acidemia encompasses a spectrum of diseases that vary in clinical presentation. It is a common inherited metabolic disorder characterized by a defect in methylmalonyl-CoA mutase or the synthesis of its cofactor adenosylcobalamin (Fig. 1). Primary methylmalonic acidemia may present as severe life-threatening disease in infancy with lethargy, vomiting, acidosis, hyperammonemia and respiratory distress; less severe forms exhibit failure to thrive, hypotonia, and developmental delay. Patients managed with these disorders are at risk for renal failure and chronic basal ganglia injury that impacts motor function, growth, and immune function.

The algorithm for diagnosing methylmalonic acidemia generally involves elevated propionyl acylcarnitine (C3-acylcarnitine) in newborn screening and subsequent positive follow-up testing using plasma acylcarnitine and urine organic acid profile analysis [1–4]. Furthermore, molecular testing is often pursued to determine the underlying genetic cause of the disorder. Once diagnosed, acute metabolic decompensation is managed by fluid balance and restoration of ammonia, acid-base, and electrolyte balance. Long-term management includes supplementation with carnitine, cobalamin (vitamin B12), and low protein nutritional regime. Without proper management, patients with methylmalonic acidemia are at risk for developing life-threatening metabolic decompensation and progressive encephalopathy.

Serum/plasma methylmalonic acid (MMA) concentration is frequently used in combination with blood free carnitine, C3-acylcarnitine, and amino acids to assess the clinical status and the need for changes in dietary management. At our pediatric hospital, we have observed elevations of MMA ranging from 0.4 to 3000 μM in patients with methylmalonic acidemia (reference interval < 0.4 μM) [5] due to the heterogeneous genetic etiology and the patient's state of decompensation. Beyond monitoring methylmalonic acidemia, MMA is also a functional marker of vitamin B12 nutritional status. Specifically, deficiency of vitamin B12 can result in the reduction of adenosylcobalamin, leading to the accumulation of MMA. Distinguishing between methylmalonic acidemia caused by cobalamin defects and nutritional vitamin B12

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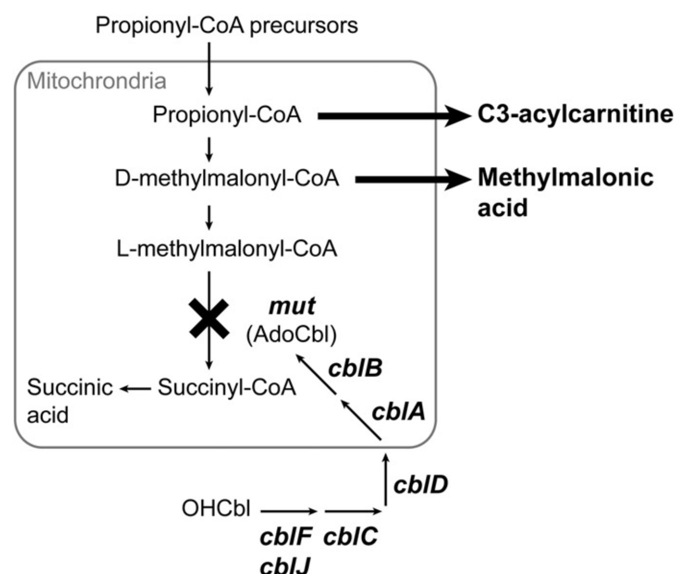

Fig. 1. Methylmalonic acidemia can be caused by defect in methylmalonyl-CoA mutase (mut) or enzymes that synthesize its cofactor, adenosylcobalamin (AdoCbl), from hydroxycobalamin (OHCbl). Deficiency in cobalamin can also inhibit isomerization of L-methylmalonyl-CoA to succinyl-CoA and cause accumulation of MMA and C3-acylcarnitine.

deficiency can be challenging. Maternal MMA levels are often necessary in evaluation of an elevated C3 propionylcarnitine and MMA in an infant, especially if breastfeeding [6].

The current literature describes multiple LC-MS/MS methods to quantify MMA [5,7–14], but the majority of these methods focus on using MMA for nutritional analysis (vitamin B12 status). To date, many sample preparations have been established, ranging from simple deprotonation using low molecular weight ultracentrifugation filter to derivatization and solid phase extraction. This paper reports the evaluation of SLE for monitoring MMA for pediatric patient populations. To our knowledge, this is the first description of MMA sample preparation using SLE.

2. Materials and methods

2.1. Specimens

Residual clinical testing specimens from Seattle Children's Hospital were used for the validation studies. All specimens were de-identified and stored at −80 °C. Use of anonymous patient data and specimens has been approved by the Institutional Review Board at Seattle Children's Hospital (IRB number 15946).

2.2. Reagents and chemicals

MMA, 99% purity, was purchased from Sigma-Aldrich (St. Louis, MO) and deuterium-labeled MMA (d_3-MMA), 98% purity, was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). SeraCon II plasma and SKML Special Assays in Serum were obtained from SeraCare Life Sciences (Milford, MA) and European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM), respectively. SKML refers to the Dutch Foundation for Quality Assessment in Medical Laboratories that provide ERNDIM external quality assessment or proficiency testing materials to assess the quality of the laboratory. Biotage 96-well ISOLUTE SLE+ 200 plates were purchased from Biotage (Sweden). Round bottom, 1 mL collection plates were purchased from Beckman Coulter, Inc. (catalog # 267006; Brea, CA) and pre-slit resealable 96-well septa plate covers were purchased from Thermo Fisher Scientific (catalog # 4412614; Waltham, MA). All reagents were of HPLC grade or better and procured from Sigma or Fisher Scientific.

2.3. Calibrators and controls

MMA and d_3-MMA stock solutions, 8.4 mM and 883 μM, respectively, were prepared in water. Working internal standard was prepared by diluting d_3-MMA stock solution with 4.6 M formic acid to 4.16 μM. Stock solutions and working IS solution were stored at 4 °C for up to a minimum of one year. Calibrators were prepared by spiking MMA stock solution into SeraCon II serum at 0.1, 1, 5, 20, 50, 100, and 500 μM. The expected calibrator concentrations were established using the mean of six measurements. Controls at three different levels were purchased from ERNDIM. Aliquots of the calibrators and controls were stored at −80 °C for up to two years.

2.4. Sample preparation and LC-MS/MS

Calibrator, control, or patient serum (30 μL) was mixed with d_3-MMA solution (30 μL) and 4.6 M formic acid (200 μL). Formic acid was added to ensure that MMA and d_3-MMA remains protonated to improve the SLE extraction efficiency. After 5 min of vigorous vortexing, mixture (200 μL) was loaded onto a Biotage 96-well ISOLUTE SLE+ 200 plate and incubated for 5 min to allow equilibration of analytes in small droplets with diatomaceous earth materials. To improve the seal between the SLE plate and the round bottom, 1 mL collection plate, a modified plate cover was placed in between the two plates. Specifically, the resealable tips of the 96-well plate cover were cut off to reveal the pre-existing openings that would better secure the leur tips of the extraction plate to wells of the collection plate. Analyte and its internal standard were then eluted with MTBE (500 μL) by gravity flow for 5 min before a gentle pressure at 2–3 psi was applied using Biotage pressure + 96 positive pressure manifold. MTBE was subsequently evaporated under a nitrogen stream at 80 °C for 30 min with the mat cover on. Once the organic solvent had completely evaporated, the dried sample was reconstituted in water (100 μL) and vortexed for 5 min to resuspend MMA and d_3-MMA.

Reconstituted specimen (10 μL) was analyzed with Waters Alliance 2795 HPLC coupled with triple quadruple tandem mass spectrometer (Waters QuattroMicro). Separation of MMA from endogenous isobaric interferent, succinic acid (SA), was achieved on Phenomenex Gemini C18 analytical column (100 × 3.00 mm, 3 μm particle size) by gradient method at 0.6 mL/min using mobile phase A (water) and phase B (methanol with 2 mM ammonium acetate, 0.1% formic acid). Chromatographic method was as follows: 15% B to 95% B from 0 to 1.5 min, 95% B from 1.5 to 2.5 min, 95% B to 15% B from 2.5 to 2.55 min, and 15% B from 2.55 to 7 min. Electrospray ionization was set at negative mode and multiple reaction monitoring (MRN) was used to monitor MMA ($117 > 73$) and d_3-MMA ($120.1 > 76$). Nitrogen was used as the nebulizer and desolvation gas (700 L/h at 400 °C) and argon was used as the collision gas (4.2 e^{-3} mbar).

2.5. Method validation

The intra-day and inter-day imprecision were estimated with three control levels. Assay bias was assessed by 47-paired specimen comparison with a national reference laboratory LC-MS/MS MMA assay and re-analysis of 11 previous proficiency test samples provided by ERNDIM. Linearity and analytical measurement range (AMR) were established using six in-house calibrators over a range of 0–500 μM. Manual dilution was validated using 1000 and 2000 μM MMA in SeraCon II serum and a patient sample with 1013 μM MMA measured by the reference laboratory. These three specimens were diluted with SeraCon II serum prior to extraction and measured in triplicates. Dilution recovery is calculated as: (corrected [MMA] − expected [MMA])/expected [MMA]. Lower limit of quantitation (LOQ) was

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