1 Läs bifogad artikel och besvara följande fråga:

Hur diagnotiseras metylmalonsyreuri (methylmalonic acidemi)?

Välj ett alternativ:

- Man detekterar sänkta nivåer av kobalamin.
- Man detekterar förhöjda nivåer av kobalamin.
- Man detekterar förhöjda nivåer av succinyl-CoA.
- Man detekterar sänkta nivåer av propionyl acylkarnitin.
- Man detekterar förhöjda nivåer av propionyl acylkarnitin.

Totalpoäng: 1

2 Läs bifogad artikel och besvara följande fråga:

Vilket av följande påståenden är korrekt:

Välj ett alternativ:

- Obehandlad metylmalonsyreuri leder till metabol kompensation utan svårare bieffekter.
- En vanlig behandling av metylmalonsyreuri är proteinrik kost.
- Metylmalonsyreuri orsakas inte av kobalmindefekter.
- Vitamin B12 brist leder till förhöjda kalcium nivåer.
- MMA kan användas som en markör för att detektera vitamin B 12 brist.

Totalpoäng: 1

3 Läs bifogad artikel och besvara följande fråga:

Vilket var syftet med studien?

Välj ett alternativ:

- Att verifiera nya kalibratorer för analys av MMA.
- Att utvärdera en ny vätskebaserad extraktionsmetod för upparbetning av prov.
- Att kvantifiera MMA med LC-MS/MS.
- Att diagnosticera barn med metylmalonsyreuri.
- Att utveckla användning av ultracentrifugering.

Totalpoäng: 1

4 Läs bifogad artikel och besvara följande fråga:

Vad användes som internstandard?
Välj ett alternativ:

- Vatten
- 4,6M Myrsyra
- 2mM ammoniumacetat
- d_3-MMA
- MTBE

Totalpoäng: 1

5 Läs bifogad artikel och besvara följande fråga:

Vilken joniseringsteknik användes för masspektrometrin?
Välj ett alternativ:

- elektron-impact
- desolvation
- trippel-quadropol
- elektrospray
- HPLC

Totalpoäng: 1

6 Läs bifogad artikel och besvara följande fråga:

Vilket av följande påståenden är korrekt?
Välj ett alternativ:

- Den kromatografiska upplösningen mellan succinat och MMA beräknas till 73.
- Retentionstiden för MMA är kortare än för succinat.
- Hos en frisk individ finns en mindre mängd succinat än MMA.
- Retentionstiden för MMA är längre än för succinat.
- Hos en patient med metylmalonsyreuri finns en större mängd succinat än MMA.

Totalpoäng: 1

7 Läs bifogad artikel och besvara följande fråga:

På vilket sätt kunde kontamination mellan prover minimeras?
Välj ett alternativ:

- Genom att byta från plattor med runda brunnar till fyrkantiga brunnar
- Genom tre injektioner med vatten samt tre nåltvättar.
- Genom att minska avståndet mellan brunnarnas öppningar.
- Genom tre injektioner med vatten samt fem nåltvättar.
- Genom tre injektioner med vatten samt nio nåltvättar.

Totalpoäng: 1

8 Läs bifogad artikel och besvara följande fråga:

Vilket av följande är **KORREKT**?
Välj ett alternativ:

- Metoden var linjär mellan 0,3 till 526 microM.
- Metoden var linjär mellan 0,08 till 1013 microM.
- Variationskoefficienten för daglig analys av kontroller varierade mellan 93 till 125%.
- Variationskoefficienten för daglig analys av kontroller varierade mellan 4,1 till 13,2%.
- Variationskoefficienten för daglig analys av kontroller varierade mellan 5,0 till 15,7%.

Totalpoäng: 1

9 Läs bifogad artikel och besvara följande fråga:

Vilket av följande är **KORREKT**?
Välj ett alternativ:

- Det fanns patientspecifik korrelation mellan MMA och C3-Acylkarnitin.
- Nivåerna av MMA och C3-Acylkarnitin var oberoende av koncentrationen av fritt karnitin.
- Nivåerna av MMA varierade obetydligt mellan olika individer.
- Det var ett strikt linjärt förhållande mellan koncentration MMA och C3-Acylkarnitin.
- Nivåerna C3-Acylkarnitin varierar med mängden fritt karnitin.

Totalpoäng: 1

10 Läs bifogad artikel och besvara följande fråga:

Vilket av följande är **KORREKT**?
Välj ett alternativ:

- Supported-liquid-extraction kräver en större provvolym men är en snabbare analys.
- Supported-liquid-extraction är inte lämplig för diagnostik av metylmalonsyreuri.
- Supported-liquid-extraction kräver en mindre provvolym och är inte lämplig för automation.
- Supported-liquid-extraction kräver en mindre provvolym men är inte lämplig på grund av carry-over problematik.
- Supported-liquid-extraction kräver en mindre provvolym och är en snabbare analys.

Totalpoäng: 1

Question 10
Attached

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

Sheng-Ying Lo [a], Cindy Gordon [b], Katerina Sadilkova [b], Rhona M. Jack [b], Jane A. Dickerson [b,*]

[a] Department of Laboratory Medicine, Chemistry Division, University of Washington, Seattle, WA, United States
[b] Department of Laboratories, Seattle Children's Hospital, Seattle, WA, United States

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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.

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

* Corresponding author at: Seattle Children's Hospital, P.O. Box 5371, Seattle, WA 98145, United States.
E-mail address: jane.dickerson@seattlechildrens.org (J.A. Dickerson).

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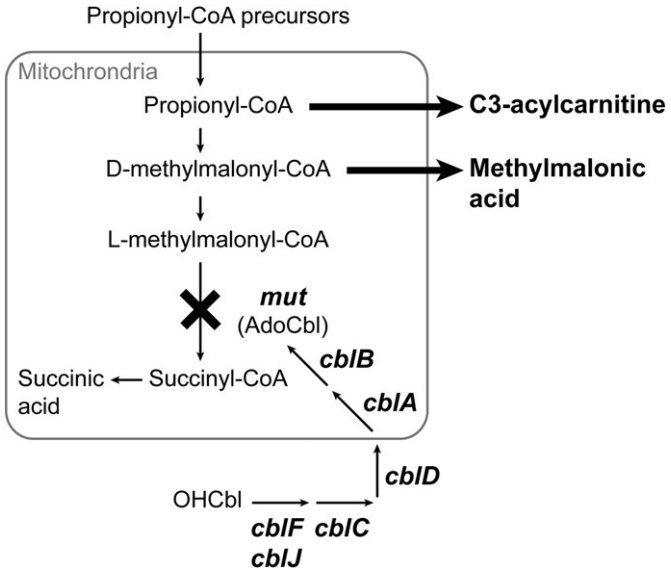

Fig. 1. Methylmalonic acidemia can be caused by defect in methylmalonyl-CoA mutase (mut) or enzymes that synthesize its cofactor, adenosylcobalamin (AdoCbl), from hydroxycobalamin (OHCbl). Deficiency in cobalamin can also inhibit isomerization of L-methylmalonyl-CoA to succinyl-CoA and cause accumulation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

determined by diluting an ERNDIM sample with 0.1% BSA to 0.1 μM and analyzed in quintuplet over three days. Analytical recovery was determined by comparing the averaged peak area of d_3-MMA added pre and post SLE (N = 3). Matrix effect was evaluated by comparing the averaged peak areas of the post extraction sample with unextracted MMA solution (0.92 μM in water). Experimental detail is illustrated in Fig. 2. Ion suppression or enhancement was also examined by post-column infusion of d_3-MMA with injection of SLE extracted patient specimens (N = 4) [15]. Reference interval was verified using 20 patient specimens with normal C3-acylcarnitine [16].

2.6. Carryover

Two types of carryover were studied. First, MMA carryover from the injection needle was assessed with a water injection (blank) followed by an injection of a high MMA sample (1000 μM MMA in SeraCon II serum) and four subsequent blanks. For each analysis, a new water sample was used, with the number of needle wash cycles set to 1, 3, 6, or 9 throughout the runs. Carryover between each analysis was minimized by three blank injections of a new water sample and nine needle wash cycles per injection. Peak area obtained from a blank run prior to the high MMA sample was used for baseline correction. Carryover was calculated as: Carryover (μM) = blank peak area/high sample peak area * 1000 μM. Second, evaporative carryover by MTBE during sample preparation was examined using nine wells (3 × 3) test area. For square wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and SeraCon II serum without MMA was extracted in the surrounding wells. MMA concentrations were quantified using calibration curve. For round wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and 500 μL of MTBE was added to the eight surrounding wells of the collection plate prior to nitrogen evaporation. All wells were then reconstituted in 100 μL of water and analyzed. Carryover for round wells was calculated as: carryover (μM) = surrounding well peak area/center well peak area * 1000 μM.

2.7. Correlation between serum MMA and C3-acylcarnitine

Historical results for paired MMA and plasma acylcarnitine analysis generated between November 1, 2011 and September 31, 2015 were pulled from the laboratory information system (Cerner Millennium Pathnet), N = 111. Results were grouped based on diagnosis then de-identified prior to correlation analysis.

2.8. Statistical analysis

Statistical analyses were performed using Microsoft Excel, GraphPad Prism 5, and EP Evaluator.

3. Results

3.1. Chromatography

Chromatographic separation of 0.81 min between MMA and its major endogenous isobaric interferent, succinic acid (SA) was achieved (Fig. 3). The run-to-run time was 8 min. The chromatographic resolution between SA and MMA is estimated to be 14.

3.2. Method validation

The intra-day CV for three control levels were 13.2% at 0.3 μM, 6.0% at 13 μM, and 4.1% at 526 μM (N = 20–22). The inter-day CV for three control levels over three months were 15.7% at 0.3 μM, 7.5% at 10.2 μM, and 5.0% at 233 μM (N = 14–15). The assay was linear between 0.1 and 500 μM with analyte recovery ranging from 93 to 125% (Supplemental Fig. 1A). A 10-fold manual dilution was validated, with corrected dilution results agreeing within 15% of the expected neat results (Supplemental Fig. 1B). With this validation, the upper limit of the reportable range is theoretically extended to 5000 μM, recognizing that in clinical practice we have not seen levels of this magnitude. The LOQ was established at 0.1 μM with a CV of 18.1%. The extraction efficiency of the Biotage plate was determined to be 72%. The matrix effect study showed no ion-suppression, but ion-enhancement of 160% was noted. These observations were consistent with qualitative matrix effect evaluation by post-column infusion of d_3-MMA after injecting 4 extracted patient specimens without internal standards. The assay was compared to a national reference lab LC-MS/MS method with analyte concentrations ranging from 0.08 to 1013 μM. The correlation coefficient was determined to be 0.9987 and the Deming regression analysis showed a slope of 1.026 and intercept of −1.335 (Supplemental Fig. 1C). Comparison to ERNDIM peer group mean showed a correlation coefficient of 0.9996 and a Deming regression slope and intercept of 1.094 and 0.630, respectively. Reference interval of <0.4 μM was verified using 20 patient specimens, which ranged from 0.0 to 0.3 μM.

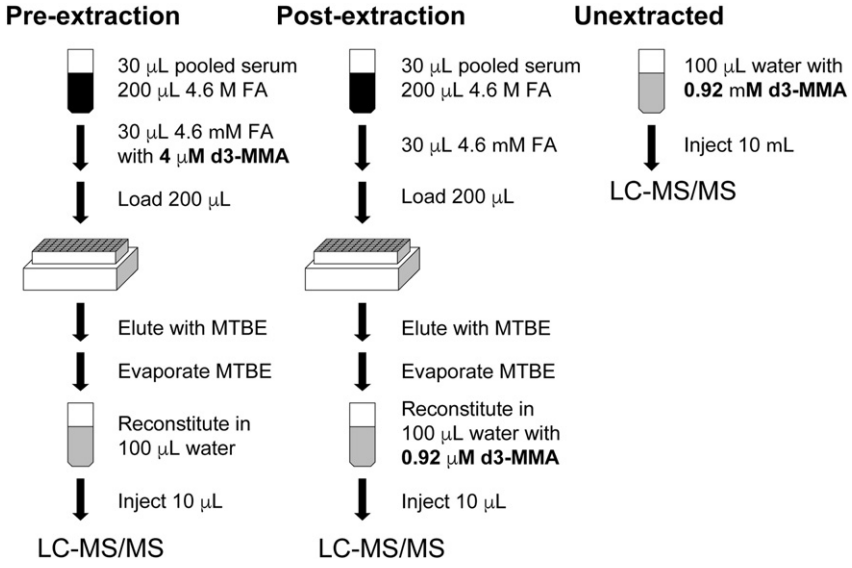

Fig. 2. Synopsis of sample preparation for analytical recovery and matrix effect.

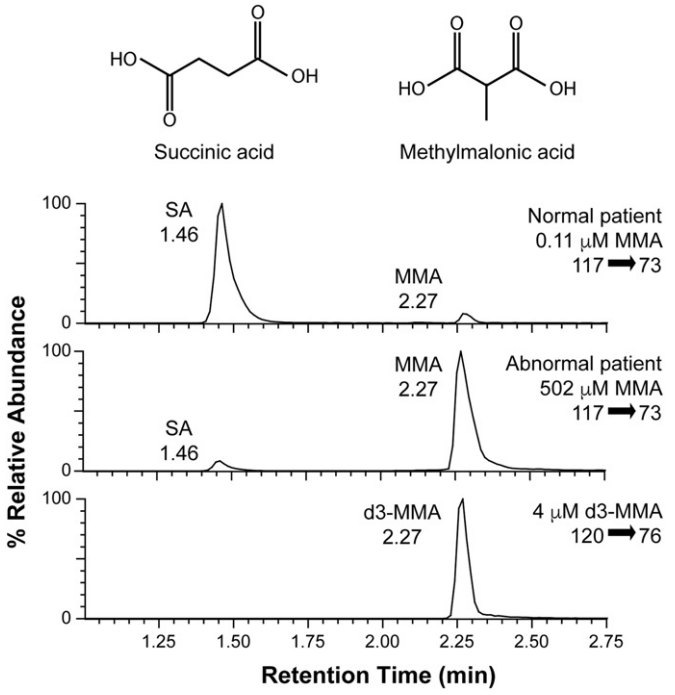

Fig. 3. Structures of succinic acid and methylmalonic acid (top). Representative chromatographic separations of succinic acid and methylmalonic acid: a normal patient and a patient with known methylmalonic acidemia (bottom).

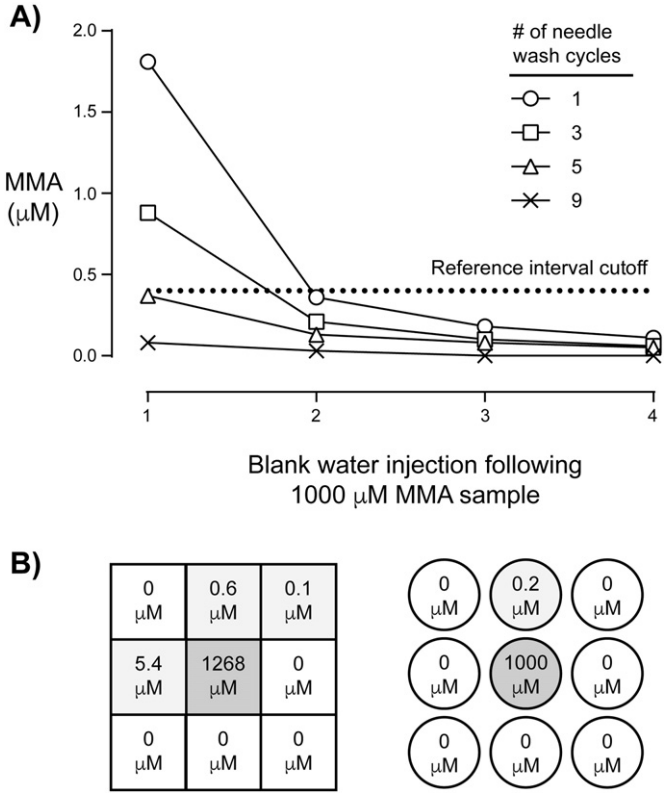

Fig. 4. Carryover minimization studies for injection needle (A) and MTBE evaporation (B). B shows a representative 3 × 3 test area for 96 square well collection plate (left) and 96 round bottom, 1 mL collection plate with modified cover (right).

3.3. Carryover

To maximize the analytical measurement range, carryover was minimized in addition to optimizing chromatographic separation. First, the wash cycle number for the injection needle was increased to nine cycles, the maximum number of wash cycles for a 7 min run. The wash buffer for the injection needle was also optimized to 40% methanol to increase the solubility of MMA. The carryover from the injection needle was determined to be 0.008% at 1000 μM MMA and was eliminated with 3 blank injections following a 1000 μM MMA sample injection (Fig. 4A). Second, evaporative carryover from MTBE was reduced to below 0.4 μM through changing the collection plate from square to round well shape and increasing the spacing of the openings between wells with a modified plate cover (Fig. 4B).

3.4. Correlation between C3-acylcarnitine and MMA

Paired MMA and plasma acylcarnitine profile analysis results were analyzed to understand the relationship between MMA and C3-acylcarnitine. These paired results were generated from 51 unique patients; 9 with primary methylmalonic acidemia (methylmalonyl CoA mutase deficiency), 1 with Cobalamin A, 1 with Cobalamin B, 10 with Cobalamin C, and 30 patients who were either undergoing screening for metabolic disease or nutritional monitoring. Comparison between MMA and C3-acylcarnitine concentrations revealed two populations with linear correlations of varying slopes (Fig. 5A). Assessment of free carnitine results indicated no association between the free carnitine concentrations and the observed populations. Plotting the results by patient, instead, showed a patient-specific relationship between MMA and C3-acylcarnitine concentrations (Fig. 5B).

4. Discussion

Analysis of MMA concentration can be used to aid the clinical diagnosis and management of methylmalonic acidemia. In order to accommodate the wide range of MMA observed in our pediatric patient population, the primary objective of our in-house LC-MS/MS MMA assay development and validation was to obtain a broad AMR while maintaining a low specimen volume requirement. Maintaining accuracy throughout the entire dynamic range was also of primary importance, along with eliminating any possibility of cross contamination. Concurrently, our secondary objectives were to establish an assay with a simple sample preparation that would allow us to minimize the turn-around-time for reporting out MMA.

In order to achieve a broad AMR spanning several orders of magnitudes, we focused on eliminating potential interference from SA by obtaining a robust baseline separation between MMA and SA. After evaluating multiple chromatography conditions and sample preparations described in the literature, we elected to use SLE and the analytical separation method described above. Optimal baseline separation, with a chromatographic resolution >10, between MMA and SA was achieved. The robustness of the chromatography, however, strongly depended on the pH of the sample. Degradation of the peak shape and resolution was noted when samples were not completely dried down to remove the formic acid.

With the robust baseline separation between MMA and SA, we were able to attain an AMR from 0.1 to 500 μM. We discovered that isotopic contribution of M + 3 MMA significantly contaminated the d_3-MMA internal standard signal above 500 μM MMA. Consequently, the internal standard concentration was increased to 4.2 μM from 0.4 μM and the upper limit of AMR was reduced to 500 μM. Through these modifications, we were able to diminish the effects of M + 3 and maintain accurate quantitation of serum MMA. It is possible that a broader AMR may be obtainable from lesser isotopic contribution of M + 4 MMA using a d_4-MMA I.S.

During the assay development, we also encountered two carryover issues not previously emphasized in the literature. If present, both types of carryover have the potential to alter the clinical diagnosis and management of patients. First, we observed carryover from the injection

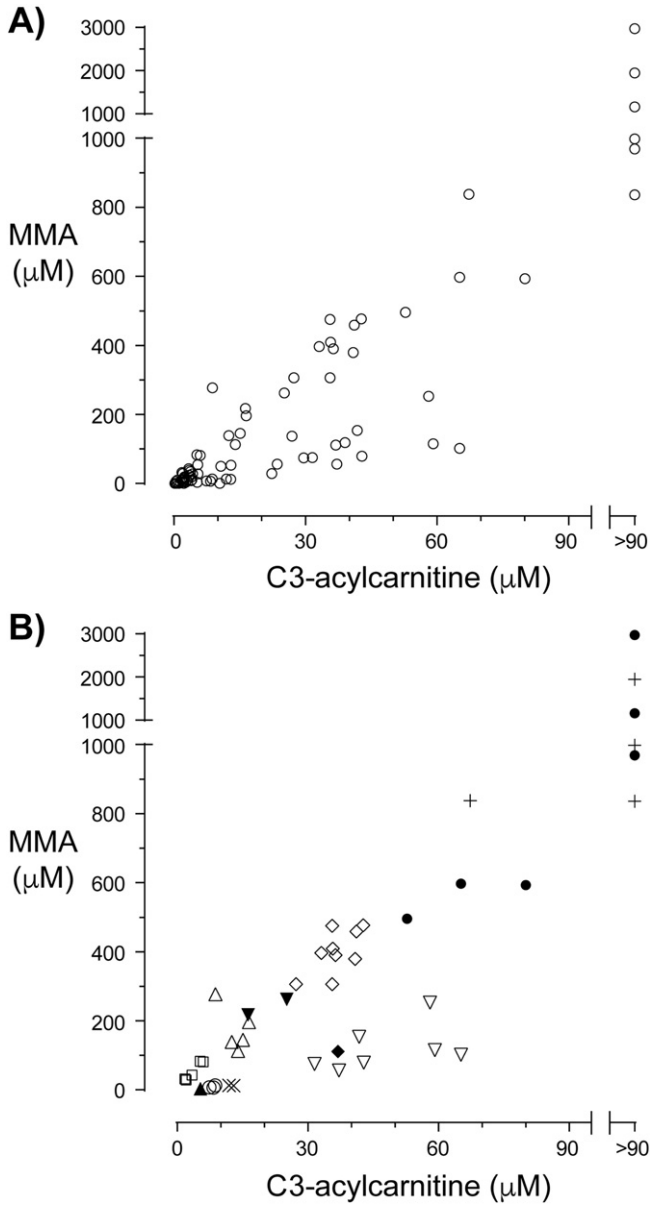

Fig. 5. Serum MMA and C3-acylcarnitine correlation data. (A) Paired serum MMA and C3-acylcarnitine reported during a 50-month period. (B) Patients with known MMA and Cobalamin A and B (N = 11), each symbol represents a unique patient.

needle, requiring the maximum number of injection wash cycles to minimize this effect. Second, and more challenging to discover, we identified a random evaporative carryover (also known as "cross-talk") where analyte from one well of a plate can flow to the neighboring well by the volatilized solvent [17]. In particular, when the difference in concentrations between two neighboring wells is >10 fold, the carryover can become significant. This second issue was difficult to resolve, and ultimately, we implemented the use of round bottom, 1 mL 96-well collection plates with a modified plate cover to increase the spacing between the openings of the wells during solvent evaporation. Furthermore, a modified plate cover was also used to improve the seal between the SLE plate and collection plate during sample elution to prevent contamination caused by splashing and sputtering.

To improve our understanding of the utility of quantifying MMA for the diagnosis and management of methylmalonic acidemia, we evaluated the relationship between MMA and C3-acylcarnitine. Our retrospective analysis unexpectedly revealed a patient-specific correlation between the two analytes that was independent of free carnitine concentration. This observed biological variation between individuals with methylmalonic acidemia strongly supports the necessity for quantifying MMA in addition to acylcarnitine analysis. Further investigation to better understand this observed phenomena and clinical utility is needed.

5. Conclusion

We encountered challenges during various stages of development of this SLE LC-MS/MS assay for serum MMA. With this work, we hope that these challenges can be carefully assessed and mitigated by laboratories prior to implementing a SLE LC-MS/MS assay for an analyte that requires a broad AMR. After resolving these hurdles, SLE's many advantages can be realized. Specifically, we were able to use minimal sample volume (30 μL) with fast extraction time that required minimal manual intervention (approximately 2 h for a full 96 well plate). The method is also adaptable to scale with semi-automation using a liquid handler to further increase laboratory efficiency [12].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2016.05.010.

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

Sheng-Ying Lo [a], Cindy Gordon [b], Katerina Sadilkova [b], Rhona M. Jack [b], Jane A. Dickerson [b,*]

[a] Department of Laboratory Medicine, Chemistry Division, University of Washington, Seattle, WA, United States
[b] Department of Laboratories, Seattle Children's Hospital, Seattle, WA, United States

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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.

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

* Corresponding author at: Seattle Children's Hospital, P.O. Box 5371, Seattle, WA 98145, United States.
E-mail address: jane.dickerson@seattlechildrens.org (J.A. Dickerson).

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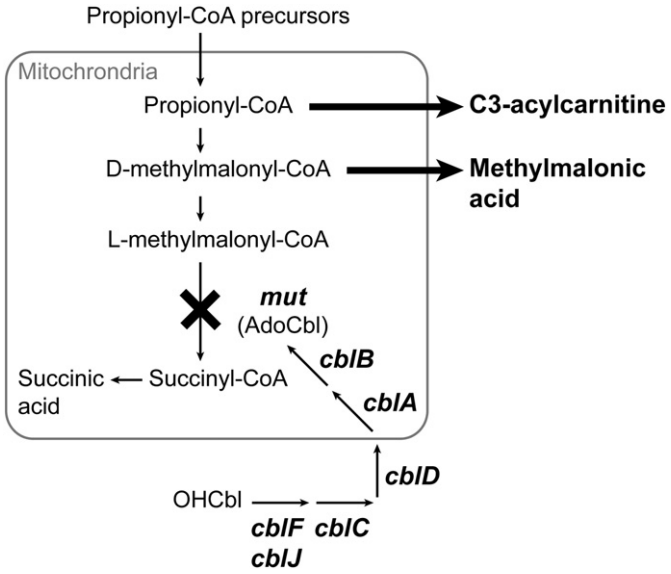

Fig. 1. Methylmalonic acidemia can be caused by defect in methylmalonyl-CoA mutase (mut) or enzymes that synthesize its cofactor, adenosylcobalamin (AdoCbl), from hydroxycobalamin (OHCbl). Deficiency in cobalamin can also inhibit isomerization of L-methylmalonyl-CoA to succinyl-CoA and cause accumulation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

determined by diluting an ERNDIM sample with 0.1% BSA to 0.1 μM and analyzed in quintuplet over three days. Analytical recovery was determined by comparing the averaged peak area of d_3-MMA added pre and post SLE (N = 3). Matrix effect was evaluated by comparing the averaged peak areas of the post extraction sample with unextracted MMA solution (0.92 μM in water). Experimental detail is illustrated in Fig. 2. Ion suppression or enhancement was also examined by post-column infusion of d_3-MMA with injection of SLE extracted patient specimens (N = 4) [15]. Reference interval was verified using 20 patient specimens with normal C3-acylcarnitine [16].

2.6. Carryover

Two types of carryover were studied. First, MMA carryover from the injection needle was assessed with a water injection (blank) followed by an injection of a high MMA sample (1000 μM MMA in SeraCon II serum) and four subsequent blanks. For each analysis, a new water sample was used, with the number of needle wash cycles set to 1, 3, 6, or 9 throughout the runs. Carryover between each analysis was minimized by three blank injections of a new water sample and nine needle wash cycles per injection. Peak area obtained from a blank run prior to the high MMA sample was used for baseline correction. Carryover was calculated as: Carryover (μM) = blank peak area/high sample peak area * 1000 μM. Second, evaporative carryover by MTBE during sample preparation was examined using nine wells (3 × 3) test area. For square wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and SeraCon II serum without MMA was extracted in the surrounding wells. MMA concentrations were quantified using calibration curve. For round wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and 500 μL of MTBE was added to the eight surrounding wells of the collection plate prior to nitrogen evaporation. All wells were then reconstituted in 100 μL of water and analyzed. Carryover for round wells was calculated as: carryover (μM) = surrounding well peak area/center well peak area * 1000 μM.

2.7. Correlation between serum MMA and C3-acylcarnitine

Historical results for paired MMA and plasma acylcarnitine analysis generated between November 1, 2011 and September 31, 2015 were pulled from the laboratory information system (Cerner Millennium Pathnet), N = 111. Results were grouped based on diagnosis then de-identified prior to correlation analysis.

2.8. Statistical analysis

Statistical analyses were performed using Microsoft Excel, GraphPad Prism 5, and EP Evaluator.

3. Results

3.1. Chromatography

Chromatographic separation of 0.81 min between MMA and its major endogenous isobaric interferent, succinic acid (SA) was achieved (Fig. 3). The run-to-run time was 8 min. The chromatographic resolution between SA and MMA is estimated to be 14.

3.2. Method validation

The intra-day CV for three control levels were 13.2% at 0.3 μM, 6.0% at 13 μM, and 4.1% at 526 μM (N = 20–22). The inter-day CV for three control levels over three months were 15.7% at 0.3 μM, 7.5% at 10.2 μM, and 5.0% at 233 μM (N = 14–15). The assay was linear between 0.1 and 500 μM with analyte recovery ranging from 93 to 125% (Supplemental Fig. 1A). A 10-fold manual dilution was validated, with corrected dilution results agreeing within 15% of the expected neat results (Supplemental Fig. 1B). With this validation, the upper limit of the reportable range is theoretically extended to 5000 μM, recognizing that in clinical practice we have not seen levels of this magnitude. The LOQ was established at 0.1 μM with a CV of 18.1%. The extraction efficiency of the Biotage plate was determined to be 72%. The matrix effect study showed no ion-suppression, but ion-enhancement of 160% was noted. These observations were consistent with qualitative matrix effect evaluation by post-column infusion of d_3-MMA after injecting 4 extracted patient specimens without internal standards. The assay was compared to a national reference lab LC-MS/MS method with analyte concentrations ranging from 0.08 to 1013 μM. The correlation coefficient was determined to be 0.9987 and the Deming regression analysis showed a slope of 1.026 and intercept of −1.335 (Supplemental Fig. 1C). Comparison to ERNDIM peer group mean showed a correlation coefficient of 0.9996 and a Deming regression slope and intercept of 1.094 and 0.630, respectively. Reference interval of <0.4 μM was verified using 20 patient specimens, which ranged from 0.0 to 0.3 μM.

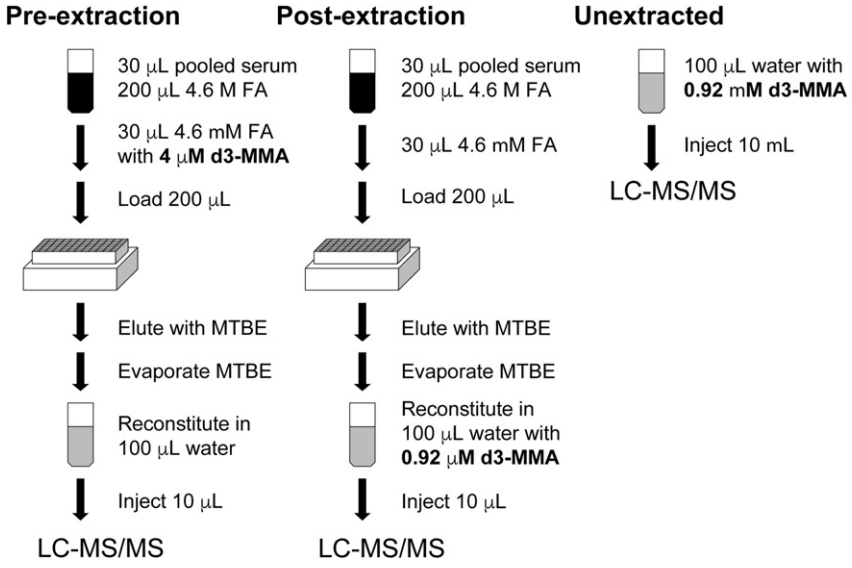

Fig. 2. Synopsis of sample preparation for analytical recovery and matrix effect.

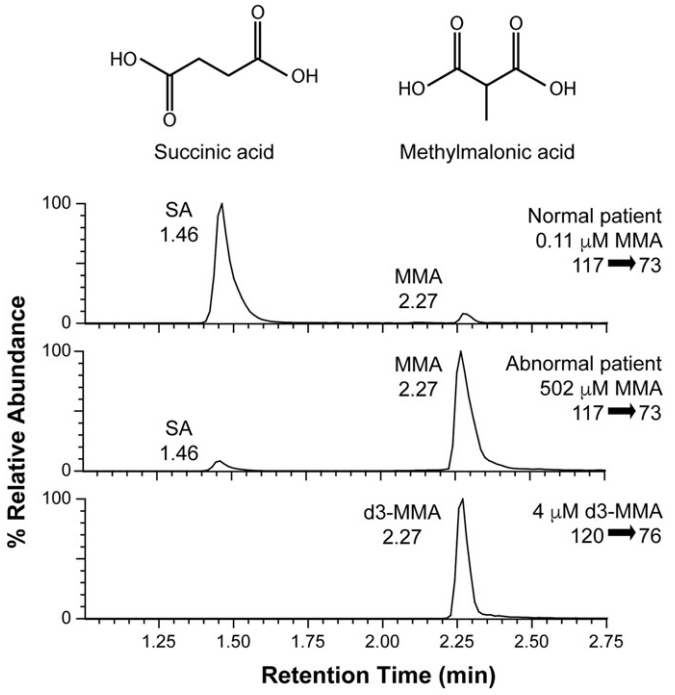

Fig. 3. Structures of succinic acid and methylmalonic acid (top). Representative chromatographic separations of succinic acid and methylmalonic acid: a normal patient and a patient with known methylmalonic acidemia (bottom).

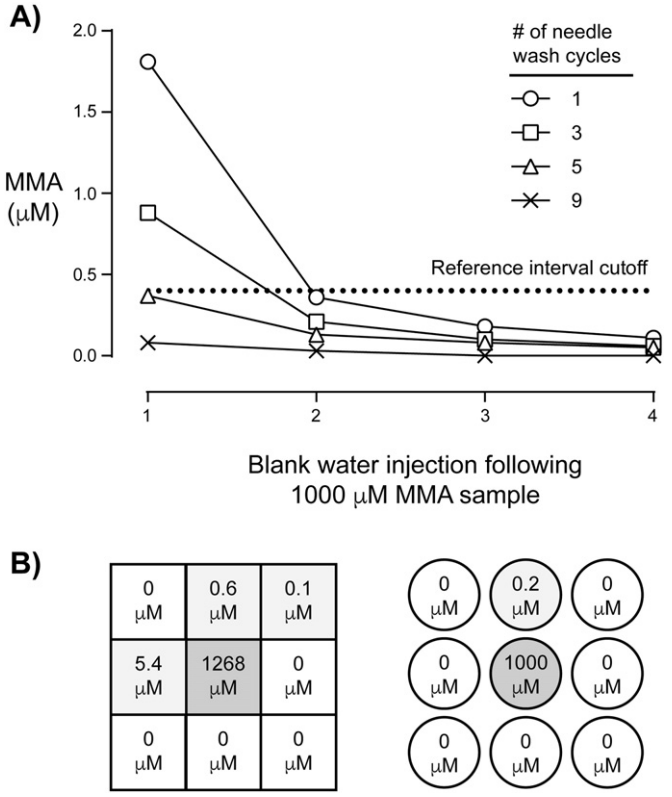

Fig. 4. Carryover minimization studies for injection needle (A) and MTBE evaporation (B). B shows a representative 3 × 3 test area for 96 square well collection plate (left) and 96 round bottom, 1 mL collection plate with modified cover (right).

3.3. Carryover

To maximize the analytical measurement range, carryover was minimized in addition to optimizing chromatographic separation. First, the wash cycle number for the injection needle was increased to nine cycles, the maximum number of wash cycles for a 7 min run. The wash buffer for the injection needle was also optimized to 40% methanol to increase the solubility of MMA. The carryover from the injection needle was determined to be 0.008% at 1000 μM MMA and was eliminated with 3 blank injections following a 1000 μM MMA sample injection (Fig. 4A). Second, evaporative carryover from MTBE was reduced to below 0.4 μM through changing the collection plate from square to round well shape and increasing the spacing of the openings between wells with a modified plate cover (Fig. 4B).

3.4. Correlation between C3-acylcarnitine and MMA

Paired MMA and plasma acylcarnitine profile analysis results were analyzed to understand the relationship between MMA and C3-acylcarnitine. These paired results were generated from 51 unique patients; 9 with primary methylmalonic acidemia (methylmalonyl CoA mutase deficiency), 1 with Cobalamin A, 1 with Cobalamin B, 10 with Cobalamin C, and 30 patients who were either undergoing screening for metabolic disease or nutritional monitoring. Comparison between MMA and C3-acylcarnitine concentrations revealed two populations with linear correlations of varying slopes (Fig. 5A). Assessment of free carnitine results indicated no association between the free carnitine concentrations and the observed populations. Plotting the results by patient, instead, showed a patient-specific relationship between MMA and C3-acylcarnitine concentrations (Fig. 5B).

4. Discussion

Analysis of MMA concentration can be used to aid the clinical diagnosis and management of methylmalonic acidemia. In order to accommodate the wide range of MMA observed in our pediatric patient population, the primary objective of our in-house LC-MS/MS MMA assay development and validation was to obtain a broad AMR while maintaining a low specimen volume requirement. Maintaining accuracy throughout the entire dynamic range was also of primary importance, along with eliminating any possibility of cross contamination. Concurrently, our secondary objectives were to establish an assay with a simple sample preparation that would allow us to minimize the turn-around-time for reporting out MMA.

In order to achieve a broad AMR spanning several orders of magnitudes, we focused on eliminating potential interference from SA by obtaining a robust baseline separation between MMA and SA. After evaluating multiple chromatography conditions and sample preparations described in the literature, we elected to use SLE and the analytical separation method described above. Optimal baseline separation, with a chromatographic resolution >10, between MMA and SA was achieved. The robustness of the chromatography, however, strongly depended on the pH of the sample. Degradation of the peak shape and resolution was noted when samples were not completely dried down to remove the formic acid.

With the robust baseline separation between MMA and SA, we were able to attain an AMR from 0.1 to 500 μM. We discovered that isotopic contribution of M + 3 MMA significantly contaminated the d_3-MMA internal standard signal above 500 μM MMA. Consequently, the internal standard concentration was increased to 4.2 μM from 0.4 μM and the upper limit of AMR was reduced to 500 μM. Through these modifications, we were able to diminish the effects of M + 3 and maintain accurate quantitation of serum MMA. It is possible that a broader AMR may be obtainable from lesser isotopic contribution of M + 4 MMA using a d_4-MMA I.S.

During the assay development, we also encountered two carryover issues not previously emphasized in the literature. If present, both types of carryover have the potential to alter the clinical diagnosis and management of patients. First, we observed carryover from the injection

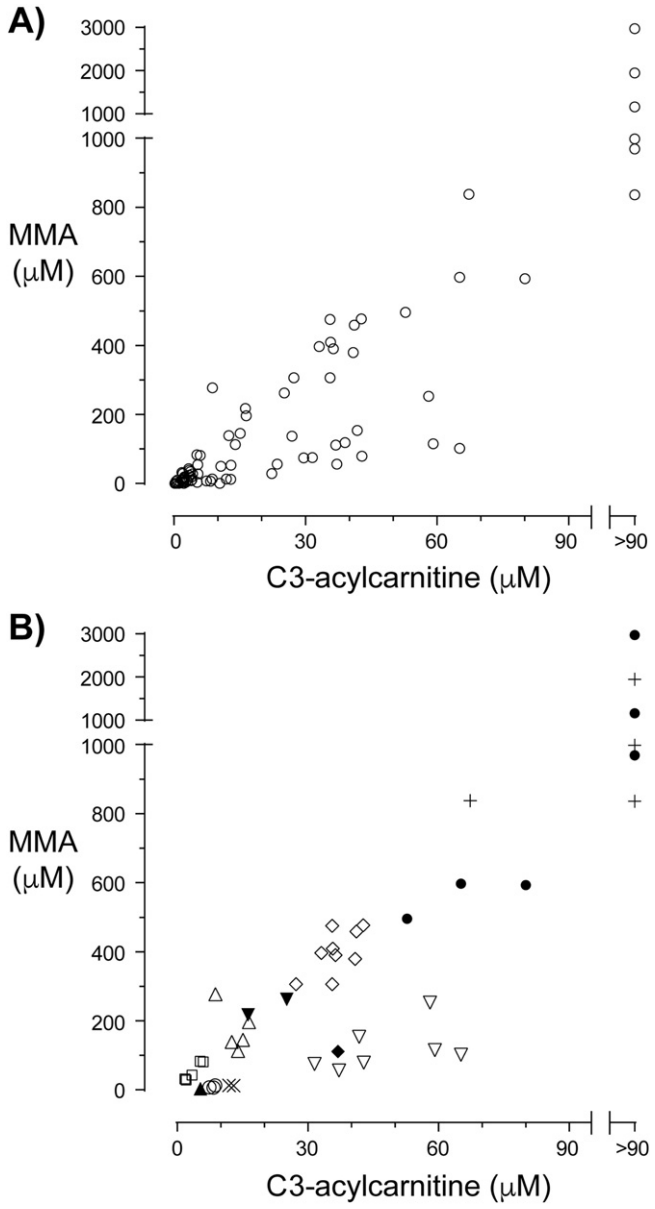

Fig. 5. Serum MMA and C3-acylcarnitine correlation data. (A) Paired serum MMA and C3-acylcarnitine reported during a 50-month period. (B) Patients with known MMA and Cobalamin A and B (N = 11), each symbol represents a unique patient.

needle, requiring the maximum number of injection wash cycles to minimize this effect. Second, and more challenging to discover, we identified a random evaporative carryover (also known as "cross-talk") where analyte from one well of a plate can flow to the neighboring well by the volatilized solvent [17]. In particular, when the difference in concentrations between two neighboring wells is >10 fold, the carryover can become significant. This second issue was difficult to resolve, and ultimately, we implemented the use of round bottom, 1 mL 96-well collection plates with a modified plate cover to increase the spacing between the openings of the wells during solvent evaporation. Furthermore, a modified plate cover was also used to improve the seal between the SLE plate and collection plate during sample elution to prevent contamination caused by splashing and sputtering.

To improve our understanding of the utility of quantifying MMA for the diagnosis and management of methylmalonic acidemia, we evaluated the relationship between MMA and C3-acylcarnitine. Our retrospective analysis unexpectedly revealed a patient-specific correlation between the two analytes that was independent of free carnitine concentration. This observed biological variation between individuals with methylmalonic acidemia strongly supports the necessity for quantifying MMA in addition to acylcarnitine analysis. Further investigation to better understand this observed phenomena and clinical utility is needed.

5. Conclusion

We encountered challenges during various stages of development of this SLE LC-MS/MS assay for serum MMA. With this work, we hope that these challenges can be carefully assessed and mitigated by laboratories prior to implementing a SLE LC-MS/MS assay for an analyte that requires a broad AMR. After resolving these hurdles, SLE's many advantages can be realized. Specifically, we were able to use minimal sample volume (30 μL) with fast extraction time that required minimal manual intervention (approximately 2 h for a full 96 well plate). The method is also adaptable to scale with semi-automation using a liquid handler to further increase laboratory efficiency [12].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2016.05.010.

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Question 2
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Quantifying MMA by SLE LC-MS/MS: Unexpected challenges in assay development

Sheng-Ying Lo [a], Cindy Gordon [b], Katerina Sadilkova [b], Rhona M. Jack [b], Jane A. Dickerson [b,*]

[a] Department of Laboratory Medicine, Chemistry Division, University of Washington, Seattle, WA, United States
[b] Department of Laboratories, Seattle Children's Hospital, Seattle, WA, United States

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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.

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

* Corresponding author at: Seattle Children's Hospital, P.O. Box 5371, Seattle, WA 98145, United States.
E-mail address: jane.dickerson@seattlechildrens.org (J.A. Dickerson).

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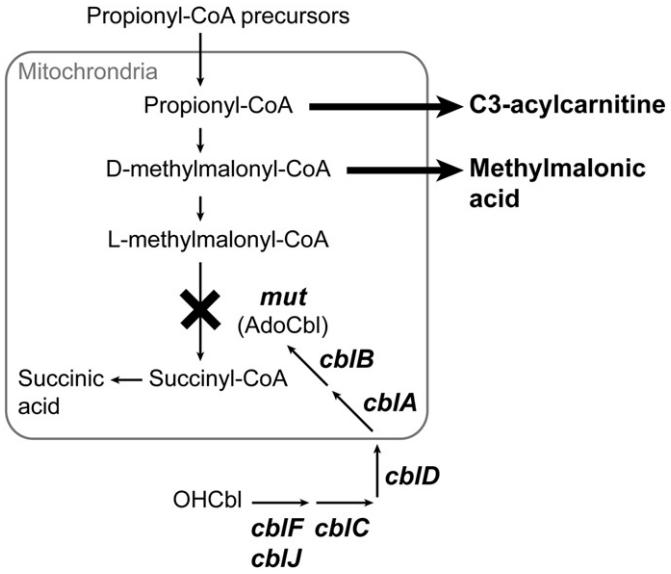

Fig. 1. Methylmalonic acidemia can be caused by defect in methylmalonyl-CoA mutase (mut) or enzymes that synthesize its cofactor, adenosylcobalamin (AdoCbl), from hydroxycobalamin (OHCbl). Deficiency in cobalamin can also inhibit isomerization of L-methylmalonyl-CoA to succinyl-CoA and cause accumulation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

determined by diluting an ERNDIM sample with 0.1% BSA to 0.1 μM and analyzed in quintuplet over three days. Analytical recovery was determined by comparing the averaged peak area of d_3-MMA added pre and post SLE (N = 3). Matrix effect was evaluated by comparing the averaged peak areas of the post extraction sample with unextracted MMA solution (0.92 μM in water). Experimental detail is illustrated in Fig. 2. Ion suppression or enhancement was also examined by post-column infusion of d_3-MMA with injection of SLE extracted patient specimens (N = 4) [15]. Reference interval was verified using 20 patient specimens with normal C3-acylcarnitine [16].

2.6. Carryover

Two types of carryover were studied. First, MMA carryover from the injection needle was assessed with a water injection (blank) followed by an injection of a high MMA sample (1000 μM MMA in SeraCon II serum) and four subsequent blanks. For each analysis, a new water sample was used, with the number of needle wash cycles set to 1, 3, 6, or 9 throughout the runs. Carryover between each analysis was minimized by three blank injections of a new water sample and nine needle wash cycles per injection. Peak area obtained from a blank run prior to the high MMA sample was used for baseline correction. Carryover was calculated as: Carryover (μM) = blank peak area/high sample peak area * 1000 μM. Second, evaporative carryover by MTBE during sample preparation was examined using nine wells (3 × 3) test area. For square wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and SeraCon II serum without MMA was extracted in the surrounding wells. MMA concentrations were quantified using calibration curve. For round wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and 500 μL of MTBE was added to the eight surrounding wells of the collection plate prior to nitrogen evaporation. All wells were then reconstituted in 100 μL of water and analyzed. Carryover for round wells was calculated as: carryover (μM) = surrounding well peak area/center well peak area * 1000 μM.

2.7. Correlation between serum MMA and C3-acylcarnitine

Historical results for paired MMA and plasma acylcarnitine analysis generated between November 1, 2011 and September 31, 2015 were pulled from the laboratory information system (Cerner Millennium Pathnet), N = 111. Results were grouped based on diagnosis then de-identified prior to correlation analysis.

2.8. Statistical analysis

Statistical analyses were performed using Microsoft Excel, GraphPad Prism 5, and EP Evaluator.

3. Results

3.1. Chromatography

Chromatographic separation of 0.81 min between MMA and its major endogenous isobaric interferent, succinic acid (SA) was achieved (Fig. 3). The run-to-run time was 8 min. The chromatographic resolution between SA and MMA is estimated to be 14.

3.2. Method validation

The intra-day CV for three control levels were 13.2% at 0.3 μM, 6.0% at 13 μM, and 4.1% at 526 μM (N = 20–22). The inter-day CV for three control levels over three months were 15.7% at 0.3 μM, 7.5% at 10.2 μM, and 5.0% at 233 μM (N = 14–15). The assay was linear between 0.1 and 500 μM with analyte recovery ranging from 93 to 125% (Supplemental Fig. 1A). A 10-fold manual dilution was validated, with corrected dilution results agreeing within 15% of the expected neat results (Supplemental Fig. 1B). With this validation, the upper limit of the reportable range is theoretically extended to 5000 μM, recognizing that in clinical practice we have not seen levels of this magnitude. The LOQ was established at 0.1 μM with a CV of 18.1%. The extraction efficiency of the Biotage plate was determined to be 72%. The matrix effect study showed no ion-suppression, but ion-enhancement of 160% was noted. These observations were consistent with qualitative matrix effect evaluation by post-column infusion of d_3-MMA after injecting 4 extracted patient specimens without internal standards. The assay was compared to a national reference lab LC-MS/MS method with analyte concentrations ranging from 0.08 to 1013 μM. The correlation coefficient was determined to be 0.9987 and the Deming regression analysis showed a slope of 1.026 and intercept of −1.335 (Supplemental Fig. 1C). Comparison to ERNDIM peer group mean showed a correlation coefficient of 0.9996 and a Deming regression slope and intercept of 1.094 and 0.630, respectively. Reference interval of <0.4 μM was verified using 20 patient specimens, which ranged from 0.0 to 0.3 μM.

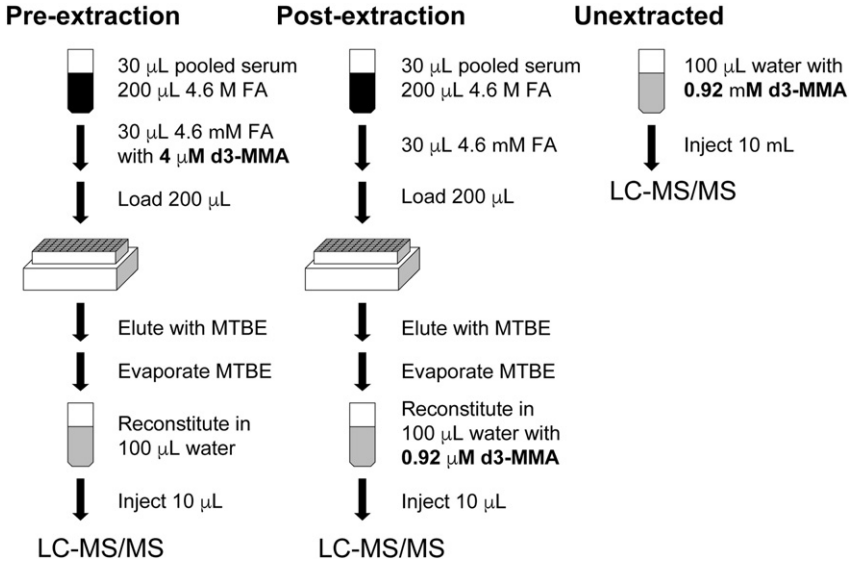

Fig. 2. Synopsis of sample preparation for analytical recovery and matrix effect.

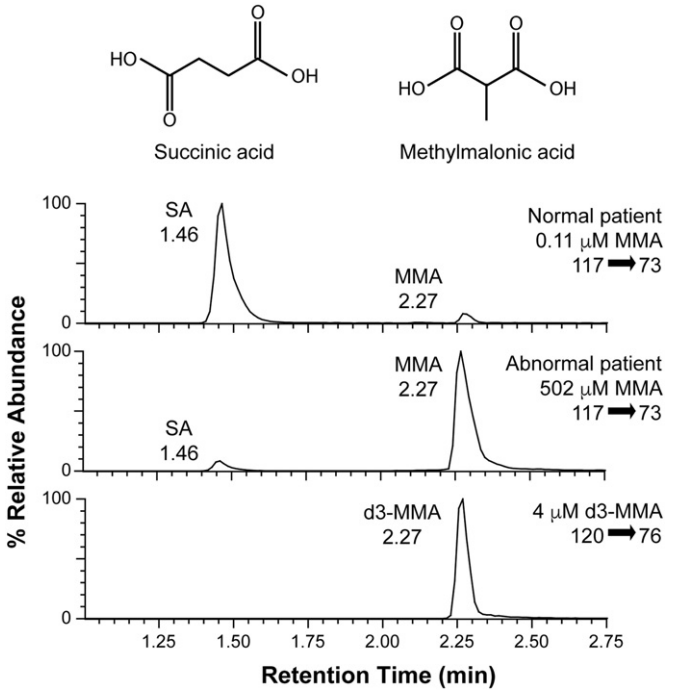

Fig. 3. Structures of succinic acid and methylmalonic acid (top). Representative chromatographic separations of succinic acid and methylmalonic acid: a normal patient and a patient with known methylmalonic acidemia (bottom).

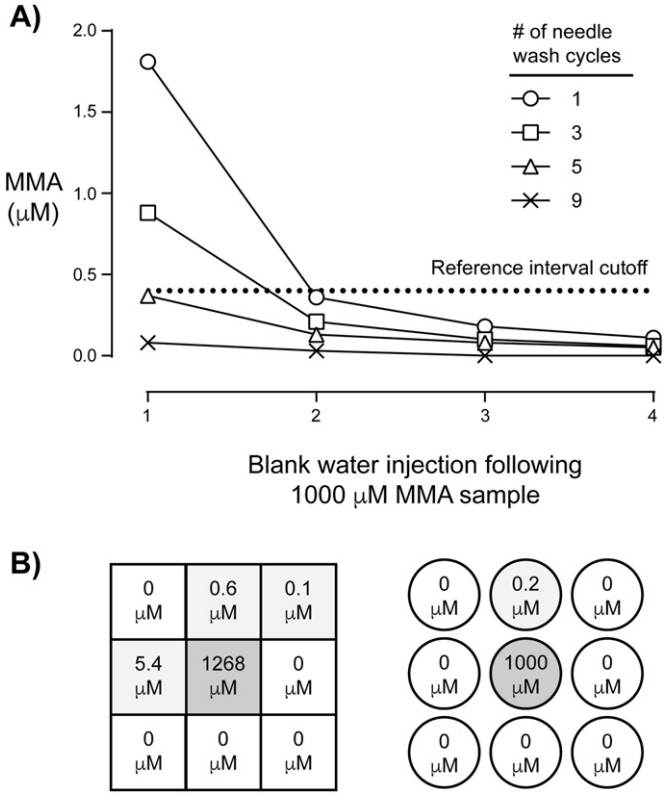

Fig. 4. Carryover minimization studies for injection needle (A) and MTBE evaporation (B). B shows a representative 3 × 3 test area for 96 square well collection plate (left) and 96 round bottom, 1 mL collection plate with modified cover (right).

3.3. Carryover

To maximize the analytical measurement range, carryover was minimized in addition to optimizing chromatographic separation. First, the wash cycle number for the injection needle was increased to nine cycles, the maximum number of wash cycles for a 7 min run. The wash buffer for the injection needle was also optimized to 40% methanol to increase the solubility of MMA. The carryover from the injection needle was determined to be 0.008% at 1000 μM MMA and was eliminated with 3 blank injections following a 1000 μM MMA sample injection (Fig. 4A). Second, evaporative carryover from MTBE was reduced to below 0.4 μM through changing the collection plate from square to round well shape and increasing the spacing of the openings between wells with a modified plate cover (Fig. 4B).

3.4. Correlation between C3-acylcarnitine and MMA

Paired MMA and plasma acylcarnitine profile analysis results were analyzed to understand the relationship between MMA and C3-acylcarnitine. These paired results were generated from 51 unique patients; 9 with primary methylmalonic acidemia (methylmalonyl CoA mutase deficiency), 1 with Cobalamin A, 1 with Cobalamin B, 10 with Cobalamin C, and 30 patients who were either undergoing screening for metabolic disease or nutritional monitoring. Comparison between MMA and C3-acylcarnitine concentrations revealed two populations with linear correlations of varying slopes (Fig. 5A). Assessment of free carnitine results indicated no association between the free carnitine concentrations and the observed populations. Plotting the results by patient, instead, showed a patient-specific relationship between MMA and C3-acylcarnitine concentrations (Fig. 5B).

4. Discussion

Analysis of MMA concentration can be used to aid the clinical diagnosis and management of methylmalonic acidemia. In order to accommodate the wide range of MMA observed in our pediatric patient population, the primary objective of our in-house LC-MS/MS MMA assay development and validation was to obtain a broad AMR while maintaining a low specimen volume requirement. Maintaining accuracy throughout the entire dynamic range was also of primary importance, along with eliminating any possibility of cross contamination. Concurrently, our secondary objectives were to establish an assay with a simple sample preparation that would allow us to minimize the turn-around-time for reporting out MMA.

In order to achieve a broad AMR spanning several orders of magnitudes, we focused on eliminating potential interference from SA by obtaining a robust baseline separation between MMA and SA. After evaluating multiple chromatography conditions and sample preparations described in the literature, we elected to use SLE and the analytical separation method described above. Optimal baseline separation, with a chromatographic resolution >10, between MMA and SA was achieved. The robustness of the chromatography, however, strongly depended on the pH of the sample. Degradation of the peak shape and resolution was noted when samples were not completely dried down to remove the formic acid.

With the robust baseline separation between MMA and SA, we were able to attain an AMR from 0.1 to 500 μM. We discovered that isotopic contribution of M + 3 MMA significantly contaminated the d_3-MMA internal standard signal above 500 μM MMA. Consequently, the internal standard concentration was increased to 4.2 μM from 0.4 μM and the upper limit of AMR was reduced to 500 μM. Through these modifications, we were able to diminish the effects of M + 3 and maintain accurate quantitation of serum MMA. It is possible that a broader AMR may be obtainable from lesser isotopic contribution of M + 4 MMA using a d_4-MMA I.S.

During the assay development, we also encountered two carryover issues not previously emphasized in the literature. If present, both types of carryover have the potential to alter the clinical diagnosis and management of patients. First, we observed carryover from the injection

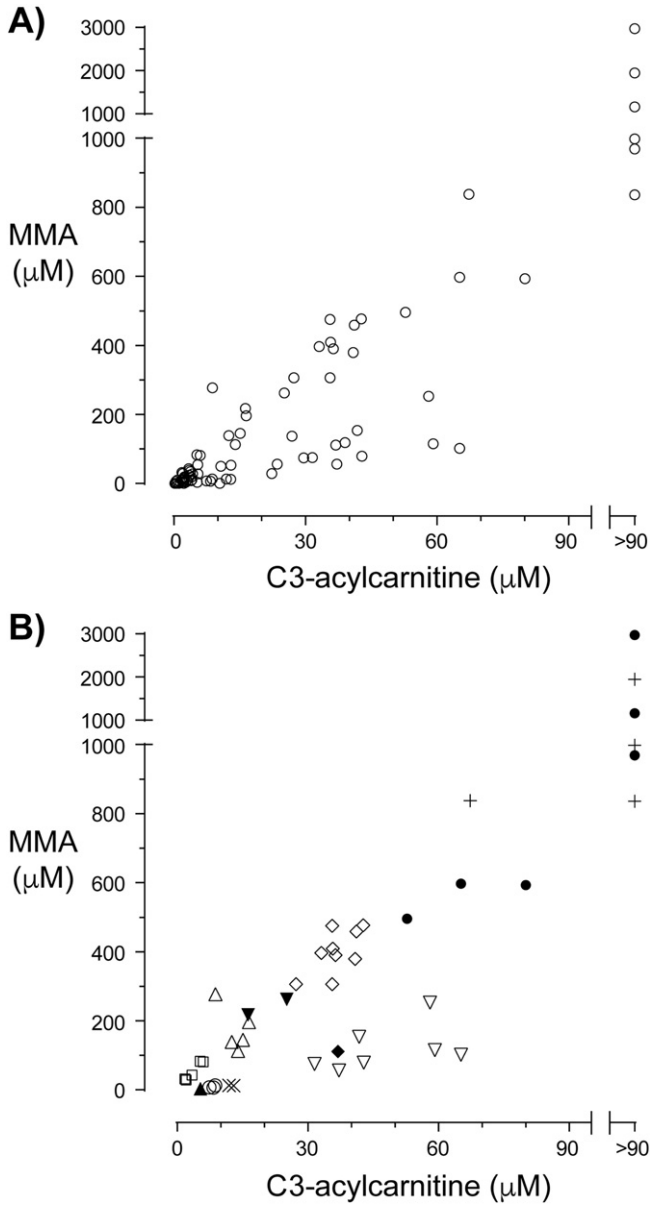

Fig. 5. Serum MMA and C3-acylcarnitine correlation data. (A) Paired serum MMA and C3-acylcarnitine reported during a 50-month period. (B) Patients with known MMA and Cobalamin A and B (N = 11), each symbol represents a unique patient.

needle, requiring the maximum number of injection wash cycles to minimize this effect. Second, and more challenging to discover, we identified a random evaporative carryover (also known as "cross-talk") where analyte from one well of a plate can flow to the neighboring well by the volatilized solvent [17]. In particular, when the difference in concentrations between two neighboring wells is >10 fold, the carryover can become significant. This second issue was difficult to resolve, and ultimately, we implemented the use of round bottom, 1 mL 96-well collection plates with a modified plate cover to increase the spacing between the openings of the wells during solvent evaporation. Furthermore, a modified plate cover was also used to improve the seal between the SLE plate and collection plate during sample elution to prevent contamination caused by splashing and sputtering.

To improve our understanding of the utility of quantifying MMA for the diagnosis and management of methylmalonic acidemia, we evaluated the relationship between MMA and C3-acylcarnitine. Our retrospective analysis unexpectedly revealed a patient-specific correlation between the two analytes that was independent of free carnitine concentration. This observed biological variation between individuals with methylmalonic acidemia strongly supports the necessity for quantifying MMA in addition to acylcarnitine analysis. Further investigation to better understand this observed phenomena and clinical utility is needed.

5. Conclusion

We encountered challenges during various stages of development of this SLE LC-MS/MS assay for serum MMA. With this work, we hope that these challenges can be carefully assessed and mitigated by laboratories prior to implementing a SLE LC-MS/MS assay for an analyte that requires a broad AMR. After resolving these hurdles, SLE's many advantages can be realized. Specifically, we were able to use minimal sample volume (30 μL) with fast extraction time that required minimal manual intervention (approximately 2 h for a full 96 well plate). The method is also adaptable to scale with semi-automation using a liquid handler to further increase laboratory efficiency [12].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2016.05.010.

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[a] Department of Laboratory Medicine, Chemistry Division, University of Washington, Seattle, WA, United States
[b] Department of Laboratories, Seattle Children's Hospital, Seattle, WA, United States

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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.

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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

* Corresponding author at: Seattle Children's Hospital, P.O. Box 5371, Seattle, WA 98145, United States.
E-mail address: jane.dickerson@seattlechildrens.org (J.A. Dickerson).

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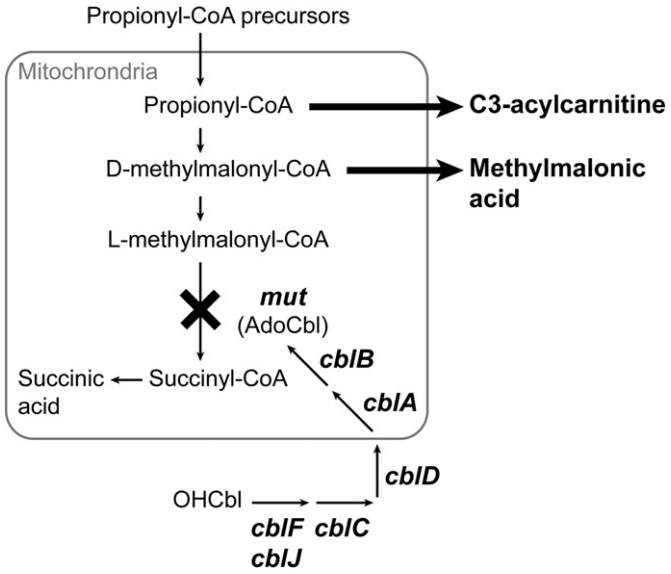

Fig. 1. Methylmalonic acidemia can be caused by defect in methylmalonyl-CoA mutase (mut) or enzymes that synthesize its cofactor, adenosylcobalamin (AdoCbl), from hydroxycobalamin (OHCbl). Deficiency in cobalamin can also inhibit isomerization of L-methylmalonyl-CoA to succinyl-CoA and cause accumulation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

determined by diluting an ERNDIM sample with 0.1% BSA to 0.1 μM and analyzed in quintuplet over three days. Analytical recovery was determined by comparing the averaged peak area of d_3-MMA added pre and post SLE (N = 3). Matrix effect was evaluated by comparing the averaged peak areas of the post extraction sample with unextracted MMA solution (0.92 μM in water). Experimental detail is illustrated in Fig. 2. Ion suppression or enhancement was also examined by post-column infusion of d_3-MMA with injection of SLE extracted patient specimens (N = 4) [15]. Reference interval was verified using 20 patient specimens with normal C3-acylcarnitine [16].

2.6. Carryover

Two types of carryover were studied. First, MMA carryover from the injection needle was assessed with a water injection (blank) followed by an injection of a high MMA sample (1000 μM MMA in SeraCon II serum) and four subsequent blanks. For each analysis, a new water sample was used, with the number of needle wash cycles set to 1, 3, 6, or 9 throughout the runs. Carryover between each analysis was minimized by three blank injections of a new water sample and nine needle wash cycles per injection. Peak area obtained from a blank run prior to the high MMA sample was used for baseline correction. Carryover was calculated as: Carryover (μM) = blank peak area/high sample peak area * 1000 μM. Second, evaporative carryover by MTBE during sample preparation was examined using nine wells (3 × 3) test area. For square wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and SeraCon II serum without MMA was extracted in the surrounding wells. MMA concentrations were quantified using calibration curve. For round wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and 500 μL of MTBE was added to the eight surrounding wells of the collection plate prior to nitrogen evaporation. All wells were then reconstituted in 100 μL of water and analyzed. Carryover for round wells was calculated as: carryover (μM) = surrounding well peak area/center well peak area * 1000 μM.

2.7. Correlation between serum MMA and C3-acylcarnitine

Historical results for paired MMA and plasma acylcarnitine analysis generated between November 1, 2011 and September 31, 2015 were pulled from the laboratory information system (Cerner Millennium Pathnet), N = 111. Results were grouped based on diagnosis then de-identified prior to correlation analysis.

2.8. Statistical analysis

Statistical analyses were performed using Microsoft Excel, GraphPad Prism 5, and EP Evaluator.

3. Results

3.1. Chromatography

Chromatographic separation of 0.81 min between MMA and its major endogenous isobaric interferent, succinic acid (SA) was achieved (Fig. 3). The run-to-run time was 8 min. The chromatographic resolution between SA and MMA is estimated to be 14.

3.2. Method validation

The intra-day CV for three control levels were 13.2% at 0.3 μM, 6.0% at 13 μM, and 4.1% at 526 μM (N = 20–22). The inter-day CV for three control levels over three months were 15.7% at 0.3 μM, 7.5% at 10.2 μM, and 5.0% at 233 μM (N = 14–15). The assay was linear between 0.1 and 500 μM with analyte recovery ranging from 93 to 125% (Supplemental Fig. 1A). A 10-fold manual dilution was validated, with corrected dilution results agreeing within 15% of the expected neat results (Supplemental Fig. 1B). With this validation, the upper limit of the reportable range is theoretically extended to 5000 μM, recognizing that in clinical practice we have not seen levels of this magnitude. The LOQ was established at 0.1 μM with a CV of 18.1%. The extraction efficiency of the Biotage plate was determined to be 72%. The matrix effect study showed no ion-suppression, but ion-enhancement of 160% was noted. These observations were consistent with qualitative matrix effect evaluation by post-column infusion of d_3-MMA after injecting 4 extracted patient specimens without internal standards. The assay was compared to a national reference lab LC-MS/MS method with analyte concentrations ranging from 0.08 to 1013 μM. The correlation coefficient was determined to be 0.9987 and the Deming regression analysis showed a slope of 1.026 and intercept of −1.335 (Supplemental Fig. 1C). Comparison to ERNDIM peer group mean showed a correlation coefficient of 0.9996 and a Deming regression slope and intercept of 1.094 and 0.630, respectively. Reference interval of <0.4 μM was verified using 20 patient specimens, which ranged from 0.0 to 0.3 μM.

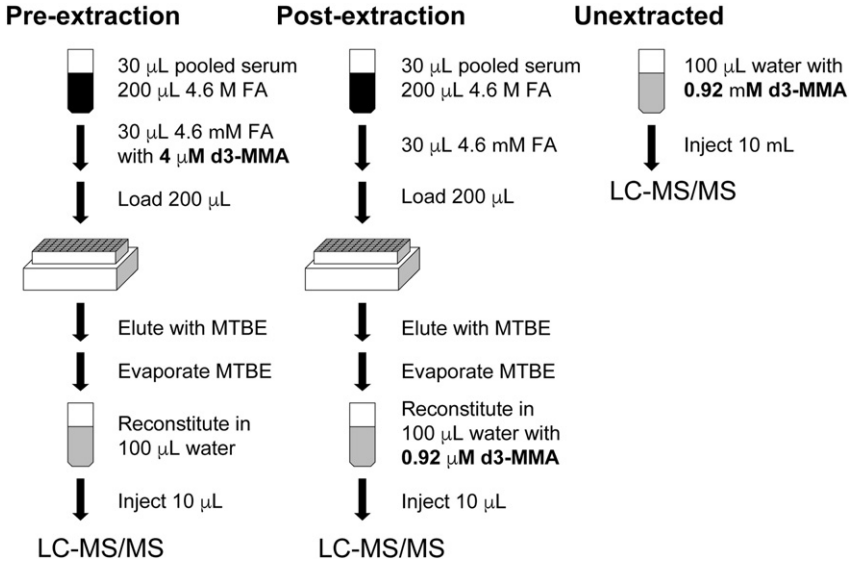

Fig. 2. Synopsis of sample preparation for analytical recovery and matrix effect.

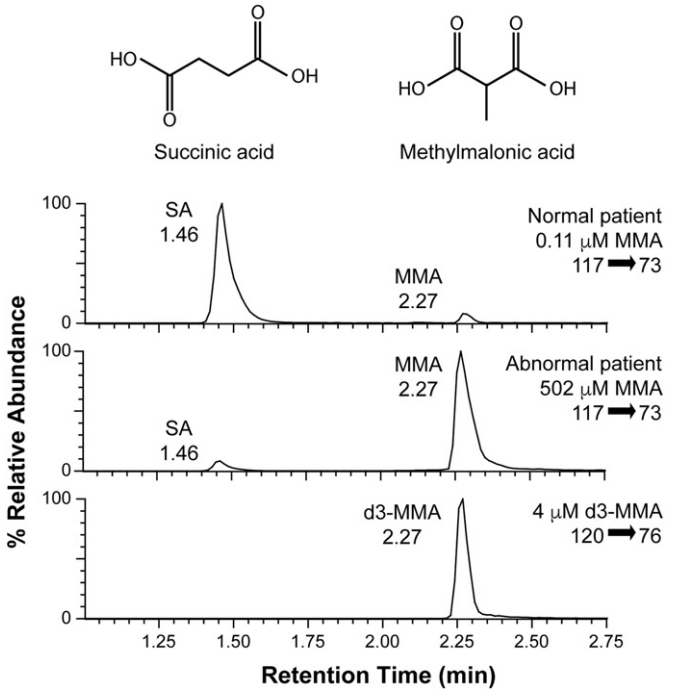

Fig. 3. Structures of succinic acid and methylmalonic acid (top). Representative chromatographic separations of succinic acid and methylmalonic acid: a normal patient and a patient with known methylmalonic acidemia (bottom).

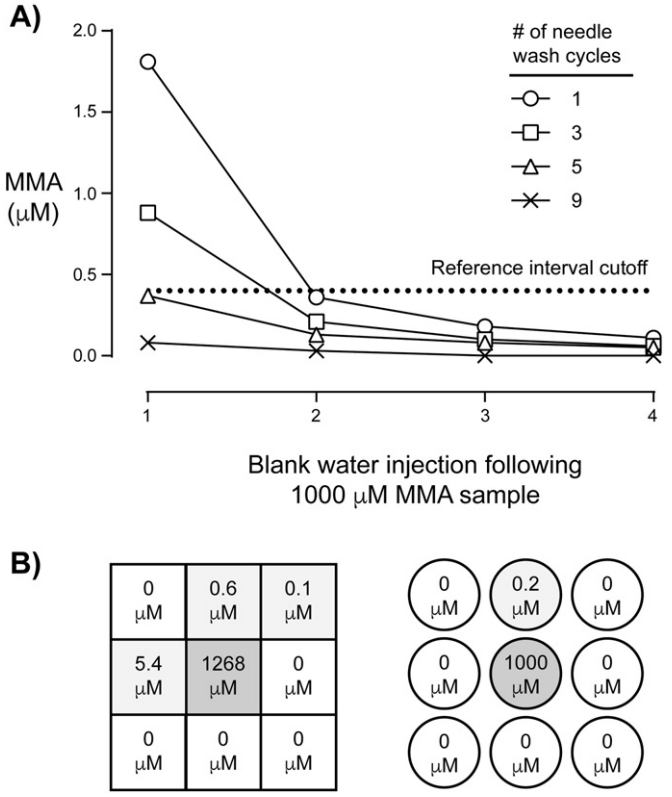

Fig. 4. Carryover minimization studies for injection needle (A) and MTBE evaporation (B). B shows a representative 3 × 3 test area for 96 square well collection plate (left) and 96 round bottom, 1 mL collection plate with modified cover (right).

3.3. Carryover

To maximize the analytical measurement range, carryover was minimized in addition to optimizing chromatographic separation. First, the wash cycle number for the injection needle was increased to nine cycles, the maximum number of wash cycles for a 7 min run. The wash buffer for the injection needle was also optimized to 40% methanol to increase the solubility of MMA. The carryover from the injection needle was determined to be 0.008% at 1000 μM MMA and was eliminated with 3 blank injections following a 1000 μM MMA sample injection (Fig. 4A). Second, evaporative carryover from MTBE was reduced to below 0.4 μM through changing the collection plate from square to round well shape and increasing the spacing of the openings between wells with a modified plate cover (Fig. 4B).

3.4. Correlation between C3-acylcarnitine and MMA

Paired MMA and plasma acylcarnitine profile analysis results were analyzed to understand the relationship between MMA and C3-acylcarnitine. These paired results were generated from 51 unique patients; 9 with primary methylmalonic acidemia (methylmalonyl CoA mutase deficiency), 1 with Cobalamin A, 1 with Cobalamin B, 10 with Cobalamin C, and 30 patients who were either undergoing screening for metabolic disease or nutritional monitoring. Comparison between MMA and C3-acylcarnitine concentrations revealed two populations with linear correlations of varying slopes (Fig. 5A). Assessment of free carnitine results indicated no association between the free carnitine concentrations and the observed populations. Plotting the results by patient, instead, showed a patient-specific relationship between MMA and C3-acylcarnitine concentrations (Fig. 5B).

4. Discussion

Analysis of MMA concentration can be used to aid the clinical diagnosis and management of methylmalonic acidemia. In order to accommodate the wide range of MMA observed in our pediatric patient population, the primary objective of our in-house LC-MS/MS MMA assay development and validation was to obtain a broad AMR while maintaining a low specimen volume requirement. Maintaining accuracy throughout the entire dynamic range was also of primary importance, along with eliminating any possibility of cross contamination. Concurrently, our secondary objectives were to establish an assay with a simple sample preparation that would allow us to minimize the turn-around-time for reporting out MMA.

In order to achieve a broad AMR spanning several orders of magnitudes, we focused on eliminating potential interference from SA by obtaining a robust baseline separation between MMA and SA. After evaluating multiple chromatography conditions and sample preparations described in the literature, we elected to use SLE and the analytical separation method described above. Optimal baseline separation, with a chromatographic resolution >10, between MMA and SA was achieved. The robustness of the chromatography, however, strongly depended on the pH of the sample. Degradation of the peak shape and resolution was noted when samples were not completely dried down to remove the formic acid.

With the robust baseline separation between MMA and SA, we were able to attain an AMR from 0.1 to 500 μM. We discovered that isotopic contribution of M + 3 MMA significantly contaminated the d_3-MMA internal standard signal above 500 μM MMA. Consequently, the internal standard concentration was increased to 4.2 μM from 0.4 μM and the upper limit of AMR was reduced to 500 μM. Through these modifications, we were able to diminish the effects of M + 3 and maintain accurate quantitation of serum MMA. It is possible that a broader AMR may be obtainable from lesser isotopic contribution of M + 4 MMA using a d_4-MMA I.S.

During the assay development, we also encountered two carryover issues not previously emphasized in the literature. If present, both types of carryover have the potential to alter the clinical diagnosis and management of patients. First, we observed carryover from the injection

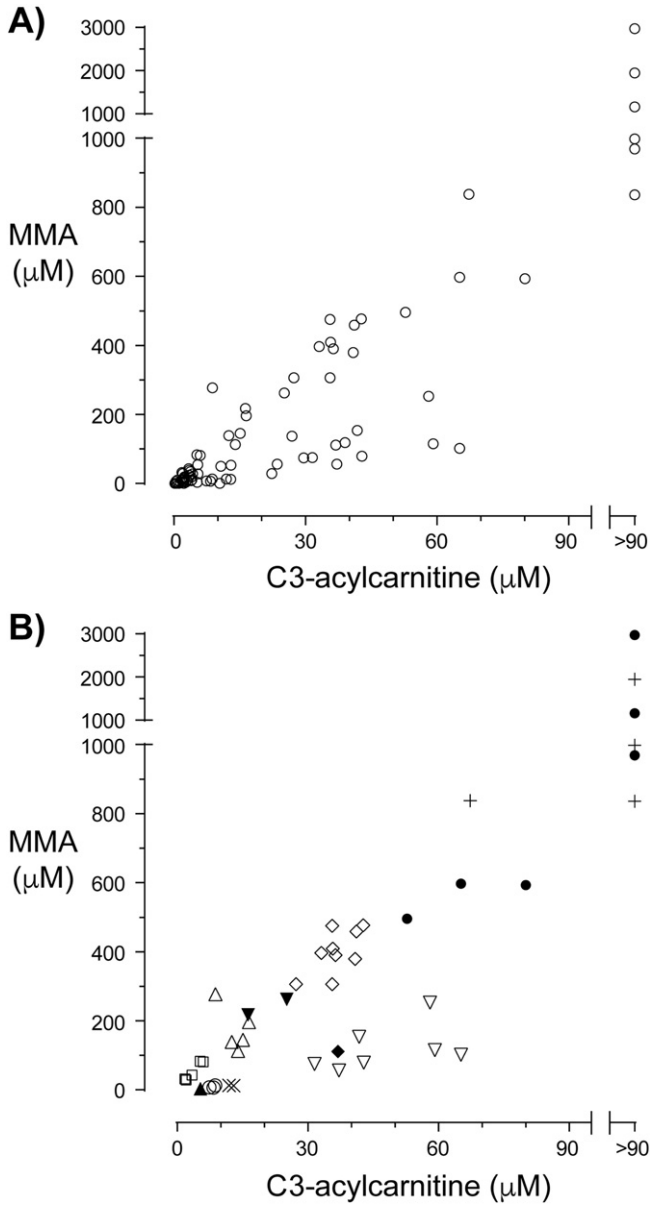

Fig. 5. Serum MMA and C3-acylcarnitine correlation data. (A) Paired serum MMA and C3-acylcarnitine reported during a 50-month period. (B) Patients with known MMA and Cobalamin A and B (N = 11), each symbol represents a unique patient.

needle, requiring the maximum number of injection wash cycles to minimize this effect. Second, and more challenging to discover, we identified a random evaporative carryover (also known as "cross-talk") where analyte from one well of a plate can flow to the neighboring well by the volatilized solvent [17]. In particular, when the difference in concentrations between two neighboring wells is >10 fold, the carryover can become significant. This second issue was difficult to resolve, and ultimately, we implemented the use of round bottom, 1 mL 96-well collection plates with a modified plate cover to increase the spacing between the openings of the wells during solvent evaporation. Furthermore, a modified plate cover was also used to improve the seal between the SLE plate and collection plate during sample elution to prevent contamination caused by splashing and sputtering.

To improve our understanding of the utility of quantifying MMA for the diagnosis and management of methylmalonic acidemia, we evaluated the relationship between MMA and C3-acylcarnitine. Our retrospective analysis unexpectedly revealed a patient-specific correlation between the two analytes that was independent of free carnitine concentration. This observed biological variation between individuals with methylmalonic acidemia strongly supports the necessity for quantifying MMA in addition to acylcarnitine analysis. Further investigation to better understand this observed phenomena and clinical utility is needed.

5. Conclusion

We encountered challenges during various stages of development of this SLE LC-MS/MS assay for serum MMA. With this work, we hope that these challenges can be carefully assessed and mitigated by laboratories prior to implementing a SLE LC-MS/MS assay for an analyte that requires a broad AMR. After resolving these hurdles, SLE's many advantages can be realized. Specifically, we were able to use minimal sample volume (30 μL) with fast extraction time that required minimal manual intervention (approximately 2 h for a full 96 well plate). The method is also adaptable to scale with semi-automation using a liquid handler to further increase laboratory efficiency [12].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2016.05.010.

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

Sheng-Ying Lo [a], Cindy Gordon [b], Katerina Sadilkova [b], Rhona M. Jack [b], Jane A. Dickerson [b,*]

[a] Department of Laboratory Medicine, Chemistry Division, University of Washington, Seattle, WA, United States
[b] Department of Laboratories, Seattle Children's Hospital, Seattle, WA, United States

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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.

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.

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* Corresponding author at: Seattle Children's Hospital, P.O. Box 5371, Seattle, WA 98145, United States.
E-mail address: jane.dickerson@seattlechildrens.org (J.A. Dickerson).

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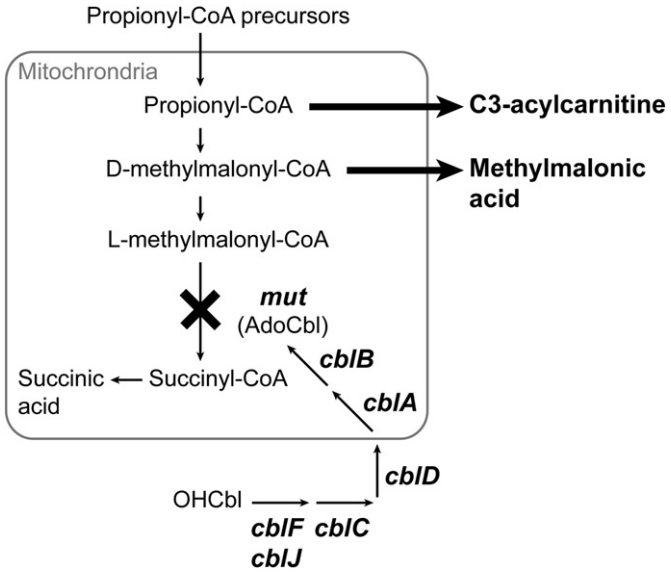

Fig. 1. Methylmalonic acidemia can be caused by defect in methylmalonyl-CoA mutase (mut) or enzymes that synthesize its cofactor, adenosylcobalamin (AdoCbl), from hydroxycobalamin (OHCbl). Deficiency in cobalamin can also inhibit isomerization of L-methylmalonyl-CoA to succinyl-CoA and cause accumulation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

determined by diluting an ERNDIM sample with 0.1% BSA to 0.1 μM and analyzed in quintuplet over three days. Analytical recovery was determined by comparing the averaged peak area of d_3-MMA added pre and post SLE (N = 3). Matrix effect was evaluated by comparing the averaged peak areas of the post extraction sample with unextracted MMA solution (0.92 μM in water). Experimental detail is illustrated in Fig. 2. Ion suppression or enhancement was also examined by post-column infusion of d_3-MMA with injection of SLE extracted patient specimens (N = 4) [15]. Reference interval was verified using 20 patient specimens with normal C3-acylcarnitine [16].

2.6. Carryover

Two types of carryover were studied. First, MMA carryover from the injection needle was assessed with a water injection (blank) followed by an injection of a high MMA sample (1000 μM MMA in SeraCon II serum) and four subsequent blanks. For each analysis, a new water sample was used, with the number of needle wash cycles set to 1, 3, 6, or 9 throughout the runs. Carryover between each analysis was minimized by three blank injections of a new water sample and nine needle wash cycles per injection. Peak area obtained from a blank run prior to the high MMA sample was used for baseline correction. Carryover was calculated as: Carryover (μM) = blank peak area/high sample peak area * 1000 μM. Second, evaporative carryover by MTBE during sample preparation was examined using nine wells (3 × 3) test area. For square wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and SeraCon II serum without MMA was extracted in the surrounding wells. MMA concentrations were quantified using calibration curve. For round wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and 500 μL of MTBE was added to the eight surrounding wells of the collection plate prior to nitrogen evaporation. All wells were then reconstituted in 100 μL of water and analyzed. Carryover for round wells was calculated as: carryover (μM) = surrounding well peak area/center well peak area * 1000 μM.

2.7. Correlation between serum MMA and C3-acylcarnitine

Historical results for paired MMA and plasma acylcarnitine analysis generated between November 1, 2011 and September 31, 2015 were pulled from the laboratory information system (Cerner Millennium Pathnet), N = 111. Results were grouped based on diagnosis then de-identified prior to correlation analysis.

2.8. Statistical analysis

Statistical analyses were performed using Microsoft Excel, GraphPad Prism 5, and EP Evaluator.

3. Results

3.1. Chromatography

Chromatographic separation of 0.81 min between MMA and its major endogenous isobaric interferent, succinic acid (SA) was achieved (Fig. 3). The run-to-run time was 8 min. The chromatographic resolution between SA and MMA is estimated to be 14.

3.2. Method validation

The intra-day CV for three control levels were 13.2% at 0.3 μM, 6.0% at 13 μM, and 4.1% at 526 μM (N = 20–22). The inter-day CV for three control levels over three months were 15.7% at 0.3 μM, 7.5% at 10.2 μM, and 5.0% at 233 μM (N = 14–15). The assay was linear between 0.1 and 500 μM with analyte recovery ranging from 93 to 125% (Supplemental Fig. 1A). A 10-fold manual dilution was validated, with corrected dilution results agreeing within 15% of the expected neat results (Supplemental Fig. 1B). With this validation, the upper limit of the reportable range is theoretically extended to 5000 μM, recognizing that in clinical practice we have not seen levels of this magnitude. The LOQ was established at 0.1 μM with a CV of 18.1%. The extraction efficiency of the Biotage plate was determined to be 72%. The matrix effect study showed no ion-suppression, but ion-enhancement of 160% was noted. These observations were consistent with qualitative matrix effect evaluation by post-column infusion of d_3-MMA after injecting 4 extracted patient specimens without internal standards. The assay was compared to a national reference lab LC-MS/MS method with analyte concentrations ranging from 0.08 to 1013 μM. The correlation coefficient was determined to be 0.9987 and the Deming regression analysis showed a slope of 1.026 and intercept of −1.335 (Supplemental Fig. 1C). Comparison to ERNDIM peer group mean showed a correlation coefficient of 0.9996 and a Deming regression slope and intercept of 1.094 and 0.630, respectively. Reference interval of <0.4 μM was verified using 20 patient specimens, which ranged from 0.0 to 0.3 μM.

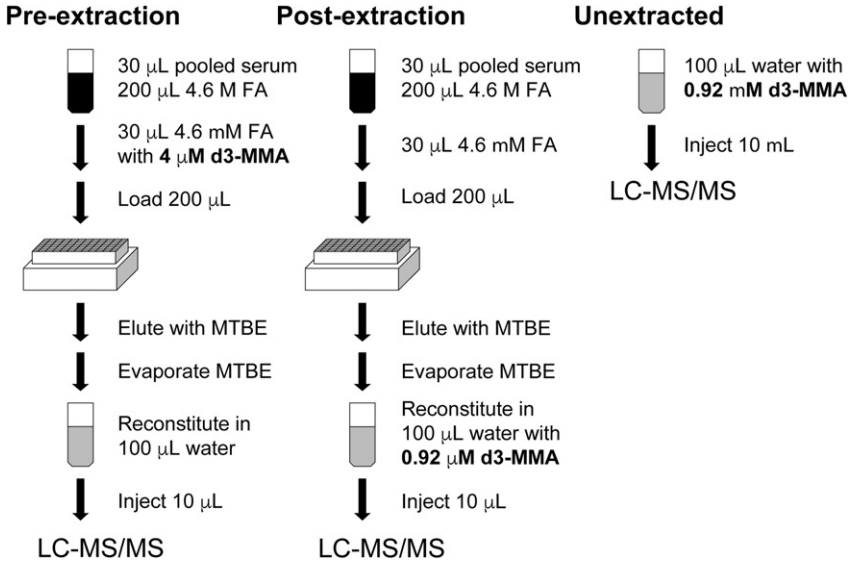

Fig. 2. Synopsis of sample preparation for analytical recovery and matrix effect.

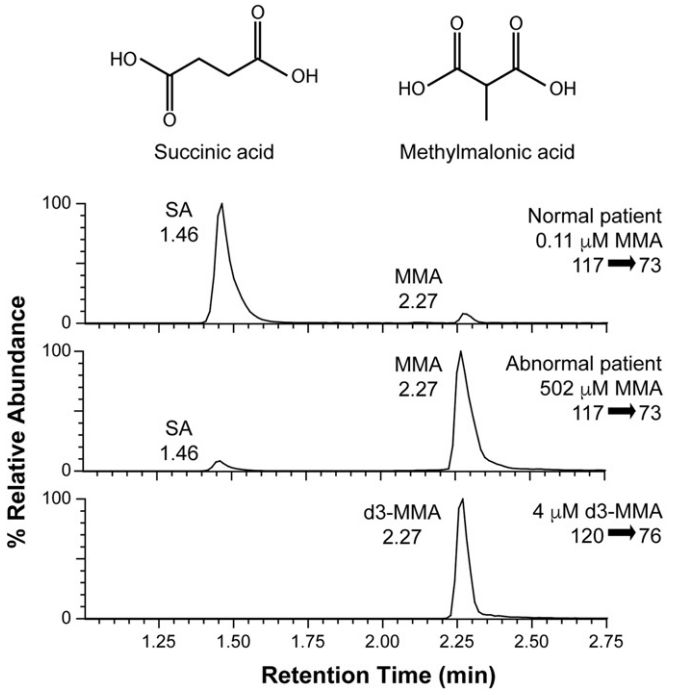

Fig. 3. Structures of succinic acid and methylmalonic acid (top). Representative chromatographic separations of succinic acid and methylmalonic acid: a normal patient and a patient with known methylmalonic acidemia (bottom).

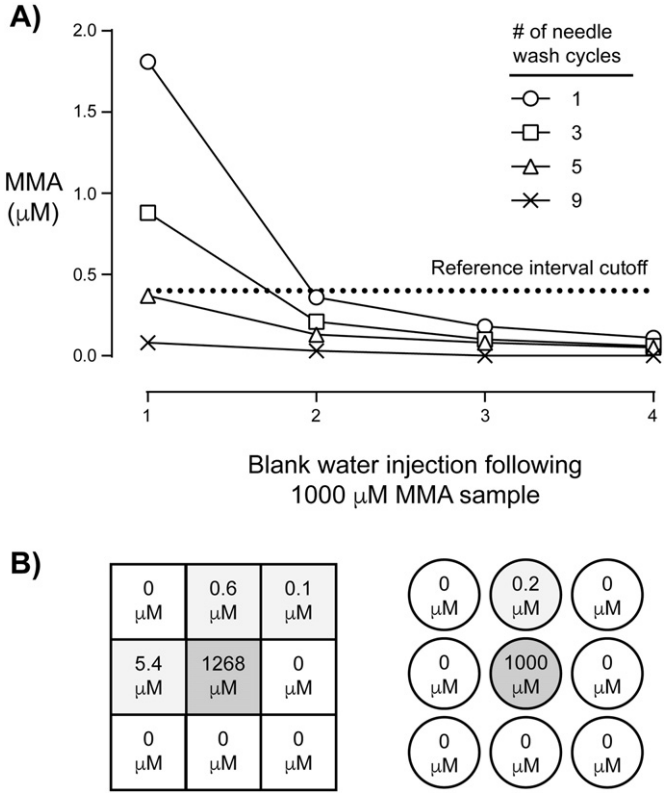

Fig. 4. Carryover minimization studies for injection needle (A) and MTBE evaporation (B). B shows a representative 3 × 3 test area for 96 square well collection plate (left) and 96 round bottom, 1 mL collection plate with modified cover (right).

3.3. Carryover

To maximize the analytical measurement range, carryover was minimized in addition to optimizing chromatographic separation. First, the wash cycle number for the injection needle was increased to nine cycles, the maximum number of wash cycles for a 7 min run. The wash buffer for the injection needle was also optimized to 40% methanol to increase the solubility of MMA. The carryover from the injection needle was determined to be 0.008% at 1000 μM MMA and was eliminated with 3 blank injections following a 1000 μM MMA sample injection (Fig. 4A). Second, evaporative carryover from MTBE was reduced to below 0.4 μM through changing the collection plate from square to round well shape and increasing the spacing of the openings between wells with a modified plate cover (Fig. 4B).

3.4. Correlation between C3-acylcarnitine and MMA

Paired MMA and plasma acylcarnitine profile analysis results were analyzed to understand the relationship between MMA and C3-acylcarnitine. These paired results were generated from 51 unique patients; 9 with primary methylmalonic acidemia (methylmalonyl CoA mutase deficiency), 1 with Cobalamin A, 1 with Cobalamin B, 10 with Cobalamin C, and 30 patients who were either undergoing screening for metabolic disease or nutritional monitoring. Comparison between MMA and C3-acylcarnitine concentrations revealed two populations with linear correlations of varying slopes (Fig. 5A). Assessment of free carnitine results indicated no association between the free carnitine concentrations and the observed populations. Plotting the results by patient, instead, showed a patient-specific relationship between MMA and C3-acylcarnitine concentrations (Fig. 5B).

4. Discussion

Analysis of MMA concentration can be used to aid the clinical diagnosis and management of methylmalonic acidemia. In order to accommodate the wide range of MMA observed in our pediatric patient population, the primary objective of our in-house LC-MS/MS MMA assay development and validation was to obtain a broad AMR while maintaining a low specimen volume requirement. Maintaining accuracy throughout the entire dynamic range was also of primary importance, along with eliminating any possibility of cross contamination. Concurrently, our secondary objectives were to establish an assay with a simple sample preparation that would allow us to minimize the turn-around-time for reporting out MMA.

In order to achieve a broad AMR spanning several orders of magnitudes, we focused on eliminating potential interference from SA by obtaining a robust baseline separation between MMA and SA. After evaluating multiple chromatography conditions and sample preparations described in the literature, we elected to use SLE and the analytical separation method described above. Optimal baseline separation, with a chromatographic resolution >10, between MMA and SA was achieved. The robustness of the chromatography, however, strongly depended on the pH of the sample. Degradation of the peak shape and resolution was noted when samples were not completely dried down to remove the formic acid.

With the robust baseline separation between MMA and SA, we were able to attain an AMR from 0.1 to 500 μM. We discovered that isotopic contribution of M + 3 MMA significantly contaminated the d_3-MMA internal standard signal above 500 μM MMA. Consequently, the internal standard concentration was increased to 4.2 μM from 0.4 μM and the upper limit of AMR was reduced to 500 μM. Through these modifications, we were able to diminish the effects of M + 3 and maintain accurate quantitation of serum MMA. It is possible that a broader AMR may be obtainable from lesser isotopic contribution of M + 4 MMA using a d_4-MMA I.S.

During the assay development, we also encountered two carryover issues not previously emphasized in the literature. If present, both types of carryover have the potential to alter the clinical diagnosis and management of patients. First, we observed carryover from the injection

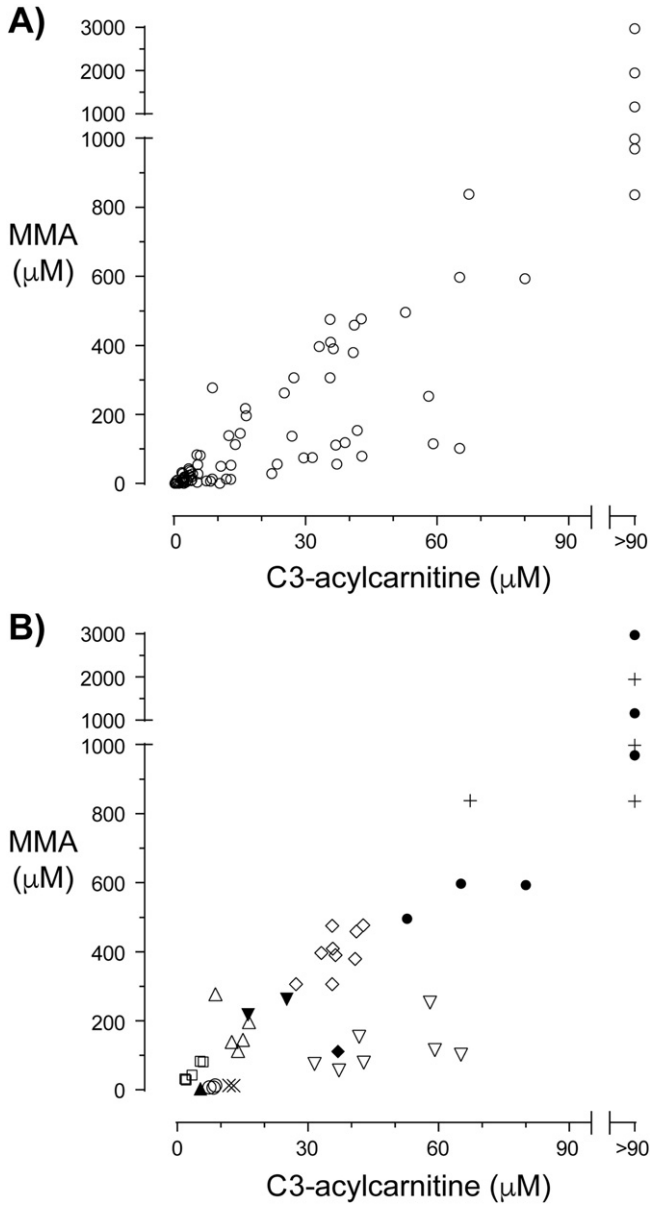

Fig. 5. Serum MMA and C3-acylcarnitine correlation data. (A) Paired serum MMA and C3-acylcarnitine reported during a 50-month period. (B) Patients with known MMA and Cobalamin A and B (N = 11), each symbol represents a unique patient.

needle, requiring the maximum number of injection wash cycles to minimize this effect. Second, and more challenging to discover, we identified a random evaporative carryover (also known as "cross-talk") where analyte from one well of a plate can flow to the neighboring well by the volatilized solvent [17]. In particular, when the difference in concentrations between two neighboring wells is >10 fold, the carryover can become significant. This second issue was difficult to resolve, and ultimately, we implemented the use of round bottom, 1 mL 96-well collection plates with a modified plate cover to increase the spacing between the openings of the wells during solvent evaporation. Furthermore, a modified plate cover was also used to improve the seal between the SLE plate and collection plate during sample elution to prevent contamination caused by splashing and sputtering.

To improve our understanding of the utility of quantifying MMA for the diagnosis and management of methylmalonic acidemia, we evaluated the relationship between MMA and C3-acylcarnitine. Our retrospective analysis unexpectedly revealed a patient-specific correlation between the two analytes that was independent of free carnitine concentration. This observed biological variation between individuals with methylmalonic acidemia strongly supports the necessity for quantifying MMA in addition to acylcarnitine analysis. Further investigation to better understand this observed phenomena and clinical utility is needed.

5. Conclusion

We encountered challenges during various stages of development of this SLE LC-MS/MS assay for serum MMA. With this work, we hope that these challenges can be carefully assessed and mitigated by laboratories prior to implementing a SLE LC-MS/MS assay for an analyte that requires a broad AMR. After resolving these hurdles, SLE's many advantages can be realized. Specifically, we were able to use minimal sample volume (30 μL) with fast extraction time that required minimal manual intervention (approximately 2 h for a full 96 well plate). The method is also adaptable to scale with semi-automation using a liquid handler to further increase laboratory efficiency [12].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2016.05.010.

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Question 5
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Quantifying MMA by SLE LC-MS/MS: Unexpected challenges in assay development

Sheng-Ying Lo [a], Cindy Gordon [b], Katerina Sadilkova [b], Rhona M. Jack [b], Jane A. Dickerson [b,*]

[a] Department of Laboratory Medicine, Chemistry Division, University of Washington, Seattle, WA, United States
[b] Department of Laboratories, Seattle Children's Hospital, Seattle, WA, United States

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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.

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

* Corresponding author at: Seattle Children's Hospital, P.O. Box 5371, Seattle, WA 98145, United States.
E-mail address: jane.dickerson@seattlechildrens.org (J.A. Dickerson).

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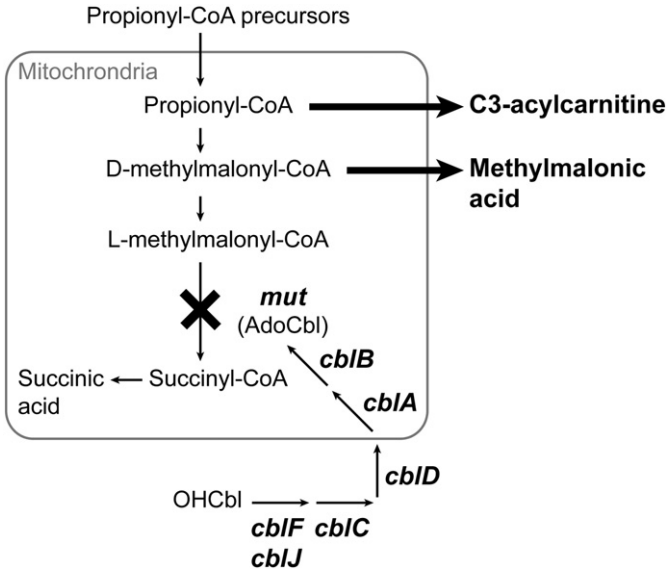

Fig. 1. Methylmalonic acidemia can be caused by defect in methylmalonyl-CoA mutase (mut) or enzymes that synthesize its cofactor, adenosylcobalamin (AdoCbl), from hydroxycobalamin (OHCbl). Deficiency in cobalamin can also inhibit isomerization of L-methylmalonyl-CoA to succinyl-CoA and cause accumulation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

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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

determined by diluting an ERNDIM sample with 0.1% BSA to 0.1 μM and analyzed in quintuplet over three days. Analytical recovery was determined by comparing the averaged peak area of d_3-MMA added pre and post SLE (N = 3). Matrix effect was evaluated by comparing the averaged peak areas of the post extraction sample with unextracted MMA solution (0.92 μM in water). Experimental detail is illustrated in Fig. 2. Ion suppression or enhancement was also examined by post-column infusion of d_3-MMA with injection of SLE extracted patient specimens (N = 4) [15]. Reference interval was verified using 20 patient specimens with normal C3-acylcarnitine [16].

2.6. Carryover

Two types of carryover were studied. First, MMA carryover from the injection needle was assessed with a water injection (blank) followed by an injection of a high MMA sample (1000 μM MMA in SeraCon II serum) and four subsequent blanks. For each analysis, a new water sample was used, with the number of needle wash cycles set to 1, 3, 6, or 9 throughout the runs. Carryover between each analysis was minimized by three blank injections of a new water sample and nine needle wash cycles per injection. Peak area obtained from a blank run prior to the high MMA sample was used for baseline correction. Carryover was calculated as: Carryover (μM) = blank peak area/high sample peak area * 1000 μM. Second, evaporative carryover by MTBE during sample preparation was examined using nine wells (3 × 3) test area. For square wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and SeraCon II serum without MMA was extracted in the surrounding wells. MMA concentrations were quantified using calibration curve. For round wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and 500 μL of MTBE was added to the eight surrounding wells of the collection plate prior to nitrogen evaporation. All wells were then reconstituted in 100 μL of water and analyzed. Carryover for round wells was calculated as: carryover (μM) = surrounding well peak area/center well peak area * 1000 μM.

2.7. Correlation between serum MMA and C3-acylcarnitine

Historical results for paired MMA and plasma acylcarnitine analysis generated between November 1, 2011 and September 31, 2015 were pulled from the laboratory information system (Cerner Millennium Pathnet), N = 111. Results were grouped based on diagnosis then de-identified prior to correlation analysis.

2.8. Statistical analysis

Statistical analyses were performed using Microsoft Excel, GraphPad Prism 5, and EP Evaluator.

3. Results

3.1. Chromatography

Chromatographic separation of 0.81 min between MMA and its major endogenous isobaric interferent, succinic acid (SA) was achieved (Fig. 3). The run-to-run time was 8 min. The chromatographic resolution between SA and MMA is estimated to be 14.

3.2. Method validation

The intra-day CV for three control levels were 13.2% at 0.3 μM, 6.0% at 13 μM, and 4.1% at 526 μM (N = 20–22). The inter-day CV for three control levels over three months were 15.7% at 0.3 μM, 7.5% at 10.2 μM, and 5.0% at 233 μM (N = 14–15). The assay was linear between 0.1 and 500 μM with analyte recovery ranging from 93 to 125% (Supplemental Fig. 1A). A 10-fold manual dilution was validated, with corrected dilution results agreeing within 15% of the expected neat results (Supplemental Fig. 1B). With this validation, the upper limit of the reportable range is theoretically extended to 5000 μM, recognizing that in clinical practice we have not seen levels of this magnitude. The LOQ was established at 0.1 μM with a CV of 18.1%. The extraction efficiency of the Biotage plate was determined to be 72%. The matrix effect study showed no ion-suppression, but ion-enhancement of 160% was noted. These observations were consistent with qualitative matrix effect evaluation by post-column infusion of d_3-MMA after injecting 4 extracted patient specimens without internal standards. The assay was compared to a national reference lab LC-MS/MS method with analyte concentrations ranging from 0.08 to 1013 μM. The correlation coefficient was determined to be 0.9987 and the Deming regression analysis showed a slope of 1.026 and intercept of −1.335 (Supplemental Fig. 1C). Comparison to ERNDIM peer group mean showed a correlation coefficient of 0.9996 and a Deming regression slope and intercept of 1.094 and 0.630, respectively. Reference interval of <0.4 μM was verified using 20 patient specimens, which ranged from 0.0 to 0.3 μM.

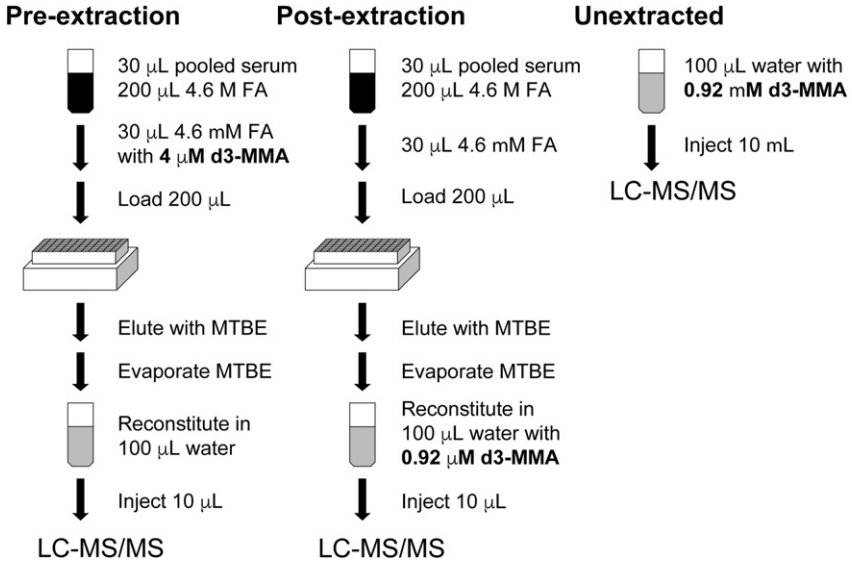

Fig. 2. Synopsis of sample preparation for analytical recovery and matrix effect.

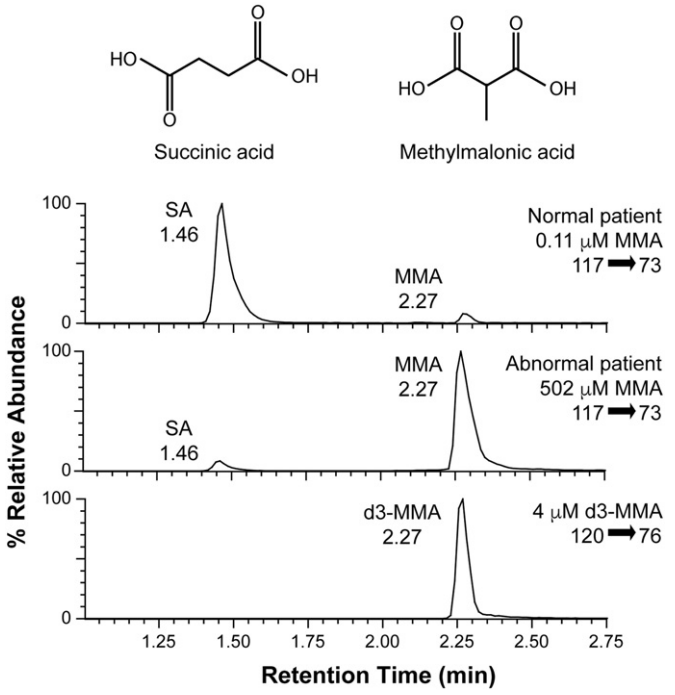

Fig. 3. Structures of succinic acid and methylmalonic acid (top). Representative chromatographic separations of succinic acid and methylmalonic acid: a normal patient and a patient with known methylmalonic acidemia (bottom).

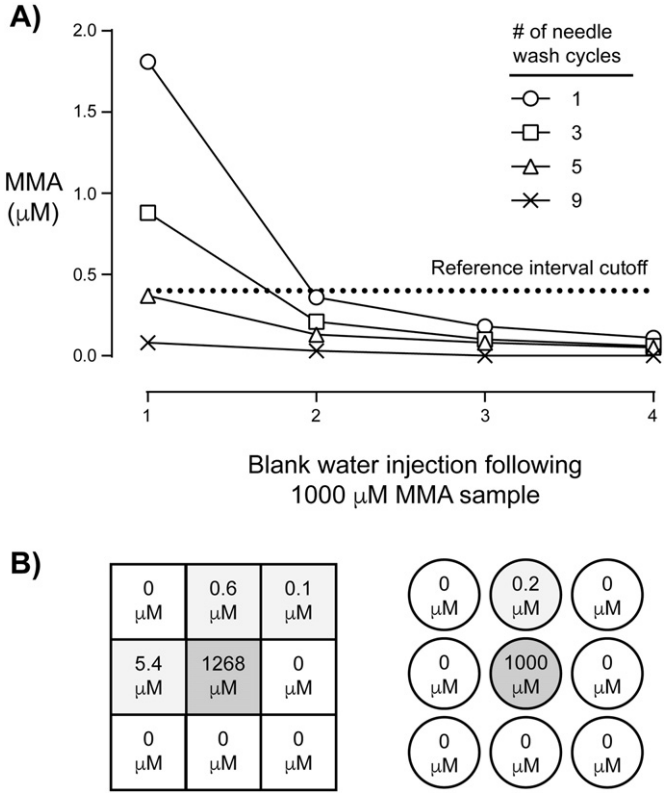

Fig. 4. Carryover minimization studies for injection needle (A) and MTBE evaporation (B). B shows a representative 3 × 3 test area for 96 square well collection plate (left) and 96 round bottom, 1 mL collection plate with modified cover (right).

3.3. Carryover

To maximize the analytical measurement range, carryover was minimized in addition to optimizing chromatographic separation. First, the wash cycle number for the injection needle was increased to nine cycles, the maximum number of wash cycles for a 7 min run. The wash buffer for the injection needle was also optimized to 40% methanol to increase the solubility of MMA. The carryover from the injection needle was determined to be 0.008% at 1000 μM MMA and was eliminated with 3 blank injections following a 1000 μM MMA sample injection (Fig. 4A). Second, evaporative carryover from MTBE was reduced to below 0.4 μM through changing the collection plate from square to round well shape and increasing the spacing of the openings between wells with a modified plate cover (Fig. 4B).

3.4. Correlation between C3-acylcarnitine and MMA

Paired MMA and plasma acylcarnitine profile analysis results were analyzed to understand the relationship between MMA and C3-acylcarnitine. These paired results were generated from 51 unique patients; 9 with primary methylmalonic acidemia (methylmalonyl CoA mutase deficiency), 1 with Cobalamin A, 1 with Cobalamin B, 10 with Cobalamin C, and 30 patients who were either undergoing screening for metabolic disease or nutritional monitoring. Comparison between MMA and C3-acylcarnitine concentrations revealed two populations with linear correlations of varying slopes (Fig. 5A). Assessment of free carnitine results indicated no association between the free carnitine concentrations and the observed populations. Plotting the results by patient, instead, showed a patient-specific relationship between MMA and C3-acylcarnitine concentrations (Fig. 5B).

4. Discussion

Analysis of MMA concentration can be used to aid the clinical diagnosis and management of methylmalonic acidemia. In order to accommodate the wide range of MMA observed in our pediatric patient population, the primary objective of our in-house LC-MS/MS MMA assay development and validation was to obtain a broad AMR while maintaining a low specimen volume requirement. Maintaining accuracy throughout the entire dynamic range was also of primary importance, along with eliminating any possibility of cross contamination. Concurrently, our secondary objectives were to establish an assay with a simple sample preparation that would allow us to minimize the turn-around-time for reporting out MMA.

In order to achieve a broad AMR spanning several orders of magnitudes, we focused on eliminating potential interference from SA by obtaining a robust baseline separation between MMA and SA. After evaluating multiple chromatography conditions and sample preparations described in the literature, we elected to use SLE and the analytical separation method described above. Optimal baseline separation, with a chromatographic resolution >10, between MMA and SA was achieved. The robustness of the chromatography, however, strongly depended on the pH of the sample. Degradation of the peak shape and resolution was noted when samples were not completely dried down to remove the formic acid.

With the robust baseline separation between MMA and SA, we were able to attain an AMR from 0.1 to 500 μM. We discovered that isotopic contribution of M + 3 MMA significantly contaminated the d_3-MMA internal standard signal above 500 μM MMA. Consequently, the internal standard concentration was increased to 4.2 μM from 0.4 μM and the upper limit of AMR was reduced to 500 μM. Through these modifications, we were able to diminish the effects of M + 3 and maintain accurate quantitation of serum MMA. It is possible that a broader AMR may be obtainable from lesser isotopic contribution of M + 4 MMA using a d_4-MMA I.S.

During the assay development, we also encountered two carryover issues not previously emphasized in the literature. If present, both types of carryover have the potential to alter the clinical diagnosis and management of patients. First, we observed carryover from the injection

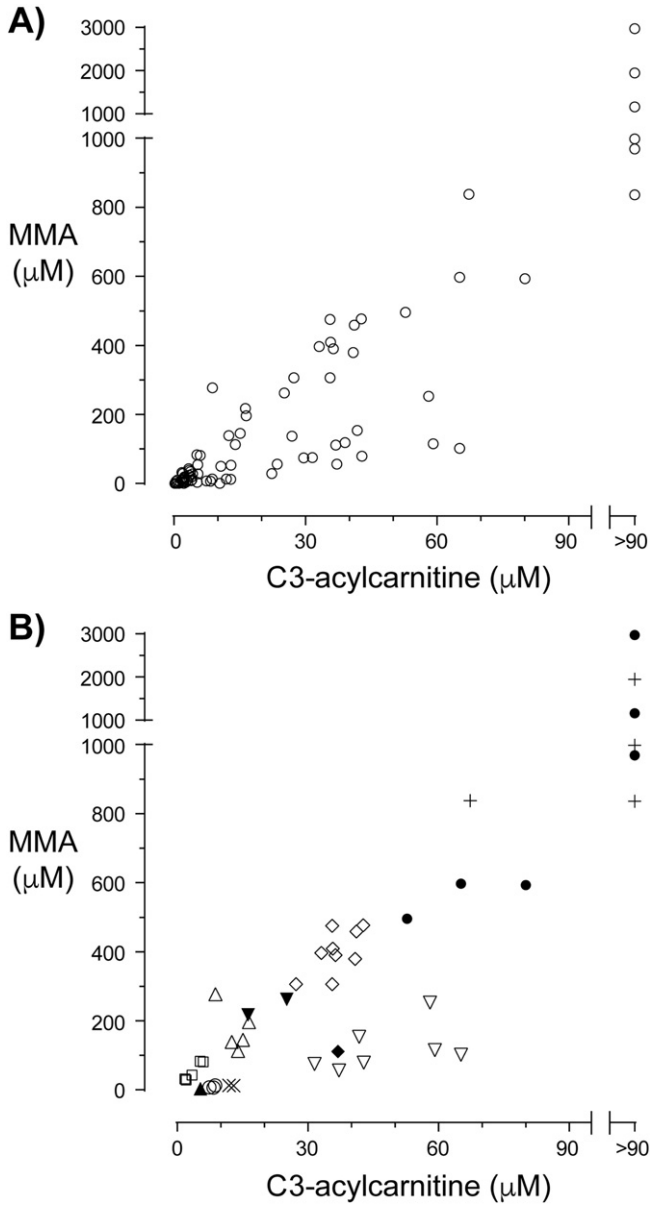

Fig. 5. Serum MMA and C3-acylcarnitine correlation data. (A) Paired serum MMA and C3-acylcarnitine reported during a 50-month period. (B) Patients with known MMA and Cobalamin A and B (N = 11), each symbol represents a unique patient.

needle, requiring the maximum number of injection wash cycles to minimize this effect. Second, and more challenging to discover, we identified a random evaporative carryover (also known as "cross-talk") where analyte from one well of a plate can flow to the neighboring well by the volatilized solvent [17]. In particular, when the difference in concentrations between two neighboring wells is >10 fold, the carryover can become significant. This second issue was difficult to resolve, and ultimately, we implemented the use of round bottom, 1 mL 96-well collection plates with a modified plate cover to increase the spacing between the openings of the wells during solvent evaporation. Furthermore, a modified plate cover was also used to improve the seal between the SLE plate and collection plate during sample elution to prevent contamination caused by splashing and sputtering.

To improve our understanding of the utility of quantifying MMA for the diagnosis and management of methylmalonic acidemia, we evaluated the relationship between MMA and C3-acylcarnitine. Our retrospective analysis unexpectedly revealed a patient-specific correlation between the two analytes that was independent of free carnitine concentration. This observed biological variation between individuals with methylmalonic acidemia strongly supports the necessity for quantifying MMA in addition to acylcarnitine analysis. Further investigation to better understand this observed phenomena and clinical utility is needed.

5. Conclusion

We encountered challenges during various stages of development of this SLE LC-MS/MS assay for serum MMA. With this work, we hope that these challenges can be carefully assessed and mitigated by laboratories prior to implementing a SLE LC-MS/MS assay for an analyte that requires a broad AMR. After resolving these hurdles, SLE's many advantages can be realized. Specifically, we were able to use minimal sample volume (30 μL) with fast extraction time that required minimal manual intervention (approximately 2 h for a full 96 well plate). The method is also adaptable to scale with semi-automation using a liquid handler to further increase laboratory efficiency [12].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2016.05.010.

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

Sheng-Ying Lo [a], Cindy Gordon [b], Katerina Sadilkova [b], Rhona M. Jack [b], Jane A. Dickerson [b,*]

[a] Department of Laboratory Medicine, Chemistry Division, University of Washington, Seattle, WA, United States
[b] Department of Laboratories, Seattle Children's Hospital, Seattle, WA, United States

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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.

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

* Corresponding author at: Seattle Children's Hospital, P.O. Box 5371, Seattle, WA 98145, United States.
E-mail address: jane.dickerson@seattlechildrens.org (J.A. Dickerson).

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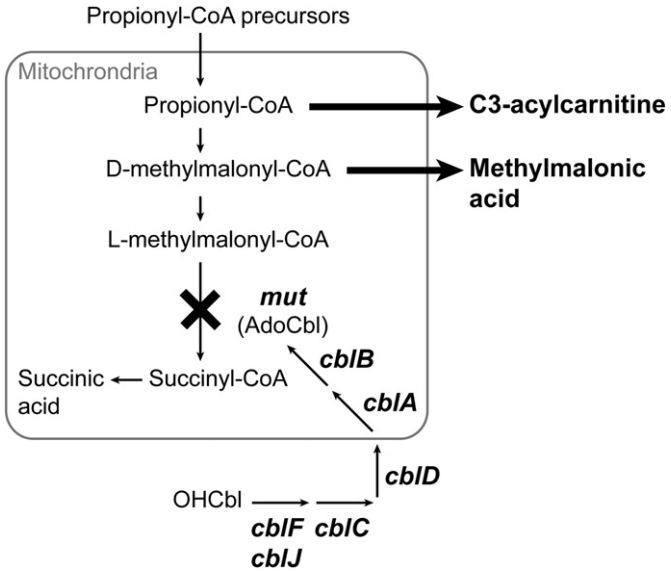

Fig. 1. Methylmalonic acidemia can be caused by defect in methylmalonyl-CoA mutase (mut) or enzymes that synthesize its cofactor, adenosylcobalamin (AdoCbl), from hydroxycobalamin (OHCbl). Deficiency in cobalamin can also inhibit isomerization of L-methylmalonyl-CoA to succinyl-CoA and cause accumulation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

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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

determined by diluting an ERNDIM sample with 0.1% BSA to 0.1 μM and analyzed in quintuplet over three days. Analytical recovery was determined by comparing the averaged peak area of d_3-MMA added pre and post SLE (N = 3). Matrix effect was evaluated by comparing the averaged peak areas of the post extraction sample with unextracted MMA solution (0.92 μM in water). Experimental detail is illustrated in Fig. 2. Ion suppression or enhancement was also examined by post-column infusion of d_3-MMA with injection of SLE extracted patient specimens (N = 4) [15]. Reference interval was verified using 20 patient specimens with normal C3-acylcarnitine [16].

2.6. Carryover

Two types of carryover were studied. First, MMA carryover from the injection needle was assessed with a water injection (blank) followed by an injection of a high MMA sample (1000 μM MMA in SeraCon II serum) and four subsequent blanks. For each analysis, a new water sample was used, with the number of needle wash cycles set to 1, 3, 6, or 9 throughout the runs. Carryover between each analysis was minimized by three blank injections of a new water sample and nine needle wash cycles per injection. Peak area obtained from a blank run prior to the high MMA sample was used for baseline correction. Carryover was calculated as: Carryover (μM) = blank peak area/high sample peak area * 1000 μM. Second, evaporative carryover by MTBE during sample preparation was examined using nine wells (3 × 3) test area. For square wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and SeraCon II serum without MMA was extracted in the surrounding wells. MMA concentrations were quantified using calibration curve. For round wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and 500 μL of MTBE was added to the eight surrounding wells of the collection plate prior to nitrogen evaporation. All wells were then reconstituted in 100 μL of water and analyzed. Carryover for round wells was calculated as: carryover (μM) = surrounding well peak area/center well peak area * 1000 μM.

2.7. Correlation between serum MMA and C3-acylcarnitine

Historical results for paired MMA and plasma acylcarnitine analysis generated between November 1, 2011 and September 31, 2015 were pulled from the laboratory information system (Cerner Millennium Pathnet), N = 111. Results were grouped based on diagnosis then de-identified prior to correlation analysis.

2.8. Statistical analysis

Statistical analyses were performed using Microsoft Excel, GraphPad Prism 5, and EP Evaluator.

3. Results

3.1. Chromatography

Chromatographic separation of 0.81 min between MMA and its major endogenous isobaric interferent, succinic acid (SA) was achieved (Fig. 3). The run-to-run time was 8 min. The chromatographic resolution between SA and MMA is estimated to be 14.

3.2. Method validation

The intra-day CV for three control levels were 13.2% at 0.3 μM, 6.0% at 13 μM, and 4.1% at 526 μM (N = 20–22). The inter-day CV for three control levels over three months were 15.7% at 0.3 μM, 7.5% at 10.2 μM, and 5.0% at 233 μM (N = 14–15). The assay was linear between 0.1 and 500 μM with analyte recovery ranging from 93 to 125% (Supplemental Fig. 1A). A 10-fold manual dilution was validated, with corrected dilution results agreeing within 15% of the expected neat results (Supplemental Fig. 1B). With this validation, the upper limit of the reportable range is theoretically extended to 5000 μM, recognizing that in clinical practice we have not seen levels of this magnitude. The LOQ was established at 0.1 μM with a CV of 18.1%. The extraction efficiency of the Biotage plate was determined to be 72%. The matrix effect study showed no ion-suppression, but ion-enhancement of 160% was noted. These observations were consistent with qualitative matrix effect evaluation by post-column infusion of d_3-MMA after injecting 4 extracted patient specimens without internal standards. The assay was compared to a national reference lab LC-MS/MS method with analyte concentrations ranging from 0.08 to 1013 μM. The correlation coefficient was determined to be 0.9987 and the Deming regression analysis showed a slope of 1.026 and intercept of −1.335 (Supplemental Fig. 1C). Comparison to ERNDIM peer group mean showed a correlation coefficient of 0.9996 and a Deming regression slope and intercept of 1.094 and 0.630, respectively. Reference interval of <0.4 μM was verified using 20 patient specimens, which ranged from 0.0 to 0.3 μM.

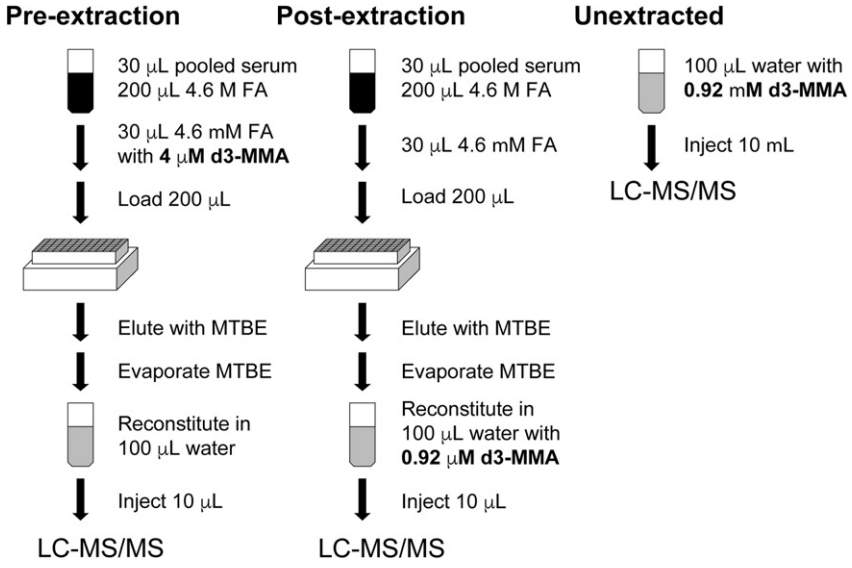

Fig. 2. Synopsis of sample preparation for analytical recovery and matrix effect.

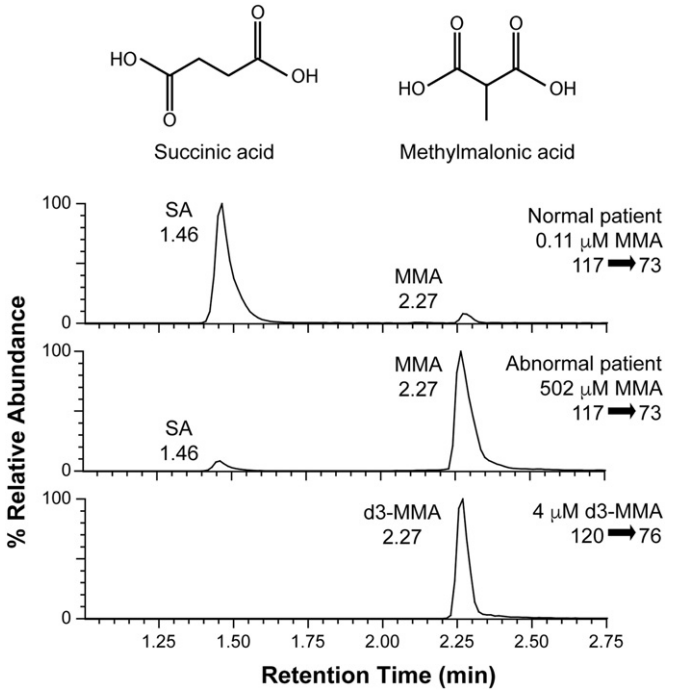

Fig. 3. Structures of succinic acid and methylmalonic acid (top). Representative chromatographic separations of succinic acid and methylmalonic acid: a normal patient and a patient with known methylmalonic acidemia (bottom).

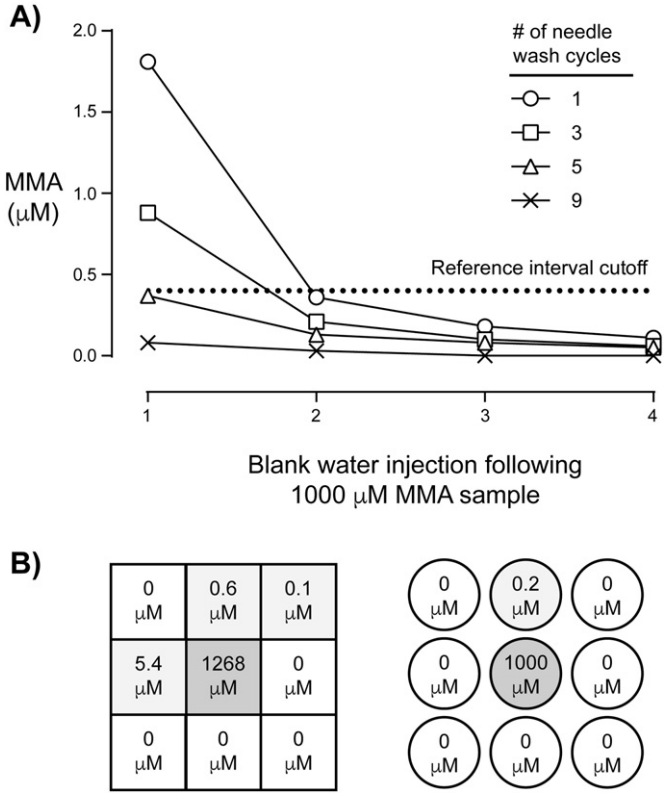

Fig. 4. Carryover minimization studies for injection needle (A) and MTBE evaporation (B). B shows a representative 3 × 3 test area for 96 square well collection plate (left) and 96 round bottom, 1 mL collection plate with modified cover (right).

3.3. Carryover

To maximize the analytical measurement range, carryover was minimized in addition to optimizing chromatographic separation. First, the wash cycle number for the injection needle was increased to nine cycles, the maximum number of wash cycles for a 7 min run. The wash buffer for the injection needle was also optimized to 40% methanol to increase the solubility of MMA. The carryover from the injection needle was determined to be 0.008% at 1000 μM MMA and was eliminated with 3 blank injections following a 1000 μM MMA sample injection (Fig. 4A). Second, evaporative carryover from MTBE was reduced to below 0.4 μM through changing the collection plate from square to round well shape and increasing the spacing of the openings between wells with a modified plate cover (Fig. 4B).

3.4. Correlation between C3-acylcarnitine and MMA

Paired MMA and plasma acylcarnitine profile analysis results were analyzed to understand the relationship between MMA and C3-acylcarnitine. These paired results were generated from 51 unique patients; 9 with primary methylmalonic acidemia (methylmalonyl CoA mutase deficiency), 1 with Cobalamin A, 1 with Cobalamin B, 10 with Cobalamin C, and 30 patients who were either undergoing screening for metabolic disease or nutritional monitoring. Comparison between MMA and C3-acylcarnitine concentrations revealed two populations with linear correlations of varying slopes (Fig. 5A). Assessment of free carnitine results indicated no association between the free carnitine concentrations and the observed populations. Plotting the results by patient, instead, showed a patient-specific relationship between MMA and C3-acylcarnitine concentrations (Fig. 5B).

4. Discussion

Analysis of MMA concentration can be used to aid the clinical diagnosis and management of methylmalonic acidemia. In order to accommodate the wide range of MMA observed in our pediatric patient population, the primary objective of our in-house LC-MS/MS MMA assay development and validation was to obtain a broad AMR while maintaining a low specimen volume requirement. Maintaining accuracy throughout the entire dynamic range was also of primary importance, along with eliminating any possibility of cross contamination. Concurrently, our secondary objectives were to establish an assay with a simple sample preparation that would allow us to minimize the turn-around-time for reporting out MMA.

In order to achieve a broad AMR spanning several orders of magnitudes, we focused on eliminating potential interference from SA by obtaining a robust baseline separation between MMA and SA. After evaluating multiple chromatography conditions and sample preparations described in the literature, we elected to use SLE and the analytical separation method described above. Optimal baseline separation, with a chromatographic resolution >10, between MMA and SA was achieved. The robustness of the chromatography, however, strongly depended on the pH of the sample. Degradation of the peak shape and resolution was noted when samples were not completely dried down to remove the formic acid.

With the robust baseline separation between MMA and SA, we were able to attain an AMR from 0.1 to 500 μM. We discovered that isotopic contribution of M + 3 MMA significantly contaminated the d_3-MMA internal standard signal above 500 μM MMA. Consequently, the internal standard concentration was increased to 4.2 μM from 0.4 μM and the upper limit of AMR was reduced to 500 μM. Through these modifications, we were able to diminish the effects of M + 3 and maintain accurate quantitation of serum MMA. It is possible that a broader AMR may be obtainable from lesser isotopic contribution of M + 4 MMA using a d_4-MMA I.S.

During the assay development, we also encountered two carryover issues not previously emphasized in the literature. If present, both types of carryover have the potential to alter the clinical diagnosis and management of patients. First, we observed carryover from the injection

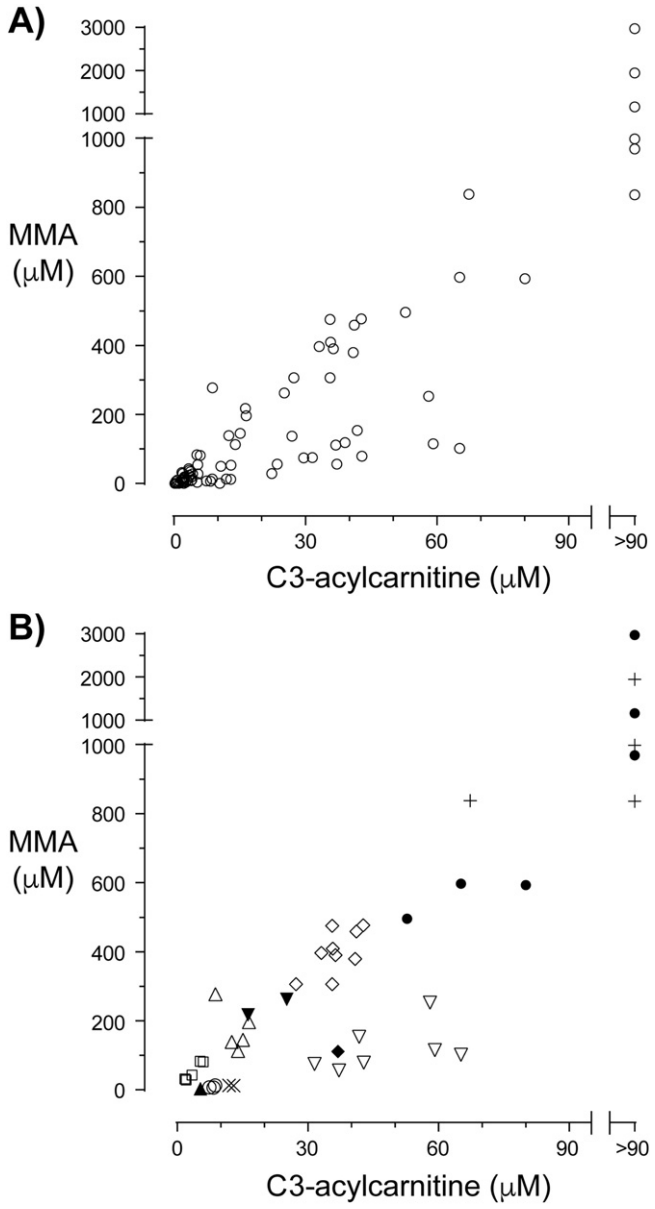

Fig. 5. Serum MMA and C3-acylcarnitine correlation data. (A) Paired serum MMA and C3-acylcarnitine reported during a 50-month period. (B) Patients with known MMA and Cobalamin A and B (N = 11), each symbol represents a unique patient.

needle, requiring the maximum number of injection wash cycles to minimize this effect. Second, and more challenging to discover, we identified a random evaporative carryover (also known as "cross-talk") where analyte from one well of a plate can flow to the neighboring well by the volatilized solvent [17]. In particular, when the difference in concentrations between two neighboring wells is >10 fold, the carryover can become significant. This second issue was difficult to resolve, and ultimately, we implemented the use of round bottom, 1 mL 96-well collection plates with a modified plate cover to increase the spacing between the openings of the wells during solvent evaporation. Furthermore, a modified plate cover was also used to improve the seal between the SLE plate and collection plate during sample elution to prevent contamination caused by splashing and sputtering.

To improve our understanding of the utility of quantifying MMA for the diagnosis and management of methylmalonic acidemia, we evaluated the relationship between MMA and C3-acylcarnitine. Our retrospective analysis unexpectedly revealed a patient-specific correlation between the two analytes that was independent of free carnitine concentration. This observed biological variation between individuals with methylmalonic acidemia strongly supports the necessity for quantifying MMA in addition to acylcarnitine analysis. Further investigation to better understand this observed phenomena and clinical utility is needed.

5. Conclusion

We encountered challenges during various stages of development of this SLE LC-MS/MS assay for serum MMA. With this work, we hope that these challenges can be carefully assessed and mitigated by laboratories prior to implementing a SLE LC-MS/MS assay for an analyte that requires a broad AMR. After resolving these hurdles, SLE's many advantages can be realized. Specifically, we were able to use minimal sample volume (30 μL) with fast extraction time that required minimal manual intervention (approximately 2 h for a full 96 well plate). The method is also adaptable to scale with semi-automation using a liquid handler to further increase laboratory efficiency [12].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2016.05.010.

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Question 7
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Quantifying MMA by SLE LC-MS/MS: Unexpected challenges in assay development

Sheng-Ying Lo [a], Cindy Gordon [b], Katerina Sadilkova [b], Rhona M. Jack [b], Jane A. Dickerson [b,*]

[a] Department of Laboratory Medicine, Chemistry Division, University of Washington, Seattle, WA, United States
[b] Department of Laboratories, Seattle Children's Hospital, Seattle, WA, United States

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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.

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

* Corresponding author at: Seattle Children's Hospital, P.O. Box 5371, Seattle, WA 98145, United States.
E-mail address: jane.dickerson@seattlechildrens.org (J.A. Dickerson).

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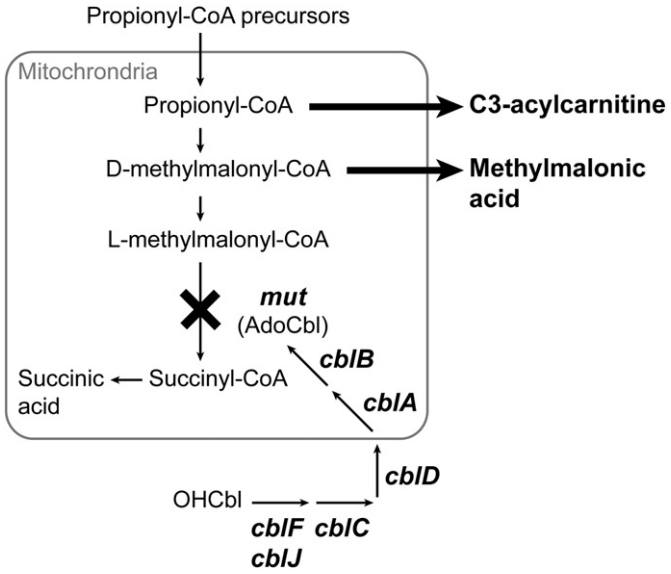

Fig. 1. Methylmalonic acidemia can be caused by defect in methylmalonyl-CoA mutase (mut) or enzymes that synthesize its cofactor, adenosylcobalamin (AdoCbl), from hydroxycobalamin (OHCbl). Deficiency in cobalamin can also inhibit isomerization of L-methylmalonyl-CoA to succinyl-CoA and cause accumulation 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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Chromatographic separation of 0.81 min between MMA and its major endogenous isobaric interferent, succinic acid (SA) was achieved (Fig. 3). The run-to-run time was 8 min. The chromatographic resolution between SA and MMA is estimated to be 14.

3.2. Method validation

The intra-day CV for three control levels were 13.2% at 0.3 μM, 6.0% at 13 μM, and 4.1% at 526 μM (N = 20–22). The inter-day CV for three control levels over three months were 15.7% at 0.3 μM, 7.5% at 10.2 μM, and 5.0% at 233 μM (N = 14–15). The assay was linear between 0.1 and 500 μM with analyte recovery ranging from 93 to 125% (Supplemental Fig. 1A). A 10-fold manual dilution was validated, with corrected dilution results agreeing within 15% of the expected neat results (Supplemental Fig. 1B). With this validation, the upper limit of the reportable range is theoretically extended to 5000 μM, recognizing that in clinical practice we have not seen levels of this magnitude. The LOQ was established at 0.1 μM with a CV of 18.1%. The extraction efficiency of the Biotage plate was determined to be 72%. The matrix effect study showed no ion-suppression, but ion-enhancement of 160% was noted. These observations were consistent with qualitative matrix effect evaluation by post-column infusion of d_3-MMA after injecting 4 extracted patient specimens without internal standards. The assay was compared to a national reference lab LC-MS/MS method with analyte concentrations ranging from 0.08 to 1013 μM. The correlation coefficient was determined to be 0.9987 and the Deming regression analysis showed a slope of 1.026 and intercept of −1.335 (Supplemental Fig. 1C). Comparison to ERNDIM peer group mean showed a correlation coefficient of 0.9996 and a Deming regression slope and intercept of 1.094 and 0.630, respectively. Reference interval of <0.4 μM was verified using 20 patient specimens, which ranged from 0.0 to 0.3 μM.

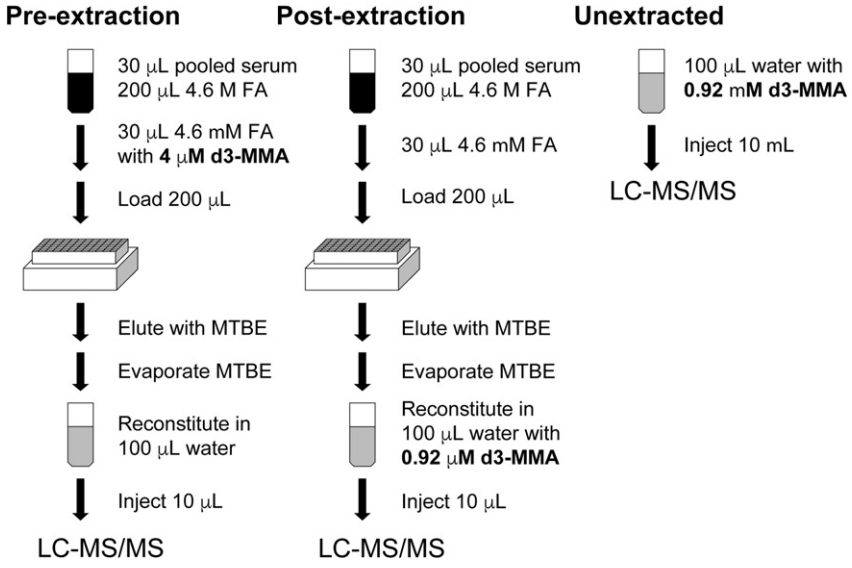

Fig. 2. Synopsis of sample preparation for analytical recovery and matrix effect.

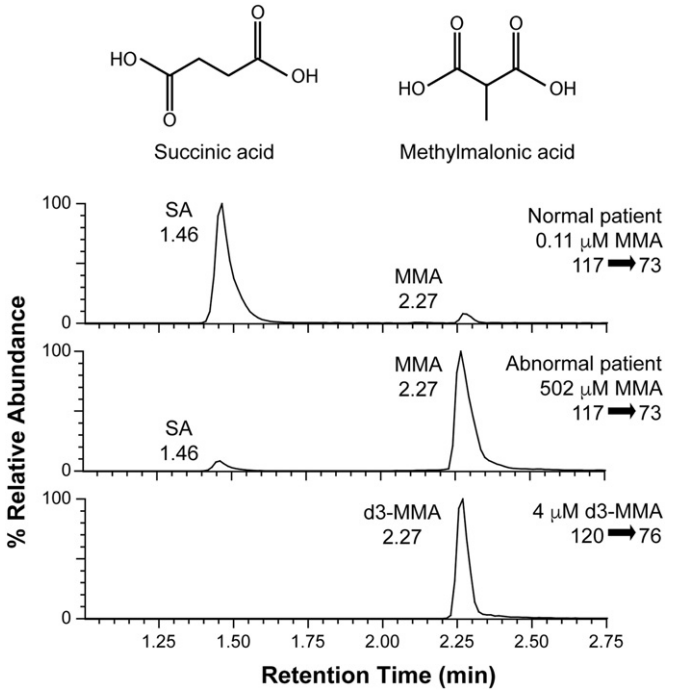

Fig. 3. Structures of succinic acid and methylmalonic acid (top). Representative chromatographic separations of succinic acid and methylmalonic acid: a normal patient and a patient with known methylmalonic acidemia (bottom).

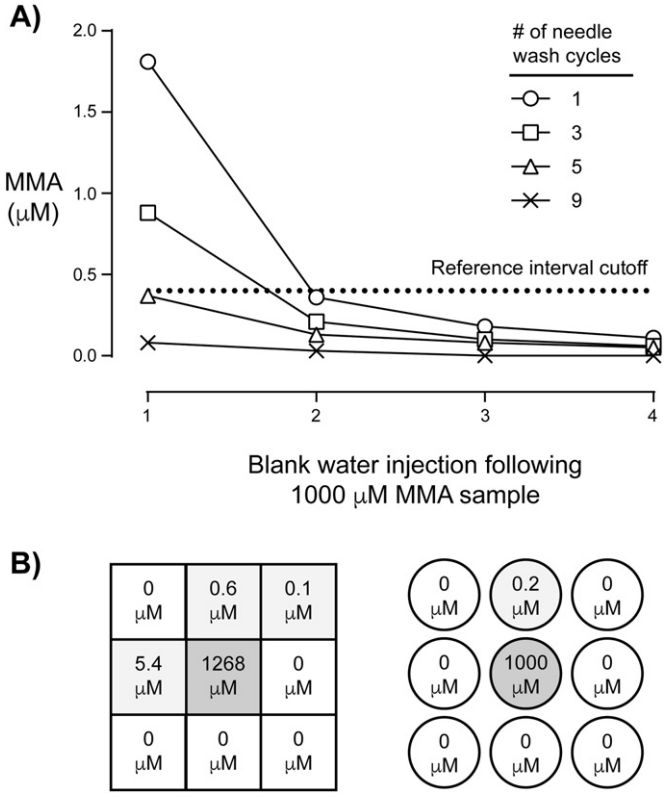

Fig. 4. Carryover minimization studies for injection needle (A) and MTBE evaporation (B). B shows a representative 3 × 3 test area for 96 square well collection plate (left) and 96 round bottom, 1 mL collection plate with modified cover (right).

3.3. Carryover

To maximize the analytical measurement range, carryover was minimized in addition to optimizing chromatographic separation. First, the wash cycle number for the injection needle was increased to nine cycles, the maximum number of wash cycles for a 7 min run. The wash buffer for the injection needle was also optimized to 40% methanol to increase the solubility of MMA. The carryover from the injection needle was determined to be 0.008% at 1000 μM MMA and was eliminated with 3 blank injections following a 1000 μM MMA sample injection (Fig. 4A). Second, evaporative carryover from MTBE was reduced to below 0.4 μM through changing the collection plate from square to round well shape and increasing the spacing of the openings between wells with a modified plate cover (Fig. 4B).

3.4. Correlation between C3-acylcarnitine and MMA

Paired MMA and plasma acylcarnitine profile analysis results were analyzed to understand the relationship between MMA and C3-acylcarnitine. These paired results were generated from 51 unique patients; 9 with primary methylmalonic acidemia (methylmalonyl CoA mutase deficiency), 1 with Cobalamin A, 1 with Cobalamin B, 10 with Cobalamin C, and 30 patients who were either undergoing screening for metabolic disease or nutritional monitoring. Comparison between MMA and C3-acylcarnitine concentrations revealed two populations with linear correlations of varying slopes (Fig. 5A). Assessment of free carnitine results indicated no association between the free carnitine concentrations and the observed populations. Plotting the results by patient, instead, showed a patient-specific relationship between MMA and C3-acylcarnitine concentrations (Fig. 5B).

4. Discussion

Analysis of MMA concentration can be used to aid the clinical diagnosis and management of methylmalonic acidemia. In order to accommodate the wide range of MMA observed in our pediatric patient population, the primary objective of our in-house LC-MS/MS MMA assay development and validation was to obtain a broad AMR while maintaining a low specimen volume requirement. Maintaining accuracy throughout the entire dynamic range was also of primary importance, along with eliminating any possibility of cross contamination. Concurrently, our secondary objectives were to establish an assay with a simple sample preparation that would allow us to minimize the turn-around-time for reporting out MMA.

In order to achieve a broad AMR spanning several orders of magnitudes, we focused on eliminating potential interference from SA by obtaining a robust baseline separation between MMA and SA. After evaluating multiple chromatography conditions and sample preparations described in the literature, we elected to use SLE and the analytical separation method described above. Optimal baseline separation, with a chromatographic resolution >10, between MMA and SA was achieved. The robustness of the chromatography, however, strongly depended on the pH of the sample. Degradation of the peak shape and resolution was noted when samples were not completely dried down to remove the formic acid.

With the robust baseline separation between MMA and SA, we were able to attain an AMR from 0.1 to 500 μM. We discovered that isotopic contribution of M + 3 MMA significantly contaminated the d_3-MMA internal standard signal above 500 μM MMA. Consequently, the internal standard concentration was increased to 4.2 μM from 0.4 μM and the upper limit of AMR was reduced to 500 μM. Through these modifications, we were able to diminish the effects of M + 3 and maintain accurate quantitation of serum MMA. It is possible that a broader AMR may be obtainable from lesser isotopic contribution of M + 4 MMA using a d_4-MMA I.S.

During the assay development, we also encountered two carryover issues not previously emphasized in the literature. If present, both types of carryover have the potential to alter the clinical diagnosis and management of patients. First, we observed carryover from the injection

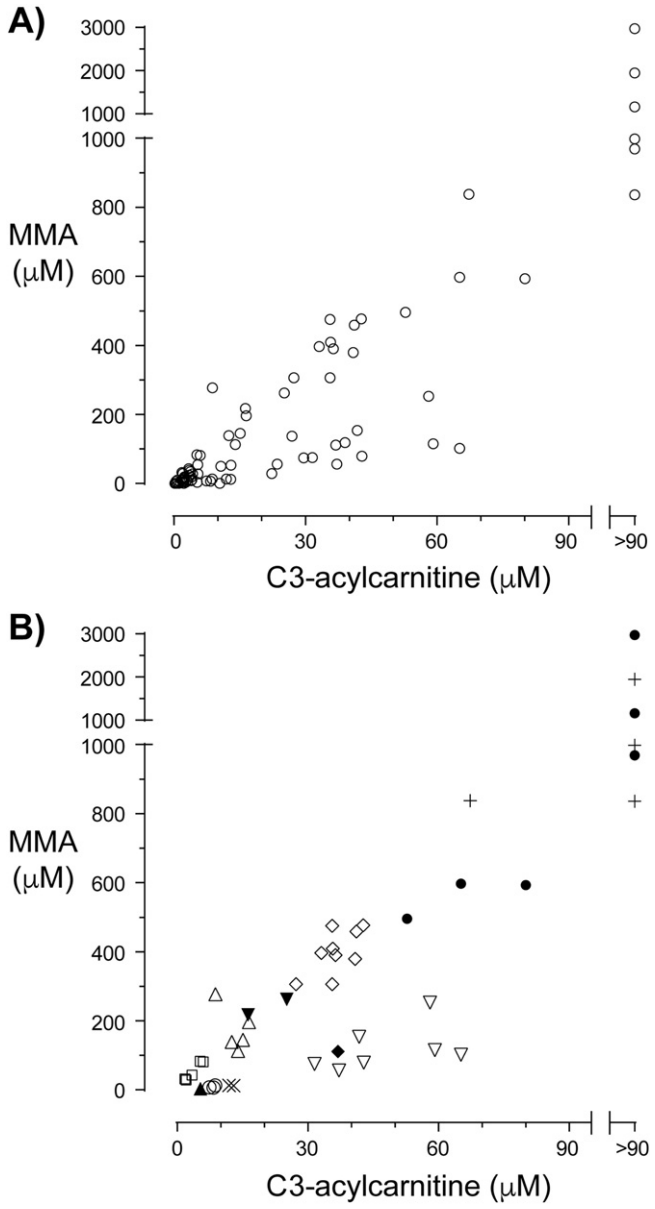

Fig. 5. Serum MMA and C3-acylcarnitine correlation data. (A) Paired serum MMA and C3-acylcarnitine reported during a 50-month period. (B) Patients with known MMA and Cobalamin A and B (N = 11), each symbol represents a unique patient.

needle, requiring the maximum number of injection wash cycles to minimize this effect. Second, and more challenging to discover, we identified a random evaporative carryover (also known as "cross-talk") where analyte from one well of a plate can flow to the neighboring well by the volatilized solvent [17]. In particular, when the difference in concentrations between two neighboring wells is >10 fold, the carryover can become significant. This second issue was difficult to resolve, and ultimately, we implemented the use of round bottom, 1 mL 96-well collection plates with a modified plate cover to increase the spacing between the openings of the wells during solvent evaporation. Furthermore, a modified plate cover was also used to improve the seal between the SLE plate and collection plate during sample elution to prevent contamination caused by splashing and sputtering.

To improve our understanding of the utility of quantifying MMA for the diagnosis and management of methylmalonic acidemia, we evaluated the relationship between MMA and C3-acylcarnitine. Our retrospective analysis unexpectedly revealed a patient-specific correlation between the two analytes that was independent of free carnitine concentration. This observed biological variation between individuals with methylmalonic acidemia strongly supports the necessity for quantifying MMA in addition to acylcarnitine analysis. Further investigation to better understand this observed phenomena and clinical utility is needed.

5. Conclusion

We encountered challenges during various stages of development of this SLE LC-MS/MS assay for serum MMA. With this work, we hope that these challenges can be carefully assessed and mitigated by laboratories prior to implementing a SLE LC-MS/MS assay for an analyte that requires a broad AMR. After resolving these hurdles, SLE's many advantages can be realized. Specifically, we were able to use minimal sample volume (30 μL) with fast extraction time that required minimal manual intervention (approximately 2 h for a full 96 well plate). The method is also adaptable to scale with semi-automation using a liquid handler to further increase laboratory efficiency [12].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2016.05.010.

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

Sheng-Ying Lo [a], Cindy Gordon [b], Katerina Sadilkova [b], Rhona M. Jack [b], Jane A. Dickerson [b,*]

[a] Department of Laboratory Medicine, Chemistry Division, University of Washington, Seattle, WA, United States
[b] Department of Laboratories, Seattle Children's Hospital, Seattle, WA, United States

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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.

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

* Corresponding author at: Seattle Children's Hospital, P.O. Box 5371, Seattle, WA 98145, United States.
E-mail address: jane.dickerson@seattlechildrens.org (J.A. Dickerson).

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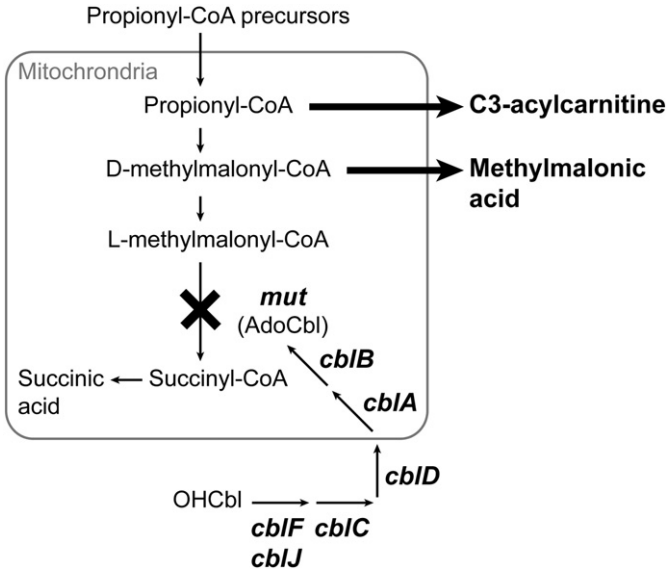

Fig. 1. Methylmalonic acidemia can be caused by defect in methylmalonyl-CoA mutase (mut) or enzymes that synthesize its cofactor, adenosylcobalamin (AdoCbl), from hydroxycobalamin (OHCbl). Deficiency in cobalamin can also inhibit isomerization of L-methylmalonyl-CoA to succinyl-CoA and cause accumulation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

determined by diluting an ERNDIM sample with 0.1% BSA to 0.1 μM and analyzed in quintuplet over three days. Analytical recovery was determined by comparing the averaged peak area of d_3-MMA added pre and post SLE (N = 3). Matrix effect was evaluated by comparing the averaged peak areas of the post extraction sample with unextracted MMA solution (0.92 μM in water). Experimental detail is illustrated in Fig. 2. Ion suppression or enhancement was also examined by post-column infusion of d_3-MMA with injection of SLE extracted patient specimens (N = 4) [15]. Reference interval was verified using 20 patient specimens with normal C3-acylcarnitine [16].

2.6. Carryover

Two types of carryover were studied. First, MMA carryover from the injection needle was assessed with a water injection (blank) followed by an injection of a high MMA sample (1000 μM MMA in SeraCon II serum) and four subsequent blanks. For each analysis, a new water sample was used, with the number of needle wash cycles set to 1, 3, 6, or 9 throughout the runs. Carryover between each analysis was minimized by three blank injections of a new water sample and nine needle wash cycles per injection. Peak area obtained from a blank run prior to the high MMA sample was used for baseline correction. Carryover was calculated as: Carryover (μM) = blank peak area/high sample peak area * 1000 μM. Second, evaporative carryover by MTBE during sample preparation was examined using nine wells (3 × 3) test area. For square wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and SeraCon II serum without MMA was extracted in the surrounding wells. MMA concentrations were quantified using calibration curve. For round wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and 500 μL of MTBE was added to the eight surrounding wells of the collection plate prior to nitrogen evaporation. All wells were then reconstituted in 100 μL of water and analyzed. Carryover for round wells was calculated as: carryover (μM) = surrounding well peak area/center well peak area * 1000 μM.

2.7. Correlation between serum MMA and C3-acylcarnitine

Historical results for paired MMA and plasma acylcarnitine analysis generated between November 1, 2011 and September 31, 2015 were pulled from the laboratory information system (Cerner Millennium Pathnet), N = 111. Results were grouped based on diagnosis then de-identified prior to correlation analysis.

2.8. Statistical analysis

Statistical analyses were performed using Microsoft Excel, GraphPad Prism 5, and EP Evaluator.

3. Results

3.1. Chromatography

Chromatographic separation of 0.81 min between MMA and its major endogenous isobaric interferent, succinic acid (SA) was achieved (Fig. 3). The run-to-run time was 8 min. The chromatographic resolution between SA and MMA is estimated to be 14.

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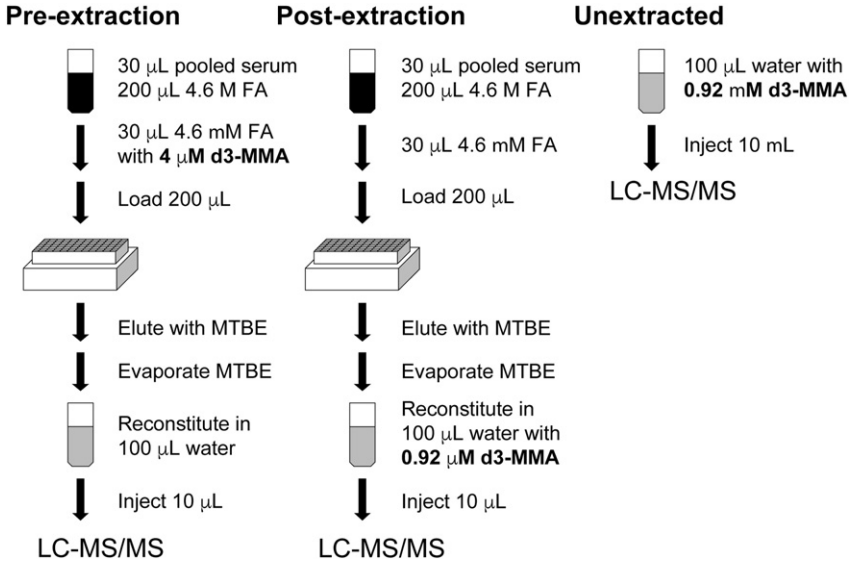

Fig. 2. Synopsis of sample preparation for analytical recovery and matrix effect.

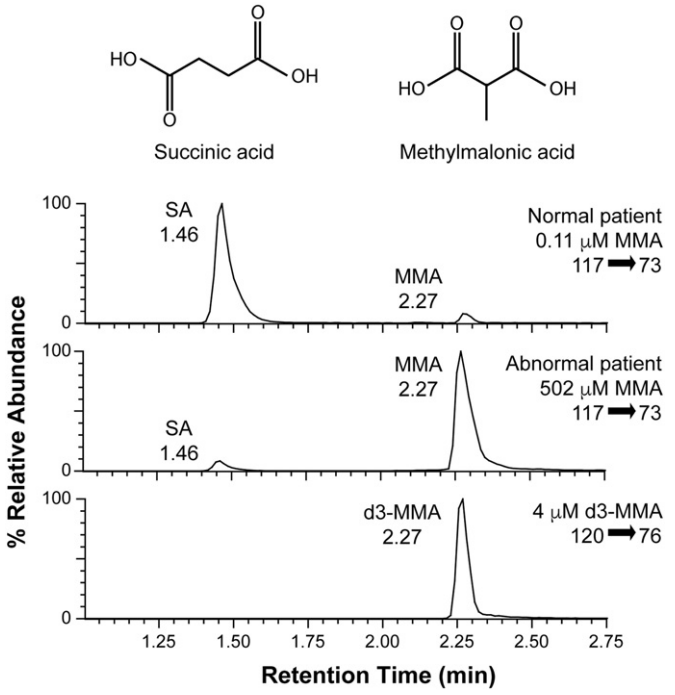

Fig. 3. Structures of succinic acid and methylmalonic acid (top). Representative chromatographic separations of succinic acid and methylmalonic acid: a normal patient and a patient with known methylmalonic acidemia (bottom).

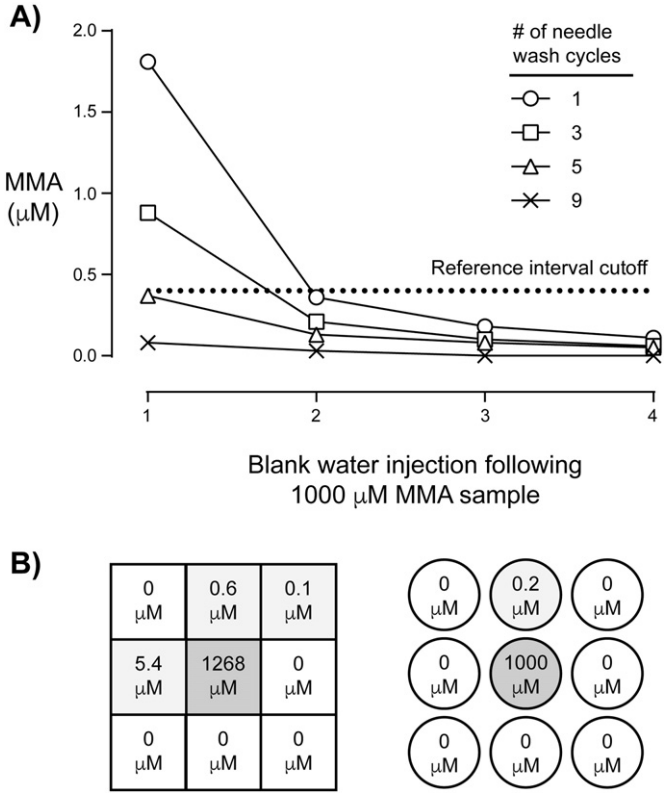

Fig. 4. Carryover minimization studies for injection needle (A) and MTBE evaporation (B). B shows a representative 3 × 3 test area for 96 square well collection plate (left) and 96 round bottom, 1 mL collection plate with modified cover (right).

3.3. Carryover

To maximize the analytical measurement range, carryover was minimized in addition to optimizing chromatographic separation. First, the wash cycle number for the injection needle was increased to nine cycles, the maximum number of wash cycles for a 7 min run. The wash buffer for the injection needle was also optimized to 40% methanol to increase the solubility of MMA. The carryover from the injection needle was determined to be 0.008% at 1000 μM MMA and was eliminated with 3 blank injections following a 1000 μM MMA sample injection (Fig. 4A). Second, evaporative carryover from MTBE was reduced to below 0.4 μM through changing the collection plate from square to round well shape and increasing the spacing of the openings between wells with a modified plate cover (Fig. 4B).

3.4. Correlation between C3-acylcarnitine and MMA

Paired MMA and plasma acylcarnitine profile analysis results were analyzed to understand the relationship between MMA and C3-acylcarnitine. These paired results were generated from 51 unique patients; 9 with primary methylmalonic acidemia (methylmalonyl CoA mutase deficiency), 1 with Cobalamin A, 1 with Cobalamin B, 10 with Cobalamin C, and 30 patients who were either undergoing screening for metabolic disease or nutritional monitoring. Comparison between MMA and C3-acylcarnitine concentrations revealed two populations with linear correlations of varying slopes (Fig. 5A). Assessment of free carnitine results indicated no association between the free carnitine concentrations and the observed populations. Plotting the results by patient, instead, showed a patient-specific relationship between MMA and C3-acylcarnitine concentrations (Fig. 5B).

4. Discussion

Analysis of MMA concentration can be used to aid the clinical diagnosis and management of methylmalonic acidemia. In order to accommodate the wide range of MMA observed in our pediatric patient population, the primary objective of our in-house LC-MS/MS MMA assay development and validation was to obtain a broad AMR while maintaining a low specimen volume requirement. Maintaining accuracy throughout the entire dynamic range was also of primary importance, along with eliminating any possibility of cross contamination. Concurrently, our secondary objectives were to establish an assay with a simple sample preparation that would allow us to minimize the turn-around-time for reporting out MMA.

In order to achieve a broad AMR spanning several orders of magnitudes, we focused on eliminating potential interference from SA by obtaining a robust baseline separation between MMA and SA. After evaluating multiple chromatography conditions and sample preparations described in the literature, we elected to use SLE and the analytical separation method described above. Optimal baseline separation, with a chromatographic resolution >10, between MMA and SA was achieved. The robustness of the chromatography, however, strongly depended on the pH of the sample. Degradation of the peak shape and resolution was noted when samples were not completely dried down to remove the formic acid.

With the robust baseline separation between MMA and SA, we were able to attain an AMR from 0.1 to 500 μM. We discovered that isotopic contribution of M + 3 MMA significantly contaminated the d_3-MMA internal standard signal above 500 μM MMA. Consequently, the internal standard concentration was increased to 4.2 μM from 0.4 μM and the upper limit of AMR was reduced to 500 μM. Through these modifications, we were able to diminish the effects of M + 3 and maintain accurate quantitation of serum MMA. It is possible that a broader AMR may be obtainable from lesser isotopic contribution of M + 4 MMA using a d_4-MMA I.S.

During the assay development, we also encountered two carryover issues not previously emphasized in the literature. If present, both types of carryover have the potential to alter the clinical diagnosis and management of patients. First, we observed carryover from the injection

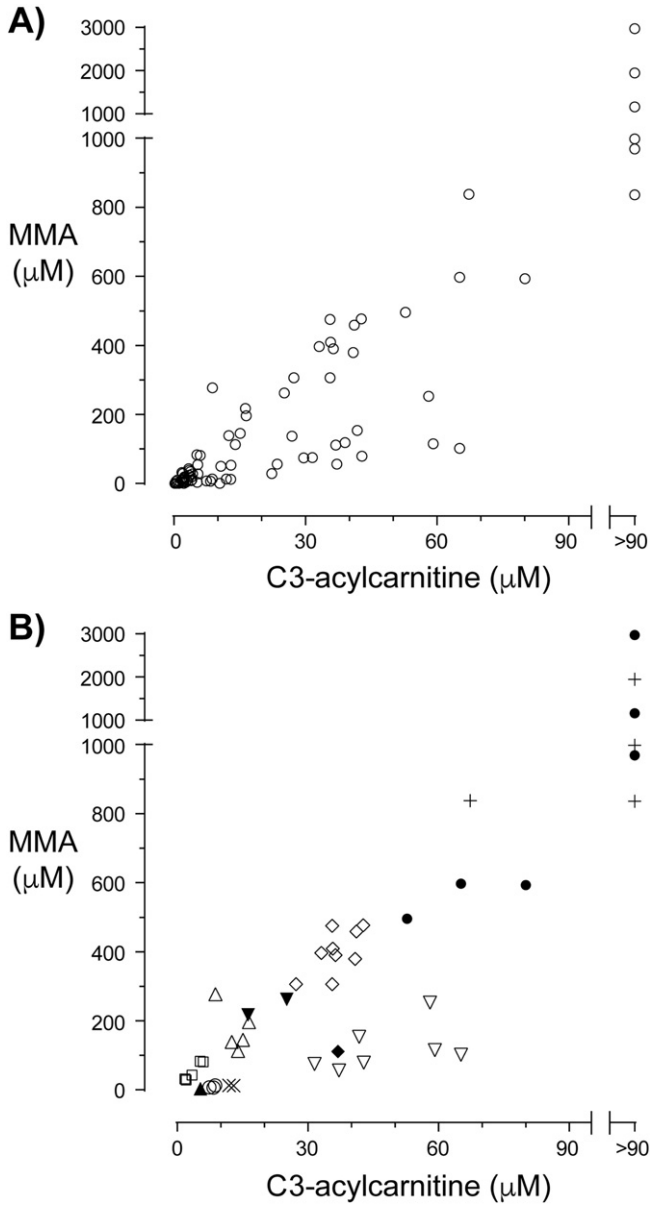

Fig. 5. Serum MMA and C3-acylcarnitine correlation data. (A) Paired serum MMA and C3-acylcarnitine reported during a 50-month period. (B) Patients with known MMA and Cobalamin A and B (N = 11), each symbol represents a unique patient.

needle, requiring the maximum number of injection wash cycles to minimize this effect. Second, and more challenging to discover, we identified a random evaporative carryover (also known as "cross-talk") where analyte from one well of a plate can flow to the neighboring well by the volatilized solvent [17]. In particular, when the difference in concentrations between two neighboring wells is >10 fold, the carryover can become significant. This second issue was difficult to resolve, and ultimately, we implemented the use of round bottom, 1 mL 96-well collection plates with a modified plate cover to increase the spacing between the openings of the wells during solvent evaporation. Furthermore, a modified plate cover was also used to improve the seal between the SLE plate and collection plate during sample elution to prevent contamination caused by splashing and sputtering.

To improve our understanding of the utility of quantifying MMA for the diagnosis and management of methylmalonic acidemia, we evaluated the relationship between MMA and C3-acylcarnitine. Our retrospective analysis unexpectedly revealed a patient-specific correlation between the two analytes that was independent of free carnitine concentration. This observed biological variation between individuals with methylmalonic acidemia strongly supports the necessity for quantifying MMA in addition to acylcarnitine analysis. Further investigation to better understand this observed phenomena and clinical utility is needed.

5. Conclusion

We encountered challenges during various stages of development of this SLE LC-MS/MS assay for serum MMA. With this work, we hope that these challenges can be carefully assessed and mitigated by laboratories prior to implementing a SLE LC-MS/MS assay for an analyte that requires a broad AMR. After resolving these hurdles, SLE's many advantages can be realized. Specifically, we were able to use minimal sample volume (30 μL) with fast extraction time that required minimal manual intervention (approximately 2 h for a full 96 well plate). The method is also adaptable to scale with semi-automation using a liquid handler to further increase laboratory efficiency [12].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2016.05.010.

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

Sheng-Ying Lo [a], Cindy Gordon [b], Katerina Sadilkova [b], Rhona M. Jack [b], Jane A. Dickerson [b,*]

[a] Department of Laboratory Medicine, Chemistry Division, University of Washington, Seattle, WA, United States
[b] Department of Laboratories, Seattle Children's Hospital, Seattle, WA, United States

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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.

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

* Corresponding author at: Seattle Children's Hospital, P.O. Box 5371, Seattle, WA 98145, United States.
E-mail address: jane.dickerson@seattlechildrens.org (J.A. Dickerson).

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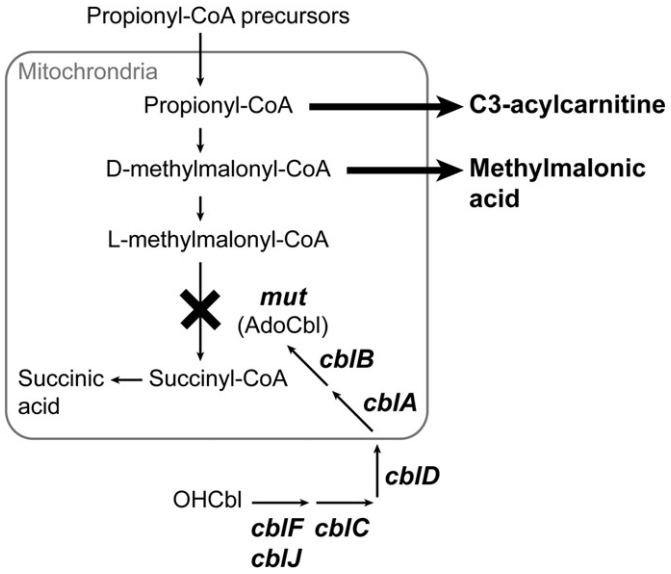


Fig. 1. Methylmalonic acidemia can be caused by defect in methylmalonyl-CoA mutase (mut) or enzymes that synthesize its cofactor, adenosylcobalamin (AdoCbl), from hydroxycobalamin (OHCbl). Deficiency in cobalamin can also inhibit isomerization of L-methylmalonyl-CoA to succinyl-CoA and cause accumulation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

determined by diluting an ERNDIM sample with 0.1% BSA to 0.1 μM and analyzed in quintuplet over three days. Analytical recovery was determined by comparing the averaged peak area of d_3-MMA added pre and post SLE (N = 3). Matrix effect was evaluated by comparing the averaged peak areas of the post extraction sample with unextracted MMA solution (0.92 μM in water). Experimental detail is illustrated in Fig. 2. Ion suppression or enhancement was also examined by post-column infusion of d_3-MMA with injection of SLE extracted patient specimens (N = 4) [15]. Reference interval was verified using 20 patient specimens with normal C3-acylcarnitine [16].

2.6. Carryover

Two types of carryover were studied. First, MMA carryover from the injection needle was assessed with a water injection (blank) followed by an injection of a high MMA sample (1000 μM MMA in SeraCon II serum) and four subsequent blanks. For each analysis, a new water sample was used, with the number of needle wash cycles set to 1, 3, 6, or 9 throughout the runs. Carryover between each analysis was minimized by three blank injections of a new water sample and nine needle wash cycles per injection. Peak area obtained from a blank run prior to the high MMA sample was used for baseline correction. Carryover was calculated as: Carryover (μM) = blank peak area/high sample peak area * 1000 μM. Second, evaporative carryover by MTBE during sample preparation was examined using nine wells (3 × 3) test area. For square wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and SeraCon II serum without MMA was extracted in the surrounding wells. MMA concentrations were quantified using calibration curve. For round wells, 1000 μM MMA in SeraCon II serum was extracted in the center well and 500 μL of MTBE was added to the eight surrounding wells of the collection plate prior to nitrogen evaporation. All wells were then reconstituted in 100 μL of water and analyzed. Carryover for round wells was calculated as: carryover (μM) = surrounding well peak area/center well peak area * 1000 μM.

2.7. Correlation between serum MMA and C3-acylcarnitine

Historical results for paired MMA and plasma acylcarnitine analysis generated between November 1, 2011 and September 31, 2015 were pulled from the laboratory information system (Cerner Millennium Pathnet), N = 111. Results were grouped based on diagnosis then de-identified prior to correlation analysis.

2.8. Statistical analysis

Statistical analyses were performed using Microsoft Excel, GraphPad Prism 5, and EP Evaluator.

3. Results

3.1. Chromatography

Chromatographic separation of 0.81 min between MMA and its major endogenous isobaric interferent, succinic acid (SA) was achieved (Fig. 3). The run-to-run time was 8 min. The chromatographic resolution between SA and MMA is estimated to be 14.

3.2. Method validation

The intra-day CV for three control levels were 13.2% at 0.3 μM, 6.0% at 13 μM, and 4.1% at 526 μM (N = 20–22). The inter-day CV for three control levels over three months were 15.7% at 0.3 μM, 7.5% at 10.2 μM, and 5.0% at 233 μM (N = 14–15). The assay was linear between 0.1 and 500 μM with analyte recovery ranging from 93 to 125% (Supplemental Fig. 1A). A 10-fold manual dilution was validated, with corrected dilution results agreeing within 15% of the expected neat results (Supplemental Fig. 1B). With this validation, the upper limit of the reportable range is theoretically extended to 5000 μM, recognizing that in clinical practice we have not seen levels of this magnitude. The LOQ was established at 0.1 μM with a CV of 18.1%. The extraction efficiency of the Biotage plate was determined to be 72%. The matrix effect study showed no ion-suppression, but ion-enhancement of 160% was noted. These observations were consistent with qualitative matrix effect evaluation by post-column infusion of d_3-MMA after injecting 4 extracted patient specimens without internal standards. The assay was compared to a national reference lab LC-MS/MS method with analyte concentrations ranging from 0.08 to 1013 μM. The correlation coefficient was determined to be 0.9987 and the Deming regression analysis showed a slope of 1.026 and intercept of −1.335 (Supplemental Fig. 1C). Comparison to ERNDIM peer group mean showed a correlation coefficient of 0.9996 and a Deming regression slope and intercept of 1.094 and 0.630, respectively. Reference interval of <0.4 μM was verified using 20 patient specimens, which ranged from 0.0 to 0.3 μM.

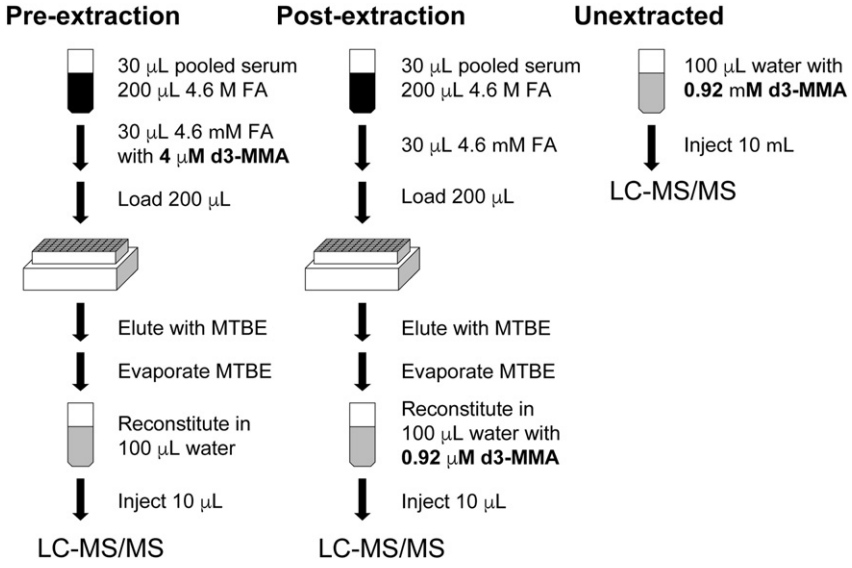
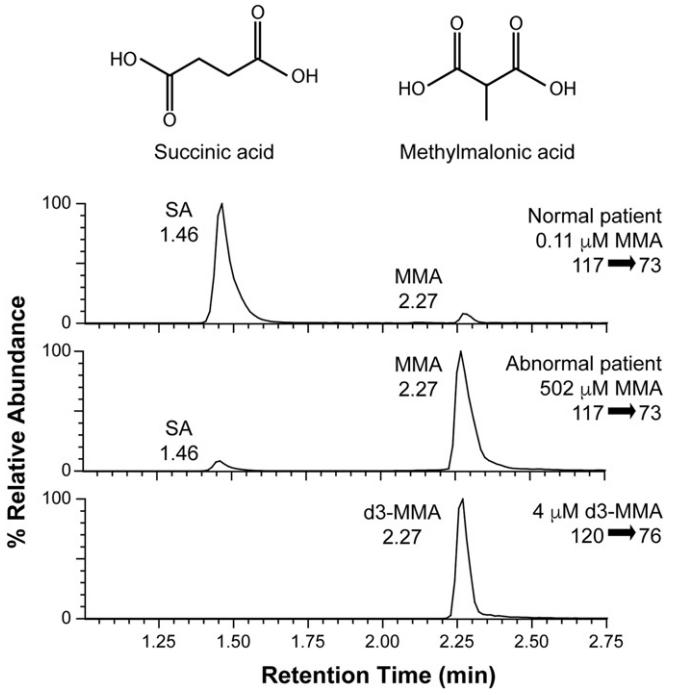


Fig. 2. Synopsis of sample preparation for analytical recovery and matrix effect.



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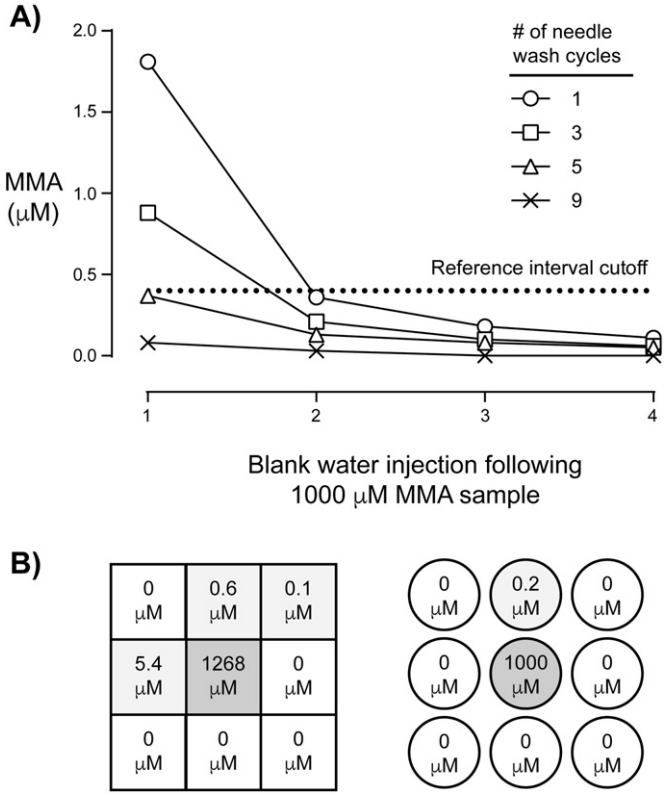

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4. Discussion

Analysis of MMA concentration can be used to aid the clinical diagnosis and management of methylmalonic acidemia. In order to accommodate the wide range of MMA observed in our pediatric patient population, the primary objective of our in-house LC-MS/MS MMA assay development and validation was to obtain a broad AMR while maintaining a low specimen volume requirement. Maintaining accuracy throughout the entire dynamic range was also of primary importance, along with eliminating any possibility of cross contamination. Concurrently, our secondary objectives were to establish an assay with a simple sample preparation that would allow us to minimize the turn-around-time for reporting out MMA.

In order to achieve a broad AMR spanning several orders of magnitudes, we focused on eliminating potential interference from SA by obtaining a robust baseline separation between MMA and SA. After evaluating multiple chromatography conditions and sample preparations described in the literature, we elected to use SLE and the analytical separation method described above. Optimal baseline separation, with a chromatographic resolution >10, between MMA and SA was achieved. The robustness of the chromatography, however, strongly depended on the pH of the sample. Degradation of the peak shape and resolution was noted when samples were not completely dried down to remove the formic acid.

With the robust baseline separation between MMA and SA, we were able to attain an AMR from 0.1 to 500 μM. We discovered that isotopic contribution of M + 3 MMA significantly contaminated the d_3-MMA internal standard signal above 500 μM MMA. Consequently, the internal standard concentration was increased to 4.2 μM from 0.4 μM and the upper limit of AMR was reduced to 500 μM. Through these modifications, we were able to diminish the effects of M + 3 and maintain accurate quantitation of serum MMA. It is possible that a broader AMR may be obtainable from lesser isotopic contribution of M + 4 MMA using a d_4-MMA I.S.

During the assay development, we also encountered two carryover issues not previously emphasized in the literature. If present, both types of carryover have the potential to alter the clinical diagnosis and management of patients. First, we observed carryover from the injection

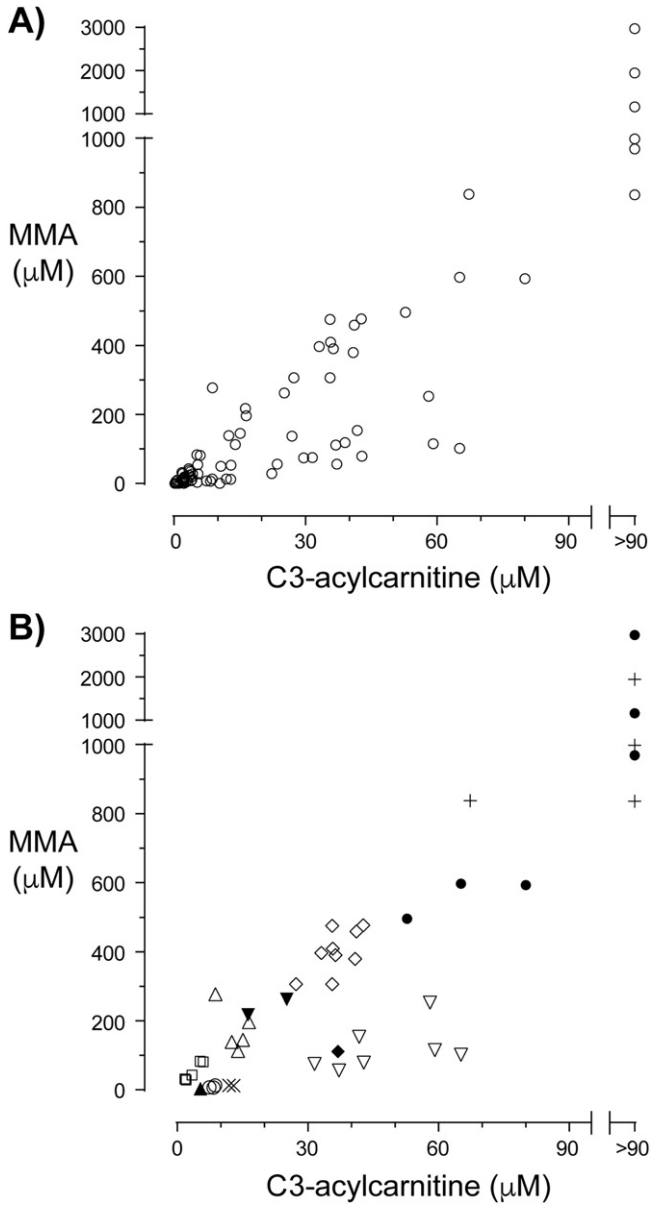

Fig. 5. Serum MMA and C3-acylcarnitine correlation data. (A) Paired serum MMA and C3-acylcarnitine reported during a 50-month period. (B) Patients with known MMA and Cobalamin A and B (N = 11), each symbol represents a unique patient.

needle, requiring the maximum number of injection wash cycles to minimize this effect. Second, and more challenging to discover, we identified a random evaporative carryover (also known as "cross-talk") where analyte from one well of a plate can flow to the neighboring well by the volatilized solvent [17]. In particular, when the difference in concentrations between two neighboring wells is >10 fold, the carryover can become significant. This second issue was difficult to resolve, and ultimately, we implemented the use of round bottom, 1 mL 96-well collection plates with a modified plate cover to increase the spacing between the openings of the wells during solvent evaporation. Furthermore, a modified plate cover was also used to improve the seal between the SLE plate and collection plate during sample elution to prevent contamination caused by splashing and sputtering.

To improve our understanding of the utility of quantifying MMA for the diagnosis and management of methylmalonic acidemia, we evaluated the relationship between MMA and C3-acylcarnitine. Our retrospective analysis unexpectedly revealed a patient-specific correlation between the two analytes that was independent of free carnitine concentration. This observed biological variation between individuals with methylmalonic acidemia strongly supports the necessity for quantifying MMA in addition to acylcarnitine analysis. Further investigation to better understand this observed phenomena and clinical utility is needed.

5. Conclusion

We encountered challenges during various stages of development of this SLE LC-MS/MS assay for serum MMA. With this work, we hope that these challenges can be carefully assessed and mitigated by laboratories prior to implementing a SLE LC-MS/MS assay for an analyte that requires a broad AMR. After resolving these hurdles, SLE's many advantages can be realized. Specifically, we were able to use minimal sample volume (30 μL) with fast extraction time that required minimal manual intervention (approximately 2 h for a full 96 well plate). The method is also adaptable to scale with semi-automation using a liquid handler to further increase laboratory efficiency [12].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2016.05.010.

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