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Methylmalonic acid quantification in low serum volumes by UPLC–MS/MS

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ABSTRACT

Methylmalonic acid (MMA) is a metabolic intermediate transformed to succinic acid (SA) by a vitamin B_{12}-dependent catalytic step, and is broadly used as a clinical biomarker of functional vitamin B12 status. However, reported methods use between 100 and 1000 μL of serum or plasma making them sub-optimal for sample-limited studies, including those with neonates and infants. LC–MS/MS based protocols to measure MMA as n-butyl esters in the presence of tri-deuterated MMA (MMA-d_3) were modified for use with 25 μL of human serum by scaling down sample processing volumes and analysis by UPLC–MS/MS. Plasma-based calibration solutions were found to be unnecessary, and chromatographic resolution and peak shape of SA and MMA was optimized in <4 min with isocratic 53:47 methanol/1.67 mM (pH 6.5) ammonium formate. Additionally, 1-cyclohexyl-urido-3-dodecanoic acid (CUDA) was included as internal standard allowing direct assessment of MMA recovery. Sample concentrations in the low normal range produced a signal:noise of >100:1. MMA intra- and inter-assay variability was under 10%. MMA-d_3 surrogate recovery averaged 93 ± 14%. MMA stability exceeded three years in frozen samples and was unaffected by up to five freeze/thaw cycles. In conclusion, we report that methylmalonic acid can be measured with 25 μL of serum using water based standards. The assay signal:noise per concentration indicates that the method could perform as implemented with as little as 5 μL of serum. The reported method is applicable for studies of functional B12 status in sample limited experiments including investigations of nutritional status in neonates and in studies where low normal MMA levels are expected.

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1. Introduction

Methylmalonic acid (MMA) is a metabolic intermediate in the mitochondrial conversion of propionic acid to succinic acid (SA). Cobalamin (*i.e.* vitamin B_{12}) is an essential cofactor for the methylmalonyl mutase-dependent carbon rearrangement of MMA to SA. Vitamin B_{12} deficiency therefore results in high circulating levels of MMA [1]. The facts that serum/plasma vitamin B_{12} concentrations may not reflect tissue vitamin B_{12} status, especially in infants [2] that MMA is more stable than vitamin B_{12} and that MMA concentrations increase before serum vitamin B_{12} concentrations fall, make circulating MMA an attractive and broadly used biomarker of vitamin B_{12} functional status [1,3–5].

Multiple analytical approaches for the analysis of methylmalonic acid have been reported using a range of sample extraction and analytical techniques, including gas chromatography mass spectroscopy and liquid chromatography tandem mass spectroscopy (LC–MS/MS), with or without sample derivatization [6–10]. While each of these approaches is amenable to clinical research, the reversed phase LC–MS/MS methods yield shorter analytical run times and derivatization appears to enhance analytical sensitivity facilitating the reduction of sample volume requirements [7,8,10].

Minimizing sample size has advantages for applications in population studies, where a variety of assays are often run on a limited amount of collected sample. This issue can be compounded if the study is conducted in neonates and infants, where there is a need for such assays to assess vitamin B_{12} status worldwide [11]. To address this issue in the clinical nutrition research field where there is a need to accurately quantify concentrations in the normal range, such as in supplementation studies, we have validated procedural modifications to the methods reported by Kushnir et al. in 2001 [7] and present a robust method for quantifying MMA as dibutyl esters

Abbreviations: MMA, methylmalonic acid; SA, succinic acid; MMA-$d_3$tri, deuterated methylmalonic acid; CUDA, 1-cyclohexyluriedo-3-dodecanoic acid; LC–MS/MS, liquid chromatography tandem mass spectroscopy; MTBE, methyl tert-butyl ether; UPLC, ultra performance liquid chromatography; ESI, electrospray ionization; API, atmospheric pressure ionization.

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across normal to pathological concentration ranges. The reported 4 min method reduces serum sample requirements to 25 μL.

2. Materials and methods

2.1. Chemicals

Methylmalonic acid was purchased from Sigma–Aldrich (St. Louis, MO) and the tri-deuterated analog MMA-d_3 from Cambridge Isotope Laboratories (Cambridge Isotope Laboratories, Andover, MA). 1-Cyclohexyluriedo-3-dodecanoic acid (CUDA) was obtained from Cayman Chemical (Ann Arbor, MI). HPLC-grade methanol, ammonium formate, succinic acid, phosphoric acid and methyl tert-butyl ether (MTBE) were purchased from Fisher Scientific (Fisher Scientific; Fair Lawn, NJ). Butylation reactions were carried out with reagent grade 3N HCl in n-butanol purchased (Regis Technologies Inc, Morton Grove, IL). 18 MΩ deionized water was obtained from a Barnstead E-Pure deionization system (Barnstead International, Dubuque, Iowa).

2.2. LC–MS/MS instrumentation

Liquid chromatography was performed on an Acquity ultra performance liquid chromatography (UPLC) unit (Waters Corp., Milford, MA) with an Acquity BEH C_{18} column (1.0 mm × 50 mm, 1.7 μm particle size) and inline 0.2 μm stainless steel frit filter (Waters Corp.) held at 60 °C. Samples were maintained at 10 °C within the instrument until injection during all runs. Injection volumes were 5 μL in partial loop mode. Mass spectral ionization, fragmentation, and acquisition parameters were optimized on a tandem quadrupole mass spectrometers using positive mode electrospray ionization (ESI). Initial work was performed on a Micromass Quattro Micro™ atmospheric pressure ionization (API) triple quadrupole tandem mass spectrometer (Waters Corp), and later transferred to an API 4000 QTrap (AB SCIEX, Foster City, CA). Optimized analyte specific and global mass spectrometer parameters for the Quattro-Micro and 4000 QTRAP are shown in Tables S1and S2 in supplemental materials.

2.3. Standards

Stock solutions of MMA and MMA-d_3 were prepared in deionized water and diluted to calibration levels to bracket expected endogenous human MMA values. Calibration standards were aliquoted into polypropylene tubes and stored frozen at −20 °C. An additional set of 1 mL 100 nM MMA-d_3 internal standard subaliquots were also prepared and stored at −20 °C. This solution is referred to as the surrogate spike solution for the remainder of this manuscript. Through the course of method development, the concentration of the surrogate spike solution was reduced from 500 to 100 nM, to limit the introduction of background MMA present in the neat MMA-d_3. For each sample preparation batch, a set of frozen calibrators and an aliquot of surrogate spike solution were thawed. Post-derivatization extract residues were reconstituted in an internal standard solution containing 100 nM CUDA in mobile phase consisting of 53% methanol/47% 1.67 mM (pH 6.5) ammonium formate in deionized water and stored at −20 °C until analysis.

2.4. Quality control

Six point MMA calibration standards were processed with each batch of 60–90 samples and analyzed throughout the data acquisition for that batch. An aliquot of deionized water was used as a reagent blank and aliquots of laboratory reference material Blank Normal Serum (UTAK Laboratories; Inc., Valencia, CA) were prepared as replicate controls, with each batch of samples, and over all studies in our laboratory. The MMA-d_3 surrogate spike solution was analyzed with each batch as a zero level calibration standard, to measure the inherent MMA concentration found in the neat MMA-d_3 received from the manufacturer, and to control for spike solution integrity over time.

The quantification of MMA was performed using the ratio response to MMA-d_3 as a control for extraction and derivatization efficiency, volumetric changes, and ion suppression. The MMA-d_3 surrogate was measured in ratio response to internal standard CUDA. CUDA is in use in our laboratory as a universal internal standard for all LC–MS/MS analyses, including metabolic profiling [12]. This compound is detectable in both positive and negative mode ESI and serves a variety of quality control purposes. These include not only surrogate recovery calculations, but also tracking sample specific ion suppression, volumetric changes, and shot-to-shot drift in instrument sensitivity. In addition, this disubstituted acidic urea is chromatographically sensitive to pH, and thus indicates maintenance of appropriate mobile phase and sample pH. In particular, we have observed shifts in ammonium formate mobile phase pH over time without observable changes in mobile phase volume.

2.5. Sample preparation

Samples were prepared based on methods developed by Magera et al. [13] and modified by Kushnir et al. [7]. Briefly, combinations of a total of 60–90 standards and samples per batch were prepared for analysis by enrichment with MMA-d_3, extracted with acidified MTBE, and derivatized in acidic n-butanol, yielding dibutyl esters. Derivatized samples were dried, reconstituted in mobile phase containing CUDA and 0.1 μm filtered prior to UPLC–MS/MS analysis. Specifically, two sets of pre-labeled 1.5 mL conical polypropylene tubes, 0.1 μm Durapore® PVDF spin filters (Millipore, Billerica, MA) and 2 mL amber vials were prepared for all samples, controls, and standards in each batch. Tubes receiving samples, replicates, and reagent blanks were spiked with 25 μL of 100 nM MMA-d_3 surrogate. A 25 μL aliquot of calibration standard, serum sample, or control was aliquoted into its respective tube. All tubes were vortexed to mix. A 400 μL aliquot of 0.5 M H_3PO_4 in MTBE was added to each tube, which were vortexed for 2 min followed by a 3 min centrifugation to separate residual water. A 300 μL aliquot of the supernatant, taken from the top layer was transferred to the second set of polypropylene tubes and the solvent was evaporated to residue (10–15 min) by centrifugal vacuum evaporation in a Savant SVC200H SpeedVac (Savant Instrument Inc., Holbrook, NY). Residues were reconstituted in 40 μL of 3 N HCl in n-butanol, vortexed to mix, and incubated in a 60 °C water bath for 30 min to produce n-butyl esters of extracted free acids. Solvent was removed by centrifugal vacuum evaporation in an acid resistant system, as above. Residues were then reconstituted in 100 μL of 100 nM CUDA in mobile phase and vortexed to assist dissolution. Extracts were transferred to spin filter tubes, spun for 3 min at 4500 rcf, transferred to glass inserts in amber 2 mL autosampler vials, capped, and stored at −20 °C until analysis.

2.6. Blood sampling and storage

Venous blood was collected from rural Mexican women (n = 87) selected to have similar proportions of subjects with B12 deficiency (<150 pmol/L), marginal status (150–221 pmol/L) and adequate status (>221 pmol/L). The subjects were all treated with a single i.m. dose of vitamin B12 (1 mg) followed by 500 μg/d oral supplementation for 3 months. Fasting serum samples were collected at baseline and 3 months, serum was isolated and stored at <−20 °C until analysis. All subjects gave written consent and the human subjects research protocols were authorized by the University of Califor-

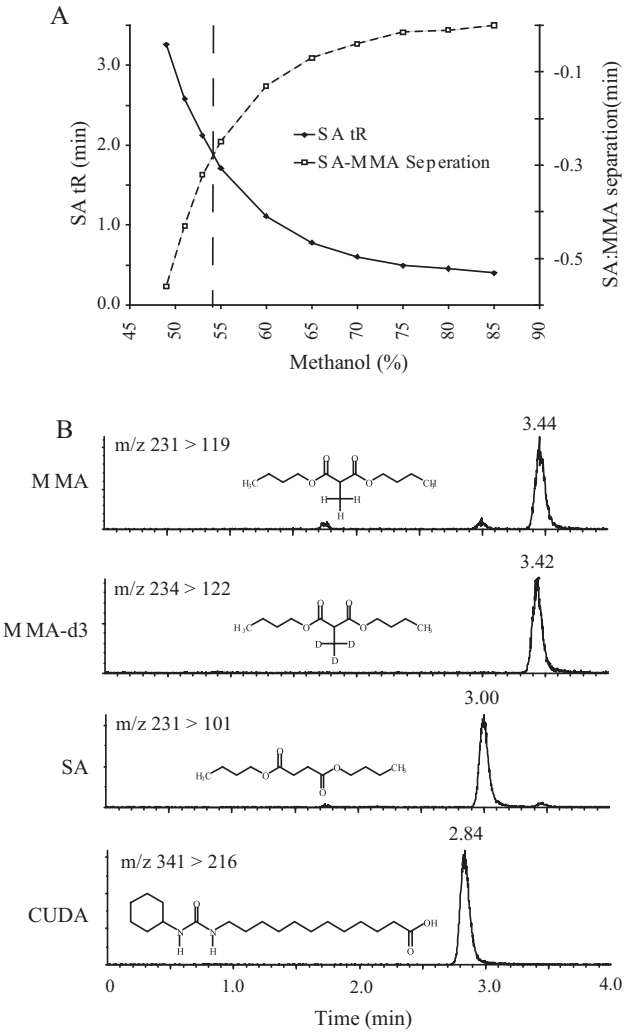

Fig. 1. Optimization of chromatographic conditions. Chromatographic resolution of succinic acid (SA) and methylmalonic acid (MMA) n-butyl esters can be achieved using mobile phase methanol concentrations below 55% (A) resulting in optimal chromatographic resolution (B) of these compounds and the associated deuterated surrogate (MMA-d_3) and internal standard (CUDA).

nia, Davis Institutional Review Board. Only randomized subjects receiving vitamin B12 supplementation are considered here.

3. Results

3.1. Derivatization conditions

Derivatization time and temperature parameters were assessed to establish procedure robustness. Derivatization by 3 N HCl in n-butanol was complete after 30 min incubation in a 60 °C water bath. Extending the time to 60 min showed no increase in final sample concentration, while a time of 15 min reduced recovery.

3.2. Chromatographic resolution of SA and MMA

Succinic acid and methylmalonic acid are closely eluting geometric isomers with confounding spectral fragmentation patterns, such that SA co-elution with MMA will artificially elevate MMA measurements. Chromatographic resolution of SA and MMA was achieved using a methanol/ammonium formate mobile phase. Decreasing methanol concentration improved SA and MMA resolution, while increasing chromatographic run time (Fig. 1). A final

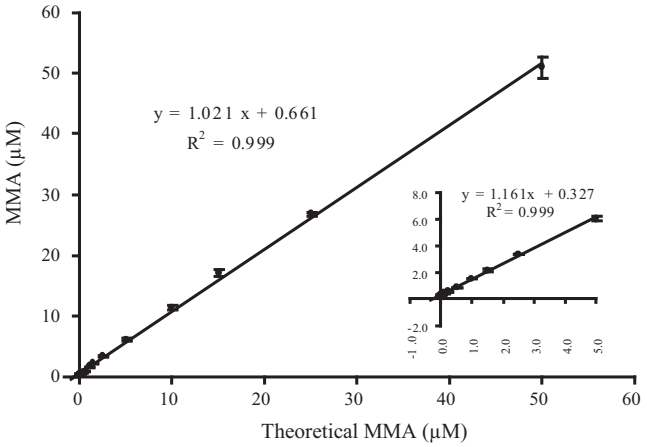

Fig. 2. Standard addition for 0–50 μM MMA in serum quantified through water based standards and plotted against the theoretical concentrations. The insert shows regression through the *x*-axis.

mobile phase composed of 53% methanol + 47% 1.67 mM (pH 6.5) ammonium formate offered the most efficient separation, with baseline resolution in the shortest time and narrowest analyte peak widths. These results showed a slight improvement in resolution and run time, reported by Schmedes and Brandslund, using a formic acid/acetonitrile gradient on a 3.5 μm C18 column of equivalent dimensions [14]. Optimal chromatographic separations without UPLC over-pressuring occurred with a flow rate of 0.2 mL/min isocratic and a column temperature of 60 °C. A final run time of 4 min was achieved as shown in Fig. 1.

3.3. Water- verses serum-based calibration solutions

Multiple methods published for MMA analyses use calibration standards prepared in dialyzed or diluted plasma [6–9,14]. To evaluate the need for this procedure we conducted a standard addition experiment in UTAK reference serum and quantified this with a water-based calibration at the same concentrations. Serum was enriched with MMA at 13 concentrations (0–50 μM) and MMA-d_3 surrogate, at a 1:1 matrix to water dilution reflecting the matrix dilution in our sample work-up. Calibration standards were prepared in deionized water at concentrations equivalent to the serum enrichment. Each standard and standard addition was injected 3 times. MMA concentrations in the standard addition solutions were quantified through the water-based calibration standard curve in ratio response to MMA-d_3 and plotted against the MMA theoretical concentrations on a linear curve (Fig. 2). The correlation coefficient was $R^2 = 0.9988$, showing a high degree of linearity over the concentration range 0–50 μM. The slope of 1.0213 indicates a negligible matrix affect on response, or subsequently quantitation of MMA in serum from the use of water-based standards. To avoid bias due to weight of high calibration standards we only used the 0–1.5 μM standard addition values for determining UTAK serum concentration. Regression through the *x*-axis of triplicate standard addition curves from 0 to 5000 nM yielded a back-calculated value of 141 ± 21 nM MMA in UTAK serum.

3.4. Signal to noise and surrogate background

The range of signal to noise measurement in 100 random samples ranging in concentration from 109–8130 nM was 157–19,100. Fig. 3 shows these results overlaid with calibration standards, which were nicely nested within the sample scatter plot. The lowest calibration standards with an effective concentration of 50 nM MMA have an average signal to noise of 31. Therefore, the

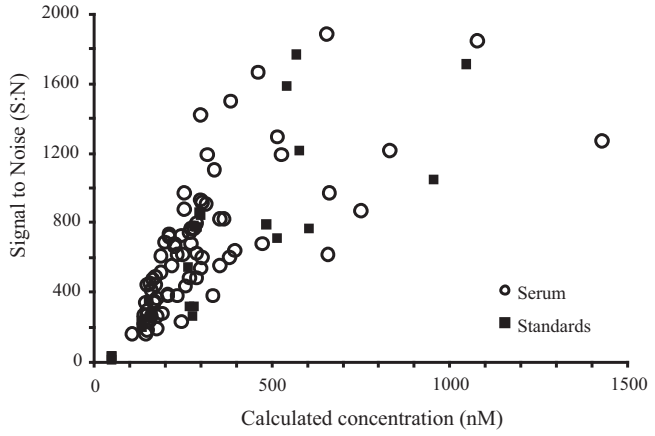

Fig. 3. Sample and standard signal-to-noise measurements versus concentration.

reported method is suitable for use in samples with low MMA concentrations. Traces of MMA were detected in surrogate solutions prepared from neat MMA-d_3, as documented by the presence of an MMA background in our zero concentration level calibration standard. This background contamination was determined at 10% of the molar surrogate spike concentration. To reduce the introduced MMA background to less than 10% of our UTAK serum MMA concentration, surrogate spike concentrations were set at 100 nM.

3.5. Assay performance, precision and accuracy

Shot-to-shot variance in CUDA area response over an analytical run of 100 injections showed a CV of 12%. Overall MMA-d_3 recovery was 93% with a CV = 14%. The MMA assay variability assessments for replicate aliquots of the UTAK serum reference material run in different sample batches over 3 yrs are shown in Table 1. Intra-assay CV ranged from 3 to 8% with average concentrations between 118 and 148 nM. Inter-assay and long-term variability were statistically equivalent with concentrations of 138 and 140 nM, respectively, each with a CV of 9%. These measurements show high method accuracy when compared with the 140 ± 21 nM concentration obtained by standard addition experiments.

3.6. Freeze/thaw stability

The effect of multiple thaws on MMA concentrations in UTAK serum stored at −20 °C was tested with 5 thaws in triplicate. No significant difference was observed and an overall CV for the experiment was 6.1% with an overall average of 150 ± 9.1 nM.

3.7. Application to human samples

Prior to supplementation, 61% of the rural Mexican women had serum MMA concentrations in the normal range reported for individuals in the United States. Supplementation shifted the frequency distribution, such that serum MMA concentrations were normal in 92% of the subjects (Fig. 4).

Table 1
Variance in UTAK replicate analyses over 3 yrs.

UTAK REPs	MMA (nM)	CV (%)	Accuracy (%)
Intra-assay variability			
Batch 1 (n = 7)	148 ± 7.7	5	105
Batch 2 (n = 8)	147 ± 3.8	3	105
Batch 3 (n = 5)	143 ± 11	8	102
Batch 4 (n = 5)	118 ± 4	3	84
Batch 5 (n = 5)	135 ± 6.3	5	97
Inter-assay variability (n = 5)	138 ± 12	9	99
Long-term variability (n = 30)	140 ± 12	9	100
Standard addition (n = 3)	140 ± 21	15	

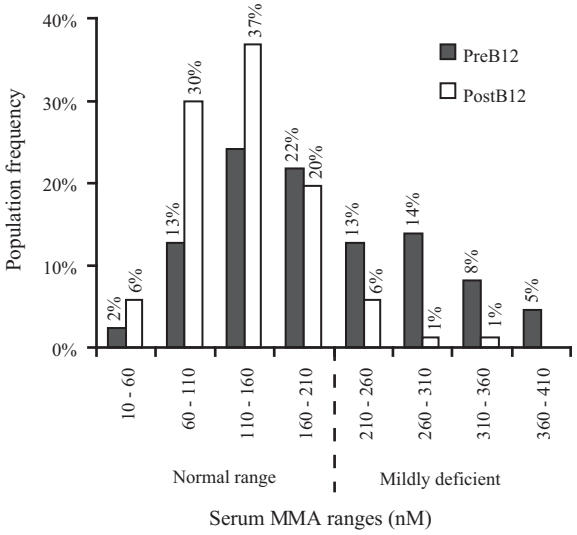

Fig. 4. Vitamin B12 supplementation shifted the serum MMA frequency distribution in a rural Mexican population with a high prevalence of mild to moderate vitamin B12 deficiency into the normal range of the US population.

4. Discussion

The clinical measurement of functional vitamin B12 status is an important aspect of nutritional assessment. Serum methylmalonic acid is an important means of making this evaluation and a variety of methods have been developed for MMA measurement. Many factors impact the utility and selection of an analytical method, including resource demand and available instrumentation. In our case the sample volume is the limiting factor.

Previous methods for MMA analysis in fresh serum or plasma have used sample volumes of between 100 and 1000 μL [6–9,13–15]. In this report, we have concentrated our efforts to refine accepted protocols and produce a robust method to measure MMA in a minimum sample volume with maximum data quality. Using LC–MS Sample reduction is limited by sample heterogeneity, measurement precision, and analytical signal to noise. To accomplish our goal to produce a robust method for the analysis of MMA levels in the normal range in sample limited human studies, we focused our efforts on measurement precision and noise reduction, while simplifying sample workup and expanding method quality control measures. Specifically, noise was reduced by optimizing the chromatographic resolution of MMA from spectral interferences and increasing the dilution of eluted analytes by 15-fold over the foundation method [7]. Similar reverse phase chromatographic resolution has been achieved by others using an alternative solvent system [14]. Moreover, we found that calibrants could be prepared in water and stored at −20 °C rather than dialyzed plasma, which not only simplified method implementation but also reduced analytical noise. Water-based calibrants stored at −80 °C (data not shown) showed unacceptable variability in measurement as reported by Schmedes and Brandslund for −20 °C [14]. As hypothesized by these researchers, we believe this may be due to adsorption to storage container surfaces. Similar difficulties are not observed in plasma samples stored at these temperatures.

Data quality can be compromised through variance associated with sample collection, storage, processing and analysis. Therefore, robust quality control procedures are needed. Storage-dependent changes in analyte concentrations are difficult to identify after the fact. Therefore, during the course of this study, we evaluated the

impact of freeze/thaw cycling as well as long term (>3 yrs) storage of serum at −20 °C on MMA concentrations. We found concentrations to be stable, arguing that samples retrieved from long term storage are amenable for accurate MMA measurements. Similarly, a recent report by Reineks et al. found that up to 1 week storage of serum at room temperature and without light protection did not impact MMA concentrations [16]. This is an especially important finding with regards to sample handling in remote locations. While the use of analytical surrogates allow for the correction of procedural variances, additional internal standards are needed to track surrogate recoveries, *i.e.* method performance. We have found that CUDA provides an ideal internal standard for a wide variety of assays, and its inclusion here allowed documentation of MMA-d_3 recoveries of 93% with a CV of 14%, providing further support of the method robustness.

A driving factor in Kushnir's method development was to reduce analytical run time. To this end, a MMA and SA spectral deconvolution approach was developed using a "branching ion ratio" method, which allowed the compression of the analysis runtime from 3 min [13] to 60 s, without chromatographic separation of MMA and SA [7]. The branching ion ratio approach corrects for the interfering spectral peak of SA with MMA and had a reported success rate of 100% for samples with MMA concentrations ≥1500 nM [13]. However, 10.8% of all samples analyzed by Kushnir failed due to 'unacceptable qualitative ion ratios' and 'needed to be reanalyzed by a method that chromatographically resolves MMA and SA.' [7] Evaluating the branching ion ratio method versus the optimized chromatographically resolved SA and MMA method reported here, the unresolved approach leads to a systematic overestimate of ∼46% in the UTAK laboratory reference material, which contains 140 ± 12 nM MMA (Table 1). According to a recent review circulating MMA concentrations as low as 400 nM indicate a mild vitamin B_{12} deficiency [17] while the Center for Disease Control has reported the normal range serum MMA to be 60–210 nM [18]. These levels also compare well with reference ranges reported across subjects from 0 to >71 yrs of age [19]. Therefore, it is clear that for nutritional assessment and intervention studies of populations with marginal vitamin B12 status chromatographic resolution of MMA and SA, as reported by us and others using reverse phase [10,14] or HILIC hydrophilic interaction liquid chromatography [9] is necessary. Applying the current method to the analysis of samples from a population with mild to moderate vitamin B12 deficiency before and after supplementation shows its ability to perform in the low normal range of human serum MMA concentrations.

5. Conclusion

Existing methods were optimized to allow the reliable and efficient measurement of MMA with as little as 25 μL of serum, a 4–40-fold reduction in sample use as compared to similar methods. The resulting signal:noise indicates that with precision in sample aliquoting, such as achievable with robotic liquid handlers, this method can be used with as little as 5 μL of serum. With proper handling, water-based calibration standards stored at −20 °C reliably quantify samples, suggesting that the common practice of preparation of calibrants in diluted or dialyzed plasma is unnecessary. An internal standard, CUDA, was included to allow determination of MMA-d_3 surrogate recoveries and method performance. The chromatographic overlap of the dibutyl esters of succinate and methylmalonate was resolved in a 4 min analysis. Consequently, there was no need to deconvolute the spectral overlap of these compounds, a procedure which gave systematically inaccurate values in the normal range. The longitudinal analysis of sub-aliquots of a single laboratory reference material over a 3 yr period shows the method to be robust. The reported method is highly suitable for the investigation of populations with normal to deficient levels of vitamin B12, especially where sample volumes are limited.

Conflict of interest statement

The authors do not have associations of a commercial or other nature that might pose a conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.03.039.

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