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An LC-MS/MS method for serum methylmalonic acid suitable for monitoring vitamin B12 status in population surveys¹

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Abstract

Methylmalonic acid (MMA), a functional indicator of vitamin B₁₂ insufficiency, was measured in the U.S. population in the National Health and Nutrition Examination Survey (NHANES) from 1999–2004 using a GC/MS procedure that required 275 μ L of sample and had a low throughput (36 samples/run). Our objective was to introduce a more efficient, yet highly accurate LC-MS/MS method for NHANES 2011–2014. We adapted the sample preparation with some modifications from a published isotope-dilution LC-MS/MS procedure. The procedure utilized liquid-liquid extraction and generation of MMA di-butyl ester. Reversed-phase chromatography with isocratic elution allowed baseline resolution of MMA from its naturally occurring structural isomer succinic acid within 4.5 min. Our new method afforded an increased throughput (160 samples/run) and measured serum MMA with high sensitivity (LOD = 22.1 nmol/L) in only 75 μ L of sample. Mean (\pm SD) recovery of MMA spiked into serum (2 days, 4 levels, 2 replicates each) was 94 \pm 5.5%. Total imprecision (41 days, 2 replicates each) for three serum quality control pools was 4.9–7.9% (97.1–548 nmol/L). The LC-MS/MS method showed excellent correlation ($n=326$, $r=0.99$) and no bias (Deming regression, Bland-Altman analysis) compared to the previous GC/MS method. Both methods produced virtually identical mean (\pm SD) MMA concentrations [LC-MS/MS: 18.47 \pm 0.71 ng/mL ($n=17$), GC/MS: 18.18 \pm 0.67 ng/mL ($n=11$)] on a future plasma reference material compared to a GC/MS method procedure from the National Institute of Standards and Technology [18.41 \pm 0.70 ng/mL ($n=15$)]. No adjustment will be necessary to compare previous (1999–2004) to future (2011–2014) NHANES MMA data.

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Keywords

MMA; method comparison; GC/MS; anticoagulant types; NHANES; NIST SRM 1950; vitamin B₁₂

Introduction

Cobalamin (vitamin B₁₂) is an essential cofactor for 2 enzymes involved in one-carbon metabolism: methylmalonyl CoA mutase and methionine synthetase [1]. Reduced function of the former enzyme results in increased serum methylmalonic acid (MMA) concentrations. Untreated vitamin B₁₂ deficiency leads to megaloblastic anemia and/or irreversible central nervous system degeneration [2]. Serum or plasma vitamin B₁₂ concentrations, particularly in the low-normal range, may not adequately reflect tissue vitamin B₁₂ status [3]. Increased MMA concentrations are specific for and believed to be the first indication of vitamin B₁₂ deficiency [4]. MMA in serum or plasma was therefore found to be a useful indicator of vitamin B₁₂ deficiency, especially in patients with few or no hematological abnormalities, normal results for the Schilling test, or normal or only slightly depressed serum vitamin B₁₂ concentrations [5].

Various analytical methods for the measurement of MMA in biological fluids have been reported, mainly since the 1980s. Different approaches were taken for sample preparation (e.g., ultrafiltration, solid-phase or liquid-liquid extraction) and analytical measurement (e.g., GC/MS, electrophoresis, and more recently LC-MS/MS) [6–19]. The analysis of serum MMA presents several challenges: this dicarboxylic acid has a small molecular weight, is hydrophilic, non-volatile, and its concentration in serum is relatively low, especially in the generally healthy population with a low prevalence of vitamin B₁₂ deficiency. This makes direct analysis by GC/MS challenging and various types of derivatization (silylation, cyclohexanol, chloroformate and butanol) have been reported to improve detection [6–9]. LC-MS/MS methods [10–18], yield shorter run times and better sensitivity compared to GC/MS procedures. These two factors are critical when a method is used for population monitoring because they translate to higher throughput and lower patient sample volume.

The NHANES has measured plasma MMA concentrations in the U.S. population from 1999–2004 using a GC/MS procedure that required 275 μ L of sample and had a low throughput (36 samples/run) [19]. No MMA data are available for 2005–2010. A 2010 expert roundtable discussing “NHANES Monitoring of Biomarkers of Folate and Vitamin B-12 Status” recommended that serum MMA measurements be reinstated in future NHANES in conjunction with serum vitamin B₁₂ measurements [20]. Our primary objective was to introduce a more efficient, yet highly accurate LC-MS/MS method for NHANES 2011–2014. Our secondary objective was to compare this new method to the previously used GC/MS procedure in order to provide continuity in interpreting NHANES data. Lastly, we aimed to assess how these two procedures compare to the GC/MS measurement procedure used by the National Institute of Standards and Technology (NIST).

Materials and methods

Chemicals, reagents, and samples

MMA, succinic acid, *tert*-butyl methyl ether (MTBE) and 3 M hydrochloric acid in *n*-butanol were purchased from Sigma-Aldrich (St. Louis, MO). Deuterium-labeled MMA (d_3 -MMA) was purchased from CDN Isotopes (Quebec, Canada). ACS grade methanol, phosphoric acid (H_3PO_4), glacial acetic acid and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Purified water (18 M Ω) was obtained from an Aqua Solutions water purification system (Aqua Solutions, Inc., Falmouth, ME) and was used to prepare all samples, calibrators and reagents. Quality control (QC) pools (low, medium and high) were prepared in-house from pooled human serum purchased from a U.S. blood bank. All units were screened for MMA and spiked with MMA standard solution as needed to achieve desired concentrations. All specimens were stored at $-70\text{ }^\circ\text{C}$ when not in use.

Sample preparation and analysis by LC-MS/MS

Samples were prepared by a modified procedure reported by Pedersen *et al.* [16]. Master stock solutions of MMA and d_3 -MMA were prepared in water (50 $\mu\text{mol/L}$). Six individual MMA working calibrators were prepared from the stock solution in water (25–2500 nmol/L) and stored (as 400 μL aliquots) at $-70\text{ }^\circ\text{C}$ until analysis. We prepared a fresh working internal standard (ISTD) solution (1 $\mu\text{mol/L}$) from the d_3 -MMA stock solution on the day of analysis in 50% methanol/water. The sample preparation steps involved liquid-liquid extraction and generation of MMA di-butyl ester (Fig. 1). MMA was separated from succinic acid and from other compounds on an Accela UHPLC system (Thermo Fisher Scientific, Waltham, MA) by isocratic elution (0.1% acetic acid:methanol 40:60; 0.4 mL/min) within 4.5 min on a Hypersil Gold C18 analytical column (2.1 mm \times 50 mm, 1.9 μm particle size) (Thermo Fisher Scientific) held at $35\text{ }^\circ\text{C}$. The total run time was 6 min. Samples were maintained at $7\text{ }^\circ\text{C}$ within the instrument during the run. Injection volume was 3 μL in a partial loop mode. Mass detection was carried out on a TSQ Vantage (Thermo Fisher Scientific, Waltham, MA) under multiple-reaction monitoring (MRM) conditions in positive electrospray ionization (ESI) mode. The mass spectrometer parameters were as follows: spray voltage 4500 V, vaporizer temperature $350\text{ }^\circ\text{C}$, nitrogen sheath gas pressure 30 arbitrary units, auxiliary gas flow 5 arbitrary units, ion transfer capillary temperature $290\text{ }^\circ\text{C}$, argon collision gas pressure 1.2 mTorr, scan time 200 ms, scan width 0.1 Da, mass resolution in Q1 and Q3 0.7 full width at half maximum. We monitored two transitions each for MMA (m/z 231 \rightarrow 119 and 175.1) and for d_3 -MMA (m/z 234.1 \rightarrow 122.1 and 178.1) and used the sum of the two signals for quantitation, MRM mode. We also used the two individual transitions in selected reaction monitoring (SRM) mode to calculate confirmation ion ratios. In this calculation, the more abundant transition (m/z 231 \rightarrow 119) was the quantitation ion (Q_{SRM}), while the less abundant transition (m/z 231 \rightarrow 175.1) was the confirmation ion (CI_{SRM}). We established confirmation ion ratio ($CI_{\text{SRM}}/Q_{\text{SRM}}$) limits (mean \pm 2 SD) for MMA from repeated analysis of the calibrators ($n = 20$). Quantitation was based on peak area ratios [analyte/internal standard (ISTD)] interpolated against a six-point aqueous calibration curve (1 \times weighting), using XCalibur software (Thermo Fisher Scientific, Waltham, MA). We reinjected the calibrators at the end of each run to assess drifting. Each run consisted of a reagent blank (no ISTD), a blank (with ISTD), a set of

calibrators, three serum QC samples prepared in duplicate (bracketing the unknown samples) and 1 blind QC sample for every 20 unknown samples (see Electronic Supplementary Material **Fig. S1**).

Method validation

We used the FDA “Bioanalytical Method Validation” and IUPAC validation guidelines [21, 22] as well as our internal division document “Policies and Procedures Manual for Bioanalytical Measurements” to design our method validation experiments. We evaluated calibrator accuracy for 10 runs by calculating the proportion of measured to nominal calibrator value. We assessed the effect of the presence of matrix on the calibration curve from a 10-point calibration curve (0–2500 nmol/L, 3 independent calibration curves) prepared both in water and in human serum. Slopes that agreed within $\pm 5\%$ were considered to be equivalent. We determined method imprecision using 3 levels of serum QC pools: within-run, 10 replicates/level in one run; total and between-run, 2 replicates/level over 10 runs. We assessed method accuracy through spike recovery: a serum pool was spiked with MMA at 4 levels (2 runs, 2 replicates/level: 50, 250, 700, and 1000 nmol/L). We also measured the unspiked serum pool for endogenous MMA concentration (2 runs, 2 replicates each). To assess spike recovery, we added the ISTD to the samples and carried them through the extraction process. To assess extraction efficiency, we added the ISTD after extraction was completed. The spike recovery and the extraction efficiency were calculated as the measured concentration difference between the spiked and the unspiked sample divided by the nominal concentration of the spike. We estimated the limit of detection (LOD) by serially diluting the low QC pool with charcoal stripped serum (Aalto Scientific, Carlsbad, CA) and calculating the SD at concentrations of zero (σ_0) from the extrapolation of repeat measurements (3 runs, 3 replicates/dilution) [23]. We defined the LOD as $3\sigma_0$.

MMA stability

We exposed 3 serum QC pools (97.1, 247 and 548 nmol/L) to room temperature for 1, 2, 3 and 7 days to assess short-term MMA stability. The vials were returned to $-70\text{ }^\circ\text{C}$ storage and later all samples were processed and analyzed together with pristine serum QC pools kept at $-70\text{ }^\circ\text{C}$ as a reference. We also subjected the same QC pools to repeated freeze-thawing (up to 3 cycles, 2 hours/cycle at room temperature in closed vials). We studied the stability of processed serum extracts for the above set of serum QC pools kept in the autosampler compartment at $7\text{ }^\circ\text{C}$ for 3 days. In a separate experiment we kept the 96-well plate containing processed serum QC pools and 25 unknown samples at $-20\text{ }^\circ\text{C}$ for 1 week and compared the results to the initial results. We assessed mid-term (6 month and 1 year) MMA stability using three plasma QC pools (179, 515 and 2007 nmol/L, 3 runs, 1 replicate/condition) stored at suboptimal temperatures ($5\text{ }^\circ\text{C}$ and $-20\text{ }^\circ\text{C}$). The vials were returned to $-70\text{ }^\circ\text{C}$ storage and later all samples were processed and analyzed together with pristine plasma QC pools kept at $-70\text{ }^\circ\text{C}$ as a reference. Long-term (14 years) MMA stability at $-70\text{ }^\circ\text{C}$ was assessed from six plasma QC pools (120, 137, 1079, 1825, 10230 and 10295 nmol/L, 2 runs, 5 replicates/level).

Effect of specimen type and anticoagulant

To study the effect of different anticoagulants, we used matched serum and plasma samples from 14 anonymous blood donors [serum, serum separator, K₂EDTA plasma, Na citrate plasma and Na heparin plasma (5-mL blood collection tubes with 0.5 mL anticoagulant), Tennessee Blood Bank Services (Memphis, TN)]. Plasma was obtained within 2–4 hours of blood collection (blood held at room temperature), serum after overnight clotting (leading to higher serum yield and less residual fibrinogen clots) at room temperature through centrifugation at 4 °C, following standard operating procedures. All specimens were refrigerated, shipped on cold packs and frozen at –70 °C within 48 hours of blood collection. Serum was used as a reference. Because of the diluent in the plasma citrate tubes, we multiplied results for this specimen type by 1.1.

Method comparison studies

We performed three comparison studies to assess how the new LC-MS/MS method compared to other methods. First, we subjected randomly selected residual NHANES plasma samples ($n = 336$; MMA: 60–2320 nmol/L) previously analyzed with our GC/MS method to analysis by our new LC-MS/MS method. We excluded seven extreme outlier results (difference between the two procedures > 40%) and three samples with MMA concentrations above 1500 nmol/L to avoid an undue influence of very high concentrations. The final set ($n = 326$; MMA: 60–1180 nmol/L) was evaluated for Pearson correlation, Deming regression, and Bland-Altman analysis using Analyse-it, a statistical plug-in for Microsoft Excel (Analyse-it version 2.20, Analyse-it Software, LTD, Leeds, U.K.). Second, we subjected eight serum and 11 plasma samples (stored at –70 °C for 14 years) from a previous international round-robin study [24] to analysis by our new LC-MS/MS method and compared the results to the consensus mean of all 13 participating laboratories and to the our previous GC/MS results. Third, we characterized the plasma-based NIST Standard Reference Material (SRM) 1950 “Metabolites in Human Plasma” by our LC-MS/MS and GC/MS procedures (replicate analyses of 6 vials over 3 days). This material was also characterized by a NIST GC/MS measurement procedure that has been used in value assignment of NIST reference materials. For all three methods we calculated the mean \pm SD and the coefficient of variation (CV). This evaluation was another accuracy assessment of the new LC-MS/MS method.

Results and discussion

The sample preparation procedure (adapted from Pedersen *et al.* with some minor modifications) involved liquid-liquid extraction of MMA from serum, followed by derivatization of MMA to dibutyl ester and analysis by positive ion ESI-LC-MS/MS.

We optimized and tested critical sample preparation steps. Because the addition of acid to the organic solvent improves the transfer of organic acid from the aqueous to the organic phase, we tested extraction efficiency with different concentrations of o-phosphoric acid (0.1, 0.25 and 0.5 M) on a set of calibrators and QC samples. We obtained poor MMA extraction with 0.1 M o-phosphoric acid in MTBE and selected the 0.5 M acid concentration because it provided optimum MMA extraction from the aqueous phase (data not shown).

Placing the tubes into a dry ice/ethanol bath was a fast and efficient way to separate the organic from the aqueous layer by simply decanting the top (MTBE) layer after the bottom (aqueous) layer froze. This process happened very quickly and small variations in the amount of time the samples were kept in the ice bath (1, 2, or 3 min) did not influence the MMA areas in a set of calibrators and QC samples. Because the derivatization of extracted MMA to the dibutyl ester (at 60 °C) is a critical step, we studied the timing on the heating block to monitor reaction completion (20, 30, and 40 min). The use of excess of derivatization reagent and a 30 min incubation time assured that even samples with high MMA concentrations (above 10,000 nmol/L) completed the reaction. The derivatized samples have to be subjected to evaporation to remove the excess of the high boiling derivatization reagent (3 M HCl in *n*-butanol). In our experience longer evaporation times, involving vacuum and heat, can reduce the recovery of the volatile MMA dibutyl ester (optimum evaporation time is 30 min at 45 °C under vacuum, data not shown).

One important challenge in the analysis of serum MMA is the potential interference from other low-mass carboxylic acids, especially the structural isomer succinic acid, which is present in serum in high concentrations. Because the mass spectra of the two acids are nearly identical, MMA and succinic acid have to be separated chromatographically. To achieve the best resolution, we tested several reversed phase analytical columns (pentafluorophenyl, cyano, and C18). We selected the C18 UHPLC column because it allowed excellent resolution between MMA and succinic acid with relatively fast isocratic chromatography, narrow analyte peaks and excellent column life (typically ~2000 injections) (Fig. 2). Our LC-MS/MS method offers an additional degree of specificity because it features data collection from two specific MMA transitions (QI_{SRM} and CI_{SRM}). We obtained fairly low ($\pm 8\%$) variation of the confirmation ion ratio when we monitored these two ions in all 6 calibrators over 20 runs, particularly compared to common “industry standards” (FDA: $\pm 10\%$ [25]; Society of Forensic Toxicologists: $\pm 20\%$ to $\pm 30\%$ [26]; European Commission: $\pm 20\%$ to $\pm 50\%$ depending of the abundance of CI_{SRM} vs. QI_{SRM} [27]). When we applied the confirmation ion ratio limits to MMA results > 150 nmol/L obtained from 600 serum samples, 99% of samples met the predetermined limits. This indicates that our confirmation ion ratio limits, while narrow, can be used in routine applications to allow identification of a potentially interfering compound that may be present in a sample.

Our previous GC/MS procedure [19], while performing reliably for the measurement of thousands of NHANES samples, was not an efficient method. The sample volume requirement was 275 μ L/test, the GC/MS run time was 15 min, the throughput was limited to 36 samples/run, and the sample preparation time for one run was ~12 hours (Table 1). With the new LC-MS/MS method we achieved our main goal to improve the method efficiency. The LC-MS/MS method used a much reduced sample volume (75 μ L), allowed a notably faster sample preparation time (~4 hours) and LC-MS/MS run time (6 min), and had a much higher sample throughput (up to 160 unknown samples/run).

We subjected our LC-MS/MS method to complete method validation. Ion suppression was evaluated by post-column infusion (using a T-connection) of 50 nmol/L MMA-dibutyl solution while injecting a reagent blank, a blank, standards, unknown and QC samples. No

baseline drifts were observed around the elution time of MMA upon injection of matrix and non-matrix samples indicating lack of ion suppression. We verified and confirmed that aqueous calibration produced an equivalent slope (0.0025, $r^2 = 0.999$) compared to calibration in serum (0.0024, $r^2 = 0.994$). The average slopes differed by 4.1%. Due to matrix equivalency, we prepared many aliquots of aqueous calibrators, stored them at -70 °C, and used a new set with each run, which greatly simplified method implementation and daily routine preparations for the analyst. The aqueous calibration curve showed linearity over two orders of magnitude (25–2500 nmol/L) with a high correlation coefficient ($r^2 = 0.999$). Some investigators reported storage at -20 °C as preferred over storage at -80 °C (for aqueous calibrators [16] or for serum-based calibrators [12]) due to possible MMA absorption to the storage container surface. We have not observed any change in method performance with aqueous calibrators stored in 2-mL Nalgene vials for up to 2 years at -80 °C (data not shown).

Our LC-MS/MS method achieved good precision with a total CV of 4.9–7.9% for three serum QC pools analyzed over 22 runs (Table 2), similar to the precision of the GC/MS method (CV of 5.4 % for three plasma QC pools analyzed over 56 runs, [19]) and to other LC-MS/MS methods [10–18]. We can rate our method precision more objectively by using generally applicable quality goals based on biologic variation [28]. As such, the analytical variation CV_a should be a fraction of the within-person biologic variation CV_w : optimum performance, $CV_a = 0.25 \times CV_w$, desirable performance, $CV_a = 0.5 \times CV_w$, minimum performance, $CV_a = 0.75 \times CV_w$. Lacher *et al.* reported a within-person biologic variation of 18.7% for MMA using data from NHANES 1999–2002 [29]. Therefore, the optimum, desirable, and minimum precision performance criteria for MMA are $< 4.7\%$, $< 9.4\%$, and $< 14\%$, respectively. Our LC-MS/MS method achieved desirable precision.

We evaluated method accuracy through spike recovery. The use of an isotopically-labeled ISTD is expected to correct for any loss of analyte during sample handling. The high accuracy assessed through spike recovery for the LC-MS/MS method ($94\% \pm 5.5\%$) was comparable to that reported for the GC/MS method ($96\% \pm 1.9\%$, [19]) and to other LC-MS/MS methods [10–18]. Our LC-MS/MS method also achieved a good extraction efficiency of 81%, as assessed from post-extraction addition of ISTD.

The LC-MS/MS method sensitivity, as expressed by the LOD (22.1 nmol/L), was improved compared to our previous GC/MS method (50 nmol/L) and commensurate or better compared to other LC-MS/MS methods [11, 13, 14, 15]. The use of MRM in our LC-MS/MS method allowed us to monitor a cumulative signal for quantitation (sum of quantitation and confirmation ion areas). This produced a notable increase in signal, while keeping the background approximately the same. The confirmation ion area contributed ~60–85% of the quantitation ion area. Thus, MRM helped to improve our method detection limit. The improved sensitivity of our new LC-MS/MS method and the small sample volume requirement (75 μ L) allowed the accurate measurement of MMA at normal concentrations encountered in population surveys. Low-volume samples (< 100 μ L) that require confirmation can be diluted with deionized water and reanalyzed.

Plasma can form micro-fibrinogen clots during long-term storage, therefore serum is generally preferred. After we adjusted for the dilution in the plasma citrate tube, we found interchangeable results among the matched serum (plain and serum separators tubes) and plasma (EDTA, Na heparin, and citrate) samples ($n = 14$) with a mean difference of $\pm 3\%$ (Fig. 3). This was generally consistent with other reports [11, 18], however Kushnir *et al.* reported an interference with the citrate tube, causing a significant increase of the confirmation ion ratio [11].

We assessed the short-term stability of MMA in serum and in extracted samples, mid- and long-term stability of MMA in refrigerated and/or frozen serum, and the stability of MMA during repeated freeze/thawing of serum, and the short-term stability of MMA in extracted samples. We confirmed previous reports [16, 18] that MMA was stable for up to three freeze/thawing cycles and for up to one week of serum storage at room temperature (data not shown). Given these results, it was not surprising that the short-term stability of MMA in extracted samples (3 days at 7 °C and 1 week at -20 °C) was also very good ($\pm 3\%$ and $\pm 1\%$, respectively from the original value). Furthermore, storage of serum at -20 °C for one year also showed excellent stability with no significant change in MMA concentrations from baseline [mean percent difference (95% CI) among the three serum QC pools: 0.98% (-1.46% to -3.47%), $P = 0.42$]. Storage of serum at 5 °C for one year showed a small but significant increase in MMA [6.59% (2.19% to 11.2%), $P = 0.0048$], possibly due to slight sample evaporation. However, the 6 month time point for storage at 5 °C showed no significant changes. This excellent MMA stability makes it possible to ship serum at ambient temperature to laboratories around the world participating in external quality assessment schemes. Lastly, the long-term (up to 14 years) stability of frozen (-70 °C) plasma QC pools that were originally value assigned by our GC/MS method was also excellent with MMA concentrations being within $\pm 1\%$ of the original target values.

The second objective of this report was to compare our new LC-MS/MS method to the previous GC/MS method as to provide continuity in interpreting NHANES data. The mean \pm SD (nmol/L) MMA concentration in the final set of 326 residual NHANES samples was 236 ± 191 nmol/L by LC-MS/MS compared to 235 ± 186 nmol/L by GC/MS. The two methods showed excellent correlation (Pearson $r = 0.99$). Deming regression analysis showed a significant but small deviation from a slope of 1 and an intercept of 0 and a non-significant bias (Table 3 and Fig. 4). Therefore no adjustments will be needed to compare the NHANES 1999–2004 GC/MS data to the new NHANES 2011–2014 LC-MS/MS data. Using a much smaller sample set from a previously conducted international round-robin ($n = 19$, concentration range: 70–10500 nmol/L, [24]), we also found excellent correlation (Pearson $r = 1.0$) and no bias between our LC-MS/MS method and the earlier generated GC/MS or consensus results from 13 laboratories (Table 3).

At present, there is no serum-based reference material available with certified MMA concentrations. As an additional accuracy evaluation of the new LC-MS/MS method and as part of our third objective, we compared our results by both the LC-MS/MS and the GC/MS method to those obtained by NIST on a future NIST SRM material. SRM 1950 “Metabolites in Human Plasma” is the first reference material that has been characterized for MMA by the NIST GC/MS and CDC GC/MS and LC-MS/MS measurement procedures. The mean \pm

SD (ng/mL) MMA concentrations obtained by the three procedures were virtually identical: CDC LC-MS/MS, 18.47 ± 0.71 ($n = 17$); CDC GC/MS, 18.18 ± 0.67 ($n = 11$); NIST GC/MS, 18.41 ± 0.70 ($n = 15$). The variability (CV) of the three procedures was also very comparable: 3.8%, 3.7%, and 3.8%, respectively.

In summary, with this new LC-MS/MS method we have implemented important features for methods used in large population surveys: low sample volume and high throughput, high accuracy, low imprecision and good sensitivity. Time will show how stable the method will perform long-term. However, so far an intermittent use of the method did not pose any problems in bringing the method back online after several months of pausing. The excellent comparability and bias-free agreement between this method and the NIST GC/MS measurement procedure as well as our previous GC/MS method will facilitate future time trend analyses using NHANES data.

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References

1. Institute of Medicine, Food and Nutrition Board. Washington, D.C.: National Academy Press; 1998. Dietary reference intakes: Thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline.
2. Baik HW, Russel RM. Vitamin B12 deficiency in the elderly. *Annu Rev Nutr.* 1999; 19:357–377. [PubMed: 10448529]
3. Allen LH. Causes of vitamin B12 and folate deficiency. *Food Nutr Bull.* 2008; 29(2 Suppl):S20–S34. [PubMed: 18709879]
4. Moelby L, Rasmussen K, Jensen MK, Pedersen KO. The relationship between clinically confirmed cobalamin deficiency and serum methylmalonic acid. *J Intern Med.* 1990; 228:373–378. [PubMed: 2266346]
5. Rasmussen K, Moller J, Lyngbak M, Holm Pedersen A-M, Dybkjaer L. Age- and gender-specific reference intervals for total homocysteine and methylmalonic acid in plasma before and after vitamin supplementation. *Clin Chem.* 1996; 42(4):630–636. [PubMed: 8605683]
6. Stabler S, Marcell PD, Podell ER, Allen RG, Lindenbaum J. Assay of methylmalonic acid in the serum of patients with cobalamin deficiency using capillary gas chromatography-mass spectrometry. *J Clin Invest.* 1986; 77:1606–1612. [PubMed: 3700655]
7. Rasmussen K. Solid-phase sample extraction for rapid determination of methylmalonic acid in serum and urine by a stable-isotope-dilution method. *Clin Chem.* 1989; 35(2):260–264. [PubMed: 2644059]
8. Windelberg A, Årseth O, Kvalheim G, Ueland PM. Automated assay for the determination of methylmalonic acid, total homocysteine, and related amino acids in human serum or plasma by means of methylchloroformate derivatization and gas chromatography-mass spectrometry. *Clin Chem.* 2005; 51(11):2103–2109. [PubMed: 16123148]
9. Chen J, Bennett M. Quantitation of methylmalonic acid in serum or plasma using isotope dilution-selected ion gas chromatography-mass spectrometry. *Clin Appl Mass Spec Methods Mol Biol.* 2010; 603:365–370.
10. Magera MJ, Helgeson JK, Matern D, Rinaldo P. Methylmalonic acid measured in plasma and urine by stable-isotope dilution and electrospray tandem mass-spectrometry. *Clin Chem.* 2000; 46(11): 1804–1810. [PubMed: 11067816]

11. Kushnir MM, Komaromy-Hiller G, Shushan B, Urry FM, Roberts W. Analysis of dicarboxylic acids by tandem mass spectrometry. High-throughput quantitative measurement of methylmalonic acid in serum, plasma and urine. *Clin Chem.* 2001; 47(11):1993–2002. [PubMed: 11673368]
12. Schmedes A, Brandslund I. Analysis of methylmalonic acid in plasma by liquid chromatography-tandem mass spectrometry. *Clin Chem.* 2006; 52(4):754–757. [PubMed: 16595826]
13. Blom HJ, van Rooij A, Hogeveen M. A simple high-throughput method for determination of plasma methylmalonic acid by liquid chromatography-tandem mass spectrometry. *Clin Chem Lab Med.* 2007; 45(5):645–650. [PubMed: 17484628]
14. Hempen C, Wanschers H. A fast liquid chromatographic tandem mass spectrometric method for the simultaneous determination of total homocysteine and methylmalonic acid. *Anal Bioanal Chem.* 2008; 391:263–270. [PubMed: 18368393]
15. Lakso H-A, Appelblad P, Scgneede J. Quantification of methylmalonic acid in plasma with hydrophilic interaction liquid chromatography separation and mass spectrometric detection. *Clin Chem.* 2008; 54(12):2028–2035. [PubMed: 18845771]
16. Pedersen TL, Keyes WR, Shahab-Ferdows S, Allen LH, Newman JW. Methylmalonic acid quantification in low serum volumes by UHPLC-MS/MS. *J Chrom B.* 2011; 879:1502–1506.
17. Ouweland JMW, Kema IP. The role of liquid chromatography-tandem mass spectrometry in the clinical laboratory. *J Chrom B.* 2012; 883–884:18–32.
18. Yaun C, Gabler J, El-Khoury JM, Spatholt R, Wang S. Highly sensitive and selective measurement of underivatized methylmalonic acid in serum and plasma by liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem.* 2012; 404:133–140. [PubMed: 22618327]
19. Pfeiffer CM, Gunter EW. Automated assay for methylmalonic acid (MMA) in plasma. 51st Annual Meeting of the American Association of Clinical Chemistry (1999). *Clin Chem.* 45(S6):A166. (abstr.593).
20. Yetley EA, Pfeiffer CM, Phinney KW, Bailey RL, Blackmore S, Bock JL, Brody LC, Carmel R, Curtin LR, Durazo-Arvizu RA, et al. Biomarkers of vitamin B-12 status in NHANES: a roundtable summary. *Am J Clin Nutr.* 2011; 94:313S–321S. [PubMed: 21593512]
21. Food and Drug Administration. Rockville: Food and Drug Administration, US Department of Health and Human Services; 2011. Guidance for industry: bioanalytical method validation. <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070336.pdf>
22. International Union of Pure and Applied Chemistry. Harmonized guidelines for single laboratory validation of method of analysis (IUPAC Technical Report). 2002 <http://www.iupac.org/publications/pac/2002/pdf/7405x0835.pdf>.
23. Taylor, JK. Quality assurance of chemical measurement. Michigan: Lewis Publishers; 1987.
24. Pfeiffer CM, Smith SJ, Miller DT, Gunter EW. Comparison of serum and plasma methylmalonic acid measurements of 13 laboratories: an international study. *Clin Chem.* 1999; 45(12):2236–2242. [PubMed: 10585358]
25. Food and Drug Administration. Rockville: Food and Drug Administration, US Department of Health and Human Services; 2003. Guidance for industry: mass spectrometry for confirmation of the identity of animal drug residues. <http://www.fda.gov/cvm/Guidance/guide118.pdf>
26. Forensic toxicology laboratory guidelines. Washington (DC): Society of Forensic Toxicology/ American Academy of Forensic Science; 2002.
27. European Union Decision. 2002/657/EC 17.08.2002: commission decision laying down performance criteria for the analytical methods to be used for certain substances and residues thereof in live animals and animal products. *Off J Eur Commun.* 2002; (221):8–32.
28. Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. *Crit Rev Clin Lab Sci.* 1989; 27:409–437. [PubMed: 2679660]
29. Lacher, DA.; Hughes, JP.; Carroll, MD. National Health Statistics Reports. Vol. 21. Atlanta: Centers for Disease Control and Prevention; 2010. Biological variation of laboratory analytes based on the 1999–2002 National Health and Nutrition Examination Survey.

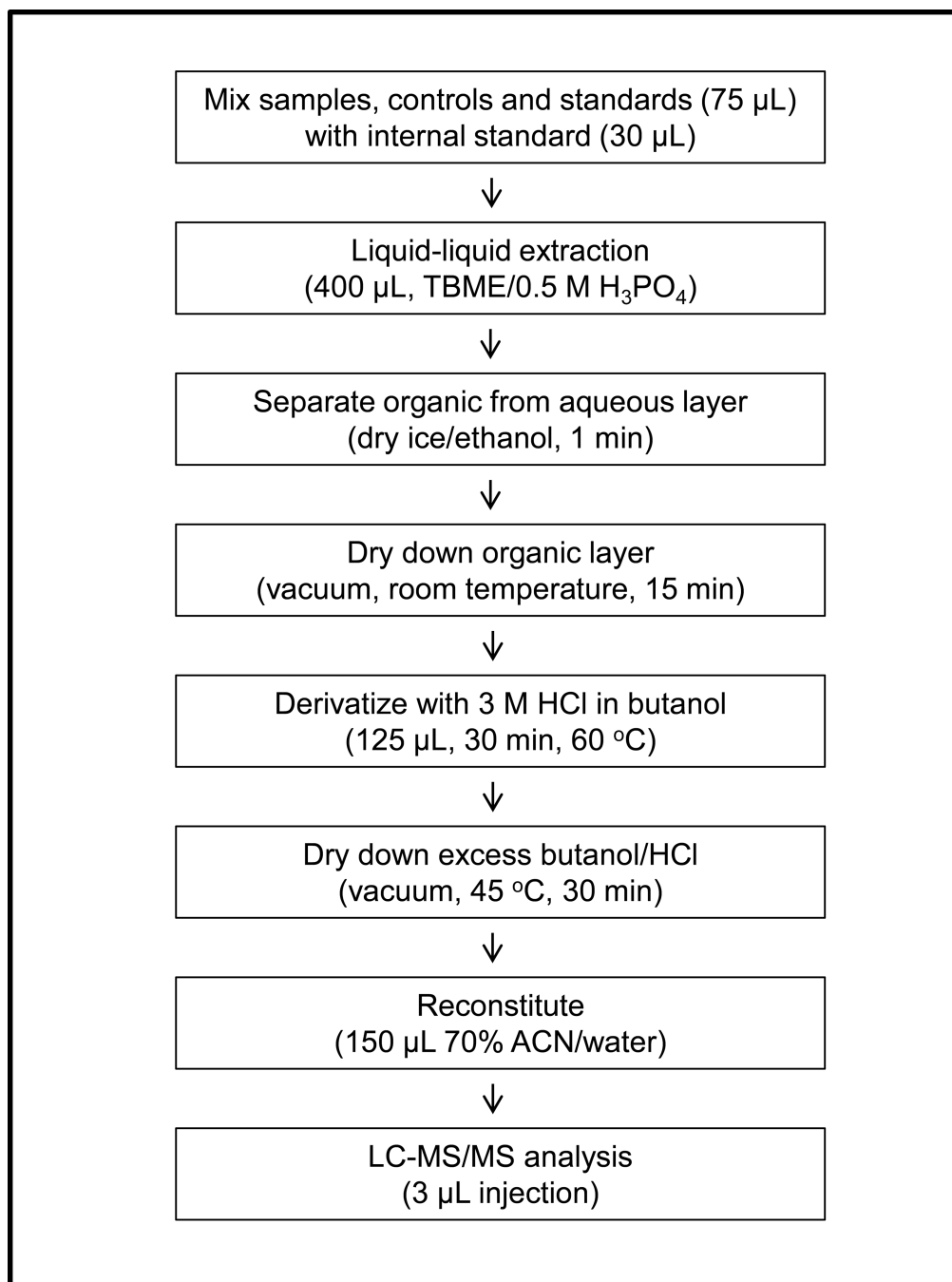


Fig. 1.
Sample preparation steps for MMA LC-MS/MS method

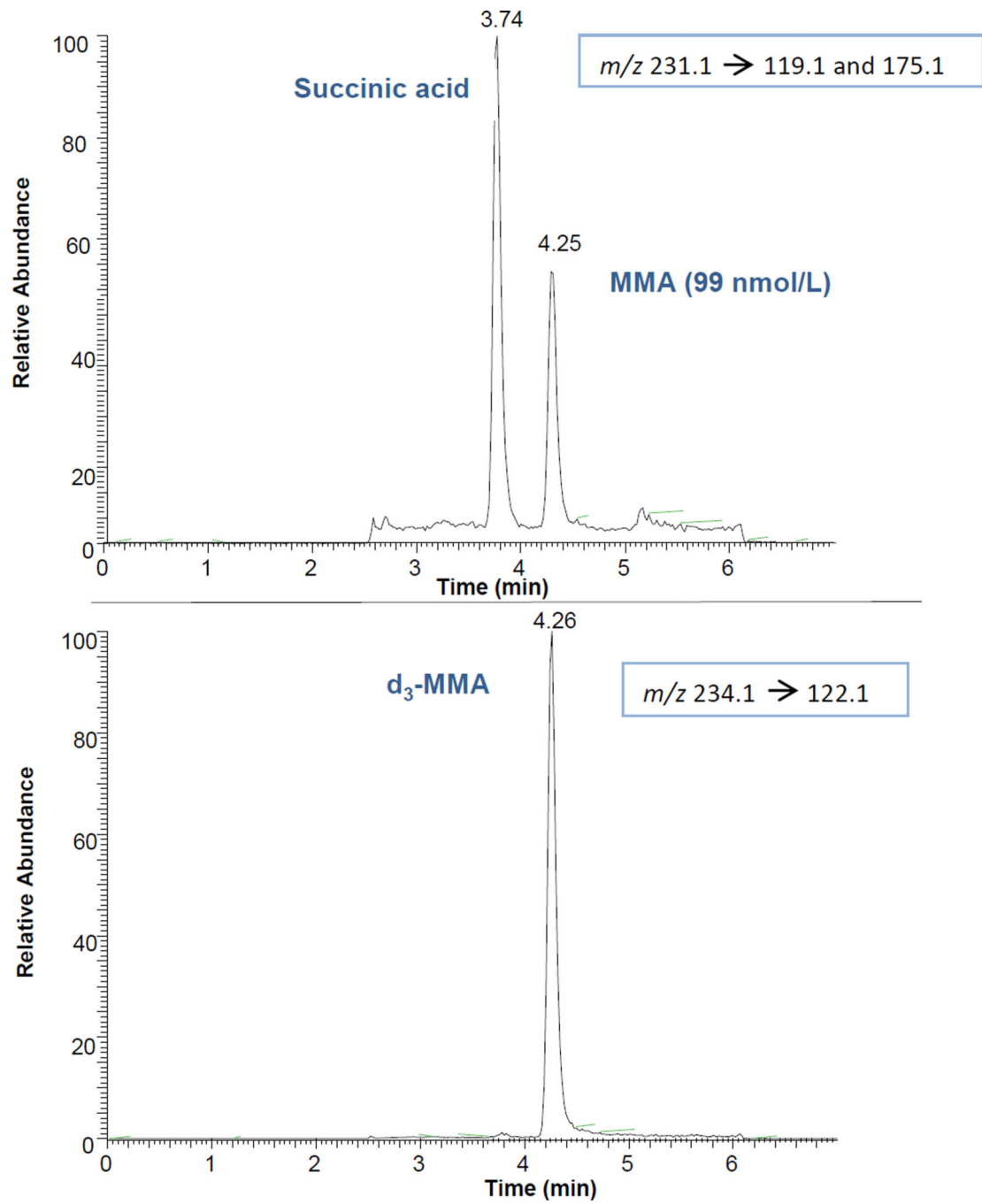


Fig. 2.
Typical chromatogram of the low quality control serum

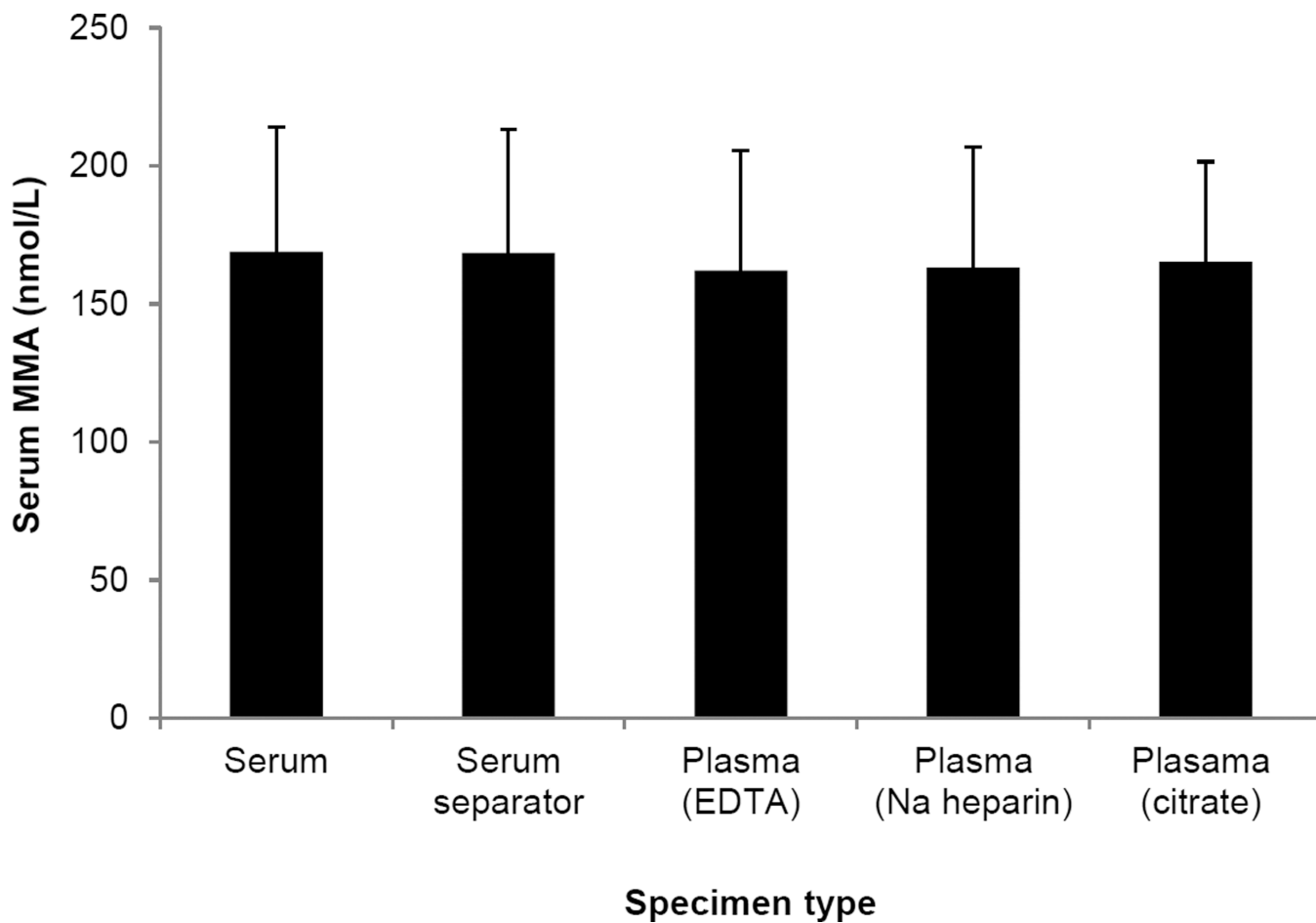


Fig. 3. Effect of specimen type and anticoagulant on MMA. Fourteen matched serum and plasma samples were analyzed with the LC-MS/MS method. Error bars represent standard error. Because Na citrate was a liquid (0.5 mL/5 mL vacutainer tube) anticoagulant, MMA results were multiplied by 1.1 to correct for the dilution.

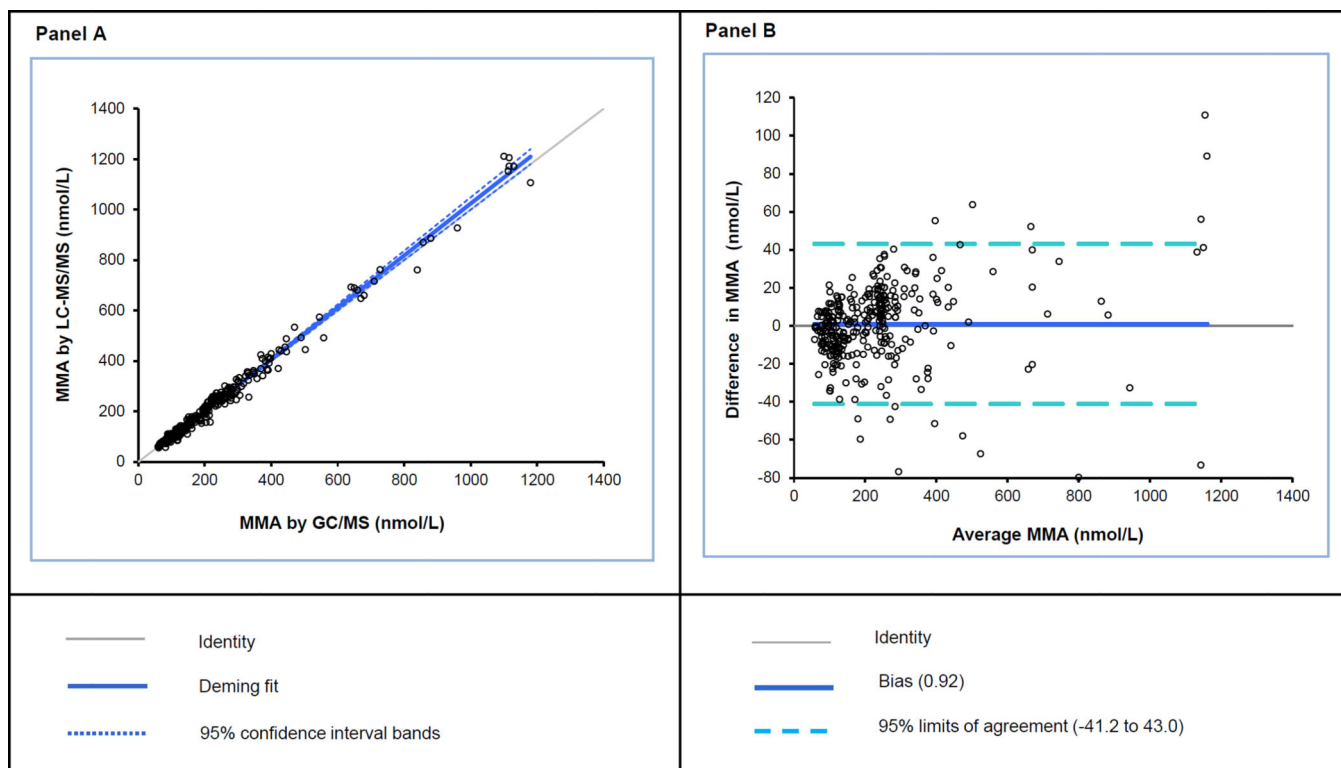


Fig. 4. Comparison of LC-MS/MS to GC/MS results obtained for MMA. Method comparison consisted of 326 residual plasma samples. Deming regression (Panel A). Bland-Altman plot of differences (Panel B).

Table 1

Sample preparation and analysis steps, comparing the CDC LC-MS/MS and GC/MS methods for MMA.

Step	LC-MS/MS	GC/MS
Sample volume/test, μL	75	275
Calibration range, nmol/L	25–2500	0–20000
Number of calibrators	6	1
Specimen type	Serum	Plasma
Derivative analyzed	Dibutyl ester	Dicyclohexyl ester
Derivative used for analysis, (amount, μL)	3M HCl in BuOH (125)	1.5 M HCl in cyclohexanol (200)
Derivatization time/temperature, min/ $^{\circ}\text{C}$	30/60	15/115
Sample extraction technique	Liquid-liquid extraction	Solid-phase extraction
Sample preparation time, h	4	12
Injection volume, μL	3	1
Throughput (samples/run)	160	36
Chromatographic run time, min	6	15

Table 2

Analytical performance of the LC-MS/MS method

Level (nmol/L)	Calibration ^a		Imprecision ^b			Accuracy ^c			Sensitivity ^d	
	Accuracy (%)	CV (%)	QC pool (nmol/L)	Total CV (%)	Within-run CV (%)	Between-run CV (%)	Spike (nmol/L)	Recovery (%)	CV (%)	LOD (nmol/L)
25	98	3.8	97.1	7.9	4.1	7.1	50	89	2.9	22.1
50	99	5.5	246.5	6.5	2.6	5.8	250	92	4.0	
150	102	4.2	547.7	4.9	1.9	4.2	700	102	1.5	
400	101	2.3					1000	92	0.7	
1000	100	1.5								
2500	100	0.6								

^a Calibration was performed over 10 runs, and the calculation accuracy was calculated as the percent difference between the measured and the nominal calibrator value. The calibrator imprecision is reported as between-run coefficient of variation (CV).

^b Method imprecision was assessed by analyzing three QC pools over 22 runs (two replicates per run, between-run) and over 1 run (10 replicates per run, within-run) and by calculating coefficients of variation (CV).

^c Method accuracy was assessed through spike recovery; serum pool was spiked with a calibrator mixture containing MMA at four levels (two replicates per level, two runs) and also measured unspiked (two replicates per level, two runs) for endogenous MMA concentrations; the spike recovery was calculated as the measured concentration difference between the unspiked and spiked samples divided by the nominal concentration of the MMA spike.

^d Method sensitivity was estimated as the LOD by diluting low QC sample with charcoal stripped serum and calculating the SD at concentrations of zero (σ_0) from the extrapolation of repeat measurements (three replicates per dilution, three runs) made near the detection limit in these dilutions, the LOD was defined as $3\sigma_0$.

Table 3

Comparison of MMA results in serum and plasma samples obtained by different methods.

Method	Pearson correlation coefficient (95% CI)	Deming slope (95% CI)	Deming intercept (95% CI)	Bland-Altman bias (95% CI)
			<i>nmol/L</i>	
LC-MS/MS vs. GC/MS ^a	0.99 (0.99 to 1.0)	1.03 (1.00 to 1.06)	-6.34 (-12.4 to 0.32)	0.92 (-1.42 to 3.26)
LC-MS/MS vs. GC/MS ^b	1.0 (1.0 to 1.0)	1.03 (0.94 to 1.10)	13.3 (-6.92 to 33.5)	-22.5 (-84.3 to 39.3)
LC-MS/MS vs. consensus ^b	1.0 (1.0 to 1.0)	0.99 (0.93 to 1.05)	-10.8 (-24.0 to 2.44)	-75.9 (-148.8 to -2.9)

^aPlasma samples ($n = 326$) were used for analysis^bEight serum and 11 plasma samples were used for analysis