Methylmalonic Acid Measured in Plasma and Urine by Stable-Isotope Dilution and Electrospray Tandem Mass Spectrometry

Mark J. Magera, Janice K. Helgeson, Dietrich Matern, and Piero Rinaldo*

Background: Liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization is robust and allows accurate measurement of both low- and high-molecular weight components of complex mixtures. We developed a LC-MS/MS method for the analysis of methylmalonic acid (MMA), a biochemical marker for inherited disorders of propionate metabolism and acquired vitamin B12 deficiency.

Methods: We added 1 nmol of the internal standard MMA-d₃ to 500 µL of plasma or 100 µL of urine before solid-phase extraction. After elution with 18 mol/L formic acid, the eluate was evaporated, and butyl ester derivatives were prepared with 3 mol/L HCl in n-butanol at 65 °C for 15 min. For separation, we used a Supelcosil LC-18, 33 × 4.6 mm column with 60:40 (by volume) acetonitrile:aqueous formic acid (1 g/L) as mobile phase. The transitions m/z 231 to m/z 119 and m/z 234 to m/z 122 were used in the selected reaction monitoring mode for MMA and MMA-d₃, respectively. The retention time of MMA was 2.2 min in a 3.0-min analysis, without interference of a physiologically more abundant isomer, succinic acid.

Results: Daily calibrations between 0.25 and 8.33 nmol in 0.5 mL exhibited consistent linearity and reproducibility. At a plasma concentration of 0.12 µmol/L, the signal-to-noise ratio for MMA was 40:1. The regression equation for our previous gas chromatography-mass spectrometry (GC-MS) method (y) and the LC-MS/MS method (x) was: y = 1.030x – 0.032 (S_y|x = 1.03 µmol/L; n = 106; r = 0.994). Inter- and intraassay CVs were 3.8–8.5% and 1.3–3.4%, respectively, at mean concentrations of 0.13, 0.25, 0.60, and 2.02 µmol/L. Mean recoveries of MMA added to plasma were 96.9% (0.25 µmol/L), 96.0% (0.60 µmol/L), and 94.8% (2.02 µmol/L). One MS/MS system used only overnight (7.5 h) replaced two GC-MS systems (30 instrument-hours/day) to run 100–150 samples per day, with reductions of total cost (supplies plus equipment), personnel, and instrument time of 59%, 14%, and 75%, respectively.

Conclusions: This method is well suited for large-scale MMA testing (>100 samples per day) where a shorter analytical time is highly desirable. Reagents are less expensive than the anion-exchange/cyclohexanol-HCl method, and sample preparation of batches up to 100 specimens is completed in less than 8 h and is automated.

© 2000 American Association for Clinical Chemistry

Methylmalonic acid (MMA) is a specific diagnostic marker for a group of inherited disorders collectively known as methylmalonic acidemias, which include at least eight different entities identified according to biochemical phenotype and somatic cell complementation analysis (1–3). Their collective incidence is ~1:48 000 live births. Two disorders (mut0 and mut2) reflect a deficiency of the apoenzyme portion of the enzyme methylmalonyl-CoA mutase; three others (cblA, cblB, and cblH) are associated with abnormalities of the adenosylcobalamin synthesis pathway. cblC and cblD are caused by impaired synthesis of both adenosylcobalamin and methylcobalamin, whereas CblF is a defect of lysosomal cobalamin efflux and is inconsistently associated with mild accumulation of MMA in plasma and urine.

Acquired causes of methylmalonic acidemia are much more common. Increased MMA in plasma and urine is found in patients with cobalamin (vitamin B₁₂) deficiency as a consequence of intestinal malabsorption, impaired digestion, or poor diet (4). Other conditions, such as renal

1 Nonstandard abbreviations: MMA, methylmalonic acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry; SPE, solid phase extraction; and SRM, selected reaction monitoring.
insufficiency, hypovolemia, and bacterial overgrowth of the small intestine, also are possible causes of mild methylmalonic acidemia or aciduria. Elderly patients with cobalamin deficiency may present with peripheral neuropathy, ataxia, loss of position and vibration senses, memory impairment, depression, and dementia in the absence of anemia (5). Therefore, the determination of MMA in serum and urine is considered an important biochemical screening for the differential diagnosis of signs and symptoms associated with a variety of causes of cobalamin deficiency.

Several studies have suggested that the determination of serum MMA could represent a marker of cobalamin deficiency more reliable than direct cobalamin determination (6). Following our recent development of a method for the determination of total homocysteine (7), we have focused our efforts on developing a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the routine determination of plasma and urine MMA in a high-volume testing laboratory environment, replacing a gas chromatography-mass spectrometry (GC-MS) procedure.

Materials and Methods

MATERIALS
MMA and methyl-d₃-malonic acid (MMA-d₃) were purchased from Sigma and Cambridge Isotope Laboratories, respectively. Working solutions (20 μmol/L) of MMA and MMA-d₃ were prepared by diluting stock solutions prepared in methanol with deionized water. HCl (3 mol/L) in n-butanol was purchased from Regis. Human serum for recovery, precision, and stability studies was obtained from the blood bank within our Institution. Strong anion-exchange solid-phase extraction (SPE) columns with 1-mL reservoirs containing 100 mg of packing material (Accu-Bond™) were obtained from J and W. All other chemicals and solvents were of the highest purity available from commercial sources and used without further purification.

SAMPLE PREPARATION
A 600-μL serum or plasma sample was placed in a 10 × 75 mm glass culture tube in a tray on the processor and mixed with 600 μL of internal standard solution (1.2 nmol MMA-d₃). For pediatric patients, sample volume could be reduced to a minimum of 50 μL and brought to volume with distilled water. A 1-mL aliquot of the mixture was extracted on a Gilson ASPEC automated SPE sample processor. After the SPE column was preconditioned with 1 mL of methanol followed by 1 mL of water, 1000 μL of the diluted sample was added to the column, which was then washed with 1 mL of deionized water. MMA and MMA-d₃ were eluted from the SPE column in 300 μL of 18 mol/L formic acid in water. The elution tubes were removed from the processor, and the eluate was evaporated to dryness in 25 min in a water bath at 30 °C under dry nitrogen. The residue was then transferred to 1-mL glass screw-cap reaction vials using two 200-μL portions of methanol. The methanol was evaporated in ~5 min on a 40 °C dry heating block under a gentle flow of dry nitrogen. After 200 μL of 3 mol/L HCl in n-butanol was added to each vial, the reaction vials were capped and heated in a dry heating block at 65 °C for 15 min to form n-butyl ester derivatives. The excess reagent was evaporated in 10 min at 40 °C under nitrogen, and the residue was dissolved in 100 μL of 80:20 (by volume) acetonitrile: deionized water. Centrifugation for 2 min at 3000g yielded a clear supernatant that was transferred for analysis to a sealed glass autosampler vial. The preparation for urine differed from that for plasma/serum only in that 100 μL of sample was mixed with 500 μL of deionized water and placed in the processor sample tube. Calibrators were prepared in deionized water by the addition of working solution (20 μmol/L) corresponding to a blank and MMA concentrations of 0.25, 0.50, 1.00, 2.00, 5.00, and 8.33 nmol in 0.5 mL.

METHODS
A triple-quadrupole mass spectrometer API 3000 (Perkin-Elmer Scieux) operated in ion-evaporation mode (source voltage, 5500 V) was used. Peripherals included two Perkin-Elmer Series 200 M pumps and an autosampler. Separation of MMA and MMA-d₃ from the bulk of the specimen matrix was achieved by use of a short column (LC-18; 33 mm × 4.6 mm; Supelco). Autosampler injections of 10 μL were made, using a mobile phase composed of acetonitrile in 1 mL/L formic acid (60:40, by volume). The column was directly connected to the TurbolonSpray ionization probe operating with the turbo gas on (6 L/min; sensor temperature, 300 °C) and the LC column effluent flow split to deliver 100 μL/min to the instrument. MMA and MMA-d₃ coeluted with a retention time of 2.2 min. Total instrument time was 3 min per sample.

All results were generated in positive-ion mode with the orifice voltage set at 26 V, automatically optimized using the protonated MMA di-n-butyl ester ion. For all MS/MS experiments, mass calibration and resolution adjustments (at 0.7 amu full width half height) on both the resolving quadrupoles were automatically optimized using a poly(propylene) glycol solution introduced via an infusion pump. Collisionally activated decomposition MS/MS was performed through the closed-design Q₂ collision cell, operating with nitrogen as collision gas. The 16 eV (lab frame) collision energy was adjusted automatically by the AutoTune algorithm.

MS/MS product ion scans of MMA and succinate standards were collected in continuous flow mode by connecting an infusion pump directly to the TurbolonSpray probe. For MS/MS optimization, a 20 μmol/L MMA di-n-butyl ester solution was prepared in acetonitrile:water (50:50, by volume) containing 2.5 mL/L formic acid and infused at a flow rate of 10 μL/min. In the selected reaction monitoring (SRM) mode, the instrument was optimized automatically by the built-in algorithm to
monitor the \(m/z\) 231.0 to \(m/z\) 119.1 and \(m/z\) 234.1 to \(m/z\) 122.0 transitions for MMA and MMA-\(d_3\), respectively. Data were acquired and processed using the MassChrom software (Ver. 1.1.1; Perkin-Elmer Sciex) including Multi-View, Ver. 1.4, for chromatographic and spectral interpretation and TurboQuan for Apple Macintosh (Ver. 1.0; Perkin-Elmer Sciex) for the quantitative processing. For the purpose of method comparison, we compared the MS/MS method with the GC/MS procedure (8, 9), which had been used previously in our laboratory and elsewhere (10). This GS-MS method is based on automated SPE and derivatization of MMA and MMA-\(d_3\) with acidified cyclohexanol.

Results

The MS/MS product ion scans obtained by infusion of 20 \(\mu\)mol/L succinic acid and MMA di-\(n\)-butyl esters are shown in Fig. 1. Both of these spectra were acquired by \(Q_1\) transmission of the protonated molecular ion, \(m/z\) 231, and scanning the second resolving quadrupole (\(Q_3\)) for products resulting from fragmentation in the collision cell. Loss of the \(n\)-butyl moieties from the protonated molecular ion of MMA (\(m/z\) 231) yielded the fragment at \(m/z\) 119, which was not detected in the product ion scan of 20 \(\mu\)mol/L succinic acid but became apparent at higher concentrations. These results were used to design the SRM experiment to sequentially transmit the \(m/z\) 231 protonated molecular ion and the \(m/z\) 119 fragment via \(Q_1\) and \(Q_3\), respectively. Although a signal for succinic acid was detected at higher concentrations, the addition of succinic acid at concentrations up to 2000 \(\mu\)mol/L to serum samples produced no interference with the MMA peak. The separation of MMA from succinic acid is shown in Fig. 2.

Four SRM ion chromatograms obtained from serum and urine samples at normal [serum, 0.25 \(\mu\)mol/L (reference interval, 0–0.4 \(\mu\)mol/L); urine, 2.67 mmol/mol creatinine (reference interval, 0–3.6, mmol/mol creatinine)] and increased (serum, 18 \(\mu\)mol/L; urine, 36.4 mmol/mol creatinine) concentrations, respectively, are shown in Fig.
3. Serum specimens with MMA concentrations up to 23 µmol/L have been analyzed accurately without dilution. At low concentrations, a serum specimen in which the calculated MMA concentration was 0.12 µmol/L exhibited a signal-to-noise ratio for the MMA-extracted SRM signal of 40:1 (injected amount equal to 1.2 pmol).

LINEARITY
Interassay variability of calibration data obtained over concentrations of 0.25–8.33 nmol/0.5 mL was monitored on 7 consecutive days. The average slope, intercept, and coefficient of linear regression ($r^2$) were 0.9050 (95% confidence interval, 0.7991–1.0109), −0.0117 nmol/0.5 mL (−0.0962 to 0.0728 nmol/0.5 mL), and 0.9998 (0.9992–1.0003), respectively.

RECOVERY, PRECISION, AND STABILITY
Recovery and precision data are summarized in Table 1. These experiments were conducted using a pool of human serum from healthy donors. To 144 aliquots (500-µL) of pooled serum, we added either 15 µL of a 5 µmol/L MMA solution, or 15 or 60 µL of a 20 µmol/L MMA solution, corresponding to final MMA concentrations of 0.125, 0.500, and 2.000 µmol/L. Six aliquots of each set were prepared as described above and analyzed in single determinations on each of 6 consecutive days. Good quantitative recovery and precision were obtained, demonstrating the accuracy of the method for the quantitative determination of MMA.

The stability of prepared specimens was investigated by repeat injection of 10 serum samples (selected over a range from 0.09 to 1.95 µmol/L) with interim storage at 4 °C. The data obtained are shown in Table 2. ANOVA (single factor) showed variances $\approx$0.007 (1.3% at 1.99 µmol/L), indicating that MMA and MMA-$d_3$ as di-$n$-butyl esters were stable during refrigeration for at least 72 h after sample preparation.

Fig. 2. SRM extracted ion chromatograms of mixtures of the internal standard MMA-$d_3$ and variable concentrations of MMA and succinic acid. Peaks: 1, succinic acid; 2, MMA-$d_3$ (internal standard); 3, MMA. Specific amounts are as follows: (A), 1.0 nmol MMA, 1.0 nmol succinic acid; (B), 10 nmol MMA, 10 nmol succinic acid; (C), 10 nmol MMA, 100 nmol succinic acid; and (D), 10 nmol MMA, 1000 nmol succinic acid.
Method Comparison

Unused portions of 106 specimens routinely analyzed by GC-MS were retested using the new LC-MS/MS method. The correlation between the MS/MS method and the GC-MS method was:

\[ y = 1.030x + 0.032 \]  

\( n = 106; r = 0.994; S_{\text{rxy}} = 1.03 \mu\text{mol/L} \), with an average \( \Delta \) of 0.01 \( \mu\text{mol/L} \). A Bland-Altman plot indicated equivalence of the methods.

Table 1. Performance of the LC-MS/MS assay.

<table>
<thead>
<tr>
<th>No. of aliquots</th>
<th>Added, ( \mu\text{mol/L} )</th>
<th>Detected, ( \mu\text{mol/L} )</th>
<th>Recovery, %</th>
<th>CV, % (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0</td>
<td>0.13 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.125</td>
<td>0.25 ± 0.01</td>
<td>96.9</td>
<td>3.4</td>
</tr>
<tr>
<td>6</td>
<td>0.50</td>
<td>0.60 ± 0.04</td>
<td>96.0</td>
<td>1.6</td>
</tr>
<tr>
<td>6</td>
<td>2.00</td>
<td>2.02 ± 0.08</td>
<td>94.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>

\( ^a \) Pooled serum.

\( ^b \) Mean ± SD.

Table 2. Stability of MMA after derivatization.

<table>
<thead>
<tr>
<th>Serum samples</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.09</td>
<td>0.06</td>
<td>0.11</td>
<td>0.10</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>0.14</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15 ± 0.005</td>
</tr>
<tr>
<td>3</td>
<td>0.21</td>
<td>0.21</td>
<td>0.17</td>
<td>0.21</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>0.26</td>
<td>0.27</td>
<td>0.26</td>
<td>0.25</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>0.31</td>
<td>0.30</td>
<td>0.29</td>
<td>0.30</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>6</td>
<td>0.36</td>
<td>0.36</td>
<td>0.30</td>
<td>0.34</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>7</td>
<td>0.40</td>
<td>0.44</td>
<td>0.38</td>
<td>0.42</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>8</td>
<td>0.48</td>
<td>0.47</td>
<td>0.44</td>
<td>0.50</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td>9</td>
<td>1.04</td>
<td>1.01</td>
<td>1.01</td>
<td>1.12</td>
<td>1.04 ± 0.05</td>
</tr>
<tr>
<td>10</td>
<td>1.95</td>
<td>1.92</td>
<td>1.97</td>
<td>2.11</td>
<td>1.99 ± 0.08</td>
</tr>
</tbody>
</table>

\( ^a \) Samples selected from a batch of serum specimens submitted for MMA determination.

\( ^b \) Samples were analyzed immediately after preparation (time 0 h) and at the times indicated above. Between analyses, samples were stored at 4 °C in the autosampler vials.
MMA results over the concentration range found in routine specimen analysis (Fig. 4) (11). A group of urine specimens was retrospectively analyzed by LC-MS/MS and GC-MS methods, and results similar to those presented for plasma were obtained: \( y = 0.956x - 0.121 \) (n = 49; \( r^2 = 0.8981 \)).

**Discussion**

Applications of LC-MS/MS in clinical laboratory practice take advantage of this technique’s ability to target individual components of complex mixtures, with the potential of improved efficiency compared with more conventional methods. Following the development of a MS/MS method for the determination of total homocysteine in plasma and urine (7), we have focused our efforts on MMA, a compound clinically related to homocysteine that is also a high-volume procedure in our practice.

The first application of MS/MS to the rapid qualitative detection of MMA in urine was reported in 1984 (12). Product ion analysis of \( m/z \ 119 \) [M+H]+ and \( m/z \ 101 \) [M-OH]+ of underivatized MMA calibrator (100 µg) and of a urine extract of a patient with methylmalonic acidemia, collected during an acute episode of metabolic decompensation, showed an excellent match of the two spectra without any apparent interference despite the complexity of the matrix. The excretion of MMA in that sample, however, was extremely high (11 959 mmol/mol creatinine by GC-MS), and there was only an approximate correlation between the MS/MS and GC-MS results. More recently, MMA was included in a study of the application of GC-MS/MS to the screening of organic acidurias (13). Processing of urine specimens included SPE, oximation, and derivatization as trimethylsilyl esters. The chromatographic run was 10 min, and the MS/MS analysis was based on ion-trap (tandem-in-time) configuration where the precursor ion of MMA was \( m/z \ 247 \) [M-CH₃]+ and the product ion was \( m/z \ 231 \). According to the authors, the advantages of this method over conventional GC-MS were greater recovery, a shorter run time (10 min), and simpler data analysis. On the other hand, the applicability of this method to plasma was not investigated, low-end sensitivity was not established (the lowest calibration point was equivalent to 0.8 µmol/L), and there was no indication of whether succinic acid could interfere in the determination of MMA. Reference intervals were expressed in arbitrary units, and the extraction efficiency was on average 10% lower than the SPE used in our method. For these reasons, we believe that our method represents an innovative contribution to the determination of MMA in plasma and urine.

To achieve an accurate determination of MMA without GC separation, we had to overcome the interference from succinic acid, an isomer physiologically present in plasma (0–32 µmol/L) and urine (adult reference interval, 0.5–16 mmol/mol creatinine) (14) at concentrations higher than MMA (plasma, 0–0.4 µmol/L; urine, 0–3.6 mmol/mol creatinine). Because satisfactory separation of underivatized calibrators was not achievable, we tested different derivatization procedures. Butylation, which is also the method of choice for derivatization of acylcarnitines (15), amino acids (16), and acylglycines (17) before MS/MS analysis, offered the best combination of chromatographic behavior and fragmentation pattern, allowing complete separation of the two isomers at succinate concentrations up to 2000 µmol/L.

Using our previous experience with the comparison of MS/MS and HPLC methods for the analysis of total homocysteine (7), we compared MMA values obtained by the GC-MS and LC-MS/MS methods, using the technique described by Bland and Altman (11), and found a very favorable agreement between the proposed method and the GC-MS method used in our laboratory (8). The mean difference for MMA values obtained by both methods was 0.01 µmol/L, and acceptable agreement was demonstrated over a concentration range up to 25 µmol/L. Although this GC-MS method is widely utilized, a recent comparative study of 13 laboratories, including ours, showed mean among-laboratory CVs of 19% and 21% for serum and plasma samples, respectively (10). The authors concluded that significant among-laboratory imprecision is an issue of concern and that improvements are needed to reduce analytical imprecision by GC-MS methods. Although we agree that introduction of high-quality reference materials and institution of external quality assessment programs could be beneficial, we propose that the introduction of an alternative method could also have a positive impact, similar to what we have observed at our institution when homocysteine analysis was moved from an HPLC to a MS/MS platform (7).

The advantages of this method over the GC-MS procedure can be summarized as follows: one MS/MS system used only overnight (7.5 h) can replace two GC-MS
systems (30 instrument-hours/day) to run 100–150 samples per day, with reductions of total cost (supplies plus equipment), personnel, and instrument time of 59%, 14%, and 75%, respectively. Under these circumstances, we have developed an alternative MMA method that not only displays comparable or greater precision of its GC-MS counterpart, but is also faster and overall less expensive.

References