Solid-Phase Sample Extraction for Rapid Determination of Methylmalonic Acid in Serum and Urine by a Stable-Isotope-Dilution Method

Karsten Rasmussen

Methylmalonic acid concentrations are increased in serum in vitamin B₁₂ (cobalamin) deficiency. Here I demonstrate the successful use of anion-exchange extraction for improving newly developed analytical procedures and describe well-documented, reliable performance of this method for rapid determination of methylmalonic acid. The sorbent counter ion is formate, and the elution solvent is formic acid. The dicyclohexyl derivative is measured by selected ion monitoring. For serum, the assay curve is linear from 0.026 to 200 μmol/L. The normal reference interval is 0.08 to 0.56 μmol/L. Added methylmalonic acid is accurately quantified. The sensitivity and the precision exceed those of the current method by three orders of magnitude. The total and within-day CVs are 4.6% to 7.9% and 2.6% to 4.7%, respectively. Similar figures were obtained for urine. This convenient method is useful for evaluation of cobalamin deficiency, especially in patients with normal or moderately depressed cobalamin concentrations in serum.

Additional Keyphrases: cobalamin • vitamin B₁₂ • nutritional status

In 1962, Cox and White (1) reported excretion of methylmalonic acid (MMA) in the urine from cobalamin-deficient patients with classic pernicious anemia.¹

L-Methylmalonyl-CoA is a normal intermediate in the biochemical pathway from propionyl-CoA to succinyl-CoA. Precursors of propionyl-CoA include isoleucine, valine, threonine, methionine, cholesterol, and odd-carbon-number fatty acids. 5'-Deoxyadenosylcobalamin is an essential cofactor in the enzymatic conversion of L-methylmalonyl-CoA into succinyl-CoA.

Increased concentrations of MMA in serum and excessive urinary excretion of MMA are believed to be direct measures of tissue stores of cobalamin (2) and to be the first indication of cobalamin deficiency (3-5). Recently, however, the concentration of MMA in serum was found to be a useful indicator of cobalamin deficiency, especially in patients with few or no hematological abnormalities, normal results for the Schilling test, or normal or only slightly depressed serum cobalamin concentrations (5).

Colorimetry and thin-layer or gas chromatographic (GC) methods for determining MMA have been unreliable for establishing or excluding the diagnosis of cobalamin deficiency (1, 6, 7). The use of gas chromatography–mass spectrometry (GC-MS) combined with the sensitive, selected-ion monitoring (SIM) mode for determination of urinary MMA was first described by Norman et al. (7). They derivatized the MMA with cyclohexanol without purifying the urine samples. More recent GC-MS methods for urinary MMA have involved partial sample purification by solvent extractions (4, 8, 9).

Only one method (10) has been described for quantifying MMA in blood from normal humans, a GC-MS-SIM method for serum and urine, involving a cumbersome sample preparation: laborious extraction steps at alkaline and acidic pH's, clean-up by HPLC, further extractions with diethyl ether, and derivatization of the MMA followed by extraction of the tert-butyldimethylsilyl derivative. The authors reported that 80% to 90% of the MMA was lost during sample preparation, and their CV for a pooled normal serum, assayed on 17 different occasions during a 10-month period, was 26%.

The aim of the present study was to develop and validate a less labor-intensive procedure that would be suitable for prospective clinical evaluation of cobalamin deficiency. For a procedure to be useful as a routine diagnostic method, one should be able to process several samples at a time with reasonable precision and speed. To avoid the disadvantages of solvent extraction techniques, such as lack of speed and use of potentially toxic organic solvents, I optimized a solid-phase extraction system to give the necessary degree of partial purification required for analysis of serum samples. Substituting the dicyclohexyl derivative for the tert-butyldimethylsilyl derivative of MMA yielded more satisfactory reproducibility.

I have validated the method by the analysis of both normal and pathological serum and urine specimens.

Materials and Methods

Apparatus

For mass spectral identification and quantification I operated in the electron-impact mode a HP 5985 B quadrupole GC-MS system equipped with SIM analysis capability and a split-mode capillary injection port (Hewlett-Packard, Palo Alto, CA). The chromatographic column, a 25 m × 0.20 mm (i.d.) Hewlett-Packard fused silica capillary coated with a 0.33-μm film of 5% phenylmethyl-silicone, was inserted directly into the ion source. The GC-MS interface and the ion source were maintained at 250 °C. The ionization voltage was 70 eV with an emission current of 300 μA. The acceleration potential was set at 2200 V. Mass spectral resolution with peak widths of 0.5 atomic mass unit was used.

Reagents

Strong anion-exchange solid-phase extraction columns, Bond Elute® SAX, with a sample capacity of 1 mL and containing 100 mg of sorbent (trimethylamino propyl) bonded to silica in chloride form, were manufactured by Analytichem International, Harbor City, CA. I changed the sorbent counter ion to formate by passing 25 mL of 1 mol/L formic acid through the columns at a flow rate of 5 mL/min. A VAC

Reference:
¹ Nonstandard abbreviations: MMA, methylmalonic acid; GC, gas chromatography; MS, mass spectrometry; SIM, selected ion monitoring; d₆-MMA, methyl-d₆ malonic acid; and m/e, mass/charge ratio.
ELUT® system from Analytichem International was used to accelerate the flow and facilitate the extraction.

Organic solvents and other chemicals were of analytical grade unless stated otherwise. De-ionized, freshly doubly distilled water was used throughout. MMA ("puriss" grade; purity >99%) and other dicarboxylic acids were from Fluka AG, Buchs, Switzerland. The internal standard, methyl-d₃-malic acid (d₃-MMA, 99.5 atom % D) was purchased from MSD Isotopes, Montreal, Canada, and used as a 20 μmol/L solution. 2-[(4)C]-MMA (purity 92–95%; Amersham International, Bucks., U.K.) was purified by ion-exchange chromatography. Cyclohexanol containing hydrogen chloride, 1.5 mol/L, was provided by Mikrolab A/S, Hoejbjerg, Denmark, and stored under nitrogen at −20 °C.

**Specimens**

Blood and 24-h urine collections were obtained from apparently healthy adults and patients with clinical cobalamin deficiency. Completeness of the urine collection was assessed by measuring the creatinine in each specimen. Blood collected without anticoagulant was allowed to clot, and the serum was separated by centrifugation. Urinary creatinine content was determined by the Technicon automated alkaline picrate method (11). Specimens were stored without additives at 4 °C for analysis the same day. Storage for longer periods was at −20 °C.

**Procedures**

**Preparation of serum and urine samples.** Prepare columns for analysis by washing sequentially with 1 mL of 1.0 mol/L formic acid, 1 mL of methanol (solvent agent), and 2 mL of water. Carry out these initial and the following extraction steps under reduced pressure so as to aspirate the solutions through the columns at a flow rate of 1 mL/min.

**Serum:** To 550 μL of serum add 55 μL of internal standard solution and 495 μL of water. Vortex-mix for 20 s and apply 1000 μL (equivalent to 500 μL of serum and 1 nmol of d₃-MMA) to a solvated column.

**Urine:** Adjust the pH of the urine specimen to 7.8–8.5 with 0.1 mol/L sodium hydroxide and dilute with water to give a creatinine content of 1 mmol/L. Mix 1200 μL of the diluted sample and 300 μL of internal standard solution for 20 s. Apply 1250 μL (equivalent to 1 μmol of creatinine and 5 nmol of d₃-MMA) to a solvated column.

**Strong anion-exchange extraction.** Aspirate the diluted serum or urine with added d₃-MMA through the sorbent. The column should not be allowed to run dry before the dilution is applied. Wash the column with two 1-mL portions of water to eliminate neutral and basic compounds, and discard the effluents. Elute the retained acids with 250 μL of 18 mol/L formic acid. Remove the solvent from the resulting extract under a stream of nitrogen at room temperature and dissolve the residue in 250 μL of methanol. Transfer to a reaction vial, together with a 250-μL methanol rinse. Remove all solvent from the sample under a stream of nitrogen.

**Derivatization.** To the reaction vial add 200 μL of 1.5 mol/L hydrogen chloride in cyclohexanol, seal the containers with Teflon-lined septum caps, and incubate them at 115 °C for 15 min. Evaporate the cyclohexanol to near dryness at 70 °C under a gentle stream of nitrogen and dissolve the residue in 100 μL of methanol by vortex-mixing for 20 s. The samples can be stored capped at 4 °C for at least one month without loss or decomposition.

**GC-MS-SIM.** Using a split-mode of operation (ratio 50:1), manually inject 1 μL of derivatized sample into the inlet system, maintained at 225 °C. Use helium as carrier gas at a column pressure of 1 kg/cm², which gives a capillary flow rate of about 1.2 mL/min. Operate the column from 120 to 350 °C at 15 °C/min. Keep the temperature at 350 °C for 3 min to clean the column. Record mass spectra from mass 50 to 350, with a scan-cycle time of 1 s.

Perform SIM with 100-ms dwell time on the mass-fragmentographic peaks at m/e 119.1 and 122.1 for the dicyclohexyl esters of MMA and d₃-MMA, respectively. Collect data from 8.2 to 8.6 min. Integrate the areas of the peaks, using the data system with operator selection of the baseline points. If an area value exceeds the maximum capacity of the data system (45 000 arbitrary counts; see Results), dilute the derivatized sample with methanol, and re-assay.

**Quantification of MMA.** Correct the area counts of the m/e 119.1 peak for the counts contributed to it by the internal standard (see Results) as follows:

Corrected peak-area ratio = (peak area at m/e 119.1 − (0.024 × the peak area at m/e 122.1))/peak area at m/e 122.1.

Calculate the concentration of MMA in serum (μmol/L) by multiplying the corrected peak-area ratio by 2. Urinary MMA is quantified similarly, multiplying the ratio by 5 to give results in millimoles of MMA per mole of creatinine.

**Evaluation of the method.** I assessed analytical recovery of the column extraction procedure by adding 2-[(4)C]-MMA to specimens, which were then subjected to full clean-up procedure. The residues from the vials were quantified for recoveries by counting radioactivity in a liquid scintillation counter. The sensitivity and linearity of the GC-MS-SIM apparatus were evaluated by injection of 0.1 fmol to 100 pmol onto the column. The lowest corrected peak-area ratio limit was tested by analyzing samples from a serum pool and a urine specimen, diluted with increasing volumes of water under standard conditions, with use of a constant amount of d₃-MMA. Similarly, the linearity of the method was tested on samples supplemented with increasing amounts of MMA. Total and within-run precision, measured on serum at three concentrations and on urine at two, was assessed by an accepted protocol (12) for one run per day. Accuracy was tested by interference studies, and by the methods of dilution, mixture, and addition (analytical recovery).

**Results and Discussion**

Optimizing Assay Conditions

The main task was to find the best combination of rapid, efficient sample preparation and GC-MS-SIM conditions that would give, reproducibly, a clean mass chromatogram with sharp peaks from derivatives of MMA and d₃-MMA. Selection of counter ion and elution solvent. Strong anion-exchange columns in hydroxide, chloride, or acetate form have been used to extract organic acids from plasma (13–15) and urine (16, 17), usually with sulfuric acid as the elution solvent. Owing to marked baseline disturbances on GC caused by interfering compounds in the eluate, especially with analysis performed on serum, I tested various combinations of different anion-exchange sorbents and elution solvents. With use of formate ion and formic acid, virtually no concurrent extraction of interfering compounds occurred, and the collected, volatile eluate from the column was easily evaporated to dryness. As pHₖₐ for MMA is 5.76, the pH of the specimen was adjusted to 7.8–8.5, because optimal
retention can only be achieved with completely dissociated 
MMA (pH > pK_a + 2). A pH higher than 8.5 will adversely 
affect the sorbent stability (17). For serum and urine specimens 
from 10 healthy subjects, the mean extraction recoveries 
of added radioactive MMA were 97.4% (SD 2.5%) and 
98.5 (SD 2.1%), respectively. Recoveries were similar for 
serum or urine supplemented with MMA to yield final 
concentrations ranging from 5 to 100 μmol/L.

Derivatization. In my hands, an initial attempt to measure 
the tert-butyldimethylsilyl derivative of MMA was 
unsatisfactory for quantitative work because of interference 
during GC from co-eluting compounds in the serum. 
Furthermore, double peaks appeared sometimes probably 
because of silylation of the ester enols. Such a phenomenon 
has previously been described for another silyl derivative of 
MMA (18). The final choice of dicyclohexyl derivatives was 
a good one, because there was no such interference from co-
eluting compounds. Furthermore, chromatography of the 
dicyclohexyl esters of MMA and d3-MMA gave single peaks 
in a reproducible manner. The derivatization is simple and 
rapid (7). The time dependence of the yield of dicyclohexylation 
of MMA at 115 °C was examined; a constant amount of the 
ester was reached after 12–14 min. The ester, stored at 
4 °C, was stable for at least a month.

GC column. Capillary columns with different liquid 
phases were tested. A 5% phenylmethyl-silicone was chosen 
because it gave the best combination of baseline separation 
between derivatives of other compounds and the MMA 
overview, short retention time, and optimal peak shape for 
SIM.

Performance of the Apparatus

GC. The dicyclohexyl esters of aliphatic dicarboxylic acids 
have excellent chromatographic properties on the chosen 
capillary column. Sharp peaks were obtained after 8.25 
(malonic acid), 8.40 (MMA), 8.85 (ethylenmalonic acid), 9.15 
(succinic acid), and 9.85 min (glutaric acid). Peak widths 
were equivalent to approximately 3.5 s at baseline.

MS. The esters give characteristic mass spectra (7). MMA 
and d3-MMA derivatives show equally intense ions at m/e 
119.1 and 122.1, respectively, corresponding to a loss of 163 
atomic mass units from the molecular ions. This loss repre-
sents a common fragmentation pattern for dicyclohexyl 
esters of dicarboxylic acids. However, the d3-MMA derivative 
also exhibited an ion at m/e 119.1, amounting to 2.4% of 
the relative abundance of the m/e 122.1 ion, and probably 
attributable to loss of both dicyclohexyl groups.

SIM. The prominent m/e 119.1 and 122.1 peaks from the 
derivatives of MMA and d3-MMA, respectively, provide 
extcellent sensitivity during SIM (Figure 1). The m/e 122.1 
peak appeared 0.6 s before the m/e 119.1 peak.

GC-MS-SIM. I tested the linearity of the detection by 
the apparatus by injecting amounts of dicyclohexyl ester of 
MMA ranging from 0.1 fmol to 100 pmol onto the column. 
The limit of detection was 1–2 fmol, with a signal/noise ratio 
of 5. The relation between analyte concentration and peak-
area counts was linear up to 10 pmol (45 000 arbitrary 
counts). This performance test indicates that the data sys-
tem SIM program was linear over four decades.

Evaluation of the Method

Specificity. The MMA peak was identified on SIM on the 
basis of the retention time and the almost simultaneous 
appearance of the d3-MMA peak. When it emerged from the 
GC capillary, MMA was well separated from other dicarbox-

![Retention Time (MIN)](chart.png)

Fig. 1. Capillary GC-MS chromatogram of aliquots of dicyclohexyl- 
derivatized extracts of 500 μL of pooled normal serum (a) and of a 
volume of normal urine containing 1 μmol of creatinine (b)

Injection volume: 1 μL. TIM: total-ion monitoring. SIM: selected-ion monitoring of the MMA derivative at m/e 119.1 and the trideuterated internal standard derivative at 122.1, each normalized to full-scale deflection. The areas integrated are indicated by the dotted lines. The peak-area ratios (119.1/122.1) were determined to be 0.184 (a) and 0.290 (b), yielding calculated concentrations of MMA as 0.32 μmol/L (a) and 1.33 mmol/mmol of creatinine (b) 

ylic acids. Whereas solvent extraction also extracts several 
nonacidic compounds having related structure or polarity, 
only organic acids are found in the strong anion-exchange 
extract (14, 17). This gain in selectivity reduces the chance 
of overlapping peaks. Monitoring specific ions ensures fur-
ther selectivity. No impurities were encountered in the 
elution region of MMA on SIM with this highly specific 
method.

Analytical sensitivity. Sensitivity, defined as the least 
concentration of MMA in diluted serum that could be 
distinguished from zero, was 31 nmol/L (mean ± 2 SD; 
n = 10). An assay curve was drawn: corrected peak-area 
ratios vs the concentration of MMA. Sensitivity, measured 
by determining the corrected peak-area ratio at which the 
assay curve deviated from linearity (8, 10), was 26 nmol/L. 
This sensitivity is threefold that possible by the current 
method (10), and undoubtedly is attributable to the five- 
to-10-fold higher extraction recovery of the present method. 
With a split ratio of 50:1, this sensitivity corresponds to 
about 3–4 fmol actually passing into the column. The 
sensitivity for urinary MMA was 0.08 mmol of MMA per 
mole of creatinine.

Accuracy. As already mentioned, no interfering comp-
ounds were encountered in the m/e 119.1 channel, where 
the derivative of MMA was monitored. Furthermore, analysis 
of serum and urine with no added d3-MMA proved the
absence of a possible interfering compound with the same retention time as d₇-MMA that might have contributed to the peak in the m/e 122.2 channel. The assay curve was linear up to 200 μmol/L for serum and 500 mmol per mole of creatinine for urine. Specimens with higher concentrations must be diluted with water and re-assayed. Table 1 shows data for dilution of serum and urine; the dilutions were quantitative. When serum or urine containing abnormally high concentrations of MMA, obtained from patients with cobalamin deficiency, were mixed with an equal volume of a normal specimen and assayed, the expected values were found (data not shown). Analytical recovery was assessed (Table 2) by supplementing a serum pool and a urine specimen obtained from apparently healthy adults, and assaying. The mean recovery was 101.4%. Furthermore, from the figures in Table 3 it can be calculated that the recoveries from the serum pool supplemented with 1.60 and 40.0 μmol of MMA per liter were 101.7% (n = 40) and 99.7% (n = 40), respectively. I conclude that the accuracy of the method is acceptable.

Precision. The amounts of d₇-MMA added to serum and urine specimens were chosen so as to result in the smallest imprecision and analytical inaccuracy at borderline MMA concentrations, where the method might provide most useful clinical information, namely from high normal to moderately above normal. Table 3 summarizes the results of my evaluation of imprecision at different MMA concentrations in serum and urine, each measured twice during routine analyses for 20 days. The imprecision of the method is satisfactory, only a third of that obtained by the current method for serum. This improvement is probably ascribable to the current method's sample-extraction procedure, which is simple when compared with clean-up by a series of extraction and HPLC steps.

Practicability. Apparently, measurements of serum and urine correlate well with one another as diagnostic tests for cobalamin deficiency (19). The main advantage of determination of MMA in serum over its determination in urine is that the former is routinely collected in the process of evaluating patients for cobalamin deficiency, so serum specimens usually are available for additional studies in the clinical laboratory. If necessary, the amounts of serum used can be scaled down to 55 μL, owing to the favorable sensitivity of the present method, especially if the amount of added d₇-MMA is equally decreased. In addition, the speed with which a result can be obtained is a major advantage of the present method. The entire procedure can be completed in 3.5 h by a practiced laboratory technician, who can process at least 15 specimens during a working day by use of batch-wise processing. The past four months have proved it to be a robust and effective method, suitable for routine analysis.

To compare my data with those previously reported for concentrations of MMA in serum by Marcell et al. (10), I chose consecutive blood donors such that specimens of

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<th>Table 1. Dilution Data for the Method*</th>
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<td>MMA in serum, μmol/L</td>
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<td>Dilution ratio b</td>
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<tr>
<td>1:8</td>
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<td>1:4</td>
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*Specimens were obtained from a cobalamin-deficient patient. Each value is the mean of duplicate determinations. Diluted with water. These derivatized samples were diluted 10-fold in methanol before measurements (see Procedures).

<table>
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<th>Table 2. Analytical Recovery of Methylmalonic Acid</th>
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<td>Added</td>
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Mean recovery:

100.8

102.0

*Each value is the mean of duplicate determinations for samples from apparently healthy subjects. These derivatized samples were diluted 10-fold in methanol before measurements (see Procedures). These enriched specimens were diluted 100-fold in water before analysis (see Accuracy).

| Table 3. Precision of Methylmalonic Acid Measurements* |
|------------------|------------------|------------------|
| Specimen | Total | Within-day |
| | Imprecision | component of |
| | SD | CV, % | SD | CV, % |
| Serum pool | 0.318 | 0.025 | 7.9 | 0.015 | 4.7 |
| +MMA, 1.60 μmol/L | 1.95 | 0.09 | 4.6 | 0.05 | 2.6 |
| +MMA, 40.0 μmol/L | 40.2 | 2.1 | 5.2 | 1.4 | 3.5 |
| Urine b | 1.20 | 0.08 | 6.7 | 0.03 | 2.5 |
| Urine c | 179 | 10 | 5.6 | 4. | 2.2 |

*Specimens were assayed in duplicates for 20 days. Mean and SD are expressed in μmol/L for serum and in mmol/mol of creatinine for urine. b 24-h specimen from healthy adult. c 24-h specimen from cobalamin-deficient patient.
serum were obtained from five men and five women in each of the following age groups: 18–26, 27–35, 36–45, 46–55, and 56–65 y, for a total of 50 specimens. My values for the actual normal range of 0.08 to 0.56 μmol/L, calculated as the mean ± 2 SD after log transformation to correct for skewness towards higher values, compare well with those reported: 0.16–0.64 μmol/L. In further agreement with Marcell et al., no age- or sex-related differences were apparent. The concentrations of MMA were measured in 24-h urine specimens from 20 healthy adults among the hospital personnel, ages 22–56 y. The values ranged from 0.58 to 3.56 mmol per mole of creatinine. This normal reference interval is in the same range reported by Marcell et al.: 0.78–3.37 mmol/mol creatinine.

Finally, the utility of the method was demonstrated for quantification of MMA in serum and 24-h urine specimens from 10 patients with clinical cobalamin deficiency. In serum and urine from five patients with moderately depressed serum cobalamin concentrations (141–191 pmol/L; normal range: 200–800 pmol/L), the concentrations of MMA ranged from 2.03 to 38.5 μmol/L and from 8.67 to 179 mmol per mole of creatinine, respectively. These values agree with values reported in the literature (3, 9, 10, 19). More importantly, in five patients with normal cobalamin concentrations (220–356 pmol/L), the concentrations of MMA ranged from 1.98 to 6.6 μmol/L and from 10.8 to 39.2 mmol per mole of creatinine, respectively. Lindenbaum et al. (5) most recently reported that cobalamin deficiency with normal or only moderately depressed concentrations of cobalamin in the serum may be more common than has previously been realized.

In conclusion: I have developed the first method with well-documented reliable performance for rapid, accurate, and precise determination of MMA in serum and urine, suitable for routine use in the clinical laboratory. This convenient method combines the speed, simplicity, and reproducibility of solid-phase extraction with the sensitivity, specificity, and accuracy of the stable-isotope-dilution GC-MS-SIM. The obvious advantage of the MMA assay is that where measurement of serum cobalamin concentration alone might be misleading, the answer provided by the former may give a diagnostic clue. This method would be useful for studies designed to evaluate patients for cobalamin deficiency and to assess the diagnostic sensitivity and specificity of serum cobalamin assays.

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References