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A simple high-throughput method for the determination of plasma methylmalonic acid by liquid chromatography-tandem mass spectrometry

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Abstract

Background: Cobalamin (Cbl) deficiency is a common clinical phenomenon, in particular among the elderly and possibly also among infants. Methylmalonic acid (MMA) is the most sensitive and specific marker of intracellular Cbl status, but its application is hindered by limited methods available for accurate and high-throughput MMA determination.

Methods: We developed a non-laborious method for determination of MMA without the need for prior derivatization using HPLC combined with liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Stable isotope-labeled methyl-d_3-malonic acid (MMA-d_3) was added to 100 μL of plasma as an internal standard. After deproteinization by ultrafiltration, an acidified aliquot of the eluate was injected into the HPLC system and analyzed by LC-ESI-MS/MS monitoring of the carbonyl loss of MMA and MMA-d_3.

Results: Calibrations between 0.1 and 1.0 μM exhibited consistent linearity and reproducibility. The lower limit of detection for plasma MMA was 0.1 μM (signal-to-noise ratio ≥10). The intra- and inter-assay CVs of ten determinations of a plasma sample were 1.5% and 6.7%, respectively, at a mean concentration of 0.29 μM. Inter-assay CVs for 25 determinations of low, medium and high concentrations (0.22, 0.45 and 0.94 μM MMA) were 8.3%, 5.9% and 4.6%, respectively. The mean recovery of MMA added to plasma was 100%.

Conclusions: By avoiding derivatization, we developed a new, non-laborious, simple and reliable high-throughput method for the determination of MMA that is suitable for automation.

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Introduction

Cobalamin (Cbl) is utilized as a cofactor in two reactions: as methylcobalamin in homocysteine remethylation to methionine by methionine synthase (homocysteine-methyltetrahydrofolate methyltransferase) and as adenosylcobalamin in the conversion of L-methylmalonyl-CoA to succinyl-CoA by methylmalonyl-CoA mutase (Figure 1) (1). As a consequence, impaired Cbl status results in elevated plasma total homocysteine (tHcy) and methylmalonic acid (MMA) concentrations, which make them sensitive markers of intracellular Cbl status (2–6). tHcy is not a specific marker of Cbl deficiency since it is utilized in different metabolic pathways and is influenced by a variety of environmental factors, such as vitamin B_{12}, vitamin B_6 or folate status, renal failure, age, sex, lifestyle and drugs, and by genetic factors. Plasma MMA is influenced by renal failure or thyroid disease only (7–9).

There are a number of advantages of determining MMA instead of plasma B_{12}. First, despite evidence of functional Cbl deficiency, plasma Cbl concentrations can be normal. This is explained at least partly by the fact that approximately 80% of total plasma Cbl is bound to haptocorrin, which is not taken up by cells. The remaining 20% of total plasma Cbl is bound to transcobalamin and this complex is responsible for transport and cellular uptake of Cbl. Plasma Cbl measurement shows the sum of both bound forms of Cbl, and thus does not necessarily reflect intracellular Cbl status (10). Therefore, the determination of holotranscobalamin rather than Cbl may well be more favorable. Second, Cbl is less stable, e.g., it is sensitive to light, whereas MMA is very stable (11). Furthermore, plasma MMA concentrations are several orders of magnitude higher than Cbl levels (nM vs. pM), and thus elevated concentrations of MMA are easier to detect than decreased levels of Cbl. A fourth argument favoring MMA determination is that plasma MMA concentrations can be used in follow-up monitoring of metabolic Cbl status during Cbl administration, which results in high plasma Cbl.

Cbl deficiency is a common clinical problem in the elderly (12, 13). Recent studies suggest that Cbl deficiency is more common among neonates and infants

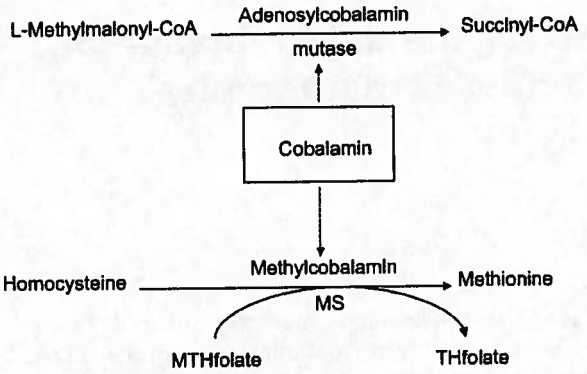

Figure 1 Role of cobalamin in metabolism.
Cobalamin is a cofactor as adenosylcobalamin in the conversion of methylmalonyl-CoA to succinyl-CoA by L-methylmalonyl-CoA-mutase (mutase) and as methylcobalamin in the remethylation reaction of homocysteine to methionine by methionine synthase (MS).

than previously recognized, with incidences as high as 15% (14–17).

As MMA appears to be the most sensitive and specific marker of Cbl deficiency and because MMA determination is likely to be requested in large populations, the aim of this study was to develop an easy, accurate and high-throughput method for plasma MMA measurement.

Materials and methods

Materials

MMA and methyl-tri-deuterated MMA (MMA-d_3; purity >95%) were purchased from Fluka (Zwijndrecht, The Netherlands) and Cambridge Isotope Laboratories (Apeldoorn, The Netherlands), respectively. Human EDTA-plasma for recovery, precision and stability studies was obtained from the blood bank of our institution. Plasma deproteinization was performed by ultrafiltration using Microcon centrifugal filter tubes with a molecular mass cutoff of 30 kDa purchased from Millipore (Amsterdam, The Netherlands). The HPLC column (2.1 mm×100 mm 3.5 μm Symmetry C18 column) was purchased from Waters (Etten-Leur, The Netherlands). Methanol and formic acid were purchased from Merck (Amsterdam, The Netherlands).

Preparation of standards

Standards of MMA and MMA-d_3 were prepared in deionized water and kept at −20°C. MMA in plasma is stable at room temperature for at least 2 days. MMA in deionized water can be kept at room temperature for more than 2 weeks before any significant change becomes apparent (data not shown). It can be kept at −20°C for more than 10 years, based on comparison of plasma MMA determined with our new method with results from gas chromatography-mass spectrometry (GC-MS) analysis (see also the Results section).

Sample preparation

An aliquot of 100 μL of plasma was pipetted onto the Microcon ultrafilter, followed by 100 μL of 0.8 μM MMA-d_3 internal standard solution. After vortexing, the tube was centrifuged for 30 min at 14,000×*g*. Then 100 μL of the resulting ultra filtrate was acidified with 10 μL of 4% formic acid and 10 μL of the resulting sample was injected into the liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) system.

Preparation of calibrators

Aliquots of 100 μL of MMA standards (0, 0.1, 0.2, 0.4, 0.6, and 1.0 μM) were pipetted into Eppendorf cups (Eppendorf, Hamburg, Germany), followed by 100 μL of 0.8 μM MMA-d_3 internal standard solution. After vortexing, a 100-μL aliquot was acidified with 10 μL of 4% formic acid. After vortexing again, 10 μL was injected into the LC-ESI-MS/MS system. The use of Microcon centrifuge filters had no effect on MMA detector response, showing no binding of MMA by these filters.

LC-ESI-MS/MS system

The LC-ESI-MS/MS system consisted of a Micromass Quattro LC (Waters) connected to an Agilent HP1100 HPLC (Amsterdam, The Netherlands) and a Gilson 232 XL auto sampler (Den Haag, The Netherlands). Using the auto sampler 10-μL aliquots of prepared samples were injected onto the column. The mobile phase consisted of 15% methanol/0.4% formic acid and elution was isocratic at a flow rate of 200 μL/min. The effluent was split, directing only 40 μL into the tandem MS. For routine diagnostics and research, the HPLC columns (Waters) lasted for at least 800 injections.

A Micromass Quattro LC (Waters) triple-quadrupole tandem mass spectrometer was fitted with an electrospray ionization probe operated in negative mode. The capillary voltage was set to 3 kV and the cone voltage to 15 V. Nitrogen was used as the drying gas at a flow rate of 250 L/h with ion block and source temperatures of 80°C and 150°C, respectively. The collision cell was operated at 9 eV at a pressure of 0.18 Pa Argon. The mass resolution of the Q1 and Q3 quadrupoles is 1 Da. The tandem MS was set to monitor the carbonyl loss of MMA and MMA-d_3 by recording the transitions m/z 117→73 and m/z 120→76, respectively. Masslynx 3.2 (Micromass; Manchester, UK) was used for HPLC and autosampler control, data acquisition and data processing. Quantification was based on peak areas. The total injection-to-injection time was 5 min.

Normal values

We determined plasma MMA concentrations in 100 healthy individuals from a general practitioner's practice (18). We used SPSS software (version 12.2; Gorinchem, The Netherlands) for statistical analysis and Microsoft Excel for constructing graphs.

Results

Chromatographic profile

Figure 2 shows a typical chromatogram for a control subject and a patient with vitamin B_{12} deficiency. The deuterated and the naturally occurring MMA coelute from the column at 2.5 min with a peak width of <0.20 min. In the ESI source, conditions for MMA and its deuterated analog will therefore be similar within the sample, which is important in terms of possible ion suppression. Under the MS/MS conditions used, the physiologically abundant isomer succinic acid will also be detected. However, its retention time is

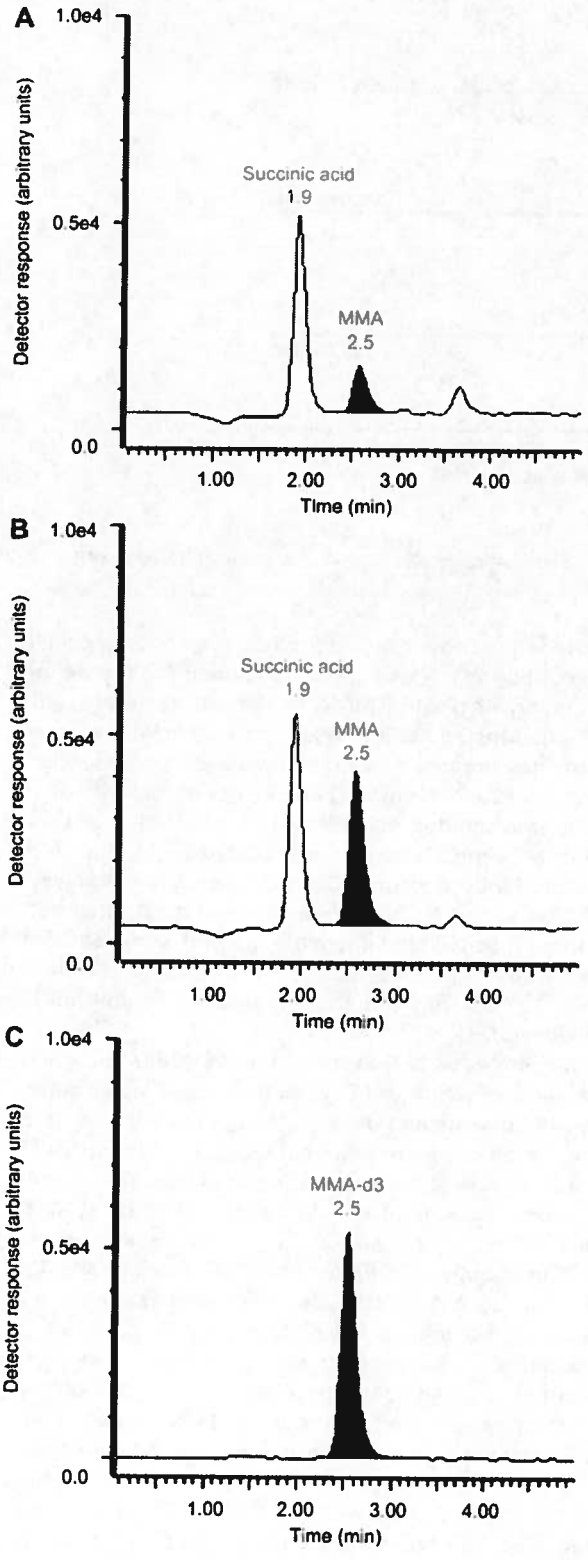

Figure 2 LC-MS/MS chromatograms using MRM ES 116.8 → 72.9 for MMA and MRM ES 119.8 → 75.9 for MMA-d_3. (A) Control subject (plasma MMA 0.18 μM). (B) Patient with a cobalamin deficiency (plasma MMA 0.54 μM). (C) MMA-d_3 standard. The retention time of MMA and MMA-d_3 is 2.5 min and that of the isomer succinic acid is 1.9 min.

1.9 min and therefore it is efficiently separated from MMA. We have not observed any interference effect by succinic acid on the MMA results in more than 4000 samples.

Calibration, linearity, recovery, intra- and inter-assay CVs and detection limit

To evaluate the linearity and recovery of MMA, standards of 0, 5, 10, 20, 50, 100 and 200 μM were diluted 100-fold with deionized water or human plasma by adding 40 μL of standard to 4 mL of deionized water or 10 μL of standard to 1 mL of plasma. There was excellent linearity up to at least 2 μM MMA in both water and plasma (coefficient of correlation >0.999 in linear regression analysis). The slopes of the calibration lines in the two matrices were equal (water 1.006 ±0.004, plasma 1.039±0.009). Analysis of 25 calibrator curves in a 12-month period resulted in mean±SD slope and intercept of 0.993±0.031 and 0.001±0.009, respectively. The recovery of MMA added to plasma samples amounted to 98%–101% in the concentration range up to 2 μM. Adequate recovery is also confirmed by the similar slopes of the correlation lines in water and plasma matrices.

A plasma pool with a mean MMA content of approximately 0.29 μM was processed, resulting in a 1-day mean (n=10) of 0.292 and an overall (4-month period) mean (n=10) of 0.278 μM with intra- and inter-assay CVs of 1.5% and 6.7%, respectively. Inter-assay CVs were further evaluated by 25 determinations over 25 different days of three different plasma samples containing low, medium or high plasma MMA concentrations of 0.22, 0.45 and 0.94 μM MMA, respectively. This resulted in inter-assay CVs of 8.3%, 5.9% and 4.6%, respectively. The lower limit of detection for plasma MMA was 0.1 μM (signal-to-noise ratio ≥10).

Ion suppression was also investigated by continuous infusion of eluent containing 15 μM MMA, which was possible because MMA and MMA-d_3 have the same retention time. Injection of processed plasma or water samples showed no ion suppression in the testing phase. When large series of plasma samples (>500) were analyzed using the same HPLC column, we observed fluctuations of the internal standard MMA-d_3 in general between 75% and 125%, but in some series even more, but never <50%. This indicates likely ion suppression of substances slowly eluting from the column. Our standard curves were not influenced by this probable ion suppression (data not shown), demonstrating the same effect of proposed ion suppression on MMA as well as MMA-d_3.

Comparison with GC-MS method

Until the development of the method described here, a GC-MS method (13, 19) was employed for the determination of MMA in plasma in our laboratory. To verify if our new LC-ESI-MS/MS method shows good correlation with the existing GC-MS method, the GC-MS quality control, a plasma pool with a mean MMA content of 0.29 μM and well-established upper and lower limits of 0.33 and 0.25 μM, respectively, was tested using the LC-ESI-MS/MS method. Results

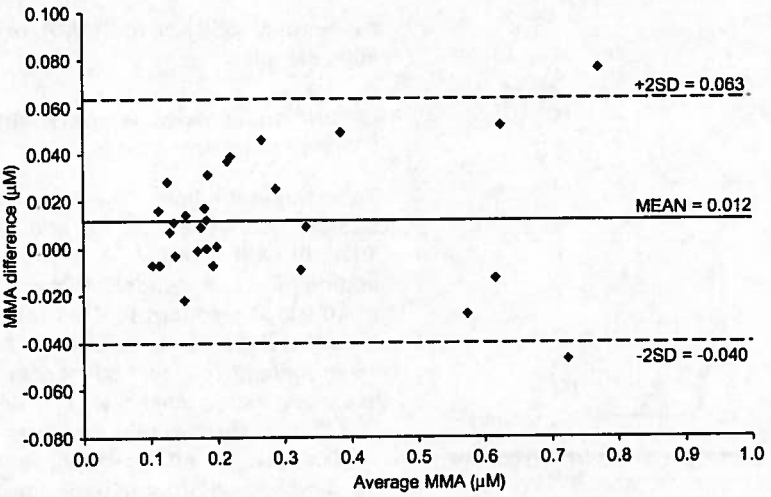

Figure 3 Correlation of LC-MS/MS with GC-MS.
The correlation of LC-MS/MS and GC-MS (n=29) is depicted in a Bland-Altman plot showing a mean difference of 0.012 µM.

showed a 1-day mean (n=10) of 0.292 µM and an overall (2 years, n=25) mean of 0.275 µM, with intra- and inter-assay CVs of 1.5% and 5.3%, respectively. In addition, we used the LC-ESI-MS/MS method to test plasma samples from 29 individuals for which the MMA concentration had already been determined by GC-MS. Comparison of the results by means of a Bland-Altman plot showed a mean difference of 0.012 µM (Figure 3).

Normal values

A total of 100 subjects (42 male, 58 female) were randomly selected from a large study group (18). The median age was 53.7 years (range 21.8–78.4 years). Since the distribution of MMA concentrations was skewed, we calculated the logarithmically transformed MMA concentrations (lnMMA) and geometric means where appropriate. The geometric mean (GM) for MMA was 0.185 µM (range 0.09–0.76 years), with 10th and 90th percentiles of 0.12 and 0.29 µM, respectively. We observed a significant correlation for lnMMA vs. lnB_{12} only ($\rho = -0.244$, $p = 0.014$). Neither age nor creatinine was significantly correlated with plasma MMA concentrations. The GM for MMA was 0.18 µM for males and 0.19 µM for females (not significantly different).

Discussion

We consider plasma MMA to be the most specific and sensitive marker of Cbl deficiency and therefore developed a new, non-laborious and effective method for MMA determination. To achieve a suitable method, MMA had to be separated by HPLC from its isomer succinic acid before MS/MS detection.

The feasibility and characteristics of dicarboxylic acid determination using MS-MS has been described previously (20). Different analytical techniques, including HPLC, MS-MS, stable isotope dilution gas chromatography mass spectrometry and capillary electrophoresis, have been developed for the determination of plasma MMA in the normal range (19, 21–26). Most, if not all, HPLC methods include extraction into organic solvent followed by derivatization prior to HPLC injection (22). Mills et al. described an HPLC-MS method suitable only for detection of very high concentrations of accumulated MMA due to a classical inborn error of Cbl metabolism (21). Analysis of GC-MS has probably been applied most often but, although considered a reliable method, some authors report a large inter-laboratory difference. Furthermore, a relatively low throughput (3–6 samples/h) is achieved (24).

Magera et al. (27) described an LC-MS/MS method for determination of MMA as the n-butyl ester derivative in plasma and urine. Although the analysis time was faster than conventional GC-MS methods, time-consuming and laborious sample preparation is still involved. Kushnir et al. (23) developed an LC-MS/MS method with high selectivity for dicarboxylic acids and the ability to differentiate MMA from its isomer succinic acid. Again, derivatization was applied. Very recently, Schmedes et al. developed an LC-MS/MS method that also requires derivatization and an overnight incubation (28). The high-throughput capillary electrophoresis method described by Schneede et al. (26) also requires derivatization. Our new method does not require derivatization owing to the optimal sensitivity and specificity of LC-MS/MS and because succinate can be efficiently separated from MMA on the C18 column. Sample preparation is extremely simple and is limited to addition of the internal standard followed by centrifugation and injection, allowing automation of this method.

A possible pitfall in determining MMA by MS/MS methods is the presence of its isomer succinic acid at concentrations higher than for MMA. Like MMA, succinic acid loses a carbonyl moiety in the collision cell under the conditions used, resulting in a fragment

with the same m/z as MMA. We used HPLC on a C18 column to separate MMA from succinic acid with a mobile phase of 15% methanol/0.4% formic acid, resulting in a reliable difference in retention time of 0.7 min.

A technical consideration is the potential occurrence of ion suppression (29). As MMA and its deuterated analogue coelute in this method, conditions in the ESI system are the same within the same sample. Any ion suppression that occurs is therefore likely to affect both compounds equally, and the deuterated MMA internal standard thus automatically compensates for this effect. Ion suppression may vary between samples due to matrix effects, which was indeed observed. If we assume the area under the curve for the internal standard in water to be 100%, this area varied considerably in a series of 29 plasma samples (range 53%–189%). In this same series of samples, correlation between the current method and the independent GC-MS method was excellent (Figure 3). Thus, our data show that the deuterated internal standard is suitable for correction of the resulting MMA concentration for this effect of variable ion suppression. In a larger series of over 4000 plasma samples measured with the new method, the area under the curve for the deuterated standard was never lower than 50%. In cases for which this area is <50% because of the specific sample matrix, the MMA concentration cannot be determined reliably using the method to our opinion.

In summary, we have validated the method by confirming its linearity and by obtaining acceptable intra- and inter-assay CVs and full recovery of MMA spikes. The method showed excellent correlation with an independent GC-MS method and the reference values obtained for plasma are in line with literature data (13, 25).

Conclusions

We developed a new, non-laborious and reliable method for determination of MMA that does not require any derivatization. Centrifugation after addition of the internal standard is the only step required prior to LC-MS/MS injection, resulting in reliable and quickly available results suited for automation and high throughput.

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