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Comparison of two methods for measuring methylmalonic acid as a marker for vitamin B12 deficiency

Abstract

Background: Methylmalonic acid (MMA) is a functional marker of vitamin B12 status and a valuable diagnostic tool. The gas chromatography mass spectrometry (GCMS) MMA assay has been used for decades in clinical studies.

Methods: In this study, we compared a newly developed liquid chromatography tandem mass spectrometry assay for MMA (ClinMass® Complete Kit) with the GCMS method and further measured other relevant vitamin B12 markers (homocysteine, total B12 and holotranscobalamin) to validate the new liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) method. For this purpose, 138 samples that were sent to our routine laboratory total B12 assay were used.

Results: The GCMS and LC-MS/MS assays showed a strong correlation ($r=0.92$, $p<0.001$) particularly at low holoTC levels (deficiency is more probable). Forty six cases had MMA $>300$ nmol/L with both methods. Only five subjects showed MMA GCMS $>300$ nmol/L, but MMA LC-MS/MS $\leq 300$ nmol/L. However, the LC-MS/MS method showed a slightly better correlation with other B12 markers (holoTC, total B12). In addition, the LC-MS/MS method offers several advantages over the GCMS method, such as saving time and costs, precision, flexibility and popularity in modern labs.

Conclusions: The new LC-MS/MS assay for MMA showed an excellent correlation to the GCMS method. The two methods showed similar agreements with other vitamin B12 markers.

Keywords: LC-MS/MS; methylmalonic acid; vitamin B12.

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Introduction

Mild to moderate vitamin B12 deficiency without anemia or neurological symptoms is common [1, 2] and is related to age associated diseases [3–5]. Holotranscobalamin (holoTC) is considered the earliest marker that declines when vitamin B12 depletion starts. Concentrations of plasma total homocysteine (tHcy) and that of methylmalonic acid (MMA) increase when vitamin B12 deficiency progresses. The concentration of MMA is more sensitive and specific marker for vitamin B12 deficiency as compared to tHcy that also increases in case of folate deficiency. Concentrations of MMA and tHcy increase in relation to age and renal function which limit their specificity in those cases.

The determination of total serum vitamin B12 has been routinely used as a first line and sole marker to diagnose vitamin B12 deficiency. The vitamin B12 test is cheap, but has a low diagnostic sensitivity and specificity. Only approximately 20% of total serum vitamin B12 is active. The active vitamin B12 (holoTC) can be measured using specific antibodies [6]. We recently suggested an algorithm for vitamin B12 diagnosis that depends on holoTC as a first line marker and MMA as a second line marker when holoTC levels are below 75 pmol/L [7].

Plasma concentrations of MMA have been used to validate the performance of holoTC and total vitamin B12 assays [8, 9]. The majority of clinical studies that tested holoTC in comparison to total serum vitamin B12 have confirmed that holoTC has a better performance in detecting an elevated plasma MMA value [7]. However, holoTC alone is not sufficient to firmly diagnose vitamin B12 deficiency.
MMA has a convenient sensitivity and specificity to detect vitamin B12 deficiency, at least in cases without renal insufficiency. The routine measurement of serum or plasma MMA was not common because of methodological and economic reasons. An established method for measuring MMA was a gas chromatography mass spectrometry (GCMS) method, first described in 1990 [10, 11]. This method is expensive and requires a long sample preparation procedure that starts with a solid-liquid chromatography to clean out matrix components, a drying step, and a derivatization step. Each sample requires 30 min run time on the GCMS system. A technique for MMA determination based on liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) has been introduced [12–15]. The LC-MS/MS method for MMA testing offers many advantages; it is fast, highly sensitive, less expensive, reproducible, and shows lower day-to-day variations [15]. Most routine clinical laboratories are using the LC-MS/MS device that allow high throughput analysis with minimal sample volume and sample preparation procedure [13]

Information on comparability of LC-MS/MS with GCMS has been published recently, but the performance of the LC-MS/MS assay against other vitamin B12 markers (holoTC, tHcy and total B12) has not been studied. In the current study, we compared the recently launched LC-MS/MS test kit for MMA from RECIPE Chemicals+Instruments GmbH with an in-house GCMS method. The study included samples that were referred for routine vitamin B12 testing. In addition, we measured total vitamin B12, holoTC, and tHcy in the same samples to investigate the relationship to the MMA that was measured by the GCMS and LC-MS/MS devices.

Materials and methods

The study comprised of samples that were referred to the Department of Clinical Chemistry, Saarland University Hospital for vitamin B12 testing. Samples were included in the study (n=136) only if volume of the blood was sufficient for performing additional B12 markers (holoTC, MMA, and tHcy). Information on age and sex [mean age=61 (SD=12.8) years, 51 (37%) male] and creatinine was available. Venous blood was drawn under fasting conditions. The blood samples were centrifuged within 30 min after venous puncture and serum and plasma were separated from blood cells immediately. Serum concentrations of vitamin B12 were tested on the same day. The rest of the samples were kept at –70°C until other laboratory markers were measured. The study protocol complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects. The study was approved by the Ethical Committee of the Saarland. None of the patients had anemia. Other clinical data were not available.

The assay of MMA in serum using GCMS was according to a slightly modified method as previously described [11, 16]. The method depends on using (methyl-D3) methylmalonic acid as an internal standard (CDN Isotope®). The sample volume is 200 μL (n=20 samples in one run). A cleaning step was performed using Poly-Prep chromatography columns filled with anionic Resin (Bio-RAD®). After elution of MMA, samples (n=20) were taken to dryness in a vacuum centrifuge. Dried eluates were then derivatized by adding 30 μL of a mix of 20 μL acetonicitrile and 10 μL of N-methyl-butyl-dimethylsilyl-tri-fluoracetamide (MBDSTFA, Machery and Nagel®). This was followed by 5 min incubation in the microwave (550 volt) and 1 μL was injected into the GCMS system. Gas chromatography 6890 N connected with mass spectrometry 5973 (both from Agilent Technologies®) were used. The total run time was 30 min/ per sample. The retention time for MMA was approximately 9.5 min. The quantification of MMA depended on ions (m/z) 292.2 and 289.2 (methyl-D3) methylmalonic acid and endogenous MMA, respectively. The between day coefficient of variations (CV) for the assay were <5% and <7% at 220 and 380 mmol/L, respectively.

The concentrations of tHcy were measured in plasma using a similar procedure by the same GCMS device and DL-homocysteine (3,3,3′,3′,4,4,4′,4′,D-8) as internal standard. Concentrations of holoTC and total vitamin B12 in serum were measured using Axsym and Centaur analyzers, respectively.

The concentrations of MMA from the second aliquots of the same sample were measured using LC-MS/MS. A 1290 UHPLC coupled with a G6460A triple-quadrupole MS/MS system (Agilent Technologies®) was used. The system was operated in ESI negative mode (Capillary Voltage -1500 V). The commercially available "ClinMass® Complete Kit (RECIPE Nr. MS5100, CE IVD certified) was used to measure MMA on LC-MS/MS. The serum calibrators (4 levels) and controls (2 levels) were provided by the manufacturer. A short sample clean-up was performed in order to remove the sample matrix. Samples (100 μL) were added to 400 μL of acetonitril-containing internal standard (d3-MMA) as precipitant. Samples were thoroughly mixed and centrifuged for 5 min. The supernatants were collected and 5 μL of it were injected into the LC-MS/MS system. Samples were prepared in duplicate. The chromatographic separation was performed in 2 min on a modified reversed phase analytical column RP-C12 (100×2.1 mm) using a pH-gradient to separate the MMA from potentially interfering isobaric substances, including succinic acid (Supplemental Material, Figure 1, that accompanies the article http://www.degruyter.com/view/j/dx.2015.2.issue-1/dx-2014-0030/dx-2014-0030.xml?format=INT). The column was held at 25°C and a stepwise gradient of 0, 30, 60, 100% of the mobile phase B over 1.3 min was used to achieve separation from the matrix. Mobile phases A and B constitute a mixture of acetonitrile/water containing formic acid. The entire run time including column equilibration was 2 min. Preparation of 20 samples was completed in 15 min. The chromatographic separation on LC-MS/MS for 20 samples was achieved in 60 min. The interpretation of the chromatograms was automatically done by using the software Masshunter (Agilent Technologies®). The same assay can be applied to serum, EDTA-plasma or urine samples.

Statistical analyses were performed by using IBM SPSS Statistics (Version 22). Results are shown as median (10–90th) percentiles or percentage. Correlations between continuous variables were obtained using Spearman test. The costs for biomarkers are according to the fee regulation in medicine [Gebührenordnung für Ärzte (GOÄ)] in Germany.
Results

The recovery rate (between 91% and 116%) was calculated according to the method described by Matuszewski et al. [17]. The MMA LC-MS/MS assay was linear between 25 and 67,700 nmol/L. The limit of detection was 15 nmol/L and that of quantification was 25 nmol/L. The between day coefficient of variations (CV) for the assay were <6% at 186, 479, and 686 nmol/L (n=16 independent value per level).

Figure 1 of the Supplementary Material shows examples of LC-MS/MS chromatograms from a serum sample with normal MMA and a serum with elevated MMA. The new LC-MS/MS assay for MMA has been compared with a GCMS method. The results of the two assays were studied in relation to other markers of vitamin B12 deficiency (holoTC, total B12, and tHcy) in 138 serum samples. Table 1 shows the concentrations of vitamin B12 markers and the percentage of samples with elevated or lowered concentrations. Several cut-off values have been employed in epidemiological studies for lowered total B12 and holoTC or elevated MMA [18, 19]. We considered the following cut-off values: low holoTC <35 pmol/L; low total vitamin B12 ≤ 148 pmol/L; elevated MMA >300 nmol/L; elevated tHcy >12 μmol/L. We consider the selection of the cut-off values in the current study not crucial for the interpretation of the results, because the aim was to study the MMA methods agreements in relation to other B12 markers rather than detecting a prevalence of deficiency in the studied population sample.

The concentrations of MMA that were measured by using GCMS strongly correlated with the concentrations that were measured by using the LC-MS/MS (r=0.92, p<0.001, n=138). The correlation between levels that were measured by the two methods was studied according to plasma holoTC (≥35 or <35 pmol/L). In the lower holoTC range, the correlation between MMA levels was stronger (r=0.965, p<0.001, n=58) than in the upper holoTC range (r=0.823, p<0.001, n=80) (Figure 1). Table 2 shows the correlation between MMA GCMS or LC-MS/MS and other vitamin B12 markers. LC-MS/MS showed a slightly better correlation with the concentrations of total serum vitamin B12 and that of holoTC compared to GCMS.

The mean difference (SD) of the LC-MS/MS and GCMS methods was 4 (56) (±2SD=+113, –106) nmol/L. The differences ranged from –203 to +198 nmol/L. The differences between the methods (GCMS-LC-MS/MS) were plotted against the value of the two methods according to Bland-Altman analysis (Figure 2). There was no significant correlation between the mean MMA ([GCMS+LC-MS/MS/2]) and the difference between the methods.

The concentrations of MMA were elevated (>300 nmol/L) with both methods in 46 samples [mean (SD) MMA-LC-MS/MS = 782 (661) nmol/L, mean (SD) MMA-GCMS = 781 (629) nmol/L, mean (SD) holoTC = 27 (15) pmol/L, mean (SD) tHcy = 18.4 (13.7) μmol/L].

Table 1  Age, sex and concentrations of vitamin B12 markers.

| Age, sex and concentrations of vitamin B12 markers. All (n=138) |
|---|---|
| Age, years | 64 (41–74) |
| Male, n (%) | 51 (37%) |
| MMA GCMS, nmol/L | 267 (153–766) |
| MMA LC-MS/MS, nmol/L | 261 (150–782) |
| holoTC, pmol/L | 40 (16–69) |
| Total vitamin B12, pmol/L | 215 (121–380) |
| tHcy, μmol/L | 12.8 (8.3–20.7) |
| HoloTC < 35 pmol/L, n (%) | 58 (42%) |
| tHcy < 12 μmol/L, n (%) | 83 (60%) |
| Total vitamin B12 ≤ 148 pmol/L, n (%) | 35 (25%) |
| MMA GCMS > 300 nmol/L, n (%) | 51 (37%) |
| MMA LC-MS/MS > 300 nmol/L, n (%) | 54 (39%) |
| MMA GCMS & LC-MS/MS > 300 nmol/L, n (%) | 46 (33%) |
| Creatinine, μmol/L | 70.7 (53.0–88.4) |

Data are median (10–90th) percentiles. Normal ranges: for MMA<300 nmol/L, for tHcy<12 μmol/L, for holoTC≥35 pmol/L, total vitamin B12≥148 pmol/L.

Figure 1  The correlation between MMA GCMS and LC-MS/MS among two holoTC ranges. The correlation coefficients are according to Spearman test. The regression lines (95%CI) are shown. The cutoff values (300 nmol/L) are shown.
Table 2  The correlation between serum MMA and other B12 markers.

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<thead>
<tr>
<th></th>
<th>GCMS</th>
<th>LC-MS/MS</th>
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<tbody>
<tr>
<td>Total vitamin B12</td>
<td>-0.54</td>
<td>-0.61</td>
</tr>
<tr>
<td>HoloTC</td>
<td>-0.61</td>
<td>-0.66</td>
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<tr>
<td>tHcy</td>
<td>0.35</td>
<td>0.30</td>
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All p values <0.001 (Spearman test).

Figure 2  Bland-Altman plot of the difference between the methods (y-axis) against the mean of the methods (x-axis). The horizontal lines indicate mean differences between the methods and the other two lines represent (mean±2SD). The regression line and the 95% confidence intervals are shown.

MMA showed normal MMA (≤300 nmol/L) with both methods [mean (SD); MMA LC-MS/MS=216 (59), MMA-GCMS=222 (58) nmol/L, holoTC=51 (32) pmol/L, tHcy=12.6 (3.9) μmol/L]. Five samples showed elevated MMA-GCMS [mean (SD)=325 (24) nmol/L], but normal MMA-LCMSMS [mean (SD)=254 (43) nmol/L]. However, the mean difference between the methods in the 5 samples was [mean (SD) GCMS-LCMSMS=71 (35) nmol/L, holoTC=39 (12) pmol/L, and tHcy=12.6 (5.3) μmol/L, confirming that vitamin B12 markers are not in a critical deficiency range in these five samples.

Discussion

The described LC-MS/MS method for MMA assay has many advantages compared to the traditional GCMS method. This technology saves working time required for sample preparation and chromatogram interpretation; allows a high throughput and an automation of the test [15]. Despite high costs of the analytical equipment, the high throughput of the LC-MS/MS method together with the low expenses of the sample preparation procedure are expected to reduce the costs per test significantly. The new LC-MS/MS method (RECIPE) showed a strong correlation to MMA-GCMS. The results were very consistent with the GCMS method in 90% of samples with serum MMA-GCMS >300 nmol/L. In addition, the LC-MS/MS method showed slightly better correlations to other vitamin B12 markers (total B12, and holoTC).

The concentration of MMA is a specific and a sensitive marker that increases in blood and urine of vitamin B12 deficient subjects [20]. When the concentration of holoTC in serum is low, MMA can serve as a valuable marker for distinguishing vitamin B12-depletion from an intracellular deficiency. Furthermore, MMA levels measured before and after vitamin B12 treatment (within 2–4 weeks) can be used to diagnose B12 deficiency in renal patients [21]. The significant lowering of MMA (by approximately >270 nmol/L) after vitamin B12 treatment has been suggested as a simple and reliable way to judge a pre-treatment B12 deficiency in patients with renal dysfunction [7, 21]. One further diagnostic role for MMA is in patients with diabetes type 2 treated with metformin. Circulating serum vitamin B12 levels (holoTC and total B12) are low in metformin-treated patients [22, 23]. Metformin has long been thought to cause vitamin B12 deficiency [24]. However, lowering vitamin B12 or holoTC in blood of metformin treated subjects was not associated with changes in the functional vitamin B12 markers (i.e., MMA and tHcy) [24–26]. Therefore, MMA measurement helps verifying vitamin B12 deficiency in patients with diabetes in general and in those who are treated with metformin, in particular [24–26].

One perspective of measuring MMA level is for monitoring the success of vitamin B12 therapy and optimizing the dose and route of administration of vitamin B12 [20, 27]. Measuring the functional marker (i.e. MMA) makes more sense than measuring serum vitamin B12 or holoTC in case of monitoring the response to treatment. In line with this, cyanocobalamin injection as a single does (1 mg) to severely deficient patients caused a continuous progressive decline in tHcy and MMA over few days (from 5.8 nmol/L to 1.0 nmol/L) [27]. Moreover, a dose-response relationship between oral vitamin B12 dose and reduction of plasma tHcy and MMA has been reported thus confirming a quantitative conversion to methylcobalamin and adenosylcobalamin [28]. Lowering the metabolic marker, MMA, after B12 treatment specifically indicates that the B12 is available for the cell and...
that B12-dependent biochemical reactions are corrected. Treatment of manifested vitamin B12 deficiency starts usually with administering the vitamin as injections. Long-term supplementation is required in most cases, but because many patients can absorb vitamin B12, oral treatment may offer a cost-effective alternative to long term injections [29]. In this case, monitoring MMA concentrations (i.e., every 6 months) can help monitoring the success of treatment.

We recently suggested using holoTC as a first line marker followed by MMA as a second line marker when holoTC is below 75 pmol/L [7]. MMA was analyzed in a group of individuals with holoTC between 25 and 50 pmol/L [30]. The prevalence of elevated MMA (>280 and 360 nmol/L for patients ≤65 or >65 years) was 26% in patients with holoTC of (45–50 pmol/L) versus 41% in patients with holoTC in the lower range (25–29 pmol/L) [30]. Therefore, current evidence suggests that the determination of MMA is essential to verify low vitamin B12 status particularly in patients with intermediate holoTC in the range of <50 pmol/L (or <75 pmol/L in older people or renal patients) [7, 30].

Testing for total plasma vitamin B12 is cheap and therefore, widely used, but is not sensitive in identifying the majority of asymptomatic deficient individuals [7]. The costs for holoTC and MMA are currently 3- and 4-fold, respectively higher than that for total vitamin B12. However, the number of cases that will be detected and treated with vitamin B12 after holoTC and MMA tests is considerably higher than that after total vitamin B12 [7]. A further cost-saving potential has been suggested by a recent study that estimated a saving of approximately 131 € per patient per year when MMA testing is included in a later step to optimize the treatment [29]. Finally, cost-effectiveness studies should also consider the huge costs for clinical complications and the safety and very low costs of vitamin B12 supplements. Obviously, spending on expensive diagnostic tests can reduce spending on inappropriate and expensive therapy [29]. In conclusion, the RECIPE assay for MMA on a LC-MS/MS platform (MS5100 MMA assay) is comparable to the GCMS method and has several additional advantages. The method shows agreement with the GCMS method in detecting samples with low B12 status as confirmed by other B12 markers. The LC-MS/MS is ready to substitute the GCMS and to be used for routine testing of MMA.

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References


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