Methylmalonic Acid Concentrations in Serum of Normal Subjects: Biological Variability and Effect of Oral L-Isoleucine Loads before and after Intramuscular Administration of Cobalamin

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The clinical value of measuring concentrations of methylmalonic acid in serum (S-MMA) as an aid in the diagnosis of cobalamin deficiency has recently aroused interest. In 58 healthy subjects, ages 40–68 years, we found a 0.95 reference interval of 0.05–0.37 μmol/L (mean 0.21, SD 0.094). In 33 of the subjects, who were studied further, day-to-day variation (SD) was 0.031 μmol/L. Intake of food had no effect. Weekly and three-monthly intra-individual variations were both 0.038 μmol/L. In all seven subjects with S-MMA >0.30 μmol/L, the concentrations declined significantly after intramuscular administration of cobalamin. No significant difference was found between mean serum cobalamin concentrations in these seven and in the remaining subjects. We have also established the normal range of S-MMA to standardized oral loading of L-isoleucine: 100 mmol caused a significant average S-MMA increase of 0.072 μmol/L before cobalamin administration vs 0.013 μmol/L after cobalamin, without significant relation to initial S-MMA values. Our results provide a necessary background for interpretation of S-MMA measurements in clinical studies.

Additional Keyphrases: intra-individual variation • reference interval • nutritional status

Currently, measurement of cobalamin in serum is essentially the only diagnostic tool in clinical chemistry generally available for use in determining whether a patient is cobalamin deficient. However, the clinical value of cobalamin estimations is uncertain. Serum concentrations of cobalamin do not necessarily reflect the cobalamin status of the body. The use of RIAs with purified intrinsic factor to analyze for cobalamin has reduced, but not eliminated, the incidence of falsely normal concentrations of cobalamin measured in serum of subjects with cobalamin deficiency. Current findings in the literature suggest that subtle cobalamin deficiency is indeed clinically significant, and the limitations in using only the serum cobalamin concentration to detect cobalamin deficiency have been widely recognized (1–7). The need for an ancillary diagnostic test in patients with low or low-normal serum cobalamin has been emphasized, and testing for methylmalonic acid (MMA) has been recommended (8–16).

Patients with cobalamin deficiency have increased concentrations of MMA in their serum (S-MMA) because 5'-deoxyadenosylcobalamin is required for the enzymatic conversion of L-methylmalonyl-CoA to succinyl-CoA by methylmalonyl-CoA mutase (EC 5.4.99.2). An increased concentration of MMA in serum and its excessive urinary excretion are believed to be direct measures of tissue stores of cobalamin (17) and thus provide an indication of functional cobalamin deficiency (8, 9, 18). Recently, Rasmussen et al. (14) reported that, in contrast to S-MMA, the urinary excretion of MMA in normal subjects is significantly influenced by previous intake of food and that distinction between patients with evidence of cobalamin deficiency and normal subjects may best be achieved by measurements of MMA in serum.

For three reasons, previous studies of MMA in humans (14, 19) led us to believe that the published reference intervals for S-MMA were too high. First, the range (0.05–0.28 μmol/L) noted (14) in serum from 28 healthy volunteers, ages 22–86 years, who all had a normal concentration of cobalamin in serum, were lower than the normal reference ranges reported, e.g., from 0.16 to 0.64 μmol/L in the U.S.A. (20) and from 0.08 to 0.56 μmol/L in Denmark (21), both calculated on the basis of determinations on 50 blood donors; however, the donors had not been assessed for cobalamin status. Second, the finding in normal subjects that S-MMA was not appreciably increased by stressing the biochemical pathway with added protein, fat, and sugar (19) makes it unlikely that the high values encountered in some of the blood donors were due to previous intake of food. Third, Stabler et al. (8), Lindenbaum et al. (9), and Moelby et al. (data to be published elsewhere) have observed high-normal or only slightly increased values of S-MMA in some patients with definite evidence of cobalamin deficiency. These findings raise the question: are the current normal reference values for S-MMA too high?

In the present work, we have attempted to define the upper limit for MMA in serum of non-cobalamin-deficient subjects. We examined intra- and interindividual variation and long-term biological variation. We also measured the response of S-MMA to standardized oral loading with L-isoleucine in subjects with normal and high-normal values of S-MMA, so as to establish a normal reference interval for the response to this loading. Finally, we investigated the effect of intramuscular cobalamin both on S-MMA and on the response of S-MMA to isoleucine load.

Specimens and Methods

Subjects

Selection. All 54 members of the laboratory staff, ages 40 years or older, and 31 chief physicians at our hospitals, selected without conscious bias, were asked to participate in the investigation; 64 volunteered. Exclusion criteria were abnormal concentrations of cobalamin or creatinine in serum, or an abnormal erythrocyte mean-cell volume. Four candidate subjects were thus rejected. In the remaining 60 subjects, erythrocyte counts, leucocyte counts, differential leucocyte counts, and platelet counts were all within normal limits, as were results of liver-function tests. Blood hemoglobin was subnormal in one woman with sideropenia but normal in the remainder. All were apparently healthy and in good nutritional state. None took drugs known to interfere with cobalamin metabolism.

CLINICAL CHEMISTRY, Vol. 36, No. 7, 1990 1295

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Reference individuals. Blood was obtained (sample 0) from the 60 volunteers on a normal working-day before lunchtime, approximately 5 h after an everyday breakfast. No restrictions were put on intake of snacks, coffee, tea, or other liquid. In two of the 60 subjects, however, we missed the samples. Consequently, this group comprises 58 healthy subjects (age range: 40–68 (median 53) years): 37 women, ages 40–64 (median 48) years, and 21 men, ages 43–68 (median 53) years.

Participants in the experiments. After informed consent, and under protocols approved by the Ethical Committee of Aarhus County, 33 of the 58 subjects participated in the succeeding experiment [ages 40–68 (median 48) years]: 18 women, ages 40–64 (median 49) years, and 15 men, ages 43–68 (median 53) years. The first 22 subjects considered entered the experiment consecutively; the final 11 subjects were selected among the remaining 36 subjects on the basis of having the highest S-MMA values. In advance, we recruited four (two women, ages 40 and 53 years, and two men, ages 43 and 53 years) of the 22 subjects to participate in the long-term study of intra-individual variation.

Experimental Procedure

The experiment started from three days to two weeks after the initial blood collection for determination of S-MMA (sample 0).

Day 1. Blood was collected (sample 1) under circumstances similar to those under which sample 0 was obtained.

Day 2. The protocol began with an overnight (12 h) fast. The first blood sample (sample 2) was drawn at the beginning of a working-day immediately before the subjects were given an oral load of 100 mmol of L-isoleucine (19). After 5 h (± 0.5) the next blood sample was collected (sample 3) and the subject was given 1 mg (1 mL) of intramuscular cyanocobalamin.

Day 5. The subjects were administered 1 mg of intramuscular cyanocobalamin.

Day 8. Sample 4 was obtained under circumstances similar to those on days 0 and 1.

Day 9. The procedure from day 2 was repeated (samples 5 and 6), except that no more cyanocobalamin was administered to the subjects.

Specimens

Blood samples for determination of MMA were taken from an antecubital vein. After coagulation at room temperature for 1 h, serum was separated by centrifugation and stored at −20 °C.

Determinations

MMA in serum was measured by stable-isotope dilution with solid-phase extraction of the sample (21). MMA from 1650 μL of serum was quantitatively extracted, together with added methyl-d₆-malic acid (internal standard), onto a small, disposable, strong-anion-exchange column (Bond Elut®, SAX; Analytichem International, Harbor City, CA). The sorbent counter-ion was formate. Neutral and basic compounds were washed out with water, and the retained organic acids were eluted with 18 mol/L formic acid reagent. The formic acid was removed by evaporating the resulting extract under a stream of nitrogen at room temperature. After a methanol rinse, the residue was dissolved in hydrogen chloride, 1.5 mol/L in cyclohexanol, and incubated at 115 °C for 15 min. The cyclohexanol was evaporated almost to dryness at 70 °C under a gentle stream of nitrogen, and the residue was dissolved in methanol. The dicyclohexyl derivatives were measured by gas chromatography–mass spectrometry with the mass spectrometer in the selected-ion monitoring mode (21). All specimens were assayed in duplicate.

Serum cobalamin concentrations were determined with a radioisotope cobalamin/folate method from Amersham International, Bucks., U.K., involving denaturation of endogenous binding proteins at strongly alkaline pH and purified hog intrinsic factor as binding protein. By this method, the normal 0.95 reference interval for cobalamin is 135–590 pmol/L.

Statistical analysis of data. We used the χ²-test for normality, analysis of variance, t-test for paired and unpaired comparisons, and F-test in the statistical analysis.

Results

Analytical Variation

Total analytical variation was assessed during the entire nine-month period. The SD for MMA in a pooled serum kept at −18 °C, assayed on 67 different occasions, was 0.032 at 0.35 μmol/L (CV 9.0%). The within-day component of imprecision, as measured on the basis of duplicate determinations and calculated by analysis of variance, was 0.019 μmol/L (for the mean of two determinations) for 264 samples.

Biological Variation before Intramuscular Cobalamin

Intra-individual variation. The day-to-day variation (SD) as calculated by analysis of variance for samples 0 and 1 from 33 subjects was 0.031 μmol/L.

For five to seven weeks, three subjects had blood sampled every week (19 samples); their weekly intra-individual variation (SD) was 0.038 μmol/L.

Four subjects had three-monthly (quarterly) samples taken two to four times (12 samples); the three-monthly intra-individual variation (SD) was 0.038 μmol/L.

No significant difference was found between day-to-day, weekly, and three-monthly intra-individual variations (F-test, P >0.05). The variation (SD) between an ordinary sample and a sample taken after an overnight fast (sample 2) was 0.031 μmol/L, i.e., equal to the intra-individual day-to-day variation. Thus fasting vs nonfasting did not introduce any significant component of variation (F-test).

Interindividual variation. Figure 1 shows the S-MMA (sample 0) measured in 58 subjects. A χ² goodness-of-fit test did not prove any deviation from the normal distribution (0.90 > P >0.80), and no age- or sex-related differences could be demonstrated. The mean value for S-MMA was 0.21 μmol/L (SD 0.094 μmol/L, 0.95 confidence interval 0.05–0.37 μmol/L).

Response to oral L-isoleucine. Primarily, we undertook this part of the study to define the normal response of S-MMA to a standardized oral isoleucine load. In the 33 subjects who underwent the loading test, the 100 mmol of L-isoleucine caused a significant (P <0.001, t-test for paired increase) increase in S-MMA (see Table 1). No significant relation with sex, age, or initial S-MMA concentration was found.

Effect of Intramuscular Cobalamin

Concentration of MMA in serum. Table 1 shows that administration of intramuscular cobalamin caused a significant decrease (sample 5 vs sample 2) in S-MMA (t-test).
for paired comparisons, \( P < 0.0001 \). The decrease correlated significantly with the S-MMA before the cobalamin injections \( (r = 0.68, \ P < 0.01) \). The seven subjects with S-MMA >0.30 \( \mu \text{mol/L} \) before cobalamin manifested the largest decrease \( (0.060-0.173 \ \mu \text{mol/L}) \) (Figure 2). The remaining 26 subjects exhibited neither a significant decrease nor any significant correlation between initial S-MMA and decrease. No significant difference between mean serum cobalamin concentrations in the seven subjects and in the 26 subjects (Table 1) was found \( t \)-test, unpaired (Welch's correction), \( 2F > 0.10 \). The seven subjects had an age- and sex-distribution corresponding to that of the complete set of probands.

**Table 1. Mean Concentrations of Cobalamin and MMA in 33 Healthy Subjects and Investigated Subgroups, Correlation between Cobalamin and MMA, and Average Effect of Intramuscular Cobalamin on Serum MMA and on Response to Oral L-isoleucine Loads**

<table>
<thead>
<tr>
<th>Conc of MMA, ( \mu \text{mol/L} )</th>
<th>All subjects</th>
<th>(&lt;0.30)</th>
<th>(&gt;0.30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>33</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>Serum cobalamin, pmol/L</td>
<td>277</td>
<td>286</td>
<td>242</td>
</tr>
<tr>
<td>(21)*</td>
<td>(25)</td>
<td>(14)</td>
<td></td>
</tr>
<tr>
<td>Serum MMA, ( \mu \text{mol/L} )</td>
<td>0.230(\text{p})</td>
<td>0.190</td>
<td>0.375</td>
</tr>
<tr>
<td>(0.018)</td>
<td>(0.012)</td>
<td>(0.022)</td>
<td></td>
</tr>
<tr>
<td>Decrease after i.m.cobalamin</td>
<td>0.031</td>
<td>0.017</td>
<td>0.091</td>
</tr>
<tr>
<td>(0.009)</td>
<td>(0.008)</td>
<td>(0.010)</td>
<td></td>
</tr>
<tr>
<td>Response to oral L-isoleucine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increase before i.m.cobalamin</td>
<td>0.073</td>
<td>0.072</td>
<td>0.077</td>
</tr>
<tr>
<td>(0.011)</td>
<td>(0.012)</td>
<td>(0.030)</td>
<td></td>
</tr>
<tr>
<td>Increase after i.m.cobalamin</td>
<td>0.010</td>
<td>0.013</td>
<td>0.000</td>
</tr>
<tr>
<td>(0.011)</td>
<td>(0.010)</td>
<td>(0.013)</td>
<td></td>
</tr>
</tbody>
</table>

* SEM values given in parentheses.

Significantly correlated with concentrations of cobalamin in serum \( (r = \ -0.33, \ P < 0.05) \).

**Discussion**

A prerequisite for the interpretation of S-MMA measurements is knowledge of analytical variation, intra- and interindividual variation, long-term biological variation, and the influence of food intake.

**Analytical variation.** The results of our evaluation of the assay performance compare reasonably well with those originally reported from our laboratory \( (21) \).

**Intra-individual variation.** This first investigation of intra-individual variation of S-MMA showed consistency of individual S-MMA. In agreement with a previous study \( (19) \) involving a limited number of subjects, our results further showed no evidence of significant influence of previous intake of food on S-MMA. One practical consequence is that S-MMA can be validly determined in serum specimens from fasting as well as nonfasting patients. Recently, Rasmussen et al. \( (14) \) reported that in healthy subjects a linear relationship exists between S-MMA and urinary MMA excretion rates; their data were consistent with glomerular filtration and passive reabsorption by the tubules. Furthermore, the excretion rate in any one individual is relatively constant \( (19) \). Therefore, the consistency of individuals' S-MMA most probably reflects a stable rate of MMA production in the same individuals over a long time.

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**Fig. 1.** S-MMA in 58 healthy subjects: 37 women and 21 men (ages 40–68 years)

**Fig. 2.** Effect of intramuscular cobalamin on S-MMA (mean \( \pm \) SEM) in all seven healthy subjects presenting with initial concentrations above 0.30 \( \mu \text{mol/L} \) (upper line), and in the remaining 26 healthy subjects (lower line)

**Response to oral L-isoleucine after cobalamin.** As Table 1 shows, the response to L-isoleucine decreased significantly after cobalamin administration \( (t \)-test for paired comparisons, \( P < 0.0001) \), but no significant relation to sex, age, or initial S-MMA was found. Figure 3 illustrates the response to loading with L-isoleucine before and after cobalamin supplementation (95% confidence range: \( -0.043 \) to \( 0.187 \) and \( -0.083 \) to \( 0.109 \) \( \mu \text{mol/L} \), respectively) in contrast to the response in a 67-year-old patient with neurological symptoms caused by cobalamin deficiency in whom the concentration of cobalamin in serum was normal as measured both by the S-binder assay (358 pmol/L) and by an R-binder assay (743 pmol/L; normal range: 303–933 pmol/L).

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**CLINICAL CHEMISTRY, Vol. 36, No. 7, 1990 1297**
suspected that "high-normal" values of S-MMA reported for blood donors in the U.S.A. and in Denmark might come from cobalamin-deficient subjects. Recently Lindenbaum et al. (9) concluded that measurements of S-MMA (and total homocysteine in serum) both before and after treatment with cobalamin provide useful confirmatory evidence of a deficiency of cobalamin. Therefore, our finding is interesting that values of S-MMA just above 0.30 μmol/L, encountered in seven subjects (Figure 2), declined significantly after administration of cobalamin. What this suppression of high-normal values of S-MMA might account for is at present unclear. The amount of 5'-deoxadenosylocobalamin in the tissues in normal humans may be insufficient to saturate the methylmalonyl-CoA mutase, as indicated for liver tissue in rat (24) and rabbit (25) in studies on intracellular binding of radioactive cobalamin to cobalamin-dependent apoenzymes. Another possibility is that the phenomenon may have been caused by mild cobalamin deficiency in some tissues (e.g., muscle cells); if so, these responses to cobalamin indicate that increased S-MMA is an early event in the development of cobalamin deficiency. However, the response of the above seven subjects to L-isoleucine loading, an average S-MMA increase of 0.077 μmol/L (range: −0.058 to 0.146 μmol/L), which does not exceed the response observed in the complete set of probands, does not support a hypothesis of early cobalamin deficiency.

In all 33 probands, response to oral L-isoleucine almost disappeared after cobalamin administration (Figure 3); however, this finding does not prove tissue cobalamin deficiency. A lack of 5'-deoxadenosylocobalamin in mitochondria in some cells to convert a short-lived surplus of L-methylmalonyl-CoA to succinyl-CoA may indicate that the individual has no excess of 5'-deoxadenosylocobalamin at the sites of biochemical utilization, but it does not necessarily reflect cobalamin deficiency.

Ongoing studies of healthy elderly people (15 thus far) support our present findings for middle-aged subjects. Four of the 15 had S-MMA values above 0.30 μmol/L (mean 0.37, range 0.32–0.40), which declined to an average of 0.23 range (0.20–0.28) after intramuscular cobalamin. In the remaining 11, the mean concentrations were 0.24 and 0.23 μmol/L, before and after cobalamin. The average response to oral L-isoleucine in the four and 11 was 0.146 and 0.105 μmol/L, respectively, and 0.046 and 0.000 μmol/L, respectively, before and after intramuscular cobalamin. The correlation between serum cobalamin and S-MMA was −0.59 (P <0.025, n = 15). The mean serum cobalamin concentrations in the four (167 pmol/L, SEM 16 pmol/L) was significantly lower than that in the 11 (318 pmol/L, SEM 27 pmol/L) subjects; although these data are only preliminary, they hint at the possibility of impaired MMA metabolism early in the development of cobalamin deficiency.

As for the future of this area of investigation, subjects with S-MMA >0.30 μmol/L should be studied over a long time to clarify whether they do eventually develop clinical cobalamin deficiency.

In conclusion, our main finding is that the observed upper level of the normal reference values may not be the ideal value for differentiating patients with early tissue cobalamin deficiency from normal subjects. The results of this study provide a necessary background for clinical studies that must include subjects with only slightly increased concentrations of MMA and patients with possible
cobalamin deficiency to clarify whether measurements of S-MMA before and after injections of cobalamin, or before and after loading with L-isoleucine, provide the best distinction between cobalamin deficiency and normality.

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References