Traditional reference values for serum vitamin B₁₂ and folate are not applicable to automated serum vitamin B₁₂ and folate assays: comparison of value from three automated serum vitamin B₁₂ and folate assays

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Summary  Because dietary reference intakes of vitamin B₁₂ and folate are set based on the amount needed for the maintenance of serum concentrations of these vitamins above a lower reference value (200 pg/mL for vitamin B₁₂ and 3 ng/mL for folate by microbiologic assays), we investigated whether these values are applicable to automated competitive protein binding (CPB) assays. In serum specimens collected from volunteers, vitamin B₁₂ and folate were determined by three automated CPB methods, i.e., Access, Advia Centaur and Elecsys. Observed values of serum folate and vitamin B₁₂ were significantly correlated among each method. There is a significant difference in mean folate concentrations in serum among the biologic assay and the three automated CPB methods, and the values of vitamin B₁₂ by Elecsys and those of folate by Advia Centaur were significantly higher. Because the bias around 200 pg/mL for vitamin B₁₂ and 3 ng/mL for folate was too large to directly compare observed values by each method, traditional reference values for serum vitamin B₁₂ and folate are not applicable to automated CPB methods.

Key words: Competitive protein binding (CPB) methods, Folic acid, Standardization, Access, Advia Centaur, Elecsys
1. Introduction

The functions of vitamin B12 and folate are interwoven metabolically. Vitamin B12 acts on the reaction of the conversion of methylmalonyl CoA to succinyl CoA and on the methylation reaction of homocysteine. Since vitamin B12 is also required for the enzymatic removal of the methyl group from methylfolate which regenerates tetrahydrofolate, the lack of vitamin B12 produces a folate deficiency. Folate is an essential coenzyme involved in single carbon transfers; five of the major reactions are conversion of serine to glycine, catabolism of histidine, and synthesis of thymidylate, methionine, and purine\(^7\). The major known result of folate deficiency is megaloblastic anemia, and in the case of vitamin B12, pernicious anemia. In addition, a lack of sufficient dietary intake of folate has recently been reported to increase the prevalence of neural tube defects at birth\(^6\), and to increase the risk of vascular disease in the elderly\(^7\).

Nowadays, sufficient dietary intake, i.e., dietary reference intakes, of vitamin B12 and folate are set in Canada, the United States and Japan based on the amount needed for the maintenance of hematological status and on the serum concentrations of these vitamins above lower reference values\(^4,5\) of 200 pg/mL for vitamin B12, and 3 ng/mL for folate\(^1,6,7,9\). These values were obtained by microbiologic assays using *Lactobacillus* sp., however, serum vitamin B12 and folate are now commonly measured by competitive protein binding (CPB) assay utilizing commercial automated methods. The aim of this study is to investigate if the values of 200 pg/mL for vitamin B12, and 3 ng/mL for folate are applicable to different automated CPB methods.

2. Materials and methods

1. Materials

Cyanocobalamin (CNCbl: 226-00343), methylcobalamin (MeCbl: M-9756), pteroylglutamic acid (PGA: F-7876), and 5-methyltetrahydrofolic acid, barium salt (SMTHF: M-7754) were all purchased from Sigma-Aldrich, Tokyo, Japan.

2. Study design

Fasting venous blood collected in evacuated tubes from 58 healthy young female university students (18-26 years) were subjected to analyses of complete blood counts, reticulocyte, serum iron, total-iron binding capacity, serum ferritin, plasma homocysteine, and serum vitamin B12 and folate. All the volunteers provided a 3-day dietary history prior to examination to verify that they were free of habitual smoking, drinking or dietary supplements. We chose young women as subjects, because they are aware that they need to take folate prior to conception as well as during pregnancy. In this study, written informed consent was obtained from all volunteers, and the protocol was approved by the Protection of Human Subjects Committee of Showa Women’s University.

3. Analyses

Serum concentrations of vitamin B12 and folate were determined by three automated methods based on the CPB assay, i.e., Access (Beckman Coulter Inc., Fullerton, CA, USA), Advia Centaur (Bayer Diagnostics, Tarrytown, NY, USA), and Elecsys (Roche Diagnostics GmbH, Mannheim, Germany) according to the instructions of automated methods and the manufacturer’s reagents. Although the assay principle of these three instruments for vitamin B12 and folate was based on the CPB assay, they have different detection methods; i.e., chemiluminescence detection in Advia Centaur, electrochemiluminescence detection in Elecsys, and detection of chemiluminescence produced from the enzymatic reaction in Access. Analytical precisions and linearity ranges of the three instruments on vitamin B12 and folate analyses are summarized in Table 1. Although more automated CPB methods are being developed including radiometric CPB assay around the world, the above three instruments are exclusively available in Japan.

To compare the results obtained employing the automated CPB method, the serum folate concentration was also measured by microbiologic assay using the assay organism *Lactobacillus rhamnosus* ATCC 27773 (*L. casei*)\(^1,10\). *L. casei* is used most commonly because it responds to a wide variety of folate derivatives such as PGA, SMTHF, 5-formyltetrahydro-
Table 1 Analytical precision and linearity ranges of three automated CPB methods on vitamin B\(_\text{12}\) and folate assays

<table>
<thead>
<tr>
<th>Assay for vitamin B(_\text{12})</th>
<th>Access</th>
<th>Advia Centaur</th>
<th>Elecsys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference material (and method)</td>
<td>CNCl</td>
<td>CNCl (RA)</td>
<td>CNCl (RA)</td>
</tr>
<tr>
<td>Within-day CV (%)</td>
<td>6</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Between-day CV (%)</td>
<td>9</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Linearity (pg/mL)</td>
<td>50—1,500</td>
<td>16—2,000</td>
<td>30—2,000</td>
</tr>
<tr>
<td>Method-specific reference value (pg/mL)</td>
<td>180—914</td>
<td>233—914</td>
<td>197—866</td>
</tr>
<tr>
<td>Assay for folate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference material (and method)</td>
<td>PGA</td>
<td>5MTHF (RA)</td>
<td>PGA (RA)</td>
</tr>
<tr>
<td>Within-day CV (%)</td>
<td>5</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Between-day CV (%)</td>
<td>10</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Linearity (ng/mL)</td>
<td>0.5—20</td>
<td>0.5—20</td>
<td>0.5—20</td>
</tr>
<tr>
<td>Method-specific reference value (ng/mL)</td>
<td>&gt;3.0</td>
<td>3.6—12.9</td>
<td>2.0—9.1</td>
</tr>
</tbody>
</table>

CV: coefficient of variation around lower reference value.
RA: radiometric CPB assay (Quantaphase B\(_\text{12}\) and Folate, Bio-Rad Laboratories, USA).

folic acid (5-HCO-HPGA), 10-formyltetrahydrofolate (10-HCO-HPGA), and 5,6,7,8-tetrahydrofolate (H\(_\text{PGA}\)). This microbiologic assay using \textit{L. casei} has been commonly used to assess the folate nutritional status in humans as to all folates present in serum. In the microbiologic assay, PGA was used as a calibration material.

For determination of reactivities to vitamin B\(_\text{12}\) with the automated CPB methods, 100 mg/L standard solutions were prepared by dissolving CNCl or MeCl in distilled water. They were then diluted 10-fold with distilled water, and their concentrations were confirmed spectrophotometrically. CNCl has a molar absorptivity of 15,600 at 278 nm, and for MeCl, this is 19,900 at 266 nm\(^\text{11}\). After 100 mg/L standard solutions of CNCl and MeCl were certified, they were diluted 10,000-fold with distilled water followed by a 10- to 40-fold dilution with the manufacturer's diluent, and the concentrations were measured by the three automated methods.

For folate, 100 mg/L standard solutions were prepared by dissolving 5MTHF or PGA in distilled water. They were then diluted 10-fold with 0.1 mol/L, pH 7.0 phosphate buffer, and their concentrations were confirmed spectrophotometrically. PGA has a molar absorptivity of 27,000 at 282 nm, and for 5MTHF, this is 31,700 at 290 nm\(^\text{11}\). After 100 mg/L standard solutions of PGA and 5MTHF were certified, they were diluted 1,000-fold with distilled water followed by a 10- to 40-fold dilution with the manufacturer's diluent, and the concentrations were measured by the three automated methods.

4. Statistical analysis of data
Data differences were analyzed by the Wilcoxon signed-rank test for non-Gaussian variables. Statistical significance was defined as \(p<0.05\).

3. Results

1. Studies on volunteers
Serum concentrations of vitamin B\(_\text{12}\) and folate in 58 healthy young Japanese women were investigated using three automated methods. Among the 58 women, serum vitamin B\(_\text{12}\) values ranged from 210 pg/mL (assessed by Access) to 1,359 pg/mL (assessed by Elecsys), and serum folate values ranged from 1.82 ng/mL (assessed by Elecsys) to 19.53 ng/mL (Advia Centaur). Although all volunteers had vitamin B\(_\text{12}\) values higher than 200 pg/mL regardless of the method, seven had folate values less than 3.0 ng/mL when assessed by Elecsys. Of the seven, only one volunteer had a serum folate value (1.82 ng/mL) below the method-specific reference value of Elecsys (<2.0 ng/mL; Table 1).

Values of vitamin B\(_\text{12}\) and folate observed by the automated methods were significantly correlated among the three methods (correlation coefficients
were 0.959 to 0.977 for vitamin B₁₂ and 0.866 to 0.977 for folate, p<0.001). Observed values by the three methods were plotted against their mean values, and a bias was calculated from the mean values (Fig. 1). Vitamin B₁₂ values observed by Elecsys were significantly higher than those by Access and Advia Centaur (Second column in Table 2, p<0.001). Folate values observed by Advia Centaur were significantly higher than those by Access and Elecsys (p<0.001).

2. Reactivities to vitamin B₁₂ and folate

We investigated reactivities to vitamin B₁₂ and folate with the three automated CPB methods as described in the Materials and Methods. For CNB, reactivities against expected values were 76.4% in Access, 91.0% in Advia Centaur, and 86.1% in Elecsys; for MeCbl they were 85.0% in Access, 89.5% in Advia Centaur, and 125.3% in Elecsys (Table 3). For PGA, reactivities against expected values were 162.5% in Access, 316.9% in Advia Centaur, and 176.2% in Elecsys; for 5MTHF they were 39.8% in Access, 84.6% in Advia Centaur, and 50.1% in Elecsys.

3. Correction of assay values

To reduce any bias in the three automated CPB methods, we corrected their values by reactivates obtained from an analysis of standard solutions of vitamin B₁₂ and folate (see Table 3). For example, observed values of vitamin B₁₂ by Access were corrected when multiplying by 1.308 and 1.177, because Access reacted 76.4% and 85.0% to the certified concentration of CNB and MeCbl, respectively. When observed values of vitamin B₁₂ by the three methods were corrected by MeCbl, bias among them was reduced by one-half. A correction of folate values by PGA, a minor component in human serum, significantly (p<0.05) reduced the bias among the
three methods, whereas a correction by 5MTHF did not (see third and fourth columns in Table 3).

4. Comparison with values by microbiologic assay

The mean (SD) concentration of serum folate in our volunteers was 17.60 (9.52) ng/mL (5.3 ng/mL to 41.3 ng/mL) by the microbiologic assay using \textit{L. casei}. Fig. 2 shows the correlation of the folate concentration determined by biologic assay and by the automated CPB methods. There was a significant difference in the mean folate concentrations in serum between the biologic assay and the automated CPB methods [6.09 (2.35) ng/mL for Access, 9.73 (3.43) ng/mL for Advia Centaur, and 5.27 (2.33) ng/mL for

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig1.png}
\caption{Comparison of three automated CPB methods for serum vitamin B12 and folate with their mean values. (Top, left), linear regression analysis of vitamin B12 by Access (\textbullet{}) gave a slope of 0.992, an intercept of -8.1 pg/mL, and r= 0.989; Advia Centaur (\textsquare) gave a slope of 0.766, an intercept of 53.2 pg/mL, and r= 0.985; and Elecsys (\texttriangle) gave a slope of 1.316, an intercept of -49.0 pg/mL, and r= 0.995. (Top, right), linear regression analysis of folate by Access (\textbullet{}) gave a slope of 0.848, an intercept of 0.140 ng/mL, and r= 0.941; Advia Centaur (\textsquare) gave a slope of 1.292, an intercept of 0.682 ng/mL, and r= 0.989; and Elecsys (\texttriangle) gave a slope of 0.868, an intercept of -0.828 ng/mL, and r= 0.972. (Bottom, left), difference plots of vitamin B12 between values of three automated methods and their mean value of Access (\textbullet{}) gave a mean difference (and ±2SD range) of -51 pg/mL (-108 pg/mL to 6 pg/mL), Advia Centaur (\textsquare) gave a mean difference of -75 pg/mL (-169 pg/mL to 19 pg/mL), and Elecsys (\texttriangle) gave a mean difference of 124 pg/mL (4 pg/mL to 244 pg/mL). (Bottom, right), difference plots of folate between values of three automated methods and their mean value of Access (\textbullet{}) gave a mean difference of -0.93 ng/mL (-2.70 ng/mL to 0.85 ng/mL), Advia Centaur (\textsquare) gave a mean difference of 2.73 ng/mL (0.89 ng/mL to 4.56 ng/mL), and Elecsys (\texttriangle) gave a mean difference of -1.75 ng/mL (-3.05 ng/mL to -0.45 ng/mL).}
Fig. 2 Analysis of serum folate by microbiologic assay for 58 young Japanese women. (Left), linear regression analysis of folate by Access (●) gave a slope of 0.203, an intercept of 2.528 ng/mL, and \( r = 0.820 \); Advia Centaur (□) gave a slope of 0.315, an intercept of 4.254 ng/mL, and \( r = 0.859 \); and Elecsys (△) gave a slope of 0.205, an intercept of 1.657 ng/mL, and \( r = 0.830 \). (Right), difference plots between folate values by microbiologic assay and three automated CPB methods. Access (●) gave a mean difference (and ±2SD range) of -11.51 ng/mL (-26.93 ng/mL to 3.92 ng/mL), Advia Centaur (□) gave a mean difference of -8.04 ng/mL (-22.71 ng/mL to 6.63 ng/mL), and Elecsys (△) gave a mean difference of -12.33 ng/mL (-27.68 ng/mL to 3.02 ng/mL).

Elecsys]. However, the serum folate concentrations determined by the biologic assay were strongly correlated with those determined by the automated CPB methods (\( r = 0.820 \), \( p < 0.001 \) in Access; \( r = 0.859 \), \( p < 0.001 \) in Advia Centaur; \( r = 0.836 \), \( p < 0.001 \) in Elecsys). For folate concentration of 3.0 ng/mL by the microbiologic assay corresponded to 3.1, 1.9 and 7.9 ng/mL, respectively before and after correction by PGA and 5MTHF in Access, 5.2, 1.6 and 6.1 ng/mL in Advia Centaur, 2.3, 1.3 and 4.6 ng/mL in Elecsys.

4. Discussion

We determined the vitamin B₁₂ and folate concentrations in the sera from 58 young Japanese women using three automated CPB methods. Although observed values were significantly (\( p < 0.001 \)) correlated among the three methods, Elecsys for vitamin B₁₂ and Advia Centaur for folate represented significantly higher values (Fig. 1). We concluded that the bias was too large to directly compare observed values by each method with previously established lower reference values for 200 pg/mL of vitamin B₁₂ and 3.0 ng/mL of folate. Because poor agreement among values by each of the automated methods has also been recognized in a non-Japanese population\(^{19}\), the acute need for the improved standardization of the folate assay has now been proposed\(^{19}\) in addition to our study.

We considered that the bias present here would be likely to arise from the different calibration processes employed in each automated CPB method. Before non-isotopic CPB assay was developed, serum vitamin B₁₂ and folate were determined by the radiometric CPB method. Advia Centaur and Elecsys adjusted their values to those obtained by a radiometric CPB assay (Quantaphase B₁₂ and Folate, Bio-Rad Laboratories, USA). Roche Diagnostics GmbH measured serum specimens simultaneously by Elecsys and Quantaphase, and then the observed regression line (Passing-Bablok regression) was stored in the reagent bar code. In Quantaphase B₁₂ and Folate, CNCbl and 5MTHF were used as the respective calibrators. Bayer Diagnostics measured their calibrators of CNCbl and 5MTHF by Quantaphase to assign their calibrator values. Access was calibrated against
known concentrations of CNCbl and PGA solutions, while McCbl is the predominant vitamin B\textsubscript{12} in human serum, and 5'-deoxyadenosyl cobalamin (coenzyme B\textsubscript{12}) is exclusively present in cytosol. For folate, the predominant folate vitamer in serum was 5MTHF, while folate within cells exists as a complex mixture of analogues in various oxidation states and polyglutamate tails of varying lengths\textsuperscript{14}. In serum, 80-90\% of the total folate was 5MTHF, and the remainder was 5-HCO-H\textsubscript{2}PGA and PGA\textsuperscript{15}.

We tried to reduce the bias present among the three methods with the use of McCbl and 5MTHF, respectively, as control materials for the serum analysis of vitamin B\textsubscript{12} and folate. Although the bias in vitamin B\textsubscript{12} was successfully reduced, unfortunately the bias in folate values tended to increase even further after correction by 5MTHF (Table 3). The same trials were performed in folate analysis by Wilson et al.\textsuperscript{19} and Pfeiffer et al.\textsuperscript{20} No trial was conducted for vitamin B\textsubscript{12} except the one in our study. Wilson et al.\textsuperscript{19} calibrated Architect (Abbot Laboratories, IL, USA: one of the automated CPB methods not available in Japan) using different concentrations of 5MTHF as standard solutions, and obtained good comparability around 3 ng/mL to the results by Quantaphase Folate\textsuperscript{21}. The use of PGA did not improve the bias. They selected the radiometric CPB assay traditionally as their reference method. However, Pfeiffer et al.\textsuperscript{20} employed an isotope-dilution tandem mass spectrometric method coupled to liquid chromatography (LC/MS/MS) for their serum folate assay, and compared their observed values to those by Quantaphase Folate and microbiologic assay. They recommended the use of 5MTHF solution (1, 2, 10 and 50 ng/mL) as a reference material employing the LC/MS/MS method as a candidate reference method for serum folate analysis. They obtained 2.9-33.4 ng/mL of folate concentrations (mean±SD, 15.7 ± 7.9 ng/mL) from 42 serum specimens by the LC/MS/MS method. Difference plots showed that the average bias between the LC/MS/MS method and a microbiologic assay was 4.8 ng/mL, while that between the LC/MS/MS method and a radiometric CPB assay was 1.2 ng/mL. The highest values were observed by a microbiologic assay followed by the LC/MS/MS method, and then by a radiometric CPB assay.

The Joint Committee for Traceability in Laboratory Medicine (JCTLM in which Professor Totani was the representative from Japan) has considered LC/MS/MS\textsuperscript{19} or the solid-phase extraction-electrospray ionization mass spectrometry (SPE-LC/MS/MS) method\textsuperscript{10} as a reference method. JCTLM has considered 5MTHF as a reference material after its concentration was verified by gravimetry or spectrophotometry. The present study showed that gravimetry was less accurate than the 5MTHF concentration spectrophotometrically certified at 290 nm. Despite the use of certified 5MTHF, we could not achieve good agreement among the three automated methods. Incompatibility likely arose from the value of 5MTHF used to harmonize assay values among the three methods\textsuperscript{10}. It would be necessary to prepare several concentrations of 5MTHF solution in addition to the 7.79 ng/mL solution (Table 3) for a standardization of serum folate value by automated CPB methods. However, we failed to compare the folate values of the automated methods to those of the LC/MS/MS method, and also failed to compare the vitamin B\textsubscript{12} values of the automated methods to those of microbiologic assay. Before reliable reference materials and methods were available, reference values were method-dependent; lower reference values of 200 pg/mL for vitamin B\textsubscript{12} and 3 ng/mL for folate as determined by microbiologic assays were not uniformly applicable to automated CPB methods. We should establish our own reference values using our own selected automated CPB method of serum vitamin B\textsubscript{12} and folate.

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