〈Original Article〉

Traditional reference values for serum vitamin B_{12} and folate are not applicable to automated serum vitamin B_{12} and folate assays: comparison of value from three automated serum vitamin B_{12} and folate assays

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Summary Because dietary reference intakes of vitamin B_{12} 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 B_{12} 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 B_{12} and folate were determined by three automated CPB methods, i.e., Access, Advia Centaur and Elecsys. Observed values of serum folate and vitamin B_{12} 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 B_{12} by Elecsys and those of folate by Advia Centaur were significantly higher. Because the bias around 200 pg/mL for vitamin B_{12} and 3 ng/mL for folate was too large to directly compare observed values by each method, traditional reference values for serum vitamin B_{12} and folate are not applicable to automated CPB methods.

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

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1. Introduction

The functions of vitamin B_{12} and folate are interwoven metabolically. Vitamin B_{12} acts on the reaction of the conversion of methylmalonyl CoA to succinyl CoA and on the methylation reaction of homocysteine. Since vitamin B_{12} is also required for the enzymatic removal of the methyl group from methylfolate which regenerates tetrahydrofolate, the lack of vitamin B_{12} 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[1]. The major known result of folate deficiency is megaloblastic anemia, and in the case of vitamin B_{12}, 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[2], and to increase the risk of vascular disease in the elderly[3].

Nowadays, sufficient dietary intake, i.e., dietary reference intakes, of vitamin B_{12} 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 B_{12} and 3 ng/mL for folate[1, 6, 7, 8]. These values were obtained by microbiologic assays using *Lactobacillus sp.*, however, serum vitamin B_{12} 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 B_{12} 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 (5MTHF: 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 B_{12} 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 B_{12} 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 B_{12} 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 B_{12} 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*)[9, 10]. *L. casei* is used most commonly because it responds to a wide variety of folate derivatives such as PGA, 5MTHF, 5-formyltetrahydro-

Table 1 Analytical precision and linearity ranges of three automated CPB methods on vitamin B_{12} and folate assays

Assay for vitamin B_{12}	Access	Advia Centaur	Elecsys
Reference material (and method)	CNCbl	CNCbl (RA)	CNCbl (RA)
Within-day CV (%)	6	4	3
Between-day CV (%)	9	5	7
Linearity (pg/mL)	50－1,500	16－2,000	30－2,000
Method-specific reference value (pg/mL)	180－914	233－914	197－866

Assay for folate	Access	Advia Centaur	Elecsys
Reference material (and method)	PGA	5MTHF (RA)	PGA (RA)
Within-day CV (%)	5	7	4
Between-day CV (%)	10	7	7
Linearity (ng/mL)	0.5－20	0.5－20	0.5－20
Method-specific reference value (ng/mL)	>3.0	3.6－12.9	2.0－9.1

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

folic acid (5-HCO-H_4PGA), 10-formyltetrahydrofolate (10-HCO-H_4PGA), and 5,6,7,8-tetrahydrofolate (H_4PGA). This microbiologic assay using *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_{12} with the automated CPB methods, 100 mg/L standard solutions were prepared by dissolving CNCbl or MeCbl in distilled water. They were then diluted 10-fold with distilled water, and their concentrations were confirmed spectrophotometrically. CNCbl has a molar absorptivity of 15,600 at 278 nm, and for MeCbl, this is 19,900 at 266 nm[11]. After 100 mg/L standard solutions of CNCbl and MeCbl 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[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_{12} and folate in 58 healthy young Japanese women were investigated using three automated methods. Among the 58 women, serum vitamin B_{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_{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_{12} and folate observed by the automated methods were significantly correlated among the three methods (correlation coefficients

Table 2 Assay values of vitamin B_{12} and folate in 58 female volunteers by three automated methods

Assay for vitamin B_{12}	Observed value (pg/mL)	Corrected values (pg/mL) by	
		CNCbl	MeCbl
Access	472 (243-906)	617 (318-1,186)	556 (286-1,067)
Advia Centaur	462 (257-804)	508 (283-884)	517 (288-900)
Elecsys	643 (313-1,270)	747 (364-1,474)	513 (250-1,013)

Assay for folate	Observed value (ng/mL)	Corrected values (ng/mL) by	
		PGA	5MTHF
Access	5.50 (2.98-11.10)	3.38 (1.83-6.82)	13.82 (7.49-27.89)
Advia Centaur	8.90 (4.83-17.59)	2.81 (1.53-5.56)	10.52 (5.71-20.79)
Elecsys	4.64 (2.08-11.20)	2.63 (1.18-6.36)	9.25 (4.15-22.35)

All values are median (central 95% distribution range).

Table 3 Reactivity with aqueous standard solutions of vitamin B_{12} and folate by three automated methods

Standard solution of vitamin B_{12}	Certified concentration (pg/mL)	Observed values (pg/mL) by		
		Access	Advia Centaur	Elecsys
CNCbl	1,209	924 (76.4)	1,100 (91.0)	1,041 (86.1)
MeCbl	966	821 (85.0)	863 (89.3)	1,210 (125.3)

Standard solution of folate	Certified concentration (ng/mL)	Observed values (ng/mL) by		
		Access	Advia Centaur	Elecsys
PGA	8.74	14.2 (162.5)	27.7 (316.9)	15.4 (176.2)
5MTHF	7.79	3.1 (39.8)	6.59 (84.6)	3.9 (50.1)

All values are means of three different measurements.
Recovery (%) against certified concentration is in parentheses.

were 0.959 to 0.977 for vitamin B_{12} 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 B_{12} 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 B_{12} and folate

We investigated reactivities to vitamin B_{12} and folate with the three automated CPB methods as described in the Materials and Methods. For CNCbl, 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, 89.3% 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 B_{12} and folate (see Table 3). For example, observed values of vitamin B_{12} 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 CNCbl and MeCbl, respectively. When observed values of vitamin B_{12} 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 *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

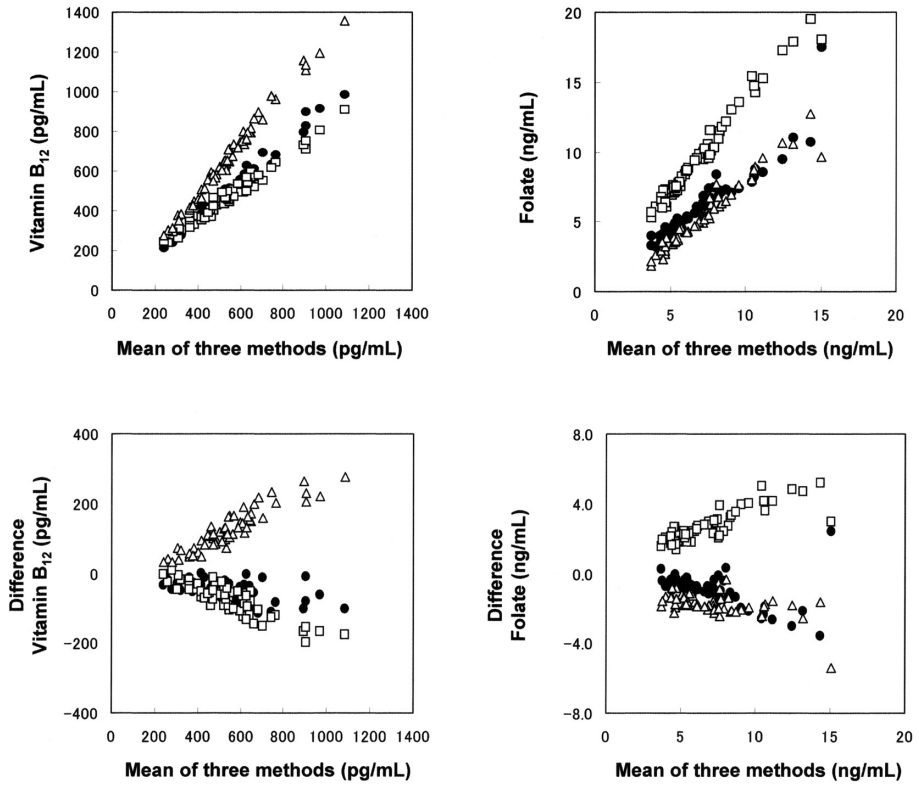

Fig. 1 Comparison of three automated CPB methods for serum vitamin B_{12} and folate with their mean values.
(Top, left), linear regression analysis of vitamin B_{12} by Access (●) gave a slope of 0.992, an intercept of -8.1 pg/mL, and r= 0.989; Advia Centaur (□) gave a slope of 0.766, an intercept of 53.2 pg/mL, and r= 0.985; and Elecsys (△) 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 (●) gave a slope of 0.848, an intercept of 0.140 ng/mL, and r= 0.941; Advia Centaur (□) gave a slope of 1.292, an intercept of 0.682 ng/mL, and r= 0.989; and Elecsys (△) gave a slope of 0.868, an intercept of -0.828 ng/mL, and r= 0.972.
(Bottom, left), difference plots of vitamin B_{12} between values of three automated methods and their mean value of Access (●) gave a mean difference (and ±2SD range) of -51 pg/mL (-108 pg/mL to 6 pg/mL), Advia Centaur (□) gave a mean difference of -75 pg/mL (-169 pg/mL to 19 pg/mL), and Elecsys (△) 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 (●) gave a mean difference of -0.93 ng/mL (-2.70 ng/mL to 0.85 ng/mL), Advia Centaur (□) gave a mean difference of 2.73 ng/mL (0.89 ng/mL to 4.56 ng/mL), and Elecsys (△) gave a mean difference of -1.75 ng/mL (-3.05 ng/mL to -0.45 ng/mL).

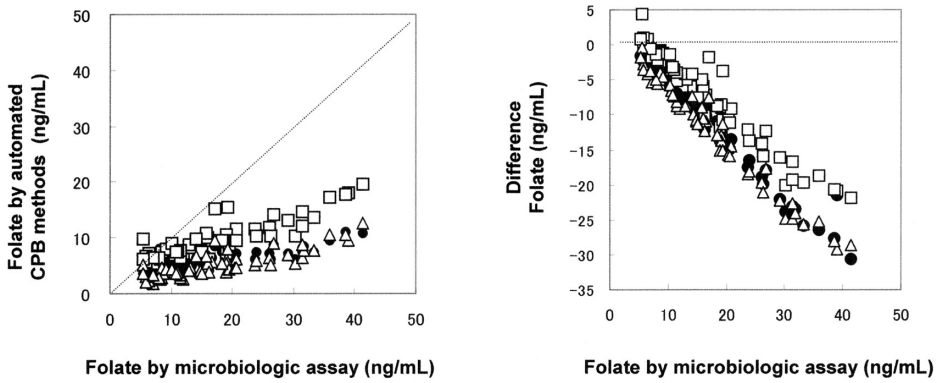

Fig. 2 Analysis of serum folate by microbiologic assay for 58 young Japanese women.
(Left), linear regression analysis of folate by Access (●) gave a slope of 0.203, an intercept of 2.528 ng/mL, and r= 0.820; Advia Centaur (□) gave a slope of 0.315, an intercept of 4.254 ng/mL, and r= 0.859; and Elecsys (△) 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 B_{12} 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 B_{12} 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 B_{12} 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[12], the acute need for the improved standardization of the folate assay has now been proposed[13] 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 B_{12} 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 B_{12} 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 B_{12} 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 MeCbl is the predominant vitamin B_{12} in human serum, and 5'-deoxyadenosyl cobalamin (coenzyme B_{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[14]. In serum, 80-90% of the total folate was 5MTHF, and the remainder was 5-HCO-H_4PGA and PGA[15].

We tried to reduce the bias present among the three methods with the use of MeCbl and 5MTHF, respectively, as control materials for the serum analysis of vitamin B_{12} and folate. Although the bias in vitamin B_{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.*[13] and Pfeiffer *et al.*[15] No trial was conducted for vitamin B_{12} except the one in our study. Wilson *et al.*[13] 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[12]. The use of PGA did not improve the bias. They selected the radiometric CPB assay traditionally as their reference method. However, Pfeiffer *et al.*[15] 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$\pm$SD, 15.7$\pm$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[15] or the solid-phase extraction-electrospray ionization mass spectrometry (SPE-LC/MS/MS) method[16] 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[13]. 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_{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_{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_{12} and folate.

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