

**Comparison of Serum Folate Species Analyzed by LC-MS/MS with Total Folate Measured by Microbiologic Assay and Bio-Rad Radioassay, Zia Fazili, Christine M. Pfeiffer,\* and Mindy Zhang** (Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia; \*address correspondence to this author at: Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, 4770 Buford Highway, NE, Mail Stop F55, Atlanta, GA 30345; fax 770-488-4139, e-mail CPfeiffer@cdc.gov)

**Background:** The Bio-Rad QuantaPhase II radioassay (BR), used for 25 years to measure total folate (TFOL) concentrations for the National Health and Nutrition Examination Survey (NHANES), will be discontinued in 2007. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) or a microbiologic assay (MA) will be used in the future.

**Methods:** We measured folate species by LC-MS/MS and TFOL by MA and BR in 327 serum samples.

**Results:** LC-MS/MS measured 5-methyltetrahydrofolic acid (5CH<sub>3</sub>THF; 82%), folic acid (FA; 8%), 5-formyltetrahydrofolic acid (5CHOTHF; 6%), tetrahydrofolic acid (THF; 4%), and 5,10-methenyltetrahydrofolic acid (5,10CH=THF; 0%). The sum of the folate species correlated well with TFOL measured by MA ( $R^2 = 0.97$ ) and BR ( $R^2 = 0.91$ ). Compared with LC-MS/MS results, MA and BR values were significantly lower (–6% and –29%, respectively); however, these differences were concentration dependent. The MA almost completely recovered folates added to serum samples except for FA [69% (3%)] and THF [36% (10%)]. The BR underrecovered 5CH<sub>3</sub>THF [61% (9%)] and 5CHOTHF [38% (14%)] and overrecovered 5,10CH=THF [234% (32%)]. Multiple linear regression models with log-transformed data yielded a good fit for converting BR data to MA or LC-MS/MS data and MA data to LC-MS/MS data.

**Conclusions:** The good correspondence between the sum of folate species determined by LC-MS/MS and TFOL determined by MA makes these 2 assays interchangeable. The BR produces much lower results, on average, probably because of 5CH<sub>3</sub>THF underrecovery. The conversion equations provided could be used for future NHANES time trend analyses.

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The serum concentration of folate is an important marker of nutritional status and has been measured for >30 years as part of the National Health and Nutrition Examination Survey (NHANES). The Bio-Rad QuantaPhase II radioassay (BR) has been used since 1991 but will be discontinued in 2007. The introduction of mandatory folic acid (FA) fortification in 1998 has contributed to appreciable increases in serum folate concentrations in the US population (1); continued monitoring through NHANES is required. Two assays have been discussed for future

surveys: the new isotope-dilution liquid chromatography–tandem mass spectrometry method (LC-MS/MS) (2, 3) and the traditional *Lactobacillus casei* microbiologic assay (MA) (4, 5). The LC-MS/MS measures 5 folate species: 5-methyltetrahydrofolic acid (5CH<sub>3</sub>THF), FA, 5-formyltetrahydrofolic acid (5CHOTHF), tetrahydrofolic acid (THF), and 5,10-methenyltetrahydrofolic acid (5,10CH=THF). The MA and BR measure total folate (TFOL). The 1st objective of this study was to compare the LC-MS/MS method with the traditional MA. The 2nd objective was to provide equations for converting results from the assay currently used for the NHANES (BR) to the new assay for future analyses (MA or LC-MS/MS).

Pristine serum samples ( $n = 237$ ) collected as part of the NHANES from 1999 to 2004 and stored at  $-70^\circ\text{C}$  were analyzed between December 2005 and February 2006 for folate species by the LC-MS/MS and for TFOL by the MA. The BR has analyzed aliquots of the same samples for TFOL from 1999 to 2004. These samples are not representative of the US population. They were selected to cover a wide range of TFOL concentrations, as measured by the BR assay. The second set of samples consisted of 100 pristine serum samples obtained from a blood bank from January to March 2006. These samples were analyzed by all 3 assays in March and April 2006.

Data were analyzed with SAS (version 9; SAS Institute) and Microsoft Excel with a clinical statistical analysis plug-in (Analyse-it; Analyse-it Software). Two NHANES samples were excluded as outliers because of an extremely high concentration of either 5CHOTHF or FA. The sample sets were analyzed first separately and then together. We used the sum of the folate species determined by the LC-MS/MS and TFOL determined by the MA or BR to compare the methods. Results are presented only for the combined set because we found no differences between the 2 data sets other than the concentration ranges (see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at <http://www.clinchem.org/content/vol53/issue4>). Methods were compared by least-squares regression after log-transformation of the data to account for nongaussian distribution. The concentration-dependent relationship between these assays prompted us to develop multiple linear regression models that used a dummy binary variable (0, 1), IND, to account for intercept differences and an interaction variable ( $\text{IND} \times \log_{10} \text{BR}$  or  $\text{IND} \times \log_{10} \text{MA}$ ) to account for differences in slope over 2 discrete concentration ranges ( $\leq 45$  vs  $> 45$  nmol/L for the BR and  $< 50$  vs  $\geq 50$  nmol/L for the MA). The fit of the models was evaluated by comparing the sum of squared residuals (SSR) to the predicted residual sum of squares (PRESS). We tested the recoveries of the MA and BR methods ( $n = 2$  days) by adding each of the 5 folate calibrators (Merck Eprova) at 10 nmol/L to a serum pool (21.8 nmol/L TFOL by LC-MS/MS).

The mean concentrations for TFOL, 5CH<sub>3</sub>THF, and FA were higher in the NHANES set than in the blood bank

set, whereas the mean 5CHOTHF concentration was higher in the blood bank set (Table 1). In the combined set, the major folate forms were 5CH<sub>3</sub>THF (82%) and FA (8%). Most samples had only low FA (<2.5 nmol/L) and 5CHOTHF (<10 nmol/L) concentrations. Approximately half the samples had detectable THF concentrations; only 10% had THF concentrations >10 nmol/L.

Mean and median TFOL concentrations measured by LC-MS/MS and MA were generally in agreement, but BR values were much lower. The sum of folate species and TFOL determined by the LC-MS/MS and the MA, respectively, were highly correlated ( $R^2 = 0.97$ ), but the latter produced slightly lower results, corresponding to a small but significant negative concentration-dependent difference of  $-5.94$  nmol/L (95% confidence interval,  $-7.49$  to  $-4.39$  nmol/L) and a relative difference of  $-6\%$ . The multiple linear regression model was developed for an MA cutoff of 50 nmol/L because of appreciably increased FA concentrations above this concentration. The FA concentration [mean (SD)] at <50 nmol/L was 0.8 (1.4) nmol/L, corresponding to 3% of the TFOL, whereas it was 38 (71) nmol/L at  $\geq 50$  nmol/L, corresponding to 20% of the TFOL (see Fig. 2A in the online Data Supplement). After log-transformation,  $R^2$  was 0.96 for the multiple linear regression equation:  $\log_{10} \text{LC-MS/MS} = 0.1439 + (0.9193 \times \log_{10} \text{MA}) + (0.0048 \times \text{IND}) + (0.029 \times \text{IND} \times \log_{10} \text{MA})$ , with  $\text{IND} = 0$  for MA results <50 nmol/L and  $\text{IND} = 1$  for MA results  $\geq 50$  nmol/L (Fig. 1A). The SSR (1.4042) agreed with the PRESS (1.4377).

We also obtained good correlation between TFOL obtained by LC-MS/MS or MA and TFOL obtained by the BR ( $R^2 = 0.91$  for both comparisons); the BR produced much lower results than either the LC-MS/MS or the MA. The mean negative concentration-dependent difference was 29%. The multiple linear regression model was developed for a 45 nmol/L cutoff for the BR, mainly because this value represents the upper end of the calibration curve, but also because of appreciably increased FA

concentrations greater than  $\sim 50$  nmol/L TFOL (see Fig. 2B in the online Data Supplement). After log-transformation, the  $R^2$  was 0.95 for both method comparisons. The conversion equations were as follows:  $\log_{10} \text{LC-MS/MS} = 0.1436 + (1.0435 \times \log_{10} \text{BR}) + (0.51 \times \text{IND}) - (0.3218 \times \text{IND} \times \log_{10} \text{BR})$ , (SSR, 2.0119; PRESS, 2.0682);  $\log_{10} \text{MA} = 0.0504 + (1.0958 \times \log_{10} \text{BR}) + (0.6358 \times \text{IND}) - (0.4105 \times \text{IND} \times \log_{10} \text{BR})$ , (SSR, 1.7889; PRESS, 1.8432). These equations were with  $\text{IND} = 0$  for BR results  $\leq 45$  nmol/L and with  $\text{IND} = 1$  for BR results >45 nmol/L (Fig. 1, B and C).

In 2005 the NIST released a new standard reference material for homocysteine and folate in human serum, SRM 1955 (6). We found similar good agreement for TFOL between our LC-MS/MS and MA methods (level 1, 6.0 vs 5.6 nmol/L; level 2, 13 vs 14 nmol/L; level 3, 41 vs 44 nmol/L). TFOL concentrations obtained by the BR were 25%–40% lower (level 1, 4.5 nmol/L; level 2, 10 nmol/L; level 3, 25 nmol/L).

The lower response of the BR compared with the MA has been known for many years (7). The BR was initially marketed (QuantaPhase I) with calibrator concentrations to match MA performance, but questions raised by Levine (8) prompted introduction of the QuantaPhase II assay in 1993 with spectrophotometrically verified FA calibrator concentrations, resulting in a 30% downward shift in TFOL concentrations. One potential cause for lower TFOL concentrations by the BR is underrecovery of certain folate forms. Our recovery experiments with serum samples showed satisfactory recovery of FA [91% (10%)] and THF [106% (27%)], underrecovery of 5CH<sub>3</sub>THF [61% (9%)] and 5CHOTHF [38% (14%)], and overrecovery of 5,10CH=THF [234% (32%)]. Others have also reported lower BR recoveries of 5CH<sub>3</sub>THF (60%) (9). We obtained satisfactory MA recoveries for 5CH<sub>3</sub>THF [88% (9%)], 5CHOTHF [120% (9%)], and 5,10CH=THF [101% (7%)]. FA and THF recoveries were 69% (3%) and 36% (10%), respectively. Because we calibrate the MA with 5CH<sub>3</sub>THF,

**Table 1. Descriptive statistics for serum folate concentrations measured by LC-MS/MS, MA, and BR methods.<sup>a</sup>**

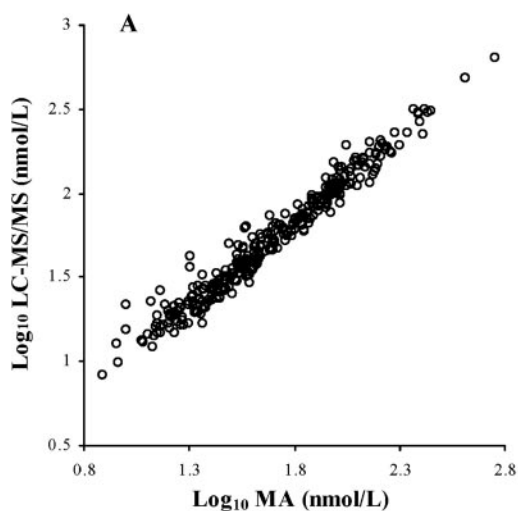
	Folate species by LC-MS/MS, nmol/L					Total folate, nmol/L		
	5CH <sub>3</sub> THF	FA	5CHOTHF	THF	5,10CH=THF	LC-MS/MS	MA	BR
Combined set (n = 325)								
Mean (SD)	54.4 (40.3)	13.5 (45.2)	2.54 (3.60)	2.54 (4.49)	<LOD	73.0 (70.9)	67.0 (62.6)	56.5 (70.0)
Median	40.9	0.81	1.20	0.49	<LOD	47.4	43.5	29.2
Range	5.86–266	0.00–561	<LOD–33.4	<LOD–28.9	<LOD	8.20–642	7.78–572	6.94–642
NHANES set (n = 225)								
Mean (SD)	67.0 (42.0)	18.9 (53.4)	1.87 (3.65)	3.54 (5.07)	<LOD	91.3 (78.0)	82.9 (68.8)	72.4 (78.8)
Median	62.3	1.04	0.96	1.83	<LOD	71.0	66.8	42.9
Range	5.86–266	<LOD–561	<LOD–33.4	<LOD–28.9	<LOD	8.20–642	7.78–572	6.97–642
Blood bank set (n = 100)								
Mean (SD)	26.0 (12.4)	1.40 (4.72)	4.04 (2.97)	0.30 (0.47)	<LOD	31.7 (15.2)	31.4 (15.7)	20.8 (11.0)
Median	23.7	0.55	3.59	<LOD	<LOD	27.9	27.8	18.8
Range	7.61–72.0	0.15–43.6	0.29–13.0	<LOD–2.39	<LOD	12.8–102	9.00–99.0	6.94–93.0

<sup>a</sup> Limit-of-detection (LOD) values are as follows: FA, 0.07 nmol/L; 5CHOTHF, 0.05 nmol/L; THF, 2.5 nmol/L; and 5,10CH = THF, 0.7 nmol/L.

which produces a slightly higher response curve than FA, we expect to underrecover FA. The lower recovery of THF, probably due to oxidative loss of THF during the 42-h incubation, might be improved by increasing the ascorbic acid concentration of the medium. Differential recovery of different folate forms by the BR is likely the reason for the concentration-dependent relationship be-

tween this assay and the LC-MS/MS or MA. The lower MA recovery of FA is probably the reason for the increased difference between LC-MS/MS and MA results at higher TFOL concentrations.

We conclude that the new LC-MS/MS method agrees well with the traditional MA method. The small difference in

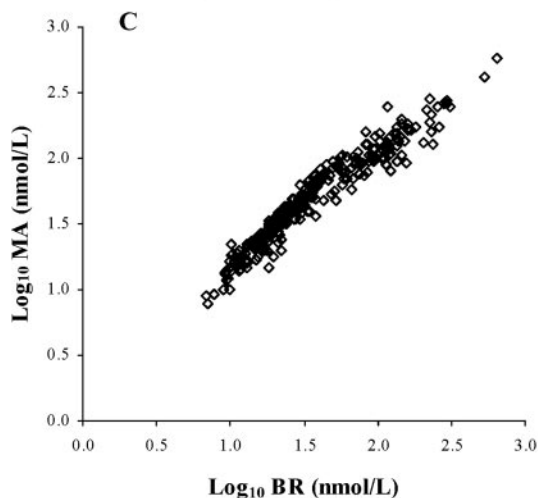


**Multiple linear regression model:**

$$\text{Log}_{10} \text{LC-MS/MS} = 0.1439 + (0.9193 \times \text{log}_{10} \text{MA}) + (0.0048 \times \text{IND}) + (0.0029 \times \text{IND} \times \text{log}_{10} \text{MA}).$$

IND = 0 for MA <50 nmol/L, and IND = 1 for MA ≥50 nmol/L.

$$R^2 = 0.9637; \text{SSR} = 1.4042; \text{PRESS} = 1.4377.$$

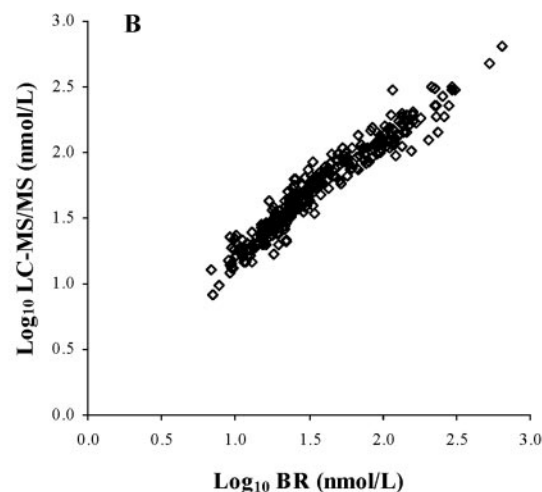


**Multiple linear regression model:**

$$\text{Log}_{10} \text{MA} = 0.0504 + 1.0958 \times \text{log}_{10} \text{BR} + 0.6358 \times \text{IND} - 0.4105 \times \text{IND} \times \text{log}_{10} \text{BR}$$

IND = 0 for BR ≤45 nmol/L and IND = 1 for BR >45 nmol/L

$$R^2 = 0.9524; \text{SSR} = 1.7889; \text{PRESS} = 1.8432$$



**Multiple linear regression model:**

$$\text{Log}_{10} \text{LC-MS/MS} = 0.1436 + (1.0435 \times \text{log}_{10} \text{BR}) + (0.51 \times \text{IND}) - (0.3218 \times \text{IND} \times \text{log}_{10} \text{BR}).$$

IND = 0 for BR ≤45 nmol/L, and IND = 1 for BR >45 nmol/L.

$$R^2 = 0.9480; \text{SSR} = 2.0119; \text{PRESS} = 2.0682.$$

Fig. 1. Least-squares regression plots for TFOL measured in the combined sample set by 3 methods: LC-MS/MS vs MA (A), LC-MS/MS vs BR (B), and MA vs BR (C).

The BR or MA is used as the reference point ( $n = 325$ ). The concentration-dependent relationship between these assays prompted our development of a multiple linear regression model that accounts for a change in slope and/or intercept over 2 discrete concentration ranges ( $\leq 45$  vs  $> 45$  nmol/L by the BR and  $< 50$  vs  $\geq 50$  nmol/L for the MA). The fit of the model was evaluated by comparing the SSR with the PRESS.

results between these 2 methods is not clinically relevant. Irrespective of the assay used for future NHANES monitoring, population reference ranges will change to higher values; however, MA-determined cutoff values for deficiency (10) could be directly applied to the LC-MS/MS because of its excellent agreement with the MA. Some advantages of the LC-MS/MS compared with the MA are that it provides information on the different folate species in addition to TFOL and it is less prone to interferences such as antibiotics. BR underrecovery of 5CH<sub>3</sub>THF, the main circulating form of folate, is likely the major reason for its lower results. A model will be required to convert results from the old or the new NHANES method for time trend analysis. Our model provides an excellent fit over a wide concentration range. This information may also be useful to the international community in that national data generated with the MA can now potentially be compared with US reference ranges.

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**Background:** C-peptide measurement has been widely used as a marker of insulin secretion in patients with diabetes. We assessed the comparability of C-peptide results obtained with different methods and by different laboratories and determined whether C-peptide results could be harmonized by normalization with a WHO reference reagent or with plasma.

**Methods:** We sent 16 different heparin plasma samples to 15 laboratories in 7 countries. The samples were analyzed with 10 different assay methods. A WHO C-peptide standard was also sent to each laboratory and used to determine the feasibility of normalizing results. To assess the impact of calibrator matrix on the comparability of results, we also used the mean results of all laboratories for 4 of the samples to normalize the remaining sample results.

**Results:** Between-laboratory variability increased with increasing C-peptide concentrations. Normalization of results with WHO reference reagents did not improve comparability, but normalization with samples significantly improved comparability among laboratories and methods. The 95% confidence interval estimate for the SD for the lab/method effect (0.0–0.061) using sample-normalized values did not overlap with the 95% CI estimate with the raw data (0.090–0.225).

**Conclusions:** C-peptide results generated by different methods and different laboratories do not always agree, especially at higher concentrations of C-peptide. These data support the concept of using a single laboratory for multisite studies and support efforts to harmonize C-peptide measurements by use of calibrators prepared in the sample matrix.

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Human C-peptide provides an accurate assessment of residual beta-cell function and thus has been widely used as a marker of insulin secretion in patients with diabetes (1,2). Some studies have also suggested that C-peptide is