Measurement of Folate in Fresh and Archival Serum Samples as *p*-Aminobenzoylglutamate Equivalents

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BACKGROUND: The development of accurate and precise folate assays has been difficult, mainly because of folate instability. Large interassay and interlaboratory differences have been reported. We therefore developed a serum folate assay that measures folate and putative degradation products as *p*-aminobenzoylglutamate (pABG) equivalents following oxidation and acid hydrolysis.

METHODS: Serum was deproteinized with acid in the presence of 2 internal calibrators ($[^{13}C_2]$pABG and $[^{13}C_5]$5-methyltetrahydrofolate). 5-Methyltetrahydrofolate and other folate species in serum were converted to pABG by oxidation and mild acid hydrolysis. pABG and its internal calibrators were quantified by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

RESULTS: The limit of quantification was 0.25 nmol/L, and the assay was linear in the range 0.25–96 nmol/L, which includes the 99.75 percentile for serum folate concentrations in healthy blood donors. Within- and between-day imprecision was ≤5%. We detected no residual folate in serum samples after sample preparation. Folate concentrations in fresh serum samples obtained with the pABG assay and with a microbiologic assay showed good agreement ($r = 0.96$). In stored samples containing low folate concentrations due to folate degradation, the pABG assay yielded substantially higher folate concentrations than the microbiologic assay.

CONCLUSIONS: The pABG assay combines automated sample preparation with LC-MS/MS analysis. It allows measurement of folate not only in fresh samples of serum/plasma but also in stored samples in which the folate has become oxidized and degraded to an extent that it cannot be assayed with traditional folate assays.

Folate is the generic term for a group of compounds that serve as carriers of one-carbon units used in maintaining and repairing DNA and in remethylating homocysteine to methionine *(1)*. These compounds have similar chemical structures, consisting of a reduced pteridine moiety coupled through a methylene group to *p*-aminobenzoic acid (pABA)[4], to which glutamic acid is attached via an amide bond. Folate species differ with respect to the oxidation state of the pteridine ring and the number of connected glutamate residues. In addition, different one-carbon substituents can be present, including methyl and formimino groups at position N-5, formyl groups at either N-5 or N-10, or a methenyl or methylene bridge that connects these 2 positions *(1)*.

Because of the associations of folate status with several chronic diseases with major impacts on public health *(2–7)*, accurately measuring folate in blood has become important in the clinical setting and in epidemiologic studies. Serum folate has been measured by a variety of different methods *(8)*. The classic microbiologic assay based on *Lactobacillus casei* has been automated and improved *(9)*. It measures a variety of biologically active folates that support bacterial growth. Different growth responses have been reported for different folate species *(10)*, however, and there is interference from growth inhibitors, including some antibiotics. Most commercial methods for measuring serum folate, including radioassays, chemiluminescence assays, and ion-capture assays, are binding assays that use folate-binding protein. There has been concern about the variation in the binding properties of folate-binding proteins from different sources and their different affinities for different folate species *(11)*. Chromatographic methods measure the separate folate forms, including 5-methyltetrahydrofolate, and are based on HPLC *(12, 13)*, liquid chromatography–mass spectrometry (LC-MS) *(14–17)*, or LC-MS/MS

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[4] Nonstandard abbreviations: pABA, *p*-aminobenzoic acid; LC-MS/MS, liquid chromatography–tandem mass spectrometry; pABG, *p*-aminobenzoylglutamate; apABG, *p*-acetamidobenzoylglutamate.

(18, 19). Despite the efforts to establish reliable methods for evaluating folate status, large interassay and interlaboratory differences have been reported *(20–22)*.

A loss of folate occurs in serum samples stored at room temperature or at $-20\,°C$ *(23)*. This disappearance is due to the instability of serum folate species, which undergo oxidative cleavage of the C9-N10 bond and oxidation of the pterin ring *(24)*. Folate instability complicates the standardization and calibration of folate assays and may cause random errors in epidemiologic studies based on stored samples.

We encountered extensive folate degradation in serum samples from the JANUS biobank, which were collected about 30 years ago *(25)*. We therefore established and validated a method for measuring serum folate in archival samples as *p*-aminobenzoylglutamate (pABG) equivalents. The method involves the conversion of serum folate species and their degradation products via controlled oxidation and limited acid hydrolysis to pABG, which is then quantified by LC-MS/MS.

Materials and Methods

CHEMICALS
pABG, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, folic acid, pABA, ascorbic acid, and hydrogen peroxide were purchased from Sigma-Aldrich. Tetrahydrofolate, dihydrofolate, and 5,10-methylenetetrahydrofolate were kind gifts from Merck Eprova (Schaffhausen, Switzerland). $[^{13}C_5]$5-methyltetrahydrofolate was obtained from Merck Eprova, and $[^{13}C_2]$pABG was from Larodan Fine Chemicals. *p*-Acetamidobenzoylglutamate (apABG) was prepared from pABG as previously described *(26)*. Acetic acid, perchloric acid, and potassium permanganate were purchased from Merck, and methanol was from LabScan.

SOLUTIONS AND SAMPLES
All folate and pABG stock solutions were prepared in 20 mmol/L phosphate buffer, pH 7.2, containing 100 mL/L acetonitrile and 1 g/L ascorbic acid. These stock solutions were stored at $-80\,°C$.

We prepared folate-free serum by treating serum with activated charcoal (8 g/L serum; Merck). The charcoal was kept in suspension by continuous mixing for 10 min at room temperature. The suspension was then centrifuged, and the clear supernatant was removed by gentle pipeting. The centrifugation and pipeting steps were repeated 3 times. The serum samples so treated contained no detectable folate (<0.1 nmol/L) as measured with microbiologic assay *(9)*.

We collected 5 sets of serum and plasma samples for this study. Serum samples were obtained from 400 healthy blood donors (63% male) with a mean age of 44 years (range, 29–69 years). Both serum and EDTA-treated plasma samples were obtained from 48 healthy fasting blood donors (47% male) with a mean age of 40 years (range, 20–62 years). We obtained 223 surplus serum samples in which folate had been measured in routine analyses in the clinical chemistry laboratory at Haukeland University Hospital. We also obtained 40 routine serum samples with increased serum creatinine concentrations (range, 130–957 μmol/L). Finally, we retrieved 83 serum samples that had been collected between 1973 and 1980 and stored in the JANUS biobank at $-25\,°C$ *(25)*. Serum from blood donors was obtained by collecting blood into Vacutainer Tubes with no additive (BD Medical Systems). Blood was allowed to clot at room temperature for 30 min before centrifugation. EDTA-treated plasma was obtained from blood donors by collecting the blood into Vacutainer Tubes; the final EDTA concentration in the samples was 4 mmol/L. The EDTA-treated blood samples were centrifuged within 60 min of collection. Serum and plasma samples were stored at $-80\,°C$ until analysis.

All samples were anonymized and could not be linked back to the donor's identity.

SAMPLE PROCESSING
One hundred microliters of serum or plasma sample was mixed with 15 μL of the internal calibrator, $[^{13}C_5]$5-methyltetrahydrofolate (300 nmol/L). The samples were deproteinized by adding 33 μL of perchloric acid (1.6 mol/L), which contained 200 nmol/L $[^{13}C_2]$pABG. After centrifugation at 5796*g* for 3 min, 90 μL of the supernatant was mixed with 34 μL of a solution containing 1.44 mol/L KOH and 1.2 mol/L potassium bicarbonate. The potassium perchlorate was allowed to precipitate, and 7 μL of 0.13 mol/L potassium permanganate was added to 80 μL of the supernatant. After 20 min of incubation at room temperature, 10 μL of 30 g/L hydrogen peroxide was added, and the solution was mixed and allowed to incubate at room temperature for another 20 min. We mixed 80 μL of this solution with 15 μL of 3.0 mol/L perchloric acid to adjust the pH to about 1. All pipeting was carried out in a 96-well microtiter plate by a robotic liquid handler (Plato 7; RoSys Anthos).

INSTRUMENTATION
We used an Agilent series 1100 HPLC system equipped with a degasser, a column oven, a quaternary pump for solvent delivery, and a thermostated autosampler for sample introduction. The HPLC system was coupled to an API 4000 triple-quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex) outfitted with an electrospray ion source (TurboIonSpray™; Applied Biosystems). A column switcher from Valco Instruments Company (type VMHA) was used to divert the flow to waste or to the ion source. Analyst software

Table 1. Mass transitions monitored, retention times, and instrument settings.					
Analyte	Transition, *m/z*	t_r,[a] min	DP, V	CE, V	CXP, V
pABG	267.2→120.0	3.30	42	22	8
[$^{13}C_2$]pABG	269.2→120.0	3.30	42	22	8
[$^{13}C_5$]pABG	272.2→120.0	3.30	42	22	8
5-Methyltetrahydrofolate	460.4→313.2	3.32	55	22	10
10-Formylfolic acid	470.3→295.4	3.79	50	25	10
Folic acid	442.4→295.2	3.87	55	22	10
pABA	138.2→120.2	3.92	50	15	7

[a] t_r, retention time; DP, declustering potential; CE, collision energy; CXP, collision cell exit potential.

(Applied Biosystems/MDS Sciex) was used for HPLC system control, data acquisition, and processing.

LC-MS/MS

Processed serum or plasma samples were placed in a cooled (4 °C) sample tray and injected into a ZORBAX StableBond C8 reversed-phase column (150 mm × 4.6 mm inner diameter; particle size, 3.5 μm) from Agilent Technologies. The column was mounted in the thermostated column compartment set at 20 °C. The flow rate was 0.9 mL/min. The 2 mobile phases used in the assay were 520 mmol/L acetic acid (solution A) and methanol (solution B). The column was equilibrated with solution A, and chromatography was performed according to the following timetable: 0–0.2 min, 100% A; 0.3 min, 60% A; 2.8 min, 30% A; and 2.9–5.0 min, 100% A. All gradient steps were linear. The column effluent was directed into the mass spectrometer in the time interval of 2.8–4.2 min; otherwise it was directed to waste.

The acquisition settings of the mass spectrometer for each compound are listed in Table 1. The ion-source temperature (500 °C), the ion-spray voltage (5 000 V), the curtain gas (15 psig), the collision gas (4 psig), ion-source gases 1 and 2 (80 psig), and the activated interface heater were identical for all analytes. The analytes were detected by multiple-reaction monitoring in the positive-ion mode of the tandem mass spectrometer.

After sample preparation was completed, matrix effects were assessed by adding 20 nmol/L pABG to 9 different serum samples and to an aqueous solution. The matrix effect was obtained by comparing peak intensities in serum and in aqueous solution and was calculated as:

Matrix effect (%) =

$$\frac{\text{Peak area (added)} - \text{Peak area (endogenous)}}{\text{Peak area (aqueous solution)}} \times 100$$

LINEARITY AND ASSAY CALIBRATION

We assessed the limit of quantification and the linear range of the assay by adding pABG or 5-methyltetrahydrofolate to folate-free serum at final concentrations of 0.125–256 nmol/L. The peak-area ratios of pABG to [$^{13}C_2$]pABG were measured and plotted against the concentrations of added pABG or 5-methyltetrahydrofolate. Using the signal-to-noise script supplied by Applied Biosystems (Analyst Version 1.4.1), we determined the limit of quantification from the linearity data as the lowest concentrations that gave peaks with signal-to-noise ratios of 10.

For routine 3-point assay calibration, we used a serum pool with 17.0 nmol/L folate, to which we added 20 nmol/L and 60 nmol/L pABG. The calibration samples were aliquoted and stored at −80 °C.

RECOVERY AND IMPRECISION

Pooled serum containing 5.0 nmol/L folate was divided into 15 portions, 14 of which were spiked with one of 2 different concentrations (medium and high) of one of 7 analytes (i.e., pABG, 5-methyltetrahydrofolate, tetrahydrofolate, 5-formyltetrahydrofolate, folic acid, dihydrofolate, or 5,10-methylenetetrahydrofolate) (Table 2). The remaining portion was left unspiked. Ten replicates were analyzed at each concentration [i.e., low (unspiked), medium, and high] in one analytical run. Percent recovery was calculated as:

[(Measured concentration − Endogenous
    concentration) × 100]/(Added concentration).

We determined within-day imprecision of the assay by measuring folate as pABG equivalents in 20 replicates of 3 different samples containing a low (5.0 nmol/L), medium (14.1 nmol/L), or high (43.0 nmol/L) folate concentration. Between-day imprecision was measured by assaying the same samples on 20 different days over a 3-week period.

Table 2. Analytical recovery.[a]

Analyte	Added, nmol/L		Recovery, %[b]	
	Medium	High	Medium	High
pABG	15.0	45.0	103.0 (13.2)	98.8 (6.0)
5-Methyltetrahydrofolate	14.0	42.0	101.9 (12.9)	91.0 (9.6)
Folic acid	14.3	39.5	94.7 (13.4)	90.6 (3.8)
5-Formyltetrahydrofolate	15.0	45.0	52.6 (6.8)	43.8 (5.7)
Tetrahydrofolate	15.0	45.0	97.2 (8.4)	98.8 (7.8)
Dihydrofolate	15.0	45.0	91.6 (10.2)	83.6 (6.1)
5,10-Methylenetetrahydrofolate	15.9	45.0	106.0 (8.3)	98.8 (7.2)

[a] n = 10 for all concentrations.
[b] Analyte recoveries are given as pABG equivalents and presented as the mean (SD).

Results and Discussion

CONVERSION OF SERUM FOLATE TO pABA

We initially investigated the possibility of quantifying serum folate as pABA following strong acid hydrolysis (0.6 mol/L perchloric acid at 100 °C for 5 h). pABA was measured with an LC-MS/MS method internally calibrated with $[^{13}C_6]$pABA. A similar strategy has been successfully used by Dueker et al. to measure folate concentrations in whole blood (27). Fig. 1 compares folate concentrations in fresh serum samples obtained with the pABA assay and with a microbiologic assay (9). The scatter plot (Spearman $r = 0.65$) shows
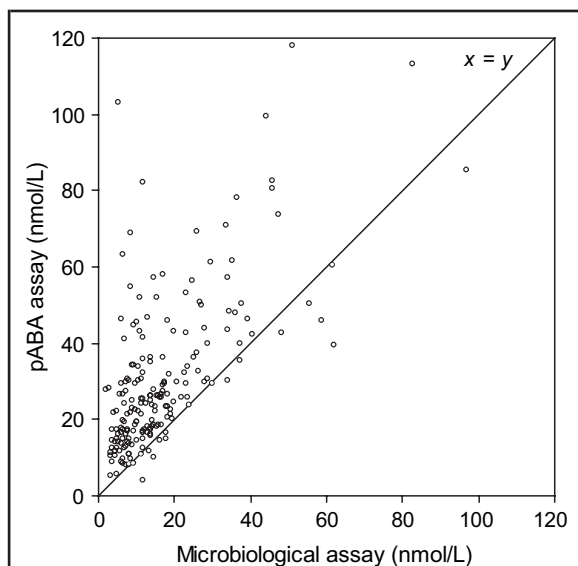

Fig. 1. Scatter plot of serum folate concentrations measured by a microbiologic method and the pABA method (n = 214).

that the pABA concentration was much higher than the folate concentration obtained with the microbiologic assay for a large portion of the samples. This finding suggests that some serum samples contain pABA precursors that are not microbiologically active folate species. Thus, we concluded that measurement of serum folate as pABA equivalents after strong acid hydrolysis is not feasible.

SAMPLE PROCESSING

The amounts of pABG recovered from serum and serum samples spiked with pABG, 5-methyltetrahydrofolate, or folic acid were independent of the potassium permanganate concentration in the range of 0.06–0.25 mol/L. The pABG recovered was also constant during incubation periods ranging from 5–240 min (at 0.13 mol/L potassium permanganate; data not shown).

LC-MS/MS

The LC-MS/MS method we used detects $[^{13}C_2]$pABG, $[^{13}C_5]$pABG, 5-methyltetrahydrofolate, $[^{13}C_5]$5-methyltetrahydrofolate, 10-formylfolic acid, folic acid, and pABA, in addition to pABG. The limits of quantification were 0.1 nmol/L for 5-methyltetrahydrofolate, 1.2 nmol/L for 10-formylfolic acid, 0.7 nmol/L for folic acid, and 1.5 nmol/L for pABA. These detection limits enabled us to monitor both the decline in native folate and the absence of residual folate in samples during and after sample processing involving oxidation and acid hydrolysis (Fig. 2).

The matrix effect [mean (SD)] for 9 different serum samples was 61% (7%). When we included labeled pABG as an internal calibrator, the calculated serum concentration of the added pABG was 99% (6%) of the concentration measured after adding the same amount to an aqueous solution. Thus, including the stable iso-
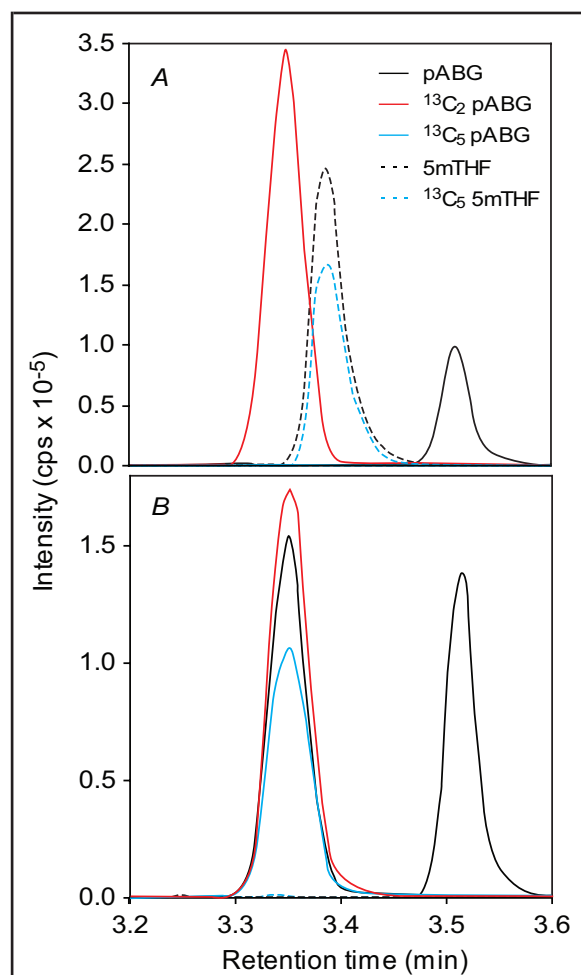
Fig. 2. LC-MS/MS chromatograms of human serum before and after sample processing involving controlled oxidation and limited acid hydrolysis.

(A), Chromatograms of a serum sample containing 28.2 nmol/L 5-methyltetrahydrofolate (5mTHF) and the internal calibrators [$^{13}C_2$]pABG and [$^{13}C_5$]5mTHF. The serum sample was not subjected to oxidation and acid hydrolysis. (B), The same specimen treated with potassium permanganate, hydrogen peroxide, and perchloric acid, as described in Materials and Methods. 5mTHF and [$^{13}C_5$]5mTHF are absent because of conversion to pABG and [$^{13}C_5$]pABG, respectively. The compound with a retention time of 3.52 min has not been identified.

tope as internal calibrator corrected for the matrix effect.

INTERNAL CALIBRATORS

The pABG method for measuring serum folate includes 2 internal calibrators labeled with a stable isotope, [$^{13}C_2$]pABG and [$^{13}C_5$]5-methyltetrahydrofolate.
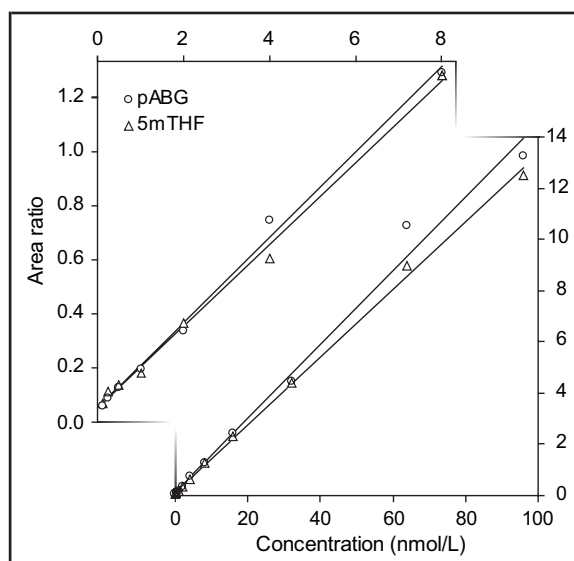

Fig. 3. Linear dynamic range for the pABG method.

The area ratio, pABG to [$^{13}C_2$]pABG, was plotted against pABG or 5-methyltetrahydrofolate concentration. The plots for the entire range and the lower range of concentrations are shown in the lower-right and upper-left parts of the figure, respectively.

Both internal calibrators were present during sample processing, chromatography, and detection by MS. This internal calibration corrects for the variation in most analytical steps. In addition, the complete degradation of [$^{13}C_5$]5-methyltetrahydrofolate to [$^{13}C_5$]pABG indicates a quantitative conversion of 5-methyltetrahydrofolate in serum to pABG.

PERFORMANCE OF THE ASSAY

To estimate the linear dynamic range, we constructed calibration curves for pABG and 5-methyltetrahydrofolate in the range of 0.125–256 nmol/L (Fig. 3). A linear dynamic range was demonstrated for 0.25–96 nmol/L, which includes the 99.75 percentile for the folate concentrations found in the sera of healthy blood donors. The equations for the regression lines were: $y = 0.14x + 0.13$ ($r^2 = 0.990$; $S_{y \cdot x} = 0.50$, Sa = 0.0051, and Sb = 0.20, where $S_{y \cdot x}$, Sa, and Sb are the SE of the y estimate, the SE of the slope, and the SE of the y intercept, respectively) for pABG and $y = 0.13x + 0.14$ ($r^2 = 0.998$; $S_{y \cdot x} = 0.19$; Sa = 0.0019; Sb = 0.074) for 5-methyltetrahydrofolate. The limit of quantification was 0.25 nmol/L for both compounds.

Recoveries ranged from 83.6%–106.0%, with the exception of 5-formyltetrahydrofolate, which had a low recovery, about 50% (Table 2). We observed that the 5-formyltetrahydrofolate that was not recovered

as pABG was converted to 10-formylfolic acid. This result could be explained by the interconversion of 5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate that occurs at the low pH *(28)* during protein precipitation in the presence of perchloric acid. Under alkaline conditions, 5,10-methenyltetrahydrofolate is converted to 10-formyltetrahydrofolate, which may be oxidized to 10-formylfolic acid, which is stable at low pH *(29, 30)*; however, because 5-formyltetrahydrofolate is a minor folate species in human serum/plasma *(19)*, the measurement error associated with low 5-formyltetrahydrofolate recovery is expected to be minimal.

The within-day CVs were 4.1%–4.6%, and between-day CVs were 4.9%–5.0%. CVs were essentially independent of folate concentration, which ranged from 5.0–43.0 nmol/L.

ASSAY VALIDATION AND INTERFERENCE

A key criterion for measuring serum folate as pABG equivalents is the absence of pABG in native serum or the presence of only trace amounts. We therefore screened for pABG in 400 serum samples from blood donors and 223 routine serum samples (with typical creatinine concentrations of <100 μmol/L) from the clinical chemistry laboratory. In 621 of these samples, we detected no pABG (<0.125 nmol/L). Two serum samples contained 4.8 and 7.1 nmol/L pABG. Notably, both of these samples contained a supraphysiological folate concentration (142.0 and 126.0 nmol/L, respectively), suggesting an intake of high doses of folic acid supplements.

We also screened for pABG in 48 serum samples with increased serum creatinine concentrations (130–957 μmol/L). pABG was detected in 9 of these samples (2.0–12.4 nmol/L), and these samples also contained high folate concentrations (>94.7 nmol/L).

Our observation of the absence of pABG in native serum is in disagreement with a recent report that stated a mean pABG concentration in human serum of about 10 nmol/L *(31)*. These results were obtained from serum samples that had been acidified with HCl (final concentration, 0.12 mol/L) in the absence of antioxidant, and we were able to confirm the presence of similar amounts of pABG in serum samples treated with 0.12 mol/L HCl. Thus, the presence in serum of pABG at a concentration similar to that of folate seems to be an artifact related to the partial conversion of folate to pABG at low pH.

We detected no residual 5-methyltetrahydrofolate, other folate species, or pABA in 400 processed serum samples from healthy individuals. This observation demonstrates the essentially complete conversion of 5-methyltetrahydrofolate and no further degra-
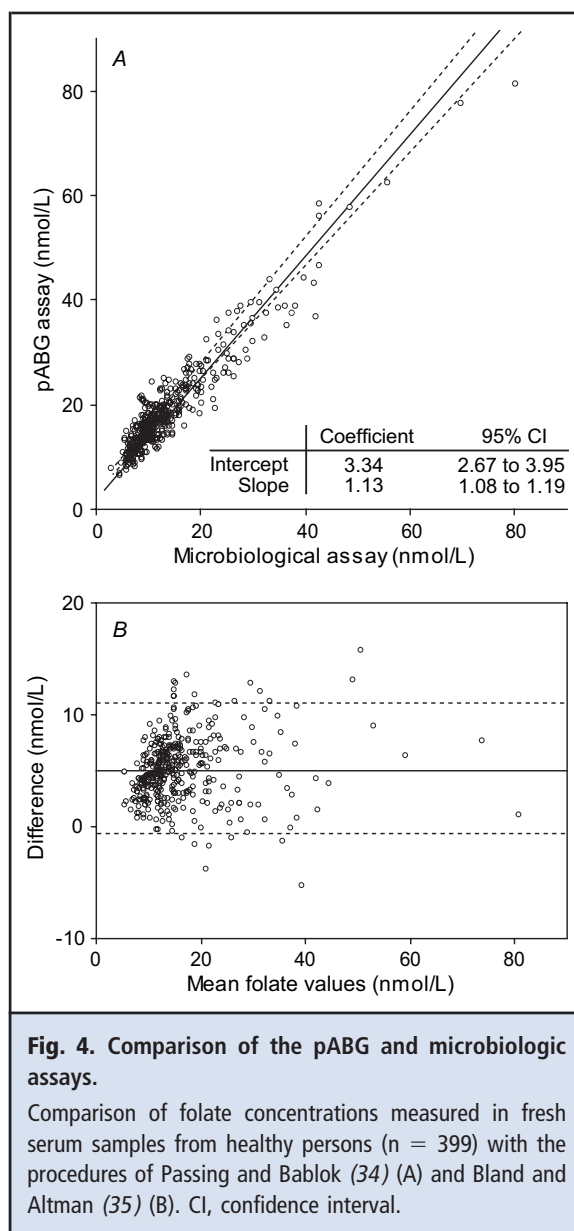

Fig. 4. Comparison of the pABG and microbiologic assays.

Comparison of folate concentrations measured in fresh serum samples from healthy persons (n = 399) with the procedures of Passing and Bablok *(34)* (A) and Bland and Altman *(35)* (B). CI, confidence interval.

dation of pABG to pABA under the conditions we established for oxidation and acid hydrolysis.

We tested for assay interference in the presence of the folate catabolite apABG *(32)* and the antifolate drug methotrexate, both of which have a *p*-aminobenzoyl-glutamyl moiety *(33)*. Both compounds were added to serum at a concentration of 100 nmol/L. apABG did not interfere with the assay, whereas about 40% of the methotrexate was recovered as pABG (data not shown). Therefore, the pABG method cannot be used to assess folate status in patients receiving methotrexate.

We compared folate concentrations obtained with the pABG assay in serum and in EDTA-treated

plasma (n = 48). The concentrations of folate measured in serum and EDTA-treated plasma were similar [15.7 (9.1) nmol/L and 16.2 (9.1) nmol/L, respectively]. Thus, serum and EDTA-treated plasma may be used interchangeably for measuring folate with the pABG assay.

METHOD COMPARISON AND SERUM FOLATE CONCENTRATIONS IN FRESH AND AGED SAMPLES

Measurements of folate concentrations in 399 fresh serum samples from healthy blood donors obtained with the pABG method and with a microbiologic assay showed good agreement (Fig. 4, A and B).

We used serum samples (n = 83) collected between 1973 and 1980 that we had retrieved from the JANUS biobank (25) to investigate whether the pABG assay measured folate in stored serum samples containing low folate concentrations due to folate degradation. These samples had been stored for 27–34 years at −25 °C in sealed polyethylene vials. The mean (SD) folate concentration was 4.1 (1.8) nmol/L (range, 0.7–8.9 nmol/L) according to the microbiologic assay and 10.7 (3.7) nmol/L (range, 3.4–20.7 nmol/L) with the pABG assay. The correlation between the serum folate concentrations obtained with the 2 methods was significant (Spearman $r = 0.70$; $P < 0.001$). Thus, the pABG assay yielded substantially higher folate concentrations than the microbiologic assay in old serum samples, but mean concentrations were still only 55% of the folate concentrations measured in 400 fresh samples from healthy individuals. One may speculate about whether the difference reflects a general improvement in folate status during the last 30 years because of increased public awareness of the health benefits from a diet rich in folate and the increased use of folic acid supplements (36). Alternatively, a portion of the folate may have become degraded during storage to the extent that it could not be recovered as pABG.

In summary, serum folate can be measured with a method that is based on the quantitative conversion of folate to pABG by oxidation and mild acid hydrolysis, whereas a method that involves strong acid hydrolysis of serum folate to pABA was not feasible because of the presence in serum of pABA precursors not related to folate. The pABG assay measured typical folate concentrations in serum samples in which most of the microbiologically active folate had become degraded during storage for about 30 years. The pABG assay for serum folate allows the assessment of folate status in epidemiologic studies based on stored samples and may also serve as a reference method for the measurement of serum folate.

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