Clinical Chemistry 50:2
423–432 (2004)

Nutrition

Determination of Folate Vitamers in Human Serum by Stable-Isotope-Dilution Tandem Mass Spectrometry and Comparison with Radioassay and Microbiologic Assay

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Background: **Current clinical methods for folate give different results and cannot measure the various forms of folate. We developed an isotope-dilution tandem mass spectrometric method coupled to liquid chromatography (LC/MS/MS) as a candidate reference method for 5-methyltetrahydrofolic acid (5MeTHF), 5-formyltetrahydrofolic acid (5FoTHF), and folic acid (FA) in human serum.**

Methods: **We quantitatively isolated folates from 275 μL of serum with a phenyl solid-phase extraction cartridge, then detected and quantified them in stabilized serum extracts by positive-ion electrospray ionization LC/MS/MS. We used an isocratic mobile phase of acetic acid in organic solvent on a C_8 analytical column. ^{13}C-labeled folates were used as internal standards.**

Results: **Limits of detection in serum were 0.13 (5MeTHF), 0.05 (5FoTHF), and 0.07 (FA) nmol/L. Within- and between-run imprecision (CV) was <7% for 5MeTHF and <10% for 5FoTHF at concentrations >0.5 nmol/L, and <10% for FA at concentrations >2.0 nmol/L. Total folate (TFOL) concentrations determined by competitive protein binding radioassay were ~9% lower than results obtained with LC/MS/MS. The microbiologic assay gave ~15% higher TFOL results with FA calibrator and no difference with 5MeTHF calibrator. The mean (SD) [range] TFOL in 42 sera was 35.5 (17.8) [6.5–75.6] nmol/L. Thirty-two samples with TFOL <50 nmol/L had, on average, 93.3% 5MeTHF, 2.3% FA, and 4.4% 5FoTHF. Ten samples with TFOL >50 nmol/L had, on average, 81.7% 5MeTHF, 15.7% FA, and 2.5% 5FoTHF.**

Conclusions: **This stable-isotope-dilution LC/MS/MS method can quantify 5MeTHF, 5FoTHF, and FA in serum. Currently used clinical assays agree with this candidate reference method.**

As part of the water-soluble B-vitamin group, folates have great nutritional importance as coenzymes in the transfer and utilization of one-carbon groups and in the regeneration of methionine from homocysteine *(1)*. Inadequate folate status can increase the risk of offspring with neural-tube defects in women of childbearing age *(2)*; be associated in the general population with certain cancers *(3)* and in the elderly with Alzheimer disease *(4, 5)*; and produce increased homocysteine concentrations in plasma, an independent risk factor for cardiovascular disease *(6)*. Although increasing dietary intake of folate helps reduce plasma homocysteine concentrations *(7)* and prevent many cases of neural-tube defects *(8)*, whether it also benefits the clinical manifestation of cardiovascular disease and Alzheimer disease is not yet clear.

After the mandated fortification of cereal-grain products with folic acid (FA)[1] in January 1998 *(9)*, folate status has improved dramatically in women of childbearing age *(10)* and in certain populations *(11–14)* beyond what was expected *(15)*. The predominant form of the vitamin circulating in serum is 5-methyltetrahydrofolic acid (5MeTHF), and the fasting serum folate concentration is used as a measure of folate status. If FA is ingested in large quantities in one meal (>300 μg), it can appear

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Received September 3, 2003; accepted November 25, 2003.

Previously published online at DOI: 10.1373/clinchem.2003.026955

[1] Nonstandard abbreviations: FA, folic acid; 5MeTHF, 5-methyltetrahydrofolic acid; THF, tetrahydrofolic acid; IS, internal standard; LC/MS, liquid chromatography–mass spectrometry; SPE, solid-phase extraction; MS/MS, tandem MS; 5FoTHF, 5-formyltetrahydrofolic acid; PteGlu, pteroylmonoglutamic acid; MRM, multiple-reaction monitoring; QC, quality control; LOQ, limit of quantification; LOD, limit of detection; and TFOL, total folate.

unmetabolized in serum *(16)*. Unlike 5MeTHF, FA can bypass the vitamin B_{12}-dependent conversion into tetrahydrofolic acid (THF) and enter the main folate metabolic cycle. Consequently, anemia associated with folate deficiency is less likely to develop, and any inherent vitamin B_{12} deficiency, with its potential consequences for irreversible nerve damage, could be masked *(17)*. Thus, whether these large increases in serum folate concentrations after fortification are accompanied by unmetabolized FA in serum is of great interest.

The microbiologic assay using *Lactobacillus casei* is believed by some investigators to be a gold standard method for folate measurement. Typical methods for measuring serum folate used in clinical laboratories are variations of immunoassays or competitive protein binding assays. Although sensitive, these methods lack specificity to distinguish among different forms of folate. They also agree poorly *(18, 19)*. In academic research laboratories, various HPLC methods for measurement of folates in biological matrices have been developed *(20–24)*. Although these chromatographic methods have better specificity than the microbiologic assay and immunoassay, they often lack sensitivity for the low concentrations of folate in serum and cannot be considered as high-order reference methods because they lack an appropriate internal standard (IS). The gas chromatography–mass spectrometry method used to analyze whole-blood folates showed greater sensitivity than previous chromatographic methods; however, gas chromatography–mass spectrometry requires complex sample preparation, including chemical derivatization, that introduces new sources of experimental error and increases the probability of folate degradation *(25, 26)*.

Recently, the first liquid chromatography–mass spectrometry (LC/MS)-based methods were developed to measure folates in food items *(27–30)*. Methods to analyze 5MeTHF in human serum and plasma have also been reported, but these methods either did not use stable-isotope dilution analysis *(31)* or were single-quadrupole LC/MS methods *(32–35)*. This report represents an adaptation and application of current solid-phase extraction (SPE) and chromatography technologies. It is the first report of an isotope-dilution LC-tandem MS (LC/MS/MS) method for accurate and simultaneous determination in human serum of the folates 5MeTHF, FA, and 5-formyltetrahydrofolic acid (5FoTHF). Being highly specific and sensitive, this method can measure three folate forms in only 275 μL of serum. A throughput of 36 samples/day is possible and can be increased to 96 samples/day through automation of the SPE step.

Materials and Methods

REAGENTS AND MATERIALS

FA [pteroylmonoglutamic acid (PteGlu) free acid], 5MeTHF calcium salt [(6*S*)-5-CH_3-H_4PteGlu-Ca], 5FoTHF calcium salt [(6*S*)-5-CHO-H_4PteGlu-Ca], THF calcium salt [(6*S*)-H4PteGlu-Ca], ^{13}C-labeled FA ($^{13}C_5$-FA), ^{13}C-labeled 5MeTHF {(6*S*)-5-CH_3-H_4Pte[$^{13}C_5$]Glu-Ca; $^{13}C_5$-5MeTHF}, and ^{13}C-labeled 5FoTHF {(6*S*)-5-CHO-H_4Pte[$^{13}C_5$]Glu-Ca; $^{13}C_5$-5FoTHF} were purchased from Merck Eprova AG. The purity of these compounds was confirmed by ultraviolet spectrophotometry, HPLC with ultraviolet and fluorescence detection, and MS analysis. Each labeled folate analog contained five ^{13}C atoms on the glutamic acid moiety. A 110 nmol/L calibrator of ^{13}C-labeled folate was analyzed by LC/MS/MS to assess the contribution of unlabeled 5MeTHF, 5FoTHF, or FA from the stable-isotope-labeled analogs. By multiple-reaction monitoring (MRM) of mass transition *m/z* 460→*m/z* 313 in a preparation of labeled 5MeTHF, *m/z* 474→*m/z* 327 in a preparation of labeled 5FoTHF, and *m/z* 442→*m/z* 295 in a preparation of labeled FA, the amount of unlabeled 5MeTHF, 5FoTHF, or FA in the stable-isotope-labeled analog was <0.25%. A 12-port vacuum manifold (Supelco) equipped with 1-mL phenyl SPE cartridges (100-mg BondElut; Varian) was used for manual sample extraction. Purified water (18 mΩ) from an AquaSolutions water purification system was used to prepare all samples and calibrators. All other chemical reagents and solvents were of ACS reagent grade unless stated otherwise.

When not in use, crystalline folate calibrators and folate stock solutions were stored at −70 °C. All reagents and calibration solutions were prepared in filtered (0.45-μm pore nylon filters; Millipore Corp.) purified water that was degassed with nitrogen. Extracted serum samples were filtered through a 0.45 μm polyvinylidene difluoride 1-mL syringe filter (Millipore) before LC/MS/MS analysis. Buffers containing ascorbic acid were prepared in small quantities freshly before use by diluting concentrated stock solutions that were free of ascorbic acid and adding the appropriate amount of ascorbic acid. All handling was done under gold-fluorescent light.

SERUM SAMPLES

Serum samples were residual aliquots of pools prepared in-house for other purposes or residual aliquots prepared for a folate interlaboratory comparison study (round robin) that the CDC performed in 2000 *(19)*. All aliquots were stored at −70 °C when not in use. For quality-control (QC) purposes, we prepared four serum QC pools at different concentrations of 5MeTHF, 5FoTHF, and FA: low, medium, high 1 (high 5MeTHF concentration), and high 2 (high FA concentration). The first three pools were native serum, whereas 11.3 nmol/L FA was added to the high 2 pool. These QC pools were analyzed in each run in two replicates. Aliquots (1 mL) of these serum QC pools were stored at −70 °C for at least 1 year, and vials were discarded after a single use to avoid repeated freezing and thawing. Aliquots of a base serum pool with a 5MeTHF concentration of ~14 nmol/L were used for calibration in serum and recovery experiments.

PREPARATION OF FOLATE STOCK SOLUTIONS AND CALIBRATORS

Stock solution I (~440 μmol/L or ~200 μg/mL). Stock solutions for reduced folates (5MeTHF, 5FoTHF, THF) were prepared by dissolving ~5 mg in 20 mmol/L phosphate buffer (pH 7.2) containing 1 g/L cysteine in a 25-mL volumetric flask. A small aliquot (<1 mL) of this stock solution was removed to determine the concentration by ultraviolet spectrophotometry. To the remaining stock solution ascorbic acid powder was added immediately to a final concentration of 10 g/L. FA stock solutions were prepared similarly, except that 20 mmol/L phosphate buffer (pH 7.2) was used without addition of 1 g/L cysteine or 10 g/L ascorbic acid. The actual concentration of each primary stock solution was determined within 10 min of preparation by measuring the ultraviolet absorbance of a 1:20 dilution of each stock solution against phosphate buffer as a blank at 290 nm (5MeTHF), 285 nm (5FoTHF), 298 nm (THF), and 282 nm (FA) *(36)*. The ratio of absorbance at 290/245 nm was monitored for 5MeTHF and its labeled analog to ensure that no oxidation to the dihydrofolate took place. This ratio is expected not to exceed 3.3 *(37)*.

Stock solution II (~220 μmol/L or 100 μg/mL). On the basis of the actual folate concentration in stock solution I, the solution was diluted to yield 25 mL of a ~220 μmol/L stock solution (stock solution II; 217.9 μmol/L 5MeTHF, 211.4 μmol/L 5FoTHF, 224.2 μmol/L THF, and 226.8 μmol/L FA; reduced folates were diluted with 10 g/L ascorbic acid solution; FA was diluted with water). Aliquots (1 mL) of stock solution II were kept frozen at −70 °C for at least 1 year without a change in concentration.

Stock solution III (~22 μmol/L or 10 μg/mL). One vial of stock solution II was freshly diluted 1:10 monthly (reduced folates; diluted with 1 g/L ascorbic acid solution) or bimonthly (FA; diluted with water) to yield 25 mL of a ~22 μmol/L stock solution (stock solution III). This final stock solution was aliquoted in 500-μL portions into microcentrifuge tubes for daily preparation of calibration curves and stored at −70 °C. Each vial of stock solution III was discarded after one use. When we analyzed stock solution III aliquots prepared and stored over 9 months together as unknowns, we found no difference in concentration exceeding the typical analytical variability.

PREPARATION OF AQUEOUS CALIBRATORS AND SERUM SAMPLES FOR SPE

A mixture of 5MeTHF, 5FoTHF, and FA (2.2, 1.1, and 1.1 μmol/L) was prepared in 1 g/L ascorbic acid from stock solution III. From this mixture, four mixed aqueous calibration solutions were prepared in SPE sample buffer (10 g/L ammonium formate, 1 g/L ascorbic acid, pH 3.2) corresponding to 2.2, 4.4, 22, and 110 nmol/L 5MeTHF (with an IS concentration of 10.9 nmol/L); 1.1, 2.3, 11.3, and 56.7 nmol/L FA (with an IS concentration of 5.7 nmol/L); and 0.53, 1.1, 2.3, and 11.3 nmol/L 5FoTHF (with an IS concentration of 5.3 nmol/L), respectively. For aqueous calibration samples, we mixed the following in 1.5-mL microcentrifuge tubes: 275 μL of aqueous calibration mixture, 495 μL of SPE sample buffer, 55 μL of IS mixture (217.9 nmol/L $^{13}C_5$-MeTHF, 211.4 nmol/L $^{13}C_5$-FoTHF, and 113.4 nmol/L $^{13}C_5$-FA, prepared in 1 g/L ascorbic acid), and 275 μL of water to give a final volume of 1.1 mL. For serum calibration samples, we mixed the following: 275 μL of aqueous calibration mixture, 495 μL of SPE sample buffer, 55 μL of IS mixture, and 275 μL of base serum. For serum QC or patient samples, we mixed the following: 275 μL of QC or patient serum, 770 μL of SPE sample buffer, and 55 μL of IS mixture. The sample mixture was then incubated at 4 °C for a minimum of 20 min to ensure equilibration between the labeled IS and the unlabeled endogenous folates. The SPE cartridges were conditioned with 2 mL each of acetonitrile, methanol, and SPE sample buffer. One milliliter of the above sample mixture was loaded on the conditioned cartridges and equilibrated for 1 min at room temperature. The cartridges were washed with 3 mL of SPE wash buffer (0.5 g/L ammonium formate, 0.05 g/L ascorbic acid, pH 3.4), and folates were eluted with 1 mL of SPE elution buffer (400 mL/L methanol, 100 mL/L acetonitrile, 10 mL/L acetic acid, 1 g/L ascorbic acid). The extracted samples were vortex-mixed and filtered before analysis. If not analyzed immediately, samples were stored at −70 °C until LC/MS/MS analysis. The concentration of each analyte in serum was calculated by interpolation of the observed analyte/IS peak-area ratio into the linear regression line for the calibration curve, which was obtained by plotting peak-area ratios vs analyte concentrations.

LC-ELECTROSPRAY IONIZATION-MS/MS CONDITIONS

All analyses were performed on a Sciex API 4000 triple-quadrupole MS system (Applied Biosystems). Chromatographic instrumentation consisted of a HP1100 LC system (Agilent) with a binary pumping system, thermostated autosampler, in-line mobile phase degasser, photodiode array detector, and fluorescence detector. The samples were chromatographed on a Luna C-8 *(2)* analytical column [150 × 3 mm (i.d.); 5 μm bead size; Phenomenex] with an isocratic mobile phase (400 mL/L methanol–100 mL/L acetonitrile–10 mL/L acetic acid) at a flow rate of 250 μL/min, a total run time of 10 min, and a 20-μL injection volume. The autosampler was set at 10 °C and the column temperature at 30 °C. The column was connected directly to the turbo ion electrospray operated in positive-ion mode. Tuning and mass calibration of quadrupole 1 and quadrupole 3 was performed by infusing a solution of polypropylene glycol (0.1 mmol/L) at 10 μL/min. The mass spectrometric system was automatically optimized for folates by constant infusion and flow injection at a flow rate of 10 μL/min of a ~5 μmol/L calibration solution in sample solvent. The optimization

included collisionally activated dissociation MS/MS, which was performed in the second quadrupole with nitrogen at 0.06 kPa as collision gas. The method conditions for each analyte transition are listed in Table 1. We continuously monitored the protonated $[M + H]^+$ molecular ion and the protonated most predominant fragment ion for each compound. Data were acquired and processed with Analyst for Windows NT software (Ver. 1.2).

stability of folates in extracted serum samples

To determine the stability of folates in aqueous calibrators and in extracted serum samples, we subjected the calibrators and the four serum QC pools to SPE as described above and analyzed the extracted samples by LC/MS/MS daily over 3 consecutive days while keeping the samples in the autosampler at 10 °C.

effect of freeze/thaw cycles on serum folate

To determine the effect of freezing/thawing on analyte stability in serum samples we conducted two experiments. The experiment to determine the effect of multiple freeze/thaw cycles with only brief exposure to room temperature was performed as follows: Three sets of serum high 1 and high 2 QC samples were subjected to one, two, or three subsequent freeze/thaw cycles over 1 day, in which we kept samples at room temperature for 1 h before returning them to −70 °C. The following day all three sets of serum samples were extracted in three replicates each and analyzed by LC/MS/MS. The results were compared with data obtained with unstressed serum high 1 and high 2 QC samples. The experiment to determine the effect of one freeze/thaw cycle with extended exposure to room temperature was similar to the one above, except that samples underwent only one freeze/thaw cycle during which they were kept at room temperature for 5 h.

Table 1. MS/MS method conditions for ^{13}C-labeled and unlabeled 5MeTHF, 5FoTHF, THF, and FA.[a]

Analyte (transition)	DP[b]	CE	CXP	EP
5MeTHF (*m/z* 460→*m/z* 313)	40	27	22	10
$^{13}C_5$-5MeTHF (*m/z* 465→*m/z* 313)	56	29	20	10
FA (*m/z* 442→*m/z* 295)	40	30	15	10
$^{13}C_5$-FA (*m/z* 447→*m/z* 295)	45	30	20	10
5FoTHF (*m/z* 474→*m/z* 327)	41	27	22	10
$^{13}C_5$-5FoTHF (*m/z* 479→*m/z* 327)	60	29	22	10
THF (*m/z* 446→*m/z* 299)	81	27	20	10

[a] The general instrument settings used for LC/MS/MS detection and quantification of all four analytes in MRM were as follows: resolution, Q1 and Q3; unit dwell time, 200 ms; ion spray voltage, 5200 V; source temperature, 350 °C; curtain gas, 20 psi; gas 1, 20 psi; gas 2, 40 psi; collisionally activated dissociation gas, 4.0 psi.

[b] DP, declustering potential; CE, collision energy; CXP, collision cell exit potential; EP, entrance potential.

comparison of LC/MS/MS with the Bio-Rad radioassay and the microbiologic assay

The microbiologic assay we used in-house was a 96-well microtiter plate assay based on the procedures of O'Broin and Kelleher *(38)* and Molloy and Scott *(39)*. The assay was calibrated in the range 0–22 nmol/L with FA from Sigma. For comparison purposes, we also calibrated the assay with FA, 5MeTHF, and 5FoTHF from Merck Eprova. The variability of this assay over 10 days was 8.7–11.1% for concentrations of 7.3–43.1 nmol/L.

The Bio-Rad QuantaPhase II assay we used in-house was a competitive protein binding radioassay *(40)*. Calibrators covering the range 0–23 nmol/L were provided as part of the assay reagent set and consisted of FA in human serum albumin base with 1 g/L sodium azide as a preservative. The variability of this assay over 20 days was 4.1–8.8% for concentrations of 3.5–22.0 nmol/L.

Results

optimization of LC-electrospray ionization-MS/MS conditions

The mass spectrometer was used in MRM mode for highest selectivity and lowest limit of quantification (LOQ). For each folate, one intense fragmentation loss occurs and can be logically rationalized as the neutral loss of the glutamic acid moiety (see Table 1 in the text for MS/MS method conditions and Figs. S1–S4 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol50/issue2/ for product ion scans of each folate form). Signal intensities were ~10 times higher in single-ion monitoring and MRM mode when the mobile phase was composed of acetic acid in organic solvent than when the mobile phase contained a buffer combination such as acetic acid/ammonium acetate or formic acid/ammonium formate. Acetic acid gave better intensities than formic acid, and intensities were highest at 7–10 g/L acetic acid. Positive-ion mode was ~10 times more sensitive than negative-ion mode in single-ion monitoring and MRM for 5MeTHF, whereas negative-ion mode was almost twice as sensitive for FA. Because 5MeTHF is the main folate form in serum, we decided to use positive-ion mode ionization. Substitution of a portion of the methanol with acetonitrile (100 mL/L) reduced the background for all transitions during LC/MS/MS while maintaining strong signal responses.

LC/MS/MS determination of 5MeTHF, 5FoTHF, and FA in serum was achieved by use of isocratic mobile phase conditions on a reversed-phase C_8 analytical HPLC column. Variability of the peak-area ratio of analyte to IS under these conditions was <3% between injections. Similar chromatographic behavior was also found with a reversed-phase phenylhexyl HPLC column of the same dimensions as the C_8 column. Fig. 1 shows representative tandem MRM profiles for 5MeTHF, 5FoTHF, FA, and ^{13}C-labeled 5MeTHF for the medium concentration serum QC pool obtained with the C_8 analytical HPLC column. Optimum chromatographic retention was achieved at an

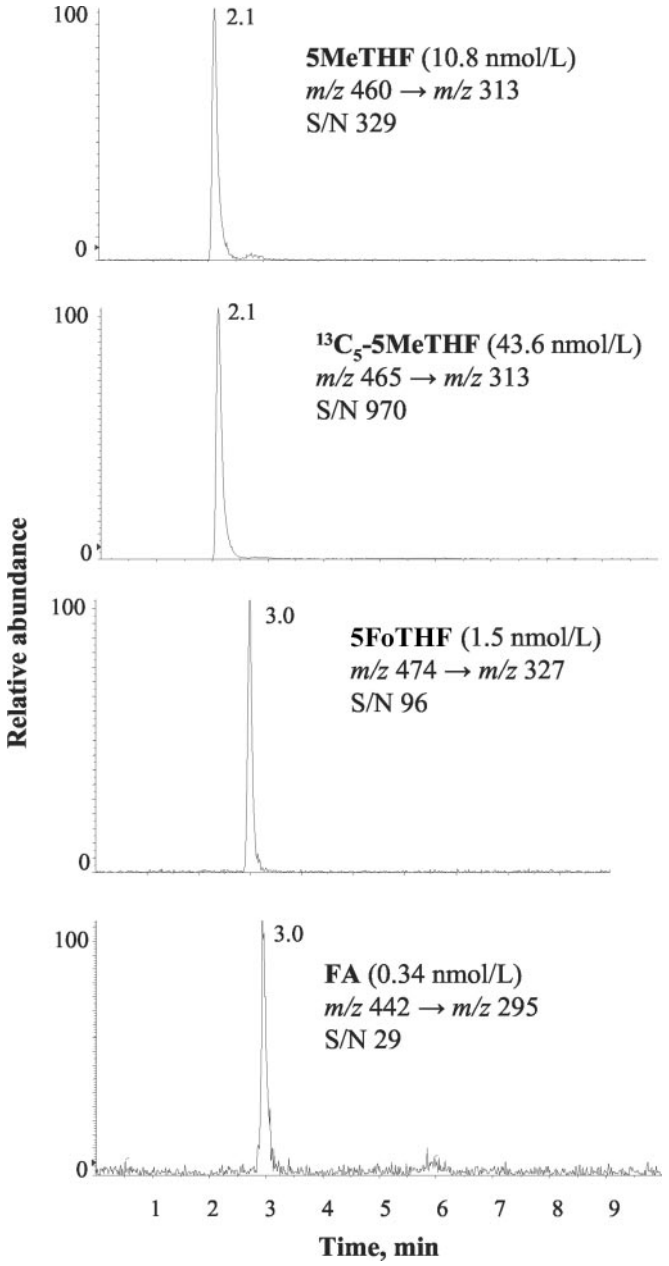

Fig. 1. Tandem MRM profiles for 5MeTHF, 5FoTHF, FA, and ^{13}C-labeled 5MeTHF for the extracted medium-concentration serum QC pool.
S/N, signal-to-noise ratio.

apparent mobile phase pH of 3.2, at which the folate α- and γ-glutamate carboxyl groups exist largely in the unionized forms (pK_a values of 3.5 and 4.8, respectively) *(41)*. 5MeTHF and FA were well resolved from each other, whereas 5FoTHF coeluted with FA at 3.0 min. THF coeluted with 5MeTHF at 2.1 min, but we did not detect any THF in serum samples. Because of the outstanding specificity of LC/MS/MS, chromatographic coelution of two or more analytes of interest can be resolved by their mass transitions using MRM, providing that no interferences occur in any monitored mass/charge transition.

Using the technique of postcolumn infusion *(42)*, we evaluated the extent of ion suppression in several serum samples. Although some ion suppression was observed at or near the retention of the analytes, the effects were not sufficient to prevent reliable quantification within the defined calibration ranges.

Optimization of SPE Conditions and Recovery of Folates Added to Serum

Dilution of serum with 10 g/L ammonium formate buffer, pH 3.2, enables release of protein-bound folates *(43)* and protonation of the α- and γ-glutamate carboxyl groups of folate, so that the folates are retained by the SPE cartridge. The ascorbic acid in the SPE sample buffer protects the folates from oxidation. Folates can be released from the SPE cartridge by eluting with a high organic content solvent. With this SPE procedure, we were able to prepare 36 samples within 3 h and analyze them by LC/MS/MS within 6 h.

A stable-isotope-labeled IS, having physical properties virtually identical to those of the analyte, is expected to correct for any potential losses during sample preparation. In an addition experiment, we confirmed that each IS indeed corrected for losses during sample preparation; we obtained complete recovery of 5MeTHF, 5FoTHF, and FA if calculations were based on the analyte/IS peak-area ratio. We also examined the recovery of folates during the SPE procedure independent of the IS. Six identical pooled serum samples were split into two groups: three serum samples to which the IS mixture was added before the SPE procedure and three serum samples to which the IS mixture was added to the eluate of the SPE procedure. The mean (SD) SPE recoveries independent of the IS, expressed as the percentage of recovered analyte, were 74.7 (3.2)% for 5MeTHF, 72.2 (3.8)% for 5FoTHF, and 76.8 (5.9)% for FA (Table 2).

Evaluation of Different Calibration Types

When a stable-isotope-labeled IS is used, matrix effects should not influence the final results, which are based on ion ratios. To confirm this, we constructed calibration curves in serum and in water. On the basis of analyte/IS peak-area ratios of 5MeTHF, slopes obtained through either direct LC/MS/MS analysis (aqueous calibration) or LC/MS/MS analysis after SPE analysis (aqueous or serum calibration) were not significantly different (0.076, 0.075, and 0.076). Calculated 5MeTHF concentrations in three serum samples were also not significantly different if based on an aqueous vs serum calibration curve (5.0 vs 5.7, 11.1 vs 11.5, and 32.9 vs 33.1 nmol/L). Thus, for all subsequent routine experiments, we used aqueous calibrators subjected to SPE analysis. Increasing folate concentrations in the calibration curve were matched by the expected proportional increase in peak-area ratios, i.e., if the concentration of the analyte was doubled, the peak-area ratio doubled. This was true for all three folates,

Table 2. Recovery of folates added to serum.

Sample	Added,[a] nmol/L	Area ratio[b] Group 1	Area ratio[b] Group 2	Mean (SD) SPE recovery,[c] %
5MeTHF added to serum				
Serum	0	0.7	0.5	73.5 (1.5)
Serum + concentration 1	10.9	1.3	1.1	77.9 (3.8)
Serum + concentration 2	109.0	6.5	4.7	72.8 (1.4)
5FoTHF added to serum				
Serum	0	0.3	0.2	74.6 (1.0)
Serum + concentration 1	5.3	1.5	1.1	74.7 (0.6)
Serum + concentration 2	52.9	12.2	8.2	67.3 (2.1)
FA added to serum				
Serum	0	0.2	0.1	83.1 (6.1)
Serum + concentration 1	5.7	1.4	1.1	75.1 (2.8)
Serum + concentration 2	56.7	12.4	9.0	72.3 (1.6)

[a] The analysis was performed in triplicate for each added concentration in each group.

[b] Group 1 consisted of three serum samples to which the IS mixture was added before the SPE procedure; group 2 consisted of three serum samples to which the IS mixture was added to the eluate of the SPE procedure.

[c] SPE recovery was calculated as the mean of the observed analyte/IS peak-area ratios for group 2 divided by the mean of the observed analyte/IS peak-area ratios for group 1.

5MeTHF, 5FoTHF, and FA, at all points of the calibration curve.

LINEAR DYNAMIC RANGE, LIMITS OF DETECTION, AND LIMITS OF QUANTIFICATION

Aqueous calibration curves for 5MeTHF, 5FoTHF, and FA were constructed based directly on LC/MS/MS results and showed excellent linearity over three orders of magnitude (0.22–220 nmol/L). The least-squares regression calibration curve for each folate was as follows: 5MeTHF, mean (SE) slope, 3506.0 (23.1); intercept, 95.2 (981.8) nmol/L; r^2 = 0.9995 (3080.0); residual sum of squares = 1.14×10^8; FA, slope, 8133.6 (31.5); intercept, 1991.0 (1336.0) nmol/L; r^2 = 0.9998 (4191.4); residual sum of squares = 2.11×10^8; 5FoTHF, slope, 31 755.9 (465.9); intercept, −24 434.9 (17 436.3) nmol/L; r^2 = 0.9966 (64 620.8); residual sum of squares = 6.68×10^{10}.

To determine the limit of detection (LOD) and LOQ for each folate in serum, we serially diluted a serum sample containing ~5.5 nmol/L 5MeTHF up to 1:20, a serum sample containing ~11.3 nmol/L FA up to 1:40, and a serum sample containing ~2.66 nmol/L 5FoTHF up to 1:10 with water and processed each sample by SPE. Using a signal-to-noise ratio of 5 as the cutoff, we found LOD of 0.13 nmol/L for 5MeTHF (0.65 fmol on column), 0.05 nmol/L for 5FoTHF (0.25 fmol on column), and 0.07 nmol/L for FA (0.35 fmol on column). With a signal-to-noise ratio of 10 as the cutoff, the LOQ were 0.26 nmol/L for 5MeTHF, 0.10 nmol/L for 5FoTHF, and 0.14 nmol/L for FA. Comparison of the obtained and expected folate concentrations in the diluted samples showed good proportionality with ratios of ~110% (5MeTHF), ~93% (5FoTHF), and ~80% (FA).

WITHIN- AND BETWEEN-RUN IMPRECISION

To assess within-run imprecision, we analyzed six different serum samples in three and/or four replicates each within 1 day. Within-run imprecision was 0.6–4.5% at 5.8–39.8 nmol/L 5MeTHF; 1.4–14% at 0.4–3.0 nmol/L 5FoTHF; and 3.0–13% at 0.5–10.8 nmol/L FA. To assess between-run imprecision, we analyzed the four QC pools in two replicates each over 18 days (for 5MeTHF and FA) and 8 days (for 5FoTHF). The mean of the two replicates from each day was used for the between-run imprecision assessment. We also analyzed the three serum pools from the CDC interlaboratory comparison study (round robin) over 11 days (for 5MeTHF and FA) and 3 days (for 5FoTHF) in one replicate (CDC round robin low, medium, and high). Between-run imprecision was 4.4–6.9% at 5.5–44 nmol/L 5MeTHF. For 5FoTHF, the between-run imprecision was ≤10% at >0.5 nmol/L and ~20% below that concentration. For FA, the between-run imprecision was <10% at >2.0 nmol/L and <20% at 0.9–2.0 nmol/L. Between-run imprecision for FA <0.9 nmol/L exceeded 20%. The imprecision for FA was higher than that of 5MeTHF and 5FoTHF. Because the between-run imprecision was ≥20% for 5FoTHF at <0.4 nmol/L and FA at <0.9 nmol/L, these concentrations were considered the practical LOQ for 5FoTHF and FA, below which results would not be reported for patient samples.

STABILITY OF FOLATES IN EXTRACTED SERUM SAMPLES AND STOCK SOLUTIONS

Folates, especially the reduced forms, are sensitive to oxidation and decomposition. We observed no signs of degradation during storage of extracted calibrators and serum QC samples in the autosampler at 10 °C over 3 days. The folate concentrations of the serum QC pools were within the 2 SD limits of the determined between-run imprecision. The ascorbic acid in the sample solvent (1 g/L) seemed to protect the folates adequately over at least 3 days. The procedure devised for preparation and storage of folate stock solutions has the advantage that the primary and secondary stock solutions have to be prepared only once a year, whereas stock solution III can be prepared fresh monthly or bimonthly to ensure uncompromised results. When we followed this procedure, the variability of the calibration curve slopes over multiple preparations of stock solution III was <10% for all three folates.

EFFECT OF FREEZE/THAW CYCLES ON SERUM FOLATE

Folates are sensitive to freezing and thawing. Multiple freeze/thaw cycles (n = 3) with only brief exposure to room temperature (1 h) did not cause any noticeable loss of folates. However, one freeze/thaw cycle with extended exposure to room temperature (5 h) caused a ≤10% loss of folate.

DISTRIBUTION OF FOLATE FORMS IN NATIVE SERUM

We analyzed 42 native serum samples by LC/MS/MS (Fig. 2). In serum samples with total folate (TFOL) <50 nmol/L, 5MeTHF, FA, and 5FoTHF constituted 93.3%, 2.3%, and 4.4%. In samples with TFOL >50 nmol/L, the amount of FA was significantly increased; 5MeTHF, FA, and 5FoTHF constituted 81.7%, 15.7%, and 2.5%. Although 7 of 10 samples with TFOL >50 nmol/L had FA concentrations between 7 and 25 nmol/L, 3 of the samples had FA concentrations <0.6 nmol/L. The mean (SD) TFOL concentration in these 42 serum samples was 35.5 (17.8) nmol/L, with a range of 6.5–75.6 nmol/L.

COMPARISON OF LC/MS/MS WITH BIO-RAD RADIOASSAY AND MICROBIOLOGIC ASSAY

Results obtained by LC/MS/MS and the Bio-Rad radioassay or microbiologic assay for ~50 serum samples were correlated by least-squares regression (see Fig. S5 in the online Data Supplement). As expected, good correlations were obtained in both cases: radioassay vs LC/MS/MS, mean (SE) slope, 0.97 (0.04); intercept, −1.67 (1.70) nmol/L; r^2 = 0.92 (5.12); residual sum of squares = 1152; microbiologic assay vs LC/MS/MS, slope, 1.38 (0.03); intercept, −2.02 (1.14) nmol/L; r^2 = 0.98 (3.63); residual sum of squares = 618. Although results obtained by radioassay were very close to the line of ideality compared with LC/MS/MS, results obtained by microbiologic assay were higher than the line of ideality compared with LC/MS/MS. This is reflected by a slope of 1.38, which is significantly different from 1.

Bland–Altman difference plots for results obtained for the same serum samples with the three methods are shown in Fig. 3. The mean difference for radioassay vs LC/MS/MS was −2.8 nmol/L, representing a relative difference of 8.9%. This small negative difference was statistically significant (the 95% confidence interval corresponding to the mean difference ± 2 SE was −4.3 to −1.3). The limits of agreement (mean difference ± 2 SD; representing the range into which 95% of all radioassay results fell) were 12.9 nmol/L lower and 7.4 nmol/L higher for the lower and higher limits, respectively, than results obtained by LC/MS/MS. The difference was not concentration-dependent.

The mean difference for microbiologic assay vs LC/MS/MS was 10.9 nmol/L, representing a relative difference of 30.2%. This large difference was statistically significant (95% confidence interval, 8.7–13.1 nmol/L) and concentration dependent ($y = 0.33x - 2.19$ nmol/L; r^2 = 0.84); it increased with increasing TFOL concentration.

After performing numerous experiments with the microbiologic assay in which we tested the recovery, the influence of serum matrix, and various calibrators, we found that different folate calibrators produced slightly different calibration curves. Whereas FA, 5MeTHF, and 5FoTHF from Merck Eprova gave similar, although not identical, calibration curves (used as calibrators in the LC/MS/MS assay), FA from Sigma (used to calibrate the microbiologic assay) gave a lower calibration curve (see Fig. S6 in the online Data Supplement for testing of

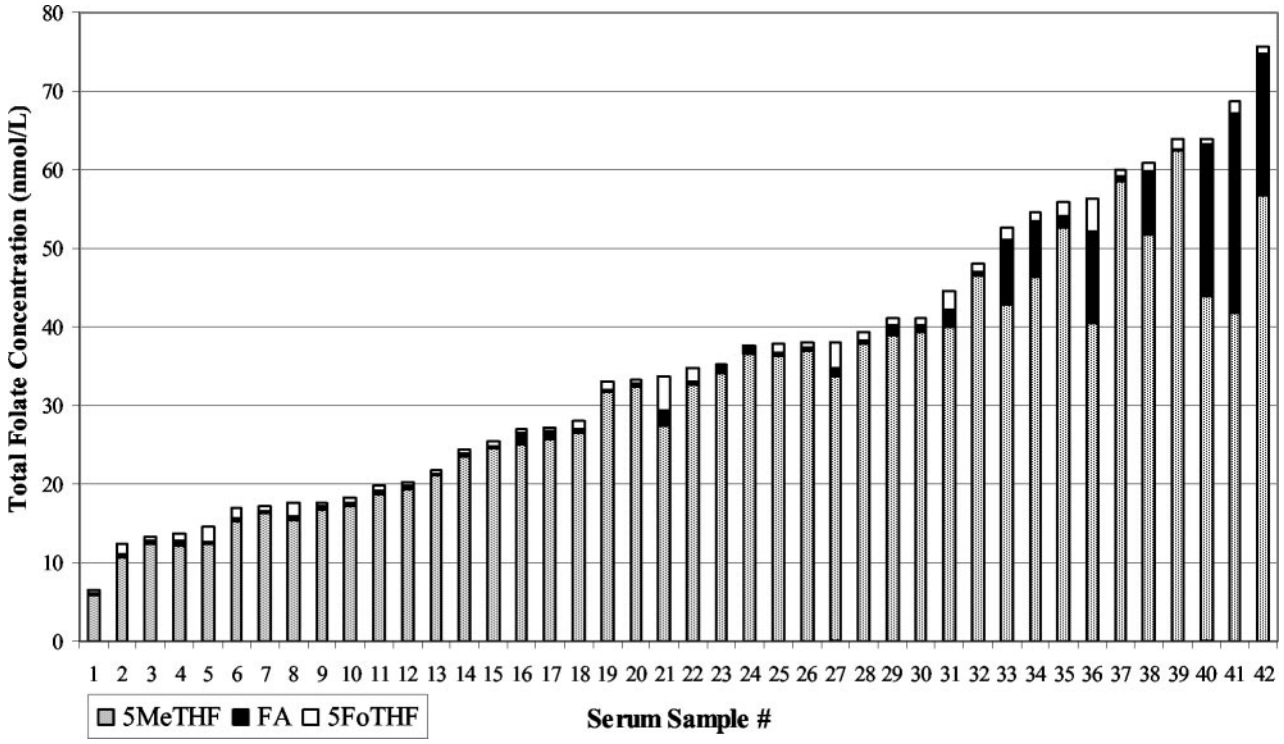

Fig. 2. Folate forms in native serum samples analyzed by LC/MS/MS.

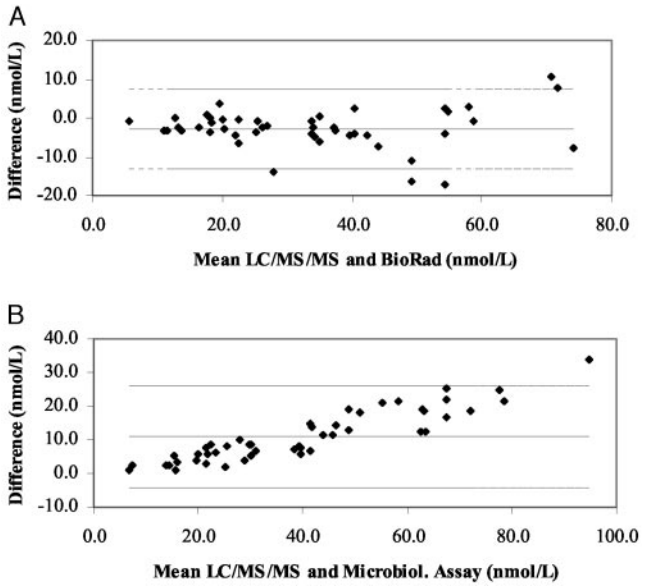

Fig. 3. Bland–Altman difference plots between LC/MS/MS and Bio-Rad radioassay (*A*) or microbiologic assay (*B*) for ~50 serum samples.

Solid line represents the mean difference between the two methods. The LC/MS/MS method is used as the comparison method. *Dashed lines* represent the limits of agreement or central 0.95 intervals of the differences between the two methods (mean difference ± 2 SD). A total of 46 serum samples were used in the analysis in *A* and 49 for the analysis in *B*.

different folate calibrators with the *L. casei* microbiologic assay). When we recalculated the concentrations of the serum samples using the Merck Eprova FA calibrator, the difference between the microbiologic assay and the LC/MS/MS decreased to ~15%, but the difference was still significant. When we recalculated the concentrations of the serum samples using the Merck Eprova 5MeTHF calibrator, the difference between the two methods disappeared completely; the microbiologic assay gave results that were, on average, 0.6% lower than the LC/MS/MS method. At this point it is unclear why the calibration curve with the Sigma FA calibrator is lower than the calibration curve with the Merck Eprova FA calibrator. Spectrophotometric determinations of the concentrations of both calibrators confirmed their purity to be ≥97%.

Comparison of the Presented SPE-LC/MS/MS Method with an Affinity Chromatography-LC/MS/MS Method

Concurrent with the method development presented here, the National Institute of Standards and Technology developed an isotope-dilution LC/MS/MS method to measure 5MeTHF in plasma or serum after sample cleanup through affinity chromatography with folate-binding protein *(44)*. We shared serum from samples 8, 27, and 36 (Fig. 2) with the investigators to compare results. Our results for 5MeTHF in these three pools (15.3, 33.8, and 41.6 nmol/L) were not significantly different from their results [15.5, 33.6, and 40.7 nmol/L; Table 2 in Ref. *(44)*].

Discussion

Although a few LC/MS-based methods for the analysis of serum 5MeTHF have been published recently *(31–35)*, none displays all of the features we sought: use of stable-isotope-labeled IS, MS/MS, and simultaneous measurement of other folate forms in addition to 5MeTHF.

The accuracy of the presented LC/MS/MS method was demonstrated by the proportional increase of signal in response to added folate with complete recovery when taking the IS into account. The measurements of all three forms of folate, 5MeTHF, 5FoTHF, and FA, were linear over a wide range of concentrations. Excellent sensitivity, as shown by the low LOD measured in diluted serum samples, allowed the quantification of serum folate in patients with folate deficiency and adequate status from only 275 μL of serum. If 5MeTHF is the only compound of interest, this method can easily be scaled down to use even less serum. This is especially true for populations that are exposed to staple foods fortified with folic acid. The simplicity of this method with regard to folate extraction and analysis, avoiding protein precipitation and post-SPE sample concentration, enhances its ruggedness. Although serum is generally preferred over plasma for the analysis of folate, we collected serum vs plasma treated with different anticoagulants from one individual. We found comparable 5MeTHF concentrations in serum and heparin-treated plasma, but ~15% lower concentrations in EDTA- and acidic citrate-treated plasma. This should be investigated further with a larger number of participants. Until such an investigation has been completed, the use of serum is recommended.

With an imprecision of ~5%, the variability of the 5MeTHF measurement in the presented LC/MS/MS method is comparable to that of automated assays used in clinical laboratories. Typically, chromatography-based assays display somewhat larger imprecision than automated assays. The MS-based methods published to date do not present detailed information on precision: Garbis et al. *(31)* reported within-run variability of 3.7–6.5%, and Pawlosky et al. *(32)* reported between-run variability for a midpoint test sample analyzed over 5 days of 7.6%. These variations were slightly higher than in our method.

On the basis of a preliminary set of data obtained from 42 apparently healthy individuals, the mean TFOL in serum was ~36 nmol/L, with a range of 6.5–75.6 nmol/L. The distribution of folate forms varied with the TFOL concentration in serum. A TFOL concentration of 50 nmol/L appeared to be the cutoff above which 70% of the samples had highly increased FA concentrations (up to 25 nmol/L), corresponding to a mean of 16% FA in the sample (see Fig. S7 in the online Data Supplement for the relationship between FA or 5FoTHF and TFOL in native serum samples analyzed by LC/MS/MS). Below the cutoff of 50 nmol/L, no sample had FA concentrations exceeding 2 nmol/L, corresponding to a mean of 2.3% FA in the sample. Because fasting was not a requirement when we obtained these samples, the results indicate that

as TFOL concentrations increase, the likelihood of absorbing unmetabolized dietary FA is higher. This could be especially relevant in a population consuming staple foods fortified with FA, such as the US population. Overall, we found that circulating 5MeTHF concentrations represent 82–93% of TFOL. We found no detectable THF in serum, even if freshly collected blood was processed immediately and prepared for LC/MS/MS measurement to avoid degradation of THF. The highest 5FoTHF concentration we encountered in serum was ~5 nmol/L, and the highest relative contribution of 5FoTHF was ~15% of the TFOL concentration. It should be noted that although it is likely that some or all of the formylfolate in serum is in the form of 10-formyltetrahydrofolic acid, this folate slowly converts to 5FoTHF at room temperature and is stable only at neutral and alkaline pH. At acidic conditions it converts to 5,10-methenyltetrahydrofolic acid with complete conversion below pH 2.0 *(41)*. Because of these interconversions we do not expect to measure any 10-formyltetrahydrofolic acid with this method. To check for the presence of 5,10-methenyltetrahydrofolic acid in extracted serum, we monitored the transition of *m/z* 458→*m/z* 311, but did not obtain any signal.

Comparison of this new LC/MS/MS candidate reference method with the Bio-Rad competitive protein binding radioassay showed good correlation and agreement between the two methods. The radioassay displayed a small negative difference of 9%. This can potentially be explained by the relatively low binding capacity of the folate binding protein for 5FoTHF *(41)*, which would give incomplete recovery of 5FoTHF in the radioassay.

Comparison of the LC/MS/MS method with the microbiologic assay showed good correlation. The size of the difference was dependent on the calibrator used. The largest difference (30%) was observed with the Sigma FA calibrator. Because this difference decreased to 15% when the Merck Eprova FA calibrator was used, it is likely that the 30% difference represented both an analytical difference and a reagent difference. Interestingly, the difference totally disappeared when 5MeTHF from Merck Eprova was used as calibrator. The finding that different folate calibrators produce slightly different calibration curves is not new. Phillips and Wright *(45)* found similar response curves for FA and 5FoTHF, but a much lower response curve for 5MeTHF. Only when the authors increased the buffering capacity of the medium and altered the starting pH of the medium were the response curves for all three folates almost identical. Although it is conceivable that the microbiologic assay displays some unspecific growth that could account for higher values compared with LC/MS/MS, our results indicate that any apparent assay differences are attributable to a calibration bias. Because of ongoing efforts to resolve the question about assay differences, any final conclusions about method differences are unwarranted at this time. It appears, however, as if common calibrators and/or standard reference materials could standardize these different assays and lead to more comparable results.

For several years, clinical assays used to measure serum folate have been known to agree poorly, and the use of assay-specific reference intervals has been suggested *(18, 19)*. Recent advances in technology finally made possible the introduction of LC/MS/MS into clinical laboratories *(46, 47)*. Isotope-dilution MS/MS is considered more specific than single-quadrupole MS, which differentiates only on the basis of molecular weight. Because of unique fragmentation patterns for each analyte in tandem MS, this technique typically offers unambiguous analyte identification and confirmation, which is required for a reference method. Our purpose was to use this technique to develop a candidate reference method to measure 5MeTHF and other folate forms in serum. The determination of 5MeTHF, 5FoTHF, and FA in serum has been shown to be accurate, precise, and sensitive enough to be performed with a small amount of serum. The method is amenable to automation for high-throughput analysis in a clinical laboratory. It can serve as a candidate high-order reference method and as a basis for future standardization efforts. It can also be applied to large-scale population studies and clinical intervention trials and be used as a tool to assess the impact of folate fortification in the US population, specifically with regard to the amount of unmetabolized FA in serum.

We thank Irene Williams and Lily Tao Jia (both from CDC, Atlanta, GA) for technical assistance with the Bio-Rad radioassay. We also express our appreciation to Bryant Nelson (Genzyme Corp., Framingham, MA) for a critical and thorough review of the manuscript during its preparation.

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