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Quantitation of Total Folate in Whole Blood Using LC-MS/MS

JANEL E. OWENS,† DIRK M. HOLSTEGE,‡ AND ANDREW J. CLIFFORD*,†

Department of Nutrition and Division of Agricultural and Natural Resources Analytical Laboratory, University of California, Davis, California 95616

An accurate method for measuring whole blood total folate using liquid chromatography with tandem mass spectrometry is described and compared to GC/MS and a chemiluminescence assay. Whole blood from normal adults ($n = 15$) was fortified with a [$^{13}C_6$]para-aminobenzoic acid (*p*ABA) internal standard and treated with 12.1 N hydrochloric acid at 110 °C for 4 h to hydrolyze all folates to *p*ABA. Contaminants in the hydrolysate were adsorbed onto a C18 SPE cartridge. The eluate containing the folate catabolite *p*ABA was partitioned into ethyl acetate and methylesterified with trimethylsilyldiazomethane. The methyl-*p*ABA derivatives were quantified by positive-ion atmospheric pressure chemical ionization (APCI)LC-MS/MS. An isocratic mobile phase of acetonitrile−water (70:30) (v/v) on a C18 analytical column was used with a postcolumn reagent of 0.025% formic acid. The limit of quantitation for folate was 56.6 nmol/L RBC, and the limit of detection was 22.6 nmol/L RBC. Folate levels as determined by LC-MS/MS correlated well with the chemiluminescence assay and a GC/MS method. This new LC-MS/MS method provides enhanced sample throughput ($n = 36$ per day) as compared to GC/MS methods. LC-MS/MS will enable accurate measurements of red blood cell (RBC) folate in nutrition surveys and clinical trials.

KEYWORDS: Folate; erythrocyte; mass spectrometry; para-aminobenzoic acid

INTRODUCTION

Adequate intake of folate is important to reduce the risk of neural tube defects (NTDs), (*1*, *2*), cardiovascular disease (*3*), some cancers (*4*), and hypertension in women (*5*). Since January 1998, when the fortification of cereal-grain products with folic acid became mandatory (*6*), the folate status has improved greatly in women of childbearing age (*7*). A higher intake of food folates and/or folic acid fortificants/supplements can raise red blood cell (RBC) folate levels and reduce the risk of NTDs by ∼70% (*8*). The RBC folate status, or the total folate concentration in red blood cells (*9*), is rated a good biomarker for nutritional status because it correlates with liver folate, a major tissue store (*10*). Additionally, amelioration of NTD susceptibility with an increase in folate intake may indeed be due to intracellular accumulation of folate (*11*).

Folate concentrations in plasma or serum are commonly determined to evaluate folate nutritional status and how it changes over time. Although RBC folate is considered to be a better indicator of body stores and hence nutritional status (*12*), uncertainty about the reliability of the analytical methods for RBC folate exists (*13*, *14*). An interlaboratory comparison of standard methods for quantifying RBC folate indicated significant differences among these laboratories (*13*). These results of the interlaboratory study then prompted the recent development of new analytical methods that included liquid chromatography coupled to mass spectrometric and tandem mass spectrometric detection (LC-MS and LC-MS/MS) for plasma and serum folate (*15*−*20*) and gas chromatography (GC/MS) for RBC folate (*21*−*24*). A recent report (*25*) also utilized stable-isotope dilution LC-MS/MS to measure intact folate monoglutamates in whole blood lysates and serum. Comparability of this method with other standard methods for RBC folate analysis has not been reported.

The GC/MS method (*23*) correlates well with the *Lactobacillus casei* method (*26*), and in the GC/MS method, the presence of neither methotrexate nor sulfonamides (folate analogues) affected the quantification of folate, unlike the assays based upon competitive binding technologies. The GC/MS method requires three derivatization steps, limiting sample throughput (*23*). A LC-MS/MS method is described here that uses acid hydrolysis to para-aminobenzoic acid (*p*ABA) coupled to only a single chemical derivatization step to increase throughput. We compared results obtained from the LC-MS/MS, chemiluminescence, and GC/MS methods using whole blood specimens from 15 normal adult volunteers.

MATERIALS AND METHODS

Chemicals and Reagents. Reagent grade *p*ABA, folic acid (FA) [pteroylmonoglutamic acid (PteGlu)], and formic acid were purchased from Sigma (St. Louis, MO). The folates 5-methyltetrahydrofolate (5MTHF) calcium salt [(6S)-5-CH_3-H_4PteGlu-Ca] and 5-formyltetrahydrofolate (5FTHF) calcium salt [(6S)-5-CHO-H_4PteGlu-Ca] and the antifolate analogue methotrexate (MTX) were purchased from

* Corresponding author. Tel.: (530) 752-3376. Fax: (530) 752-8966. E-mail: ajclifford@ucdavis.edu.
† Department of Nutrition.
‡ Division of Agricultural and Natural Resources Analytical Laboratory.

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Schircks Laboratories (Jona, Switzerland). The internal standard of [$^{13}C_6$]-*p*ABA was purchased from Beta Chem (Leakwood, KS). The purity of the previous chemicals was confirmed by ultraviolet spectrophotometry or by HPLC-DAD (*27*). Trimethylsilyldiazomethane (2 M in hexanes) was obtained from Fluka of Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile was purchased from Fisher Scientific (Fairlawn, NJ), and 18 mΩ deionized water (Millipore, Bedford, MA) was used. All other chemicals were purchased from Fisher Scientific (Fairlawn, NJ).

Preparation of Standards. Standards of *p*ABA were prepared in methanol, and vitamers 5MTHF, 5FTHF, MTX, and FA were prepared in 100 mM KH_2PO_4 dibasic buffer system (pH 7.0) containing 10% acetonitrile and 0.1% ascorbic acid. Stock solutions (1000 μg/mL) of folate vitamers were prepared with 1% ascorbic acid and stored at −20 °C, and dilute aliquots were prepared when needed.

Ten microliters of [$^{13}C_6$]-para-aminobenzoic acid was utilized as an internal standard (IS) at 5.6 μg/mL prepared in 2-propanol. It was purified by HPLC, and its UV spectrum was compared to *p*ABA from Sigma-Aldrich (*23*).

For the preparation of calibration standards, a stock solution of 5 μg/mL *p*ABA in methanol was methylesterified with 100 μL of trimethylsilyldiazomethane in the presence of ethyl acetate. The standard was shaken vigorously for 30 min at room temperature and then evaporated to dryness under a stream of argon at 50 °C. The methylated standard was resuspended in acetonitrile and serially diluted with acetonitrile to prepare standards ranging from 1 to 0.025 μg/mL. IS was added to each standard at a constant volume to maintain an equal concentration throughout with ratios of analyte to internal standard ranging from 1.8 to 0.05. Stock solutions of methyl-*p*ABA and calibrators were stored at −20 °C when not in use.

Sample Collection and Preservation. The study was approved by the University of California, Davis Institutional Review Board, and it was conducted according to the Good Clinical Practice Guidelines and the Declaration of Helsinki, version 1989. Informed consent was discussed and obtained from each subject before study participation. Users of multivitamin supplements were not excluded. Blood was drawn from each subject into three separate 4 mL tubes that contained spray-dried K_2EDTA and were shaken to prevent clotting. One tube was stored on ice for the determination of complete blood count (CBC) and packed cell volume (PCV). Blood samples for folate analysis were immediately wrapped in aluminum foil to exclude light and placed on ice for transit and stored at −80 °C until analysis.

Folate Analysis by Chemiluminescence. Whole blood was analyzed for folate using chemiluminescence technology (Bayer Diagnostics of ADVIA Centaur, Bayer Corporation Diagnostics Division, Tarrytown, NY) at the UCDMC Pathology Department Laboratory (technical procedure 3832.T). The chemiluminescence assays were performed on whole blood lysates (prepared with ascorbic acid). Briefly, folates from a whole blood specimen competed with acridinium ester-labeled folate for a limited amount of biotin-labeled folate-binding protein. Whole blood and specified kit reagents were incubated before the addition of acridinium ester-labeled folate. Kit reagents were added to initiate the chemiluminescent reaction. An inverse relationship existed between amount of folate present and amount of light detected by the system.

Folate Analysis by GC/MS. Samples were analyzed by GC/MS as previously described (*23*). Briefly, whole blood (100 μL) was hydrolyzed with 100 μL of 12.1 N HCl to hydrolyze the folates to *p*ABA. The *p*ABA was partitioned into ethyl acetate after C18 SPE cleanup and was derivatized with trimethylsilydiazomethane before being heptafluorobutylated. The final derivatization step converted the analyte to a *t*-butyldimethylsilyl derivative analyzed by GC/MS. [$^{13}C_6$]*p*ABA (1.40 μg/mL in 10 μL of 2-propanol) was used as an internal standard.

Folate Analysis by LC-MS/MS. Sample Preparation. To a 4 mL borosilicate glass tube (Alltech, Deerfield, IL), the [$^{13}C_6$]*p*ABA internal standard (5.6 μg/mL in 10 μL of 2-propanol) was added, followed by 100 μL of whole blood and 100 μL of 12.1 N hydrochloric acid. The headspace of the tubes was filled with argon gas for a nonoxidative environment and sealed with a polypropylene screw cap with a TFE liner (Alltech, Deerfield, IL). The samples were heated in a dry block for 4 h at 110 °C, allowed to cool to 23 °C, and diluted with 200 μL of 18 mΩ water. The diluted hydrolysate was loaded onto a 100 mg C18 SPE cartridge (Varian Sample Preparation Products, Harbor City, CA) that had been conditioned with 500 μL of methanol and 1 mL of 1 N HCl before addition of the sample. The eluate was collected in an 8 mL borosilicate glass tube (Alltech, Deerfield, IL). The cartridge was further eluted with 600 μL of 1 N HCl, which was also collected.

The pH of the eluate was adjusted with 500 μL of citric acid buffer (1.25 mol/L, pH 5.0), 500 μL of saturated sodium chloride, and 95 μL of 50% NaOH (w/w) such that the final pH was between 4.5 and 5.5. The sample was extracted with 4 mL of ethyl acetate by vigorous shaking for 1 min. The tubes were centrifuged at 3000 × *g* on an International Clinical Centrifuge (International Clinical Co., Needham Heights, MA) for 5 min. The top ethyl acetate layer (3.8 mL) was removed and added to 200 μL of methanol in an 8 mL conical vial. Thirty microliters of trimethylsilyldiazomethane was added. The tubes were capped and shaken vigorously at room temperature for 30 min. The extract was carefully evaporated just to dryness at 50 °C on a dry block using argon following the addition of two drops of 5% (v/v) decanol in an acetone keeper solution. The residue was resuspended in 100 μL of acetonitrile by vortexing and transferred to a 300 μL volume PolySpring (Fisher Scientific, Fairlawn, NJ) glass insert. The samples were stored at −20 °C until analysis.

Instrumental Conditions. Quantification of CH_3-*p*ABA was performed on a Sciex API 2000 triple-quadrupole MS system (Perkin-Elmer, Shelton, CT) controlled by Analyst 1.3.1 software (Applied Biosystems, Foster City, CA). HPLC instrumentation consisted of a Perkin-Elmer Series 200 LC system with a quaternary pumping system, autosampler, and in-line mobile phase degasser. The samples were separated using a Synergi 4 μ Hydro-RP column (150 × 4.6 mm i.d.; Phenomenex, Torrance, CA) with an isocratic mobile phase of acetonitrile/water (70:30 v/v) at 500 μL/min for a total run time of 6 min and a 20 μL injection volume. The autosampler and column were operated at room temperature. The methylated $^{12}C_6$- and $^{13}C_6$-*p*ABA eluted at 3.65 min. A postcolumn reagent of 0.025% formic acid in acetonitrile was added at 0.2 mL/min just prior to entry into the source. The column eluate with postcolumn reagent was split 1:1 to the ionization source and waste.

The mass spectrometer was tuned according to manufacturer specifications. MS data were acquired with positive ion atmospheric pressure ionization (APCI) utilizing the multiple-reaction monitoring (MRM) mode. Settings were as follows: temperature was 425 °C, the resolution settings of the first quadrupole (Q1) and third quadrupole (Q3) were low and unit, respectively, and the dwell time was 400 ms. The nebulizing current was set to 4 kV and the curtain gas, gas 1, gas 2, and collision induced dissociation (CID) gases were set at 20, 80, 60, and 10 psi, respectively. The declustering potential, focusing potential, entrance potential, collision energy, and collision cell exit potential were set at 20, 400, 10, 29, and 5 V, respectively.

Transitions for methyl-$^{12}C_6$-*p*ABA were monitored at *m*/*z* 152−120 (loss of methoxy group) and to *m*/*z* 93 (loss of methylester). IS transitions were monitored from *m*/*z* 158−126 and to *m*/*z* 99. Peak areas were from chromatograms generated using *m*/*z* 152−93 and *m*/*z* 158−99 transitions. Quantitation was by comparison to a six-point calibration curve using the ratio of the analyte response to the internal standard plotted against the amount of analyte injected. Calibrations were performed 3 times before the analysis of samples, once in the middle of the sample analysis, and at the end of each run of samples. The calibration curve used weighted (1/*x*) second-order regression. Concentrations of folate were divided by the PCV (fraction) and expressed as nmol of folate/L of erythrocytes.

Method Validation. The method was validated by determining the recovery of 5MTHF, 5FTHF, FA, or MTX added at two different concentrations ($n = 5$) (**Table 1**). Recoveries were calculated as $R = ((C_{\text{spikedsample}} - C_{\text{control}})/(C_{\text{added}})) \times 100\%$, where $C_{\text{spikedsample}}$ is the concentration of the spiked sample, C_{control} is the average concentration from five replicates of a control sample, and C_{added} is the concentration of the folate vitamers added to the whole blood before hydrolysis. The reported recoveries for all experiments were corrected by the internal standard. The internal standard recovery was determined to be 85%.

Table 1. Summary of Validation Study Results of Fortifications with Three Folate Vitamers and Methotrexate Added to Whole Blood[a]

folate	*N*	added vitamer (nmol/L)	av % recovery	CV (%)
5MTHF	5	5170	99	6.1
	5	2580	93	1.7
5FTHF	5	4960	92	5.6
	5	2490	99	7.1
FA	5	3800	66	13
	5	2380	62	5.8
MTX	2	3520	0	3.6
control	5	1140		9.4

[a] Fortification levels, average percent recovery, and percent coefficient of variation are provided for each level. The average value for the control (no spike added) is also included.

RESULTS AND DISCUSSION

The method quantitatively extracted and recovered the natural folate vitamers at the two concentrations studied (**Table 1**). Recoveries of 5MTHF at two fortification levels were 99 and 93%, while recoveries for 5FTHF were 92 and 99%. Folic acid recoveries (66 and 62%) were the lowest of the three vitamers. 5MTHF, the most prevalent form found in blood (*26*), gave excellent recovery with good precision. Naturally occurring folate vitamers were added at concentrations twice that of background level; and for these added concentrations, the method gave an excellent recovery with coefficient of variation values ranging from 1.7 to 7.1%, indicating little variability. Folic acid is only found in serum shortly after ingestion of a large dose (*19*) and is unlikely to make significant contributions to total folate values of whole blood. Thus, these folic acid recoveries are acceptable.

Sample Preparation Optimization. The sample preparation method for GC/MS (*23*) calls for samples to be hydrolyzed for a minimum of 6 h using 12.1 N hydrochloric acid. To optimize this step for LC-MS/MS, we hydrolyzed blood samples ($n = 3$ for each time point) for 2, 4, 8, 10, 14, 20, and 24 h. The hydrolysis time of 4 h with 12.1 N HCl gave the optimum tradeoff between complete hydrolysis of folate vitamers to *p*ABA and minimum degradation of *p*ABA (data not shown). Additionally, we investigated the effectiveness of 12.1 N hydrochloric acid, 15 M sulfuric acid, and 8 M methanesulfonic acid (MSA) in recovering spiked folate vitamers. All three acids studied, 12.1 N HCl, 15 M H_2SO_4, and 8 M MSA, yielded quantitative recoveries of *p*ABA (≈101%) and 5-MTHF (≈103%), the predominant vitamer in RBCs. Approximately 100% 5FTHF and about 65% FA was recovered using 12.1 N HCl or 8 M MSA. Our recovery of added FA is lower than another report (*18*), but because folic acid is not found in red blood cells, we did not optimize this recovery. The 12.1 N HCl was employed because less than 50% NaOH (w/w) was needed to adjust the pH before ethyl acetate extraction.

The initial C18 SPE cleanup was necessary to remove all the hydrolyzed heme and other contaminants from the whole blood and gave a clear extract afterward. To optimize the recovery from the SPE cartridge, the sorbent should not be allowed to become dry. Decanol was added to avoid potential losses during the evaporation of ethyl acetate.

Finally, blood samples from one volunteer showed no statistical change in folate concentration over a 107 day period when measured by LC-MS/MS ($n = 3$ for three different aliquots) when stored at −80 °C until analysis. The CV(%) for these nine samples over the 107 day period was 10.3% ($P = 0.7329$), so the day-to-day variability of this method is minimal. Aliquots of the original sample were frozen at −80 °C and analyzed periodically throughout the 107 day storage period. Storage times greater than 4 months were not evaluated.

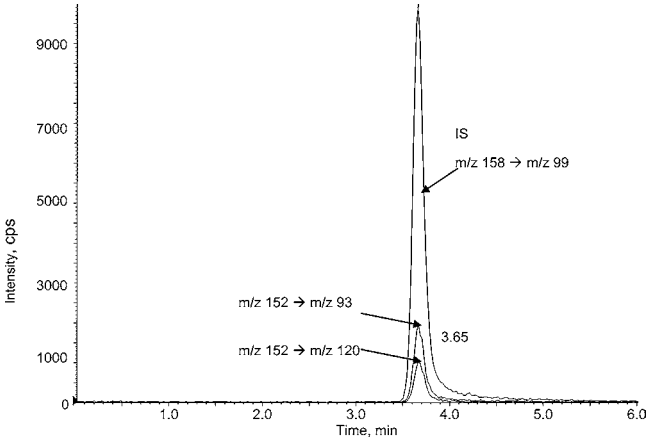

Figure 1. LC-MS/MS chromatogram of methyl-*p*ABA standard at 0.1 μg/mL.

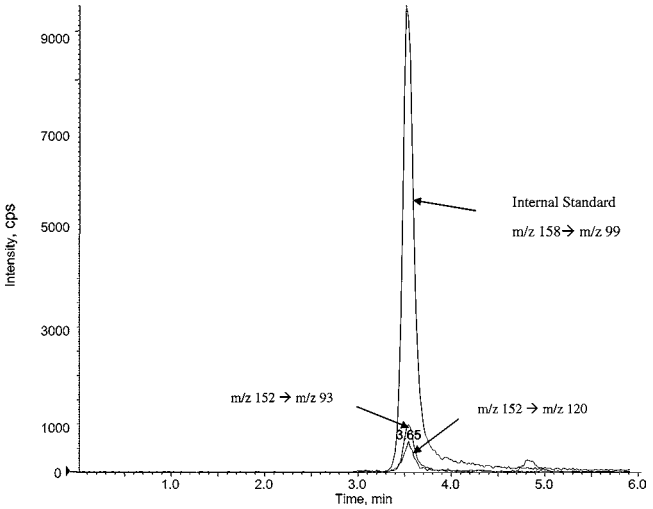

Figure 2. LC-MS/MS chromatogram of whole blood sample from a normal adult volunteer.

LC-MS/MS Optimization. The analytical conditions were optimized to produce a rapid and selective analysis. The HPLC run time was only 6.0 min, with methyl-*p*ABA eluting at 3.65 min. The *p*ABA isotopomers were methylated to improve sensitivity and to simplify chromatography because ion-pair chromatography is not feasible with the MS detection. The selectivity of MS/MS was able to produce chromatograms with only one peak despite the complexity of the matrix (**Figures 1** and **2**). Addition of the antifolate methotrexate (3520 nmol/L) did not interfere with the quantification of RBC folate. A product ion spectrum obtained via multiple reaction monitoring (MRM) of methyl-*p*ABA showed the precursor ion at *m*/*z* 152 $[M + H]^+$ and product ions at *m*/*z* 120, 107, and 93. The transition from *m*/*z* 152 to 93 was the most sensitive and was used for quantification. The *m*/*z* 152−120 transition was used for confirmation because the *m*/*z* 152−107 transition had a low relative peak abundance. The limit of detection ($S/N = 3$) and the limit of quantification ($S/N = 10$) were 22.6 nmol/L RBC and 56.6 nmol/L RBC, respectively. The folate deficiency is described as a RBC folate value of less than 305 nmol/L RBC (*28*). Thus, this method attained limits of quantitation well below the cutoff level for folate deficiency. The calibration curve was linear over a range from 56.6 to 9000 nmol/L RBC with typical R^2 values of 0.999.

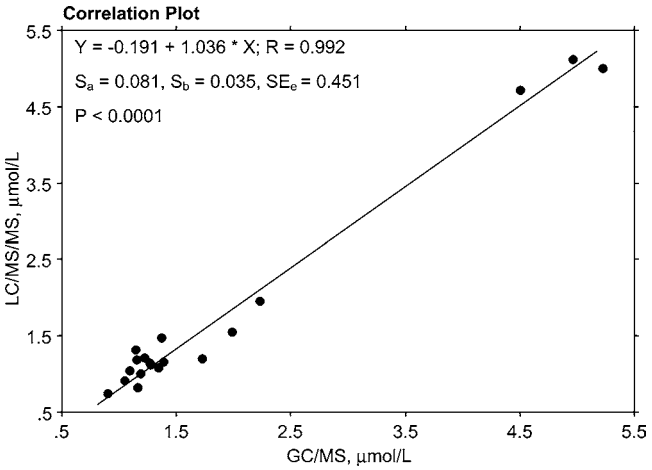

Figure 3. Plot of RBC folate results of GC/MS analysis vs LC-MS/MS analysis of 19 samples, including volunteer blood samples, QC samples, and fortifications. S_a, S_b, and S_e represent the standard errors of intercept, slope, and estimate, respectively.

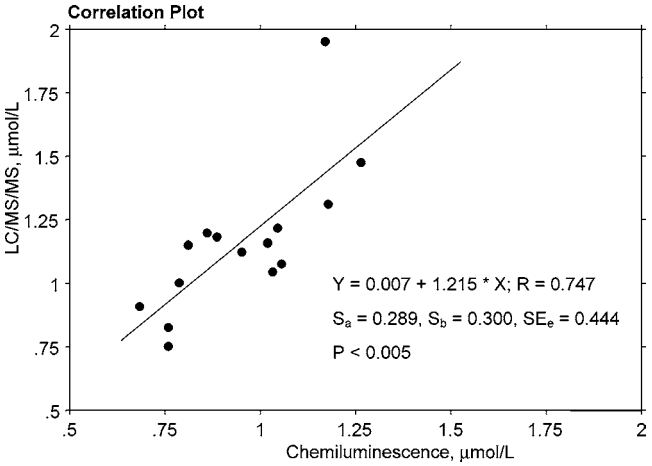

Figure 4. Plot of RBC folate results of chemiluminescence assay vs LC-MS/MS analysis of 15 samples, including volunteer blood samples and QC samples.

Comparison of LC-MS/MS and GC/MS. Blood samples analyzed by LC-MS/MS were analyzed by GC/MS, and the correlation of these two methods is shown in **Figure 3**. The slope and intercept are not statistically different from 1 and 0, respectively. These methods were correlated, with $r = 0.992$ at $P < 0.0001$, for 13 blood samples, three control samples, and three samples fortified with 1970 nmol/L 5MTHF (average recovery of 100% ± 6 by LC-MS/MS and 99% ± 10 for GC/MS methods). When the upper right data points are deleted from the correlation plot, the r value drops to 0.880. Two subjects of the 15 were dropped from the study because their CBC values were not determined. The LC-MS/MS method gave the better recovery (100%) and precision (CV of 6%).

Comparison of LC-MS/MS and Chemiluminescence Assay. RBC folate as determined by LC-MS/MS and a chemiluminescence assay showed good correlation with a correlation coefficient of 0.747 at $P < 0.005$ (**Figure 4**). The slope and intercept are not significantly different than 1 and 0, respectively. The r value of 0.747 compares very well to our previously reported correlation between chemiluminescence and radioassay ($r = 0.730$ and $P < 0.001$ for 120 samples), both of which are based upon protein-binding technologies (*29*). Of the 15 volunteers that were recruited for the study, three were dropped because they had folate values undetected by the chemiluminescence method, while LC-MS/MS and GC/ MS analyses gave quantifiable RBC folate levels for these three subjects and/or CBC values were not determined.

Method Characteristics. There is no endogenous generation of *p*ABA, and it is not normally found in blood. Our method would not be appropriate for individuals who use *p*ABA with regularity. *p*ABA is rapidly cleared, however, and excretion is almost complete within 6 h after ingestion (*30*). The drug is rapidly absorbed and excreted and in normal humans, and very little is excreted as free *p*ABA. Most of it is excreted as *p*-aminohippuric acid or as glucuronate (*31*). If individuals abstain from using *p*ABA for a few days, total folate could be assessed using the LC-MS/MS method.

Our method can also reliably determine total folate in patients currently using methotrexate, one of the most common antifolates. For MTX to raise *p*ABA levels, the methyl group at the N10 position must be lost during hydrolysis. This reaction does not normally proceed without oxidative catalysis. Like *p*ABA, MTX is rapidly cleared within 24 h of administration (*32*). Total folate could again be assessed using the LC-MS/MS method 2 days after the last dosing of MTX (*21*).

Conclusion. Our LC-MS/MS method for quantification of total RBC folate is sensitive (LOQ of 56.6 nmol/L RBC) and selective due to the use of tandem mass spectrometric detection. It allows for a sample throughput of about 36 samples per 12 h. The LC-MS/MS method showed good correlation with the GC/MS method ($R = 0.992$ and $P < 0.0001$) and with a chemiluminescence assay ($R = 0.747$ and $P < 0.005$). The values determined by GC/MS and LC-MS/MS correlate highly with one another. The case to verify with the chemiluminescence method is less strong. At the same time, it is widely used and works well for plasma/serum but less so for the determination of erythrocyte folate. Therefore, it is appropriate to include a comparison of chemiluminescence and LC-MS/MS methods for folate analysts interested in research and clinical applications. The use of tandem MS detection with two product ions offers unambiguous identification and confirmation. Thus, this LC-MS/MS method is a good candidate reference check method for quantification of RBC folate. Future work will include adaptation to a high-throughput system.

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