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High-Throughput Method for the Quantitation of Total Folate in Whole Blood Using LC-MS/MS

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A high-throughput liquid chromatography tandem mass spectrometry (HT LC-MS/MS) method for red blood cell (RBC) folate analysis was developed from a previously described manual (M) LC-MS/MS method. The HT LC-MS/MS method used 96-well plates in which RBC folates were hydrolyzed with concentrated HCl in the presence of the [$^{13}C_6$]*p*ABA internal standard (IS). The pH of the hydrolysate was adjusted to 5.0 before cleanup using 96-well plate OASIS HLB SPE cartridges. The analyte and IS were eluted with ethyl acetate/hexane (95:5, v/v) and methylated with methanol and trimethylsilyldiazomethane. The methylated analyte and IS were quantified with LC-MS/MS as previously described. The HT LC-MS/MS method was validated by determining the recovery of six different folate vitamers, which were quantitatively recovered (84−105% with CV < 9.0%). RBC folate concentrations in whole blood samples correlated between HT and M LC-MS/MS methods ($r = 0.922$, $p < 0.0001$ for $n = 43$ samples) and between the HT LC-MS/MS method and a chemiluminescence assay ($r = 0.664$, $p < 0.001$ for $n = 325$ samples). Comparison of the results between HT LC-MS/MS and chemiluminescence methods with Bland−Altman difference plots and by ROC curve analysis indicates that the chemiluminescence assay underreports RBC folate concentrations. The HT LC-MS/MS method allows for high-throughput sample preparation for the analysis of RBC folate.

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

INTRODUCTION

An inadequate folate nutritional status is associated with vascular disease (*1*), hypertension in women (*2*), dementia of Alzheimer's type (*3*), hyperhomocysteinemia, certain types of cancer (*4*), and increased risk of neural tube defects (NTDs) (*5*, *6*). To increase the folate intake of women of child-bearing age, to lower the incidence of NTDs, the FDA mandated that by 1998, most enriched grain and cereal products in the U.S. be fortified with folic acid at a level of 140 μg/100 g (*7*). Folate status is best reflected by the red blood cell (RBC) concentration because of the correlation with the liver, a significant tissue store (*8*). The post-fortification reference ranges (fifth to 95th percentiles) of RBC folate for the U.S. population were 347−1167 nmol/L, and a recent paper (*9*) documenting the rise in RBC folate levels post-fortification revealed an increase of 204−272 nmol/L across all age groups sampled in the National Health and Nutrition Examination Survey 1999−2000 (NHANES). The prevalence of low RBC folate concentrations (<317 nmol/L) (*10*) decreased from 31 to 3% in the adult U.S. population and from 38 to 5% for women of child-bearing age, thus illustrating the effectiveness of fortification on improving folate nutritional status in the general population. However, there remain differences among ethnic groups surveyed for the NHANES: 2% of Mexican-American, 4% of non-Hispanic white, and 11% of non-Hispanic black women of child-bearing age had RBC folate levels of <317 nmol/L (*9*).

The recent studies that revealed assay dependence for the quantitation of RBC folate (*11*−*13*) prompted development of new analytical methods, including liquid chromatography coupled to mass spectrometric and tandem mass spectrometric detection (LC-MS and LC-MS/MS) for plasma and serum folate (*14*−*19*) and for whole blood folate (*20*−*23*). A newly developed method (*20*) showed good agreement with microbiological assay results for whole blood and an ability to speciate folate forms in a 96-well plate platform to enhance throughput (*21*). However, this method relied on biological deconjugation; consequently, it could be affected by the presence of chemotherapeutic drugs such as methotrexate.

A recently described manual (M) LC-MS/MS method (*22*) for the analysis of total folate in whole blood relied on a hydrolysis step to form the folate catabolite *para*-aminobenzoic acid (*p*ABA). This LC-MS/MS method is sensitive, selective, and has an easy sample preparation.

This paper describes the adaptation of the M LC-MS/MS method for the analysis of total folate in whole blood to a high-throughput (HT) method using 96-well plates, increasing the

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sample preparation throughput from 36 samples to potentially 192 samples per day. The HT LC-MS/MS method described here uses acid hydrolysis to form *p*ABA with hydrolysate cleanup and phase partitioning in a single step through a 96-well plate OASIS HLB SPE cartridge. The method was validated by determining recoveries of six folate vitamers added to whole blood. The HT LC-MS/MS and M LC-MS/MS methods were compared using whole blood specimens from 43 normal adult volunteers. Also, the HT LC-MS/MS method and a chemiluminescence assay were compared using whole blood samples from 325 normal adult volunteers.

MATERIALS AND METHODS

Chemicals and Reagents. Chemicals and reagents obtained for the sample preparation have been previously described (*22*). Additional chemicals and reagents included dihydrofolic acid (DHF; CAS no. 207226-40-2) from Sigma (St. Louis, MO), and tetrahydrofolate (THF; CAS no. 71963-69-4) calcium salt [H_4PteGlu-Ca], 5,10-methenyltetrahydrofolate (5,10-CH=THF; CAS no. 7444-29-3) calcium salt [(6R,S)-5,10-CH=H_4PteGlu-Ca] from Schircks Laboratories (Jona, Switzerland). The citric acid buffer was prepared from citric acid monohydrate (CAS no. 5949-29-1) dissolved in water before its pH was adjusted to 5.0 with 50% sodium hydroxide in water (w/w).

Preparation of Standards. Folate vitamer standards, including 5-methyltetrahydrofolate (5MTHF), 5-formyltetrahydrofolate (5FTHF), folic acid (FA), DHF, THF, and 5,10-CH=THF were prepared in 20 mM phosphate buffer system (pH 7.2), while *p*ABA standards were prepared in methanol. UV−vis spectrophotometry was used to confirm gravimetric concentrations before the addition of ascorbic acid. Dilute aliquots were prepared as needed in the same buffer system and with 1% ascorbic acid (w/w). [$^{13}C_6$]*p*ABA was utilized as an internal standard (IS), prepared in 2-propanol at 28 μmol/L.

CH_3-*p*ABA calibration standards were prepared by treating 1 mL of working stock solution (365 μmol/L of *p*ABA in methanol) with 100 μL of trimethylsilyldiazomethane as previously described (*22*). After reacting at room temperature for 30 min, the working stock solution was evaporated to dryness at 50 °C under argon. The standard was resuspended in 1 mL of acetonitrile, and then a second working stock solution (14.6 μmol/L of CH_3-*p*ABA) was prepared from this 365 μmol/L working stock solution described previously to prepare the CH_3-*p*ABA standards (prepared in acetonitrile) ranging from 0.15 to 7.3 μmol/L. A constant volume of the derivatized IS (prepared like the working standards described previously) was added to each calibration standard to provide ratios of analyte to IS ranging from 0.05 to 2.5 after derivatization.

Additionally, aqueous calibration standards of underivatized *p*ABA with added underivatized IS were processed with whole blood samples through all the steps in the method (hydrolysis, SPE cleanup, and derivatization) before LC-MS/MS analysis to determine the calibration by accounting for losses of *p*ABA through the protocol. Stock solutions and calibrators were stored at −20 °C when not in use.

Sample Collection. The study was approved by the University of California, Davis IRB, and blood samples were collected from normal adult volunteers as previously described (*22*).

Folate Analysis by Chemiluminescence and M LC-MS/MS. A chemiluminescence assay was utilized to determine concentrations of RBC folate in whole blood as previously described (*22*). An aliquot of freshly thawed whole blood was added to an ascorbic acid kit reagent before analysis. The RBC folate results were reported as ng of folate/mL of whole blood/packed cell volume (PCV).

Samples were analyzed by the M LC-MS/MS method as previously described (*22*). Briefly, in the presence of [$^{13}C_6$]*p*ABA IS, whole blood (100 μL) was mixed 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 pH adjustment, derivatized with trimethylsilyldiazomethane, and then analyzed by LC-MS/MS.

HT LC-MS/MS. Sample Preparation. Samples were hydrolyzed in a 1.2 mL borosilicate glass removable insert tube in an aluminum 96-well base plate (Hirschmann Inc., Louisville, KY). Ten μL of [$^{13}C_6$]-*p*ABA IS (28 μmol/L) was added, followed by 100 μL of whole blood and 100 μL of 12.1 N HCl. The headspace of the tubes was flushed with argon using a high-throughput concentrator/evaporator for 96-well plates (Glas-Col, Terre Haute, IN), and the tubes were covered with a silicon 96-well cover mat (Hirschmann Inc., Louisville, KY) that was weighted down with a 2 kg ring-stand base to prevent the cover mat from opening during heating. The samples were heated in the aluminum base plate atop a dry block heater for 4 h at 110 °C and then allowed to cool to 23 °C. The hydrolysate was treated with 500 μL of a solution that was 85% of 1.25 M citric acid buffer (pH 5.0) and 15% of a 50% (w/w) sodium hydroxide solution. The diluted hydrolysate was loaded onto a 60 mg OASIS HLB SPE 96-well plate (Waters Corporation, Milford, MA) that had been conditioned with 1 mL each of methanol and water before addition of the hydrolysate. Vacuum was applied for 5 min after loading the hydrolysates to remove residual water from the sorbent bed. The *p*ABA analyte and IS were eluted with 1 mL of ethyl acetate/hexane (95:5, v/v) and collected into a 96-well plate containing removable 1.2 mL borosilicate glass inserts (Hirschmann Inc., Louisville, KY). The top layer (800 μL of ethyl acetate) was clearly distinguishable from the bottom layer, and this facilitated maximum removal of the top layer (to maximize the absolute recovery of the IS) with a multi-channel pipettor. The top ethyl acetate layer was transferred to a clean 1.2 mL borosilicate glass insert tube containing 100 μL of methanol and 20 μL of trimethylsilyldiazomethane. The tube (with mix) was allowed to react for 30 min at 23 °C and then slowly evaporated to dryness using argon and heating to 50 °C in a high-throughput concentrator/evaporator for 96-well plates (Glas-Col, Terre Haute, IN). The residue was resuspended in a 100 μL aliquot of acetonitrile by pipet mixing and then transferred to small volume microtiter autosampler plates to fit a two-plate autosampler tray (Perkin-Elmer, Shelton, CT). The samples were stored at −20 °C until analysis.

Instrument Conditions. A Perkin-Elmer Series 200 LC system (Perkin-Elmer) was combined with a Sciex API 2000 triple-quadrupole MS system (Perkin-Elmer) controlled by Analyst 1.3.1 software (Applied Biosystems, Foster City, CA) for quantitation of CH_3-*p*ABA as previously described (*22*). Additionally, the Perkin-Elmer LC autosampler was equipped with a low volume two-plate microtiter autosampler tray to hold two 96-well plates. The samples were separated using a chromatographic system and mixed with a post-column reagent before ionization as previously described (*22*). Briefly, a Synergi 4 μ Hydro-RP column (150 mm × 4.6 mm i.d.; Phenomenex, Torrance, CA) with an isocratic mobile phase of acetonitrile/water (70:30, v/v) at 0.5 mL/min was utilized for analyte separation. A post-column reagent of 0.025% formic acid in acetonitrile was added at 0.2 mL/min prior to entry into the source.

Optimized settings for the sample ionization by positive ion atmospheric pressure chemical ionization (APCI) were described (*22*), and the multiple reaction monitoring (MRM) mode was utilized for data acquisition. Briefly, these settings include a temperature of 425 °C, resolution settings of unit and low for first and third quadrupoles, respectively, and a dwell time of 400 ms. The nebulizing current was set to 4 kV, and the curtain gas, gas 1, gas 2, and collision induced dissociation gases were set at 20, 80, 60, and 10 psi, respectively. The declustering potential, focusing potential, collision energy, and collision cell exit potential were set as before (*22*), and the respective settings were as follows: 20, 400, 10, 29, and 5 V. Two transitions (*m*/*z* 152 to 120 and *m*/*z* 153 to 93) were monitored for *p*ABA as well as a transition (*m*/*z* 158 to 99) for the IS.

A six-point calibration curve was prepared by plotting the ratio of the analyte response (*m*/*z* 152 to 93 transition) to the IS (*m*/*z* 158 to 99 transition) against the amount of analyte injected using 1/*x* second-order regression. Calibration curves were prepared in triplicate and were run before, during, and at the end of each set of samples. Additionally, different calibration standards were analyzed after sets of five or six samples and were included in the final calibration curve. Finally, folate concentrations from hydrolyzed samples of whole blood were normalized by PCV and expressed as nmol of folate/L of RBCs.

Method Validation. The method was validated, and recoveries were calculated as described previously (*22*) with a few modifications. Briefly, six different folate vitamers (5MTHF, 5FTHF, FA, DHF, THF,

Table 1. Validation Study Results of Fortifications with Six Folate Vitamers Added to Whole Blood (1070 ± 70 nmol/L)[a]

folate form added	replications	amount added (nmol/L)	mean recovery (%)	CV (%)
5-methyltetrahydrofolate	5	199	95	4.6
	5	523	94	2.2
5-formyltetrahydrofolate	5	199	100	7.2
	5	487	87	6.8
folic acid	5	199	94	3.9
	5	510	84	6.5
tetrahydrofolate	5	205	92	5.9
	5	516	85	6.0
dihydrofolate	3	212	91	6.8
	3	529	87	6.3
5,10-methenyltetrahydrofolate	5	450	97	6.6
	4	1130	105	9.0

[a] Spike levels, mean percent recovery, and coefficient of variation (%) are provided for each level. Recovery of folates added to whole blood (1070 ± 70 nmol/L before additions).

Table 2. Within- and Between-Day Variability (CV, %) for Calibration Standards, Pooled Control Whole Blood Samples, and 5MTHF Spike (1830 nmol/L) That Were Included in Each Batch of Whole Blood Samples Collected from Adult Volunteers

	within-day variability (n = 1 day)	between-day variability (n = 8 days)
1 μg/mL	1.1 (n = 3)	7.1 (n = 21)
0.5 μg/mL	1.1 (n = 3)	4.3 (n = 27)
0.25 μg/mL	2.0 (n = 3)	5.2 (n = 22)
0.1 μg/mL	3.5 (n = 3)	7.7 (n = 23)
0.05 μg/mL	2.0 (n = 3)	7.0 (n = 21)
0.02 μg/mL	4.7 (n = 3)	8.4 (n = 15)
pooled control sample of whole blood (1070 nmol/L)	6.5 (n = 5)	11 (n = 27)
5MTHF spike (1830 nmol/L)	3.8 (n = 5)	8.0 (n = 20)

and 5,10-CH═THF) were individually added to a pooled control sample of whole blood at two different concentrations (**Table 1**) with a minimum of three replicates to validate the method. Recoveries of the spiked folate vitamers were determined by subtracting the mean concentration of exogenous folates in the control sample from the spiked sample and normalized by the concentration of spike added. Within-day and between-day variabilities were calculated for all calibration standards, pooled control whole blood samples, and 5MTHF spike (1830 nmol/L) that was included with all sets of whole blood samples (**Table 2**).

Additionally, samples from 43 normal adult volunteers were analyzed using the M LC-MS/MS method and the HT LC-MS/MS described here, and the agreement between the methods was determined. Whole blood samples from a large population (n = 325) of normal adults analyzed by both chemiluminescence and HT LC-MS/MS were compared using receiver operating curve (ROC) analysis as described by previous investigators comparing folate methods (*24*).

Calculations and Data Analysis. Regression analyses were conducted using the software StatView (SAS Institute, Cary, NC). Agreement between methods was determined using Bland−Altman difference plots (*25*). ROC curve analyses were completed using an on-line calculator (*26*), and the statistical analyses to determine the significance of the difference between methods were completed (*27*). ROC plots enable the predictive effectiveness of methods or assays to be evaluated (*28*). These three different modes of method comparison, which included regression analyses, Bland−Altman difference plots, and ROC curve analyses, were completed to assess the following: (i) how well the methods compared (regression analyses); (ii) the agreement between the two methods (Bland−Altman difference plots); and (iii) which method was more effective at predicting the low RBC folate status (ROC curve analyses).

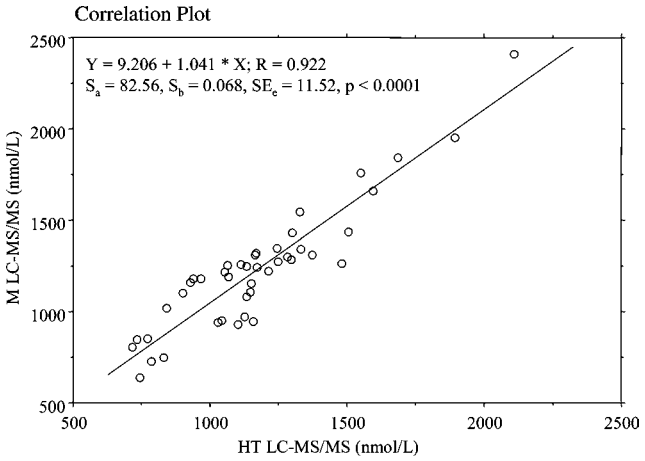

Figure 1. Plot of RBC folate results of high-throughput LC-MS/MS vs manual LC-MS/MS of samples and quality control samples. S_a, S_b, and S_e represent the standard errors of intercept, slope, and estimate, respectively.

RESULTS AND DISCUSSION

Method Validation. Recovery of folates added to pooled control whole blood (1070 ± 70 nmol/L before additions) is summarized in **Table 1**. Folate vitamers were spiked at concentrations of ∼500 and ∼200 nmol/L. Recoveries for the folate vitamers ranged from 84 to 94% when the addition level was ∼500 nmol/L and from 91 to 100% when the addition level was ∼200 nmol/L. The 5,10-CH═THF had recoveries of 105% at an addition of 1130 nmol/L and a recovery of 97% at an addition of 450 nmol/L.

Recovery of added FA was 84% (at 510 nmol/L) and 94% (at 199 nmol/L) in the present report, as compared to recoveries of 66% (*22*) and 76% (*29*) reported previously. Because the reduction of FA to THF is a facile process, the key to increasing recovery of the added FA was the addition of ascorbic acid to facilitate the reduction of FA to THF.

The within-day variability was <5%, and between-day variability was <9% (**Table 2**). Within-day variability for pooled control whole blood samples and 5MTHF spikes (1830 nmol/L) was 6.5 and 3.8%, respectively. Between-day variability for the control and spiked samples was ≤11%.

Analyte Losses through the Method. Samples of pooled control whole blood were spiked with *p*ABA at each step of the method so as to determine the absolute recoveries through each step of the protocol. Thirteen percent of the *p*ABA spike was lost during hydrolysis, 17% during SPE cleanup, and 0% during derivatization and drying. As the *p*ABA was not well-retained on the SPE, the water/methanol (95:5, v/v) washing step (recommended by the manufacturer prior to elution) was omitted. Overall losses of *p*ABA totaled 30% with an absolute mean recovery of 70% (n = 2). Losses were consistent over a concentration range of 0.3−6 μmol/L calibrators, demonstrating that the IS method of calibration was appropriate. Differences in slopes between external calibration standards (that were added just prior to analysis) versus the aqueous calibration standards (that were processed through the method) were not significant (p = 0.745), and the same was true for the zero intercepts (p = 0.902).

Comparison of HT LC-MS/MS and M LC-MS/MS Methods. RBC folate concentrations as determined by the M LC-MS/MS method and the HT LC-MS/MS method compare well (**Figure 1**) for the 43 whole blood samples that were analyzed (p < 0.0001). The excellent agreement between these methods

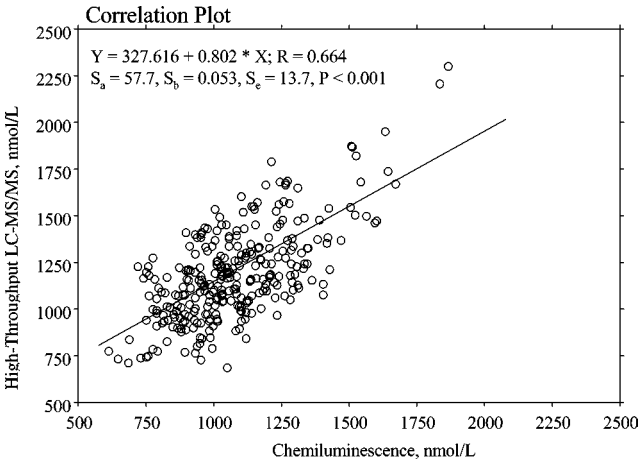

Figure 2. Relationship of a chemiluminescence assay to a high-throughput LC-MS/MS analytical method for the analysis of RBC folate. S_a, S_b, and S_e refer to the standard error of the intercept, slope, and estimate, respectively.

is demonstrated by the zero intercept, slope of 1, and a correlation value of 0.922 ($p < 0.0001$).

Comparison of HT LC-MS/MS and Chemiluminescence Assay. Whole blood samples were analyzed for RBC folate concentration by both an HT LC-MS/MS method and a chemiluminescence assay for 325 subjects. The correlation between the two methods was low ($r = 0.664$) but strong ($p < 0.001$). The regression line had a slope of 0.802 ± 0.053 (mean $\pm$ SE) and an intercept of 327.6 ± 57.7 (**Figure 2**). The mean RBC folate value was 1108 ± 13.7 nmol/L (mean $\pm$ SE) and ranged from 612 to 2075 nmol/L as determined by the chemiluminescence assay. The mean RBC folate by HT LC-MS/MS was 1178 ± 14.1 nmol/L and ranged from 524 to 2301 nmol/L.

The present results are in excellent agreement with those of our previous study (*22*), in which the M LC-MS/MS method was compared with the popular chemiluminescence assay for RBC folate in blood from only 15 subjects. In that study, the correlation between the M LC-MS/MS method and the chemiluminescence assay was also low ($r = 0.747$) but strong ($p < 0.005$). Furthermore, in our previous study (*22*), we also compared the M LC-MS/MS with a GC/MS method for RBC folate. We found that the correlation between the M LC-MS/MS and the GC/MS method was both high ($r = 0.992$) and strong ($p < 0.0001$). In another study (*13*), the GC/MS method was compared with the popular chemiluminescence assay for RBC folate in blood from 24 pregnant and 29 non-pregnant women before and after each received a folic acid supplement (1814 nmol/day) for 30−60 days. The correlation between the GC/MS and chemiluminescence methods was low ($r = 0.4679$) but strong ($p < 0.0001$). So, in our hands, the correlations between HT LC-MS/MS and M LC-MS/MS and between M LC-MS/MS and GC/MS were each high ($r \geq 0.922$) and strong ($p < 0.0001$). The correlations between HT LC-MS/MS and chemiluminescence, M LC-MS/MS and chemiluminescence, and GC/MS and chemiluminescence were all low ($r \leq 0.747$) but strong ($p \leq 0.005$). Furthermore, the correlation between chemiluminescence and radioassay (two competitive binding assays) was low ($r = 0.7299$) and strong ($p < 0.0001$). Finally, the rise in RBC folate following supplementation was the same when measured by GC/MS and *Lactobacillus casei*, but this was not the case when measured by chemiluminescence and radioassay (*13*).

In the present study, the RBC folate concentrations determined by chemiluminescence assay were ~10% lower than the values determined by HT LC-MS/MS. Similar results were already reported for serum folate concentrations (*15*). While the chemiluminescence assay is popular for clinical purposes, it may be hormone sensitive (*13*), and it may be affected by the presence of antifolates that bind to the folate-binding protein contained in these kits. The HT LC-MS/MS, M LC-MS/MS, and GC/MS methods are not subject to these limitations (*22*), and because these assays measure the *p*ABA moiety of the folate, they can be accurate for samples stored for a long time (*23*) as was determined for a storage period of >100 days (*22*).

The agreement between HT LC-MS/MS method and chemiluminescence assay was determined through use of the Bland−Altman difference plots (*25*). From this analysis, it was determined that the relative mean difference between these methods (~10%) was significant ($p < 0.001$). The limits of agreement (mean difference $\pm$ 2 SD, the range into which 95% of all chemiluminescence assay values fell) were 517 nmol/L lower and 288 nmol/L higher than the lower and upper limits, respectively, of results obtained by HT LC-MS/MS (*25*). So, the chemiluminescence assay tends to underreport RBC folate as compared to the HT LC-MS/MS method.

The basis for the ROC analysis was the premise that the HT LC-MS/MS method gave the correct result (definitive standard). This was assumed because of the agreement and high correlations of sample folate concentrations as determined between this method and the M LC-MS/MS method ($r = 0.922$), which also agreed well with the GC/MS method ($r = 0.992$) (*22*). Second, the HT LC-MS/MS method was validated by determining the recovery of six folate vitamers, all of which were recovered (≥84%) with high precision (CV < 9.0%), and the method had low within-day (≤6.5%) and between-day variabilities (≤11%). Assuming that the HT LC-MS/MS method provided accurate RBC folate values, the area under the ROC curve (AUC) for the HT LC-MS/MS method was 0.964 when the RBC folate cutoff level was 800 nmol/L (*26*). However, when a chemiluminescence assay was assumed to provide accurate RBC folate values, the AUC was only 0.788 at an RBC folate cutoff level of 800 nmol/L (*26*). This difference (0.964 vs 0.788) was significant ($z_{crit} = 3.41$, $p < 0.01$) (*27*). The HT LC-MS/MS method was a sensitive (85.3%) and specific (98.3%) assay, meaning that it was capable of identifying positive cases of RBC folate levels from 56.6 nmol/L (the LOQ as previously reported (*22*)) to ≤800 nmol/L (sensitivity) and at identifying individuals in the population with RBC folate >800 nmol/L (specificity), while the chemiluminescence method had low sensitivity (33.3%) and high specificity (98.3%). Thus, the chemiluminescence assay incorrectly identifies more individuals as having low RBC folate values. This finding confirms a previous observation that the chemiluminescence assay underestimates RBC folate (*30*).

The results of these modes of method comparisons, including regression analyses, Bland−Altman difference plots, and ROC curve analyses, indicate that the HT LC-MS/MS and chemiluminescence assay are comparable but not equal. The methods compare ($r = 0.664$, $p < 0.001$ for the strength of comparison), but the relative difference between these methods (~10%) is significant ($p < 0.01$). The chemiluminescence method tends to underreport RBC folate concentrations as was determined by the Bland−Altman difference plots. Because there is a significant difference between these methods, ROC curve

analyses were conducted to determine which method was more effective at predicting low RBC folate. Because of both high sensitivity (85.3%) and specificity (98.3%) and a statistically higher AUC value (0.964), the HT LC-MS/MS method is a better tool for predicting low RBC folate status.

Conclusion. The HT LC-MS/MS method for quantitation of total RBC folate is instrumentally sensitive and selective. It also has a high-throughput of samples in two 96-well plates in 8 h of preparation followed by 21 h of automated instrument analysis. Results from the HT LC-MS/MS correlated well with the M LC-MS/MS method ($r = 0.922$, $p < 0.0001$), which compared well to a GC/MS method. The HT LC-MS/MS method is accurate and precise as determined by high folate recovery ($\geq 84\%$) and low CV values ($<9.0\%$). It not only compares to an established clinical chemiluminescence assay ($r = 0.664$, $p < 0.01$), it is also a better predictive tool than the chemiluminescence assay (ROC curve analysis, $p < 0.01$). Additional work will include comparison of the HT LC-MS/MS method of measuring RBC folate concentrations with folate intake assessments as determined by the Block dietary folate equivalents screener and a longer Block food frequency questionnaire. The relation between folate intake and RBC folate is of interest to monitor folate intake and to identify suboptimal folate status.

SAFETY

The HT LC-MS/MS method requires hydrolysis of the whole blood samples with concentrated hydrochloric acid (100 μL), which should only be handled in a proper fume hood. The pH of the citric acid buffer was adjusted by 50% sodium hydroxide, and the beaker containing the buffer should be kept on ice. The derivatizing reagent trimethylsilyldiazomethane (CAS no. 18107-18-1) should be handled in a proper fume hood only.

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