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Folate forms in red blood cell lysates and conventionally prepared whole blood lysates appear stable for up to 2 years at −70°C and show comparable concentrations

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Abstract

Background: The use of red blood cell lysates (RBC-Lys) eliminates the need for serum folate and hematocrit measurement to calculate RBC folate. Information on the long-term frozen storage stability of RBC-Lys is missing.

Objective: We aimed to assess the comparability of RBC folate forms in whole blood lysates (WB-Lys) and RBC-Lys and the folate stability in both matrices.

Methods: We prepared conventional WB-Lys (1/11 dilution with 1% ascorbic acid) and RBC-Lys (1/11 dilution of washed and saline diluted RBCs with 1% ascorbic acid) from EDTA blood (n=60 donors) and stored lysates at −70°C until analysis at baseline (1 wk), 3, 6, 12, and 24 mo. Prior to analysis by HPLC-MS/MS, we incubated the WB-Lys (4 h at 37°C) and treated the RBC-Lys with human recombinant γ-glutamyl hydrolase (~30 min at room temperature) for folate polyglutamate deconjugation. We analyzed RBC-Lys samples for hemoglobin (same aliquot) to normalize for the pre-analytical dilution; hemoglobin-folate was converted to RBC folate for each folate form using the mean corpuscular hemoglobin concentration. We analyzed folate forms and hematocrit in matching serum samples for traditional RBC folate calculation.

Results: At baseline, results for individual RBC folate forms derived from WB-Lys *vs* RBC-Lys samples showed excellent correlation (Pearson $r \geq 0.97$). Concentrations (mean±SD, nmol/L) compared well for total folate (886±255 *vs* 899±271), 5-methyltetrahydrofolate (831±258 *vs* 843±276), and non-methyl folate (53.3±74.4 *vs* 52.9±70.7), but were 17% higher in RBC-Lys for MeFox (147±44.1 *vs* 172±53.5). Frozen storage of WB-Lys and RBC-Lys samples for ≤24 mo showed ≤5% change in total folate and 5-methyltetrahydrofolate, ≤13% change in non-methyl folate, and ≤11% change in MeFox.

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Conclusion: Erythrocyte folate forms appear to be stable in RBC-Lys samples stored frozen at −70°C for ≤2 y. The relatively small changes in folate concentrations over time were comparable between RBC-Lys and conventionally prepared WB-Lys samples.

Keywords

Polyglutamates; deconjugation; exogenous γ-glutamyl hydrolase; RBC folate; mean corpuscular hemoglobin concentration (MCHC)

Introduction

Red blood cell (RBC) folate measurement is an important diagnostic tool and a marker of long-term folate status (1). Traditional RBC folate determination is complex because it requires whole blood folate, serum folate, and hematocrit (Hct) measurements to calculate RBC folate. Furthermore, the folate polyglutamyl side chain residue needs to be deconjugated by a γ-glutamyl hydrolase (GGH) enzyme (2). Conventionally, slightly acidic whole blood lysates (WB-Lys) are prepared in 1% ascorbic acid to activate the endogenous human GGH present in plasma. For the gold-standard microbiologic assay (MBA), which captures folates with up to 3 glutamate residues, a short 30-min incubation (at room temperature or 37°C) is sufficient to assess total folate (tFOL) (2, 3). On the other hand, the measurement of individual folate forms by HPLC-MS/MS methods requires longer incubation times (typically several hours at room temperature or 37°C) to deconjugate folate polyglutamates to monoglutamates (4-10), which can result in the degradation of labile folate forms. To provide an alternative with a short incubation time, our group recently published an HPLC-MS/MS method for conventionally prepared WB-Lys using a commercially available human recombinant GGH protein as an exogenous enzyme source [exoGGH] (11). This method achieved deconjugation to folate monoglutamates in only 30 min at room temperature.

In order to enable an in-depth and accurate look at RBC folate status, the actual folate forms need to be directly measured in RBCs. That is, because inaccuracies in calculated RBC folate form concentrations from WB-Lys values can occur when the plasma concentration is large, exceeding that of the RBC concentration (11). To correct for any residual moisture that may be part of the packed RBCs, the hemoglobin (Hb) concentration in the same RBC-Lys in which the folate forms are measured needs to be determined (11). The resulting Hb-folate for each folate form can then be easily converted to RBC folate using the mean corpuscular hemoglobin concentration (MCHC).

The NHANES 2019–2020 survey introduced a new laboratory component that directly measures folate forms in RBCs by HPLC-MS/MS to characterize RBC folate status in the US population in addition to the traditional RBC total folate (tFOL) calculated from the WB-Lys values measured by MBA. We conducted the current study to generate comprehensive information on a matrix comparison for folate forms measured in RBC-Lys treated with exoGGH as compared to conventionally prepared WB-Lys for a set of 60 samples from US blood donors. We also assessed the long-term frozen stability of folate forms in these 2 matrices over a period of 2 y. Finally, we assessed how well tFOL measured

by HPLC-MS/MS agreed with tFOL measured by MBA for these 2 matrices. The findings from this study are important to help bridge and interpret past, present, and future data on folate status from NHANES.

Methods

Reagents and materials

Folate monoglutamate calibrators (5-methyltetrahydrofolate [5-methylTHF], folic acid, 5-methylTHF oxidation product [MeFox; pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF], 5-formyltetrahydrofolate [5-formylTHF], tetrahydrofolate [THF], and 5,10-methenyltetrahydrofolate [5,10-methenylTHF]) together with their respective $^{13}C_5$-labeled analogs were purchased from Merck & Cie. Recombinant His-tagged human γ-glutamyl hydrolase protein (exoGGH) was purchased from Novus Biologicals (11). All reagents and solvents were ACS grade and other reagents or materials used were described previously (5, 6,11,12). EDTA whole blood was purchased from a commercial US blood bank (Bioreclamation IVT). Saline (0.9% sodium chloride) was used for RBC washing and subsequent dilution. All sample handling was done under gold-fluorescent light.

Blood processing

We obtained freshly collected EDTA whole blood and matching serum specimens from 60 blood donors. The blood collection tubes were gently mixed for ≥10 min upon arrival. An aliquot from each sample was used to determine the Hct (using the micro-centrifuge capillary tube method) and Hb (using the HemoCue Hb 201^+ system). To prepare conventional WB-Lys, we diluted an aliquot of WB 1/11 with 1% ascorbic acid, thoroughly mixed the contents, and stored samples at −70°C until analysis. The remainder of the blood was centrifuged (1100 *g* at 4°C) for 10 min, the plasma layer was removed, and an equivalent volume of cold saline was added to the RBCs in the blood collection tubes. After gentle mixing of the contents, the tubes were centrifuged again (1100 g at 4°C) for 10 min and the saline layer was discarded. This step was repeated twice to isolate the packed RBCs. Finally, an approximately equal volume of cold saline was added to the washed/packed RBC samples and contents were gently mixed. To prepare RBC-Lys, an aliquot of the saline diluted RBCs (1 part RBCs + 1 part saline) was further diluted 1/11 with 1% ascorbic acid for a final approximate 1/22 dilution of RBCs, which is about the same dilution as in WB-Lys samples. Samples were vortex-mixed for about 5 sec and stored at −70°C until analysis.

Analysis of folate forms by HPLC-MS/MS

Different folate monoglutamates (5-methylTHF, folic acid, MeFox, 5-formylTHF, THF, and 5,10-methenylTHF) in serum, WB-Lys, and RBC-Lys samples were analyzed by HPLC-MS/MS after sample clean-up by SPE according to previously published procedures (1-3)5,6,11,12). Briefly, to deconjugate folate polyglutamates, we incubated WB-Lys aliquots in the presence of an internal standard mixture for 4 h at 37°C (conventional procedure) and treated RBC-Lys aliquots with exoGGH (5 μg enzyme/mL sample added to the internal standard mixture) for 30 min at room temperature, which is the standard time to equilibrate the internal standards with the endogenous folates. We also treated an aliquot of

WB-Lys for each sample with exoGGH (30 min at room temperature; no 4-h incubation at 37°C) to assess whether the presence of added enzyme beyond the endogenous enzyme led to higher folate concentrations compared to the conventional procedure. A freshly prepared 5-point calibration curve (5-methylTHF: 1 to 100 nmol/L; all other folate forms: 0.5 to 50 nmol/L) and 3 serum or WB-Lys quality control (QC) pools in duplicate, bracketing the unknown samples, were included in each experiment. All unknown samples were prepared in duplicate over a period of 2 d for a total of 4 results per sample.

Analysis of tFOL by MBA

To allow a comparison between tFOL measured by HPLC-MS/MS and MBA, we used matching aliquots of serum, conventionally prepared WB-Lys, and RBC-Lys treated with exoGGH at the baseline condition. We prepared each sample for the MBA in duplicate over a period of 2 d for a total of 4 results per sample. We did not incubate the WB-Lys samples prior to the MBA analysis and we treated the RBC-Lys samples with exoGGH in 0.1% ascorbic acid. The samples for the MBA were processed in parallel on the same day as the samples for the HPLC-MS/MS analysis.

Analysis of Hb in RBC-Lys samples

To normalize for the residual saline remaining in the RBC pellet from the washing and for the approximate pre-analytical dilution, we measured Hb in the same sample as folate and calculated Hb-folate (folate divided by Hb). We used a modification of a published sodium lauryl sulfate procedure (13,14) that we validated for a 96-well microplate to measure Hb-SLS in RBC-Lys samples. We measured each baseline sample in duplicate over 2 d for a total of 4 results per sample.

Storage stability of frozen blood samples

To assess long-term frozen storage stability of folate forms, we stored multiple aliquots of WB-Lys and RBC-Lys samples for the same 60 samples at −70°C and analyzed an aliquot each at baseline (1 wk frozen storage at −70°C), 3, 6, 12, and 24 mo by HPLC-MS/MS.

Additionally, we assessed long-term frozen storage stability of folate forms in 2 WB-Lys (prepared in 2011) and RBC-Lys (prepared in 2018) QC pools each. At the time of pool preparation, the materials were spiked with the minor folate forms 5-formylTHF, folic acid, and 5,10-menthenylTHF to obtain moderate folate concentrations. Materials were stored frozen at −70°C and analyzed periodically as part of our internal quality assurance process. Towards the end of 2020, we analyzed these pools in replication in 1 analytical run (WB-Lys pool: 8 replicates; RBC-Lys pool: 3 replicates) and compared the results to the initial pool limits.

Calculation of RBC folate concentrations

Folate results with a concentration below the limit of detection were used in calculations as imputed values defined as limit of detection divided by square root of 2. Serum tFOL by HPLC-MS/MS was calculated as the sum of 5-methylTHF, folic acid, 5-formylTHF, THF, and 5,10-methenylTHF (using the average of the 4 replicates and excluding MeFox), which was used to compare to the serum tFOL measured by MBA. We used different calculations

to determine the concentrations of RBC folate forms in the 2 matrices, as described below, and then calculated RBC tFOL as the sum of individual folate forms, both including and excluding MeFox.

For conventionally prepared WB-Lys samples, we calculated the RBC concentration for each folate form from the measured WB-Lys concentration (average of 4 replicates) multiplied by the dilution factor, corrected for the serum contribution (average of 4 replicates), and normalized to the Hct, according to the following formula: RBC folate form A = [WB-Lys folate form A*11–serum folate form A*(1–Hct)]/Hct. Folate was expressed in nmol/L and Hct as a fraction (e.g., 0.4).

For RBC-Lys samples, we calculated the RBC concentration for each folate form from the measured RBC-Lys concentration (average of 4 replicates) divided by the Hb-SLS concentration (average of 4 replicates) measured in the same aliquot, and multiplied by the MCHC (calculated as measured Hb [in g/L] divided by measured Hct [as a fraction] in freshly collected EDTA blood), according to the following formula: RBC folate form A = RBC-Lys folate form A/Hb-SLS*MCHC. Folate was expressed in nmol/L, Hb-SLS in g/L, and MCHC as g/L.

Statistical analysis

All statistical analyses were conducted using Analyse-it (version 4.96.2), a software plug-in for Microsoft Excel. We calculated the mean±SD and median (IQR) concentrations for each folate form by HPLC-MS/MS and for tFOL by HPLC-MS/MS and MBA. To compare the 2 methods, we reviewed the Pearson correlation and Lin's concordance for each sample matrix. We used Bland-Altman analysis to assess for non-constant difference and non-constant variance and to calculate the limits of agreement (mean difference ± 2SD). Because we observed a slight non-constant difference for serum and more pronounced non-constant difference the 2 blood-based sample matrices which was normalized through log transformation, we interpreted the relative Bland-Altman plots and conducted weighted Deming regression analysis. We calculated the mean relative difference (95% CI) between HPLC-MS/MS and MBA. To assess long-term frozen storage stability of folate forms, we calculated the mean relative change (95% CI) for each storage time point compared to the baseline. We also visualized the agreement between each storage time point and the baseline with scatter plots that showed the weighted Deming regression line and the line of identity. Lastly, we stratified the data based on samples with low *vs* high concentrations of non-methyl folate and evaluated changes in minor folate forms (particularly THF) during long-term frozen storage.

Results

Comparability of serum and RBC tFOL by HPLC-MS/MS vs MBA.

The mean (range) serum tFOL concentration by HPLC-MS/MS excluding MeFox (representing the sum of biologically active folate forms) showed excellent agreement with the MBA: 28.6 (8.68 to 70.7) *vs* 28.8 (9.18 to 72.5) nmol/L (Table 1). The results were highly correlated (Pearson $r = 0.99$), showed excellent concordance (Lin's *rho* = 0.99), and

the weighted Deming regression line was nearly superimposed on the line of identity (slope [95% CI]: 0.992 [0.964 to 1.02]; intercept [95% CI]: 0.064 [−0.542 to 0.669]) (Figure 1, **panel A**). The relative difference (95% CI) showed a negligible bias of −0.40% (−1.83% to −1.03%) and the relative Bland-Altman plot revealed a constant variance and constant difference (Figure 1, **panel B**). As expected, serum tFOL concentrations by HPLC-MS/MS including MeFox were higher compared with the MBA, on average by 15.4% (Table 1).

While the correlation and concordance between the 2 methods was also high for RBC tFOL concentrations excluding MeFox for WB-Lys ($r = 0.96$; *rho* = 0.75) or RBC-Lys ($r = 0.97$; *rho* = 0.86) samples, we found less agreement (Table 1). Concentrations for WB-Lys samples showed a relative difference (95% CI) of −18.6% (−20.3% to −16.9%) for HPLC-MS/MS (mean [range]: 886 [412 to 1605] nmol/L) *vs* MBA (1089 [469 to 1966] nmol/L). Concentrations for RBC-Lys samples showed a relative difference of −13.5% (−15.2% to −11.8%) for HPLC-MS/MS (899 [419 to 1668] nmol/L) *vs* MBA (1037 [478 to 1849] nmol/L). While the weighted Deming regression slopes (95% CI) were significantly different from 1 (WB-Lys: 0.824 [0.767 to 0.882]; RBC-Lys: 0.901 [0.845 to 0.958]), intercepts (95% CI) were not significantly different from 0 (WB-Lys: −12.8 [−71.5 to 45.9]; RBC-Lys −36.5 [−91.2 to 18.1]) (Figure 1, **panels C and E** and Table 1). The relative Bland-Altman plot revealed a constant variance and constant difference (Figure 1, **panels D and F**).

RBC folate forms by HPLC-MS/MS for conventionally treated WB-Lys, WB-Lys treated with exoGGH, and RBC-Lys treated with exoGGH.

Using baseline samples, we assessed whether the presence of added enzyme beyond the endogenous GGH led to higher folate concentrations in WB-Lys treated with exoGGH (30 min incubation at room temperature) compared with the conventional procedure (4 h incubation at 37°C) and compared with treating RBC-Lys with exoGGH (30 min incubation at room temperature) (Table 2). For 5-methylTHF and tFOL excluding MeFox, WB-Lys samples treated with exoGGH produced higher RBC folate concentrations compared with conventionally treated WB-Lys samples (relative difference [95% CI]: 4.4% [3.7% to 5.1%] for 5-methylTHF and 4.2% [3.4% to 4.9%] for tFOL) and compared with RBC-Lys samples treated with exoGGH (3.1% [1.6% to 4.5%] for 5-methylTHF and 2.7% [1.3% to 4.2%] for tFOL). For non-methyl folate, we found no difference between WB-Lys samples treated with exoGGH compared with conventionally treated WB-Lys samples (relative difference [95% CI]: −1.5% [−3.4% to 0.4%]) and compared with RBC-Lys samples treated with exoGGH (−4.0% [−8.2% to 2.2%]). Lastly, for MeFox, WB-Lys samples treated with exoGGH produced higher RBC folate concentrations compared with conventionally treated WB-Lys samples (relative difference [95% CI]: 12.2% [10.6% to 13.8%]), but lower concentrations compared with RBC-Lys samples treated with exoGGH (−4.2% [−5.5% to −2.8%]).

Long-term frozen stability of RBC folate forms in WB-Lys and RBC-Lys samples.

Baseline concentrations of most RBC folate forms were similar between RBC-Lys and WB-Lys (reference) samples (Table 2). The relative difference (95% CI) was small and non-significant: 1.22% (−0.58% to 3.02%) for 5-methylTHF, 2.27% (−1.91% to 6.44%) for

non-methyl folate, and 1.37% (−0.43% to 3.18%) for tFOL excluding MeFox. This was supported by mostly non-significant weighted Deming regression slopes [95% CI] (1.063 [1.006 to 1.12] for 5-methylTHF, 1.029 [0.956 to 1.102] for non-methyl folate, and 1.044 [0.986 to 1.102] for tFOL excluding MeFox) and intercepts [95% CI] (−40.0 [−80.7 to 0.59] for 5-methylTHF, −0.51 [−2.38 to 1.37] for non-methyl folate, and −26.6 [−71.9 to 18.6] for tFOL excluding MeFox). However, baseline MeFox concentrations were 16.8% (14.8% to 18.8%) higher in RBC-Lys compared with WB-Lys samples (slope [95% CI] of 1.197 [1.126 to 1.268]; intercept [95% CI] of −4.31 [−13.8 to 5.18]). The relative differences between WB-Lys and RBC-Lys folate forms remained similar regardless of the storage time point (within ±5% for 5-methylTHF, non-methyl folate [except for the aberrant 3-mo time point], and tFOL excluding MeFox, and within ~10-20% for MeFox) (Table 2). The similarity between the 2 sample matrices across storage time points was supported by weighted Deming regression lines with non-significant slopes and mostly non-significant intercepts for 5-methylTHF and tFOL excluding MeFox, slopes slightly lower than 1 or non-significant and mostly non-significant intercepts for non-methyl folate, and slopes slightly higher than 1 and non-significant intercepts for MeFox (data not shown).

Next, we evaluated changes compared to baseline in RBC folate forms from WB-Lys and RBC-Lys samples stored frozen at −70°C for up to 24 mo (Table 2). We observed small (±5%), but mostly significant relative changes in both sample matrices for 5-methylTHF and tFOL excluding MeFox. For non-methyl folate, we observed no change in WB-Lys samples at 3 mo, with losses gradually increasing to −13% at 24 mo; in RBC-Lys samples, we observed losses of ~8–15% regardless of the storage time point. For MeFox, we observed increases of ~7–11% in WB-Lys samples regardless of the storage time point; in RBC-Lys samples, we observed a minimal increase at 3 mo (1.62%), with ~8% increases at the longer storage time points. The excellent correspondence between each storage time point and the baseline for 5-methylTHF (Supplementary Figure 1) and non-methyl folate (Supplementary Figure 2) in both sample matrices can be observed by the nearly superimposed weighted Deming regression lines with the line of identity.

Long-term frozen stability of RBC folate forms stratified by non-methyl folate concentration.

To assess the potential impact of the folate form composition on storage stability, we stratified our data for each lysate type into samples with low (n=49) and high (n=11) non-methyl folate concentrations (sum of minor folate forms 5-formylTHF, 5,10-methenylTHF, and THF) (Table 3). In high non-methyl folate samples, baseline concentrations (nmol/L) of non-methyl folate were much higher (WB-Lys: 175±111; RBC-Lys: 169±105) compared with low non-methyl folate samples (WB-Lys: 25.9±7.76; RBC-Lys: 26.8±9.35). On the other hand, baseline concentrations of 5-methylTHF were slightly lower in high non-methyl folate samples (WB-Lys: 788±252; RBC-Lys: 769±238) compared with low non-methyl folate samples (WB-Lys: 840±261; RBC-Lys: 860±283). Baseline concentrations of MeFox were similar between high non-methyl folate samples (WB-Lys: 140±49.7; RBC-Lys: 159±56.5) and low non-methyl folate samples (WB-Lys: 149±43.2; RBC-Lys: 175±53.0). The non-methyl folate portion in the high non-methyl folate samples was mainly made up of THF (WB-Lys: 129±83 nmol/L; RBC-Lys: 115±67 nmol/L) and 5,10-methenylTHF (WB-

Lys: 37.5±31.3 nmol/L; RBC-Lys: 50.3±40.7 nmol/L), with 5-formylTHF concentrations mostly around the limit of detection. The relative contribution (mean±SD) of each folate form to tFOL in low *vs* high non-methyl folate samples in RBC-Lys were: 81%±1.7% *vs* 69%±11% for 5-methylTHF, 2.6%±0.7% *vs* 17%±12.8% for non-methyl folate, and 17% ±1.6% *vs* 14%±2.7% for MeFox, respectively.

The stability of folate forms stratified by the non-methyl folate concentration (Supplementary Figure 3 and Table 3) was mostly a mirror image of what we observed in the unstratified data set (Table 2). The only difference was for non-methyl folate: the relative change compared to baseline was higher in low compared with high non-methyl folate samples, likely because the lower concentrations made the relative change appear larger.

Long-term frozen stability of RBC folate forms in QC materials.

The repeat analysis of 2 WB-Lys QC materials stored frozen over 7 y showed only small relative changes compared to the initially assigned target values: 5-methylTHF ≤1%; MeFox and 5,10-methenylTHF ≤5%; THF and 5-formylTHF <10% (data not shown). The repeat analysis of 2 RBC-Lys QC materials stored frozen over nearly 2 y showed similar relative changes: 5-methylTHF and THF <3%; 5-formylTHF and 5,10-methenylTHF ≤4%; MeFox <10% (data not shown). All results were within the predetermined QC acceptability limits. The stable QC performance of folate forms in WB-Lys QC materials stored frozen and analyzed periodically over a period of 7 y can also be observed in the Shewhard plots (Supplementary Figure 4).

Discussion

The present study deals with 4 important aspects of RBC folate measurements. First, we conducted a method comparison between HPLC-MS/MS and MBA for tFOL and found excellent correlation and good concordance between the 2 methods, but 18.6% and 13.5% lower concentrations by HPLC-MS/MS for WB-Lys and RBC-Lys samples, respectively. Second, we assessed the effect of 2 GGH enzymes (endogenous plasma enzyme and exogenous recombinant enzyme for 30 min at room temperature) compared with only 1 enzyme (endogenous plasma enzyme for 4 h at 37°C) and found 5-methylTHF (4.4%) and MeFox (12.2%) concentrations in WB-Lys samples treated with 2 enzymes to be higher than in conventionally prepared WB-Lys samples. Third, we performed a sample matrix comparison between conventionally prepared WB-Lys samples and RBC-Lys samples treated with exoGGH and found highly agreeable results except for MeFox concentrations, which were 16.8% higher in RBC-Lys samples. And fourth, we assessed the long-term frozen stability of folate forms in these 2 matrices and found small (≤5%) changes in 5-methylTHF and tFOL and moderate (≤15%) changes in non-methyl folate and MeFox over a period of 2 y, with WB-Lys and RBC-Lys samples showing mostly similar changes.

To our knowledge, this is the first report that investigated the comparison of RBC tFOL between HPLC-MS/MS and the gold-standard MBA in 2 blood matrices in addition to serum. In this study we confirmed with a larger sample size (n=60 compared with n=23) the excellent correlation and agreement between these 2 methods for serum shown before

(15). The lower agreement between these 2 methods in WB-Lys samples has been reported before (16). However, the current study provides a potential explanation. The fact that we found no difference between RBC tFOL concentrations excluding MeFox in WB-Lys and RBC-Lys samples measured by HPLC-MS/MS (1.4% [95% CI: −0.43% to 3.2%]), yet a higher difference between HPLC-MS/MS and MBA in WB-Lys samples (18.6%) compared with RBC-Lys samples (13.5%), suggests that at least part of this method difference may be due to the MBA showing a matrix effect or responding to some folate like compounds that were eliminated during the RBC washing steps. However, the question about which method measures tFOL concentrations in RBC-Lys samples correctly remains unanswered until matrix-based reference materials exist and have been value assigned with an accepted higher-order reference method.

We re-visited the question of the effect of 2 GGH enzymes (endogenous and exogenous) compared with only 1 using a larger sample size (n=60 compared with n=15). As shown previously (11), we found higher (12.2%) MeFox concentrations in WB-Lys treated additionally with exoGGH compared to conventionally treated WB-Lys and no difference for non-methyl folate. However, possibly due to the larger sample size in the current study, we found slightly higher (4.4 %) 5-methylTHF concentrations in the WB-Lys treated additionally with exoGGH. This is likely due to a small amount of residual 5-methylTHF diglutamate after the conventional 4 h incubation at 37°C (11) and confirms the superiority of the exoGGH treatment.

We also extended our previous pilot investigation (11) comparing concentrations of RBC folate forms between WB-Lys and RBC-Lys samples (n=60 compared with n=4). As expected, the 2 sample matrices were highly correlated and showed excellent agreement for 5-methylTHF and non-methyl folate. In addition to finding higher MeFox concentrations in the 60 RBC-Lys samples treated with exoGGH (and in WB-Lys samples treated with exoGGH beyond the endogenous enzyme), we also observed ~20% higher MeFox concentrations in 2 WB-Lys QC pools treated with exoGGH compared with the pre-determined QC target values generated without the additional exoGGH (n=73 data points per QC level; data not shown). Together, these findings indicate that treatment with exoGGH results in efficient deconjugation of MeFox polyglutamates, probably due to the excess of added enzyme. This may reduce the substrate competition between biologically active folate forms and biologically inactive MeFox for binding to the active sites of the endogenous GGH enzyme, which otherwise would occur in conventionally treated WB-Lys samples. Our findings confirmed that the extra MeFox found in samples treated with exoGGH was not due to oxidation or degradation of methyl folate during sample processing. This supports the notion that MeFox may already be present in RBCs *in vivo*. Otherwise, the MeFox polyglutamates would have to be produced via *in vitro* oxidation of 5-methylTHF polyglutamates during venous blood collection/processing and/or the frozen storage, which seems unlikely.

The use of RBC-Lys samples for folate measurements has several advantages over the use of conventional WB-Lys samples. It allows the exact calculation of concentrations for RBC folate forms, while eliminating the need to measure serum folate or Hct. Furthermore, the folate polyglutamate deconjugation with exoGGH is faster (30 min at room temperature)

compared with the conventional procedure (4 h at 37°C) and thus protects labile folate forms such as THF from degradation and/or oxidation which is inevitable during longer incubation times despite using labeled internal standards. However, this procedure also requires a few additional steps. To avoid a potential gradient in RBC packed cells after centrifugation (17), we diluted (1/2) and gently mixed the packed cells with cold saline prior to taking an aliquot to generate the RBC-Lys. Furthermore, we measured the Hb concentration in the same RBC-Lys aliquot in which we measured folate forms to normalize for the dilution step and for any residual moisture in the packed cells. Lastly, the procedure requires the addition of exoGGH to deconjugate polyglutamates, which incurs an additional cost.

This study provides novel findings on the long-term frozen stability of RBC folate forms in RBC-Lys and WB-Lys samples. While a few studies have tested the short-term folate stability in intact whole blood and/or WB-Lys samples (14, 18,19), there is little information on the long-term frozen storage stability. WB-Lys samples have been reported to be stable at −20°C for 12 mo (20) and 26 mo (21), while intact whole blood stored at −70°C incurred 10–25% loss of folate from 6 to 30 mo (22). In this study, the small (≤5%) losses of 5-methylTHF and tFOL, small to moderate (≤10%) gains in MeFox, and moderate (≤15%) losses of non-methyl folate (given their relatively low concentrations) over a period of 2 y were comparable between WB-Lys and RBC-Lys samples and seemed acceptable when compared to objective criteria derived from biologic variation (23). For whole blood tFOL, the quality specifications for bias, expressed as a fraction of the combination of intra-individual and inter-individual variation, are 4.6% (desirable), 9.2% (optimum), and 13.9% (minimum). It was reassuring to see that when we stratified WB-Lys and RBC-Lys samples into those with low *vs* high non-methyl folate concentrations, both groups showed similar changes for 5-methylTHF, tFOL, and MeFox during frozen storage. Furthermore, despite the presence of substantial amounts of THF in samples with high non-methyl folate concentrations, the change over time was <10%.

In conclusion, this is to our knowledge the first report that studied multiple important questions related to RBC folate forms and tFOL, such as method comparisons between HPLC-MS/MS and MBA, matrix comparisons between conventionally prepared WB-Lys samples and RBC-Lys samples treated with exoGGH, and frozen storage stability over a period of 2 y. We found excellent agreement between the 2 sample matrices for folate form concentrations measured by HPLC-MS/MS and good agreement between the 2 methods for tFOL measured in both sample matrices. Furthermore, RBC folate forms appear to be stable in RBC-Lys samples when kept frozen at −70°C for up to 2 y. This provides an opportunity to use washed RBCs for folate measurements and it eliminates the need for serum folate and Hct measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The findings and conclusions in this manuscript are those of the authors and do not necessarily represent the official views or positions of the Centers for Disease Control and Prevention/Agency for Toxic Substances and Disease Registry.

Abbreviations:

exoGGH	exogenous γ-glutamyl hydrolase
5-formylTHF	5-formyltetrahydrofolate
5-methylTHF	5-methyltetrahydrofolate
5,10-methenylTHF	5,10-methenyltetrahydrofolate
MeFox	pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF
THF	tetrahydrofolate
MCHC	corpuscular hemoglobin concentration
RBC	red blood cell
RBC-Lys	red blood cell lysate
SLS	sodium lauryl sulfate
WB-Lys	whole blood lysate

References

1. Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory JF 3rd, Mills JL, Pfeiffer CM, Fazili Z, Zhang M, Ueland PM, et al. Biomarkers of Nutrition for Development – Folate review. J Nutr 2015;145:1636S–80S. [PubMed: 26451605]
2. Pfeiffer CM, Fazili Z, Zhang M. Folate analytical methodology. In: Bailey LB, editor. Folate in Health and Disease. 2nd ed. CRC Press. Taylor & Francis Group. 2010.
3. O'Broin S, Keller B. Microbiological assays on microtiter plate of folate in serum and red cells. J Clin Pathol 1992;45:344–7. [PubMed: 1577973]
4. Molloy AM, Scott JM. Microbiological assay for serum, plasma and red cell folate using cryopreserved, microtiter plate method. Meth Enzymol 1997;281:43–53.
5. Fazili Z, Pfeiffer CM. Measurements of folates in serum and conventionally prepared whole blood lysates: application of an automated 96-well plate isotope-dilution tandem mass spectrometry method Clin Chem 2004;50:2378–81. [PubMed: 15459090]
6. Fazili Z, Pfeiffer CM, Zhang M, Jain R. Erythrocyte folate extraction and quantitative determination by liquid chromatography-tandem mass spectrometry: comparison of results with microbiologic assay. Clin Chem 2005;51:2318–25. [PubMed: 16214826]
7. Huang Y, Khartulyari S, Morale ME, Stanislawska-Sachadyn A, Von Feldt JM, Whitehead AS, Blair IA. Quantitation of key red blood cell folates from subjects with defined MTHFR 677C>T geneotypes using stable isotope dilution liquid chromatography/mass spectrometry. Rapid Comm Mass Spec 2008;22:2403–12.
8. Smith DE, Kok RM, Teerlink T, Jakobs C, Smulders YM. Quantitative determination of erythrocyte folate vitamer distribution by liquid chromatography-tandem mass spectrometry. Clin Chem Lab Med 2006;44:450–9. [PubMed: 16599840]
9. Mönch S, Netzel M, Netzel G, Rychlik M. Quantitation of folates and their catabolites in blood plasma, erythrocytes and urine by stable isotope dilution assays. Anal Biochem 2010;398:150–60. [PubMed: 19903444]

10. Kirsch S, Herrmann W, Geisel J, Obeid R. Assay of whole blood (6S)-5-CH3-H4folate using ultra performance liquid chromatography tandem mass spectrometry. Anal Bioanal Chem 2012;404:895–902. [PubMed: 22729356]
11. Stamm R, Fazili Z, Pfeiffer CM. Addition of exogenous γ-glutamyl hydrolase eliminates the need for lengthy incubation of whole blood lysates for quantitation of folate vitamers by HPLC-MS/MS. Curr Dev Nutr 2018;2:1–9.
12. Fazili Z, Whitehead R Jr., Paladugula N, Pfeiffer CM. A high-throughput LC-MS/MS method suitable for population biomonitoring measures five serum folate vitamers and one oxidation product. Anal Bioanal Chem 2013;405:4549–60. [PubMed: 23462981]
13. Oshiro I, Takenaka T, Maeda J. New method for hemoglobin determination by using sodium lauryl sulfate (SLS) 1982. Clin Biochem 1982;15(1):83–8. [PubMed: 7094292]
14. O'Broin SD, Kelleher BP, Davoren A, Gunter EW. Field-study screening of blood folate concentrations: Specimen stability and finger-stick sampling. Am J Clin Nutr 1997;66:1398–405. [PubMed: 9394692]
15. Zhang M, Sternberg MR, Pfeiffer CM. Harmonizing the calibrator and microorganism used in the folate microbiological assay increases the comparability of serum and whole-blood folate results in a CDC Round-Robin study. J Nutr 2018;148:807–17. [PubMed: 30053280]
16. Yetley EA, Pfeiffer CM, Phinney KW, Fazili Z, Lacher DA, Bailey RL, Blackmore S, Bock JL, Brody LC, Carmel R, et al. Biomarkers of folate status in the National Health and Nutrition Examination Survey (NHANES): a roundtable summary. Am J Clin Nutr 2011;94:303S–12S. [PubMed: 21593502]
17. Chu RC, Begley JA, Hall CA. The relationship between erythrocyte age and cell content of micronutrients and levels of related enzymes. Nutr Res 1990;10:31–7.
18. O'Broin JD, Temperly IJ, Scott J. Erythrocyte, plasma and serum folate: Specimen stability before microbiological assays. Clin Chem 1980;26:522–4. [PubMed: 6767573]
19. Zemlin A, Essack Y, Rensburg M, Keller T, Brinkmann T. Stability of red cell folate in whole blood and hemolysate. Clin Lab 2010;56:391–6. [PubMed: 21086784]
20. Chen H, Sternberg MA, Schleicher RL, Pfeiffer CM. Long-term stability of 18 nutritional biomarkers stored at −20°C and 5°C for up to 12 months. J Appl Lab Med 2018;03:011–9.
21. Kelleher B, O'Broin S. Choice of materials for long-term quality control of blood folate assays. Clin Chem 1996;42:652–4. [PubMed: 8605693]
22. Fazili Z, Sternberg MR, Pfeiffer CM. Assessing the influence of 5,10-methylenetetrahydrofolate reductase polymorphism on folate stability during long-term frozen storage, thawing, and repeated freeze/thawing of whole blood. Clin Chim Acta 2012;413:966–72. [PubMed: 22342879]
23. Fraser CG, Hyltoft PP, Libeer JC, Rocos C. Proposals for setting generally accepted quality goals solely based on biology. Ann Clin Biochem 1997;34:8–12. [PubMed: 9022883]

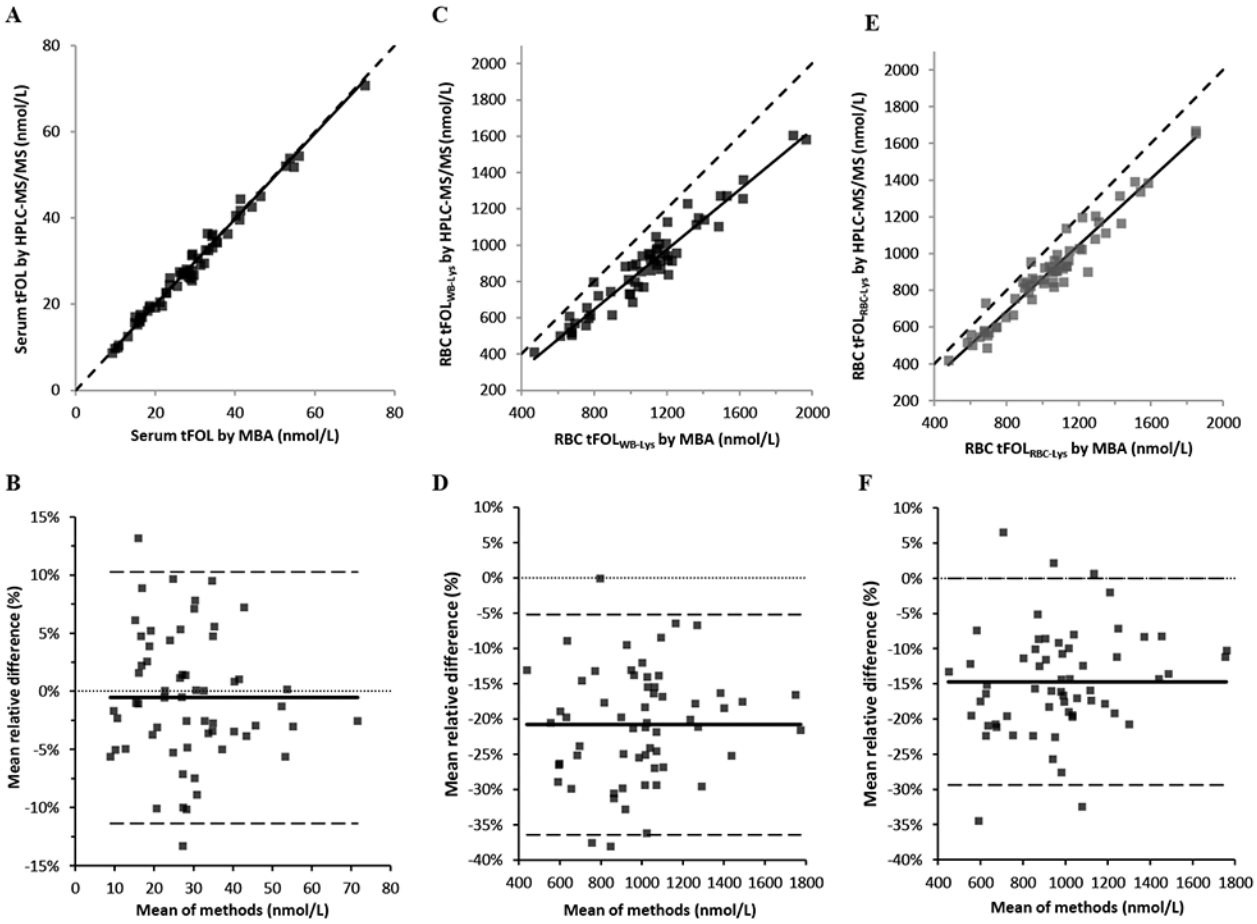

FIGURE 1.

Method comparison between HPLC-MS/MS and MBA for serum tFOL (panels A and B), RBC tFOL derived from WB-Lys samples (panels C and D), and RBC tFOL derived from RBC-Lys samples (panels E and F) at baseline (1 wk). Scatter plots (panels A, C, and E) show ordinary least squares regression line (solid line) compared to the line of identity (dashed line). Bland-Altman plots (panels B, D, and F) show the mean relative difference (solid horizontal line), 95% limits of agreement (long dashed horizontal lines), and zero difference (short dashed horizontal line). WB-Lys samples were conventionally prepared (4 h incubation at 37°C) prior to sample analysis by HPLC-MS/MS and analyzed by MBA without additional incubation. RBC-Lys samples were treated with exoGGH (30 min at room temperature) prior to sample analysis by HPLC-MS/MS and MBA. Sum of serum or RBC folate forms represents tFOL by HPLC-MS/MS. For each sample matrix, 60 samples were analyzed in 4 replicates per sample (duplicates over 2 d). exoGGH, exogenous γ-glutamyl hydrolase; MBA, microbiologic assay; RBC-Lys, red blood cell lysate; tFOL, total folate; WB-Lys, whole blood lysate.

Table 1.

Comparison of total folate measured at baseline by HPLC-MS/MS and MBA for various matrices[1]

Sample Matrix	Descriptive Statistic	HPLC-MS/MS$_{\text{Excluding MeFox}}$	HPLC-MS/MS$_{\text{Including MeFox}}$	MBA
Serum[2]	Mean±SD, nmol/L	28.6±12.5	32.3±12.7	28.8±12.8
	Median (IQR), nmol/L	27.2 (19.2 to 35.2)	30.44 (23.0 to 37.9)	28.40 (18.5 to 34.3)
	Pearson correlation *r*	0.993	0.978	n/a
	Relative difference (95% CI)[3], %	−0.4 (−1.83 to −1.03)	15.4 (11.5 to 19.4)	n/a
	Weighted Deming regression slope (95% CI)	0.992 (0.964 to 1.020)	1.017 (0.950 to 1.083)	n/a
	Weighted Deming regression intercept (95% CI)	0.064 (−0.542 to 0.067)	3.07(1.247 to 4.898)	n/a
WB-Lysate[4]	Mean±SD, nmol/L	886±255	1033±295	1089±300
	Median (IQR), nmol/L	893 (724 to 996)	1028 (855 to 1165)	1109 (893 to 1205)
	Pearson correlation *r*	0.963	0.970	n/a
	Relative difference (95% CI)[3], %	−18.6 (−20.3 to −16.9)	−5.12 (−6.85 to −3.40)	n/a
	Weighted Deming regression slope (95% CI)	0.824 (0.767 to 0.882)	0.958 (0.899 to 1.017)	n/a
	Weighted Deming regression intercept (95% CI)	−12.81(−71.51 to 45.9)	−11.4 (−69.7 to 46.9)	n/a
RBC-Lysate[5]	Mean±SD, nmol/L	899±271	1071±319	1037±295
	Median (IQR), nmol/L	900 (739 to 1019)	1051 (852 to 1186)	1052 (839 to 1180)
	Pearson correlation *r*	0.971	0.975	n/a
	Relative difference (95% CI)[3], %	−13.5 (−15.2 to −11.8)	3.13 (1.36 to 4.89)	n/a
	Weighted Deming regression slope (95% CI)	0.901 (0.845 to 0.958)	1.066 (1.005 to 1.127)	n/a
	Weighted Deming intercept (95% CI)	−36.5 (−91.2 to 18.1)	−35.4(−91.9 to 21.2)	n/a

[1]Total folate by HPLC-MS/MS is the sum of folate forms (5-methyltetrahydrofolate, 5-formyltetrahydrofolate, tetrahydrofolate, 5,10-methenyltetrahydrofolate, excluding or including MeFox); MBA, microbiologic assay

[2]Serum samples (*n*=60); aliquots processed and analyzed as duplicates by HPLC-MS/MS and MBA over 2 days (*n*=4 results per sample)

[3]Calculated as HPLC-MS/MS minus MBA divided by MBA (expressed as percent) and then averaged across all 60 samples

[4]WB-Lysate samples (*n*=60); conventionally prepared as follows: whole blood aliquot from each sample diluted 1/11 with 1% ascorbic acid, then frozen; prior to analysis by HPLC-MS/MS, WB-Lysate aliquots were incubated for 4 h at 37°C; WB-Lysate aliquots for MBA were not incubated prior to analysis; aliquots processed and analyzed as duplicates by each assay over 2 days (*n*=4 results per sample).

[5] RBC-Lysate samples (n=60); washed RBC samples diluted approximately ½ with saline prior to 1/11 dilution with 1% ascorbic acid, then frozen; prior to analysis, RBC-Lysate aliquots were treated with exoGGH enzyme (5 μg/mL of lysate) for 30 min at room temperature; aliquots processed and analyzed as duplicates by each assay over 2 days (n=4 results per sample).

Table 2.

Concentrations of RBC folate forms and total folate in conventionally prepared WB-Lys and RBC-Lys samples stored at −70°C for ≤2 y

	Concentration[1], nmol/L			Relative difference[2], %	Relative change compared to baseline[3], %	
Storage time (mo)	WB-Lys[4]	RBC-Lys[5]	WB-Lys + exoGGH[6]	RBC-Lys *vs* WB-Lys	WB-Lys	RBC-Lys
5-MethylTHF[7]						
Baseline	831±258	843±276	867±268	1.22 (−0.58 to 3.02)	n/a	n/a
3	840±259	818±265	n.m.[8]	−2.88 (−4.88 to −0.88)	1.24 (0.70 to 1.78)	−2.93 (−3.50 to −2.36)
6	830±257	812±265	n.m.	−2.58 (−4.23 to −0.94)	0.07 (−0.73 to 0.87)	−3.68 (−4.30 to −3.07)
12	825±256	818±266	n.m.	−0.97 (−2.82 to 0.87)	−0.72 (−1.15 to −0.30)	−2.90 (−3.32 to −2.48)
24	807±252	800±261	n.m.	−0.95 (−3.02 to 1.11)	−2.94 (−3.36 to −2.52)	−5.10 (−5.63 to −4.56)
Non-methyl folate[9]						
Baseline	53.3±74.4	52.9±70.7	53.0±75.6	2.27 (−1.91 to 6.44)	n/a	n/a
3	53.0±74.5	46.9±67.4	n.m.	−14.8 (−17.8 to −11.8)	0.32 (−2.43 to 3.08)	−15.3 (−19.1 to −11.5)
6	51.5±75.5	49.5±67.5	n.m.	1.49 (−2.90 to 5.89)	−6.60 (−9.47 to −3.67)	−7.66 (−10.1 to −5.21)
12	50.9±72.7	49.0±67.8	n.m.	−1.25 (−4.64 to 2.14)	−6.47 (−8.55 to −4.39)	−9.23 (−11.8 to −6.67)
24	48.4±72.9	48.5±69.6	n.m.	2.82 (−1.41 to 7.04)	−13.0 (−15.8 to −10.2)	−12.6 (−15.2 to −10.0)
$tFOL_{Excluding\ MeFox}$[10]						
Baseline	886±255	899±271	922±263	1.37 (−0.43 to 3.18)	n/a	n/a
3	895±255	867±259	n.m.	−3.15 (−5.13 to −1.17)	1.16 (0.61 to 1.70)	−3.42 (−3.9 to −2.91)
6	884±253	864±260	n.m.	−2.44 (−4.12 to −0.75)	−0.08 (−0.76 to 0.60)	−3.83 (−4.38 to −3.29)
12	878±253	870±260	n.m.	−0.88 (−2.68 to 0.92)	−0.92 (−1.32 to −0.51)	−3.12 (−3.53 to −2.72)
24	857±248	851±255	n.m.	−0.58 (−2.63 to 1.48)	−3.28 (−3.74 to −2.82)	−5.22 (−5.67 to −4.77)
MeFox[11]						
Baseline	147±44.1	172±53.5	165±50.8	16.8 (14.8 to 18.8)	n/a	n/a
3	160±47.9	175±55.1	n.m.	9.26 (6.94 to 11.6)	8.66 (7.65 to 9.67)	1.62 (0.10 to 3.14)
6	163±48.9	185±56.7	n.m.	13.9 (11.2 to 16.5)	10.6 (9.46 to 11.8)	7.61 (6.75 to 8.47)
12	162±48.2	185±57.3	n.m.	14.1 (11.7 to 16.5)	10.2 (9.14 to 11.3)	7.54 (6.81 to 8.28)
24	157±47.9	185±56.2	n.m.	18.2 (15.7 to 21.9)	6.60 (4.84 to 8.40)	7.96 (7.24 to 8.68)

[1]Concentrations are reported as mean±SD for n=60 samples (4 results per sample and storage time point)

[2] Calculated as RBC-Lys minus WB-Lys divided by WB-Lys (expressed as percent) and then averaged across all 60 samples; 95% CI is shown in parenthesis

[3] Calculated as time point x minus baseline divided by baseline (expressed as percent) and then averaged across all 60 samples; 95% CI is shown in parenthesis

[4] WB-Lys, whole blood lysate; WB-Lys samples were conventionally prepared by 1/11 dilution with 1% ascorbic acid, then frozen; prior to analysis by HPLC-MS/MS, WB-Lys aliquots were incubated for 4 h at 37°C (in duplicate over 2 days for n=4 results per sample)

[5] RBC-Lys, red blood cell lysate; RBC-Lys samples were washed RBC diluted ½ with saline prior to 1/11 dilution with 1% ascorbic acid, then frozen; prior to analysis by HPLC-MS/MS, RBC-Lys aliquots were treated with exoGGH enzyme (5 μg/mL of lysate) for 30 min at room temperature (in duplicate over 2 days for n=4 results per sample)

[6] exoGGH, exogenous gamma-glutamyl hydrolase; WB-Lys samples were conventionally prepared by 1/11 dilution with 1% ascorbic acid, then frozen; prior to analysis by HPLC-MS/MS, exoGGH was added to WB-Lys aliquots (5 μg enzyme/mL sample added to the internal standard mixture), and samples were incubated for 4 h at 37°C (in duplicate over 2 days for n=4 results per sample)

[7] 5-MethylTHF, 5-methyltetrahydrofolate; major circulating folate form

[8] n.m., not measured

[9] Non-methyl folate; sum of minor folate forms (5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate)

[10] tFOL, total folate; sum of biologically active folate forms (5-methylTHF and non-methyl folate)

[11] MeFox is a pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF (an oxidation product of 5-methylTHF)

Table 3.

Concentrations of RBC folate forms and total folate stratified by non-methyl folate concentrations in conventionally prepared WB-Lys and RBC-Lys samples stored at −70°C for up to 2 y

	Concentration[1], nmol/L		Relative change compared to baseline[2], %	
Storage time (mo)	WB-Lys[3]	RBC-Lys[4]	WB-Lys[3]	RBC-Lys[4]
Samples with low non-methyl folate concentration				
5-MethylTHF[5]				
Baseline[6]	840±261	860±283	n/a	n/a
3	853±262	835±271	1.59 (1.01, 2.17)	−2.75 (−3.36, −2.14)
6	842±259	827±270	0.36 (−0.55, 1.28)	−3.64 (−4.33, −2.96)
12	835±259	835±271	−0.57 (−1.04, −0.10)	−2.82 (−3.26, −2.39)
24	818±255	817±266	−2.69 (−3.14, −2.23)	−4.89 (−5.46, −4.31)
Non-methyl folate[7]				
Baseline[6]	25.9±7.76	26.8±9.35	n/a	n/a
3	26.1±8.41	22.0±8.42	0.93 (−2.38, 4.24)	−17.2 (−21.7, −12.7)
6	24.0±8.25	24.5± 8.94	−7.61 (−11.1, −4.14)	−8.35 (−11.3, −5.39)
12	24.1±8.17	23.9±8.42	−7.39 (−9.85, −4.94)	−10.2 (−13.2, −7.06)
24	22.4±7.85	22.9±8.35	−13.9 (−17.1, −10.7)	−14.5 (−17.4, −11.5)
$tFOL_{Excluding\ MeFox}$[8]				
Baseline[6]	868±266	889±289	n/a	n/a
3	880±268	860±276	1.52 (0.96, 2.09)	−3.24 (−3.78, −2.69)
6	868±265	855±276	0.11 (−0.70, 0.92)	−3.81 (−4.42, −3.21)
12	861±264	861±277	−0.75 (−1.21, −0.29)	−3.08 (−3.51, −2.65)
24	842±260	843±272	−3.04 (−3.49, −2.58)	−5.20 (−5.71, −4.69)
MeFox[9]				
Baseline[6]	149±43.2	175±53.0	n/a	n/a
3	161±47.1	177±53.4	8.81 (7.63, 9.99)	1.74 (0.08, 3.39)
6	164±48.6	188±55.4	10.6 (9.36, 11.9)	7.89 (6.95, 8.83)
12	164±47.8	188±56.0	10.1 (8.99, 11.3)	7.79 (7.01, 8.58)
24	159±48.0	189±54.9	6.68 (4.76, 8.59)	8.23 (7.39, 9.06)
Samples with high non-methyl folate concentration				
5-MethylTHF[5]				
Baseline[6]	788±252	769±238	n/a	n/a
3	786±251	742±236	−0.31 (−1.45, 0.83)	−3.74 (−5.49, −2.00)
6	780±252	741±237	−1.23 (−2.71, 0.25)	−3.86 (−5.53, −2.19)
12	777±246	746±237	−1.42(−2.49, −0.34)	−3.23 (−4.63, −1.83)
24	757±244	724±230	−4.08 (−4.96, −3.19)	−6.05 (−7.53, −4.56)
Non-methyl folate[7]				

Storage time (mo)	Concentration[1], nmol/L		Relative change compared to baseline[2], %	
	WB-Lys[3]	RBC-Lys[4]	WB-Lys[3]	RBC-Lys[4]
Baseline[6]	175±111	169±105	n/a	n/a
3	173±114	158±99.8	−2.38 (−5.62, 0.85)	−7.17 (−8.92, −5.41)
6	174±115	160±99.3	−1.91 (−4.12, 0.29)	−4.55 (−6.31, −2.80)
12	170±108	161±100	−2.38 (−4.44, −0.32)	−5.18 (−7.13, −3.23)
24	164±114	163±103	−8.89 (−14.8, −3.01)	−4.50 (−6.30, −2.70)
$tFOL_{Excluding\ MeFox}$[8]				
Baseline[6]	966±188	941±169	n/a	n/a
3	961±187	902±172	−0.46 (−1.84, 0.91)	−4.24 (−5.83, −2.65)
6	957±185	905±174	−0.92 (−1.92, 0.07)	−3.94 (−5.51, −2.38)
12	950±184	910±171	−1.64 (−2.53, −0.75)	−3.33 (−4.70, −1.96)
24	924±182	891±162	−4.37 (−5.91, −2.83)	−5.31 (−6.32, −4.30)
MeFox[9]				
Baseline[6]	140±49.7	159±56.5	n/a	n/a
3	151±52.8	162±63.5	8.01 (6.00, 10.0)	1.11 (−3.40, 6.62)
6	154±52.1	169±62.7	10.6 (7.09, 14.2)	6.36 (4.02, 8.69)
12	154±51.8	170±63.5	10.7 (7.05, 14.2)	6.42 (4.22, 8.62)
24	148±49.0	170±62.3	6.38 (0.88, 11.9)	6.76 (5.58, 7.94)

[1] Concentrations are reported as mean±SD for low non-methyl folate samples (n=49) and high non-methyl folate samples (n=11); each sample was analyzed in 4 replicates per storage time point

[2] Calculated as time point x minus baseline divided by baseline (expressed as percent) and then averaged across all samples; 95% CI is shown in parenthesis

[3] WB-Lys, whole blood lysate; WB-Lys samples were conventionally prepared by 1/11 dilution with 1% ascorbic acid, then frozen; prior to analysis by HPLC-MS/MS, WB-Lys aliquots were incubated for 4 h at 37°C (in duplicate over 2 days for n=4 results per sample)

[4] RBC-Lys, red blood cell lysate; RBC-Lys samples were washed RBC diluted ½ with saline prior to 1/11 dilution with 1% ascorbic acid, then frozen; prior to analysis by HPLC-MS/MS, RBC-Lys aliquots were treated with exoGGH enzyme (5 μg/mL of lysate) for 30 min at room temperature (in duplicate over 2 days for n=4 results per sample)

[5] 5-MethylTHF, 5-methyltetrahydrofolate; major circulating folate form

[6] Baseline aliquots of WB-Lysate and RBC-Lysate were stored at −70°C immediately after preparation for up to 1 week prior to analysis by LC-MS/MS.

[7] Non-methyl folate; sum of minor folate forms (5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate)

[8] tFOL, total folate; sum of biologically active folate forms (5-methylTHF and non-methyl folate)

[9] MeFox is a pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF (an oxidation product of 5-methylTHF)