Addition of exogenous \(\gamma\)-glutamyl hydrolase eliminates the need for lengthy incubation of whole blood lysate for quantitation of folate vitamers by high performance liquid chromatography-tandem mass spectrometry\(^1\)-\(^7\)

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\(^6\) Running head: Exogenous hydrolase to deconjugate whole blood folates

\(^7\) Abbreviations: 5-formylTHF, 5-formyltetrahydrofolate; 5-methylTHF, 5-methyltetrahydrofolate; 5,10-methenylTHF, 5,10-methenyltetrahydrofolate; exoGGH, exogenous \(\gamma\)-glutamyl hydrolase; LOD, limit of detection; MeFox, pyrazino-s-triazine derivative of 4\(\alpha\)-hydroxy-5-methylTHF; RBC, red blood cell; THF, tetrahydrofolate; WBL, whole blood lysate

Key words: deconjugation, human recombinant protein, monoglutamate, diglutamate, RBC folate, washed erythrocytes
Abstract

**Background:** Measurement of folate monoglutamates by HPLC-MS/MS in whole blood lysate (WBL) requires lengthy incubation prior to analysis, risking degradation of labile folate vitamers.

**Objective:** We explored whether addition of a commercially available recombinant exogenous \( \gamma \)-glutamyl hydrolase (exoGGH) enzyme reduced the required incubation time of WBL for measurement of folate as monoglutamates.

**Methods:** For conventional deglutamylation of polyglutamates, WBL was incubated 4 h at 37°C. Alternatively, we added exoGGH to WBL at varying concentrations (1–10 \( \mu g/mL \)) and incubation times (0–90 min). We also investigated modifications to the sample diluent (pH, ascorbic acid vs. sodium ascorbate, and ascorbate concentration). Finally, we tested the effect of the enzyme in different sample types: WBL from frozen whole blood vs. frozen WBL or vs. frozen washed red blood cells (RBC).

Samples (\( n \leq 15 \) per experiment) were analyzed by HPLC-MS/MS for 6 folate monoglutamates and 5-methyltetrahydrofolate diglutamate.

**Results:** Optimal deconjugation of folate polyglutamates was achieved using 1% ascorbic acid and 5 \( \mu g \) enzyme/mL WBL, requiring ≤30 min incubation time to achieve complete folate recovery as monoglutamates. This treatment resulted in similar folate concentrations as conventional deglutamylation (4 h at 37°C). The exoGGH enzyme was effective in samples stored frozen as whole blood and as WBL. However, extended thaw time of whole blood resulted in 5-methyltetrahydrofolate loss and unacceptable changes to non-methyl folate concentration. Total folate (with exoGGH) measured in washed RBC was ~15% lower than RBC folate calculated from WBL concentrations (conventional deglutamylation).

**Conclusions:** Use of exoGGH minimized incubation time and thus may avoid degradative losses of labile folate forms during sample preparation. The lower folate results in washed RBC may be due to inadequate packing of RBC among other unidentified factors. A larger study is required to confirm lack of differences in folate concentrations determined with and without the use of exoGGH.
Introduction

Both serum and red blood cell (RBC) folate are often used as indicators for population monitoring of folate status (1). The measurement of serum folate is simpler because it does not require dilution or incubation of samples to yield folate monoglutamates from polyglutamates. However, serum folate concentration only indicates short-term status, is confounded by fasting status, and there is no established cut-off for serum folate as an indicator of neural tube defect risk. RBC folate is a marker of long-term folate status and can be used to infer risk of neural tube defects in women of reproductive age (2). A RBC folate concentration <305 nmol/L is often applied as a cut-off for risk of megaloblastic anemia (3), while a concentration ≥906 nmol/L is considered optimal for reducing the risk of neural tube defects (2).

Folate in RBCs is polyglutamylated and requires the deconjugation of glutamyl chain residues by the enzyme γ-glutamyl hydrolase (GGH) (EC number 3.4.19.9) prior to measurement (4). Typically, whole blood is diluted 1/10 or 1/11 in 1% ascorbic acid to activate the endogenous human GGH present within the plasma portion of whole blood (5). After a short 30-min incubation at 37°C or a freeze-thawing cycle, total folate can then be measured by the microbiologic assay, which responds to folates with 3 or fewer glutamate residues (4). Analysis of folate by HPLC-MS/MS requires longer incubation times at 37°C for up to 4 h to recover monoglutamate folates (6), however long incubation times increase the likelihood of degradation of labile folate forms. While the microbiologic assay is the historically preferred method for measurement of total folate, HPLC-MS/MS offers several advantages: quantitation of individual folate vitamers, such as 5-methyltetrahydrofolate (5-methylTHF), folic acid, and non-methyl folate forms; and improved specificity and precision compared to the microbiologic assay. Although HPLC-MS/MS procedures employing stable isotopes as internal standards theoretically account for losses of folate forms, the utility of the internal standard may reach its limit when too little internal standard is left after a long incubation time to generate a reliable area ratio with the compound of interest. Thus, a reduction in incubation time may improve the analytical performance of HPLC-MS/MS methods.
Most procedures measure folate forms in whole blood lysates (WBL) utilizing the endogenous GGH enzyme. These methods calculate RBC folate concentration by subtracting the serum folate portion and normalizing to the hematocrit value. Procedures that directly quantitate RBC folate concentration do not require the additional measurement of serum folate or hematocrit values, however, they require the addition of an exogenous source of GGH (exoGGH). Several procedures have been published employing the use of exoGGH (rat plasma, human plasma, or chicken pancreas) in the measurement of human tissue samples, including in RBC (7-11). In one study RBC folate concentrations with added exoGGH substituted from low folate plasma compared well with calculated folate concentrations from whole blood in traditionally prepared lysates (11).

The objectives of this study were to investigate whether the additional use of exoGGH can reduce the incubation time of WBL for the measurement of folate monoglutamates by HPLC-MS/MS and to explore the direct measurement of RBC folate in enzyme treated washed RBCs.

Methods

Reagents and materials

Folate monoglutamate calibrators [(6S)-5-methyltetrahydrofolate (5-methylTHF), folic acid, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF (MeFox), (6S)-5-formyltetrahydrofolate (5-formylTHF), (6S)-tetrahydrofolate (THF), and (6R)-5,10-methenyltetrahydrofolate (5,10-methenylTHF)] together with their respective 13C5-labeled analogs were purchased from Merck & Cie. (6R,S)-5-Methyltetrahydropteroyldi-γ-L-glutamic acid (5-methylTHF diglutamate) was purchased from Schircks Laboratories. All other reagents were ACS grade. Recombinant His-tagged human γ-glutamyl hydrolase protein was purchased from Novus Biologicals. EDTA whole blood was purchased from a commercial blood bank (Bioreclamation IVT).

Blood processing
Whole blood and WBL were prepared for frozen storage in vapor phase liquid nitrogen from EDTA whole blood after gentle mixing of the blood collection tubes for $\geq 10$ min (Figure 1). WBL was prepared by dispensing 2.5 mL whole blood into 25 mL 1% ascorbic acid, vortex mixing, and aliquoting 1 mL each into cryovial tubes. These samples were used to test the conditions for optimal functioning of the exoGGH enzyme ($n=4$ in experiment 1a and $n=15$ in experiment 1b) and to compare the performance of WBL (stored frozen) vs. whole blood (stored frozen; WBL prepared prior to analysis) ($n=15$ in experiment 2).

To test the direct measurement of RBC folate from washed packed RBC we obtained additional EDTA whole blood samples ($n=5$, experiment 3). The blood collection tubes were gently mixed for $\geq 10$ min upon arrival before filling a microhematocrit tube and centrifuging it at 12,000 g for 5 min to determine the hematocrit (Figure 1). Only 4 of the 5 samples were used for testing because no valid hematocrit was obtained for 1 sample. Whole blood (300 µL aliquots) was dispensed into cyrovial tubes and the remainder of the blood was centrifuged at 1,811 g and 4°C for 15 min. EDTA plasma (1 mL) was dispensed into cryovial tubes and an approximate equivalent volume of cold refrigerated phosphate buffered saline was added to the RBCs in the blood collection tubes. The tubes were centrifuged again at 1,811 g and 4°C for 15 min and the phosphate buffered saline discarded. This step was repeated twice more to isolate the RBC before dispensing washed packed RBC into cryovial tubes (200 µL aliquots). Samples were stored at -70°C until analysis.

**Analysis of folate forms by HPLC-MS/MS**

Folate monoglutamates, inclusive of 5-methylTHF, folic acid, MeFox, 5-formylTHF, 5,10-methenylTHF, and THF, were analyzed by HPLC-MS/MS in plasma, WBL and RBC lysate samples according to previously published procedures (12-14). In addition, 5-methylTHF diglutamate (m/z 589/313) concentration was determined using $^{13}$C$_5$-labeled 5-methylTHF monoglutamate (m/z 465/313) as an internal standard. A 5-point calibration curve ranging from 1 nmol/L to 100 nmol/L for 5-methylTHF and from 0.5 to 50 nmol/L for all other monoglutamate analytes and for 5-methylTHF diglutamate,
corresponding to diluted sample concentrations, was included in each experiment. Three quality control pools (WBL and/or serum) with previously characterized folate concentrations were included in each analytical run in duplicate bracketing the unknown samples to assess the validity of the analytical run.

Conditions for optimal functioning of exogenous recombinant human GGH

In experiment 1a (Figure 1), whole blood samples (n=4) were thawed and diluted 1/11 in 1% ascorbic acid (conventional diluent), or in 1% ascorbic acid with 10 µg/mL exoGGH, 1% sodium ascorbate (pH 3.8) with either 10, 5, 2, or 1 µg/mL exoGGH, and 0.5% ascorbic acid with either 10, 5, 2, or 1 µg/mL exoGGH. WBL samples (150 µL) were incubated with internal standard (60 µL) containing each of the 13C5-labeled folate analogues made up in 0.1% ascorbic acid for 0 h (all samples), 0.5 h at room temperature (enzyme treated samples with the exception of those diluted in 1% ascorbic acid), and 4 h at 37°C (conventionally prepared samples).

In experiment 1b (Figure 1), WBL samples (n=15) were thawed and 150 µL of each sample was mixed with 60 µL internal standard mix containing either 25 µg/mL exoGGH (equivalent to 10 µg enzyme/mL WBL), 12.5 µg/mL exoGGH (equivalent to 5 µg enzyme/mL WBL) or no added enzyme (conventional). Samples were incubated for 0 h (10 µg/mL and 5 µg/mL enzyme treated samples), 0.5 h at room temperature (5 µg/mL enzyme treated samples), and 4 h at 37°C (conventionally prepared samples).

After their respective incubation periods, all samples were stored frozen overnight before undergoing reversed-phase solid phase extraction (SPE) prior to batch analysis by HPLC-MS/MS.

Comparison of samples stored frozen as whole blood or WBL

In experiment 2 (Figure 1), matched frozen whole blood and WBL samples (n=15) were removed from frozen storage and after thawing (~30 min) were exposed to extended thaw time for an additional 0 h, 1 h, and 4 h (samples were removed from frozen storage at staggered intervals to allow experimental set-up at a singular time point). Whole blood was diluted 1/11 in 1% ascorbic acid. For each of the samples, 150
µL WBL was mixed with 60 µL internal standard mix using both the conventional procedure and the procedure employing 5 µg/mL enzyme (25 µg/mL exoGGH added to the internal standard mixture). Conventionally prepared samples were incubated for 4 h at 37°C prior to SPE and analysis by HPLC-MS/MS, while enzyme treated samples were processed for SPE after 20 min equilibrating with the internal standard mix.

**Comparison of RBC folate measured directly or calculated from WBL concentrations**

In experiment 3 (Figure 1), whole blood was diluted 1/11 in 1% ascorbic acid and thawed washed packed RBC were diluted 1/21 in 0.5% ascorbic acid (n=4). Plasma and WBL folate were measured by conventional procedure (4 h incubation at 37°C for WBL), each with their own calibration curve. RBC lysate samples were treated with exoGGH at a concentration of 10 µg/mL and no incubation. The exoGGH was mixed with RBC lysate through the addition of internal standard mix containing exoGGH, as described previously. Immediately after their respective incubation periods, all samples were frozen overnight prior to SPE and batch analysis by HPLC-MS/MS. This experiment was carried out twice on 2 separate days and results were averaged.

**Statistical analysis**

We used Microsoft Excel to calculate the mean ± SD concentration for each folate form, except when the proportion of results < LOD exceeded 40% (for 5-methylTHF diglutamate), in which case we calculated the median and interquartile range (25th–75th percentile). We calculated the HPLC-MS/MS total folate as the sum of the individual folate forms, using an imputed value of LOD divided by square root of 2 for a folate form result < LOD. We used a 2-tailed paired t-test to assess statistical differences between treatment conditions in experiments 1b and 2. P values <0.05 were considered significant.

**Results**

**Conditions for optimal functioning of exogenous recombinant human GGH**
The effects of sample diluent, incubation time and temperature, and enzyme concentration on folate concentrations were tested in 4 samples that were stored frozen as whole blood (Table 1). In samples made up in 1% ascorbic acid, the pH of the lysate was 3.8 and in samples made up in 1% sodium ascorbate (pH 3.8) and in 0.5% ascorbic acid, the pH of the lysate was 4.2. In all of the diluents, use of 10 µg enzyme/mL WBL resulted in fast deconjugation and no measurable 5-methylTHF diglutamate remained even without incubation. Overall, comparable folate concentrations were obtained between the conventional procedure and the conditions where enzyme was added. An exception was noted for non-methyl folate (in samples with high non-methyl), where higher concentrations were measured in enzyme treated samples under particular conditions, including when the enzyme concentration was 10 µg/mL and when samples diluted in 0.5% ascorbic acid with enzyme were incubated for 0.5 h at room temperature. When 1% sodium ascorbate (pH 3.8) was used as a diluent, incomplete deconjugation was observed and lower concentrations of monoglutamate folate were obtained with lower concentrations of the enzyme. Regardless of enzyme concentration, a half hour incubation period appeared to increase recovery of monoglutamate folate when 0.5% ascorbic acid was used as a diluent. The concentrations observed after the half hour incubation period in a diluent of 0.5% ascorbic acid were not distinguishable from concentrations observed in enzyme treated samples in 1% ascorbic acid without incubation.

In a larger number of samples (n=15) stored frozen as WBL (Table 2), there were no statistically significant differences in folate concentrations when samples were diluted in 1% ascorbic acid with 10 µg enzyme/mL WBL compared with 5 µg/mL (either no incubation or 0.5 h incubation; all P values were >0.2). The only significant differences were found for 5-methylTHF diglutamate and MeFox. A small amount of residual 5-methylTHF diglutamate was found even after the conventional 4 h incubation, while either of the enzyme treated conditions resulted in non-detectable 5-methylTHF diglutamate (P=0.007). MeFox was slightly but statistically significantly higher in 2 of the 3 enzyme treated conditions compared to the conventional 4 h incubation (P=0.046 for 10 µg enzyme/mL and P=0.009 for 5 µg enzyme/mL and 0.5 h incubation).
Comparison of samples stored frozen as whole blood or WBL

Thawed WBL was more stable than thawed whole blood for all forms of folate. Total folate in WBL (enzyme treated samples) did not change over an extended thaw period of 4 h (mean ± SD: 502 ± 238 at 0 h, 504 ± 235 at 1 h, 501 ± 231 at 4 h), while total folate concentration in thawed whole blood decreased approximately 10% over 1 h and 25% over 4 h (mean ± SD: 506 ± 230 at 0 h, 455 ± 232 at 1 h, 372 ± 203 at 4 h). At 0 h, there was no statistically significant difference between total folate from thawed WBL and thawed whole blood (P=0.663). Results were similar in conventionally prepared samples (data not shown). Figure 2 shows the effect of extended thaw time on various folate forms in enzyme treated samples. The effect of extended thaw time on 5-methylTHF in WBL and whole blood paralleled the effect for total folate, however in whole blood a larger portion of 5-methylTHF was lost in low non-methyl compared to high non-methyl folate samples (Figure 2, panels A and B). In contrast, MeFox concentration remained unchanged with extended thaw time (Figure 2, panels C and D). We also observed differences in whole blood samples with low vs. high non-methyl folate (Figure 2, panels E and F). High non-methyl folate concentrations decreased with longer thaw time (mean ± SD: 120 ± 34 nmol/L at 0 h vs. 46 ± 30 nmol/L at 4 h), while the opposite effect was observed in low non-methyl folate samples (mean ± SD: 18 ± 10 nmol/L at 0 h vs. 57 ± 27 nmol/L at 4 h). There were no statistically significant differences in any of the folate forms between samples stored as WBL and whole blood when thaw time was minimized (0 h after thawing), except for non-methyl folate in the low non-methyl folate samples, which was marginally higher in thawed whole blood (P=0.024).

Comparison of RBC folate measured directly or calculated from WBL concentrations

Plasma total folate concentrations in 4 blood donors showed positive skew with a median (range) of 45.0 (21.6 to 95.1) nmol/L (Table 3). The 2 donors with the highest plasma total folate concentrations also had the highest plasma folic acid concentrations of 6.0 and 68.8 nmol/L. Folic acid was detected in WBL only in the 2 samples with the highest plasma folic acid concentrations and was not detected in any of the washed RBC samples. For each of the donors, the directly measured values in washed packed RBCs were
lower compared with calculated values using WBL folate, plasma folate, and hematocrit values (conventional procedure) for RBC total folate (13% on average), RBC 5-methylTHF (17% on average), and RBC non-methyl folate (37% on average). MeFox values for 3 samples compared well between the direct and conventional procedures, however resulted in a 20% difference for 1 sample.

Discussion

These results demonstrate for the first time a procedure that eliminates the requirement for a lengthy sample incubation time in order to measure individual folate vitamers as monoglutamate folate. Previous research from our laboratory has shown that a 4-h incubation period in 1% ascorbic acid is required prior to HPLC-MS/MS analysis of folates as monoglutamates (15). In contrast, the microbiologic assay responds to di- and tri-glutamate folates, requiring only a 30-min incubation or exposure to a single freeze-thaw cycle (5). These differences in incubation time lead to uncertainty in the sources of bias existing between the 2 procedures due the lability of folate forms. Even though the HPLC-MS/MS procedure implemented in our laboratory utilizes stable isotopes, thereby providing an adjustment for losses of folate over the incubation period, the stable isotopes may not completely adjust for losses of folate forms. By minimizing the incubation time of the samples prior to analysis by HPLC-MS/MS we are able to eliminate potential biases caused by losses of labile folate forms during this process, warranting further investigation into the comparison between HPLC-MS/MS and the microbiologic assay.

We previously reported on the stability of folate during prolonged thawing of whole blood samples (16). In the current study we have expanded on these results by including more samples (n=15 vs. n=4), using updated methodology that separates 5-formylTHF and MeFox, and including a comparison with WBL. We observed similar reductions of approximately 25% for 5-methylTHF and total folate over a similar time period compared to the previous report. An additional and considerably important finding unable to be observed in the previous study was the discrepant changes in high and low non-methyl folate-containing samples occurring during extended thaw time. At 0 h, a clear distinction was observed between low non-methyl folate samples (mean ± SD; 18 ± 10) and high non-methyl samples (120 ± 34).
However, with increasing thaw time the mean and SD of the low non-methyl folate samples expanded and the mean of the high non-methyl samples decreased until there was a considerable overlap (57 ± 27 and 46 ± 30, respectively). This observation together with the decrease in 5-methylTHF concentration in whole blood samples with low non-methyl folate is consistent with a previous report showing that freezing and thawing resulted in a change of folate vitamer distribution from methyl to non-methyl folate, albeit in samples from the rat (17). We did not genotype individuals in the current study, however, it is likely that those classified as high non-methyl folate were of the T/T genotype for the 5,10-methenylTHF reductase C677T polymorphism. These results show a clear potential to misrepresent folate vitamer distribution in RBCs when stored whole blood samples are not handled adequately. In contrast, WBL was considerably stable over a period of 4 h for all folate forms and should be considered for storage preferable to whole blood because the sample handling during thawing is less sensitive to changes in methyl and non-methyl folates.

Total folate concentrations were approximately 15% lower on average in packed RBCs compared with concentrations calculated from WBL using the conventional procedure. While the direct measurement of RBC folates, made possible through addition of exogenous enzyme seems advantageous, several logistical concerns exist in obtaining a RBC sample that may account for some of the discrepancy observed. We separated washed RBC from PBS by centrifugation at a force of 1,811 g for 15 min. Under similar centrifugal conditions, in previous studies, approximately 7% of the volume of unwashed red cells consisted of trapped plasma (18-20). Increasing the centrifugal force sufficiently to obtain close to 100% packing of RBCs is not possible because it leads to cell rupturing. However, if the hemoglobin concentration is determined in the same RBC lysate in which the folate forms are measured, folate results can be normalized to hemoglobin, which makes the result independent of any residual moisture. This would be similar to the procedure of determining hemoglobin-folate in dried blood spots by the microbiologic assay (21). Another concern is that centrifugation may result in a gradient of denser, older cells at the bottom of the packed cell volume and less dense, and younger cells at the top (22). Folate concentrations vary substantially with age, with older RBCs containing significantly lower concentrations...
than younger cells (22). This complicates the process of obtaining a homogeneous sample of RBC representative of average RBC folate concentration, but could be solved by using the entire yield of washed RBCs to generate a RBC lysate rather than taking an aliquot of washed RBCs. Both of these approaches will have to be tested and validated in a larger set of samples.

Large inaccuracies in calculated RBC concentrations of an analyte from WBL values are an issue when the plasma concentration is large, exceeding that of the RBC concentration (23). In contrast, the calculation of total RBC folate concentration is subject only to minor amounts of bias due to the approximate 10-fold higher concentration of folate in RBC compared with plasma. This holds true also for most minor forms of folate given that 5-formylTHF and 5,10-methenylTHF are infrequently detected and THF is close to the LOD in the majority of serum samples (24). One exception may be in individuals consuming high-dose folinic acid (5-formylTHF) supplements, where high serum 5-formylTHF concentration increases the likelihood of inaccurate calculation of RBC non-methyl folate. The use of the calculation for folic acid presents misleading non-zero values as the occurrence of unmetabolized folic acid in blood is limited to serum (25). This was confirmed in our small sample set, where we did not detect folic acid in washed RBC samples. Therefore, calculation of folic acid in RBC is not recommended.

This article is to our knowledge the first report that uses a recombinant human GGH protein to deglutamylate folate polyglutamates in RBCs for subsequent measurement by HPLC-MS/MS. The use of recombinant plant GGH from the Arabidopsis thaliana species has been utilized previously for folate deglutamylation in complex food matrices (26). We obtained a small quantity of the plant GGH as a gift from the investigators and conducted a few pilot experiments using WBL samples. The plant GGH requires a higher pH (6.0) for deglutamylation compared to the human GGH (pH 4.5). The results were encouraging and similar to what we report in this article in terms of enzyme conditions and speed of deglutamylation. However, the non-methyl folate concentration (THF) appeared to increase with increasing length of incubation, which we did not observe with the human GGH. It is possible that differing GGH enzymes may result in changes to the distribution of folate forms (17). A comparison of
the folate vitamer distribution obtained using other sources of GGH, for example from rat plasma, human
plasma, or chicken pancreas, is warranted. Ultimately, our preference was to have a commercial source of
a recombinant GGH protein, ideally of human origin. The cost of the additional exoGGH (approximately
US$ 2/sample) has to be weighed with the advantages of time savings during sample processing and the
potential to more accurately characterize which folate forms are present in RBCs if washed RBCs are
used. However, given the tendency of pH-dependent interconversions of non-methyl folate forms, one
should not interpret the subcomponents (5-formylTHF, 5,10-methenylTHF, and THF) of this folate group
regardless of whether WBL or washed RBCs are used.

In summary, we have determined that addition of an exogenous source of human GGH eliminates
the requirement for incubation of WBL prior to analysis of folate monoglutamates by HPLC-MS/MS. The
enzyme works efficiently in samples stored frozen both as whole blood and WBL, although it is
preferable to process samples as WBL if possible. Direct measurement of RBC folate is possible using
exoGGH and eliminates the requirement for analysis of hematocrit and serum folate concentrations,
however, current protocols to isolate RBC may be inadequate. Further work using a larger number of
samples is required to establish the comparability of results determined using conventional procedures
with results produced using exogenous recombinant human GGH.
Acknowledgments

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References


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1 Values are reported either as mean ± SD or median (25th–75th percentile), n=4. AA, ascorbic acid; diglu, diglutamate; MeFox, pyrazino-s-triazine derivative of a 5-methylTHF oxidation product; monoglu, monoglutamate; NaAsc, sodium ascorbate; RT, room temperature; 5-methylTHF, 5-methyltetrahydrofolate

2 1% sodium ascorbate solution was pH adjusted with HCl to 3.8, resulting in a whole blood lysate pH equivalent with use of 0.5% AA of 4.25

3 Conventional prepared samples were incubated up to 4 h and enzyme treated samples were incubated up to 0.5 h

4 Sum of 5-methylTHF, 5-methylTHF diglu, MeFox, 5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate

5 Sum of 5-methylTHF, MeFox, 5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate

6 Includes n=2 individual samples with high non-methyl folate; non-methyl folate is the sum of 5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate

7 Includes n=2 individual samples with low non-methyl folate; non-methyl folate is the sum of 5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate
TABLE 2. Whole blood folate concentrations in samples prepared from thawed whole blood lysate with varying exogenous enzyme concentrations

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Time</th>
<th>1% AA, 37°C Conventional</th>
<th>1% AA, RT 10</th>
<th>1% AA, RT 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total folate</td>
<td>0</td>
<td>515 ± 234</td>
<td>514 ± 242</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 / 0.5</td>
<td>512 ± 246</td>
<td>514 ± 240</td>
<td></td>
</tr>
<tr>
<td>Total monoglu folate</td>
<td>0</td>
<td>512 ± 234</td>
<td>511 ± 242</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 / 0.5</td>
<td>499 ± 241</td>
<td>511 ± 240</td>
<td></td>
</tr>
<tr>
<td>5-methylTHF</td>
<td>0</td>
<td>380 ± 213</td>
<td>381 ± 221</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 / 0.5</td>
<td>373 ± 219</td>
<td>380 ± 218</td>
<td></td>
</tr>
<tr>
<td>5-methylTHF diglu</td>
<td>0</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td></td>
<td>4 / 0.5</td>
<td>7 (7–13)</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>MeFox</td>
<td>0</td>
<td>69 ± 35</td>
<td>68 ± 36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 / 0.5</td>
<td>65 ± 36</td>
<td>69 ± 37</td>
<td></td>
</tr>
<tr>
<td>Non-methyl folate</td>
<td>0</td>
<td>119 ± 34</td>
<td>120 ± 41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 / 0.5</td>
<td>115 ± 31</td>
<td>118 ± 34</td>
<td></td>
</tr>
<tr>
<td>Non-methyl folate</td>
<td>0</td>
<td>12 ± 5</td>
<td>12 ± 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 / 0.5</td>
<td>13 ± 5</td>
<td>13 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are reported either as mean ± SD or median (25th–75th percentile), n=15. AA, ascorbic acid; diglu, diglutamate; MeFox, pyrazino-s-triazine derivative of a 5-methylTHF oxidation product; monoglu, monoglutamate; RT, room temperature; 5-methylTHF, 5-methyltetrahydrofolate

2 Conventional prepared samples were incubated for 4 h only and enzyme treated samples were incubated for up to 0.5 h

3 Sum of 5-methylTHF, 5-methylTHF diglu, MeFox, 5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate

4 Sum of 5-methylTHF, MeFox, 5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate

5 Includes n=7 individual samples with high non-methyl folate; non-methyl folate is the sum of 5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate

6 Includes n=8 individual samples with low non-methyl folate; non-methyl folate is the sum of 5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate
TABLE 3. Folate concentrations in plasma and red blood cells, either calculated from whole blood lysate folate, serum folate and hematocrit (conventional procedure), or measured directly in washed red blood cells

<table>
<thead>
<tr>
<th>Donor</th>
<th>5-MethylTHF</th>
<th>MeFox</th>
<th>Non-methyl</th>
<th>Folic acid</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma folate (nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>17.9</td>
<td>1.00</td>
<td>1.44</td>
<td>1.3</td>
<td>21.6</td>
</tr>
<tr>
<td>2</td>
<td>18.7</td>
<td>1.45</td>
<td>1.04</td>
<td>0.4</td>
<td>21.6</td>
</tr>
<tr>
<td>3</td>
<td>57.6</td>
<td>2.01</td>
<td>2.75</td>
<td>6.0</td>
<td>68.3</td>
</tr>
<tr>
<td>4</td>
<td>22.1</td>
<td>3.43</td>
<td>0.81</td>
<td>68.8</td>
<td>95.1</td>
</tr>
<tr>
<td>Mean</td>
<td>29.1</td>
<td>1.97</td>
<td>1.51</td>
<td>19.1</td>
<td>51.6</td>
</tr>
<tr>
<td>Median</td>
<td>20.4</td>
<td>1.73</td>
<td>1.24</td>
<td>3.65</td>
<td>45.0</td>
</tr>
<tr>
<td>WBL folate measured (nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>21.8</td>
<td>4.01</td>
<td>0.92</td>
<td>&lt;LOD</td>
<td>n.c.</td>
</tr>
<tr>
<td>2</td>
<td>23.9</td>
<td>4.31</td>
<td>0.97</td>
<td>&lt;LOD</td>
<td>n.c.</td>
</tr>
<tr>
<td>3</td>
<td>59.1</td>
<td>9.23</td>
<td>1.58</td>
<td>0.45</td>
<td>n.c.</td>
</tr>
<tr>
<td>4</td>
<td>22.9</td>
<td>4.59</td>
<td>0.87</td>
<td>3.73</td>
<td>n.c.</td>
</tr>
<tr>
<td>Mean</td>
<td>31.9</td>
<td>5.53</td>
<td>1.08</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>23.4</td>
<td>4.45</td>
<td>0.94</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>RBC lysate folate measured (nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>25.3</td>
<td>6.01</td>
<td>0.73</td>
<td>&lt;LOD</td>
<td>n.c.</td>
</tr>
<tr>
<td>2</td>
<td>24.6</td>
<td>5.63</td>
<td>0.80</td>
<td>&lt;LOD</td>
<td>n.c.</td>
</tr>
<tr>
<td>3</td>
<td>63.0</td>
<td>13.3</td>
<td>1.14</td>
<td>&lt;LOD</td>
<td>n.c.</td>
</tr>
<tr>
<td>4</td>
<td>22.6</td>
<td>6.32</td>
<td>0.66</td>
<td>&lt;LOD</td>
<td>n.c.</td>
</tr>
<tr>
<td>Mean</td>
<td>33.9</td>
<td>7.82</td>
<td>0.83</td>
<td>&lt;LOD</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>25.0</td>
<td>6.16</td>
<td>0.76</td>
<td>&lt;LOD</td>
<td></td>
</tr>
<tr>
<td>RBC folate calculated (nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>634</td>
<td>121</td>
<td>25.5</td>
<td>-1.35</td>
<td>779</td>
</tr>
<tr>
<td>2</td>
<td>599</td>
<td>111</td>
<td>23.9</td>
<td>0.20</td>
<td>734</td>
</tr>
<tr>
<td>3</td>
<td>1435</td>
<td>233</td>
<td>36.9</td>
<td>3.53</td>
<td>1709</td>
</tr>
<tr>
<td>4</td>
<td>661</td>
<td>134</td>
<td>25.2</td>
<td>-8.33</td>
<td>812</td>
</tr>
<tr>
<td>Mean</td>
<td>832</td>
<td>150</td>
<td>27.9</td>
<td>-1.49</td>
<td>1008</td>
</tr>
<tr>
<td>Median</td>
<td>648</td>
<td>127</td>
<td>25.3</td>
<td>-0.58</td>
<td>796</td>
</tr>
<tr>
<td>RBC folate measured (nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>532</td>
<td>126</td>
<td>15.3</td>
<td>&lt;LOD</td>
<td>674</td>
</tr>
<tr>
<td>2</td>
<td>516</td>
<td>118</td>
<td>16.8</td>
<td>&lt;LOD</td>
<td>652</td>
</tr>
<tr>
<td>3</td>
<td>1322</td>
<td>280</td>
<td>23.9</td>
<td>&lt;LOD</td>
<td>1626</td>
</tr>
<tr>
<td>4</td>
<td>475</td>
<td>133</td>
<td>13.9</td>
<td>&lt;LOD</td>
<td>622</td>
</tr>
<tr>
<td>Mean</td>
<td>711</td>
<td>164</td>
<td>17.5</td>
<td>&lt;LOD</td>
<td>893</td>
</tr>
<tr>
<td>Median</td>
<td>524</td>
<td>129</td>
<td>16.1</td>
<td>&lt;LOD</td>
<td>663</td>
</tr>
<tr>
<td>Difference RBC folate measured to RBC folate calculated (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-16</td>
<td>4</td>
<td>-40</td>
<td>n.c.</td>
<td>-14</td>
</tr>
<tr>
<td>2</td>
<td>-14</td>
<td>7</td>
<td>-29</td>
<td>n.c.</td>
<td>-11</td>
</tr>
<tr>
<td>3</td>
<td>-8</td>
<td>20</td>
<td>-35</td>
<td>n.c.</td>
<td>-5</td>
</tr>
<tr>
<td>4</td>
<td>-28</td>
<td>-1</td>
<td>-45</td>
<td>n.c.</td>
<td>-23</td>
</tr>
<tr>
<td>Mean</td>
<td>-17</td>
<td>7</td>
<td>-37</td>
<td></td>
<td>-13</td>
</tr>
</tbody>
</table>
Hematocrit values were: donor #1: 36%; donor #2: 42%; donor #3: 43%; donor #5: 36%. McFox, pyrazino-s-triazine derivative of a 5-methylTHF oxidation product; n.c., not calculated; RBC, red blood cell; WBL, whole blood lysate; 5-methylTHF, 5-methyltetrahydrofolate

1 Whole blood diluted 1/11 in 1% ascorbic acid
2 Washed packed RBC diluted 1/21 in 0.5% ascorbic acid
3 Formula used: ((WBL * 11) – plasma folate * (1 – hematocrit/100)) / (hematocrit/100)
4 Formula used: (RBC lysate * 21)
5 Calculated as the sum of individual folate forms for plasma folate and RBC folate
Figure Legends

**FIGURE 1.** Schematic diagram of the experimental procedures. AA, ascorbic acid; exoGGH, exogenously added γ-glutamyl hydrolase; NaAsc, sodium ascorbate; RBC, red blood cell; RT, room temperature; SPE, solid-phase extraction; WB, whole blood; WBL, whole blood lysate.

**FIGURE 2.** Effect of extended thaw time on thawed whole blood (represented by dotted line) and whole blood lysate (represented by solid line) concentrations of 5-methylTHF, MeFox, and non-methyl folate. Values (means ± SD, nmol/L) are from enzyme treated samples (5 µg/mL); n=8 samples with low non-methyl folate concentration (panels A, C and E) and n=7 samples with high non-methyl folate concentration (panels B, D and F). Asterisks represent a significant difference between thawed whole blood and thawed whole blood lysate (*P* <0.05). MeFox, pyrazino-s-triazine derivative of a 5-methylTHF oxidation product; non-methyl folate is the sum of 5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate; 5-methylTHF, 5-methyltetrahydrofolate.
**Experiment 1:**
Optimal functioning of exoGGH

- **a) n=4 WB samples**
  - WB aliquots
  - Aliquots frozen in vapor phase liquid nitrogen for ~1 y
  - Thawing for 30 min at RT
  - Dilute WB 1/11 to WBL
  - Different diluents
    - 1% AA diluent
      - No exoGGH (conventional)
      - 4 h at 37°C
    - 1% AA diluent
      - 10 µg/mL exoGGH
      - 0 h at 37°C
    - 1% NaAsc diluent
      - 10, 5, 2, 1 µg/mL exoGGH
      - 0 h and 0.5 h at 37°C
    - 0.5% AA diluent
      - 10, 5, 2, 1 µg/mL exoGGH
      - 0 h and 0.5 h at 37°C
  - Overnight storage at -70°C
  - SPE and HPLC-MS/MS analysis

- **b) n=15 WB samples**
  - WB aliquots
  - Aliquots frozen in vapor phase liquid nitrogen for ~1 y
  - Thawing for 30 min at RT
  - Dilute WB 1/11 to WBL
  - WBL in 1% AA diluent
  - No exoGGH (conventional)
    - 4 h at 37°C
  - 1% AA diluent
    - 10 µg/mL exoGGH
    - 0 h at RT
  - 1% NaAsc diluent
    - 5 µg/mL exoGGH
    - 0 h and 0.5 h at RT
  - 0.5% AA diluent
    - 10, 5, 2, 1 µg/mL exoGGH
    - 0 h and 0.5 h at 37°C
  - SPE and HPLC-MS/MS analysis

**Experiment 2:**
WB vs. WBL stored frozen

- **n=15 WB samples**
  - WB aliquots
  - Aliquots frozen in vapor phase liquid nitrogen for ~1 y
  - Thawing for 30 min at RT, then extended thawing for 0 h, 1 h, and 4 h
  - Dilute WB 1/11 in 1% AA to WBL
  - WBL in 1% AA diluent
  - No exoGGH (conventional)
    - 4 h at 37°C
  - 10 µg/mL exoGGH
    - 0 h at RT
  - 5 µg/mL exoGGH
    - 0 h and 0.5 h at RT
  - WBL
    - 5 µg/mL exoGGH
    - 20 min at RT
  - SPE and HPLC-MS/MS analysis

**Experiment 3:**
Measured vs. calculated RBC folate

- **n=4 WB samples**
  - Determine HCT
  - WB aliquots
  - Centrifuge at 1,811 g and 4°C for 15 min
  - Plasma aliquots
    - Washed RBCs
  - Aliquots frozen at -70°C for ~1 mo
  - Thawing for 30 min at RT
  - Dilute WB 1/11 to WBL
  - WB aliquots
    - Dilute RBC 1/21 to RBC lysate
    - 1% AA diluent
      - No exoGGH (conventional)
      - 4 h at 37°C
    - 0.5% AA diluent
      - 10 µg/mL exoGGH
      - 0 h at RT
    - 0.5% AA diluent
      - 5 µg/mL exoGGH
      - 20 min at RT
  - SPE and HPLC-MS/MS analysis
  - Over night storage at -70°C