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Research Methodology and Study Design

# 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

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## Abstract

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

**Objective:** We explored whether the 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 for 4 h at 37°C. Alternatively, we added exoGGH to WBL at varying concentrations (1–10 μg/mL) and incubation times (0–90 min). We also investigated modifications to the sample diluent (pH, ascorbic acid compared with sodium ascorbate, and ascorbate concentration). Finally, we tested the effect of the enzyme in different sample types: WBL from frozen whole blood compared with frozen WBL or with frozen washed RBCs. Samples ($n \leq 15$/experiment) were analyzed by HPLC-MS/MS for 6 folate monoglutamates and 5-methyltetrahydrofolate diglutamate.

**Results:** Optimal deconjugation of folate polyglutamates was achieved by using 1% ascorbic acid and 5 μg 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, the extended thaw time of whole blood resulted in 5-methyltetrahydrofolate loss and unacceptable changes to the non-methyl folate concentration. Total folate (with exoGGH) measured in washed RBCs was ~15% lower than RBC folate calculated from WBL concentrations (conventional deglutamylation).

**Conclusions:** The 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 RBCs may be due to inadequate packing of RBCs, among other unidentified factors. A larger study is required to confirm the lack of differences in folate concentrations determined with and without the use of exoGGH. *Curr Dev Nutr* 2018;2:nzx003.

## Introduction

Both serum and 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 and is confounded by fasting status, and there is no established cutoff 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 the risk of neural tube defects in women of

**Keywords:** deconjugation, human recombinant protein, monoglutamate, diglutamate, RBC folate, washed erythrocytes



Manuscript received September 15, 2017. Initial review completed October 17, 2017. Revision accepted November 19, 2017. Published online November 22, 2017.
The authors reported no funding received for this study.
Author disclosures: RAS, ZF, and CMP, no conflicts of interest.

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

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Abbreviations used: exoGGH, exogenous $\gamma$-glutamyl hydrolase; GGH, $\gamma$-glutamyl hydrolase; HPLC-MS/MS, HPLC–tandem mass spectrometry; LOD, limit of detection; MeFox, pyrazino-s-triazine derivative of 4$\alpha$-hydroxy-5-methylTHF; SPE, solid-phase extraction; THF, tetrahydrofolate; WBL, whole-blood lysate; 5-formylTHF, 5-formyltetrahydrofolate; 5-methylTHF, 5-methyltetrahydrofolate; 5-methylTHF diglutamate, 5-methyltetrahydropteroyldi-$\gamma$-L-glutamic acid; 5,10-methenylTHF, 5,10-methenyltetrahydrofolate.

reproductive age (2). An RBC folate concentration <305 nmol/L is often applied as a cutoff for the risk of megaloblastic anemia (3), whereas 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 no. 3.4.19.9) before 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 microbiological assay, which responds to folates with ≤3 glutamate residues (4). Analysis of folate by HPLC–tandem MS (HPLC-MS/MS) requires longer incubation times at 37°C for ≤4 h to recover monoglutamate folates (6); however, long incubation times increase the likelihood of degradation of labile folate forms. Although the microbiological 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 with the microbiological assay. Although HPLC-MS/MS procedures that use 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 (WBLs) by 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 that used exoGGH (rat plasma, human plasma, or chicken pancreas) in the measurement of human tissue samples, including in RBCs (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 [(6*S*)-5-methylTHF, folic acid, pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF (MeFox), (6*S*)-5-formyltetrahydrofolate (5-formylTHF), (6*S*)-tetrahydrofolate (THF), and (6*R*)-5,10-methenyltetrahydrofolate (5,10-methenylTHF)] together with their respective $^{13}C_5$-labeled analogs were purchased from Merck & Cie. (6*R*,*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 GGH 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 ≥10 min (**Figure 1**). WBL was prepared by dispensing 2.5 mL whole blood into 25 mL 1% ascorbic acid, mixing on a vortex, and placing 1-mL aliquots 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) with whole blood (stored frozen; WBL prepared before analysis) ($n = 15$ in experiment 2).

To test the direct measurement of RBC folate from washed, packed RBCs, we obtained additional EDTA whole-blood samples ($n = 5$; experiment 3). The blood collection tubes were gently mixed for ≥10 min upon arrival before filling a microhematocrit tube and centrifuging it at 12,000 × *g* at room temperature 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 cryovial tubes, and the remainder of the blood was centrifuged at 1811 × *g* at 4°C for 15 min. EDTA plasma (1 mL) was dispensed into cryovial tubes and an approximately equivalent volume of cold, refrigerated PBS was added to the RBCs in the blood collection tubes. The tubes were centrifuged again at 1811 × *g* at 4°C for 15 min and the PBS was discarded. This step was repeated 2 more times to isolate the RBCs before dispensing washed, packed RBCs 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 by using $^{13}C_5$-labeled 5-methylTHF monoglutamate (*m/z* 465/313) as an internal standard. A 5-point calibration curve ranging from 1 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 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 exoGGH/mL; 1% sodium ascorbate (pH 3.8) with either 10, 5, 2, or 1 μg exoGGH/mL; and 0.5% ascorbic acid with either 10, 5, 2, or 1 μg exoGGH/mL. WBL samples (150 μL) were

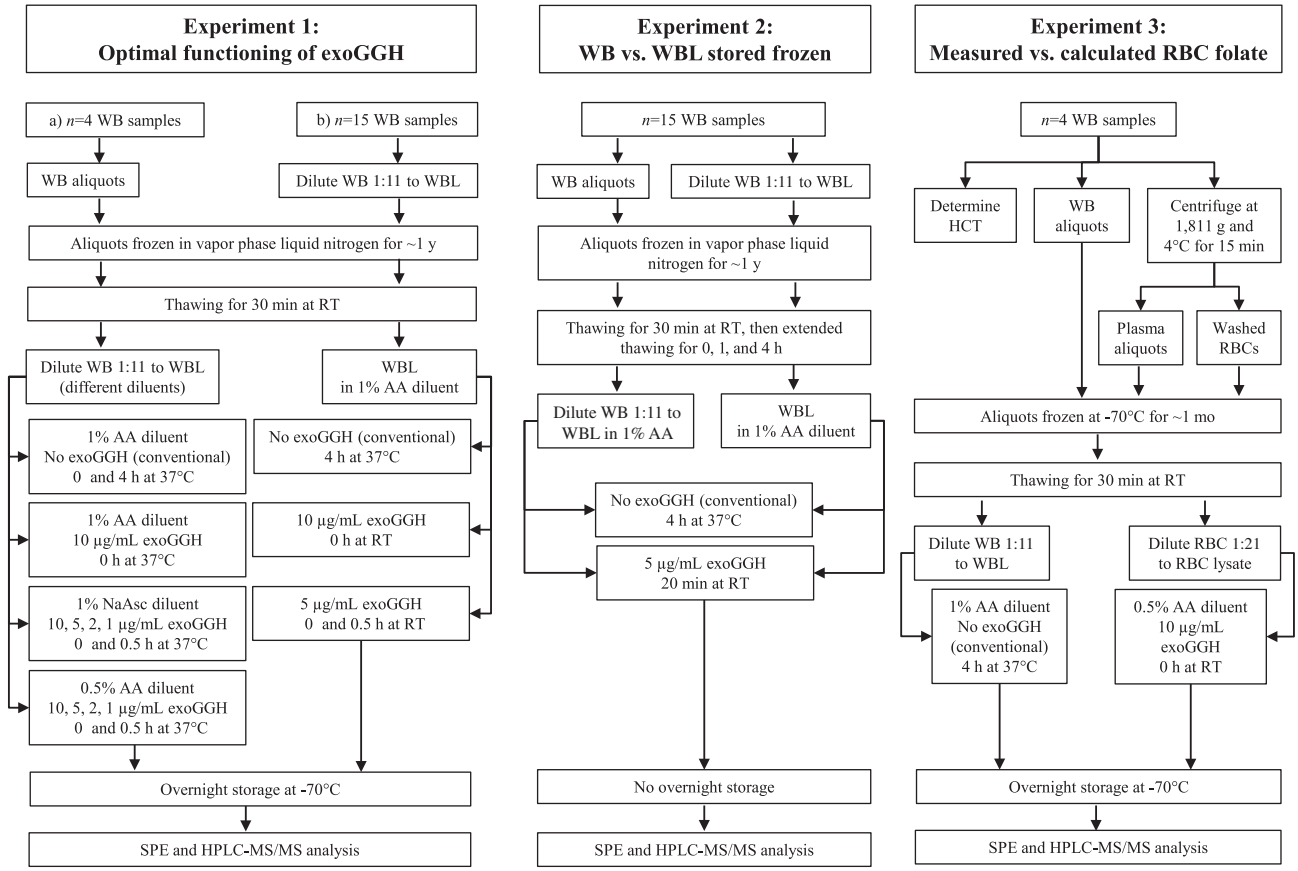


**FIGURE 1** Schematic diagram of the experimental procedures. AA, ascorbic acid; exoGGH, exogenously added $\gamma$-glutamyl hydrolase; HCT, hematocrit; HPLC-MS/MS, HPLC–tandem MS; NaAsc, sodium ascorbate; RT, room temperature; SPE, solid-phase extraction; WB, whole blood; WBL, whole blood lysate.

incubated with internal standard (60 µL) containing each of the $^{13}C_5$-labeled folate analogs 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 exoGGH/mL (equivalent to 10 µg enzyme/mL WBL), 12.5 µg exoGGH/mL (equivalent to 5 µg enzyme/mL WBL), or no added enzyme (conventional). Samples were incubated for 0 h (10 µg and 5 µg enzyme-treated samples/mL), for 0.5 h at room temperature (5 µg enzyme-treated samples/mL), and for 4 h at 37°C (conventionally prepared samples).

After their respective incubation periods, all of the samples were stored frozen overnight before undergoing reverse-phase solid-phase extraction (SPE) before 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 an extended thaw time for an additional 0, 1, and 4 h (samples were removed from frozen storage at staggered intervals to allow experimental set-up at a single 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 by using both the conventional procedure and the procedure that used 5 µg enzyme/mL (25 µg exoGGH/mL added to the internal standard mixture). Conventionally prepared samples were incubated for 4 h at 37°C before SPE and analysis by HPLC-MS/MS, whereas enzyme-treated samples were processed for SPE after 20 min of 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 RBCs 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 of the samples were frozen overnight before 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 concentrations for each folate form, except when the proportion of results less than the limit of detection (LOD) exceeded 40% (for 5-methylTHF diglutamate), in which case we calculated the median and IQR (25th–75th percentile). We calculated the HPLC-MS/MS total folate as the sum of the individual folate forms by using an imputed value of LOD divided by the square root of 2 for a folate form result less than the 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, the 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 in which enzyme was added. An exception was noted for non-methyl folate (in samples with high non-methyl folate), in which 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

**TABLE 1** Whole-blood folate concentrations in samples prepared from thawed whole blood with varying incubation time and temperature, diluent composition, and exogenous enzyme concentration[1]

| | Folate concentration, nmol/L | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Analyte | 37°C | RT | | | | | | | | |
| and | 1% AA | 1% AA | 1% NaAsc[3] | | | | 0.5% AA | | | |
| time,[2] h | Conv. | 10 μg/mL | 10 μg/mL | 5 μg/mL | 2 μg/mL | 1 μg/mL | 10 μg/mL | 5 μg/mL | 2 μg/mL | 1 μg/mL |
| Total folate[4] | | | | | | | | | | |
| 0 | 299 ± 112 | 437 ± 124 | 422 ± 118 | 384 ± 110 | 370 ± 110 | 357 ± 107 | 420 ± 110 | 416 ± 124 | 424 ± 120 | 411 ± 100 |
| 4/0.5 | 441 ± 140 | — | 433 ± 110 | 415 ± 110 | 415 ± 117 | 377 ± 110 | 445 ± 126 | 439 ± 128 | 422 ± 113 | 395 ± 97 |
| Total folate monoglu[5] | | | | | | | | | | |
| 0 | 237 ± 85 | 434 ± 134 | 419 ± 118 | 374 ± 103 | 345 ± 99 | 319 ± 90 | 418 ± 110 | 413 ± 124 | 422 ± 120 | 408 ± 00 |
| 4/0.5 | 431 ± 138 | — | 430 ± 110 | 411 ± 109 | 405 ± 114 | 356 ± 102 | 442 ± 126 | 436 ± 128 | 419 ± 113 | 392 ± 97 |
| 5-MethylTHF | | | | | | | | | | |
| 0 | 186 ± 87 | 296 ± 139 | 292 ± 137 | 268 ± 121 | 259 ± 115 | 244 ± 103 | 284 ± 123 | 280 ± 140 | 289 ± 140 | 278 ± 116 |
| 4/0.5 | 303 ± 151 | — | 293 ± 130 | 290 ± 131 | 296 ± 140 | 266 ± 117 | 299 ± 143 | 298 ± 144 | 282 ± 128 | 305 ± 152 |
| 5-MethylTHF diglu | | | | | | | | | | |
| 0 | 65 (39–88) | <LOD | <LOD | 6 (<LOD–13) | 21 (19–27) | 36 (26–48) | <LOD | <LOD | <LOD | <LOD |
| 4/0.5 | 8 (7–11) | — | <LOD | <LOD | 8 (7–11) | 20 (16–24) | <LOD | <LOD | <LOD | <LOD |
| MeFox | | | | | | | | | | |
| 0 | 22 ± 11 | 65 ± 35 | 61 ± 31 | 48 ± 26 | 38 ± 20 | 33 ± 17 | 65 ± 33 | 63 ± 32 | 64 ± 32 | 63 ± 30 |
| 4/0.5 | 61 ± 33 | — | 63 ± 32 | 57 ± 30 | 48 ± 25 | 41 ± 22 | 68 ± 36 | 65 ± 35 | 64 ± 34 | 68 ± 35 |
| Non-methyl folate[6] | | | | | | | | | | |
| 0 | 49 ± 23 | 132 ± 42 | 118 ± 25 | 102 ± 20 | 84 ± 19 | 71 ± 11 | 123 ± 36 | 125 ± 32 | 126 ± 36 | 120 ± 33 |
| 4/0.5 | 117 ± 27 | — | 134 ± 32 | 115 ± 18 | 110 ± 22 | 87 ± 23 | 135 ± 38 | 131 ± 34 | 130 ± 33 | 131 ± 35 |
| Non-methyl folate[7] | | | | | | | | | | |
| 0 | 8 ± 1 | 15 ± 3 | 14 ± 2 | 13 ± 2 | 12 ± 1 | 11 ± 1 | 15 ± 2 | 15 ± 2 | 13 ± 0 | 14 ± 3 |
| 4/0.5 | 17 ± 7 | — | 15 ± 2 | 13 ± 0 | 12 ± 1 | 11 ± 1 | 16 ± 2 | 15 ± 3 | 17 ± 5 | 15 ± 4 |

[1]Values are means ± SDs or medians (25th–75th percentiles); $n = 4$. AA, ascorbic acid; Conv., conventional; diglu, diglutamate; MeFox, pyrazino-*s*-triazine derivative of 4-$\alpha$-hydroxy-5-methylTHF; monoglu, monoglutamate; NaAsc, sodium ascorbate; RT, room temperature; 5-methylTHF, 5-methyltetrahydrofolate.
[2]Conventionally prepared samples were incubated ≤4 h, and enzyme-treated samples were incubated ≤0.5 h.
[3]1% Sodium ascorbate solution was pH-adjusted with HCl to 3.8, resulting in a whole-blood lysate pH of 4.2, equivalent to the use of 0.5% AA.
[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 incubation time and temperature, and exogenous enzyme concentrations[1]

| | Folate concentration, nmol/L | | |
|---|---|---|---|
| | 1% AA (37°C) | 1% AA (RT) | |
| Analyte and time,[2] h | Conventional | 10 μg/mL | 5 μg/mL |
| Total folate,[3] | | | |
| 0 | — | 515 ± 234 | 514 ± 242 |
| 4/0.5 | 512 ± 246 | — | 514 ± 240 |
| Total monoglu folate[4] | | | |
| 0 | — | 512 ± 234 | 511 ± 242 |
| 4/0.5 | 499 ± 241 | — | 511 ± 240 |
| 5-MethylTHF | | | |
| 0 | — | 380 ± 213 | 381 ± 221 |
| 4/0.5 | 373 ± 219 | — | 380 ± 218 |
| 5-MethylTHF diglu | | | |
| 0 | — | <LOD | <LOD |
| 4/0.5 | 7 (7–13) | — | <LOD |
| MeFox | | | |
| 0 | — | 69 ± 35 | 68 ± 36 |
| 4/0.5 | 65 ± 36 | — | 69 ± 37 |
| Non-methyl folate[5] | | | |
| 0 | — | 119 ± 34 | 120 ± 41 |
| 4/0.5 | 115 ± 31 | — | 118 ± 34 |
| Non-methyl folate[6] | | | |
| 0 | — | 12 ± 5 | 12 ± 4 |
| 4/0.5 | 13 ± 5 | — | 13 ± 5 |

[1] Values are means ± SDs or medians (25th–75th percentiles); $n = 15$. AA, ascorbic acid; diglu, diglutamate; LOD, limit of detection; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; monoglu, monoglutamate; RT, room temperature; 5-methylTHF, 5-methyltetrahydrofolate.
[2] Conventionally prepared samples were incubated for 4 h only, and enzyme-treated samples were incubated for ≤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.

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 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, whereas both of the enzyme-treated conditions resulted in nondetectable 5-methylTHF diglutamate ($P = 0.007$). MeFox was slightly but significantly higher in 2 of the 3 enzyme-treated conditions compared with 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 nmol/L at 0 h, 504 ± 235 nmol/L at 1 h, and 501 ± 231 nmol/L at 4 h), whereas total folate concentration in thawed whole blood decreased by ∼10% over 1 h and by 25% over 4 h (mean ± SD: 506 ± 230 nmol/L at 0 h, 455 ± 232 nmol/L at 1 h, and 372 ± 203 nmol/L at 4 h). At 0 h, there was no 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 than in high non-methyl folate samples (Figure 2A, B). In contrast, MeFox concentrations remained unchanged with extended thaw time (Figure 2C, D). We also observed differences in whole-blood samples with low compared with high non-methyl folate (Figure 2E, F). High non-methyl folate concentrations decreased with longer thaw time (mean ± SD: 120 ± 34 nmol/L at 0 h compared with 46 ± 30 nmol/L at 4 h), whereas the opposite effect was observed in low non-methyl folate samples (mean ± SD: 18 ± 10 nmol/L at 0 h compared with 57 ± 27 nmol/L at 4 h). There were no 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 (21.6–95.1) 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 than calculated values with the use of 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, they resulted in a 20% difference for 1 sample.

## Discussion

These results show, for the first time to our knowledge, 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 before HPLC-MS/MS analysis of folates as monoglutamates (15). In contrast, the microbiological assay responds to di- and triglutamate folates, requiring only a 30-min

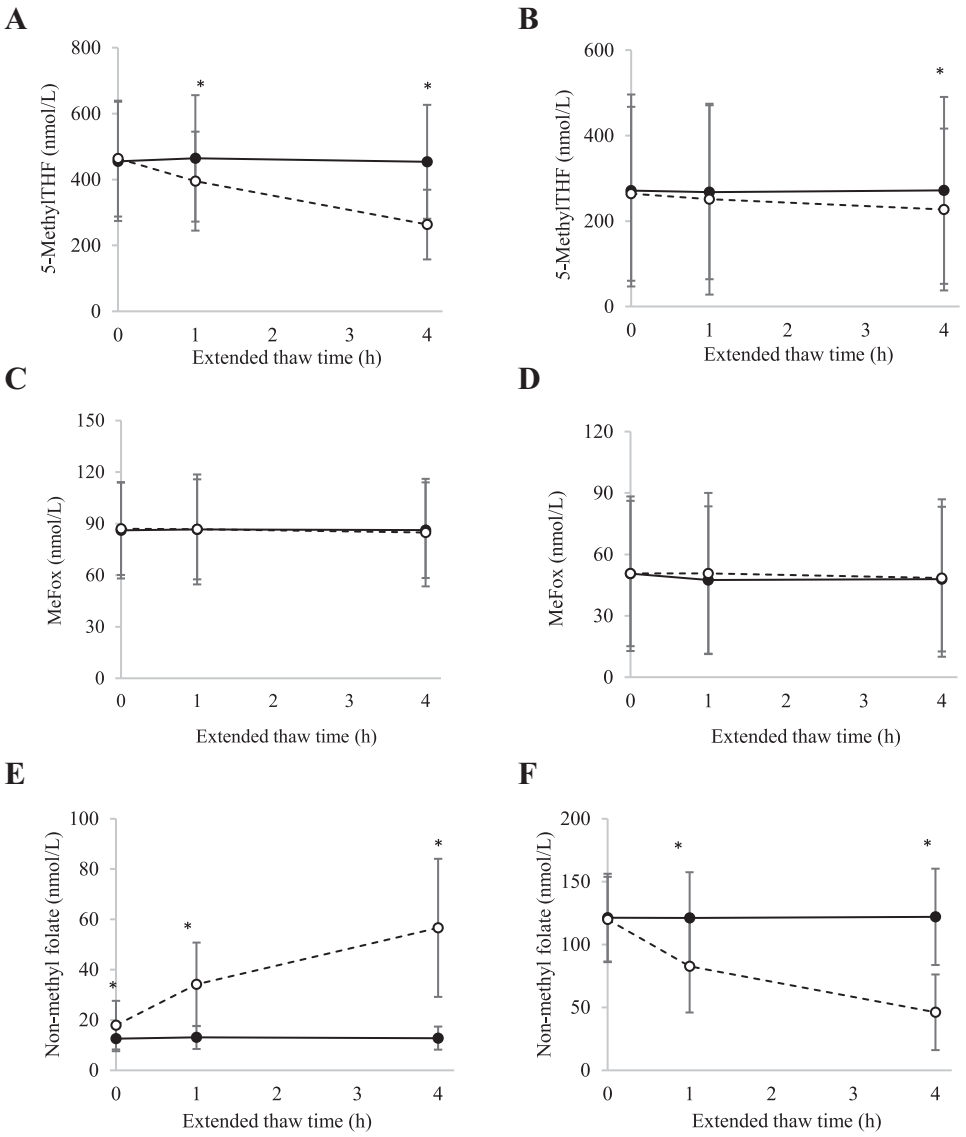


**FIGURE 2** Effect of extended thaw time on thawed whole-blood (represented by dotted lines) and whole-blood lysate (represented by solid lines) concentrations of 5-methylTHF, MeFox, and non-methyl folate. Values are means ± SDs from enzyme-treated samples (5 μg/mL); $n = 8$ samples with low non-methyl folate concentrations (A, C, E) and $n = 7$ samples with high non-methyl folate concentrations (B, D, F). Non-methyl folate is the sum of 5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate. *Difference between thawed whole blood and thawed whole blood lysate, $P < 0.05$. MeFox, pyrazino-s-triazine derivative of 4$\alpha$-hydroxy-5-methylTHF; 5-methylTHF, 5-methyltetrahydrofolate.

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 before analysis by HPLC-MS/MS, we were 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 microbiological 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 ($n = 15$ compared with $n = 4$) by using updated methodology that separates 5-formylTHF and MeFox, and including a comparison with WBL. We observed similar reductions of ~25% in 5-methylTHF and total folate over a similar time period compared with the previous report. An additional and important finding that was unobservable in the previous study was the discrepant changes in high and low non-methyl folate-containing samples occurring during an extended thaw time. At 0 h, a clear distinction was observed between low non-methyl folate samples (mean ± SD: 18 ± 10 nmol/L) and high non-methyl folate samples (120 ± 34 nmol/L). However, with increasing thaw time, the means ± SDs of the low non-methyl folate samples expanded and the means of the high non-methyl folate samples decreased until there was a

**TABLE 3** Folate concentrations in plasma and RBCs, calculated from WBL folate, serum folate and hematocrit (conventional procedure), or measured directly in washed RBCs[1]

| Donor | 5-Methyl THF | MeFox | Non-methyl folate | Folic acid | Total[2] |
|---|---|---|---|---|---|
| Plasma folate, nmol/L | | | | | |
| 1 | 17.9 | 1.00 | 1.44 | 1.3 | 21.6 |
| 2 | 18.7 | 1.45 | 1.04 | 0.4 | 21.6 |
| 3 | 57.6 | 2.01 | 2.75 | 6.0 | 68.3 |
| 4 | 22.1 | 3.43 | 0.81 | 68.8 | 95.1 |
| Mean | 29.1 | 1.97 | 1.51 | 19.1 | 51.6 |
| Median | 20.4 | 1.73 | 1.24 | 3.65 | 45.0 |
| WBL folate measured,[3] nmol/L | | | | | |
| 1 | 21.8 | 4.01 | 0.92 | <LOD | NC |
| 2 | 23.9 | 4.31 | 0.97 | <LOD | NC |
| 3 | 59.1 | 9.23 | 1.58 | 0.45 | NC |
| 4 | 22.9 | 4.59 | 0.87 | 3.73 | NC |
| Mean | 31.9 | 5.53 | 1.08 | 1.06 | — |
| Median | 23.4 | 4.45 | 0.94 | 0.24 | — |
| RBC lysate folate measured,[4] nmol/L | | | | | |
| 1 | 25.3 | 6.01 | 0.73 | <LOD | NC |
| 2 | 24.6 | 5.63 | 0.80 | <LOD | NC |
| 3 | 63.0 | 13.3 | 1.14 | <LOD | NC |
| 4 | 22.6 | 6.32 | 0.66 | <LOD | NC |
| Mean | 33.9 | 7.82 | 0.83 | <LOD | — |
| Median | 25.0 | 6.16 | 0.76 | <LOD | — |
| RBC folate calculated,[5] nmol/L | | | | | |
| 1 | 634 | 121 | 25.5 | −1.35 | 779 |
| 2 | 599 | 111 | 23.9 | 0.20 | 734 |
| 3 | 1435 | 233 | 36.9 | 3.53 | 1709 |
| 4 | 661 | 134 | 25.2 | −8.33 | 812 |
| Mean | 832 | 150 | 27.9 | −1.49 | 1008 |
| Median | 648 | 127 | 25.3 | −0.58 | 796 |
| RBC folate measured,[6] nmol/L | | | | | |
| 1 | 532 | 126 | 15.3 | <LOD | 674 |
| 2 | 516 | 118 | 16.8 | <LOD | 652 |
| 3 | 1322 | 280 | 23.9 | <LOD | 1626 |
| 4 | 475 | 133 | 13.9 | <LOD | 622 |
| Mean | 711 | 164 | 17.5 | <LOD | 893 |
| Median | 524 | 129 | 16.1 | <LOD | 663 |
| Difference between RBC folate measured and RBC folate calculated, % | | | | | |
| 1 | −16 | 4 | −40 | NC | −14 |
| 2 | −14 | 7 | −29 | NC | −11 |
| 3 | −8 | 20 | −35 | NC | −5 |
| 4 | −28 | −1 | −45 | NC | −23 |
| Mean | −17 | 7 | −37 | — | −13 |

[1]Hematocrit values were as follows: donor 1, 36%; donor 2, 42%; donor 3, 43%; donor 4, 36%. LOD, limit of detection; MeFox, pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF; NC, not calculated; WBL, whole-blood lysate; 5-methylTHF, 5-methyltetrahydrofolate.
[2]Calculated as the sum of individual folate forms for plasma folate and RBC folate.
[3]Whole blood diluted 1:11 in 1% ascorbic acid.
[4]Washed packed RBCs diluted 1:21 in 0.5% ascorbic acid.
[5]Formula used: [(WBL × 11) − plasma folate × (1 − hematocrit/100)]/(hematocrit/100).
[6]Formula used: (RBC lysate × 21).

considerable overlap (57 ± 27 nmol/L and 46 ± 30 nmol/L, 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 in folate vitamer distribution from methyl to non-methyl folate, albeit in samples from rats (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 ∼15% lower, on average, in packed RBCs compared with concentrations calculated from WBL by using the conventional procedure. Although the direct measurement of RBC folates, made possible through the addition of exogenous enzymes, seems advantageous, several logistical concerns exist in obtaining an RBC sample, which may account for some of the discrepancy observed. We separated washed RBCs from PBS by centrifugation at a force of 1811 × *g* for 15 min. Under similar centrifugal conditions in previous studies, ∼7% of the volume of unwashed RBCs 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 microbiological 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 RBCs representative of average RBC folate concentration, but could be solved by using the entire yield of washed RBCs to generate an 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 ∼10-fold higher concentration of folate in RBCs compared with plasma. This also holds true 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 who consume 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 nonzero values because 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, the calculation of folic acid in RBCs is not recommended.

This article is, to our knowledge, the first report to use 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 used 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 with the use of WBL samples. The plant GGH requires a higher pH (6.0) for deglutamylation than does 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 by 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 (~$2/sample) has to be weighed against 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 the addition of an exogenous source of human GGH eliminates the requirement for incubation of WBL before 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 by using exoGGH and eliminates the requirement for analysis of hematocrit and serum folate concentrations; however, current protocols to isolate RBCs may be inadequate. Further work with the use of a larger number of samples is required to establish the comparability of results determined by using conventional procedures with results produced by using exogenous recombinant human GGH.

## Acknowledgments

We thank Rocío Díaz de la Garza for providing a small amount of the recombinant plant enzyme (AtGGH2). The authors' responsibilities were as follows—RAS: conducted the research and analyzed the data and wrote the first draft of the manuscript; CMP: had primary responsibility for the final content; and all authors: designed the research, contributed to critical revisions, and read and approved the final manuscript.

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