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Serum folate forms are stable during repeated analysis in the presence of ascorbic acid and during frozen sample storage

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Abstract

Background: Serum folate forms and particularly tetrahydrofolate are sensitive to oxidation.

Methods: Using a repeated measures design, we investigated the stability of folate forms in convenience samples with added ascorbic acid (AA, 5 g/L) analyzed initially and after variable (~1–33 weeks) storage time at -70°C. We examined the recovery of tetrahydrofolate added at different spiking levels to serum with and without AA (5 g/L). We also assessed the long-term frozen storage stability of folate forms.

Results: Repeat analysis produced consistent results with the initial analysis; the mean relative change (95% CI; Lin's concordance correlation between initial and repeat result; sample size) was 0.08% (-0.24% to 0.39%; r_c=0.999; n=301) for 5-methyltetrahydrofolate; 4.23% (2.44% to 6.05%; r_c =0.984; n=211) for pyrazino-s-triazine derivative of 4α-hydroxy-5-methyltetrahydrofolate (MeFox); -0.22% (-1.90% to 1.49%; r_c =0.986; n=214) for folic acid; and 1.49% (-2.71% to 5.88%; r_c =0.889; n=81) for tetrahydrofolate. Linear regression testing for a time trend indicated an estimated average percent change of <±5% for samples retested after 4 months: 5-methyltetrahydrofolate P_{trend}=0.0007, folic acid P_{trend} <0.0001, MeFox P_{trend} =0.38, and tetrahydrofolate P_{trend} =0.0256. The mean±SD tetrahydrofolate spiking recovery was 96.7%±9.4% for serum with added AA, but <50% for serum without added AA. We observed ≤10% loss for most serum folate forms during 4 years of storage at -70°C.

Conclusions: Serum containing added AA showed acceptable stability of folate forms during repeat analysis from the same vial within 4 months, complete spiking recovery of tetrahydrofolate during sample processing, and long-term frozen storage stability of folate forms.

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Keywords

Oxidation; antioxidant; folate degradation; 5-methyltetrahydrofolate; MeFox; folic acid; tetrahydrofolate; HPLC-MS/MS

Introduction

Serum folate is an important biomarker of folate status and has been measured as part of the U.S. National Health and Nutrition Examination Survey and other national surveys (1). For accurate serum folate measurements, optimum sample storage conditions are critical to prevent folate losses. Reduced serum folate forms readily undergo oxidative degradation. Adding either ascorbic acid (AA) or sodium ascorbate to serum (5 g/L) greatly improved folate stability, particularly for long-term storage (2–4). Reducing agents also helped stabilize free tetrahydrofolate (THF); ascorbate (5 g/L) increased the stability of THF 53-fold at 4°C in acetate buffer, pH 5 (5). In this study we used readily-available convenience samples with added AA to investigate the stability of serum folate forms measured by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) during repeat analysis from the same vial within an 8-month period. We also investigated the stability of neat THF added to serum or stored in 0.5% AA at room temperature. Lastly, we assessed the long-term frozen storage stability of folate forms in multiple serum pools.

Methods

Reagents, materials, and serum samples

Folate calibrators [(6*S*)-5-methylTHF, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF (MeFox), (6*S*)-THF, folic acid, (6*S*)-5-formyltetrahydrofolate (5-formylTHF), and (6*R*)-5,10-methenyltetrahydrofolate (5,10-methenylTHF)] and their respective $^{13}C_5$-labeled analogs were purchased from Merck & Cie. All other reagents and solvents were of ACS grade. We used in-house prepared quality control (QC) pools (low, medium, and high based on 5-methylTHF concentration) using serum purchased from a U.S. blood bank. Other serum samples were from a CDC study (convenience samples) and were prepared with AA (5 g/L) added to freshly-prepared serum and stored at -70°C until HPLC-MS/MS analysis (6–8). The addition of AA lowers the pH of serum from a mean±SD of 7.3±0.2 to 5.8±0.1. Study participants provided informed consent. The CDC Research Ethics Review Board approved the study protocol.

Experimentation

Frozen sample stability during repeat analysis.—We used available results from the initial and repeat analysis of CDC study samples within an 8-month period to assess serum folate stability (Figure 1). The repeat analysis of CDC study samples was carried out for 1 of 3 reasons: subset repeat for quality assurance purposes, because 1 of the folate forms was outside the reportable range, or because a folate form result failed pre-specified QC acceptability limits. Thus, we had a variable length of time between the initial and repeat measurements and a different number of valid paired results for each folate form (5-methylTHF, n=301; MeFox, n=211; folic acid, n=214; THF, n=81). We limited our analysis

to samples with 2 valid results within the reportable range (limit of detection [LOD] to highest calibrator) and excluded samples where the initial or repeat result was lower than the limit of quantitation (LOQ): 5-methyltetrahydrofolate (5-methylTHF) 0.43 nmol/L; pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF (MeFox) 0.33 nmol/L; folic acid 0.47 nmol/L; and THF 0.83 nmol/L. Results for 5-formylTHF and 5,10-methenylTHF were lower than the LOD for the majority of samples and were therefore not included.

Stability of neat THF.—We performed 3 experiments to investigate the stability of neat THF. First, we conducted a THF spiking recovery experiment in 2014 where we added 1, 2, 10, and 50 nmol/L of THF stock solution to a serum pool prepared in 2013 with additional 5 g/L added AA. We used each day a fresh QC specimen and freshly prepared THF stock solution, and we processed samples immediately (within 1 hour) for analysis by HPLC-MS/MS. We analyzed each spiking level in 3 replicates over 3 days (n=9). Second, we conducted a THF spiking recovery experiment in 2017 using 2 types of serum pools (AA added – pools prepared in 2013 – or not added – pools prepared in 2006 – at the time of pool preparation. We added solid AA (5 g/L) to half of the vials from each pool after thawing. Then we added 20 nmol/L of THF stock solution to all 4 types of samples and processed samples immediately for analysis by HPLC-MS/MS (n=3 replicates per pool and condition). The spiking recovery in both experiments was calculated as the measured concentration difference between the spiked and unspiked sample divided by the nominal concentration of the spike. We also monitored whether there was an increase in folic acid concentration in the THF-spiked samples. In the third experiment we tested the degradation of neat THF (2 μmol/L) in 0.5% AA in a closed vial kept at room temperature for ≤24 hours (hourly measurements up to 8 hours and measurement at 24 hours). At each time point we prepared a 100 nmol/L calibrator from the THF stock solution in sample solvent (1% acetic acid, 40% methanol, 10% acetonitrile, 0.5% AA) and immediately measured the area for THF and the other folate forms by direct HPLC-MS/MS analysis. We prepared each time point in 3 replicates and injected each replicate 3 times (n=9).

Long-term frozen QC pool stability.—To assess the long-term frozen storage stability of folate forms, we reanalyzed in late 2017 serum pools prepared in 2006, 2009, 2011, and 2013. Serum QC pools prepared in 2006 and 2009 did not contain added AA, while 5 g/L of AA was added to pools prepared in 2011 and 2013 at the time of preparation. Some of the pools were spiked at the time of pool preparation with minor folate forms (THF, 5-formylTHF, and 5,10-methenylTHF) to obtain moderate concentrations. All pools were stored at -70°C until analysis.

Statistical analysis.—All statistical analyses were conducted after transforming the measurements to a logarithmic scale (SAS version 9.3 and Analyse-it version 4.60 plug-in for Microsoft Excel). The back transformed means (95% CI) provide estimates of the geometric mean (95% CI); the back transformed mean change (95% CI) between the initial and repeat analysis can be used to obtain an estimate of the relative percent change between the geometric means. We used a paired t-test to assess serum folate stability in samples with added AA and calculated Lin's concordance correlation coefficient for each folate form between the initial and repeat analysis to evaluate reproducibility. This correlation

coefficient calculates a scaled version of the expected squared deviation from the line of identity (9). Using the relative percent change in concentrations, we generated a box-and-whisker plot for each folate form. Because the time between the initial and repeat analysis varied, we examined the percent change in concentration over time by plotting the percent change as a function of number of weeks between the initial and repeat analysis. To determine whether there is a time trend from the initial to the repeat result, we regressed the percent change from initial result (y) against the time in weeks between the initial and repeat measurement (x). The test for trend is based on the slope coefficient of the linear regression line. The estimated regression equation was used to calculate the expected average percent change (95% CI) at a specified time point (e.g., 16 weeks) and the 95% prediction interval. The latter indicates the expected range of percent changes for a single future sample. *P* values <0.05 were considered significant.

Results

Stability of serum folate forms during repeat analysis.

Repeat analysis over a period of ~8 months produced consistent results with the initial analysis. We observed no statistically significant change for 5-methylTHF (P=0.63), folic acid (P=0.80), and THF (P=0.49); only MeFox showed 4.2% (2.4% to 6.1%) higher concentrations (P<0.0001) (Table 1). We used box-and-whisker plots to visualize the distribution of the relative change among samples between the initial and repeat analysis for each folate form (Figure 2). The range of relative change was smallest for 5-methylTHF (-10% to 8%), largest for THF (-35% to 77%), and intermediate for MeFox (-31% to 52%) and folic acid (-60% to 36%). The initial and repeat results were highly correlated (Lin's concordance coefficient $r_c \geq 0.889$). The estimated linear regression between the 2 results was superimposed on the line of equality (slope=1 and intercept=0) (Supplemental Figure 1).

About 50% of the repeat analyses were performed within ~5 weeks after the initial analysis, the remaining repeat analyses were evenly spread over a period of 32 weeks (Table 1 and Figure 3). We found a statistically significant time trend for 5-methylTHF (P_{trend}=0.0007), folic acid (P_{trend} <0.0001), and THF (P_{trend} =0.0256), but not for MeFox (P_{trend} =0.38). Based on the estimated regression equation for the percent change from the initial result, samples retested after 16 weeks (4 months), will have an estimated average percent change (95% CI) of 0.52% (0.14, 0.91) for 5-methylTHF, 4.68 (2.69, 6.68) for MeFox, -2.36 (-4.23,-0.49) for folic acid, and 0.63 (-4.36, 5.62) for THF. Thus, the estimated average percent changes are <±5% for all the folate forms at 4 months. The 95% prediction intervals for the expected relative change after 16 weeks from the initial result for a single future sample are understandably much wider than the 95% CIs: -4.85 to 5.89 for 5-methylTHF, -21.6 to 30.9 for MeFox, -24.4 to 19.6 for folic acid, and -29.1 to 40.4 for THF. This means that for THF, for example, 95% of future samples may have -29.1% lower to 40.4% higher results compared to the initial result when retested after 16 weeks.

Stability of neat THF.

We found complete THF recovery (mean±SD) in serum containing AA of 96.7%±9.4% (Table 2). The serum folic acid concentration remained mostly unchanged in the spiked samples at the 3 lower spiking levels (up to 10 nmol/L THF). The small increase (~0.2 nmol/L) at the highest spiking level (50 nmol/L THF) represents the amount of folic acid found in freshly prepared THF calibrator (~0.5%), indicating no notable THF oxidation to folic acid during sample processing. We recovered <50% of spiked THF in serum pools prepared without added AA, but addition of AA at the time of sample preparation resulted in nearly complete THF recovery (Supplemental Table 1). On the other hand, the addition of AA at the time of sample preparation to serum pools already containing AA from the time of pool preparation did not appear to provide additional benefit. Neat THF in 0.5% AA showed reasonable stability at room temperature for ≤8 hours (≤20% loss), but a substantial loss at 24 hours (~60%) (Supplemental Figure 2). The amount of folic acid in these samples corresponded to ~0.5% of the THF amount up to 7 hours (similar folic acid amount as found in freshly prepared THF calibrator), but increased slightly to ~0.8% of the THF amount at 24 hours, potentially indicating a small oxidation of THF to folic acid.

Long-term folate stability in frozen serum pools.

We observed ≤11% change (within pre-determined QC acceptability limits) in 5-methylTHF, MeFox, and folic acid in serum pools with (2011 and 2013 pools) or without (2006 and 2009 pools) added AA stored frozen for ≤11 years (Figure 4 and Supplemental Table 2). THF showed good stability in pools prepared with added AA during the first 2 years of frozen storage (Supplemental Figure 3), but then showed ~10% and ~20% loss after 4 and 6 years of storage, respectively (Supplemental Table 2). Concentrations of the 2 minor folate forms 5-formylTHF and 5,10-methenylTHF were also within pre-determined QC acceptability limits (≤12%) for pools stored for ≤6 years at -70°C.

Discussion

Limited data exist on serum total folate stability with and without added AA during <1 week storage at room temperature (2,3); without added AA during <1 month at room temperature or refrigerated (10,11); and without added AA during 1–4 years at ≤-20°C (12,13). Samples stored without AA for 1 year at -20°C showed 20–40% total folate loss (12,13). The current study is to our knowledge the first report that investigated the stability of serum folate forms measured by HPLC-MS/MS in samples with added AA after repeat analysis from the same vial for up to 8 months of storage at -70°C.

While the average percent change in THF between the initial and repeat result was not statistically significant, we observed a significant trend leading to minimally higher (0.63%) THF concentrations at 4 months. In 2 additional convenience data sets (samples with added AA) where repeat analyses were conducted within weeks to months, we found similarly good THF stability (±3%) and a reasonably good concordance between the 2 results (Lin's coefficient $r_C = 0.661$ and $r_C = 0.823$) (data not shown). The presence of ascorbate as an antioxidant to help protect labile serum folates is likely responsible for the good stability of THF we observed during repeat analysis from the same vial, as has been shown elsewhere

(3). The poor spiking recovery of THF we observed in the absence of AA reinforces this. However, once AA has been added at pool preparation time, additional AA added at sample preparation time does not provide further benefit. The complete THF spiking recovery in the presence of AA in this study, and the good stability of neat THF in 0.5% AA for ~1 hour at room temperature, the time needed to prepare daily calibrators, attest to the validity of our analytical method measuring serum THF.

Despite the significant trend leading to minimally higher (0.52%) 5-methylTHF and slightly lower folic acid (-2.36%) concentrations at 4 months, there was no significant difference on average between the initial and repeat results for these 2 analytes, suggesting good stability during repeat analysis of samples with added AA. While MeFox did not show a significant trend, we observed on average a small increase in MeFox (0.06 nmol/L corresponding to 4%). This slight increase during repeat analysis was observed regardless of time point, in the presence of AA. Previously, we also found stable 5-methylTHF with increasing MeFox (<1 nmol/L) for 3 serum pools not containing AA exposed to ≤3 freeze-thawing cycles in closed vials, and slightly decreasing 5-methylTHF (<4%) and increasing MeFox (<0.4 nmol/L) for 30 pooled serum samples not containing AA exposed to ≤3 freeze-thawing cycles in open vials (14). The good stability of 5-methylTHF, MeFox, and folic acid in this study was also apparent in the recent reanalysis of serum pools with and without added AA stored at -70°C for ≤11 years.

The main strength of our study is its repeated measures design which allowed us to estimate a time trend and avoided repeat analysis to be confined to only 1 or a few analytical runs. This reflects typical lab practice. Another strength is that we measured serum folate forms by a precise, sensitive, and specific HPLC-MS/MS method in a moderately large sample set with added AA. This allowed us to investigate relatively small changes in folate concentrations, which was particularly important for THF because of low endogenous concentrations and a somewhat weaker correlation between the 2 time points of analysis. However, our study was retrospective using a convenience sample instead of a tightly designed controlled experiment. We did not systematically control for the reasons or the timing of the repeat analysis, nor did we control for whether the same initial or repeat measurements were in the same run. Therefore, our estimates of the effect of the timing may be confounded with other elements of the re-measurement process. Another shortcoming was that we did not have paired samples with and without added AA. It would have been desirable to generate multiple aliquots of the same sample set with and without AA that could be analyzed in parallel at different time periods. It would also be interesting to investigate whether folate forms in serum with AA show better storage stability when stored at higher temperature (e.g., -20°C) compared to serum without AA.

In conclusion, serum with added AA showed acceptable stability of folate forms, including THF, during repeat analysis from the same vial for ≤4 months and during multi-year frozen storage of serum pools. Thus, the addition of AA to serum for long-term storage seems warranted. We believe that our findings are transferable to other analytical methods as long as the method in question measures folates accurately.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

ZF and CMP designed the research, NP and GW conducted the research. MRS, NP, ZF and CMP performed the statistical analysis. NP, ZF, MRS, and CMP performed the data interpretation. NP wrote the first manuscript draft, which was modified after feedback from ZF, CMP and MRS. All authors contributed to critical revision of the manuscript. ZF has primary responsibility for content. All authors have read and approve the final manuscript.

Abbreviations:

5-methylTHF	5-methyltetrahydrofolate
AA	ascorbic acid
HPLC-MS/MS	high performance liquid chromatography coupled to tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
MeFox	pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF
QC	quality control
THF	tetrahydrofolate

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Impact statement

Testing nutritional biomarkers for short-term and long-term storage stability is critical in epidemiological research. Information on optimal sample storage conditions that maintain stability of serum folate forms during frozen storage and analytical measurements is highly valuable to scientists in the area of folate research. The data on serum samples stored at -70°C with added ascorbic acid (antioxidant) showed good storage stability of folate forms including tetrahydrofolate, the least stable folate form.

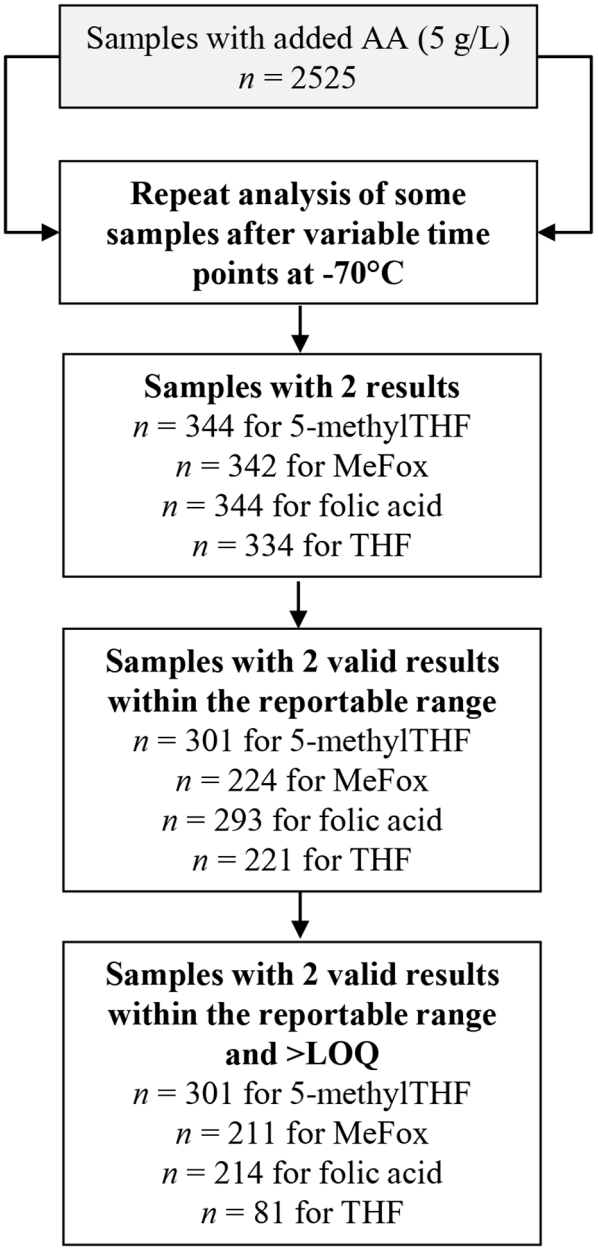

Figure 1.

Sampling scheme for serum folate analysis using convenience samples from a large CDC study. Samples contained 5 g/L L-ascorbic acid added at the time the serum was prepared. Repeat analysis was conducted after variable time points within an 8-month period and different numbers of samples for each folate form had 2 valid results within the reportable range, with the initial and repeat result exceeding the limit of quantitation (LOQ). 5-MethylTHF, 5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; THF, tetrahydrofolate.

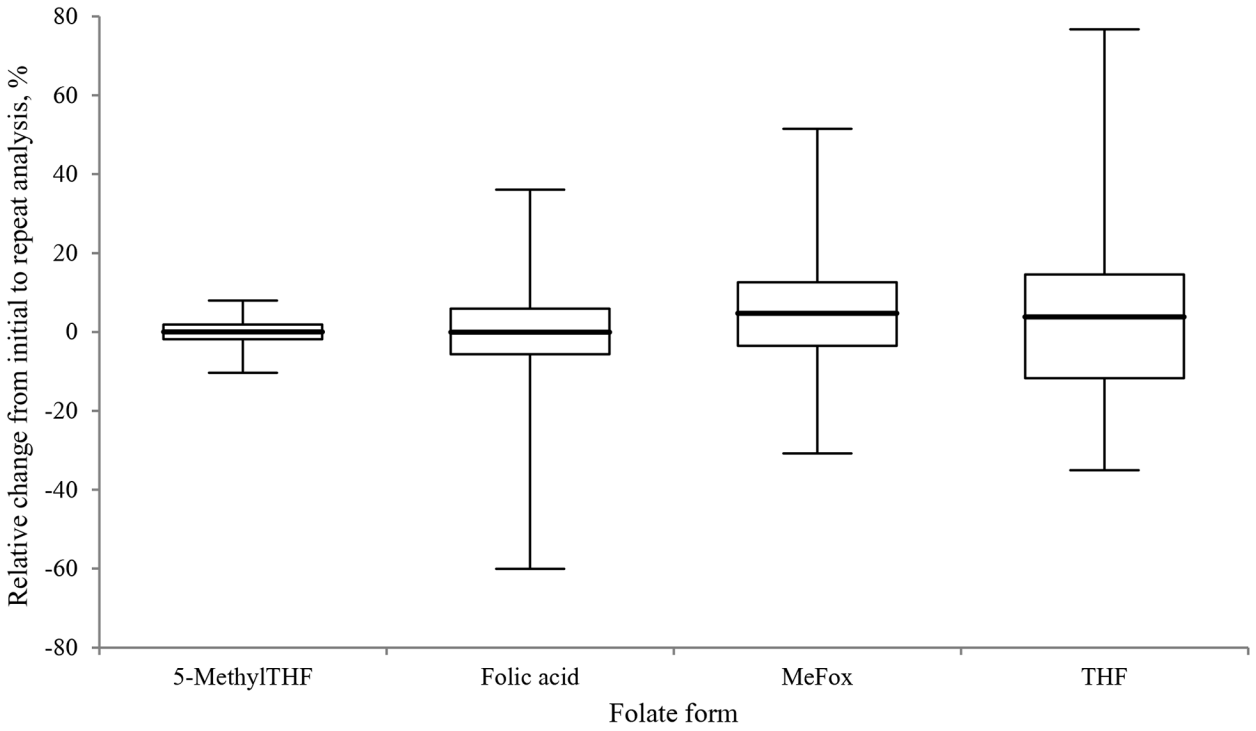

Figure 2.

Box-and-whisker plots of the relative change in serum folate form concentrations between the initial and repeat analysis within an 8-month period measured in convenience samples from a large CDC study. Samples contained 5 g/L L-ascorbic acid added at the time the serum was prepared. The line and box represent the median and the 1st to 3rd quartiles, respectively; the whiskers represent the range of values. 5-MethylTHF, 5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; THF, tetrahydrofolate.

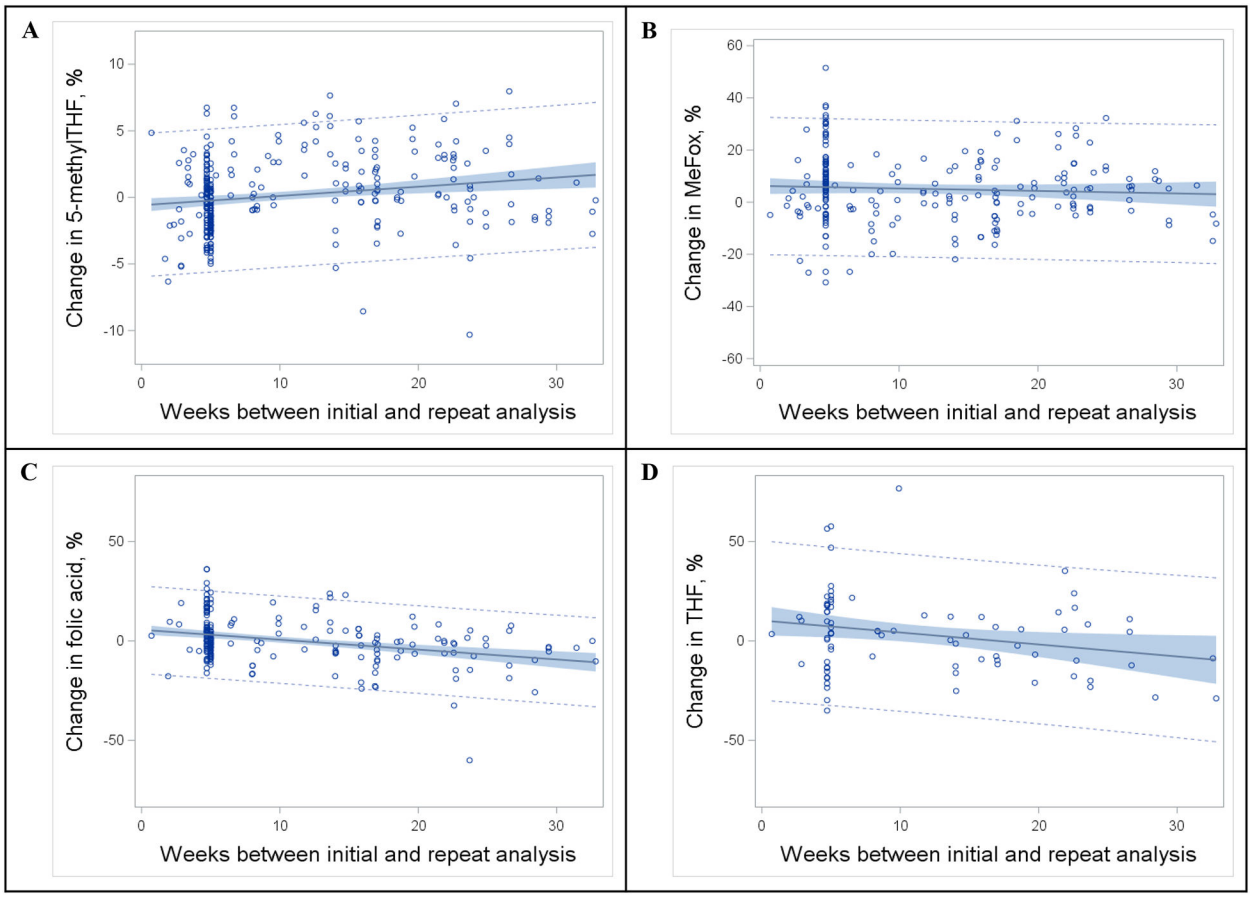

Figure 3.

Scatter plot of the relative change in concentrations between the initial and repeat analysis for each serum folate form as a function of the time between the 2 measurements in convenience samples from a large CDC study. The estimated regression line for the percent change from the initial result, the 95% confidence bands for the mean percent change (shaded area), and the 95% prediction bands for the expected percent change for a single future sample (dashed lines) are shown. Regression slope (SE, *P*-value) and intercept (SE, *P*-value): 5-methylTHF 0.07 (0.02, 0.0007) and -0.59 (0.26, 0.0231); MeFox -0.10 (0.11, 0.38) and 6.23 (1.60, 0.0001); folic acid -0.50 (0.10, <0.0001) and (5.61 (1.26, <0.0001); and THF -0.60 (0.27, 0.0256) and 10.3 (3.75, 0.0076). Samples contained 5 g/L L-ascorbic acid added at the time the serum was prepared. Sample size was 301 for 5-methylTHF (panel A), 211 for MeFox (panel B), 214 for folic acid (panel C), and 81 for THF (panel D). 5-MethylTHF, 5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; THF, tetrahydrofolate.

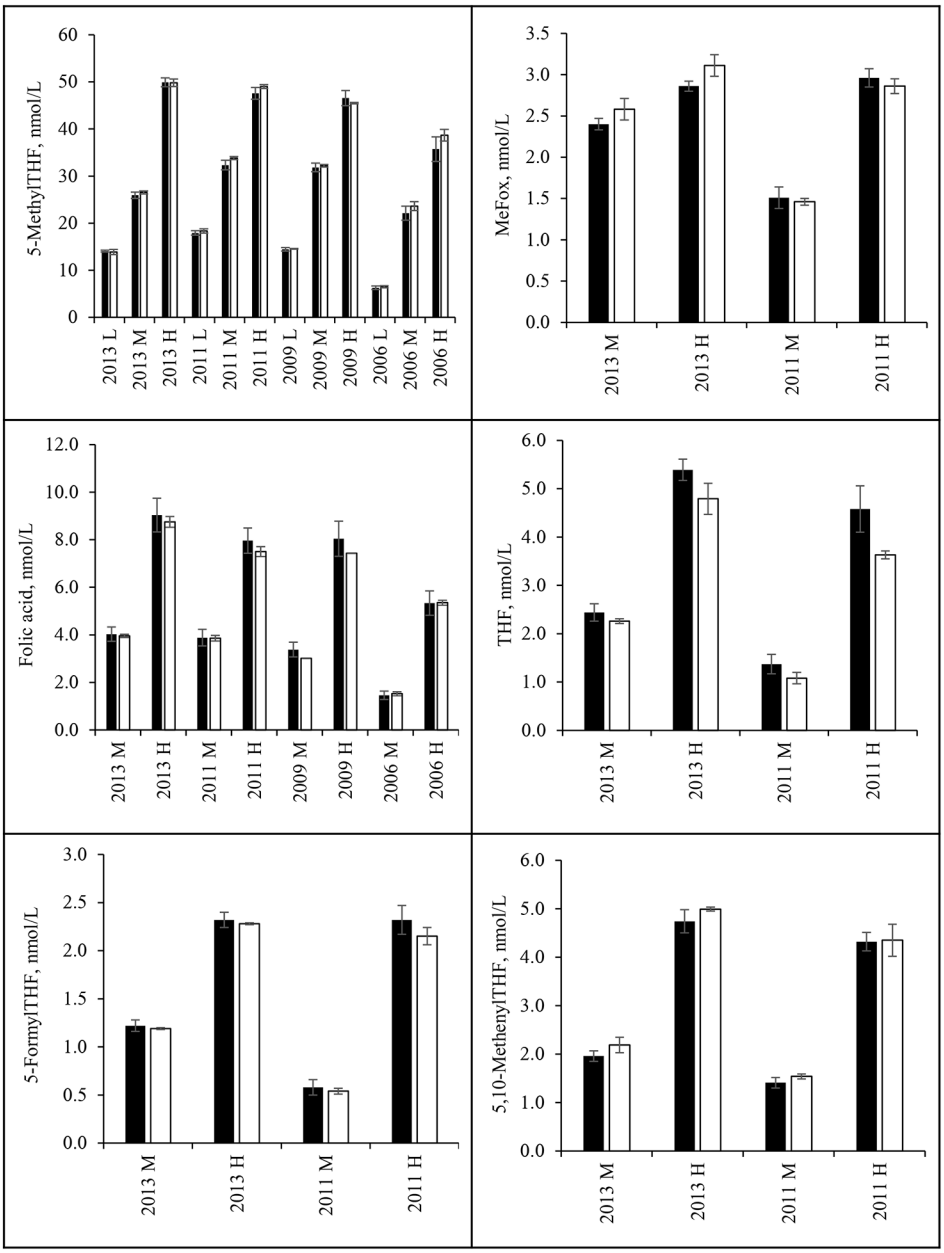

Figure 4.

Concentrations of folate forms measured in multiple serum QC pools at the time of pool preparation (black bars) and in late 2017 (white bars) to assess long-term storage stability at -70°C. Pool names indicate the year the material was prepared and the level of the pool (L, low; M, medium; H, high). Error bars represent SD. See Supplemental Table 2 for *n* and for information on spiking levels for minor folate forms by pool.

Table 1.

Concentration of serum folate forms in samples with added ascorbic after initial and repeat analysis[a]

Analyte	Sample size	Initial analysis[b] (nmol/L)	Repeat analysis[b] (nmol/L)	Relative change[c] (%)	*P*-value[d]	Median [range] time[e] (wk)	Lin's concordance coefficient r_c[f]
5-MethylTHF	301	32.7 (30.5, 35.1)	32.7 (30.5, 35.1)	0.08 (-0.24, 0.39)	0.63	5.0 [0.7 to 32.8]	0.999 (0.999, 0.999)
MeFox	211	1.29 (1.17, 1.43)	1.35 (1.22, 1.49)	4.23 (2.44, 6.05)	<0.0001	8.6 [0.7 to 32.8]	0.984 (0.979, 0.988)
Folic acid	214	1.00 (0.90, 1.11)	1.00 (0.90, 1.10)	−0.22 (-1.90, 1.49)	0.80	5.0 [0.7 to 32.8]	0.986 (0.982, 0.989)
THF	81	1.30 (1.19, 1.42)	1.32 (1.20, 1.44)	1.49 (-2.71, 5.88)	0.49	6.5 [0.7 to 32.8]	0.889 (0.831, 0.927)

[a] Samples stored at -70°C between analyses; repeat analysis was conducted at different time points after the initial analysis; samples contained 5 g/L L-ascorbic acid added at the time the serum was prepared; 5-methylTHF, 5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; THF, tetrahydrofolate

[b] Values represent least squares geometric mean (95% Cl)

[c] Values represent percent change (95% Cl) compared to initial analysis

[d] Comparison between initial and repeat analysis is based on a paired *t*-test on log transformed concentrations

[e] Time between initial and repeat analysis

[f] Lin's concordance correlation between initial and repeat analysis is based on log transformed concentrations; calculates a scaled version of the expected squared deviation from the line of identity

Table 2.

Recovery of tetrahydrofolate added to a serum QC pool prepared with ascorbic acid and relative change in serum folic acid concentration

		Tetrahydrofolate		Folic acid	
Sample[a]	**Spike (nmol/L)**	**Measured**[b] **(nmol/L)**	**Recovery**[c] **(%)**	**Measured**[b] **(nmol/L)**	**Change**[d] **(%)**
Serum	0	0.20[e] ± 0.09	-	1.09 ± 0.22	-
Serum + spike 1	1	1.16 ± 0.14	96 ± 15	1.12 ± 0.20	2.8
Serum + spike 2	2	2.09 ± 0.20	95 ± 10	1.04 ± 0.17	−4.5
Serum + spike 3	10	9.86 ± 0.62	97 ± 6	1.10 ± 0.15	0.8
Serum + spike 4	50	50.1 ± 1.24	100 ± 2	1.29 ± 0.17	18.9

[a] 5 g/L solid L-ascorbic acid was added to a serum QC pool at the time of pool preparation in 2013 and the pool was stored at -70°C; in 2014, an aliquot of the pool was thawed and spiked with 4 levels of tetrahydrofolate

[b] Values represent mean ± SD of 3 replicates per spike level analyzed over 3 days for $n = 9$

[c] Recovery represents mean ± SD and was calculated as the measured concentration difference between the spiked and unspiked sample divided by the nominal concentration of the spike

[d] Values represent mean relative change in folic acid concentration in spiked samples compared to unspiked serum

[e] Limit of detection (LOD) for THF: 0.25 nmol/L; measured concentrations <LOD replaced with an imputed value of LOD divided by square root of 2