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Accounting for an isobaric interference allows correct determination of folate vitamers in serum by isotope dilution-liquid chromatography-tandem mass spectrometry[1, ,2, ,3]

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Abstract

Mild and prolonged oxidative degradation of 5-methyltetrahydrofolate (5-methylTHF) leads to the biologically inactive pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF (MeFox). MeFox and the biologically active 5-formyltetrahydrofolate (5-formylTHF) are isobaric compounds that behave similarly during chromatographic and mass separation, making coelution and misidentification likely. Our published routine LC-MS/MS method did not discern between 5-formylTHF and MeFox, measuring the sum of these compounds at mass/charge ratio [*m/z*] 474→327 as 5-formylTHF. We modified this method to separate MeFox and 5-formylTHF either by chromatography or by unique mass transitions and then applied the two methods to serum specimens to determine typical concentrations of these compounds. The two unique transitions (*m/z*: 5-formylTHF 474→299; MeFox 474→284) showed good sensitivity (LOD [nmol/L]: 5-formylTHF 0.21; MeFox 0.34), selectivity (no interfering peaks), spiking recovery (mean±SD: 5-formylTHF 103%±3.4%; MeFox 94%±10%), and low imprecision (CV: 5-formylTHF 3.9% at 2.4 nmol/L; MeFox 5.1% at 2.9 nmol/L). The mass separation method detected 5-formylTHF in the same specimens as the chromatographic separation method. Analysis of several thousand serum specimens showed that the majority (~85%) contained MeFox at <3 nmol/L, but no detectable 5-formylTHF concentrations; some (~14%) contained 5-formylTHF at <0.5 nmol/L; a few specimens contained 5-formylTHF at >1 nmol/L and MeFox at >10 nmol/L. In summary, serum can contain 5-formylTHF high enough to contribute to total folate and contains MeFox that will bias total folate if not separated appropriately. Including measurements of MeFox and 5-formylTHF along with the other folate vitamers will enhance assessments of the association between biologically active folate and health effects.

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[3]Supplemental Text 1 and Supplemental Figures 1–3 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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INTRODUCTION

Folate vitamers are important cofactors in one-carbon metabolism, participating in methylation reactions and DNA synthesis; suboptimal folate status may modulate chronic diseases such as cardiovascular disease, cancer, and/or cognitive impairment (1). There is great interest in exploring associations between folate vitamers and health effects and recent advances in the application of tandem mass spectrometry to the measurement of folate vitamers make such studies possible (2). However, the analysis of folates in serum is complex because of numerous folate derivatives that exist in different oxidation states and one-carbon substitutions.

Folates are also sensitive to oxidative degradation (3,4). Serum 5-methyltetrahydrofolate (5-methylTHF), the main circulating folate form, can readily undergo mild reversible oxidation to 5-methyldihydrofolate (5-methylDHF) (3,5) (Supplemental Figure 1). Severe or prolonged oxidation can convert 5-methylTHF or 5-methylDHF to 4α-hydroxy-5-methylTHF, an intermediate product also called hmTHF (3–7). In the absence of a reducing agent, hmTHF undergoes structural rearrangement to form a pyrazino-s-triazine derivative (4–8). This stable oxidation product of 5-methylTHF is also known as MeFox, the methyl folinate oxidation product. MeFox is not biologically active (3,5) and has been reported previously in long-term frozen stored serum and plasma specimens and even in fresh frozen specimens (9–11; the authors however referred to this compound as hmTHF, even though they stated that it was the pyrazino-s-triazine derivative of hmTHF). It is not known whether MeFox is formed only *in vitro* or may already be present *in vivo*.

MeFox and 5-formyltetrahydrofolate (5-formylTHF) are isobaric compounds and as such form the same mass to charge parent to product ion pairs during ionization, making coelution and misidentification during liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis likely. Our published isotope-dilution LC-MS/MS method (12,13) did not discern between 5-formylTHF and MeFox and we reported the measured concentrations as 5-formylTHF. Only one LC-MS/MS method analyzing serum folate vitamers has been reported so far that separates these two compounds chromatographically (9). Using this method, the authors did not find any 5-formylTHF in 168 fresh frozen serum specimens. Other methods did not incorporate the analysis of MeFox as part of their measurand profile (14–16) and either reported no (14,15) or low (16) concentrations of 5-formylTHF in a rather limited number of serum specimens ($n \leq 32$).

Our first objective was to improve our published LC-MS/MS method such that it accurately captures all folate vitamers that can be present in serum, as well as MeFox, the oxidation products of the major circulating folate vitamer. To achieve that, we modified our method to allow separation and accurate quantitation of MeFox and 5-formylTHF, while maintaining the desirable features of the method, such as short runtime, isocratic separation, good sensitivity and precision. Our second objective was to apply the improved LC-MS/MS method to a large number of serum specimens to determine what the typical concentrations of MeFox in serum are and whether 5-formylTHF is present in addition to MeFox. This information will help investigators decide which folate forms to measure in studies that assess the relationship of folate and health effects.

MATERIALS AND METHODS

REAGENTS, MATERIALS, AND SERUM SPECIMENS

Folate monoglutamate standards ([6S]-5-methylTHF, [6S]-5-formylTHF, folic acid [FA], [6S]-tetrahydrofolate [THF], [6R]-5,10-methenyltetrahydrofolate [5,10-menthenylTHF], and [6S]-MeFox) together with their respective $^{13}C_5$-labeled analogues were purchased from Merck Cie. Other reagents and solvents were of ACS reagent grade unless stated otherwise. Purified water (18 MΩ/cm) from an Aqua Solutions water purification system was used for all reagents and solutions. All sample handling was performed under gold-fluorescent light. All folate calibrators were prepared as described earlier (12,13). For a detailed description of the preparation of MeFox calibrators, see Supplemental Text 1. In-house prepared serum QC pools (low, medium and high levels) and several thousand serum specimens from a CDC study and US blood bank were used to determine the presence and typical concentrations of 5-formylTHF and MeFox. All serum specimens were stored at −70°C. The serum specimens from the large CDC study contained 0.5% (w/v) L-ascorbic acid, added at the time of specimen preparation. Study participants provided informed consent. The study protocol was approved by the CDC Research Ethics Review Board.

SAMPLE PROCESSING

A six-point calibration curve (0–100 nmol/L for 5-methylTHF with 10 nmol/L $^{13}C_5$-5-methylTHF; 0–50 nmol/L for other folates including MeFox with 2.5 nmol/L each of $^{13}C_5$-labeled folate), serum quality control (QC) samples (duplicate analysis of three levels/run), and unknown serum specimens were carried through all sample processing steps, which were conducted according to an automated reversed-phase solid phase extraction (SPE) procedure as reported previously (12,13).

ANALYSIS OF NATIVE SERUM SPECIMENS BY LC-MS/MS

Extracted serum samples were analyzed by one or both modified LC-MS/MS methods that allowed quantitation of MeFox and 5-formylTHF either by chromatographic separation (method 2) or by mass separation (method 3) (Table 1). Specifics regarding the instrumentation and instrument parameters used can be found in the Supplemental Text 1. As part of the method validation process, we applied both methods to three serum QC pools that were spiked with 5-formyTHF (0.5, 2.0, and 3.5 nmol/L) at the time of pool preparation. We applied method 2 to a set of serum specimens (n = 132) to assess whether native samples contain one or both compounds. Samples found to contain 5-formylTHF, were then analyzed by method 3 for confirmation. Finally, we applied method 3 to several thousand serum specimens from a CDC study and US blood bank to assess typical concentrations of MeFox and 5-formylTHF in a large sample set. We assessed the correlation between MeFox and 5-methylTHF in this large sample set using Pearson correlation.

METHOD VALIDATION EXPERIMENTS

To assess whether the complete analytical sample preparation process including SPE leads to formation of MeFox, we used method 2 (no $^{13}C_5$-MeFox added) to measure peak areas in the MRM transition of $^{13}C_5$-MeFox (m/z 479→327) for 50 serum specimens. We compared

the areas to those found in the $^{13}C_5$-MeFox transitions of a $^{13}C_5$-5-methylTHF calibrator that didn't undergo sample processing. Increased areas in processed serum samples would indicate potential 5-methylTHF oxidation (of the internal standard) during sample preparation and analysis.

We assessed whether 5-methylTHF stock solutions prepared approximately annually and stored at −70°C over a period of up to nine years (eight 100 mg/L solutions containing 1% ascorbic acid) showed any loss of 5-methylTHF or increase in MeFox with increasing age. We performed subsequent injections of freshly prepared 500 nmol/L working solutions (69% water:1% acetic acid:30% methanol) containing 0.5% ascorbic acid of each yearly stock by method 2 (n = 3 injections per preparation) and monitored the MRM transitions for 5-methylTHF, and MeFox to assess whether areas changed from one preparation to another and for other folate forms to ensure that there were no interfering peaks appearing with older preparations.

We assessed method performance characteristics such as selectivity, sensitivity, accuracy, imprecision, and ion suppression behavior for method 3. To confirm the selectivity of the new mass transitions in method 3, we prepared individual calibrator solutions for each folate form in sample solvent (49% water:1% acetic acid:40% methanol:10% acetonitrile) containing 0.5% ascorbic acid at concentrations representing or exceeding the upper limit of the calibration range (50, 100 and 200 nmol/L) and analyzed each preparation in triplicate.

To determine method sensitivity, we calculated the limit of detection (LOD) for each analyte. We serially diluted the "low" QC pool with 0.1% ascorbic acid and estimated the standard deviation at a concentration of zero (σ_0) by extrapolating repeat analyte measurements ($n = 9$) made near the detection limit in these dilutions. The LOD was defined as 3 σ_0.

We assessed the accuracy of method 3 by spike recovery. We added a calibrator mixture of 5-formylTHF and MeFox at three concentrations (2.0, 5.0 and 10 nmol/L) in triplicate to a serum specimen that was also measured in triplicate for endogenous concentrations of these two compounds. The spike recovery was calculated as the measured concentration difference between the spiked and unspiked sample divided by the nominal concentration of the spike.

Method 3 imprecision (within-run, between-run, and total CV) was assessed by analyzing serum QC pools in duplicate (at the beginning and end of each run bracketing patient samples) for an extended period of time (122 runs over a period of one year).

We also assessed the ion suppression behavior of method 3 by using a post-column infusion procedure. We infused analyte and internal standard (1.6 μmol/L at a constant flow of 10 μL/min) to elevate the baseline of each mass transition to approximately 1×10^6 ion counts/s. We then injected various serum samples as test specimens and evaluated the degree of ion suppression as the relative deviation of the baseline observed in the elution time window for each analyte and internal standard.

RESULTS

ADAPTATION OF LC-MS/MS METHOD TO SEPARATE MEFOX AND 5-FORMYLTHF

Our published LC-MS/MS method 1 achieves rapid isocratic separation of several folate vitamers: 5-methylTHF, 5-formylTHF, THF, 5,10-methenylTHF, and FA. However, MeFox and 5-formylTHF coelute at 3.1 min and both compounds show predominant transitions at the same mass/charge ratio (*m/z*) 474→327. In method 2, we adapted the chromatography of method 1 to separate MeFox and 5-formylTHF by use of a more polar isocratic mobile phase and by diluting one part of SPE-extracted sample with one part of mobile phase to more closely match the sample composition to the mobile phase composition (Table 1). Method 2 allowed nearly baseline separation of MeFox (3.62 min) and 5-formylTHF (4.17 min) at *m/z* 474→327 (see Supplemental Figure 1), while retaining the separation of the other folate vitamers.

In method 3, we adapted the mass detection of method 1 to separate MeFox and 5-formylTHF by use of selective transitions. While both compounds showed the same protonated molecular ion $[M + H]^+$ of 474.4 and the same predominant fragment ion of 327.2, they also produced less abundant (2/3 less) unique fragment ions: 284.2 for MeFox and 299.2 for 5-formylTHF. By optimizing the MS parameters for these selective transitions (see Supplemental Text 1), we were able to accurately separate and quantitate these two compounds without changing the sample preparation or isocratic chromatography of method 1.

METHOD VALIDATION

Using method 2, we assessed whether sample processing or storage leads to formation of MeFox. We prepared serum samples without the addition of $^{13}C_5$-MeFox, carried them through the complete procedure, and monitored the transition for $^{13}C_5$-MeFox (*m/z* 474→327) to assess how much of the $^{13}C_5$-5-methylTHF was “oxidized”. We found on average 0.3% of the $^{13}C_5$-5-methylTHF area in the $^{13}C_5$-MeFox transition of processed serum samples. This amount was about the same as in $^{13}C_5$-5-methylTHF internal standard solutions that didn’t undergo sample processing (0.4%). The amount of MeFox was also very similar to what we found in 5-methylTHF stock solutions (100 mg/L) stored for up to 9 y at −70°C in the presence of 1% ascorbic acid (0.2%). We therefore concluded that our sample processing did not generate additional MeFox beyond what was present at the beginning of processing and that high concentration stock solutions are stable for many years if stored properly.

We analyzed three serum QC pools that were spiked with 5-formylTHF (0.5, 2.0, and 3.5 nmol/L) at the time of pool preparation with methods 2 and 3 to confirm that both methods separate MeFox and 5-formylTHF and achieve comparable quantitation of these two compounds. We found low MeFox concentrations (mean ± SD, nmol/L, $n = 4$ runs: 1.13 ± 0.19, 1.04 ± 0.21, and 2.70 ± 0.43 by method 2 and 0.93 ± 0.05, 1.20 ± 0.04, and 2.74 ± 0.07 by method 3) in addition to 5-formylTHF concentrations (0.41 ± 0.25, 1.69 ± 0.42, and 2.84 ± 0.62 by method 2 and 0.56 ± 0.08, 2.13 ± 0.09, and 3.67 ± 0.15 by method 3). While both methods produced comparable MeFox concentrations, the 5-formylTHF

concentrations measured by method 3 corresponded better with the spiking levels than those measured by method 2. Furthermore, the SD for both compounds in these four runs were much lower for method 3 compared to method 2. Method 2 had an additional disadvantage: the use of a more polar mobile phase resulted in peak broadening which together with the need to dilute the extracted sample led to a loss in sensitivity. Because method 3 showed better features for routine use, we proceeded to validate it for implementation as our new routine method.

When we examined the new transitions in method 3 for spurious signal contributions from other folate forms, we found good selectivity with virtually no contributing signals (< 0.01%). We also confirmed that high concentrations of MeFox (or $^{13}C_5$-MeFox) did not contribute any signal to transitions used for other folate forms (including the new 5-formylTHF transition). Considering that the new transitions in method 3 represent less abundant fragment ions, they showed good sensitivity with a limit of detection (LOD) of 0.34 and 0.21 nmol/L for MeFox and 5-formylTHF, respectively. Using a postcolumn infusion procedure we established that matrix effects and ion suppression were not present.

Due to the lack of reference materials for 5-formylTHF and MeFox we determined the accuracy of method 3 by analyte spike recovery. We found complete recovery (mean ± SD) for 5-formylTHF (103% ± 3.4%) and MeFox (94% ± 10%) added to serum (Table 2) and a nearly perfect linear relationship between the three spiking levels and the measured concentrations in the spiked sample (Supplemental Figure 3), additionally confirming the respective selectivity of the new transitions. We obtained low imprecision after using method 3 routinely for 1 y (122 runs): 4.4%, 3.9%, and 5.0% for 5-formylTHF (2.38 nmol/L) and 3.6%, 5.1%, and 5.7% for MeFox (2.93 nmol/L) for within-run, between-run, and total CV, respectively. No imprecision exceeded 10% for the low concentration QC pool (0.67 and 1.54 nmol/L for 5-formlyTHF and MeFox, respectively).

ANALYSIS OF NATIVE SERUM SPECIMENS

We applied method 2 to a set of serum specimens (n = 132) to assess whether native samples contain one or both compounds in question. The analysis showed exclusively MeFox with the exception of two specimens that had low 5-formylTHF concentrations (0.52 and 0.56 nmol/L). We confirmed the presence of 5-formylTHF in these two specimens by method 3 (1.28 and 0.47 nmol/L).

We then applied method 3 to a large set of several thousand serum specimens to assess the typical concentrations of MeFox and 5-formylTHF. We found detectable 5-formylTHF concentrations in ~15% of samples [90th percentile (95% CI), nmol/L: 0.24 (0.239–0.248)]; a few samples had 5-formylTHF concentrations > 1 nmol/L. Most samples (99%) had detectable MeFox concentrations [median (95% CI), nmol/L: 1.35 (1.32–1.39)] and < 1% of samples had MeFox concentrations > 10 nmol/L. We found a weak (r = 0.19) but highly significant correlation (P < 0.001) between MeFox and 5-methylTHF, with MeFox concentrations increasing with increasing 5-methylTHF concentrations. We observed three different patterns of these two compounds found in native serum specimens (Figure 1): the low MeFox/low 5-formylTHF pattern was encountered most frequently, while the low MeFox/high 5-formylTHF and high MeFox/low 5-formylTHF patterns were encountered

much less frequently. The figure also shows that the unselective mass transition of 474→327 captured the sum of the selective MeFox (474→284) and 5-formylTHF (474→299) transitions quite well.

Discussion

This manuscript presents an improved and validated routine isotope-dilution LC-MS/MS procedure for the measurement of key folate forms in serum. The method allows separation and accurate quantitation of the two isobaric compounds MeFox, a stable oxidation product of 5-methylTHF, and 5-formylTHF, a minor folate vitamer, in addition to other folate vitamers. The method has been applied to several thousand serum specimens over a period of 1 y, providing to our knowledge the first data from an extensive sample set that shows what the typical concentrations of MeFox and 5-formylTHF in serum are.

Only a small number of research groups have so far developed isotope-dilution LC-MS/MS methods for the analysis of folate vitamers in serum. Several investigators limited their methods to the analysis of 5-methylTHF and sometimes FA (17–21), partly because their research interests were in the area of folate bioavailability and kinetics. While some investigators expanded their methods to analyze a wider spectrum of folate vitamers including 5-formylTHF (12–16,22), none of these methods incorporated the analysis of MeFox as part of the folate profile. Hannisdal et al. reported the first and so far only LC-MS/MS method that measures the main folate vitamers in serum in addition to MeFox (9).

The present study confirms the finding by Hannisdal et al. that the isobaric compounds MeFox and 5-formylTHF have to be separated for correct quantitation of folate forms in serum (9–11). While the authors opted for chromatographic separation using gradient elution and an unselective mass transition of 474→327 for detection, we explored two different approaches to separate MeFox from 5-formylTHF: chromatographic separation using an adapted isocratic elution (method 2) and mass separation using unique mass transitions (method 3). Both approaches allowed us to separate the two isobaric compounds, however the chromatographic separation approach had several shortcomings such as impaired sensitivity due to peak broadening and poor imprecision. The newest generation tandem mass spectrometer offered an improvement in sensitivity and allowed us to further explore the second approach—using unique mass transitions—, while retaining the time-tested isocratic chromatography procedure from our published method 1. The new unique transitions for MeFox and 5-formylTHF in method 3 proved to be highly selective as well as sensitive. Compared to the LOD values reported by Hannisdal et al. (MeFox 0.20 nmol/L, 5-formylTHF 0.52 nmol/L [9]), the method 3 LOD values (MeFox 0.34 nmol/L, 5-formylTHF 0.21 nmol/L) were slightly higher for MeFox, but lower for 5-formylTHF. The other performance parameters for method 3 were also very good: we found complete spiking recoveries (MeFox 94%, 5-formylTHF 103%) and low between-run imprecision after using the assay for an entire year (MeFox 5.1% at 2.93 nmol/L, 5-formylTHF 3.9% at 2.38 nmol/L). Hannisdal et al. reported slightly lower spiking recoveries (MeFox 82%–94%, 5-formylTHF 91%–98%) and higher between-run imprecision (MeFox 7.1% at 4.0 nmol/L, 5-formylTHF 9.9% at 3.7 nmol/L) (9).

The question has been raised whether MeFox is formed only *in vitro* or may already be present *in vivo*. Hannisdal et al. observed that this compound was already present in fresh frozen serum samples that were kept in an ice bath for maximum 150 min while the whole blood was allowed to clot (9). Our experiments have added new information. We have shown that our sample processing does not generate additional MeFox beyond what is present at the beginning of processing. While this may not seem too surprising, as ascorbic acid is incorporated into all reagents used as part of sample processing to avoid folate oxidation, it is reassuring that there is no artifactual increase in MeFox as a result of sample manipulations at the bench. Furthermore, it was also reassuring to note that high concentration stock solutions of 5-methylTHF can be stored for nearly a decade without any observed loss of 5-methylTHF or increase in MeFox. However, the question whether MeFox may already be present *in vivo* has yet to be answered.

We analyzed two sets of native serum samples: a smaller set ($n = 132$) by method 2 and a large set of several thousand samples by method 3. In the smaller set we found two specimens with low 5-formylTHF concentrations that we confirmed with method 3. Hannisdal et al. analyzed a similar size sample set ($n = 168$) and did not find any 5-formylTHF, possibly because their LOD was slightly higher than ours or because folate concentrations in the non-fortified Norwegian population were lower than in the fortified US population (9). In the large sample set, we found detectable 5-formylTHF concentrations in ~15% of samples and a few samples had concentrations > 1 nmol/L. This shows that 5-formylTHF needs to be included as a potential folate vitamer when developing an LC-MS/MS method. Overall, its contribution to total folate may be small, but in selected samples it can contribute an amount that cannot be neglected. In the large sample set, we found detectable MeFox concentrations in almost all samples (99%) and very few samples (< 1%) with concentrations > 10 nmol/L. Our median concentration for MeFox of 1.35 nmol/L was comparable to the median of 2.3 nmol/L reported by Hannisdal et al. (9). While MeFox concentrations are generally low, they will bias the total folate if MeFox is not appropriately separated from 5-formylTHF.

The present study provides some much needed information on typical concentrations of MeFox and 5-formylTHF found in native serum specimens to help investigators decide which folate forms to measure in studies that investigate the relationship of folate and health effects. While it confirms previous observations that the isobaric compounds MeFox and 5-formylTHF have to be separated for correct quantitation of folate forms in serum, it provides evidence that 5-formylTHF cannot be ignored in the measurement process. It is to our knowledge the first study that analyzed a large number of specimens with a sufficiently sensitive method to show the presence of 5-formylTHF, sometimes at notable concentrations. While certified reference materials are not yet available for either MeFox or 5-formylTHF to verify absolute method accuracy, the validation experiments we performed indicate that the selective mass separation method presented here exhibits good accuracy. This new routine method also shows excellent performance characteristics, such as low long-term imprecision. These features in combination with the sample processing and chromatography procedure of the previously published method that has proven to be robust and rugged during almost 10 y of use in our laboratory, provide an invaluable improvement in the analysis of serum folate vitamers by LC-MS/MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5-formylTHF	5-formyltetrahydrofolate
5-methylDHF	5-methyldihydrofolate
5-methylTHF	5-methyltetrahydrofolate
5,10-methenylTHF	5,10-methenyltetrahydrofolate
hmTHF	4α-hydroxy-5-methyltetrahydrofolate
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOD	limit of detection
MeFox	pyrazino-s-triazine derivative of hmTHF
FA	folic acid
QC	quality control
THF	tetrahydrofolate

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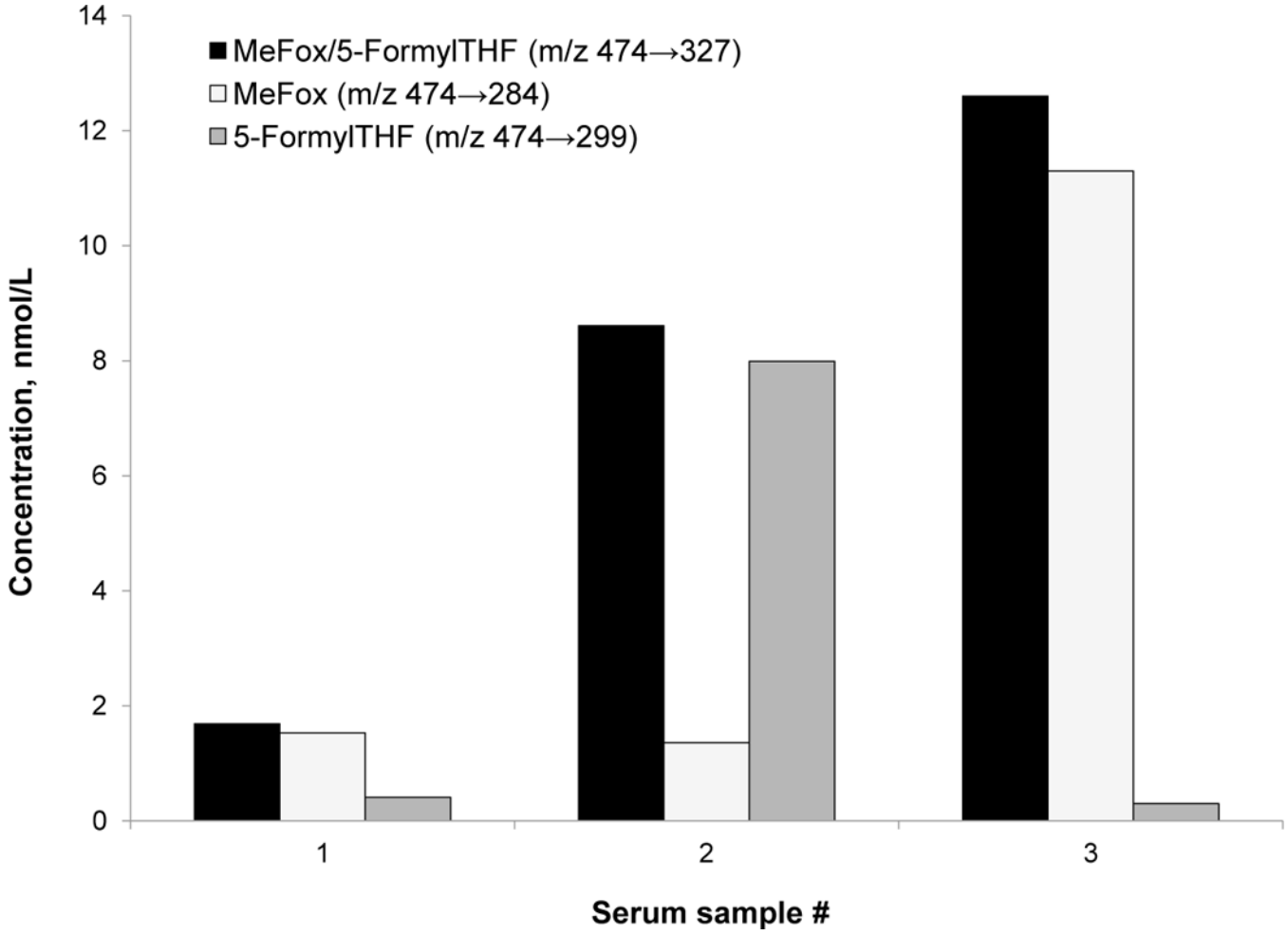

Figure 1.

Endogenous levels of MeFox and 5-formylTHF in three serum specimens that show different patterns of these two compounds (low MeFox/low 5-formylTHF, low MeFox/high 5-formylTHF, high MeFox/low 5-formylTHF) among several thousand samples analyzed. 5-formylTHF, 5-formyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methyltetrahydrofolate.

Table 1

LC-MS/MS method conditions for a published procedure (method 1) and for newly adapted chromatographic (method 2) and mass (method 3) separation methods

Parameters	Method 1	Method 2	Method 3
Sample extraction and clean-up	Automated reversed-phase SPE using 96-well phenyl plates (Bond Elute, Varian) on an 8-probe Gilson 215 processor (Gilson Inc.)		
Dilution of sample extract after SPE	None	One part sample extract + one part mobile phase	None
Isocratic mobile phase	49.5% water:0.5% acetic acid: 40% methanol:10% acetonitrile	69% water:1% acetic acid: 30% methanol	49.5% water:0.5% acetic acid: 40% methanol:10% acetonitrile
MS transitions (*m/z*)	MeFox + 5-formylTHF 474→327	MeFox + 5-formylTHF 474→327	MeFox 474→284 5-formylTHF 474→299
Chromatographic resolution	Co-elution of MeFox and 5-formylTHF	Baseline separation of MeFox and 5-formylTHF	Co-elution of MeFox and 5-formylTHF

5-formylTHF, 5-formyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methyltetrahydrofolate; SPE, solid phase extraction.

Table 2

Spiking recovery of 5-formylTHF and Mefox added to serum[1]

	Concentration			Recovery	
Spiking level[2]	**MeFox + 5-FormylTHF[3]** ***nmol/L***	**5-FormylTHF[4]** ***nmol/L***	**MeFox[5]** ***nmol/L***	**5-FormylTHF** ***%***	**MeFox** ***%***
Serum blank	3.58 ± 0.06	< LOD[6]	4.30 ± 0.07		
Serum + spike 1	7.22 ± 0.18	2.01 ± 0.07	5.95 ± 0.13	101 ± 3.4	83 ± 6.7
Serum + spike 2	13.1 ± 0.42	5.28 ± 0.16	9.09 ± 0.06	106 ± 3.2	96 ± 1.2
Serum + spike 3	22.3 ± 0.44	10.4 ± 0.25	14.6 ± 0.27	104 ± 2.5	103 ± 2.7

[1] Values represent mean ± SD; 5-formylTHF, 5-formyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methyltetrahydrofolate.

[2] Serum specimen was spiked with three levels (2, 5, and 10 nmol/L) of 5-formylTHF and MeFox and samples were processed by solid-phase extraction for LC-MS/MS analysis by method 3 (n = 3 replicates per spike level).

[3] Measured at m/z transition of 474→327.

[4] Measured at m/z transition of 474→299.

[5] Measured at m/z transition of 474→284.

[6] LOD for 5-formylTHF was 0.21 nmol/L.

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