Analytical Recovery of Folate Degradation Products Formed in Human Serum and Plasma at Room Temperature[1]

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Abstract

Folate is not stable in serum and plasma. This may impair laboratory diagnostics and distort the outcome of epidemiological studies on folate and chronic diseases. The present study was designed to determine the kinetics of folate loss in human serum and plasma (collected into tubes containing EDTA, heparin, or citrate) at room temperature and the recovery of folate as 4-α-hydroxy-5-methyltetrahydrofolate (hmTHF) or p-aminobenzoylglutamate (pABG) equivalents. Different folate species and pABG were determined by liquid chromatography-tandem MS and microbiologically active folate was measured by a *Lactobacillus rhamnosus* assay. Concentrations of 5mTHF and microbiologically active folate had a parallel and rapid decrease in EDTA plasma to ~60% of the initial concentration after 24 h. In serum, heparin plasma, and citrate plasma, folate decreased more slowly to ~50% after 192 h. The loss of 5mTHF that occurred within 48 h was totally recovered as hmTHF. Folate measured as pABG equivalents decreased slowly to ~80% in 192 h and the decline was essentially matrix independent. In conclusion, the degradation of 5mTHF and microbiologically active folate in serum and plasma at room temperature can largely be corrected for by determining hmTHF or measuring folate as pABG equivalents. Moreover, results obtained using conventional folate assays may be biased by improper sample handling or if samples contained high concentrations of hmTHF.   J. Nutr. 139: 1415–1418, 2009.

Introduction

The growing interest in folate, a micronutrient belonging to the B-vitamin group, is related to its role in numerous biochemical reactions that are critical for human health. Folate serves as a carrier of methyl, formyl, and other 1-carbon units, which are used for the synthesis of purines and pyrimidines and in methylation reactions. Poor folate status is a risk factor for neural tube defects (1) and has been associated with risk of cancer (2), cardiovascular diseases (3), and cognitive impairment (4).

The concentration of folate in serum or plasma has been used as a marker of folate status in humans. 5-Methyltetrahydrofolate (5mTHF)[5] is the predominant form (> 90%), but folic acid (FA) may be present after ingestion of supplements or food fortified with FA (5,6). Low concentrations of 5-formyltetrahydrofolate (5,7) and 4-α-hydroxy-5-methyltetrahydrofolate (hmTHF) (8) are also found.

A number of methods have been developed for the determination of serum and plasma folate (9). A commonly used microbiological assay measures folate that supports growth of *Lactobacillus rhamnosus* (10). Most routine methods for measuring serum folate, including chemiluminescence assays, radioassays, and ion capture assays, use folate binding proteins, but there has been some concern about their specificity due to variable binding affinities for different folate species (11). With recent chromatographic methods based on liquid chromatography (LC) combined with tandem MS (LC-MS/MS), most folate species in serum or plasma can be identified and quantified (5,12). LC-MS/MS is therefore suitable for the study of folate stability and interconversion.

Folate instability increases preanalytical variability in routine laboratory diagnostics and epidemiological studies. Folate concentrations in serum and plasma decrease at room temperature. This decline seems to proceed at a faster rate in EDTA plasma than in serum, and the folate loss may approach 50% after 6 d (13). Folate is more stable at low temperatures, but there is still a substantial loss of folate in samples stored for years at −20°C (14,15).

Data on folate degradation in serum and plasma under ordinary storage and sampling conditions are sparse. Folate species differ in their resistance to oxidative degradation (16). In serum and plasma, 5mTHF can be converted to 5-methyl-5,6-dihydrofolate (5mDHF) under aerobic conditions, but 5mDHF can easily be reconverted to 5mTHF by a reducing agent such as ascorbic acid. Under more severe oxidative conditions, e.g. in

the presence of hydrogen peroxide, 5mDHF is transformed to a compound that was initially identified as hmTHF (17) but was later assigned a pyrazino-s-triazine structure (18).

We recently showed that folate species and their degradation products may be recovered as p-aminobenzoylglutamate (pABG) by subjecting the samples to controlled oxidation and limited acid hydrolysis. This is the basis of an assay measuring folate in fresh and stored samples as pABG equivalents (8). In the present work, we investigated the stability, loss, or formation of folate species in serum or plasma (EDTA, heparin, and citrate) during storage for up to 8 d at room temperature. Folate was measured by LC-MS/MS [5mTHF, hmTHF, FA, and 5-formyltrahydrofolate (5fTHF)] (19) by a microbiological assay (10) and as pABG equivalents by LC-MS/MS (8).

Materials and Methods

Sample collection. We obtained serum and plasma (collected into tubes containing EDTA, heparin, or citrate) from 16 healthy humans. Whole blood was collected into plain silicon-coated tubes (Becton Dickinson Vacutainer) and allowed to clot at room temperature (23°C), in the dark for 30 min before centrifugation at 2100 × g for 10 min at 4°C. The serum fraction was separated and care was taken to avoid any admixture of buffy-coat. The material was then divided into 10 different 2-mL polypropylene tubes (Sarstedt AG&Co). One tube (T0, baseline) was immediately stored at −80°C and the others were kept at room temperature in the dark for 1, 2, 4, 8, 12, 24, 48, 96, and 192 h before storage at −80°C. Plasma was obtained by collecting whole blood into tubes (Terumo Venoject) containing tripotassium EDTA, lithium heparin, or trisodium citrate (0.5 mL, 0.129 mol/L). The tubes were turned upside down 5 times and centrifuged before the plasma fractions were isolated. The plasma samples were then aliquoted and handled in the same way as the serum samples.

The study was examined by the Institutional Review Board (REK Vest) and found to be of the quality control category that, under the current Norwegian regulations, is exempt from full review by the Board. The Board had no objection to publication of the results.

Biochemical analyses.

The samples were analyzed using 3 different methods. Four different folate species, 5mTHF, hmTHF, FA, and 5fTHF, were determined by LC-MS/MS, as described (19). The second method was a microbiological assay using *L. rhamnosus* (10). Finally, folate was determined as pABG equivalents after quantitative conversion using oxidation and mild acid hydrolysis (8). Samples from each donor were analyzed together and further grouped by matrix and analyzed in an order that was determined by incubation time (0, 192, 1, 96, 2, 48, 4, 24, 8, and 12 h). This was done to avoid any bias related to variable time of sample processing.

Statistical methods.

Medians with ranges were used for descriptive statistics. The change in folate concentrations during 8 d (192 h) of storage was depicted in relative terms with baseline concentrations set at 100%. Changes in the concentrations of folate and folate species over time were further analyzed by segmented linear regression with a single breakpoint. Model fits and parameter estimates for the first and second line slopes, the breakpoint (time at which a slope change appears to occur), and their 95% CI were calculated using GraphPad Prism (GraphPad Software).

Results

We measured folate and folate species in aliquots of serum and plasma stored at room temperature from 0 to 192 h. At baseline (T0), the median concentrations of 5mTHF (15.6 nmol/L), hmTHF (1.6 nmol/L), microbiologically active folate (10.4 nmol/L), and folate measured by the pABG assay (15.6 nmol/L) were essentially the same in serum, EDTA plasma, and heparin

plasma and ~15% lower in citrate plasma (**Table 1**). FA and 5fTHF were below their limits of detection (0.27 and 0.52 nmol/L, respectively) in all samples.

In serum, the 5mTHF concentration was essentially stable for 48 h and thereafter decreased to ~50% after 192 h (**Fig. 1***A*). In EDTA plasma, the reduction in 5mTHF was more pronounced, with a rapid initial decrease followed by a slow decline (Fig. 1*A*). After 192 h, 5mTHF was reduced to 20%. Notably, the decline in 5mTHF was essentially mirrored by an accumulation in hmTHF, which had a slow increase in serum and a rapid burst phase for the first 48 h in EDTA plasma (Fig. 1*B*). The kinetics of 5mTHF decrease and hmTHF accumulation in heparin plasma and citrate plasma were essentially the same as in serum (**Table 2**).

We calculated the kinetics for the decrease of the sum of 5mTHF plus hmTHF (total folate) as measured by LC-MS/MS. In serum, the reduction in 5mTHF was totally recovered as hmTHF after 96 h and total folate was reduced to 80% after 192 h. In EDTA plasma, a smaller percentage of 5mTHF was recovered as hmTHF and total folate reached 60% after 192 h (**Fig. 2***A*). The declines in folate in serum and EDTA plasma measured by the microbiological assay (Fig. 2*B*) were similar to that of 5mTHF (Fig. 1*A*), with a faster and more pronounced reduction in EDTA plasma than in serum. Folate measured as pABG equivalents had a slow and moderate reduction, which was similar in serum and EDTA plasma and which reached ~80% after 192 h (Fig. 2*C*). Again, the decrease in folate in heparin plasma and citrate plasma measured by the microbiological and pABG assays showed kinetics similar to those observed in serum (Table 2).

Discussion

We studied the degradation of folate species at room temperature in serum and in different types of plasma (EDTA, heparin, and citrate) and found that 5mTHF and microbiological active folate rapidly decreased in EDTA plasma and more slowly in serum and the other matrices. Loss of 5mTHF within the first 48 h was almost totally recovered as hmTHF in all 4 matrices. After 48 h, the sum of 5mTHF plus hmTHF decreased in EDTA plasma, whereas it was stable for up to 96 h in serum and citrate plasma and 192 h in heparin plasma. Folate measured as pABG equivalents decreased slowly and at a similar rate in all matrices and was the most stable folate marker after 192 h.

The study had a longitudinal design and determined the loss of folate in serum and plasma stored in the dark at room temperature. These are conditions that may mimic sampling, processing, and transportation of serum or plasma to the routine

TABLE 1 Folate indices in human serum and plasma at baseline[1,2]

	LC-MS/MS folate assay		Folate	
			Microbiological assay[3]	pABG assay
Matrix	5mTHF	hmTHF		
	nmol/L			
Serum	16.5 (8.7–30.5)	1.9 (0.4–5.2)	11.2 (6.7–21.9)	18.6 (8.4–36.7)
EDTA plasma	16.8 (8.7–27.2)	1.5 (0.3–4.8)	10.4 (6.5–20.9)	16.6 (9.5–28.6)
Heparin plasma	15.7 (6.3–30.8)	1.7 (0.2–4.9)	11.2 (7.2–22.2)	16.0 (8.8–29.1)
Citrate plasma	13.4 (6.7–24.2)	1.1 (0.2–3.3)	9.9 (6.0–18.5)	14.0 (7.6–23.8)

[1] Values are median (range), n = 16.
[2] Baseline concentrations were measured in the samples that were stored immediately at −80°C (T0).
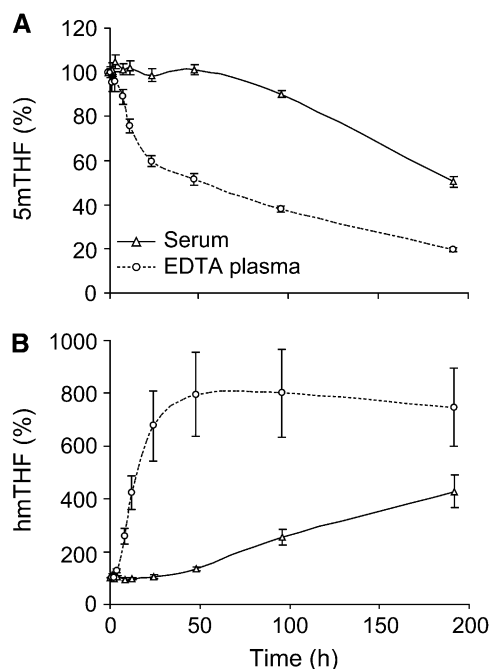[3] *L. rhamnosus.*

FIGURE 1 Degradation of 5mTHF (*A*) and accumulation of hmTHF (*B*) in human serum and EDTA plasma at room temperature. The changes in folate species are plotted as a percentage of concentrations at the start of incubation. Values are means ± SEM, *n* = 16.

clinical chemistry laboratory and field collection of samples used in epidemiological studies. Samples for epidemiological studies are usually stored in repositories at −20 or −80°C or in liquid nitrogen. A significant degradation of folate has been demonstrated in serum samples at –20°C (14) and folate measured as pABG equivalents is substantially higher than microbiological active folate in serum samples stored at −25°C for 27–34 y (8). Further studies are needed to assess folate stability in frozen samples.

Median concentrations at baseline determined by the LC-MS/MS folate assay and by the pABG assay were somewhat higher than those obtained with the microbiological method. This could be related to method calibration; for LC-MS/MS, this is carried out with the actual analyte, whereas FA is used to calibrate the microbiological assay. Lower concentrations of folate species in citrate plasma might be attributed to dilution by the citrate solution.

The kinetics of folate and folate degradation products were not linear but fitted a 2 segmented linear regression model including a time point of change in slope. The kinetics did not obey first order, suggesting that folate degradation is not a simple unimolecular event. This may imply that conditions that promote degradation change over time or it may indicate the presence of different folate fractions, possibly related to folate protein binding (20). Folate is degraded in the presence of reactive oxygen species (21) and it is possible that the concentration of oxygen or reactive oxygen species in closed tubes containing serum or plasma may change during storage at room temperature. Folate may be formed or degraded by bacteria growing in the sample tubes. However, we observed the highest rate of decline in folate during the initial hours of storage in plasma containing EDTA, which is reported to inhibit bacterial growth (22). These observations make it unlikely that loss of folate is related to bacterial growth in the sample tubes.

The most notable observation in this study is that the LC-MS/MS method, which we used to determine 5mTHF and hmTHF, allows almost complete correction for folate degradation that occurs during the first 48 h. Longer incubation or storage at room temperature seems to cause further degradation of folate to unknown species and 80% of folate was recovered in serum after 192 h. Thus, determination of total folate (5mTHF plus hmTHF) by LC-MS/MS seems to be an adequate strategy to obtain reliable folate levels in specimens subjected to moderate degradation.

Folate determined by the microbiological assay based on the growth of *L. rhamnosus* measures 5mTHF, but not hmTHF (23). This explains the parallel decline of microbiological active folate and 5mTHF. Our results suggest that the microbiological assay does not provide a reliable measure of folate status in EDTA

TABLE 2 Changes in folate indices in human serum and plasma by segmented linear regression[1]

Index	Matrix	Slope 1	Slope 2	Breakpoint[2]
		%/h		*h*
5mTHF	Serum	−0.02 (−0.13 to 0.09)	−0.41 (−0.49 to −0.34)	72.0 (48.6 to 95.4)
	EDTA plasma	−1.92 (−2.44 to −1.39)	−0.23 (−0.28 to −0.19)	22.6 (16.3 to 28.8)
	Heparin plasma	−0.05 (−0.28 to 0.18)	−0.36 (−0.51 to −0.21)	64.8 (5.1 to 124.6)
	Citrate plasma	−0.10 (−0.21 to 0.00)	−0.37 (−0.44 to −0.30)	54.8 (22.9 to 86.7)
hmTHF	Serum	0.03 (−2.07 to 2.14)	2.02 (1.59 to −2.43)	27.5 (−4.54 to 59.6)
	EDTA plasma	25.7 (16.6 to 34.9)	−0.37 (−2.20 to 1.47)	28.9 (18.1 to 39.7)
	Heparin plasma	1.01 (−0.71 to 2.73)	3.51 (2.42 to 4.61)	70.7 (14.3 to 127)
	Citrate plasma	1.79 (−0.95 to 4.52)	2.08 (1.53 to 2.62)	48.0 (37.7 to 58.3)
Folate, microbiological assay	Serum[3]	−0.16 (−0.41 to 0.10)		
	EDTA plasma	−1.61 (−1.96 to −1.26)	−0.21 (−0.28 to −0.14)	25.3 (18.1 to 32.5)
	Heparin plasma	−0.08 (−0.25 to 0.09)	−0.41 (−0.51 to −0.30)	83.2 (38.1 to 128)
	Citrate plasma	−0.11 (−0.24 to 0.03)	−0.35 (−0.44 to −0.27)	65.1 (21.1 to 109)
Folate, pABG assay	Serum[3]	−0.09 (−0.17 to −0.02)		
	EDTA plasma	−0.24 (−0.33 to −0.14)	0.01 (−0.05 to 0.08)	62.8 (31.9 to 93.8)
	Heparin plasma	0.16 (−0.21 to 0.53)	−0.12 (−0.15 to −0.09)	17.6 (−3.3 to 38.5)
	Citrate plasma	−0.05 (−0.15 to 0.04)	−0.15 (−0.21 to −0.09)	42.0 (−34.3 to 130)

[1] Values are means (95% CI), *n* = 16.
[2] Time when slope changes.
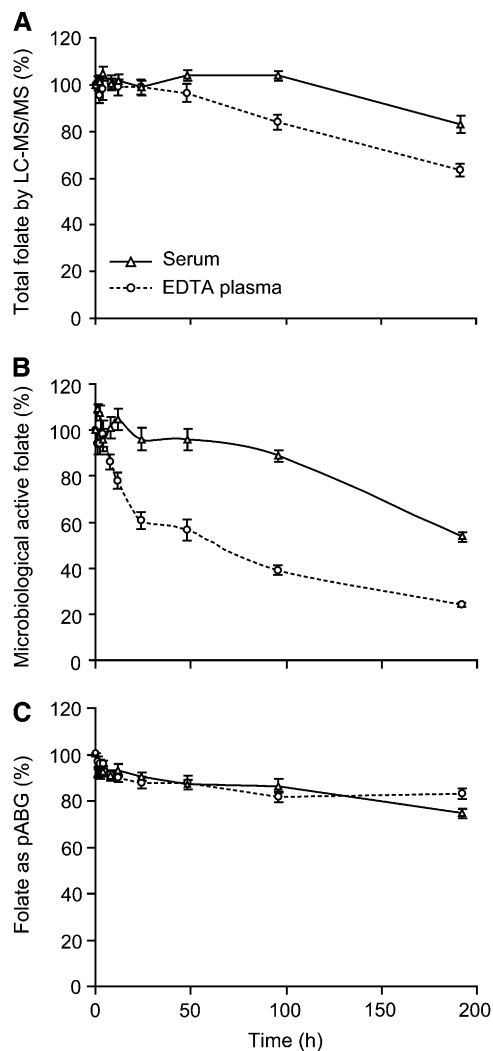[3] Data fitted to a linear model with a single slope.

FIGURE 2 Changes in folate in human serum and EDTA plasma at room temperature by assay format. Folate was determined as total folate (5mTHF plus hmTHF) by LC-MS/MS (A), by the microbiological assay (B), and as pABG equivalents by LC-MS/MS (C). Values are plotted as a percentage of concentrations at the start of incubation and are means ± SEM, $n = 16$.

plasma samples left at room temperature for hours. The rapid initial decrease in folate implies that short but variable storage may cause substantial preanalytical variability.

Folate determined as pABG equivalents decreased slowly and ~80% of folate was recovered after 192 h. Notably, the results were not influenced by matrix, as the same concentrations were measured in serum and plasma with various additives. This would make it the preferred assay for samples subjected to prolonged storage, in particular for EDTA plasma and for studies including both serum and EDTA samples. Lack of knowledge about specimen matrices and unintended admixture of matrices may occur when sampling blood for clinical diagnostics and epidemiological studies.

In conclusion, the present study shows that 5mTHF decreases rapidly during storage of serum and plasma and can to a large extent be recovered as hmTHF, which is not microbiologically active (23) but can be measured by LC-MS/MS. Folate determined as pABG equivalents decreases more slowly and the decline is essentially matrix independent. These observations point to strategies to correct for loss of folate in serum or plasma caused by delayed freezing of samples. They also motivate

studies on the feasibility of measuring folate as hmTHF or pABG equivalents to obtain reliable folate indices for blood specimens subjected to long-term freezing.

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