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Analytical Recovery of Folate and Its Degradation Products in Human Serum Stored at –25°C for up to 29 Years[1,2]

Rita Hannisdal,[3,4,9]* Randi E. Gislefoss,[5,6,9] Tom K. Grimsrud,[6] Steinar Hustad,[3,8] Lars Mørkrid,[5] and Per Magne Ueland[3,7]

[3]Section for Pharmacology, Institute of Medicine, University of Bergen, Bergen 5021, Norway; [4]National Institute of Nutrition and Seafood Research, Bergen 5004, Norway; [5]Institute of Clinical Biochemistry, Faculty of Medicine, University of Oslo and Department of Medical Biochemistry, Rikshospitalet-Radiumhospitalet Medical Centre, 0022 Norway; [6]Cancer Registry of Norway, Institute of Population-Based Cancer Research, Oslo 0166, Norway; and [7]Laboratory of Clinical Biochemistry, and [8]Hormone Laboratory, Haukeland University Hospital, Bergen 5021, Norway

Abstract

Epidemiological studies on folate and chronic diseases often involve the use of frozen serum stored in biorepositories for decades. Folate instability may attenuate associations between folate status and study endpoints. In this cross-sectional study, we retrieved serum samples stored at –25°C for 0, 4, 6, 17, or 29 y in the Janus biobank. Samples were obtained from a total of 650 men aged 40–49 y at the time of blood collection and were evenly distributed according to storage time. Folate was determined by a liquid chromatography tandem MS (LC-MS/MS) assay that measures 5-methyltetrahydrofolate (5mTHF), its oxidation product 4-α-hydroxy-5-methyltetrahydrofolate (hmTHF), and other folate species; by a *Lactobacillus rhamnosus* microbiological assay; and by LC-MS/MS as p-aminobenzoylglutamate (pABG) equivalents after oxidation and mild acid hydrolysis of the folate species. Concentrations of 5mTHF and microbiologically active folate were lower in samples that had been subjected to long-term storage and the data were consistent with a decrease of 3.2 and 2.8%/y, respectively. hmTHF was detected in all specimens but did not accumulate upon long-term storage (>4 y). Folate measured as pABG declined at a slow rate of 0.98%/y and ~80% of the folate was recovered after 29 y of storage. B-vitamin status did not differ between individuals delivering samples at different time points, as assessed by measuring total homocysteine, methylmalonic acid, and serum vitamin B-12. In conclusion, folate is substantially degraded in serum frozen for decades but can to a large extent be recovered as pABG equivalents. The pABG assay appears to be the method of choice for the determination of folate status in archival serum samples. J. Nutr. 140: 522–526, 2010.

Introduction

Epidemiological studies have demonstrated associations between low concentrations of folate in blood and an increased risk of adverse birth outcomes (1), cardiovascular diseases (2), and different forms of cancers (3). These associations are biologically plausible given the role of folate in homocysteine remethylation to methionine, purine, and pyrimidine formation (DNA synthesis) and methylation reactions involving DNA and other molecules (4).

Plasma total homocysteine (tHcy)[10] and serum or plasma folate have been used as markers of folate status in epidemiological studies. tHcy is thought to be stable during storage (5–7), but concentrations reflect the status of other B-vitamins than folate, including vitamin B-12, and therefore has low specificity (8). Concentration of folate in serum and plasma is a more specific marker of folate status. However, there is a substantial degradation of folate in serum and EDTA plasma kept at room temperature for days (9) and in frozen samples stored for 1 y (10).

5-Methyltetrahydrofolate (5mTHF) is the predominant folate species in human serum (11,12). It undergoes reversible oxidation to 5mTHF and is oxidized to 4-α-hydroxy-5-methyltetrahydrofolate (hmTHF) upon prolonged storage of serum samples (9) or in the presence of a strong oxidant (13). We recently measured hmTHF, 5mTHF, and other folate species in serum by liquid chromatography tandem MS (LC-MS/MS) (11) and demonstrated that folate lost in samples stored for up to 48 h at room temperature could be recovered as hmTHF (9). Folate was thereafter further degraded to unidentified species, which could be collectively detected by LC-MS/MS as p-aminobenzoylglutamate (pABG) equivalents after oxidation and mild hydrolysis (14).

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[9] These authors contributed equally to the work.
[10] Abbreviations used: 5mTHF, 5-methyltetrahydrofolate; FA, folic acid; hmTHF, 4-α-hydroxy-5-methyltetrahydrofolate; MMA, methylmalonic acid; pABG, p-aminobenzoylglutamate; tHcy, total homocysteine.
* To whom correspondence should be addressed. E-mail: rita.hannisdal@nifes.no.

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Similar studies have not been performed on the degradation of folate in frozen serum samples. In serum repositories used for prospective epidemiological studies, frozen specimens are stored for years, but longitudinal investigation covering a long time frame is an impractical undertaking in terms of design and logistics.

In this cross-sectional study, we used samples from the Janus Serum Bank, which was established in 1973 (15) and contains serum samples stored at –25°C from >330,000 participants. The samples were collected, processed, and stored under standardized conditions. We included samples stored for up to 29 y to investigate folate degradation in frozen serum and the recovery of folate as hmTHF (11) and as pABG equivalents (14). We monitored possible differences in B-vitamin status according to time of collection of serum samples by measuring tHcy, methylmalonic acid (MMA), and vitamin B-12 in serum.

Materials and Methods

Participants and sample handling. Samples from a total of 650 men aged 40–49 y at the time of blood collection were obtained from the Janus Serum Bank of Norway. There were 130 samples from each of the years 1979, 1991, 2002, 2004, and 2008. The samples were analyzed in 2008 and, accordingly, storage times were 0, 4, 6, 17, and 29 y. Routines for sample collection and processing were essentially the same throughout the 29-y period. Whole blood was collected into tubes without additives or separating gel. The samples were left to coagulate at room temperature for 1 h and were then stored at 4°C for 15–30 h, before centrifugation and collection of the serum fraction. For 2008, parallel samples were collected from each donor and stored for 15–24 h and 21–30 h at 4°C before separation of the serum fraction. Serum was aliquoted into polypropylene tubes and stored at –25°C in cardboard boxes covered with lids. Direct exposure to light occurred only during collecting, retrieval, and aliquoting of samples. The study was approved by the National Committees for Research Ethics in Norway.

Methods. We measured folate in serum with 3 different methods. Four folate species, 5mTHF, hmTHF, folic acid (FA), and 5-formyltetrahydrofolate, were measured by LC-MS/MS (11). Limits of detection of the assay were between 0.07 and 0.52 nmol/L and the assay was linear up to 140 nmol/L for all analytes. Imprecision varied between 4 and 12%. We also measured folate by a microbiological assay using *Lactobacillus rhamnosus* (16). The method had an imprecision of ~6%. The 3rd assay measured folate as pABG equivalents after oxidation and mild acid hydrolysis (14). pABG was quantified by LC-MS/MS. The limit of detection was 0.13 nmol/L, the impression was ≤5%, and linearity was observed up to 96 nmol/L. tHcy and MMA were measured by GC-MS (17), whereas vitamin B-12 was measured by a microbiological assay (18).

To investigate the long-term stability of tHcy, 126 plasma samples from the Hordaland Homocysteine Study (19) were reanalyzed after ~15 y of storage at –25°C and results were compared.

Statistics. Ages of participants in the 5 storage groups were compared by ANOVA and differences were assessed by the Student Neuman-Keul test. To obtain normal distribution of data for parametric tests, data were log-transformed. Visual inspection of Z-score plots was used to check the distribution and Tukey's fence with factor 1.5 was used to exclude outliers in F-tests and residual outliers in linear regression analyses. Possible differences in dispersion (e.g. variances) compared with the reference group (2008 samples) were examined by means of an in house Excel F-test algorithm. We used linear regression analysis (SPSS version 16) to estimate the relation between vitamer level and storage time. Both locations of central tendencies (median) and dispersions were used to evaluate the stability. A significance level of 0.05. was chosen except for the variance ratio test, where Bonferroni's correction was applied ($P < 0.0125$). Values in the text are means ± SD unless otherwise noted.

Results

The ages of the men delivering the samples were 44.5 ± 3.1, 45.0 ± 2.9, 45.5 ± 2.9, 43.6 ± 2.7, and 44.4 ± 3.0 y for samples collected in 1979, 1991, 2002, 2004, and 2008, respectively. The age distribution was slightly heterogeneous with men delivering samples in 2004 younger than those from the other cohorts ($P = 0.03$).

We initially measured the folate concentration in serum samples collected in 2008 with a mean difference of 6 h of storage at 4°C between duplicates of whole blood before separation of the serum fraction. Storage time did not affect the concentration of any of the folate species in these samples.

We determined concentrations (median, 2.5–97.5 percentile) of 5mTHF and hmTHF, microbiologically active folate, and folate measured as pABG-equivalents in serum specimens stored for 0, 4, 6, 17, and 29 y (**Table 1**). In samples collected in 2008, the median concentration of 5mTHF was somewhat higher than the median concentration of microbiologically active folate but lower than folate measured as pABG. A large amount of hmTHF, but no FA or 5-formyltetrahydrofolate, was detected in these samples.

Differences in folate concentration by storage time were calculated by linear regression analysis (**Table 2**). 5mTHF and microbiologically active folate had a similar decline of 3.2% and 2.8%/y, respectively, corresponding to a folate loss of ~60% after 29 y of storage. Concentrations of hmTHF were highest in samples stored for 4 y. Total folate, which is calculated as the sum of 5mTHF and hmTHF, decreased by 2.3%/y, whereas folate determined as pABG equivalents was the most stable folate index (0.98%/y) (Table 2).

The estimated change in total folate, microbiologically active folate, and pABG equivalents over time were corroborated by using Z-score plots, which demonstrated normal distribution of the folate indices after log transformation and a time-dependent symmetrical shift of values across the whole distribution with no tail effect (**Fig. 1**). Variance ratio tests demonstrated essentially equal dispersion for the folate indices (except for hmTHF) according to time of storage (**Table 3**).

Notably, both tHcy and MMA (a function marker of cobalamin status) were stable over the time period investigated. Serum vitamin B-12 levels were highest in samples with the longest storage time (P-trend = 0.025). We validated the long-term stability of plasma tHcy in samples from the Hordaland Homocysteine Study and compared measurements carried out in 1994 and in 2009, i.e. after 15 y of storage at –25°C. Concentrations were 10.4 ± 3.7 μmol/L and 9.7 ± 3.5 μmol/L for the first and second measurements, respectively, which correspond to a 7% decrease ($P < 0.001$).

Discussion

We studied folate degradation and the recovery of folate as hmTHF and as pABG equivalents in serum samples that had been stored in the Janus biobank at –25°C for up to 29 y. The samples originated from 5 different years and from a total of 650 men aged 40–49 y at the time of blood collection and were equally distributed according to storage time. We observed lower concentrations folate in serum samples stored at –25°C for up to 29 y compared with fresh samples. For 5mTHF and microbiologically active folate, the concentration difference was consistent with 3% degradation per year corresponding to a folate loss of 60% over 29 y. For serum folate measured as pABG equivalents, the corresponding values were <1% degradation per year and only 21% over 29 y.

TABLE 1 Concentrations of B-vitamin indices in human serum by duration of sample storage[1,2]

	Storage, *y*				
Index	0	4	6	17	29
5mTHF, *nmol/L*	11.8 (5.4–25.7)	12.6 (5.1–31.0)	11.2 (5.1–24.4)	8.0 (3.3–19.5)	4.9 (2.1–11.7)
n	128	130	128	128	128
hmTHF, *nmol/L*	4.3 (1.6–11.4)	6.4 (2.7–15.3)	4.8 (2.3–10.2)	4.0 (1.5–10.6)	4.2 (9.4–21.0)
n	128	130	129	128	125
5mTHF + hmTHF, *nmol/L*	16.7 (8.6–32.2)	19.5 (9.9–38.3)	16.2 (7.9–33.1)	12.6 (6.1–26.0)	9.3 (4.9–17.6)
n	128	130	128	127	126
Microbiologically active folate, *nmol/L*	9.6 (4.8–19.4)	9.1 (4.4–18.7)	8.0 (4.3–15.1)	6.2 (3.1–12.5)	4.2 (1.9–9.1)
n	125	129	126	124	127
Folate as pABG equivalents, *nmol/L*	15.2 (8.3–27.5)	15.8 (9.5–26.3)	14.3 (8.5–24.1)	12.8 (7.0–23.2)	12.0 (6.9–20.9)
n	126	128	126	129	127
tHcy, *μmol/L*	13.4 (9.5–18.9)	13.8 (9.3–20.76)	14.0 (10.3–18.8)	13.4 (8.5–20.1)	14.0 (9.4–21.0)
n	128	127	126	127	125
MMA, *μmol*/L	0.16 (0.1–0.26)	0.17 (0.09–0.31)	0.16 (0.09–0.29)	0.16 (0.09–0.26)	0.16 (0.09–0.3)
n	128	129	130	128	127
Vitamin B-12, *pmol/L*	346 (207–577)	371 (222–620)	354 (197–634)	388 (224–669)	377 (213–668)
n	126	126	128	128	127

[1] Data are given as median (2.5–97.5 percentile).
[2] Outliers are excluded.

The study is based on cross-sectional data obtained by studying serum samples from a large research biobank. An advantage of the study is that the individuals who delivered the samples are healthy and homogeneous with respect to sex and age. The procedures for handling of serum samples collected for the Janus biobank have been standardized and were essentially the same throughout the study period. However, storage of whole blood samples at 4°C before separation of the serum fraction varied from 15 to 30 h. We observed the same concentrations of folate species in parallel serum samples with a difference of 6 h in storage time at 4°C. Sublimation has been found to be minor (0.015%/y) (20). Thus, variable sample handling before freezing and sublimation are not likely to have had a major impact on the results.

The interpretation of the results rests on the assumption that the folate status of the study population has not changed over the years. In Norway, there is no mandatory folate fortification of flour and FA supplements are recommended for women of childbearing age (21) but are not widely used in the general population. More comprehensive data on folate intake are lacking. We determined tHcy in the study participants to assess possible changes in folate status over the 29-y study period and included measurements of serum vitamin B-12 and MMA to ensure that the tHcy results were not influenced by differences in vitamin B-12 status. Data on tHcy stability published are sparse (5,6), although the view prevails (22) that tHcy concentrations do not change during long-term storage of frozen samples. The reanalysis of plasma samples from the Hordaland Homocysteine Study after storage at –25°C for 15 y indicates that plasma tHcy is stable and corroborates the utility of tHcy as a long-term indicator of B-vitamin status.

In fresh serum samples and in samples stored for ≤6 y, folate measured as total folate by LC-MS/MS was 10–23% higher than folate measured as pABG equivalents. This observation is in agreement with results from comparison of the methods in fresh samples (11) and is probably explained by different calibration procedures for the 2 methods (14).

We found hmTHF in all samples and the median concentration of hmTHF in samples collected in 2008 was 4.3 nmol/L and accounted for 26% of total folate in these samples. A previous study detected hmTHF at a median concentration of 1.9 nmol/L in fresh human serum samples (9). Significant amounts of hmTHF may therefore exist in vivo or are formed rapidly by oxidation of 5mTHF after sampling.

Longitudinal data on folate stability in frozen samples (–20°C) are sparse and cover only a short storage period. One study reported that folate decreased 14 and 39% in serum samples stored for 1 and 2 y, respectively (23). In EDTA plasma, a 20% reduction in folate was observed after 1 y of storage and thereafter the folate concentration was stable for up to 3 y (10).

We have previously determined loss of folate in serum and EDTA-plasma stored for 8 d at room temperature (9). 5mTHF was quantitatively recovered in serum and EDTA plasma as hmTHF within the first 48–96 h but not after further incubation. Folate measured as pABG equivalents was more stable (9). Although the time frame and storage conditions differ, these

TABLE 2 Differences in B-vitamin indices in human serum according to storage time by linear regression analysis[1]

Index	a	b	*P*-value	Differences/y, %
5mTHF	1.122	−0.0140	<0.0001	−3.2
hmTHF	NA[2]	NA	NA	NA
5mTHF + hmTHF	1.276	−0.0101	<0.0001	−2.3
Microbiologically active folate	0.997	−0.0125	<0.0001	−2.8
Folate as pABG equivalents	1.194	−0.0042	<0.001	−0.98
tHcy	1.140	$-1.7 \cdot 10^{-5}$	0.96	0.004
MMA	−0.788	$-3.0 \cdot 10^{-5}$	0.95	0.01
Vitamin B-12	2.559	0.0016	0.025	+0.25

[1] Model: lg component = a + b × time (y).
[2] NA, Not applicable due to nonlinearity.

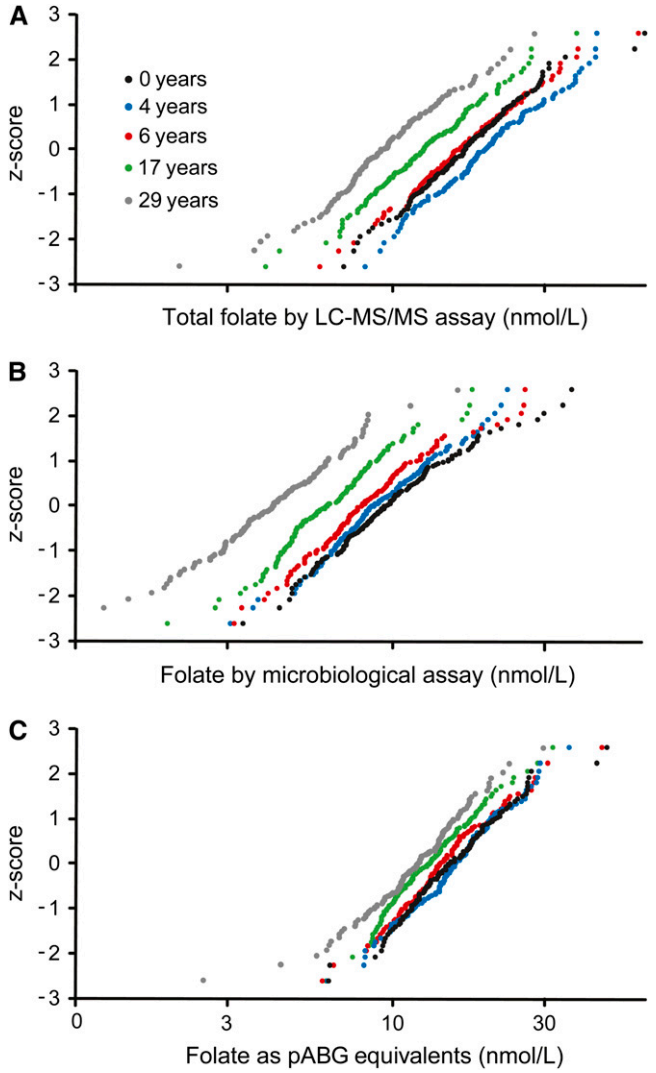

FIGURE 1 Z-score plots for folate concentration (nmol/L) in stored human serum samples by assay format. Folate was determined as total folate (5mTHF plus hmTHF) by LC-MS/MS (*A*), by the microbiological assay (*B*), and as pABG equivalents (*C*). Folate concentrations are log-transformed.

observations resemble results from the present study, which demonstrates a maximal hmTHF concentration in serum stored for 4 y, a marked decrease (44%) of total folate (5mTHF plus hmTHF) and a moderate decrease (20%) in pABG equivalents after 29 y of storage. Thus, during storage of samples, folate is oxidized to hmTHF, which may be further degraded to compounds that are recovered as pABG but also to compounds that are not recoverable. The latter seem to accumulate upon prolonged storage. This may explain the discrepancy between results obtained with the folate LC-MS/MS assay and the pABG assay in the oldest samples.

In conclusion, folate degradation is substantial in serum samples that have been frozen for decades. Folate instability may increase preanalytical variability, particularly if sample storage time varies or if samples have otherwise received nonuniform treatment, and this may distort associations between folate indices and endpoints in epidemiological studies. Folate measured as pABG equivalents is the most stable folate index in serum samples stored at both room temperature and as frozen specimens and the pABG assay may be the method of choice for the determination of folate status in archival serum samples.

TABLE 3 Dispersion differences expressed as the variance ratio with reference to human serum samples collected in 2008[1–3]

	Storage time, *y*			
Index	4	6	17	29
5mTHF	1.50 (0.02)	1.21 (0.3)	1.30 (0.14)	1.45 (0.04)
hmTHF	0.63 (0.009)	0.64 (0.01)	0.78 (0.16)	0.65 (0.014)
5mTHF + hmTHF	1.22 (0.26)	1.17 (0.38)	1.08 (0.67)	1.02 (0.90)
Microbiologically active folate	1.01 (0.95)	0.82 (0.27)	0.78 (0.17)	1.16 (0.4)
Folate as pABG equivalents	1.05 (0.75)	1.01 (0.96)	1.06 (0.74)	1.06 (0.73)
tHcy	1.45 (0.04)	1.03 (0.86)	1.40 (0.06)	1.42 (0.05)
MMA	1.25 (0.2)	1.30 (0.13)	0.94 (0.73)	1.36 (0.09)
Vitamin B-12	1.10 (0.58)	1.18 (0.36)	0.98 (0.9)	1.21 (0.3)

[1] Data are given as variance ratio (*P*-value).
[2] F-test for log-transformed data.
[3] Bonferroni's correction with $P < 0.0125$ was used.

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