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Kinetic Modeling of Storage Effects on Biomarkers Related to B Vitamin Status and One-Carbon Metabolism

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BACKGROUND: Biomarkers and metabolites related to B vitamin function and one-carbon metabolism have been studied as predictors of chronic diseases in studies based on samples stored in biobanks. For most biomarkers, stability data are lacking or fragmentary.

METHODS: Degradation and accumulation kinetics of 32 biomarkers were determined at 23 °C in serum and plasma (EDTA, heparin, and citrate) collected from 16 individuals and stored for up to 8 days. In frozen serum (−25 °C), stability was studied cross-sectionally in 650 archival samples stored for up to 29 years. Concentration vs time curves were fitted to monoexponential, biexponential, linear, and nonlinear models.

RESULTS: For many biomarkers, stability was highest in EDTA plasma. Storage effects were similar at room temperature and at −25 °C; notable exceptions were methionine, which could be recovered as methionine sulfoxide, and cystathionine, which decreased in frozen samples. Cobalamin, betaine, dimethylglycine, sarcosine, total homocysteine, total cysteine, tryptophan, asymetric and symmetric dimethyl argenine, creatinine, and methylmalonic acid were essentially stable under all conditions. Most B vitamins (folate and vitamins B2 and B6) were unstable; choline increased markedly, and some amino acids also increased, particularly in serum. The kynurenines showed variable stability. For many biomarkers, degradation (folate and flavin mononucleotide) or accumulation (pyridoxal, riboflavin, choline, amino acids) kinetics at room temperature were non–first order.

CONCLUSIONS: Data on stability and deterioration kinetics for individual biomarkers are required to optimize procedures for handling serum and plasma, and for addressing preanalytical bias in epidemiological and clinical studies.

Samples for laboratory diagnostics or biorepositories are often kept at room temperature for hours or days during sample collection and transportation. A centralized approach for processing and aliquoting repository samples causes delayed processing and transport, which may lead to reduced sample quality *(1)*. Storage in freezers at −80 °C or below is recommended to maintain long-term integrity of biomarkers *(2)*, but some biorepositories *(3)*, including the Janus Serum Bank *(4)*, were established decades ago, when regulations regarding collection and storage of samples were less standardized. The typical concern is that biomarker concentrations decay over time, but the concentrations of some biomarkers may even increase during storage *(5)*. Instability of biomarkers will increase preanalytical variability, which may translate into failure to detect associations between biomarkers and disease risk *(6)*.

Biomarkers and metabolites related to B vitamin function and one-carbon metabolism have been related to risk of chronic diseases, such as cardiovascular disease and cancer *(7)*. These biomarkers include plasma/serum concentrations of B vitamins (folate, cobalamin, vitamins B2 and B6) *(8)*, methylamines (choline, betaine, dimethylglycine) *(9)*, metabolites (total homocysteine, methylmalonic acid) reflecting B vitamin status in tissues *(10)*, methylarginines linked to endothelial function *(11)*, and tryptophan metabolites along the kynurenine pathway *(12)*, which depends on both vitamin B6 and B2.

Data on the stability of B vitamins and related metabolites during storage are scarce and fragmentary. No data exist on several biomarkers, for which methods have not

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been commonly available. In the present work, we investigated the stability of 32 biomarkers and determined their degradation or accumulation kinetics in serum/plasma for up to 8 days (192 h) at 23 °C. To determine long-term stability at −25 °C, we used samples from the Janus Serum Bank *(4)* stored for up to 29 years.

Methods

SAMPLE COLLECTION

Short-term stability at room temperature was studied in serum and plasma (EDTA, heparin, and citrate) from 16 healthy human study participants (50% male) ages 32–72 years. To obtain serum, whole blood was collected into plain silicon-coated tubes (Becton Dickinson Vacutainer) and allowed to clot at room temperature in the dark for 30 min before centrifugation at 2100*g* for 10 min at 4 °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)]. Before the plasma fractions were isolated, the tubes were turned upside down 5 times and centrifuged. Care was taken to avoid any admixture of buffy coat during collection of the serum and plasma. Each sample was then divided into 10 different 2-mL polypropylene tubes (Sarstedt). One tube (baseline) was immediately stored at −80 °C and the others were kept at 23 °C in the dark for 1, 2, 4, 8, 12, 24, 48, 96, and 192 h before storage at −80 °C *(13)*.

This part of the study was examined by the institutional review board (Regionale Komiteer for Medisinsk og Helsefaglig Forskningsetikk, Western Norway) and found to be in the QC category, which under the current Norwegian regulations is exempt from full review by the board. The board had no objection to publication of the results.

We investigated stability at −25 °C by collecting 650 serum samples from the Janus Serum Bank in Norway *(4)*. All participants were men 40–49 years old at the time of blood collection, which was carried out in 1979, 1991, 2002, 2004, and 2008. A total of 130 samples was obtained for each year. The samples were analyzed together in 2008, and the duration of storage was 0, 4, 6, 17, and 29 years. Routines for sample collection and processing were essentially the same throughout the 29-year period *(4)*. 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 isolation of the serum fraction. Serum was transferred to polypropylene tubes and stored at −25 °C in cardboard boxes covered with lids. Direct exposure to light occurred only during collection, retrieval, and pipetting of samples.

This part of the study was approved by the National Committees for Research Ethics in Norway.

BIOCHEMICAL ANALYSES

Folate (5-methyltetrahydrofolate), riboflavin, flavin mononucleotide (FMN),[7] FAD, pyridoxal 5′-phosphate (PLP), pyridoxal (PL), pyridoxic acid, free choline, betaine, dimethylglycine, creatinine, methionine sulfoxide, arginine, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), tryptophan, kynurenine, kynurenic acid, anthranilic acid, 3-hydroxykynurenine, xanthurenic acid, 3-hydroxyanthranilic acid, and formiminoglutamic acid, were determined by liquid chromatography–tandem mass spectrometry *(14–17)*, and methylmalonic acid, total homocysteine, total cysteine, methionine, serine, glycine, cystathionine, and sarcosine by gas chromatography–tandem mass spectrometry *(15, 18)*. Cobalamin (vitamin B12) was determined by a microbiological method *(19)*. All analyses were carried out in the laboratories of Bevital AS (www.bevital.no).

STATISTICAL METHODS AND KINETIC MODELING

The distributions of biomarkers in serum/plasma are presented as medians and 10th to 90th percentiles. We calculated changes in concentrations during storage at room temperature after we normalized the data to percentage of the baseline (time = 0) concentration, which was defined as 100%. For the cross-sectional data (−25 °C) absolute concentrations were used. The level of significance was assessed by use of the Kruskal–Wallis test. For analytes that were unstable, the kinetics was determined by appropriate curve fitting.

Degradation kinetics was fitted to regression models according to published guidelines *(20)*. The models tested included exponential decline [single first order (SFO)], sum of biexponential decline functions [double first order in parallel (DFOP)], and 2 exponential decline functions with a breakpoint [hockey stick (HS)]. The goodness of fit was expressed as error level in percent, which is the relative error that has to be assumed for the measurement method so that the residuals pass a χ^2 test *(20)*. The output estimates of the model with the best fit were reported in terms of a first order rate constant (k) and degradation half-life or time required for disappearance of 50% of the initial

[7] Nonstandard abbreviations: FMN, flavin mononucleotide; PLP, pyridoxal 5′-phosphate; PL, pyridoxal; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; SFO, single first order kinetics, DFOP, double first order in parallel kinetics; HS, hockey stick kinetics; k, first order rate constant; DT_{50}, degradation half-life; k_1, first kinetic constant; k_2, second kinetic constant; g, fraction of the starting value declining according to the first kinetic constant in a DFOP model; t_b, breakpoint in an HS model; SEG, segmented regression.

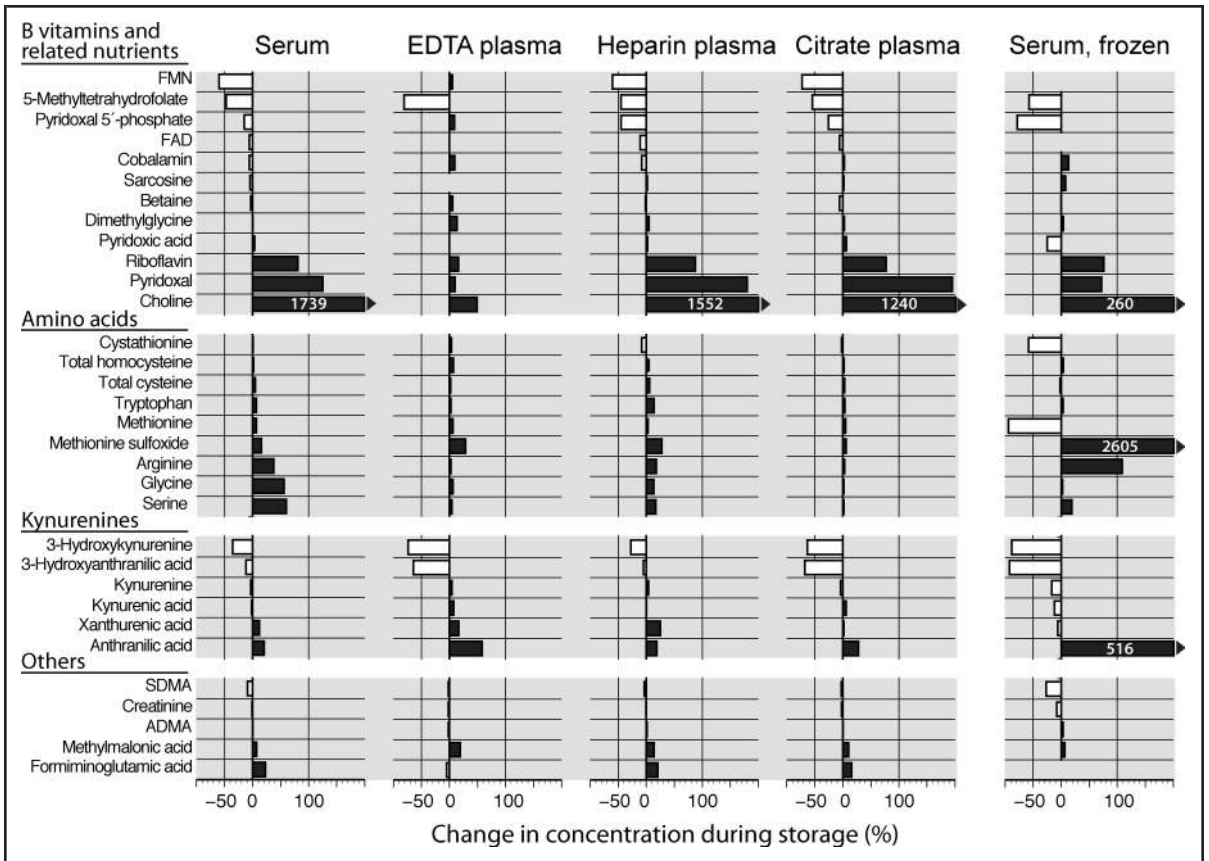

Fig. 1. Changes in biomarker concentrations during storage at room temperature and at −25 °C.

Biomarkers are sorted within each category according to the magnitude of change in serum at room temperature. Decay (open bars) and accumulation (closed bars) are shown as percentage change across extremes of storage times (8 days at 23 °C; 29 years at −25 °C).

concentration (DT_{50}) for an SFO model; first (k_1) and second (k_2) kinetic constants, fraction (g) of the starting value declining according to the first kinetic constant, and DT_{50} for a DFOP model; and k_1, k_2, breakpoint (t_b), and DT_{50} for an HS model.

Increase in biomarker concentration was modeled by segmented regression (SEG) with 1 breakpoint, with our selecting the starting point for iteration by inspecting a GAM (generalized additive model) curve *(21)*; if the algorithm failed to converge on a breakpoint (t_b), linear regression was used. The output estimates of the model were slope 1 and 2 (% change per unit time) and t_b for a SEG model and slope for a linear regression model; model fit was given as multiple R^2.

The package "kinfit" was used for fitting kinetic models to degradation data and the package "segmented" to estimate biomarker increases with 1 or more segmented relationships; both were run on the program R version 2.12.1 *(22)*.

Results and Discussion

PRINCIPAL FINDINGS

We investigated the stability of 32 biomarkers related to one-carbon metabolism and B vitamin function in serum and plasma (EDTA, heparin, and citrate) stored at room temperature for up to 8 days, and 30 of these analytes were also measured in frozen (−25 °C) serum stored for up to 29 years. For many biomarkers, stability was highest in EDTA plasma; important exceptions were folate and some kynurenines (Fig. 1). Storage effects were similar at room temperature and at −25 °C; the most notable exception was methionine, which could be recovered as methionine sulfoxide in frozen samples (Figs. 1 and 2). Cobalamin, betaine, dimethylglycine, sarcosine, total homocysteine, total cysteine, tryptophan, ADMA, SDMA, creatinine, and methylmalonic acid were essentially stable under all conditions. Most B vitamins (folate and vitamins vitamin B2

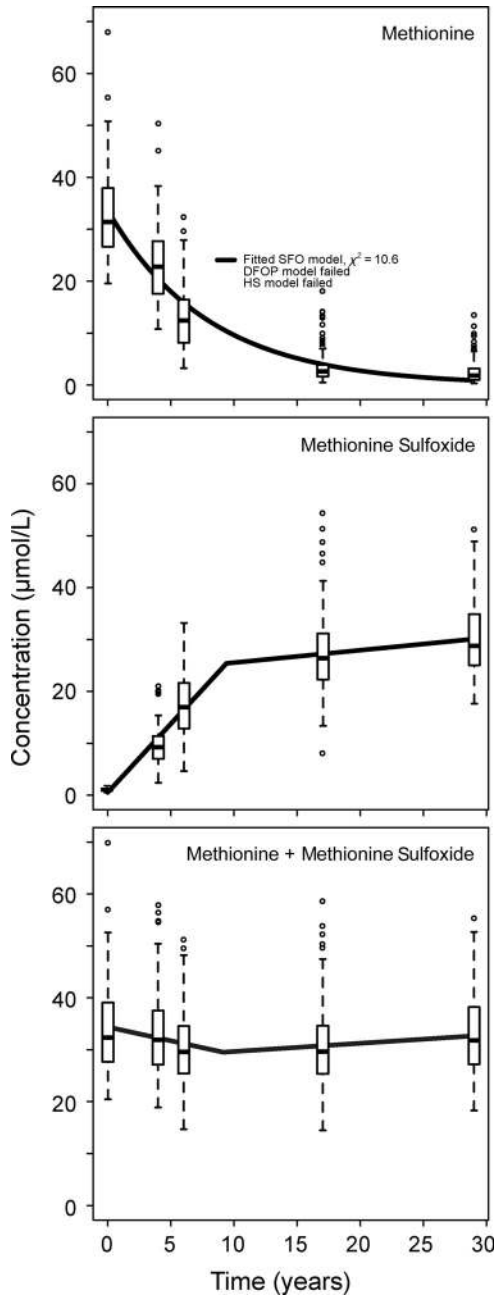

Fig. 2. Recovery of methionine as methionine sulfoxide in serum samples stored at −25 °C.

Methionine degradation (upper panel) was analyzed by SFO, DFOP, and HS models *(20)*, and the model with the best fit is shown. The concurrent accumulation of methionine sulfoxide (middle panel) was analyzed by SEG, which demonstrated a 2-segmented relationship. The sum of methionine and methionine sulfoxide (lower panel) was essentially stable (lower panel).

and B6) were unstable. Choline increased markedly, and some amino acids also increased, particularly in serum. For many biomarkers, degradation (folate and FMN) or accumulation (PL, riboflavin, choline, amino acids) kinetics at room temperature were non–first order.

STRENGTHS AND WEAKNESSES

The study included analytes that provide complementary information on nutritional and metabolic status, and are often analyzed together *(15)*. All analytes but one were determined by assays based on mass spectrometry, which are highly selective and therefore well suited to study the true stability of chemical compounds in biological samples. The short-term study conducted at room temperature was based on longitudinal measurements, whereas the cross-sectional design of the long-term biobank study was a limitation, because the interpretation of the results rests on the assumption that B vitamin status and metabolite concentrations in the study population have not changed over the years. However, total homocysteine, which is a functional marker of vitamin B status, did not vary according to year of sampling. In several European countries *(23)*, including Norway, there is no mandatory fortification for any of the compounds that were measured, and voluntary fortification is uncommon. The individuals from whom samples were taken at the Janus biobank were healthy and homogeneous with respect to ethnicity, age, and sex.

BIOMARKER CONCENTRATIONS AT BASELINE

The concentrations of the biomarkers in fresh and frozen samples (time = 0) are summarized in Supplemental Table 1 (in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol58/issue2). Concentrations of biomarkers (PL, riboflavin, choline, serine, glycine, and anthranilic acid) that showed a marked increase during storage at room temperature (Fig. 1) were higher in the biobank samples than in samples collected under optimal conditions (see online Supplemental Table 1). This finding probably reflects delayed preanalytical processing and transport of biobank samples, which may also explain the higher concentration of total homocysteine, known to increase in whole blood before separation of the blood cells *(24)*.

STABILITY OF BIOMARKERS ACCORDING TO STORAGE CONDITIONS

During storage at room temperature, many biomarkers were more stable in EDTA plasma than in other matrices, and stability was similar in serum, heparin plasma, and citrate plasma. Notable exceptions were amino acids, which were generally stable in both citrate and EDTA

Table 1. Degradation of biomarkers at room temperature in serum and plasma.

Biomarker	Matrix[a]	Kinetics						Error level, %[b]
		k/k_1, h^{-1}	k_2 (h^{-1})	g	t_b (h)	DT_{50} (h)	Model	
B vitamins and related nutrients								
Folate (5-methyltetrahydrofolate)	S	0.0028**[c]				>192	SFO	5.1
	E	0.062***	0.0058*	0.38		45.2	DFOP	3.3
	H	0.0027*				>192	SFO	4.2
	C	0.0034**				>192	SFO	4.1
Pyridoxal 5′-phosphate	H	0.0024*				>192	SFO	1.1
	C	0.0015*				>192	SFO	1.2
FMN	S	0.075*	0.0013****		9.0	24.2	HS	2.3
	H	0.23**	0.0041	0.54		10.9	DFOP	2.8
	C	0.13*	0.0009	0.63		11.1	DFOP	3.7
Kynurenines								
3-Hydroxykynurenine	S	0.00057	0.0025*		46.6	>192	HS	1.6
	E	0.0059*				117.3	SFO	3.1
	H	0.00050	0.0026*		45.1	>192	HS	1.1
	C	0.0040*				173	SFO	7.2
3-Hydroxyanthranilic acid	E	−0.0015	0.0059*		36.4	163	HS	2.3
	C	0.0026*				>192	SFO	13

[a] Sample matrix is S, serum; E, EDTA plasma; H, heparin plasma; C, citrate plasma.
[b] Error level in percentages is the relative error that has to be assumed for the measurement method so that the residuals pass a χ^2 test *(20)*.
[c] *P <0.0001, **P <0.001, ***P <0.01, ****P <0.05.

plasma, and the kynurenines and folate, which were most stable in serum and heparin plasma (Fig. 1; also see online Supplemental Table 2). Details on folate degradation in EDTA-plasma have been published previously *(13)*.

The stability of biomarkers in serum was similar during short-term storage at room temperature and long- term storage at −25 °C, except that cystathionine and methionine were extensively degraded in frozen samples but not at room temperature. The degradation of methionine was associated with a parallel increase in methionine sulfoxide (Fig. 2), which was essentially absent in samples stored at room temperature (Fig. 1; also see online Supplemental Table 2).

Concentrations of pyridoxic acid, FAD (not measured in frozen samples), cobalamin, betaine, dimethylglycine, sarcosine, total homocysteine, total cysteine, ADMA, methylmalonic acid, and creatinine were stable or increased only slightly (<13.7%) in all matrices both at room temperature and at −25 °C (Fig. 1; also see online Supplemental Table 2).

The most pronounced declines (92%–12%) in serum concentrations during storage at room temperature or in frozen samples were observed for FMN (not analyzed at −25 °C), folate (5-methyltetrahydrofolate), PLP, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid, and the most pronounced increases (1700%–19%) were observed for choline, PL, riboflavin, serine, and arginine (Fig. 1; also see online Supplemental Table 2).

These data provide comprehensive information about the stability of biomarkers in different sample matrices and during short-term and long-term storage. They are essentially in agreement with stability data previously reported for folate *(25)*, vitamin B2 *(3, 26)*, vitamin B6 *(3, 27, 28)*, cobalamin *(3, 25)*, choline *(14, 29)*, betaine and dimethylglycine *(14)*, total homocysteine *(24)*, methionine and other amino acids *(30, 31)*, methylmalonic acid *(32)*, ADMA, SDMA *(33)*, and creatinine *(34)*.

DEGRADATION KINETICS

Degradation kinetics were determined for biomarkers that decreased 15% or more during storage, by testing both monoexponential and biexponential models according to FOCUS (FOrum for Coordination of pesticide fate models and their USe) guidelines *(20)*. The results are expressed as rate constants ($time^{-1}$) and half-life DT_{50} and are summarized in Tables 1 and 2.

At room temperature, FMN degraded at the highest rate in heparin and citrate plasma; the decline

Table 2. Degradation of biomarkers in frozen serum samples.

Biomarker	Kinetics					Error level, %[a]
	k/k_1, year^{-1}	k_2, year^{-1}	g	DT_{50}, years	Model	
B vitamins and related nutrients						
Folate (5-methyl-THF)	0.029*[b]			23.6	SFO	5.5
Pyridoxal 5′-phosphate	0.047*			14.7	SFO	5.4
Pyridoxic acid	0.0035			197	SFO	2.8
Amino acids						
Cystathionine	0.035*			19.6	SFO	7.2
Methionine	0.13*			5.5	SFO	11
Kynurenines						
Kynurenine	0.0055*			126	SFO	1.7
3-Hydroxykynurenine	0.28***	0.042**	0.47***	5.9	DFOP	5.2
3-Hydroxyanthranilic acid	0.20*			3.5	SFO	18
Others						
SDMA	0.010*			64	SFO	1.0

[a] Error level in percentages is the relative error that has to be assumed for the measurement method so that the residuals pass a χ^2 test *(20)*.
[b] *P <0.0001, **P <0.01, ***P <0.05.

showed an initial rapid phase followed by a slow decline, with a DT_{50} of about 11 h (Table 1). Also, folate degraded rapidly at room temperature, in particular in EDTA plasma, where it displayed a biphasic pattern with an initial rapid phase and a DT_{50} of 45.2 h. Similar results have been previously been reported by us *(13)* and others *(3)*. 3-Hydroxykynurenine degradation was also fastest in EDTA plasma and showed a monoexponential decline, corresponding to a DT_{50} of 117 h (Table 1).

In frozen serum samples, 3-hydroxyanthranilic acid (DT_{50} = 3.5 years), methionine (DT_{50} = 5.5 years), 3-hydroxykynurenine (DT_{50} = 5.9 years), PLP (DT_{50} = 14.7 years), cystathionine (DT_{50} = 19.6 years), and folate (DT_{50} = 23.6 years) showed the fastest degradation, and most showed a monoexponential decline (Table 2).

ACCUMULATION KINETICS

Accumulation kinetics were determined for biomarkers that increased 15% or more during storage, and expressed as percentage increase per unit time of storage. The data were fitted to linear models with a 2-segmented relationship, or a linear model if the breakpoint was not significantly different from zero by SEG. The results are summarized in Tables 3 and 4.

At room temperature, free choline in serum, heparin plasma, and citrate plasma accumulated at a rate of 3%–5% per h during the first 47–70 h and 5.6%–11% per h thereafter. In serum, heparin plasma, and citrate plasma, both riboflavin and PL increased, but followed a segmented kinetics with an initial burst (3%–4% per h), which leveled off after 13–28 h. Similar burst kinetics were observed for the accumulation of some amino acids (serine, glycine, and arginine) in serum (Table 3).

In frozen serum samples, methionine sulfoxide increased rapidly (247% or 2.6 μmol/L per year) for the first 9.4 years, and then leveled off (Table 4) when methionine was essentially depleted (Fig. 2). Segmented kinetics with an initial burst was also observed for the massive increase in anthranilic acid (38.6% per year for the first 11.7 years), whereas choline (8.68% per year), arginine (2.52% per year), and serine (0.52% per year) increased in a linear fashion (Table 4).

BIOMARKER RECOVERY BY MEASUREMENT OF DEGRADATION PRODUCTS

At room temperature, the decrease in FMN mirrored the increase in riboflavin, and the sum of FMN plus riboflavin was essentially stable for up to 8 days. Furthermore, degraded PLP was recovered as PL, and PLP plus PL was stable (see online Supplemental Fig. 1). Likewise, the extensive degradation of methionine in frozen serum samples was paralleled by accumulation of methionine sulfoxide, and the sum was stable for up to 29 years (Fig. 2). Thus, the combined measurement of the degradation product and parent compound may correct for biomarker decomposition in archival sam-

Table 3. Increase in biomarkers at room temperature in serum and plasma.

Biomarker	Matrix[a]	Kinetics				
		Slope 1, %/h	Slope 2, %/h	t_b, h	Model	R^2
B vitamins and related nutrients						
Pyridoxal	S	3.8*[b]	0.41*	14.2*	SEG	0.73
	H	3.0*	0.53*	24.2*	SEG	0.73
	C	3.5*	0.49*	27.8*	SEG	0.75
Riboflavin	S	3.5*	0.09	13.3*	SEG	0.43
	E	0.089*			LM[c]	0.15
	H	3.8*	0.07	12.7*	SEG	0.39
	C	3.7*	0.07	13.1*	SEG	0.38
Choline	S	5.0*	10.8*	65.8*	SEG	0.94
	E	0.66**	0.20*	14.1*	SEG	0.74
	H	4.0****	9.9*	47.0**	SEG	0.92
	C	3.1*	5.59*	70.1*	SEG	0.93
Amino acids						
Methionine sulfoxide	S	0.12*			LM	0.14
	E	0.14*			LM	0.18
	H	0.0037	0.12***	41.3	SEG	0.09
	C	−1.90****	0.09****	8.25***	SEG	0.08
Serine	S	2.2*	0.15*	12.5*	SEG	0.82
	H	0.047***	0.123*	58.8***	SEG	0.80
Glycine	S	1.5*	0.10*	26.0*	SEG	0.80
Arginine	S	2.8*	0.074*	8.0*	SEG	0.63
	E	0.068	0.012	25.8	SEG	0.14
	C	0.023*			LM	0.14
Kynurenines						
Xanthurenic acid	H	0.42	0.035	20.2	SEG	0.02
	C	0.011			LM	0.004
Anthranilic acid	S	0.11*			LM	0.31
	E	0.11	0.28*	58.3****	SEG	0.63
	H	0.089*			LM	0.30
	C	0.027	0.14**	48.4	SEG	0.31
Others						
Formiminoglutamic acid	S	0.69	0.32****	33.6	SEG	0.13
	H	0.26****	0.11	26.6	SEG	0.18
	C	0.73**	0.02	34.2***	SEG	0.28

[a] Sample matrix is given as S, serum; E, EDTA plasma; H, heparin plasma; C, citrate plasma.
[b] *P <0.0001, **P <0.001, ***P <0.01, ****P <0.05.
[c] LM, linear regression.

ples, as we have previously demonstrated for folate content in such samples *(13, 35)*.

POSSIBLE MECHANISMS

The degradation of some biomarkers at room temperature (folate and 3-hydroxyanthranilic acid in EDTA plasma and FMN) or in frozen samples (3-hydroxykynurenine) does not obey first order kinetics, suggesting that the decomposition is not a simple unimolecular event in a stable or homogeneous matrix. Conceivably, kinetics may be influenced by protein binding, changing concentration of reactive oxygen species, inactivation of degrading enzymes, or ex vivo increase in pH *(36)*. The massive accumulation of

Table 4. Increase in biomarkers in frozen serum samples.

	Kinetics				
Biomarker	Slope 1, %/year	Slope 2, %/year	t_b, years	Model	R^2
B vitamins and related nutrients					
Pyridoxal	3.40*[a]			LM[b]	0.14
Riboflavin	7.24**	1.02	8.0***	SEG	0.07
Choline	8.68*			LM	0.78
Amino acids					
Methionine sulfoxide	247*	22.5*	9.4*	SEG	0.78
Serine	0.52*			LM	0.11
Arginine	2.52*			LM	0.44
Kynurenines					
Anthranilic acid	38.6*	4.5***	11.7	SEG	0.57

[a] *P <0.0001, **P <0.001, ***P <0.05.
[b] LM, linear regression.

free choline in all matrices except ETDA plasma may be explained by the conversion of phosphatidylcholine to choline *(14, 29)* catalyzed by phospholipase D, a calcium-dependent enzyme *(37)* inhibited by EDTA. Precursor degradation also explains the accumulation of riboflavin, PL, and methionine sulfoxide (in frozen samples), and the increase levels off when the precursor is depleted (Fig. 2; also see online Supplemental Fig. 1). The increase in amino acids like serine, glycine, and arginine may be explained by proteolysis *(30)* that is partly inhibited by anticoagulants like EDTA *(38)*, heparin *(39)*, and citrate *(40)*.

Conclusions

Of 32 biomarkers related to one-carbon metabolism and B vitamin function, 11 were stable (cobalamin, betaine, dimethylglycine, sarcosine, total homocysteine and cysteine, tryptophan, ADMA, SDMA, creatinine, and methylmalonic acid). For the remaining biomarkers, large differences in degradation or accumulation rates were observed, and stability differed between sample matrices. The results may be useful for optimizing procedures for handling and storage of serum and plasma specimens for biobanks and clinical diagnostics and for addressing preanalytical bias in epidemiological studies involving these biomarkers.

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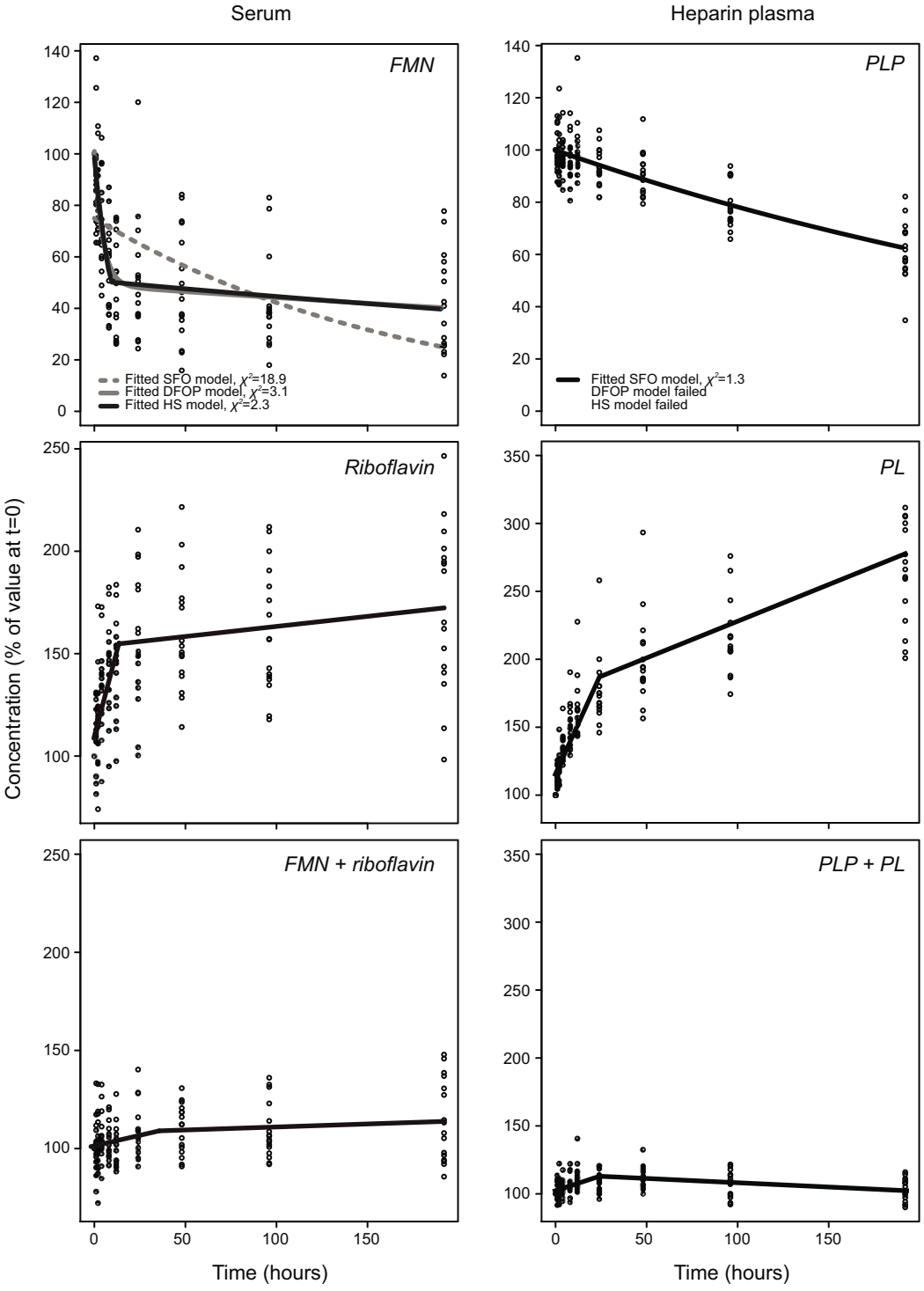

Supplemental Fig. 1. Recovery of FMN as riboflavin and PLP as PL at 23°C in serum and heparin plasma.

Degradation data (upper panels) were analyzed by SFO (single first order), DFOP (double first order in parallel) and HS (hockey stick) (20) models, and the models with the best fit are shown. The concurrent accumulation of riboflavin and PL (middle panels) was analyzed by segmented regression, which demonstrated a two-segmented relationship for both compounds. The sums of FMN and riboflavin and of PLP and PL were essentially stable (lower panels). FMN, flavin mononucleotide; PLP, pyridoxal 5´-phosphate; PL, pyridoxal.

Supplemental Table 1. Concentrations of biomarkers in fresh and frozen samples.				
Biomarker	**23°C[a]**		**-25°C**	
	Median	**(10-90 perc)**	**Median**	**(10-90 perc)**
B vitamins and related nutrients				
Folate (5-methyl-THF), nmol/L	16.2	(9.3-26.4)	11.7	(7.49-20.6)
Pyridoxal 5´-phosphate, nmol/L	51.7	(38.2-75.4)	45.6	(27.0-76.0)
Pyridoxal, nmol/L	10.6	(8.4-19.7)	19.4	(13.3-28.8)
Pyridoxic acid, nmol/L	24.3	(18.6-34.3)	20.8	(13.7-30.2)
Riboflavin, nmol/L	9.74	(5.80-98.1)	19.2	(12.2-42.5)
FMN, nmol/L	8.47	(6.01-23.4)		
FAD, nmol/L	63.3	(51.0-79.1)		
Cobalamin, pmol/L	441	(346-569)	337	(250-512)
Choline, µmol/L	7.92	(6.99-10.2)	20.6	(16.0-24.1)
Betaine, µmol/L	40.5	(29.2-54.7)	40.3	(31.2-53.6)
Dimethylglycine, µmol/L	3.39	(2.52-10.80)	3.86	(2.75-5.17)
Sarcosine, µmol/L	1.33	(0.90-1.72)	1.62	(1.14-2.50)
Amino acids				
Total homocysteine, µmol/L	8.61	(7.25-12.8)	13.5	(11.1-17.2)
Total cysteine, µmol/L	288	(262-339)	268	(232-304)
Cystathionine, µmol/L	0.22	(0.15-0.38)	0.24	(0.14-0.47)
Methionine, µmol/L	28.7	(21.9-38.7)	31.4	(24.8-43.3)
Methionine sulfoxide, µmol/L	0.73	(0.51-1.09)	1.06	(0.75-1.55)
Serine, µmol/L	107	(94-134)	178	(148-205)
Glycine, µmol/L	239	(177-282)	323	(270-373)
Arginine, µmol/L	53.4	(44.2-62.0)	85.7	(64.8-114)
Tryptophan, µmol/L	66.9	(58.3-81.3)	76.0	(62.0-92.5)
Tryptophan metabolites (kynurenines)				
Kynurenine, µmol/L	1.29	(1.15-1.64)	1.53	(1.25-1.90)
3-Hydroxykynurenine, nmol/L	26.5	(21.8-39.2)	32.0	(21.7-50.2)
Kynurenic acid, nmol/L	49.0	(40.4-63.5)	55.8	(38.9-79.3)
Xanthurenic acid, nmol/L	15.1	(11.0-23.4)	19.6	(11.0-30.78)
Anthralinic acid, nmol/L	9.79	(7.52-14.2)	15.2	(10.6-23.7)
3-Hydroxyanthralinic acid, nmol/L	36.9	(26.7-50.7)	44.3	(28.7-63.7)
Others				
Methylmalonic acid, µmol/L	0.16	(0.13-0.25)	0.16	(0.12-0.23)
ADMA, µmol/L	0.72	(0.64-0.80)	0.59	(0.45-0.69)
SDMA, µmol/L	0.62	(0.51-0.80)	0.58	(0.44-0.75)
Formiminoglutamic acid, nmol/L	566	(363-655)		
Creatinine, µmol/L	77.7	(61.6-94.4)	82.5	(70.9-94.0)

[a] Biomarker concentrations in serum, EDTA, and heparin plasma at base-line showed no difference ($P>0.05$ by by Kruskal-Wallis test) and results are shown for the pooled data.
ADMA, asymmetric dimethylarginine; FMN, flavin mononucleotide; SDMA, symmetric dimethylarginine.

Supplemental Table 2. Median (%) change in biomarker concentrations between extremes of storage time (0 vs. 8 days at 23°C and 0 vs. 29 years at -25°C) by sample matrix.

Biomarker	23°C[a]				-25°C
	S[b]	E	H	C	S
B vitamins and related nutrients					
Folate (5-methyl-THF)	-47.3[*c]	-80.8[*]	-44.9[***]	-54.2[*]	-57.2[*]
Pyridoxal 5´-phosphate	-14.8	+8.9[*]	-44.8[**]	-25.6[*]	-78.4[*]
Pyridoxal	+125[*]	+9.6[*]	+180[*]	+195[*]	+72.1[*]
Pyridoxic acid	+3.3	-0.29	1.9	6.4	-24.9[*]
Riboflavin	+80.9[*]	+16.1[****]	+87.4[*]	+76.9[*]	+76.0[*]
FMN	-59.6[*]	+5.2	-60.2[*]	-72.8[*]	
FAD	-5.9	-0.16	-10.9	-5.9	
Cobalamin	-5.8	+9.4[****]	-8.1	2.5	+13.2[***]
Choline	+1739[*]	+49.0[*]	+1552[*]	+1240[*]	+260[*]
Betaine	-3.3[****]	+5.8	-1.3	-5.9	-0.24
Dimethylglycine	+0.3	+13.4[*]	+4.6	+2.8	+3.6
Sarcosine	-4.5		+1.6	+1.6	+7.7
Amino acids					
Total homocysteine	+1.6[****]	+7.1[*]	+4.6[*]	+2.0[***]	+3.8[****]
Total cysteine	+4.8[***]	+2.2	+5.5[*]	+3.2[*]	-2.3[****]
Cystathionine	+0.7	+3.3	-8.1	-2.5	-58.3[*]
Methionine	+6.6[*]	+6.3[*]	+2.8[*]	+4.9[****]	-94.2[*]
Methionine sulfoxide	+15.8[****]	+28.5[***]	+27.6[****]	5.6	2605[*]
Serine	+59.9[*]	+4.3[**]	+16.9[*]	+1.6[*]	+19.0[*]
Glycine	+56.0[*]	+6.3[**]	+13.2[*]	+1.9[*]	+2.2[***]
Arginine	+37.4[*]	+2.7[**]	+17.9[*]	+3.0[*]	+109[*]
Tryptophan	+6.4[****]	+2.3	+13.6[***]	+3.4	+3.3
Kynurenines					
Kynurenine	-3.2	4.5[**]	3.8	-4.0	-17.0[*]
3-Hydroxykynurenine	-35.6[*]	-73.6[*]	-27.7[*]	-63.6[*]	-88.4[*]
Kynurenic acid	-2.0	+7.6	0.2	+5.6	-12.0[***]
Xanthurenic acid	+12.0	+16.4	+25.0	+1.65	-6.3
Anthranilic acid	+20.6[*]	+58.2[*]	+18.8[*]	+27.9[*]	+516[*]
3-Hydroxyanthranilic acid	-11.8[*]	-64.3[*]	-5.0	-68.0[*]	-92.3[*]
Others					
Methylmalonic acid	+7.1[*]	+19.0[*]	+13.7[*]	+10.2[*]	+6.3
ADMA	+0.5	-1.8	+1.4	+0.4	+3.1
SDMA	-9.3[*]	-1.9[*]	-3.9[***]	-2.7[****]	-26.8[*]
Formiminoglutamic acid	+22.7[***]	-5.1	+20.3[*]	+15.8[*]	
Creatinine	-0.81	-1.8	-0.8	-2.4[***]	-8.1[*]

[a] Concentrations for samples stored at 23°C were normalized individually to a percentage of the baseline concentration, which was defined as 100%. Absolute concentrations were used for frozen (-25°C) samples.
[b] Sample matrix is given as S, serum; E, EDTA plasma; H, heparin plasma; C, citrate plasma.
[c] [*]P<0.0001, [**]P<0.001, [***]P<0.01, [****]P<0.05, by Kruskal-Wallis test.
FMN, flavin mononucleotide; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine.