Can Whole-Blood Samples Be Stored over 24 Hours without Compromising Stability of C-Reactive Protein, Retinol, Ferritin, Folic Acid, and Fatty Acids in Epidemiologic Research?

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Ideally, blood samples for biomarker measurement are collected centrally and processed immediately to avoid any unwanted changes in concentrations that could affect validity. In large-scale epidemiologic and clinical studies, however, this theoretical goal must give way to a more pragmatic approach *(1, 2)*. The Amsterdam Born Children and their Development (ABCD) study is a clinically based cohort study of pregnant women in Amsterdam (The Netherlands) in which we use, for practical and ethical reasons, blood collection in conjunction with existing schemes of care, with samples subsequently sent to a central laboratory by mail or courier. As a consequence, delay times between collection and processing may exceed 24 h, to an incidental maximum of 96 h. Although the stabilities of the biomarkers of interest, i.e., C-reactive protein (CRP), retinol, ferritin, folic acid, and fatty acids (FAs), have been studied previously *(1–9)*, the variety of designs (e.g., storage ≤24 h) hampers the applicability of existing results to our research and similar studies. We therefore investigated the appropriateness of our standardized, practice-based approach of blood collection by assessing stability in samples stored up to 96 h (the maximum delay time possible when samples are sent by mail) with a focus on the first 28 h (the maximum delay time allowed in the ABCD study).

Blood samples were collected from 41 generally healthy female volunteers, 22–56 years of age. One woman was at that time 16 weeks pregnant, but because her measurements did not deviate, we decided not to exclude her from analysis. Written informed consent was obtained from all participants. For 20 women, 28 mL of blood was collected in seven 4-mL Vacuettes (Greiner BV) for the preparation of serum. For the other 21 women, 28 mL of blood was collected in seven 4-mL Vacutainer EDTA tubes (Becton and Dickinson BV) for the preparation of plasma. One tube from each woman was centrifuged ($1600g$ for 10 min) between 1 and 2 h after blood collection (t_0; baseline), and the remaining tubes were stored in a cabinet at room temperature (~21 °C) and centrifuged 2 (t_2), 4 (t_4), 24 (t_{24}), 26 (t_{26}), 28 (t_{28}), or 96 (t_{96}) h after baseline. Time points were chosen to mimic courier and postal conditions seen in the ABCD study, 4 h being the anticipated maximum delay time when blood is sent by courier, 28 h being the anticipated maximum delay time when blood is sent by overnight mail, and 96 h being the anticipated maximum delay time when blood is sent by mail but over the weekend.

Aliquots (1 mL) of plasma and serum were stored at −80 °C and transferred on dry ice to collaborating laboratories for analysis within 1 week. In serum, CRP concentrations were measured by use of the Dade-Behring high-sensitivity assay *(10)*. Ferritin and folic acid concentrations were measured by immunoassay with chemiluminescence detection on the Advia Centaur System (Bayer Group) *(11)*, and retinol was measured with an isocratic reversed-phase HPLC method (Waters Ltd.) *(12)*. Intra- and interassay CVs, as determined previously, were 4.1% and 5.2%, respectively, for CRP; 2.5% and 5.4% for ferritin; 7.9% and 6.1% for folic acid; and 3.3% and 7.3% for retinol.

The FA composition of plasma phospholipids was determined by capillary gas chromatography with flame ionization detection (HP5890 series II; Hewlett Packard) as described by Al et al. *(13)*. Absolute (mg/L of plasma) and relative (percentage of total phospholipid-associated FAs) amounts were calculated for the major saturated FAs and the major mono- and polyunsaturated FAs of the n-3, n-6, and n-9 families, each constituting ≥0.1% of total FAs. CVs varied from 1.3% for arachidonic acid (C20:4n-6) to 5.6% for Mead acid. For FA analysis, all paired samples were assayed in the same run.

After verification of the homogeneity of variance and identification of outliers [two measurements of folic acid processed at t_{26}, one baseline measurement of linoleic acid (C18:2n-6), one baseline measurement of Mead acid (C20:3n-9), and one measurement of adrenic acid (C22:4n-6), all excluded from analysis], we performed repeated-measures ANOVA to explore time effects over 96 h and to calculate the mean percentage change per time point. Because we assumed that the stability depended on both reliability and validity (because true changes over time can be established only if measurement error is small), we assessed stability at 28 h by calculating intraclass correlation coefficients *(5)* (two-way mixed-effect model with single-measure reliability; SPSS), and by computing Spearman rank correlations for a (n = 50) bootstrap sample. In the latter procedure, we took for all women random samples from all values and calculated correlation coefficients with baseline values. Because these correlations depend on both the distribution of the individual values and the stability, this measure provides a nonparametric estimate of the "noise" to be expected because of unknown variance of delay, in particular in regression-like analyses. Additional analyses of within- and between-subject CVs, assessing the magnitude of the variation attributable to time in relation to the variation between individuals *(1)*, can be viewed in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue1/.

The changes in concentration, reliability coefficients, and Spearman rank correlations for measured analytes are shown in Table 1. CRP was excluded from the reliability and validity analyses because we considered

Table 1. Stability of serum analytes and FAs in plasma phospholipids: changes in concentration after storage for 2 to 96 h, and 28-h reliability and validity measures.

	Mean (SE) baseline value	Change from baseline, %						*P*, time effect[a]	28-h Reliability (ICC)[b]	28-h Validity (Spearman)[c]
		2 h	4 h	24 h	26 h	28 h	96 h			
Serum analytes										
CRP,[d] mg/L (n = 6)	18.4 (14.2)	−3.0	−4.4	−5.5	−5.2	−2.9	−3.0	0.370		
CRP,[e] mg/L (n = 5)	4.2 (0.9)	−10.0	−1.4	−6.2	−3.8	1.4	2.8	0.105		
Retinol, µmol/L	3.01 (0.2)	2.7	0.8	1.7	0.4	1.8	1.1	0.311	0.97	0.96
Ferritin, µg/L	45 (11)	−0.4	−1.4	0.5	2.1	3.1	2.6	0.110	0.99	0.99
Folic acid, nmol/L	19.7 (2.9)	−8.7	−10.8	−13.6	−18.7	−11.5	−16.5	0.037	0.97	0.87
FAs in plasma phospholipids, % by weight of total FAs										
C16:0	29.16 (0.40)	−0.5	−0.2	0.7	0.7	0.6	2.4	<0.001	0.98	0.93
C18:0	12.41 (0.27)	−0.4	0.1	2.0	2.0	1.7	6.0	<0.001	0.98	0.96
C18:1n-9	8.39 (0.24)	−0.8	−1.0	−1.9	−1.7	−2.2	−3.8	<0.001	0.97	0.96
C20:3n-9	0.38 (0.02)	6.1	7.1	2.3	0.1	0.5	4.9	0.256	0.81	0.78
C18:2n-6	20.97 (0.59)	−0.4	−1.1	−4.7	−4.9	−5.3	−14.0	<0.001	0.99	0.97
C20:3n-6	3.00 (0.14)	−0.4	−0.3	1.0	1.1	1.5	4.0	<0.001	0.99	0.98
C20:4n-6	8.73 (0.30)	−0.2	−0.3	−0.1	0.0	0.2	1.4	0.021	0.99	0.98
C22:4n-6	0.30 (0.01)	−0.2	0.9	3.1	4.1	4.8	16.2	<0.001	0.99	0.99
C22:5n-6	0.21 (0.02)	0.2	−0.2	0.5	1.7	2.7	6.8	<0.001	0.99	0.97
C18:3n-3	0.25 (0.01)	−0.9	−1.7	−4.7	−3.5	−4.9	−9.7	<0.001	0.97	0.95
C20:5n-3	1.03 (0.17)	−0.2	−1.0	−1.9	−2.0	−1.7	−5.2	0.004	0.99	0.99
C22:5n-3	0.78 (0.04)	0.5	0.9	3.0	3.6	4.3	9.6	<0.001	0.99	0.98
C22:6n-3	3.56 (0.22)	0.0	0.1	1.7	2.0	2.6	5.8	<0.001	0.99	0.98

[a] Huynh–Feldt correction in repeated-measures ANOVA (accounting for unequal variances of differences across groups).

[b] ICC, intraclass correlation coefficient.

[c] Mean Spearman rank correlation for bootstrap sample of n = 50.

[d] Only six individuals with values above the detection limit (0.5 mg/L); power too low for reliability and validity measurements.

[e] One of the six individuals had extremely high values (baseline value, 89 mg/L); analyses repeated with five individuals.

the number of observations above the clinically significant detection level (0.5 mg/L) insufficient (insufficient power). A graphic representation of changes across all time points is presented in the online Data Supplement. For CRP, retinol, and ferritin, changes in concentration during the 96-h storage period were small and not significant (≤10%; *P* ≥0.1). For folic acid, the mean concentration changed significantly over time (*P* = 0.037), with the sharpest decrease (8.7%) in the first 2 h of storage. Reliability and validity measures were high for all serum analytes (intraclass correlation coefficient ≥0.96; Spearman rank correlation coefficient ≥0.80).

Significant changes over time were observed for most FAs, but only for linoleic acid and adrenic acid were changes ≥10%. The linoleic acid concentration decreased 14% over the 96-h storage period (*P* ≤0.001), and the concentration of adrenic acid increased 16% (*P* ≤0.001). For the majority of FAs, reliability coefficients were ≥0.97. For Mead acid (C20:3n-9), the reliability coefficient was lower (0.81). Spearman rank correlations were ≥0.93 for all FAs except Mead acid (0.78).

Our results demonstrate that a pragmatic approach for data collection does not affect the stability of measurements of CRP, retinol, ferritin, and most FAs because these markers showed limited variance within a 96-h storage period. For folic acid, linoleic acid, and adrenic acid, significant changes (≥10%) over time were observed, but these did not significantly affect measures of 28-h validity.

Although previous studies on the stability of CRP and ferritin showed results similar to ours (*3–6, 8*), studies on retinol are conflicting. Both Key et al. (*1*) and Hankinson et al. (*2*) have reported small decreases (≤5.0%), whereas Mejia et al. (*7*) observed no change in concentration. We also observed no significant changes, possibly as a result of the storage of our samples in the dark to protect vitamin A from photodestruction (*14*).

Folic acid is known for its sensitivity for both heat and light, and substantial decreases (>10%) during storage have been reported (*8, 9*). Zhang et al. (*9*), who found a 15% decrease after 24 h of storage, concluded that this is within acceptable limits for clinical analysis. Similarly, we observed no relevant time-related changes in the 28-h stability analyses.

To our knowledge, no previous studies have reported on the stability of FAs in the plasma phospholipid fraction. Generally, we observed only small changes in concentrations, which did not significantly affect coefficients of correlation. For Mead acid, however, reliability and validity coefficients were more influenced by variability in concentration, possibly because of the small magnitude of the individual values (<1%) (*5*). Surprisingly, we ob-

served not only decreases in FA concentrations (assumingly attributable to FA peroxidation), but also increases. A second process may be ongoing during storage: the formation of erythrocyte microparticles. In vitro, glycolysis will lower the glucose concentration in erythrocytes, causing depletion of ATP. Consequently, the erythrocytes cannot maintain membrane integrity, and microparticles will be released (15). Over time, the concentration of plasma phospholipid-associated FAs may thus become enriched with erythrocyte membrane phospholipids containing relatively high amounts of polyunsaturated FAs.

Our study has some limitations. One limitation is that we did not measure in duplicate to adjust for intraassay variation. In addition, serum samples were not analyzed within one run to minimize interassay variability. However, standardized procedures were used for analysis, with CVs ≤7.9% for serum analytes and ≤5.6% for plasma phospholipid-associated FAs. In addition, the reliability analyses showed high agreement between follow-up and baseline values. Another limitation is that samples were not randomized before analysis. Possible bias from order of draw, although unlikely, can therefore not be ruled out. A third limitation is that we were not able to test the reliability and validity of CRP over the 28-h storage period. However, changes in concentration were small (≤5.5%), which, as mentioned before, is in line with results from other studies (3, 5).

In conclusion, this study shows that in the context of epidemiologic studies investigating (nutritional) status during routine care, a pragmatic approach to blood collection may validly be applied to determine CRP, retinol, ferritin, folic acid, or FA status. Although storage will diminish the precision of estimates, standard (correlational) epidemiologic analyses will not be compromised in samples stored for a maximum of 28 h.

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Development and Validation of an Automated Chemiluminometric Immunoassay for Human Intrinsic Factor Antibodies in Serum, *Elizabeth A. Gomez,[1] Daniel D. Ehresmann,[1] Lisa K. Ledebuhr,[2] Mary L. Eastvold,[2] Ravinder J. Singh,[2] George G. Klee,[2] and Stefan K.G. Grebe[2,3*]* ([1] Beckman Coulter Inc., Chaska, MN; Departments of [2] Laboratory Medicine and Pathology and [3] Medicine, Mayo Clinic, Rochester, MN; * address correspondence to this author at: Endocrine Laboratory, Hilton 730C, Mayo Clinic, 200 1st Street SW, Rochester, MN 55905; fax 507-284-9758, e-mail grebs@mayo.edu)

The cobalamins, also referred to as vitamin B_{12}, are a group of closely related enzymatic cofactors involved in the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A and in the synthesis of methionine from homocysteine (1–3). Vitamin B_{12} deficiency can lead to megaloblastic anemia and neurologic deficits (4). The latter may exist without anemia or precede it. Adequate replacement therapy will generally improve or cure cobalamin deficiency. Unfortunately, many other conditions, which require different interventions, can mimic the symptoms and signs of vitamin B_{12} deficiency (4). Moreover, even when cobalamin deficiency has been established, clinical improvement may require different dosages or routes of vitamin B_{12} replacement, depending on the underlying cause (3, 4). In particular, patients with pernicious anemia, possibly the commonest type of cobalamin deficiency in developed countries, require either massive doses of oral vitamin B_{12} or parenteral replacement therapy (3–5). The reason is that in pernicious anemia, patients suffer from gastric mucosal atrophy, which leads to diminished or absent gastric acid, pepsin, and intrinsic factor (IF) production. Because gastric acid,