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Stabilization of Homocysteine in Unseparated Blood over Several Days: A Solution for Epidemiological Studies,

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Increased blood homocysteine is a potentially modifiable risk factor for cardiovascular disease. In a recent meta-analysis of individual participant data from prospective epidemiologic studies, a 25% lower homocysteine concentration was associated with an 11% lower risk for ischemic heart disease and a 19% lower risk for stroke *(1)*. Blood homocysteine is easily lowered by folic acid supplementation, and several large-scale randomized trials are currently underway to assess the effects of homocysteine-lowering vitamin supplements on the risk of vascular disease. If such trials demonstrate benefit, there will be increasing interest in homocysteine determinations to assess vascular disease risk. In addition, further large-scale epidemiologic studies may be required to investigate the association between homocysteine and cardiovascular disease in a wider range of populations. These would be facilitated by simple and cost-effective methods for blood collection and analysis.

One of the chief constraints in homocysteine measurements is the continuing production and release of homocysteine by red blood cells after venipuncture, which causes an artificial increase in plasma concentration of ~10% per hour *(2, 3)*. It has been recommended, therefore, that blood samples for homocysteine measurements be drawn into tubes containing EDTA, chilled, or placed on ice immediately after collection and that the plasma be separated from the red cells within 1 h. Such procedures can be difficult to implement in large-scale epidemiologic studies or other situations in which samples have to be collected remotely (e.g., in multiple clinics or in people's homes) and transported to a central laboratory. Use of NaF or acidic citrate has been advocated for stabilization of homocysteine in whole blood at ambient temperature for several hours before plasma separation, but there are few data available on longer-term stability *(4, 5)*. Using commercially available Vacutainers, we evaluated the stability of homocysteine in whole blood over several days, using EDTA with or without NaF as preservative and with storage at room temperature or chilled conditions.

Participants in the study were healthy volunteers (two males and eight females; age range, 25–60 years) from our department who consented to have their blood drawn. No ethics committee approval was gained for this study. We collected blood from each of the volunteers into two 5-mL Vacutainers containing EDTA and two 7-mL Vacutainers containing disodium EDTA (7.0 mg) and NaF (17.5 mg; EDTA + NaF; final NaF concentration, 60 mmol/L; Becton Dickinson UK Limited). The Vacutainers were centrifuged immediately (2100*g* for 15 min at 4 °C), and a 300-μL aliquot of plasma was taken from each one and stored at −80 °C. The Vacutainers were then gently mixed to recombine the cells with the remaining plasma. For each individual's samples, one EDTA and one EDTA + NaF Vacutainer were stored at room temperature (21 °C) and the other EDTA and EDTA + NaF Vacutainers were stored under chilled conditions (4 °C). At 1, 2, 3, 4, and 7 days after collection, the Vacutainers were recentrifuged, a 300-μL aliquot of plasma was taken and stored at −80 °C, and the Vacutainers were mixed and replaced. The plasma aliquots corresponding to each Vacutainer type, temperature condition, and time point for each volunteer were analyzed together in one analytical run to avoid run-to-run variability.

Before analysis by HPLC, plasma samples underwent chemical reduction of the sulfur bonds by the addition of tris(2-carboxy-ethyl)phosphine (TCEP), protein precipitation with tricholoroacetic acid, and reaction with 4-aminosulfonyl-7-fluorobenzo-2-oxa-1,3-diazole (ABD-F) to form fluorescent adducts. We injected 20 μL of the resulting extract onto a C_{18} column (Bio-Rad Laboratories Ltd.) on a Waters Alliance chromatography system (Model 2690; Waters Ltd.). The system was equipped with a Waters scanning fluorescence detector (Model 474) operating at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. Data collection, integration, and quantification were performed by the Waters Millennium 32 software. Within-batch CVs were <2% at homocysteine concentrations of 12.8, 22.5, and 31.9

Table 1. Percentage change in homocysteine concentration from fresh (immediately separated) samples and within-person correlation coefficients for each time point in EDTA or EDTA + NaF Vacutainers stored at 21 or 4 °C.

	Time from blood collection to separation, days				
Storage conditions	**1**	**2**	**3**	**4**	**7**
EDTA at 21 °C					
Mean (SE) increase, %	37.3 (10.6)	127.2 (26.3)	217.9 (43.0)	306.8 (55.6)	381.2 (94.5)
Correlation coefficient[a]	0.90	0.58	0.47	0.53	0.54
EDTA at 4 °C					
Mean (SE) increase, %	4.6 (3.2)	21.0 (4.6)	34.0 (7.3)	50.4 (11.4)	114.8 (21.7)
Correlation coefficient[a]	0.98	0.95	0.95	0.87	0.83
EDTA + NaF at 21 °C					
Mean (SE) increase, %	16.9 (5.9)	20.0 (6.5)	13.8 (7.2)	12.3 (8.3)	13.8 (7.9)
Correlation coefficient[a]	0.97	0.97	0.96	0.90	0.94
EDTA + NaF at 4 °C					
Mean (SE) increase, %	8.2 (3.1)	9.6 (2.5)	8.1 (2.5)	4.8 (4.4)	8.4 (6.1)
Correlation coefficient[a]	0.97	0.99	1.00	0.92	0.95

[a] Spearman correlation coefficients for values recorded at baseline and subsequent time points.

μmol/L (n = 6), and between-batch CVs were <2.5% at homocysteine concentrations of 11.5, 16.2, and 27.8 μmol/L (n = 6).

Individual homocysteine concentrations in the samples that had been separated and frozen immediately varied between 6 and 22 μmol/L. The mean (SE) of these "fresh" homocysteine concentrations was 14.6 (1.0) μmol/L in EDTA Vacutainers and 13.4 (1.0) μmol/L in EDTA + NaF Vacutainers. The stability of homocysteine for each preservative and temperature condition was determined by calculating the percentage change in concentration from the fresh value in the particular preservative at each time point for each individual and then calculating the mean percentage change from fresh at each time point.

Shown in Table 1 is the percentage increase in plasma homocysteine concentrations over time for each preservative and temperature condition. In EDTA Vacutainers stored at 21 °C, mean homocysteine concentrations increased by ~40% during the first 24 h after blood collection and had increased almost fivefold after 7 days. After 2 days, the correlation with the fresh values was only ~0.6. In EDTA Vacutainers stored at 4 °C, homocysteine concentrations had changed by only ~5% at 24 h after collection, but they increased steadily thereafter and had more than doubled by 7 days. The addition of NaF to EDTA attenuated the increase in homocysteine concentration to <20% for up to 7 days after collection, even when the samples were stored at 21 °C. Stability in EDTA + NaF at 21 °C was superior to EDTA alone at 4 °C when samples were stored for longer than 2 days. The combination of chilling and the addition of NaF to EDTA limited the increase in homocysteine concentration to <10% for up to 7 days after collection, and the correlation with fresh values remained >0.9.

Previous studies on the use of fluoride to stabilize homocysteine have assessed the effects of delaying separation for up to only 8 h after blood collection *(4, 6–8)*, with the exception of two studies that evaluated stability up to 24 h or longer *(9, 10)*. Ubbink et al. *(9)* found that the average homocysteine concentration at 24 h after collection in EDTA tubes increased by 188% at 25 °C and 15% at 4 °C, whereas the concentration in NaF tubes increased by 20% at 25 °C and by 12% at 4 °C. Al-Khafaji et al. *(10)* found that at room temperature, the average homocysteine concentration in EDTA tubes increased by 124% at 24 h and by 257% at 72 h, whereas in NaF/potassium oxalate tubes, the average concentration increased by 17% at 24 h but was 21% lower than the fresh value at 72 h after collection. We found that mean homocysteine concentrations were 8% lower in EDTA + NaF compared with EDTA Vacutainers, which is consistent with some previous reports *(6–8)* but not all *(9, 10)*. Differences in mean homocysteine concentrations in Vacutainers with different preservatives will not, however, affect comparisons between measures made using the same type of Vacutainer within a study.

The present study demonstrates that plasma homocysteine concentrations can be measured reliably in blood collected into EDTA + NaF Vacutainers that are kept chilled for up to 7 days before plasma separation. Furthermore, the use of EDTA + NaF provides better long-term stability of homocysteine at room temperature than does EDTA alone even when such samples are kept chilled. These findings should facilitate homocysteine measurements in a wide range of settings.

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