Effects of Anticoagulant and Time of Plasma Separation on Measurement of Homocysteine, Natalia Louise Duarte,1 Xing Li Wang,2 and David Emil Leon Wilcken1* (1 Cardiovascular Genetics Laboratory, Department of Medicine, Prince of Wales Hospital, Randwick, NSW 2031 Australia; 2 Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX 78245-0549; * author for correspondence: fax 612-9382-4921, email d.wilcken@unsw.edu.au)

Current interest in plasma total homocysteine (tHcy) measurements has increased with evidence that even mild hyperhomocysteinemia may be an independent risk factor for vascular disease (1) and that increases occur with vitamin deficiencies and reduced renal function. This has led to the investigation of problems associated with measurement, in particular the stability of tHcy after blood collection (2, 3). Transfer of homocysteine from red cells to plasma after venesection may occur and produce a 10% per hour increase in plasma tHcy concentrations (4), and thus influence assessments of the relative risk of disease (5). To reduce this increase, samples may be stored on ice and centrifuged within 1 h of collection. In epidemiologic surveys or even routine collection, this may not always be feasible.

We studied the stability of plasma tHcy measured by a fluorescence polarization immunoassay (FPIA) in samples from 9 cystathionine β-synthase (CβS)-deficient homocystinuric patients and 13 healthy individuals. Blood was collected into both EDTA (1.8 g/L) and NaF (1.8 g/L EDTA and 3 g/L NaF) tubes. For six of the healthy individuals, we also used lithium heparin (14 kIU/L), sodium citrate (32 g/L trisodium citrate; 3.2% solution), and ACD-B (13.2 g/L trisodium citrate and 3 g/L NaF) tubes. The CβS-deficient patients were diagnosed and treated as described previously (6, 7), and the controls were healthy staff. Informed consent was obtained from all participants.

Within 15 min of collection, samples were divided into five aliquots. One aliquot from each sample was centrifuged (10 000g for 5 min) 15 min after blood collection, and the plasma was separated. Aliquots from the remaining samples were incubated at room temperature (25 ± 3 °C, mean ± SD) and centrifuged after specified lengths of time. Plasma was stored at −70 °C before analysis with the Abbott (Abbott Diagnostics) IMx analyzer (8). Our interassay CVs were 5.7%, 4.7%, and 4.6% at 7.0, 12.5, and 25.0 μmol/L tHcy, respectively.

tHcy concentrations at different time points were com-

| Table 1. Changes of plasma tHcy (μmol/L) in 9 CβS-deficient patients and 13 healthy controls over time from venesection to separation in blood collected into EDTA and NaF tubes. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Time to separation, h |
| 0 | 1 | 2 | 3 | 4 |
| EDTA | NaF | EDTA | NaF | EDTA | NaF | EDTA | NaF |
| Patients | | | | | | | |
| 90 ± 32 | 91 ± 31 | 88 ± 31 | 91 ± 31 | 94 ± 33 | | |
| 79 ± 28 | 79 ± 29 | 77 ± 29 | 84 ± 34 | 80 ± 30 | | |
| Control | | | | | | | |
| 9.7 ± 2.6 | 10.6 ± 2.8 | 11.2 ± 3.0 | 11.8 ± 3.2 | 12.3 ± 3.2 | | |
| 8.3 ± 2.2 | 8.9 ± 2.2 | 9.1 ± 2.1 | 9.6 ± 2.2 | 10.2 ± 2.7 | | |

* Values are mean ± SD, with percentage change (SD) shown below each value.

b P <0.001.
pared by repeated-measures ANOVA. The overall effects of anticoagulants on concentrations were assessed in the same model as a between-sample factor, and Bonferroni-corrected \( P \) values were reported. The differences in tHcy concentrations between time points were compared by the Student \( t \)-test. Percentage differences were calculated by comparison with baseline concentrations.

For the controls, baseline concentrations of plasma tHcy were significantly lower in NaF tubes than in EDTA tubes (\( P < 0.001 \); Table 1). In EDTA tubes, tHcy increased significantly at 1 and 4 h, by 9% ± 4% and 27% ± 9%, respectively (\( P < 0.001 \)), for the controls, but did not increase significantly in the patients. Similar changes were seen with NaF tubes (Table 1).

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**Fig. 1.** tHcy concentrations in blood collected from six healthy individuals into different tubes and separated at 0–4 h post venesection (A) and effect of time of plasma separation on blood collected from nine CJS-deficient patients into EDTA and NaF tubes (B).

Whole blood samples were maintained at room temperature (25 ± 3 °C). (A), thick line, mean ± SE. (B), ———, EDTA tubes; ———, NaF tubes.
A general linear model of repeated-measures ANOVA was applied to data for both controls and patients to evaluate the effects of storage on tHcy concentrations. The repeated measurements of tHcy at five different time points were the within-subject factors. In the controls, the within-subject factor, i.e., the duration of storage, significantly affected tHcy concentrations ($F = 77.489; n = 13; P = 0.0001$). For patients, the duration of storage was not significantly predictive of the tHcy concentrations ($F = 0.783; P = 0.555$).

With the same analytical model, we evaluated the effects of anticoagulants on tHcy during storage. Baseline concentrations in the six control individuals for the samples in EDTA, lithium heparin, sodium citrate, and ACD-B tubes were $8.1 \pm 2.6, 7.9 \pm 2.5, 7.7 \pm 2.6,$ and $7.3 \pm 2.6 \mu mol/L,$ respectively (EDTA vs ACD-B, $P = 0.014$). There was no interaction between the duration of storage and anticoagulant used for sample collection ($F = 1.296; P = 0.221$). After Bonferroni correction, the differences in tHcy concentrations among different anticoagulants were not statistically significant ($P > 0.05; n = 6$ for all comparisons), and there was also no interactive effect between duration of storage and anticoagulants ($F = 0.566; P = 0.858$). After Bonferroni correction, the effects of anticoagulants on measured tHcy concentrations were not statistically significant. Percentage increases over the next 4 h were similar for each: at 1 h, $8\% \pm 2\%$; at 2 h, $12\% \pm 2\%$; at 3 h, $18\% \pm 2\%$; and 4 h, $24\% \pm 2\%$. Fig. 1A shows the tHcy concentrations with each anticoagulant over 4 h in each individual. tHcy measurements were corrected for the ratio of anticoagulant to whole blood (1:4 for the ACD-B and 1:10 for the citrate collection tube) (9). (EDTA in the crystalline form in tubes is assumed to produce no dilution, ignoring water shifts between cells and plasma.) There was a uniform pattern of increased concentrations with delayed separations in the samples from controls.

The tHcy values in the patient samples collected into EDTA and NaF over 4 h from separation are shown in Fig. 1B. Concentrations were consistently lower in samples collected into NaF tubes. With both collection methods, tHcy concentrations ranging from 25 to 120 \mu mol/L were unaffected by the delay in separation.

When tHcy concentrations are within the reference interval, our results are consistent with a release of homocysteine in the tested types of collection tubes. These findings differ from those of Palmer-Toy et al. (9) and Willems et al. (10), who found that tHcy concentrations were stable in blood collected into sodium citrate tubes and maintained at room temperature for 6 h. They also differ from the observations of Moller and Rasmussen (11), who reported that collection into NaF tubes hinders homocysteine release for 2 h at room temperature. However, they are consistent with the findings of Caliskan et al. (12) and Salazar et al. (2).

In the CBS-deficient patients, we found no significant increase over 4 h in measured plasma tHcy with concentrations $\geq 25.0 \mu mol/L$. Our findings suggest that at these concentrations plasma and circulating blood cell concentrations are in equilibrium, but not when tHcy is within the reference interval.

Plasma collected into NaF tubes had tHcy concentrations $16.6\% \pm 1.6\%$ lower than in the EDTA tubes at baseline, and the increases in concentrations with time in healthy individuals for NaF tubes were $\sim 2\%$ lower than for the EDTA tubes. This may be attributable to a slower rate of tHcy release from red blood cells, perhaps by inhibition of glycolysis, or to some interaction with the assay.

We conclude that with the Abbott method, measured tHcy concentrations within the reference interval are lower for blood collected into NaF ($P < 0.001$) and ACD-B tubes than for blood collected into EDTA, lithium heparin, and sodium citrate tubes. Although delayed separation increases tHcy when concentrations are within the reference interval, markedly increased tHcy concentrations ($\geq 25 \mu mol/L$) remain stable at room temperature for up to 4 h, suggesting that concentrations within red cells and plasma are then in equilibrium and that further tHcy production in red cells is not occurring. It is also possible that the release of tHcy from red blood cells is independent of plasma tHcy concentrations. When a fixed amount of tHcy is released into plasma, the concentration changes produced by this addition could be analytically significant when the base plasma concentrations are low, as in the healthy controls, but have minimal effects when base concentrations are high, as in the patients. Thus, reference values for tHcy need to be defined in relation to the method of sample collection, the time to separation, and the assay system used.

References

Detection of Cardiac Troponin I Early after Onset of Chest Pain in Six Patients, David A. Colantonio,1 William Pickett,2 Robert J. Brison,2 Christine E. Collier,3 and Jennifer E. Van Eyk1,4* (Departments of 1 Physiology, 2 Emergency Medicine, 3 Pathology, and 4 Biochemistry, Queen’s University, Kingston, Ontario, K7L 3N6 Canada; * author for correspondence: Fax 613-533-6880, e-mail JVE1@post.queensu.ca)

Patients presenting to the emergency departments (ED) with symptoms of acute coronary syndrome (ACS) and with a nondiagnostic electrocardiogram (ECG) pose a management challenge (1). Cardiac troponins [(cTns), troponin I (cTnI) and troponin T (cTnT)], creatine kinase (CK), and CK-MB are frequently used in the assessment of ACS. cTns are superior in their analytical specificity and diagnostic sensitivity and specificity for myocardial injury (2, 3). Findings from both animal and clinical studies show that cTnI is released into the blood in various cardiac conditions, including angina, acute myocardial infarction (1, 4, 5), congestive heart failure (6), and myocarditis (7). Because cTns in serum represent myocardial damage and increased risk of future adverse outcomes (8), improving the detection of serum cTns has implications for better diagnosis of myocardial damage and better risk stratification for patients with ACS.

With current clinical assays, cTns are detectable in the circulation 4–6 h after the onset of pain in acute myocardial infarction, peaking within 12–24 h and remaining increased for a few days (9). However, a recently developed Western blot method, WB-DSA (10), detected minute amounts of cTnI in serum of patients undergoing bypass surgery within 10 min after reperfusion (11), suggesting increased detection of TnI by the WB-DSA method. Although WB-DSA does not permit analysis of troponin’s quaternary structure, it does allow accurate assessment of the chemical status of individual troponin subunits, such as the extent and pattern of cTnI degradation. cTnI is specifically degraded in ischemic/reperfused injured rat myocardium (4, 12), and TnI degradation products are detected in myocardium of patients undergoing coronary artery bypass surgery. Because ACS represents a spectrum of cardiac pathophysiology, unique patterns of cTnI degradation may be present in serum at various points along this spectrum and detectable by the WB-DSA. This study presents a series of cases to evaluate the potential clinical applicability of this novel method for the detection of cTnI and any of its degradation products in serum from patients presenting for emergency medical care with symptoms of ACS.

Serum samples were obtained from patients presenting within 4 h of onset of symptoms of ACS to the Kingston General Hospital ED. We recruited patients between May and August 2000 and selected those with no or mild increases in CK, CK-MB, or cTnI as measured by clinical methods. Patients were excluded if they had renal impairment or skeletal injury. Patient selection was meant to be illustrative.

Patients underwent a history and clinical examination, a 12-lead ECG was recorded, and blood samples were obtained at presentation and at 1, 2, 4, 6, and 16–24 h for routine clinical testing of biochemical cardiac markers and for analysis by the WB-DSA (10). Samples were stored at −80 °C until analyzed. This study was approved by the Queen’s University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board. All participants gave written informed consent.

Total CK was assayed on the CX7 (Beckman Instruments); CK-MB and cTnI were assayed on the Immulon (Bayer Corporation). The reference intervals for CK were 55–197 U/L (men) and 35–155 U/L (women), with precision estimates (CV) from daily quality-control samples of 2.9% at 131 U/L and 2.7% at 480 U/L. A 20% increase in CK above the upper limit was considered meaningful. CK-MB was interpreted as positive when >8 μg/L with a relative index [(CK-MB × 100)/CK] > 3%. The CVs of the CK-MB assay were 3.2% (4.5 μg/L) and 2.9% (18.2 μg/L). The minimum detectable cTnI concentration reported by the manufacturer for the Immulon assay was 0.1 μg/L. The CV of the cTnI assay was 10% at 0.5 μg/L, 7.0% at 3.0 μg/L, and 5.7% at 27 μg/L.

Detection of serum cTnI by WB-DSA was performed under denaturing and reducing conditions (10). Serum was diluted 12-fold in sample buffer containing, per liter, 3.3 g of sodium dodecyl sulfate, 3.3 g of CHAPS, 3.3 g of Nonidet P-40, 0.1 mol of dithiothreitol, 1 mol of urea, 50 mmol of Tris-HCl (pH 6.8), and 500 mL of glycerol. We used 2 μL of serum per lane. Proteins were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (14 cm × 14 cm × 0.75 mm) in electrode buffer containing, per liter, 25 mmol of Tris, 192 mmol of glycine, and 1 g of SDS, at 130 V for 4 h. Gel-resolved proteins were then transferred to nitrocellulose (45 μm; Micton Separation) in the presence of 10 mmol/L CAPS, pH 11, for 1 h at 100 V, using a Trans-Blot Cell apparatus (Bio-Rad). Membranes were blocked overnight at 4 °C in 10% blocking reagent (Boehringer Mannheim) and probed separately with monoclonal antibody 8I-7 (epitope amino acids 136–154; Spectral Diagnostics), which detects most forms of cTnI (4, 11), and polyclonal antibody P3 (epitope amino acids 26–58; BiosPacific). Although the exposure times of the Western blots were optimized for better visual interpretation of results, all blots shown are in the linear range of detection. Sera were analyzed three times with each antibody. Results were consistent each time. Multiple exposures were conducted for each Western blot, ensuring that blots were in the linear range of detection.

For comparison, human recombinant intact cTnI1–209 and cTnI1–192 [the primary cTnI degradation products observed in stunned myocardium from isolated rat hearts (4, 12, 13)] in human serum were resolved alongside each