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References


Stabilization of Homocysteine Concentration in Whole Blood, Reiner Probst,1 Richard Brandl,2 Matthias BlГіnke,1 and Dieter Neumeier1 (1 Klinikum rechts der Isar, Institute for Clinical Chemistry and Pathobiochemistry and 2 Klinikum rechts der Isar, Institute for Vascular Surgery, Ismaninger Str. 22, 81675 Munich, Germany; * author for correspondence: fax 49-89-41408475)

Homocysteine (Hcy) is a cytotoxic (1), sulfur-containing amino acid that increasingly appears to be an independent risk factor for coronary artery disease, stroke, and peripheral occlusive arterial disease (POAD) (2–4) when present at high plasma concentrations. In addition to several physiological factors that can increase Hcy concentration in blood such as vitamin B12, B6, or folate (5) deficiency and renal insufficiency (6), there is an artificial increase of Hcy concentration in whole blood after blood collection. This severe preanalytical problem is due to the well-known, time-dependent release of Hcy from erythrocytes (7) in the isolated blood sample, even when stabilization is attempted with different additives such as EDTA, NaF, or heparin/NaF (8–10). The result of this release is a falsely increased plasma Hcy concentration, unless plasma is separated from blood cells immediately after blood collection. We describe a method to overcome this problem for making the routine determination of Hcy possible for institutions where sample transport is critical.

For this purpose, we used a specially prepared blood collecting system, where whole blood is lysed and Hcy generating and converting enzymes are inhibited at the time of venipuncture, resulting in a stabilization of Hcy for 2 days at room temperature. Preparation of the blood collecting system for Hcy determination in lysate was performed by adding Nonidet P40 (25 μL, pure), 50 μL of EDTA disodium salt dihydrate (75 g/L), and 25 μL of citric acid monohydrate (610 g/L) to a 2.7-mL EDTA-Monovette (EDTA = 1 g/L blood; Sarstedt), which was then used for blood collection. These concentrations of additives enabled sufficient stabilization and caused minimal dilution of the blood sample. After venipuncture, we recommend vigorously shaking the blood collecting system for 5 s to lyse blood cells. Complete lysis was checked microscopically in several samples.

EDTA blood was used for plasma Hcy determination. Centrifugation of blood at 3000 g for 10 min and the separation of plasma from the cellular fraction were performed within 30–60 min of blood collection. Sample preparation of EDTA plasma and lysate was performed as described by Vester and Rasmussen (11). The HPLC method was also carried out according to their protocol after minor modification. HPLC measurements of plasma and lysate Hcy were performed by using a C18 reversed-phase 125–4 mm column (Merck LiChrosphere 100, 5-μm particle) with a guard column [RP-18 (5 μm) LiChrosphere 100, 4–4 mm], a gradient pump (Merck, L-6200), an autosampler (Merck, AS–2000), and a fluorescence detector (Merck, FL1080). The gradient started with 100% A (acetate buffer, pH 4.2, 20 mL MeOH), which was decreased to 92% over 3 min and then to 88% over the next 2 min at a flow rate of 0.9 mL/min. After an additional 3 min, the system reached its initial conditions; the whole run required 15 min. Eluent B was methanol.

A calibration curve was plotted by using three different concentrations (5, 15, and 40 μmol/L) of homocysteine dissolved in 5 mmol/L EDTA (pH 7) and added to three lysate or plasma samples (standard addition). Stability measurements were performed by aliquoting blood immediately after venipuncture. These aliquots were stored for different time intervals (0 1, 2, 4, 6, 24, and 48 h) at room temperature. Then the samples were frozen at –30 °C. Measurements were performed in triplicate. The
CV was <3%. Initial (0 h) lysate Hcy concentrations ranged from 4 to 32 µmol/L.

We determined Hcy concentrations in samples from 136 individuals presenting with carotid stenosis and symptomatic POAD, all of them classified as having 70–99% stenosis by ultrasound and angiography. A group of 39 apparently healthy individuals was recruited from hospital employees. They gave no history of POAD, heart disease, thrombosis, or cerebrovascular disease. Informed consent was given from all individuals and approved by our institution’s responsible committee.

The comparison of lysate and plasma homocysteine concentrations was performed with regression analysis according to Passing and Bablok (12). The correlation coefficients were calculated with linear regression. Astute®, a statistics program running under Microsoft Excel®, version 5.0, was used for statistical analyses.

With the above-mentioned reagent mixture, we succeeded in achieving a sufficient stabilization of lysate Hcy concentration over 48 h at room temperature, and citric acid was necessary to prevent a time-dependent decline of Hcy ranging from 10% to 20% during 48 h. Time-dependent deviations of lysate Hcy concentrations from the initial Hcy concentration ranged from −3.3% to 6.4% over 24 (n = 13) and from −6.1% to 9% over 48 h (n = 10).

To verify the suitability of lysate homocysteine measurements with HPLC, we determined and compared Hcy concentrations measured in EDTA plasma and lysed blood from 136 patients with POAD (Fig. 1).

Hcy concentrations in lysed blood are lower than in plasma, because of a lower intracellular Hcy concentration, but increase parallel to increasing plasma Hcy concentrations. The squared correlation coefficients resulting from linear regression of plasma and lysed whole blood Hcy concentrations were 0.93 (n = 92) in men and 0.90 (n = 44) in women. The slope of the correlation for women’s samples was 0.60 [95% confidence interval (CI), 0.53 to 0.69] and the intercept = −0.06 (95% CI, −1.3 to −0.9). For men, the slope was 0.6 (95% CI, 0.54–0.65), and the intercept was −0.38 (95% CI, −1.2 to 0.4). Although an accepted reference range has not yet been defined (13), we chose to use the commonly discussed upper plasma Hcy cutoff value for an adult population (14) of 15 µmol/L. Because there is general agreement that women have lower Hcy values than men (15), we used 15 µmol/L for men and 14 µmol/L for women to calculate the respective lysate Hcy cutoffs from the linear regression equation of the comparison of plasma and lysate Hcy concentrations. The result was 8.3 for women and 8.6 µmol/L for men, which fits well with the 95% CI of our reference group, which is 8.8 and 8.3 µmol/L for women and men, respectively. The mean lysate and plasma Hcy levels for women and men presenting with POAD were 9.0 and 9.5 µmol/L and 15.1 and 16.5 µmol/L, respectively. The women (n = 18) and men (n = 23) from our reference group showed mean values of 6.0 (4.4–10 µmol/L) and 5.9 µmol/L (3.7–9.6 µmol/L) in lysate and 10.3 (4.8–17.4 µmol/L) and 11.6 µmol/L (6.4–15.9 µmol/L) in plasma.

The approximate correction of lysate Hcy concentrations for hematocrit [c(Hcy)/1 − hematocrit] resulted in only a slight improvement of the squared correlation coefficient for the male patient group (0.96) and was slightly poorer in the female patient group (0.87). Thus, we conclude that correction for hematocrit for the interpretation of lysate Hcy values is not necessary for clinical purposes.

In conclusion, an improvement of inter- and intralaboratory comparability of the results and therefore a more reliable atherosclerotic risk assessment might be achieved with this method. The unproblematic handling of blood samples after venipuncture is beneficial for clinicians, general practitioners, and clinical chemists. For this reason, we hold the opinion that the determination of Hcy in lysate is clinically more relevant than the hitherto performed determination of Hcy in plasma. Nevertheless, to verify the above statement, it is necessary to perform prospective studies with a greater number of patients.

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**Fig. 1.** Correlation of plasma Hcy concentrations and lysed whole blood Hcy concentrations from 92 men (A) and 44 women (B) with POAD. The linear regression equations for men and women are \( y = -0.38 + 0.6x \) and \( y = -0.06 + 0.6x \). The squared correlation coefficients were 0.93 and 0.90 for men and women, respectively.
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References


Chiron 800 System CO-oximeter Module Overestimates Methemoglobin Concentrations in Neonatal Samples Containing Fetal Hemoglobin, Patrick L.M. Lynch,1* David E. Bruns,1 James C. Boyd,1 and John Savory1,2 (Departments of 1Pathology and 2Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA 22908; *author for correspondence: fax 804-924-2574, e-mail pl8n@avery.med.virginia.edu)

Inhaled nitric oxide (NO) is an established treatment for persistent pulmonary hypertension of the newborn (1). NO can oxidize erythrocyte hemoglobin Fe(II) to Fe(III), however, and can lead to potentially toxic accumulation of methemoglobin (MetHb) (2). Because MetHb fractions >5% can lead to toxicity, monitoring of MetHb throughout therapy is required. In one exceptional case, excessive dosing of NO led to a MetHb fraction of 40% (3). Because neonatal blood contains a high proportion of fetal hemoglobin (HbF), typically 75–90% at term (4), accurate measurement of MetHb in the presence of HbF is essential. We wished to ascertain the accuracy of MetHb determination by the Chiron 800 system CO-oximeter module (Chiron Diagnostics), part of their 865 analyzer, in the presence of high HbF. A similar study of the Corning 270 CO-oximeter (Ciba-Corning) was performed recently (5). The Chiron 800 CO-oximeter is an overdetermined system that uses 10 wavelengths to directly measure total hemoglobin, oxyhemoglobin, carboxyhemoglobin, deoxyhemoglobin, and MetHb. For comparison, MetHb concentrations were also measured with the Corning 270 CO-oximeter, an overdetermined system that uses seven wavelengths, and the Evelyn-Malloy KCN addition manual method (6) on a Shimadzu UV-1201 spectrophotometer (Shimadzu Scientific Instruments).

Two ~40-ML pools of blood were collected into lithium heparin tubes: adult blood and fetal umbilical cord blood with 85.5% HbF. By preparing 100% MetHb samples with potassium nitrite, as outlined previously (5), samples with 0%, 5%, 10%, 15%, 25%, 50%, 75%, and 100% MetHb were produced for each pool. These samples were then analyzed by the Chiron 800, the Corning 270, and the manual method. The results are shown in Table 1.

Analysis of covariance of the results was carried out using the SAS general linear model procedure with a homogeneity of slopes model (SAS Institute) to compare results for adult and umbilical cord blood. For MetHb concentrations of 0–50%, the Corning 270 showed only a small, although statistically significant, proportional error (P = 0.0013), and the Chiron 800

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<th>Table 1. MetHb results (%) for adult and umbilical cord blood samples treated with potassium nitrite.</th>
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* Both the 100.0% Chiron 800 are cutoff values.