Chronic Lymphocytic Leukemia Cells May Interfere in a Glycated Hemoglobin Assay Based on Fluorescence Quenching, Ludvig N.W. Daas and Trine Andreassen (Clinical Chemical Laboratory, Diakonhjemmets Hospital, 0319 Oslo, Norway; * address correspondence to this author at: Box 23 Vinderen, 0319 Oslo, Norway; fax 47 22 45 16 06)

Recently, we observed falsely low results by the Abbott IMx Glycated Hemoglobin assay apparently caused by a high number of leukocytes in patients with chronic lymphocytic leukemia. Because chronic lymphocytic leukemia and diabetes mellitus (monitored by glycohemoglobin measurements) occur more frequently in the elderly, we examined this interference further.

The IMx Glycated Hemoglobin assay (1) is based on ion-capture technology. A glass-fiber matrix is precoated with a positively charged quaternary ammonium compound that, after hemolysis of the blood sample, electrostatically captures the negatively charged glycohemoglobin complex. A dihydroxyboronate/high-molecular weight polyacrylic acid affinity reagent specifically captures the glycohemoglobin, whereas the nonglycohemoglobin constituents are removed by a washing procedure. Glycohemoglobin is quantified by measurement of fluorescence quenching.

In 20 consecutive blood samples from patients with known chronic lymphocytic leukemia, a total leukocyte count and a differential were done in a Coulter Counter STKS, and glycohemoglobin was measured simultaneously in the Abbott IMx (ion capture technology, fluorescence quenching), the Bayer DCA 2000 (immunologic latex agglutination), and the Bio-Rad DiaSTAT (low pressure liquid chromatography). The results, arranged in ascending leukocyte order, are shown in Table 1.

Constant systematic differences were observed among the three methods. Compared with the two other methods, however, the Abbott IMx results seemed to be affected by high total leukocyte counts, with falsely decreased values at leukocyte concentrations in the range $67.9 \times 10^9$/L to $100 \times 10^9$/L. At higher leukocyte counts, asterisks appeared as an error warning.

After the patients’ blood samples were mixed manually with the hemolyzing and other reagents for the IMx glycohemoglobin assay in the same proportions as are used in this analyzer, numerous intact leukocytes (lymphocytes) could be observed by microscopy. The draining velocities through the glass fiber matrix were comparable for solutions of the patients’ samples and for blood from healthy subjects. Hence, the length of time that solutions remained on the matrix during the reading does not appear to contribute to interference. None of these patients had a monoclonal gammopathy, some of which can react with quaternary amines (D. Bruns, personal communication, October 5, 1998). Aspiration of the buffy coat in one patient’s blood sample, which reduced the lymphocyte count from $154 \times 10^9$/L to $93.7 \times 10^9$/L, avoided the false lowering of the glycohemoglobin result. Hence, interference from intact leukocytes in the glycohemoglobin fluorescence quenching is the most likely explanation of the falsely low results obtained in these samples. As suggested by the results in the leukocyte range $67.9 \times 10^9$/L to $100 \times 10^9$/L, individual differences may exist in leukocyte (lymphocyte) resistance to the hemolytic agents used.

Interference by high numbers of leukocytes in a fluorescence quenching assay for glycohemoglobin has not been reported. We suggest the possibility of such interference in other fluorescence quenching assays based on “hemolyzed” whole blood.

References

Fully Automated Measurement of Total Homocysteine in Plasma and Serum on the Abbott IMx Analyzer, Aila Leino (Research and Development Centre of Social Insurance Institution and Department of Clinical Chemistry, Central Laboratory, University Hospital, Turku, Finland; address for correspondence: University Hospital of Turku, Kinnamyllynkatu 4-8, FI-N20520 Turku, Finland; fax 358-2-2613920, e-mail aila.leino@tyks.fi)

Increasing evidence indicates that high total homocysteine (tHcy) may be causally related to several clinical
situations, such as cardiovascular disease, birth defects, and folate and vitamin B<sub>12</sub> deficiency (1–4). Accordingly, the interest in tHcy determinations in blood has increased in routine and research laboratories. Several methods, such as HPLC, stable isotope dilution, and enzyme immunoassay, have been used for the determination of tHcy, but they are time-consuming and require highly skilled technical staff. Recently, an automated tHcy assay has come on the market. This IMx Homocysteine assay (Abbott Laboratories) is a fluorescence polarization immunoassay for the quantitative measurement of tHcy in human plasma and serum with no manual sample pre-treatment (5). For the evaluation of the method, EDTA plasma and serum samples were collected simultaneously from 32 men, ages 45–67 years. After collection, the samples were kept on ice, centrifuged within 1 h, and stored at −70 °C until analysis.

The analytical performance of the automated IMx Homocysteine assay was compared with the manual Hcy enzyme immunoassay kit (Axis EIA Homocysteine; Axis Biochemicals) on microtiter plates with spectrophotometric measurement of peroxidase activity (6). Both assays are based on enzymatic conversion of Hcy to S-adenosyl-L-homocysteine (SAH) by the action of SAH hydrolase (SAHase; EC 3.3.1.1), followed by quantification of SAH in a competitive immunoassay with the use of a monoclonal anti-SAH antibody.

The comparison of the IMx Homocysteine assay with the Axis EIA Homocysteine assay yielded the following equation: IMx = (1.0753 ± 0.042) × EIA − (1.2949 ± 0.459); S<sub>dy</sub> = 0.54; n = plasma and serum samples from 16 individuals (Fig. 1A). When plasma and serum samples were considered separately, the equation for plasma was: IMx = (1.0738 ± 0.032) × EIA − (1.3994 ± 0.332); S<sub>dy</sub> = 0.28; r = 0.9877. The equation for serum was: IMx = (1.0597 ± 0.080) × EIA − (1.0057 ± 0.885); S<sub>dy</sub> = 0.71; r = 0.9261. The mean (± SD) values for plasma and serum samples were 11.3 (± 3.58) and 12.4 (± 3.76) μmol/L on the IMx and 10.1 (± 2.23) and 10.8 (± 2.30) μmol/L on the EIA, respectively. The parallel measurement of plasma and serum Hcy with IMx Homocysteine assay gave the equation: plasma = (0.9571 ± 0.010) × serum − (0.5163 ± 0.147); S<sub>dy</sub> = 0.34; r = 0.997; n = 32 individuals (Fig. 1B). Correspondingly, the equation with Axis EIA Homocysteine assay was: plasma = (0.9245 ± 0.008) × serum + (0.1107 ± 0.832); S<sub>dy</sub> = 0.67; r = 0.9153; n = 16 individuals.

The imprecision of the IMx Homocysteine assay was evaluated using NCCLS guidelines (7). Two IMx assays were performed on 5 days with serum controls of 7.0, 12.5, and 25.0 μmol/L. Hcy assayed in replicates of two. The within-assay CVs were 1.8%, 1.9%, and 1.3%; the between-assay CVs were 0.8%, 0.3%, and 0.7%; and the total CVs were 1.9%, 1.8%, and 1.4%, respectively.

The detection limit for the assay was 0.44 μmol/L, which was measured by assaying the zero calibrator in replicates of 10 in four IMx assays and calculated as 3 SD of the mean response of the zero calibrator (0.165 ± 0.09).

The sample volume requirement was 50 μL, the stated measuring range was 0.5–50 μmol/L, and the throughput was 20 samples per hour.

The observed results indicate that the IMx Homocysteine assay provides a precise and easy-to-use quantitative measurement of tHcy in plasma and serum for routine use. The automation of cumbersome manual steps in currently used methods of Hcy measurement may reduce analytical variability between studies.

I thank Aila Sjöholm and Anja Ilmanen for technical assistance and Abbott Diagnostics Division for providing the IMx Homocysteine assay kits for this study.

References
3. Allen RH, Stabler SP, Savage DG. Diagnosis of cobalamin deficiency. I.
Phenylalanine and Tyrosine Quantification by Stable Isotope Dilution Liquid Chromatography–Mass Spectrometry from Filter Paper Blood Spots, Mendel Tuchman* and Mark T. McCann (Department of Pediatrics, University of Minnesota, Minneapolis, MN 55455; * address correspondence to this author at: Department of Pediatrics, University of Minnesota Hospitals, Box 400, 420 Delaware St SE, Minneapolis, MN 55455; fax 612-624-2682, e-mail tuchm001@tc.umn.edu)

Phenylketonuria (PKU) is one of the more common inborn errors of metabolism, affecting 1 in 10,000–15,000 individuals (1). Newborn screening for PKU is performed in all 50 states and started with the development of the bacterial inhibition screening assay by Guthrie and Susi (2). Frequent monitoring of blood Phe concentrations is required to maintain the concentration as close as possible to the reference interval [60–120 μmol/L (1–2 mg/dL)] to prevent damage to the brain from increased Phe concentrations and to prevent a negative nitrogen balance from an overly Phe-restricted diet. Moreover, the high risk of birth defects for the fetus of a PKU mother requires extremely frequent monitoring of blood Phe concentrations and would greatly benefit from a rapid and accurate blood spot method. Phe determination from dried blood spots is feasible by various methods: bacterial (2), fluorometric (3), liquid chromatography (LC) (4), and tandem mass spectrometry (MS) (5). Of these methods, only tandem MS allows use of a stable isotope as internal standard. Although tandem MS is the most rapid method available, the extremely high equipment cost and the need for sample derivatization are disadvantages.

We developed a rapid and precise blood-spot method for Phe and Tyr determination using stable isotope dilution reversed-phase LC-MS. The advantages of this method are the very short sample preparation without derivatization, a short chromatographic run, the use of selective detection to eliminate interference, and the use of ideal internal standardization (stable isotope). The method described herein is now used routinely in our PKU clinic.

l-Phe (M, 165.19) and l-Tyr (M, 181.19) were purchased from Sigma Chemical Co. Stable isotopes [ring-d5]-l-phenylalanine (M, 170.23) and [ring-d4]-l-tyrosine (M, 185.22) were purchased from Cambridge Isotope Labora-
tories. S & S 903 specimen collection paper was purchased from Schleicher & Schuell. Acetonitrile (HPLC grade) was purchased from EM Industries. Trifluoroacetic acid (TFA; photometric grade) was purchased from Aldrich Chemical Co.

The blood-spot sample consisted of a single paper disc, 6.3 mm in diameter, punched out from a blood spot dried onto the paper; based on previous studies (6), the disc contains 10.6 μL of blood. When smaller size spots were submitted to the laboratory, the method was sensitive enough to analyze a single 3-mm disc (2.4 μL of blood). The blood was eluted from the paper disc by sonication for 5 min into 500 μL of deionized water containing 5.0 μg of [ring-d5]-phenylalanine (29.4 nmol) and 5.0 μg of [ring-d4]-tyrosine (27.0 nmol). The blood-spot extract was then filtered using an Amicon Centrifree YM-30 filter (Millipore), yielding a clear filtrate of which a 10-μL aliquot was injected into the HPLC column. We also tried an alternative method of extraction that consisted of autoclaving the sample for 5–15 min (to fix the protein to the paper) followed by sonication. Both extraction methods are suitable for analysis. Separation of Phe and Tyr was achieved by HPLC (Hewlett-Packard 1100 series) using a Hypersil C-18 4 mm × 100 mm column containing 3 μm particles (Hewlett-Packard). The column temperature was held at 24 °C. The mobile phase was isocratic, at 93% solvent A (1 mL/L TFA in H2O) and 7% solvent B (1 mL/L TFA in 100 mL/L H2O–900 mL/L acetonitrile); the flow rate was 0.6 mL/min. Detection was by mass-spectrum detector (1100 MSD; Hewlett-Packard) using selected ion monitoring in atmospheric pressure chemical ionization mode with positive polarity. The flow rate of the drying gas (nitrogen) was 6.0 L/min, and its temperature was 350 °C; the nebulizer pressure was 413 kPa, and the vaporizer temperature was 410 °C. Capillary voltage was 4000 V, and the corona current was 6.0 μA. A fragmentor voltage ramp was used with mass 50 at 130 V, mass 100 at 90 V, and mass 150 at 50 V. The mass ions of the compounds of interest were monitored as follows: isotopic Tyr-186 m/z, native Tyr-182 m/z, isotopic Phe-171 m/z, native Phe-166 m/z. The elution time for Tyr was 3.9 min; the elution time for Phe was 8.7 min for the isotope and 9.0 min for the native compound. The calibration curves consisted of injected amounts of native amino acids (x-axis) vs the ratio between native amount and fixed amount of the respective stable isotope (y-axis). A small number of comparisons were made between simultaneous collections of paper blood spots and liquid plasma samples (analyzed by an automated amino acid analyzer) from patients with PKU. Seven blood spot analyses were performed using external standard quantification, and five were performed using stable isotope.

A typical chromatogram obtained from a blood spot sample of a patient with PKU is shown in Fig. 1. There was no apparent interference from other compounds present in the sample because of the selectivity of selected ion monitoring. The calibration curves were linear up to at least 1 nmol of injected Phe and Tyr (Phe, y = 0.004x + 0.017; r² = 1.00; Tyr, y = 0.005x + 0.002; r² = 1.00). The