Falsely Low Direct HDL-Cholesterol Results in a Patient with Dysbetalipoproteinemia, William L. Roberts,1* Elizabeth T. Leary,2,3 Thomas L. Lambert,4 Linda Moulton,3 and Janice L. Goestch2

Direct methods for measuring HDL-cholesterol (HDL-C) are becoming more widely used because of their labor savings and favorable performance characteristics. We investigated three methodologies for direct HDL-C measurements. All three contain an initial reagent to block measurement of cholesterol in lipoproteins other than HDL, followed by a second reagent to measure HDL-C.

The performance of each of these assays has been evaluated previously (1–8). All of these direct assays appear to be relatively free of common interferences; however, triglycerides >10 g/L may affect recoveries. There are minimal published data on these new direct methods with patients who have a dyslipidemia or are receiving lipid-modifying drugs. In this report, we describe a patient with type III hyperlipidemia for whom three direct HDL-C assays yielded erroneously low results compared with traditional HDL-C methods.

We analyzed a specimen from a 69-year-old man, using a direct HDL-C assay, and obtained a value that was less than the 40 mg/dL detection limit of the assay. The patient had no signs or symptoms such as large orange tonsils, corneal opacities, or relapsing polyneuropathy, compatible with primary hypoalphalipoproteinemia. His medical history was remarkable for chronic obstructive pulmonary disease, peripheral vascular disease, recurrent deep vein thrombosis, congestive heart failure, coronary artery disease, hypertension, and an unspecified hyperlipidemia. His current medications included aspirin, coumadin, digoxin, diltiazem, furosemide, albuterol/ipratropium inhaler, and simvastatin, a hydroxymethylglutaryl-CoA reductase inhibitor. He had consumed no alcohol for at least 1 year. A review of results for hepatic disease tests for the past 4 years revealed serum concentrations of aspartate aminotransferase, alanine aminotransferase, and total bilirubin within the appropriate reference intervals. Alkaline phosphatase values were 1.3- to 1.9-fold higher than the upper limit of normal over this same time period. Additional testing was performed to determine the true nature of his lipid abnormality.

The Liquid N-geneous HDL-C assay (Genzyme Diagnostics) uses a polyanion that selectively complexes LDL, VLDL, and chylomicrons followed by a detergent that selectively solubilizes HDL. The HDL-C Plus assay (Roche/Boehringer Mannheim) uses sulfated α-cyclodex-

trin, which reduces the reactivity of chylomicrons and VLDL, followed by polyethylene glycol-modified enzymes that are selective for HDL. The EZ HDL-C assay (Sigma Diagnostics) uses an anti-human β-lipoprotein antibody to block reactions with LDL, VLDL, and chylomicrons. Liquid N-geneous HDL-C and HDL-C Plus assays were performed on an Hitachi 917 analyzer (Roche/BMC). EZ HDL direct HDL-C measurements were performed on a Paramax analyzer (Dade Behring). All methods were performed according to the manufacturers’ instructions.

In addition to the direct methods described above, HDL-C was quantified by five other methods. The first method was a CDC designated comparison method (DCM) for HDL-C certification by the Cholesterol Reference Method Laboratory Network. The DCM uses direct dextran sulfate/magnesium precipitation followed by Abell-Kendall cholesterol analysis of the supernate. The DCM was performed by Pacific Biometrics Research Foundation, a member of the Cholesterol Reference Method Laboratory Network. The second method was a modified CDC reference method that uses ultracentrifugation (UC) followed by heparin/manganese precipitation on the density >1.006 kg/L fraction and an Abell-Kendall cholesterol determination of the supernatant. The third HDL-C method was UC followed by dextran sulfate/magnesium precipitation on the density >1.006 kg/L fraction and enzymatic cholesterol determination of the supernatant. The fourth method was direct dextran sulfate/calcium precipitation followed by enzymatic cholesterol determination of the supernatant. All four HDL-C methods were standardized to the CDC method. β-VLDL-cholesterol (β-VLDL-C) and HDL-C were also measured in whole serum and fractions from UC by lipoprotein electrophoresis using a REP instrument (Helena Laboratories). LDL-cholesterol (LDL-C) and VLDL-C were determined by UC followed by Abell-Kendall determination of cholesterol. Total cholesterol and triglycerides were quantified by enzymatic measurements standardized to the CDC methods. Apolipoprotein (apo) A-I was quantified on an Immage nephelometer (Beckman Coulter). Lipoprotein(a) [Lp(a)] was quantified by mass, using a Lp(a) AutoKit (Wako Chemicals) on an Hitachi 917 and by molar basis with the ApoTek ELISA method (Sigma).

Three samples for lipoprotein analysis were obtained from this patient over the course of 2 months. The results of HDL-C analyses are shown in Table 1. The direct HDL-C methods evaluated consistently gave substantially lower results than the chemical precipitation, electrophoresis, or UC/precipitation methods. Results from the precipitation, electrophoresis, and UC/precipitation methods showed good agreement.

Additional lipid testing performed on sample 3, which was obtained after an overnight fast, gave the following results: triglycerides, 2820 mg/dL; VLDL-C by UC, 919 mg/dL; LDL-C by UC, 654 mg/dL; VLDL-C/total triglycerides ratio, 0.33; and a distinct β-VLDL band in the d <1.006 kg/L fraction compatible with a type III phenotype. The concentration of Lp(a) was 800 mg/L (>300
mg/L indicates risk) by the Wako method and 292 nmol/L (48 nmol/L indicates risk) by the ApoTek method. The concentration of apo A-I was 1090 mg/L (reference interval, 940-1780 mg/L), and the HDL-C measured by UC followed by precipitation was 300 mg/L (0.78 mmol/L), yielding an HDL-C/apo A-I ratio of 0.71 [apo A-I units are g/L and HDL-C units are mmol/L for this ratio, in accordance with the convention established previously (9)].

Reaction kinetics monitor plots (Fig. 1) for the N-geneous HDL-C assay on a randomly selected control sample with HDL-C of 700 mg/L (Fig. 1A) and sample 3 from this case report patient (Fig. 1B) are shown. A high background absorbance produced by the addition of reagent 1 was clearly visible for our patient but not the control sample.

A previous report indicated that the β-VLDL fraction from patients with type III hyperlipoproteinemia interferes with the determination of HDL-C when the polyethylene glycol-modified enzymes/sulfated α-cyclodextrin method is used (10). These investigators found that HDL-C measured by this method could be overestimated by 23–114% in four patients with type III hyperlipoproteinemia. This is in marked contrast to the underestimation of HDL-C we observed. Another study examined the same three homogeneous HDL-C methods that we compared, using samples from patients with liver cirrhosis (9). These authors found that when compared with a reference UC method, a polyethylene glycol-modified enzyme method underestimated HDL-C by an average of 49%, a polyanion-detergent method underestimated HDL-C by an average of 32%, and a β-lipoprotein antibody method underestimated HDL-C by an average of 14%. The recovery in each of these methods relative to one another was the same as the recovery we observed for our patient. However, the absolute recoveries calculated for our patient for each of the three methods were substantially lower than the average recoveries observed previously for cirrhotic patients. This study also found that the negative bias of the direct HDL-C method increased with increases in the HDL-C/apo A-I ratio (9). The maximal bias was observed for ratios >2.0 and was minimal for ratios <1.0. Our patient had a ratio of 0.7, suggesting that apo-A-poor nascent HDL, which is underestimated by direct HDL-C methods in patients with liver cirrhosis, was not responsible for the effect seen in this patient.

One explanation for differences between the N-geneous and precipitation HDL-C methods seen in this patient may be the high background absorbance seen after the

![Fig. 1. Plots of absorbance vs measurement time point for N-geneous direct HDL-C assay. Each time point equals 17.6 s. The first absorbance reading was made at point 16. Reagent 2 was added after point 16. The final absorbance reading was made at point 34. (A), randomly selected sample with an HDL-C concentration of 700 mg/L; (B), sample 3 from the patient with an HDL-C result of 70 mg/L.](https://academic.oup.com/clinchem/article-abstract/46/4/560/5641265)
addition of reagent 1. This high background may result from unusual interactions between the polyanion in reagent 1 and lipoprotein particles in the patient’s serum. However, the low values observed for the other two direct HDL-C methods, which use different methodologies and whose reaction kinetics were not analyzed in this study, may require another explanation. The heterogeneous nature of HDL particles, which is still not well understood, may also account, at least in part, for the differences observed (11). Results obtained by direct dextran sulfate precipitation appear to be accurate.

Further studies of direct HDL-C methods on a larger number of patients with type III hyperlipidemia are required to determine how frequently results similar to those we observed in this single patient occur. One precipitation method and one immunoprecipitation method for LDL-C have been evaluated previously using a limited number of samples from patients with type III hyperlipidemia (12, 13). To our knowledge, no data have been published on direct LDL-C methods using such samples. Therefore, a study also needs to be performed using currently commercially available direct LDL-C methods and samples from patients with type III hyperlipidemia. This case again demonstrates that laboratory results that do not match the clinical picture should always be repeated and, if possible, verified by an alternate or reference method.

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References


Fluorimetric Measurement of Plasma α-L-Fucosidase Activity with a Centrifugal Analyzer: Reference Values in a Healthy French Adult Population, Maryvonne Cuer,1 Anne Barinier,1 Pauline de La Salmoniere,2 Geneviève Durand,1 and Nathalie Seta1 (1) Biochemical Laboratory A, Bichat-Claude Bernard Hospital, 75877 Paris Cedex 18, France; 2 Department of Medical Biostatistics, Saint Louis Hospital, 75475 Paris Cedex 10, France; * address correspondence to this author at: Laboratoire de Biochimie A, Hôpital Bichat-Claude Bernard, 46, Rue Henri Huchard, 75877 Paris Cedex 18, France; fax 33-1-40-25-88-21, e-mail labo.bioa@bch.ap-hop-paris.fr)

Release of α-L-fucosidase (AFU; EC 3.2.1.51) from the cells to the extracellular fluid can reflect an inherited or acquired disease involving the lysosome apparatus or a nonspecific lesion leading to cell lysis. Increased activities of AFU have been observed in the sera of patients with diabetes mellitus, alcoholic cirrhosis, and gastric carcinoma (1). Deugnier et al. (2) first proposed that serum AFU activity is a useful marker in the diagnosis of hepatocellular carcinoma (3). Its use as a diagnostic test, however, is limited by the absence of well-defined reference values and the lack of a sensitive and rapid automated enzyme assay.

In most cases, AFU activity is determined by a modification of the method of Zielke et al. (4). This method is limited by its long incubation time (30–60 min), which reflects the poor affinity of the enzyme for the colorimetric substrate used (4-nitrophenyl-α-L-fucoside; 4-NPF). Wood (5) observed that specific activities obtained with fluorescent 4-methylumbelliferyl-α-L-fucoside (4-MUF) substrate are more than twice those obtained with 4-NPF. However, adaptation of this method as described by Lombardo et al. (6) on the CLA 1500 continuous-flow analyzer (Carlo Erba) leads to a large expense for substrate. Here we describe the optimization of the fluorometric assay and its micromethod adaptation to the Monarch 2000 centrifugal automated analyzer (Instrumentation Laboratory) fitted with a spectrofluorimeter. We further describe the distribution of plasma AFU activity on a healthy French adult population.

All chemicals were purchased from Sigma except the 4-methylumbellif erone (4-MU) calibrator, which was obtained from Sebia. The working substrate solution, 0.25 mmol/L 4-MUF, was obtained by dilution of a 5 mmol/L 4-MUF solution (in methanol-water; 1:2, by volume) in an
acidic buffer (0.1 mol/L citrate-phosphate buffer, pH 5.0). The working 50 and 100 μmol/L 4-MU calibrator solutions were obtained by dilution of a 10 mmol/L 4-MU methanolic solution in the acidic buffer and confirmed by measurement of their absorbance at 321 nm ($\varepsilon_{321} = 16720$). The present investigation was carried out with plasma samples, according to the recommendations of Lombardo et al. (7). Plasma samples (n = 274) were obtained from healthy blood donors (age range, 18–60 years) with written informed consent. We used only noninfectious (negative for hepatitis B, hepatitis C, and HIV virus) samples and excluded plasma with alanine aminotransferase ≥56 U/L and/or γ-glutamyltransferase activities ≥35 U/L (measured on a Hitachi 747 analyzer; Boehringer-Mannheim). Some studies (8,9) have reported differences in enzymatic properties between low- and high-activity plasma variants. Rather than study conditions for maximal AFU activity on a plasma pool (7), we performed optimization studies using plasma selected from two healthy adult variants expressing low and high activity. For the determination of precision, plasma pools were obtained from samples with low, medium, and high AFU activity. Aliquots were stored at −20 °C for at least 6 months. For the determination of linearity, plasma samples (n = 21) were obtained from patients with hepatocellular carcinoma.

Before AFU measurement, all samples (plasma, calibrators, and controls) were first diluted automatically (1:10) with 0.15 mol/L NaCl containing 10 g/L bovine serum albumin (BSA) to prevent saturation of the fluorescence intensity. Diluted plasma and calibrators (10 μL) were mixed with 75 μL of acidic buffer with or without 4-MUF. After a 15-min incubation at 37 °C, enzymatic activity was stopped by the addition of 75 μL of a basic buffer (0.3 mol/L glycine-NaOH, pH 9.75). The fluorescence ($\lambda_{\text{excitation}}$ = 360 nm; $\lambda_{\text{emission}}$ = 450 nm) of the reaction product (4-MU) was recorded in the linear region delimited by the reference (10 μmol/L 4-MU in the basic buffer). A two-point calibration procedure was used to measure the corrected fluorescence intensity (reaction–blank) of each sample and to evaluate the enzymatic activity (1 U = 1 μmol · L$^{-1}$ · min$^{-1}$). The colorimetric method of Zielke et al. (4) was also adapted to the Monarch 2000 centrifugal analyzer. In that case, undiluted plasma samples and calibrators were mixed with the acidic buffer containing the 4-NPF substrate (reaction) or not (blank), and the absorbance of the reaction product (4-nitrophenol) was recorded at 405 nm. Correlation analysis and linear regression analysis were performed with the Statview software for Macintosh. Distribution of the plasma AFU activity was analyzed with the FASTCLUST procedure of SAS software (SAS Institute).

The slope obtained after serial dilution of a high-activity plasma sample in a diluent of 0.15 mol/L NaCl containing 10 g/L BSA was 1.25-fold higher than the slope with 0.15 mol/L NaCl as diluent. Citrate-phosphate buffer was preferred to acetate-phosphate buffer to prevent the formation of a cloudy mixture obtained with some plasma samples. The effects of pH (4.0–6.3) and 4-MUF concentration (0.01–1 mmol/L) on AFU activity were examined using low- and high-activity plasma variants. We measured slight differences in the pH activity profile and in $K_v$ values (low variant, pHmax = 5.0; $K_v = 0.035$ mmol/L; high variant, pHmax = 4.8; $K_v = 0.022$ mmol/L) as reported by Willems et al. (8). Working conditions (pH 5.0, 0.25 mmol/L 4-MUF) were chosen in favor of the low-activity variant.

The detection limit (mean + 3 SD of the method was 0.026 U/L (n = 35), as determined by measuring the fluorescence intensity of the diluent solution. The upper limit of linearity of the assay, assessed by serial dilutions (from 1:2 to 1:200 in 0.15 mol/L NaCl containing 10 g/L BSA) of high-activity plasma samples was 35 U/L. None of abnormal samples (n = 21; AFU = 10.2 ± 5.5 U/L, mean ± SD) included in this study exhibited AFU values >35 U/L. Within-batch CVs ranged from 2.5% (low-activity samples, n = 20) to 3.0% (high-activity samples, n = 20), and the between-batch CV was 5.1% (n = 41). Plasma AFU activity was not affected by storage at 4 °C for 48 h and at −20 °C for 6 months. Analytical recovery was studied by the addition of serial dilutions (0.15–15 U/L) of human placental AFU to a plasma pool. The mean recovery of AFU activity was 101% (97–107%). We found no interference with bilirubin up to 500 μmol/L or with hemoglobin up to 1.5 g/L. Beyond these concentrations, bilirubin and hemoglobin reduced fluorescence intensities. Assay of AFU activity was unaffected by opalescent solutions (triglycerides >10 mmol/L). Plasma AFU activities measured in 41 samples from different healthy individuals correlated with those determined with the adapted colorimetric method of Zielke et al. (4): $y = 3.003x + 0.240; r = 0.914$.

The frequency distribution of plasma AFU values exhibited positive skewness and suggested a trimodality (Fig. 1). Taken all together, the mean (± SD) plasma AFU value for healthy adults was estimated at 4.73 ± 2.42 U/L (0.66–10.53 U/L). Using the FASTCLUST procedure, we partitioned plasma AFU into the three activity ranges described in Fig. 1. Fifteen percent of individuals expressed low activity (1.23 ± 0.47 U/L), 45% expressed medium activity (3.69 ± 0.82 U/L), and 40% expressed high activity (7.27 ± 1.20 U/L).

The use of AFU as a routine clinical enzymology marker requires optimization of the assay reaction conditions and improvement of its reliability and practicability by automation. The conditions that provided maximal activity (0.1 mol/L citrate-phosphate, pH 5.0, 0.25 mmol/L 4-MUF, 15-min incubation time at 37 °C) were close to those used by Lombardo et al. (6) on a continuous flow analyzer. The sensitivity of the fluorimeter of the Monarch 2000 centrifugal analyzer allowed us to reduce incubation time and plasma sample volume (1:10 dilution). The use of rotors equipped with microcups (280 μL total volume) allowed us to reduce the volume of the expensive selected substrate (4-MUF) 10-fold. Automation by Monarch 2000 yielded precision near that obtained by the AFU colorimetric measurement with a Cobas Bio
In the physiologic range, the fluorometric assay correlated with the colorimetric assay, but the high specific activities obtained with the 4-MUF substrate yielded AFU values threefold higher than those obtained with the 4-NPF substrate. The fluorometric assay correlated with the colorimetric assay, and parameters influencing the assay. Clin Chim Acta 1980;108:337–46.

References

Lipid and Carbohydrate Metabolism in Acatalasemia

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Oxidative stress appears to be involved in degenerative diseases, including atherosclerosis and some forms of diabetes mellitus. The free radicals that damage cellular macromolecules are scavenged by a range of antioxidant enzymes. Superoxide dismutase catalyzes the conversion of superoxide anion into hydrogen peroxide, and catalase (EC 1.11.1.6) provides the main defense against the toxic hydrogen peroxide (1, 2). Despite interest in antioxidant capacity, determination of antioxidants is not routine (3–5).

In acatalasemia, a genetic deficiency of erythrocyte catalase inherited as an autosomal recessive trait, and in hypocalcitasemia, heterozygosity of the acatalasemia gene, the defense system against hydrogen peroxide is diminished; however, no biochemical changes have been reported for this syndrome (6, 7). Furthermore, recent findings concerning the connection of decreased antioxidant enzyme activities and diabetes mellitus lack clarification of the mechanism (8, 9).

We have reported on two acatalasemic sisters in the first Hungarian acatalasemic family (10) and nine hypocatalasemic families (11) with 37 hypocatalasemics. The frequencies for acatalasemia and hypocatalasemia in Hungary are 0.05 in 1000 and 1.8 in 1000.
We describe here the first comprehensive study of the biochemical markers of lipid and carbohydrate metabolism in acatalasemia and hypocatalasemia. We studied one acatalasemic and five hypocatalasemic Hungarian families with 2 acatalasemic females, 28 hypocatalasemic family members (15 females and 13 males), and 28 normocatalasemic family members (15 males and 13 females).

Serum glucose was determined by a glucose oxidase-peroxidase method (glucose test; Reanal), serum fructosamine (Roche Fructosamine Test; Hoffmann-La Roche), blood hemoglobin A$_1C$ (DIAMAT; Bio-Rad), triglycerides (triglyceride GPO-PAP Test; Boehringer Mannheim), cholesterol (cholesterol CHOD-PAP Test; Boehringer Mannheim) on a Boehringer/Hitachi 717 analyzer. LDL-cholesterol was calculated according to the Friedewald formula. Serum apolipoprotein (Apo) A1, Apo B, and lipoprotein(a) [Lp(a)] were measured on a COBAS MIRA analyzer (Hoffmann-La Roche). Oxidative modification of LDL was measured by a microassay of the oxidative resistance of LDL based on the hemin-catalyzed oxidation of LDL (12).

Blood catalase activity was determined spectrophotometrically (10, 11), with a reference mean ± SD of 113.3 ± 16.5 MU/L (n = 1756). We selected a normocatalasemic family member age-matched to each hypocatalasemic family member. The Student t-test was used to evaluate the statistical significance of difference between the two groups.

The results are shown in Table 1. The increased glucose, hemoglobin A$_1C$, and fructosamine in the patients reflected the higher incidence of diabetes in the affected members (n = 8) than in controls (n = 0). Seven of the affected members had type 2 diabetes, and one had type 1. Although decreased blood catalase activity in type 1 and type 2 diabetes mellitus has been reported (8, 9, 13–15), the high frequency (23%) of diabetes mellitus we found in hypocatalasemic and acatalasemic patients is a new finding. The toxic effect of increased hydrogen peroxide concentrations on either the pancreatic cells or the peripheral tissues may be involved in the pathogenesis of the disease. A study using pancreatic insulin-producing cells from rats showed that catalase plays a critical importance for the removal of reactive oxygen species (16).

Significant (P <0.048) changes were detected in cholesterol, LDL-cholesterol, Apo A1, Lp(a), and Apo B concentrations, and LDL oxidative resistance. These values in the diabetic patients did not show a significant (P >0.88) change when compared with those of the control group (cholesterol, 4.79 ± 1.13 vs 4.56 ± 0.89 mmol/L; LDL-cholesterol, 3.04 ± 0.38 vs 2.83 ± 0.97 mmol/L; Apo A1, 1.55 ± 0.29 vs 1.51 ± 0.38 g/L; Apo B, 1.09 ± 0.16 vs 1.05 ± 0.23 g/L; Lp(a), 259 ± 144 vs 212.1 ± 193.8 mg/L). These data suggest that the hypocatalasemia is the main contributor of the lipid abnormalities. The triglyceride and HDL-cholesterol concentrations were similar in the two groups.

In the two acatalasemias (both diabetic), increases (compared with their age- and gender-matched pairs) were also seen in cholesterol (6.39 vs 4.18 mmol/L), LDL-cholesterol (4.14 vs 2.46 mmol/L), Apo A1 (1.69 vs 1.53 g/L), Apo B (1.35 vs 1.10 g/L), and Lp(a) (294 vs 222 mg/L), with lower LDL oxidative resistance (2580 vs 4480 s) and increased glucose (8.6 vs 5.4 mmol/L), fructosamine (256 vs 210 μmol/L), and hemoglobin A$_1C$ (7.1% vs 4.5%). Changes in cholesterol, LDL-cholesterol, Apo A1, Apo B, Lp(a), and LDL oxidative resistance have not been reported for acatalasemic and hypocatalasemic patients and could be attributed to the increased oxidation of cholesterol, especially of LDL-cholesterol. The connection between lipid peroxidation and catalase activity has been reported in other diseases (4, 9, 13, 14). The change in conventional (cholesterol, LDL-cholesterol, and Apo B) and in nonconventional [Lp(a), LDL oxidative resistance] risks may mean a higher risk for these patients.

In addition to the condition itself, hypocatalasemia is seen with increased frequency in other disorders [e.g., anemia, tumors, schizophrenia, and atherosclerosis (4)], yielding a prevalence of ~1%. We conclude that these acatalasemic and hypocatalasemic subjects are at increased risk of diabetes mellitus and atherosclerosis.

### Table 1. Markers (mean ± SD) of carbohydrate and lipoprotein metabolism in five hypocatalasemic families in Hungary.

<table>
<thead>
<tr>
<th>Analyte or condition</th>
<th>Hypocatalasemia (n = 28)</th>
<th>Controls (n = 28)</th>
<th>P</th>
<th>Reference values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/L</td>
<td>5.40 ± 1.02</td>
<td>5.10 ± 0.69</td>
<td>0.193</td>
<td>3.6–6.0</td>
</tr>
<tr>
<td>Fructosamine, μmol/L</td>
<td>216.1 ± 28.2</td>
<td>214.0 ± 17.1</td>
<td>0.705</td>
<td>&lt;285</td>
</tr>
<tr>
<td>Hemoglobin A$_1C$, %</td>
<td>5.40 ± 1.01</td>
<td>5.10 ± 0.69</td>
<td>0.225</td>
<td>4.2–6.1</td>
</tr>
<tr>
<td>Diabetes</td>
<td>17.8%</td>
<td>0%</td>
<td>&lt;0.001</td>
<td>2–4%</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.35 ± 1.28</td>
<td>2.24 ± 1.26</td>
<td>0.758</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.58 ± 0.97</td>
<td>4.56 ± 0.89</td>
<td>0.001</td>
<td>&lt;5.2</td>
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<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>3.44 ± 0.96</td>
<td>2.83 ± 0.97</td>
<td>0.003</td>
<td>&lt;3.4</td>
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<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>1.42 ± 0.30</td>
<td>1.38 ± 0.39</td>
<td>0.664</td>
<td>&gt;1.2</td>
</tr>
<tr>
<td>Apo A1, g/L</td>
<td>1.76 ± 0.10</td>
<td>1.51 ± 0.38</td>
<td>0.003</td>
<td>&lt;1.15</td>
</tr>
<tr>
<td>Apo B, g/L</td>
<td>1.29 ± 0.25</td>
<td>1.05 ± 0.23</td>
<td>0.001</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Lp(a), mg/L</td>
<td>368.7 ± 214.0</td>
<td>212.1 ± 193.8</td>
<td>0.048</td>
<td>&lt;300</td>
</tr>
<tr>
<td>LDL oxidative resistance, s</td>
<td>4130 ± 1467$^a$</td>
<td>5441 ± 1828$^a$</td>
<td>0.020</td>
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<tr>
<td>Catalase, MU/L</td>
<td>48.9 ± 15.8</td>
<td>106.3 ± 18.5</td>
<td>&lt;0.001</td>
<td>80.3–146.3</td>
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<td>Age, year</td>
<td>44.7 ± 19.9</td>
<td>42.7 ± 17.8</td>
<td>0.778</td>
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</table>

$^a$n = 18.
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References

Effect of Prolonged Storage on the Activities of Superoxide Dismutase, Glutathione Reductase, and Glutathione Peroxidase, Clifford Abiaka,1,2 Farida Al-Awadi,2 and Samuel Olusi3 (1 Department of Medical Laboratory Science, Faculty of Allied Health Sciences, Kuwait University, P.O. Box 31470, Kuwait; Departments of 2 Biochemistry and 3 Pathology, Faculty of Medicine, Kuwait University, Kuwait; 4 author for correspondence: fax 965-4830937, e-mail clifford@hsc.kuniv.edu.kw)

Reactive oxygen species such as superoxide and hydrogen peroxide react in human tissues to form hydroxyl free radicals, especially when catalyzed by transition metals, e.g., iron [Fe(II)] and copper [Cu(I)]. The product is highly electrophilic and damaging to surrounding tissues and is implicated in the pathology of debilitating human diseases such as atherosclerosis (1), Parkinson disease and other neurodegenerative disorders (2), and cancer (3). Copper zinc-superoxide dismutase (CuZn-SOD; EC 1.15.1.1), glutathione peroxidase (GPX; EC 1.11.1.9), and glutathione reductase (GR; EC 1.6.4.2) are cellular antioxidants that can protect cells from the potentially harmful effects of reactive oxygen species (4–6). Assays of the activities of these mainly intracellular enzymes form part of the indirect determination of the activity of free radicals.

Measurements of erythrocyte CuZn-SOD, GPX, and GR activities usually are performed in the assessment of antioxidant status, and there has been speculation on the stability of these enzymes over a long period of storage. In this study, we investigated the long-term stability of CuZn-SOD, GPX, and GR in washed erythrocyte hemolysates by comparing the activities in stored hemolysates with those in hemolysates from freshly drawn blood. Subjects were recruited from blood donors (n = 24) with the consent of the ethics committee. The blood donors fulfilled the health requirements of the blood transfusion center, i.e., absence of contagious disease, medication, or history of viral hepatitis, malaria, and drug addiction. Venous blood (5 mL) was collected from each donor into a potassium-EDTA Vacutainer Tube by means of a cannula inserted into the arm of the recipient donor. The blood was centrifuged within 4 h of sampling at 1620g for 10 min in a refrigerated centrifuge at 4 °C to separate the plasma. The buffy coat was removed, and the remaining erythrocytes were drawn from the bottom, washed three times in cold saline (9.0 g/L NaCl), and hemolyzed by the addition of an equal volume of ice-cold demineralized ultrapure water (MilliQ plus reagent grade; Millipore) to yield a 50% hemolysate. Aliquots of the hemolysates (500 µL) were prepared for immediate assay of CuZn-SOD, GPX, and GR. Additional aliquots (500 µL) of the remaining hemolysates were dispensed into 1.5-mL capacity Beckman microfuge tubes (Beckman Instruments), capped, and frozen at −80 °C; these aliquots were assayed for CuZn-SOD, GPX, and GR after storage for 5, 12, and 21 months. To the 50% hemolysates (500 µL), was added 1 mL of demineralized water; 10 µL of each diluted hemolysate was brought to a final volume of 1.25 mL with Ransod sample diluent (a solution of 0.01 mol/L phosphate buffer, pH 7.0), thus bringing the final dilution of the hemolysates assayed for the activity of CuZn-SOD to 1.500. This dilution gives a 40–60% inhibition by hemolysate CuZn-SOD of the generation of superoxide anion free radical (O2·) by the reagent xanthine and xanthine oxidase (XOD). Measurement of CuZn-SOD activity was performed using Ransod reagents (Randox Laboratories) and is based on the method developed by McCord and Fridovich (7) coupling O2· generators (xanthine and XOD) with an O2· detector [2-(4-iophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride]. Absorbance was monitored in a Beckman DU 7500 spectrophotometer (Beckman Instruments).
ments) for 30 s (for the initial reading, $A_1$) after the addition of XOD (125 μL) as the starting reagent, and subsequently for 3 min for the final reading ($A_2$). The final reaction volume was 1 mL. All rates for the calibrators and diluted samples were converted into percentages of the rate for the sample diluent (uninhibited) and subtracted from 100% to give a percentage of inhibition. The unit of activity of the assay is defined as the amount of CuZn-SOD that inhibits the rate of formazan dye formation by 50%.

Measurement of GPX activity was performed using Ransel reagents (Randox) and is based on the method of Paglia and Valentine (8). GPX catalyzes the oxidation of reduced glutathione (GSH) by cumene hydroperoxide. In the presence of GR and NADPH, the oxidized glutathione (GSG) is immediately converted to the reduced form (GSH) with concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm is measured.

Drabkin cyanide-ferricyanide solution, pH 7.0–7.4, was prepared by dissolving potassium ferricyanide (200 mg), potassium cyanide (50 mg), and potassium dihydrogen phosphate (140 mg) in distilled water, making the volume up to 1 liter, and mixing after the addition of 0.5 mL of 250 g/L Brij 35.

For the GPX assay, hemolysates (50 μL) were diluted with 1 mL of Ransel diluting agent (Ransel reagent; 1:100) and incubated for 5 min, followed by the addition of 1 mL of the Drabkin reagent and mixing (total dilution of the hemolysates before assay, 1:82). The diluting agent reduces any GSSG present in the hemolysates to GSH because the cyanide in the Drabkin reagent rapidly inactivates GSSG. The cyanide in the Drabkin reagent inhibits other peroxidases that may be present in human blood and prevents falsely high results.

GR activity was assayed using reagent from Randox Laboratories; the assay was adapted from the method of Goldberg and Spooner (9). GR catalyzes the reduction of GSSG in the presence of NADPH, which is oxidized to NADP+. The decrease in absorbance at 340 nm is measured.

For the GR assay, the 50% hemolysates were centrifuged to remove stroma, and 100 μL was diluted with 1.9 mL of 9 g/L NaCl (total dilution of the hemolysates for the GR assay, 1:40).

Statistical analyses were performed using SPSS 9.0 for Window software (SPSS). Differences in the means between groups were analyzed by one-way ANOVA. Two-tailed $P$ values were used, and statistical significance was set at $P < 0.05$.

The activities of erythrocyte CuZn-SOD, GPX, and GR (mean ± SE, in kU/L) obtained for the fresh hemolysates were compared with the activities of enzymes in hemolysates after storage at −80 °C for 6, 12, and 21 months (Table 1). There were no significant changes in the activities of the enzymes between fresh and frozen hemolysates.

Previous studies generally compared the activities of cytoprotective enzymes for groups of patients with matched controls. The observed differences in enzyme activities in most of these studies could constitute a contributory cause of the disease, an effect of drug treatment, drug overdose, or xenobiotics. However, we are unaware of any published study on the stability of these enzymes in washed human erythrocytes. The uncertainty about their stability often necessitated the assay of the cytoprotective enzymes shortly after hemolysate preparation. We have demonstrated that CuZn-SOD, GPX, and GR in washed erythrocyte hemolysates are stable at −80 °C for close to 2 years. We believe that our observations will be useful in large-scale epidemiological studies involving cytoprotective enzymes by providing the knowledge that these enzymes are stable over a prolonged period at −80 °C.

### Table 1. Comparison of the activities* of SOD, GPX, and GR in fresh and frozen hemolysates (n = 24).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fresh</th>
<th>6 months</th>
<th>12 months</th>
<th>21 months</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD, kU/L</td>
<td>270 ± 8</td>
<td>270 ± 8</td>
<td>271 ± 8</td>
<td>267 ± 7</td>
<td>0.982</td>
</tr>
<tr>
<td>GPX, kU/L</td>
<td>10.9 ± 0.6</td>
<td>10.7 ± 0.6</td>
<td>10.9 ± 0.7</td>
<td>10.8 ± 0.7</td>
<td>0.996</td>
</tr>
<tr>
<td>GR, kU/L</td>
<td>1.26 ± 0.09</td>
<td>1.28 ± 0.09</td>
<td>1.27 ± 0.09</td>
<td>1.24 ± 0.08</td>
<td>0.990</td>
</tr>
</tbody>
</table>

*Mean ± SE.

### References


### Response to Acute Osteoclast Activity Inhibition Assessed by the Determination of C-Telopeptide of Type I Collagen in Serum, Isabella Villa, Barbara Saccon, and Alessandro Rubinacci* (Bone Metabolic Unit, Scientific Institute H San Raffaele, Via Olgettina 60, 20132 Milan, Italy; * author for correspondence: e-mail a.rubinacci@hsr.it)

Several degradation products of bone matrix are released in serum by osteoclasts when they resorb bone. The most critical molecular fragments of type I collagen that have clinical utility as sensitive and specific markers of bone resorption contain the nonreducible pyridinium cross-link, deoxypyridinoline (Dpd), which is excreted in the urine in free and peptide-bound forms (1). Immunoassays

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have been developed recently to measure free Dpd, but two factors limit their large application in clinical settings: (a) the cleavage of the peptide-bound to the free form appears to be a rate-limiting, saturable process occurring in the kidney, which is in agreement with the observation that osteoclasts release the peptide-bound form and not the free form; and (b) the fractions of free and peptide-bound pyridinoline are not constant, being affected by disease states, osteoclast activity, and treatments. In a previous study, when short-term inhibition of osteoclast activity was induced by oral calcium load, measurements of the total Dpd fraction in the urine were able to distinguish the effects of the physiological perturbation of calcium homeostasis from the circadian variations, whereas measurements of free Dpd did not, suggesting that free Dpd was not as sensitive as total Dpd to detect acute osteoclast inhibition.

A new assay (Serum CrossLaps™ One Step ELISA) for the determination of serum concentrations of the C-telopeptide of type I collagen (β-CTx) has been developed recently and applied clinically; therefore, we found it interesting to determine whether it can detect the inhibition of osteoclast activity that follows oral calcium loading. This assay is performed as a monoclonal sandwich assay in a one-step procedure and is based on a monoclonal antibody that exclusively recognizes an isomerized β-aspartate (d) form of the EKAHDGGR epitope of the α1 chain of human type I collagen. This sequence contains the lysine residue that participates in the intermolecular cross-linkage of the collagen triple chains. Although the metabolic clearance of the AHD-β-GGR is largely unknown, it is conceivable that the isoaspartyl bonds may contribute to the resistance of the telopeptide sequence to proteases. When measured in serum, β-CTx theoretically is less prone to the drawbacks that have limited free Dpd measured in the urine and does not require correction for creatinine, which adds imprecision.

In this study, we measured β-CTx in the serum aliquots collected for a previous study of calcium loading, and compared β-CTx performance to that described previously for urinary total Dpd. All technical details have been described previously. Briefly, two groups of subjects, one taking calcium (test group; n = 16) and the other not (control group; n = 9), underwent the measurement of markers of bone and mineral metabolism (serum calcium, parathyroid hormone, and calcitonin, as well as urinary total Dpd, free Dpd, and calcium corrected for creatinine) at sequential times after the loading in the test group and at the corresponding times in the control group not taking calcium [before loading (t0), and 1 (t1) and 3 h (t2) after loading for serum samples; and before loading (t0), and 2 (t1) and 4 (t2) h after loading for urine samples].

Under these experimental conditions, serum β-CTx concentrations decreased significantly (P < 0.01, t0 vs t1 and t0 vs t2, one-way repeated-measures ANOVA with
the Dunnett multiple comparison test) in the test group (Fig. 1A), whereas they did not change significantly in the control group (Fig. 1B). The reduction was highest at $t_2$ when most of calcium was absorbed by the gut. The results obtained indicate that $\beta$-CTx in the serum is able to detect the perturbation of calcium homeostasis induced by calcium loading. The dynamics of the changes observed strongly confirm the view that osteoclasts are involved in the short-term error correction mechanism of plasma calcium homeostasis (5). It is in fact conceivable that the decrease in serum $\beta$-CTx concentrations after calcium loading could be ascribed only to the inhibition of osteoclast activity induced by the calcemic increment: any possible calcium-related changes in the degradative pattern of collagen are unlikely because of the short-term nature of the experiment, and further degradation of $\beta$-CTx could be hindered by the presence of the isoaspartyl bonds. This view is confirmed by the observation that the individual decrements of the $\beta$-CTx concentrations in serum were inversely correlated to the amplitude of the calcemic increase ($r = -0.60; P = 0.018$; Fig. 1C), and to their relevant basal values ($r = -0.90; P < 0.0001$; Fig. 1D).

By dividing the subjects taking calcium according to basal bone turnover into two subgroups, one with $t_0$ $\beta$-CTx concentrations higher than the mean value (2901 pmol/L) and the other with concentrations lower than the mean value, we found a highly significant difference ($P < 0.0001$, two-tailed Student $t$-test) between the amplitude of the decrements (2904 ± 268 pmol/L vs 862 ± 220 pmol/L, respectively). Two metabolic factors, therefore, appear to modulate the decrement observed in the $\beta$-CTx serum concentration as they did with total Dpd in the urine: one is the amplitude of the perturbation in plasma calcium homeostasis, and the other is the osteoclast activity. Any circadian daylight variations in the $\beta$-CTx concentrations in serum during the experimental time cannot be taken into account to explain the changes observed because serum $\beta$-CTx values did not change significantly in the control group. When the clear-cut difference in the pattern of the changes observed in the subjects taking calcium or not (Fig. 1, A and B) is considered, it can be inferred that serum measurement of $\beta$-CTx may better discriminate parathyroid hormone-dependent (test group) and -independent (control group) changes of osteoclast activity than urinary total Dpd (5).

We can therefore conclude that in our experimental conditions, the clinical performance of the serum $\beta$-CTx is comparable to that obtained by total urinary Dpd. In the clinical setting, it could be advantageous to measure the cross-links and related peptides in the serum rather than in the urine, thus avoiding possible bias related to creatinine correction and renal handling.

We thank Dr. C. Nassivera (Cis Diagnostici S.p.A.) for the kind gift of the Serum CrossLaps One Step ELISA assays.

References

Effects of Nine Hemoglobin Variants on Five Glycohemoglobin Methods, William L. Roberts,1 Elizabeth L. Frank,2 Linda Moulton,3 Christine Papadea,3 Jimmie K. Noffsinger,4 and Ching-Nan Ou 5

Most studies of the effects of variant hemoglobins (Hbs) on specific glycohemoglobin (gHb) methods have been case reports of a single variant Hb and one or two analytical methods (1–4). Few studies have systematically examined multiple Hb variants with several widely used analytical methods. In this report, we describe the effects of nine heterozygous Hb variants on five gHb methods. A boronate affinity method was chosen as the comparative method because it has high specificity for glycated Hb and negligible interference by variant Hbs (5).

Over 10 months, we studied 40 samples with nine variant Hbs; 38 were detected during routine gHb analysis by a cation-exchange method. Two samples (Hb E trait) were identified during routine Hb phenotype analysis. Samples were stored at 2–8 °C until analysis within 10 days of collection.

Cation-exchange chromatography was performed on a Variant system with the Hb A1c program (Bio-Rad Labo-
Hb phenotype analysis was performed on all samples using a PVS99 system (Primus). This HPLC system uses a poly-aspartic acid cation-exchange column and a complex nonlinear salt and pH gradient at 40°C. Rare phenotypes, including Grady, Hope, and Raleigh were identified using a poly-aspartic acid column with a nonlinear salt and pH gradient (6) and comparison with the authentic Hb variants.

The effects of variant Hbs on each gHb method are summarized in Table 1. Neither immunoassay method showed significant effects with any of the Hb variants tested. One sample containing Hb Camden trait was investigated. The Variant method poorly resolved Camden from Hb A and underestimated gHb (Fig. 1A).

Eight samples with Hb D Los Angeles trait were investigated. Three different chromatographic patterns were observed for the Variant method. In the first pattern (Fig. 1B), Hb A was resolved from glycated Hb D (Hb D1c) and Hb D. However, the instrument printout included the area of Hb D in the total for Hb A and consequently underestimated gHb. Manipulation of the peak areas by summing Hb A1c and Hb D1c and dividing by the total area or by dividing the Hb A1c peak area by the Hb A peak area failed to yield results within 1% of the comparative method. In the second pattern (Fig. 1C), Hb D was not resolved from Hb A, with consequent underestimation of gHb. The A1c 2.2 Plus method showed good agreement with the comparative method for all Hb D samples tested.

Two samples from nondiabetic patients with Hb E trait were analyzed. Neither chromatographic method resolved Hb A from Hb E. The Variant method did not resolve Hb A1c from Hb E1c, and results agreed well with the comparative method. The A1c 2.2 Plus method resolved Hb A1c from Hb E1c, but did not resolve Hb A from Hb E, with consequent underestimation of gHb.

Six samples containing Hb G Philadelphia trait were analyzed. The Variant method produced three chromatographic patterns resembling those seen for Hb D in Fig. 1, B–D. gHb was underestimated. The A1c 2.2 Plus method overestimated gHb compared with the comparative method because of coelution of Hb A1c and Hb G1c, whereas Hb A and G were resolved.

Two samples containing Hb Grady trait were evaluated. The chromatograms were virtually identical to Fig. 1C. However, the Variant results agreed with the comparative method without correction. The A1c 2.2 Plus showed good agreement with the comparative method.

Five samples containing Hb Hope trait were evaluated. Hb Hope comigrated with Hb A1c in the Variant method, with a large overestimation of gHb. The A1c 2.2 Plus method resolved Hb Hope from Hb A1c and Hb A (Fig. 1E) and yielded gHb values comparable to the comparative method. Fourteen samples containing Hb J Baltimore trait were evaluated. A Variant chromatogram (Fig. 1F) showed a peak eluting just before Hb A1c1c, which presumably is Hb J Baltimore1c. A single peak at 1.56 min indicated coelution of Hb J Baltimore and A. Consequently, gHb was underestimated. When gHb was recalculated as the sum of the peak areas of glycated Hbs A and J Baltimore divided by the total area, the value yielded was low. Inspection of the chromatogram revealed that the method of drawing the baseline (an inverted “v”) under the Hb J Baltimore1c and A1c peaks was responsible for an underestimation of the true areas of both of these peaks. Of the 14 Variant results for Hb J Baltimore samples, only 2 showed flat baselines and gHb

### Table 1. Summary of variant Hb effects on Hb A1c results by method.

<table>
<thead>
<tr>
<th>Hb trait</th>
<th>A1c 2.2 Plus</th>
<th>Variant</th>
<th>DCA 2000</th>
<th>Tina-quant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camden</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Falsely low&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No effect&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>D Los Angeles</td>
<td>No effect</td>
<td>Falsely low</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>E</td>
<td>Falsely low</td>
<td>Falsely low</td>
<td>No effect</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>G Philadelphia</td>
<td>Falsely high&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Falsely low</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Grady</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Hope</td>
<td>No effect</td>
<td>Falsely high</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>J Baltimore</td>
<td>Falsely low</td>
<td>Falsely high</td>
<td>No effect</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Raleigh</td>
<td>Falsely high</td>
<td>Falsely high</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Russ</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND, not determined.

<sup>b</sup> For the effect of a Hb variant to be considered falsely low, the average difference of the percentage of Hb A1c between the test and comparative methods had to be less than 1%.

<sup>c</sup> For the effect of a Hb variant to be considered falsely high, the average difference between the test and comparative methods had to be greater than 1%.

<sup>d</sup> ND, not determined.
values that agreed closely with the comparative method after recalculation. Inverted “v” baselines were seen for the other 12. A chromatogram obtained by the A1c 2.2 Plus method (Fig. 1G) showed an extra peak, which corresponded to Hb J Baltimore1c. Hb A and J Baltimore coeluted, with consequent underestimation of gHb.

One sample with Hb Raleigh trait was investigated. This variant Hb comigrated with Hb A1c in both the Variant and A1c 2.2 Plus methods (Fig. 1H), leading to a large overestimation of gHb. The DCA 2000 and comparative methods agreed.

One Hb Russ sample was evaluated. This variant eluted after Hb A in both the Variant (Fig. 1I) and A1c 2.2 Plus methods. The elution position of Hb Russ1c was not...
The A1c 2.2 Plus method. However, only Hb E and J Baltimore have this limitation with D Los Angeles, G Philadelphia, and J Baltimore underestimated of gHb. Several of the Hb variants tested, including D Los Angeles, G Philadelphia, and J Baltimore showed this limitation with the Variant method. However, only Hb E and J Baltimore have this limitation with the A1c 2.2 Plus method. (b) The Hb variant can comigrate with HbA1c, leading to an overestimation of gHb. Hb Hope had this effect in the Variant method, and Hb Raleigh had this effect in both cation-exchange methods. These Hb variants generally account for 35–45% of the total Hb and therefore cause gHb to be >20%, a concentration seldom seen in diabetic patients. (c) The Hb variant can comigrate with HbA1c, leading to an overestimation if the Hb variant is resolved from Hb A. This seems to occur for G Philadelphia with the A1c 2.2 Plus system.

The effects of variant Hb traits on Hb A1c results produced by the Variant and DCA 2000 methods had been examined previously (7). These investigators concluded that if appropriate calculations were performed, samples containing Hb D and Hb J Baltimore traits could be accurately analyzed using the Variant system. In contrast, we found a great deal of variability in the chromatograms generated by the Variant system with these two Hb variants. Rarely, gHb could be correctly estimated by summing the peak areas of the glycated Hb A and Hb variant and dividing by the total area. However, in most cases this approach did not work.

The practice of correcting results for samples containing Hb variants by summing the peak areas of glycated A and the glycated variant and then dividing by the total area can be problematic not only when chromatographic resolution is poor, as discussed above, but also when the specific variant is not known. This is best illustrated for samples containing Hb Grady trait.

The two immunoassay methods evaluated produced results that agreed well with the boronate affinity comparative method. Only Hb Raleigh has an amino acid substitution in the six N-terminal amino acids of the β chain. It previously has been shown that the presence of Hb C or S trait, both of which have substitutions at position six of the β chain, can affect the accuracy of some immunoassay results (8). Other variant Hbs with substitutions or deletions in the first six amino acids of the β chain, including Deer Lodge, Fukoka, C150Leiden, Long Island, Machida, Marseille, Okayama, Raleigh, South Florida, and Warwickshire have the potential to adversely affect the accuracy of immunoassay gHb methods (9). Herein, the DCA 2000 result (5.4%) was within 1% of the boronate affinity result (6.3%) for one sample containing Hb Raleigh. A previous study of Hb Raleigh, whose β-chain NH2 terminus is acetylated, suggested that both immunoassay methods might underestimate glycated Hb Raleigh (and that boronate affinity methods may underestimate mean blood glucose because the acetylated N-terminal amino acid cannot be glycated) (10).

In conclusion, several variant Hbs can produce variable interferences with both of the cation-exchange chromatography gHb methods that we evaluated. The DCA 2000 and Tina-quant methods agreed well with boronate affinity chromatography.

A DCA 2000 Analyzer and reagents were provided by Bayer Corporation. Tina-quant HbA1c II reagents were provided by Roche Diagnostics.

References

Preliminary Evaluation of the Vitros ECI Cardiac Troponin I Assay, Fred S. Apple, Brenda Koplen, and Mary Ann M. Murakami (Hennepin County Medical Center, Clinical Laboratories MC 812, 701 Park Ave., Minneapolis, MN 55425; * author for correspondence: fax 612-904-4229, e-mail fred.apple@co.hennepin.mn.us)

The recently published standards of practice for the use of cardiac marker testing for ruling in and ruling out acute myocardial infarction (AMI) recommend the implementation of cardiac troponin I (cTnI) or T (cTnT) as appropriate markers (1). The purpose of this preliminary study was to analytically and clinically evaluate the OrthoClinical Diagnostics (Rochester, NY) Vitros Troponin I immunodiagnostic assay.

The Vitros ECI system uses reagents containing bionylated monoclonal anti-cTnI antibody and goat polyclonal anti-cTnT antibody labeled with horseradish peroxidase. After an 8-min incubation for reagents and sample in a well precoated with streptavidin, the well was washed and
a signal reagent containing horseradish peroxidase substrates is added to detect peroxidase bound to the well by utilizing an enhanced chemiluminescence reaction (2).

The imprecision (as the CV, %) within the same calibration performed according to NCCLS guidelines (3) showed the following results: between-run (n = 80) means over 31 days on 20 occasions were 0.347 µg/L (10%), 0.768 µg/L (5.9%), 4.10 µg/L (4.3%), and 15.5 µg/L (3.2%). The analytical sensitivity, determined using 2 SD after 20 replicates of the zero calibration material were measured, was 0.018 µg/L. The lowest concentration giving rise to a between-assay CV ≤10% was 0.351 µg/L. Deming relationships, obtained from 36 fresh plasma samples across the linear range (0–100 µg/L) of the Vitros cTnI assay compared with the Dade-Behring RxL cTnI assay, showed the following: Vitros cTnI = 0.556 Dade cTnI + 0.039; r = 0.972. The 95% confidence intervals for slope and intercept were 0.509–0.602 and −0.394 to 0.472, respectively. The difference plot (Exstat Ver. 9; DDU Software) shown in Fig. 1A demonstrates a proportional, increasing negative bias across the range of concentrations tested for by the ECI cTnI assay compared with the Dade RxL cTnI assay. The bias is likely a result of lack of standardization between cTnI assays (4). Deming regression analysis for the Vitros cTnI assay for fresh plasma samples (n = 50) compared with the same frozen, thawed plasma samples showed no significant differences (frozen cTnI = 0.965 fresh cTnI − 0.005; r = 0.997) over a cTnI range of 0–11.3 µg/L. Deming regression analysis between paired fresh serum and plasma (heparinized) samples showed a bias for plasma as follows: plasma Vitros cTnI = 0.760 serum Vitros cTnI − 0.201; r = 0.998 (n = 17). Based on 200 apparently healthy male and female blood donors, the upper reference 97.5th percentile was 0.1 µg/L in serum and 0.08 µg/L in plasma samples. Fig. 1B shows a comparison of ROC curves for the ECI and RxL assays determined from 74 chest pain patients admitted to rule in or rule out AMI. A modified WHO criterion, using a cTnI clinical decision cutpoint at 0.8 µg/L (Dade Behring RxL assay) for the biochemical criterion (predetermined at Hennepin County Medical Center), was used (5). Peak cTnI values, from the three to four plasma samples obtained serially within 24 h of presentation to the hospital, were used to construct the ROC curves. Thirty percent (22 of 74) of patients were diagnosed with AMI. The ECI cTnI cutoff of 0.40 µg/L demonstrated a sensitivity of 100% and a specificity of 79%. In comparison, the RxL cTnI cutoff of 0.55 µg/L demonstrated a sensitivity of 100% and a specificity of 77%. There were no statistical differences in the areas under the ROC curves between assays (0.9038 for ECI; 0.9008 for RxL). Patients with clinical diagnoses involving myocardial damage, including acute coronary syndromes (unstable angina), congestive heart failure, and cardiac surgery, but excluding AMI, were responsible for the 77% and 79% specificities of both cTnI assays at 100% sensitivities. Outcome studies involving risk assessment, however, were not part of this study design.

In summary, the Vitros ECI Troponin I immunodiagnostic assay demonstrated acceptable analytical performance. The impression around the upper reference cutpoints was clinically acceptable (<10%). The 97.5th
Cystinosis is an autosomal recessive disease caused by impaired transport of cystine across lysosomal membranes. The subsequent lysosomal storage of the poorly soluble cystine produces crystal formation and cellular damage in many tissues. The earliest involvement occurs in the renal tubules and causes Fanconi syndrome, with polyuria, dehydration, acidosis, rickets, and failure to thrive. In untreated cystinosis, the progression of renal glomerular dysfunction leads to uremia and death by 9–10 years of age unless dialysis or renal transplantation is initiated (1). The therapy of nephropathic cystinosis involves treatment with the cystine-depleting agent cysteamine (2).

The most direct diagnostic method for cystinosis is measurement of leukocyte cystine content by an Escherichia coli cystine-binding protein assay. This assay is time-consuming and involves competition between non-radioactive cystine and [14C]cystine for a cystine-binding protein. The protein-bound radioactivity is then trapped on nitrocellulose filters and is inversely correlated to the protein. The protein-bound radioactivity is then determined by analyzing 10 replicates of the biological samples (4). The method is reproducible, sensitive, and requires no radioactive reagents.

Blood (4.5 mL) was drawn by venipuncture and was collected into a heparin-containing tube. PMN leukocyte separations were carried out as described by Smolin et al. (6). After sonication of the PMN leukocytes three times for 2 s (each) in 0.1 mL of 0.1 mol/L phosphate buffer, pH 7.2, containing 5 mmol/L N-ethylmaleimide (NEM), 50 µL of 120 g/L sulfosalicylic acid was added, and the cystine content in the acid-soluble fraction was determined. The protein pellet was then dissolved in 150 µL of 0.1 mol/L NaOH, and the protein concentration was determined with the BCA protein assay (Pierce).

The derivatization and chromatography procedures were performed, with little modification, as reported previously (4). Briefly, the autosampler collected 3 µL of 4 mol/L NaBH₄, 2 µL of 2 mmol/L EDTA-dithiothreitol, 1 µL of 1-octanol, and 2 µL of 1.8 mol/L HCl and placed the mixture in the derivatization vial containing 10 µL of sample. After the mixture was incubated for 3 min, 10 µL of 1.5 mol/L N-ethylmorpholine buffer, pH 8.0, 40 µL of distilled water, and 2 µL of 25 mmol/L bromobimane were added. Following an additional 3-min incubation, 4 µL of acetic acid was added, and 40 µL of this mixture was injected into the column.

Known concentrations of cystine were added to samples. The concentrations in samples with added cystine were determined in five replicates, and analytical recoveries were calculated. The intraassay precision was obtained by analyzing 10 replicates of the biological samples on the same day. The interassay precision was determined by analyzing the same biological samples on 10 different days over 1 month.

Calibration curves for cystine (0.3–10 µmol/L) were prepared in duplicate by diluting the stock solutions with 0.1 mol/L HCl containing 100 µmol/L dithiothreitol. The linearity of the assays was also studied in the following range: 0–10 µmol/L cystine. The limit of detection, defined as the concentration that produces a signal-to-noise ratio >3, was ~0.4 pmol in the assay.

In a typical HPLC-FD chromatogram of a sample from a healthy subject (Fig. 1A), the retention time for cysteine-S-bimane was 3.7 min. The cysteine peak at 3.7 min in Fig. 1A was given by a sample concentration of 0.08 nmol half-cysteine/mg protein. In a typical chromatogram from an individual with cystinosis (Fig. 1B), the cysteine-
S-bimane peak gave a sample concentration of 3.5 nmol half-cysteine/mg protein. A linear relationship was obtained between the peak area and cysteine concentration in the ranges studied in an aqueous matrix (data not shown). The correlation coefficient was >0.99. The equation for the regression line (n = 8) was: y = 0.03x + 0.16, where y is the peak area, and x is the concentration of the analyte. The lowest concentrations in the linearity studies were above the limit of detection of 0.4 pmol in the assay. No significant matrix effect was observed when linearity studies were performed in samples diluted up to 1:32 with water containing 100 µmol/L dithiothreitol (data not shown). The intraassay CV for the cysteine measurements was 2%; the interassay CV, determined by assaying on 10 different days over 1 month, was 4.7%. The mean recovery was 99.6%.

A discontinuous gradient of Ficoll-Hypaque (Sigma) was used to separate PMN leukocytes from blood. The PMN cells were 78–96% pure as determined by cell counting with a hemocytometer. The residual leukocyte material contained <4% PMN cells. The PMN leukocyte cystine concentrations in 20 healthy children (Fig. 1C) were 0.01–0.19 nmol half-cysteine/mg protein (mean ± SD, 0.08 ± 0.06), consistent with other studies (1). As reported previously (5), the PMN leukocyte cystine concentrations in subjects before treatment with cysteamine were 3.5–3.8 nmol half-cysteine/mg protein (n = 3; age range, 6 months to 1 year); the cysteine-depleting effect of cysteamine was shown during treatment, with PMN leukocyte cystine concentrations ranging from 0.7 to 1.23 nmol half-cysteine/mg protein (n = 16; age range, 1–20 years).

In obligate heterozygotes, PMN leukocyte cystine concentrations were intermediate between those in homozygotes and healthy individuals (n = 24; mean ± SD, 0.9 ± 0.6 nmol half-cysteine/mg protein); this finding is consistent with other studies (1, 6). Early diagnosis of cystinosis is essential for timely treatment, and follow-up of a patient in treatment is essential for a correct therapy. Moreover, determination of PMN leukocyte cystine concentrations in individuals with a family history of nephropathic cystinosis is important for identifying heterozygotes for this heritable disease.

The method presented here is based on the measurement of cystine with a simple, fully automated HPLC-FD assay after isolation of PMN leukocytes and sonication of cells. We measured the cystine concentrations in PMN leukocytes fraction because Smolin et al. (6) demonstrated that the use of this fraction provides an accurate method for detecting heterozygotes. Our results in obligate heterozygotes agree with the report of Smolin et al. (6) in that >90% of individuals who are heterozygotes will have values that fall above the reference interval; in the present study, none of the values for heterozygotes were within 2 SD of the mean for the healthy subjects (Fig. 1C).

In all experimental systems where both cysteine and

![Fig. 1. HPLC-FD chromatograms obtained for PMN leukocytes of a healthy subject (A) and a cystinotic patient before treatment with cysteamine (B), and cystine content of leukocytes (C).](https://academic.oup.com/clinchem/article-abstract/46/4/560/5641265)
cystine are present, care must be taken to prevent the spontaneous oxidation of cysteine to cystine, which would spuriously increase the results for cystine. This is accomplished using NEM, which forms an irreversible adduct with free thiols such as cysteine.

The current method measures cysteine after reduction of cystine with NaBH₄; before this reduction, free cysteine and other free thiols are blocked by the use of 5 mmol/L NEM in the sonication mixture to prevent oxidation of free cysteine and the disulfide exchange reaction of cystine. The use of high NEM concentrations is justified by the presence in the cell of high concentrations (1–2 mmol/L) of reduced glutathione, which also react with NEM (7). It was demonstrated that excess NEM has essentially no effect on the determination of oxidized thiols with this method (8). Moreover, samples are at acid pH before and during the first step of derivatization (reduction), and NEM is almost inactive at acid pH (9).

In conclusion, this HPLC assay is sensitive and precise and provides high sample throughput; moreover, the cost of the procedure is lower than the cystine-binding protein assay. This assay thus enables reliable determination of intracellular cystine in both homozygous and heterozygous subjects, which allows simple and rapid diagnosis of cystinosis in newborns as well as identification of heterozygous individuals.

References