Glycosylated Hemoglobins: Hematologic Considerations Determine Which Assay for Glycohemoglobin Is Advisable

To the Editor:

Hemoglobin A1c assay is now well established as an indicator of the effectiveness of carbohydrate control in diabetics, particularly in those who are insulin dependent (1, 2). However, current methods (2) for glycosylated hemoglobin (short- and long-column cation-exchange chromatography, “high-performance” chromatography, immunogenic methods, isoelectric focusing, colorimetry) measure different “moieties” of the process of nonenzymatic posttra-

tional glycosylation of hemoglobin. Such differences were brought into perspective by the following experiences.

Indians of southern Africa are subject to a high incidence of diabetes mellitus. In a group of newly diagnosed diabetics, glycosylated hemoglobin formed 4 to 16% (normal subjects, 2 to 6%) of total hemoglobin as measured by cation-resin long-column chromatography (2) or thiobarbituric acid colorimetry (3). Results were in reasonable agreement (r = 0.76), with three exceptions. These indicated 7.6, 7.2, and 9.4% glycohemoglobin (fast-moving hemoglobins A1a+he) by short-column resin chromatography, and 6.8, 6.9, and 8.3% by isoelectric focusing and densitometric scan of the gel for Hb A1a (KLB-Pro-
dukter, Bromma, Sweden), compared with 9.8, 10.6, and 13.9%, respectively, by the colorimetric method (A445 mm, thiobarbituric acid complex with 5-
hydroxymethylfurfural, liberated from hydrolysis of glycohemoglobins). He-
matologic investigations identified these diabetics as belonging to the sickle cell trait, with 42, 40, and 53% hemoglobin S, respectively. Isolated sickle erythrocytes, incubated in isotonic saline containing 25 mmol of glucose per liter, formed a glycosylated hemoglobin S1, separable from Hb S by isoelectric focusing, while both Hb S and S2 co-eluted with slow-moving Hb A in the cation-
resin chromatograph. Thus the short-
column chromatographic procedure, with collection of the fast-moving frac-
tions of hemoglobin on elution, will only determine the extent of glycosylation of the hemoglobin A component in dia-
betics with sickle hemoglobin (Negro, East Indians, and Mediterraneans).

Presumably, other slow-eluting hemoglobin variants such as Hb C (West African and U.S. Negroes) and Hb E (Southeast Asians) would result in similar errors. Recently, Spicer et al. (4) have shown that incubation of cord blood (about 90% Hb F) and Hb SC with 250 mmol/L glucose for 12 h produced minor bands on isoelectric focusing, considered to represent glycosylation of hemoglobins F, S, and C.

The reverse situation occurred when a diabetic of Greek extraction was re-
ported, from another laboratory, to have 19.2% glycohemoglobin by short-column elution of fast-moving hemoglobins (Isolab, Akron, OH 44321), and 8.6% by isoelectric focusing and 10.3% by color-

imetric assay. The discrepancy was re-

solved, when it was recollected that the subject was a case of hereditary persis-
tence of fetal hemoglobin (HPFH) with 18% Hb F (discovered in 1972 when Head, Human Biochemical Genetics Unit, South African Institute for Med-
cal Research, Johannesburg). In the short-column resin method, Hb F co-

 chromatographs with fast-moving he-

moblobins, including those glycosylated, and will thus artefactually increase the calculated glycohemoglobin. Normally, fetal hemoglobin approximates 0.5–0.8% of total hemoglobin in children and adults; it produces a minor error in the fast-column estimation, approaching a 10% increase in healthy subjects and 5% or less in diabetics. HPFH represents the extreme effect of the presence of Hb F, since it may be present at 4 to 40% (average 20%) in the heterozygous car-
rier (Negro and Mediterranean peoples).

It should be kept in mind that increased Hb F occurs in pernicious anemia, thalassemias, hereditary spherocytosis, sickle cell anemia, some leukemias, and feto-maternal perfusion.

In addition to hemoglobin variants producing systematic errors in chromo-
matographic determinations (short column) for fast-moving hemoglobins, lactescence (5), resulting from hyperli-
pemia, a prominent feature in diabetic keto-acidosis, and preparative faults (presence of erythrocyte ghosts and stroma, and hemoglobin breakdown products) produce a problem in the estimation. Essentially, application of anticoagulated whole blood to the col-
umn is to be avoided. Erythrocytes from a fresh specimen should be washed free of plasma with 10 g/L saline, extracted with tolue

  ne or carbon tetrachloride, and centrifuged, and a Millipore-filtered (0.45 μm; Millipore Corp., Bedford, MA 01730) or dialyzed hemolysate of fixed volume and hemoglobin concentration applied to the column. Elution buffers and the column should be kept at a constant temperature, or a temperature correction factor should be used. Estima-
tion as cyanmethemoglobin, A540 nm, is superior to other methods of Hb de-
termination.

Diabetics are particularly prone to a wide variety of infections, certain of which are associated with a hemolytic anemia of some severity. The result is a circulating population of relatively young erythrocytes. Because the for-
mation of the irreversible ketoamine link of glucose with hemoglobin is a comparatively slow process, the pro-
portion of glycosylated hemoglobin in such circumstances will not reflect the degree of efficacy in metabolic control. In three nondiabetic individuals with hemolytic anemia or chronic blood loss, fractionation of erythrocyte samples on the basis of increasing density of the erythrocyte population with age (6), revealed 1.1, and 5% glycohemoglobin in the top fraction (young erythrocytes); 4, 6, and 6% in the bottom fraction (mature erythrocytes); with 2.0, 2.8, and 3.4% for the total erythrocyte popula-
tion. In a diabetic with a 12% reticulo-
cytosis, total glycohemoglobin was 5.8%, compared with 3.1% for the young erythrocyte component and 12.2% for the fraction of mature erythrocytes. Reliance on the assay value for the total

References
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("Vacutainer", "SMA II", and "oca" are registered trademarks of Becton-Dickinson, Technicon Instruments, and Du Pont In-

struments, respectively.)

This letter was referred for comment to Becton Dickinson and Co. For the four analytes for which Dr. Steindel
found differences, they also found “clinically insignificant” differences. They comment farther: "We also ex-

tracted the contents of the tube with distilled water and determined calcium and LDH. The range of calcium ex-

tracted from the tube measured by atomic absorption [spectroscopy] was 0.4 to 0.5 mg/L, which is of negligible

level to introduce any contamination. Levels of lactate dehydrogenase assayed with a centrifugal analyzer ranged from 1 to 2 U/L with the distilled water ex-
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tracted, similar to levels obtained by extracting the contents of a non-additive tube. Levels of potassium and chloride are too low to measure with the distilled water extract. Therefore, it is our opinion that the thrombin tube is suitable for stat blood collection for routine clinical chemistry procedures.”
erythrocyte population would have given, in this situation, a false idea of the effectiveness of diabetic control.

References

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Collection and Storage of Serum Lactic Acid Samples at Room Temperature without Deproteinization

To the Editors:
The role of lactic acid (lactate) as a frequent cause of acute metabolic acidosis is well established. The diagnosis of lactic acidosis should be considered whenever there is an increased "anion gap" between serum electrolytes that cannot be explained by uremia or a strongly positive test for plasma ketones (1).

Lactic acid methodology has improved considerably since the first automated assay was described in 1972 (2); with development of a kinetic enzymatic assay, analytical time has been reduced to less than 4 min (3, 4).

Although these improvements have made plasma lactic acid measurements more accessible for care of acutely ill patients, the collection, transport, and storage of blood samples remain major factors in limiting the use of the assay in many emergency clinical situations. As soon as blood is withdrawn from the body, lactic acid levels increase rapidly, as much as 70% in 30 min at room temperature (5). Numerous attempts have been made to retard or prevent this increase before analysis (6–10).

We compare lactic acid concentrations in plasma samples collected in sodium heparin, with strict attention to temperature and separation conditions, and values in samples collected in 0.5 g/L sodium iodoacetate solution stored at room temperature for various intervals before separation. Free-flowing venous blood was collected in either 10-mL heparinized tubes (143 USP units of Na heparin, "Monoject"; Sherwood Medical Industries, St. Louis, MO 63103) or 7-mL Na iodoacetate tubes (3.5 mg of Na iodoacetate; Kimble-Terumo, Elkhorn, MD 21921) from nonfasting, apparently healthy volunteers. We collected blood in two heparinized tubes (A and B) and two iodoacetate tubes (C and D) for each subject. One heparinized tube (A) was kept at 4 °C for 10 min before collection; the other tubes were at room temperature.

After collection of the blood, the two heparinized tubes and one iodoacetate tube (C) were given identical treatment: immediate positioning in iced water (4 °C), separation of plasma within 30 min of collection in a refrigerated centrifuge, and storage of the plasma in sealed tubes at 4 °C until batch analysis. The second iodoacetate tube (D) was stored and plasma separated at room temperature within 30 min after collection of the blood, and the resulting serum held at 4 °C until batch analysis. The lactate values were as follows (mmol/L, mean ± SD, n = 8): Tube A, 2.3 ± 0.9; Tube B, 2.1 ± 0.7; Tube C, 1.5 ± 0.9; Tube D, 2.0 ± 0.9.

In a different study, blood was collected in heparinized tubes, previously at room temperature, and then placed in ice water (4 °C) for 120 min before plasma separation, as described previously. In addition, four iodoacetate tubes were collected from each subject. These samples were stored at room temperature for periods of 30, 60, 90, and 120 min before serum separation. The serum was then kept at 4 °C until batch analysis. We assayed for serum and plasma lactate in duplicate with the Centrifichem (Union Carbide Corp., Rye, NY 10580), using a modified method of Pesce et al. (3). The assay, run as an endpoint analysis at 30 °C with a 4-min reaction time, was calibrated with use of a 2.5 mmol/L aqueous lactate standard. The reaction gave a linear response between a lactate concentration of 0.02 and 5 mmol/L.

We assessed within-run and between-run, within-day precision by using reconstituted lyophilized control sera (Hyland, Div. of Travenol Laboratories, Inc., Costa Mesa, CA 92626) and a selected patient's sample. The within-run and between-run coefficients of variation (CV) of the reconstituted control sera were 0.7% (n = 20) and 2.7% (n = 10), respectively, at lactate concentrations of 10.4 and 1.8 mmol/L. The between-run CV for the patient's sample was 10.5% (n = 10).

Values for samples collected in the heparinized tubes with and without precollection cooling to 4 °C showed no statistically significant difference. Similar results were obtained for statistical difference between values for samples collected in heparin and iodoacetate tubes under identical conditions, nor between those samples collected in iodoacetate tubes and stored and separated at either 4 °C or room temperature. Regression analysis of 30 lactate values of patients' samples collected in heparin (2.5 ± 0.9 mmol/L) (2.3 ± 1.0 mmol/L) showed a close correlation (r = 0.944) between the two methods of collection.

No significant difference was seen in serum lactate between samples collected in iodoacetate and stored at room temperature for 30, 60, 90, or 120 min before centrifugation. Serum lactate after storage in iodoacetate for 30 min was 2.4 ± 0.7 mmol/L; after 60 min, 2.5 ± 0.8 mmol/L; after 90 min, 2.5 ± 0.8 mmol/L; and after 120 min, 2.4 ± 0.9 mmol/L. Samples collected in heparin at the same time and stored in ice water (4 °C) for 120 min before separation of cells and plasma showed a slightly higher (p < 0.05) lactate value (2.9 ± 0.6 mmol/L) than the samples in iodoacetate.

The search for a suitable system to prevent glycolysis and an increase in lactic acid content of drawn blood has continued for almost 30 years since Boeding and Goldfarb (6) recommended a combination of 10 g of Na fluoride and 10 g of Na iodoacetate per liter of blood as a preservative. During that time, despite a number of detailed studies (3, 6, 7) involving various concentrations and combinations of Na fluoride, Na iodoacetate, heparin, and potassium oxalate, no satisfactory reagent has been reported that did not require immediate storage at 4 °C. Even with immediate storage of heparinized samples at 4 °C, a significant increase in lactic acid has been reported after 1 h (3), and was also seen in this study after 2 h at 4 °C. The alternative methods of immediate deproteinization with either trichloroacetic acid or sodium hydroxide–zinc sulfate are cumbersome and require analytical skill (3, 9).

In a study by Savory and Kaplan (7), use of sodium iodoacetate in a concentration of 10 g/L of blood decreased lactic acid by 7–11%. They could not explain the reduction, which was seen only with iodoacetate added to whole blood and not aqueous standard solutions. A recent article by Stascopo et al. (11) demonstrated that in diabetic patients administered 304 g of sodium dichloroacetate, plasma lactate concentrations decreased 73% from baseline values. The dichloroacetate is believed