Rapid Method for Eliminating Labile Glycosylated Hemoglobin from the Assay for Hemoglobin A$_1$

David M. Nathan, Eric Avezzano, and John L. Palmer

The irreversible formation of stable glycosylated hemoglobin proceeds through a labile intermediate that is indistinguishable, by most methods, from the stable glycosylated product. The inclusion of the labile intermediate, which changes acutely with acute blood glucose changes, detracts from the utility of the assay as an index of chronic glucose concentration. We have developed a rapid, reliable chemical method for eliminating the labile intermediate: a 30-min incubation of whole-blood samples with semicarbazide (30 mmol/L) and aniline (12 mmol/L) at pH 5 and 38 °C. The semicarbazide serves as a glucose trap; the transfer of glucose from the labile glycosylated hemoglobin to the semicarbazide is catalyzed by the acidic pH and aniline. This treatment is effective in the three most commonly used assays: “high-performance” liquid chromatography, electrophoresis, and a minicolumn kit.

Additional Keyphrases: diabetes - glycohemoglobin - chromatography, liquid - electrophoresis - “kit” methods

The measurement of glycosylated proteins in patients with diabetes mellitus is an important index of chronic metabolic control and has been proposed as a pathogenetic model for diabetic complications (1–3). The reaction and kinetics of formation of glycosylated hemoglobin (hemoglobin A$_1$, hemoglobin A$_1c$, “fast hemoglobin”), the most commonly used index of chronic metabolic control, have been studied in detail (4). The slow, irreversible, nonenzymatic formation of the ketoamine, hemoglobin A$_1c$, proceeds through a labile aldimine or Schiff base (4). The formation of the aldimine is reversible and 60-fold faster than the rate-limiting formation of the final product. Although the measurement of hemoglobin A$_1$ reflects the mean blood glucose concentration integrated over the preceding two months, the inclusion of the labile fraction in the measurement reflects acute blood glucose fluctuations and detracts from the assay as an index of chronic metabolic control (5–7). We have previously demonstrated that liquid-chromatographic and electrophoretic methods of measuring glycosylated hemoglobin concentration do not distinguish between hemoglobin A$_1$ and the labile fractions (7). Therefore, elimination of the labile fraction is necessary to preserve the utility of the assay. Incubation of erythrocyte samples in isotonic saline (7, 8) is time consuming (5 h at 38 °C or 14 h at room temperature) and can produce hemolysis. In this paper we present a chemical means of removing the labile glycosylated fraction that is fast and convenient, does not cause hemolysis, and does not interfere with the measurement of the ketoamine adduct.

Materials and Methods

[1-C$^{14}$]Glucose and Aquasol were purchased from New England Nuclear, Boston, MA 02118; semicarbazide - HCl from Eastman Kodak Co., Rochester, NY 14650; hydroxylamine - HCl and methoxyamine - HCl from Aldrich Chemical Co., Milwaukee, WI 53233; and aniline free base and p-anisidine from Sigma Chemical Co., St. Louis, MO 63178. Semicarbazide - HCl was recrystallized from aqueous ethanol (water/ethanol, 10/90 by vol) before use.

Hemoglobin A$_1$ concentrations were determined with three separate methods. For the “high performance” liquid-chromatographic (HPLC) method adapted from Cole et al. (9) we used a cation-exchange resin column, a gradient liquid chromatograph (Model 332; Altex Scientific Inc., Berkeley CA 94710), and a spectrophotometer (Model 100-40; Hitachi Instruments, Tokyo, Japan). The elution buffers were as previously published and temperature was maintained at 25 °C. A flow-through radioactivity monitor (RAM 7500; Packard Instrument Co., Downers Grove, IL 60515) was used to measure [14C]glucose incorporation.

Electrophoresis on agar gels was performed in an electrophoresis chamber (Corning ACI, Corning Medical and Scientific, Palo Alto, CA 94306), and the bands were quantitated with a Corning 720 densitometer, according to the manufacturer’s specifications.

We also used a commercially available “minicolumn” assay for hemoglobin A$_1$ (Quik-SEP; Isolab, Inc., Akron, OH 44321), according to the manufacturer’s directions at 23 °C. The principle of separation of the glycosylated from nonglycosylated hemoglobin fractions is the same as the HPLC method and relies on the charge difference between the glycosylated and nonglycosylated fractions. The coefficients of variation for each of the three assays are listed in Table 1.

To prepare “artificially” glycosylated samples, whole blood from research volunteers with no history of diabetes was collected into EDTA-containing tubes. As published previously, increased concentrations of the labile glycosylated fraction were generated by incubating erythrocytes in 10 g/L glucose at 37 °C for 3 h (7). After glucose incubation, the erythrocytes were centrifuged (1000 × g, 5 min, 4 °C) and the packed erythrocytes were either hemolyzed directly and assayed or (a) incubated in 10 volumes of isotonic saline for 14 h at 22 °C (saline incubation) or (b) incubated for 30 min at 38 °C, pH 5.0, with 1.5 volumes of, per liter, 50 mmol of semicarbazide.

Table 1. Coefficients of Variation for the Hemoglobin A$_1$ Assays

<table>
<thead>
<tr>
<th>Method</th>
<th>Control</th>
<th>Diabetes’ samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>2.0%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>5.6%</td>
<td>2.4%</td>
</tr>
<tr>
<td>Minicolumn</td>
<td>7.8%</td>
<td>4.6%</td>
</tr>
</tbody>
</table>

* Based on five to eight repeated assays.

---

* Diabetes Unit and Medical Services, Massachusetts General Hospital, and Department of Medicine, Harvard Medical School, Boston, MA 02114.

Received Oct. 12, 1981; accepted Dec. 15, 1981.
20 mmol of aniline, and 90 mmol of NaCl (final concentration, 30 mmol of semicarbazide and 12 mmol of aniline per liter) (chemically treated). The saline-incubated and chemically treated samples were then centrifuged, and the erythrocytes hemolyzed and assayed.

A different means of hemolyzing the packed erythrocytes was used for each assay method. For the HPLC method, three volumes of de-ionized water and then three volumes of toluene were vigorously mixed with the packed erythrocytes. After the hemolysate was separated from glycolipids by centrifugation at 1000 × g (30 min, 4 °C) we diluted the hemolysate in 80 mmol/L phosphate buffer, pH 6.82 (prepared from NaH2PO4 and Na2HPO4), containing 16 mmol of KCN per liter, and assayed 10 μg of hemoglobin. Preparation of hemolysate for the electrophoretic method was according to the manufacturer's directions: add five volumes of hemolyzing buffer (saponin, 1 g/L in water) to the packed erythrocytes and assay 1 μL. Hemolysate for the minicolumn method was also prepared according to the manufacturer's directions; whole blood was mixed with four volumes of hemolyzing buffer (surfactant/water, 1/1000 by vol).

To further examine the effect of semicarbazide/aniline treatment on the reduction of labile hemoglobin, packed erythrocytes (6 μL) obtained from a nondiabetic volunteer were incubated for 3 h at pH 7.0, 38 °C with [1-14C]glucose (specific activity 55.8 Ci/mol) and 4 μL of NaPO4 buffer, 10 mmol/L, to make an isotonic solution containing 16 g of [14C]glucose per liter. The ethanol/water solution was evaporated from the [14C]glucose with a gentle nitrogen stream before making the isotonic solution. After incubation, 100 μL of phosphate-buffered saline (per liter, 10 mmol of NaPO4, 140 mmol of NaCl, pH 7) was added and the solution was centrifuged (1000 × g, 4 min, 4 °C). The supernatant liquid was aspirated, the erythrocytes were suspended in 100 μL of phosphate-buffered saline, and the erythrocyte suspension was divided into two equal aliquots. The erythrocytes were centrifuged again, and one aliquot of packed cells was immediately prepared for assay by electrophoresis. The other aliquot was chemically treated; then the erythrocytes were collected by centrifugation, hemolysed, and assayed by electrophoresis. Equal amounts of hemoglobin (assessed by absorbance at 410 nm) from the untreated and semicarbazide/aniline-treated aliquots were assayed. The incorporation of [14C]glucose into the hemoglobin fractions was determined by cutting 1-mm fractions from the electrophoresis agar gels and scintillation counting in 3 mL of Aquasol. Greater than 95% of the total [14C]glucose counts on the gel were in the hemoglobin A1 peaks.

Patients' samples were whole blood collected with EDTA from 31 diabetic patients of the Diabetes Clinic of the Massachussetts General Hospital. Each sample was divided; one-half was saline-incubated, the other half chemically treated.

**Results**

Incubation of erythrocytes in increasing concentrations of glucose produced an increase in labile glycosylated hemoglobin proportional to the glucose concentration as previously demonstrated (7); but saline incubation effectively reduced the artificially increased hemoglobin A1 concentration to baseline in all assays (Figure 1).

Incubation of glucose-treated erythrocytes with semicarbazide in concentrations up to a final concentration of 36 mmol/L (30 min, 38 °C) decreased the labile fraction, but not back to baseline values. Increasing the incubation period to 60 min decreased the labile fraction further, but still not to baseline concentrations. The addition of aniline at pH 5 to the semicarbazide further decreased the labile hemoglobin, but aniline concentrations greater than 24 mmol/L caused hemolysis. A dose–response curve of semicarbazide in the presence of aniline, 12 mmol/L, showed that 30 mmol/L semicarbazide eliminated the labile fraction effectively without causing hemolysis during 30-min incubation (Figure 2). The semicarbazide/aniline solution was most effective at pH 5 (Figure 3); at pH 4.5 or less, hemolysis occurred.

We also investigated the temperature dependence of the semicarbazide/aniline treatment. Incubation of erythrocytes with 1.5 volumes of semicarbazide, 50 mmol/L, aniline, 20 mmol/L, and NaCl, 90 mmol/L, pH 5, for 30 min eliminated the labile fraction at 38 °C but not at 22 or 4 °C (data not shown). The chemical treatment of erythrocyte samples was as effective as overnight saline incubation in eliminating the labile hemoglobin (Figure 1).
Fig. 3. Effect of pH on the reduction of labile hemoglobin, as measured by electrophoresis.
Solutions of, per liter, 50 mmol of semicarbazide and 20 mmol of aniline were buffered with aniline for pH 4, 4.5, and 5; with 2-(4-morpholino)ethanesulfonic acid, 10 mmol/L, for pH 5.5, 6, and 6.5; and with 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 10 mmol/L, for pH 7 and 7.5. WB indicates HbA1 concentration of whole blood.

To determine whether semicarbazide/aniline treatment specifically removed labile glucose, we incubated erythrocytes with [14C]glucose (3 h, 38 °C) (see Methods). Ninety-five percent of the incorporated [14C]glucose was in the hemoglobin A1 peak. Semicarbazide/aniline treatment of the 14C-labeled hemoglobin decreased the amount of glycosylated hemoglobin (measured electrophoretically) and the incorporated [14C]glucose to the same extent (Figure 4).

The correlation of the hemoglobin A1c results for patients' samples treated in parallel with saline incubation and semicarbazide/aniline solution was excellent (Figure 5).

Discussion

The glycosylated hemoglobin assay is a reliable index of blood glucose concentrations integrated over the two months prior to the assay (7, 8). Unfortunately, all of the assay methods except the colorimetric method of Fluckinger and Winterhalter (10) fail to distinguish the labile from stable products. Therefore, samples must be pretreated to preserve the utility of the assay as an index of chronic metabolic control. The pretreatment method used in previous studies, incubation of erythrocytes in isotonic saline, is effective but time consuming and can lead to sample hemolysis and spurious results.

We eliminated the labile fraction by treating erythrocytes with semicarbazide and aniline, pH 5.0, for 30 min at 38 °C. Semicarbazide, present in molar excess of glucose, acts both as a glucose trap and, we believe, as a nucleophile in a trans Schiffification reaction. Other a-effect amines such as hydrazine, hydroxylamine, and thiosemi carbazide, which form stable glucose imines, have a similar effect and can be used in this procedure.

Treatment of erythrocytes with semicarbazide alone, however, did not completely eliminate the labile fraction within a 30-min incubation time. The aminolysis of amines reportedly is subject to general-base, specific-acid catalysis (11, 12). Of note, catalysis by aniline, substituted anilines, and secondary amines is considerably better than would be expected for general bases of their pK a. This phenomenon has been investigated in detail (11, 12) and reportedly involves covalent catalysis by aniline, in which a transient, unstable aniline imine is rapidly formed.

Fig. 5. Comparison of semicarbazide/aniline (y) and saline (x) treatment of samples from diabetic patients assayed with HPLC (r = 0.948, p < 0.001)

Portions of EDTA-anticoagulated blood samples obtained from 31 diabetic patients were treated with 10 volumes of isotonic saline (4 h, 22 °C) or with 1.5 volume of semicarbazide/aniline solution (see text).
We used these known features of amine breakdown to develop the semicarbazide/aniline treatment. Specific-acid catalysis is provided by lowering the pH of the pretreatment solution to pH 5.0. As shown in Figure 3, increasing proton concentration increases the efficiency of our pretreatment procedure, providing evidence that specific-acid catalysis is involved in the aminolysis of hemoglobin–glucose imines. The use of pH below 5 should be avoided, however, as this leads to considerable hemolysis. The addition of aniline to pH 5 semicarbazide greatly increased the rate of removal of labile hemoglobin. An aniline dose–response curve in the presence of 36 mmol of semicarbazide per liter (data not shown) indicated that the effect occurred with as little as 3.6 mmol of aniline per liter and was maximum at 8 mmol of aniline per liter.

A growing body of evidence supports the hypothesis that the vascular complications of diabetes are a direct result of the chronic increase of blood glucose concentration. Excessive glycosylation of a variety of blood and membrane proteins in diabetes has been demonstrated, and it is likely that the same two-step reaction that forms hemoglobin A₁ is involved. The rapid and reliable chemical means we have developed for removing the labile glycosylated hemoglobin will aid the further study of protein glycosylation as well as simplify and preserve the utility of the hemoglobin A₁c assay in diabetes.

We are grateful to Corning Medical and Scientific, Medfield, MA, for their support and to Mrs. C. Bovest for her expert help in preparing the manuscript. Dr. Nathan is the Capps Scholar of Harvard University and Dr. Palmer the recipient of a New Investigator Research Award from the National Institutes of Health AM 28041.

References