Simple Method for Estimating Glycosylated Hemoglobin, and Its Application to Evaluation of Diabetic Patients

Jingshyen Chou, C. Andrew Robinson, Jr., and Abraham L. Siegel

Recent reports have suggested that determination of glycosylated hemoglobin may serve as a clinical aid for long-term blood glucose control in diabetes mellitus. We describe a simple procedure for measuring it by ion-exchange chromatography. Hemolysates were subjected to Bio-Rex 70 chromatographic separation on small columns. Percentages in the normal group ranged from 4.7 to 8.8% of total hemoglobin; the mean ± standard error was 6.61 ± 0.31%. Values in the diabetic group ranged from 6.9 to 17.4%; the mean was 10.63 ± 0.34. Plasma glucose concentrations after fasting, plotted vs. the percent of glycosylated hemoglobin, revealed a linear relationship at normal or moderately high glucose concentrations. However, the values for glycosylated hemoglobin approached a plateau with grossly higher plasma glucose concentrations after fasting. Our results support the view that, due to its long half-life, the estimation of glycosylated hemoglobin reflects the integrated glucose concentrations to which the erythrocytes have been previously exposed.

Additional Keyphrases: methods for the small laboratory • control of the diabetic patient • clinical aids • normal values • protein glycosylation

Hemoglobin A\textsubscript{1c} (HbA\textsubscript{1c}), a minor component of normal human hemoglobins, may show a twofold increase in diabetic patients (1). According to a study of its biosynthesis, the concentration of HbA\textsubscript{1c} in the diabetic patient should be proportional to the time-averaged concentration of glucose within the erythrocytes (2, 3). Data on the concentration of HbA\textsubscript{1c} could provide the clinician with information concerning the degree of control of the diabetic patient. It has been shown by column chromatography that both the individual fast-moving fractions and the total percentage of glycosylated hemoglobin were correlated with the degree to which blood glucose was under control (4, 5). Of the various methods proposed for estimating the glycosylated hemoglobins, the one most frequently cited is that of Trivelli et al. (6), which calls for an overnight dialysis of hemolysates before the column-chromatographic steps and requires the collection of both the glycosylated hemoglobin and the hemoglobin A fraction. This process is time-consuming and required a 6-hour equilibration with buffer solution. The estimation of the glycosylated hemoglobins by high-performance liquid chromatography has also been proposed (7), but this method offers no significant time-saving over the proposed procedure. In this paper we describe a method modified from that of Trivelli et al. (6) and Knoch and Lehmann (8) that requires only inexpensive equipment, occupies little space, and appears to be more appropriate for the small hospital laboratory.

Materials and Methods

Reagents

Isotonic saline solution: 9 g of NaCl per liter.
Buffer, pH 6.7 (9): Dissolve 4.59 g of monosodium phosphate monohydrate, 1.18 g of disodium phosphate, and 0.65 g of potassium cyanide in 1 liter of distilled water.
Buffer, pH 6.4 (9): Dissolve 6.52 g of anhydrous disodium phosphate and 14.35 g of monosodium phosphate monohydrate in 1 liter of distilled water.
Bio-Rex 70, 200-400 mesh, sodium form, from Bio-Rad Laboratories, Richmond, Calif. 94801.

Experimental Arrangements

Apparatus: Beckman Model 25 Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).
Column preparation: Prepare 10-ml columns in 20-ml syringe barrels, using a slurry of the resin equilibrated in pH 6.7 buffer. Close the top of the column with a rubber stopper, pierced with a No. 22 gauge needle. Bend the needle point upward so that the solution coming through the needle will not disturb the surface of the resin bed.

Chromatographic system: (see Figure 1). With transmission tubing, connect each of three columns to a Technicon three-way glass cactus fitting. Connect the delivery end of the glass fitting to a three-way valve which can select eluent from a reservoir of either buffer placed on the shelf 60 cm above the columns.

Procedure

Collect blood samples in tubes with ethylenediaminetetraacetic acid anticoagulant. Wash the erythrocytes with isotonic saline two times after removing the plasma by centrifugation. Hemolyze the washed cells with pH 6.7 buffer (about one volume of cells to six volumes of buffer). Centrifuge to remove all traces of debris. The final hemoglobin concentration in the hemolysate should be 15 to 30 g/liter. Deliver a 0.5-ml aliquot of hemolysate to the top of the column as described by Schneek and Schroeder (9) without disturbing the resin layer. Open the valve under the column and start collecting the eluate in a 100-ml volumetric flask. When the hemolysate has drained into the column, carefully add 4 ml of pH 6.7 buffer to the top of the resin bed with a 10-ml syringe fitted with a fine tube. Place a rubber stopper with the delivery
needle on the column and open the valve connecting the column with the pH 6.7 buffer reservoir. Collect 100 ml of eluate and mix well. Measure the absorbance (A) of the eluate at 540 nm (fast fraction). To obtain the A of the total hemoglobin, put a 0.5-ml aliquot of the original hemolysate into a 100-ml volumetric flask, dilute to volume with pH 6.7 buffer, and measure the absorbance of the solution at 540 nm.

Calculation: The percentage of glycosylated hemoglobin is calculated as follows:

\[
\text{GHB} = \left( \frac{A_{\text{fast fraction}}}{A_{\text{total hemoglobin}}} \right) \times 100\%
\]

Note: The following procedure is recommended for the rapid re-equilibration of the columns: Aspirate the supernate from the resin. Add about 4 ml of pH 6.4 buffer to the column. Use a medicine dropper to stir the top layer of resin. The hemoglobin A which remains in the top layer of the column will be dissolved in the solution. Aspirate and discard this solution. Repeat this step several times. The column can now undergo re-equilibration with pH 6.7 buffer from the reservoir. (This process is usually completed in 30 min.)

Patients Studied

We studied a group of 52 male diabetic patients, 40 to 80 years of age. Forty-two individuals in this group were being treated with insulin. We also studied 18 normal men, in the same age bracket. The normal patients were selected on the basis of having no past history of diabetes and having normal plasma glucose concentrations when measured after a short fast.

Results

Table 1 shows our results. A regression analysis was performed on the data and revealed the relationship between glycosylated hemoglobins (GHB) and fasting plasma glucose concentrations (FPG) illustrated in Figure 2. This curve is represented by GHB = ln FPG × 4.9 - 14.96, r = 0.75, and indicates that in the region of normal or high-normal FPG, the percent of GHB varies linearly; but at high glucose values, a plateau is approached.

We examined the stability of GHB in stored hemolysates and found it to be stable in the pH 6.7 buffer at 4°C for at least a month. Precision (CV%) was found to be 2.3% (n = 9) within-run and 3.4% (n = 7) run-to-run.

Discussion

Our method for determining the percentage of GHB relative to the total hemoglobin is reproducible and simple to perform. The entire procedure takes 40 min for each run and 30 min for re-equilibration. The relatively shortened analysis time is the result of eliminating the overnight dialysis, the elution of hemoglobin A with a second buffer, and shortening the time for re-equilibration. The procedure does not require a continuous-flow analyzer (4) or a "high-performance" liquid-chromatographic system (7). The set-up described can be run with a group of 12 to 24 columns or for an individual column by use of three-way valves. Although the flow rate of each column shows some variation, the process can be easily monitored visually. Table 2 compares the results of our method to those of others (4, 6–8).

Because a standard for GHB is not yet available, the absolute accuracy of this procedure cannot be properly evaluated. However, the mean and range of values of GHB in 18 normal and 52 diabetic groups reported here are comparable to the

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Table 1. Comparison of Two Groups (Diabetics and Non-Diabetics) with Respect to GHB and FPG.

<table>
<thead>
<tr>
<th>GHB, %</th>
<th>Diabetics</th>
<th>Normal</th>
<th>FPG, g/liter</th>
<th>Diabetics</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SE</td>
<td>10.83 ± 0.34</td>
<td>6.61 ± 0.31</td>
<td>1.94 ± 0.11</td>
<td>0.99 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>No. samples</td>
<td>52</td>
<td>18</td>
<td>51</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>t-test</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

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Fig. 1. Chromatographic system; columns prepared in 20-ml syringe barrels fitted with stop-cocks
One reservoir contained GHB elution buffer. The other reservoir was used to re-equilibrate the column

Fig. 2. Correlation between plasma glucose concentrations (fasting subjects) and percent GHB
Correlation coefficient r = 0.75; GHB = 4.9 ln FPG - 14.96, --- = theoretical line; A = actual values obtained
results obtained by others. The plateauing effect of GHB at higher FPG has also been observed by Graf et al. (10), who suggested that GHB behaves as a saturable system. If this is the case, then a more appropriate expression to represent this phenomenon might be:

\[
\text{GHB} = 15.0 - 739.7 \frac{1}{\text{FPG}}; \quad r = 0.74.
\]

Although FPG appears to be a major predictor of GHB, the former is not always reliable. In several cases in our study, only minimum changes in GHB were observed, in contrast to marked fluctuations of serial FPG values. This is in keeping with the view that the GHB provides an index of the patient’s average blood glucose over an extended time period.

The value of close blood glucose control in avoiding the complications in diabetic disease has been controversial (3). Engerman (11) has found that diabetic dogs under good control exhibited a significantly reduced incidence and severity of microvascular lesions. If the main cause of the complications of diabetes is associated with prolonged periods of hyperglycemia, the periodic determination of GHB could provide the clinician with an estimation of the degree of the control of the disease.

The information on GHB may also provide a model for the possible glycosylation of other proteins, the function of which may be altered, such as insulin receptors, membrane proteins, enzymes, and structures in nervous tissue.

We thank Dr. Seng-Jaw Song for providing assistance in statistical analysis.

**References**