Measurement of Glycosylated Hemoglobins Using Boronate Affinity Chromatography

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ABSTRACT

A boronate affinity column method for the measurement of glycosylated hemoglobins was evaluated. In the procedure the glycosylated hemoglobins were bound by immobilized boronic acid to separate them from nonglycosylated hemoglobins. Elution of bound glycosylated hemoglobins was carried out with sorbitol buffer, and the absorbance was read at 414 nm. The method was linear to a glycosylated hemoglobin concentration of at least 20 percent. The precision of the method ranged from 1.2 to 2.8 percent (C.V.) within-run, and 3.4 to 5.3 percent day-to-day. The reference interval was 4.8 to 6.4 percent. The method correlated with a cation exchange resin mini-column method ($r = 0.94$) and a colorimetric method ($r = 0.93$) but results from the boronate affinity method were higher in diabetic patients. The measured glycosylated hemoglobin was significantly correlated with estimated one-day-mean plasma glucose in diabetic patients ($r = 0.54$, $n = 52$, $p < 0.002$). The affinity method provides an attractive alternative to earlier methods for measuring glycosylated hemoglobins.

Introduction

The measurement of glycosylated hemoglobins is a well established tool for monitoring the degree of metabolic control in diabetes mellitus. Major problems, however, have been encountered with the various techniques used to perform the assay. Standardization has been difficult and quality control materials have not been satisfactory. The tech-
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niques of electrophoresis, isoelectric focusing, radioimmunoassay, spectrophotometry, high performance liquid chromatography, and cation exchange chromatography all have been proposed. The most widely used of these assay procedures has been cation exchange chromatography, especially using mini-columns which are now available commercially. Schellekens and co-workers have described the many variables affecting the cation exchange chromatographic methods. These workers emphasize the difficulties in obtaining consistent results using the mini-columns. Temperature control, column load, pH, ionic strength, and sample storage all contribute to variations in the analysis.

Recently, an affinity chromatographic method has been reported for the quantitation of glycosylated hemoglobins using a boronic acid affinity support. The principle of the assay is that the co-planar cis-diol groups of the ketoamine moiety of glycosylated hemoglobins (such as hemoglobins A\textsubscript{le}, A\textsubscript{ld}) bind to immobilized boronic acid. Non-glycosylated hemoglobins, such as hemoglobins A, F, S, or C, do not bind and are eluted in the column buffer. The glycosylated hemoglobins can be eluted by adding sorbitol to the buffer or by lowering the pH of the buffer. The glycosylated and non-glycosylated hemoglobins can then be quantitated by spectrophotometric measurement.

This general method has been investigated by several workers using different gels and procedures. In our laboratory, an investigation of this technique was initiated using a system which has been recently introduced commercially. The objectives of this study were to perform a technical evaluation of the technique and a long-term evaluation in a clinical laboratory setting, and to establish the relationship of the measured glycosylated hemoglobin to indicators of glucose control. The results of this evaluation and an 18-month clinical laboratory experience are reported.

Materials and Methods

Blood Specimens

Whole blood was collected into EDTA-containing vacutainers from laboratory employees and from known diabetic patients. The reference interval was based on study of 142 medical students, 109 males and 33 females, with a mean age of 25.3 years. The relationship of glycosylated hemoglobin and mean plasma glucose was estimated in 52 insulin-dependent diabetic patients about to begin a home glucose monitoring program. The mean plasma glucose in these patients was estimated from five measurements performed on each patient during a single day. Whole blood specimens were stored at 4°C pending centrifugation and hemolysate preparation. Hemolysates were prepared from the red blood cells within 24 hours of collection and were stored at −70°C if not analyzed immediately.

Reagents

Columns and reagents for the affinity chromatographic method were obtained commercially. The prepacked columns contained 1.0 ml of gel which consisted of m-aminophenyl-boronic acid immobilized on a support of cross-linked six percent beaded agarose. The buffer for elution of the non-glycosylated hemoglobins contained per liter, 250 mmol ammonium acetate, 50 mmol magnesium chloride and 0.2 g sodium azide, pH 8.0 (ammonium acetate buffer). A second buffer used to elute the boronic acid-bound glycosylated hemoglobins, contained per

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liter, 300 mmol sorbitol, 100 mmol tris (hydroxymethyl)aminomethane, and 0.2 g sodium azide, pH 8.5 (sorbitol buffer). Compositions of the gel and the buffers are similar to those described by Mallia et al.\textsuperscript{11} and to that reported by Klenk et al.\textsuperscript{10}

**Glycosylated Hemoglobin Procedure**

The procedure that follows was described previously.\textsuperscript{11} Hemolysates of blood samples were prepared by diluting packed red blood cells in 20 volumes of deionized water followed by centrifugation. Aliquots of two hemolysates were stored at $-70^\circ$C and used routinely as quality control samples. Hemolysate (100 $\mu$l) was applied to the columns which had been equilibrated with ammonium acetate buffer. Non-glycosylated hemoglobins were eluted with 20 ml of ammonium acetate buffer dispensed from a semi-automated dispenser;\textsuperscript{‡} in four 5-ml aliquots; the absorbance of the eluate was measured at 414 nm in a spectrophotometer.\textsuperscript{§} Glycosylated hemoglobins were eluted by adding five ml of sorbitol buffer and again measuring the absorbance at 414 nm. Control experiments indicated that the $A_{414}$ of hemolysate was identical in the two column buffers. Thus, glycosylated hemoglobins were calculated as a percentage of the total hemoglobins from the two absorbance measurements using the formula:

$$\text{Percent glycosylated hemoglobin} = \frac{5.0 \cdot A_{414}B}{20.1 \cdot (A_{414}NB + 5.0 \cdot A_{414}B)} \times 100$$

where: $A_{414}NB$ = Absorbance at 414 nm of the bound or glycosylated hemoglobin fraction. (Total volume = 5 ml).

**Other Methods**

Results of the new method were compared with both a cation exchange resin mini-column procedure (8) (Glycosylated Hemoglobin Quick Column Method, Helena Laboratories, Beaumont, TX 77704) and the thiobarbituric acid method described by Pecoraro et al.\textsuperscript{14}

Plasma glucose was measured, within 60 min of collecting fluoride-preserved samples, by a fixed-time kinetic method using glucose oxidase (Beckman Instruments, Fullerton, CA 92634).

**Results**

**Linearity**

The linearity of the method was assessed in two ways. First, a blood specimen containing 18.9 percent glycosylated hemoglobins was diluted with one containing only 4.8 percent glycosylated hemoglobins. The dilutions used were undiluted high sample, 2:1, 1:1, 1:2, and undiluted low sample. Five replicate analyses of each dilution indicated linearity of the method with respect to percentage of glycosylated hemoglobin (figure 1). In a second experiment, the method was found to be linear with respect to total hemoglobin concentration over at least a two-fold range (data not shown).

**Precision Studies on Fresh and Regenerated Columns**

The precision of the assay was determined by performing replicate analyses of hemolysates (stored at $-70^\circ$C) of a specimen obtained from a normal volunteer and one taken from a patient with

\[\text{\textcopyright Dispensette, Brinkman Instruments, Inc., Westbury, NY 11590.}\]

\[\text{\textsuperscript{§} Model 35, Beckman Instruments, Inc., Irvine, CA 92713.}\]
diabetes. These precision studies were performed on fresh columns and on columns regenerated with five ml 0.1N HCl followed by 0.001N HCl according to the manufacturer's instructions. The results of these studies are given in table I. Within-run precision and day-to-day precision over six months were excellent using fresh and once-generated columns.

**Correlation Studies**

Blood specimens \((n = 108)\) from 51 laboratory employees and 57 patients with diabetes were analyzed by both the boronate affinity method and cation exchange chromatography (figure 2). The affinity method was also compared with the thiobarbituric acid method\(^{14}\) in a separate study of 53 specimens from a different group of laboratory personnel and diabetics (figure 3). The correlation coefficients in these studies were 0.94 and 0.93, respectively.

**Interferences**

Paired blood samples were collected from eight volunteers in EDTA-containing vacutainers and in vacutainers containing NaF and potassium oxalate. Results using the latter samples were higher by 0.4 to 1.5 percent of total hemoglobin (mean, 1.0 percent).

Artificially increased glycosylated...
hemoglobin results were not seen in patients with heterozygous and homozygous atypical hemoglobins such as hemoglobins S and C. In one homozygous patient with hemoglobin SS and one patient with SC phenotype, the glycosylated hemoglobins were 2.8 percent and 3.1 percent, below the reference range and consistent with the expected shortened red cell survival times in these patients.

LOT-TO-LOT VARIABILITY

Columns from a 1981 lot were compared with columns from a 1982 lot. Results were slightly but significantly lower with the newer columns both for a high control (17.64 percent ± 0.50 percent vs. 16.57 percent ± 0.60 percent, p < 0.001) and for a low control (5.91 percent ± 0.22 percent vs. 5.56 percent ± 0.27 percent, p < 0.005).

REFERENCE INTERVAL

The reference interval for the boronate affinity method was estimated based upon analysis of blood from 142 apparently healthy medical students with fasting blood glucose concentrations ranging from 65 to 111 mg per dl (figure 4). The glycosylated hemoglobin values ranged from 4.6 percent to 6.8 percent and were significantly correlated with the fasting glucose concentrations (r = 0.22, p < 0.01). Because the distribution of glycosylated hemoglobin values for the medical students was found to be non-Gaussian by the Cramer-Von Mises W^2 statistic17 the 95 percent reference interval was estimated using a transformed-parametric approach3 and was found to be 4.8 percent to 6.4 percent.

CORRELATION OF GLYCOXYLATED HEMOGLOBIN AND PLASMA GLUCOSE

Glycosylated hemoglobin was measured in each of 43 insulin-dependent diabetic patients who were about to begin a program5 of home glucose monitoring. On the first day of each patient’s program, plasma glucose concentrations were measured at 0700, 1000, 1130, 1400, and 1600 hours. The mean of these
values was compared with the glycosylated hemoglobin result for the same day (figure 5). The correlation coefficient found \((r = 0.54)\) was slightly higher than the correlation \((0.50)\) of glycosylated hemoglobin with fasting plasma glucose in the same patients.

**Discussion**

The evaluation of the boronate affinity method described here demonstrates the performance characteristics of the technique. Linearity is achieved to at least 20 percent glycosylated hemoglobin and the coefficients of variation over six months were 3.4 percent to 5.4 percent for fresh and once-regenerated columns. Similar coefficients of variation have been noted with other control materials, each analyzed for periods of one to three months during the past 18 months. The method was highly correlated with both the cation exchange mini-column procedure and the thiobarbituric acid method. A factor in the higher results by the affinity method may be the difficulty of achieving complete separation of glycosylated and non-glycosylated hemoglobins using small ion-exchange columns.\(^{15}\) The method described here offers no improvement in convenience over the ion-exchange mini-column approach. Both procedures require several steps and cannot be automated. However, it has been found by us that certain steps in the boronate affinity method can be streamlined by using an automatic dispenser. A small but statistically significant difference was noted in the results obtained from different lots of columns and new lots should be evaluated before use.

The mean value \((±SD)\) for glycosylated hemoglobin in an apparently healthy population was 5.52 ± 0.53 percent in the present investigation compared to 6.36 ± 0.55 percent obtained by Klenk et al,\(^{10}\) 7.31 ± 0.92 percent by Gould et al,\(^{6}\) and 5.4 ± 1.2 percent by Bouriotis et al.\(^{2}\) Klenk et al\(^{10}\) used the same materials and procedure as reported by us and the difference can be explained readily by the populations utilized for the two studies. Klenk et al used samples taken from 124 non-diabetic volunteers (57 males and 67 females) between the ages of 9 and 62 years. Our study used 142 medical students (109 males and 33 females) with a mean age of 25.3 years. Graf et al\(^{7}\) have reported increased glycosylated hemoglobin levels with age in normal subjects. This is not unexpected since it has been shown that there is an increase of fasting plasma glucose and a deterioration of glucose tolerance with age. The other workers used different gels and methods. Gould et al\(^{6}\) used one ml of Glycogel, but used only eight ml of ammonium acetate wash buffer at pH 8.5 and three ml of sorbitol elution buffer as compared to our use of 20 mls of ammonium acetate at pH 8 and five ml of sorbitol elution buffer. Bouriotis et al\(^{2}\) used PBA-30 gel and buffers from a different supplier.

A variety of indicators of blood glucose control have been found to be significantly correlated with hemoglobin A\(_c\) or glycosylated hemoglobins as measured by various methods such as ion-exchange chromatography.\(^{13}\) The present study
demonstrates that fasting plasma glucose, in both normals and Type I diabetics, is significantly correlated with glycosylated hemoglobin measured by the affinity method. The mean of five plasma glucose measurements in a single day was also correlated with the glycosylated hemoglobin. These findings suggest that the results of the affinity method are useful clinical indicators of blood glucose control.

The boronate affinity procedure for the measurement of glycosylated hemoglobins has advantages over existing methods. The separation of glycosylated from non-glycosylated fractions is much better than with the cation exchange columns. The method does not require complicated equipment such as a high performance liquid chromatograph. Also, time-consuming than the thiobarbituric acid comparison method. Moreover, the method appears to be free of interference from labile Schiff-base intermediates which do not reflect long-term control of glyceria. These factors and the results of the present study suggest that the new affinity chromatography method provides an attractive alternative to previous methods for estimating glycosylated hemoglobin.

References