Improved Colorimetric Assay for Glycosylated Hemoglobin

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This colorimetric assay for glycosylated hemoglobin can be performed in 120 min with equipment available in most clinical laboratories. The glucose moiety of glycosylated hemoglobin is converted to 5-hydroxymethylfurfural by heating with oxalic acid for 60 min in an autoclave at 124 °C and 124 kPa (18 lb/in.²). The adduct formed by reacting 2-thiobarbituric acid with hydroxymethylfurfural is measured photometrically and results are expressed either as nanomoles of hydroxymethylfurfural or as fructose equivalents. Within-assay and between-assay coefficients of variation were <2% and <3%, respectively. Comparison of results for 50 patients' specimens as measured by the present assay and as analyzed for hemoglobin A₁c by liquid chromatography showed excellent correlation (r = 0.98).

Additional Keyphrase: diabetes mellitus

Glycosylated hemoglobin is now generally considered to be a useful index of blood glucose control in patients with diabetes mellitus (1). Chromatographic, electrophoretic, and colorimetric procedures have been developed to measure glycosylated hemoglobin. Each of these has disadvantages (1, 2) that limit its usefulness in clinical laboratories.

In the most widely used colorimetric approach (3) the hexose moiety of glycosylated hemoglobin is converted to 5-hydroxymethylfurfural (HMF) by heating at 100 °C in the presence of a weak acid, the hydroxymethylfurfural is reacted with 2-thiobarbituric acid, and the resulting color is measured photometrically. Most manual methods for quantitating glycosylated hemoglobin based on this approach have required incubation times of 3-16 h for the production of hydroxymethylfurfural (4-6). Here we report an improved colorimetric procedure that is more rapid and reproducible than those methods and correlates well with a liquid-chromatographic method.

Materials and Methods

Apparatus

For liquid chromatography we used an Analyst Series 7800 Laboratory Data Liquid Chromatographic System (Laboratory Data Control, Riviera Beach, FL 33404), as described elsewhere (7). We used a Cyclostatic Control Autoclave (American Sterilizer Co., Erie, PA 16500) for the autoclave step in our colorimetric procedure. All samples and reagents were pipetted with Pipetman Micropipettes (Rainin Instrument Co., Inc., Woburn, MA 01801).

Reagents

De-ionized water is used throughout.

**Oxalic acid, 0.5 mol/L:** Dissolve 6.3 g of oxalic acid in water and dilute to 100 mL.

**Thiobarbituric acid, 0.05 mol/L:** Dissolve 0.721 g of 2-thiobarbituric acid in water and dilute to 100 mL. Adjust to pH 6.0 ± 0.1 with 5 mol/L NaOH.

**Trichloroacetic acid, 400 g/L:** Dilute 40 g of trichloroacetic acid to 100 mL with water.

**Saline, 0.15 mol/L:** Dissolve 8.76 g of sodium chloride in water and dilute to 1 L.

**Hydroxymethylfurfural (stock), 100 μmol/L:** Dissolve 12.6 mg of 5-hydroxymethylfurfuraldehyde (Sigma Chemical Co., St. Louis, MO 63178) in saline and dilute to 1 L. Verify the concentration of this stock solution by measuring the absorbance at 284 nm (the molar absorptivity is 16 700 (8)). Dilute this stock solution with saline to give 50 and 25 μmol/L solutions.

**Fructose (stock), 1 mmol/L:** Dissolve 0.18 g of fructose (J. T. Baker Chemical Co., Phillipsburg, NJ 08865) in saline and dilute to 1 L. Dilute with saline to prepare 40, 50, 60, 70, and 80 μmol/L standards.

Reagents for the total hemoglobin determination by a modified cyanmethemoglobin procedure (9) were used according to the manufacturer's instructions (Fisher Scientific Co., Pittsburgh, PA 15219).

Blood Samples

Blood specimens were drawn by venipuncture into evacuated tubes containing EDTA. Hemolysates of the washed, packed erythrocytes were prepared by a modification of the method of Trivelli et al. (10).

Centrifuge the blood (800 × g, 10 min, 4 °C), remove the plasma, and wash the packed cells at 20 °C with five volumes of saline. Repeat this wash procedure two additional times. Lyse the packed cells with two volumes of water. Add one volume of carbon tetrachloride to the hemolysate, mix, and refrigerate at 4 °C overnight. Centrifuge (27 000 × g, 30 min, 4 °C) and store the aqueous supernate at −70 °C until analysis. For the colorimetric procedure, prepare an appropriate aqueous dilution to adjust the hemoglobin concentration to 10 g/L.

Pooled specimens of 12 normal and 12 diabetic hemolysates were prepared as described above.

Assay Procedure

Combine in screw-capped tubes (13 mm × 100 mm) with Teflon-lined caps 1 mL of diluted hemolysate containing 10 mg of hemoglobin or fructose standard with 1 mL of oxalic acid reagent. Incubate the tightly sealed tubes for 60 min in the autoclave at 124 ± 1 °C and 124 ± 3 kPa (18.0 ± 0.5 lb/in.²). Allow to cool to room temperature, mix the contents of the tube by inversion, add 1 mL of the trichloroacetic acid reagent to each tube, mix, and filter through a 15 cm × 0.5 cm (i.d.) glass column with a glass wool plug in the bottom. To 1.5 mL of the eluate, add 0.5 mL of the thiobarbituric acid reagent. Heat all tubes in a water bath at 40 °C for 30 min, allow to cool to room temperature for 15 min, and measure the absorbance at 443 nm.

Each assay includes the five fructose standards, a sample of the normal hemolysate pool, one of the diabetic hemolysate pool, and hemolysates prepared from patients' specimens. For each hemolysate, prepare an appropriate blank (omitting the color development step with thiobarbituric acid), take it through the procedure, and subtract its absorbance at 443 nm from that of the corresponding sample.

To convert measured absorbances for the hemolysates to
nanomoles of hydroxymethylfurfural, we used a relative absorption of 0.009 for the adduct formed between HMF and thiobarbituric acid. Alternatively, convert absorbances to fructose equivalents by using a fructose standard curve.

Rate Studies

We studied the rate of formation of hydroxymethylfurfural for both the fructose standards and the hemolysate pools. Assay tubes were removed from the autoclave after 10, 20, 30, 60, 120, 180, 240, and 300 min and the assay procedure was completed.

![Figure 1](image1.png)

**Fig. 1.** Rate of net production of hydroxymethylfurfural for the normal and diabetic hemolysate pools (x ± 2 SD, n = 4)

We studied the rate of destruction of hydroxymethylfurfural in the autoclave. Assay tubes containing HMF standards were removed from the autoclave after 30, 60, 90, 120, and 180 min and the assay procedure was completed.

![Figure 2](image2.png)

**Fig. 2.** Rate of net production of hydroxymethylfurfural for the fructose standards (μmol/L)

**Fig. 3.** Fructose standard curve for our colorimetric assay (x ± 2 SD, n = 4)

Results

Figure 1 illustrates the net increase in hydroxymethylfurfural for the normal and diabetic hemolysate pools as a function of heating time in the autoclave. For the normal pool 71% of the net absorbance present after 5 h in the autoclave was present after 60 min. For the diabetic pool 84% of the net absorbance present after 5 h in the autoclave was reached within 60 min.

Both hemolysate pools were analyzed by the colorimetric procedure of Pecorado et al. (4), in which the reaction with oxalic acid was carried out for 5 h in a 100 °C water bath. Comparison of the results from the autoclave incubation with those from the water-bath procedure indicates that for the normal pool 85% of the net absorbance developed after 5 h in the boiling water bath was reached after 60 min of autoclave incubation. Similarly, for the diabetic pool 102% of the net absorbance developed after 5 h in the boiling water bath was present after only 60 min of autoclave incubation.

Figure 2 shows for the fructose standards the net increase in hydroxymethylfurfural with incubation time in the autoclave. The initial rate of increase in absorbance was similar to that seen with the hemolysate pools; however, a maximum absorbance was reached at 120 min, followed by a decrease. When this study was repeated in the presence of protein (10 mg of albumin per assay tube), the same results were obtained for the fructose standards. The net production of hydroxymethylfurfural after 60 min in the autoclave is proportional to the fructose concentration (Figure 3).

The rate of destruction of the hydroxymethylfurfural standards in the autoclave is shown in Figure 4. Initial destruction was greatest, resulting in a 23% loss of hydroxymethylfurfural by 30 min; thereafter, approximately 15% of the remaining HMF was destroyed per 30 min of heating. After 60 min in the autoclave, the concentration of hydroxymethylfurfural decreased by 35%. When HMF standards were
heated for 5 h at 100 °C, there was a comparable decrease in hydroxymethylfurfural (30%). The HMF standards were added to the normal hemolysate pool so that the hemoglobin concentration in each tube was 10 g/L and the study was repeated. Even in the presence of this protein, HMF was destroyed at a similar rate.

The within-assay and between-assay variability of our colorimetric procedure was estimated by using the fructose standards and the hemolysate pools. Table 1 summarizes these results.

To compare our colorimetric and liquid-chromatographic procedures, we analyzed 50 blood samples by both methods. Regression analysis of the data (Figure 5) for the colorimetric (y) vs liquid chromatographic (x) assays gave the following results: y-intercept = 4.42, slope = 2.60, r = 0.98, and standard error of the estimate = 1.60.

**Discussion**

Recently, it has been shown that the proportion of glycosylated hemoglobin in a single blood specimen is an accurate and reliable measure of the glycemic status of a diabetic patient over a two- to four-month period. The clinical evaluation of glycosylated hemoglobin determinations as an indication of long-term control has been paralleled by the development of numerous methods for measuring hemoglobin A1c and total glycosylated hemoglobin. We describe here an inexpensive, precise, and relatively simple method for measuring glycosylated hemoglobin. The procedure requires equipment that is readily available in most laboratories and a total time of approximately 120 min, about one-third the time required to perform the colorimetric procedure of Pecoraro et al. (4). The increased pressure and temperature attained by incubation in the autoclave has increased the reaction rate and thereby allowed a significant reduction in assay time.

The amount of hydroxymethylfurfural present after the heating step is apparently related to both the production and destruction of HMF. Heating the reaction in the autoclave seems to increase both processes. The destruction of HMF upon heating in the autoclave was consistent with the report of Singh et al. (8), and was not significantly greater after 60 min in the autoclave than after 5 h in the boiling water bath. We selected 60 min as the length of the autoclave step because both the normal and diabetic hemolysate pools had by then produced an absorbance reading great enough to minimize the relative photometric error and at this time the fructose standards reacted similarly to the pooled hemolysate.

The substitution of filtration through a glass wool plug for the centrifugation step to remove precipitated protein represents a significant improvement in the assay procedure. We found that centrifugation frequently failed to sediment the protein precipitate and that filtration was much more effective in this regard. The speed with which the filtration step can be accomplished further reduces the total assay time.

We recommend the analysis in each assay of a hemolysate blank in which the thiobarbituric acid color reagent is omitted. We found that the amount of color generated in the blank is independent of the hemolysate when the hemoglobin concentration was adjusted to 10 g/L (i.e., the normal and diabetic pools produced comparable amounts of color), but is dependent on the length of incubation time in the autoclave. After 60 min in the autoclave a background color was produced from 10 mg of hemoglobin that would have led to a constant error of approximately 4 nmol of hydroxymethylfurfural. Failure to subtract the blank reading will increase the final results for the hemolysates by this amount.

A standard curve can be constructed from the fructose data and the results from the hemolysates converted to fructose equivalents. This offers the advantage of using a readily

### Table 1. Precision of the Colorimetric Assay, as Assessed from Replicates of Fructose Standards and Pooled Hemolysate Specimens

<table>
<thead>
<tr>
<th>Fructose, μmol/L</th>
<th>Within-assay*</th>
<th>Between-assay*</th>
<th>Within-assay**</th>
<th>Between-assay***</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>13.61 (0.22)</td>
<td>13.62 (0.31)</td>
<td>27.34 (0.40)</td>
<td>18.38 (0.30)</td>
</tr>
<tr>
<td>60</td>
<td>20.19 (0.14)</td>
<td>20.46 (0.30)</td>
<td>27.84 (0.42)</td>
<td>27.27 (0.39)</td>
</tr>
<tr>
<td>80</td>
<td>26.98 (0.20)</td>
<td>27.34 (0.40)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Hemolysate**

<table>
<thead>
<tr>
<th></th>
<th>Within-assay*</th>
<th>Between-assay*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal pool</td>
<td>18.38 (0.30)</td>
<td>18.67 (0.49)</td>
</tr>
<tr>
<td>Diabetic pool</td>
<td>27.32 (0.39)</td>
<td>27.84 (0.42)</td>
</tr>
</tbody>
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* n = 15, *b* n = 10, *c* n = 13, *d* n = 14.

Fig. 4. Rate of destruction of hydroxymethylfurfural standards in the autoclave

![Graph showing the rate of destruction of hydroxymethylfurfural standards in the autoclave](image)

Fig. 5. Comparison of glycosylated hemoglobin measured by the colorimetric assay with HbA1c measured by liquid chromatography

![Graph comparing glycosylated hemoglobin measured by colorimetric assay with HbA1c measured by liquid chromatography](image)
available and relatively pure material to monitor both the autoclave-incubation and the color-development steps in the procedure. Fructose is not an intermediate in the formation of hydroxymethylfurfural from glycosylated hemoglobin; however, the results indicate that under our assay conditions it is converted to HMF at a rate similar to that for the pooled hemolysates and that the amount of HMF present after 60 min is linearly related to the concentration of the fructose standards.

Earlier reports indicated that the colorimetric assay, involving the production of hydroxymethylfurfural and the subsequent color reaction with thiobarbituric acid, was imprecise (1) and did not correlate with chromatographic procedures (11). More recently, modifications of the colorimetric assay showing improved precision and correlation have been reported (4, 12). Our colorimetric assay offers additional modifications that further improve both the precision and correlation, as shown by a between-assay coefficient of variation of less than 3% for the hemolysate pools and a correlation coefficient of 0.98 for our colorimetric and liquid-chromatographic procedures.

The colorimetric assay appears to be uniquely suited to the following applications. First, it is now appreciated that protein glycosylation is a rather widespread phenomenon, in which the formation of glycosylated hemoglobin is but one example (13). Most of the methods currently used to measure glycosylated hemoglobin cannot be applied to the measurement of other glycosylated proteins, but the particular chemical reactions used in our colorimetric procedure can also be used to measure other glycosylated proteins. Second, we have reported (7) that there are labile and stable forms of glycosylated hemoglobin that are not easily distinguished by cation-exchange chromatography, electrophoresis, or other colorimetric methods. However, it is the stable form that best indicates long-term glycemic control. We have reported (14) that the reactions of our colorimetric assay occur only with the stable ketoamine forms of glycosylated hemoglobin. This suggests that our colorimetric assay may be particularly well suited to assess long-term glycemic control in the diabetic patient.

References