of the operator, each of these procedures should give results that are of greater value than measurement of urine sugar in the control of diabetes.

This evaluation was supported by Bio-Dynamics Inc., Indianapolis, IN 46250.

References
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Evaluation of a Colorimetric Method for Determination of Glycosylated Hemoglobin
Jim C. Standefer1 and R. Phillip Eaton2

We evaluated a colorimetric assay for glycosylated hemoglobin to determine the effects of several variables—oxalic acid concentration, extraneous glucose, hemoglobin concentration, hydrolysis interval, and 5-hydroxymethylfurfural destruction—and the precision. The interference seen when the blood glucose concentration exceeds 2.0 g/L (11 mmol/L) can be eliminated by washing the erythrocytes with 9 g/L saline. The accuracy of this assay is not influenced by hemoglobin concentrations from 80 to 150 g/L. The background nonspecific color, although substantial, is similar from sample to sample. After a 5-hydrolysis at 100 °C, about 85% of the hexose is released, and the analytical recovery of 5-hydroxymethylfurfural is constant over a wide range of glycosylated hemoglobin concentrations. The 5th to 95th percentile reference interval for a population of 65 nondiabetic individuals was 4.6 to 6.1 mol per 100 mol of total hemoglobin. The range of values for a population of 85 diabetic patients was 6.9 to 20.4 mol per 100 mol.

Additional Keyphrases: reference interval • variation, source of • diabetes

Because of the increasing interest in developing some method for monitoring therapeutic control of blood glucose concentrations in diabetic patients, considerable attention is being directed toward methods for assaying glycosylated hemoglobin (Gly-Hb).3 Chromatographic methods for isolating and assaying hemoglobin A1c (HbA1c) have been widely adopted (7–5), but these methods require strict control of several analytical variables (6–8). Several colorimetric assays of Gly-Hb have been reported (9–13); in general, they require heating a sample of hemolyzed blood with a weak acid such as oxalic acid to hydrolyze the hexose off of the hemoglobin and convert it to 5-hydroxymethylfurfural (5-HMF). This hydrolysis step is followed by adding thiobarbituric acid, which couples with 5-HMF to produce a color that is measured with a spectrophotometer. The advantages of colorimetric methods include (a) reliable assay of stored, frozen blood samples (14); (b) adaptability of the hydrolysis procedure to measurement of other glycosylated proteins in blood, such as glycosylated albumin (15, 16); (c) lack of interference from the labile glucose adduct (pre-A1c) that forms between glucose and hemoglobin during recent acute increases in blood glucose (10, 17); and (d) lack of interference from other “fast” variant forms of hemoglobin, which may increase the apparent HbA1c assayed by column chromatography. These advantages make hydrolysis/colorimetric methods attractive for routine clinical laboratory assay of Gly-Hb as well as assay of other glycosylated serum proteins. However, the reported disadvantages of this approach include destruction of 5-HMF during the hydrolysis step, lack of a reasonable primary standard, a variable background spectral interference, and an apparent dependence on the protein content of the sample.

Our objectives in this study were to try to overcome reported disadvantages of a colorimetric Gly-Hb procedure and evaluate the clinical utility of this method for monitoring diabetic control. We chose to investigate the method of Fluckiger and Winterhalter (9), with the following changes: (a) the concentration of oxalic acid is increased from 0.3 to 0.5 mol/L, (b) the hydrolysis time is extended from 1 to 5 h, and (c) we express our results as mol %, i.e., moles of 5-MHF per 100 mol of hemoglobin.

Materials and Methods

Reagents

Oxalic acid, 0.5 mol/L. Dissolve 31.5 g of oxalic acid in 500 mL of de-ionized water. This reagent is stable for two weeks at room temperature but, because some degradation of the crystalline oxalic acid occurs during long storage, it should be stored at −20 °C for not longer than six months.

Oxalic acid, 0.25 mol/L. Mix 100 mL of 0.5 mol/L oxalic acid with 100 mL of de-ionized water. This reagent is stable for two weeks at room temperature.

Sodium chloride, 9 g/L. Dissolve 9 g of sodium chloride in 1 L of de-ionized water. This reagent is stable for three months at room temperature.

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Trichloroacetic acid, 400 g/L. Place 400 g of trichloroacetic acid in a 1-L flask. Dissolve and adjust the volume to 1 L with de-ionized water. This reagent is stable for three months at 4 °C.

5-HMF standard. Dissolve 126.0 mg of 5-hydroxymethylfurfural (Aldrich Chemical Co., Milwaukee, WI 53201) in 100 mL of 0.25 mol/L oxalic acid. This stock solution is diluted 100-fold with 0.25 mol/L oxalic acid to obtain a 100 μmol/L working standard. This working standard is diluted with 0.25 mol/L oxalic acid to obtain other standards ranging in concentration from 12.5 to 100 μmol/L. The stock standard, stored at −20 °C, is stable for at least three months.

Thiobarbituric acid, 50 mmol/L. Dissolve 0.72 g of 2-thiobarbituric acid (Aldrich Chemical Co.) in 100 mL of de-ionized water. Adjust pH to 6.0 with 1 mol/L NaOH. This reagent is stable for a week at room temperature.

D-[3-3H]glucose, 11.5 kCi/mol, lot no. 1360-144; New England Nuclear, Boston, MA 02118.

Procedures

Sample preparation. Collect whole blood anticoagulated with EDTA in Vacutainer Tubes (Becton Dickinson, Rutherford, NJ 07070) and centrifuge (1000 × g, 4 °C, 10 min). Remove the plasma and wash the packed erythrocytes twice with saline (sodium chloride, 9 g/L), then store them at −4 °C. Gly-Hb in blood prepared and stored in this way is stable for at least 15 days (14). Just before assay, warm the sample to room temperature, dilute an aliquot with an equal volume of saline, and measure its hemoglobin content (17).

Hydrolysis. Add 100 μL of packed erythrocytes to 1.4 mL of de-ionized water and vortex-mix. Include one sample of pooled blood in duplicate to use as an assay blank. Mix 0.5 mL of 0.5 mol/L oxalic acid with 1.0 mL of this hemolsate in a 13 × 100 mm glass test tube. Seal each tube with a rubber stopper that has been penetrated with a 20-gauge hypodermic needle and incubate the stopped tube at 100 °C for 5 h. Remove the hypodermic needle after the first 10 min of heating. Remove the tubes from the heating block and cool them in an ice/water bath for 10 min, then add 1.0 mL of the ice-cold trichloroacetic acid reagent. Vortex-mix, then centrifuge at 1000 × g for 10 min.

Preparation of the standard curve. Mix 1 mL of each 5-HMF working standard with 0.5 mL of de-ionized water and 1.0 mL of the trichloroacetic acid reagent. Include one tube containing 1.0 mL of 0.25 mol/L oxalic acid, 0.5 mL of water, and 1.0 mL of the trichloroacetic acid reagent as a zero standard.

Colorimetric reaction. Mix 0.5 mL of the thiobarbituric acid reagent with 1.5 mL of each standard and each supernate from the hydrolysis step, except for the assay blank, in which 0.5 mL of de-ionized water is substituted for the thiobarbituric acid reagent. Adjust the spectrophotometer to zero with the zero standard. Incubate all tubes at 40 °C for 30 min, then measure the absorbance of each solution at 443 nm. The final absorbance will remain constant for at least 2 h.

Calculations. Plot the absorbance of each standard with its corresponding 5-HMF concentration on linear graph paper and read the 5-HMF concentrations of the unknowns from the graph. Divide the value for micromoles of 5-HMF per liter of hemolysate by the value for hemoglobin concentration (g/L) and multiply by 48 to obtain the final value expressed as mol %.

Results

Analytical Variables

Oxalic acid concentration. Several concentrations of oxalic acid have been used for hydrolyzing glucose from hemoglobin, the final oxalic acid concentrations in the reaction vessel ranging from 0.1 mol/L (9) to 0.5 mol/L (14). To determine the optimal oxalic acid concentration, we used three concentrations—0.085, 0.17, and 0.34 mol/L—to assay aliquots of pooled blood, an aqueous 5-HMF standard, and pooled specimens of blood supplemented with 5-HMF. (Those are the final oxalic acid concentrations in the hydrolysis mixtures.) Less 5-HMF was released from hemoglobin in pooled blood when 0.085 mol/L rather than 0.17 mol/L oxalic acid was used (Table 1), but there was no significant increase in 5-HMF release by increasing the oxalic acid concentration to 0.34 mol/L. The analytical recovery of 5-HMF added to blood is unchanged for oxalic acid concentrations between 0.085 and 0.34 mol/L, but with aqueous solutions recovery decreases at higher oxalic acid concentrations. Thus, we chose 0.17 mol/L oxalic acid (final concentration in the hydrolysis mixture) as providing the optimal 5-HMF release. Moreover, in the presence of hemoglobin, 0.17 mol/L oxalic acid does not degrade 5-HMF during the hydrolysis procedure noticeably more than does 0.085 mol/L oxalic acid.

Effect of glucose. Colorimetric methods for glycosylated hemoglobin may be influenced by high concentrations of glucose in the blood sample (19). To test this possibility, we supplemented whole-blood samples with glucose to give final glucose concentrations between 2.0 and 15.0 g/L (11.1 to 83.3 mmol/L), then determined glycosylated hemoglobin as described above. As Figure 1 shows, extraneous glucose may increase the apparent Gly-Hb value if the plasma glucose concentration exceeds 2.0 g/L (11 mmol/L). This increase in Gly-Hb is eliminated if the supplemented blood samples are washed twice with the saline solution before they are assayed for Gly-Hb.

Table 1. Effect of Oxalic Acid Concentration on Analytical Recovery of 5-HMF

<table>
<thead>
<tr>
<th>Sample</th>
<th>Oxalic acid concn, mol/L</th>
<th>5-HMF measured, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled blood</td>
<td>0.085</td>
<td>21.5</td>
</tr>
<tr>
<td>Pooled blood + 5-HMF, 20 μmol/L</td>
<td>0.17</td>
<td>22.8</td>
</tr>
<tr>
<td>Aqueous 5-HMF, 20 μmol/L</td>
<td>0.34</td>
<td>23.0</td>
</tr>
</tbody>
</table>

![Graph showing effect of extraneous glucose on the Gly-Hb assay](image-url)
This interference by glucose could result from a glucose hydrolysis product that couples with thiobarbituric acid or from a more direct influence of glucose. To test this latter possibility, we added glucose to a blood sample after hydrolysis but before adding thiobarbituric acid reagent. We observed no direct effect of glucose on the color development in the hydrolyzed sample. Then, to determine whether the effect of glucose is mediated through an interaction with hemoglobin, we added glucose to aqueous 5-HMF standards and assayed them for Gly-Hb. The increase in 5-HMF equaled or exceeded the increase due to endogeneous glucose concentrations in blood.

Analytical recovery of 5-HMF. A potential problem for colorimetric assays of glycosylated hemoglobin is incomplete and variable production of 5-HMF from Gly-Hb during the hydrolysis procedure (11). To study this, we mixed whole blood with radiolabeled glucose and unlabeled glucose to obtain a final glucose concentration of 33 mmol/L with a [3H]glucose specific activity of 4.2 mCi/mol. We stored the blood at room temperature for three weeks, long enough to produce the stable ketoamine (20). The mixture was not sterile, but we included ethylmercurithiosalicylate (Sigma Chemical Co., St. Louis, MO 63178) as a preservative to prevent glucose metabolism. After dialyzing the sample in saline for 48 h, the hemoglobin was hydrolyzed according to the procedure for Gly-Hb. The incorporation of [3H]glucose was calculated to be 2.2 mol/mol of hemoglobin. Approximately 97% of the tritiated material was recovered in the infranate after 5 h of hydrolysis, treatment with trichloroacetic acid reagent, and centrifugation.

We also investigated the recovery of 5-HMF from Gly-Hb by observing the release of chromogen at various times during hydrolysis. The production of 5-HMF chromogen from Gly-Hb is essentially complete after 15 h of incubation with 0.15 mol/L oxalic acid (12). If we assume that our 15-h hydrolysis value represents 100% of the available 5-HMF chromogen, then an average of 81% ± 5% of the chromogenic material is released from blood samples representing normal, slightly increased, and greatly abnormal Gly-Hb values after 5 h of hydrolysis (Figure 2).

To test the linearity of 5-HMF release from Gly-Hb, we mixed portions of two blood samples to obtain pooled blood samples with Gly-Hb values between 5.5 and 19.0 mol%. A plot of the chromogen absorbance vs 5-HMF concentration from these samples yields a curve identical to a curve obtained from a series of aqueous standards (Figure 3). Analytical recovery of 5-HMF from Gly-Hb was linearly related to concentration to at least 19.0 mol%.

Stability of 5-HMF. To study the stability of 5-HMF during the hydrolysis, we added 5-HMF to either whole blood or oxalic acid to obtain concentrations between 15 and 80 μmol/L. These blood samples were assayed for Gly-Hb as usual except that the hydrolysis was stopped at 1.0, 3.0, and 5.0 h. The results (Table 2) suggest that 5-HMF recovery declines after 1 h of hydrolysis; after 5 h of hydrolysis approximately 70% remains in the absence of blood and 80% remains when blood is present.

A part of the radioactive hydrolysate from the recovery experiment described above was applied with 5-HMF onto thin-layer silica gel G and chromatographed in the solvent system ethyl acetate/methanol/ammonium hydroxide (17/2/1 by vol). Sections of the silica gel were scraped into vials containing liquid scintillation fluid and their radioactivity was measured. Eighty percent of the radioactive material migrated the same (Rf 0.52) as the 5-HMF standard.

Hemoglobin concentration. The production of 5-HMF from

![Fig. 2. Release of 5-HMF chromogen during hydrolysis](image)

**Fig. 2. Release of 5-HMF chromogen during hydrolysis**

Pooled blood samples were assayed as described, except that the hydrolysis interval was varied as indicated. The 5-HMF concentrations measured after 5 h of hydrolysis are A, 57; B, 36; and C, 25 μmol/L.

![Fig. 3. Assay of 5-HMF in a series of aqueous standards](image)

**Fig. 3. Assay of 5-HMF in a series of aqueous standards (● — ●) and in dilutions of a blood specimen from a diabetic patient (O — O)**

<table>
<thead>
<tr>
<th>5-HMF added, μmol/L</th>
<th>% recovery after incubation interval of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0 h</td>
</tr>
<tr>
<td>To blood</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>98</td>
</tr>
<tr>
<td>40</td>
<td>96</td>
</tr>
<tr>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>To oxalic acid (0.25 mol/L solution)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>105</td>
</tr>
<tr>
<td>40</td>
<td>102</td>
</tr>
<tr>
<td>80</td>
<td>101</td>
</tr>
</tbody>
</table>
hemoglobin may vary with the amount of hemoglobin or protein in the hydrolysis mixture, but this variation may be accounted for by applying a correction factor derived from the amount of hemoglobin in the sample (13, 14). To determine the effect of hemoglobin content on the yield of 5-HMF from this hydrolysis procedure, we diluted the packed cells with saline to obtain samples having hemoglobin concentrations between 155 and 310 g/L. We chose packed cells—and hence twofold-increased hemoglobin concentrations—because we routinely use packed cells in our procedure. Each sample was assayed according to the usual protocol and each Gly-Hb concentration was calculated by using the appropriate hemoglobin value (Table 3). The results suggest that original blood hemoglobin concentrations between 80 and 150 g/L will cause no significant change in Gly-Hb values.

Nonspecific absorbance. Some of the absorbance measured at 443 nm in colorimetric Gly-Hb assays may be due to nonspecific color contributed by the sample (23). To determine whether (a) this nonspecific contribution to the final color is significant and (b) whether the contribution varies from sample to sample, we assayed 15 blood samples in three separate assays together with their respective "blanks." These blanks contained the sample plus all reagent except thiobarbituric acid reagent. The average absorbance of the blanks at 443 nm was 0.018 (SD 0.003), and did not vary with Gly-Hb concentration.

In another approach to evaluating spectral interferences from sample composition we scanned the absorbance between 370 and 500 nm for a 5-HMF standard and two blood samples that had been processed for Gly-Hb (one of the blood samples had been supplemented with 5-HMF). These scans (Figure 4) indicate good agreement between the absorbance curves for 5-HMF and processed blood samples in this wavelength region.

Precision. For evaluation of within-assay and between-assay precision, blood samples containing low, medium, and high concentrations of Gly-Hb were pooled, aliquoted, and stored at -20 °C. These aliquots were assayed several times in one assay and in consecutive assays spanning a period of 10 weeks (Table 4).

Reference Interval

Blood samples were collected from 85 patients who were being treated for diabetes mellitus and whose blood glucose regulation was under various degrees of control. We also collected blood samples for 65 nondiabetic individuals. By review of available medical history, we excluded from this group diseased individuals, especially those with carbohydrate intolerance. All samples were assayed for Gly-Hb and all values for both populations are shown in Figure 5. The mean Gly-Hb value (± SD) for the nondiabetic population is 5.51 ± 0.62 mol %. Thus, an expected 2 SD reference interval for Gly-Hb in a nondiabetic population of mixed sex and age is 4.3 to 6.7 mol %. The expected reference interval for this population derived from the 5th and 95th percentiles is 4.6 to 6.1 mol %.

Clinical Utility

To illustrate that this colorimetric Gly-Hb assay can be used

![Fig. 4. Spectral scan of 5-hydroxymethylfurfural and the Gly-Hb hydrolysis product after reaction with thiobarbituric acid. Two aliquots of a blood sample and an aqueous 5-HMF standard were assayed according to the procedure for Gly-Hb; 5-HMF was added to one aliquot before assay.](image)

![Fig. 5. Reference interval study for Gly-Hb. Data on 160 blood samples from diabetic and nondiabetic individuals, assayed for Gly-Hb. The mean Gly-hb value for nondiabetic individuals is 5.5 (SD 0.60) mol %](image)
to follow the progress of diabetic patients during therapy to control their blood glucose, we graphed the sequential Gly-Hb values in four diabetic patients and one healthy individual (Figure 6). Two of the diabetic patients required vigorous insulin therapy. Patient A was placed on a therapeutic protocol involving self-monitoring of blood glucose at home and variable, split-dose insulin therapy. Patient C received an insulin pump implant, which delivered insulin as prescribed by a rigid treatment protocol. The other two diabetic patients, B and D, were treated first with eight weeks of carbohydrate-restricted diet followed by treatment with an oral hypoglycemic agent. The data for the nondiabetic patient are given as an example of the type of variation in values one might expect to see when a patient in good, constant glucose control is being monitored. The expected range shown in Figure 6 (4.3–6.7 mol %) is the 2 SD range derived from this study.

Discussion

Certain analytical problems are associated with colorimetric methods for Gly-Hb and it is important to understand their limitations before the assay is routinely used in the clinical laboratory. Our results indicate that these analytical problems can be solved to produce an assay that is reliable and reasonably convenient for routine use.

Because colorimetric methods for Gly-Hb are based on the assay of 5-HMF produced when glucose is released from glycosylated hemoglobin, one must define the relation between the amount of 5-HMF assayed and the Gly-Hb concentration. Mild acid hydrolysis does not remove all of the glucose from its binding sites on the hemoglobin beta-chain, but stronger hydrolysis, to obtain more complete release of glucose, increases the rate of 5-HMF destruction. Thus, the amount of 5-HMF assayed is less than the theoretical amount of glucose attached to hemoglobin because (a) analytical recovery of glucose is incomplete and (b) part of the 5-HMF is lost during the hydrolysis step, owing to its liability in the hydrolysis environment. Consequently, the measured 5-HMF will not be stoichiometrically related to glycosylated hemoglobin. However, a stoichiometric relationship between assayed 5-HMF and glycosylated hemoglobin is not essential to reliable assay of Gly-Hb, if the following analytical conditions of the assay are well defined and controlled: the effect of extraneous glucose (19), the destruction of 5-HMF during the hydrolysis (11), the linearity of 5-HMF recovery with glycosylated hemoglobin content (13), the variable nonspecific color development during hydrolysis (23), and the protein content of the hydrolysis mixture (13, 14).

Abnormally high glucose concentrations in blood samples can significantly affect the apparent 5-HMF content, an effect that may vary from sample to sample, but can be eliminated by washing the blood cells before hydrolysis.

Just how glucose interferes with the Gly-Hb assay is not clear, but glucose can interfere in the absence of hemoglobin, so a probable explanation is that glucose is directly converted to 5-HMF during the hydrolysis procedure rather than being converted through a glucose–hemoglobin intermediate. Clearly, glucose does not participate directly in the color reaction.

With respect to the problem of 5-HMF destruction during the hydrolysis step, our results show that approximately 20% of added 5-HMF is destroyed during 5 h of hydrolysis in the presence of hemoglobin. Estimates of 5-HMF destruction made by examining samples of blood supplemented with it may be too high, because the presumably more labile 5-HMF is free during the entire hydrolysis interval rather than being gradually produced from glycosylated hemoglobin throughout the hydrolysis. So a better estimate of 5-HMF destruction may be obtained from hydrolysis of radiolabeled Gly-Hb. However, estimates of recovery of labeled 5-HMF will be distorted slightly by the incomplete release of 5-HMF from Gly-Hb. In any case, our estimated 20% loss of 5-HMF is similar to the 30% loss reported by Parker et al. (11) and is consistent with our finding that 80% of the hydrolyzed material co-chromatographed with 5-HMF on thin-layer chromatography. The rate of 5-HMF destruction varies with the vigor of hydrolysis conditions, which must be well controlled with respect to temperature, acid concentration, and incubation time (12).

Although analytical recovery of 5-HMF from glycosylated hemoglobin is not complete after 5 h of hydrolysis, the percentage recovery is consistent and does not change significantly with Gly-Hb concentration. Our data suggest that 80 to 85% of the glucose is recovered as 5-HMF from Gly-Hb during hydrolysis, which agrees well with the 80% recovery reported by others (14, 19).

If the recovery of 5-HMF were well defined and the exact extent of 5-HMF destruction known, then the calculated Gly-Hb concentration could be adjusted upward to account for these losses. Because these losses, although constant and controlled, are not known exactly, we have chosen not to adjust our calculated values: the percentage recovery is important, but a reproducible recovery of 5-HMF is more critical. Under the hydrolysis conditions in this procedure, the precision of the 5-HMF assay is acceptable (Table 4), and the amount of 5-HMF liberated from Gly-Hb is linearly related to Gly-Hb concentration (Figure 3). Although recovery of hexose from glycosylated hemoglobin is incomplete after 5 h of hydrolysis at 100 °C, it is consistent and the destruction of 5-HMF is limited and consistent.

Another problem reported for colorimetric assays of Gly-Hb is the requirement to adjust the final hemoglobin concentration in the sample before hydrolysis; moreover, a correction factor has been suggested to compensate for deviations from the optimal protein concentration (14). Our results indicate that this correction factor can be eliminated if the final results are expressed as mol %. We see no significant difference in the calculated values for released 5-HMF in samples in which the packed cell hemoglobin concentration ranges from 155 to 310 g/L.

Bunn reported that the nonspecific background color developed during the hydrolysis step may interfere with accurate
Gly-Hb assay (23). We find that this background color is significant but relatively nonvariable among specimens. The mean absorbance value of the blank, 0.018 (SD 0.003), is equivalent to a 5-HMF value of 1.5 (SD 0.3) μmol/L, or a Gly-Hb value of 0.6 (SD 0.01) mol %, if a hemoglobin concentration of 140 g/L is assumed. Because the absorbance of the blanks is relatively constant from sample to sample, we recommend including one blank in the assay and subtracting its absorbance value from the absorbance of each blood sample before calculating the concentration of 5-HMF.

Because our 65 nondiabetic individuals is a relatively small study population and was unselected except for the elimination of glucose intolerance, the reference interval we report must be considered tentative. Additionally, we report no information regarding the sensitivity or specificity of this assay. We observed no overlap between results for the diabetic and non-diabetic populations, but such overlap would not be surprising as more data become available. An overlap would be compatible with the notion that a wide range of carbohydrate intolerance exists as well as a wide range of responses to diabetic therapy. As indicated in Figure 6, effective treatment of diabetic patients will lower their Gly-Hb value to near the reference interval, which would also lead to an overlap of values.

From these results we can make the following observations about this colorimetric Gly-Hb assay: (a) the release of glucose from Gly-Hb is not complete but, with well-controlled analytical conditions, it is reproducible and linear to high Gly-Hb concentrations; (b) blood samples may be collected and stored without unusual precautions except that excess glucose must be washed from the sample before storage or analysis; (c) the nonspecific background color, although substantial, is relatively nonvariable from sample to sample, and an appropriate correction should be applied by including a blank in each assay; (d) an expected reference interval can be delineated sufficiently to identify individuals with abnormal blood glucose; and (e) colorimetric assays for Gly-Hb are sufficiently reliable for monitoring therapy in diabetic patients.

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