Standardization of Glycohemoglobin Determinations in the Clinical Laboratory: Three Years of Experience

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Measurement of glycohemoglobin has been recommended for the long-term assessment of glycemic control in diabetic patients. Because different analytical methods measure different glycohemoglobin species, it has been difficult to compare results between laboratories. Here we report 3 years of experience with calibration of an affinity chromatography method for measuring total glycohemoglobin (GHB). Calibration was achieved by including in each assay three hemolysate calibrators for which values for HbA₁c and GHB had been determined by repeated analyses by high-performance liquid chromatography (HPLC) and affinity chromatography, respectively. Calibration improved interassay precision (CV = 3.20–7.90% and <5.0% before and after the introduction of calibration, respectively) and eliminated lot-to-lot variability. In 91 samples, HbA₁c was estimated by the calibrated affinity chromatography assay and measured by an ion-exchange HPLC method. Estimated and HPLC-measured HbA₁c showed no clinically significant differences during 36 months. The high degree of long-term precision, the disappearance of lot-to-lot variability, and the excellent comparability between analytical methods measuring different species of glycated hemoglobins demonstrate the advantages of calibration.

Additional Keyphrases: diabetes · chromatography, boronate affinity · chromatography, ion-exchange · variation, source of · calibration

Glycohemoglobins, sometimes called glycosylated hemoglobins, were first described by Rahbar in 1968 as "diabetic hemoglobins" (1). Their production depends on blood glucose concentration and occurs by way of a post-translational nonenzymatic process called glycation, in which sugar is attached to the amino groups of the hemoglobin (Hb) molecule (2–6). Glycation of the N-terminal amino acids of the α and β chains as well as on the ε-amino groups of lysine residues in the hemoglobin molecule (5, 7) results in a variety of glycated hemoglobins, including HbA₁c, which is the β-chain N-terminal valine-glycated species, and total glycated hemoglobin (GHB) (8). Several analytical methods have been developed for the measurement of glycohemoglobins; those most frequently used are ion-exchange chromatography for HbA₁c and affinity chromatography for GHB (reviewed in reference 9).

Measurement of glycohemoglobin in the form of HbA₁c or HbA₁ is recommended by the American Diabetes Association (10) and others (11) for monitoring diabetic patients, but determination of total GHB is also acceptable (12). Patients should be tested at the initial evaluation and at least semiannually (all diabetic patients) or quarterly (insulin-treated patients) for the long-term assessment of average blood glucose and for the assessment of reliability of self-glucose monitoring (10). It has also been proposed that determination of glycohemoglobins could benefit the diabetic patient by promoting better glycemic control (13) and, consequently, longer-term stability of the patient’s condition (14). A strong relation between increased glycohemoglobin concentration and proliferative retinopathy (15) underlines the clinical importance of glycohemoglobin measurement.

Measurement of glycohemoglobin, either as HbA₁c or as GHB, places a great demand on the clinical laboratory. To be clinically useful, the method should measure glycohemoglobin with a great degree of precision and should be economical and simple to perform. Accuracy of the measurement is particularly important in predicting the clinical outcome of diabetes, but it is difficult to verify. Because of the multitude of methods used, comparison of results from different laboratories is difficult or even impossible (16, 17).

It has been accepted laboratory practice to use calibrators or standards, or both, to increase the accuracy of analytical methods. Indeed, to overcome the difficulties of glycohemoglobin measurement in the clinical laboratory, Peterson et al. proposed standardization in 1984 (18). The need for standardization was also recognized by the National Diabetes Data Group of the National Institutes of Health (14) and the College of American Pathologists (19). At present, however, there is no accepted standard nor is there an acknowledged reference method. The feasibility of calibrating the measurement of HbA₁c by using a highly precise HPLC method to assign values to the calibrators (hemolysate standards) was reported previously (20). Here we present data from 3 years of experience in calibrating measurements of GHB in the clinical laboratory.
Materials and Methods

Calibrators

Primary calibrators were prepared at the University of Missouri (Columbia, MO) from the blood of single donors at three different conditions of glycemic control (low, medium, and high) as described previously (21). Briefly, packed erythrocytes were washed and incubated overnight in saline to remove the labile aldime component, and the cells were lysed with water. The hemolysates were then incubated with carbon tetrachloride and the aqueous supernates were used as calibrators. HbA1c values were assigned to the primary calibrators (Table 1) on the basis of the mean of multiple determinations (n = 11 runs) by HPLC (21). Aliquots of these calibrators were sent to Barnes Hospital Clinical Chemistry Laboratory (St. Louis, MO) before the study.

Table 1. Assigned Values for the Three Calibrators

<table>
<thead>
<tr>
<th>Method</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>% HbA1c by HPLC*</td>
<td>5.39</td>
<td>8.49</td>
<td>13.52</td>
</tr>
<tr>
<td>% GHb by AC°</td>
<td>5.25</td>
<td>10.34</td>
<td>19.93</td>
</tr>
</tbody>
</table>

* Determined by HPLC (comparison method) at the University of Missouri (Columbia, MO).

° Determined by boronate affinity chromatography at Barnes Hospital Clinical Chemistry Laboratory (St. Louis, MO) before the study.

Affinity Chromatography Method

GHb assays were performed with a commercially available boronic acid affinity chromatography kit (GlycoTest™; Pierce Chemical Co., Rockford, IL). The glycated hemoglobin was separated from nonglycated hemoglobin on the basis of the affinity of m-aminophenyl boronic acid for the cis-diol groups on the glucose portion of the glycated species. The assay was performed as recommended by the manufacturer. Briefly, 50 μL of hemolyzed anticoagulated whole blood (10-fold dilution) was loaded onto each preequilibrated column. Each column was washed with 5.5 mL of ammonium acetate–magnesium chloride buffer (equilibration/wash buffer), pH 8.05, to elute the nonbound, nonglycated Hb fraction. The glycated hemoglobin fraction was then eluted from each column with 3.0 mL of sorbitol–Tris buffer, pH 8.50 (GHb elution buffer). The amount of Hb in each fraction was determined by measuring absorbance (A) at 414 nm. The percentage of GHb was calculated as follows:

\[ \% \text{GHb} = \frac{\text{GHb} \times 3.0 \times 10^4}{\text{GHb Values}} \]

Values of GHb were determined by affinity chromatography at Barnes Hospital (n = 24 runs) at the beginning of the study (Table 1). The primary calibrators were used for the first 24 months of the clinical study, after which secondary calibrators were developed at Barnes Hospital and had HbA1c and GHb values similar to the primary ones. The secondary calibrators were made from pooled whole-blood hemolysates; both the HbA1c and GHb values were assigned from calibrated (with primary calibrators) affinity chromatography assays.

Quality-Control Samples

Three pools of whole-blood hemolysates were prepared at Barnes Hospital Clinical Chemistry Laboratory and used as quality controls: low (QC I), medium (QC II), and high (QC III) quality-control samples. Samples of whole blood, anticoagulated with EDTA and assayed at the clinical laboratory, were selected for pooling depending on their GHb concentration. Those with GHb < 6%, between 6% and 8%, and > 13% of total Hb were selected for the QC I, QC II, and QC III pool, respectively. After pooling the blood, we made hemolysates by mixing one volume of whole blood with 10 volumes of 10 mL/L Triton X-100 detergent solution. The pools were divided into ~500-μL portions and stored at −70 °C for long-term study. GHb values of the individual pools were measured by affinity chromatography.

Calibration Procedures

Calibrators were included in each assay and a new calibration curve was generated in every run by plotting the assigned GHb values (affinity chromatography, Barnes Hospital) of the calibrators against the measured GHb values of the calibrators and connecting the three points with two straight lines (piecewise linear interpolation). The measured GHb value of an unknown sample was compared with the measured GHb value of the medium calibrator to select the appropriate portion of the calibration curve. The selected portion of the curve was used to calculate the calibrated GHb value of the unknown sample.

Another curve was generated by plotting affinity-measured GHb values against the assigned HbA1c values (HPLC comparison method, University of Missouri) of the three calibrators and connecting the points by piecewise linear interpolation. This curve was used to convert affinity-measured GHb values to HbA1c equivalents (estimated HbA1c). The calculations were performed by a computer program written in-house that accepted measured absorbance data as input and printed out noncalibrated GHb, calibrated GHb, and estimated HbA1c values. Patients' results were routinely reported as "standardized %GHb." The estimated HbA1c values were used to compare the affinity-measured (estimated) HbA1c results with the HPLC-measured HbA1c.

Study Samples and Research Design

To estimate the reference interval of our calibrated method and to investigate the influence of calibration on the reference interval of the method, we collected blood samples from 60 apparently healthy non diabetic laboratory workers and analyzed them by affinity chroma-
both calibrated GHb and estimated HbA\textsubscript{1c} reference intervals were calculated as the mean ± 2 SD of the 60 determinations.

Intra- and interassay precision of standardized and nonstandardized affinity chromatography measurements were estimated by using the GHb values of the three QC samples. Thirty-two results per QC sample were randomly selected from all routine determinations for each of the four reagent lots and were evaluated for variability by calculating the CV of the determinations for each lot of reagents before and after calibration of the assay.

To further study the effect of calibration on results obtained with two different lots of affinity chromatography materials (lot-to-lot variability), we selected, from clinical samples submitted to Barnes Hospital Clinical Chemistry Laboratory, samples from 38 patients whose GHb concentrations included low and high values. The samples were analyzed with two lots of affinity chromatography reagents. The results were compared by linear least-squares regression analysis and paired t-tests to assess whether the two reagent lots gave similar results with and without calibration.

To study long-term agreement of the calibrated GHb assay with the HPLC method, we used 91 clinical samples submitted for GHb analysis to Barnes Hospital Clinical Chemistry Laboratory. Aliquots were sent to the University of Missouri in Columbia over a period of 36 months for measurement of HbA\textsubscript{1c} by HPLC. The estimated and measured results of the 91 determinations were compared by linear least-squares regression analysis and paired t-tests. The mean difference between the results obtained at two laboratories was also calculated.

**Results**

**Precision**

The mean GHb and CV for the three QC samples, calculated from the noncalibrated assay data obtained with the same reagent lot in one day (intra-assay precision), were 5.82% and 2.10%, 9.65% and 1.30%, and 15.9% and 1.30% for QC I, II, and III, respectively (n = 9). Interassay precision of noncalibrated and calibrated GHb determinations calculated separately for each of the four reagent lots is shown in Table 2. Interassay precision of measured GHb varied between 3.2% and 7.9%, depending on the GHb concentration of the QC sample and the reagent lot. The interassay precision calculated from the same determinations but after calibration improved substantially. (Table 2): All CVs became <5% (range, 1.8–5.0%).

**Lot-to-Lot Variability**

We also investigated the influence of lot-to-lot variability before and after calibration of the affinity chromatography reagents. We analyzed 38 patients' samples with two consecutive lots of reagents. Linear least-squares regression analysis (lot A vs lot B) showed a 15% proportional bias without calibration (Figure 1). We derived the following regression equation (SDs of the slope and intercept are in parentheses): GHb\textsubscript{lot B} = 0.850 (± 0.029)·GHb\textsubscript{lot A} + 0.891 (± 0.323); \( S_\text{SE} = 0.855, r^2 = 0.9610 \). Paired t-tests showed that the two lots of reagents produced statistically different results \( P = 0.001 \). After

### Table 2. Interassay Precision of Noncalibrated and Calibrated Affinity Chromatography GHb Measurements

<table>
<thead>
<tr>
<th></th>
<th>Noncalibrated GHb assay</th>
<th>Calibrated GHb assay</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>QC I</td>
<td>QC II</td>
</tr>
<tr>
<td>% GHb*</td>
<td>5.76</td>
<td>9.30</td>
</tr>
<tr>
<td>CV, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent lot 1</td>
<td>6.70</td>
<td>3.50</td>
</tr>
<tr>
<td>Reagent lot 2</td>
<td>6.10</td>
<td>4.50</td>
</tr>
<tr>
<td>Reagent lot 3</td>
<td>7.90</td>
<td>4.60</td>
</tr>
<tr>
<td>Reagent lot 4</td>
<td>4.38</td>
<td>3.98</td>
</tr>
</tbody>
</table>

n = 32, each lot.

*Average of the four reagent lots.
calibration, the regression equation showed the following relationship (Figure 1): \( \text{GHb}_{ \text{tot} } = 0.992 \times \text{GHb}_{ \text{tot} } + 0.089 \) (± 0.220); \( S_{\text{SE}} = 0.599, r^2 = 0.9864 \). Paired t-tests showed no statistically significant differences among the calibrated GHb results (\( P = 0.09 \)) for the same 38 patients’ samples.

Comparison of Calculated and Measured HbA_{1c}

HbA_{1c} calculated from calibrated affinity chromatography data in the clinical laboratory (Barnes Hospital, estimated HbA_{1c}) was compared with HbA_{1c} measured by HPLC in the research laboratory (University of Missouri, reference HbA_{1c}). Ninety-one clinical samples were selected to cover both the low and high ranges of HbA_{1c} concentrations; each sample was analyzed in both laboratories over a 36-month period. No more than five samples were included in each group of comparison samples at one time. The linear least-squares regression equation showed the following relationship (Figure 2): estimated HbA_{1c} = 0.948 (± 0.016) \times \text{HPLC HbA}_{1c} + 0.677 (± 0.141); \( S_{\text{SE}} = 0.437, r^2 = 0.9940 \). The mean difference between the estimated and the HPLC-measured HbA_{1c} was 0.23% HbA_{1c}, statistically significant (\( P < 0.001 \)) by paired t-test.

Reference Interval

The reference interval for the calibrated GHb, calculated as the mean ±2 SD from 60 healthy laboratory workers, was 4.45–6.27%. This is markedly narrower than the noncalibrated reference range provided by the kit’s manufacturer (3.8–6.0%). The reference range for estimated HbA_{1c} by calibrated affinity chromatography was 4.9–6.0% from the same group of healthy volunteers. The clinically important upper reference limit of our estimated HbA_{1c} thus equals the reference range that was calculated from data obtained by the HPLC method used for comparison (4.0–6.0%).

Discussion

The affinity chromatography method for measuring GHb has several advantages over other methods (22). For instance, it is less dependent on temperature and is not affected by hemoglobinopathies when erythrocyte turnover is normal. However, this method is sensitive to changes in gel or buffer properties (23), which can lead to substantial lot-to-lot variation and thus limit the usefulness of the method in the long-term follow-up of a single patient. Our study shows that GHb can be measured routinely in a clinical laboratory with a high degree of long-term precision by using the calibrated affinity chromatography method. Depending on the reagent lot used, interassay CVs during our study ranged from 3.2–7.9% without calibration, but improved to 5% with calibration. This low CV was maintained for the duration of the study in spite of our using four different lots of affinity columns and in spite of the ambient temperature ranging from 19.2 to 25.6 °C (mean temperature: 21.7 °C). Calibration made the assay results independent of temperature changes. The precision attained through calibration meets the recommendation of the Diabetes Data Group Committee on Glucosylated Hemoglobin (15).

The measured GHb results showed a proportional bias of −15% when 38 patients’ samples were assayed with two different lots of reagents without calibration. That there was no difference after calibration was indicated by the near-zero intercept and the slope of −1 for the linear least-squares regression equations, as well as by the statistically nonsignificant difference between the two groups of results. Because the 38 samples were assayed during four consecutive days and the calibrated and noncalibrated GHb results were calculated from the same affinity chromatography assays, we can state with confidence that the observed difference was due to lot-to-lot variability of the affinity reagents. Our calibration procedure eliminated the influence of this variability. The demonstrated lot-to-lot variation of results would translate into a wider reference interval for the GHb values. With improved precision one would expect a narrowing of the reference interval, which would better separate normal from diabetic GHb values and increase the potential for use of GHb measurement in diabetes screening.

The feasibility of standardizing glycohemoglobin measurements between laboratories was shown previously (20, 24, 25) when results were compared from two laboratories in one institution, both using ion-exchange chromatography. Little et al. showed that standardization between ion-exchange and affinity chromatography methods is possible when one uses results from a limited number of patients (20). In those studies a standard curve was constructed by linear regression analysis from raw assay values of the standards and their reference method-assigned values.

The different amino groups of the hemoglobin molecule have different affinities for glucose (4, 7). Therefore, their glycation should proceed at different rates (4). Indeed, at a given blood glucose concentration, GHb and
HbA1c are formed in different proportions, resulting in reports of a nonlinear relation between GHB and HbA1c concentrations (26, 27). This has been confirmed in our clinical laboratory (data not shown). This essentially nonlinear relation between GHB measured by affinity chromatography and HbA1c, as well as the ease and speed of calculation, prompted this clinical laboratory to adopt the piecewise linear interpolation for the calculation. With the appropriate selection of the three calibrators, the resulting two line segments would more closely approximate the nonlinear relation between the two glycated hemoglobin species than first-order linear regression, although the differences would be small and might not be clinically significant. Our experience showed good agreement between affinity-derived (estimated) HbA1c and HPLC-measured HbA1c results for 91 patients during 36 months of clinical evaluation. Although the results obtained with the two methods were statistically different by paired t-test, the mean difference between the methods was 0.23% HbA1c, which we feel is clinically not significant.

HbA1c concentrations are correlated with mean plasma glucose concentrations (28, 29). Because the results of affinity chromatography can be directly expressed as HbA1c through our calibration, the mean plasma glucose concentration can be estimated with GHB values measured by affinity chromatography. Reference ranges obtained by one method can be directly compared with those from another method. Most important, our study shows that direct comparisons can be made between results from various clinical laboratories, thus improving the prospects for long-term care of the mobile patient.

References