Interlaboratory Standardization of Measurements of Glycohemoglobins


The diversity of methods used to measure glycohemoglobins (GHb) makes it difficult to compare patients' results among laboratories. We reported previously the feasibility of providing comparable results from different assays by use of common calibrators. We here compare results from seven different GHb methods calibrated by use of hemolysates assayed by a precise ion-exchange high-performance liquid-chromatographic (HPLC) method for hemoglobin A_1c (HbA_1c). Thus, regardless of the GHb species measured by the seven methods, results were referenced to the HbA_1c content of the calibrators. Without this calibration, GHb values for single samples varied, e.g., from 4.0% to 8.1% and from 10% to 14.2% in the normal and high ranges, respectively. Calibration decreased between-method variability (single sample ranges of, e.g., 4.8% to 5.4% and 9.4% to 10.2% in the normal and high ranges, respectively) and improved interassay precision. We conclude that this approach to calibration of GHb measurements allows direct comparison of results obtained by different methods and improves precision.

Additional Keyphrases: diabetes · calibration · intermethod comparison

Diabetes mellitus is associated with serious complications of the eyes, kidneys, heart and blood vessels, and other organ systems. The relation between the development and progression of microvascular complications of diabetes and glycemic control has been debated for many years, in part because of the inadequacy of methods to assess glycemic control over the long term. Traditional methods of measuring glucose in blood and urine are of limited value for this purpose, and it is only with the development of glycohemoglobin (GHb) testing that accurate, objective measurement of long-term glycemic status has been possible. Routine determination of GHb is now widely used in clinical practice to monitor glycemia in persons with diabetes. Studies indicate that, under most circumstances, GHb is a reliable measure of average blood glucose concentration during the preceding 2 to 3 months (1-5).

Assay methods for GHb can be categorized on the basis of (a) the manner in which glycated and nonglycated hemoglobin components are separated, e.g., charge differences or structural characteristics, or (b) the GHb component measured, e.g., HbA_1c, HbA_1, or total GHb. The most commonly used methods quantify HbA_1c by ion-exchange chromatography, HbA_1 by ion-exchange chromatography or electrophoresis, or total GHb by affinity chromatography.

Because different assays measure different proportions of GHb and do so in different ways, and because there is no consensus on either a standard or a reference method for GHb, results are not comparable among methods (6, 7). We reported previously that interlaboratory comparability of GHb determinations is feasible (8, 9). We have extended our earlier study to evaluate the comparability of seven methods in three laboratories. We propose that such calibration can be achieved in all clinical laboratories.

Materials and Methods

Study Design

The study was performed in three separate laboratories in two institutions. The reference laboratory (RL) for this study was the University of Missouri Diabetes Research Laboratory in Columbia, MO. This laboratory performed GHb analyses by an ion-exchange high-performance liquid chromatography (HPLC) method (RL HPLC method). All primary calibration materials were assigned GHb values by this RL HPLC method. Primary calibrators, with assigned values, were then analyzed by the methods to be evaluated, and calibrated GHb values for samples were obtained by a standard curve or a single-point calibration procedure. The primary calibration materials used for this study were not purified.

Two clinical laboratories performed GHb analyses by several different methods. The Diabetes Diagnostic Laboratory (Clin Lab 1), also at the University of Missouri, performed GHb analyses by Diamat™ HPLC (Bio-Rad Labs, Hercules, CA), CLC330™ affinity chromatography HPLC (Primus Corp., Kansas City, MO), GlycoTest™ affinity chromatography (Pierce Chemical Co., Rockford, IL), Abbott Vision™ Glycated Hb affinity binding assay (Abbott Labs., Abbott Park, IL), Diatrac™ HbA_1c electrophoresis (Beckman Instruments, Inc., Brea, CA), and DCA 2000™ immunoassay (Miles, Elkhart, IN). The Wilford Hall USAF Medical Center Laboratory (Clin Lab 2), Lackland Air Force Base, TX, performed GHb analyses by rapid electrophoresis (REPs Glyco-30; Helena Labs., Beaumont, TX), Glyc-Affin™ affinity chromatography (Isolab Inc., Akron, OH), and DCA 2000 immunoassay.

We used patients' blood samples submitted routinely
for glycohemoglobin measurement or blood drawn from nondiabetic volunteers as our study samples. Samples containing abnormal hemoglobins, as demonstrated by characteristic Diamat chromatograms, were excluded. Samples showing extraneous degradation peaks on the Diamat chromatogram, which are known to interfere with accurate quantification of HbA1c by this method, also were excluded.

Primary Calibration Materials

We used a variety of calibration materials (Table 1); all were erythrocyte hemolysates with the labile (Schiff base) fraction (10, 11) removed. For some methods, the calibrator was supplied by the manufacturer of the particular GbH assay method. For other methods, the calibrator was either prepared at the RL by a previously described method (12) and stored at −70 °C in small, single-use aliquots [EH (RL), −70 °C] or purchased from Sherwood Medical (St. Louis, MO) as lyophilized hemolysate [LEH (Sherwood), 4 °C].

Quality-Control Materials

Various materials were used for quality controls (QCs) (Table 1). For some methods, either lyophilized or liquid erythrocyte hemolysates were provided as QCs by the manufacturer specifically for a particular assay system. For other methods, lyophilized GbH controls were purchased from Sherwood Medical [LEH (Sherwood)]. For the remaining methods, controls were prepared in the RL either as an erythrocyte hemolysate with the labile fraction removed [EH (RL), −70 °C] (12) or as a whole-blood hemolysate [WBH (RL), −70 °C]. For several methods, the same type of material was used both as a calibrator and a QC sample; in these cases the calibrators and controls were from different batches or lots, i.e., they were derived from different individuals and had different GbH values.

Assignment of Values to Calibrators

The semiautomated RL HPLC method was used to assign values to the primary calibrators used in this study. This two-buffer, step-gradient HPLC assay for HbA1c was described previously (12). The column consisted of a BioRex 70, 400-mesh resin (Bio-Rad, Richmond, CA) packed in a glass column that was temperature-controlled. A liquid-chromatographic system was used to separate, measure (at 410 nm), and quantify the differentially eluted hemoglobin fractions. The prepared hemolysate (labile fraction removed) was injected onto the column, which had been equilibrated with sodium phosphate buffer (80 mmol/L, pH 6.8). After the HbA1c fraction was eluted, a more concentrated sodium phosphate buffer (300 mmol/L, pH 6.40) was used to elute the HbA2 fraction, and the relative concentration of HbA1c was calculated with an integrating recorder. A single calibrator was included in each run and the system’s variables were adjusted, if necessary, so that the value was within acceptable limits of a previously established value (±1 SD). Two QC specimens were included in each run. This assay system, which has been on-line since 1978, is rigorously controlled and can deliver reproducible results over the long term; the interassay CV for this method has been consistently <3%. Assigned values were based on the mean of multiple determinations (n >10 different runs).
Comparison HPLC Method

The Diamat HPLC was used as the comparison HPLC method because it is suitable for large-scale sample comparisons and because our laboratory has had long-term experience with this method. The Diamat is a dedicated, automated HPLC system used to quantify HbA1c. HbA1c is separated from HbE0 and other minor hemoglobin components by step-gradient elution on a cation-exchange column. Samples were analyzed according to the manufacturer's instructions for an 8-min run. The labile fraction was removed by a 30-min incubation at 37°C with the hemolyzing reagent provided by the manufacturer.

For each run, the instrument was calibrated with a single lyophilized sample supplied by the manufacturer. If the observed value of the calibrator was outside acceptable limits, which were based on values assigned by the RL HPLC method, then calibration parameters were adjusted, according to the manufacturer's recommendations, in the microprocessor.

Other Methods

*Affinity chromatography minicolumn assays.* Total GHB was measured by boronate affinity chromatography column test kits from two manufacturers (Pierce GlycoTest; Isolab Glyc-Affin). Assays were performed according to the manufacturers' instructions. Four calibrators prepared in the RL were included in each assay (EH (RL), −70°C). For each assay, a calibration curve was constructed by linear regression analysis of the measured values of the calibrators and their RL HPLC method-assigned values (8, 9) (Figure 1).

*Affinity binding assay.* GHB was measured by the Abbott Vision Glycated Hb test according to the manufacturer's instructions. The method is a fully automated boronate affinity binding assay that measures total GHB. All reagents are contained in individual disposable test packs (13). Four calibrators, consisting of whole-blood samples, were used. These samples were divided and prepared for analysis on either the Vision assay system (whole blood, stored at −70°C) or by the RL HPLC method (hemolysates with the labile fraction removed, stored at −70°C). Using the Abbott Vision system, we constructed a linear calibration curve by correlating the observed percentage decrease in absorbance (Abbott Vision signal) with the RL HPLC-assigned value. This primary calibration curve was then used to analyze the three-concentration secondary calibrators (bovine hemolysates, Abbott) and to assign them GHB values. The assignment of values to the secondary calibrators was performed at Abbott Laboratories for each new lot of secondary calibrators. These secondary calibrators were then analyzed in Clin Lab 1 to establish a calibration curve for each lot of test packs used. This curve was stored in the instrument and calibrated results were reported.

*Affinity HPLC.* GHB was measured by the Primus CLC33O glycated hemoglobin analyzer, an automated HPLC system with a boronate affinity resin and a two-point calibration system. RL HPLC values were assigned to the two lyophilized hemolysates provided by the manufacturer. The final GHB result was obtained automatically by a two-point calibration. These calibrators were run only when a new column was used, after 500 injections, or when a new computer disk was being used.

*HbA1c, electrophoresis.* HbA1c was measured by the REP Glyco-30 electrophoresis system according to the manufacturer's instructions. Three lyophilized hemolysates (Sherwood Medical) were included in each assay. For each assay, a calibration curve was constructed by linear regression analysis of the assay values of the calibrators and their RL HPLC method-assigned values (8) (Figure 1).

*HbA1c, electrophoresis.* HbA1c was measured by the Beckman Diatrac HbA1c electrophoresis kit according to the manufacturer's instructions. Calibrated liquid hemolysates (stored at −20°C) supplied by the manufacturer were included with each gel. A calibration curve was constructed by linear regression analysis of the assay values of the calibrators and their RL HPLC method-assigned values (8) (Figure 1).

*Immunoassay.* HbA1c was measured by an immunoassay method (14) with a Miles DCA 2000 HbA1c system. The assay, based on an HbA1c-specific monoclonal antibody, was run according to the manufacturer's instructions. This method was calibrated in the laboratory by using a bar-coded calibration card with factory preset parameters specific for each lot of reagents. These calibration parameters were obtained from a calibration curve generated for 12 different values of HbA1c (12 primary calibrators). A total of 288 immunoassays was
performed and calibration parameters were generated from a four-parameter curve fit by using immunoassay values and RL HPLC method-assigned values (15).

Data Analysis

Results from each method were compared with results from either the RL HPLC or the Diamat HPLC methods by correlation and linear regression analyses. Statistical analyses were performed with the use of SAS programs (SAS Institute, Cary, NC). Imprecision was expressed as interassay CV. CVs were calculated separately from both unstandardized and standardized GHb values where appropriate.

Results

Relation between RL and Diamat HPLC Methods

The long-term correlation (1984–91) between the RL HPLC and the Diamat HPLC methods has been established over an 8-year period (Figure 2) and is monitored continually to ensure comparability of values. The regression line is close to the line of identity (n = 1739; y = 1.01x + 0.045; r = 0.98).

Relation between Uncalibrated GHb Methods and RL HPLC and Diamat HPLC Methods

Figure 3 shows regression analyses between values from uncalibrated affinity chromatography assays (Pierce GlycoTest minicolumns) and values from either the RL HPLC method or the Diamat HPLC method. These regression lines were derived from six different groups of samples analyzed over 8 years. All assays between 1984 and 1990 were performed by using the original GlycoTest columns; data from 1991 were derived from assays with GlycoTest II columns (GlycoTest II assay kits replaced the original GlycoTest kits). As expected, there were substantial differences between the GHb and HbA1c values, especially in the range found in diabetic patients (>6% HbA1c by HPLC). Furthermore, the affinity chromatography values tended to more closely agree with the RL HPLC values over time (1984–91).

In Figure 4 we show regression analyses between GHb values obtained by uncalibrated assays and values for the Diamat HPLC. The five different methods used included two affinity chromatography minicolumn methods, an affinity HPLC method, an HbA1c electrophoresis method, and an HbA1c electrophoresis method. The Abbott Vision Glycated Hb method and the Miles DCA 2000 immunoassay method are both calibrated internally and display only calibrated values. Although there is good agreement in absolute values for the two affinity chromatography methods, there are large differences in values among other methods. For example, a patient with an HbA1c value of 10% by the Diamat method could be reported as having 10.3% total GHb by the Pierce GlycoTest II method or 14.2% HbA1 by REP electrophoresis.

Relation between Calibrated GHb Methods and RL HPLC and Diamat HPLC Methods

Figure 5 shows regression analyses between GHb values from the calibrated affinity chromatography method (the same six groups of samples as shown in Figure 3) and values from either the RL HPLC method or the Diamat HPLC method. There is considerably less variability in GHb values obtained by affinity chromatography between the six groups of samples and better comparability with the reference methods.

In Figure 6 we show regression analyses between GHb values from seven different calibrated methods and the Diamat HPLC. An HbA1c value of 10% by the Diamat HPLC would measure between 9.5% and 10.2% by the other standardized methods, a substantially narrower range (0.7% GHb) than the range obtained by using uncalibrated values (4.2% GHb).
Interassay Imprecision with and without Calibration

Figure 7 shows a comparison of interassay imprecision, with and without calibration, for those GHb methods for which uncalibrated values were available. The imprecision estimates for the REP and Diatrac electrophoresis methods and the Isolab affinity chromatography method reflect imprecision over a relatively short period of time (∼5 months). In contrast, the imprecision estimates for the Pierce affinity method reflect a much longer time (5 years) and include not only different reagents and column lots but two different affinity resin formulations (GlycoTest I and GlycoTest II). Long-term precision is improved substantially by calibration; e.g., CV = 10.1% vs 4.3% for uncalibrated vs calibrated assays when the low-concentration QC samples are used (see Figure 7). Imprecision estimates for the two affinity minicolumn methods over a comparable period (e.g., 5 months) and more similar conditions are more comparable, with CVs all <5%. Precision for the Primus CLC300 HPLC instrument was excellent (CV <3%); even uncalibrated assay precision is excellent, presum-
ably because calibration occurs within method. CVs for the Diamat, Vision GHB, and DCA 2000 were <3%.

Discussion

At present, it is estimated that routine determinations of GHB are performed on only a small percentage of diabetic patients. The American Diabetes Association has recommended routine use of the test in all patients with diabetes: first to document the degree of glycemic control at initial assessment, then as part of continuing care (16). Currently, however, the clinical usefulness of GHB measurements is limited by variability of the results between laboratories and between assay methods. This variability may be almost 10% (7). Because patients may obtain care in various clinical settings, each of which may use different laboratories or assay methods, the patient and physician are often presented with an array of results that can only obfuscate determination of the level of glycemia. Recent survey data from the College of American Pathologists confirm that a wide variety of GHB methods are being used and that interlaboratory variability is quite large (7).

Most practitioners (and patients with diabetes) do not know how to relate their own laboratory’s GHB results to average blood glucose concentration. Because there is no standardization, results in the literature relating blood glucose and GHB concentrations (17) cannot be used to help the medical practitioner and diabetic patient interpret the GHB test results, except in a very general way.

A few commercially available methods include some type of within-method calibration. For example, one manual method (Bio-Rad HbA1c column test) includes three concentrations of hemolysates, called HbA1c calibrators, in each assay: the calibrator values are assigned by the manufacturer for that particular method at 24 °C. Some newer automated methods, such as those described in this study, either provide calibrators or have an internal calibration curve. However, several of the most widely used methods do not include calibrators.

A major obstacle to national standardization has been the lack of appropriate standard materials. Most commercially available QC materials do not give representative results with all methods (7). Compounding this problem is the fact that different methods may measure different GHB fractions (e.g., HbA1c vs HbA1c vs total GHB). Even when all methods are designed to measure the same component, e.g., HbA1c, the specificity of the assays is not constant. Another obstacle to standardization has been the lack of a consensus on an appropriate reference method and measured component.

The National Reference System for the Clinical Laboratory (NRSCL) has as its objective “the development and maintenance of a system for clinical laboratory measurement in which the results from all clinical laboratories are compatible with each other and with high quality medical needs” (18) or, restated, “to assure that one may assume that the results from any clinical laboratory are interchangeable with those of any other” (19). The NRSCL has been successful in achieving standardization of several clinical analytes. The basic approach of this organization is to recognize a hierarchy in the accuracy of the methods it approves for assigning values to reference materials (Definitive and Reference Methods), as well as a hierarchy in the reference materials used (from pure, certified substances to patient-specimen-simulating materials). The process of accepting proposed reference methods and materials involves review by the NRSCL Council and approval, which is based on the scientific merit of the proposal as well as consistency with NRSCL guidelines.

Here we have demonstrated a practical approach to calibrating measurements of GHB. The high precision of the RL HPLC method we used for measuring HbA1c has been well documented over the long term (12). We chose crude hemolysates as our calibration material because of the lack of availability of pure material. The materials chosen, although not chemically defined, can be analyzed by the RL HPLC method and provide results comparable with those for patients' samples in all of the comparison methods.

As a first step toward national standardization of GHB measurements, we are proposing the RL HPLC method, used in the present study to assign calibrator values, as a candidate reference method for GHB measurement. The primary reference standard would consist of highly purified glycated HbA1c and nonglycated HbA0. This highly purified material would then be used to calibrate the Reference Method, which would be performed in a few approved laboratories. Approved laboratories would assign values to calibrators (such as the materials used in the present study). These secondary calibrators, with Reference Method-assigned values, would be available to all laboratories performing GHB measurements. In accordance with the goals of the NRSCL, one advantage of the procedure we are proposing is that laboratories would not have to change methods to standardize their assays to the Reference Method.

There are a few potential difficulties, both political and technical, with this approach to standardization. For example, various groups might have a vested interest in a particular method and may resist standardization, hoping that their particular method would be selected as the only accepted method. Other groups may think that some method other than the one described here may be more appropriate as a reference method or that HbA1c may not be the appropriate reference analyte. It is also possible that one of the new GHB methods may prove to be a superior candidate reference method. We chose HbA1c as the reference analyte because, at present, it is the most chemically specific GHB component we can measure.

Another potential problem is that the linearity of the relationships between total GHB vs HbA1c vs HbA1c has not been proved. Some reports suggest that these relations may not be linear, especially at high values for GHB (20–22). However, this deviation from linearity, if real, does not appear to be clinically significant.

In the present study, we used calibration methods
that allowed for direct comparison of GHb measurements between laboratories and between methods. Such a system of standardization, in which measurements were compared with those obtained by an accepted HPLC method, could improve the surveillance of assay quality and facilitate the development of goals for uniform patient care and glycemic control in diabetes.

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References

10. Svendsen PA, Christiansen JS, Soegaard U, Welinger BS, Nerup J. Rapid changes in chromatographically determined hae-