Interlaboratory Standardization of Glycated Hemoglobin Determinations

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As the clinical utility of glycated hemoglobin (gHb) measurement increases, so does the need for standardization of values between different methods and different laboratories. Using three different methods, we examined the feasibility of interlaboratory standardization of gHb measurement. A liquid-chromatographic (HPLC) system from our research laboratory was designated the reference method. For gHb standards we used erythrocyte hemolysates prepared from blood samples from nondiabetic and diabetic subjects. Values assigned to each standard were based on the mean of multiple gHb determinations by the HPLC method. A clinical laboratory routinely prepared hemolysates and gHb by commercially available ion-exchange ("mini column") and affinity chromatographic methods. For each assay a standard curve was constructed and gHb values were derived from these curves. Samples analyzed in the clinical laboratory were also analyzed in the research laboratory and the curve-derived values were compared with the HPLC-measured values, to determine the accuracy of our interlaboratory standardization procedure. Correlations were excellent (r = 0.99). The lack of significant differences between calculated and HPLC-measured values indicates that interlaboratory standardization is feasible.

Additional Keyphrases: glycohemoglobin · diabetes · quality control · chromatography: liquid, ion-exchange, affinity

Determination of glycated hemoglobin (gHb) is considered a useful index of long-term glycemic control, and the test is now routinely used to monitor diabetic control.1 Unfortunately, because each of the wide variety of assay methods available measures gHb in a slightly different way, the data generated in one laboratory are difficult to relate to those generated in another. For example, at one laboratory a report that 9% of the total hemoglobin is gHb might indicate that the concentrations of glucose in blood have been near the normal range, whereas a result of 9% gHb at another laboratory might indicate that, on the average, the glucose concentrations were high. The diversity of methods, combined with the lack of a stable standard for use with many types of assays, has made it difficult to carry out multicenter studies, and to compare results directly from studies carried out at different locations, even when the same assay method is used at each location. The need for a standard has, in fact, been considered a major priority by the NIH Diabetes Data Group (1).

We have previously reported the development of a "high-performance" liquid-chromatographic system (HPLC) for determination of gHb that offers good long-term assay precision (2, 3) and yields results that correlate well with those by various other assay methods used in our laboratory (4, 5). Here we present data showing the feasibility of standardizing gHb determinations between two laboratories. We used our HPLC system as the reference method and distributed frozen hemolysates of pooled diabetic and nondiabetic blood specimens as standards to a second laboratory that used two different assay methods.

Materials and Methods

Blood samples. The 333 blood samples used in this study were part (20%) of a larger group of samples routinely submitted for glycated hemoglobin measurement to the Clinical Chemistry Laboratory of the University of Missouri Hospital and Clinics ("Lab I") during three years. Specimens were collected by venipuncture into EDTA-containing evacuated blood-collection tubes and kept at 4 °C before and during transport to the laboratory within 24 h of collection.

Hemolysate preparation. Sample hemolysates were prepared as previously described (2). Briefly, packed erythrocytes were washed and incubated overnight (37 °C) in isotonic saline, to facilitate removal of the labile aldime components, then lysed with water. Hemolysates were incubated overnight at 4 °C with an equal volume of carbon tetrachloride. The aqueous supernate was centrifuged (2 000 × g, 30 min, 4 °C) and stored at −70 °C until assay.

HPLC assay. A semi-automated HPLC ion-exchange method (the "reference method") was used to measure gHb (as %HbA1c) in all samples. The assay was performed in the University of Missouri Juvenile Diabetes Research Laboratory ("Lab II"). The chromatographic apparatus consisted of a Laboratory Data Control Analyst Series 7800 HPLC system (Milton Roy Co., Riviera Beach, FL 33404), with a pulse-free Consta Metric I1G pump (Milton Roy Co.) and a Model 7125 (Rheodyne Inc., Cotati CA 94923) sample-injection valve. The flow cell monitor was a Laboratory Data Control UV monitor III (Model 1203) with a fixed 410-nm filter. Absorbance was recorded by a Model 3402 Laboratory Data Control dual-pen recorder. The 9 × 250 mm glass column contained Bio Rex 70 <400-mesh ion-exchange resin (Bio Rad Labs., Richmond, CA 94804) and was maintained at a constant temperature of 22 °C. The two-buffer step-gradient HPLC assay has been described in detail previously (3).

We chose this particular method as the reference method because of its previously demonstrated high long-term precision (6). During the period of the study, the interassay CV for this method was 1.79% and 4.9% for diabetic and nondiabetic controls, respectively. The normal range (mean ±2 SD) for gHb by this method, which has been in continuous operation for the past seven years, is 5.42% ± 0.68% (3). Details of the HPLC calibration and quality-control procedures have been described previously (6).

Ion-exchange mini-column assay. Total "fast" glycated
hemoglobin, HbA1 (HbA1c+HbF), was measured in Lab I with a commercially available ion-exchange mini-column kit (Isolab, Inc., Akron, OH 44321). Each hemolysate sample (prepared as above) was diluted threefold with the diluting fluid provided in the kit; 50 μL of the diluted hemolysate was applied to each column and assayed according to the manufacturer's instructions. Assay temperature was precisely controlled with a water jacket and a constant-temperature water bath set at 22°C. The interassay CV was 5.1% and 6.6% for diabetic and nondiabetic controls, respectively.

Affinity chromatography assay. The coplanar cis-diol groups of gHb were measured in 38 of the above samples by Lab I, with use of a commercially available affinity chromatography assay kit (Glyco-Gel test kit; Pierce Chemical Co., Rockford, IL 61105). All samples were assayed in duplicate. The interassay CV for this method was 4.83% and 4.96% for diabetic and nondiabetic controls, respectively.

Standardization and data analysis. gHb "standards" at four different concentrations spanning the desired test range were prepared in Lab II in the same manner as the clinical samples from blood obtained from diabetic and nondiabetic volunteers. Standards were divided into small aliquots, color coded, and stored at -70°C for use by both participating laboratories.

Each standard was analyzed by HPLC in Lab II at least 12 times to establish the concentrations of HbA1c; these were designated the "standard values." Moreover, these standards were assayed routinely by HPLC throughout the study to document the stability of the standard during storage and the high precision of the reference method over time (6). Three or four of these standards were included in each ion-exchange mini-column and affinity chromatography assay performed by Lab I.

Standard curves based on linear regression analysis of results by the ion-exchange mini-column and affinity chromatography assays were prepared. The value of a sample obtained by comparison with the linear regression curve was reported as calculated gHb. Randomly selected samples were also analyzed by HPLC to obtain an actual measured value for HbA1c (designated "HPLC-measured gHb") for each sample. We then compared the calculated gHb with the HPLC-measured gHb value, to determine the accuracy of our standardization procedure.

Results

As shown in Figure 1, the calculated gHb values (mini-column), and the HPLC-measured gHb values for samples analyzed over a three-year period was excellent. The mean values (Table 1) were nearly identical (mean difference = 0.009% gHb). When we separated results into diabetic and nondiabetic groups, based on the normal reference interval of our reference method (mean ± 2 SD), we found that the small difference between paired calculated gHb and HPLC-measured gHb values was statistically significant in the normal range (p < 0.01, paired t-test) but not in the diabetic range (p < 0.26).

The relationship between calculated gHb values from the affinity chromatographic method (y) and the HPLC-measured gHb values (x) was similar; \( y = 1.05x - 0.51 \) (r = 0.99, \( p < 0.0001, S_y = 0.51 \)). Again, mean values were nearly identical (mean difference = 0.11% gHb). There was no significant difference between paired HPLC-measured and calculated gHb values (p < 0.20).

Discussion

For optimal clinical usefulness of measuring gHb, we believe that an internationally recognized reference method or reference material must be developed. At present, however, there is neither. Results of currently available methods usually correlate well (4, 5) even though they may measure different gHb species. For example, results from HPLC (HbA1c), HbA1 ion-exchange mini-columns, thiobarbituric acid colorimetry (ketamines), and affinity chromatography (total glycated species, including glycated HbA1) show excellent intercorrelation, although each measures a different array of glycated hemoglobin components. From this we conclude that any of these methods alone will give clinically useful gHb results if the methods are carefully controlled and precision is adequate. The problem arises when results from laboratory A are to be compared with results from laboratory B. The comparison is especially difficult if, e.g., laboratory A results are expressed as nanomoles of hydroxymethylfurfural per 10 mg of Hb and laboratory B results are expressed as %HbA1c. In the few interlaboratory comparisons reported (7, 8), results appear to depend on the design of the study and the methods being compared. Absolute values for gHb, even when measured by similar methods, cannot be directly compared.
Here we have shown the feasibility of standardizing gHb measurements by using the HPLC method as a reference method, with hemolyzate standards prepared from whole-blood samples. Probably any precise method can be a suitable reference method, and numerous other methods can be used to prepare standards. We chose our HPLC method because of its demonstrated high degree of long-term precision (6).

Ideally, a standard should be a highly purified material. As others have shown, however, use of a pure reference standard does not always eliminate interassay variability (9). In ferritin determinations, for example, variability was in part due to the heterogeneity of the protein itself and to the differing sensitivities of each method to the various ferritin species (9). gHb presents a similar problem in that different gHb assays measure different gHb species (e.g., HbA1c, HbA1b, coplanar cis-diol groups, ketoamines). It would be difficult to prepare a combination of purified gHb's for use with all gHb assays.

We obtained excellent results by using crude hemolyzate standards with proper long-term storage conditions (−70 °C). Even though each of the three methods used here measured a different combination of gHb components, the standardized values were remarkably close to the actual measured HPLC values. We conclude from this that each of the components measured increases in proportion to the others over the range of concentrations studied. For the inter-exchange chromatographic method, the standardized values were slightly lower than the HPLC values only in the normal range; although statistically significant, these small differences were not clinically significant. Thus, in contrast to values in the diabetic range, in the normal range the different gHb species may not all increase proportionately. Additional trials applying the standardisation procedure described here need to be conducted with other gHb methods such as agar-gel electrophoresis and thiobarbituric acid colorimetry.

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References