Calibration of ion-exchange HPLC measurements of glycohemoglobin: effect on interassay precision

Erik Gerlo* and Frans Gorus

To investigate the effect of calibration with lyophilized calibrators on the interassay precision of glycohemoglobin (glyHb) measurements, we used an ion-exchange HPLC system equipped with a Pharmacia Mono S® HR 5/5 column. Calibration of analytical runs substantially increased interassay variation (CV), from 1.7% to 4.4% and from 0.9% to 3.2% for control samples with low (6.5%) and high (14%) glyHb percentages, respectively. Standardization of glyHb results, though essential for interlaboratory comparisons, should not be done at the expense of assay precision, as may occur with thoughtless use of lyophilized calibrators. We therefore recommend the use of carefully determined conversion factors for standardization of glyHb results obtained with ion-exchange HPLC systems that are capable of excellent long-term interassay precision.

The Diabetes Control and Complications Trial (DCCT) confirmed the direct relationship between the degree of glycemic control, as estimated by glycohemoglobin (glyHb) determinations, and the development and progression of long-term complications such as retinopathy, nephropathy, and neuropathy in insulin-dependent diabetes mellitus (IDDM) [1]. On the basis of these findings, it was recommended that “most patients with IDDM be treated with closely monitored intensive regimens, with the goal of maintaining their glycemic status as close to the normal range as is safely possible” [1]. Measurement of glyHb is considered the most reliable index of average blood glucose concentration during the preceding 6 to 8 weeks [2]. GlyHb is a heterogeneous group of molecules, formed by nonenzymatic reactions of hemoglobin (Hb) with sugars. The most widespread assay methods are based on ion-exchange HPLC, affinity chromatography with phenylboronate matrices, electrophoretic separation, or immunoassays that utilize monoclonal antibodies directed to the N-terminal peptide of the β-chain [3, 4]. Because these different assays measure different fractions of glyHb, the results are not interchangeable between methods [5]. Also, for a given chromatographic method, the glyHb results can be affected by numerous factors, e.g., type of stationary phase, column dimensions, mobile phase, and the integration algorithm. Standardization of glyHb results is therefore strongly recommended [6] and is achievable among a wide variety of assay methods [7].

Excellent assay precision is also a prerequisite to meaningful glyHb measurements. Although the National Diabetes Data Group Expert Committee’s recommendation that assays of glyHb should have CVs of <5% [8] is still widely accepted, more-stringent analytical precision goals have been proposed, based on biological variation or clinical relevance [9–11]. Calibration has been shown to improve the interassay precision of glyHb measurements made by affinity chromatography, a method sensitive to changes in gel or buffer properties and subject to substantial lot-to-lot variability [12]. However, the effect of calibration on ion-exchange HPLC procedures, which are capable—without calibration—of excellent long-term precision (with interassay CVs <3%), has not been adequately studied so far. The aim of the present study was to evaluate the effect of standardization with commercially available calibrators on the between-run precision of glyHb measurements made with an HPLC system equipped with a Pharmacia Mono S® HR 5/5 column (Pharmacia-LKB Technology).

Materials and Methods

ASSAY METHOD
An automated gradient ion-exchange method, adapted from the method described by Stenman et al. [13], was used to measure glyHb in all samples. Buffer A (low ionic strength, pH 6.5) contained NaH₂PO₄ (18 mmol/L), Na₂HPO₄ (3 mmol/L), and 0.25 g of KCN (4 mmol/L); buffer B (high ionic strength, pH 6.3) contained NaH₂PO₄...
(260 mmol/L) and Na₂HPO₄ (120 mmol/L). The chromatographic apparatus consisted of a Model 305 pump combined with a Model 306 pump, a Model 805 monometric module, and a Model 811B dynamic mixing chamber (all from Gilson). The flow rate was 1.4 mL/min. The volume percentage of buffer B was varied as follows: linearly increased from 0% to 3% (0–0.2 min), held at 3% (0.2–2 min), linearly increased to 7% (2–4 min), held at 7% (4–6 min), linearly increased to 40% (6–9 min), held at 40% (9–10 min), and then linearly decreased to 0% (10–11 min). Samples (20 μL) were injected at 20-min intervals by use of a WISP Model 712 autosampler with a refrigerated (4 °C) sample compartment (Waters Associates). The Mono S HR 5/5 column was maintained at 37 °C. The eluent was monitored with a Uvicord SD detector (time constant = 1 s) with use of a 405-nm interference filter (Pharmacia-LKB Technology). Integration was performed with a Model SP4270 integrator (Spectra Physics). The baseline was drawn forward horizontally as illustrated in Fig. 1. The measured glyHb values (glyHb meas) correspond to the Hb A₁c peak area, expressed as a percentage of the total peak area:

\[
glyHb\text{meas} (%) = 100 \times \frac{\text{Hb A₁c peak area}}{\text{total peak area}}
\]  

Within-run and total SDs, determined according to the ANOVA procedure recommended by NCCLS [14], were respectively 0.11% and 0.12% at a mean glyHb content of 6.5%, and 0.08% and 0.12% at a mean glyHb of 14%.

QUALITY-CONTROL SAMPLES

Two control samples with low (<7%) and high (>12%) glyHb values were prepared. Erythrocytes (2 mL) were washed three times with 9 g/L NaCl and lysed in water (1 mL). Cell debris was removed by delipidation with CCl₄ (0.8 mL). The clear erythrolysate was diluted 1:31 with acid phosphate buffer (25 mmol/L NaH₂PO₄) adjusted to pH 4.85 with phosphoric acid, plus 4 mmol/L KCN). After 2 h at room temperature to remove the labile adduct, small, single-use aliquots were stored at −70 °C for long-term study. Under these conditions, the controls were stable for at least 1 year, as judged by the reproducibility of the measured glyHb values: Typical between-run CVs determined over a 19-month period (n = 280) were 2.0% and 1.2% for samples with mean glyHb values of 6.4% and 13.6%, respectively. Because the same procedure (except for storage at −70 °C) is used in our laboratory for preparation of hemolysates from routine patients’ samples, we considered the data obtained for the precision of the glyHb measurements on the control samples to be representative of the precision of the measurement of real patients’ samples.

CALIBRATORS

Vials containing lyophilized hemolysates with low, medium, and high glyHb content (batch no. 9545; assigned values of 4.6%, 7.7%, and 10.8% glyHb, respectively) were obtained from the Stichting Kwaliteitsbewaking klinisch chemische Ziekenhuis Laboratoria (SKZL, Winterswijk, The Netherlands) and stored at −20 °C. The SKZL calibrator values are assigned with the HPLC method used in the DCCT study. Vial contents were reconstituted with 200 μL of demineralized water and then diluted with 6 mL of acid phosphate buffer and extracted with CCl₄ (2.4 mL). The diluted, delipidated solutions were stored at −70 °C as small, single-use aliquots (n = 23).

We chose to use stored frozen aliquots for the daily calibrations, rather than the reconstituted fresh material recommended by the manufacturer, for the following reasons: (a) Storage at −70 °C has been successfully used for calibrators or quality-control samples for glyHb measurements in previous studies [3, 12]; (b) erythrocyte hemolysates treated and stored in the same way showed excellent long-term stability (see above); (c) daily reconstitution of lyophilized material would substantially increase the cost per test and would not be feasible in routine practice.

CALIBRATION PROCEDURES

Calibrators were included in every run during 3 months (2 runs per week), and for each daily run a calibration curve was constructed by linear regression analysis, with the assigned calibrator values on the x-axis and the measured glyHb values on the y-axis:

\[
y (%) = a \times x (%) + b
\]

where a corresponds to the slope and b to the intercept of the daily calibration curve.

Standardized glyHb values for the control samples were calculated from the measured values (glyHb meas) by two different procedures:

1. Daily calibration procedure,

\[
glyHb\text{cal} = (\text{glyHb meas} - b)/a
\]

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\]
in which $\text{glyHb}_{\text{meas}}$ values are standardized against the daily calibration curve to yield calibrated glyHb values ($\text{glyHb}_{\text{cal}}$), and a conversion factor procedure,

$$\text{glyHb}_{\text{conv}} = (\text{glyHb}_{\text{meas}} - \beta) / \alpha,$$  \hspace{1cm} (4)

in which $\text{glyHb}_{\text{meas}}$ values are converted to standardized values ($\text{glyHb}_{\text{conv}}$) by using the mean values of the slope ($\alpha$) and intercept ($\beta$) of the 23 daily calibration curves (Table 1). The CV determined for the converted values ($\text{CV}_{\text{conv}}$) is related to that for the measured values ($\text{CV}_{\text{meas}}$) by the equation

$$\text{CV}_{\text{conv}} = \text{CV}_{\text{meas}} 	imes (M - \beta) / M,$$ \hspace{1cm} (5)

where $M$ is the mean of the measured glyHb values and thus is readily predictable (Fig. 2).

STATISTICAL METHODS

One-sample $t$-tests (2-tailed) were used to compare the mean measured values for the SKZL calibrators with their assigned values. The results for the control samples obtained with the two calibration procedures were compared by Student’s paired $t$-test (2-tailed). The variances of the glyHb results obtained without calibration were compared with those obtained with the different calibration procedures by use of the $F$-test. Linear regression analysis was used to construct calibration curves and to evaluate the stability of the stored calibrator aliquots.

Results

Table 1 summarizes the data of the daily calibrations. Linear regression analysis of the values measured for the three calibrators and of the slopes and intercepts of the calibration curves as a function of time did not reveal any statistically significant drift during the time period considered ($r^2 < 0.03$). The absence of significant changes for the calibrator values and for the slope and intercept of the calibration curves suggests that the chromatographic properties of the Hb molecules are not affected by storage at $-70 \degree C$ or by the freezing and thawing process, and justifies the use of frozen aliquots instead of freshly reconstituted material for the daily calibrations.

The mean measured value for the medium-content calibrator corresponded closely to the assigned value ($P = 0.26$ by one-sample $t$-test). The measured values for the low- and high-content values differed significantly ($P < 0.0001$) from their assigned value. This resulted in calibration curves with slopes significantly different from 1 and a marked intercept effect. The mean values of the slopes (0.66) and intercepts (2.54%) of the daily calibration curves were used as the $\alpha$ and $\beta$ coefficients to calculate standardized glyHb values for the control samples by the conversion factor procedure (Eq. 4).

Table 2 summarizes the results for the control samples, as measured without calibration and as derived by the daily calibration and the conversion factor procedures. The glyHb values obtained with the two calibration procedures were not significantly different ($P > 0.8$ by paired $t$-test). However, the daily calibration procedure produced significantly greater assay imprecision: the CV rose from 1.7% to 4.4% for the low-content sample, and from 0.9% to 3.2% for the high-content control sample ($P < 0.001$). The CVs obtained with the conversion factor procedure differed only slightly—as predicted by Eq. 5—from those obtained without calibration ($P > 0.05$).

| Table 1. Interassay precision of calibrator measurements and calibration curves$^a$. |
|---------------------------------|-----------------|-----------------|
| **SKZL calibrator, % glyHb**   | **Calibration curve$^b$** |
| **Low** | **Medium** | **High** | **Slope** | **Intercept, % glyHb** |
| Assigned value | 4.6 | 7.7 | 10.8 | 0.66 | 2.54 |
| Mean measured value | 5.56 | 7.65 | 9.65 | 0.02 | 1.7 |
| SD | 0.12 | 0.21 | 0.19 | 3.7 | 6.8 |
| CV, % | 2.2 | 2.8 | 2.0 | 0.0093 | 0.004 |
| $r^2c$ | 0.029 | 0.007 | 0.009 | 0.0003 | 0.004 |

$^a$ From 23 determinations over a 3-month period.

$^b$ Measured value (% glyHb) - slope $\times$ assigned value (% glyHb) + intercept.

$^c$ Coefficient of determination of linear regression analysis as a function of time.
Table 2. Interassay precision of noncalibrated and calibrated glyHb measurements of control samples*.  

<table>
<thead>
<tr>
<th>Calibration procedure</th>
<th>None†</th>
<th>Daily calibration procedure</th>
<th>Conversion factor procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low control sample</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean value, %glyHb</td>
<td>14.03</td>
<td>17.44</td>
<td>17.42</td>
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<tr>
<td>SD, %glyHb</td>
<td>0.13</td>
<td>0.56</td>
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<tr>
<td>CV, %</td>
<td>0.9</td>
<td>3.2</td>
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<tr>
<td><strong>High control sample</strong></td>
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<td></td>
</tr>
<tr>
<td>Mean value, %glyHb</td>
<td>6.50</td>
<td>6.01</td>
<td>6.00</td>
</tr>
<tr>
<td>SD, %glyHb</td>
<td>0.11</td>
<td>0.26</td>
<td>0.17</td>
</tr>
<tr>
<td>CV, %</td>
<td>1.7</td>
<td>4.4</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*See text for descriptions of the daily calibration procedure (Eq. 3) and the conversion factor procedure (Eq. 4).
†From 23 determinations over a 3-month period.
‡glyHbmean(%) = 100 × Hb A1c peak area/total peak area.

Discussion

In the absence of a primary reference material and a definitive reference method, both the AACC Subcommittee on Glycohemoglobin Standardization and the IFCC Working Group on Standardization of Hb A1c propose that the chromatographic method used in the DCCT as an interim "designated comparison method," against which other methods should be standardized. The development of conversion factors for the standardization of glyHb assays to the DCCT method is best based on fresh sample comparisons, as recommended by the US National Glycohemoglobin Standardization Program. Method comparison with use of lyophilized materials is not recommended, as these materials may have matrix effects and behave differently from patients' specimens in analytical processes. Artifacts caused by denaturation during preparation or storage could change physicochemical properties of the hemoglobin molecules, resulting in altered reaction kinetics in immunological reactions or disturbed patterns in chromatographic or electrophoretic methods. Weykamp et al. [7] investigated the effect of calibration with lyophilized calibrators on whole-blood glyHb results in 103 laboratories that were using 20 different methods. Deviating results, indicating possible matrix effects, were observed for two affinity methods (Abbott Vision Test and Abbott IMx1c Test; Abbott Labs.). Results from other methods, including the Pharmacia ion-exchange method used in this study, were consistent and demonstrated the feasibility of standardization with lyophilized calibrators. The use of lyophilized calibrators with these methods can thus be considered a pragmatic approach (though less ideal than split-sample comparisons) to the standardization of glyHb results in terms of the DCCT method.

Previous studies [3, 7] clearly demonstrated that calibration of glyHb measurements decreased intermethod and interlaboratory variations. The effect of calibration on the intralaboratory precision for different assay methods is less well documented, and the results of such studies have not always been concordant. Affinity methods appear to benefit most from calibration. Bodor et al. [12] obtained CVs of 3.2–7.9% and 1.5–5.0% for the GlycoTest 228 (Pierce Chemical Co.) before and after introduction of calibration, respectively. Little et al. [3] demonstrated a substantial improvement by calibration for the long-term precision of the same affinity test: CV = 10.1% vs 4.3% for the uncalibrated vs the calibrated assays. The precision of the Diatrac 228 electrophoresis method (Beckman Instruments) was not affected over a 5-month study period: CVs were ~5% for both uncalibrated and calibrated assays. Weykamp et al. [19] evaluated the effect of calibration on the dispersion of glyHb measurements in 111 laboratories that used 21 methods. The most striking decrease in intralaboratory variation was observed for the modified Pierce affinity method with mean intralaboratory CVs of 8% vs 2% for uncalibrated vs calibrated assays. Other methods that showed a statistically significant, though less pronounced, decrease in mean intralaboratory CV were the Diatrac 228 electrophoretic method (from 11% to 6%), and the group of the Pharmacia Mono S HR 5/5 column users (from 6% to 3%).

Ion-exchange HPLC assays of glyHb have shown excellent long-term assay precision. Between-run CVs from 0.7% to 2.5% have been reported for systems equipped with the Pharmacia Mono S HR 5/5 column [15–17]. In our laboratory, interassay CVs for noncalibrated glyHb values have consistently been <3% since we started using the Mono S column in 1991. Such a high precision is essential for meaningful diabetes monitoring, especially in tightly controlled patients [9–11, 18].

The use of calibrators for glyHb measurements (as a percentage of total Hb) introduces additional variables to the assay procedure. It will decrease interassay precision only in procedures that are subject to considerable between-run variation, as is the case for affinity chromatographic methods that show significant matrix lot-to-lot variability [12]. The use of calibrators will inevitably increase the interassay variation of procedures with low between-run variance. In our study, the between-run CV increased from 1.7% to 4.4% for a mean measured glyHb value of 6.5%, and from 0.9% to 3.2% for a mean measured value of 14.0%, i.e., to a value exceeding that required for meaningful monitoring. Although lower CVs for the calibrated glyHb values might perhaps have been obtained if triplicate or duplicate measurements of the daily calibrators had been made, the replicate calibrator measurements would have increased the total analysis time and thus were not considered in the present study. We also note that a single batch of calibrators was used in this study; long-term precision is likely to deteriorate even further when different batches of calibrators are used. The procedure of including calibrators in every analytical run is therefore not recommended for high-precision ion-exchange glyHb assays such as the one used in this study.

However, harmonization is essential when results from different laboratories are to be compared, even if these
laboratories use similar ion-exchange HPLC methods. Indeed, the glyHb results obtained with ion-exchange HPLC methods may be influenced by many assay variables, e.g., mobile-phase composition, flow rate, gradient profile, protein load, column length, temperature, detection wavelength, and last but not least, baseline allocation procedure. Harmonization of such methods can be achieved by the use of conversion factors. The increase of assay imprecision (as estimated by the CV) associated with the use of conversion factors depends on the glyHb percentages present and the intercept effect observed in the correlation between measured and assigned calibrator values (β in Eq. 4), and is readily predictable (Eq. 5). With the described HPLC procedure (β = 2.5%), the CVs for the converted values were less than the critical 3% value for control samples with normal and above-normal glyHb contents (Table 2). The increase of imprecision will be more (less) important for procedures with a larger (smaller) intercept effect, as illustrated in Fig. 2. The data in Fig. 2 allow each laboratory that is considering the use of conversion factors to evaluate whether the increase in imprecision is acceptable or not. This ultimately depends on the intrinsic assay imprecision, i.e., the CV of the measured values.

In conclusion, because thoughtless use of a daily calibration procedure may be associated with a significant and unacceptable increase in imprecision, we recommend the use of carefully determined conversion factors for the standardization of glyHb measurements by methods with excellent long-term reproducibility (i.e., interassay CVs ≤ 2%) and a small to moderate intercept effect (β ≤ 2.5% glyHb). The effect on interassay precision for such procedures can be readily predicted from Fig. 2. Conversion factors should ideally be determined by using fresh blood samples for comparisons. Alternatively, the conversion factors may be obtained from replicate measurements (n ≥ 20) of lyophilized materials (n ≥ 3), provided this material is guaranteed to be free from matrix-effects in the given assay procedure.

References