Three Assays for Glycohemoglobin Compared

Ursula Turpeinen, Ulla Karjalainen, and Ulf-Håkan Stenman

Using 123 specimens, we compared the concordance of three different methods for determining glycohemoglobin (GHB): the Diamat™ (Bio-Rad Laboratories), an automated analyzer measuring HbA1c by cation-exchange chromatography; an assay with the IMx analyzer (Abbott Laboratories), based on boronate affinity binding; and an HPLC method measuring HbA1c by cation-exchange chromatography on a PolyCAT A column (PolyLC Inc.). The Pearson's correlation coefficient between PolyCAT A and Diamat was 0.900 ± 0.038 (mean ± 2 SD) and between PolyCAT A and IMx, 0.857 ± 0.042. However, up to twofold differences were seen in some samples. The proportion of GHB was consistently lower with the PolyCAT A method than with the other two assays, apparently because of better separation of HbA1c from nonglycated eluting forms of Hb. The difference in glycation percentage between the PolyCAT A and Diamat methods is 2–3% over the whole concentration range. These results point to the limitations of Diamat as a reference method to be used to calibrate other methods for determining HbA1c. Further, a switch from one method to another is likely to cause considerable problems in the clinical follow-up of certain patients.

Indexing Terms: HPLC/cation-exchange chromatography/boronate affinity binding assay

The percentage of glycohemoglobin (GHB) reflects blood glucose concentrations of the previous 2–3 months, and is therefore considered a valuable indicator of long-term diabetic control (1). However, the methods currently used for its measurement in clinical chemistry laboratories show large differences between reported values, and comparison of results from different laboratories is difficult (2). Various methods differ in their sensitivity to interferences from the labile intermediate of GHB, fetal Hb, and other Hb variants such as adducts formed between Hb and urea, aldehydes, or acetylsalicylic acid (3, 4). Lack of standardization of GHB measurements remains a source of interlaboratory variation. At present there is no accepted standard or acknowledged reference method. Recently, calibration based on a cation-exchange HPLC method has been shown to increase the comparability between various analytical methods (5, 6). This principle has also been used to calibrate some recently developed nonchromatographic methods based on boronate affinity binding (7) and immunoassay (8).

In this study we compare our own high-resolution HPLC cation-exchange method (PolyCAT A) with two other assays: a boronate affinity binding assay (IMx) and an automated system for GHB analysis by cation-exchange chromatography (Diamat™).

Materials and Methods

Preparation of Samples

Blood samples were drawn into evacuated tubes containing EDTA as an anticoagulant. For the PolyCAT A analysis the blood cells were sedimented by centrifugation at 1000g for 10 min at room temperature and washed three times with 0.15 mol/L NaCl. One volume of packed red cells was lysed by addition of one volume of distilled water. To remove the labile fraction of GHB, the sample was diluted 40-fold with 25 mmol/L sodium phosphate buffer, pH 4.8, and incubated for 15 min at 37°C. The samples were then diluted 10-fold with buffer A (see below) and analyzed within 5 h.

For the IMx method (Abbott Laboratories, N. Chicago, IL) EDTA blood was used without pretreatment. Before analysis with the Diamat analyzer, 5 μL of the whole-blood sample was diluted with 1.25 mL of hemolysis reagent (Bio-Rad Laboratories, Munich, Germany), 1 mL/L polyoxymethylene ether in a borate buffer, and incubated at 37°C for 30 min to remove the labile fraction.

In vitro acetylation of Hb was performed for 1 h at 37°C as previously described (4).

Samples

For method comparison we used 123 blood samples obtained mainly from diabetics sent to our routine laboratory for HbA1c analysis. Reference values for the Diamat and PolyCAT A methods were determined by using 60 freshly drawn blood samples from healthy controls who were considered nondiabetic on the basis of their known medical history and fasting blood glucose analysis.

Apparatus

We quantified HbA1c according to the manufacturer's instructions with the Diamat automated GHB analyzer (Bio-Rad Laboratories), involving cation-exchange HPLC. Three phosphate buffers of increasing ionic strength are used for stepwise elution of the Hbs. The detection of Hbs is based on the absorbance at 415 and 690 nm.

The Abbott IMx GHB assay is a boronate affinity binding assay that is calibrated to report both the percentage of total GHB and the percentage of HbA1c. It is based on specific complex formation between GHB...
and a polyanion affinity reagent composed of 3-amino-phenylboronic acid. A positively charged reaction cell coated with a quaternary ammonium compound captures through electrostatic interaction the negatively charged polyanion–analyte complexes. GBHb is quantified by measuring the fluorescence quenching of Hb.

The cation-exchange chromatography on PolyCAT A involves a chromatographic system consisting of two Model 2150 HPLC pumps combined with a high-pressure mixer and a Model 2152 HPLC controller (LKB-Produkter AB, Bromma, Sweden), a Spectroflow 783 detector (Kratos Analytical, Ramsey, NJ) and a C-R3A integrator (Shimadzu, Kyoto, Japan). We use a 200 × 4.6 mm PolyCAT A column, 5-μm-diameter particle size with 100-nm pores (PolyLC, Columbia, MD). Disposable filter units, Millex-HVLP 0.45-μm (average pore size), were from Millipore, Bedford, MA.

Reagents

2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol (“bistris”) was from Sigma Chemical Co., St. Louis, MO. Buffer A (pH 6.87) contained 10 mmol of bistris and 1.0 mmol of KCN per liter. Buffer B (pH 6.57) contained buffer A plus 200 mmol of NaCl per liter. These buffers were filtered through a 0.45-μm (average pore size) filter before use. All other chemicals were of analytical grade. All reagents for the Diamat and IMx methods were from Bio-Rad and Abbott Laboratories, respectively.

Chromatographic Conditions on PolyCAT A

We injected 10 μL of the filtered hemolysate onto the column. Elution was achieved with a linear gradient of buffers A and B at a flow rate of 1.1 mL/min at room temperature. The gradient was: 0–2 min, 21% B; 16 min, 47% B; 22 min, 100% B; 24 min, 100% B; and 26 min, 21% B. The detection wavelength was 415 nm, integrator attenuation 3 (corresponding to 0.008 A/full scale), peak detection slope 1000 μV/min, and chart speed 5 mm/min. Integration was performed by the valley-to-valley method (9) as shown in Fig. 1.

Method Comparison

The regression lines between each pair of assay methods were calculated by the Deming method (10, 11). This method allows for variation in both the x- and y-axes at the same time.

The confidence limits for the slope and intercept values were calculated with the jackknife method (12), a nonparametric error estimation method. Its central idea is to discard each observation point in turn and to fit the model to the rest of the data points. The robustness of the correlation coefficient was calculated with bootstrap analysis (12), a computer-intensive method that is useful for estimating the standard error of any statistical parameter. It repeatedly resamples the original data set to produce a distribution of the parameters of interest. We used 500 bootstrap replicates. Both algorithms were programmed in Matlab™ (The MathWorks, Natick, MA) by one of us (U.K.).
Po1yCAT assay runs are 1.032 ± 0.0006, shows that the PolyCAT A method reflects differences in GHB in the same way as the Diamat method.

A nonspecific background is also observed in the IMx assay when we compare it with PolyCAT A (PolyCAT A = 0.881 IMx - 1.90). However, the magnitude of the background, ~2% of total Hb, is somewhat smaller than with the Diamat method (Fig. 2C). Interestingly, the correlation with the IMx method (r = 0.857) is slightly inferior to that with the Diamat method (r = 0.900).

The differential plots show that the negative bias of ~2–3% of total Hb is seen at all values of HbA1c when PolyCAT A is compared with Diamat (Fig. 3B) and IMx assays (Fig. 3C).

The correlation between IMx, calculated as %HbA1c, and the Bio-Rad Diamat (Fig. 2A) gave the following results: IMx = 1.16 Diamat - 0.98 (r = 0.922). The good correlation is explained by the fact that the IMx assay has been standardized with an ion-exchange HPLC method, with the Diamat assay as a secondary reference HPLC system maintained in close calibration to the primary reference HPLC assay (7). The bias observed (Fig. 3A) suggests that the IMx method gives slightly higher results than the Diamat method at high amounts but similar results at normal amounts of HbA1c. The correlation obtained by Wilson et al. (7), IMx = 0.98 Diamat - 0.07 (r = 0.973), was somewhat better than that in our study. The larger range of their values, 2–17% (7) vs 4.8–13.8% in this study, explains part but not all of the difference. With all methods the differences observed in individual samples could be so large that a switch from one method to the other might cause misinterpretations in some patients.

A large bias has been observed in comparative studies involving cation-exchange (x) and affinity (y) chromatography. In two studies the correlations between the methods were: y = 1.40x - 2.19 (13) and y = 1.45x + 0.03 (14). The difference in slope is explained by the fact that affinity methods measure glycated components other than HbA1c. The negative y-intercept may be explained by the fact that nonglycated components of HbA are measured by the ion-exchange chromatography method used. It would be interesting to know the correlation between the IMx and Diamat methods before recalibration.

For IMx a reference range of 4.5–5.5% has been reported (7). Our estimates the reference values for HbA1c with the PolyCAT A and Diamat methods with samples from 60 healthy controls were, for Diamat, mean (± SD) 5.13% ± 0.33%, the reference range (mean ± 2SD) 4.5–5.8%, and total range 4.4–6.1%. For the PolyCAT A method the mean (± SD) was 3.43% ± 0.47%, the reference range 2.5–4.4%, and the range 2.6–5.0%. Interestingly, the difference in mean values between the PolyCAT A and Diamat methods was only 1.7%, compared with the 2.8% bias estimated by regression analysis. This suggests that the nonspecific

during storage, which can be separated from the HbA1c peak by using methods with higher resolution. Our PolyCAT A assay has been optimized to separate different Hb variants from HbA1c. This apparently partly explains the lower results obtained by this method. However, the difference between Diamat method and PolyCAT A assay cannot be explained only by carbamylated and acetylated Hbs, for which concentrations <0.4% have been reported (3). The slope of the curve, 1.032 ± 0.0006, shows that the PolyCAT A method reflects differences in GHB in the same way as the Diamat method.

Fig. 2. Correlation between HbA1c values obtained by different assay methods: (A) IMx (y) and Diamat (x); (B) PolyCAT A (y) and Diamat (x); and (C) PolyCAT A (y) and IMx (x).

Corresponding regression equations and Pearson’s correlation coefficients are: (A) \( y = (1.161 \pm 0.009)x - (0.963 \pm 0.003), r = 0.922 \pm 0.022; \) (B) \( y = (1.032 \pm 0.006)x - (2.832 \pm 0.004), r = 0.900 \pm 0.036; \) and (C) \( y = (0.861 \pm 0.008)x - (1.895 \pm 0.006), r = 0.857 \pm 0.042. \) See Materials and Methods for details of the calculation.
background is overestimated by regression analysis. It might also indicate that some of the peaks not included in the HbA1c peak obtained with PolyCAT A represent other glycation products of Hb.

Our reference range is lower than that observed with our original HPLC method (4.2–6.6%) involving the MonoS column (15) and that reported by Jeppsson et al. (16), 3.9–5.3%, who used the same column but a different gradient. For use of PolyCAT A with other gradients, reference ranges of 2.8–5.5% (14) and 3.9–5.7% (17) have been reported. Slightly higher reference ranges of 5.0–7.6% (13) and 4.8–6.4% (18) have been reported for boronate affinity chromatography methods. Of the GHb adsorbed to boronate affinity columns, 52% elutes in ion-exchange chromatography as HbA1c, 38% in the HbA0 peak, and 10% as HbA1a+b (19). The values for the PolyCAT A method can be estimated to correspond to ~60–70% of those obtained by boronate affinity chromatography. This suggests that the PolyCAT A method fairly specifically measures the glycated component of the HbA1c peak.

A certain change in blood glucose causes a larger change in GHb measured by affinity chromatography than in HbA1c. This is demonstrated by the slope of the correlation, ~1.4–1.5 (13, 14), in combination with similar reference values. Thus methods based on boronate affinity (IMx) or immunological detection of the glycated amino terminus of the β-chains of Hb (8, 19) may be expected to reflect blood glucose control more accurately than ion-exchange chromatography methods, which also measure coeluting nonglycated Hb. However, whether this actually is the case in a true clinical setting remains to be determined.

Being a boronate affinity method, IMx, like boronate affinity chromatography, may also measure other GHbs. Therefore, although calibrating IMx against the Diamat has practical advantages, it is theoretically questionable. Alternative methods of calibration need to be considered.

Although there is no general agreement on standardization of methods for determination of GHbs, ion-exchange chromatographic methods measuring HbA1c have been used to calibrate both boronate affinity methods and some new immunochemical methods (8, 20) probably measuring, in addition to HbA1c various other forms of GHb. Even if this simplifies interpretation of the results for the clinicians, it should be borne in mind that the results are systematically biased.

References