Glycohemoglobin Measured by Automated Affinity HPLC Correlates with Both Short-Term and Long-Term Antecedent Glycemia

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We evaluated glycohemoglobin (GHB) and glycated plasma protein (GPP) by automated affinity HPLC for their ability to monitor both short-term and long-term antecedent glycemia in 70 diabetic subjects. We placed 30 subjects on an intervention protocol in which insulin and (or) dietary changes were made twice weekly to acutely decrease glycemia. We monitored 40 subjects at 6-week intervals; changes in the clinical regimen were made at that time only. Despite weekly changes in mean blood glucose in the subjects who received more intensive intervention, GHB concentrations correlated significantly with the weekly (r = 0.66, P < 0.001), 2-week (r = 0.70, P < 0.001), 3-week (r = 0.72, P < 0.001), and 6-week (r = 0.83, P < 0.001) mean glucose concentrations. GPP correlated significantly with measured glycated albumin determined by boronate affinity columns (r = 0.83, P < 0.001) and correlated best with the 1-week (r = 0.66, P < 0.001), 2-week (r = 0.64, P < 0.001) and 3-week (r = 0.60, P < 0.001) mean antecedent glucose concentration. Thus, GHB, traditionally considered a marker for only long-term diabetic control, correlated significantly with both short-term and long-term antecedent glycemia.

Indexing Terms: diabetes mellitus/blood glucose monitoring/glycated proteins

Recent evidence has conclusively related the development of diabetic complications to clinical hyperglycemia and demonstrated that control of blood glucose, with use of intensive insulin therapy, is associated with a significant reduction in complications such as retinopathy, nephropathy, and neuropathy (1–4). Even before this association was scientifically validated, the measurement of glycohemoglobin (GHB), an objective marker for antecedent glycemic control, was an integral part of clinical diabetes management (5–8). Because of its high level of precision, ion-exchange HPLC has been recommended as the “gold standard” for measurement of GHB. However, this method is still less than ideal because of potential interference by Hb variants with altered charge (e.g., HbS or HbC) by many glucose adducts migrating with HbA1 species (e.g., carbamylated Hb), and by problems arising from artifacts induced under various storage conditions (9–13). The boronate affinity chromatography method involving minicolumns has been gaining wide acceptance because it is not affected by Hb variants, slight temperature changes, carbamylated Hb, the labile fraction (aldimine or Schiff base), or ionic strength of the buffer (9, 14–19). However, in some laboratories, precision has been a problem with these small columns. The application of HPLC used with affinity methods has markedly improved precision and run-time while maintaining the major advantage of the boronate affinity method (17–20): Its applicability to Hb and to soluble blood proteins in serum and plasma samples allows objective glycation assessment for all blood proteins of interest with a single method. This may allow objective determination of antecedent glycemic control for the previous 2–3 months (e.g., via GHB) and short-term diabetic control for the preceding 1–2 weeks (e.g., via glycated plasma proteins (GPP), especially glycated albumin) in the same specimen.

To clinically evaluate the utility of measurements of GHB and GPP by affinity HPLC in assessing durations of antecedent glycemia, we studied a diabetic outpatient population undergoing both intensive and conventional clinical intervention. We evaluated these analytes for correlation with mean glucose concentrations for short-term (1–3 weeks) and long-term (6 weeks) periods.

Patients and Methods

Subjects. We studied 278 subjects, 70 of whom were diabetic. The 208 subjects evaluated to establish control ranges were considered nondiabetic on the basis of their known medical history and repeated fasting blood-glucose analysis. The diabetic subjects were grouped according to treatment intervention. Group I consisted of 30 non-insulin-independent diabetes mellitus (NIDDM) patients (16 women, 14 men, mean age 53 ± 3 years, range 34–69), considered to be moderately uncontrolled (GHB >10%), for which clinical intervention consisted of dietary changes and (or) insulin adjustments to decrease mean weekly glucose significantly over 8 weeks. These patients were monitored with a home glucose-monitoring system with electronic log books as performed with Accu-Check II glucose meters (Merlin Diabetes Data Management System; Boehringer Mannheim Diagnostics, Indianapolis, IN). In this aspect of study, patients performed capillary blood glucose (CBG) determinations before meals and at bedtime. Patients reported to the clinic weekly, at which time mean CBG was determined, and insulin and (or) dietary adjustments were...
made twice weekly (via clinic and phone contact) on the basis of the glucose recorded at home to decrease the previous weeks' glucose. Insulin therapy consisted of twice-daily injections of intermediate-acting insulin (e.g., NPH) with mixed short-acting insulin (e.g., Regular) involving sliding scale at breakfast and supper. We refer to the Group I patients as the "intensive clinical intervention" group for the purpose of this study only; we do not intend to misrepresent the subjects as having intensive insulin therapy as administered in the Diabetes Control and Complications Trial (4). Group II consisted of 40 diabetic subjects (22 women, 18 men; 24 NIDDM, 16 IDDM; mean age 49 ± 3 years, range 25–73). These patients also recorded CBG values before meals and at bedtime and reported to the clinic at 6-week intervals for dietary and (or) medication adjustments. All group II subjects were on a split/mixed NPH/Regular insulin regimen, except for eight IDDM patients who were on either a long-acting insulin (e.g., Ultralente based) or insulin-pump regimen. The correlations of GHb and GPP with the weekly, 2-, 3-, and 6-week glucose means were determined for Group I subjects and with 6-week glucose means for Group II subjects. All patients gave informed consent and the study was approved by the Clinical Research Practices Committee of the Bowman Gray School of Medicine.

Sample preparation. Whole blood collected by venipuncture with EDTA as anticoagulant was centrifuged at 1500g for 10 min; the plasma was removed and diluted 10-fold with GPP diluent (Primus, Kansas City, MO); the packed erythrocytes were diluted with 200-fold GHb diluent containing an aqueous hemolysis reagent and preservative (Primus). The samples were diluted directly in the vials used for the assay.

Instrumentation. The Primus CLC330 high-performance liquid chromatograph determines both GHb and GPP by automated HPLC boronate affinity methods. Equipped with an ultraviolet/visible light detector, autosampler, computing integrator, and two disk drives, the system is controlled through automation software provided by Primus. Both the analytical system and the automation software are described in detail elsewhere (21).

HPLC analysis. Sample vials were loaded into the autosampler, identified as to sample type, and analyzed by the automation software. The analytical sequence is as follows: Whole-blood hemolysate (5 µL) or diluted plasma (10 µL) is automatically injected onto a boronate affinity column (Primus). The 0.5 × 5 cm glass analytical column used for the separation of GHb from other Hb species contains m-aminophenyl boronic acid covalently bound to a 10-µm-thick vinyl polymer solid support, producing >1200 theoretical plates per column. Glycated species are retained by the column while non-glycated species (peak 1) are eluted through the column and detector with elution reagent 1 (mobile phase from injection to 0.2 min): ammonium acetate buffer (250 mmol/L), sodium chloride (500 mmol/L) magnesium chloride (30 mmol/L), ethanol (45 mL/L), methanol (2.5 mL/L), isopropanol (2.5 mL/L), pH 9. Elution reagent 2 (0.2–1.3 min) contains a competing polyol designed to release the boronate-bound glycated protein fraction (peak 2). The reagent contains sodium chloride (150 mmol/L), ethanol (45 mL/L), methanol (2.5 mL/L), isopropanol (2.5 mL/L), and mannitol (100 mmol/L). Elution reagent 1 is then added (1.3–2.0 min) to prepare for the next sample sequence. There is ~0.7-min lag time between switching to each mobile phase and its arrival at the detector. All reagents were supplied by Primus.

The flow rate was 1.8 mL/min; the column oven was kept at 40°C. GHb was detected at 413 nm, GPP at 280 nm. Quantification was by peak-area analysis with a calibration curve derived from calibrator samples. The computing integrator calculates the percentage of the glycated species by the following formula, as previously described (19): % glycated species = 100 (peak 1 area/peak 1 area + peak 2 area)]. Intra- and interassay CVs were 1.2% and 2.1%, respectively (n = 10, mean = 5.7%).

Calibration. The Primus CLC330 uses a two-point calibration system. Calibrators are prepared from human whole blood (for GHb) or from human plasma (for GPP) obtained from nondiabetic and diabetic donors. Samples are lyophilized and stored refrigerated in sealed vials until use. Values for the calibrators were determined by the manufacturer. Data obtained in a calibration run were used to correct for variations in assay conditions.

Glycated serum albumin (inter- and intraassay CVs 4.5% and 3.4%, respectively) was determined with boronate affinity chromatography with minicolumns, as previously described (22, 23).

Data were analyzed with Pearson correlation coefficients and repeated-measures analysis of variance where appropriate.

Results

The range of GHb results for the nondiabetics (n = 208, mean ± SD fasting glucose 4.6 ± 1.2 mmol/L) was 4.0–8.0% (mean ± SD 5.55 ± 0.74%). Using the 95th percentile as the upper limit, we determined the upper range of normal GHb for this assay to be 7.0%. The range of GPP results for nondiabetics was 13.4–25.0% (mean 19.2%; SD 2.2%), with the 95th percentile (upper range of normal) at 23.5%.

Figure 1A represents the mean weekly concentrations of CBG, GHb, and GPP in the intensive intervention group. With use of repeated-measures analysis of variance, significant decreases in GHb (P < 0.0001), GPP (P < 0.003), and CBG (P < 0.0001) were noted over time. As demonstrated in Fig. 1B, no major changes in GPP, mean CBG, or GHb were seen in Group II subjects, demonstrating relatively stable glycemia over the period of observation.

The mean weekly CBG decreases for the intensively intervened subjects (Group I) at weeks 1–8 were 15.7%, 12.3%, 6.7%, 5%, 9.1%, 5.8%, 1.4%, and 0.8%, respectively. Because most the change in mean glucose (±50%) occurred after only 2 weeks of intervention, we correlated the absolute changes in mean glucose
minicolumns for all Group I and Group II patients at baseline and correlated significantly with the GPP by automated affinity HPLC \( (r = 0.83, P < 0.001) \). GHb correlated significantly with the GPP at baseline in Group I \( (r = 0.61, P < 0.001) \), but by week 8 the correlation was no longer significant \( (r = 0.26) \). In Group II, GHb maintained good correlation with GPP at each 6-week cycle \( (r = 0.65-0.72, P < 0.001) \).

**Discussion**

As would be expected, GHb determined by automated affinity HPLC correlates well with long-term glucose control in diabetic outpatients monitored at 6-week intervals. Despite weekly changes in mean blood glucose in our intensive intervention subjects, GHb, traditionally considered a marker only for long-term diabetic control, correlated significantly with the weekly, 2-week, 3-week, and 6-week mean glucose and demonstrated an ability to detect recent changes in glycemia. GHPP correlated significantly with measured glycated albumin concentrations determined by boronate affinity minicolumns and correlated best with the 1–3-week mean glucose, demonstrating validity as a measure of short-term glycemia.

Long-term prospective studies (e.g., 4) have demonstrated that the measurement of glycated blood protein markers as objective markers of antecedent glycomic control can predict the development of diabetic complications such as retinopathy, nephropathy, and neuropathy (1–4). Physicians are now encouraged to monitor the patient’s glycomic profile while minimizing the side effects of intensive therapy (4). The reduction in glycation of blood proteins, secondary to a lowered mean blood glucose concentration, is postulated to decrease tissue accumulation of nonenzymatically attached glucose, thereby preventing the advanced glycation (i.e., protein cross-linking) thought to contribute to the tissue dysfunction (24–27). Therefore, the measurement of GHb as an objective marker for antecedent glycomic control has received renewed clinical interest.

Our data demonstrate that GHb determined by automated affinity HPLC is an excellent index of antecedent long-term diabetic control (i.e., 6 weeks), showing excellent correlation in both an intensively intervened and a relatively stable outpatient population at 6-week intervals. GPP primarily showed validity in monitoring short-term control in the acute intervention patients, and had decreased correlation with mean CBG when evaluated at 6-week intervals in this group. This suggests that the most appropriate antecedent glycomic window for GPP may be the preceding 1–3 weeks. However, in the Group II patients, GPP maintained high correlation with the mean outpatient glucose concentration over a 6-week interval. Group II did not have major changes in mean blood glucose, so the GPP would be expected to maintain excellent correlation with both mean CBG and GHb. However, GPP correlated with 1-, 2-, and 3-week mean glycemia, and the change in GHb concentrations, especially during the first weeks of therapy, correlated significantly with the change in mean glycemia. These findings cannot be attributed to inter-
Table 1. Correlation of antecedent glycemia with glycated blood proteins.*

<table>
<thead>
<tr>
<th>Clinical Group</th>
<th>Duration of Antecedent Glycemia, Weeks</th>
<th>r²</th>
<th>Slope</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHB, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1</td>
<td>0.68 (0.39–0.82)</td>
<td>0.87</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.70 (0.47–0.84)</td>
<td>0.89</td>
<td>−0.31</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.72 (0.49–0.85)</td>
<td>0.91</td>
<td>−0.28</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.83 (0.68–0.91)</td>
<td>0.98</td>
<td>−0.36</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.84 (0.72–0.91)</td>
<td>0.74</td>
<td>1.51</td>
</tr>
<tr>
<td>GPP, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1</td>
<td>0.68 (0.39–0.82)</td>
<td>0.85</td>
<td>−10.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.64 (0.33–0.81)</td>
<td>0.78</td>
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<tr>
<td></td>
<td>3</td>
<td>0.60 (0.31–0.79)</td>
<td>0.74</td>
<td>−7.9</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.49 (0.16–0.72)</td>
<td>0.66</td>
<td>−5.4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.64 (0.41–0.79)</td>
<td>0.47</td>
<td>−2.1</td>
</tr>
</tbody>
</table>

* All correlations significant at P <0.001.

The relation between GHB concentrations and recent glycemic excursions has been reported recently (28). Furthermore, two other studies support our findings that GHB may reflect short-term changes. Lee et al. (29), using boronate affinity methodology, demonstrated that GHB and GPP assays provide the most useful clinical indicators of short-term changes in glucose control. Mortensen and VeLund (30) concluded that weekly determination of HbA1c could be used as a supplement to preprandial glucose concentrations to evaluate the effectiveness of intensive treatment regimens. Our data agree with the observation noted in previous reports and suggest that the GHB assay we used may provide difference from the Schiff base reflecting recent or current glycemia, because the Schiff base is not detected by this method. The results suggest that GHB determined by automated affinity HPLC may serve as a marker of short-term control if evaluated at frequent intervals in an intensive monitoring program.
the same clinical information as the GPP assay. Therefore, one may have little need to measure GPP if a highly precise GHb assay is available. We note, however, that an acute decrease in glyemia in this report was accompanied by significant changes in an increased GHb (our patients were considered in poor control initially). Whether GHb at a normal or near-normal value on initial measurements can detect glycemic excursions as well as short-lived proteins do need to be addressed.

In conclusion, this boronate affinity HPLC method for the determination of GHb and GPP is rapid (run time <2 min) and precise (CV ≤2%), and provides a valid objective measure to assess both short- and long-term antecedent control.

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References