Aminophenylboronic Acid Affinity Chromatography and Thiobarbituric Acid Colorimetry Compared for Measuring Glycated Albumin

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Two techniques originally developed for measurement of glycated ("glycosylated") hemoglobin but also applicable to determination of glycated albumin are the thiobarbituric acid colorimetric technique (I) and the aminophenylboronic acid affinity chromatographic procedure (II). The latter reliably distinguishes diabetics from nondiabetics, and concentrations of glycated hemoglobin and glycated albumin are linearly correlated. I is nonspecific; it neither correlates with diabetic status nor with values derived via the affinity technique. Most of the chromogenic material is present in the fraction of albumin that does not bind to aminophenylboronic acid. Glucose interferes significantly with I but only slightly with II. Prolonged incubation of plasma with glucose dramatically increases the II-determined glycated albumin. Reactivity with thiobarbituric acid increases much less, and mainly in the II-bound fraction. This fraction contains a high proportion of nonspecifically reactive material. The percentage of glycated albumin determined in crude plasma samples by II differs only slightly from the value determined by purifying the albumin from the plasma. This technique appears more promising than I for eventual clinical applications.

Glucose can irreversibly interact with all body proteins, a process called glycation. Because of the differential electrophoretic mobility of one subtraction of glycated hemoglobin, HbA1c, various assays have been developed (1) for reliably and accurately measuring this protein, which serves as a long-term index of the concentration of glucose in blood, given the 120-day life span of the erythrocyte. However, glucose in blood also undergoes relatively short-term changes in concentration, for which glycated albumin, with a 14-day half life, provides a better index. Only two of the techniques for measuring glycated hemoglobin, the thiobarbituric acid (TBA) colorimetric procedure and aminophenylboronic acid affinity chromatography, are suitable for measuring glycated albumin. Although theoretically straightforward (2, 3) the TBA technique is cumbersome in practice and it has not become standard clinical procedure.

The newer affinity procedure (4, 5), which accurately and reliably measures glycated hemoglobin, is not affected by many potentially interfering substances, including glucose (6). Here we report our assessment of an affinity-chromatographic technique for determining glycated albumin, as compared with the TBA technique.

Materials and Methods

Blood samples: Specimens were collected by venipuncture into EDTA-containing evacuated blood-collection tubes. To prepare hemolysates, we lysed one volume of unwashed packed cells with 20 volumes of distilled water. After centrifugation (800 × g, 10 min) to settle cell debris, we froze the supernates, storing them at −20 °C for as long as a week or at −70 °C for as long as six months before analysis. Plasma samples were similarly centrifuged (800 × g, 10 min), and the supernates removed, frozen, and stored. We pooled plasma and hemoglobin samples from at least 25 diabetics (glycated hemoglobin >8%) and at least 25 nondiabetic (glycated hemoglobin <8%) patients.

Measurement of glycated hemoglobin: We determined glycated hemoglobin by affinity chromatography as previously described (6), using 1-mL columns of m-aminophenylboronic acid ("Glycogel"; Pierce Chemical Co., Rockford, IL 61105).

Measurement of glycated albumin: We mixed 1-mL plasma samples with 4 mL of pH 8.0 wash buffer (per liter, 250 mmol of ammonium acetate, 50 mmol of MgCl2, and 0.2 g of sodium azide) to adjust sample pH and ionic strength. All 5 mL was then applied to the m-aminophenylboronic acid columns that had previously been equilibrated with the wash buffer. After passing the sample through the column and collecting the effluent in a 16 × 150 mm test tube, we passed 15 mL of wash buffer through the column, collecting it in the same test tube. We then eluted the bound plasma protein from the column into a 16 × 100 mm test tube, the eluent being 3 mL of sodium citrate buffer (0.2 mol/L, pH 4.5).

We then determined the concentration of albumin in the wash (nonbound) and eluted (bound) fractions by reaction with brom cresol green and measurement of the absorbance at 630 nm, subtracting the appropriate column-blank values. We further quantified the protein in each fraction with Coomassie Brilliant Blue (Quant-T; Quantimetrix Corp., Hawthorne, CA 90250), or by measuring the ultraviolet absorbance at 280 nm, and subtracting the appropriate column-blank values.

After either of the procedures the total amount of albumin or protein in each fraction was then calculated as the elution volume of each fraction (3 or 20 mL) times the blank-corrected absorbance times the molar absorptivity of albumin or protein. The percentages of glycated albumin (glycated albumin) and glycated plasma protein were then calculated as follows:

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\% = \frac{300 \times A_{bound}}{(20 \times A_{non-bound}) + (3 \times A_{bound})}
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Thiobarbituric acid (TBA) reaction for serum protein: Because of the high interassay variability of color development inherent in this procedure, various concentrations of 5-hydroxymethylfurfural or fructose are used to establish a standard curve, rather than expressing results directly in absorbance units (7). The technique we used was as described by McFarland et al. (3), except we used fructose standards rather than 5-hydroxymethylfurfural. Results
were expressed either as the fructose concentration providing same absorbance as the sample at 443 nm (fructose equivalents in nanomoles per milliliter) or as fructose equivalents (nmol/mL) divided by the total protein in each sample.

Purification of serum albumin: Reactive Blue Sepharose (Affigel Blue; Sigma Chemical Co., St. Louis, MO 63178) was used to isolate albumin as described by Travis and Pannell (8) and McFarland et al. (3). Albumin was further concentrated and purified by repeated washing and ultrafiltration on selectively permeable ultrafiltration membranes (YM 50; Amicon Corp., Dovers, MA 01923).

In vitro glucose incubation: We incubated plasma at 37 °C with glucose in a final concentration of 5 g/L in the presence of sodium azide, 1 L/L. To stop the reaction at appropriate time points, we removed the incubation medium by ultrafiltration, then reconstituted the samples in buffer and stored them frozen until assay.

Sodium borohydride incubation: After incubating samples at 0 °C for 30 min in a 100-fold molar excess of sodium borohydride over total protein, we removed the borohydride by ultrafiltration, reconstituted the samples in wash buffer, and determined the percent glycated albumin and protein.

Statistical analysis: Statistical significance was determined by analysis of variance or by the Mann-Whitney nonparametric test for variables not expected to be necessarily normally distributed.

Results

In vitro glycosylation of plasma: When plasma from nondiabetics was incubated for 14 days with glucose, 5 g/L final concentration, affinity chromatography showed a linear fivefold increase in glycation of both albumin and total protein during the incubation (Figure 1, left).

Parallel determination of TBA reactivity showed a much smaller increase, with a nonlinear course of glycation: the TBA method detected an early rapid increase, followed by a much slower climb to a final value about 60% greater than baseline after the 14 days. Testing the bound and nonbound fractions from the affinity separation for TBA reactivity (Figure 1, right) showed considerable TBA reactivity in the nonbound fractions, suggesting that the aminophenylboronic acid column does not bind all species of glycated proteins. The nonbound fraction showed an early rapid increase in glycation, again followed by a slow climb to about 60% over baseline; results for the bound fraction by the TBA procedure generally paralleled those by affinity chromatography.

Glucose interacting with protein forms freely reversible aldime linkages, which then undergo Amadori rearrangement to form irreversible ketoamine linkages. To determine whether aldime groups contributed to the fraction bound to the column, we reduced the samples with sodium borohydride. Sodium borohydride accelerates glycation by converting freely reversible Schiff base as well as ketoamine-linked glucose to the irreversible open-chain hexiroylysine form, which does not react with TBA (9). Therefore, results for the TBA methods after NaB₄H₆ treatment are used as an index of nonspecific TBA reactivity.

Borohydride reduction of aliquots from the plasma samples incubated with glucose for 0 and 14 days dramatically increased the percentages of glycated albumin and glycated total plasma protein, tripling the values in the day 0 sample (Figure 2, left). The absolute increase in both analytes was duplicated in the day 14 sample. We conclude that rapidly reversible aldime linkages are evidently present in much larger concentration in nondiabetic serum than are the ketoamine moieties, but the affinity procedure, under our conditions, does not retain them in the bound fraction unless they are converted to hexiroylysine groups.

The TBA reactivity measured after borohydride treatment was between 60 and 70% of the value for nonborohydride-treated (Figure 2, right). Furthermore, about 80% of the TBA reactivity of the nonbound fraction was due to material that was not affected by borohydride treatment. Therefore much of the TBA reactivity of the nonbound fraction was nonspecific, i.e., caused by something other than glycated protein. Because the protein concentration is much greater in the NaB₄H₆-treated bound fraction than in the untreated one, the TBA values both before and after incubation were substantially higher after borohydride treatment.

Characterization of affinity separation: We measured by affinity chromatography the percentage of glycated albumin in two plasma pools, one from nondiabetics and the other from diabetics (samples pooled from about 40 different individuals in each). For the normal pool the mean percentage of glycated albumin was 1.50 (SE 0.06)% and it was 5.15 (SE 0.14)% for the diabetic pool (n = 10 each). Electro-

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Fig. 1. In vitro glycation of protein in non-diabetic plasma as determined by affinity chromatography (left) or reaction with TBA (right).

Plasma from non-diabetes was incubated with glucose, 5 g/L until ultrafiltration on the days indicated to remove free glucose. Left: percent glycated albumin; total glycated plasma protein (Percent Gly P.P.). Bars indicate SE (n = 6 each). Right: TBA reactivity (fructose equivalents/mL) in the plasma samples (8) and in the non-bound (C) and bound (E) fractions eluted from the column. Bars indicate SE (n = 4 each).

Fig. 2. Effect of borohydride reduction on glycation determined by aminophenylboronic acid chromatography (left) or by TBA method (right).

On days 0 and 14 of the glucose incubation, glycated albumin and glycated plasma protein percentages (left) and TBA reactivity (right) were determined in duplicate with (solid bar) and without (open bar) subsequent exposure to NaB₄H₆. B, bound; NB, nonbound.
phoresis on cellulose acetate showed albumin bands of similar size in the normal and diabetic nonbound fractions. The bound protein, however, showed an albumin peak and a broad globulin peak, mostly in the gamma fraction (Figure 3). The globulin peak in the bound fractions from both diabetic and nondiabetic samples was considerably greater than in corresponding nonbound fractions, reflecting the high glycoprotein content of the globulin fraction. The bound albumin peak was considerably larger for the diabetic plasma than that for the nondiabetic sample. The globulin peaks were similar in the diabetic and non diabetic samples, presumably due to the high normal glycoprotein content of gamma globulins.

To verify that the percent of glycated albumin in plasma samples did not differ from the results obtained with purified albumin, we compared results for samples processed as described in Materials and Methods with those for samples concentrated and reconstituted with distilled water three times on YM 50 membranes (washed samples), and with those for purified albumin samples from both pools. There was no significant difference in the percentage of glycated albumin obtained with the three different methods of preparation. To rule out the possibility that dye binding could be influenced by glycation of protein or other interfering ligands, we assessed percent glycation in purified albumin by dye binding (bromocresol green, Coomassie Blue) and ultraviolet absorbance; the latter obviously is not affected by glycosylation. Results for percentage glycation of albumin were identical by all these techniques.

Sequential fractionation of the two pools with 2 mL of wash buffer and 1 mL of the sodium citrate elution buffer, followed by reaction with bromocresol green, Coomassie Brilliant Blue, or TBA showed that the albumin peak was well separated, the absorbance of bromocresol green dropping to zero after the first five aliquots of wash buffer (Figure 4, left). The bound albumin fraction was completely eluted in the first 3 mL of elution buffer. Separation of total plasma protein was not quite as clean, as shown by Coomassie dye reactivity in the final 2-mL fractions eluted by the wash buffer. Most of the TBA-reactive protein was obviously not bound to the column, and was eluted with the wash buffer. Similarly, purified albumin from each pool was subjected to sequential fractional chromatography (Figure 4, right). Again, most of the TBA-reactive material was not bound to the column. The TBA-bound reactivity per milligram of protein of both diabetic and non diabetic samples was about fourfold greater than in the nonbound fractions, but the total amount of protein in the nonbound fraction was much greater than in the bound fraction. The TBA reactivity of the nonbound fractions of diabetics and non-diabetics was similar, whereas that in the bound fractions was three- to fourfold greater in diabetic samples than in the non-diabetics (Figure 5). The apparent lack of difference in the TBA reactivity of nonbound fractions from diabetics and non-diabetics suggests that plasma from either contains TBA-reactive albumin species that were not bound to the column and so did not distinguish between diabetics and non-diabetics, whereas the albumin species bound to the columns did differentiate samples from these two types of subjects.

**Plasma samples:** The correlation of the two techniques with state of diabetic control was further evaluated with three different plasma pools, one from non-diabetic patients (glycated hemoglobin 6–8%), one from fairly well-controlled diabetic patients (glycated hemoglobin in the range 8–10%), and one from patients with poorly controlled blood sugar (glycated hemoglobin >15%). As expected, there was an increasing level of affinity-determined percentage of glycated albumin, correlating with the increasing percentage of glycated hemoglobin (Figure 6, bottom). There was no apparent relationship between non-bound TBA reactivity and increasing glycated hemoglobin, but TBA reactivity of the bound fractions did increase (Figure 6, top). Filtration of samples to remove plasma glucose decreased the reactivity of the non-bound fractions, but did not modify the lack of correlation of non-bound TBA with glycated hemoglobin and glycated albumin. The percentage of glycated albumin as determined by the affinity procedure was not affected by filtration, nor was bound TBA reactivity.

We further studied the strong correlation between diabetic status and values for glycated albumin in a group of 40 individuals, finding a linear correlation \( r = 0.85 \) between glycated albumin and glycated hemoglobin obeying the relationship glycated albumin = 0.38 + 0.28(percent glycated hemoglobin).

**Effect of plasma glucose on the affinity procedure:** Filtration of plasma samples appeared to negligibly affect the proportion of glycated albumin as determined by the affinity procedure. To confirm this finding, we obtained pooled plasma from non-diabetic donors from the American Red Cross, which stores its plasma in a solution containing, per liter, 5 g of glucose, 3.69 g of trisodium citrate, and 0.46 mg of citric acid. Citrate is the competing ligand in our elution buffer, so the Red Cross crude plasma sample gave a slightly lower value for glycated albumin and glycated plasma protein than did a comparable non-diabetic pool without glucose storage (Figure 7, top left), but the total TBA reactivity of the Red Cross pool was much higher than that of the non-diabetic pool (Figure 7, top right) because the value for the non-bound fraction was much higher. The Red Cross bound TBA reactivity was lower than that of the non-diabetic bound fraction (Figure 7, top right). After we washed off the citrate dextrose by ultrafiltration, the Red Cross sample gave (Figure 7, bottom left) a somewhat higher percent glycalbumin \((1.73 \text{ (SE 0.08%)})\) than the filtered non-diabetic pool \((1.32 \text{ (SE 0.08%)})\). TBA reactivity measured in both the filtered plasma samples and the first filter effluent (Figure 7, bottom right) showed a higher value for the Red Cross effluent than the effluent from the non-diabetic pool.
Fig. 4. Sequential chromatographic fractionation of duplicate samples from normal and diabetic control pools (left) and of duplicate albumin samples purified from those pools (right). Brom cresol green absorbance at 630 nm, Coomassie Blue absorbance at 595 nm, and TBA reactivity (fructose equivalents/mL) were determined on each of 10 2-mL aliquots of wash buffer passed through the column, then followed by four 1-mL aliquots of elution buffer (cross-hatched).

Fig. 5. Total TBA reactivity of nonbound and bound fractions of albumin from diabetics and non-diabetics. 1-mL albumin samples were processed in the usual way. TBA reactivity was determined in the NB and B fractions and expressed as fructose equivalents/mL (left) and on a per-milligram-protein basis (right). n = 10

The affinity procedure and TBA procedure, carried out on albumin purified from these two pools, showed no difference in the values for percent glycated albumin from those obtained after simple plasma filtration (Figure 8A). The bound fractions of the Red Cross and non-diabetic albumins were identical in TBA reactivity (Figure 8C), but albumin purified from a diabetic pool gave a fourfold higher value for its bound fraction. Evidently the affinity technique is capable of correctly identifying non-diabetic plasma in the presence of considerable interfering glucose, whereas the TBA technique clearly is reliable only after considerable purification through the affinity column.

Discussion

We conclude that the aminophenylboronic acid affinity procedure has several advantages over the TBA technique for determining glycated albumin. It is simple to perform, requiring a single column separation followed by colorimetry. It shows a simple, linear kinetic time course in following
Fig. 6. Plasma pools: Effect of filtration on TBA and affinity results
Three plasma pools with increasing glycosylation (1, non-diabetic; 2, relatively well-controlled diabetics; 3, poorly controlled diabetics) were ultrafiltered, then reconstituted in normal saline. Affinity (bottom) and TBA (top) results were determined (n = 10) on the crude (open bars) and ultrafiltered (dark bars) plasma.

Fig. 7. Comparison of non-diabetic plasma pool with non-diabetic American Red Cross plasma
Non-diabetic (open bars) plasma was compared with Red Cross (stippled bars) plasma in terms of affinity percentage (left) and TBA reactivity (right). n = 5. Top: Crude, unwashed plasmas are compared. Bottom: Same samples after ultrafiltration. The effluent from the filtration of the Red Cross sample gave a much higher TBA reactivity than that from the non-diabetic plasma.

Fig. 8. Comparison of albumin from non-diabetics, Red Cross plasma, and diabetics
Albumin purified from normal plasma (open bars), Red Cross plasma (stippled bars), and diabetic plasma (cross-hatched bars) are compared for percent glycated albumin (A), percent glycated plasma protein (B), and TBA reactivity (C). n = 5.

in vitro glycation and appears to select the true ketoamine-linked glucose, allowing the aldimine intermediates to pass in the nonbound fraction. There is a well-defined linear correlation between the percentages of glycated hemoglobin and glycated albumin. Unlike the TBA procedure (10), the affinity technique is hardly affected by increases in plasma glucose or high glycoprotein content of plasma globulins, so that the percentage of glycated albumin in crude plasma can be quantified without prior purification of plasma albumin.

Aside from the potential ability to assess short-term changes in glucose control, the affinity technique has several other theoretical advantages. Patients with anemia may have altered erythrocyte life spans, which will affect the proportion of glycated hemoglobin present, whereas the half-lives of albumin...
life of albumin is relatively stable, except in coexisting liver failure or nephrotic syndrome. Moreover, because albumin is freely accessible to glucose in the blood whereas hemoglobin is enclosed in the erythrocytes, the effects of changes in erythrocyte permeability to glucose and changes in erythrocyte physiology will affect the concentration of glycated hemoglobin (II, 12) but not modify the formation of glycated albumin. Finally, and probably most important, the simple techniques presented here, unlike those of the TBA procedure, are readily amenable to use in the clinical laboratory. Determination of glycated albumin should soon be as routine as that of glycated hemoglobin.

References