The Measurement of Glycosylated Hemoglobin in Man and Animals by Aminophenylboronic Acid Affinity Chromatography

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SUMMARY

Glycosylated hemoglobin (GHb) was estimated in normal and diabetic human, rat, and dog hemolysate by m-aminophenylboronic acid (PBA) affinity chromatography and the results compared with the values determined using two ion-exchange (ION-E) methods and a colorimetric thiobarbituric acid (TBA) method. There was a good correlation between the values estimated by PBA and both ION-E chromatography methods for the human samples ($r = 0.83$, $P < 0.0002$, $r = 0.86$, $P < 0.002$). In diabetic rat and dog hemolysates, PBA chromatography demonstrated higher glycosylated hemoglobin than in normal hemolysate. In both species, there was an excellent correlation between the PBA-estimated GHb and plasma glucose levels (rat $r = 0.79$, $P < 0.001$; dog $r = 0.67$, $P < 0.003$). The ION-E and TBA methods were not as effective in separating diabetic from normal samples and correlated less well with plasma glucose levels.

PBA chromatography relies on the interaction of m-amino phenylboronate with the hydroxyl groups of the glucose residues attached to hemoglobin. It is not affected by intra- or interspecies variations in the hemoglobin moiety and should be adaptable for measurement of GHb in a number of laboratory animals and in patients with hemoglobinopathy. It is not affected significantly by temperature and may offer advantages over the ION-E method in the routine determination of human glycosylated hemoglobin. DIABETES 31:701–705, August 1982.

The increased glycosylation of hemoglobin and plasma proteins in diabetes is well established.[1–5] Measurement of glycosylated hemoglobin (GHb) is being used by many centers to monitor diabetic control. However, there are still technical difficulties encountered with the various methods of GHb measurement and none is entirely satisfactory.[1] Measurement of GHb has been reported in monkeys, mice, rabbits, and rats, but the technique has not been widely applied to the study of diabetes in laboratory animal models.[6–10] This is partly due to the absence of a method which can measure GHb in a wide variety of animal species. This report describes the use of m-aminophenylboronic acid (PBA) chromatography as a technique that should be applicable to measurement of GHb in all animal species. In this method, GHb is selectively bound by PBA immobilized on solid phase and subsequently eluted by an excess of sorbitol. PBA chromatography has been used to study human, rat, and dog hemolysate and compared with two ion-exchange (ION-E) methods and the thiobarbituric acid (TBA) method commonly used in the measurement of GHb. The PBA method is technically simple and should be a useful adjunct in the study of diabetes in laboratory animals.

MATERIALS AND METHODS

Patients and laboratory animals. Heparinized blood (2 ml) was obtained from 35 patients attending the diabetic clinic of Royal Prince Alfred Hospital. The patients were not fasted and were included irrespective of the type of treatment they were receiving. A 100-μl aliquot of the blood was used for GHb measurement by a mini-column ION-E method (Bio-Rad laboratory, Richmond, California). The remainder was used to prepare hemolysate as described below. This was stored at −80°C and analyzed by a macro-column ION-E method[2] and by PBA chromatography.

Inbred female wistar rats (weight 220 ± 10 g) were obtained from the Atomic Energy Commission of Australia (Lucas Heights). Diabetes was induced by intravenous injection of streptozotocin (65 mg/kg) and confirmed by blood glucose measurement (Dextrostix, Miles Laboratories, Elkhart, Indiana) of greater than 22 mmol/L. In total, 32 rats were studied: 8 diabetics, 15 insulin-treated diabetics (2–6 U/day), and 9 littermate controls. Animals were killed at 2–

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28 wk after onset of diabetes by an overdose of Halothane. Heparinized blood was obtained by cardiac puncture and hemolysate prepared as described below. As the animals in different groups showed similar findings the results were pooled for the purpose of the study.

Heparinized blood samples were obtained by venipuncture from seven spontaneously diabetic dogs and seven control dogs of various breeds. In nine diabetic dogs, blood samples were obtained after 3–10 mo treatment with insulin.

Preparation of hemolysate. Hemolysate for the measurement of human GHb by the mini-column ION-E method was prepared from whole blood using the hemolysing solution provided by the manufacturer.[11] For macro-column ION-E and PBA methods, human hemolysate was produced by washing centrifuged red cells three times in 10 vol of isotonic saline and then lysed in 5 vol of distilled water. Dog hemolysate used in all three methods was prepared from red cells in identical manner to that described for human. Rat hemolysate was produced similarly except that cells were washed in phosphate buffer (155 mmol/L, pH 7.6) and then lysed in 5 vol of phosphate buffer (5 mmol/L, pH 7.6). Different hemolysing conditions were chosen for rats as this method was most suitable for preparation of red cell ghosts used in another series of experiments. The manufacturer's literature indicated that results of ION-E method were independent of whether the hemolysate was produced from whole blood or washed red cells.[11] In our control experiments, the different conditions chosen for production of rat or dog hemolysate yielded similar results for all three methods of GHb measurement.

m-Aminophenylboronic acid chromatography. m-Aminophenylboronic acid gel (PBA-30) was purchased from Amicon. Except in certain control experiments, chromatography was carried out routinely at 22°C using 1 ml of gel packed in a plastic column (5 × 0.7 cm) connected to a 10-ml reservoir. The gel was pre-equilibrated with 5 ml of phosphate buffer (50 mmol/L, pH 9.2) and hemolysate in a volume of 200 μL was loaded on to the column and then washed with 10 ml of the same phosphate buffer. Non-GHb was eluted in this fraction (Peak 1) and GHb that remained attached to the column was eluted with the same phosphate buffer containing 100 mmol/L sorbitol. Eluate from this wash was collected (Peak 2). Hemoglobin concentration in Peak 1 and Peak 2 was estimated by reading the optical density at a wavelength of 430 nm. The amount of GHb present was calculated according to the formula:

$$\text{GHb}(\%) = \frac{OD_{430}\ \text{Peak 2}}{OD_{430}\ \text{Peak 1} + OD_{430}\ \text{Peak 2}} \times 100.$$

Control experiments of the PBA chromatography were performed by varying the temperature of the column, volume of gel bed, volume of eluting fluid, concentration of sorbitol, and hemoglobin concentration of the hemolysate applied to the column.

The reproducibility of this method was estimated by assaying three samples (high, middle, and low GHb) daily on 30 occasions.

ION-E chromatography. GHb was measured by cation-exchange chromatography using commercially available mini-columns (Bio-Rad Laboratory). The elution and running of the column was exactly as described by the manufacturer, except that hemolysate for the rat and dog samples was prepared from washed red cells rather than whole blood. In addition, 10 dialyzed samples (24 h) of human hemolysate were studied by both macro-column ION-E[2] and PBA methods, and the results were compared.

Thiobarbituric acid method. Measurement of GHb of rat and dog hemolysate was performed by a previously reported modification[5] of the thiobarbituric acid method described by Fluckiger and Winterhalter.[12] Rat hemoglobin concentration was standardized at 3 g/dl and dog hemoglobin concentration at 5 g/dl and the results expressed as nmol of 5-hydroxymethylfurfural generated per milligram of hemoglobin.

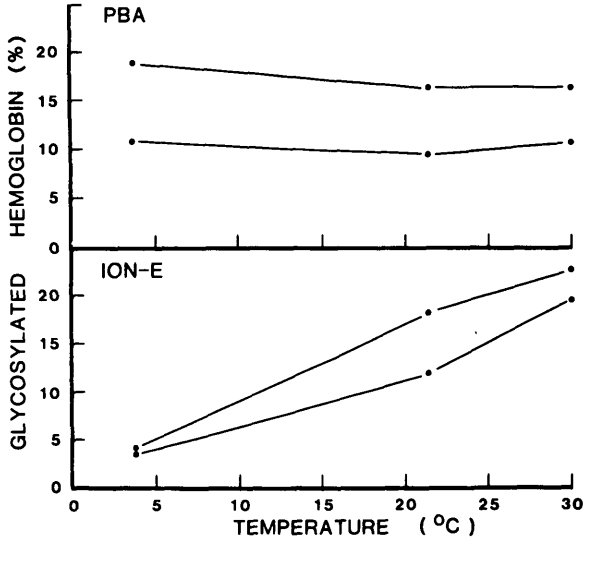
Effects of glucose on the PBA and ION-E methods. The effects of glucose on the PBA and ION-E methods were studied by incubating normal human red cells in phosphate buffer (155 mmol/L, pH 7.6) containing glucose at a concentration of 40 mmol/L or 80 mmol/L. After 3 h, incubation at 37°C or 1 min at 4°C the incubation mixtures were used to prepare hemolysate either without prewashing or after washing the cells three times in phosphate buffer. Samples of hemolysate obtained were analyzed by the PBA and ION-E methods. In addition, 16 patient samples were studied with and without dialysis (phosphate buffer, 50 mmol/L, pH 9.2, 24 h) prior to PBA chromatography.

Statistical methods. Comparison was made by Student's *t* test and Linear regression calculated by the least squares method.

RESULTS

Effects of experimental conditions on PBA chromatography. When the volume of PBA gel was increased from 0.5 to 1.5 ml there was no change in the results obtained. However, a higher result was obtained when the gel volume was increased to 2 ml. Due to leaching of GHb from the column, larger volume of elution buffer yielded lower final GHb results, e.g., a 20 ml volume of eluting fluid yielded final GHb

FIGURE 1. Temperature profiles of glycosylated hemoglobin results measured by the PBA and ION-E chromatography. Each experiment was performed three times.



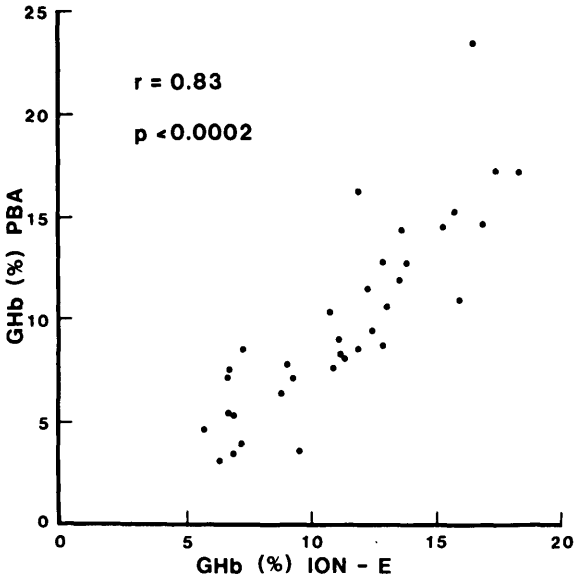


FIGURE 2. Correlation between glycosylated hemoglobin levels in human diabetic subjects measured by the PBA and ION-E chromatography.

results about 50% of that obtained if 10 ml was used. Irrespective of whether the sample was obtained from normal or diabetic subjects the results of GHb remained constant over a fourfold range of hemoglobin concentration in the hemolysate (5–20 g/dl). Recovery of hemoglobin from the column was greater than 99% when sorbitol at a concentration of 100 mmol/L was used as eluting fluid.

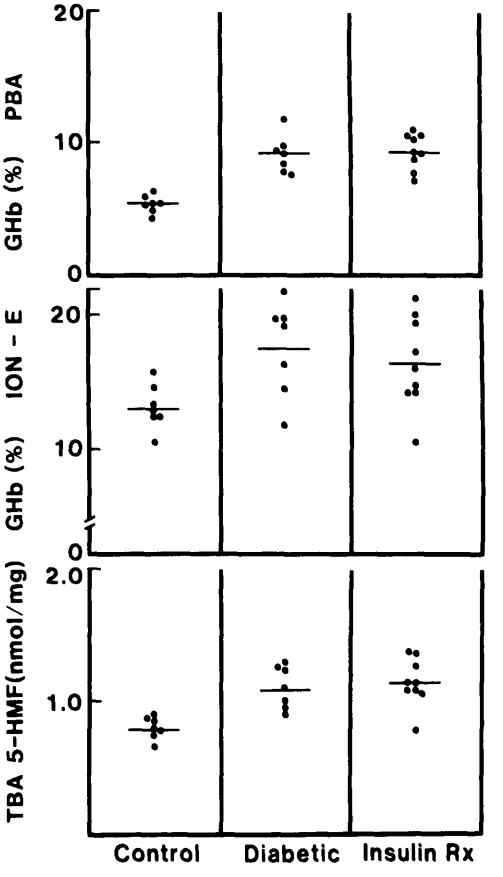
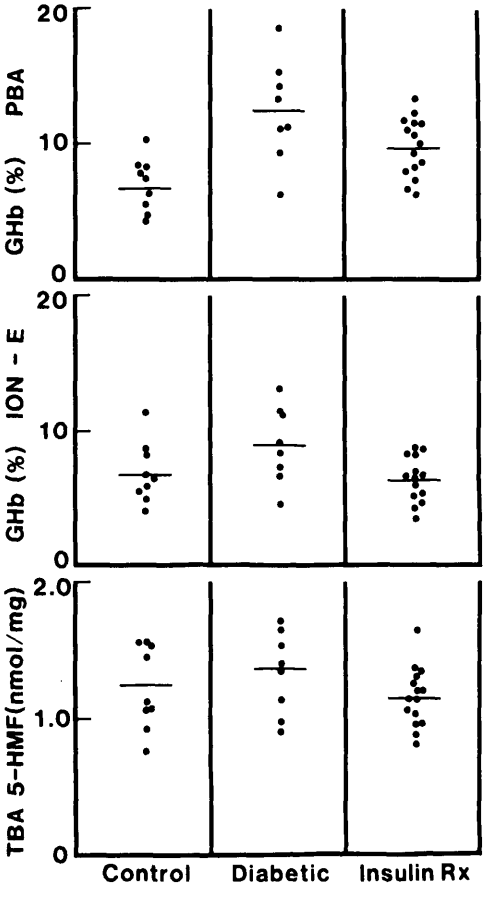


FIGURE 4. Results of dog glycosylated hemoglobin measured by PBA chromatography, ION-E chromatography, and TBA method.

FIGURE 3. Results of rat glycosylated hemoglobin measured by PBA chromatography, ION-E chromatography, and TBA method.



The effect of temperature on results obtained by PBA or mini-column ION-E method is shown in Figure 1. PBA chromatography was not appreciably affected by the temperature changes and the difference between samples with high or low GHb remained discernible from 4°C to 30°C. Using the ION-E method, the separation of the high and low GHb samples was much reduced at the extremes of temperature.

The inter-assay variations of the high, middle, and low GHb samples over 30 consecutive measurements were 10.7%, 8.4%, and 9.2%, respectively.

Measurement of human GHb. The correlation of human GHb measured by PBA and mini-column ION-E chromatography is shown in Figure 2. There was good correlation between the two methods ($r = 0.83$, $P < 0.0002$). Very similar results were obtained comparing PBA with the macro-column ION-E method ($N = 10$, $r = 0.86$, $P < 0.002$).

Measurement of rat GHb. Results of measurement of rat GHb by three different methods are shown in Figure 3. PBA chromatography gave clear separation of the normal from diabetic rats ($P < 0.05$) with partial normalization in the insulin-treated group. In contrast, the diabetic groups could not be separated readily from the control rats when GHb was measured by either the TBA method or ION-E chromatography. The correlation between GHb measured by PBA chromatography and blood glucose levels was highly significant ($r = 0.79$, $P < 0.001$). The corresponding correla-

tion between GHb measured by the other two methods and blood glucose level was not significant.

Measurement of dog GHb. The measurement of dog GHb by the three different methods is shown in Figure 4. PBA chromatography gave clear separation of the normal from diabetic dogs ($P < 0.05$) with no significant reduction in the insulin-treated group. Separation of the normal and diabetic dog groups was possible using the TBA and ION-E chromatography but there was more overlap. There was a good correlation between GHb measured by PBA chromatography and blood glucose levels ($r = 0.67$, $P < 0.003$). The corresponding correlation coefficients for the ION-E and TBA methods were 0.50 and 0.66, respectively.

When dog hemolysate was analyzed by ION-E chromatography, there was significant smearing of hemolysate through the gel after elution of the GHb fraction, suggesting some leakage of non-GHb from the column in this method.

Effects of glucose on the PBA and ION-E methods. Glucose (40 or 80 mmol/L) added to normal blood samples at 4°C and incubated for 1 min had no effect on the results obtained by the PBA or ION-E methods. However, after 3 h at 37°C, glucose in these concentrations caused significant rapid glycosylation with increase in GHb estimated by either method. Results of the percentage increase over basal sample is shown in Table 1. This interference by rapid glycosylation can be partially reversed by washing the cells three times before preparation of the hemolysate. For the study of patient samples dialysis prior to PBA chromatography did not alter the GHb results ($N = 16$, $r = 0.98$, $P < 0.001$, slope and intercept did not differ from 1.0 and 0, respectively.)

DISCUSSION

The measurement of GHb is now used routinely in many clinics to assess diabetic control. It has also been used in many research projects as a guide to diabetic severity.[1–5] In contrast, despite the use of many animal models for diabetic research, there have been very few studies that have included measurement of GHb. This may be partly due to the unavailability of a simple method that can be adapted for GHb measurement in different animal species. Measurement of GHb offers significant advantages over blood glucose determinations, especially in small laboratory animals as serial blood sampling is difficult. In addition, hyperglycemia induced by the stress of sampling may lead to falsely elevated estimations of diabetic severity. Wood and Smith[6] described the measurement of canine GHb by ion exchange chromatography using a column 1 × 30 cm in size. Quantitation of rat GHb has been reported using high pressure liquid chromatography.[9] These methods require specialized equipment or considerable running time. In this study, we have investigated the use of PBA chromatography to measure human, rat, and dog GHb and compared its efficacy with three other methods commonly used to measure GHb. The PBA method is based on the interaction of immobilized m-aminophenylboronic acid with the 1,2 cis diol groups of the glucose residue attached to the hemoglobin molecule.[13,14] The retained GHb is subsequently eluted by competitive displacement with excess sorbitol. The use of PBA chromatography to estimate GHb in man has been suggested, although a preliminary comparison showed poor correlation with the ION-E method.[13] We have found a much better correlation between the PBA and ION-E method.

It can be expected that the binding of GHb to m-aminophenylboronic acid and its subsequent elution by sorbitol should not be critically dependent on the species of hemoglobin. Our results appeared to have confirmed this theoretical prediction. The PBA method is effective in separating diabetic from normal controls in all species studied. By contrast, the TBA method and the ION-E method were less successful. In rats, the lack of sensitivity of the TBA method may be partly due to the use of hemoglobin at a relatively low concentration of 3 g/dl (a result of the small volume of blood obtainable from each animal). The heterogeneity of hemoglobin molecules known to be present in rats may contribute to the failure of ION-E chromatography to separate diabetic from normal samples.[15] In dogs, both the TBA and ION-E methods were able to separate diabetic from normal animals. However, the distinction was less clear-cut than that found with PBA chromatography. The range of normal canine GHb estimated by the ION-E method in our study was higher than that reported by Wood and Smith.[6] This could be attributed to overlapping of glycosylated and nonglycosylated hemoglobin on the mini-column. Wood and Smith used columns of 30 cm in length; overlapping of hemoglobin would be less with a column of this dimension.[6] However, the need for long columns would make the assay less suitable for routine use.

The PBA method is technically simple and a large number of samples can be processed simultaneously. The interassay reproducibility and the cost of the method is comparable to that of mini-column ION-E chromatography. The PBA method correlates well with both the mini-column and macro-column ION-E methods. It is less susceptible to changes induced by temperature variation; a factor known to have profound effects on results with the ION-E method.[1,16] In patients with hemoglobinopathy such as Hb-S disease, the PBA method is not affected by the different electrophoretic mobility of the variant hemoglobin molecule.[1] The results of the PBA method and the mini-column ION-E method are both increased by rapid glycosylation when the red cells are incubated in extremely high glucose medium. However, due to the extensive prewashing of red cells before preparation of hemolysate, the PBA method is affected to a lesser extent. Dialysis of patient samples before PBA chromatography did not alter the result. The volume of PBA-30 gel used in this method has a high binding capacity (30–50 μmol). This is supported by the observed narrow band of GHb remaining at the top of the PBA column

TABLE 1
Effects of glucose on PBA and ION-E chromatography

Glucose* concentration (mmol/L)	% increase of GHb over basal level			
	ION-E		PBA	
	No prewash†	Wash × 3	No prewash	Wash × 3†
40	32	26	25	15
80	51	33	55	25

* Incubated with red cells at 37°C for 3 h.
† Conditions routinely used in this study.

after sample application. Theoretically, it would be possible to further reduce the volume of gel used to minimize running costs. A higher volume of gel bed increases the nonspecific trapping of nonglycosylated hemoglobin and gives higher results. The reaction between m-aminophenylboronic acid and the glucose molecule is reversible, and a small but significant amount of bound GHb is continuously eluted from the column even in the absence of sorbitol. Thus, the final result is also affected by the volume of buffer used to wash the column after loading of hemolysate. However, provided the gel-bed and eluting fluid volume are kept constant, consistent results can be obtained. The PBA gel was chosen for this study due to its high molecular weight cutoff (4×10^6 daltons), making it suitable for the binding of hemoglobin. The elution of GHb was achieved by using a buffer containing excess sorbitol. Although acidification has been used for elution in PBA chromatography of other substances, it is usually not successful in eluting protein.[13]

Chromatography with PBA is a simple and reliable method to determine GHb and a suitable alternative to ION-E chromatography for the routine human GHb estimation. Its stability at different temperatures is a significant advantage. As it is not influenced by hemoglobin heterogeneity, either between or within species, it can be used to estimate GHb in a number of laboratory animals and patients with hemoglobinopathy.

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Note added in proof. After submission of this manuscript, similar work by K. Mallia et al. (Analytical Letters *14*:649, 1981) and in a Letter to the Editor by Dean et al. (Diabetologia *21*:570, 1981) was brought to our attention.

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