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Separation of glycosylated haemoglobins using immobilized phenylboronic acid

Effect of ligand concentration, column operating conditions, and comparison with ion-exchange and isoelectric-focusing

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Haemoglobins from diabetic and non-diabetic individuals have been separated by affinity chromatography using immobilized phenylboronate, which interacts specifically with diol-containing compounds such as glycosylated haemoglobin. The effects of ligand concentration, flow rate, column geometry, preincubation of sample, buffer composition and temperature have been investigated. Significant correlation was found between results from affinity-chromatography and ion-exchange and isoelectric-focusing methods. Isoelectric-focusing of the haemoglobin fractions obtained from affinity chromatography indicate that, in addition to haemoglobin A_{1c}, some haemoglobin A is also bound to immobilized phenylboronic acid. Assays of haemolysates obtained from red blood cells incubated in glucose solutions suggest that unstable pre-(haemoglobin A_{1c}) does not interfere. The assay is not affected by the presence of haemoglobin F.

Interest has recently increased in the measurement of glycosylated haemoglobin (Hb) as an indicator of diabetic control (for reviews, see Bunn, 1981*a*,*b*). The most abundant glycosylated haemoglobin is HbA_{1c}. This is formed by an initial reversible reaction between glucose and the *N*-terminus of the β-chain of haemoglobin to give an unstable Schiff base, which is converted by a slower irreversible reaction into a stable ketoamine form (Higgins & Bunn, 1981). In addition to HbA_{1c}, there are other glycosylated haemoglobins, HbA_{1a1}, HbA_{1a2} and HbA_{1b}, all of which contain sugars linked to the *N*-terminus of the β-chain (McDonald *et al.*, 1978). These haemoglobins are known collectively as HbA_1 and can be separated from normal adult haemoglobin (HbA) by ion-exchange chromatography because of their altered isoelectric points. Haemoglobin is also glycosylated at the terminus of the α-chain and at lysine residues throughout the molecule (Bunn *et al.*, 1979; Shapiro *et al.*, 1980), but since this results in little change in net charge at neutral pH, these glycosylated haemoglobins cannot be separated by ion-exchange or electrophoretic techniques.

Present methods for assaying glycosylated haemoglobins measure different species. The most widely used assays are based on ion-exchange, which separates either HbA_{1c} or the total HbA_1 fraction [as in the method described by Trivelli *et al.* (1971)]. These methods are very sensitive to experimental variables such as pH and temperature (Schellekens *et al.*, 1981) and are subject to interference from abnormal haemoglobins and the unstable Schiff base 'pre-HbA_{1c}', which is formed in the presence of high blood glucose levels (Svendsen *et al.*, 1980; Mortenson, 1980; Compagnucci *et al.*, 1981). Other methods include colorimetric assays, isoelectric focusing, electrophoresis and radioimmunoassay. The only method that measures haemoglobin glycosylation at lysine residues throughout the molecule is the colorimetric assay (Fluckiger & Winterhalter, 1976), which has proved difficult to standardize. There is a need for an assay that is easy to perform, free from interference and relatively insensitive to experimental variables such as pH and temperature.

The potential of affinity separations (Lowe &

Abbreviations used: PBA, phenylboronic acid; Hb, haemoglobin.

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Dean, 1974) led us to consider immobilized ligands capable of differentiating between glycosylated and non-glycosylated haemoglobins. The reaction of immobilized boronic acids with molecules containing *cis*-diols is well known (Weith *et al.*, 1970). Since glucose bound to haemoglobin contains diols, it seemed likely that this boron–diol interaction might be used to provide an assay for total glycosylated haemoglobin. Agarose-immobilized phenylboronic acid (PBA) has already been used to separate glycosylated from non-glycosylated proteins, e.g. cell-membrane glycoproteins (Williams *et al.*, 1981). Preliminary reports on the use of agarose-immobilized PBA to measure glycosylated haemoglobin have been encouraging (Dean *et al.*, 1980; Bouriotis *et al.*, 1981; Mallia *et al.*, 1981; Yue *et al.*, 1982), and a correlation has been found between the affinity method and ion-exchange methods.

In order to optimize this affinity assay, we examined a number of experimental variables, particularly buffer composition, which can greatly influence the performance of PBA-mediated affinity separations (McCutchen *et al.*, 1975). We chose a set of conditions to compare the performance of the affinity assay with ion-exchange and isoelectric-focusing methods. We also investigated which species of haemoglobin are bound to immobilized PBA.

Materials and methods

m-Aminophenylboronic acid immobilized on to Matrex Gel (PBA-Matrex Gel) was obtained from Amicon Corp., Lexington, MA, U.S.A. Before chromatography, PBA-Matrex Gels were washed with 0.2 M-sodium acetate/acetic acid buffer, pH 5.4 (20 column volumes), and equilibrated with starting buffer (20 column volumes) in polycarbonate (0.7 cm internal diameter) chromatography columns (from Amicon). Except where otherwise stated, Matrex Gel-PBA 30 (38 mmol of PBA/litre) was used. (Gels could be used at least three times when regenerated between use with acetate buffer.) Bio-Rex 70 cation-exchange resin (200–400 mesh) was obtained from Bio-Rad Laboratories, Watford, Herts., U.K. Other chemicals were obtained from Sigma (London) and BDH, both of Poole, Dorset, U.K.

Samples

Samples were obtained from a diabetic outpatient clinic. Non-diabetic control samples were taken from healthy volunteers.

Haemolysate preparation

(I) Red cells from anticoagulated (EDTA- or heparin-treated) whole blood (0.5 ml) were washed once with 0.15 M-NaCl (10 ml) and haemolysed with 0.8 ml of a solution containing white saponin and KCN (0.1 and 0.5 g/100 ml respectively). The haemolysate was diluted with 4.5 ml of Buffer A, pH 6.74 (33 mM-NaH_2PO_4/9 mM-Na_2HPO_4/10 mM-KCN) and centrifuged at 1000 *g* for 10 min before use. Haemolysates were stored at −20°C.

(II) Heparin- or EDTA-anticoagulated whole blood (1.0 ml) was washed with 0.15 M-NaCl (10 ml). Cells were haemolysed with 10 mM-KCN (6.0 ml), five drops of carbon tetrachloride were added, and the haemolysate was centrifuged for 10 min at 1000 *g*. Haemolysates were stored at −70°C.

Affinity chromatography

Samples of haemolysate II (0.2 ml) were diluted with starting buffer (100 mM-taurine/NaOH, 0.2 ml) and applied to columns containing pre-equilibrated PBA-Matrex Gel (0.5 ml). Columns were stoppered and left for 60 min before being washed with taurine buffer (25 ml). Bound haemoglobin was desorbed with 0.1 M-sorbitol (10 ml) in taurine buffer. The absorbance (A_{413}) of the fractions was measured at 413 nm and the amount of haemoblogin bound was calculated from the equation:

$$\text{Percentage of Hb bound} = \frac{A_{413}\text{ bound} \times 100}{A_{413}\text{ bound} + (2.5 \times A_{413}\text{ unbound})}$$

Ion-exchange chromatography

Bio-Rex 70 cation-exchange resin was equilibrated in Buffer A, and poured into columns (4.0 cm × 0.7 cm). After further washing of the columns with Buffer A (30 ml), haemolysate I (0.2 ml) was transferred to the top of the columns. Glycosylated haemoglobins were eluted with Buffer A (10 ml) ('fast' fraction). The remainder of the haemoglobin was eluted with Buffer B (10 ml), pH 7.4, containing 210 mM-Na_2HPO_4 and 50 mM-NaH_2PO_4 ('slow' fraction). The absorbance of each fraction was measured at 413 nm and the concentration of glycosylated ('fast') haemoglobin calculated as a percentage of the total.

Isoelectric focusing

Isoelectric focusing was carried out with LKB Ampholine PAG plates for determination of HbA_{1c} (LKB no. 1804-131) with an LKB Multiphor 2117 and LKB 2103 power supply, according to the LKB instruction sheet supplied and using haemolysates II. After electrofocusing, the bands containing HbA_{1c} were cut out, eluted in 0.05 M-Tris/HCl buffer, pH 8.3, containing 0.01 M-KCN and 0.1 mM-EDTA, and the absorbance measured at 413 nm.

Results

Optimization of assay conditions

Haemolysates from both diabetics and non-diabetics were separated on immobilized PBA under a variety of conditions. In every case, the diabetic

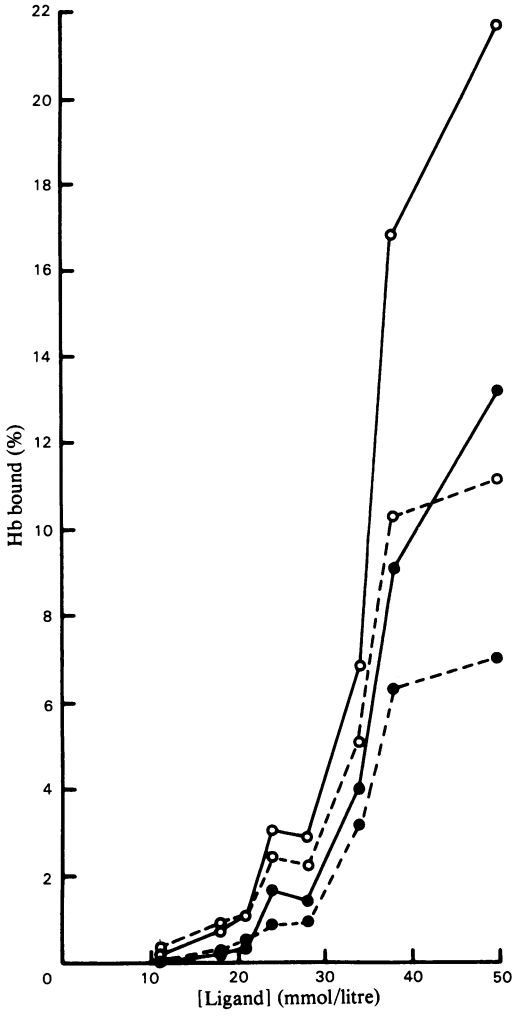

Fig. 1. *Effect of ligand concentration on binding of haemoglobin to immobilized phenylboronic acid*
Haemolysates from diabetic (O) and non-diabetic (●) subjects were separated on columns containing 1 ml of PBA-Matrex Gel, with 50 mM-taurine buffer, pH 8.70, at 20°C (——) and at 4°C (– – – –).

samples contained more PBA-retained haemoglobin than the non-diabetic controls.

Gels with ligand concentrations ranging from 11 to 50 mmol/litre were used to separate haemolysates at 4 and 20°C. The results are shown in Fig. 1. When chromatography was performed at 4°C, the amount of haemoglobin bound to the column increased with increasing ligand concentrations, approaching a constant level at the higher concentrations used. Similar results were found at 20°C, except that binding of haemoglobin continued to increase even at high ligand concentrations.

The amount of haemoglobin bound to immobilized PBA was found to be inversely related to flow rate; increasing the flow rate from 10 to 70 ml/h caused a 50% reduction in the amount bound. Preincubation of the sample on the column resulted in increased binding of haemoglobin, a maximum amount (an increase of about 70%) being bound within 30–60 min. In addition, when a preincubation step was included, changes in flow rate ceased to affect the amount of bound haemoglobin. When chromatography was carried out with columns of different dimensions (diameter 0.24–0.90 cm), with a constant volume and flow rate, little difference was found in the amount of haemoglobin binding.

Varying the sample size from 0.1 to 20 mg showed that columns remained unsaturated when large amounts of haemoglobin were applied, but with small amounts (less than 5 mg), decreased sample size resulted in increased binding. This effect was more marked at 20°C than at 4°C.

Haemolysates were separated by using serine, glycine, pyrophosphate, barbitone Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], ammonium acetate, morpholine, glycylglycine and taurine buffers at pH values from 7.8 to 9.6. For all buffers, the amount of haemoglobin bound to the PBA gel decreased as the pH increased, the effect being greater with some buffers than with others. Particularly low binding was found with serine buffers, whereas pyrophosphate buffers caused increased amounts of haemoglobin to bind to PBA. Results obtained using taurine buffers are shown in Fig. 2. When the same taurine buffers were used at 20°C, similar results were obtained with buffers of pH 8.70 and above. Slightly higher binding was observed at 4°C than at 20°C when the pH 8.5 buffer was used (Fig. 2). When the concentration of taurine buffer was varied from 20 to 200 mM, a constant amount of haemoglobin was bound at concentrations of 60 mM or greater. With the more dilute buffers, increased binding was observed. The addition of $MgCl_2$ to taurine buffers, pH 8.50, 8.70 and 8.90, resulted in an increased amount of bound haemoglobin, the effect being greater at lower pH values (Fig. 3). The addition of similar amounts of NaCl caused a much smaller increase in binding.

Performance of the affinity assay

Haemolysates from diabetic (97) and non-diabetic (44) subjects were assayed by the optimized method. The mean value obtained for diabetic patients was 11.8% (S.D. 2.29%), with a range from 7.9 to 19.7% The equivalent result for the non-diabetic control group was 7.1% (S.D. 0.52%), and the range was 5.5–8.2%. Within-assay precision was assessed by

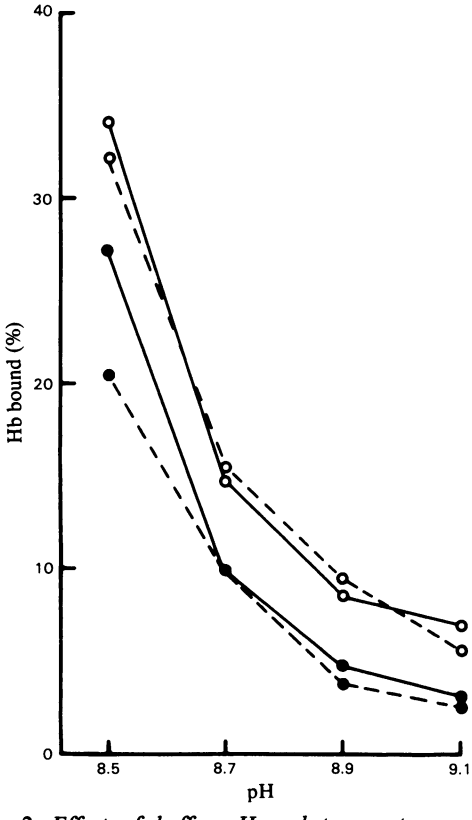

Fig. 2. *Effect of buffer pH and temperature on the binding of haemoglobin to immobilized PBA*
Haemolysates from diabetic (O) and non-diabetic (●) subjects were separated at 4°C (——) on 1 ml columns of PBA 30-Matrex Gel, with 50 mM-taurine buffers. Chromatography was then repeated, using the same buffers at 20°C (– – – –).

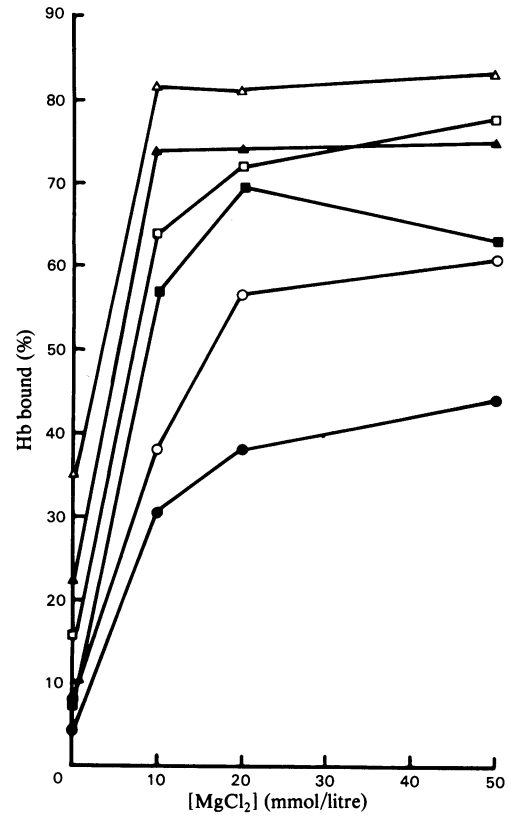

Fig. 3. *Effect of $MgCl_2$ on the binding of haemoglobin to immobilized PBA*
Haemolysates from diabetic (open symbols) and non-diabetic (closed symbols) subjects were separated at 20°C on 1 ml columns of PBA 30-Matrex Gel, with 50 mM-taurine buffers, pH 8.50 (△▲), 8.70 (□■) and 8.90(○●), supplemented with various amounts of $MgCl_2$. The percentage haemoglobin desorbed by the addition of sorbitol was calculated from the total haemoglobin applied.

using duplicate samples in the same assay, and calculating the standard deviation from the formula:

$$\text{S.D.} = \sqrt{\Sigma d^2/2N}$$

where d is the difference between duplicates and N is the number of duplicates. For the diabetic samples ($N = 15$) the mean value was 11.2% (S.D. = 0.50) and the coefficient of variation (CV) was 4.4%. For the non-diabetic group ($N = 15$) the mean value was 6.8% (S.D. = 0.47) and the CV 6.9%. Between-assay precision was measured by assaying a diabetic and a non-diabetic sample on 15 separate occasions. For the diabetic sample the mean value was 13.7% (S.D. = 0.90) and the CV was 6.6%. For the non-diabetic group the mean value was 8.1% (S.D. = 0.46) and the CV was 5.7%.

To examine the linearity of the assay, mixtures containing different proportions of diabetic and non-diabetic haemolysates were assayed. Results are shown in Table 1. When observed results were compared with expected results by linear-regression analysis (calculated with five points), the following equation was obtained:

Expected percentage bound
= (1.02 × observed percentage bound) + 0.06

(S.D. slope = 0.091, S.D. intercept = 1.03). Correlation of results obtained by using affinity chromatography with those from ion-exchange chromatography and isoelectric-focusing was investigated by using diabetic and non-diabetic haemolysates. Results are shown in Figs. 4 and 5.

In order to identify the haemoglobin species bound to PBA, haemolysates were separated with PBA-Matrex Gel, concentrated and examined by

isoelectric-focusing. For comparison, the haemolysates were also chromatographed using the ion-exchange method before electrofocusing. Results are shown in Fig. 6. The 'fast' and 'slow' fractions obtained from ion-exchange chromatography contained mainly HbA_{1c} and HbA respectively. The PBA-'unbound' fraction, like the ion-exchange 'slow' fraction, contained mainly HbA, but the 'bound' fraction contained both HbA and HbA_{1c}. A quantitative 'cross-over' experiment was carried out with the same fractions, remeasuring them by ion-exchange and affinity chromatography. Results are shown in Table 2. The PBA-'bound' fractions showed an increase in glycosylated haemoglobin as measured by ion-exchange, and the ion-exchange 'fast' fractions showed increased binding when measured by affinity chromatography. However, when PBA-'bound' fractions were remeasured by PBA chromatography, only 53.3% of the diabetic and 40.4% of the non-diabetic sample were bound. In addition, when PBA-'unbound' fractions were re-run on PBA columns, a small amount of haemoglobin was bound.

Possible interference in the method from pre-HbA_{1c} was investigated by incubating red blood cells in NaCl/glucose solutions. Blood samples (0.5 ml) from diabetic and non-diabetic subjects were washed with 0.15 M-NaCl and incubated at 37°C for 2 h in 0.15 M-NaCl (0.25 ml) containing glucose at con-

Table 1. *Assays of mixture of diabetic and non-diabetic haemolysates*

A diabetic and a non-diabetic haemolysate were mixed in different proportions and assayed by affinity chromatography as described in the text. The expected percentage of haemoglobin bound for the mixtures was calculated from the results obtained for the unmixed haemolysates.

Haemolysate in mixture (%)		Hb bound		Expected Hb bound
Diabetic	Non-diabetic	(%)*	S.D.	(%)
0	100	7.8	0.24	—
25	75	9.3	0.54	9.6
50	50	11.4	0.41	11.4
75	25	12.2	0.66	13.2
100	0	15.0	0.33	—

* Mean of five results.

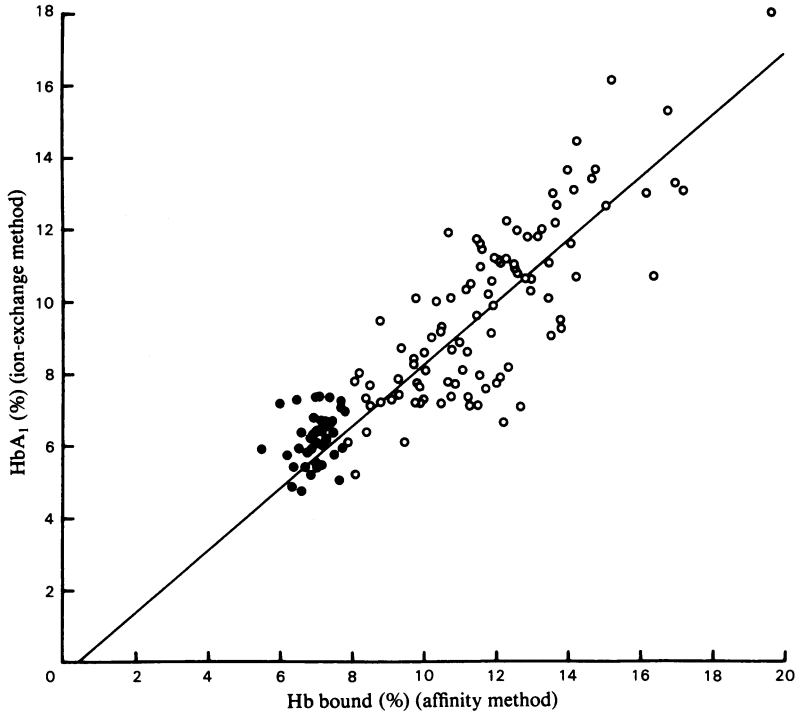

Fig. 4. *Correlation between affinity-chromatography and ion-exchange-chromatography methods*

Haemolysates from 97 diabetic (○) and 40 non-diabetic (●) subjects were assayed by affinity chromatography (x) and ion-exchange chromatography (y). Regression equation (calculated using diabetic results only): $y = 0.85x - 0.31$. (S.D. slope = 0.065, S.D. intercept = 0.78). Correlation coefficient $r = 0.80$ ($P < 0.001$).

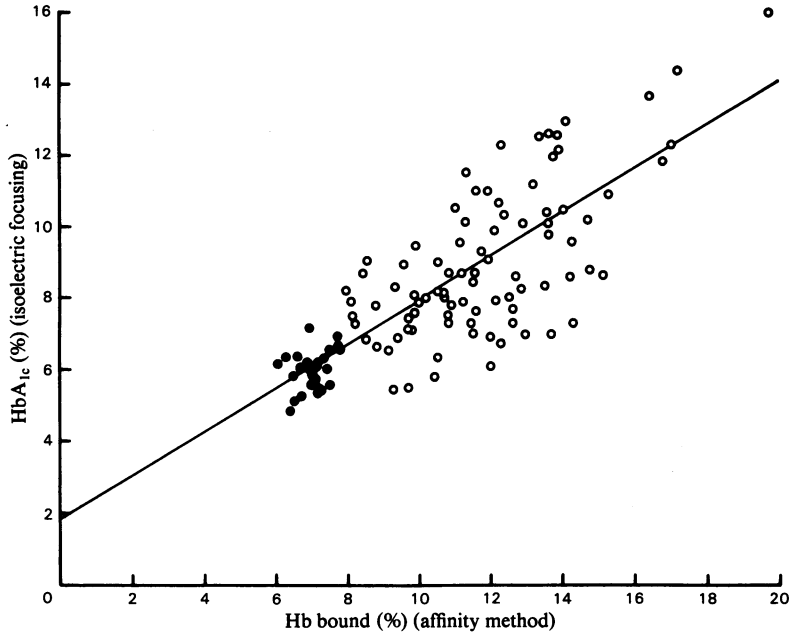

Fig. 5. *Correlation between affinity-chromatography and isoelectric-focusing methods*
Haemolysates from 87 diabetic (O) and 27 non-diabetic (●) subjects were assayed by affinity chromatography (x) and isoelectric-focusing (y). Regression equation (calculated using diabetic results only): $y = 0.61x + 1.81$. (S.D. slope = 0.073, S.D. intercept = 0.87). Correlation coefficient $r = 0.67$ ($P < 0.001$).

centrations from 0 to 100 mM. After incubation, cells were washed three times with 0.15 M-NaCl before being haemolysed as usual, and samples were examined by using ion-exchange and affinity chromatography and isoelectric-focusing. Results are shown in Table 3. Very little change was found in the amount of glycosylated haemoglobin as measured by affinity chromatography, whereas with the ion-exchange method, increased levels were observed in samples incubated at high glucose concentrations. These samples also showed double bands in the HbA_{1c} region on isoelectric-focusing. When red cells were incubated in phosphate-buffered saline (40.5 mM-Na_2HPO_4/9.5 mM-NaH_2PO_4/100 mM-NaCl), pH 7.4, containing 50 mM-glucose, at 37°C, for a longer period of 6 days, increased amounts of glycosylated haemoglobin were found when measured either by ion-exchange or by affinity chromatography. The results were 9.1% when measured by ion-exchange, and 9.6% when measured using PBA. Equivalent results for the sample incubated without glucose were 6.3 and 5.4% respectively.

The effect of raised amounts of foetal haemoglobin (HbF) was examined by using a non-diabetic haemolysate containing 24% HbF (normal adult level <0.9%). Results from affinity chromatography, isoelectric-focusing and ion-exchange chromatography were 7.34, 6.27 and >23% respectively. Whereas the affinity-chromatography and isoelectric-focusing results were within the normal range, gross interference was found with the ion-exchange assay.

Table 2. *Measurement of haemoglobin fractions by ion-exchange and affinity chromatography*
Haemolysates from diabetic and non-diabetic subjects were separated by both ion-exchange chromatography on Bio-Rex 70 and affinity chromatography. The fractions obtained were concentrated and remeasured by ion-exchange and affinity chromatography.

	Hb bound (%) (PBA assay)		Hb 'fast' (%) (ion-exchange)	
Haemoglobin fraction	Diabetic	Non-diabetic	Diabetic	Non-diabetic
Haemolysate	12.7	6.9	8.8	5.4
PBA				
'Bound'	53.3	40.4	29.0	23.0
'Unbound'	6.7	6.1	6.8	5.1
Ion-exchange				
'Fast'	36.6	26.6	71.1	72.7
'Slow'	10.8	7.6	3.8	2.8

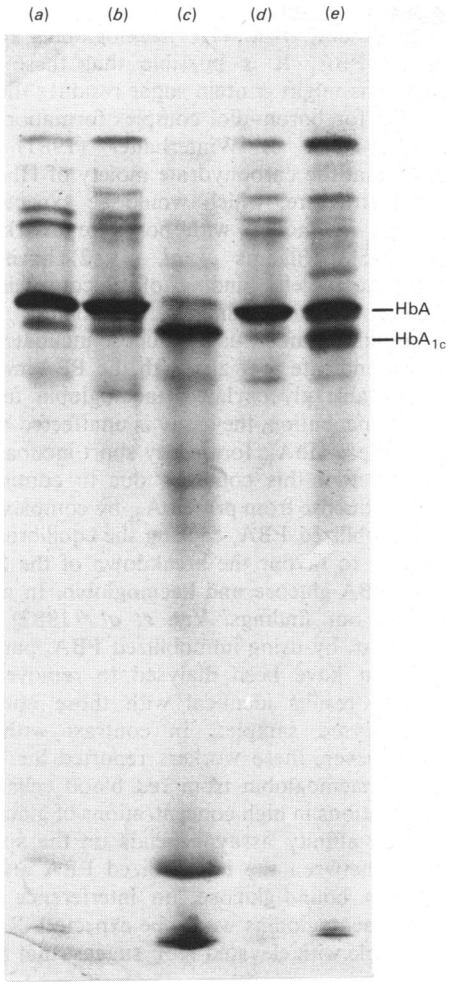

Fig. 6. *Isoelectric focusing of fractions from ion-exchange and affinity chromatography*
Haemolysates were separated by either affinity or ion-exchange chromatography. Fractions were concentrated before isoelectric focusing. (*a*) Haemolysate; (*b*) ion-exchange, 'slow'; (*c*) ion-exchange, 'fast'; (*d*) affinity chromatography, 'unbound'; (*e*) affinity chromatography, 'bound'. The gel was stained with Coomassie Brilliant Blue R250.

Table 3. *Rapid glycosylation of haemoglobin*
Red blood cells from diabetic and non-diabetic subjects were incubated for 2h at 37°C in 0.15M-NaCl containing various concentrations of glucose from 0 to 100mM. Haemolysates prepared from incubated cells were assayed by ion-exchange and affinity chromatography and examined for the presence of a double HbA_{1c} band in isoelectric-focusing.

Sample	[Glucose] (mM)	Hb bound (%) (PBA assay)	Hb 'fast' (%) (ion-exchange assay)	Presence of double band on isoelectric focusing
Non-diabetic	0	7.2	6.5	–
	20	6.7	7.4	+
	50	7.8	8.2	+
	100	7.8	9.3	+
Diabetic	0	11.4	9.6	–
	20	11.1	10.2	+
	50	11.7	11.2	+
	100	11.8	13.1	+

Discussion

The effect of varying ligand concentration suggests a weak boron–diol interaction, amplified by a hydrophobic component at high PBA concentrations and high temperature. A ligand concentration of 30–40mmol/litre was chosen for the affinity assay, since at this concentration useful differences between diabetic and non-diabetic haemolysates were found, and temperature-dependence was low. The inverse relationship found between flow rate and haemoglobin binding also reflects the weak nature of the PBA–haemoglobin interaction. It seems advisable to preincubate samples before chromatography both to promote the interaction and to exclude effects of variations in flow rate. The results obtained using different sample sizes indicate that very small sample/column ratios should be avoided because of non-specific binding. In order for diol-containing compounds to interact with PBA it is necessary that the boron be in a negatively-charged, tetrahedral form (Yurkevitch *et al.*, 1975). Since the pK_a of the immobilized PBA is 9.2 (Maestas *et al.*, 1980), or 8.86 in free solution (Yabroff *et al.*, 1934), buffer experiments were restricted to the pH range 7.8–9.6. At higher pH values, haemoglobin binding and stability were reduced, whereas at low pH values, non-specific binding increased and results were too sensitive to small changes in pH to give a useful clinical assay. This non-specific binding could include ionic effects associated with the negatively charged PBA and a hydrophobic component introduced by the phenyl ring. In considering different buffer ions, Tris and triethanolamine were avoided because, like sorbitol, they bind to PBA, excluding other diols. In contrast, amine-containing buffers can be used to strengthen the interaction between diols and PBA (Wulff *et al.*, 1982). The low haemoglobin binding obtained with serine buffers was presumed to be due to the involvement of both amino and alcohol groups of the buffer ion complexing with the boron and thus preventing glycosylated haemoglobins from binding. The high binding obtained with pyrophosphate buffers was presumably due to increased hydro-

phobic interactions caused by the substantially higher ionicity of this buffer. Among the buffers tested, the use of taurine in an affinity assay for glycosylated haemoglobin was indicated because of its suitable pK_a, its low toxicity and low cost. It seems best to conduct an assay at pH 8.70, since at this pH haemoglobin binding is less sensitive to small changes in pH than at lower pH values, and greater differences in binding are found between diabetic and non-diabetic samples than in buffers of higher pH. Since reduced buffering of the sample by dilute taurine buffers leads to increased non-specific binding, a concentration of 100mM was chosen for the assay.

Bivalent cations (Mg^{2+}) have previously been used to lower charge–charge interactions between the negatively charged borate and diol-containing molecules (Rosenberg *et al.*, 1972), but an increase in ionic strength may be expected to increase hydrophobic binding. We found that added $MgCl_2$ dramatically increased the amount of haemoglobin bound at all pH values examined (8.50 to 8.90). The smaller increase in binding after the addition of NaCl was probably due to increasing hydrophobic interactions.

The temperature-dependence of pH of taurine buffers is about 0.02 pH unit/degree Celsius. This change in pH with temperature does not appear to affect the amount of haemoglobin bound to PBA 30-Matrex Gel at pH values of 8.7 and above. We have observed that when a buffer of pH 8.7 or higher is prepared at 20°C, the same amount of haemoglobin binds irrespective of column temperature. However, as discussed above, operating columns at room temperature caused increased non-specific hydrophobic binding when using low haemoglobin concentrations or gels of high ligand concentration.

Significant correlation was found between results from affinity chromatography, ion-exchange and isoelectric focusing. However, from the isoelectric focusing of fractions obtained by ion-exchange or affinity separations (Fig. 6), it can be seen that the haemoglobin species measured by the affinity method are different from those measured in ion-exchange (HbA_1) and isoelectric focusing (HbA_{1c}). The presence of a band moving in the HbA_0 region in PBA-bound fractions may possibly be explained by the glycosylation of HbA at sites other than the β-chain terminus (Bunn *et al.*, 1979; Gabbay *et al.*, 1979). Several glycosylated forms of haemoglobin were also found by Mallia *et al.* (1981) after chromatography of haemolysates on immobilized PBA. The slopes of the regression lines in Figs. 4 and 5 (0.85 and 0.61 respectively) also suggest that PBA binds other haemoglobin species in addition to HbA_1. The results obtained in the quantitative 'cross-over' experiments (Table 2) are consistent with the isoelectric-focusing results, but also indicate that some 'fast' haemoglobins are not bound by PBA. It is possible that these 'fast' haemoglobins might contain sugar residues that are unavailable for boron–diol complex-formation. For example, Fischer & Winterhalter (1981) have suggested that the carbohydrate moiety of HbA_{1c} is in a ring structure, which would be expected to weaken the interaction with boronate (Yurkevich *et al.*, 1975). Williams *et al.* (1982) have also observed incomplete binding of glycoproteins to immobilized PBA under different conditions.

The results obtained using glucose-incubated red blood cells indicate that although the PBA method measures stable glycosylated haemoglobin formed by a 6-day incubation, the assay is unaffected by the presence of pre-HbA_{1c} formed by short incubations. We suggest that this could be due to continuous removal of glucose from pre-HbA_{1c} by complexation to the immobilized PBA, causing the equilibrium of the reaction to favour the breakdown of the Schiff base into PBA-glucose and haemoglobin. In accordance with our findings, Yue *et al.* (1982) have observed that, by using immobilized PBA, patients' samples that have been dialysed to remove pre-HbA_{1c} give results identical with those obtained from undialysed samples. In contrast with our results, however, these workers reported increased binding of haemoglobin from red blood cells after short incubations in high concentrations of glucose.

Since the affinity assay depends on the specific interaction between the immobilized PBA and the haemoglobin bound-glucose, no interference from abnormal haemoglobins would be expected. Results from a sample with elevated HbF suggest that this is the case.

In summary, the correlation between affinity chromatography using immobilized PBA and other methods of measuring glycosylated haemoglobins indicates that it provides a means of assessing diabetic control. Although the affinity method measures some HbA_0 in addition to HbA_1, the amount of bound HbA_0 (whether glycosylated or not) is proportional to the amount of HbA_1, and should also, therefore, reflect diabetic control. Although it is easier to perform than ion-exchange chromatography, being less sensitive to experimental variables such as pH and temperature, the affinity method must be conducted under carefully controlled conditions so that the rather weak boron–diol interaction is encouraged, while non-specific hydrophobic effects are kept to a minimum. Our results indicate that the assay is unaffected by the presence of either unstable pre-HbA_{1c} or HbF. It is possible that the method could also be extended to measure other glycosylated proteins such as albumin, which has been shown to reflect recent glycaemic control in diabetics (Dolhofer & Wieland, 1980).

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