Journal of Chromatography, 500 (1990) 543–553
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 119

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS ON DEFORMED NON-POROUS AGAROSE BEADS

FAST BORONATE AFFINITY CHROMATOGRAPHY OF HAEMOGLOBIN AT NEUTRAL pH

STELLAN HJERTÉN* and JIN-PING LI
Institute of Biochemistry, University of Uppsala, Biomedical Centre, P.O. Box 576, S-751 23 Uppsala (Sweden)
(First received July 11th, 1989; revised manuscript received October 26th, 1989)

SUMMARY

Aminophenylboronic acid was attached to epoxy-activated non-porous agarose beads with diameters of 12–15 μm and this boronate gel column was used for the fractionation of glycosylated from non-glycosylated haemoglobin. By varying the experimental conditions it was shown that the ratio between the second peak (glycosylated haemoglobin) and the first peak (non-glycosylated haemoglobin) was virtually independent of pH in the range 7–8, ionic strength, flow-rate and sample load (up to at least 160 μl of haemolysate on a 0.7-ml column). It is therefore not necessary to control thoroughly these parameters in order to obtain reproducible results, which is a great advantage in fast routine analyses of glycosylated haemoglobin. At a flow-rate of 4.0 ml/min an analysis was finished within 2 min on a 2.5 cm × 0.6 cm I.D. column. The total time of an analysis was also short because a sample from a droplet of blood could be applied directly onto the column after haemolysis for 1 min without removal of cell debris by time-consuming centrifugation. The experiments were performed at a pH close to the isoelectric point of haemoglobin, because at this pH haemoglobin has a negligible net charge and will therefore not interact with the charged groups of the ligands, the matrix and proteins adsorbed in previous runs.

INTRODUCTION

Boronates react reversibly with hydroxyl groups oriented in the proper geometry and particularly with *cis*-1,2-diols. This property of boronate has been utilized both in electrophoresis and chromatography for the separation of diol-containing substances. For instance, borate derivatives have been immobilized on various chromatographic supports for the fractionation of both low-molecular-weight compounds, such as nucleotides[1,2] and catecholamines[3], and high-molecular-weight substances, such as membrane proteins[4] and haemoglobins[5]. Specific elution is achieved by displacement with *cis*-diol compounds, such as sorbitol.

Haemoglobin may become modified with carbohydrate moieties post-translationally by non-enzymatic processes. There is clinical interest in these glycosylated

0021-9673/90/$03.50

haemoglobins because their concentration in diabetes mellitus reflects the mean or 'integrated' blood glucose level over the days or months that the erythrocytes have been in circulation[6–8].

Some of the methods for assaying glycosylated haemoglobin are based on ion exchange and are very sensitive to experimental variables, such as pH and temperature[9], and are subject to interference from abnormal haemoglobins and the labile HbA_{1c} which is formed in the presence of high blood glucose levels[10].

Boronate affinity chromatography competes favourably with ion-exchange chromatography for the analysis of glycosylated haemoglobin, as the method is simple and free from the above interference and relatively insensitive to variations in the experimental conditions[6,11].

Recently, we have shown that compressed beds of non-porous agarose beads can be used with advantage in high-performance liquid chromatography (HPLC) based on hydrophobic interaction[12,13], ion exchange[14], chromatofocusing[15] and affinity of proteins to dyes[16]. A common and important feature of these columns is that they give a high resolution also at high flow-rates (a prerequisite is, of course, that adsorption and desorption proceed fast). The purpose of the experiments described here was to study whether compressed beds of non-porous beads of boronate–agarose have the same attractive property (we chose to study the separation of glycosylated from non-glycosylated haemoglobin because this fractionation is of clinical interest) and to compare the results obtained with those previously reported for non-compressed, macroporous boronate–agarose beds[17]. We were also interested to know whether the separation could be achieved at a pH close to the isoelectric point of haemoglobin, which is about 7. This would be an advantage, as at this pH haemoglobin has a negligible net charge and therefore will not react with the charged groups of the ligands, the traces of sulphate and carboxylic groups in the agarose matrix and proteins adsorbed in previous runs, which is of importance in avoiding the above disadvantages associated with the ion-exchange chromatography of haemoglobin (that there is no electrostatic interaction between a protein at its isoelectric point and charged groups in the stationary phase is evident from chromatofocusing experiments). As one cannot exclude non-specific hydrophobic interaction between haemoglobin and the phenyl group of the ligand, this possible interaction was studied by performing the experiments at different ionic strengths. Such experiments also give information on non-specific electrostatic interactions.

EXPERIMENTAL

m-Aminophenylboronic acid hemisulphate and boron trifluoride diethyl etherate were purchased from Serva (Heidelberg, F.R.G.), D-sorbitol from Kebo Lab. (Stockholm, Sweden), 1,4-butanediol diglycidyl ether from Sigma (St. Louis, MO, U.S.A.) and sodium borohydride from Merck–Schuchardt (Darmstadt, F.R.G.). Heparin-anticoagulated blood from healthy volunteers and diabetic patients (with elevated levels of glycosylated haemoglobin) was obtained from the University Hospital (Uppsala, Sweden). A 20-ml volume of the blood was centrifuged at 1000 *g* for 30 min and plasma was withdrawn. The stacked erythrocytes were washed with isotonic saline three times by centrifugation at this *g*-value and were then lysed in three volumes of distilled water at 4°C for 1 h. Debris was removed by centrifugation

at 4000 *g* for 30 min at 4°C. The haemolysates were divided into 1.0-ml portions and stored at −20°C.

The chromatographic system consisted of the following units: a Model 2150 HPLC pump and a Model 2152 LC controller from LKB (Bromma, Sweden), a Model C-RIA integrator from Shimadzu (Kyoto, Japan), an injection valve from Rheodyne (Cotati, CA, U.S.A.) and a Model 1306 variable-wavelength monitor from Bio-Rad Labs. (Richmond, CA, U.S.A.). Column tubes of Plexiglas[18] were packed at a flow-rate of 5 ml/min. The plunger was then immediately pushed down to make contact with the gel bed, which prevented it from expanding when lower flow-rates were used in subsequent experiments. The effluent was monitored at 415 nm for the specific determination of the haemoglobin content.

Preparation of non-porous agarose beads

Macroporous 12% agarose beads were prepared essentially as described previously[19]. By wet sieving, beads with a diameter of 15–20 μm were collected. The shrinkage and cross-linking of the beads have been described elsewhere[12,13]. Briefly, 5 g of 12% agarose beads were washed with distilled water by centrifugation at 1000 *g* for 2 min (these centrifugation conditions were used in all subsequent washings) and then transferred to dioxane by washing three times with water–dioxane (1:1) and three times with dioxane. Finally, 5 ml of dioxane–chloroform (1:1) were added. This last step was repeated twice. The volume of the beads was then about one third of the original volume. The shrunken beads were suspended in 25 ml of chloroform, then 3.2 ml of 1,4-butanediol diglycidyl ether was added with stirring, followed by 0.3 ml of boron trifluoride diethyl etherate in 15 ml of chloroform as catalyst for activation of the epoxide groups in the cross-linker. After cross-linking for 30 min, the beads were transferred back to water by washing with dioxane, dioxane–water (1:1) and water. These shrunken, cross-linked beads had a diameter of about 12–15 μm and were impermeable (non-porous) to proteins[12–14].

Coupling of m-aminophenylboronic acid to non-porous agarose beads

The coupling method, based on the oxirane method, was that described by Sundberg and Porath[20] with some modifications. First, 2 g of non-porous agarose beads, prepared as described in the previous section, was suspended in 2.5 ml of 1 *M* sodium carbonate solution (pH 10) containing 20 mg of sodium borohydride and 2.0 ml of 1,4-butanediol diglycidyl ether was added dropwise. The suspension was stirred at room temperature for 24 h. After this epoxy activation, the gel was washed with water repeatedly by centrifugation at 1000 *g* and 800 mg of *m*-aminophenylboronic acid hemisulphate, dissolved in 3 ml of 1 *M* sodium carbonate solution (pH 10), was added. The gel suspension was stirred for 72 h at room temperature. The gel was washed three times by centrifugation with 1 *M* sodium chloride solution and then three times with water. The reaction scheme is shown in Fig. 1.

For the quantitative determination of glycosylated haemoglobin a calibration graph should be constructed[11] for each new column, as the ligand density may vary from batch to batch.

Standard conditions

In all experiments the dimensions of the packed bed were 2.5 cm × 0.6 cm I.D.

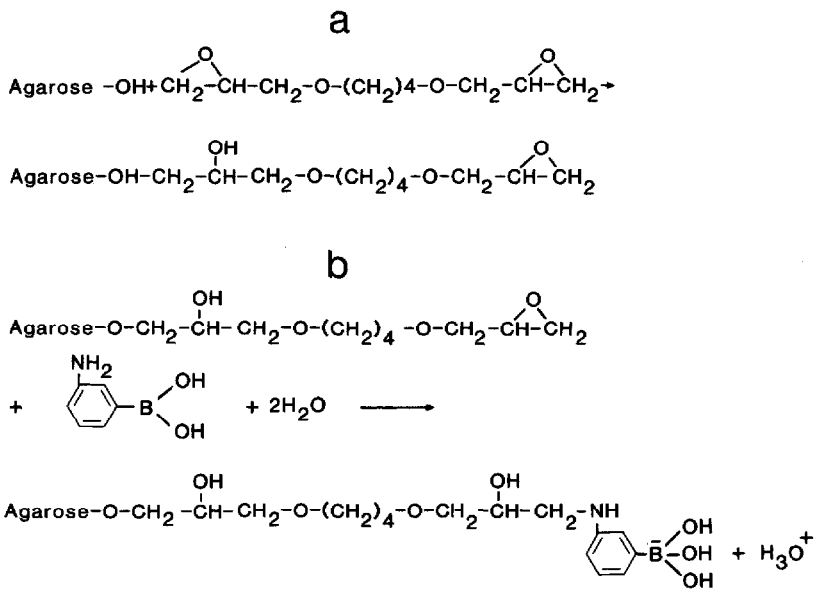

Fig. 1. Reaction scheme for the synthesis of boronate agarose. (a) Activation of agarose with 1,4-butanediol diglycidyl ether. (b) Coupling of *m*-aminophenylboronic acid to the activated agarose.

If not stated otherwise, we used the following conditions. The bed was equilibrated with about 5 ml of 0.05 *M* sodium phosphate buffer (pH 7.1). After application of the sample, the column was washed with 4–5 ml of the equilibration buffer for elution of non-glycosylated haemoglobin. The glycosylated haemoglobin was desorbed with this buffer containing 0.1 *M* sorbitol (the carbohydrate moiety of the glycosylated haemoglobin reacts with the boronate ligands; the bond is specifically broken by sorbitol). The flow-rate in all steps was 2.0 ml/min.

RESULTS

Pressure–flow-rate relationship

The experiment was conducted in 0.05 *M* sodium phosphate buffer (pH 7.1) at flow-rates from 0.5 to 5.0 ml/min in steps of 0.5 ml/min. The pressures were read and plotted against flow-rate. A linear relationship was obtained (maximum pressure 32 bar).

Preliminary experiment for the separation of glycosylated from non-glycosylated haemoglobin at pH 7.1

Fig. 2a shows an analysis of 10 µl of a haemolysate from a non-diabetic individual. When the experiment was repeated with the haemolysate from a diabetic patient, the area of the second peak was considerably larger (Fig. 2b) than that in Fig. 2a. When the sample volume of the haemolysate from the diabetic patient was doubled, the ratio remained the same. The experiment indicated that glycosylated haemoglobin was adsorbed efficiently also at a pH as low as 7.1. This finding was surprising as a pH of above 8 is generally used because it has been assumed that the ionic form of the

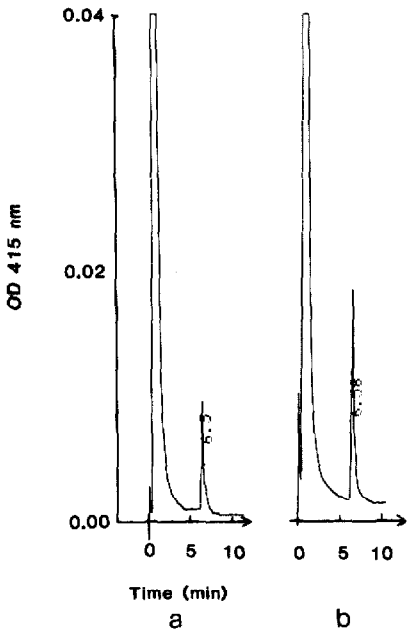

Fig. 2. Separation of non-glycosylated (first peak) from glycosylated (second peak) haemoglobin. Column, 2.5 cm × 0.6 cm I.D.; flow-rate, 2.0 ml/min; equilibration buffer, 0.05 *M* sodium phosphate (pH 7.1); elution buffer, equilibration buffer containing 0.1 *M* sorbitol. (a) 10 μl of haemolysate from a non-diabetic individual. (b) 10 μl of haemolysate from a diabetic individual.

phenylboronic acid is active in forming complexes with *cis*-diols[3] (the p*K* value of phenylboronic acid is about 8.9[3]). It was accordingly important to study in more detail the pH dependence of the adsorption (see Fig. 4 and corresponding experiments).

Although if it is well known that haemoglobin released by sorbitol is glycosylated (the second peak in Fig. 2), we demonstrated it explicitly by running diabetic haemolysates both on the columns described here and on the ion-exchange columns used routinely for the analysis of glycosylated haemoglobin at the Tunåsen Hospital in Uppsala (there was a linear relationship between the concentrations of glycosylated haemoglobin obtained by these two methods).

Effect of sorbitol concentration on the ratio between glycosylated and non-glycosylated haemoglobin

The sample (20 μl of haemolysate from a diabetic patient) was chromatographed under standard conditions with the exception that glycosylated haemoglobin (the second peak) was eluted with the equilibration buffer containing 1.0 *M* instead of 0.1 *M* sorbitol. . The experiment was repeated with the difference that the eluting buffer contained 0.8 *M* sorbitol and then 0.6, 0.4, 0.2, 0.1 and 0.01 *M* sorbitol. The result is shown in the top curve in Fig. 3 (the second peak was often larger when haemoglobin from other patients was analysed). A similar set of experiments was then performed with haemolysate from a non-diabetic individual (see the bottom curve in Fig. 3). Both curves in Fig. 3 show that the ratio between glycosylated and

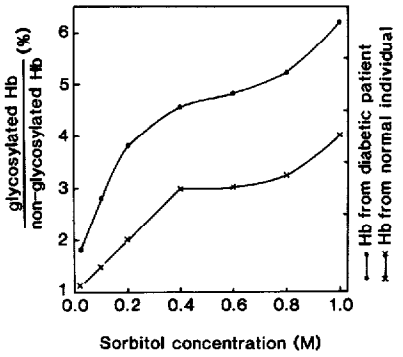

Fig. 3. Ratio between glycosylated (second peak) and non-glycosylated (first peak) haemoglobin as a function of the concentration of sorbitol in the elution buffer. Column and flow-rates as in Fig. 2; samples, 20 μl of haemolysate from a diabetic patient and 20 μl from a non-diabetic individual. Elution was first performed with the equilibration buffer (0.05 *M* sodium phosphate, pH 7.1) and then with the equilibration buffer containing various concentrations of sorbitol.

non-glycosylated haemoglobin increased with increase in the sorbitol concentration. However, the quotients between these ratios were 1.6, 1.9, 1.8, 1.5, 1.5, 1.6 and 1.6 for sorbitol concentrations of 0.01, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 *M*, respectively. Therefore, using 0.05 *M* sodium phosphate buffer (pH 7.1), a sorbitol concentration in the range 0.1–0.2 *M* gives the highest accuracy in the discrimination between diabetic and non-diabetic states, provided that the amount of haemoglobin applied is not so small that the area of the peak corresponding to glycosylated haemoglobin is unduly small.

A practical disadvantage of using high sorbitol concentrations is that the attendent higher viscosity causes an increase in back-pressure. For instance, at a flow-rate of 2.0 ml/min and a sorbitol concentrations of 0.1 and 1.0 *M*, the back-pressures were 12 and 50 bar, respectively.

Effect of pH on the ratio between glycosylated and non-glycosylated haemoglobin

Variation of pH of the equilibration buffer with constant pH in the elution buffer. For equilibration of the column, 0.05 *M* sodium phosphate buffer of pH 5.1 was used. The sample was 20 μl of haemolysate from a diabetic patient. The non-adsorbed haemoglobin was washed out with the buffer used for equilibration. Specific elution of the glycosylated haemoglobin was carried out with 0.05 *M* phosphate buffer containing 0.1 *M* sorbitol (pH 7.1). The column was again equilibrated with 0.05 *M* phosphate buffer, but its pH was now 6.1; the same sample was applied and the same eluting procedure at pH 7.1 was used. The experiment was repeated although the pH of the 0.05 *M* phosphate buffer used for equilibration was 7.1, 8.1 and 9.1 for three more runs. Fig. 4a shows that the ratio between glycosylated and non-glycosylated haemoglobin increased continuously from pH 5.1 to 8.1, but then decreased when the pH was increased to 9.1.

Constant pH in the equilibration buffer with variation of pH in the elution buffer. The conditions used in this experiment were the same as above except that the equilibration buffer was 0.05 *M* sodium phosphate (pH 7.1) and the pH in the elution buffer (0.05 *M* phosphate buffer containing 0.1 *M* sorbitol) was varied from 5.1 to 9.1.

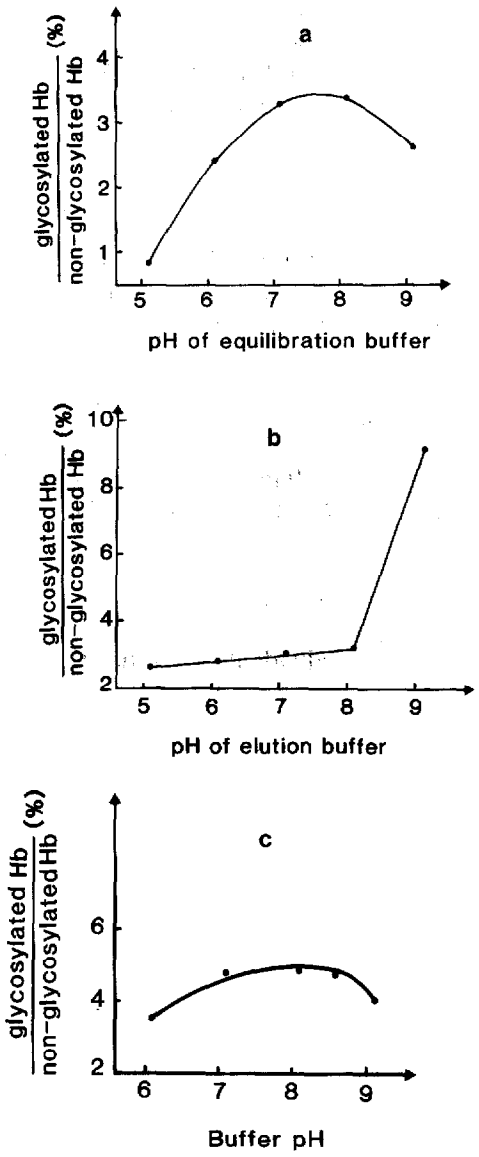

Fig. 4. Ratio between glycosylated (second peak) and non-glycosylated (first peak) haemoglobin as a function of pH. Column, flow-rate and sample as in Fig. 3. (a) Variation of pH in equilibration buffer. (b) Variation of pH in elution buffer. (c) Variation of pH in both elution and equilibration buffers.

Fig. 4b shows that the ratio increased only slightly when the pH was increased from 5.1 to 8.1, but increased considerably when the column was eluted with a buffer of pH 9.1, probably owing to desorption of accumulated non-specifically adsorbed haemoglobin. Measurements of the absorption at 415 nm of haemoglobin solutions of different pH showed that the higher ratio is not caused by a larger absorption coefficient of haemoglobin at pH 9.1.

The same pH in the equilibration and elution buffers. Both the equilibration buffer (0.05 *M* sodium phosphate) and the elution buffer (0.05 *M* sodium phosphate containing 0.1 *M* sorbitol) had first a pH of 6.1 and then 7.1, 8.1, 8.5 and 9.1. Fig. 4c shows that the ratio between glycosylated and non-glycosylated haemoglobin did not change significantly in the pH range 7.1–8.5.

Effect of flow-rate on the ratio between glycosylated and non-glycosylated haemoglobin

The sample consisted of 10 μl of haemolysate from a non-diabetic individual. The experiment was performed under standard conditions (described under Experimental) with the difference that the flow-rate was 3.0 ml/min. The experiment was repeated with flow-rates of 2.0, 1.0 and 0.5 ml/min. The ratio between glycosylated and non-glycosylated haemoglobin was virtually constant and independent of flow-rate.

The ratio between the areas of the second and first peaks was 0.0149 and 0.0147 when the specific elution was started 1.0 and 3.0 min, respectively, after the elution with the equilibration buffer (Fig. 5). The flow-rate was 4.0 ml/min. The ratios were accordingly very similar, but the total time for the latter experiment was less than half that of the first (1.5 and 4 min).

Effect of amount of haemolysate applied on the ratio between glycosylated and non-glycosylated haemoglobin

A 10-μl volume of haemolysate from a healthy individual was applied to the

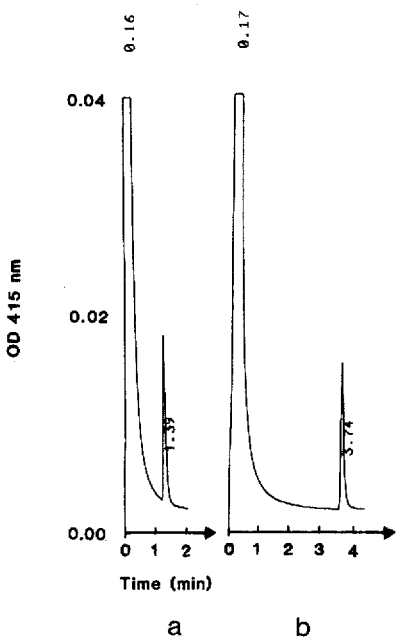

Fig. 5. Ratio between glycosylated (second peak) and non-glycosylated (first peak) haemoglobin at two different analysis times. Column, 2.5 cm × 0.6 cm I.D.; flow-rate, 4.0 ml/min; sample, 20 μl of a diabetic haemolysate. The elution times were (a) 1.39 min and (b) 3.74 min. The ratios between the peak areas were 1.49% and 1.47%, respectively.

column and the analysis was performed under standard conditions. The experiment was repeated with an increase in the sample volume to 20 μl and then to 40, 80 and 160 μl. The ratio between the areas of the second and first peaks was constant and independent of the volume of haemolysate applied in the range 10–160 μl.

Effect of salt on the ratio between glycosylated and non-glycosylated haemoglobin

A diabetic haemolysate (10 μl) was analysed as described under Experimental and the ratio between the areas of the peaks corresponding to glycosylated and non-glycosylated haemoglobin was determined. The experiment was repeated with the difference that the elution buffer contained 0.1, 0.2, 0.3, 0.4 and 0.5 *M* sodium chloride. The ratio between the peak areas was virtually constant and independent of the concentration of sodium chloride.

Using the same column, we varied the concentration of sodium phosphate in the equilibration buffer from 0.01 to 0.05 *M* in steps of 0.01 *M* and eluted with 0.05 *M* sodium phosphate buffer (pH 7.1) containing 0.1 *M* sorbitol. The results are shown in Fig. 6. Again, the ionic strength of the buffers had no great influence on the ratio between the areas of the second and first peaks.

Recovery

An experiment was performed under standard conditions (see Experimental). The total recovery of haemoglobin was calculated to be 98% from the volumes and the absorption values at 415 nm (measured in a spectrophotometer) of both the applied sample and of the two peaks corresponding to non-glycosylated and glycosylated haemoglobin. When the first peak was rechromatographed, 99% of the applied material appeared in the void volume; the second small peak (probably caused by the presence of sorbitol in the buffer) contained no haemoglobin. Rechromatography of the second peak gave an analogous result (no haemoglobin in the void volume and a recovery of 100%).

Determinations of glycosylated haemoglobin from whole blood

A 1-ml volume of blood was collected from each of ten healthy volunteers and divided into two 0.5-ml samples. One set of the samples from each of the volunteers

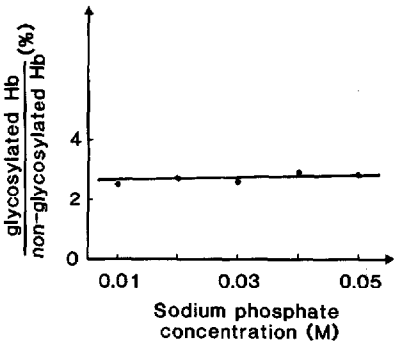

Fig. 6. Ratio between glycosylated (second peak) and non-glycosylated (first peak) haemoglobin as a function of the concentration of sodium phosphate in the equilibration buffer. 10 μl of a diabetic haemolysate was applied onto the column (2.5 cm × 0.6 cm I.D.) and eluted at a flow-rate of 3.0 ml/min.

was haemolysed and centrifuged as described under Experimental and the other set was only lysed with distilled water in a ratio of 1:3, resulting in a whole blood lysate (membrane and plasma were not removed). A 20-μl volume of each sample in the two sets was applied to the column and eluted with 0.05 *M* phosphate buffer containing 0.1 *M* sorbitol (pH 7.1) at a flow-rate of 3.0 ml/min. The ratios between the areas of the second and first peaks were calculated. A *t*-test showed that there was no significant statistical difference in the ratios for the two sets of samples ($p < 0.05, n = 10$). Haemolysed blood can accordingly be applied with advantage directly onto the boronate column without time-consuming centrifugation steps. However, we recommend washing the column every twentieth run with 2 ml of 0.05 *M* phosphate buffer (pH 9) containing 6 *M* urea to remove non-specifically adsorbed material (the favourable effect of high pH for desorption of adsorbed haemoglobin is mentioned under *Constant pH in the equilibration buffer with variation of pH in the elution buffer*). This also prolongs the usable life of the column.

DISCUSSION

It is highly desirable to suppress electrostatic interactions between haemoglobin and the charged groups in the ligand and non-specifically adsorbed proteins (and the traces of sulphate and carboxylic groups in the agarose matrix). This can be accomplished by performing the experiments at pH 7.1, which is close to the isoelectric point of haemoglobin (Figs. 2 and 5). However, at this pH one could expect a weak interaction between haemoglobin and the boronate ligand (see *Preliminary experiment for the separation of glycosylated from non-glycosylated haemoglobin at pH 7.1*). It was therefore satisfactory to find (see Fig. 4a–c) that the interaction at this pH was as strong as that at pH 8.0, a widely used pH in boronate chromatography[3]. Equilibration of the column at a pH as low as 5.1 should give a very weak interaction, which was verified experimentally (Fig. 4a).

An increase in ionic strength diminishes the electrostatic interaction, but unfortunately increases the hydrophobic interaction to the phenyl group in the ligand. Therefore, we also studied the salt dependence of the adsorption. The ratio between glycosylated (second peak) and non-glycosylated haemoglobin (first peak) was not significantly affected by addition of sodium chloride to the elution buffer (0.05 *M* sodium phosphate, pH 7.1, containing 0.1 *M* sorbitol) when the column was equilibrated with the elution buffer without sorbitol. Nor did a decrease in the phosphate concentration in the equilibration buffer from 0.05 to 0.01 *M* influence the ratio between glycosylated and non-glycosylated haemoglobin (Fig. 6). The desorption of haemoglobin with sorbitol is accordingly highly specific without significant disturbances caused by electrostatic or hydrophobic interactions.

The finding that the ratio between the areas of the second peak (glycosylated haemoglobin) and the first peak (non-glycosylated haemoglobin) is virtually independent of ionic strength (Fig. 6), pH in the range 7–8 (Fig. 4), flow-rate and sample load up to at least 160 μl of haemolysate facilitates the possibilities of obtaining reproducible chromatograms also at high flow-rates (short run times), which makes boronate chromatography on compressed non-porous agarose beds an attractive analytical method. For general information on the quantitative determination of glycosylated haemoglobin, see ref. 11.

We have previously described the preparation of macroporous boronate–agarose beads for HPLC separations of glycosylated from non-glycosylated haemoglobin at pH 8.5[17]. Those beads and the non-porous beads in this study have similar chromatographic properties. The latter beads have, however, the important advantage of permitting a much higher flow-rate and accordingly considerably shorter analysis times.

To summarize, the non-porous compressed boronate–agarose beds can be used with advantage for the determination of glycosylated haemoglobin in blood under conditions similar to those used in the experiment shown in Fig. 5. When very rapid analyses and a very small volume of sample are desirable, the following experimental conditions are recommended. A droplet of blood from a finger is mixed with three drops of distilled water. After haemolysis for about 1 min, 10 or preferably 20 μl of the haemolysate is injected onto the column, equilibrated with 0.03 or 0.05 *M* sodium phosphate (pH 7.1). After a 2-min elution with this buffer at a flow-rate of 4 ml/min, the glycosylated haemoglobin is specifically eluted with the same phosphate buffer containing 0.2 *M* sorbitol.

ACKNOWLEDGEMENTS

This work was financially supported by the Swedish Natural Science Research Council and the Carl Trygger and the Knut and Alice Wallenberg Foundations.

REFERENCES

1 J. H. Hageman and G. D. Kuehn, *Anal. Biochem.*, 80 (1977) 547–554.
2 S. Higa, T. Suzuli, A. Hayashi, I. Tsuge and Y. Yamamura, *Anal. Biochem.*, 77 (1977) 18–24.
3 A. Bergold and W. H. Scouten, *Chem. Anal. (New York)*, 66 (1983) 149–187.
4 G. T. Williams, A. P. Johnstone and P. D. G. Dean, *Biochem. J.*, 205 (1982) 167–171.
5 F. A. Middle, A. Bannister, A. J. Bellingham and P. D. G. Dean, *Biochem. J.*, 209 (1983) 771–779.
6 D. E. Goldstein, K. M. Parker, J. D. England, H.-S. Wiedmeyer, S. S. Rawlings, R. Hess, R. R. Little, J. F. Simonds and R. P. Breyfogle, *Diabetes*, 31, Suppl. 3 (1982) 70–78.
7 D. M. Nathan, D. E. Singer, K. Hurxthal and J. D. Goodson, *N. Engl. J. Med.*, 310 (1984) 341–346.
8 D. E. Goldstein, B. Walker, S. S. Rawlings, R. Hess, J. D. England, S. B. Peth and J. E. Hewett, *Diabetes Care*, 3 (1980) 503–507.
9 A. P. M. Schellekens, G. T. B. Sanders, W. Thornton and T. V. Groenestein, *Clin. Chem.*, 27 (1981) 94–99.
10 P. A. Svendsen, J. S. Christaiansen, U. Soegaard, B. S. Welinder and J. Nerup, *Diabetologia*, 19 (1980) 130–135.
11 D. C. Klenk, G. T. Hermanson, R. I. Krohn, E. K. Fujimoto, A. Krishna Mallia, P. K. Smith, J. D. England, H.-M. Wiedmeyer, R. R. Little and D. E. Goldstein, *Clin. Chem.*, 28 (1982) 2088–2094.
12 S. Hjertén, K. Yao and J.-L. Liao, *Makromol. Chem., Macromol. Symp.*, 17 (1988) 349–357.
13 S. Hjertén and J. L. Liao, *J. Chromatogr.*, 457 (1988) 165–174.
14 J. L. Liao and S. Hjertén, *J. Chromatogr.*, 457 (1988) 174–182.
15 S. Hjertén and J. P. Li, *J. Chromatogr.*, 475 (1989) 177–185.
16 J. P. Li and S. Hjertén, in preparation.
17 S. Hjertén and D. Yang, *J. Chromatogr.*, 316 (1984) 301–309.
18 S. Hjertén, in M. T. W. Hearn (Editor), *HPLC of Proteins, Peptides and Polynucleotides*, VCH, Weinheim, in press.
19 S. Hjertén, *Biochim. Biophys. Acta*, 79 (1964) 393–398.
20 L. Sundberg and J. Porath, *J. Chromatogr.*, 90 (1974) 87–98.