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Coupling of *m*-aminophenylboronic acid to *s*-triazine-activated Sephacryl: use in the affinity chromatography of glycated hemoglobins

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ABSTRACT

An improved process is described for covalent coupling of *m*-aminobenzeneboric acid to *s*-triazine-activated Sephacryl matrices. The derivatized Sephacryl gel contained up to 150–200 μmol boronate per ml. It has been applied to the separation of glycated and non-glycated hemoglobins (Hbs) present in red-cell hemolysate. The new bioaffinity support was evaluated by the analysis of 67 diabetic patients and 20 normal adults. The mean value for glycated Hb was 6.6 ± 0.8% for non-diabetics and 11.2 ± 2.9% for diabetics. The method effects group-specific separation between glycated and non-glycated Hbs even in presence of foetal Hb and abnormal Hb variants. There is an excellent correlation between the glycated Hb levels obtained by the new method and two established procedures, namely high-performance liquid chromatography (r = 0.933) and affinity Merckotest (r = 0.991). The inter-assay and intra-assay coefficient of variations of less than 3.0% suggest that the method is reproducible. The results indicate that the method may serve as an alternative procedure for the study of glycated proteins. The *s*-triazine-activated Sephacryl could also be used for immobilizing enzymes and for preparing biospecific absorbents.

INTRODUCTION

Boronate matrices have been found to be very useful for the separation and quantitation of glycated and non-glycated forms of hemoglobin (Hb) and other proteins [1–6]. So far, most investigations have involved boronic acid covalently bound to carriers such as aminoethyl cellulose [7], polyacrylamide and inorganic supports, including controlled pore glass. These boronate matrices are limited in different ways by analytical parameters, such as non-specific adsorption flow characteristics, residual charge, binding capacity, hydrophobic groups and molecular mass exclusion. The aminoethyl cellulose showed poor flow characteristics, low binding capacity and high residual charge. Polyacrylamide gels have obvious disadvantages of swelling or shrinking with changes in pH or ionic strength [8–10]. Inorganic supports are unsatisfactory, because of non-specific binding of substrate and low ligand concentration.

Several other boronate matrices have also been prepared using cellulose, Agarose or Sepharose [11]. Singhal *et al.* [12] described a reversed-phase boronate matrix for the purification of RNA and nucleotides. This method consisted of synthesizing a nine-carbon aliphatic phenyl boronate, which was bound to polychlorotrifluoroethylene beads. Because of its high molecular mass exclusion and low non-specific adsorption, cross-linked Agarose matrices are most widely used with various affinity ligands, including phenylboronate amines. Relatively little attention has been paid to the use of Sephacryl (cross-linked co-polymer of allyl dextran and N,N-methylene-bisacrylamide) support, which has advantages over Agarose, becuase it is more stable and per-

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mits greater pressure handling capacity for fast protein liquid chromatography (FPLC) applications.

This report describes the coupling of *m*-aminophenyl boronate to Sephacryl activated with *s*-triazine (cyanuric chloride) and the use of this derivative for the measurement of glycated Hbs.

EXPERIMENTAL

Activation of Sephacryl gel

A 10-g sample of *s*-triazine (Merck, Darmstadt, Germany) was dissolved in 100 ml of chloroform and filtered. After removal of the solvent in a rotatory evaporator, the solid was redissolved in 100 ml of acetone and cooled to *ca.* -5°C in an ice–salt bath. The Sephacryl gel (Pharmacia, Freiburg, Germany) was washed extensively with water on a sintered-glass funnel and activated under various sodium hydroxide concentrations (0.25–3 *M*). After removal of the water by suction, 10 g of the gel were suspended in 50 ml of sodium hydroxide solution for 15 min at room temperature. The sodium hydroxide was filtered off, the gel was resuspended in 10 ml of cold sodium hydroxide (4°C) and then cooled to 0°C in an ice-bath. A 20-ml aliquot of cold *s*-triazine acetone solution was added to the mixture under continuous stirring for 70 min in an ice-bath, followed by an additional 30-min stirring at room temperature, and then filtered. The activated gel was rinsed first three times with 50 ml of 50% (v/v) acetic acid–water, then with water (250 ml). It was stored at 4°C in water.

Coupling of m-aminophenylboronic acid to s-triazine-activated Sephacryl

The coupling buffer (pH 7.6) was prepared by mixing 8.5 volumes of 0.2 *M* K_2HPO_4 and 1.5 volumes of 0.2 *M* KH_2PO_4. A 10-g amount of *s*-triazine-activated gel was suspended in 10 ml of coupling buffer. To this slurry, 20 ml of coupling buffer containing 10 mg/ml *m*-aminophenylboronic acid hemisulphate (Sigma, St. Louis, MO, USA) were added. The mixture was stirred for 10–12 h at room temperature. The gel was washed with the coupling buffer until the washing solution showed no absorbance at 293 nm. The derivatized gel was then resuspended in 20 ml of coupling buffer containing 0.25 *M* ethanolamine and stirred overnight at room temperature. It was again washed with the coupling buffer following with 0.2 *M* sodium acetate buffer (pH 5.4) containing 0.02% sodium azide (w/v), and stored in this buffer.

Determination of boronate content of gel

About 0.500 ml of gel was dried using a centrifugal concentrator, and 10 mg of dry derivatized gel were hydrolysed with 1 ml of 6 *M* hydrochloric acid for 1 h at 60°C. The suspension was filtered and washed with 20 ml of water. The resultant filtrate was neutralized and diluted to 50 ml with water for boron determination. The boron concentration was determined according to the method described in ref. 13.

Glycated hemoglobin assay

Column. Disposable polystyrene minicolumns, 102 mm × 8 mm I.D., were obtained form Pierce (Rockford, IL, USA, Article No. 29920). A porous polyethylene disc was fitted at the bottom, and 0.8 ml of slurry (50% suspension) of the derivatized gel was transferred to the column.

Reagents. The washing buffer (first fraction elution agent) contained 50 m*M* APS [N-tris(hydroxymethyl)methyl-3-amino-propanesulphonic acid], 0.05 *M* magnesium chloride and 0.02% sodium azide (pH 8.5).

The second fraction buffer contained the same ingredients as the washing buffer, except for 0.2 *M* sorbitol instead of the magnesium chloride.

Sample preparation. Blood samples were collected in EDTA tubes. Plasma and cells were separated by centrifugation. Packed cells were washed three times with cold saline, then hemolysed with 20 volumes of distilled water.

Separation and quantitation of non-glycated Hb (N-GHb) and glycated Hb (GHb). After removal of the storage buffer, the column was equilibrated with 1.5 ml of wash buffer. A 30-μl sample of the lysate prepared as described above was applied to the column, followed by an equal volume of the wash buffer. After 5 min the column was

placed over a test-tube (10 ml) and the N-GHb fraction was eluted with 0.5 ml of wash buffer followed by a further 4 ml of wash buffer.

The GHb was then eluted with 2.5 ml of second fraction buffer. The absorbance of each fraction was read at 415 nm, and the percentage of GHb was calculated using following equation:

$$\%\mathrm{GHb} = \frac{2.5\, A_B}{2.5\, A_B + 4.5\, A_A}$$

where A_B is the absorbance of the GHb fraction, and A_A is the absorbance of the N-GHb fraction.

Regeneration and storage of affinity columns. As soon as possible after elution of GHb (second fraction) the affinity gel was regenerated by washing with 5 volumes of 0.2 *M* sodium acetate buffer (storing buffer, pH 5.4). The columns were stored at 4°C in storing buffer, containing sodium azide (0.02%, w/v).

RESULTS

Fig. 1 shows the effect of sodium hydroxide concentration on the coupling yield of *m*-aminophenylboronate-Sephacryl. The alkali treatment from 1.5 to 3 *M* sodium hydroxide gave usable derivatives, with a high capacity and specific affinity interaction with the GHb fraction. The maximum amount of boronate (150–200 μmol boronate per ml gel) was immobilized to Sephacryl that had been treated with 3 *M* sodium hydroxide. The *s*-triazine-activated Sephacryl retained up to 80% of its initial activity for a year when stored in distilled water at 4°C. The following results were obtained with the gel derivatized after treatment of Sephacryl with 2 *M* sodium hydroxide.

A frontal uptake analysis of Hb on *s*-triazine-activated Sephacryl (control column) and on boronate gel (affinity column) is illustrated in Fig. 2. The frontal volume (1.5 ml) was the difference in elution volume between the 50% saturation point for the control and affinity columns.

Fig. 3 depicts the use of Sephacryl-boronate gel in the separation of GHb and N-GHb fractions.

The percentage of Hb bound to the affinity gel was increased on lowering the pH from 9.0 to 7.8. Below pH 8.0 the Hb was non-specifically bound to the gel. Above pH 8.1 a variation of 0.1 pH unit introduces no significant changes in the per-

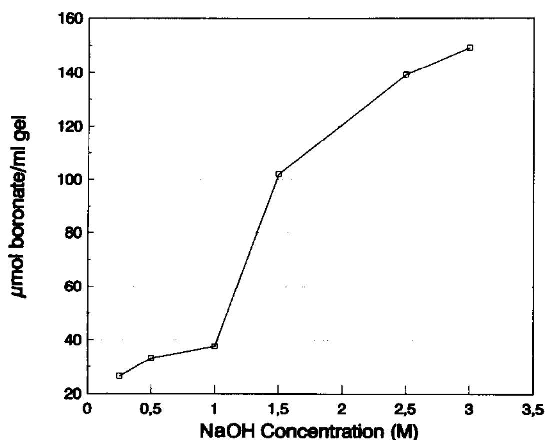

Fig. 1. *m*-Aminophenylboronic acid bound to *s*-triazine-activated Sephacryl under various sodium hydroxide concentrations (1 ml of gel corresponds to 0.236 g dry weight).

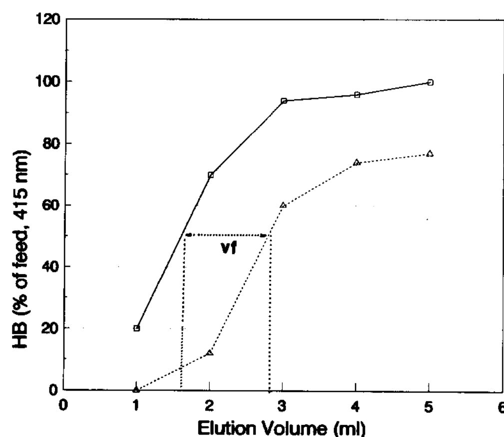

Fig. 2. Frontal uptake analysis of hemoglobin on Sephacryl-boronate gel. Sephacryl derivative gel (1 ml) was packed in a HR (Pharmacia) column (HR5/5, 5.0 cm × 0.5 cm I.D.). The column was connected to the HPLC system [20] and equilibrated with the first fraction buffer at a flow-rate of 9 ml/h. The same buffer containing 10 mg Hb per dl was applied continuously to the column until saturation was reached, and 1-ml fractions were collected and read at 415 nm. Similar experiments were performed with triazine-activated Sephacryl (control column). vf = frontal volume. (——) Unsubstituted activated Sephacryl control. (- - - -) Boronate Sephacryl affinity column.

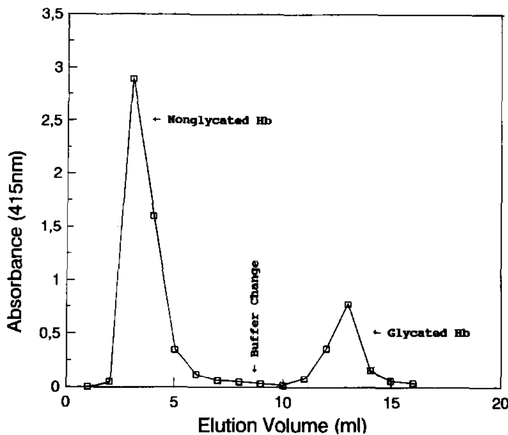

Fig. 3. Separation of glycated Hb and non-glycated Hb on a 3-ml boronate Sephacryl column. Chromatographic conditions were as described in Experimental; 100 μl of sample were applied to the column, and 1-ml fractions were collected.

centage of Hb that remained bound to the affinity column. The interaction of GHb with the Sephacryl-boronate gel was obviously independent of the hydrogen ion concentration in the pH range 8.7 ± 0.2. Within this pH interval the coefficient of variation (C.V.) was less than 1.5%. The pH value of 8.5 was selected for the GHb assay.

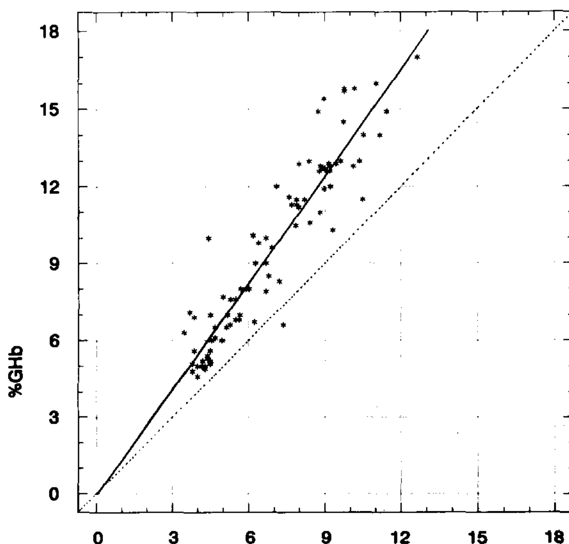

Fig. 4. Correlation between glycated Hb (GHb) levels obtained by the new bioaffinity method (y-axis) and by HPLC (x-axis); $y = 1.38x - 0.04$, $r = 0.933$ ($n = 87$).

The procedure was evaluated through analysis of blood samples from 67 diabetic and 20 non-diabetic volunteers. The mean values and standard deviations found for GHb in normal and diabetic subjects were 6.6 ± 0.8 and 11.2 ± 2.9%, respectively. When patients were divided into juvenile ($n = 27$) and adult ($n = 47$) onset diabetes, the mean levels of GHb were 12.2 ± 2.7 and 10.1 ± 3.1%, respectively. The results for these groups were significantly different from those for a non-diabetic group. Figs. 4 and 5 display the covariance of 87 paired determinations of GHb by three methods. The results obtained by the new affinity gel and HPLC correlated well. The regression line ($y = 1.38x - 0.04$) has a correlation coefficient of $r = 0.933$. There is also a highly correlated linear relationship between the new method and the affinity Merckotest (Merck). The regression line (Fig. 5) and the correlation coefficient were $y = 0.94x + 0.21$ and $r = 0.991$. The intra-assay C.V. was 2.6% ($n = 10$).

The gel is easy to regenerate. After seven regenerations the C.V. for the affinity column did

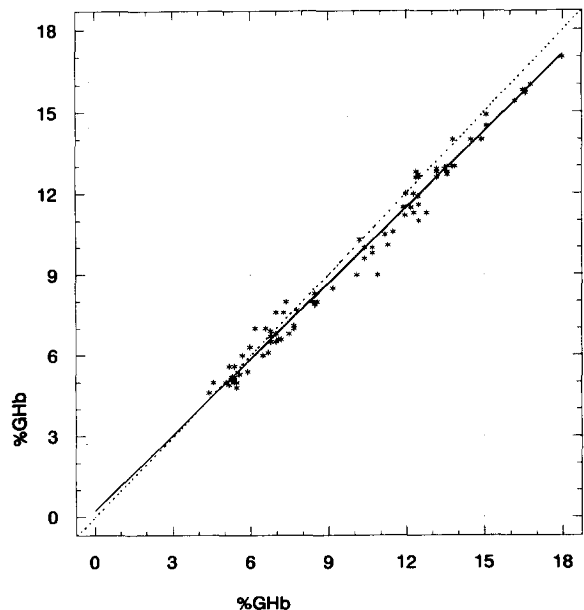

Fig. 5. Comparison of GHb values determined by the boronate Sephacryl bioaffinity method (y-axis) and Merckotest (x-axis): $y = 0.94x + 0.21$, $r = 0.991$ ($n = 87$).

not exceed 3.0%. The affinity columns were stored at 4°C in storing buffer for six months without detectable loss in the binding capacity.

DISCUSSION

We have described the synthesis and application of a new kind of boronate chromatography matrix. The activation of polysaccharide carriers with cyanuric chloride has been described by many other authors [14–18]. To our knowledge, this is the first report of affinity chromatography using a Sephacryl-boronate matrix. The chemical steps involved in this coupling chemistry have been previously elucidated in detail [15,18] and are shown in Fig. 6. The high reaction yield is characteristic of this coupling procedure. It was possible to attach up to 200 μmol of boronate per ml of activated Sephacryl gel; this is at least twice the yield reported for Agarose derivatives [2]. The boronate Agarose affinity support obtained via carbodiimide suffers from oxidative degradation and has to be stored in the dark at 4°C. In contrast, the Sephacryl derivative is chemically inert and is remarkably stable to pressure handling when used in FPLC aplications. No discoloration or loss of binding capacity was detectable when the affinity columns were exposed to light for six weeks at room temperature. When the gel was used under FPLC conditions the incubation time of the sample for the binding of GHb was less than 1 min, and GHbs and N-GHbs were clearly separated (data not shown). This indicates that the Sephacryl-boronate gel allows rapid formation of 1,2-*cis*-diol complex. The high coupling of boronate to Sephacryl permitted the use of mini-columns, and only 0.40 ml of gel was required for the separation and quantitation of GHbs. There was excellent correlation of GHb values determined by boronate Sephacryl gel and two established procedures. Our method provided excellent discrimination between diabetic and non-diabetic populations.

The intra-assay C.V. of 2.8% reflects the precision of the new affinity procedure. The quantitation of GHb was possible even in the presence of foetal Hb and other abnormal Hbs. These findings are in agreement with results previously re-

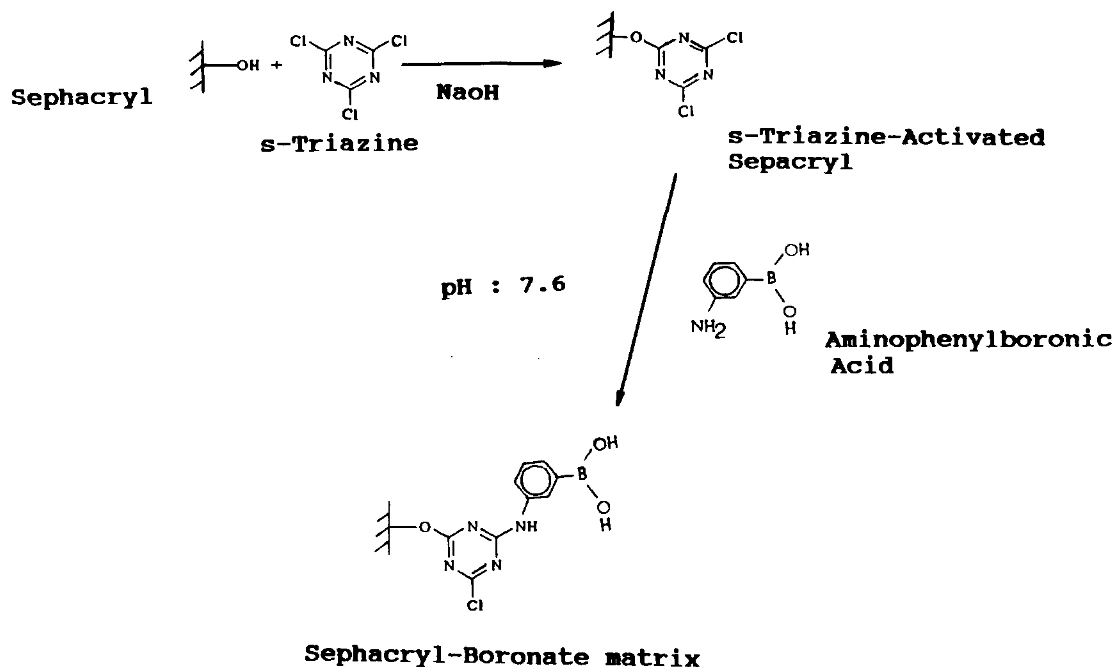

Fig. 6. Sephacryl activation with *s*-triazine and *m*-aminophenylboronic acid coupling.

ported [4,19], and demonstrate that the boronate Sephacryl affinity medium effects group-specific separation between GHbs and N-GHbs. So far, we used *s*-triazine-activated Sephacryl only for coupling *m*-aminophenylboronic acid. However, it could be used for the preparation of other bio-specific absorbents and enzyme immobilization. Boronate Sephacryl affinity chromatography may also be applied to the study of other proteins known to undergo glycation.

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