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Synthesis and application of boronic acid-immobilized porous polymer particles: a novel packing for high-performance liquid affinity chromatography

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Abstract

A preparation method of a novel type of packing materials for high-performance liquid affinity chromatography to determine glycated proteins was studied, and a fundamental study on its application to diabetic serum was conducted. To quantify glycated proteins such as glycated serum albumin, a new hydrophilic and durable porous polymer particle recently developed in our laboratory was used as the basic matrix. The matrix was activated with 1,1′-carbonyldiimidazole (CDI), and optimization of the coupling reaction between these CDI-activated matrix and *m*-aminophenylboronic acid hemisulfate (APBA) as affinity ligand was investigated. The optimum value for the APBA coupling yield was found to be obtained under acidic conditions very different from the data reported by previous workers. Using this APBA-immobilized matrix an affinity column was prepared, and its usefulness in HPLC separation of glycated serum proteins was investigated. Also, the fundamental and preliminary results for diagnosis of diabetes mellitus are discussed in this paper.

Keywords: Proteins; Boronic acid

1. Introduction

Methods on quantitative determination of glycated proteins, such as glycohemoglobin or glycated serum albumin, for diabetes diagnosis have been widely investigated [1]. For example, they include colorimetric measurement with thiobarbituric acid following separation of albumin by Affi-Gel Blue chromatography [2,3], APBA affinity chromatography followed by quantitation with immunoturbidimetry [4], radioimmunoassay [5] and fluorometric determination using dansylated phenylboronic acid [6]. These clinical assay methods however are not yet practicable because of their laboriousness and tediousness. The commercially available affinity chromatography mini-column kit for separation of these glycated proteins also has the critical problem of fast saturation of the binding sites. In addition, the usual gels used in this affinity chromatography are made from a cross-linked agarose gel of polysaccharides or Sepharose gel composed of polyacrylamide, the so-called "soft-gel" type, so that such gels cannot be used as high-performance liquid affinity chromatographic column material. The use of a HPLC method for monitoring these proteins now has been playing an important role as the more

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practical approach. For instance, Yasukawa et al. have reported an automated but somewhat complicated HPLC system consisting of an anion-exchange column and a boronate affinity column using a polyvinylalcohol–triallylisocyanulate-type gel [7]. In fact, only a few porous polymer gels suitable as packing for affinity HPLC columns are known to date. Thus, the aim of this study was the preparation and the application of affinity HPLC packings for the monitoring of glycated proteins. In the present study, we deal with a hydrophilic porous polymer particle as the basic matrix for this affinity HPLC. This basic matrix has three main merits for affinity HPLC; firstly, the high mechanical strength of more than 500 kg/cm^2; secondly, almost no non-specific adsorption of blood proteins; and thirdly, the existence of many functional hydroxy groups available for ligand coupling, as described in our previous paper [8]. Note that glycated albumin produced non-enzymatically in human blood serum has now been recognized as a more useful index than glycohemoglobin, because the serum albumin has a much shorter half-life (10–20 days) and is more sensitive to changes in serum glucose than that of red blood cells (90–120 days) [9]. Therefore, we will discuss in this paper both the preparation of a new affinity sorbent and the separation and quantitation of the glycated serum proteins using an affinity HPLC column packed with the above sorbent.

2. Experimental

2.1. Materials

Porous polymer gels prepared in our laboratory were used, which were composed of 2,3-dihydroxypropylmethacrylate (GLM) monomer, poly(oxyethylenedimethacrylate) (PEG23) and dipentaerythritolhexaacrylate (DPH) as cross-linkers. The preparation method was described in detail in a previous paper [8]. Typical physical properties of the gel used in this study were as follows: average particle size, 14 μm; average pore radius, 150 Å; OH value, 6.0 mmol/dry g. Sodium carbonate, ammonium acetate, magnesium chloride, sorbitol and glucose were all of first-class grade purchased from Wako (Osaka, Japan) and used without further purification. 1,1′-Carbonyldiimidazole (CDI) of peptide synthesis grade and *m*-aminophenyl boronic acid (APBA) hemisulfate salt were obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and Aldrich (Milwaukee, WI, USA), respectively. Special grade dioxane, acetic acid, sodium dodecyl sulfate (SDS), Coomassie brilliant blue and Bromphenol blue were also obtained from Wako.

Human serum albumin and globulin were obtained from Sigma (St. Louis, MO, USA) and dithiothreitol, polyacrylamide gel, molecular mass standard markers and buffer gel for SDS-PAGE analyses were obtained from Pharmacia (Uppsala, Sweden). Ready-made gel and standard p*I* markers for isoelectric focusing were also obtained from Pharmacia. Abnormal human serum (Consera A) as commercially available glycated serum albumin was obtained from Nissui Pharmaceutical (Tokyo, Japan). Another glycated serum albumin as a standard sample was non-enzymatically prepared by incubating an aqueous solution which consisted of 2 g of lyophilized human serum albumin (from Sigma) and 3.25 g of special grade glucose (from Wako) in 50 ml of highly purified distilled water at 37°C for 1 month. Blood samples from patients with nondiabetes (non-DM) and diabetes mellitus (DM) were kindly supplied from Takeda General Hospital (Hukushima Prefecture, Japan). The serum from each sample was separated by centrifugation at 1500 *g* at room temperature and stored at −20°C prior to use.

2.2. Activation of porous polymer gels

The porous polymer gel described above was activated with CDI using a modified Bethell et al. method [10,11]. Typical procedure was as follows: 10 g of wet porous polymer gel (OH group content 6 mmol/dry g, which corresponds to 2.78 g dry weight) was washed on glass filter (G-2) sequentially with 100 ml of water, 200 ml of 30% dioxane aqueous solution, 200 ml of 70% dioxane and finally 200 ml of dioxane gradually, and then suspended in 166 ml of dioxane. To this suspension, 0.96 g of CDI was added, and dispersed by sonication for ca. 15 s. Then the suspension was shaken at room temperature for the desired time interval. After that, the obtained

powdery product was washed with 200 ml of dioxane on glass filter (G-2) and stored in a refrigerator.

2.3. Analysis of the activated gels

Quantitative analysis of the CDI-activated gel was carried out in principle by the retitration method described by Ayers et al. [12]. The typical procedure used in this study is as follows: about 0.5 g of the CDI-activated gel obtained above was hydrolyzed overnight at room temperature in 50 ml 0.1 *M* NaOH. A 25-ml portion of the supernatant liquid was then titrated with 0.1 *M* HCl under nitrogen from pH 9 to pH 4. This titration gives the total amounts of CO_2 and imidazole formed from the active groups. The CO_2 was then expelled by flushing with nitrogen for 30 min at pH 2, and the sample was retitrated between the same pH values to obtain the amount of imidazole present. The number of active groups present was calculated from the difference between the two figures, which is based on the amount of CO_2 expelled. All titrations were carried out using an automatic pH titrator Comtite-900 (Hiranuma, Japan) at room temperature.

2.4. Immobilization of APBA

The typical coupling procedure is depicted as follows: 0.75 g of the activated gel with 2.5 mmol/g of the CDI-activated concentration sucked to a moist cake, was added to 20 ml 0.146 *M* APBA in aqueous solution consisting of 1 *M* acetic acid and 1 *M* sodium acetate buffer, pH 4.0. The suspension obtained was shaken for 96 h at room temperature (25°C). After the coupling reaction, the brownish powdery product was washed on the glass filter (G-2) with purified water and methanol, and then stored in a refrigerator. The other experimental conditions set up for optimization of this reaction will be described in detail below.

2.5. Analysis of immobilized matrix

Several methods for precise quantification of immobilized APBA, such as UV spectroscopy, HPLC determination using Shodex RS pak DS-613 columns and boron atomic absorption spectroscopy, were carried out but unfortunately all failed because of the poor reproducibility. After that, we found that the method of elemental analysis using an inductively coupled plasma (ICP) Instrument developed by Kyoto Kohken Industries (Japan) was most suitable for the quantitative analysis of these boron atoms. The typical procedure is as follows: 50 mg of the prepared APBA-immobilized gel which was dried in vacuo overnight was taken into a Teflon vessel covered with stainless steel jacket and 10 ml of the mixed acid solution prepared from 60% $HClO_4$ and 60% HNO_3 (1:5, v/v), was added to the vessel and closed tightly. The jacket was then heated to 100°C for 1 h and then heated to decompose at 200°C for 2 h. After cooling, the solution was diluted to 50 ml using a volumetric flask with the mixed acid solution, and this diluted solution was analyzed by using the ICP instrument for quantitative determination of APBA.

2.6. Column packing

A typical affinity packing procedure used in this study is as follows: about 8 ml of the 14-μm wet APBA-immobilized gel and 30 ml of stationary solvent prepared from glycerol and methanol (35:65, v/v) were sonicated for ca. 30 s and centrifuged at 1100 *g* followed by removal of superfine particles in the supernatant. This operation was repeated several times to remove superfine particles thoroughly and then the obtained slurry was placed in a 15-ml reservoir connected to a 4×150 mm stainless steel column and was packed using methanol as packing solvent at a flow-rate of 2 ml/min under a gauge-controlled pressure between 200 and 500 kgf/cm^2. After packing, the column was well washed with methanol and sealed with a column stopper.

2.7. Affinity HPLC

Affinity HPLC experiments used in this study were conducted using a Shimadzu LC-6A equipped with a system controller (Shimadzu SCL-6B), UV detector and autosampler. All experiments were carried out at room temperature and with UV detection at 280 nm. The mobile phase system for adsorption and desorption of glycated proteins in affinity chromatography as described in the literature

[13] was used. However, we found that the use of such a system with third and fourth eluents which consisted of 0.2 *M* ammonium acetate and 0.2 *M* sorbitol for washing of the column and 0.1 *M* HCl for regeneration of the affinity matrix, respectively, increased the analysis time considerably and, furthermore, resulted in a slightly unfavorable elimination of APBA from the column due to the high concentration of HCl (0.1 *M*) needed for regeneration. Considering these experimental results, we used a simple mobile phase system (0.01 *M* HCl) instead of both the third and the fourth eluents. The mobile phase system used in this study is summarized in Table 1.

A typical procedure for affinity HPLC experiments is as follows; the affinity column was equilibrated with constant flow of eluent I. After application of 20 μl of blood sample by the autosampler, the column was flushed with eluent I for 10 min to elute non-glycated proteins and adsorb glycated proteins, respectively. Then, the glycated proteins adsorbed in the affinity column were desorbed by the acidic eluent II (10 min), and finally the column was washed by eluent III for 10 min. All elutions were monitored at 280 nm and the flow-rate in all steps was 1.0 ml/min.

The pressure–flow-rate relationship of the packed column was established, and a good linear relationship was obtained with maximum back pressure of 90 kgf/cm^2 at a flow-rate of 4.0 ml/min.

2.8. Measurement of glycated proteins

The percentage of glycated proteins was calculated from the peak area obtained from the affinity chromatograms by the following formula:

$$\text{Percent glycated protein} = \frac{\text{Peak area given by eluent II}}{\text{Total peak area given by eluent I and II}} \times 100$$

The accuracy of quantitative determination by this peak-area analysis was confirmed by the evaluation study that a calibration curve of the above percent glycated protein values obtained from a series composed of a typical non-DM serum sample and a DM serum gave a good linear relationship.

2.9. Electrophoresis

For SDS-PAGE and IEF measurements, preparative liquid affinity chromatography was carried out. Each of three fractions obtained by the boronate affinity HPLC of glycated human serum albumin or typical diabetic individuals were pooled in the fraction collector using an automatic system controller (Shimadzu SCL-6B) over several days. Three pooled fractions were stored at 4°C until they were concentrated and desalted by using centrifugal ultrafiltration. SDS-PAGE was performed on Excel Gel (Pharmacia) using a Tris–acetic acid buffer (pH 6). Plates were stained with 0.07% Coomassie brilliant blue at 60°C for 1 h. IEF was carried out using Phastsystem (Pharmacia) installing a PhastGel IEF with a pH 4 to pH 6.5 gradient. For isoelectrocfocusing, 4 μl of each desalted fraction was applied, and the p*I* was measured by using the Pharmacia calibration kit. The plates were stained with 0.02% Coomassie brilliant blue at 50°C for 10 min.

3. Results and discussion

3.1. Physical properties of the basic matrix

Porous polymer gels used as the basic matrix consist of 2,3-dihydroxypropylmethacrylate (GLM) monomer and co-polymerizable cross-linking agents as the main framework as described in Section 2, so that OH group contents in the basic matrix are generally high and (max. 6.0 mmol/dry g; this

Table 1
Mobile phase system used

Eluent	Composition	pH	
I	0.2 *M* Ammonium acetate–0.1 *M* $MgCl_2$	9.0	Adjusted with 1 *M* NaOH
II	0.2*M* Ammonium acetate	5.5	Adjusted with 1 *M* HCl
III	0.01*M* HCl	1–2	

study). Such OH values of the gel are usually known to be consistent with those calculated from the monomer compositions. However, the optimum OH value of the matrix is restricted by physical and chemical requirements such as surface morphology, functional group content, mechanical strength and so on, depending on the purpose. Degrees of swelling of the basic matrix in several organic solvents and typical pH values of aqueous solutions were measured (Table 2). As is clear from Table 2, the basic matrix was quite stable with regard to swelling in both organic solvents with various solubility parameters and aqueous solutions with a wide range of pH. Non-specific adsorption of biological materials to the basic matrix was also investigated. It was observed that a column (ca. 10 ml) packed with the basic matrix showed little adsorption from the buffer solutions of human serum albumin, globulin and blue dextran, respectively.

3.2. Activation reaction by CDI

The relationship between the yield of CDI-activated gel and reaction time in dioxane is shown in Fig. 1. As is obvious from Fig. 1, the activation reaction of the matrix with CDI was observed to be very fast and to reach a more than 60% yield based on CDI used within 1 h. Even when the reaction continued more longer, the yield of the activation reaction could not reach more than ca. 80%. Bethell et al. [10,11] have also reported that the activation reaction of cross-linked agarose with CDI was comparably fast in dioxane. The limit of the yield might be due to the hydrolysis of CDI molecules by trace amounts of water mingled in the reaction mixture, since rapid hydrolysis of CDI with water is well-known. Storage stability of the CDI activated matrix obtained in this experiment at temperatures below 10°C was good, as expected from the finding that the initial level of 80% CDI-activated yield was maintained even after 10 days storage in closely stopped vessels in a refrigerator. This property was very convenient when carrying out the immobilization reaction with APBA.

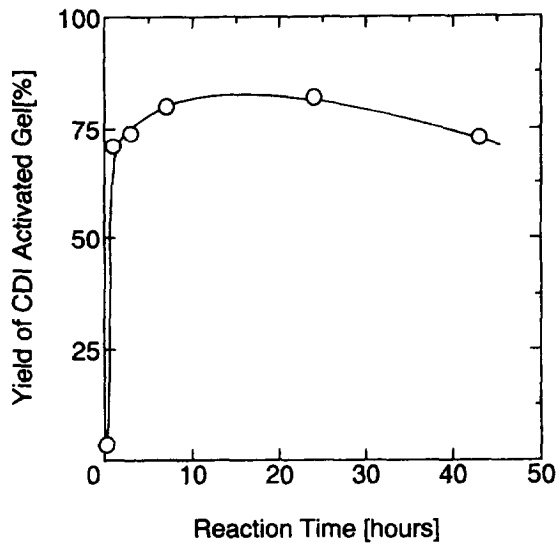

Fig. 1. Effect of reaction time on the yield of the activation reaction between porous polymer matrix and CDI at 25°C.

Table 2
Degree of swelling of porous polymer particles in various solvents

Solvent	DS[a]
THF	2.43
$CHCl_3$	2.02
CH_3CN	2.11
H_2O	3.08
0.1 *M* HCl	2.95
1.0 *M* HCl	2.91
1.0 *M* NaOH	2.82
0.1 *M* NaCl	2.93

[a]DS, degree of swelling as measured by calculating $(W-W_0)/W_0$ after 2 h immersion at room temperature, where W and W_0 represent the wet gel and dry gel weight, respectively.

3.3. Immobilization of APBA

The experimental results which were obtained on the effect of pH of immobilization reaction media on the reaction yield between the CDI-activated matrix obtained above and APBA are shown in Table 3 and Fig. 2. Entirely unexpected, it was found that coupling yields between CDI-activated matrix and APBA gave higher values in acidic media than in alkaline media. Usually the coupling reaction of N-nucleophiles such as amino acids, peptides and proteins with CDI-activated matrices like Sepharose CL-6B or polymeric polymer gels had been carried out at pH 8.5–10 using a wide variety of buffer solutions [14]. In fact, Mallia et al. have prepared an APBA affinity support using cross-linked agarose gel

Table 3
Effect of pH on APBA coupling yields[a]

pH	Yield of immobilized APBA (μmol/dry g)	Used buffer solution[b]
2	650	KCl–HCl
4	763	CH_3COOH–CH_3COONa
6	526	K_2HPO_4–KH_2PO_4
8	45	Tris–HCl
10	57	Na_2CO_3–$NaHCO_3$

[a]Reaction conditions are described in Section 2.
[b]Concentrations of these buffer solutions were all 1 *M*.

(at pH 10 with 1 *M* Na_2CO_3) in a routine manner [13]. So we tried several times to prepare the APBA affinity matrix using our CDI-activated gel under the same condition (1 *M* Na_2CO_3 solution, pH 10), but only low coupling yields of the matrix were obtained with a good reproducibility. Fig. 2 shows that the coupling reaction between CDI-activated matrix and APBA is significantly affected by the pH of the buffer solution used and it appears to be reasonable that acidic conditions are more suitable for the coupling reaction than alkaline conditions. One could easily understand that such a phenomenon is not due to the physical change of the matrix like swelling, because the basic gel matrix has a hard and rigid structure with a high mechanical strength (>500 kgf/cm^2). Furthermore, we reconfirmed from the facts that the average particle size and size distribution do not show any change, even when they were measured in situ under ultrasonic dispersion in either 0.1 *M* HCl (pH 2) or in an alkaline buffer solution (0.2 *M* ammonium acetate–0.1 *M* $MgCl_2$, pH 9). Fig. 2 also shows the experimental results of the coupling reaction between CDI-activated Sepharose CL-6B and APBA over a wide pH range in various buffer conditions. We found, even in the case of CDI-activated Sepharose CL-6B, a similar phenomenon that the APBA-immobilized matrix was obtained in much higher yield in acidic conditions than in alkaline conditions, as is seen in Fig. 2.

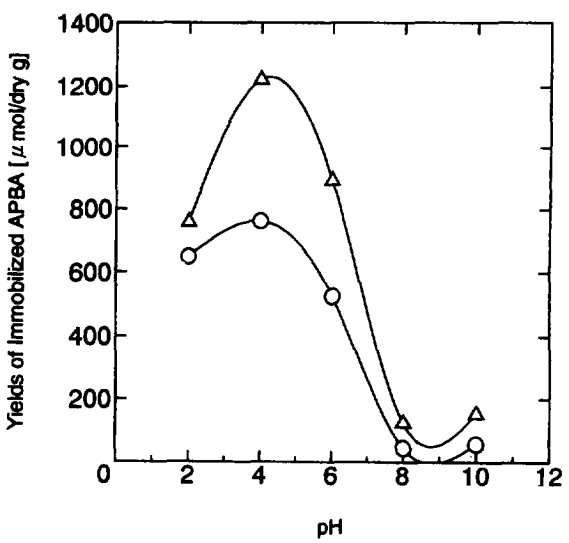

Fig. 2. Effect of pH on the yield of the immobilization reaction between APBA and CDI-activated porous polymer matrix (○), and APBA and CDI-activated Sepharose CL-6B (△).

As was already mentioned, these phenomena do not agree to either the previous observations by Mallia et al. [13] or Milton et al. [14], who suggested that the coupling reaction of CDI-activated matrix had to be carried out under alkaline conditions in order to prevent the obtained matrix from having a side reaction with further nucleophiles during the affinity chromatographic separation. In fact, hydrolysis of CDI-activated Sepharose CL-6B is known to proceed twice as fast under alkaline conditions (pH 10) than in acidic media (pH 5) [10]. In this sense, it might appear to be suitable for the coupling reaction with APBA to be carried out under alkaline conditions. However, it would be reasonable to assume that the hydrolytic reaction of CDI-activated matrix might be in competition with the coupling reaction with APBA, even in various kinds of buffer solutions with any pH. On the other hand, we have found that APBA had two pK_a values, 4.2 and 8.8, of which the first was thought to correspond with the change of the NH_2 group to the NH_3^+ cation, while the latter was thought to correspond with the change of $B(OH)_2$ to the $B(OH)_3^-$ anion group, from the titration curve of aqueous APBA solution titrated

with 1 *M* NaOH. As is shown in Fig. 2, APBA-coupled products have been obtained with the highest yield around pH 4, using two kinds of CDI-activated matrices. From these findings, it would be reasonable to assume that the pK_a value of APBA might a factor in the coupling reaction between the activated matrices and APBA. As a matter of fact, the optimum pH in the coupling reaction has been in best accordance with one of the pK_a values of APBA, suggesting that in acidic media the protonation of carbonyl oxygen makes carbonyl carbon more susceptible to nucleophilic attack by the amino group of APBA, and also fast elimination of a molecule of imidazole from the intermediate formed by above nucleophilic attack may take place.

3.4. *Affinity chromatography*

Affinity chromatograms of human serum albumin, glycated serum albumin and commercial available glycated albumin (Consera A) using a 150×4 mm stainless-steel column packed with APBA immobilized matrix are shown in Fig. 3a–c. The elution system used in this study is described in Table 1.

Generally, phenyl boronic acid is known to form a specific and reversible covalent bonding interaction with the five-membered ring between the boronate group and *cis*-diol compounds [15], as is shown in Fig. 4.

cis-Diol compounds, such as glycated proteins, are chemically adsorbed to the boronate matrix when using an alkaline eluent, and easily dissociate under acidic conditions to reconstitute the original *cis*-diol compounds. Therefore, the first peak as shown in Fig. 3 is thought to correspond to some non-glycated components which are eluted without adsorption by the alkaline eluent (pH 9), while the second peak could be assigned to the glycated serum albumin, in this case undoubtedly, since it was eluted using an acidic buffer solution (pH 5.5). On the other hand, materials involved in the third peak eluted with the 0.01 *M* HCl are assumed to be certain impurities or highly glycated proteins which were adsorbed on the boronate matrix too strongly to be desorbed by such a weak acidic buffer as the second eluent. Furthermore, compared with the chromatograms shown in Fig. 3, it is obvious that the second peak areas obtained from both incubated serum albumin with

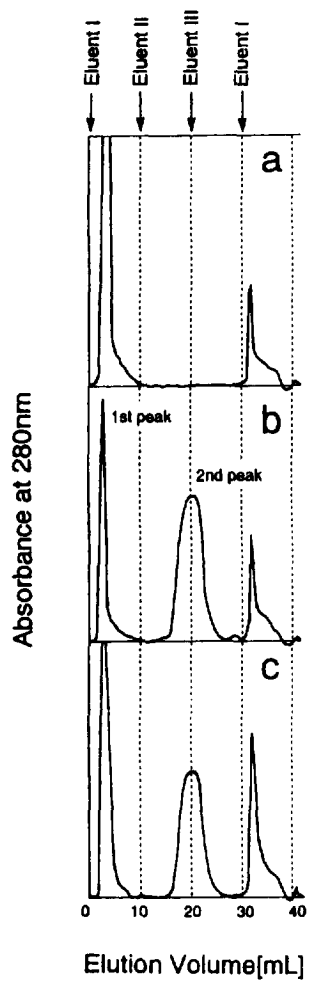

Fig. 3. HPLC of human serum albumin (a), glycated serum albumin (b) which was prepared by incubation of HSA and glucose, and commercially available glycated albumin (Consera A, c). The mobile phase system (I, II and III) used is shown in Table 1. Flow-rate in all steps is 1.0 ml/min.

glucose (Fig. 3b) and the commercially available albumin Consera A (Fig. 3c) are significantly larger than that of human serum albumin (Fig. 3a). These observations are quite valid because it is clear that the glycated serum albumin contents in the samples shown in Fig. 3b and Fig. 3c, respectively, are much higher than that in the sample shown in Fig. 3a. Such findings and the good reproducibility of these affinity chromatographic data suggest that the affinity HPLC system using a boronate column prepared in this experiment may possibily enable monitoring the

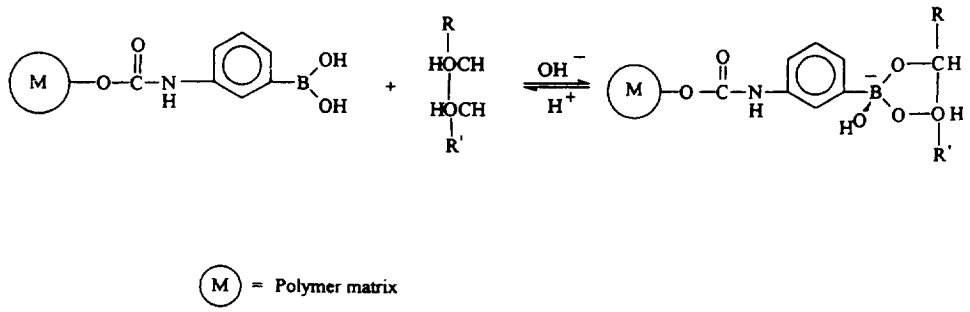

Fig. 4. Scheme of possible complex formation between the boronate group and *cis*-diol compounds.

glycated proteins in human blood and, in addition, the elution system used in this study also may be sufficiently effective to control the column for the detection of glycated proteins.

In order to make sure that the glycated proteins were well separated, each of three fractions eluted from the affinity column was taken repeatedly and condensed by centrifugal ultrafiltration at 950 *g*, after which SDS-PAGE and IEF analyses were carried out. Although well-defined separation of the glycated albumin could not be observed in SDS-PAGE experiments, a strong band centered at 67 kDa which could be assigned to serum albumin was observed in both first and second peak fractions, indicating that some serum albumin analogs were separated from the third peak fraction, whereas only a weak band at 50 kDa region observed from the third peak fraction could be assigned as α_1,β-glycoprotein analogs [16]. On the other hand, the data from IEF seem to indicate a more distinct differentiation between first and second fractions. That is, we observed that a strong band from the first peak fraction appeared around p*I* 4.7, which was assigned to serum albumin [17], while from the second peak a weak band appeared at a p*I* value somewhat lower than 4.7, suggesting the existence of glycated albumin in the second peak fraction. The slightly lower p*I* values are the result of glycation [18]. A band from the third fraction appeared near p*I* 6.5, indicating a protein quite different from the albumin component. In view of the above facts the most reasonable conclusion to be drawn from the available data is that the affinity HPLC system used in the present study may be capable of separating the glycated protein from the serum samples.

Prior to an experiment for clinical study, the effectiveness of this APBA affinity column for detection of glycated serum albumin was investigated. The glycated serum albumin used as standard material was obtained by incubation of human serum albumin and glucose as described in Section 2. The relative peak area of glycated albumin calculated from the second peak area versus total (=the first and the second) peaks area was plotted as ordinate and the mass ratio of the standard material against total albumin used was depicted as abscissa. Fig. 5 shows the relative peak area obtained as the glycated

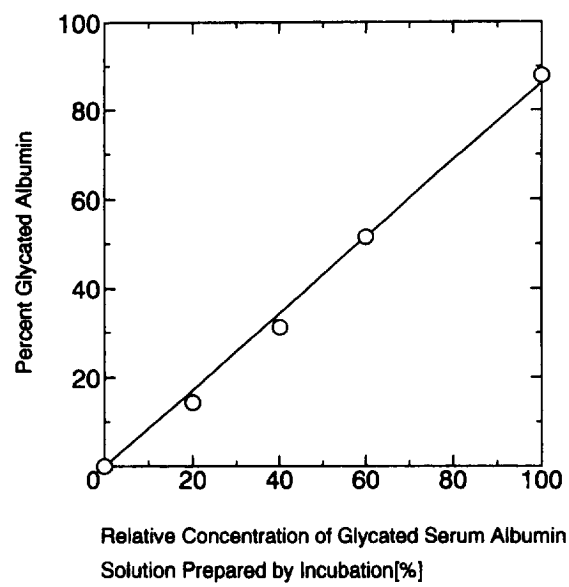

Fig. 5. Relationship between percent glycated albumin measured off a 150×4 mm APBA affinity column and relative concentration of human serum albumin glycated by incubation with glucose (see Section 2).

albumin content was found to increase linearly, in accordance with the increased proportion of the glycated albumin standard sample charged into the column, indicating that the system can be easily applied for analytical affinity HPLC purposes.

3.5. Clinical study

Quantitative analysis for the glycated proteins in serum obtained from 55 non-DM and 114 DM subjects was carried out by using our affinity HPLC system as described above. Fig. 6 shows the experimental results of relative values calculated by plotting the glycated protein content against the blood glucose contents of each subject measured in Takeda General Hospital. Regression analysis of all these glycated protein values versus the blood glucose contents yielded $Y_{GP}=0.0047X_{BG}+0.24$ ($r=0.65$). A somewhat poor correlation (0.65) between glycated protein values and the blood glucose contents was obtained. Similarly poor correlations between these two values have been reported by several other workers [19,20] using a commercially available boronate affinity kit column. We assume that such a poor correlation value could be attributable in a part to the random glucose contents used in this study instead of fasting glucose levels or mean glucose concentrations, or in part to other pathogenic effects of DM.

The frequency of subject occurrence versus the glycated protein values is shown in Fig. 7. The histogram obtained in Fig. 7 was found to be clearly divided into two distinct groups of non-DM and DM subjects. The difference between the two groups is statistically significant ($P<0.01$). Thus, the relative peak-area values of the second peak obtained using this affinity HPLC system are expected to provide a valuable index for diabetic control.

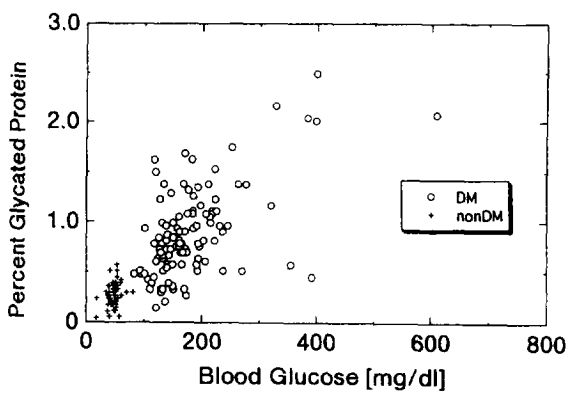

Fig. 6. Relationship between the percent glycated protein observed in this affinity system and blood glucose levels in non-DM and DM samples.

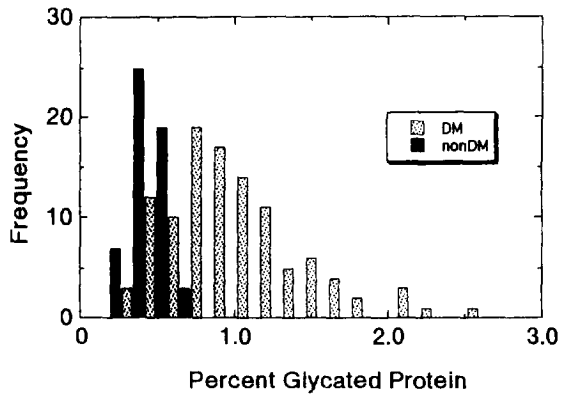

Fig. 7. Frequency histogram of the observed glycated protein values in non-DM and DM subjects.

4. Conclusion

Rapid and reliable determinations of glycated proteins in human serum were studied using a novel affinity HPLC system. The packing materials for the affinity chromatography were prepared by using a new type of hydrophilic and durable porous polymer particles developed in our laboratory, and by manufacturing CDI-activated gels, followed by the coupling reaction with aminophenyl boronic acid (APBA) as an affinity ligand. It was found in the optimization experiments on this coupling reaction that acidic conditions gave rise to better coupling yields, quite different from several cases reported previously. The affinity HPLC separations for glycated proteins in serum samples of non-DM and DM subjects using a small column (150×4 mm) packed with APBA-immobilized particles were carried out using a new elution system, and the analytical data obtained quantitatively by the present affinity HPLC system was demonstrated to be effective as an useful index for the management of DM.

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