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High-performance affinity chromatography system for the rapid, efficient assay of glycated albumin

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ABSTRACT

A high-performance affinity chromatographic system was constructed and shown to permit highly reproducible, rapid, automatic assays of serum glycated albumin (GA) by separation of albumin (Alb) on an anion-exchange column (Asahipak ES-502N) packed with a vinyl alcohol copolymer bearing diethylamino groups and consecutive separation of GA on a column packed with a vinyl alcohol copolymer bearing boronate groups. The first column selectively retained Alb free of other serum proteins and permitted at least a 95% recovery of sample Alb. The purity of the Alb peak was confirmed by two-dimensional electrophoresis. Chromatographic analyses of human serum Alb incubated with glucose on the second column showed that the peak area for GA increased in accordance with the incubation time and suggested selective adsorption of GA on the second column. Optimization of the conditions for the two-column system reduced the analysis time to 10 min. Analyses of human sera with the present system showed GA to be 16.1 ± 1.1% (mean ± S.D.) of total Alb in non-diabetic children and 39.9 ± 9.1% (mean ± S.D.) in diabetic children (0–17 years old).

INTRODUCTION

Glycated albumin (GA) is an important diabetic indicator [1] which is more sensitive than glycohaemoglobin to changes in serum glucose and, because of its shorter half-life, represens the average blood glucose level over a shorter period [2]. Routine clinical assay, however, has not been practicable because all existing assay methods are laborious and lengthy, consisting either of Affi-Gel Blue chromatography and thiobarbituric acid (TBA) spectrophotometry or furosine assay [3], or of boronate affinity chromatography and bromcresol green spectrophotometry [4].

We therefore undertook to construct an automated, high-performance affinity chromatographic (HPAC) system for GA determination which consisted principally of an anion-exchange column and a boronate affinity column, and the effectiveness of this systemm was investigated.

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EXPERIMENTAL

Materials

Anion-exchange gel (Asahipak ES-502N) and size-exclusion gel (Asahipak GS-520; protein relative molecular mass exclusion limit $9 \cdot 10^5$; particle size 9.0 μm) were products of Asahi (Kawasaki, Japan).

Epichlorohydrin was obtained from Tokyo Chemical Industry (Tokyo, Japan), *m*-aminophenylboronic acid, standard human serum proteins, α_1-acid glycoprotein, α_1-antitrypsin, α_2-macroglobulin and albumin from Sigma (St. Louis, MO, USA) and haptoglobin, transferrin immunoglobulin (Ig) G and IgA from Green Cross (Osaka, Japan).

Special-grade reagents (Wako, Osaka, Japan) were used for chromatographic experiments except for sorbitol, which was of first-class grade (Wako).

For the preparation of glycated albumin, Pentex human serum albumin (fraction V, Miles) and glucose (special grade, Wako) were used.

Preparation of affinity adsorbent

Dried Asahipak GS-250 gel (100 g) was suspended in 1 l of dimethyl sulphoxide, followed by addition of 468 ml of epichlorohydrin and 50 ml of 10 mmol/l sodium hydroxide and then by stirring at 30°C for 20 h. The activated gel (40 g) was suspended in water, followed by addition of 26 g of *m*-aminophenylboronic acid hemisulphate salt and then by stirring at 60°C for 20 h.

Chromatographic equipment for analytical studies

The equipment for evaluation of the anion-exchange and boronate affinity columns consisted of a Model 880-UP pump (JASCO, Tokyo, Japan), a Model 850 AS auto-injector (JASCO), a Model 875-UV ultraviolet detector (JASCO), a Model T-80 column oven (EYELA, Tokyo, Japan) and a Model 056 recorder (Hitachi, Japan).

Two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out by the method of Okuyama [5]; for the first dimension, isoelectric focusing was performed with a capillary tube (35 mm × 1.0 mm I.D.) and for the second dimension, continuous electrophoresis was performed with polyacrylamide gradient gel.

HPAC system for GA analysis

Anion-exchange gel and boronate affinity gel were packed in stainless-steel columns (50 mm × 7.6 mm I.D. and 100 mm × 4.6 mm I.D., respectively). Eluent A was 250 m*M* ammonium acetate containing 50 m*M* magnesium chloride and 5% ethanol (pH 8.50) and eluent B contained 200 m*M* sorbitol, 100 m*M* tris(hydroxymethyl)aminomethane (Tris) and 50 m*M* EDTA (disodium salt) (pH 8.50).

The equipment consisted of an SCL-6A system controller (Shimadzu, Kyoto, Japan) which controlled an SIL 6B auto-injector (Shimadzu), two

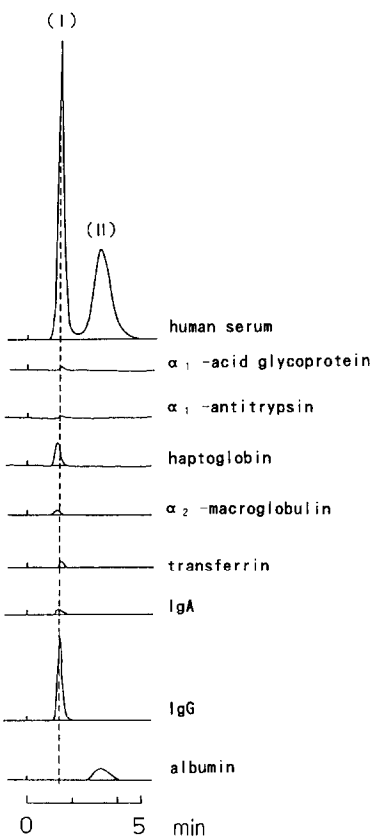


Fig. 1. Chromatograms of human serum and standard serum proteins. Column, Asahipak ES-502N (50 mm × 7.6 mm I.D.); samples, 10 μl of serum diluted by 100 times; α_1-acid glycoprotein, 0.1 μg; α_1-antitrypsin, 0.3 μg; haptoglobin, 0.15 μg; α_2-macroglobulin, 0.3 μg; transferrin, 0.3 μg; IgA, 0.27 μg; IgG, 13 μg; albumin, 45 μg; mobile phase, 250 m*M*, ammonium acetate solution containing 50 m*M* $MgCl_2$ and 5% (v/v) ethanol (pH 8.5); flow-rate, 1 ml/min; detection, UV at 280 nm; temperature, 35°C.

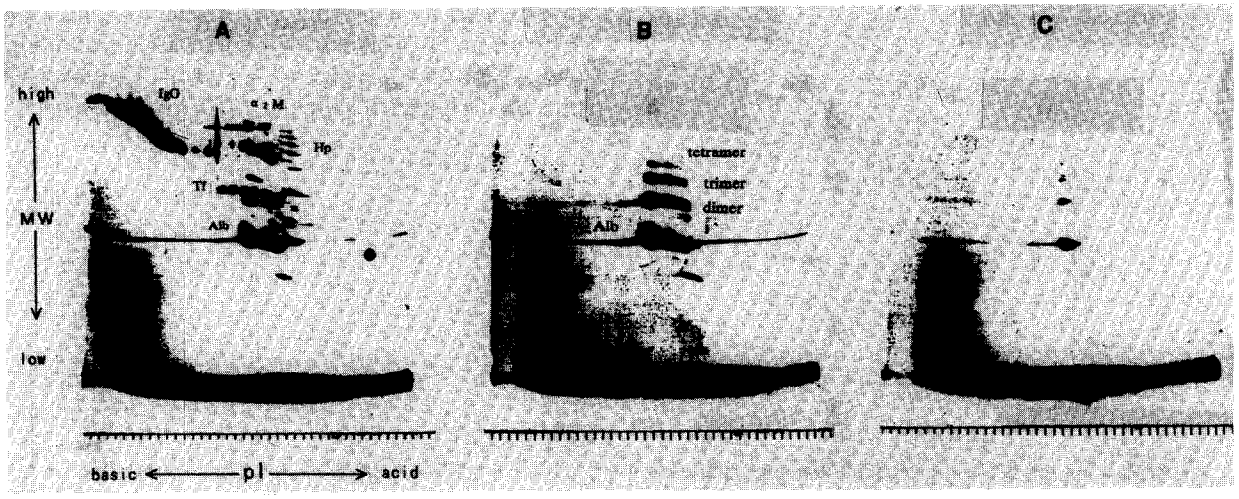


Fig. 2. Two-dimensional electrophoresis patterns of (A) human serum, (B) pure (99.8%) commercial human albumin and (C) the peak II fraction. Method: isoelectric focusing at 0.2 mA (per tube) constant current for 10 min and then at 300 V constant voltage for 80 min; second-dimension electrophoresis with 4–17% linear gradient acrylamide gel at 6 mA per gel plate; samples, human serum, 0.5 μl; commercial human albumin, 24 μg; peak II fraction, 100 μl. Alb = Albumin; α2M = α_2-macroglobulin; Hp = haptoglobin; Tf = transferrin; IgG = immunoglobulin G; MW = molecular mass.

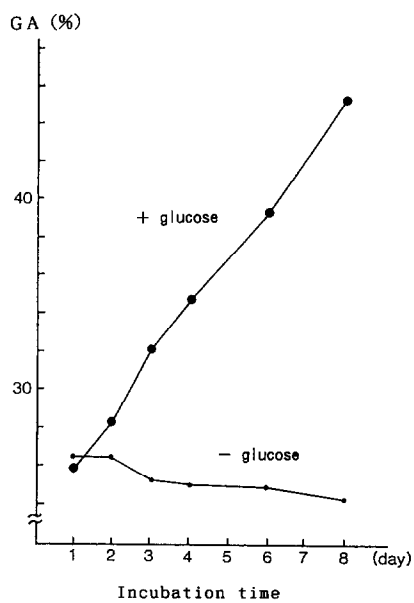
LC-6A pumps (Shimadzu) and an FCV-2AH high-pressure flow-channel selection valve (Shimadzu), an RF-530 fluorescence monitor (Shimadzu) and a DEGAS degasser (Showa Denko, Tokyo, Japan).

RESULTS AND DISCUSSION

Analytical studies

Chromatograms of human serum and standard serum proteins on the anion-exchange column are shown in Fig. 1. All major serum proteins except albumin show the same retention time (1.5 min) as the first peak (I) on the chromatogram obtained for human serum. This demonstrates that albumin was effectively separated from other serum proteins by the column. No such separation was observed on a control column packed with the same matrix devoid of ion-exchange groups. This suggests that such a separation is due in part to interaction with the ion-exchange groups. Such an interaction is not sufficient, however, to explain the separation, as haptoglobin and α_2-macroglobulin, which have lower isoelectric points than albumin, were not retained on the column and thus were eluted at the time of the first peak. It is therefore probable that an as yet unidentified interaction also occurs between the gel matrix and albumin.

The albumin recovery was found to be 95% or higher by comparing the peak areas of albumin injected on to the anion-exchange column and into a PTFE tube (10 m × 0.5 mm I.D.) in its place, under the same conditions.



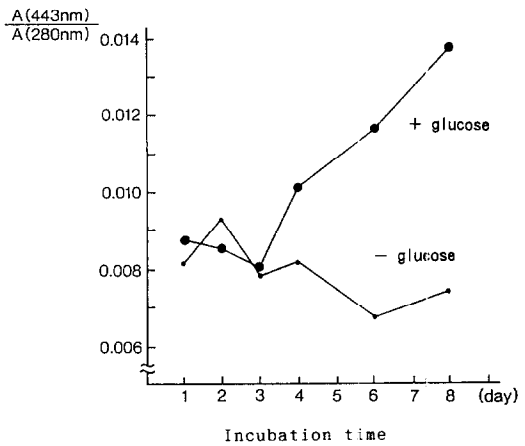


Fig. 4. Relationship between incubation time and ratio of GA (443 nm) to total Alb (280 nm) absorbance. TBA analysis based on the method of Pecoraro and Porte [6].

Analysis of peak II obtained on injection of serum by two-dimensional electrophoresis indicated that it was pure albumin, free from other proteins, as shown by comparison of the pattern obtained for the peak II fraction (Fig. 2C) with those obtained for normal human serum (Fig. 2A) and pure (99.8%) commercial human albumin (Fig. 2B). All three patterns were obtained at the same time, under identical electrophoretic conditions.

The effectiveness of the boronate affinity column for the separation of GA from non-GA (NGA) was investigated by analysing the product obtained by incubation of 5000 mg of human serum albumin (Miles) and 25 mg of glucose in 100 ml of Dulbecco's phosphate-buffered saline at 37°C for various peri-

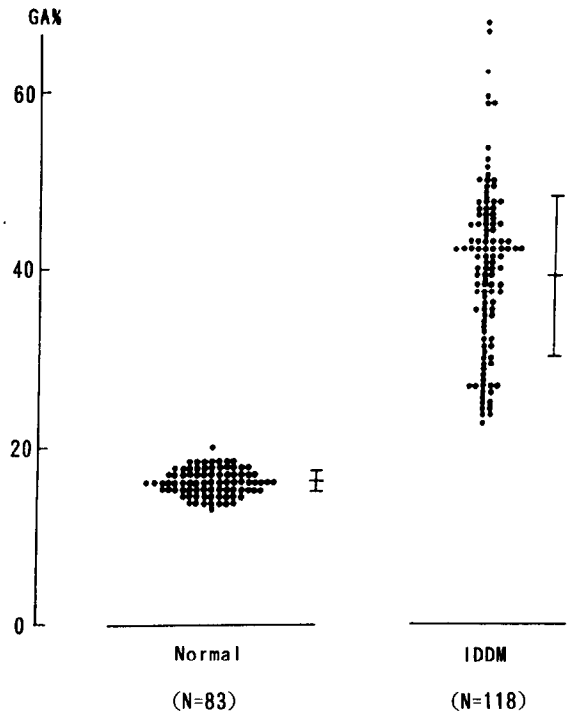


Fig. 6. Serum level of GA in non-diabetic (normal) and diabetic (IDDM) children. Sample, 5 μl of serum fraction obtained by centrifugation and stored at −20°C until analysed.

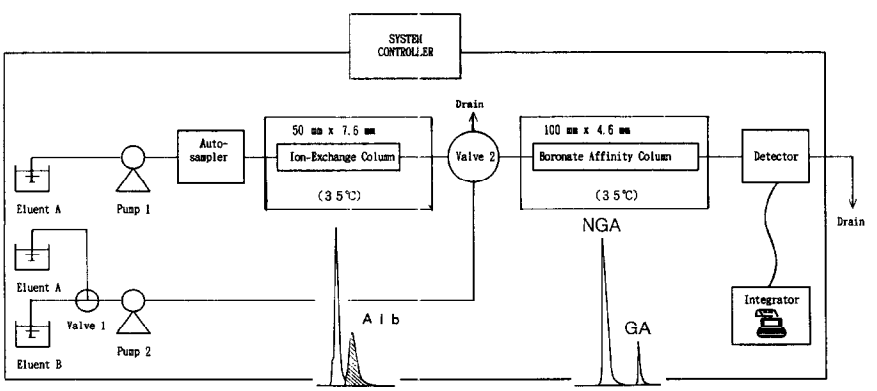


Fig. 5. HPAC system for GA analysis. Procedure: albumin is separated form other proteins on the ion-exchange column with eluent A. The albumin fraction continues into the boronate affinity column with eluent A through valve 2. Non-glycated albumin is eluted without retention. Glycated albumin is eluted with eluent B. GA values are expressed as in Fig. 3. Eluent A, 250 m*M* ammonium acetate–50 m*M* $MgCl_2$–5% ethanol (pH 8.50); eluent B, 100 m*M* Tris–200 m*M* sorbitol–50 m*M* EDTA (pH 8.50).

ods and comparing the results with those obtained by TBA spectrophotometry.

As shown in Fig. 3, the relative peak area obtained for GA on the boronate affinity column, expressed as a percentage of the total area of the NGA and GA peaks, was found to increase in accordance with the incubation time. When no glucose was present in the incubation medium, on the other hand, no increase in the GA peak area was observed.

These results were in accord with those obtained by TBA spectrophotometry [6] (Fig. 4), which is the method generally employed for the determination of GA.

Clinical study

The HPAC system, designed for automated clinical analysis of GA in human serum, is shown schematically in Fig. 5. Its effectiveness in the determination of GA values was investigated by analysis of serum from 83 non-diabetic (normal) and 118 diabetic (IDDM) children, 0–17 years of age.

The resulting GA values [7], expressed as a percentage of the glycated form in the total albumin [GA (%) = GA/(GA + NGA) × 100], are shown in Fig. 6. The GA value was found to be 16.1 ± 1.1% (mean ± S.D.) of total Alb in the non-diabetic children (n = 83) and 39.3 ± 9.1% (mean ± S.D.) in the diabetic children (n = 118). These results thus show that the GA value, as determined with the HPAC system, is significantly higher in diabetic than in non-diabetic subjects. The GA values obtained with this system are therefore expected to provide a valuable index of diabetic control.

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