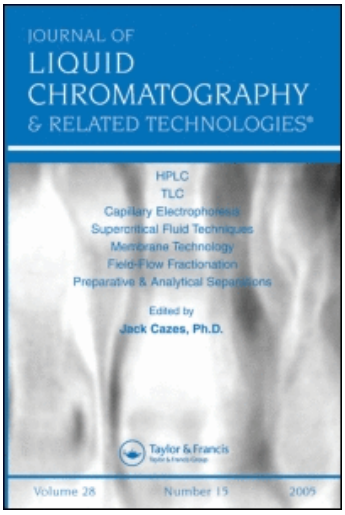

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[a] Health Research Foundation, Institut Pasteur 5F, Kyoto, Japan [b] School of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan

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SELECTIVE CHEMILUMINESCENCE ANALYSIS OF AMADORI FORM OF GLYCATED HUMAN SERUM ALBUMIN

Toshihiko Hanai,[1,*] Miyuki Uchida,[2] Michiko Amao,[2,3] Chiaki Ikeda,[2,4]
Kuniko Koizumi,[2,5] Toshio Kinoshita[2]

[1] Health Research Foundation
Institut Pasteur 5F
Sakyo-ku
Kyoto 606-8225, Japan

[2] School of Pharmaceutical Sciences
Kitasato University
Shirokane, Minato-ku
Tokyo 108-0072, Japan

[3] Ajinomoto Co. Inc.
Central Research Laboratories
Suzukicho, Kawasaki-ku
Kawasaki 210-8681, Japan

[4] Toyama Chemical Co. Ltd.
Nishishinjuku, Shinjuku-ku
Tokyo 160-0023, Japan

[5] Yokogawa Analytical Systems
Mitaka Bldg, Musashino
Tokyo 180-0006, Japan

ABSTRACT

Human serum albumin was purified by size-exclusion liquid chromatography. The glycated albumin in the effluent was selectively measured by a post-column reaction detector. The selective reaction mechanism is to measure the amount of Amadori form of

glycated albumin. According to the Maillard reaction, glycosylated amines are rearranged to Amadori form ketoamines which can produce superoxide in strong alkaline solution, and this superoxide produces chemiluminescence with lucigenin. The chemiluminescence intensity was related to the concentration of glycated albumin in albumin while the fluorescence intensity was the same.

INTRODUCTION

Glycation of proteins seems to play a role in both age-related symptoms (cataract, atherosclerosis, and modification of collagen-containing tissues) and the pathological complications of patients with diabetes mellitus.[1] The extent of nonenzymatic glucosylation of serum protein in control and diabetic subjects was measured by a chemical procedure using thiobarbituric acid. A mean value of 0.81 ± 0.21 nmol glucose per milligram serum protein was observed in the control group.

Diabetics displayed elevated levels of glucosylated serum protein, up to 4 nmol glucose per mg protein. Glucosylation of serum protein was correlated strongly with percent glycated albumin.[2]

Diabetic patients in poor glycemic control show increased glycation of total plasma proteins. The level of glycation was much higher in diabetic plasma immunoglobulins followed in descending order by albumin, complement C3, fibrinogen, transferrin, haptoglobin, and alpha-1-antitrypsin. In general, proteins with the longest biological half-lives, such as IgG and albumin showed the highest level of in vivo glycation. The relative extents of glycation of different plasma proteins are a complex function of integrated glucose concentrations over time, and of the half-life and chemical characteristics of each protein.[3]

Human serum albumin is the most abundant plasma protein, constituting approximately 60% of total serum protein. The non-enzymatic reactivity of a variety of saccharides with the amino group of toluidine indicated that deoxyribose, arabinose, mannose, ribose, and xylose were more reactive than glucose in vitro.[4] However, glucose is the major saccharide in vivo. Glucosylation of albumin in vivo occurs non-enzymatically under physiological conditions at multiple sites, Lys-525 being the principle site of modification.[5]

Serial changes in glycated blood proteins and direct measurement of glycemia were studied in 100 subjects with insulin-dependent diabetes mellitus over 6 weeks. All measures of glycemic control improved. Mean ± SEM glycated hemoglobin (HbA1) fell from 9.1 ± 0.2 to 8.0 ± 0.1%, glycated serum

albumin (GSA) from 9.8 ± 0.4 to 7.3 ± 0.3%, and fructosamine from 3.92 ± 0.08 to 3.42 ± 0.07 mM.

Fasting blood glucose levels fell from 12.5 ± 0.3 to 8.8 ± 0.3 mM. Mean percentage changes in direct measurements of glycemia (32 - 66%) and GSA (29%) were greater than for fructosamine (11%) and HbA1 (12%) levels. Glycated hemoglobin (HbA1) is the most commonly used integrated measurement of glycemic control, but measurement of glycated serum albumin would be a more sensitive indicator of short-term improvement in glycemic control and glycemic instability in insulin-dependent diabetes mellitus than fructosamine or HbA1.[6] Glycated serum albumin levels in diabetes mellitus reflect short-term glucemic control during 2 and 4 weeks levels before measurement.[7-19]

However, the amount of glycated albumin varies between different methods and reports. Glycated and unmodified albumins were separated by ion-exchange liquid chromatography. The physiological significance of these observations in vitro was confirmed by the isolation and quantification of glycated albumin from normal human serum.

Glycated albumin was reported to represent 6 to 15% (n = 6) of total serum albumin in normal adults.[9] Glycated serum albumin of normal subjects was 8.3 ± 2.2% as determined by the thiobarbituric acid procedure and 7.0 ± 1.9% as determined by chromatography as indicators of the degree of hyperglycemia in diabetes.[8] A combination of ion-exchange and affinity chromatography using a phenylboronate agarose column was performed to analyze glycated albumin in blood serum.

Diabetic subjects contained 16 ± 1.1% (n = 25) vs 8.1 ± 0.9% (n = 13) of that which is normal in plasma.[13] Serial measurements of glycated albumin were performed by affinity chromatography throughout the course of pregnancy in 14 insulin-dependent diabetic women. In patients whose glycemic control improved markedly after four weeks of good diabetic control, the glycated albumin level was 3.61 ± 1.72% compared to that of non-diabetic women, 1.65 ± 0.27%.[16]

Mean initial glycated albumin in 73 children with type L diabetes was 16.4 ± 0.6% and that in nondiabetic controls was 8.7 ± 0.3%.[15] In the normal subjects, the mean percentage of glycated albumin was 1.50 ± 0.06% (n = 10), and the value in the diabetic group (n = 10) was 5.15 ± 0.14%, as determined by affinity chromatography.[17]

The percentage of glycated plasma albumin was measured by a procedure involving ammonium sulfate precipitation and Affi-Gel-Blue and phenylboronate chromatography. The value correlated well with the amount of ketoamine-bound sugars determined by calorimetric assay. The normal mean

value is 3.9 ± 0.3% (n = 39) and varied from 3.9 to 21% in diabetics (n = 54). A good correlation was found with the mean blood glucose value for the preceding twenty days.[19]

An antibody, raised in mice immunized with non-enzymatically glycated albumin isolated from human plasma that recognized glycated epitopes in albumin but not in other plasma proteins and did not react with nonglycated albumin, was immobilized onto the wells of microliter plates, and enzyme-linked immuno sorbent assay (ELISA) was used for determination of glycated albumin.[20] The glycated albumin level (mean ± SEM) in 12 samples from normal subjects was 2.4 ± 0.22% of total albumin and that in 25 diabetic subjects ranged from 1.6 to 11.6% with a mean ± SEM of 4.5 ± 1.2%.[21] ELISA was used to study glycemic status in rats.[22]

Quantitative analysis of glycated albumin has been performed using liquid chromatography. Affinity chromatography on metha-aminophenyl boronate columns, together with albumin measurement by radioimmunoassay, was validated as to determine glycated albumin in serum and urine. Reference ranges for glycated albumin in control subjects were 0.6 - 1.8% in serum and 0.9 - 2.6% in urine, and those for subjects with diabetes mellitus were 1.4 - 10.9% in serum and 1.5 - 12.5% in urine.[23]

High performance liquid chromatography with a combination of anion-exchange and boronate affinity columns was developed. This method was not affected by the presence of glucose. The values of glycated albumin in normal subjects (20.2 ± 1.6%) were less than those in diabetic patients; 39.6 ± 5.4% in 40 Type I (insulin-dependent) and 39.4 ± 5.9% in 25 Type II (non-insulin-dependent) patients.[24]

This system was further improved for shorter analysis time, and used for analysis of glycated albumin in children. The levels were 16.1 ± 1.1% of total albumin in non-diabetic children and 39.9 ± 9.1% in diabetic children.[25]

The amounts of glycated albumin in serum determined using these analytical methods were very different. This may have been due to the alkyl chain length of ligand bound to the matrix. In the former method, phenyl boronate was directly bound to agarose gel,[23] and in the latter method phenyl boronate was bound through the propyl group.[24] Therefore, such alkyl chain length should affect the affinity effect of the phenyl boronate bonded phase with the large glycated albumin molecule. Glucose and buffer components also affected the dissociation of glycated albumin.[23]

These methods were used to measure the amount of glycated albumin. However, it may be necessary to measure the amount of Amadori form of glycated albumin as, according to the Maillard reaction, glycosylated amines are

rearranged to Amadori form ketoamines[26-28] which can produce superoxide. This superoxide may then damage cellular tissues and DNA.[29]

In this experiment, a standard Amadori rearrangement compound was first synthesized from glucose and para-toluidine, and chemiluminescence analysis was performed for analysis of glycated albumin. Glycated albumin samples were prepared from human serum albumin (Fraction V) using affinity chromatography on boronate gel, and the contents of glycated albumin were measured by liquid chromatography using a fluorescence detector and a borate column.[30]

EXPERIMENTAL

Synthesis of 1-Deoxy-1-*p*-Toluidine-D-Fructose (DTF)

1-Deoxy-1-*p*-toluidine-D-fructose was synthesized by a modification of the method reported previously.[31] A mixture of 20 g of glucose (0.112 mol) and 16 g of para-toluidine (0.112 mol) in 10 mL of water was heated at 110°C for 30 min with 3 drops of acetic acid. After addition of 100 mL of ethanol, the reaction mixture was stored overnight at ambient temperature. The crystal was filtered and washed using a mixture of ethanol and ether (2:3), then recrystalized from methanol.

The purity of white whisker type crystals was studied using thin-layer chromatography on Silica gel 60F254 (Merck KGaN, Germany). The R_f value was 0.16 and those of para-toluidine and glucose were 0.68 and 0.022, respectively. The developing solvent was a mixture of 3 mL of chloroform, 1 mL of methanol, and one drop of acetic acid.

The structure of 1-deoxy-1-*p*-toluidine-D-fructose that produced chemiluminescence with lucigenine in alkaline solution[32] was identified by infrared and nuclear magnetic resonance spectrometry and elemental analysis.

System for Flow Injection Analysis and Liquid Chromatography

A flow injection system was assembled with two model LC-9A liquid chromatography pumps from Shimadzu (Kyoto), a model 7125 Reodyne injector and a model Shodex CL2 chemiluminescence detector from ShowaDenko (Tokyo). The intensity of chemiluminescence was recorded with a Chromatocorder 12 from SIC (Tokyo, Japan).

A liquid chromatograph was constructed with two model LC-10AD pumps, a model SIL-10AXL auto-injector from Shimadzu (Kyoto), a model

ERC-3522 degasser from ERC (Tokyo) and a model Σ02column oven from IRICA (Kyoto) and a Valco 6 port column switching valve with a micro-electro two position actuator from ESD Lab. (Tokyo).

The fluorescence detector was a model RF530 from Shimadzu. The operation and chromatographic data analysis were performed with a model CLASS-LC10 workstation from Shimadzu.

RESULTS AND DISCUSSION

Flow Injection Analysis Using Lucigenine as a Chemiluminescence Reagent

The concentration of aqueous solution of lucigenine (Aldrich Chem. Co.) was 0.02 mmol/L and the flow rate was 0.1 mL/min. The concentration of sodium hydroxide (Extra Pure grade from Wako Pure Chemicals, Osaka, Japan) was 0.05 mol/L and the flow rate was 0.1 mL/min.

The purification of albumin using a preparative scale borate column and dialysis are time-consuming processes, and such purified products were not stable. 1-Deoxy-1-*p*-toluidine-D-fructose was selected as the standard Amadori rearrangement compound, as it can be easily synthesized and purified. The detection limit of 1-deoxy-1-*p*-toluidine-D-fructose (DTF) in this system was 0.3 pmol and the calibration curve was obtained from 20 pmol to 1000 pmol. Albumin solutions containing 3-90% glycated albumin 30 were analyzed to evaluate this new analytical system.

The chemiluminescence intensity was linearly correlated to concentration of glycated albumin as shown in Figure 1 ($y = 1.780\ x + 9.381$, $r^2 = 0.986$; $n = 8$). The relation between chemiluminescence intensity and concentration of glycated albumin indicated that 100% pure albumin produced chemiluminescence. The intensity was equivalent to that of cysteine in albumin. Other structural amino acids of albumin did not respond to this reaction method.

The peak area measured with a fluorescence detector was constant, but that measured with the chemiluminescence detector increased with increasing glycated albumin content as analyzed by flow injection. The content of glycated albumin was determined from the calibration curve of DTF using the chemiluminescence detector. The linear relationship between glycated albumin concentration and amount of DTF was as follows: $y = 10.414\ x - 17.486$, $r^2 = 0.975$ ($n = 6$). Generally, the amount of glycated albumin is indicated as the relative percent concentration related to total albumin, and specially controlled standard samples are used for the calibration.

In this system, glycated albumin was analyzed under simple and reproducible conditions using DTF as the standard. The total amount of albumin can

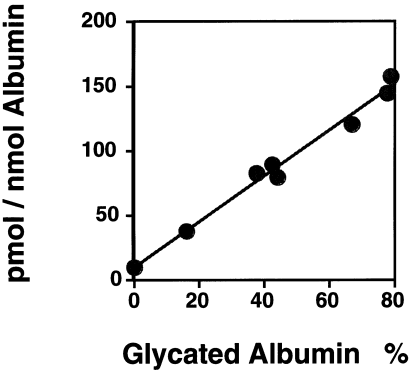

Figure 1. Concentration of glycated albumin in total albumin using 1-deoxy-1-*p*-toluidino-D-fructose as the standard measured by flow injection analysis. Concentration of lucigenine and NaOH : 20 mM and 50 mM, Flow rate : 0.1 and 0.1 mL/min, respectively. Concentration of albumin : 3.8-5.3 g/dL, Amount of albumin injected: 11.5-16 nmol.

be easily analyzed by ordinary clinical analysis or simple liquid chromatography with a fluorescence detector. Therefore, the amount of glycated albumin can be reported as relative concentration in total albumin or serum.

Analysis of Glycated Albumin Using Liquid Chromatography Combined with Chemiluminescence Detection

This chemiluminescence detection system was applied as a liquid chromatographic detector to separate glycated and non-glycated albumin using a borate column. The chromatographic system is shown in Figure 2. The impurity of sorbitol, a component of the eluent, affected the baseline shift as shown in Figure 3 where chromatogram A was monitored with a fluorescence detector (ex. 285 and em. 350 nm) and chromatogram B was monitored by chemiluminescence detection.

The content of glycated albumin was determined as 40% from this fluorescence detection where the fluorescence intensity of glycated and non-glycated albumin were the same. Even if the baseline was not linear, glycated albumin was selectively detected by this chemiluminescence detection method. Therefore, one column and isocratic elution analysis system of glycated albumin was developed using this chemiluminescence detection method.

The chemiluminescence detection can selectively detect Amadori form compounds of proteins and other small molecules in serum. Therefore, separa-

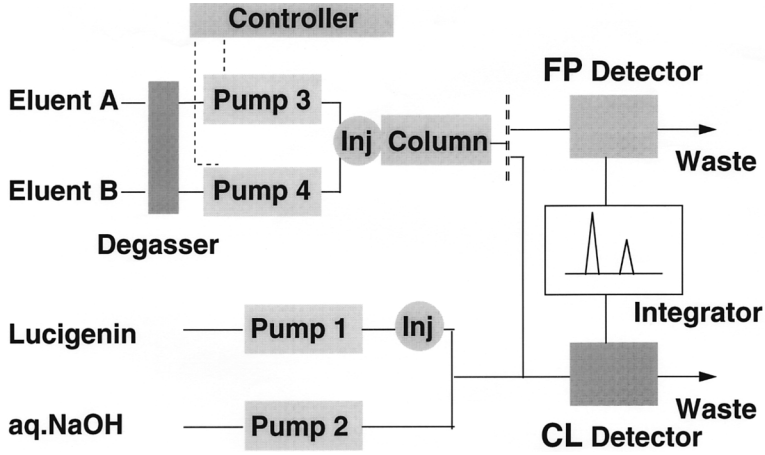

Figure 2. Analytical system for flow injection analysis and liquid chromatography. Inj: injector, FP: fluorescence, CL: chemiluminescence.

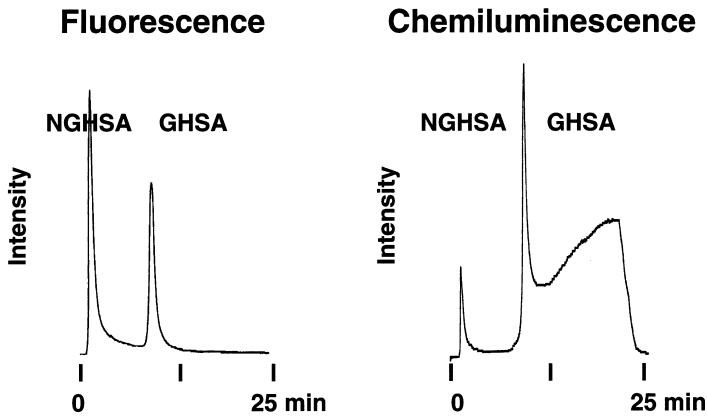

Figure 3. Separation of glycated human serum albumin from non-glycated human serum albumin using borate column with gradient elution. The column: Asahipak borate 150 x 4.6 mm I.D., Gradient elution from Eluent A to Eluent B for 15 min. Flow rate : 1.0 mL/min at ambient, Eluent A: 250 mM ammonium acetate + 50 mM magnesium chloride (pH 8.50) + 5v% ethanol, Eluent B: 200 mM D-sorbitol + 100 mM Tris + 50 mM EDTA (pH 8.50). Detector Fluorescence: ex. 285 and em. 340 nm, Chemiluminescence: The concentration of lucigenin and NaOH for liquid chromatographic detector : 0.12 mM and 1.0 M/L, Flow rate: 0.5 and 0.3 mL/min, respectively. Only the detection system was used for a flow injection analysis.

tion of the albumin fraction from other proteins and small molecules was necessary for selective analysis of glycated albumin. The original glycated albumin analyzer consisted of two columns.[24]. The first column, an anion-exchange column (Asahipak 502N), was used for purification of the albumin fraction, and the second column (Asahipak borate) was used for separation of glycated and non-glycated albumin using gradient elution. However, the components of the eluent of the first column caused a high background for chemiluminescence detection.

The ion-exchange column was, therefore, replaced with a size-exclusion column (Asahipak 501H) and the eluent components were modified to make them more suitable for chemiluminescence detection. The modified eluent was a mixture of 15% ethanol, 250 mM ammonium acetate buffer, pH 10.0, and 200 mM sorbitol.

Several albumin samples, containing 19 - 79% glycated albumin, using a glycated albumin analyzer,[32] were separated through a size-exclusion column and the eluent was monitored by the chemiluminescence detector. The peak area measured by the chemiluminescence detector and the concentration of glycated albumin are summarized in Figure 4.

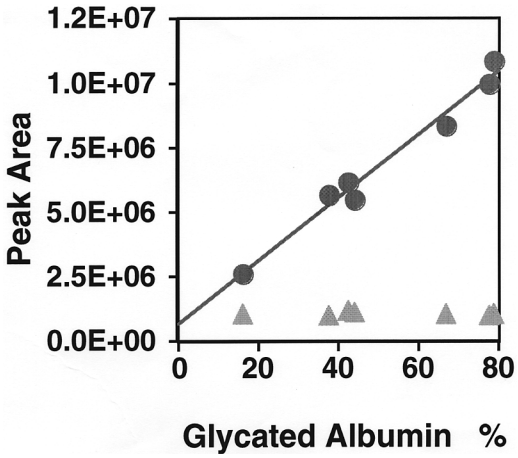

Figure 4. Comparison of peak area measured by chemiluminescence and fluorescence detection methods. Column: Asahipak 510H, 150 x 6.0 mm I.D., Eluent : 25 mM ammonium acetate + 200 mM D-Sorbitol (pH 8.5) + 5% ethanol. Flow rate : 0.5 mL/min at 37°C, Fluorescence detector: ex. 285 and em. 340 nm, Chemiluminescence detector : 0.12 M lucigenin at 0.6 mL/min and 1.0 M NaOH, 0.3 mL/min.

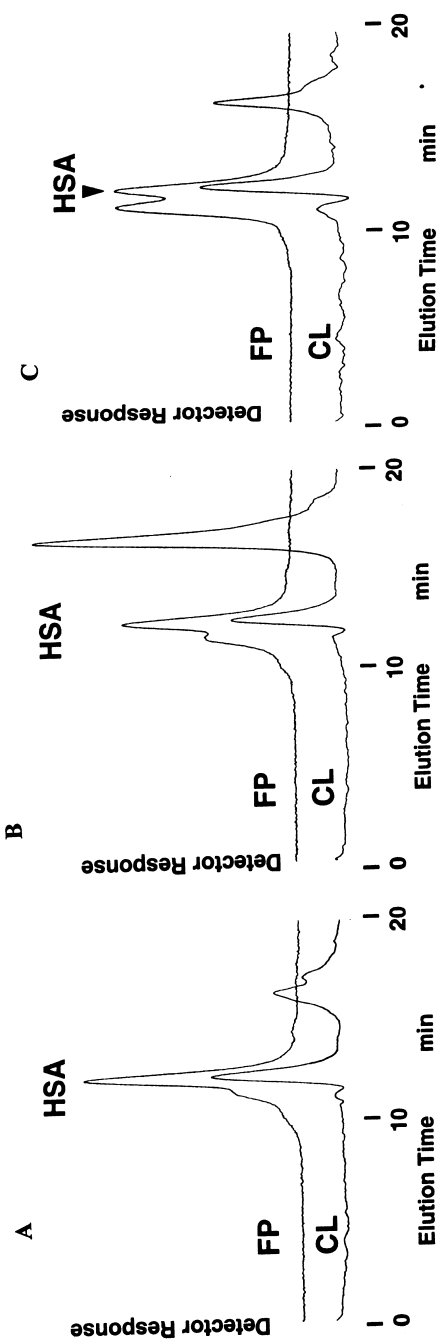

Figure 5. Size-exclusion chromatograms of human blood serum measured by fluorescence and chemiluminescence detections. Column: Asahipak 510H, 250 x 6.0 mm I.D., Eluent : 25 mM ammonium acetate + 200 mM D-Sorbitol (pH 10.0) + 15% ethanol. Flow rate : 0.5 mL/min at 37°C, Fluorescence detector: ex. 285 and em. 340 nm, Chemiluminescence detector : 0.12 M lucigenin containing 5% ethanol at 0.6 mL/min and 0.5 M NaOH containing 2.5% ethanol, 0.3 mL/min.

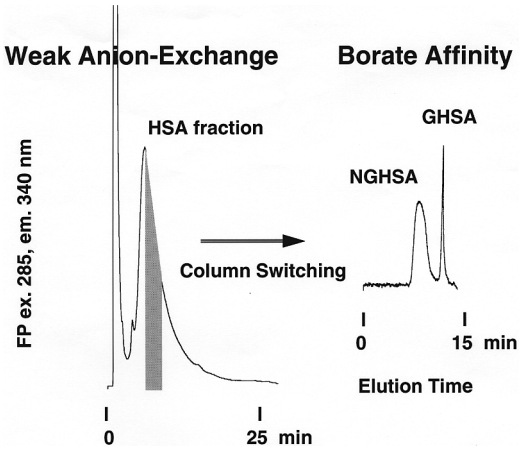

Figure 6. Chromatographic analysis of glycated and non-glycated human serum albumin in serum using two column system. This system used 3 pumps, two fluorescence detectors, and one Valco 6 port column switching valve with a micro-electro two position actuator from ESD Lab., Tokyo.

Peak area of human serum albumin samples measured by the fluorescence detector was constant as the same injection volume was used. However, the peak area measured by the chemiluminescence detector was related to concentration of glycated albumin.

This one-column chemiluminescence detection system was evaluated by analysis of blood serum of diabetic patients. The chromatograms obtained by fluorescence and chemiluminescence are shown in Figure 5A, 5B and 5C. The concentrations of glycated albumin in the samples shown in these figures were 893, 712, and 988 pmol as DTF standard, respectively. An example of a chromatogram obtained by the two-column system is shown in Figure 6, where albumin fraction in blood serum was first separated by ion-exchange liquid chromatography, then further separation was performed by affinity liquid chromatography using a borate column.[25] These chromatograms indicated that this new analytical system can be used as a glycated albumin analyzer specific for Amadori form glycated albumin.

This new chromatographic system is simple and the further development of a separation column will reduce the analytical time.

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