

Selective Detection of Human Serum Albumin Using a Fused-Silica Capillary Modified with Anti-Human Serum Albumin

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A fused-silica capillary modified with anti-human serum albumin was prepared using the glutaraldehyde method after activating the inner wall with a silane coupling reagent. The antibody selectively interacted via an immune reaction with the human serum albumin, which acted as an antigen. The analytical performance of the modified capillary was examined by capillary affinity chromatography using either absorption or chemiluminescence detection. The capillary affinity chromatography required only minimal reagents and samples due to the extremely small diameter of the capillary. For chemiluminescence detection, a peroxyoxalate reagent was used and human serum albumin was labeled with fluorescamine for competitive immunoassay. After optimization of the analytical conditions, chemiluminescence detection showed a higher sensitivity than absorption detection. Furthermore, a modified capillary was also used in capillary electrophoresis with the absorption detector to allow affinity capillary electrophoresis.

Immunoaffinity chromatography using a column packed with immunosorbents is a technique based on a specific reaction between an antigen and an antibody, and is widely used in the fields of medicine and pharmacy as well as analytical chemistry.[1,2] Affinity chromatography enables the determination of a sample easily, rapidly, selectively, and continuously. However, the reagents and samples used for an immune reaction are generally expensive, and thus we developed an immunoaffinity chromatography system consisting of immunosorbents packed into a micro-bore column (1 mm i.d. × 50 mm) manufactured using a Teflon tube and a Teflon line-filter.[3]

On the other hand, the use of capillary tubes has become increasingly sophisticated. Many studies of open tubular liquid chromatography (OTLC), open tubular capillary electrochromatography (OTCEC), as well as capillary electrophoresis (CE) have been reported, in most of which a fused-silica capillary of less than 100 μm i.d. was been used.[4–8] Clearly, these methods using capillaries have achieved greater success in the down-sizing of equipment than micro-bore liquid chromatography.

Guo et al. facilitated the fabrication of a porous silica glass layer onto the inner wall of a capillary through the sol–gel process for OTLC.[7] Francotte et al. demonstrated enantiomer separation by OTLC and OTCEC using capillaries coated with cellulose derivatives.[8] We have introduced phenylboronic acid moieties onto the inner wall of a capillary through polymerization of *m*-acrylamidophenylboronic acids.[9] The thus-obtained phenylboronic acid-modified capillary successfully separated a model mixture sample of nucleosides in OTCEC based on the interaction between *cis*-diol groups and phenylboronic acid moieties on the capillary wall. Furthermore, analytical protocols that combine CE and a competitive immunoassay have been reported.[10,11] Schmalzing et al. incubated fluorescein-labeled cortisol and anti-cortisol antibody with serum in a competitive immunoassay, and then introduced the serum into a capillary to separate free and bound labeled antigen by CE with high reproducibility for the quantitation of cortisol in serum.[10]

In the present study, we prepared an antibody-modified capillary using the glutaraldehyde method after activating the inner wall with a silane coupling reagent, and examined the capillary's performance in OTLC, also known as capillary affinity chromatography, with either absorption or chemiluminescence (CL) detection. The antibody selectively interacted with its antigen through an immune reaction. Furthermore, we also examined the modified capillary in OTCEC, or affinity capillary electrophoresis, with absorption detection. Human serum albumin (antigen) and anti-human serum albumin (antibody) were used as a model immune reaction. Human serum albumin in a serum sample was analyzed selectively through the immune reaction on the inner wall.

Experimental

Reagents. All of the reagents used were commercially available and of special grade. Ion-exchanged water was distilled for use. Human serum albumin (HSA) (M_w, 66000), rabbit anti-human serum IgG (anti-HSA) (M_w, 150000), and control human serum were purchased from Sigma Co., Wako Pure Chemical Industries, Ltd., and Sanko Junyaku Co. Ltd., respectively. Bis(2,4,6-trichlorophenyl)oxalate (TCPO), bis[2-(3,6,9-trioxadecanyloxycarbonyl)-4-nitrophenyl]oxalate (TDPO), and fluorescamine (FR) were purchased from Nacalai Tesque, Inc., Wako Pure Chemical Industries, Ltd., and Fluka Chemie AG, respectively. Solutions of 8 mM hydrogen chloride–25 mM potassium chloride (pH 2.2), 200 mM acetic acid–200 mM sodium acetate buffer (pH 4.0), 10 mM Tris–10 mM boric acid buffer (pH 7.0), and 5 mM phosphate buffer (pH 7.3 and 9.0) were used as elution or migration buffers. FR-labeled HSA was prepared by vigorously mixing a HSA solution (pH 9.0, 450 μL) with a 1×10^{-2} M FR acetone solution (100

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μL) in a vortex mixer.

Preparation of Anti-HSA-Modified Capillary. An anti-HSA-modified capillary was originally prepared as described in our previous reports of a phenylboronic acid-modified capillary[9,12] and antibody-immobilized glass beads.[13] A fused-silica capillary (75 μm i.d.) was washed with 1 M NaOH and water. Next, the alkylamino-bonded inner wall of the capillary was prepared using a 2 wt % (3-aminopropyl)triethoxysilane toluene solution (6 h). The immobilization of anti-HSA onto the alkylamino-bonded inner wall was performed using the glutaraldehyde method as follows. The inner wall of the capillary was treated with 5 wt % glutaraldehyde dissolved in a phosphate buffer solution (pH 7.3) for 24 h at room temperature. After washing with a phosphate buffer solution (pH 7.3), the activated wall was filled with an anti-HSA solution (7.7×10^{-7} M) dissolved in the phosphate buffer. The capillary was then kept at 4 °C for 48 h to give an anti-HSA-modified capillary. After that, 12 mg mL^{-1} sodium borohydride was fed into the capillary for 10 h.

Capillary Affinity Chromatography with Absorption Detection. An unmodified or modified capillary (75 μm i.d.) of 70 cm length (effective length of 55 cm) was used. A phosphate buffer (pH 7.3) for the immune reaction or a hydrogen chloride–potassium chloride solution (pH 2.2) for elution was delivered into the capillary by siphoning from a height of 30 cm (that is, only gravity flow with no electrophoresis). An aliquot of protein sample (ca. 50 nL) was injected into the capillary by siphoning for 15 s at a height of 30 cm. The sample was detected at 210 nm using a modified spectrophotometric detector (Shimadzu Co. SPD-6AV).

Capillary Affinity Chromatography with CL Detection. A batch-type CL detection cell was used. The concept of the cell was originally proposed by us in a previous paper.[14] The detection cell was made of Teflon, which had a 4 cm outer diameter, 2.5 cm height, and ca. 8 mL inner volume. An optical fiber (a core diameter of 2 mm), a fused-silica capillary (75 μm i.d.), and a platinum wire as a grounding electrode were fixed to the cell. Thus, the cell also worked as an outlet reservoir including an electrolyte solution. The optical fiber was set up straight to the capillary with a distance of ca. 0.3 mm between them. A CL reagent of 2 mM TDPO and 200 mM H_2O_2 in acetonitrile was added into the cell.

An unmodified or modified capillary of 50 cm length was used. A hydrogen chloride–potassium chloride solution (pH 2.2), acetic acid–sodium acetate buffer (pH 4.0), Tris–boric acid buffer (pH 7.0), or phosphate buffer (pH 7.3 and 9.0) was delivered into the capillary by siphoning from a height of 30 cm (that is, only gravity flow with no electrophoresis). An aliquot of protein sample (ca. 50 nL) was usually injected into the capillary by siphoning for 15 s at a height of 30 cm, while, for the competitive immunoassay sample volume of ca. 200 nL was introduced into the modified capillary. When analyte emerged from the capillary, it reacted with the CL reagent at the capillary outlet in the CL detection cell to produce visible light. The light was transported through the optical fiber to a photomultiplier tube.

Affinity CE with Absorption Detection. An unmodified or modified capillary (75 μm i.d.) of 70 cm length (effective length of 50 cm) was used. A phosphate buffer (pH 7.3) was used as a migration buffer. An aliquot of a protein sample (ca. 40 nL) was injected into the capillary by siphoning for 15 s at a height of 25 cm. A high voltage of 12 kV was applied to the capillary (an electrode in the outlet reservior worked as a cathode). The sample was detected with a modified spectrophotometric detector of Shimadzu Co. SPD-6AV (210 nm).

Results and Discussion

Capillary Affinity Chromatography with Absorption Detection. Bovine serum albumin (BSA) and HSA solutions (8.0×10^{-5} M) as samples were introduced into an unmodified or anti-HSA-modified capillary. When using an unmodified capillary, BSA and HSA similarly moved at both pH 2.2 and 7.3 and were detected at ca. 18 min. With the modified capillary, BSA was detected at both pH values at ca. 18 min and HSA was detected only at pH 2.2. HSA was never detected in the modified capillary at pH 7.3, at least for the 2-hour duration of the experiment. After the phosphate buffer (pH 7.3) was delivered for 40 min into the modified capillary to which HSA had been injected; the hydrogen chloride–potassium chloride solution (pH 2.2) was subsequently fed into the capillary, and then the HSA signal appeared with a single peak at ca. 60 min.

The immune reaction between HSA and anti-HSA immobilized on the capillary inner wall occurred at pH 7.3, and trapped HSA was eluted at pH 2.2 at the capillary outlet. The calibration curve of HSA revealed that the eluted HSA could be determined over a range of 1.6×10^{-5}–1.2×10^{-4} M with a good linear relationship (correlation coefficient, 0.999) and a slope of ca. 0.8. The detection limit of 1.6×10^{-5} M was selected to be $S/N = 3$. A comparison of the absorption of 1.0×10^{-4} M HSA obtained directly using the unmodified capillary and that obtained through immune reaction using the modified capillary revealed that the latter showed ca. 90% absorption of the former.

A control serum solution that was diluted to 20 times its volume was introduced into the modified capillary at pH 7.3 for 40 min, and proteins other than HSA were detected at ca. 18 min. Subsequently, a buffer of pH 2.2 was delivered into the capillary and then the signal due to HSA appeared at ca. 60 min (Fig. 1). Based on the HSA calibration curve, the amount of HSA in the serum was estimated to be 5.1×10^{-4} M (relative standard deviation of 3.8%; $n = 9$). The value of HSA almost corresponded to that reported by the manufacturer (5.4×10^{-4} M). Thus, the present method is applicable to the determination of a specific protein in a serum sample without experiencing interference from coexisting constituents.

Capillary Affinity Chromatography with CL Detection. Absorbance and fluorescence phenomena have been the most basic principles for the detection technique in instrumental analysis. Furthermore, CL, which has a profound relationship on their detection principles, has received much attention as an attractive detection technique. CL detection is highly sensitive, determinable over a wide range, easy to operate, and requires an inexpensive apparatus and reagents, and has been used in FIA, HPLC,[15–18] and CE.[19–23] Recently, CL detection has also been estimated to be one of the best matched detection methods for the micro-total analysis system (μ-TAS), because CL does not require any light source or spectroscopes.[24,25]

The peroxyoxalate CL system has been widely applicable to the detection of fluorescence and fluorescence-labeled compounds. In FIA and HPLC, the sensitivity of the CL detection system is higher than that of the fluorescence detection system.[26,27] TCPO and TDPO are representative peroxyoxalate CL reagents. Their performances were examined using an unmodified capillary, and compared with each other in FR-labeled

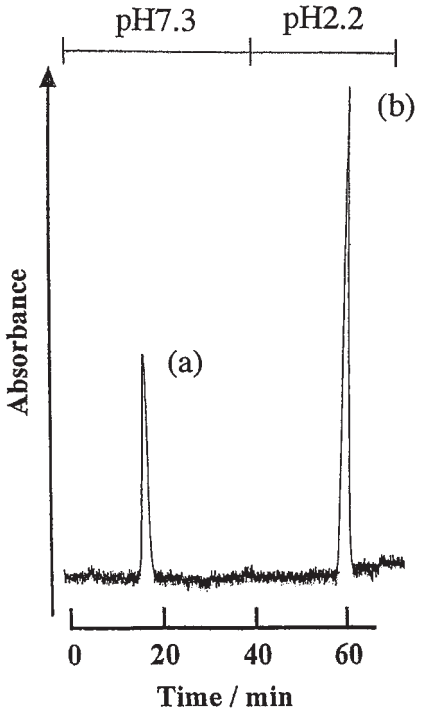

Fig. 1. Analysis of HSA in a serum using affinity capillary chromatography with absorption detection. (a) Proteins other than HSA and (b) HSA. Conditions: Capillary; anti-HSA-modified capillary of 75 μm i.d. and 70 cm length (effective length of 55 cm), solution; a phosphate buffer (pH 7.3) for immune reaction and a hydrogen chloride–potassium chloride solution (pH 2.2) for elution, detection; 210 nm, and sample; control serum solution diluted to 20 times its volume.

HSA detection. The TDPO signal featured a larger peak height than that of TCPO. Furthermore, the CL intensity of TDPO for repeated injections of the labeled HSA was more stable than that of TCPO. Based on the above results, TDPO was adopted in the present investigation.

We also examined the relationship between the standing time of FR-labeled HSA and the relative peak height (data not shown). The CL intensity did not change until ca. 30 min, and after that it gradually decreased. Thus, a measurement of FR-labeled HSA should be carried out within 30 min after preparation. Figure 2 shows the effects of the TDPO and H_2O_2 concentrations on the CL intensity, with the plots showing the average of 7–9 experiments. We found that 2 mM TDPO and 200 mM H_2O_2 provided the maximum CL intensity. The reason that the CL intensities decreased at higher concentrations of both reagents has not been clear. The production of dioxetane as an intermediate may proceed quickly at higher concentrations. The following experiments were carried out using 2 mM TDPO and 200 mM H_2O_2.

The calibration of FR-labeled HSA was examined using an unmodified capillary under the above recommended reagent concentrations. The labeled HSA indicated a good linear relationship between the concentration and the CL intensity; the intensity was reproducibly observed with a relative standard deviation of less than 3.5%. The linear determinable range was more than 2 orders of magnitude with a slope of about 1. The

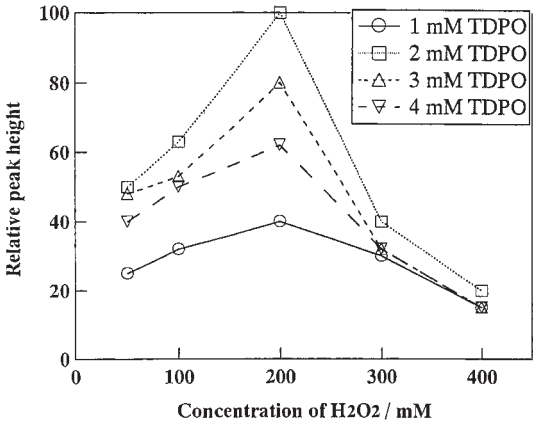

Fig. 2. The effects of TDPO and H_2O_2 concentration on the CL peak height. Conditions: Capillary; unmodified capillary of 75 μm i.d. and 55 cm length, carrier; Tris–borate buffer (pH 7.0), and sample; 1.6×10^{-6} M FR-labeled HSA.

detection limit (S/N = 3) of the labeled HSA was 8×10^{-7} M. The sensitivity of the CL detection was about 15-times as high as that of absorption detection.

In preliminary experiments, HSA, which was adsorbed on an anti-HSA modified capillary via an immune reaction at pH 7.0, was eluted at pH 2.2, 4.0, 7.0, and 9.0, and detected using absorption detection. The HSA could not be eluted with the neutral solution, but eluted well with the acidic and alkaline solutions. Next, we examined the effect of the pH of a carrier on the CL intensity of FR-labeled HSA using an unmodified capillary. Larger CL intensities were observed around pH 7–9, and the CL intensities obtained under the acidic conditions were lower than those obtained under neutral and alkaline conditions. Based on the above results, the elution of labeled HSA from the inner wall of the modified capillary was carried out at pH 9.0 for the following experiments.

The adsorption capacity of HSA by the modified capillary was examined using a 55 cm length modified capillary and 8.0×10^{-6} M FR-labeled HSA, and the CL intensities were then examined against the sample injection times (the data not shown). No peak was observed up to 60 s of injection time, after which the CL peak gradually appeared. About 1.6×10^{-12} mol HSA could be adsorbed on the inner wall of the modified capillary through an immune reaction under the present conditions. The sample injection was carried out for 90 s for the following competitive immunoassay.

A competitive immunoassay was carried out using a mixture of 8.0×10^{-6} M FR-labeled HSA and 8.0×10^{-6} or 8.0×10^{-5} M HSA. The results are shown in Fig. 3. The labeled HSA and HSA competitively reacted with anti-HSA on the inner wall at pH 7.0, and the unadsorbed labeled HSA was detected at ca. 17 min. After 40 min, an elution solution at pH 9.0 was delivered into the capillary. The labeled HSA and HSA were released from the capillary inner wall, and the labeled HSA was detected at ca. 55 min. It was reasonable based on the principles of competitive immunoassay to expect that the chromatogram obtained using 8.0×10^{-6} M HSA would indicate a smaller CL intensity in the first peak, and a larger CL intensity in the second peak, than that of 8.0×10^{-5} M HSA. However, the second peaks lacked repro-

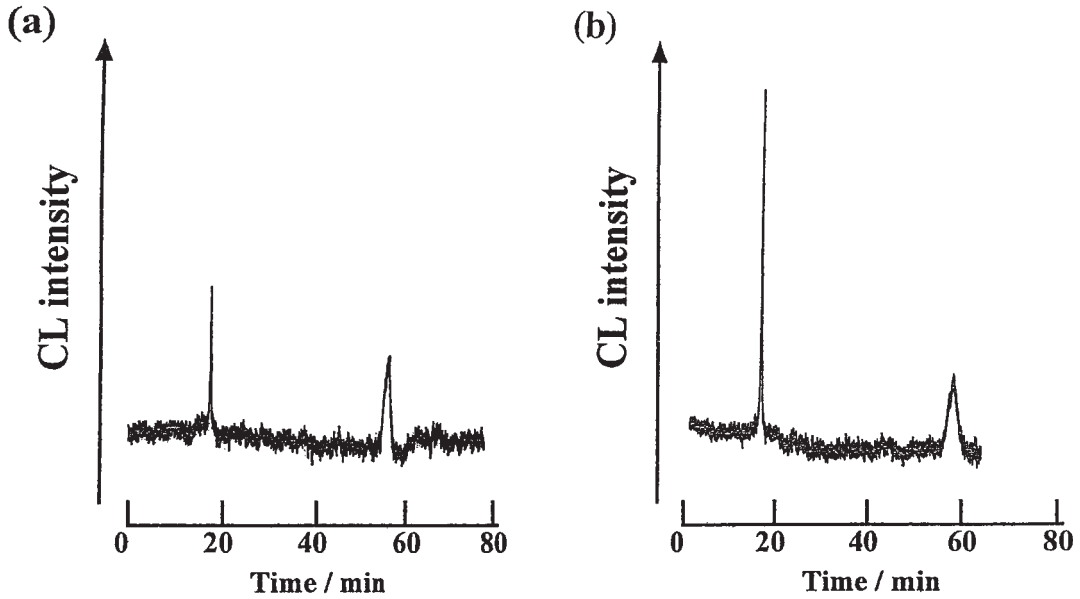

Fig. 3. Competitive immunoassay using FR-labeled HSA in capillary affinity chromatography with CL detection. (a) 8.0×10^{-6} M FR-labeled HSA + 8.0×10^{-6} M HSA (analyte) and (b) 8.0×10^{-6} M FR-labeled HSA + 8.0×10^{-5} M HSA (analyte). Conditions: Capillary; unmodified capillary of 75 μm i.d. and 55 cm length, solution; a phosphate buffer (pH 7.3) for immune reaction (0–40 min) and a phosphate buffer (pH 9.0) for elution (40 min–), and CL reagent; 2 mM TDPO and 200 mM H_2O_2 in acetonitrile.

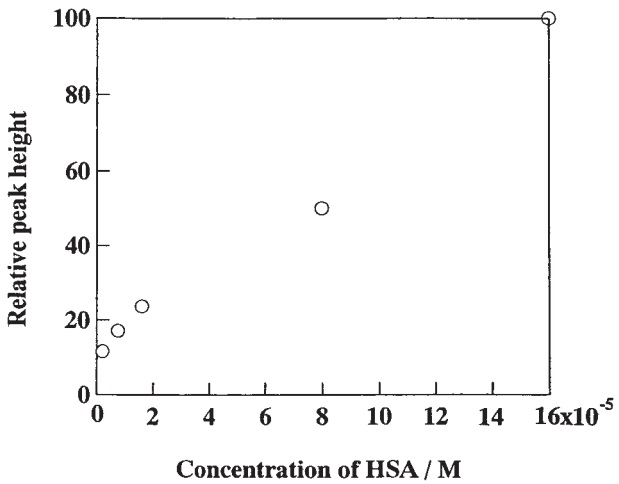

Fig. 4. The calibration curve of HSA with competitive immunoassay using FR-labeled HSA in capillary affinity chromatography with CL detection. Conditions: Capillary; unmodified capillary of 75 μm i.d. and 55 cm length, solution; a phosphate buffer (pH 7.3) for immune reaction (0–40 min) and a phosphate buffer (pH 9.0) for elution (40 min–), and CL reagent; 2 mM TDPO and 200 mM H_2O_2 in acetonitrile and 8.0×10^{-6} M FR-labeled HSA.

ducibility, because they were detected after 40 min. Judging from the data concerning the stability of CL intensity of the FR-labeled HSA mentioned above, FR-labeled HSA must be analyzed within 30 min. The calibration curve of HSA was examined using the first peak, and HSA was determined over a range of 1.6×10^{-6}–1.6×10^{-4} M with a detection limit of 1.6×10^{-6} M (Fig. 4). The detection limit was about one to tenth as small as that (1.6×10^{-5} M) obtained using capillary affinity chromatography with absorption detection.

Table 1. Migration Times of Proteins in Affinity Capillary Electrophoresis with Absorption Detection

Protein	Migration time/min	
	Unmodified capillary	Modified capillary
Ova	21.2	28.1
BSA	21.4	39.8
HSA	21.4	50.2
Control serum	21.4	25.4, 50.3

Sample concentrations: 2.0×10^{-5} M Ova, BSA, and HSA. Control serum was diluted 50 times.

In order to determine the amount of HSA in a serum by the present method, a control serum solution, diluted to 100-times its volume, was added to a microvessel including the FR-labeled HSA. The mixture was subjected to capillary affinity chromatography with CL detection. The amount of HSA obtained by the present method, 5.7×10^{-4} M (the relative standard deviation of 5.6%; $n = 7$), almost corresponded to that reported by the manufacturer (5.4×10^{-4} M). Thus, the present method is applicable to the determination of protein in a serum sample.

Affinity Capillary Electrophoresis with Absorption Detection. The migration times of proteins, such as ovalbumin (Ova), BSA, and HAS, as well as a control serum were examined in affinity capillary electrophoresis using the modified capillary, and compared with those obtained with an unmodified capillary. The results are summarized in Table 1 (the data represent the average of 7–9 experiments). All of the migration times in the unmodified capillary were the same, ca. 21 min, while the migration times in the modified capillary differed. There must be some contribution of the silanol group residues that can act effectively as negative charges, leading to an elec-

Table 2. HSA Concentration in Control Serum Estimated with Affinity Capillary Electrophoresis with Absorption Detection

Dilution[a)]	Estimated HSA concentration[b)]/M
17 times	1.0×10^{-3}
50 times	5.5×10^{-4}
100 times	5.4×10^{-4}

a) Control serum was diluted 17, 50, or 100 times for the analysis. b) HSA concentration, 5.4×10^{-4} M, was reported by the manufacturer.

troosmotic flow. These proteins may be slightly, but negatively, charged under the present conditions. However, the electrophoretic mobilities toward the capillary inlet due to the negative charges would be negligible because of the very small ratios of the charge to mass. The results meant that all proteins indicated no interaction with the unmodified inner wall, but individual affinities to the modified inner wall. The individual affinities comprised specific interaction due to either an immune reaction or non-specific hydrophobic interaction. HSA, which had the longest migration time, had an interaction with anti-HSA immobilized on the inner wall at pH 7.3 through an immune reaction. Control serum indicated two peaks, at ca. 25 and 50 min. The first peak was due to proteins other than HSA and the second peak to HSA.

In order to determine the amount of HSA in a serum sample by the present method, a control serum solution, diluted to 17, 50, and 100 times its volume, was subjected to affinity capillary electrophoresis with absorption detection. The amount of HSA was calculated using an HSA calibration curve. The results are given in Table 2 (the data represent the average of 7–9 experiments). A serum contains many coexisting constituents other than albumin, which may influence the CL intensity. As shown in Table 2, 17-times dilution could not sufficiently dilute the coexisting constituents, leading to a higher HSA concentration. However, the dilution of 50 and 100 times eliminated the influence of the coexisting constituents on the CL intensity. Consequently, the HSA concentrations in serum samples diluted by 50 or 100 times, which of the relative standard deviations were within 6% ($n = 7$), almost corresponded to that reported by the manufacturer (5.4×10^{-4} M). Thus, via dilution, the present method is applicable to the determination of protein in a serum sample without interference from coexisting constituents. We are now studying affinity capillary electrophoresis with CL detection.

The modified capillary was repeatedly used at least 30 times with good reproducibility in capillary affinity chromatography, while in affinity capillary electrophoresis it lasted for 10 experiments, perhaps due to the influence of Joule's heat on the inner wall modified with anti-HSA. Although the present immunoassay may need more detailed examinations to determine the optimal analytical conditions, it is one of the most promising methods for the determination of biological constituents, with specific selectivity, high sensitivity, trace amount of sample volume required, and easy and rapid operation. An immune reaction taking advantage of such a micro-space will be applied to the fields of μ-TAS and micro-reactors.

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