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Capillary electrophoresis immunoassay based on an on-column immunological reaction

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Abstract

An on-column immunological reaction was employed to achieve simple and rapid analysis in an immunoassay based on capillary electrophoresis using semiconductor laser-induced fluorescence detection. Human serum albumin (HSA) labeled with sulfoindocyanine succinimidyl ester (Cy5), a fluorescent compound with an absorption maximum at 649 nm, was used as a fluorescent probe for the immunoassay. In a binding assay, with anti-HSA as the analyte molecule, Cy5-HSA was injected in a capillary column followed by the injection of anti-HSA so as to form individual zones. By applying a potential, the anti-HSA reacted with Cy5-HSA at the boundary between Cy5-HSA and anti-HSA zones, since anti-HSA has a higher electrophoretic mobility than Cy5-HSA. Furthermore, the on-column method enhances the sensitivity by injecting a large volume of the sample. Free Cy5-HSA and its immunocomplex with anti-HSA were separated with less degradation in resolution than that predicted from the injection time of anti-HSA, even when the injection time for anti-HSA was increased. The ratio of the peak area of the complex to that of the total Cy5-HSA (free Cy5-HSA and the complex) increased in proportion to the injection time of anti-HSA. As a result, the detection limit was improved up to eight-fold (the concentration detection limit, 0.007 mg mL^{-1}), for an injection time of 240 s, compared to that obtained using an off-column sample preparation. Furthermore, the on-column reaction method was applicable to an immunoassay to determine native HSA, in which native HSA and Cy5-HSA react with anti-HSA stepwise. The detection limit in the stepwise reaction immunoassay was 0.005 mg mL^{-1}, which is 14 times lower than that in an off-column method, with the analysis time less than 10 min as the result of increasing the injection time of native HSA. In addition, the present on-column immunoassay was applied to the sample containing a high concentration of salts for investigating the effect of salts in the sample solution.

Keywords: Capillary electrophoresis; Immunoassay; On-column reaction; Human serum albumin; Diode laser; Laser-induced fluorometry

1. Introduction

It is well known that antigen–antibody interactions are specific. Immunoassays, which are highly sensitive and selective methods for quantitative analyses that take advantage of this interaction, are in widespread use in pharmaceutical, clinical, and environmental chemistry because of this high specificity. However, commonly used immunoassays, as represented by enzyme-linked immunosorbent assay and radio immunoassay, require a large sample volume and several hours to complete the measurement. Capillary electrophoresis (CE) has recently been shown to be a powerful technique for use in immunoassays [1–14] because it entails a short analysis time (less than 15 min), small amount of a sample (several nL), and low detection limits (nM–pM), and can be used for the simultaneous analysis of multiple components [1]. Capillary electrophoresis immunoassay (CEIA) has been applied for the determination of small molecules, including insulin [2–4], methamphetamine [5], theophylline [6], estradiol [7], cyclosporine A [8], vancomycin [9], as well as for large molecules, e.g., human IgG [10], bovine serum albumin [11,12], and lactoferrin [13], in which it is frequently difficult to separate the free antigen from its

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complex. We have also reported on CEIA of human serum albumin (HSA) [14] using semiconductor laser-induced fluorescence detection (SLIF), since semiconductor lasers have the advantage of lower cost and smaller size [15]. In fact, SLIF has been used for the determination of amino acids [16], proteins [17,18], DNA [19,20], and drugs [21] as a conventional detection method with high sensitivity. However, the sensitivity was still poor in the CEIA application so that further improvement in the sensitivity is necessary for trace analysis.

In several CEIA methods, on-line incubations are performed to achieve automation and rapid analysis [2–3,6,22]. In these methods, the separations and reactions are performed in different channels, and the reaction products are then introduced into the separation channel. However, such methods need more complicated equipment that permits two capillaries to be joined or a specifically designed microchip with reaction and separation channels. On-column reaction methods are promising for rapid analysis since the reaction and separation are carried out simultaneously. One of them has been developed by Bao and Regnier [23], in which a substrate dissolved in the running buffer is electrophoretically mixed with its enzyme on column, resulting in the product during electrophoretic migration of the enzyme. Avila and Whitesides [24] has also been demonstrated on-column enzyme assay, in which an enzyme and its substrate are introduced into a capillary as individual plugs and are electrophoretically mixed to react on column, resulting in the product. Such electrophoretic mixing has also been applied to binding assay of IgG in human serum by Relf et al. [25]. In the paper, antigen and antibody solutions were injected consecutively into the capillary to reduce sample handling, i.e., no pre-mixing was employed. While electrophoretic mixing provided some advantages, manipulation of injection volume was not performed to improve detection limit. In this study, we describe a new method which requires no sample pretreatment and enhances the sensitivity of the analysis by increasing injection time of the analyte solution. The reaction and separation are performed simultaneously in the same capillary by introducing solutions of antigen and antibody in the form of individual zones. When a potential is applied, the antigen reacts with the antibody at the boundary between the two zones, and the resulting complex accumulates at the boundary due to differences in the electrophoretic mobilities between the antigen, the antibody, and the complex. When the volume of the injected analyte solution was increased, less degradation in resolution than that predicted from the increased injection volume was observed between the free antigen and the corresponding immunocomplex. Therefore, a large amount of analyte can be introduced into the capillary, resulting in an improvement in sensitivity without the need for any special equipment. The sensitivity obtained in this study is not so high due to insufficient optimization of the optics, such as optical and spatial filters, in the detection system assembled in-house. However, the present technique is potentially applicable to any detection systems for improving sensitivity.

2. Experimental

2.1. Apparatus

A semiconductor laser, emitting at 635 nm with 1.5 mW of output power (LDA1035, ILEE Laser-Innovation, Switzerland) or 3 mW of output power (LDM 635/3LT, Roithener Lasertechnik, Vienna, Austria), was used as the excitation light source for SLIF. The laser beam was focused on the capillary by an objective lens (22405, Nikon, Tokyo, Japan). Fluorescence was collected with an objective lens (101247, Olympus, Tokyo, Japan) and a photomultiplier tube (R3896, Hamamatsu Photonics, Shizuoka, Japan) was employed to detect the fluorescence after it was passed through a pin hole, bandpass filter (CWL: 672 nm, FWHM: 5 nm), and longpass filter (cut-off: <640 nm). A high-voltage power supply (HCZE-30P, Matsusada Precision Devices, Shiga, Japan) was used for applying the high voltage (17 kV). Fused-silica capillaries (50 μm I.D. × 375 μm O.D.) were purchased from GL Science Inc. (Tokyo, Japan). The length of the capillary was 60 cm with the effective length of 50 cm from the inlet to the detection window. The sample was injected by means of a siphon method (the inlet was raised 6 cm above the outlet). Fluorescence signals were recorded using a personal computer (Macintosh PHX 100, Apple Computer, CA, USA) and an interface (Compare Module, Rainin Instrument Co., CA, USA). Peak areas of electropherograms shown in figures were normalized by the total peak area of Cy5-HSA and its immunocomplex to correct the experimental errors arising from fluctuation in the laser power and focusing conditions of the laser and fluorescence. In quantitative analyses, the ratio of the peak area of free Cy5-HSA or the complex to the total peak area of Cy5-HSA and the complex can be employed advantageously for determining native HSA (immunoassay) or anti-HSA (binding assay) with no normalization. An absorption spectrophotometer, UV-2400PC (Shimadzu, Kyoto, Japan), was employed for the determination of the concentration of HSA labeled with Cy5, a fluorescent molecule. The electric conductivity was measured by an electric conductometer, CM-409 (TOA Electronics, Hyogo, Japan).

2.2. Chemicals

Sodium hydrogen phosphate, HSA (mouse ascites fluid), and phosphate-buffered saline (PBS) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Monoclonal anti-HSA (mouse ascites fluid) was obtained from Cedarlane laboratories (Ontario, Canada). Sodium dihydrogen phosphate was purchased from Kishida (Osaka, Japan). *N*-Cyclohexyl-3-aminopropanesulfonic acid (CAPS) was obtained from Dojindo (Kumamoto, Japan). A fluorescent labeling reagent, Cy5 monofunctional dye (Cy5), was obtained from Amersham Pharmacia Biotech (Tokyo, Japan). All solutions were prepared using deionized water prepared by means of an Elix-3 purification system (Millipore, Tokyo, Japan) and were filtered with a 0.20 μm

disposable membrane filter (DISMIC-25, Advantec Toyo Kaisha, Tokyo, Japan) to remove dust in the solutions.

2.3. Preparation of Cy5-HSA

The fluorescent Cy5-HSA conjugate was prepared by the reaction between HSA and Cy5. A pack of Cy5 (∼0.2 mg) was dissolved in 500 μL of anhydrous dimethylformamide. The labeling reaction was achieved by mixing the Cy5 solution and 50 mM carbonate buffer (pH 9.3) containing 1 mg mL^{-1} of HSA in a mass ratio of 4:1 followed by standing for 2 h. After the completion of the reaction, ultrafiltration was carried out (15 min, 6000 G) to remove the unreacted labeling reagent with an ultrafree-MC (Millipore: cut-off M_r, 10,000). The labeled HSA (Cy5-HSA) on the filter was recovered by adding 400 μL of CAPS buffer (pH 9.5) in a filter cup of the ultrafiltration tube followed by mixing for 1 min by a vortex mixer (Automatic Lab-mixer HM-10H, As One Corp., Osaka, Japan). The concentration of Cy5-HSA was determined from the absorbances using extinction coefficients at 649 nm (250,000 M^{-1} cm^{-1} [21]) and at 280 nm (370,000 M^{-1} cm^{-1} [25]). The Cy5-HSA solution was placed in a microtube and stored in a dark room at a temperature of 4 °C.

2.4. Binding assay for anti-HSA

The separation conditions determined in our previous study [14] were used as optimal conditions, in which the running buffer was 100 mM CAPS buffer (pH 9.5) and the applied voltage was 17 kV (total length of the capillary: 60 cm, effective length: 50 cm, electric field: 283 V cm^{-1}). Stock solutions of anti-HSA and Cy5-HSA were diluted by CAPS buffer that is the same as the migration buffer for CE run. In the off-column method, equal volumes of 2 mg mL^{-1} Cy5-HSA and anti-HSA solutions (1.0, 0.5, 0.1 or 0.05 mg mL^{-1}) were mixed and incubated for 15 min at room temperature. These solutions were injected for 15 s into the capillary. In the on-column method, 1 mg mL^{-1} Cy5-HSA was injected for 15 s, followed by the injection of anti-HSA solution with the concentration of 1.0, 0.5, 0.1, 0.05 or 0.025 mg mL^{-1} for 15, 30, 60, 90, 120, 180 or 240 s.

2.5. Stepwise reaction immunoassay

In the immunoassay of native HSA, the concentration of anti-HSA and Cy5-HSA should be optimized. Under the conditions where the concentration of Cy5-HSA was constant at 1 mg mL^{-1} and the concentration of anti-HSA was varied, the solutions were injected for 15 s using the same method as the on-column binding assay for anti-HSA. The equivalent concentration of anti-HSA to yield only a single peak for the complex in an electropherogram was determined to be 4 mg mL^{-1} (data not shown). Therefore, 4 mg mL^{-1} of anti-HSA was utilized in the on-column immunoassay using the stepwise reaction. The solutions containing several concentrations of native HSA (2.0, 1.0, 0.5, 0.1, 0.05, 0.025, 0.01, 0.025 and 0.005 mg mL^{-1}) were prepared. The Cy5-HSA and anti-HSA solutions were injected for 15 s, and native HSA solutions were injected for 30, 60, 90, 120, 180, 240 or 300 s. The separation conditions were same as in the binding assay.

2.6. Application of on-column binding assay for the analyte dissolved in PBS buffer

In the application of an on-column binding assay to the determination of anti-HSA dissolved in PBS buffer, a phosphate buffer solution (100 or 50 mM, pH 7.4) was used as a running buffer. The applied voltage was 10 kV (56 μA). The concentration of Cy5-HSA was 0.1 mg mL^{-1}, and the concentration of anti-HSA was 0.01 mg mL^{-1}. The Cy5-HSA solution was injected for 15 s and the injection time of 15, 60, 120 or 180 s was used for the anti-HSA solution.

3. Results and discussion

3.1. Principle of on-column binding assay and stepwise reaction immunoassay

Fig. 1 depicts the scheme of the binding assay for anti-HSA (A) and stepwise reaction immunoassay for native HSA (B) using an on-column immunological reaction. In the binding assay, labeled antigen and native antibody were introduced into a capillary in the form of individual plugs. In the case of HSA, which is an anionic molecule, anti-HSA is introduced after the injection of Cy5-HSA solution (Fig. 1A-1) because anti-HSA migrates faster than Cy5-HSA in the presence of the electrophoretic flow. After applying a high voltage, a reaction is initiated between Cy5-HSA and anti-HSA due to the mixing that occurs when the zones pass through each other, resulting in the formation of a complex (Cy5-HAS–anti-HSA) at the boundary between these solutions (Fig. 1A-2). The produced complex passes through the free Cy5-HSA zone (Fig. 1A-3), and two peaks corresponding to the complex and free Cy5-HSA are then observed in the electropherogram (Fig. 1A-4). A stepwise reaction immunoassay was performed by the same strategy as was used for the binding assay, except that a native HSA solution was introduced between the Cy5-HSA and anti-HSA solutions (Fig. 1B-1). In this case, reactions would be expected to occur in two steps. Initially, anti-HSA binds to native HSA, resulting in complex formation after applying a high voltage (Fig. 1B-2). The stepwise reaction subsequently occurs so as to form the complex of Cy5-HSA with residual anti-HSA (Fig. 1B-3). Ultimately, two peaks, corresponding to the complex of Cy5-HSA with anti-HSA and free Cy5-HSA, appear in the electropherogram, in which the ratio of the peak area between free Cy5-HSA and the complex is dependent on the amount of native HSA injected.

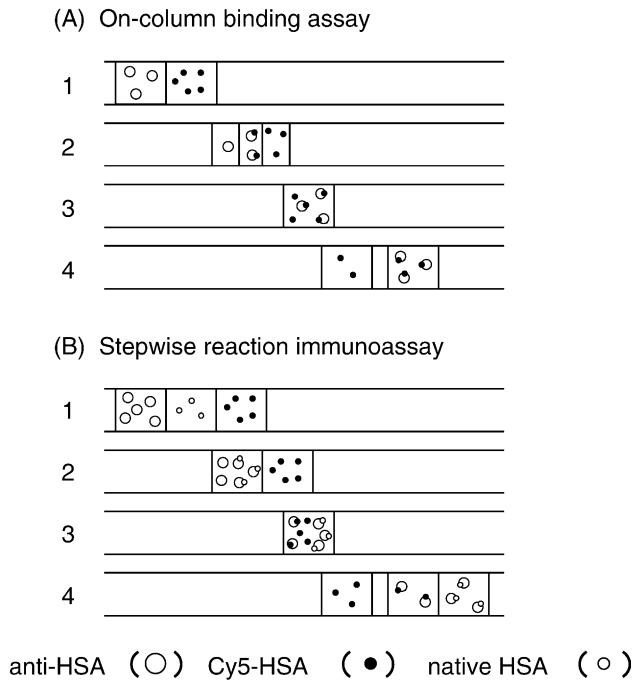

Fig. 1. Scheme for on-column immunological reaction capillary electrophoresis. Symbol: anti-HSA (○), Cy5-HSA (●) and native HSA (○). (A) On-column binding assay: (1) anti-HSA, which has a higher electrophoretic mobility, introduced into a capillary after the injection of Cy5-HSA; (2)–(3) under applied voltage, anti-HSA and Cy5-HSA react at the boundary between the two solutions; (4) complex and Cy5-HSA are separated. (B) Stepwise reaction immunoassay: (1) Cy5-HSA, native HSA, and anti-HSA are injected in the specific order indicated by the illustration; (2) initially, native HSA reacts with anti-HSA to produce a complex; (3) a portion of the Cy5-HSA binds to the antibody; (4) the complex, consisting of Cy5-HSA and anti-HSA, and free Cy5-HSA are separated.

3.2. On-column binding assay for anti-HSA

To demonstrate the on-column immunological reaction, 1.0, 0.5, 0.1 or 0.05 mg mL^{-1} of anti-HSA was injected for 15 s after injecting 1 mg mL^{-1} of Cy5-HSA for 15 s, according to the method proposed in Fig. 1A. Using the on-column immunological reaction, the immunocomplex between anti-HSA and Cy5-HSA was found to be formed during CE run and was clearly separated from free Cy5-HSA. The resolution was similar to that of the off-column method using injection time of 15 s, in which the reaction is completed in a microtube prior to the CE run.

The ratio of the peak area for the complex against the sum of the peak areas for Cy5-HSA and the complex was calculated for the determination of anti-HSA. The peak ratios obtained by the both off-column and on-column methods increase in proportion to increasing concentrations of anti-HSA (correlation coefficient, R^2, on-column method; $R^2 = 0.994$ and off-column method; $R^2 = 0.998$). Consequently, the on-column method can be successfully applied to the binding assay of anti-HSA, and the time required for the analysis was only 10 min including the immunological reaction. Unfortunately, yield of the complex in the on-column method was slightly smaller than in the off-column method as indicated by the slopes of the calibration carves (the ratio of complex decreases to 80%) under the conditions where the same amounts of anti-HSA and Cy5-HSA molecules were injected into the capillary in both the off-column and on-column methods. This should be caused by the incomplete reaction of anti-HSA and Cy5-HSA due to the short reaction time which is a result of the migration time of the species involved, i.e., shorter than 10 min. However, it should also be noted that the concentration of anti-HSA in the sample solution injected into the capillary in the off-column method is the same as that in the on-column method in this experiment. Therefore, the concentration of the anti-HSA in the analyte solution is lower in the on-column method than that in the off-column method, since the sample solution is prepared by mixing Cy5-HSA and anti-HSA solutions in the off-column method. The on-column method requires no pre-mixing, i.e., no dilution, while the concentration of the analyte becomes half in the off-column method when the analyte and probe solutions are mixed by the volume ratio of 1:1 as being carried out in the experiment. Consequently, the concentration sensitivity in the on-column method is higher than in the off-column method although the yield of the complex is 80%.

Furthermore, the injection time of the analyte (anti-HSA) was increased (15–240 s) using a constant injection time (15 s) of Cy5-HSA to improve the sensitivity of the on-column method. It is essential to eliminate stacking effects and unexpected band broadening due to the difference in electric conductivity between running and sample buffer solutions in order to clarify the mechanism of the on-column reaction. Therefore, CAPS buffer was used for dissolving anti-HSA and Cy5-HSA in this study, although 100 mM phosphate buffer was used in previous work [14]. In the preliminary study, it has been verified that reactivity between Cy5-HSA and anti-HSA in CAPS buffer (pH 9.5) was similar to that in phosphate buffer (pH 7.4). The electropherograms for the injection time of 60, 120, 180 and 240 s are shown in Fig. 2. The ratio of the peak area of the Cy5-HSA complex to the total peak area of Cy5-HSA increases proportionally with increasing injection time of the anti-HSA for all concentrations examined (correlation coefficient,

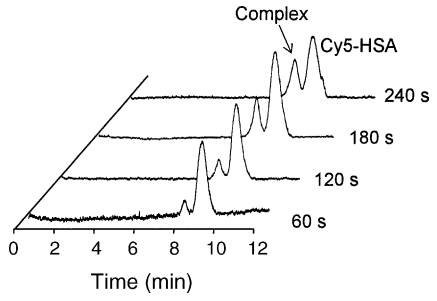

Fig. 2. Electropherograms showing the separation of an immunocomplex from Cy5-HSA in an on-column binding assay. Injection time of anti-HSA for each electropherogram is shown in the figure. The concentrations were 0.1 mg mL^{-1} for anti-HSA, and 1.0 mg mL^{-1} for Cy5-HSA.

R^2, the anti-HSA concentration of 0.5 mg mL^{-1}; R^2 = 0.997, 0.1 mg mL^{-1}; R^2 = 0.992, 0.05 mg mL^{-1}; R^2 = 0.953, and 0.025 mg mL^{-1}; R^2 = 0.976). It should be noted that the peak area of free Cy5-HSA also decreases with increasing the injection time of anti-HSA. In all cases, the relative standard deviation for each data point was less than 8%.

A linear relationship between the concentration of anti-HSA and the ratio of the peak area was observed up to 240 s of injection time of anti-HSA (correlation coefficient, R^2, the injection time of 15 s; R^2 = 0.994, 30 s; R^2 = 0.982, 60 s; R^2 = 0.998, 90 s; R^2 = 0.998, 120 s; R^2 = 0.970; 180 s, R^2 = 0.970, and 240 s; R^2 = 0.965). The detection limit of anti-HSA was improved to 0.007 mg mL^{-1} (S/N = 3 for the complex) by using 240 s of the injection time, which is eight-fold more sensitive than that obtained using the off-column method. In Fig. 2, the migration times of the components seems to be constant even when the injection time increases. Using the Poiseuille equation, a 240 s injection was calculated to correspond to 36 nL or 3.1% of the total column volume. Therefore, no significant change in migration time was observed even when the injection time was 240 s.

In the off-column method, the ratio of the complex to the total Cy5-HSA is independent of the injection time. Furthermore, the increase in injection time degrades the resolution due to band broadening. However, in the on-column method, the peak width of Cy5-HSA remains constant, because the injection volume of Cy5-HSA is constant. In addition, as shown in Fig. 2, the peak width of the complex is narrower than that expected from the increased injection time of anti-HSA. With the brief reaction time allowed, the kinetics of binding reaction may be too slow to achieve equilibrium in the binding reaction. Consequently, the zone length of the complex is similar to that of Cy5-HSA as shown in Fig. 2. The complex/free resolution was adequate up to an injection time of 240 s.

3.3. Stepwise reaction immunoassay

In a stepwise reaction immunoassay of native HSA, three species (native antigen, labeled antigen and antibody) are injected into the capillary independently. Hence, the effects of the order of injecting the solutions were investigated. Fig. 3A and B show two electropherograms, obtained using different injection orders of labeled and native HSAs. In Fig. 3A, Cy5-HSA (1 mg mL^{-1}) was injected before the injection of native HSA (2 mg mL^{-1}). On the other hand, in Fig. 3B, native HSA was initially injected. The injection time of native HSA was 15 s for both runs. The peak areas corresponding to free Cy5-HSA and the complex are affected by the injection sequence of Cy5-HSA and native HSA. The peak area of free Cy5-HSA in Fig. 3A is apparently larger than that in Fig. 3B. The effect of the order of the addition of reagents or sample is common in competitive immunoassays that operate before reaching an equilibrium in the binding systems with slow dissociation kinetics. In the stepwise reaction immunoassay, the reaction is not completely at equilibrium because of the brief reaction

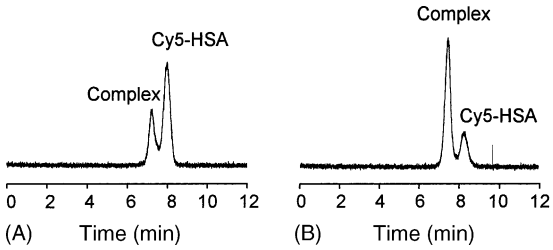

Fig. 3. Electropherograms obtained by stepwise reaction immunoassay. The injection order: (A) Cy5-HSA, native HSA, anti-HSA; (B) native HSA, Cy5-HSA, anti-HSA. The concentrations are 1 mg mL^{-1} for Cy5-HSA, 2 mg mL^{-1} for native HSA, and 4 mg mL^{-1} for anti-HSA.

time. The results of Fig. 3 indicate that no exchange reaction between HSA bound with anti-HSA and free HSA takes place during the stepwise reaction. Namely, HSA injected adjacent to the anti-HSA zone dominates complex formation. A competitive immunoassay is based on the amount of free Cy5-HSA, which cannot react with anti-HSA. Thus, the conditions in Fig. 3A are more advantageous in the immunoassay of native HSA, since the peak area of free Cy5-HSA is larger at a constant amount of native HSA. Therefore, all runs were performed using the injection order shown in Fig. 3A.

Fig. 4A shows electropherograms obtained by introducing native HSA, in which only the injection time for native HSA was varied (injection times; 90, 120, 180, 240, 300 s, the concentrations of solutions injected independently; 4 mg mL^{-1} anti-HSA, 1 mg mL^{-1} Cy5-HSA, and 0.025 mg mL^{-1} native HSA). The electropherogram obtained by the off-column method using an injection time of 240 s is also shown in Fig. 4B (the concentration of the sample; 2 mg mL^{-1} anti-HSA, 1 mg mL^{-1} Cy5-HSA, and 0.1 mg mL^{-1} native HSA). The different concentrations of Cy5-HSA and anti-HSA are used for the on-column and off-column methods because the optimum conditions for the on-column method are different in those for the off-column method. As shown in Fig. 4A, even in the immunoassay of native HSA, the improvement in sensitivity, which is determined by the peak area of free Cy5-HSA, is attained by increasing the injection time of native HSA. The peak area of free Cy5-HSA increases with increasing the injection time of native HSA in the stepwise reaction immunoassay. Even when injecting native HSA for 300 s, Cy5-HSA was clearly separated from the complex. On the other hand, the peak width in the off-column method is expanded and free Cy5-HSA is not separated from the complex. In addition, it should be noted that the concentration of 0.025 mg mL^{-1} is lower than the detection limit of the off-column method determined in this study (0.07 mg mL^{-1}). Thus, significant improvement in the sensitivity is observed as shown in Fig. 4A.

Interestingly, no significant degradation in resolution was observed in the electropherograms of Fig. 4A at least up to 300 s of the injection time, i.e., the peak widths of free Cy5-HSA and the complex are independent of the injection time of native HSA. In the step-

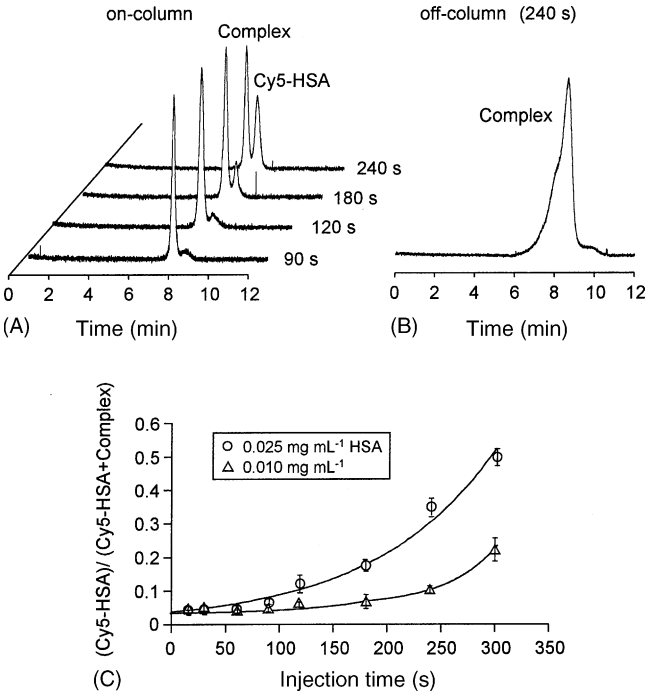

Fig. 4. Electropherograms showing the separation of an immunocomplex from Cy5-HSA and relationship between injection time and the ratio of the peak area of Cy5-HSA with different concentrations of native HSA. (A) On-column (stepwise reaction) method: the concentrations of the solutions injected into the capillary independently; 4.0 mg mL^{-1} for anti-HSA, 1.0 mg mL^{-1} for Cy5-HSA, and 0.025 mg mL^{-1} for native HSA. Injection time of native HSA for each electropherogram is given in the figure. (B) Off-column method: the concentration of a sample; 2.0 mg mL^{-1} for anti-HSA, 1.0 mg mL^{-1} for Cy5-HSA, and 0.1 mg mL^{-1} for native HSA. (C) Relationship between injection time and the ratio of the peak area of Cy5-HSA in a stepwise reaction immunoassay with different concentrations of native HSA. ◯: 0.025 mg mL^{-1}, △: 0.010 mg mL^{-1}. Other conditions are described in the text.

wise reaction immunoassay, both anti-HSA and Cy5-HSA are injected as short plugs (15 s injection times). Therefore, the reaction time for the complex formation of Cy5-HSA with anti-HSA is shorter in the stepwise reaction immunoassay than in the binding assay, independent of the injection time of the analyte (native HSA). Consequently, no degradation in resolution was observed even for the injection time of 300 s in the stepwise reaction immunoassay. Furthermore, no displacement of native HSA by Cy5-HSA seems to occur after formation of the native HSA complex. This phenomenon is also supported by the results of Fig. 3, in which the peak areas of free Cy5-HSA and the complex depend on the injection sequence of Cy5-HSA and native HSA. This means that the yield of the immunocomplex of native HSA in on-column reaction is greater than that in off-column reaction (equilibrated condition) because of no competitive reaction. Fig. 4C shows the relationship between the injection time for different concentrations, 0.010 and 0.025 mg mL^{-1}, of native HSA solutions and the ratio of free Cy5-HSA to the total area of Cy5-HSA and the complex. These results also show that the ratio of the peak area of free Cy5-HSA increases depending on the injection time of native HSA.

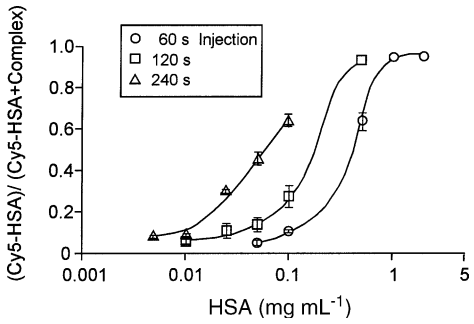

Fig. 5. Calibration curves for the stepwise reaction immunoassay. Injection time: 60, 120, and 240 s. Conditions are described in the text.

Fig. 5 shows calibration curves for the stepwise reaction immunoassay using injection times of 60, 120, and 240 s for native HSA. Sigmoidal curves are obtained in the stepwise reaction immunoassay as well as in a general competitive immunoassay. The sigmoidal curve shifts to a low concentration range with increasing injection time of native HSA. The detection limit, which is estimated from the calibration curve according to the method reported by Hayashi et al. [26], was 0.005 mg mL^{-1} for injection time of 300 s for native HSA. This value is 14 times lower than that of the off-column competitive immunoassay (0.07 mg mL^{-1}), and, surprisingly, it is better than that of the binding assay for anti-HSA.

3.4. Effect of salts contained in a sample

A sample used in clinical chemistry, such as serum and urine, contains a lot of salts, and antibodies are typically stored in PBS buffer. Therefore, to demonstrate applicability of the on-column immunological reaction to a sample containing a large amount of salts, Cy5-HSA and anti-HSA were dissolved in PBS buffer containing 137 mM sodium chloride. A phosphate buffer solution (100 mM, pH 7.4) was chosen as a running buffer. The conductivity of 100 mM phosphate buffer is similar to that of PBS (electric conductivity; 100 mM phosphate buffer, 11.81 mS/cm, PBS, 15.58 mS/cm). Fig. 6 shows the electropherograms at various injection times of anti-HSA in the binding assay. No distortion of the peaks was observed in these electropherograms, so that the present method would be applicable to selective determination of a protein in a practical sample. The relationship between the injection time and the ratio of the complex is linear (correlation coefficient, $R^2 = 0.990$). In addition, 50 mM phosphate buffer was used as the running buffer to evaluate the effect of the buffer concentration under the condition of 240 s injection. No change in resolution and reactivity of antigen and antibody was found for 50 mM phosphate buffer. Therefore, no influence on the on-column reaction, stacking, and CE separation was found at least at 50–100 mM of the buffer concentration.

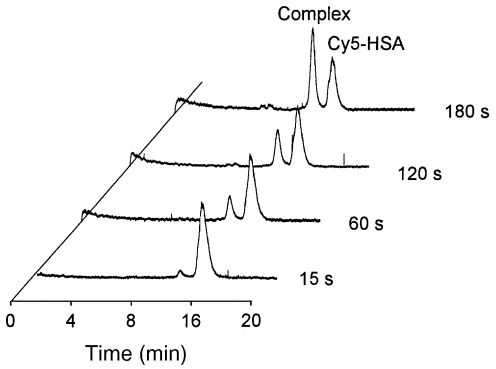

Fig. 6. Electropherograms showing the separation of an immunocomplex from Cy5-HSA in an on-column binding assay for anti-HSA dissolved in PBS buffer. The concentrations of Cy5-HSA and anti-HSA were 0.1 and 0.01 mg mL^{-1} in PBS, respectively.

4. Conclusions

An on-column immunological reaction method using CE-SLIF was investigated in a binding assay of anti-HSA and an immunoassay of native HSA. The developed method provides a rapid analysis in both the binding assay and immunoassay. Furthermore, by injecting a large volume of the sample, sensitivity is improved without band broadening. In a stepwise reaction immunoassay for native HSA, the detection limit for 300 s of injection time was 14 times more sensitive than that of the off-column method. In addition, the on-column immunological reaction is also applicable to quantitative analysis for a sample containing a large amount of salts. There are a variety of advantages in this method, i.e., expansion of the dynamic range, no sample pretreatment is needed, no dilution of the sample by mixing solutions, and saving of the sample, since the antigen reacts with the antibody in the capillary column.

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