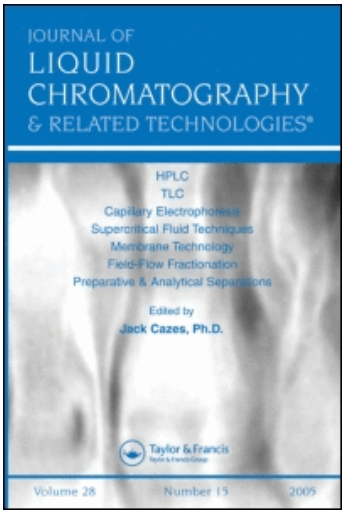

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SANDWICH IMMUNOASSAY FOR MONOCLONAL ANTIBODY USING PROTEIN G IMMUNOAFFINITY CAPILLARY CHROMATOGRAPHY AND DIODE LASER INDUCED FLUORESCENCE DETECTION

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ABSTRACT

A sandwich immunoassay for monoclonal antibody using protein G immunoaffinity capillary chromatography and LIF detection was described. Monoclonal anti-bovine serum albumin (BSA) was used as a model to test the performance of the system. Cy5 was used as the fluorescent tag and the 635 nm line of a diode laser was used as the excited source. Capillaries with inner diameter of 150 μm were packed with recombinant protein G bound perfusive support and used to capture the immunocomplexes, which were subsequently desorbed by an acidic buffer and then detected using LIF. The results demonstrated the feasibility to perform sandwich immunoassay using this system.

INTRODUCTION

With the movement toward automation in immunoassays, methods that use immunoaffinity chromatography (IAC) are becoming increasingly popular.[1-2] In a typical IAC method, an antibody or an antibody binding protein, such as protein A or protein G, is immobilized onto a small, rigid support and placed into a column. When the sample is applied to this column, the analyte will be captured while other components will elute nonretained. Subsequent to capture, the mobile phase conditions are changed to desorb the analyte from the column for quantification or collection for further use. The column is then regenerated by going back to the initial mobile phase conditions, and the process is repeated. These IAC methods can provide both selectivity and sensitivity that are competitive with any other immunoassay method currently available; meanwhile, they are usually fast and compatible with full automation.

Many kinds of IAC methods have been developed to meet different needs. The simplest format is the direct detection of the analyte after it is released from the IAC column.[3-5] The advantages of this format include relative simplicity and good precision, but it usually encounters the problem of solvent interference when UV absorbance detection is used. To solve this problem, Regnier et al. have developed a dual-column system which consist of an immunoaffinity column coupled to an analytical column via a switching valve.[6-9] However, the instrumentation of such dual-column immunoassays is rather complicated. Other solutions involve either competitive binding or sandwich immunoassays using immunological reagents labeled with enzymes or fluorophores. De Alwis and Wilson have used enzyme tags that generated an electroactive product.[10-11] Regnier et al. have demonstrated high performance protein G immunoaffinity chromatographic immunoassay with fluorescence detection. Texas Red labeled human growth hormone was used as a fluorescence probe for detecting antibodies against human growth hormone.[12] Hage and Kao have described an automated IAC method for parathyroid hormone using chemiluminescent detection.[13]

In a previous report, we described a competitive assay for BSA using protein G immunoaffinity capillary chromatography and Laser induced fluorescence (LIF) detection.[14] LIF is one of the most sensitive detection modes in capillary based separation techniques because of its low background signal.[15-16] However, there are also some problems associated with LIF detection. First, almost all lasers are difficult to use due to their high cost and complex maintenance. Second, most biological fluids are strongly luminescent when excited by the laser in the blue or green region of the spectrum. Due to the small number of compounds that demonstrate intrinsic fluorescence above 600 nm, the use of near-infrared fluorescence detection in biological fluids is a desirable alternative to visible fluorescence detection. This fact, as well as the availability of cheap diode lasers for this spectral region, has prompted current efforts to use

near-infrared dyes for biological applications.[17-21] Cy5 is an activated cyanine dye that can be readily coupled with peptides and proteins. It contains two broad absorption maxima at 630 and 655 nm, and can be excited by the 652 nm line of a diode laser or the 632.8 nm line of a red Helium-Neon laser, respectively. The fluorescence emission maximum of Cy5 in aqueous solution is at 670 nm.[22-23]

There is, now, a growing need for efficient analytical procedures for monoclonal antibodies since the use of monoclonal antibodies has expanded rapidly with the development of hybridoma technology in the late 1970s. In this paper, we describe a sandwich immunoassay for monoclonal antibody using protein G immunoaffinity capillary chromatography and LIF detection. Cy5 was used as the fluorescent tag and the 635 nm line of a diode laser was used as the excited source. The results demonstrated the feasibility of performing sandwich immunoassay using this system.

EXPERIMENTAL

Chemicals and Reagents

Monoclonal anti-BSA (mouse IgG_{2a}, 4.7 mg/mL or 32 μM assuming the molecular mass of IgG_{2a} is 146,000) were from Sigma (St. Louis, MO, USA). It was in mouse ascites fluid, and the total protein concentration was 22 mg/mL. BSA, glycine, and sodium phosphate, were also from Sigma. Urine, from a healthy man, was collected and used to prepare control samples. All solutions were prepared with deionized water obtained from a Milli-Q plus system (Millipore, Bedford, MA, USA), filtered through 0.45 μm filter, and degassed with vacuum for 20 min before use.

BSA was labeled with Cy5 using Cy5 conjugation Kit from Amersham (Pittsburgh, USA). The labeled protein was purified with column chromatography (Sephadex G-25M), and the elution buffer was 10 mM phosphate-buffered saline (PBS, 10 mM sodium phosphate, 138 mM NaCl, 27 mM KCl, pH 7.4). The purified fraction was stored at 4°C until use. The dye/protein (D/P) ratio and the final concentration were estimated to be 3.5:1 and 0.11 mg/mL, according to absorbance measurements at 280 nm and 650 nm, respectively. The corresponding molar concentration was 1.64 μM, assuming that the molecular mass of BSA is 67,000. BSA was labeled with FITC as described previously.[14]

Apparatus

All experiments were performed on an automated P/ACE Model 5510 capillary electrophoresis system (Beckman Fullerton, CA, USA) fitted with a LIF

detector. The 635 nm line of a 5 mW diode laser was utilized as the excitation source, and the emitted fluorescence was collected at 670 nm. The instrument can supply a 138 kPa pressure rinse or separation. System control, data acquisition and analysis were accomplished with P/ACE Workstation software for Windows 95 using an IBM 586 personal computer. The fluorescence was measured with a Model LS-50B fluorometer (Perkin-Elmer, USA). Polyimide-coated fused silica capillary columns (150 μm I.D., 360 μm O.D.) were from Polymicro Technologies (Phoenix, AZ, USA). The chromatographic packing material was POROS 20G (PerSeptive Biosystems, Cambrige, MA, USA). The packing method was the same as described previously.[14]

Immunoassay Protocol

Standards of anti-BSA were made up in 10 mM PBS to give final concentrations of 0, 0.4, 0.8, 2, 4, 8, 16 nM. Control samples were prepared by adding anti-BSA to urine. Cy5-BSA solution was diluted with 10 mM PBS to 16.4 nM. To perform an assay, 20 μL of anti-BSA standards or control samples were mixed with 20 μL of the diluted Cy5-BSA solution in a 0.5 mL eppendroff tube and incubated at room temperature (23°C) for 20 min before injection.

Protein G Immunoaffinity Capillary Chomatography Procedure

A schematic diagram of the principle of the assay is shown in Figure 1. The pressure separation function provided by the CE instrument was used for sample loading and elution. The loading buffer consisted of 50 mM sodium phosphate and 50 mM potassium sulfate (pH 7.0). Protein G column was first equilibrated with the loading buffer for 10 min, and the sample was then loaded onto the column for 5 min. Unbound Cy5-BSA was removed by rinsing the capillary with the loading buffer for 20 min. The bound Cy5-BSA was then desorbed from the column with an acidic buffer (100 mM glycine, 20% acetic acid, pH. 2.5) and detected with LIF.

RESULTS AND DISCUSSION

Evaluation of Cy5-BSA as the Fluorescence Probe

Figure 2 showed the effect of pH on the fluorescence identity of Cy5-BSA and FITC-BSA. It could be seen that the fluorescence of Cy5-BSA was quite insensitive to pH between 1 and 10, which was different from that of FITC-BSA. This property of Cy5 labeled immunoreagent made it more suitable to be used in IAC methods where acidic elution was very commonly employed.. The fluorescence spectra of Cy5-BSA in acidic and basic pH was shown in Figure 3. No wavelength shift could be found in the absorption and fluorescence emission maxima of Cy5-BSA over this pH range.

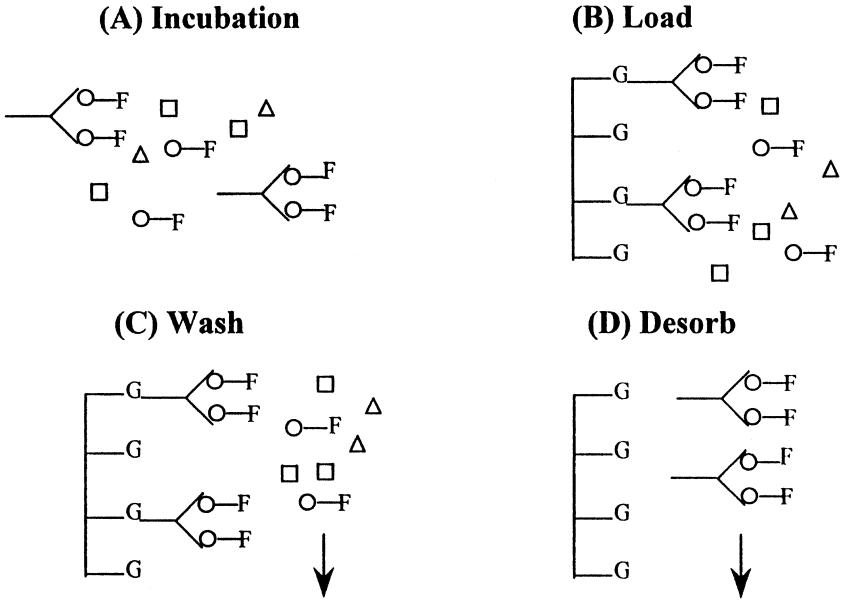

Figure 1. Diagram of protein G immunoaffinity capillary chromatographic steps.

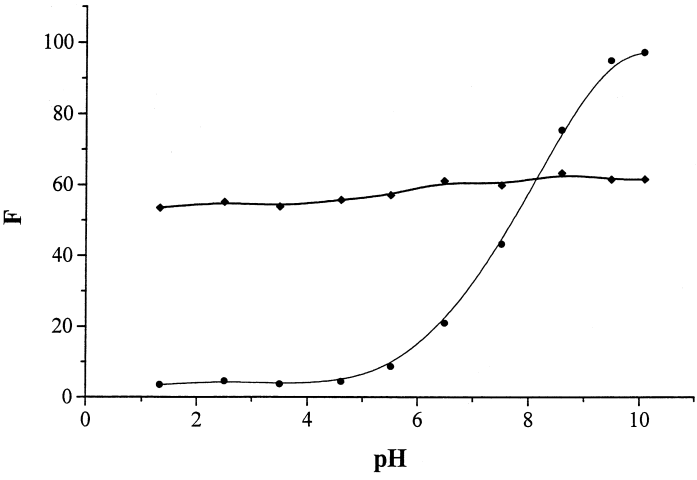

Figure 2. Effect of pH on the fluorescence identity of Cy5-BSA (♦) and FITC-BSA (•).

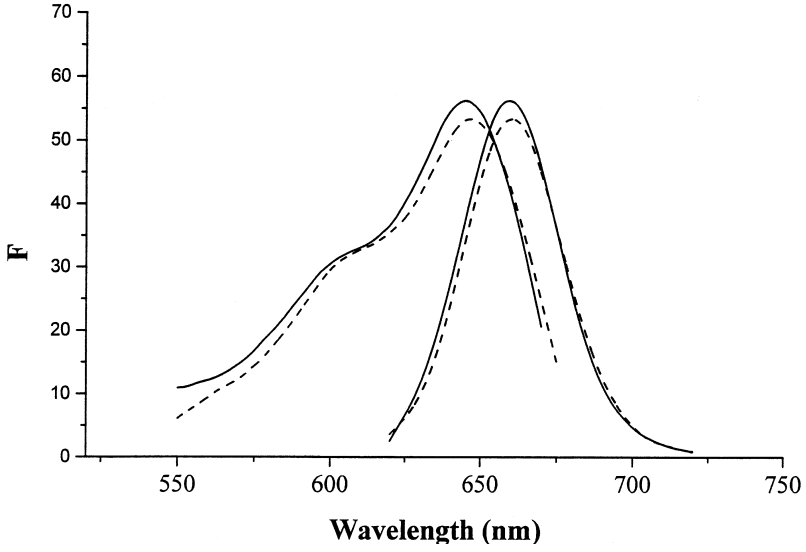

Figure 3. Absorption and emission spectra of Cy5-BSA in pH 2.5 (dash line) and pH 10.1 (solid line).

Optimization

In order to ensure that all the complexes injected were captured by the protein G column, two procedures were undertaken. First, the total amount of anti-BSA injected never exceeded 1 μg, which was less than one-twentieth of the estimated immunoglobulin binding capacity of the protein G column. Second, a slow flow rate was used to allow the binding of the immunoglobulin onto the column. When the 138 kPa pressure separation function of the instrument was used for sample loading, the linear velocity was calculated to be 9.5 cm/min, and the flow rate was 1.6 μL/min. The chromatographic support used in this study is a perfusion type particle with large diameter that allows rapid sample loading. Similar protein G columns captured all the injected antibodies without loss even when the flow rate was 10 μL/min.[24]

Quantification of Anti-BSA

Figure 4 showed typical chromatograms of the assay. The first flat peak was unbound Cy5-BSA, and the second peak was immunocomplexes. With the increase of anti-BSA in the sample, the first peak was decreased and the second peak was increased. This was due to the fact that anti-BSA was success-

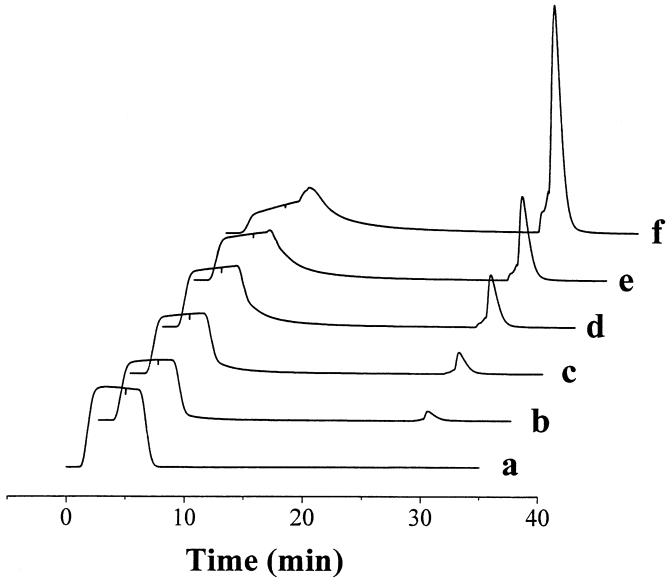

Figure 4. Typical chromatograms of Protein G immunoaffinity separation of free and anti-BSA bound Cy5-BSA. The concentration of Cy5-BSA was 16.4 nM and the concentrations of anti-BSA were: a) 0 nM, b) 0.4 nM, c) 0.8 nM, d) 1.6 nM, e) 2 nM, f) 4 nM.

fully binding with Cy5-BSA, and thus made more Cy5-BSA in complexes form. The calibration curve for anti-BSA was established by plotting the area of the immunocomplexes against concentration of anti-BSA (Figure 5). Each point represented the average of three consecutive runs, and the R.S.D. for the area was less than 4.4%. The dynamic linear range for the calibration curve was from 0.4-8 nM (r^2 = 0.999, slope = 2.82 L/nmol, intercept = -0.89). At higher concentration of anti-BSA, the curve gradually plateau'd off, as would be expected if all the available binding sites on the Cy5-BSA were saturated. The sample without anti-BSA (negative control) was analyzed five times to determine the mean and standard deviation of the blank peak area, and the concentration LOD was estimated to be about 0.2 nM (S/N=3). The injection volume was 8 μL considering the flow rate was 1.6 μL/min, and thus the mass LOD was about 1.6 fmol.

In the elution profile of the negative control, there was still a small background peak that was eluted with the acidic glycine buffer. This was probably due to nonspecific binding of Cy5-BSA to the protein G column since the amount of Cy5-BSA used was in excess. Although this background peak was less than 0.6% of the amount of Cy5-BSA injected, it dictated the LOD of the

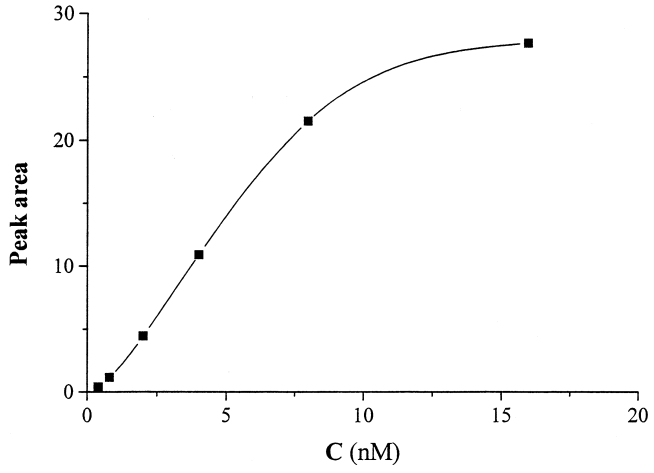

Figure 5. Calibration curve for anti-BSA.

method. The nonspecific binding can be minimized in two ways. First is by using longer washing time or high flow rate. Second is by rinsing the protein G column with sample loading buffer containing other proteins prior to the sample loading to saturate the nonspecific binding site of the column for Cy5-BSA.

In order to determine both the recovery and the precision of the assay, BSA was added to urine at three concentrations. The results were shown in Table 1. The recovery was between 94-115% and precision < 6.8% R.S.D.

Table 1

Recovery and Precision of Anti-BSA Quantification

Conc. of Anti-BSA Added (nM)	% of Anti-BSA Found	% R.S.D. (n = 5)
0.6	115	6.8
4.8	94	3.2
7.2	108	4.5

CONCLUSION

Protein G immunoaffinity capillary chromatography and LIF detection was successfully combined to perform sandwich immunoassay for monoclonal antibody. The system was compatible with automation, and only a few microliters of sample was required for the assay.

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