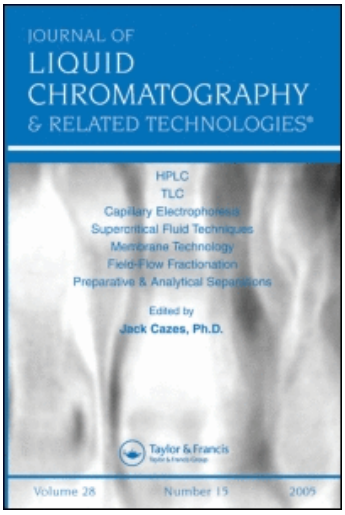

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COMPETITIVE IMMUNOASSAY USING PROTEIN G AFFINITY CAPILLARY CHROMATOGRAPHY WITH LASER INDUCED FLUORESCENCE DETECTION

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ABSTRACT

A simple approach to perform competitive immunoassay using protein G affinity capillary chromatography and laser induced fluorescence (LIF) detection was described. Bovine serum albumin (BSA) was used as a model to test the performance of the system. The assay used fluorescein isothiocyanate (FITC) labeled BSA as a tracer and an anti-BSA monoclonal antibody as the immunoreagent. 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 100 mM glycine (pH 9.0) and then detected using LIF. The results demonstrated the feasibility to perform competitive immunoassay using this system with high sensitivity and small sample size.

INTRODUCTION

Immunoassay can specifically and quantitatively determine minute amounts of analytes in complex biological matrices. Therefore, it is a powerful research tool as well as the method of choice for a wide variety of clinical, environmental and pharmaceutical analyses.[1] The primary drawback of common immunoassay formats is that they are time consuming and labor intensive. Such assays are usually performed in batches to achieve high sample throughout. However, this is not desirable when rapid feedback is required or for totally automated immunoassays.

One way to circumvent the above problem is to combine immunoassay with flow-based techniques.[2-8] In such flow systems, an antibody or an antibody binding protein, such as protein A or protein G, is immobilized in a column or cartridge that captures either a protein analyte or an antibody as it passed through the system. Some degree of analyte purification is generally associated with this capture step. Subsequent to capture, analyte is released directly to a detector [2] or transferred to a chromatography column for further separation and detection.[3] Kinetic and thermodynamic aspects of these interactions and the contribution of nonspecific interactions have also been studied.[4] Such flow-through immunological assays often encounter the problem of solvent interference with detection when UV absorbance detection mode is used.

To solve this problem, Regnier et al. have developed a dual-column system which consists of an affinity column coupled to an analytical column via a switching valve.[3, 5-7] Although compatible with automation, the instrumentation of such dual-column immunoassays are 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.[8-9] Riggin and Regnier have demonstrated high performance protein G affinity chromatographic immunoassay with fluorescence detection. Texas Red labeled human growth hormone was used as a fluorescence probe for detecting antibodies against human growth hormone.[10] Reinecke et al. have established a fast online flow injection analysis system for IgG monitoring in bioprocesses, and the IgG was detected without interference from other sample components by protein fluorescence.[11]

With the development of capillary electrophoresis, LIF detection technique has been well studied.[12-14] LIF is one of the most sensitive detection modes available for capillary-based separation presently.

In this paper, we describe a simple approach to perform immunoassay using protein G affinity capillary chromatography with LIF detection. A competitive assay for BSA was used to test the performance of the system.

EXPERIMENTAL

Apparatus

All experiments were performed with an automated P/ACE Model 5010 capillary electrophoresis system (Beckman Fullerton, CA, USA) fitted with a LIF detector or a UV detector. The 488 nm line of a 5 mW argon ion laser was utilized as the excitation source of LIF detection, and the emitted fluorescence was collected at 520 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 using an IBM 586 personal computer. Polyimide-coated fused silica capillary columns (150 μm I.D., 360 μm O.D.) were from Polymicro Technologies (Phoenix, AZ, USA).

Chemicals and Reagents

Monoclonal anti-BSA (mouse IgG_{2a}, 4.7 mg/mL or 32 μM assuming the molecular weight of IgG_{2a} is 146,000) was 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. Other chemicals were of analytical grade. Urine from a healthy man was collected and used to prepare control samples. All buffers were prepared with deionized water obtained from a Milli-Q plus system (Millipore Corp., Bedford, MA, USA), filtered through 0.45 μm filter, and degassed with vacuum for 20 min. before use.

BSA was labeled with FITC using FluoroTag FITC conjugation Kit from Sigma. 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 fluorescence/protein (F/P) ratio and the final concentration of FITC-BSA was estimated to be 2.8:1 and 0.43 mg/mL according to its absorbance at 280 nm and 495 nm. The corresponding molar concentration was 6.4 μM, assuming that the molecular weight of BSA is 67,000.

Preparation of Protein G Affinity Capillary Column

The chromatographic packing material was POROS 20G (PerSeptive Biosystems, Cambridge, MA, USA). This was a perfusive support made of beads with a diameter of 20 mm. Recombinant protein G was covalently bound onto the beads. The capillary with 150 μm I.D. was cut to a length of 20 cm, and installed with a frit by packing 1-2 mm of 10 μm spherical silica particles at

one end of the capillary under low pressure. The particles were then sintered in place by heating this section of the capillary in a flame for about 10 s. A water pressure of about 1 MPa was applied to the capillary with a syringe to test the stability of the frit.

A slurry of the POROS 20 G particles was packed into the column using a bomb and a HPLC pump with a pressure of 6.9 MPa for over 15 min. The slurry was prepared by mixing the POROS beads with deionized water in a ratio of 1:80 (g/mL). The length of the packed bed was about 15 cm.

The capillary was then cut to a length of 10 cm from the end with the frit. A second frit was made at the end of another 150 μm I.D. blank capillary with a length of 17 cm. The end of the packed capillary without the frit was connected to the end of the blank capillary with the frit by a thin PTFE tubing. A detection window was made on the blank column by burning the outer coating of the capillary at a distance of 20 cm from the inlet. The connected capillaries were then fixed into the P/ACE capillary cartridge, and mounted on the CE instrument for analysis.

Immunoassay Protocol

To construct the calibration curve, standards of BSA were made up in 10 mM PBS to give final concentrations of 0, 1, 2, 5, 10, 20, 50, 100, 200, 1000 nM. Control samples were prepared by adding BSA to urine. FITC-BSA and anti-BSA solutions were diluted with 10 mM PBS to 82 nM and 40 nM respectively. These diluted solutions were used as stock solutions in the following experiments.

Standard and control samples were prepared by mixing 40 μL of BSA standards or controls, 20 μL of FITC-BSA and 20 μL of anti-BSA from their respective stock solutions in a 0.5 mL eppendroff tube and incubated at room temperature (23°C) for 20 min before injection.

Protein G Affinity Capillary Chromatography Procedure

The pressure separation function provided by the CE instrument was used for sample loading and elution. The sample loading buffer consisted of 50 mM sodium phosphate and 50 mM potassium sulfate (pH 7.0). Protein G column was first equilibrated with the sample loading buffer for 10 min. The sample was loaded onto the column for 10 min. Unbound species were removed by rinsing the capillary with the sample loading buffer for 10 min. The bound FITC-BSA was then desorbed from the column with 100 mM glycine buffer (pH 9.0).

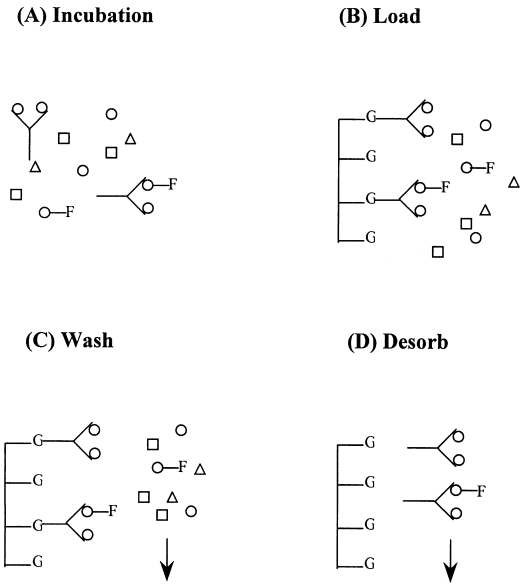

Figure 1. Diagram of Protein G affinity capillary chromatographic steps.

RESULTS AND DISCUSSION

Assay Format

The affinity chromatographic procedure used in this work for quantification of BSA involves: 1) Incubation of BSA standards or controls with FITC-BSA and anti-BSA solutions in room temperature, 2) Injection of this incubation mixture onto the protein G capillary column under neutral pH conditions, 3) Washing out the unbound species, 4) Desorbing the antigen-antibody complexes from the protein G capillary column by changing to a basic elutent, and 5) Quantification of the bound FITC-BSA by LIF detection. A schematic diagram of these affinity chromatographic steps is shown in Figure 1.

Optimization of Protein G Affinity Capillary Chromatography

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 1μg, 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

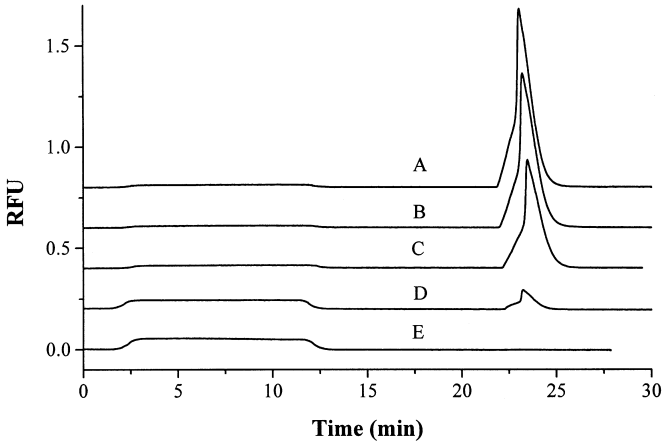

Figure 2. Protein G affinity separation of free and anti-BSA bound FITC-BSA. The concentrations of FITC-BSA and anti-BSA were 20 nM and 10 nM, respectively. The concentrations of BSA: (A) 0 nM, (B) 5 nM, (C) 20 nM, (D) 50 nM, (E) blank (no FITC-BSA added). Other conditions are described in Experimental.

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 a large diameter that allows rapid sample loading.

A similar protein G column captured all the injected antibodies without loss, even when the flow rate was 10 μL/min.[15]

Because the fluorescence of FITC is quenched at low pH conditions,[16] elution of the adsorbed antibody from the protein G column under acidic conditions can not be used in this study. Akerstrom and Bjorck reported that the binding between mouse monoclonal IgG and protein G was strongest at pH 4 and 5, and weakened with increased pH value.[17] Therefore, a series of 100 mM glycine buffers with pH value ranging from 8.0 to 9.0 were tested for their capability of desorbing the bound anti-BSA from the protein G column using UV detection at 280 nm.

The results showed that the bound anti-BSA was totally desorbed at pH 9.0. This was supported by two observations. First, the peak for desorbed zone always returned to baseline after 10 min elution with this glycine buffer. Second, subsequent elution with an acidic buffer (100 mM glycine, 20 % acetic acid, pH. 2.5) did not produce any detectable peak.

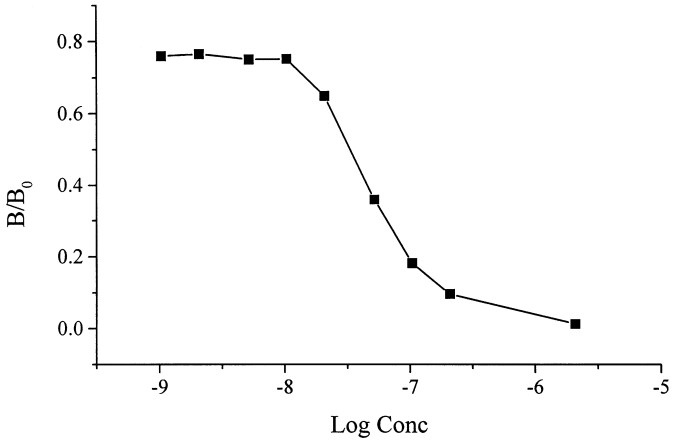

Figure 3. Calibration curve for BSA.

BSA Quantification

Figure 2 shows typical chromatograms of the assay. The first flat peak was unbound FITC-BSA, and the second peak was immunocomplexes. With the increase of unlabeled BSA in the sample, the first peak was increased and the second peak was decreased.

This was due to the unlabeled BSA successfully competing for the anti-BSA binding sites with FITC-BSA, and thus made more FITC-BSA unbound with anti-BSA.

The areas of the second peak in Fig. 2 were used to calculated B/B_0 and the calibration curve for BSA was established by plotting B/B_0 against logarithm concentration of unlabeled BSA (Figure 3). Each point represented the average three consecutive runs, and the R.S.D. for B/B_0 was less than 2.5 %. The curve showed a sigmoidal shape which was typical for competitive immunoassay. The sample without BSA (negative control) was analyzed five times to determine the mean and standard deviation of the blank peak area. The concentration LOD was estimated to be about 1.2 nM, which was the mean plus 3 times its standard deviation. The injection volume was 16 μL considering the flow rate was 1.6 μL/min, and thus the mass LOD was about 19 fmol.

The system was totally compatible with automation. Although each assay took about 30 min, this time can be easily shortened by increasing the flow rate of sample loading and washing.

Table 1

Recovery and Precision of BSA Quantification

Conc. of BSA Added (nM)	% of BSA Recovered	% R.S.D. (n = 5)
5	112	5
20	102	3
50	93	4

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 I. The recovery was between 93-112 % and precision < 5 % R.S.D. No serum samples were determined in this assay since the concentration of albumin in serum was too high. Further development of the assay on biological samples using other analytes would be performed in the following studies.

CONCLUSION

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

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