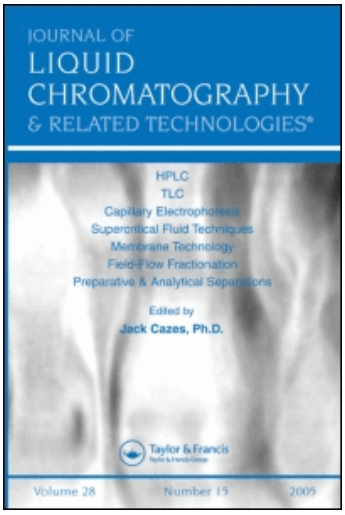

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ABSTRACT

The feasibility of performing the enzyme-amplified sandwich immunoassay with protein G capillary affinity chromatography and laser induced fluorescence (LIF) detection was investigated using the determination of human immunoglobulin G (hIgG) as a model system. The incubated mixture of samples containing hIgG and alkaline phosphatase (ALP) conjugated goat anti-human IgG $F_{(ab')2}$ fragment was loaded onto the capillary column packed with recombinant protein G bound perfusive support in neutral pH. After nonretained compounds were eluted, the fluorogenic ALP substrate, fluorescein diphosphate (FDP), was loaded onto the capillary column followed by stop-flow incubation.

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Finally, the product of ALP-mediated hydrolysis of FDP, fluorescein, was swept out of the capillary column and detected with a LIF detector using the 488 nm line of an argon ion laser as the excitation source. Chromatographic conditions were optimized. The calibration curve for hIgG was linear over the range of 0.5-50 pmol/L (r^2=0.999) with the limit of detection of 0.2 pmol/L.

INTRODUCTION

Immunoassays are the methods of choice for a wide variety of clinical, biochemical, pharmaceutical, and environmental analysis due to their ability to determine minute amounts of analytes in complex matrices quantitatively.[1] Of the various immunoassay formats available, one of the most sensitive is the sandwich immunoassay.[2]

In a typical sandwich immunoassay, two different types of antibodies that each binds to the analyte of interest are used. The first of these two antibodies is immobilized onto a solid support and is used to extract the analyte from samples. The second antibody contains an easily measured tag, such as radioisotopes, fluorescent compounds, and enzyme systems, which generate colored or electrochemically active products, and is added in solution to the analyte either before or after the extraction.

The fact that two types of antibodies are used in sandwich immunoassays tends to give this technique higher sensitivity and selectivity than competitive binding immunoassays. However, despite these advantages, the sandwich immunoassay still has some limitations. It generally requires long incubation time and many time consuming and irreproducible manipulations. It is usually carried out in antibody-coated plastic tubes, which easily suffers from irreproducible surface adsorption behavior. Moreover, the nature of the tube makes it almost impossible to totally automate the assay.

One way to circumvent the above problem is to combine the sandwich immunoassay with immunoaffinity chromatography (IAC).[2-12] 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.

There are two schemes for performing a sandwich immunoassay by IAC. In the first case, the sample and the labeled antibody are allow to incubate and

bind before injection onto an IAC column that contains immobilized antibodies for sample extraction. In the second case, the sample and the labeled antibody can be injected sequentially onto the IAC column. These IAC methods can provide both selectivity and sensitivity that are competitive with any other immunoassay methods currently available; meanwhile, they are usually fast and compatible with full automation.

In previous reports, we showed that the sandwich immunoassay could also be performed by capillary affinity chromatography.[11-12] The advantages of capillary-based sandwich immunoassay were more clearly demonstrated by Frutos[9] et al. They described an enzyme-amplified sandwich immunoassay for human IgG in fused-silica capillaries coated with protein G. By performing immunological complex before application to the column and stop-flow incubation of the complex in the column, the limit of detection (LOD) was reduced to 333 zmol, which was 3-4 orders of magnitude lower that that of conventional ELISA performed in a microtiter well.

The sensitivity of the enzyme-amplified sandwich immunoassay depends on the label enzyme and the substrate used. Alkaline phosphatase (ALP) is often chosen as a label enzyme because of its high enzymatic turnover rates over a variety of synthetic substrates. Fluorescein diphosphate (FDP, Fig. 1) is a fluorogenic ALP substrate which is colorless and nonfluorescent. Sequential ALP-mediated hydrolysis of the two phosphate groups yields weakly fluorescent fluorescein monophosphate followed by strongly fluorescent fluorescein. Huang[13] et al. have used FDP in an ALP-based ELISA for 2,4-dinitrophenol, finding that it provided a detection limit 50 times lower than that obtained with the chromogenic substrate, *p*-nitrophenyl phosphate.

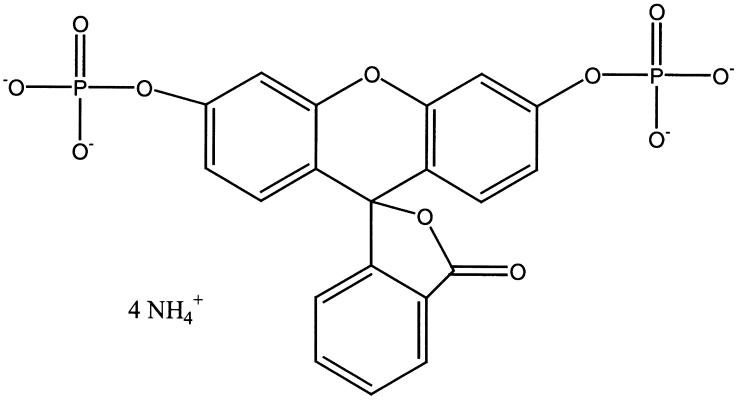

Figure 1. The structure of FDP.

In this paper, we investigated the feasibility of performing the enzyme-amplified sandwich immunoassay with protein G capillary affinity chromatography and LIF detection, using the determination of human immunoglobulin G (hIgG) as a model system. ALP conjugated goat anti-human IgG $F_{(ab')2}$ fragment was used as the immunoreagent and FDP was used as the fluorogenic ALP substrate. Chromatographic conditions were also optimized.

EXPERIMENTAL

Apparatus

All experiments were performed on an automated P/ACE Model 5500 capillary electrophoresis system (Beckman Fullerton, CA, USA) fitted with a LIF 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 for Windows 95 using an IBM 586 personal computer. Polyimide-coated fused sillica 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). This is a perfusive support made of beads with a diameter of 20 μm that has recombinant protein G covalently bound onto the beads. The capillary with 150 μm I.D. was cut to a length of 20 cm, and a frit was made by packing 1-2 mm of 10 μm spherical sillica 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 used to pack 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 blank capillary with the frit, using 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 into the CE instrument for analysis.

Chemicals and Reagents

Purified human immunoglobulin G (hIgG), ALP conjugated goat anti-human IgG $F_{(ab')2}$ fragment, bovine serum albumin (BSA), Tween 20, and Tris were from Sigma (St. Louis, MO, USA). Fluorescein diphosphate tetraammonium salt (FDP) was from Molecular Probes (Eugene, OR, USA). Other chemicals were of analytical grade.

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.

Immunoassay Protocol

Standards of hIgG were made up in buffer A (50 mmol/L Tris, 100 mmol/L NaCl, 0.1% BSA, 0.1% Tween 20, pH 7.4) to give final concentrations of 0, 0.5, 5, 25, 50 pmol/L. The blank serum was prepared by passing normal male serum through a 1 mL HiTrap protein G column from Pharmacia (Uppsala, Sweden) three times, with hIgG desorption and exhaustive washing between passes through the column. Control samples containing 1 and 10 pmol/L hIgG were prepared by adding appropriate amounts of hIgG to the blank serum.

The solution of ALP conjugated goat anti-human IgG $F_{(ab')2}$ fragment was 1/100,000 diluted with buffer A. To perform an assay, 50 μL of hIgG standards or control samples were mixed with 50 μL of diluted ALP conjugated goat anti-human IgG $F_{(ab')2}$ fragment solution in a 0.5 mL eppendroff tube, and incubated at room temperature (23°C) for 1 hr before injection.

Protein G Capillary Affinity Chromatography Procedure

The pressure separation function provided by the CE instrument was used for sample loading and elution with a flow rate of 1 μL/min. Protein G capillary column was first equilibrated with buffer A for 10 min. The incubated sample was loaded onto the column for 5 min, and the injection volume was about 5 μL. Unbound compounds were removed by rinsing the capillary column with buffer A for 10 min. After that, 5 μL of the substrate FDP (100 mmol/L in buffer B (50 mmol/L Tris, 100 mmol/L NaCl, pH 9.0)) was injected onto the column in 5 min, and the flow was stopped to incubate the substrate in the capillary column for 10 min. The LIF detector was switched on to collect data as soon as the substrate was injected. When the incubation was complete, buffer B was passed through the capillary column to sweep the product of the enzyme catalysis reaction out of

the capillary column. Buffer C (0.3 mol/L $MgCl_2$ in 2% acetic acid) was passed through the capillary column for 10 min to desorb the bounded complexes.

RESULTS AND DISCUSSION

Assay Format

The protein G capillary immunoaffinity chromatographic procedure used in this work involves: 1) Incubation of hIgG standards or controls with ALP conjugated goat anti-human IgG $F_{(ab')2}$ fragment in room temperature, 2) Injection of this incubation mixture onto the protein G capillary column under neutral pH, 3) Washing out the unbound compounds, 4) Injection of the substrate under basic pH, and incubation of the substrate in the stopped-flow condition, 5) Sweeping the product out of the capillary column and detection with LIF, 6) Desorbing of the complexes from the capillary column.

Evaluation of FDP as the Substrate

In order to evaluate FDP as the substrate of ALP, 100 μL 1/100,000 diluted ALP conjugated goat anti-human IgG $F_{(ab')2}$ fragment was added to 500 μL FDP solution (100 mmol/L). The absorbance spectra of the mixture at different time are shown in Fig. 2A. The mixture had weak absorbance at 435 nm and 480 nm in the beginning.

The absorbance at 480 nm rose up rapidly after a 2 min incubation and reached the maximum value in 20 min. The absorbance maximum wavelength also shifted from 480 nm to 488 nm. These results indicated that ALP catalyzed FDP to form fluorescein as the final product. The fluorescence spectra of FDP and its mixture with ALP conjugated goat anti-human IgG $F_{(ab')2}$ fragment after 20 min incubation, were shown in Fig. 2B. The fluorescence intensity of FDP was very weak, while that of the mixture was strong. The excitation maximum of the mixture was about 485 nm, while the emission maximum was about 525 nm. These results showed that FDP could be used as the substrate in an ALP-based enzyme-amplified immunoassay using the 488 nm line of an argon ion laser as the excitation source.

Optimization

Optimization of the chromatographic conditions was carried out in three steps. The first step was to choose a loading buffer. Because ALP was product

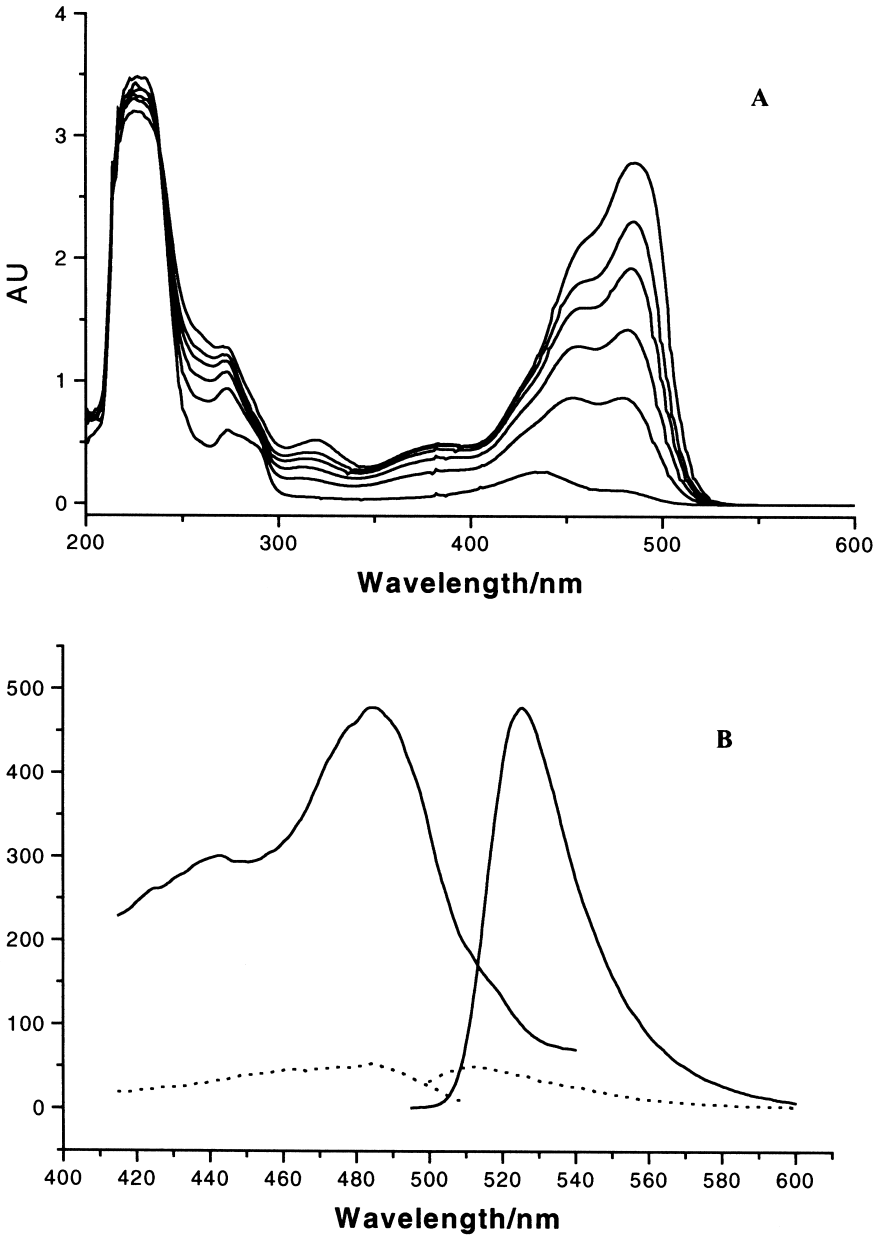

Figure 2. A) Absorbance spectra of the mixture of 500 μL 100 mmol/L FDP and 100 μL 1/100,000 diluted ALP conjugated goat anti-human IgG $F_{(ab')2}$ fragment at different times. From the bottom up, the incubation time is 0, 2.5, 5, 7.5, 10, 20 min. B) Fluorescence spectra of FDP (·····) and its mixture with ALP conjugated goat anti-human IgG $F_{(ab')2}$ fragment after 20 min incubation (——).

inhibited, the use of phosphate buffer in the assay was precluded. It was found that Tris buffer in neutral pH, with addition of 100 mmol/L NaCl, 0.1% BSA and 0.1 % Tween 20, could facilitate immunological complex formation and minimize nonspecific adsorption.

The second step was to choose the detection buffer. In order to improve the sensitivity, we need to choose conditions where both the activity of ALP and the absorbance of fluorescein were high. Since the absorbance of fluorescein was high in basic pH buffers, which were often used as the detection buffer in an ALP-based enzyme-amplified immunoassay,[13] we chose Tris buffer in basic pH with addition of 100 mmol/L NaCl as the detection buffer.

The third step was to choose the desorbing buffer. According to the manufacturer's instruction from PerSeptive Biosystems, we chose 0.3 mol/L $MgCl_2$ in 2% acetic acid as the desorbing buffer.

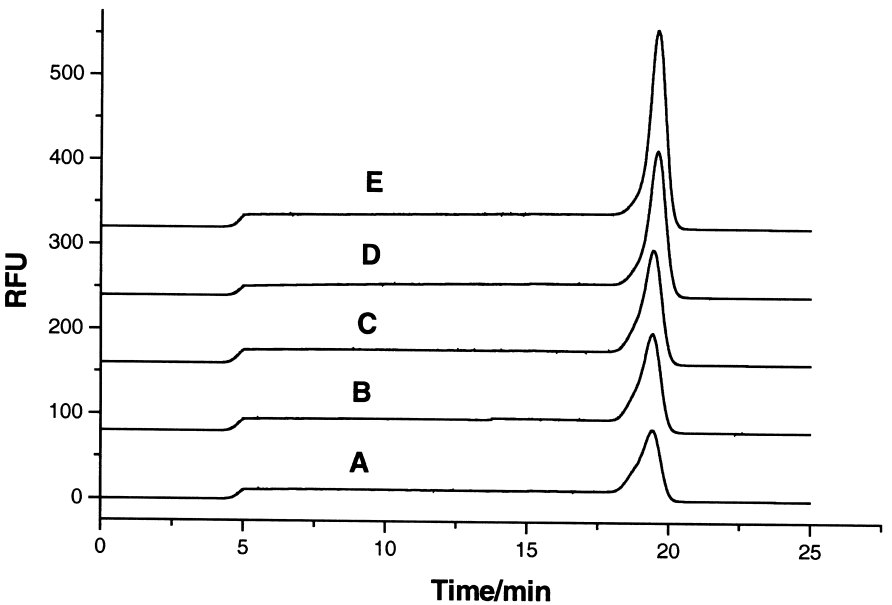

Figure 3. Typical chromatograms of the assay. Concentrations of hIgG are A) 0 pmol/L, B) 0.5 pmol/L, C) 5 pmol/L, D) 25 pmol/L, E) 50 pmol/L. Conditions are described in Experimental.

Quantification

Under the optimized conditions, typical chromatograms of the assay were shown in Fig. 3. The slight baseline increase in 5 min was probably due to the substrate hydrolysis in the continuous-flow mode, and the peak in 19.6 min was due to fluorescein accumulated during the incubation of the immunocomplex with FDP in the capillary column. With the increase of hIgG in the sample, the peak in 19.6 min was increased. The calibration curve for hIgG was established by plotting the areas of the peak in 19.6 min against concentrations of hIgG (Fig. 4). Each point represented the average of three consecutive runs, and the R.S.D. for the area was less than 3.4%. The dynamic linear range for the calibration curve was from 0.5-50 pmol/L ($r^2 = 0.999$, slope = 2.3 L/pmol, intercept = 102).

The sample without hIgG (negative control) was analyzed six times to determine the mean and standard deviation of the blank peak area. The concentration LOD was estimated to be about 0.2 pmol/L (S/N=3), and the mass LOD was about 1 pmol considering the injection volume was 5 μL. It could be seen from Fig. 3 that the LOD was dictated by the nonspecific adsorption, which could be further minimized by using longer washing time or high flow rate.

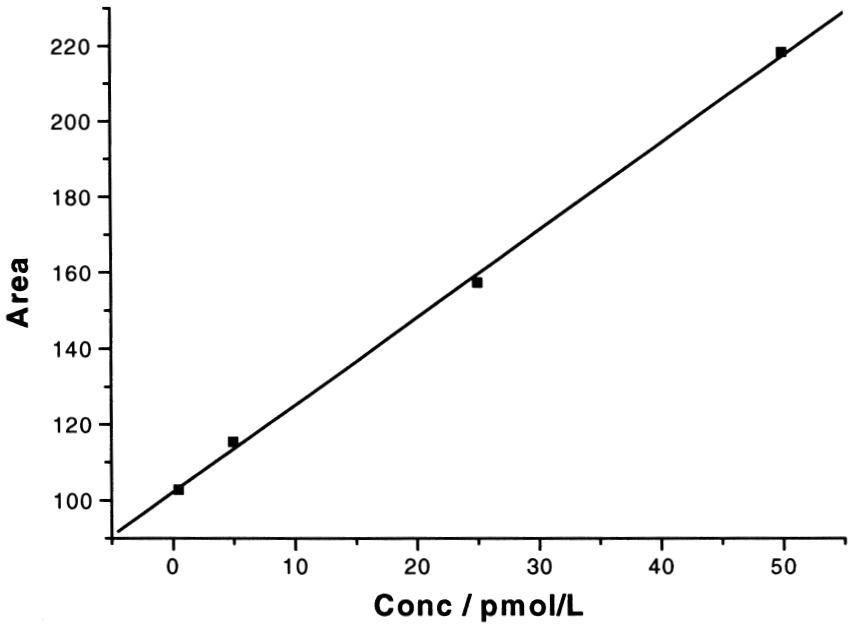

Figure 4. Calibration curve for hIgG.

Table 1. Recovery and Precision of hIgG Quantification

Concn of hIgG Added (pmol/L)	% of hIgG Recovered	% R.S.D. (n=5)	
		Intra-Assay	Inter-Assay
1	114	5.2	8.6
10	108	2.5	4.7

In order to determine both the recovery and the precision of the assay, control samples were analyzed at two concentrations. The results were shown in Table 1. The recovery was between 108-114%. Intra-assay precision was less than 5.2% R.S.D., and inter-assay precision was less than 8.6% R.S.D.

CONCLUSION

In this paper, the feasibility of performing the enzyme-amplified sandwich immunoassay with protein G capillary affinity chromatography and laser induced fluorescence (LIF) detection was investigated using the determination of human immunoglobulin G (hIgG) as a model system. ALP conjugated goat anti-human IgG $F_{(ab')2}$ fragment was used as the immunoreagent with FDP as the fluorogenic ALP substrate. The results showed that the enzyme-amplified sandwich immunoassay could be performed by protein G capillary affinity chromatography with LIF detection.

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