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Development of a Microcolumn One-Site Immunometric Assay for a Protein Biomarker: Analysis of Alpha₁-Acid Glycoprotein in Serum

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Abstract

A one-site immunometric assay based on affinity microcolumns was developed for the analysis of alpha₁-acid glycoprotein (AGP) as a model protein biomarker. In this assay, a sample containing AGP was incubated with an excess amount of a labeled binding agent, such as fluorescein-labeled anti-AGP antibodies or F_{ab} fragments. The excess binding agent was removed by passing this mixture through a microcolumn that contained an immobilized form of AGP, while the signal was measured for the binding agent-AGP complex in the non-retained fraction. Theoretical and practical factors were both considered in selecting the concentration of labeled binding agent, the incubation time of this agent with the sample, and the application conditions for this mixture onto the microcolumn. The effects of using various labeling methods and intact antibodies vs F_{ab} fragments were also considered. The final assay was performed with fluorescein-labeled anti-AGP antibodies and a 2.1 mm i.d. × 1.0 cm AGP microcolumn operated at 0.30 mL min⁻¹. This method required only 1 µL of serum, had a detection limit of 0.63 nM AGP, and gave a potential throughput of 2 min per sample. This assay was used to measure AGP in normal serum and serum from patients with systemic lupus erythematosus, giving good agreement with the literature and a reference method. The same approach and guidelines can be used to create assays for other protein biomarkers by changing the labeled binding agent and immobilized protein within the microcolumn.

Keywords

chromatographic immunoassay; one-site immunometric assay; alpha₁-acid glycoprotein; affinity microcolumn; systemic lupus erythematosus

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1 Introduction

Immunoassays are analytical techniques that use antibodies or antibody-related agents for the analysis of a given target compound [1,2]. These techniques rely on the selective and strong binding between an antibody and its target to provide assays that can often be used directly, and with minimal pretreatment steps, with complex samples such as serum or plasma [2,3]. In these methods, a column is used that contains one component of an immunoassay, making it possible to combine the binding selectivity and strength of antibodies with the precision, ease of automation, and speed of modern liquid chromatography [2–8].

A one-site immunometric assay is one format that can be used in a chromatographic immunoassay (see Figure 1) [9–17]. In this method, a sample or standard that contains the target analyte is combined and incubated with an excess amount of a labeled binding agent for the target (e.g., an intact antibody or F_{ab} fragment). The amount of non-bound labeled agent that remains after the incubation step is removed by passing this mixture through a column or support that contains an immobilized analog of the target. A signal that is related to the amount of analyte in the original sample is then obtained by measuring the amount of labeled binding agent that elutes non-retained from the column or that is captured by this column [9–17].

One-site immunometric assays have been previously used with traditional-sized columns and a number of targets with small-to-intermediate sizes (i.e., molar mass < 18 kDa) [9–16,18–21]. Examples have included assays for targets such as digoxin [9,10], thyroxine [11,15], β -estradiol [12], α -(difluoromethyl)ornithine [13], 2,4-dichlorophenoxyacetic acid [14], D-phenylalanine [16], interleukin-10, and human methionyl granulocyte colony stimulating factor [9–18]. It has only recently been shown that the same method can be used for proteins, as has been demonstrated for human serum albumin (HSA, the major protein in serum; normal concentration, 39–53 g L⁻¹; molar mass, 66.5 kDa) [19,20].

One development that has made the extension of one-site immunometric assays to protein targets feasible has been the use of affinity microcolumns (i.e., columns that contain an immobilized binding agent and with a size in the low-to-mid microliter range) [19,21]. Advantages to using microcolumns in applications such as chromatographic immunoassays include their need for only a small amount of an immobilized agent, their good capture efficiencies for an applied target or binding agent, and their ability to produce methods with analysis times on the order of only a few minutes [21]. This report will examine the development of a one-site immunometric assay that uses affinity microcolumns to measure a protein biomarker that occurs at a low-to-intermediate concentration in a biological system. This process will be illustrated by using α_1 -acid glycoprotein (AGP) as a model target.

AGP is an acute phase glycoprotein that consists of a single chain of 183 amino acids [22]. The molar mass of AGP ranges from 41 to 43 kDa, with glycan chains making up around 45% of this mass [22]. The normal serum concentration of AGP in humans is 0.5–1.0 g L⁻¹ (12–24 μ M), or up to 100-fold lower than the concentration of HSA [20]. However, AGP levels in some diseases can be increased by up to 10-fold [22,23]. The analysis of AGP in

serum and plasma has been previously done by using single radial immunodiffusion, which can take up to 48 h to perform [24,25]. Fluorescent probes such as auramine O and quinaldine red have also been used to measure AGP; however, these assays are susceptible to non-specific binding or matrix effects and require relatively large sample volumes (i.e., 100–200 μ L) [26,27]. Acidic precipitation and reversed-phase liquid chromatography have been used to measure AGP in plasma, but this approach requires 30 min to perform [28]. Capillary electrophoresis (CE) with electrophoretic injection has also been employed to measure AGP in serum, giving a method with a total analysis time of around 1 h [29]. Thus, there is still a need for a technique that can measure AGP in serum or related samples in both a rapid and selective manner.

This report will examine the development and use of a one-site immunometric assay that employs an affinity microcolumn with fluorescence detection for the analysis of AGP in serum. Several theoretical and practical factors will be examined and considered in the creation of this method. These factors will include the concentration of labeled binding agent that should be used, the incubation time needed for this binding agent with the analyte, and the application conditions for this mixture onto an affinity microcolumn. The use of intact antibodies or F_{ab} fragments will be considered, as well as various methods for labeling these binding agents. The analytical performance of the final method will then be characterized. This approach will be examined for use in the measurement of AGP in normal serum and serum from patients with systemic lupus erythematosus (SLE), a disease in which elevated serum levels of AGP may occur [22,29]. The results of this study should provide useful guidelines for the development and optimization of similar assays for other protein biomarkers or targets of biomedical interest.

2 Experimental Section

2.1 Reagents and Materials

The AGP (from pooled human plasma, 99% pure; product number G9885), polyethylene oxide (PEO; viscosity-average molar mass, 8000 kDa), Brij 35 (number-average molar mass, 1.198 kDa), Lucifer yellow CH (LyCH, dilithium salt), normal human serum (from human male AB plasma, USA origin, sterile filtered; product number H4522), oxalic dihydrazide, D-glucose, *N,N*-dimethylformamide (DMF) and Amicon Ultra-4 centrifugal filters (4 mL, 30 kDa cutoff) were from Sigma-Aldrich (St Louis, MO, USA). The Zeba spin desalting columns (0.5 mL volume, 7 kDa cutoff), immobilized papain (on agarose), cysteine HCl, and *N*-hydroxysuccinimide (NHS)-fluorescein (> 90%) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Goat anti-AGP polyclonal antibodies (catalog no. GAGP-80A, affinity-purified) were obtained from Lee Biosolutions (Maryland Heights, MO, USA). The SLE serum samples were de-identified and pre-existing samples from individuals known to have this disease, as provided by Johns Hopkins University School of Medicine (Baltimore, MD, USA); the use of such samples for this work was determined to be exempt from IRB review by the Johns Hopkins School of Medicine, according to the Code of Federal Regulations – 45 CFR 46.101 b. All aqueous solutions and samples were prepared using water obtained from a Milli-Q Advantage A10 purification system (EMD

Millipore Corporation, Billerica, MA, USA) and were filtered through 0.20 μm GNWP nylon membranes from Millipore.

2.2 Apparatus

The affinity microcolumns were packed using a HPLC slurry packer from ChromTech (Apple Valley, MN, USA). A MiniCentrifuge (Fisher Scientific, Pittsburgh, PA, USA) was used at 6600 rpm and room temperature with Zepa spin desalting columns. A 5702RH temperature-controlled centrifuge (Eppendorf, New York, NY, USA) was used at 4400 rpm and 25°C with the Amicon Ultra-4 centrifugal filters.

The one-site immunometric assays were performed with a Jasco HPLC system (Tokyo, Japan) that contained two PU-2080 isocratic pumps, an AS-2057 autosampler, a CO-2067 column oven, a UV-2075 UV absorbance detector and a FP-2020 fluorescence detector. A two position/six port valve from IDEX Health & Science (Rohnert Park, CA, USA) was used to switch the flow between the two isocratic pumps for sample loading and elution. Frontal analysis was performed with a modified setup (see Supplemental Material) of this HPLC system, which had one PU-2080 isocratic pump, a two position/six port valve with a 5.0 mL sample loop, a CO-2067 column oven, and a UV-2075 UV absorbance detector. Each configuration of the HPLC system was controlled with ChromNav software from Jasco. The chromatograms were analyzed with Peakfit 4.12 from Jandel Scientific Software (San Rafael, CA, USA).

CE was performed with a P/ACE MDQ system that was equipped with a UV absorbance detector (Beckman Instruments, Fullerton, CA, USA). This system was controlled with 32 Karat 7.0 software from Beckman and used 60.2 cm $\times$ 50 μm i.d. fused silica capillaries from Polymicro Technologies (Phoenix, AZ, USA) with an effective length to the detector of 50 cm. Each new capillary was activated by rinsing it with 1.0 M sodium hydroxide for 30 min, followed by a 10 min rinse with water. The electropherograms were analyzed using Peakfit 4.12 software.

2.3 Preparation of AGP microcolumns

The immobilization of AGP onto hydrazide-activated silica support was performed by using a modified form of a previous method [30]. The AGP was oxidized by combining 1.0 mL of 5.0 g L⁻¹ AGP with 1.0 mL of 20 mM periodic acid, with both of these solutions being prepared in 0.10 M acetate buffer (pH 5.5). This oxidation reaction was allowed to occur for 0.5 h in the dark and at room temperature. The oxidized AGP was washed five times with 0.10 M potassium phosphate buffer (pH 7.0) by using a centrifugal filter. Hydrazide-activated silica was prepared as described previously [31]; a 0.20 g portion of this activated support was combined with the purified oxidized AGP in 2.0 mL of 0.10 M potassium phosphate buffer (pH 7.0). This slurry was allowed to react for three days at 4°C with constant mixing. The non-immobilized AGP was washed away from the support by using centrifugation and 0.10 M potassium phosphate buffer (pH 7.0). A 40 mg portion of sodium cyanoborohydride was added to the support and allowed to react for 2 h to reduce the hydrazone bonds between the AGP and support to a more stable form [32]. An 18 mg portion of D-glucose was added to the slurry and allowed to react for 1 h at room

temperature to cap any unreacted hydrazide groups on the support [33]. The final AGP support was washed three times with 0.10 M potassium phosphate buffer (pH 7.0), by means of centrifugation, and stored in this same buffer at 4°C. The amount of immobilized AGP was determined in triplicate by comparing absorbance measurements made at 280 nm for portions of the AGP solution (i.e., 100 μ L diluted 10-fold with 0.10 M potassium phosphate buffer, pH 7.0) that were taken before and after the immobilization step.

The AGP support was downward slurry-packed into 2.1 mm i.d. $\times$ 1.0 cm long stainless steel microcolumns at a pressure of 3000 psi (20.7 MPa). The packing solution was 0.067 M potassium phosphate buffer (pH 7.4). A control microcolumn was prepared in the same manner with the same type of silica but with no AGP being present during the immobilization step. The microcolumns were stored in the pH 7.4 phosphate buffer at 4°C when not in use. An AGP column that was prepared in this manner was found to be stable for more than 6 months.

2.4 Preparation and labeling of F_{ab} fragments and antibodies

F_{ab} fragments were prepared by digesting the anti-AGP antibodies with immobilized papain, as per the manufacturer's instructions (see Supplemental Material for details). The F_{ab} fragments (in a solution volume of about 700 μ L) were affinity-purified at room temperature by adding 0.13 g of AGP silica, as prepared in Section 2.3. After allowing this mixture to react for 5 min, the AGP support was washed three times with 1.0 mL of 0.067 M sodium phosphate (pH 7.4). Anti-AGP F_{ab} fragments that were bound to this support were released by washing the AGP silica three times with 1.0 mL of 0.10 M phosphate buffer (pH 2.5). The purified anti-AGP F_{ab} fragments were placed into 0.067 M sodium phosphate buffer (pH 7.4) and concentrated to a volume of 1.0 mL by using a centrifugal filter. The final solution was stored at 4°C or used immediately for labeling or assays.

The anti-AGP antibodies and F_{ab} fragments were labeled with NHS-fluorescein as described previously (see Supplemental Material) [19]. The anti-AGP antibodies were reacted with a 24-fold mol excess of NHS-fluorescein, and the corresponding F_{ab} fragments were reacted with a 28- or 280-fold mol excess of the same labeling agent. The final preparations had a solution volume of $\sim$ 130 μ L and a concentration of $\sim$ 0.9 g L⁻¹ for the fluorescein-labeled anti-AGP antibodies or a concentration of $\sim$ 23 mg L⁻¹ for the fluorescein-labeled F_{ab} fragments. These and the other labeled binding agents that were prepared for this study were stored at 4°C and used within one week.

Anti-AGP antibodies were also labeled, after being oxidized with periodic acid, by using LyCH and methods adapted from the literature (see Supplemental Material) [34]. A 200-fold mol excess of LyCH was used versus the oxidized antibodies, followed by the use of sodium cyanoborohydride to reduce the hydrazone bond between the LyCH and oxidized antibodies to a more stable form. The final antibody solution was washed and concentrated by using 0.067 M sodium phosphate buffer (pH 7.4) and a centrifugal filter. The final volume of the labeled anti-AGP antibody solution was 1.0 mL and the concentration was $\sim$ 0.92 g L⁻¹.

The labeling ratio for the antibodies or F_{ab} fragments was determined in triplicate through absorbance measurements, with the results being combined with the reported molar

absorptivity of each label or binding agent. These measurements were made at 280 nm to find the antibody or F_{ab} content of the solution and at 494 or 428 nm, respectively, to determine the amount of fluorescein or LyCH label that was present.

2.5 Conditions for one-site immunometric assay

The following conditions were used for the final one-site immunometric assay that was developed in this project. A 1.0 μL sample of serum was dissolved in 2.5 mL of a 1.0 mg L^{-1} solution of normal AGP that was prepared in 0.067 M sodium phosphate buffer (pH 7.4) (Note: The purpose of adding this normal AGP to the sample was to adjust the response to fall within the linear range of the assay when using intact labeled antibodies, as discussed later in Section 3.6). A 27.5 μL aliquot of this solution was mixed with 26.8 μL of 41.1 mg L^{-1} fluorescein labeled anti-AGP antibodies in 0.067 M sodium phosphate buffer (pH 7.4) and 55.7 μL of 0.067 M sodium phosphate buffer (pH 7.4). This mixture had final concentrations for labeled anti-AGP antibodies and AGP of 10.0 mg L^{-1} and 0.25 mg L^{-1} , respectively, and a total volume of 110 μL . The original serum sample was diluted by 10,000-fold during this process (see Supplemental Material for issues to consider with regards to matrix effects when using less dilute samples).

Standard solutions of AGP were prepared by adding 26.8 μL of 41.1 mg L^{-1} fluorescein labeled anti-AGP antibodies into various volumes of a 1.0 mg L^{-1} AGP stock solution in 0.067 M sodium phosphate buffer (pH 7.4). Additional 0.067 M sodium phosphate buffer (pH 7.4) was added to bring the total solution volume up to 110 μL . The AGP concentrations in these standards typically ranged from 0.25 to 0.55 mg L^{-1} . A concentration of 0.25 mg L^{-1} AGP was used as the starting point for the calibration curve to avoid the non-linear response that occurred at lower AGP concentrations when using intact antibodies (see Sections 3.5–3.6).

The one-site immunometric assay was performed on a 2.1 mm i.d. $\times$ 1.0 cm AGP microcolumn by using 0.067 M sodium phosphate buffer (pH 7.4) as the application buffer. Sample injections were made at 0.30 mL min^{-1} . After non-retained sample components were allowed to wash from the microcolumn for 2 min, an elution buffer consisting of 0.10 M glycine-HCl (pH 2.7) was passed through the system at 0.50 mL min^{-1} for 3 min. The microcolumn was then regenerated with the original application buffer at 0.30 mL min^{-1} for 3 min. The injection volume was 20 μL and the AGP microcolumn was maintained at 25 $^{\circ}\text{C}$ throughout this study. Three replicate injections were performed for each type of sample, with a separate cycle of injection, elution and column regeneration being used per sample. An excitation wavelength of 494 nm and an emission wavelength of 518 nm were used for detecting the fluorescein-labeled antibodies or labeled F_{ab} fragments. An excitation wavelength of 428 nm and emission wavelength of 540 nm were used for LyCH-labeled binding agents.

2.6 Sample preparation and conditions for capillary electrophoresis

The sample pretreatment methods and CE analysis conditions were the same as described in a previous report [29]. A 65 μL portion of serum was combined and mixed with 130 μL of 0.50 M perchloric acid, with the latter being prepared in water and used to precipitate

abundant serum proteins while leaving AGP in the supernatant (Note: Use appropriate chemical hazard precautions when handling perchloric acid) [35]. This mixture was centrifuged at 6600 rpm for 10 min at room temperature. A 130 μ L portion of the supernatant was desalted by loading the sample into a Zeba spin desalting column and spinning the column for 2 min at 6600 rpm and room temperature. The filtrate was diluted with water to give a treated sample with total volume of 5.0 mL for CE analysis.

Each capillary used in the CE system was first cleaned by using a 5 min rinse with 1.0 M sodium hydroxide, followed by a 3 min rinse with water. A coating was applied by rinsing the capillary for 5 min with 1.0 M hydrochloric acid and then rinsing for 5 min with a 0.2% (w/v) PEO solution that contained 0.10 M hydrochloric acid [36]. The capillary was rinsed for 5 min with a running buffer that consisted of 20 mM acetate buffer (pH 4.2) that contained 0.05% (w/v) PEO and 0.1% (w/v) Brij 35. Each rinse was carried out at an applied pressure of 50 psi (0.34 MPa), and this rinsing procedure was repeated before each CE separation. The samples were electrophoretically injected by applying a potential of -5 kV to the PEO-coated capillary for 5 min. The applied voltage used for the separation of AGP glycoforms was -30 kV. The capillary temperature was 25 $^{\circ}$ C and detection was performed at 200 nm [29].

3 Results and Discussion

3.1 Development and characterization of AGP microcolumn

The AGP microcolumns that were used in this study were based on the coupling of oxidized AGP (i.e., which contained aldehyde groups in its carbohydrate chains, as generated by mild oxidation with periodate) with hydrazide-activated silica. This was carried out by using a modified form of a previously-reported procedure for the preparation of AGP silica (see Ref. [30] for details), with the addition of a step in which the hydrazone bonds formed between hydrazide groups on the support and aldehyde groups on AGP were reduced with cyanoborohydride to form a more stable bond [37,38]. It was found that final amount of AGP on the support was as $25.9 (\pm 0.1)$ mg AGP per gram of silica, which was roughly two-fold higher than obtained previously [30]. This content meant that a total of 0.40 mg (or 9.6 nmol) AGP was present in a 2.1 mm i.d. $\times$ 1.0 cm microcolumn, based on the void volume of the column and the known packing density of the silica (i.e., 0.45 g silica per cm^3).

The amount of active AGP that was present after immobilization was also estimated. This was determined by performing frontal analysis [39,40] on both an AGP microcolumn and a control column using disopyramide as a probe that has well-characterized binding with soluble AGP [41]. Some typical results from these experiments are provided in the Supplemental Material. It was found by this approach that the amount of active AGP in a 2.1 mm $\times$ 1.0 cm microcolumn, with regards to disopyramide binding, was $3.7 (\pm 0.3)$ nmol. This meant that 39% of the AGP was still fully active after immobilization as a binding agent for drugs (Note: some of the remaining AGP would still be expected to bind to a portion of the anti-AGP antibodies in a polyclonal preparation even when partially inactive). This meant the estimated binding capacity of such a column was at least 3–4 nmol (i.e., based on a 1:1 binding ratio of an anti-AGP antibody or F_{ab} fragment with the immobilized AGP). This amount was 920- to 2800-fold larger than what would be needed to fully bind 20

μL of a 10 mg L^{-1} solution of anti-AGP labeled antibodies or F_{ab} fragments, such as used later in this study. Alternatively, the same microcolumn could be used for many sample injections before the need for column elution and regeneration, as suggested previously [19]. Thus, this binding capacity was determined to be sufficient for the intended assay and this is the type of AGP microcolumn that was used in all following sections of this report.

3.2 Effect of flow rate on capture efficiency of AGP microcolumn

Another important factor to be considered in the development of a chromatographic one-site immunometric assay is the flow rate at which the labeled binding agent is applied to the column. This flow rate determines the extent to which this column can capture and remove the excess labeled binding agent from the complex of this agent with the analyte. In this study, the effect of varying the injection flow rate was evaluated by injecting LyCH-labeled anti-AGP antibodies onto a $2.1 \text{ mm i.d.} \times 1.0 \text{ cm}$ AGP microcolumn and control microcolumn at various flow rates. The results were also compared to the maximum level of capture that was obtained for the anti-AGP antibodies before labeling, which was done to correct for the fact that not all of the original antibodies could bind to AGP.

Some typical results that were obtained in these studies are shown in Figure 2. These results were obtained at 0.30 mL min^{-1} and with injected samples that contained 8.0 pmol of the labeled antibodies (i.e., $20 \mu\text{L}$ of 60 mg L^{-1} LyCH-labeled anti-AGP antibodies). This flow rate was the same as selected for use in the final one-site immunometric assay (see Section 3.6) and the amount of injected antibodies was six-fold larger than that used per injection in the final method. This amount of labeled antibodies was 450-fold lower than expected binding capacity of the AGP microcolumn, as estimated in the previous section. It can be seen that under these conditions that the non-retained peak for the labeled antibodies appeared within 30–60 s after injection. Also, more than 70% of the labeled antibodies were captured by the AGP microcolumn when compared to the total area that was measured for the same injection onto a control microcolumn. The remainder of the labeled antibodies, which eluted non-retained even from the AGP microcolumn, was approximately equal to the amount of non-labeled antibodies in the same type of preparation to have no binding activity for AGP (see Section 3.5). No significant levels of non-specific binding by the anti-AGP antibodies to the support were noted in this study, as based on the observed peak areas and peak shapes that were obtained for these antibodies on the control microcolumn. However, even if some non-specific binding had been present (i.e., decreasing the size of the non-retained antibody peak), a correction for this would have been made later in this study when AGP standards were used to generate a calibration curve for the final assay.

Table 1 shows how the amount of bound labeled antibodies changed as the injection flow rate was varied from 0.10 to 0.70 mL min^{-1} . Essentially complete capture of the antibodies that could bind to AGP was seen when using a flow rate of 0.30 mL min^{-1} or less. However, increasing the flow rate even up to 0.70 mL min^{-1} only reduced the extent of this capture by 6–12%. Based on these results, an injection flow rate of 0.30 mL min^{-1} was chosen for use in all further studies. This flow rate made it possible to effectively capture all of the active labeled antibodies in the applied samples but also making it possible to measure the non-retained peak for these labeled binding agents within only 2 min after injection.

3.3 Selection of labeling agent concentration

Another factor to consider in the use of a chromatographic one-site immunometric assay is the concentration or relative amount of the labeled binding agent that is to be added to a sample that contains the analyte. The general process by which the labeled binding agent (Ab^*) binds to a large analyte (A) can be described as shown in Eq. (1),

where $Ab^* \cdot A$ is the complex formed between the labeled agent and the analyte [6]. If a large excess of the labeled binding agent is used versus the analyte, the concentration of Ab^* in its unbound form at equilibrium ($[Ab^*]$) will be approximately equal to the total concentration of this labeled agent ($[Ab^*]_T$). When equilibrium is reached under these conditions, the fraction of the analyte (F) that is bound by the labeled binding agent can be estimated by using Eq. (2),

$$F = \frac{[Ab^* \cdot A]}{[A]_T} = \frac{[Ab^*]_T}{K_d + [Ab^*]_T} \quad (2)$$

where $[Ab^* \cdot A]$ is the concentration of the complex between Ab^* and A , and $[A]_T$ is the total concentration of the analyte in the mixture of Ab^* and A . The term K_d is the dissociation equilibrium constant for the reaction shown in Eq. (1), as given by the relationship in Eq. (3).

$$K_d = \frac{[Ab^*][A]}{[Ab^* \cdot A]} \quad (3)$$

Some of the labeled binding agent may lose its activity towards the analyte as a result of the labeling process (see Section 3.5). To correct for this, Eq. (4) can be used in this case to describe the fraction of A that is bound to Ab^* when only fraction f of the labeled agent is active,

$$F = \frac{[Ab^* \cdot A]}{[A]_T} = \frac{f[Ab^*]_T}{K_d + f[Ab^*]_T} \quad (4)$$

in which the value of f is between 0.0 (for a fully inactivated binding agent) and 1.0 (for a fully active agent).

Based on Eqs. (1–4), there are two requirements placed on the concentration of the labeled agent for a successful one-site immunometric assay. First, the total amount of active binding agent must be present in an excess when compared to the amount of the analyte that may be present in a sample. This relationship is represented by Eq. (5) for a system in which A can form a 1:1 complex with Ab^* (i.e., as can occur when antibodies or smaller F_{ab} fragments bind to a large analyte such as a protein).

$$\text{Requirement 1: } f[Ab^*]_T > [A]_T \quad (5)$$

In this study, the labeled binding agent was typically added to the pre-diluted analyte in a 20- to 50-fold mol excess, which helped to ensure that the condition stated in Eq. (5) was met even if not all the labeled agent was present in an active form.

A second requirement is that most of A will form a complex with Ab*, which maximizes the signal that is produced by [Ab*·A] and results in good linearity and a low detection limit in a one-site immunometric assay. Based on the relationship given earlier in Eq. (4), this situation occurs when the value of $f[\text{Ab}^*]_{\text{T}}$ is larger than K_{d} .

$$\text{Requirement 2: } f[\text{Ab}^*]_{\text{T}} > K_{\text{d}} \quad (6)$$

As is shown in Figure 3, the fraction of bound analyte (F) will be 0.50 when the active concentration of the labeled binding agent ($f[\text{Ab}^*]_{\text{T}}$) is equal to K_{d} . Also, F will be greater than 0.90 when $f[\text{Ab}^*]_{\text{T}}$ is at least 10-fold larger than K_{d} (i.e., more than 90% of the analyte is present in the complex Ab*·A after incubation). It is known that a typical polyclonal antibody preparation, such as that used in this study, may have dissociation equilibrium constants for the target analyte that span from 10^{-8} to 10^{-12} M [8]. Therefore, for an antibody-related binding agent with an affinity for its target, a concentration higher than 10^{-8} M (i.e., $> 1.5 \text{ mg L}^{-1}$ for an intact antibody or $> 0.5 \text{ mg L}^{-1}$ for a F_{ab} fragment) should generally be used in a one-site immunometric assay to ensure most of the analyte is complexed during incubation. The actual concentrations of labeled binding agents that were used for method development in this study were at least 3.3- to 80-fold larger than this estimated requirement (i.e., even when using binding agents that may have a moderate K_{d} of only 10^{-8} M).

3.4 Selection of incubation time

The incubation time for the labeled binding agent with a sample also needs to be considered when developing a one-site immunometric assay, as this can be a limiting factor in the throughput of such methods [19]. As stated in the last section, the binding of a labeled antibody-type agent (Ab*) and the analyte (A) is usually strong (i.e., $K_{\text{d}} < 10^{-8}$ M) [8]; this process is also essentially irreversible on a small-to-intermediate time scale (i.e., the dissociation rate constant, k_{off} , is much less than the association rate constant, k_{on}). Under these conditions, the binding of Ab* with A in the reaction given earlier in Eq. (1) can be approximated by an irreversible second-order reaction [9] with the following rate expression.

$$\frac{d[A]}{dt} = -k_{\text{on}}[A][\text{Ab}^*] \quad (7)$$

The expression in Eq. (7) can be simplified further by using the fact that the value of $f[\text{Ab}^*]$ should be much larger than $[A]$ for an effective one-site immunometric assay, as stated earlier in Eq. (5). This also means the value of $f[\text{Ab}^*]$ will be essentially constant and equal to $f[\text{Ab}^*]_{\text{T}}$ during the incubation process. Eq. (8) shows the modified form of Eq. (7) that is obtained with these further assumptions,

$$\frac{d[A]}{dt} = -k'_{\text{on}}[A] \quad (8)$$

where k_{on}' is an apparent first-order association rate constant equal to the product $k_{on} f$ $[Ab^*]_T$.

An estimate of the minimum incubation time that will be needed for a one-site immunometric assay can be obtained from Eq. (8) for selected values of k_{on} and $f[Ab^*]_T$. For instance, a typical value of k_{on} for the interaction between antibody and a target protein is around 10^6 to 10^7 $M^{-1}s^{-1}$ [9,41]. It was also determined in the previous section that $f[Ab^*]_T$ should generally be at least 1×10^{-8} M, with values of $3.3\text{--}80 \times 10^{-8}$ M actually being used in this current study. By using these values with Eq. (8), it was estimated that formation of the complex $Ab^* \cdot A$ should be over 95% complete in 0.5–5.0 min.

The incubation time needed for a one-site immunometric assay was also examined experimentally by preparing a mixture that contained a known amount of AGP (15 mg L^{-1}) and labeled anti-AGP antibodies (LyCH tag, 60 mg L^{-1} Ab^*). This mixture was allowed to incubate for various lengths of time and injected onto an AGP microcolumn. The amount of labeled antibodies that eluted non-retained was then used as a measure of how far the antibody-analyte reaction in Eq. (1) had proceeded to complex formation. The results are shown in Figure 4. It was again found under these conditions that over 95% of the maximum signal was reached within 5 min of preparing this mixture (i.e., the minimum incubation time used later in this study), with no significant changes being seen after incubation times of 10 to 60 min. Given the fact that at least five minutes were needed to prepare a series of samples and standards for analysis, it was determined that the incubation step of this particular method did not contribute significantly to the overall analysis time. These conditions also helped to provide a situation in which a strong signal due to complex formation was obtained that was consistent and stable even when longer incubation times may have been present (e.g., during the injection of replicates for the same sample mixture).

3.5 Effect of labeling method and use of intact antibodies versus F_{ab} fragments

It was shown in Eqs. (4–6) and (8) that the relative activity of the binding agent can affect both the concentration of this agent needed to provide a strong, stable signal in a one-site immunometric assay and the rate at which the analyte will complex with this agent. This section examined the effect on this relative activity of varying the labeling method and the use of intact antibodies vs F_{ab} fragments (i.e., with two potential binding sites for the target or one site, respectively). Items considered with regards to the labeling methods included the type of coupling chemistry that was used and the relative amount of labeling reagent that was used for this process. Table 2 shows the results that were obtained when various types of labeled binding agents were tested for their activity by examining their extent of capture on a 2.1 mm i.d. $\times$ 1.0 cm AGP microcolumn (i.e., by using the approach described in Section 3.2).

The first comparison made was in the use of intact antibodies vs F_{ab} fragments that were labeled with the same type of reagent, NHS-fluorescein. The results were compared to binding by the same type of anti-AGP antibodies or F_{ab} fragments to an AGP microcolumn before the labeling process. The capture efficiency, and relative activity, of the unlabeled antibodies or F_{ab} fragments was found to be $71.4 (\pm 3.0)\%$ and $65.1 (\pm 3.8)\%$, respectively. These activities were equivalent at the 95% confidence level, which indicated that the

method used to generate the F_{ab} fragments did not have a significant effect on their activity. The reason that less than 100% activity was seen for the unlabeled antibodies and F_{ab} fragments is believed to be the fact that not all of the antibodies in the original polyclonal preparation were able to bind to AGP and to be captured by the AGP microcolumn.

Although the unlabeled antibodies and F_{ab} fragments had essentially the same binding activities for AGP, this was not the case after these agents were labeled with NHS-fluorescein. The intact antibodies showed no significant loss in their activity when reacted with a 24:1 mol:mol ratio with this labeling agent. However, the F_{ab} fragments lost between 92% and almost 96% of their activity when using a 28:1 or 280:1 mol:mol ratio of NHS-fluorescein for labeling. This occurred even though the final amount of label that was coupled to each binding agent was higher for the intact antibodies (i.e., 6.3 mol mol^{-1} for the intact antibodies vs $0.8\text{--}4.2 \text{ mol mol}^{-1}$ for the F_{ab} fragments). The reason for the difference in final activity is not clear, but it suggests that the additional amine groups that were provided by the F_c region of the intact antibodies for labeling helped to protect the binding regions on these antibodies from inactivation by the NHS-fluorescein.

Figure 5 illustrates the effect of this difference in activity for the labeled antibodies vs F_{ab} fragments on a one-site immunometric assay. The change in response of these calibration curves (i.e., as the amount of AGP was varied) represented the signal that was due to the labeled antibody- or F_{ab} -AGP complexes; the background response (i.e., as obtained when no AGP was added) was due to the non-retained fraction of the labeled binding agent. Both types of labeling agents gave an essentially linear response at low-to-moderate AGP concentrations (correlation coefficients of 0.9935 and 0.9933, respectively, at $n = 5$ for the labeled F_{ab} fragments and antibodies at $0.00\text{--}0.50 \text{ mg L}^{-1}$ AGP); this similarity in behavior suggested that both the intact antibodies and F_{ab} fragments had 1:1 binding to AGP over this concentration range. A slight deviation from linearity possibly occurred at the lower end of the calibration curve for the intact antibodies ($< 0.10 \text{ mg L}^{-1}$ AGP, as addressed in Section 3.6), which may have been caused by some antibodies binding to both soluble and immobilized AGP under these conditions. However, the greater activity and higher level of labeling for the antibodies meant they had a much lower signal for their non-retained peak when no AGP was present in the sample (i.e., a difference of 4.2-fold in Figure 5). The labeled intact antibodies also had a larger slope in Figure 5 for their change in response with AGP levels (i.e., a 2.2-fold increase over the labeled F_{ab} fragments), and better precision when used in replicate measurements (see error bars in Figure 5). Based on these results, labeled intact antibodies were used in all further experiments in this study. An alternative would have been to use immobilized AGP to affinity-purify the labeled F_{ab} fragments to increase their activity [15], which would have also increased the time and effort involved in reagent preparation.

The next comparison was between the intact anti-AGP antibodies that were labeled with an amine-reactive tag (i.e., NHS-fluorescein) versus a more site-selective labeling agent (i.e., LyCH). The second of these agents was coupled through a hydrazide group to aldehyde groups that were generated in the carbohydrate chains of the antibody's F_c region [42]. The relative amount used for these labeling agents was 24:1 mol:mol antibody for the NHS-fluorescein and 200:1 mol:mol for the LyCH, based on the literature [19,34]. As is shown in

Table 2, both these reagents and labeling conditions gave essentially the same level of activity in the final antibody preparation and no significant loss in activity from the unlabeled antibodies. Labeling based on NHS-fluorescein was selected for use in further assay development due to the higher labeling ratio that was obtained with this reagent (i.e., 6.3 vs. 2.2 mol mol⁻¹ antibody for LyCH), the greater brightness of this tag and higher signal obtained due to this label [43,44], and the ~3-fold lower cost of this reagent.

3.6 Evaluation of one-site immunometric assay for AGP

In the conditions selected for the final assay, the AGP/labeled antibody mixture contained 10.0 mg L⁻¹ (or 67 nM) of the labeled anti-AGP antibodies, an added concentration of 0.25 mg L⁻¹ AGP, and additional AGP from the sample or standard. The 0.25 mg L⁻¹ AGP was added to all samples and standards to avoid the small non-linear response seen at low AGP concentrations in Figure 5. As mentioned in the previous section, this non-linearity was probably due to binding by some antibodies to both soluble and immobilized AGP through their two available binding sites to this target [9]. The overall mixture was allowed to incubate for at least 5 min and then injected in a 20 µL volume onto a 2.1 mm i.d. × 1.0 cm AGP microcolumn at 0.30 mL min⁻¹. The size of each non-retained peak was then measured and related to the AGP content.

Figure 6(a) shows some typical chromatograms and a calibration curve that was obtained with this assay. A signal for the non-retained peak was obtained within 30–45 s after injection, with the size of this peak increasing as the concentration of AGP was increased. Complete elution of the non-retained peak for even high-concentration AGP samples was obtained within 2 min. The within-day precision of the measurements for the non-retained peaks, over the range of concentrations shown in Figure 6(b), ranged from ± 0.6 to 2.0% (*n* = 3). The within-day precision of the retention times during these same injections was ± 0.4 to 0.9% (*n* = 3).

This method gave a linear response at low-to-moderate AGP concentrations; for instance, the best-fit line in Figure 6(b) had a correlation coefficient of 0.9940 (*n* = 7), with an estimated detection limit at S/N = 3.3 of 0.026 mg L⁻¹ AGP (or 630 pM). This detection limit was approximately 10⁴-fold lower than the normal concentration of AGP in human serum and was sufficient for the detection of this protein even in highly-diluted small serum volumes, as shown in the next paragraph (i.e., a 10,000-fold dilution of 1 µL serum) [22,23]. The upper end of the linear range occurred at approximately 0.5 mg L⁻¹, as shown earlier in Figure 5.

Figure 7 shows the results that were obtained when this assay was used to measure the AGP concentrations in both normal commercial serum and in individual serum samples from several patients with SLE (i.e., a condition in which elevated AGP levels often occur). Each of these measurements were made by using 1 µL portions of serum that were diluted by 10,000-fold in the final AGP/labeled antibody mixtures. Some typical chromatograms that were obtained with these samples are shown in Figure 7(a). The results were again obtained in 30–45 s after injection, with complete elution of each non-retained peak within 2 min.

It was possible from the areas of these peaks to determine the concentration of AGP in each sample. These results are summarized in Table 3. The measured concentrations of AGP in these samples ranged from 1.17 to 3.71 g L⁻¹ and had precisions of ± 2.5 to 12.3% (average, $\pm 7.4\%$). The amount of AGP in the commercial serum sample was found to be 1.17 (± 0.11) g L⁻¹ which was consistent with concentration expected in a normal serum [22]. The AGP concentrations found in the SLE serum samples were 1.5- to 3.2-fold higher than seen in the normal serum, as has been noted previously for patients who suffer from this disease, and especially when secondary infections are present [45].

The concentrations of AGP in the same serum samples were also measured by a CE method that employed a neutral capillary coating and electrophoretic injection [29]. This second method used 65 μ L of each serum sample and required precipitation of the AGP and desalting of the protein preparation prior to analysis [29]. Results were obtained within about 20 min of sample injection onto the CE system, as shown in Figure 7(b). The general trends in the SLE vs normal serum samples were similar in the CE method and the one-site immunometric assay. In the case of the CE method, the level of change for AGP in the SLE serum spanned from 1.1- to 2.4-fold. This observed change was slightly smaller than the level of increase that was seen with the one-site immunometric assay and may have been due to the additional precipitation and sample pretreatment steps that were needed in the CE technique [29]. Such steps were avoided in this report due to the selective binding of AGP to the anti-AGP antibodies and the capture of the excess labeled antibodies by the AGP microcolumn.

4 Conclusion

In this study, a one-site immunometric assay using affinity microcolumns was developed and optimized for the analysis of AGP, which was used as a model protein biomarker. The theory of chromatographic-based one-site immunometric assays was used to help design this method. Factors that were considered included the binding capacity and capture efficiency of the affinity microcolumn; the type and concentration of the labeled binding agent; and the conditions used to incubate this agent with the sample.

The final method was evaluated in terms of its response, speed, precision, and results versus other methods. Conditions were identified which provided a linear response at low-to-moderate protein concentrations and which gave a detection limit with a fluorescein-based label of only 630 pM. This limit of detection was at a level that should be suitable when extending this approach to a wide range of other low-to-moderate level proteins in serum [21]. The final method for AGP required only 1 μ L of human serum for a series of replicate injections and had a potential sample throughput of 30 samples per hour, or 2 min per sample. The affinity microcolumns that were used in this method were stable over 6 months of use and more than 430 injections. In addition, the overall method gave precise results (± 2.5 –12.3%) with good correlation vs the literature and a reference method [22,29]. Future work may examine in more detail the accuracy, precision, stability, and utility of this method with the goal of validating and further optimizing this approach for the routine clinical analysis of AGP in SLE or other disease states [20,22–29]. The same approach could be used to create assays for other protein biomarkers by changing the labeled binding agent and

immobilized protein within the microcolumn. Examples of potential applications include the analysis of tumor biomarkers and of trace proteins or modified forms of these proteins for personalized medicine [20,46].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A one-site immunometric assay using affinity microcolumns was developed and tested.
- Alpha₁-acid glycoprotein (AGP) was used a model protein biomarker for this work.
- Several theoretical and practical factors were considered in creating this assay.
- These factors included conditions used for incubation, the reagents, and the microcolumn.
- The final method required only 1 µL serum and had a detection limit of 0.63 nM AGP.
- The same approach can be easily modified for use with for other protein biomarkers.

Step 1: Combine sample with labeled binding agent

Labeled binding agent (anti-AGP)
Analyte (AGP)

Step 2: Inject sample/labeled binding agent **Step 3: Elute retained binding agent; regenerate column** **Figure 1.**

General scheme for a chromatographic one-site immunometric assay, as illustrated for the analysis of α_1 -acid glycoprotein (AGP).

Figure 2.

Non-retained peaks obtained for 20 μL injections of 60 mg L^{-1} LyCH-labeled anti-AGP antibodies (Ab^*) made onto a 2.1 mm i.d. $\times$ 1.0 cm AGP microcolumn and a control microcolumn at 0.30 mL min^{-1} . Other conditions are given in the text.

Figure 3.

Predicted fraction, F , of analyte A that will be complexed with a labeled binding agent (Ab^*) as a function of the concentration of the labeled binding agent ($[\text{Ab}^*]$), the fraction of this agent that is active (f), and the dissociation equilibrium constant for the binding of Ab^* with A (K_d). These plots are based on Eq. (4) and assume that Ab^* is present in a large excess versus A.

Figure 4.

The effect of incubation time on the binding of labeled anti-AGP antibodies with AGP.

These results are for a mixture that contained 60 mg L^{-1} (400 nM) of LyCH-labeled anti-AGP antibodies and 15 mg L^{-1} (360 nM) AGP and that was allowed to incubate for various periods of time at room temperature. Samples of this mixture were injected in $20 \text{ }\mu\text{L}$ volumes onto a $2.1 \text{ mm i.d.} \times 1.0 \text{ cm}$ AGP microcolumn at 0.30 mL min^{-1} to determine the response due to the non-retained labeled antibodies.

Figure 5.

Response obtained in a chromatographic one-site immunometric assay for a series of AGP standards that were diluted by 10,000-fold and incubated with (a) 33 nM fluorescein-labeled anti-AGP F_{ab} fragments or (b) 33 nM (5 mg L⁻¹) fluorescein-labeled anti-AGP antibodies. These mixtures were injected onto a 2.1 mm i.d. × 1.0 cm AGP microcolumn at 0.30 mL min⁻¹. The AGP concentrations shown on the *x*-axis represent the final values for the diluted standards. Other conditions are given in the text. The correlation coefficients for the best-fit lines shown in these plots (over all data points in black) were (a) 0.9935 and (b) 0.9933. The error bars represent a range of ± 1 S.D. (*n* = 3).

Figure 6.

(a) Typical non-retained peaks seen for AGP standards in a one-site immunometric assay, after correction for the blank response that was obtained at 0.0 mg L⁻¹ AGP. (b) Calibration curve obtained by the one-site immunometric assay, where the *x*-axis shows the final diluted concentration of AGP from the standard. The experimental conditions are described in the text. The error bars represent a range of ± 1 S.D. ($n = 3$).

Figure 7.

(a) Chromatograms obtained in a one-site immunometric assay for AGP in normal serum or serum from SLE patients, and (b) electropherograms acquired in a CE-based assay for the same group of samples. The chromatograms in (a) were corrected for the blank response obtained with a 0.0 mg L^{-1} AGP standard. The conditions used in (a) and (b) are given in the text.

Table 1.

Extraction efficiency for LyCH-labeled anti-AGP antibodies when applied to a 2.1 mm I.D. $\times$ 1.0 cm AGP microcolumn^a

Flow rate (mL min ⁻¹)	Amount bound by AGP microcolumn (%)	Amount bound vs. non-labeled antibodies (%) ^b
0.10	76.7 ($\pm$ 0.6)	107.4 ($\pm$ 4.6)
0.30	71.7 ($\pm$ 0.7)	100.4 ($\pm$ 4.4)
0.50	70.0 ($\pm$ 0.5)	98.0 ($\pm$ 4.3)
0.70	66.8 ($\pm$ 0.6)	94.6 ($\pm$ 4.0)

^aThe numbers in parentheses represent $\pm$ 1 S.D. ($n = 3$).

^bThese values were calculated by comparing the results for the labeled antibodies with a maximum capture level and activity of 71.4 ($\pm$ 3.0)% for the non-labeled anti-AGP antibodies on the AGP microcolumn.

Table 2.Activity of anti-AGP antibodies or F_{ab} fragments after using various labeling conditions^a

Label & binding agent	Label used per binding agent (mol:mol)	Final label ratio (mol:mol)	Relative binding activity (%)	Reduction in activity due to labeling (%) ^b
NHS-Fluorescein,	280:1	4.2:1	5.7 (± 0.9)	92.0 (± 1.5)
anti-AGP F _{ab}	28:1	0.8:1	3.0 (± 1.8)	95.8 (± 2.8)
NHS-Fluorescein, anti-AGP antibodies	24:1	6.3:1	67.0 (± 1.5)	6.2 (± 4.5) ^c
LyCH, anti-AGP antibodies	200:1	2.2:1	71.7 (± 0.7)	-0.4 (± 4.4) ^c

^aThe numbers in parentheses represent ± 1 S.D. (*n* = 3).^bThe activity of the anti-AGP F_{ab} fragments before labeling was 65.1 (±3.8)%, and the activity of the anti-AGP antibodies before labeling was 71.4 (± 3.0)%.^cThese values were not significantly different at the 95% confidence level from those obtained for the unlabeled binding agents.

Table 3.Measured concentration of AGP in serum samples^a

Sample ^b	Conc. AGP (g L ⁻¹) - One-site immunometric assay	Relative conc. vs. normal serum - One-site immunometric assay	Relative conc. vs. normal serum - CE
Normal serum	1.17 (± 0.11)	1.00	1.00
SLE serum 1	1.77 (± 0.17)	1.51 (± 0.21)	1.49 (± 0.14)
SLE serum 2	1.77 (± 0.16)	1.51 (± 0.20)	1.21 (± 0.11)
SLE serum 3	3.71 (± 0.20)	3.18 (± 0.35)	2.44 (± 0.20)
SLE serum 4	2.77 (± 0.07)	2.37 (± 0.24)	1.71 (± 0.28)
SLE serum 5	2.28 (± 0.28)	1.96 (± 0.30)	1.12 (± 0.13)
SLE serum 6	2.34 (± 0.08)	2.00 (± 0.20)	1.15 (± 0.10)

^aThe numbers in parentheses represent ± 1 S.D. (*n* = 3).^bTerms: SLE, systemic lupus erythematosus.