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Analysis of Drug-Protein Binding using On-Line Immunoextraction and High-Performance Affinity Microcolumns: Studies with Normal and Glycated Human Serum Albumin

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Abstract

A method combining on-line immunoextraction microcolumns with high-performance affinity chromatography (HPAC) was developed and tested for use in examining drug-protein interactions with normal or modified proteins. Normal human serum albumin (HSA) and glycated HSA were used as model proteins for this work. High-performance immunoextraction microcolumns with sizes of 1.0–2.0 cm $\times$ 2.1 mm i.d. and containing anti-HSA polyclonal antibodies were developed and tested for their ability to bind normal HSA or glycated HSA. These microcolumns were able to extract up to 82–93% for either type of protein at 0.05–0.10 mL/min and had a binding capacity of 0.34–0.42 nmol HSA for a 1.0 cm $\times$ 2.1 mm i.d. microcolumn. The immunoextraction microcolumns and their adsorbed proteins were tested for use in various approaches for drug binding studies. Frontal analysis was used with the adsorbed HSA/glycated HSA to measure the overall affinities of these proteins for the drugs warfarin and gliclazide, giving comparable values to those obtained previously using similar protein preparations that had been covalently immobilized within HPAC columns. Zonal elution competition studies with gliclazide were next performed to examine the specific interactions of this drug at Sudlow sites I and II of the adsorbed proteins. These results were also comparable to those noted in prior work with covalently immobilized samples of normal HSA or glycated HSA. These experiments indicated that drug-protein binding studies can be carried out by using on-line immunoextraction microcolumns with HPAC. The same method could be used in the future with clinical samples and other drugs or proteins of interest in pharmaceutical studies or biomedical research.

Keywords

Immunoextraction; Affinity microcolumn; Drug-protein binding; Human serum albumin; Glycation; High-performance affinity chromatography

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1. Introduction

Drugs, low mass hormones, and fatty acids are commonly distributed throughout the body through their binding to transport proteins such as human serum albumin (HSA) [1]. HSA is the most abundant protein in plasma (normal concentration, 35–50 g/L) and accounts for approximately 60% of the total protein content in serum [1,2]. HSA has a molecular weight of 66.5 kDa and consists of a single polypeptide chain that has 585 amino acids [3,4]. There are two major binding sites on HSA, Sudlow sites I and II [1,3–6]. Sudlow site I is found in subdomain IIA of HSA and is known to bind to anticoagulant drugs such as warfarin and anti-inflammatory drugs such as azapropazone [1,3,7]. Sudlow site II is found in subdomain IIIA and binds to drugs such as ibuprofen, as well as the essential amino acid L-tryptophan [1,3,8].

Recent studies have shown that proteins like HSA can be affected by diseases such as diabetes [9–29]. Diabetes results in elevated levels of glucose in blood and can lead to the nonenzymatic glycation of proteins, which is the result of the addition of glucose to free amine groups on a protein. This reaction initially forms a reversible Schiff base, which can later rearrange to form a more stable Amadori product [11–16,30,31]. Modifications caused by glycation can occur at or near Sudlow sites I and II [3,27–30]. Patients with diabetes can have a 2- to 5-fold increase in the amount of HSA that is glycated when compared to healthy individuals [33]. Recent studies have also found that glycation can affect the binding of sulfonylurea drugs and other solutes with HSA [18–26].

High-performance affinity chromatography (HPAC) is a liquid chromatographic technique that utilizes an HPLC support and a biologically-related binding agent as the stationary phase [34–36]. HPAC and low-performance affinity separations have been frequently used for the separation, purification or analysis of specific analytes; however, these methods can also be utilized to examine drug-protein binding and other types of biological interactions [34–36]. For example, many previous studies have shown that drug-binding parameters that are measured by HPAC can be comparable to those obtained by solution-phase reference methods (e.g., ultrafiltration or equilibrium dialysis) [34–37]. It has also been found that HPAC can be used with covalently immobilized samples of normal HSA and glycated HSA to study the effects of glycation on the interactions of drugs or other solutes with these proteins [18–26]. A number of recent reports have further examined the use of HPAC with affinity microcolumns (i.e., columns with volumes in the low microliter range, and often with lengths of 1–5 cm or less) for the analysis of drug-protein interactions [23,37–45]. The advantages of using microcolumns for such work include their low back pressures (e.g., for short microcolumns) and compatibility with miniaturized systems; their need for only small amounts of binding agents, samples and reagents; their short analysis times (e.g., for high-throughput screening); and their ability to be used in some assay formats that are not possible with traditional-sized columns [23,37–45].

Immunoextraction is a type of affinity chromatography in which immobilized antibodies are used to isolate a given target from a sample [46]. Due to their high specificity and strong binding, antibodies are often used for the purification and isolation of biologically-related targets such as proteins, peptides, and hormones [46,47]. Antibodies have recently been used

with low-performance supports and a manual immunoextraction method for the selective isolation of normal HSA and glycated HSA from serum or plasma samples, such as those acquired from patients with diabetes. The isolated proteins were then eluted, collected and covalently immobilized within affinity microcolumns to examine their binding with sulfonylurea drugs [23].

This study will seek to develop and evaluate an approach in which immunoextraction microcolumns are coupled on-line with HPAC methods for the analysis of drug-protein interactions with normal or modified proteins (see scheme in Fig. 1). First, the desired protein (e.g., normal HSA or glycated HSA) will be applied to an immunoextraction microcolumn that contains antibodies against this protein (e.g., polyclonal anti-HSA antibodies). Next, the adsorbed proteins will be used to examine their interactions with applied solutions or samples of a given drug. Finally, after one or more experiments have been conducted with the microcolumn, the adsorbed protein can be eluted and the immunoextraction microcolumn regenerated prior to the application of a fresh sample of the same protein or of a related protein.

Several factors will be considered in the development and evaluation of this approach, with normal HSA and glycated HSA being used as model proteins for this work. For instance, the content, binding capacities and extraction efficiencies of the immunoextraction microcolumns will be characterized. This will be followed by the use of these microcolumns and their adsorbed proteins in various HPAC methods for studying drug-protein interactions. These methods will include frontal analysis (e.g., for studying the overall affinity and binding capacity of the adsorbed protein for a given drug) and zonal elution competition studies (e.g., to examine site-specific interactions of a drug with the protein) [34,35]. The information that is obtained from these experiments should make it possible to determine the advantages or limitations of this combined immunoextraction/HPAC method. Based on this data, it should also be possible in the future to modify this approach for work with clinical samples and with other proteins or modified binding agents that are of interest in pharmaceutical studies or biomedical research.

Experimental

2.1. Materials

The anti-HSA fractionated antiserum (goat, primarily immunoglobulin fraction, lyophilized; catalog no. A1151, batch SLBG8437V), goat immunoglobulin G (goat IgG; reagent grade, 95% purity, lyophilized), HSA (essentially fatty acid free, 96%), gliclazide (99.9%), racemic warfarin (98%), and L-tryptophan (98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nucleosil Si-1000 (7 μ m particle diameter, 300 Å particle size) was obtained from Macherey-Nagel (Duren, Germany). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). The Econo-Pac 10DG desalting columns were from Bio-Rad Laboratories (Hercules, CA, USA) and the Slide-A-Lyzer digest 7K dialysis cassettes (7 kDa MW cutoff; 0.5–3, 3–12 or 12–30 mL sample volumes) were from Thermo Scientific (Rockford, IL, USA) for use in purifying the *in vitro* glycated HSA samples. The fructosamine assay kit was purchased from Diazyme Laboratories (San Diego, CA, USA). All aqueous solutions were prepared using water from a NANOpure

system (Barnstead, Dubuque, IA, USA) or a Milli-QAdvantage A 10 system (EMD Millipore Corporation, Billerica, MA, USA) and were filtered through 0.20 μ m GNWP nylon membranes from Millipore.

2.2. Instrumentation

The chromatographic system consisted of a DC-2080 degasser, two PU-2080 pumps, an AS-2057 autosampler, a CO-2060 column oven, and a UV-2075 absorbance detector from Jasco (Tokyo, Japan). This system also included a Rheodyne Advantage PF six-port valve (Cotati, CA, USA). EZ Chrom Elite software v.3.21 (Scientific Software, Pleasanton, CA, USA) and Jasco ChromNav software were used to control the system. The chromatographic data were analyzed by using PeakFit 4.12 (Jandel Scientific Software, San Rafael, CA, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA). Non-linear regression was carried out by using DataFit 8.1.69 (Oakdale, PA, USA).

2.3. Preparation of immunoextraction microcolumns

Nucleosil Si-1000 was converted into a diol-bonded form, as described previously [48]. Polyclonal antibodies that had been isolated from anti-HSA goat antiserum (see Supplementary Material) were immobilized onto this support by using the Schiff base method [49,50]. A control support was prepared in the same manner but with no antibodies being added during the immobilization step. The final immunoextraction support was stored in pH 7.4, 0.067 M potassium phosphate buffer and at 4°C until further use.

A BCA assay was performed in triplicate to determine the protein content of the immunoextraction support, using goat IgG as a secondary standard and the control support as the blank. This assay was carried out on portions of the immunoextraction support and control support that had been placed into pH 7.4, 0.067 M potassium phosphate buffer. The average protein content measured for two batches of the immunoextraction support was 28 ($\pm$ 8) mg antibodies/g silica, where the values in parentheses throughout this paper represent $\pm$ 1 S.D.

The immunoextraction and control supports were downward slurry packed into separate 1.0 cm or 2.0 cm $\times$ 2.1 mm i.d. microcolumns at 4000 psi (28 MPa) using pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. These microcolumns were stored at 4°C in the same pH 7.4 buffer. Each microcolumn was used for 500 sample applications or less and was routinely washed with pH 7.4, 0.067 M potassium phosphate buffer.

2.4. Preparation of glycated HSA

The glycated HSA was prepared *in vitro* by using a previously published method (see Supplementary Materials) [22,51]. A portion of this preparation was analyzed by a fructosamine assay to determine the glycation level of the modified HSA [22]. The level of glycation was 3.20 ($\pm$ 0.13) mol hexose/mol HSA, which was representative of highly-glycated HSA; a similar preparation has been employed in prior binding studies that utilized *in vitro* glycated HSA [22].

2.5. Evaluation of immunoextraction microcolumns

All solutions and samples that were used in the chromatographic studies were prepared in pH 7.4, 0.067 M potassium phosphate buffer. This buffer was also used as the application buffer for the immunoextraction microcolumns and drug-protein binding studies. All drug solutions and mobile phases were filtered using a 0.2 μ M nylon filter and degassed for 10–15 min prior to use. The warfarin and gliclazide solutions were used within one or two weeks of preparation, respectively [20,43], and the L-tryptophan solutions were prepared fresh daily [54]. All chromatographic experiments and binding studies were conducted at 37°C.

The binding capacities for the immunoextraction microcolumns were determined by frontal analysis [34,35] using solutions containing 5.0 μ M normal HSA or glycated HSA in pH 7.4, 0.067 M potassium phosphate buffer. The immunoextraction microcolumns (1.0 cm $\times$ 2.1 mm i.d.) were first equilibrated with the pH 7.4 phosphate buffer at 0.10 mL/min. A switch was then made to apply a solution of normal HSA or glycated HSA at 0.10 mL/min for up to 20 min. This resulted in the formation of a breakthrough curve, which was monitored at 280 nm [34,35]. These experiments were performed in quadruplicate, with the central location of each breakthrough curve being determined by the equal area method [34,35]. A pH 2.5, 0.10 M potassium phosphate buffer was used to elute the adsorbed normal HSA or glycated HSA, and pH 7.4, 0.067 M potassium phosphate buffer was used to regenerate the microcolumn prior to additional studies. Similar experiments were performed on a control microcolumn to correct for the void time and for any non-specific binding of the normal HSA or glycated HSA to the system. This non-specific binding made up about 20–26% of the total binding that was measured for these proteins on the immunoextraction microcolumns.

The extraction efficiencies for the immunoextraction microcolumns (1.0 cm $\times$ 2.1 mm i.d.) were measured by injecting 20 μ L of 5.0 μ M normal HSA or glycated HSA (i.e., 6.67 μ g) at 0.05 to 0.50 mL/min in the presence of the pH 7.4 application buffer. This amount of injected protein was equivalent to that found in 0.13–0.19 μ L of undiluted serum containing 35–50 g/L HSA. The elution profiles were monitored at 280 nm. The mobile phase was later switched to pH 2.5, 0.10 M potassium phosphate buffer to elute the adsorbed normal HSA or glycated HSA from the microcolumn. The microcolumn was then regenerated with the pH 7.4, 0.067 M potassium phosphate buffer prior to the injection of the next protein sample. This process was also carried out on the control microcolumn. Similar injections using 20 μ L of pH 7.4, 0.067 M potassium phosphate buffer were performed to correct for the background response of the buffer. The elution profiles for the samples were fit to exponentially-modified Gaussian curves [34,35].

2.6. Chromatographic studies of drug-protein binding

Prior to the drug-protein binding studies, normal HSA or glycated HSA was applied to an immunoextraction microcolumn under the same conditions as described in Section 2.6 for the binding capacity measurements (i.e., application of 5 μ M normal HSA or glycated HSA at 0.10 mL/min for 20 min). The studies with warfarin used 1.0 cm $\times$ 2.1 mm i.d. immunoextraction and control microcolumns, while the work with gliclazide used longer 2.0 cm $\times$ 2.1 mm i.d. microcolumns due to the weaker binding of this latter drug vs. warfarin for

normal HSA and glycated HSA [7,20,22]. In the frontal analysis experiments, the immunoextraction microcolumns containing adsorbed normal HSA or glycated HSA were placed into pH 7.4, 0.067 M potassium phosphate buffer at 0.10 mL/min. A switch was then made to a solution prepared in the same pH 7.4 buffer but that contained a known concentration of the drug of interest (e.g., warfarin or gliclazide), which was also applied at 0.10 mL/min. Once a breakthrough curve had been formed and a stable plateau had been reached, pH 7.4, 0.067 M phosphate buffer alone was passed through the column at 0.25 mL/min to elute the retained drug. The flow rate was then returned to 0.10 mL/min prior to application of the next drug solution. The adsorbed protein was released and replaced on a regular basis during these experiments (as described in Section 2.6), which typically occurred after the application of 40–48 drug solutions to the microcolumn.

The frontal analysis studies used ten drug solutions containing 0.5–50 μ M warfarin or twelve solutions containing 0.5–200 μ M gliclazide. The elution of warfarin was monitored at 308 nm, and the elution of gliclazide was detected at 250 nm. All frontal analysis experiments were carried out in quadruplicate, with the central point of each breakthrough curve being determined by the equal area method [34,35]. The same solutions were also applied to a control column to correct for the void time and any non-specific binding by the drug to the system [18–22]. Non-specific binding made up only 4.6–6.6% of the total binding seen for a 50 μ M warfarin solution and 0.3–0.9% of the total binding seen for 200 μ M gliclazide when applied to the immunoextraction columns that contained adsorbed normal HSA or glycated HSA.

Competition studies based on zonal elution experiments were also conducted on the immunoextraction columns containing adsorbed samples of normal HSA or glycated HSA. These studies were performed in quadruplicate using 0–20 μ M gliclazide as the competing agent in the pH 7.4 application buffer. These solutions were applied to the columns at 0.1 mL/min for warfarin, which was used as a probe for Sudlow site I of normal HSA or glycated HSA, and at 0.05 mL/min for L-tryptophan, which was used as a probe for Sudlow site II [5–8]. The mobile phases containing gliclazide were also used to prepare 5 μ M samples of warfarin or L-tryptophan that were used for these injections. The injection volume was 20 μ L, and the warfarin or L-tryptophan was monitored at 308 or 280 nm, respectively. Sodium nitrate was injected as a non-retained solute and was monitored at 205 nm [34,35]. The central moment for each peak was found by using a fit to an exponentially-modified Gaussian model, along with the automatic baseline correction function of PeakFit v4.12 [34,35]. Similar injections were carried out using the control column to correct for the void time and any non-specific binding by the injected probes to the system (Note: both the warfarin and L-tryptophan had negligible binding to such columns, as noted previously) [7,8]. The adsorbed proteins were released and replaced on a regular basis (i.e., after 75 sample injections), using the conditions provided in Section 2.6.

Results and Discussion

3.1. Characterization of immunoextraction microcolumns

The binding capacities of the immunoextraction microcolumns were determined by carrying out frontal analysis with both normal HSA and highly-glycated HSA. An example of such

an experiment is shown in Fig. 2(a). The binding capacities that were measured ranged from 0.34–0.42 nmol protein (23–28 μ g) for a 1.0 cm $\times$ 2.1 mm i.d. immunoextraction microcolumn. The binding capacities that were obtained for normal HSA and glycosylated HSA were statistically equivalent at the 95% confidence level, indicating that both types of protein had similar binding to the immunoextraction microcolumns. This result agreed with prior observations made during the use of low-performance immunoextraction columns containing polyclonal anti-HSA antibodies for the off-line isolation of normal HSA and glycosylated HSA [23].

When these measured binding capacities were combined with the known protein content of the immunoextraction support, it was determined that 13–16% of the immobilized antibodies were able to bind to normal HSA and glycosylated HSA. This was not a surprise, because it is likely that many of the antibodies that were isolated from the fractionated goat antiserum by using protein G Sepharose were not actually directed against HSA. In addition, some of the anti-HSA antibodies in this preparation may have been inactive or inaccessible to normal HSA and glycosylated HSA after these antibodies had been immobilized [55]. However, the binding capacity that was obtained for this support was still sufficient for the initial design and testing of an immunoextraction/HPAC system for drug binding studies involving HSA. For instance, if this system were to be used with a clinical sample, the given binding capacity would be equivalent to the total amount of HSA that would be found in 0.45–0.80 μ L of normal serum [1,2].

The extraction efficiency was measured by injecting 0.10 nmol (or $\sim$ 6.6 μ g) of normal HSA or glycosylated HSA onto a 1.0 cm $\times$ 2.1 mm i.d. immunoextraction microcolumn or control microcolumn at various flow rates (see results in Fig. 2(b) and Table 1). The column back pressures that were generated during these experiments ranged from only 14 to 160 psi (0.1 to 1.1 MPa). When injecting 0.10 nmol of normal HSA, an extraction efficiency of 82–90% was seen at 0.05–0.10 mL/min, with this value decreasing to approximately 60–70% at 0.25–0.50 mL/min. A similar trend was observed for 0.10 nmol of glycosylated HSA, with an extraction efficiency of 93% being measured at 0.05–0.10 mL/min and this value decreasing to 79–90% at 0.25–0.50 mL/min. These results were consistent with those predicted when using an adsorption-limited binding model [55] and the known binding capacity of this column, along with an association rate constant of $4.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ that has been reported for normal HSA in its binding to a comparable preparation of immobilized, polyclonal anti-HSA antibodies [56].

The results of these binding capacity and extraction efficiency experiments were used to select the conditions employed in the remainder of this study to apply solutions of normal HSA or glycosylated HSA to the immunoextraction microcolumns. This was accomplished by applying 10 nmol of these proteins to 1.0–2.0 cm $\times$ 2.1 mm i.d. immunoextraction microcolumns at 0.10 mL/min. These conditions provided good extraction efficiencies for these proteins while also ensuring that the immobilized antibodies were essentially saturated with normal HSA or glycosylated HSA. Under these conditions, the amount of protein that was applied to each immunoextraction microcolumn was 12–29 times the column binding capacity, with the application of the protein requiring 20 min at 0.10 mL/min. However, the

data obtained in this report indicate that smaller amounts of protein or higher application flow rates could also have been used for this process.

The immunoextraction microcolumns showed good stability under the application and elution conditions that were used in this report. It has been found in a number of previous studies that columns containing similar polyclonal anti-HSA antibodies can be eluted and regenerated for at least 120 cycles when using a pH 2.5–3.0 buffer for elution and a pH 7.0–7.4 application buffer [47,55,56]. Although the application of a large number of protein samples was not required in this current report (i.e., with up to 16 protein application/elution cycles being used per column), similar stability was noted for the immunoextraction microcolumns that were used to adsorb the normal HSA or glycated HSA. This was reflected in the fact that only random variations of ± 14 –16% (1 S.D.) were seen in the total binding capacities of the immunoextraction microcolumns over four application/elution cycles.

It was also observed that there was good reproducibility between these immunoextraction columns from one application of a protein to the next. For instance, binding studies conducted with warfarin on several immunoextraction microcolumns that contained normal HSA showed only random variations of 1.2–9.4% in this drug's retention under equivalent experimental conditions. As will be shown later, the stability of the adsorbed proteins was further reflected in the precision of the binding parameters that were obtained during the use of these adsorbed proteins over multiple applications or injections of drug solutions or samples.

In addition to the use of immunoextraction microcolumns for this research, the ability to reuse the adsorbed proteins within these microcolumns served to minimize the amount of these agents that was required for the drug binding studies. As an example, each application of an adsorbed protein to an immunoextraction microcolumn was used for analyzing 40–48 drug solutions during the frontal analysis experiments or 75 injections of drugs or other solutes during the zonal elution competition studies, as described in the following sections. For an immunoextraction microcolumn that contained 0.34–0.42 nmol of adsorbed HSA, this was the equivalent of using 7–11 pmol of protein per experiment in the frontal analysis studies and 4–6 pmol of protein per experiment in the zonal elution experiments.

3.2. Frontal analysis studies with warfarin

The immunoextraction microcolumns and their adsorbed proteins were next tested for use in frontal analysis-based studies of drug-protein binding. These experiments were first carried out by using warfarin, which is a drug that has well-characterized binding at a single major site on HSA (i.e., Sudlow site I) [1,3,7]. This drug was useful as a model because frontal analysis experiments have previously been conducted with this drug on columns containing covalently immobilized preparations of normal HSA or glycated HSA [7,22].

Fig. 3(a) shows some frontal analysis results that were obtained for warfarin on a 1.0 cm $\times$ 2.1 mm i.d. immunoextraction microcolumn that contained normal HSA. Each application of this drug to the microcolumns provided a breakthrough curve within 5–8 min at 0.10 mL/min. The results were then fit to a binding model to estimate the number of interaction sites

that were present between this drug and the adsorbed proteins, as well as the association equilibrium constants for these interactions.

In the case of a single-site interaction, as occurs between warfarin and HSA [7,22], Eqs. 1 or 2 can be used to describe the binding of the drug or analyte (A) to an immobilized protein or ligand (L) [34,35].

$$m_{Lapp} = \frac{m_L K_a [A]}{(1 + K_a [A])} \quad (1)$$

$$\frac{1}{m_{Lapp}} = \frac{1}{(K_a m_L [A])} + \frac{1}{m_L} \quad (2)$$

In these equations, m_{Lapp} represents the apparent moles of the applied analyte that are required to reach the central point of the breakthrough curve at a given concentration of the analyte, [A]. The term K_a is the association equilibrium constant for this interaction, and m_L is the total moles of binding sites for A that are present in the column.

Fig. 3(b) shows an example of the fit of Eq. 2 to frontal analysis data that were obtained for warfarin on immunoextraction microcolumns that contained adsorbed HSA. These particular results are for normal HSA, but similar behavior was seen for glycosylated HSA. A linear fit to Eq. 2 was obtained, as would be expected for a system with a 1:1 interaction [34,35,57], resulting in correlation coefficients between 0.9970 ($n = 6$) and 0.9979 ($n = 6$). The same behavior has been noted for warfarin with covalently immobilized normal HSA and glycosylated HSA [7,22].

Table 2 summarizes the binding parameters that were obtained by fitting these data to Eq. 2. Each set of binding parameters was obtained when using multiple applications of warfarin and a single application of normal HSA or glycosylated HSA on an immunoextraction microcolumn. The association equilibrium constant of $2.4 (\pm 0.4) \times 10^5 \text{ M}^{-1}$ that was determined by this approach for normal HSA was statistically identical, at the 95% confidence level, to an average association equilibrium constant of $2.4 (\pm 0.4) \times 10^5 \text{ M}^{-1}$ that has been previously reported for the binding of racemic warfarin to this protein [7,22]. In a previous report it was found that levels of protein glycosylation similar to those used in this study did not have any appreciable effect on the association equilibrium constants for warfarin with HSA [22]; this also agrees with the data in Table 2 for glycosylated HSA versus normal HSA.

The amount of active binding sites for warfarin on the immunoextraction microcolumns containing adsorbed HSA was also estimated from the frontal analysis data. These values were $2.4 (\pm 0.1) \times 10^{-10}$ and $2.8 (\pm 0.1) \times 10^{-10} \text{ mol}$ for the normal HSA and glycosylated HSA, respectively. The specific activities of these adsorbed proteins for warfarin were found by combining the measured binding capacities with the total amount of adsorbed HSA that was present. These specific activities ranged from 0.48–0.71 mol/mol for the normal HSA and glycosylated HSA, and were similar to values that have been obtained with covalently

immobilized preparations of normal HSA or glycated HSA in prior frontal analysis experiments [22].

The relative precision of the K_a values that were determined for warfarin on the immunoextraction microcolumns and for a single application of normal HSA or glycated HSA was ± 15 –17%. This level of variation was only slightly higher than precisions of ± 8.6 –11% that have been reported when using more traditional HPAC columns and covalently immobilized samples of HSA [7,22]. The binding capacities that have been obtained with these other columns have had relative precisions of ± 8.7 –10.1% [7,22], which also compared well with the relative precisions of ± 3.5 –4.2% that were obtained for the same parameter in this current study. However, one advantage of using HSA that had been adsorbed to an immunoextraction microcolumn, instead of being covalently immobilized, was this protein could be eluted periodically and replaced with a fresh protein sample for use in further drug binding studies. In addition, the use of immunoextraction for protein immobilization made it possible to use a single microcolumn to study more than one type of protein (e.g., normal HSA vs. glycated HSA).

3.3. Frontal analysis studies with gliclazide

Gliclazide was another drug that was used to evaluate the utilization of immunoextraction microcolumns and adsorbed proteins in drug binding studies. This drug and related sulfonylurea drugs have been shown previously to have two classes of binding sites with normal HSA and glycated HSA: 1) a set of well-defined and moderate-to-high affinity sites and 2) a larger set of weaker affinity regions [18–22]. This feature made gliclazide attractive as a second model drug for testing the immunoextraction/HPAC approach that was developed in this report.

This type of drug-protein system can be examined by frontal analysis and relationships similar to those in Eqs. 1 and 2, but which describe interactions that involve multiple binding sites. An example of such an equation is provided in Eq. 3 for a system with two groups of independent binding sites [57].

$$m_{Lapp} = \frac{m_{L1} K_{a1} [A]}{(1 + K_{a1} [A])} + \frac{m_{L2} K_{a2} [A]}{(1 + K_{a2} [A])} \quad (3)$$

The terms m_{L1} and m_{L2} in Eq. 3 represent the moles of the higher and lower affinity sites in the column, respectively, while K_{a1} and K_{a2} are the association equilibrium constants for these sites. The term m_{Ltot} is the summation of the moles of all available binding sites for the applied analyte.

Fig. 4 shows some typical frontal analysis results that were acquired for gliclazide on a 2.0 cm $\times$ 2.1 mm i.d. immunoextraction microcolumn containing normal HSA. Similar results were obtained for glycated HSA. These data were fit to both one-site and two-site models to test for the presence of multi-site interactions. In each case, the two-site model gave a better description of the results. For instance, the correlation coefficient was 0.9970 ($n = 12$) when a single-site model was fit to the data for normal HSA, while a two-site model gave a correlation coefficient of 0.9999. The residual plot for the two-site model produced a more

random distribution of the data about the best-fit line than was seen for the one-site model (see insets in Fig. 4), and the sum of the squares of the residuals were significantly smaller, at the 95% confidence level, for the two-site model (1.3×10^{-23} vs. 1.9×10^{-21}). The results for glycosylated HSA also gave a larger correlation coefficient for a fit to a two-site model as opposed to a one-site model (0.9999 vs. 0.9990, $n = 12$), a more random distribution of the results about the best-fit line, and a lower sum of the squares of the residuals for the two-site model (2.4×10^{-23} vs. 5.7×10^{-22} for the one-site model). This behavior was consistent with that seen in previous frontal analysis studies that examined the binding of gliclazide with covalently immobilized HSA [20].

Table 3 summarizes the binding parameters that were obtained for gliclazide with normal HSA and glycosylated HSA when using a two-site model. For normal HSA, association equilibrium constants of $4.1 (\pm 0.5) \times 10^4 \text{ M}^{-1}$ and $4.2 (\pm 2.9) \times 10^2 \text{ M}^{-1}$ were estimated for gliclazide at its highest affinity sites and lower affinity regions. Glycosylated HSA gave association equilibrium constants of $4.9 (\pm 1.4) \times 10^4 \text{ M}^{-1}$ and $3.6 (\pm 0.6) \times 10^3 \text{ M}^{-1}$ for gliclazide at these two groups of sites. The value of K_{a1} for the high affinity sites was also estimated by using the linear portion of a double-reciprocal plot of $1/m_{Lapp}$ and $1/[A]$ (see Supplementary Material) [57]. This second approach gave an association equilibrium constant of $4.1 (\pm 1.4) \times 10^4 \text{ M}^{-1}$ for gliclazide at its highest affinity sites on normal HSA, and a K_{a1} of $3.9 (\pm 1.1) \times 10^4 \text{ M}^{-1}$ for this drug with glycosylated HSA. These results were comparable to those from an earlier study involving more traditional HPAC columns and covalently immobilized proteins, which produced estimates of $3.4\text{--}10.0 \times 10^4 \text{ M}^{-1}$ for the average association equilibrium constant of the higher affinity sites for gliclazide with normal HSA and glycosylated HSA [20]. The agreement in these results further indicated that immunoextraction microcolumns and adsorbed samples of proteins like HSA could be used with frontal analysis to characterize drug-protein interactions.

The specific activity for gliclazide was also determined by combining the amount of each adsorbed protein with the measured binding capacity of this protein for gliclazide. In previous work with covalently immobilized samples of normal HSA or glycosylated HSA, specific activities ranging from 0.38–0.50 mol/mol HSA were obtained for gliclazide at its highest affinity sites on this protein [20]. These specific activities were consistent with the values of $0.52 (\pm 0.06)$ and $0.25 (\pm 0.08)$ mol/mol that were acquired when using the immunoextraction microcolumns and adsorbed normal HSA or glycosylated HSA.

The precisions of the binding parameters that were measured for gliclazide by using frontal analysis and adsorbed HSA were also compared to the precisions that were obtained for these parameters when using covalently immobilized HSA [20]. The association equilibrium constants that were determined through the use of immunoextraction microcolumns and adsorbed HSA had relative precisions of $\pm 12\text{--}29\%$ for the high affinity site and $\pm 17\text{--}69\%$ for the lower affinity regions of gliclazide. These values were similar to precisions of $\pm 8\text{--}27\%$ and $\pm 17\text{--}68\%$, respectively, that have been noted for the same drug when using covalently immobilized HSA [20]. The immunoextraction/HPAC method had precisions of $\pm 11\text{--}27\%$ and $\pm 10\text{--}60\%$ for the amount of high and lower affinity sites that were measured for gliclazide on normal HSA or glycosylated HSA, which were also comparable to precisions of

$\pm 31\text{--}68\%$ and $\pm 4\text{--}15\%$ that have been obtained when using covalently immobilized HSA [20].

3.4. Zonal elution competition studies

Another method that was tested for use with the immunoextraction/HPAC method was a zonal elution competition study. This type of experiment has been used with HPAC columns made through covalent immobilization to examine site-specific changes in drug-protein interactions, including changes that may occur due to HSA glycation [18–22]. For instance, previous competition studies using gliclazide and other sulfonylurea drugs and have found that these drugs can bind to both Sudlow sites I and II of normal HSA and glycated HSA, by using warfarin as a probe for Sudlow site I and L-tryptophan as a probe for Sudlow site II [18–22].

In a zonal elution competition study, a small amount of a probe for a given binding site (e.g., warfarin or L-tryptophan) is injected onto a column that contains the protein or binding agent of interest. These injections are made in the presence of various known concentrations of a possible competing agent (e.g., gliclazide) in the mobile phase. The retention factor (k) of the probe is then measured at each competing agent concentration and used to determine whether the probe and competing agent have a common binding site in the column [34,35].

If direct competition exists between the competing agent (I) and the probe (A), a decrease in the retention factor for the probe should occur as the concentration of the competing agent is increased. This change in retention is a function of the concentration of the competing agent, as is indicated in Eq. 4 for a system in which A and I have a single site of competition and A has no other types of binding sites in the column [34,35].

$$\frac{1}{k} = \frac{K_{aI}V_M[I]}{K_{aA}m_L} + \frac{V_M}{K_{aA}m_L} \quad (4)$$

In Eq. 4, the association equilibrium constants for the probe and the competing agent at their site of competition are given by the terms K_{aA} and K_{aI} . The term V_M represents the column void time, and m_L is the moles of the common binding sites in the column. Eq. 4 predicts that a system with 1:1 competition should give a linear relationship between $1/k$ and $[I]$. In addition, the ratio of the slope over the intercept for the best-fit line can be used to obtain the value of K_{aI} at the site of competition [34,35].

As is shown in Fig. 5, linear fits were obtained to Eq. 4 when $2.0\text{ cm} \times 2.1\text{ mm}$ i.d. immunoextraction microcolumns and adsorbed normal HSA or glycated HSA were used in zonal elution competition studies involving gliclazide as the analyte and warfarin or L-tryptophan as probes for Sudlow sites I and II. The best-fit lines in the competition studies with warfarin and gliclazide had correlation coefficients of 0.9787 ($n = 6$) and 0.9855 ($n = 7$) for normal HSA and glycated HSA. The best-fit lines for the experiments performed with L-tryptophan and gliclazide gave coefficient coefficients of 0.9945 ($n = 8$) and 0.9937 ($n = 7$) for the same protein preparations. The residual plots gave random variations in the data about the best-fit lines, and the sum of the squares of the residuals ranged from 0.010–0.034 for the experiments performed with warfarin and from 1.90–3.37 for the experiments

conducted with L-tryptophan. This linear behavior was similar to that seen with the same probes and gliclazide or other sulfonylurea drugs on columns containing covalently immobilized normal HSA or glycated HSA [18–22].

Table 4 lists the association equilibrium constants that were determined for gliclazide at Sudlow sites I and II in these competition studies. The association equilibrium constant measured for gliclazide with normal HSA or glycated HSA at Sudlow site I were in the range of $2.5\text{--}3.1 \times 10^4 \text{ M}^{-1}$ and the values at Sudlow site II ranged from $7.7\text{--}8.1 \times 10^4 \text{ M}^{-1}$. These results were similar to values of $2.1\text{--}3.6 \times 10^4 \text{ M}^{-1}$ and $3.8\text{--}7.6 \times 10^4 \text{ M}^{-1}$ that have been obtained at Sudlow sites I and II with covalently immobilized samples of normal HSA and glycated HSA with modification levels similar to those used in this study [20]. This information indicated that immunoextraction microcolumns and adsorbed proteins could also be successfully employed in zonal elution studies for the analysis of drug binding at specific sites on a protein such as HSA.

4. Conclusion

This report examined the combined use of immunoextraction microcolumns and HPAC for studying drug-protein interactions. Normal HSA and glycated HSA were used as model proteins to test this approach, while warfarin and gliclazide were used as model drugs. Immunoextraction microcolumns containing polyclonal anti-HSA antibodies were prepared that had extraction efficiencies up to 82–93% for normal HSA and glycated HSA (at 0.05–0.10 mL/min) and binding capacities of 0.34–0.42 nmol for a 1.0 cm $\times$ 2.1 mm i.d. microcolumn. These immunoextraction microcolumns were also found to be quite stable when used with adsorbed HSA in drug-protein binding studies.

Frontal analysis experiments were conducted with both warfarin and gliclazide and using adsorbed samples of normal HSA or glycated HSA on the immunoextraction microcolumns. Each of these model systems gave similar binding behavior and association equilibrium constants to those that have been obtained by using more conventional HPAC columns with covalently immobilized proteins [7,20,22]. Zonal elution studies were used to further examine the site-specific binding of gliclazide with adsorbed samples of normal HSA and glycated HSA at Sudlow sites I and II. These association equilibrium constants were also in the same range as previous values measured for covalently immobilized normal HSA or glycated HSA [20].

The use of immunoextraction microcolumns for this work offered several potential advantages over columns containing covalently immobilized proteins. First, it was possible for adsorbed proteins in the immunoextraction microcolumns to be eluted and replaced with a fresh protein sample, as needed. In addition, the same immunoextraction microcolumn could be utilized to examine drug interactions with more than one type of protein, as was demonstrated for normal HSA and glycated HSA. This method does require separate steps for periodically applying and eluting the adsorbed protein. However, it was found that a single application of a protein to the immunoextraction microcolumn could be used for many experiments (e.g., at least 40–48 frontal analysis or 75 zonal elution studies for adsorbed samples of HSA). This last feature helped minimize the amount of protein that was

required for such work, with the equivalent of only 4–11 pmol HSA being used in this report per experiment. The same approach could be applied to alternative drug-protein systems by using other types of antibodies to prepare the immunoextraction microcolumns. Future work will also consider the extension of this method to proteins that have been directly adsorbed from clinical samples and as a tool for biomedical research in areas such as personalized medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Immunoextraction microcolumns were used to study drug-protein interactions.
- Human serum albumin (HSA) and glycated HSA were used as model proteins.
- The microcolumns were used in both frontal analysis and zonal elution formats.
- Good agreement was seen with prior results obtained by more traditional columns.
- This approach can be adapted for work with other proteins and drugs.

Figure 1. General scheme for studying drug-protein interactions through the use of proteins that are adsorbed to immunoextraction microcolumns.

Figure 2.

Typical chromatograms obtained for (a) frontal analysis studies and (b) extraction efficiency experiments performed on 1.0 cm $\times$ 2.1 mm i.d. immunoextraction microcolumns containing anti-HSA polyclonal antibodies (dashed line) or a microcolumn containing an inert, control support (solid line). The results in (a) were obtained for a 5 μ M solution of normal HSA that was applied at 0.10 mL/min. These results in (b) are for 20 μ L solutions containing 5 μ M of normal HSA and that were injected at 0.05 mL/min.

Figure 3.

(a) Typical frontal analysis chromatograms obtained for various concentrations of warfarin that were applied at 0.10 mL/min to a 1.0 cm $\times$ 2.1 mm i.d. immunoextraction microcolumn containing adsorbed normal HSA, and (b) a plot prepared according to Eq. 2 to examine the data that resulted from such studies. The error bars for each data point in (b) represent ± 1 S.D. for the average of four replicate experiments.

Figure 4.

Fit of frontal analysis data, obtained for gliclazide on immunoextraction microcolumns containing adsorbed normal HSA, when analyzed by (a) a single-site binding model based on Eq. 1 or (b) a two-site binding model based on Eq. 3. The insets show the corresponding residual plots, where each residual value is the difference between the experimental value of m_{Lapp} and the predicted value based on the best-fit line. Each point represents the average of four experiments in which the typical relative standard deviations ranging from 3.6 to 11.5% (average, $\pm 7.5\%$).

Figure 5.

Results for zonal elution competition studies between gliclazide and (a) warfarin or (b) L-tryptophan as injected probes on immunoextraction microcolumns that contained adsorbed normal HSA ($\blacklozenge$) or glycated HSA ($\blacksquare$). These data were fit to a direct competition model, as described by Eq. 4. The error bars for each data point represent ± 1 S.D. for the average of four replicates in each experiment.

Table 1

Extraction efficiencies for normal HSA and glycated HSA on immunoextraction microcolumns containing polyclonal anti-HSA antibodies^a

Flow rate (mL/min)	Capture efficiency (%)	
	Normal HSA	Glycated HSA
0.05	90 (± 5)	93 (± 1)
0.10	82 (± 1)	93 (± 1)
0.25	70 (± 8)	90 (± 1)
0.50	60 (± 2)	79 (± 1)

^aThese results were obtained at 37 °C and in the presence of pH 7.4, 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ± 1 S.D. (*n* = 4).

Table 2

Association equilibrium constants (K_a) and moles of binding sites (m_L) measured for warfarin with normal HSA or glycated HSA adsorbed onto immunoextraction microcolumns containing polyclonal anti-HSA antibodies^a

<i>Type of HSA</i> ^b	K_a ($\times 10^5 \text{ M}^{-1}$)	m_L ($\times 10^{-10} \text{ mol}$)
Normal HSA	2.4 (± 0.4)	2.4 (± 0.1)
Glycated HSA	2.0 (± 0.3)	2.8 (± 0.1)

^aThe results were measured at 37 °C in the presence of pH 7.4, 0.067 M potassium phosphate buffer and were obtained from double-reciprocal plots that were analyzed according to a single-site binding model, as described by Eq. 2. The values in parentheses represent a range of ± 1 S.D., as based on error propagation and the precisions of the best-fit slopes and intercepts that were obtained when using Eq. 2 ($n = 6$).

^bThe amount of adsorbed normal HSA was 0.34 (± 0.06) nmol. The amount of adsorbed glycated HSA was 0.42 (± 0.06) nmol, with this protein preparation having a glycation level of 3.20 (± 0.13) mol hexose/mol HSA.

Table 3

Association equilibrium constants (K_d) and moles of binding sites (m_L) obtained for gliclazide on immunoextraction microcolumns containing adsorbed normal HSA or glycated HSA^a

<i>Type of HSA</i> ^b	$K_{d1} (\times 10^4 \text{ M}^{-1})$	$m_{L1} (\times 10^{-11} \text{ mol})$	$K_{d2} (\times 10^2 \text{ M}^{-1})$	$m_{L2} (\times 10^{-9} \text{ mol})$
Normal HSA	4.1 (± 0.5)	1.8 (± 0.2)	4.2 (± 2.9)	5.2 (± 3.1)
Glycated HSA	4.9 (± 1.4)	11 (± 3)	36 (± 6)	1.0 (± 0.1)

^aThe results were measured at 37 °C in the presence of pH 7.4, 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ± 1 S.D., as based on error propagation and the precisions of the best-fit slopes and intercepts that were obtained when using Eq. 3 ($n = 12$).

^bThe properties of these protein samples were the same as listed in Table 2.

Table 4

Association equilibrium constants (K_a) measured for gliclazide at Sudlow sites I and II on immunoextraction microcolumns containing adsorbed normal HSA or glycated HSA^a

Type of HSA ^b	K_a ($\times 10^4$ M ⁻¹)	
	Sudlow site I	Sudlow site II
Normal HSA	3.1 (± 0.3)	8.1 (± 0.4)
Glycated HSA	2.5 (± 0.2)	7.7 (± 0.4)

^aThese results were measured at 37 °C in the presence of pH 7.4, 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ± 1 S.D., as based on error propagation and the precisions of the best-fit slope and intercepts that were obtained when using Eq. 4 ($n = 6-8$).

^bThe properties of these protein samples were the same as listed in Table 2.