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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 × 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 × 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.

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 non-enzymatic 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 [11–16,30–32]. 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

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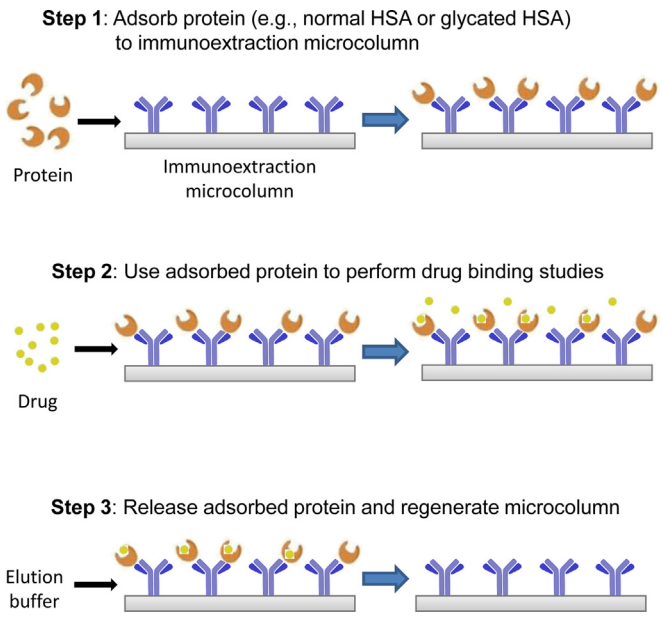

Fig. 1. General scheme for studying drug–protein interactions through the use of proteins that are adsorbed to immunoextraction microcolumns.

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.

2. 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 μm 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 μm 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, Pleasonton, 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). Nonlinear regression was carried out by using DataFit 8.1.69 (Oakdale, PA, USA).