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Analysis of multi-site drug–protein interactions by high-performance affinity chromatography: Binding by glimepiride to normal or glycated human serum albumin

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ABSTRACT

High-performance affinity chromatography (HPAC) was used in a variety of formats to examine multi-site interactions between glimepiride, a third-generation sulfonylurea drug, and normal or *in vitro* glycated forms of the transport protein human serum albumin (HSA). Frontal analysis revealed that glimepiride interacts with normal HSA and glycated HSA at a group of high affinity sites (association equilibrium constant, or K_a, 9.2–11.8 × 10^5 M^{-1} at pH 7.4 and 37 °C) and a group of lower affinity regions (K_a, 5.9–16 × 10^3 M^{-1}). Zonal elution competition studies were designed and carried out in both normal- and reversed-role formats to investigate the binding by this drug at specific sites. These experiments indicated that glimepiride was interacting at both Sudlow sites I and II. Allosteric effects were also noted with *R*-warfarin at Sudlow site I and with tamoxifen at the tamoxifen site on HSA. The binding at Sudlow site I had a 2.1- to 2.3-fold increase in affinity in going from normal HSA to the glycated samples of HSA. There was no significant change in the affinity for glimepiride at Sudlow site II in going from normal HSA to a moderately glycated sample of HSA, but a slight decrease in affinity was seen in going to a more highly glycated HSA sample. These results demonstrated how various HPAC-based methods can be used to profile and characterize multi-site binding by a drug such as glimepiride to a protein and its modified forms. The information obtained from this study should be useful in providing a better understanding of how drug–protein binding may be affected by glycation and of how separation and analysis methods based on HPAC can be employed to study systems with complex interactions or that involve modified proteins.

1. Introduction

The sulfonylureas are a class of drugs that are commonly used to treat type II diabetes. These drugs stimulate the secretion of insulin from beta cells in the pancreas to alleviate elevated levels of glucose in the blood stream [1]. These drugs are often divided into groups such as "first-generation" and "second-generation", which differ in their effectiveness for treatment and their ability to be metabolized by the body [2–4]. Glimepiride (Fig. 1) is a third-generation sulfonylurea drug that can be used at even lower dosages than second-generation drugs like gliclazide and glibenclamide [1]. The effectiveness of glimepiride is similar to that of glibenclamide; however, glimepiride can be taken only once daily, while glibenclamide and other sulfonylurea drugs are administered 1–2 times per day [1].

First- and second-generation sulfonylurea drugs are known to bind to and be transported by human serum albumin (HSA), the most abundant protein in blood plasma [5–13]. Such binding is an important function of HSA, which aids in the transportation of many endogenous and exogenous substances throughout the body (e.g., drugs, low mass hormones, and fatty acids) [14–19]. Sudlow sites I and II are the two main binding sites for drugs on HSA [14,20,21]. Bulky heterocyclic anionic drugs such as warfarin, azapropazone, phenylbutazone, and salicylate tend to bind at Sudlow site I [14,17,20,22]. Ibuprofen, ketoprofen, benzodiazepines, and L-tryptophan are examples of drugs and solutes that bind to Sudlow site II [14,17,20,23]. There are some additional sites on HSA that have been reported for drugs such as tamoxifen and digitoxin (i.e., the tamoxifen and digitoxin sites) [24–26]. A number of first- and second-generation sulfonylurea drugs have been reported to bind to Sudlow sites I and II; glibenclamide has also been found to bind at the digitoxin site [4–7,9–13].

Several recent reports have found that the binding of sulfonylurea drugs to HSA can be affected by non-enzymatic glycation

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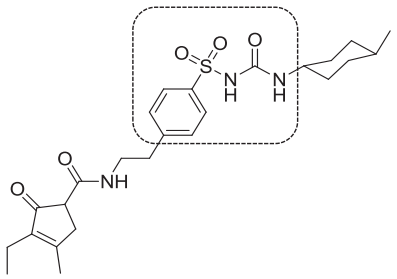

Fig. 1. Structure of glimepiride. The section in the dashed box shows the core structure of a sulfonylurea drug.

[6,7,9–13,27,28]. Glycation occurs when glucose reacts with free amine groups on a protein such as HSA [16,29–33]. A Schiff base is initially formed by this reaction but can rearrange to form a stable Amadori product, or ketoamine [16,29–33]. There is roughly a 2- to 5-fold increase in the amount of glycated HSA in patients with diabetes versus normal individuals [34]. Studies based on mass spectrometry have shown that some of these glycation-related modifications can occur at or near Sudlow sites I and II [13,35–37].

The goal of this study is to investigate the possible multi-site binding of glimepiride to HSA and *in vitro* glycated HSA through the use of high-performance affinity chromatography (HPAC). HPAC is a liquid chromatographic technique that utilizes an immobilized biological molecule (e.g., HSA) as the stationary phase [38]. One application of HPAC is as a tool for studying biological interactions [27,28,38–45]. In the use of HPAC to study drug interactions with normal HSA, it has been frequently noted that the binding parameters that can be obtained are comparable to those of traditional solution-phase techniques or reference methods (e.g., equilibrium dialysis and ultrafiltration) [38–47]. It has also been found recently that HPAC can be used to profile drug interactions with glycated HSA [6–13].

Glimepiride has a limited solubility in an aqueous solution (e.g., <1 mg/L in water) [48]. Previous work based on fluorescence spectroscopy has used relatively non-polar solvents (e.g., 2.5–10% dimethyl sulfoxide) to make it possible to investigate the interactions of this drug with normal HSA [49–51]. This current report will use HPAC to examine these interactions directly in aqueous solutions and at a physiological pH, while also expanding such studies to see how glycation affects these binding processes. A variety of HPAC methods, including frontal analysis and both normal- and reversed-role zonal elution competition studies, will be developed and used to examine the overall binding and interactions for glimepiride on normal HSA and glycated HSA. Some of these experiments will be further used to provide a detailed depiction of the interactions, including positive or negative allosteric effects, for glimepiride with probes for specific sites on HSA. A comparison between the binding by glimepiride with normal HSA and glycated HSA will also be made, as well as with previous binding parameters that have been reported for first- and second-generation sulfonylurea drugs to similar protein preparations [5–7,9–13,27]. These experiments should provide a more complete picture of how glimepiride and sulfonylurea drugs interact with HSA and of how glycation may affect these processes. In addition, the results obtained for glimepiride with various separation and analysis formats based on HPAC should provide useful information on how similar tools might be used in examining additional multi-site interactions involving other classes of drugs and normal or modified proteins.

2. Experimental

2.1. Chemicals

Glimepiride (≥96% pure) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The racemic warfarin (≥99%), *R*-warfarin (≥97%), L-tryptophan (≥97%), digitoxin (≥97%), tamoxifen (≥99%), β-cyclodextrin (>98%), D-(+)-glucose (≥99.5%), sodium azide (95%), and HSA (essentially fatty acid free, ≥96%) were from Sigma–Aldrich (St. Louis, MO, USA). Nucleosil Si-300 (7 μm particle diameter, 300 Å pore size) was purchased from Macherey–Nagel (Düren, Germany). *In vitro* glycated HSA samples were purified by using Econo-Pac 10DG desalting columns from Bio-Rad Laboratories (Hercules, CA, USA) and Slide-A-Lyzer digest 7K dialysis cassettes (7 kDa MW cutoff; 0.5–3, 3–12 or 12–30 mL sample volumes) from Thermo Scientific (Rockford, IL, USA). A fructosamine assay kit (Diazyme Laboratories, San Diego, CA, USA), was used to measure the modification levels of the *in vitro* glycated HSA samples. A bicinchoninic acid (BCA) protein assay was used to determine the protein content of the chromatographic supports; the reagents for this assay were obtained from Pierce (Rockford, IL, USA). All aqueous solutions were prepared in water purified by a Milli-Q-Advantage A 10 system (EMD Millipore Corporation, Billerica, MA, USA). The same solutions were filtered through 0.20 μm GNWP nylon membranes from EMD Millipore.

2.2. Apparatus

The HPLC system contained two PU-2080 pumps, a DG-2080 degasser, an AS-2057 autosampler, a CO-2060 column oven, and an UV-2075 absorbance detector from Jasco (Tokyo, Japan). This system also included a Rheodyne Advantage PF six-port valve (Cotati, CA, USA). Jasco LC Net and ChromNav software were used to control the system. The chromatograms were analyzed by using Peakfit 4.12 software (Jandel Scientific Software, San Rafael, CA, USA). DataFit 8.1.69 (Oakdale, PA, USA) was used for data analysis by non-linear regression.

2.3. In vitro *glycation of HSA*

In vitro glycated HSA was prepared at physiological concentrations of HSA and glucose, as described previously [8,52,53], to prepare samples that were representative of glycation levels found in patients with prediabetes or confirmed diabetes. These two samples will be referred to as "gHSA1" and "gHSA2", respectively. To prevent bacterial growth during the glycation process, all materials (e.g., glassware and spatulas) were first sterilized in an autoclave. A pH 7.4, 0.20 M potassium phosphate buffer for use in this procedure was prepared that contained 1 mM sodium azide. This buffer was also sterilized in an autoclave to prevent bacterial growth.

The *in vitro* glycated HSA was prepared by adding 840 mg of normal HSA to either a solution containing 15 mM glucose (for gHSA1) or 30 mM glucose (for gHSA2) that was prepared in the sterile pH 7.4, 0.20 M phosphate buffer. The final HSA concentration of the glycated solutions was 42 mg/L of HSA. These mixtures were then incubated for 4 weeks at 37 °C. The protein samples were later purified through the use of size exclusion chromatography by using desalting columns and pH 7.4, 0.067 M potassium phosphate buffer to remove the excess glucose [8]. The collected samples were dialyzed against water using a volume that was 200–500 times the volume of sample to remove any remaining glucose or phosphate salts [8]. The resulting protein solutions were then lyophilized and stored at −80 °C until further use.

A fructosamine assay was conducted in duplicate to determine the glycation level of the *in vitro* glycated samples, as described previously [8]. The measured glycation levels were 1.39 (±0.28) and 3.20 (±0.13) mol hexose/mol HSA for gHSA1 and gHSA2, respectively. The glycation level for the normal HSA was 0.24 (±0.13) mol hexose/mol HSA.