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CHROMATOGRAPHIC STUDIES OF CHLORPROPAMIDE INTERACTIONS WITH NORMAL AND GLYCATED HUMAN SERUM ALBUMIN BASED ON AFFINITY MICROCOLUMNS

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Abstract

Sulfonylurea drugs have significant binding to proteins in blood, with most of this binding believed to occur with human serum albumin (HSA). High performance affinity chromatography and affinity microcolumns containing immobilized HSA were used to investigate binding by the sulfonylurea drug chlorpropamide to normal HSA and glycated HSA, which is a modified form of HSA that has an increased serum concentration in diabetes. Experiments employing frontal analysis indicated that the binding by chlorpropamide gave a good fit to a two-site model for both normal HSA and glycated HSA samples that were representative of controlled or advanced diabetes. These interactions involved a set of moderate-to-high affinity sites and a set of lower affinity sites, with binding constants in the range of $6.2\text{--}9.9 \times 10^4 \text{ M}^{-1}$ and $0.18\text{--}0.57 \times 10^4 \text{ M}^{-1}$, respectively, at pH 7.4 and 37°C. Competition studies utilizing a zonal elution format demonstrated that chlorpropamide could interact at both Sudlow sites I and II of HSA, with affinities in the range expected for the moderate-to-high affinity sites of this drug. The affinity of chlorpropamide at Sudlow site I had a small increase of up to 1.2-fold when comparing the normal HSA and glycated HSA samples. Chlorpropamide gave a larger 1.4- to over 1.5-fold increase at Sudlow site II when the affinity of this drug was compared between normal HSA and the same samples of glycated HSA. These results were compared to those obtained previously with other sulfonylurea drugs to help determine how glycation can change the overall and site-selective binding strength of these drugs with HSA at levels of protein modification that are seen in patients with diabetes.

Keywords

affinity microcolumn; chlorpropamide; drug-protein binding; glycation; high-performance affinity chromatography; human serum albumin

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1. Introduction

More than 360 million people in the world suffer from diabetes, and this number is expected to grow to 438 million by 2030 [1,2]. In the United States, an estimated 29.1 million persons, or about 9% of the population, have been diagnosed with this disease [1,3]. Type I diabetes (or juvenile-onset diabetes) and type II diabetes (non-insulin dependent diabetes) account for 5-10% and 90%-95% of diabetic patients worldwide, respectively [2,3].

Sulfonylurea drugs are often used in the treatment of type II diabetes [4,5]. The basic structure of a sulfonylurea drug is composed of a phenylsulfonyl group and a urea group. Chlorpropamide is an oral first-generation sulfonylurea drug that has been used in the past to lower glucose levels in blood by stimulating insulin secretion [4-7]. The structure of this drug is given in Figure 1(a). Like many other sulfonylurea drugs [8-11], chlorpropamide is known to bind with the transport protein human serum albumin (HSA) in blood [12-14]. It is believed that this interaction occurs with a moderate affinity and may involve more than one site on HSA or the related protein bovine serum albumin (BSA); however, only a limited amount of previous work has been carried out in characterizing these interactions [5,12,14,15].

HSA has a normal concentration of 30–50 g/L and is the most abundant protein in serum or plasma. This protein consists of a single chain of 585 amino acids and has a molar mass of 66.5 kDa [13]. HSA has two main drug-binding sites: Sudlow sites I and II [13,16-18]. Sudlow site I, which is present in subdomain IIA of HSA, binds to solutes and drugs such as salicylate and warfarin. Sudlow site II is located in subdomain IIIA and binds to solutes and drugs such as L-tryptophan, ibuprofen, and ketoprofen, among many others [13,17,18]. It has been previously found that both these sites can bind to the first-generation sulfonylurea drugs acetohexamide and tolbutamide [8-11], as well as to several second- or third-generation sulfonylureas [19-22]. It has been further found through competition studies that chlorpropamide binds to at least Sudlow site I of HSA [12], although it is not known if Sudlow site II can also bind this drug.

The high levels of glucose that are present in blood during diabetes can result in the modification of proteins, including HSA [23-27]. This modification occurs through a process that is known as glycation [8,16,23-25], which is illustrated in Figure 1(b). Glycation is a non-enzymatic reaction during which glucose combines with an amine group on a protein to create a Schiff base; the Schiff base can then later rearrange to form a stable ketoamine, or Amadori product [8,16,25]. The glycation of proteins is linked to many of the long-term complications of diabetes [16]. This process has also been shown to alter the binding of some sulfonylurea drugs to HSA [8-11,25-27]. However, no previous studies have examined the effects of glycation on the interactions of chlorpropamide with HSA.

This study will combine the use of high-performance affinity chromatography (HPAC, also known as high-performance liquid affinity chromatography) with affinity microcolumns to examine the binding of chlorpropamide to normal HSA and glycated HSA. HPAC is a type of liquid chromatography that uses an immobilized binding agent (e.g., HSA) as the stationary phase [28-30]. Advantages of HPAC with regards to the study of biological

interactions include its ability to be automated and to provide relatively fast and precise results, while also allowing the same binding agent to often be used for hundreds of experiments [8-11,28-32]. HPAC has been used in prior work to examine the effects of HSA glycation on the binding of this protein to sulfonylurea drugs such as tolbutamide and acetohexamide [8-11]. It has been further shown in recent studies how affinity microcolumns with volumes in the low-to-mid microliter range can be employed with small amounts of a protein such as glycated HSA to rapidly provide a detailed characterization of how a target solute or drug interacts with this binding agent [8,33,34].

This report will use HPAC and affinity microcolumns to examine the overall binding of chlorpropamide to normal or glycated HSA and the specific interactions of this drug at Sudlow sites I and II. The results will then be compared to those obtained previously for related sulfonylurea drugs [8-11,19-22]. These data should provide a clearer picture of how glycation can alter the binding of sulfonylureas to HSA during diabetes. This work will produce fundamental information on how protein glycation, along with changes in the structure of sulfonylureas, may affect drug-protein interactions and illustrate how HPAC and affinity microcolumns can be employed as tools for a detailed analysis of these processes, as might be used for personalized medicine [8].

2. Experimental

2.1. Chemicals

The *R*-warfarin (97% pure), L-tryptophan (98%), HSA (product A1887, from human serum, essentially fatty acid free, 96%), and D-(+)-glucose (99.5%) and were from Sigma-Aldrich (St. Louis, MO, USA). The chlorpropamide (99%) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The Nucleosil Si-300 (pore size, 300 Å; particle size, 7 µm) was acquired from Macherey-Nagel (Duren, Germany). The fructosamine assay was carried out using a kit from Diazyme Laboratories (San Diego, CA, USA). The bicinchoninic acid (BCA) protein assay reagents were acquired from Pierce (Rockford, IL, USA). Water that had been purified by a Milli-Q-Advantage A 10 system (EMD Millipore, Billerica, MA, USA) was used to make all the aqueous solutions and mobile phases that were utilized in this research. These solutions were filtered by passing them through 0.20 µm GNWP nylon membranes from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Instrumentation

A Jasco (Tokyo, Japan) HPLC system was used in this report. This system included the following components: two PU-2080 pumps; an AS-2057 autosampler; a DG-2080 degasser; a CO-2067 column oven; and a UV-2080 absorbance detector. This system also employed a Rheodyne Advantage PF six-port valve (Cotati, CA, USA) and was controlled by LCNet and ChromNav software from Jasco. The data from the chromatographic system were analyzed by using PeakFit 4.12 software from Jandel Scientific (San Rafael, CA, USA). Regression and best-fit parameters for the data were acquired by using DataFit 8.169 (Oakdale, PA, USA).

2.3. Glycation and modification of HSA

Two samples of glycated HSA (i.e., gHSA1 and gHSA2) were prepared *in vitro* as described previously under sterile conditions and using 1 mM sodium azide as a buffer additive to prevent bacterial growth [9,10,35]. This was done by using reaction mixtures made in pH 7.4, 0.067 M potassium phosphate buffer that contained a typical physiological concentration of normal HSA (42 g/L, using the same type of commercial preparation as employed in Section 2.4 when making the normal HSA microcolumns) and either 15 or 30 mM of D-glucose (i.e., as used to make gHSA1 or gHSA2 samples, which represented glucose levels that might be seen during controlled or advanced diabetes, respectively) [9,35]. These mixtures were incubated for about 4 weeks at 37 °C to allow glycation to occur, followed by use of a desalting column to remove the excess and unreacted glucose. The glycated HSA samples were lyophilized and stored at –80 °C. A fructosamine assay was carried out on each HSA sample, in duplicate, to determine the level of glycation that was present [35]. The measured levels of glycation for the normal HSA, gHSA1, and gHSA2 samples were < 0.1, 1.40 ($\pm$ 0.06), and 3.24 ($\pm$ 0.07) mol hexose/mol HSA (Note: the values in parenthesis represent $\pm$ 1 S.D.).

2.4. Column preparation

The supports used to make the affinity microcolumns were prepared by first modifying Nucleosil Si-300 into a diol-bonded form [36,37], with the HSA or glycated HSA then being immobilized to this material by the Schiff base method [36-38]. A control support was prepared in the same way but with no HSA being added as part of the immobilization process. A BCA protein assay was employed, in triplicate, to determine the HSA content of each support, using soluble and normal HSA as the standard and the control support as the blank. The protein contents of these materials were found to be 89 ($\pm$ 3), 88 ($\pm$ 6), and 91 ($\pm$ 8) mg HSA/g silica for the normal HSA, gHSA1, and gHSA2 supports.

These supports were placed into individual 1.0 cm $\times$ 2.1 mm i.d. stainless steel columns by using downward slurry packing at 3500 psi (24 MPa) and by utilizing a packing buffer that consisted of pH 7.4, 0.067 M potassium phosphate buffer. These microcolumns contained 1.37-1.42 mg of normal HSA or glycated HSA, as determined by using the known packing density of the support (i.e., 0.45 g silica/cm³) and the support's measured protein content. Control microcolumns were packed in the same manner. The microcolumns and any remaining supports were stored in the pH 7.4 buffer at 4 °C until use. As has been noted in previous work [9-11,38], several hundred sample injections or applications could be made on the types of HSA supports and columns that were used in this study without appreciable changes being noted in their drug-protein binding properties. For instance, a change in retention of less than 1.1% was seen in this work for *R*-warfarin on a normal HSA microcolumn over the course of almost 300 injections and 18 months of use.

2.5. Chromatographic studies

The solutions of chlorpropamide, L-tryptophan, *R*-warfarin and sodium nitrate were prepared in pH 7.4, 0.067 M phosphate buffer, which was also used as the mobile phase for the chromatographic studies (Note: this buffer was selected because it has been routinely used in prior studies to examine binding by other sulfonylurea drugs with HSA)

[9-11,34,38]. This buffer was passed through a 0.2 μm filter and degassed for 10-20 min prior to its use. The solutions of *R*-warfarin were used within two weeks of preparation, and the L-tryptophan solutions were used within 1-2 days, as based on previous studies examining the stability of such solutions [38-40]. All of the chromatographic experiments were performed at 0.30-0.50 mL/min and 37 $^{\circ}\text{C}$. The back pressure for the microcolumns under these conditions was 2.0 MPa (290 psi) or less. As noted previously, no significant changes in the binding parameters that were measured in this work were seen when using other flow rates as long as sufficient time was still allowed for a local equilibrium to be established between the immobilized binding agent and the injected/applied drugs and probe compounds [8-11].

A six-port valve was used in the frontal analysis experiments to switch the mobile phase from the pH 7.4, 0.067 M phosphate buffer to the same buffer containing a known concentration of chlorpropamide. The elution of chlorpropamide was detected at 250 nm. After a stable plateau had been reached in a frontal analysis experiment, the valve was switched to reapply the original pH 7.4 buffer to regenerate the column. Frontal analysis was carried out on each column, including the control microcolumn and microcolumns containing normal HSA, gHSA1, or gHSA2, at ten concentrations of chlorpropamide spanning from 1.0 to 50.0 μM . These drug concentrations gave a response in the linear range of the detector, and all measurements were made four times. The mean position of each frontal analysis curve was found by using the first derivative function of PeakFit 4.12 and were corrected for the void time by using sodium nitrate as a non-retained solute (see following paragraph). Over the range of drug concentrations that were employed, between 10 and 22% of the total binding for chlorpropamide was due to non-specific binding to the support on the normal and glycosylated HSA microcolumns, as has been noted for other first-generation sulfonylurea drugs [9-11]. The amount of bound drug that was obtained for the control microcolumn at each drug concentration was either subtracted from the amount of bound drug on an HSA microcolumn at the same drug concentration or considered as part of binding models to correct for non-specific binding of chlorpropamide to the support (see Section 3.1.1 and Supplemental Material for further details) [9-11,38].

The competition studies based on zonal elution were carried out by using *R*-warfarin (i.e., a probe for Sudlow site I) and L-tryptophan (i.e., a probe for Sudlow site II) [35,38]. These studies were performed with eight different concentrations of chlorpropamide in the mobile phase, spanning from 0 to 20 μM . The same solutions of chlorpropamide were used to make samples of the desired probe at a probe concentration of 5 μM (Note: the chlorpropamide was added to minimize changes in the local concentration of this drug in the mobile phase, and the corresponding changes in the background response during sample injection, as has been used in prior studies with other sulfonylurea drugs) [9-11,38]. The injection volume for all samples was 20 μL . The probe samples were injected onto each type of microcolumn while the probe was monitored at 308 nm for *R*-warfarin or 280 nm for L-tryptophan. A 20 μM solution of sodium nitrate, which was used as a non-retained solute for the HSA and glycosylated HSA supports [38], was also injected onto each microcolumn, as well as onto the chromatographic system with no microcolumn present; this solute was monitored at 205 nm. Each zonal elution experiment was carried out in quadruplicate, and the mean retention time of each peak was determined by using PeakFit 4.12.

3. Results and discussion

3.1. Overall binding of chlorpropamide with HSA

3.1.1. General approach—The overall interactions of chlorpropamide with normal and glycosylated HSA were examined by using frontal analysis (see Supplemental Material for data) [34,38]. These studies were carried out to provide an initial estimate for the number and types of sites (e.g., high vs. low affinity) that took part in these interactions; the higher affinity sites were of particular interest, as examined in more detail later through zonal elution (Section 3.2). In the frontal analysis experiments, several solutions containing known concentrations of chlorpropamide were applied onto an HSA microcolumn or control microcolumn and used to obtain a series of breakthrough curves, as shown in Figure 2. The mean breakthrough times for these curves occurred within 4–15 min of solution application at 0.30 mL/min (i.e., applied solution volumes of 1.2–4.5 mL) and when using a 1.0 cm × 2.1 mm i.d. column containing normal HSA. The glycosylated HSA microcolumns exhibited similar behavior. Even shorter breakthrough times could be obtained with these microcolumns by using higher flow rates for sample application, but some loss in the precision of this method is known to occur as the flow rate is increased [41].

The frontal analysis results were analyzed by looking at how the moles of chlorpropamide that were needed to reach the mean breakthrough time of each curve changed as the applied concentration of chlorpropamide was varied. For instance, Eqs. (1) and (2) were used to represent a system with a single set of saturable sites and relatively fast association and dissociation between a drug (D) and an immobilized protein (or affinity ligand, L) [41–44].

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

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

In Eqs. (1–2), m_{Lapp} is the apparent binding capacity (i.e., moles of drug needed to reach the mean point of the breakthrough curve) of an immobilized protein at a given applied concentration for the drug [41]. Other terms in these equations include the association equilibrium constant (K_a) and moles of active sites for this interaction (m_L). Eq. (1) indicates that a drug and protein with a one-site interaction should give a linear response for a plot of $1/m_{Lapp}$ versus $1/[D]$; the value m_L can be acquired from the reciprocal of the intercept for this plot, and K_a can be found by using the ratio of the intercept over the slope [41]. A non-linear fit to Eq. (2) can also be used to obtain these best-fit parameters [9–11,41–43]. An advantage of using frontal analysis and expression like Eqs. (1–2) is it allows separate estimates to be obtained for both the binding constants and moles of binding sites for an applied solute with an immobilized protein or binding agent [28,29,41]. However, no information is provided on the location of these binding regions [10,11,28,41].

The presence of multiple sites that have significant differences in their affinity can be examined by using a suitably large range of drug/solute concentrations and alternative equations to those shown in Eqs. (1-2) [28,41]. An example of such an expression is shown in Eq. (3), which is a non-linear equation that describes a system with two groups of saturable sites for a drug with an immobilized binding agent (e.g., specific moderate-to-high affinity sites and non-specific lower affinity regions) [41,42].

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

The terms K_{a1} and K_{a2} in this equation are the association equilibrium constants for the drug at a set of higher and lower affinity sites on the binding agent, while the moles of these sites are given by m_{L1} and m_{L2} . The double-reciprocal form of Eq. (3) is known to give an apparent linear response for a plot of $1/m_{Lapp}$ versus $1/[D]$ when using low-to-medium concentrations of the applied drug D, with negative deviations from this linear behavior occurring at high drug concentrations, or low values of $1/[D]$ [42,43]. Furthermore, the slope from the linear region for this latter type of plot has been shown to provide a good initial estimate for K_{a1} (i.e., the binding strength for the high affinity sites) in systems that follow a two-site interaction model [42].

It is necessary when studying drug-protein binding by frontal analysis to correct for any secondary interactions the drug may have with the support [41]. This secondary binding can be examined by using frontal analysis to measure the amount of drug that is bound to a control column that contains the support but no immobilized protein. One way this data can be used is to subtract the amount of drug that is bound to the control column from the total amount of drug that is bound to the protein column at the same drug concentration [9-11]; this provides an estimate for the value for m_{Lapp} in Eqs. (1-3). However, this correction method assumes the immobilized protein does not significantly alter the amount of binding the drug has with the support [45], which may not be true for supports that have a high protein content.

An alternative approach is also possible for correcting for these secondary interactions, and that does not assume they are constant in the presence of an immobilized protein. This can be done by fitting the frontal analysis data for the control column to a binding model to estimate the total moles of sites for the drug on the support (m_S) and the association equilibrium constant of the drug at these sites (K_{aS}) (see Supplemental Material). These parameters can then be used with expanded versions of the one-site and two-site drug/protein models in Eqs. (2-3) that now include an additional binding term for the support. Examples of these expanded equations are provided in Eqs. (4-5), in which the total moles of drug that is bound to each protein column, including secondary binding to the support, is represented by the term $m_{Lapp,tot}$.

$$m_{Lapp,tot} = \frac{m_{L1}K_{a1}[D]}{(1 + K_{a1}[D])} + \frac{\alpha m_S K_{aS}[D]}{(1 + K_{aS}[D])} \quad (4)$$

$$m_{Lapp,tot} = \frac{m_{L1}K_{a1}[D]}{(1 + K_{a1}[D])} + \frac{m_{L2}K_{a2}[D]}{(1 + K_{a2}[D])} + \frac{\alpha m_S K_{aS}[D]}{(1 + K_{aS}[D])} \quad (5)$$

Because the values of m_S and K_{aS} are determined separately for the control column, the only additional fitted parameter in going from Eqs. (2-3) to Eqs. (4-5) is the term α . This term, which should have a value between zero and one, represents the fraction of the original support's surface or binding sites that is still accessible to the drug after protein immobilization.

Both of these approaches for considering secondary interactions were employed at various stages in this report. Similar trends and binding constants for chlorpropamide with HSA were obtained by each approach (see Section 3.1.2 and Supplemental Material). However, the final results given in Section 3.1.2 were obtained by using the second method. It was determined in this method that the presence of HSA did lead to a decrease in the amount of secondary binding between chlorpropamide and the support. For instance, Table 1 shows that the term α was equal to 0.40-0.42 when using Eq. (5) to fit the frontal analysis data for the HSA microcolumns to a two-site drug/protein binding model. This indicated that up to 58-60% of the support's secondary sites were no longer accessible to chlorpropamide once HSA had been immobilized to this material. The average precision for the overall moles of bound drug that were measured and used in these fits was $\pm 2.2\%$ (range, ± 0.2 -6.7%) under the concentration, column size, and flow rate conditions that were employed.

3.1.2. Interactions of chlorpropamide with normal and glycated HSA—Figure 3 shows the best-fit results that were obtained for chlorpropamide on microcolumns containing normal HSA or gHSA2 and when using a one-site model with a double-reciprocal plot based on Eq. (1) (Note: these particular results used a simple subtraction of the HSA and control column data to correct for binding by the drug to the support). The column containing gHSA1 gave similar behavior to that seen in Figure 3. These plots appeared initially to give a good fit to Eq. (1) over the range of drug concentrations that were studied, with correlation coefficients for these fits that ranged from 0.9996 to 0.9998 ($n = 10$). However, a closer examination of these results did indicate that some slight negative deviations from a linear response may have been present for the higher drug concentrations that were examined, as can be seen at the lower left portion of Figure 3(b). Thus, linear fits were also made to only the data obtained at lower drug concentrations (i.e., 1.0 to 7.5 μM , in this case), as based on Ref. [42,43]. These latter plots gave correlation coefficients that ranged from 0.9998 to 0.9999 ($n = 5$).

The linear fits of double-reciprocal plots to both the entire data set and data obtained at lower drug concentrations, with the latter emphasizing the strongest interaction sites, were

used to estimate the value of K_{a1} for the higher affinity sites of chlorpropamide on the various types of HSA microcolumns. The resulting estimates that were obtained for chlorpropamide with normal HSA from the entire data set and the data at lower drug concentrations were $4.3 (\pm 0.3) \times 10^4 \text{ M}^{-1}$ and $3.5 (\pm 0.6) \times 10^4 \text{ M}^{-1}$, respectively; this was equivalent to dissociation equilibrium constants (K_d , where $K_d = 1/K_a$) of $2.3 (\pm 0.2) \times 10^{-5} \text{ M}$ and $2.9 (\pm 0.5) \times 10^{-5} \text{ M}$. The corresponding estimates of K_{a1} that were obtained for chlorpropamide with microcolumns containing gHSA1 were $4.9 (\pm 0.2) \times 10^4 \text{ M}^{-1}$ and $4.9 (\pm 0.4) \times 10^4 \text{ M}^{-1}$, or K_d values of $2.0 (\pm 0.1) \times 10^{-5} \text{ M}$ and $2.0 (\pm 0.2) \times 10^{-5} \text{ M}$. The K_{a1} values for the microcolumn containing gHSA2 were $5.6 (\pm 0.4) \times 10^4 \text{ M}^{-1}$ and $6.8 (\pm 0.3) \times 10^4 \text{ M}^{-1}$, giving values for K_d of $1.8 (\pm 0.1) \times 10^{-5} \text{ M}$ and $1.5 (\pm 0.1) \times 10^{-5} \text{ M}$. The best estimates for the highest affinity sites for gHSA1 and gHSA2, as obtained from the lower drug concentration data, were 1.40-fold to 1.94-fold higher than the estimate of K_{a1} that was obtained for normal HSA, with each of these changes being significant at the 95% confidence level. These changes indicated that the glycation of HSA did have an effect on the binding of chlorpropamide with at least some regions on this protein.

The same data were analyzed in more detail by using non-linear regression and one-site or two-site models, with a simultaneous correction for binding the support as based on Eq. (4) or Eq. (5). Figure 4 shows how these two types of models fit with the experimental data, as demonstrated by using the results obtained for chlorpropamide on a normal HSA microcolumn. Similar trends were seen for the glycated HSA microcolumns. The one-site model gave lower correlation coefficients than the two-site model, with values that ranged from 0.9489-0.9856 and 0.9993-0.9997, respectively ($n = 11$). In addition, the two-site model gave a more random distribution of the data about the best-fit lines. The use of higher-order models did not improve the fit of these data sets any further. These results, along with the deviations from linearity seen for some of the plots made according to Eq. (1), suggested that the two-site model gave a better description than the one-site model for the interactions between chlorpropamide and normal HSA or glycated HSA. The same observation has been made for other first-generation sulfonylurea drugs [8-11] and in previous binding studies between chlorpropamide and normal HSA or BSA [14,15].

The best-fit parameters that were obtained with both the one- and two-site models for HSA, including the parameters used to correct for binding by chlorpropamide to the support, are summarized in Table 1. The overall association equilibrium constants that were obtained for chlorpropamide at pH 7.4 and 37°C with normal HSA and glycated HSA varied from 3.5 to $4.7 \times 10^4 \text{ M}^{-1}$ when using a one-site model for HSA and, for the higher affinity sites, ranged from 6.2 to $9.9 \times 10^4 \text{ M}^{-1}$ when using a two-site model for HSA. The corresponding values for K_d ranged from 2.9 to $2.1 \times 10^{-5} \text{ M}$ for the one-site model and from 1.6 to $1.0 \times 10^{-5} \text{ M}$ for the higher affinity sites in the two-site model. The overall K_a values that were obtained were consistent with a value of $0.99 \times 10^{-5} \text{ M}$ for $n_1 K_{a1}$ (where n is the number of a given type of site per protein), as calculated based on prior data reported at pH 7.4 and 37°C for the high affinity site of chlorpropamide with normal HSA in solution [14]. The range of these values for chlorpropamide also agreed with the overall or high affinities of 12 to $20 \times 10^4 \text{ M}^{-1}$ and 8.4 to $12 \times 10^4 \text{ M}^{-1}$ that have been observed for acetohexamide and tolbutamide with normal HSA and similar samples of glycated HSA [9-11]. The precisions

of these values varied from ± 8.5 -17% (average, $\pm 13.0\%$) for K_{a1} in the one-site model and from ± 6.5 -9.1% (average, $\pm 7.8\%$) for K_{a1} in the two-site model.

The number of the high affinity sites per HSA molecule (i.e., as based on the measured content of HSA in the microcolumns and the values for m_{L1} when using the two-site model) was 0.12 to 0.18 (average, 0.15). Given that not all of the immobilized HSA was active [9-11,37,38], these results indicated that only one or a few binding regions on HSA probably made up the higher affinity sites for chlorpropamide, as suggested in a prior solution-phase studies [14]. The number of lower affinity sites (e.g., with K_{a2} values of 1.8 to $5.7 \times 10^3 \text{ M}^{-1}$ and representing weak or non-specific binding regions on HSA) was less well-defined but was determined in the same manner to range from at least 2.1 to 7.0 (average, 5.0). This latter result was also consistent with previous solution-phase binding studies that have been conducted between chlorpropamide and normal HSA or BSA [14,15].

With both the one-site and two-site models, there was an increase in the overall affinity of chlorpropamide when going from normal HSA to the samples of glycated HSA, as discussed earlier. This increase was 1.29- to 1.34-fold when using the one-site model and 1.44- to 1.60-fold for the estimated binding strength of the high affinity sites in the two-site model. Each of these changes was significant at the 95% confidence level. It has been suggested in work with other sulfonylurea drugs and through structural characterization based on mass spectrometry that these changes in affinity are a result of glycation-related modifications that occur at or near the various drug binding regions of HSA, including Sudlow sites I and II [8-11,25,44-48].

3.2. Site-selective binding of chlorpropamide with HSA

3.2.1. General approach—Zonal elution was next used to provide a more detailed examination of how the binding of chlorpropamide changed at Sudlow sites I or II of HSA as a result of glycation. This was done by using a competition study, in which a site-specific probe (e.g., *R*-warfarin or *L*-tryptophan) was injected onto an HSA or glycated HSA microcolumn as a solution of chlorpropamide with a known and constant concentration was passed through the same microcolumn (see Supplemental Material for data). Figure 5 shows some typical chromatograms from these experiments. The elution of the probe compounds occurred within 6 min or less when using an injection flow rate of 0.50 mL/min. The retention time for each injected probe was measured as the concentration of the chlorpropamide was varied, and the corresponding value of the probe's retention factor (k) was calculated for each mobile phase that was employed.

It is known from prior work with this type of experiment that if the injected probe and drug in the mobile phase have a single site of competition, a plot made of $1/k$ versus the molar drug concentration, $[D]$, should follow the linear relationship that is given by Eq. (6) [28,34,41]. Examples of such plots are provided in Figure 6.

$$\frac{1}{k} = \frac{K_{aD}V_M[D]}{K_{aP}m_L} + \frac{V_M}{K_{aP}m_L} \quad (6)$$

The association equilibrium constants for the probe and drug at their site of competition are represented in Eq. (6) by K_{ap} and K_{ad} , while V_M is the void volume, and m_L is the moles of the common binding sites the probe and drug have in the column. If competition occurs between the drug and probe at a single common site, the ratio of the slope versus intercept for a plot made according to this equation should provide the local association equilibrium constant for the drug at this common site [28,34,41]. One advantage of this approach is it provides a value for K_{ad} that is independent of the moles of active protein or binding sites in the column [41]. This value is also independent of any other interaction regions the applied drug may have with the immobilized binding agent or with the support as long as the injected probe does not interact with these other regions [28,41], as has been noted to be the case in prior work with the site-selective probes that were employed in this study (i.e., *R*-warfarin and L-tryptophan) [28,35,38,40,44].

3.2.2. Competition studies at Sudlow site I—Competition studies were first carried out by using zonal elution experiments and *R*-warfarin as a probe for Sudlow site I [35,38,44,49]. This site was of interest because it has been suggested through prior competition studies to be a binding region for chlorpropamide on HSA [12]. Furthermore, this region is known to bind other first-generation sulfonylurea drugs [8-11]. The data from zonal elution competition studies that used *R*-warfarin as a probe gave a linear plot for each type of HSA microcolumn when the data were examined by using Eq. (6), as illustrated in Figure 6(a). The correlation coefficients for these lines spanned from 0.9758 to 0.9930 ($n = 8$). This behavior confirmed that chlorpropamide had direct interactions with *R*-warfarin and binding at Sudlow site I on normal HSA and glycated HSA. The average precision of the retention factors that were obtained in these measurements was $\pm 1.2\%$ (range, ± 0.2 -4.1%).

Table 2 lists the association equilibrium constants that were acquired by this method at Sudlow site I for chlorpropamide on the normal HSA and glycated HSA microcolumns. These values were in the general range of 3.9 - $4.7 \times 10^4 \text{ M}^{-1}$ at pH 7.4 and 37°C (i.e., K_d values of 2.6 to $2.1 \times 10^{-5} \text{ M}$), with precisions of ± 5.1 -10.6% (average, $\pm 8.4\%$). These binding constants were consistent with the general estimates of 3.4 - $9.6 \times 10^4 \text{ M}^{-1}$ that had been obtained by frontal analysis in Section 3.1 for the higher affinity regions of chlorpropamide with normal or glycated HSA. Previous competition studies that were conducted at pH 7.0 and a lower temperature of 25°C gave an estimated binding constant of $2.9 \times 10^5 \text{ M}^{-1}$ for chlorpropamide at Sudlow site I of normal HSA [12]; however, it is known that the binding strength of drugs with HSA tends to decrease with an increase in temperature [28]. For instance, a decrease in affinity of 20-30% has been observed at pH 7.4 for *R/S*-warfarin at Sudlow site I when going from 25 to 37°C [44], and a decrease of 21% in affinity for the strongest binding sites has been reported for chlorpropamide with BSA in going from 15 to 30°C [15].

The values shown in Table 1 for chlorpropamide were in the same general range as binding constants of 3.8 - $5.9 \times 10^4 \text{ M}^{-1}$ and 5.5 - $6.9 \times 10^4 \text{ M}^{-1}$ that have been measured at Sudlow site I at pH 7.4 and 37°C for acetohexamide and tolbutamide, respectively, with normal HSA and samples of glycated HSA like those used in this report [9-11]. Chlorpropamide gave a binding constant at Sudlow site I that was 29% weaker than seen for tolbutamide with normal HSA, with similar decreases of 28-35% being seen in the binding strength for

glycated forms of HSA [9,11]. The two differences in the structures of tolbutamide and chlorpropamide, as shown in Figure 1(a), are the change from a methyl group to a chlorine on the phenyl ring (R_1) and a change from a butyl group to a propyl group at the other end of the structure (R_2). Given that Sudlow site I is known to prefer compounds with bulky heterocyclic structures [13,17,18], it is the change on the phenyl group that is probably responsible for most of the decrease in binding strength for chlorpropamide vs. tolbutamide at this site for both normal and glycated HSA. This observation is consistent with the much smaller change noted in binding strength between chlorpropamide and acetohexamide, with a 7% decrease for chlorpropamide for normal HSA and a 13-15% increase for glycated HSA [10,11]. For these latter two compounds, there is a change from a chlorine to an acetyl group on the phenyl ring at R_1 and a change from a propyl group to a cyclohexyl group at R_2 , neither of which appears to have led to any major differences in the binding of these two drugs to Sudlow site I.

The effect of glycation on the site-specific affinities measured at Sudlow site I were consistent with the patterns seen in Section 3.1 for the overall affinities or higher affinity constants for chlorpropamide with HSA. For instance, there was an apparent 1.10-fold increase in the binding strength for chlorpropamide at Sudlow site I in going from normal HSA to gHSA1 and an increase of 1.21-fold in going from normal HSA to gHSA2. Although these differences were relatively small and approached the level of precision for the zonal elution measurements, the results for gHSA2 were significant at the 95% confidence level. As stated in Section 3.1, such changes in binding strength with glycation are believed to reflect the different amounts and types of modification that occur on HSA as its level of glycation is increased [8-11,46-48,50]. Examples of specific modification sites due to glycation that have been found to occur at or near Sudlow site I include K199, K281, and K276 [25].

3.2.3. Competition studies at Sudlow site II—Zonal elution and competition studies were further used to examine the interactions of chlorpropamide at Sudlow site II by using L-tryptophan as a site-selective probe for this region [28,38,40,51]. This site was of interest because it has been found to bind to other first-generation sulfonylurea drugs such as acetohexamide and tolbutamide [8-11]. The results for these experiments when chlorpropamide was added to the mobile phase gave a linear plot for both the normal and glycated HSA microcolumns when the data were plotted according to Eq. (6), as shown by the example in Figure 6(b). These plots had correlation coefficients that ranged from 0.8877 to 0.9546 ($n=7-8$). These results indicated that chlorpropamide had direct interactions at Sudlow site II on normal or glycated HSA, in addition to the binding that occurred at Sudlow site I (see previous section). The retention factors used to generate these plots had an average precision of $\pm 3.8\%$ (range, $\pm 0.2-18\%$).

Table 2 includes the association equilibrium constants measured at Sudlow site II during the zonal elution experiments for chlorpropamide with normal HSA or the samples of glycated HSA. These values were in the range of $2.0-3.1 \times 10^4 \text{ M}^{-1}$ at pH 7.4 and 37°C (i.e., K_d values of 5.0 to $3.2 \times 10^{-5} \text{ M}$) and had precisions of $\pm 14-20\%$ (average, $\pm 18\%$). The results were also only slightly lower than the estimated binding constants of $3.9-4.7 \times 10^4 \text{ M}^{-1}$ that were acquired in the previous section for chlorpropamide at Sudlow site I on the same

microcolumns. In addition, these values were of the same order of magnitude, but lower than, binding constants for Sudlow site II of $7.9\text{--}13 \times 10^4 \text{ M}^{-1}$ and $5.3\text{--}7.2 \times 10^4 \text{ M}^{-1}$ that have been reported at pH 7.4 and 37°C for acetohexamide and tolbutamide with normal HSA and comparable preparations of glycosylated HSA [9-11].

The binding constant for chlorpropamide at Sudlow site II was 63% lower than seen for tolbutamide with normal HSA; similar decreases of 52-61% were observed in the binding constants for glycosylated HSA [9,11]. Sudlow site II is known to interact with aromatic carboxylic acids and related compounds [13,17,18], so these changes in binding were again mostly likely due to the change from a methyl group to a chlorine group at R₁. It is interesting to note that this alteration in structure produced a much larger change than was seen at Sudlow site I, with normal and glycosylated HSA having similar patterns in their relative binding strengths for chlorpropamide vs. tolbutamide. A large decrease in relative binding strength was further seen when comparing chlorpropamide with acetohexamide, with chlorpropamide giving an 85% lower affinity at Sudlow site II for normal HSA and 74-75% lower values for glycosylated HSA [10,11]. These differences were also thought to be primarily the result of changes in structure at R₁, although some additional contributions from the changes at R₂ may have been present.

As was seen for Sudlow site I, glycosylation again resulted in an increase in the affinity of chlorpropamide at Sudlow site II and at the levels of modification employed in this report. In this case, there was a 1.40-fold increase in the affinity for chlorpropamide at Sudlow site II in going from normal HSA to gHSA1 and an increase of 1.55-fold in going from normal HSA to gHSA2, with all of these differences being significant at the 95% confidence level and larger than the relative uncertainty expected due to only random errors. These changes in binding were again probably a result of the different amounts and types of glycosylation-related modifications that occurred on these HSA samples [8-11,47,48,50]. For instance, K439 is one major glycosylation site that occurs at Sudlow site II of HSA [25].

4. Conclusion

HPAC was used in this work to investigate the binding of chlorpropamide, a first-generation sulfonylurea drug, to normal HSA and HSA with levels of glycosylation corresponding to controlled or advanced diabetes. Frontal analysis was first used to examine the overall binding of chlorpropamide with normal and glycosylated HSA. Although a one-site model gave a reasonable fit to the binding data for these interactions, a slightly better fit was obtained when using a two-site model based on a set of moderate-to-high affinity sites (i.e., the equivalent of two sites with overall binding constants around $6.2\text{--}9.9 \times 10^4 \text{ M}^{-1}$ at pH 7.4 and 37°C) and a set of lower affinity binding sites (binding constants, $1.8\text{--}5.7 \times 10^3 \text{ M}^{-1}$). This type of behavior and observed range of affinities were consistent with interaction models that have been reported for tolbutamide and acetohexamide, two other first-generation sulfonylurea drugs, with normal HSA and similar preparations of glycosylated HSA [8-11]. A net increase in the overall binding strength of chlorpropamide for the higher affinity sites of up to 1.6-fold was noted when comparing the glycosylated HSA samples to normal HSA.

Zonal elution-based competition studies were used to provide more site-specific information on the interactions between chlorpropamide at Sudlow sites I and II of normal HSA or glycated HSA. This drug was found to bind to both Sudlow sites I and II, as has been noted for other first-generation sulfonylurea drugs [6-9]. Glycation levels like those found in controlled or advanced diabetes gave a slight increase in the binding strength of chlorpropamide at Sudlow site I by up to 1.2-fold and a larger increase at Sudlow site II of 1.4- to almost 1.5-fold, when compared to the binding observed for normal HSA. These changes both contributed to the overall increase in affinity of chlorpropamide that was noted for the same glycated HSA samples. A comparison of these results with those of closely-related drugs made it further possible to examine the role played by the side groups in these compounds in altering their interactions at Sudlow sites I and II.

This report demonstrated several potential advantages in using affinity microcolumns for examining drug-protein interactions. The small size of the affinity microcolumns made it possible to carry out detailed binding studies in a matter of minutes and under isocratic conditions for interactions that had affinities up to 10^5 M^{-1} . In addition, only a small amount of HSA was required per column (i.e., about 1.4 mg), making it practical to use this approach with modified forms of HSA. Each column could also be used for many binding studies. For instance, a single affinity microcolumn was used in this study for over 300 sample injections or application cycles, which was the equivalent of using less than 5 μg HSA per experiment. The good long-term stability of these columns, the use of these microcolumns in an HPLC system, and the ability to reuse the same immobilized protein made it possible to obtain binding constants with good precisions and to examine even relatively small shifts in binding strength that occurred for chlorpropamide with glycated versus normal forms of HSA.

The approach used in this report is not limited to glycated HSA but could be employed in binding studies with other modified proteins or even proteins that have been isolated from individual clinical samples [8,52]. The advantages of affinity microcolumns in this type of research should make them useful in future interaction studies of additional solute-protein systems and as tools for personalized medicine in examining the changes in protein binding and function that may occur in diseases such as diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Wild S, Roglic G, Green A, Sicree R, King H, Global prevalence of diabetes: estimates for the year 2000 and projections for 2030, *Diabetes Care* 27 (2004) 1047–1053. [PubMed: 15111519]
- [2]. Gan D (Ed.), *Diabetes Atlas*, 2nd ed., International Diabetes Federation, Brussels, 2003.
- [3]. National diabetes fact sheet: general information and national estimates on diabetes in the United States, 2011, U.S. Centers of Disease Control, Atlanta, GA, 2011.

- [4]. Ashcroft FM, Gribble FM, ATP-sensitive K⁺ channels and insulin secretion: their role in health and disease, *Diabetologia* 42 (1999) 903–919. [PubMed: 10491749]
- [5]. Harrigan RA, Nathan MS, Beattie P, Oral agents for the treatment of type 2 diabetes mellitus: pharmacology, toxicity, and treatment, *Ann. Emerg. Med* 38 (2001) 68–71. [PubMed: 11423816]
- [6]. Graal MB, Wolffenbittel BHR, The use of sulphonylureas in the elderly, *Drugs & Aging* 15 (1999) 471–481. [PubMed: 10641958]
- [7]. Judis J, Binding of sulfonylureas to serum proteins, *J. Pharm. Sci* 61 (1972) 89–93. [PubMed: 5058652]
- [8]. Anguizola J, Joseph KS, Barnaby OS, Matsuda R, Alvarado G, Clarke W, Cerny RL, Hage DS, Development of affinity microcolumns for drug-protein binding studies in personalized medicine: interactions of sulfonylurea drugs with in vivo glycated human serum albumin, *Anal. Chem* 85 (2013) 4453–4460. [PubMed: 23544441]
- [9]. Joseph KS, Anguizola J, Jackson AJ, Hage DS, Chromatographic analysis of acetohexamide binding to glycated human serum albumin, *J. Chromatogr. B* 878 (2010) 2775–2781.
- [10]. Joseph KS, Anguizola J, Hage DS, Binding of tolbutamide to glycated human serum albumin, *J. Pharmaceut. Biomed. Anal* 54 (2011) 426–432.
- [11]. Joseph KS, Hage DS, Characterization of the binding of sulfonylurea drugs to HSA by high-performance affinity chromatography, *J. Chromatogr. B* 878 (2010) 1590–1598.
- [12]. Ascenzi P, Bocedi A, Notari S, Menegatti E, Fasano M, Heme impairs allosterically drug binding to human serum albumin Sudlow's site I, *Biochem. Biophys. Res. Commun* 334 (2005) 481–486. [PubMed: 16004963]
- [13]. Peters T, Jr., All About Albumin: Biochemistry, Genetics and Medical Applications, Academic Press, San Diego, 1996.
- [14]. Crooks MJ, Brown KF, The binding of sulphonylureas to serum albumin, *J. Pharm. Pharmacol* 26 (1974) 304–311.
- [15]. Goto S, Yoshitomi H, Kishi M, Binding of sulfonylureas to bovine serum albumin, *Yakugaku Zasshi*, 97 (1977) 1219–1227.
- [16]. Rondeau P, Bourdon E, The glycation of albumin: structural and functional impacts, *Biochimie* 93 (2011) 645–658. [PubMed: 21167901]
- [17]. Sudlow G, Birkett DJ, Wade DN, Further characterization of specific drug binding sites on human serum albumin, *Mol. Pharmacol* 12 (1976) 1052–1061. [PubMed: 1004490]
- [18]. Ascoli GA, Domenic E, Bertucci D, Drug binding to human serum albumin: abridged review of results obtained with high-performance liquid chromatography and circular dichroism, *Chirality* 18 (2006) 667–679. [PubMed: 16823814]
- [19]. Matsuda R, Anguizola J, Joseph KS, Hage DS, High-performance affinity chromatography and the analysis of drug interactions with modified proteins: binding of gliclazide with glycated human serum albumin, *Anal. Bioanal. Chem*, 401 (2011) 2811–2819. [PubMed: 21922305]
- [20]. Matsuda R, Anguizola J, Joseph KS, Hage DS, Analysis of drug interactions with modified proteins by high-performance affinity chromatography: binding of glibenclamide to normal and glycated human serum albumin, *J. Chromatogr. A*, 1265 (2012) 114–122. [PubMed: 23092871]
- [21]. Matsuda R, Li Z, Zheng X, Hage DS, Analysis of glipizide binding to normal or glycated human serum albumin by high-performance affinity chromatography, *Anal. Bioanal. Chem*, 407 (2015) 5309–5321. [PubMed: 25912461]
- [22]. Matsuda R, Li Z, Zheng X, Hage DS, Analysis of multi-site drug-protein interactions by high-performance affinity chromatography: binding by glimepiride to normal or glycated human serum albumin, *J. Chromatogr. A*, 1408 (2015) 133–144. [PubMed: 26189669]
- [23]. Garlick RL, Mazer JS, The principal site of nonenzymatic glycosylation of human serum albumin in vivo, *J. Biol. Chem* 258 (1983) 6142–6146. [PubMed: 6853480]
- [24]. Nakajou K, Watanabe H, Kragh-Hansen U, Maruyama T, Otagiri M, The effect of glycation on the structure, function, and biological fate of human serum albumin as revealed by recombinant mutants, *Biochim. Biophys. Acta* 1623 (2003) 88–97.
- [25]. Anguizola J, Matsuda R, Barnaby OS, Hoy KS, Wa C, Debolt E, Koke M, Hage DS, Review: glycation of human serum albumin, *Clin. Chim. Acta* 425 (2013) 64–76. [PubMed: 23891854]

- [26]. Tsuchiya S, Sakurai T, Sekiguchi SI, Nonenzymatic glucosylation of human serum albumin and its influence on binding capacity of sulfonylureas, *Biochem. Pharmacol* 33 (1984) 2967–2971. [PubMed: 6487349]
- [27]. Koyama H, Sugioka N, Uno A, Mori S, Nakajima K, Effects of glycosylation of hypoglycaemic drug binding to serum albumin, *Biopharm. Drug Dispos* 18 (1997) 791–801. [PubMed: 9429743]
- [28]. Hage DS, High-performance affinity chromatography: a powerful tool for studying serum protein binding, *J. Chromatogr. B* 768 (2002) 3–30.
- [29]. Hage DS, Jackson A, Sobansky M, Schiel JE, Yoo MJ, Joseph KS, Characterization of drug-protein interactions in blood using high-performance affinity chromatography, *J. Sep. Sci* 32 (2009) 835–853. [PubMed: 19278006]
- [30]. Duong-Thi M-D, Meiby E, Bergstrom M, Fex T, Isaksson R, Ohlson S, Weak affinity chromatography as a new approach for fragment screening in drug discovery, *Anal. Biochem* 414 (2011) 138–146. [PubMed: 21352794]
- [31]. Meiby E, Simmonite H, le Strat L, Davis B, Matassova N, Moore JD, Mrosek M, Murray J, Hubbard RE, Ohlson S, Fragment screening by weak affinity chromatography: comparison of established techniques for screening against HSP90, *Anal. Chem*, 85 (2013) 6756–6766. [PubMed: 23806099]
- [32]. Singh P, Madhaiyan K, Duong-Thi M-D, Dymock BW, Ohlson S, Analysis of protein target interactions of synthetic mixtures by affinity-LC/MS, *SLAS Discovery* 22 (2017) 440–446. [PubMed: 28328315]
- [33]. Zheng X, Li Z, Beeram S, Matsuda R, Pfaumiller EL, Podariu M, White CJ, II, Carter N, Hage DS, Analysis of biomolecular interactions using affinity microcolumns: a review, *J. Chromatogr. B* 968 (2014) 49–63.
- [34]. Matsuda R, Jobe D, Beyersdorf J, Hage DS, Analysis of drug-protein binding using on-line immunoextraction and high-performance affinity microcolumns: studies with normal and glycated human serum albumin, *J. Chromatogr. A* 1416 (2015) 112–120. [PubMed: 26381571]
- [35]. Joseph KS, Hage DS, The effects of glycation on the binding of human serum albumin to warfarin and L-tryptophan, *J. Pharm. Biomed. Anal* 53 (2010) 811–818. [PubMed: 20537832]
- [36]. Larsson PO, High-performance liquid affinity chromatography, *Methods Enzymol.* 104 (1984) 212–223. [PubMed: 6371445]
- [37]. Kim HS, Hage DS, Immobilization methods for affinity chromatography, In *Handbook of Affinity Chromatography*, 2nd ed., Hage DS (Ed.), Taylor & Francis, New York, 2006, Chap. 3.
- [38]. Matsuda R, Anguizola J, Hoy KS, Hage DS, Analysis of drug-protein interactions by high-performance affinity chromatography: interactions of sulfonylurea drugs with normal and glycated human serum albumin, *Methods Mol. Biol* 1286 (2015) 255–277. [PubMed: 25749961]
- [39]. Moser AC, Kingsbury C, Hage DS, Stability of warfarin solutions for drug-protein binding measurements: spectroscopic and chromatographic studies, *J. Pharm. Biomed. Anal* 41 (2006) 1101–1109. [PubMed: 16545534]
- [40]. Conrad ML, Moser AC, Hage DS, Evaluation of indole-based probes for studying drug binding to human serum albumin in high-performance affinity separations, *J. Sep. Sci* 32 (2009) 1145–1155. [PubMed: 19296478]
- [41]. Hage DS, Chen J, Quantitative affinity chromatography: practical aspects, In *Handbook of Affinity Chromatography*, 2nd ed., Hage DS, (Ed.), Taylor & Francis, New York, 2006, Chap. 22.
- [42]. Tweed SA, Loun B, Hage DS, Effects of ligand heterogeneity in the characterization of affinity columns by frontal analysis, *Anal. Chem* 69 (1997) 4790–4798. [PubMed: 9406530]
- [43]. Tong Z, Hage DS, Detection of heterogeneous drug-protein binding by frontal analysis and high-performance affinity chromatography and peak profiling, *J. Chromatogr. A* 1218 (2011) 8915–8924. [PubMed: 21612784]
- [44]. Loun B, Hage DS, Chiral separation mechanisms in protein-based HPLC columns. I. Thermodynamic studies of (*R*)- and (*S*)-warfarin binding to immobilized human serum albumin, *Anal. Chem* 66 (1994) 3814–3822. [PubMed: 7802261]
- [45]. Xuan H, Joseph KS, Wa C, Hage DS, Biointeraction analysis of carbamazepine binding to alpha 1-acid glycoprotein by high-performance affinity chromatography, *J. Sep. Sci*, 33 (2010) 2294–2301. [PubMed: 20574961]

- [46]. Wa C, Cerny RL, Clarke WA, Hage DS, Characterization of glycated adducts on human serum albumin by MALDI-TOF MS, Clin. Chim. Acta 385 (2007) 48–60. [PubMed: 17707360]
- [47]. Barnaby O, Wa C, Cerny RL, Clarke W, Hage DS, Quantitative analysis of glycation sites on human serum albumin using $^{16}\text{O}/^{18}\text{O}$ -labeling and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Clin. Chim. Acta 411 (2010) 1102–1110. [PubMed: 20394739]
- [48]. Barnaby OS, Cerny RL, Clarke W, Hage DS, Comparison of modification sites formed on human serum albumin at various stages of glycation, Clin. Chim. Acta 412 (2011) 277–285. [PubMed: 21034726]
- [49]. Petitpas I, Bhattacharya AA, Twine S, East M, Curry S, Crystal structure of warfarin binding to human serum albumin: anatomy of drug site I, J. Biol. Chem 276 (2001) 22804–22809. [PubMed: 11285262]
- [50]. Barnaby OS, Cerny RL, Clarke W, Hage DS, Quantitative analysis of glycation patterns in human serum albumin using $^{16}\text{O}/^{18}\text{O}$ -labeling and MALDI-TOF MS, Clin. Chim. Acta 412 (2011) 1606–1615. [PubMed: 21601565]
- [51]. Yang J, Hage DS, Characterization of the binding and chiral separation of D- and L-tryptophan on a high-performance immobilized human serum albumin column, J. Chromatogr 645 (1993) 241–250. [PubMed: 8408417]
- [52]. Zheng X, Li Z, Beeram S, Podariu M, Matsuda R, Pfaunmiller EL, White CJ, II, Carter N, Hage DS, Analysis of biomolecular interactions using affinity microcolumns: A review, J. Chromatogr. B, 968 (2014) 49–53.

Highlights

- Affinity microcolumns were used to study binding by chlorpropamide with albumin.
- Both normal human serum albumin and glycated forms of this protein were examined.
- Several approaches in correcting for interactions with the support were considered.
- Frontal analysis showed there was an increase in chlorpropamide binding with glycation.
- Changes were also seen in the site-specific binding of this drug at Sudlow sites I and II.
- The changes in binding were compared with those seen for related sulfonylurea drugs.

Figure 1.

(a) Structures of the first-generation sulfonylurea drugs chlorpropamide (i.e., 1-[(p-chlorophenyl)sulfonyl]-3-propylurea)), tolbutamide and acetohexamide; (b) the addition of glucose to an amine group on a protein through the process of glycation. The dashed box in (a) shows the core structure of a sulfonylurea drug. The reactions in (b) represent the formation of an early stage glycation product; further reactions can occur to form advanced glycation end-products (AGEs) [23].

Figure 2.

Typical chromatograms obtained by frontal analysis for the application of chlorpropamide to a 1.0 cm × 2.1 mm i.d. column containing normal HSA column. The concentrations of the applied drug are shown on the right. The flow rate was 0.30 mL/min. These studies were performed at 37°C and pH 7.4.

Figure 3.

Double-reciprocal plots made according to Eq. (1) to examine data obtained for the binding of chlorpropamide with (a) normal HSA and (b) glycated HSA2 during frontal analysis studies. The best-fit lines shown are for the lower concentration data (i.e., 1.0 to 7.5 μM) and had correlation coefficients of 0.9998-0.9999 ($n = 5$). The error bars represent a range of ± 1 S.D.

Figure 4.

Comparison of the fits for (a) one-site and (b) two-site models to frontal analysis data for the overall binding of chlorpropamide to a column containing normal HSA. These fits were obtained by using Eqs. (4) and (5), respectively. The insets show the corresponding residual plots. The relative standard deviations for the values of $m_{Lapp,tot}$ that are shown in these plots ranged from $\pm 0.17\%$ to $\pm 4.7\%$ (average, $\pm 2.0\%$).

Figure 5.

Typical chromatograms obtained by zonal elution competition studies on a 1.0 cm × 2.1 mm i.d. column containing normal HSA and for the injection of *R*-warfarin in the presence of application of mobile phases that contained 20 or 0 μM chlorpropamide. The flow rate was 0.50 mL/min. These studies were performed at 37°C and pH 7.4.

Figure 6.

Example of plots made according to Eq. (6) when using chlorpropamide as a competing agent in the mobile phase and (a) *R*-warfarin or (b) *L*-tryptophan as the injected probe on a column containing glycosylated HSA1. The best-fit lines in (a) and (b) had correlation coefficients of 0.9813 and 0.9546, respectively. The error bars represent ± 1 S.D.

Table 1.

Best-fit parameters based on one- or two-site models for the overall binding of chlorpropamide with normal HSA or glycosylated HSA^a

Type of HSA & binding model	K_{a1} (M^{-1}) $\times 10^4$	m_{L1} (mol $\times 10^{-9}$)	K_{a2} (M^{-1}) $\times 10^4$	m_{L2} (mol $\times 10^{-8}$)	α
<i>One-site model</i>					
Normal HSA	3.5 ($\pm$ 0.3)	16.5 ($\pm$ 0.7)	-----	-----	0.48 ($\pm$ 0.12)
gHSA1	4.5 ($\pm$ 0.6)	15.9 ($\pm$ 0.9)	-----	-----	0.49 ($\pm$ 0.19)
gHSA2	4.7 ($\pm$ 0.8)	15.7 ($\pm$ 1.3)	-----	-----	0.48 ($\pm$ 0.31)
<i>Two-site model</i>					
Normal HSA	6.2 ($\pm$ 0.4)	3.4 ($\pm$ 0.3)	0.57 ($\pm$ 0.10)	4.3 ($\pm$ 0.6)	0.42 ($\pm$ 0.02)
gHSA1	8.9 ($\pm$ 0.7)	3.7 ($\pm$ 0.3)	0.18 ($\pm$ 0.01)	12.4 ($\pm$ 7.1)	0.41 ($\pm$ 0.03)
gHSA2	9.9 ($\pm$ 0.9)	2.4 ($\pm$ 0.2)	0.19 ($\pm$ 0.06)	14.8 ($\pm$ 0.3)	0.40 ($\pm$ 0.01)

^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 $\pm$ 1 S.D. The parameters shown for the one-site and two-site binding of chlorpropamide with HSA were obtained by using Eqs. (4) and (5), respectively, along with independently-estimated values for K_{aS} and m_{LS} of $1.64 \times 10^4 M^{-1}$ and 8.6×10^{-9} mol (see Supplemental Material).

Table 2.

Site-specific association equilibrium constants (K_a) for the interactions of chlorpropamide at Sudlow sites I and II^a

Type of HSA	Sudlow site I K_a ($M^{-1} \times 10^4$)	Change vs. Normal HSA	Sudlow site II K_a ($M^{-1} \times 10^4$)	Change vs. Normal HSA
Normal HSA	$3.9 (\pm 0.2) \times 10^4$	-----	$2.0 (\pm 0.4) \times 10^4$	-----
gHSA1	$4.3 (\pm 0.4) \times 10^4$	↑ 1.10-fold (N.S.) ^b	$2.8 (\pm 0.4) \times 10^4$	↑ 1.40-fold
gHSA2	$4.7 (\pm 0.5) \times 10^4$	↑ 1.21-fold	$3.1 (\pm 0.6) \times 10^4$	↑ 1.55-fold

^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.

^bMost of the changes shown in this table for gHSA1 and gHSA2 vs. normal HSA were significant at the 95% confidence level. The exception was the change seen at Sudlow site I for gHSA1 vs. normal HSA, which was not significant (N.S.) at the 95% confidence level but was significant at the 90% confidence level.