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CHARACTERIZATION OF TOLAZAMIDE BINDING WITH GLYCATED AND NORMAL HUMAN SERUM ALBUMIN BY USING HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

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Abstract

Sulfonylurea drugs are antidiabetic drugs that are utilized in the treatment of type II diabetes and often have significant binding with human serum albumin (HSA). Immobilized samples of normal or glycated HSA in affinity microcolumns were used to investigate interactions of these proteins with the sulfonylurea drug tolazamide. HPLC and frontal analysis were used to first examine the overall binding of this drug with these samples of HSA. It was found that tolazamide had two general classes of binding sites (i.e., high and low affinity) for normal and glycated HSA. The higher affinity sites had binding constants of around $4.3\text{--}6.0 \times 10^4 \text{ M}^{-1}$ for these interactions at pH 7.4 and 37 °C, while the lower affinity sites had binding strengths of $4.9\text{--}9.1 \times 10^3 \text{ M}^{-1}$. Zonal competition studies between tolazamide and probes for Sudlow sites I and II on HSA were also performed and used to provide site-specific affinities for tolazamide at these sites. A decrease of 22% in affinity was observed for tolazamide at Sudlow site I and an increase up to 58% was seen at Sudlow site II when comparing glycated HSA with normal HSA. These observed changes were compared to those of other first-generation sulfonylurea drugs, providing information on how glycation can alter the total and local binding strength of tolazamide and related compounds with HSA under levels of glycation seen in patients with diabetes.

Keywords

tolazamide; human serum albumin; drug-protein binding; glycation; affinity microcolumn

1 Introduction

In 2014 a report from the World Health Organization stated that approximately 422 million adults suffered from diabetes [1]. About 30.3 million people were diagnosed as having diabetes in the United States in 2015 [1,2]. Diabetes is a group of metabolic disorders that pertain to the pancreas not producing enough insulin (i.e., insulin deficiency) or the body not

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effectively using the insulin it has produced (e.g., insulin resistance), with both situations resulting in an elevated glucose level in blood. The two main forms of diabetes are type I and type II. Type I diabetes (i.e., juvenile-onset diabetes) is caused by the immune system attacking the pancreatic beta cells, leading to a lack of ability of the body to produce insulin. Type II diabetes (i.e., non-insulin dependent diabetes) affects 90%–95% of diabetic patients [1–3].

Sulfonylurea drugs are frequently used to treat type II diabetes. These agents can reduce glucose levels in the circulation by stimulating pancreatic beta cells to secrete insulin. The basic structure of a sulfonylurea-type compound has a core that contains a phenylsulfonyl group and a urea group [3,4]. Tolazamide (see Figure 1) is a first-generation sulfonylurea drug that has been previously employed to treat type II diabetes [4]. Like other compounds in this group, tolazamide is known to have a moderately strong interaction with the transport protein human serum albumin (HSA) and the closely-related protein bovine serum albumin (BSA) [5–13]. Prior work has suggested that either one or two groups of sites are involved in the binding of tolazamide to serum albumin [6,7].

Glycation is a non-enzymatic process of protein modification that occurs at increased levels in the blood of diabetic patients [14–16]. The glycation process begins when free amine groups on a protein react with the aldehyde group on glucose to form a reversible Schiff base. A more stable Amadori product is later formed from this Schiff base through rearrangement [16]. Glycation is of interest among researchers because it can affect both the structure and function of serum proteins [14–16]. HSA has been recently examined in such work because the amount of its glycated form in diabetes can increased two-fold to five-fold when compared to HSA from healthy adults [16].

HSA is the most abundant protein in human blood and is composed of 585 amino acids (molar mass, 66.5 kDa) [17]. This protein has a typical concentration of 35–50 mg/mL and makes up about 50% of all the proteins in serum [17]. HSA participates in many processes, including the transportation of compounds such as hormones, fatty acids, and drugs. Sudlow sites I and II are two major binding sites for drugs on HSA [17,18]. Sudlow site I mainly binds to bulky heterocyclic compounds such as warfarin, azapropazone, phenylbutazone, and salicylate, while Sudlow site II primarily binds to aromatic carboxylic compounds such as L-tryptophan, ibuprofen, fenoprofen, and ketoprofen [17,18]. Both sites have been found in previous work to bind to several sulfonylurea drugs [8–13], although the interactions of tolazamide at these sites has not been examined in prior studies.

High-performance affinity chromatography (HPAC) will be utilized in this report to examine the interactions between tolazamide and HSA at various levels of glycation. HPAC uses immobilized biological molecules as its stationary phase and as a means to retain specific solutes or groups of solutes [19,20]. It is known from prior work that HPAC can provide good precision, fast analysis times, and good correlation with other techniques during the study of drug-protein interactions [19]. Furthermore, HPAC can be readily automated, and it can be used with the same preparation of a protein for many experiments [8–11,19]. Other advantages of HPAC, as pertaining to this study, are its capability to provide both the overall

strength of a drug-protein interaction and the local site-specific affinities for a drug with a protein [19,20].

Frontal analysis and zonal elution will be utilized in this report with HPAC to investigate the overall binding and site-specific interactions of normal and *in vitro* glycated forms of HSA with tolazamide. This study will also include a comparison between these results and prior observations made for alternative first-generation sulfonylurea drugs to see how alterations in the structure of these compounds affect their binding to HSA and glycated HSA [6–13]. The final results should provide a better understanding of the effects of HSA glycation on the transport and protein binding by sulfonylurea drugs in serum. These data should also provide useful information for future applications in the field of personalized medicine, as pertaining to the ability to adjust a drug dosage for a given patient in a disease such as diabetes [8].

2 Experimental

2.1 Chemicals

The HSA (lyophilized powder, essentially fatty acid free, 96% pure), L-tryptophan (98%), *R*-warfarin (97% pure), D- (+)-glucose (99.5%), and sodium azide (>95%) were purchased from Sigma Aldrich (St. Louis, MO, USA). The tolazamide (99% pure) was from Santa Cruz Biotechnology (Dallas, TX, USA). Modification levels of the *in vitro* glycated HSA samples were measured using a fructosamine kit purchased from Diazyme Laboratories (San Diego, CA, USA), and the protein content of each chromatographic support was measured using a bicinchoninic acid assay (BCA) from Pierce (Rockford, IL, USA). All aqueous solutions were prepared using purified water from a Milli-Q-Advantage A 10 system (EMD Millipore, Billerica, MA, USA); these solutions were filtrated through 0.20 µm GNWP nylon membranes (Fisher Scientific, Pittsburgh, PA, USA).

2.2 Instrumentation

A Jasco HPLC system (Cotati, CA, USA) was used for this work. This system consisted of 1) two PU-2080 pumps, 2) a DG-2080 degasser, 3) an AS-2057 autosampler, 4) a CO-2067 column oven, 5) a UV-2080 absorbance detector, and 5) a Rheodyne Advantage PF six-port valve (Cotati, CA, USA). Chromatographic data were processed and analyzed by using Peakfit 4.12 from Jandel Scientific Software (San Rafael, CA, USA), and nonlinear regression was performed using DataFit 8.169 (Oakdale, PA, USA).

2.3 Preparation of glycated HSA

Glycated samples of HSA (i.e., gHSA1 and gHSA2) were made *in vitro* by using a prior method [9–11]. All tools used for this procedure (e.g., glassware and spatulas) were sterilized by autoclaving [9–13,21], and 1 mM sodium azide was added to the reaction buffer to prevent further bacterial growth. An 840 mg sample of normal HSA was first dissolved into 20 mL of potassium phosphate buffer (pH 7.4, 0.067 M), giving a solution that contained a typical physiological concentration of 42 mg/mL for this protein. Moderate or high concentrations of D-glucose (i.e., 15 or 30 mM, representing levels found in serum during controlled or advanced diabetes) were then added to this mixture [9,21]. These

solutions were placed into a water bath and gently mixed for four weeks at 37 °C. The excess glucose was removed by using a desalting column, with the protein samples then being lyophilized and stored at –80 °C. The levels of glycation in these protein samples were determined in duplicate using a fructosamine assay. The measured modification levels were < 0.1, 1.40 ($\pm$ 0.06), and 3.24 ($\pm$ 0.07) mol hexose/mol HSA for the normal HSA, gHSA1, and gHSA2, where the numbers in parentheses represent $\pm$ 1 S.D.

2.4 Preparation of HSA microcolumns

Nucleosil Si-300–7 silica was used in preparing affinity microcolumns for the HPAC studies. The silica was first modified and converted into a diol-bonded form, with normal HSA or glycated HSA being immobilized onto this support via the Schiff base method [22,23]. The same approach was employed in preparing a control support without the addition of any HSA. A BCA assay was used to measure the amount of immobilized protein for each support. The control support was used as a blank and normal HSA was the standard, giving protein contents of 97 ($\pm$ 2), 87 ($\pm$ 10), and 95 ($\pm$ 4) mg HSA per g silica for normal HSA, gHSA1, and gHSA2, respectively.

Using a potassium phosphate buffer (pH 7.4, 0.067 M) as the packing solution, all supports were downward slurry packed into a 1.0 or 2.0 cm $\times$ 2.1 mm i.d. stainless steel column at 3500 psi (24 MPa). The columns and any remaining support were stored in the same buffer at 4 °C until further use. Affinity microcolumns prepared in this manner with HSA have been found to be usable over several hundred sample injections without significant changes in their binding properties [8–13,23].

2.5 Chromatographic studies

All drug solutions were prepared in phosphate buffer (pH 7.4, 0.067 M) and filtered through a 0.2 μ m membrane, followed by degassing for 10–20 min. The L-tryptophan solutions were prepared fresh daily, while the *R*-warfarin solutions were prepared and used within two weeks [23]. The chromatographic experiments were conducted at 37 °C and 0.50 mL/min, as employed in previous drug-binding studies using similar protein supports or columns [8–13].

Frontal analysis studies were conducted by applying a potassium phosphate buffer (pH 7.4, 0.067 M) onto a microcolumn until a steady baseline was obtained. A step change was then made for the application of a known concentration of tolazamide, as prepared in the same buffer and passed continuously through the microcolumn. A total of 12 solutions containing 0.5 to 50 μ M tolazamide were applied in triplicate to each HSA microcolumn and control microcolumn. After reaching a stable plateau at a given concentration of tolazamide, the original buffer was then reapplied to the microcolumn to elute the bound tolazamide and regenerate the system. The elution of tolazamide was detected at a wavelength of 254 nm, and all concentrations of this drug that were examined were within the linear range of the detector at this wavelength. The first derivative function in PeakFit 4.12 was used to find the mean position of each frontal analysis (or breakthrough) curve. Identical studies were conducted on a control column to correct for the system void time and non-specific binding of tolazamide to the support, as has been used in previous work with similar support

materials and columns [8–13,23]. The relative extent of non-specific binding ranged from 27–36% of the total measured binding on the HSA microcolumns over the range of tolazamide concentrations that were examined; these results are in line with previous data that have been acquired with another first-generation sulfonylurea drugs (i.e., chlorpropamide) on similar columns [13].

Zonal elution competition studies were conducted by employing *R*-warfarin and L-tryptophan as site specific probes for HSA [21,23]. Eight mobile phase concentrations prepared in phosphate buffer (pH 7.4, 0.067 M) and ranging from 0 to 20 μ M tolazamide were used in these experiments. Each sample contained 5 μ M of the given probe, as prepared in the mobile phase. A 20 μ L portion of each sample was injected (four times) under each set of mobile phases and microcolumn conditions. The wavelengths that were utilized for detection were 308 and 280 nm for *R*-warfarin and L-tryptophan, respectively. A 20 μ M solution of sodium nitrate in phosphate buffer (pH 7.4, 0.067 M) was used as a void volume marker and was monitored at 205 nm [23]. Zonal elution experiments were performed in replicate (four times), and an exponentially-modified Gaussian curve fit was used to find the mean retention time of each peak.

3 Results and discussion

3.1 Frontal analysis studies

Frontal analysis was used to examine the overall interactions of tolazamide with normal or glycated HSA [24]. In this method, a drug solution with a known concentration was continuously applied onto a microcolumn containing one of these protein preparations. During application of the drug solution, the amount of drug that eluted was monitored and formed a breakthrough curve. Figure 1 shows some typical breakthrough curves for several concentrations of tolazamide that were applied at 0.50 mL/min to microcolumns containing normal HSA or gHSA1 (see Supplemental Material for a detailed list of results). Similar results to those seen for gHSA1 were obtained for gHSA2. These curves reached stable plateaus within 2.5–4.0 min under the given conditions. The precision for the moles of bound tolazamide that were measured during these experiments ranged from ± 0.1 –5.5% (average, $\pm 1.8\%$).

Each frontal analysis chromatogram for tolazamide showed a small initial plateau (i.e., at about 2.2–2.5 min in Figure 1) which represented the void volume and which was created by a slight difference in the background absorbance of the tolazamide solution when compared to the application buffer. The frontal analysis results after the void volume and with normal HSA gave a set of two curves at the higher concentrations of applied tolazamide, indicating that multiple types of binding sites were present for this drug. The glycated HSA samples exhibited only one overall breakthrough curve beyond the void volume, demonstrating that some changes in the binding of tolazamide had taken place in going from normal HSA to glycated HSA. The overall breakthrough time for the combined curves in each plot, beyond the void volume, were used to determine the binding capacity for tolazamide to the immobilized HSA (see Section 2.5); this approach for data analysis made it possible to directly compare the results obtained at different concentrations of tolazamide with the same preparation of HSA or on different types of HSA microcolumns.

The overall binding capacities that were measured at different applied concentrations of tolazamide were fit to various binding models to obtain the equilibrium constants and amounts of binding sites for these interactions [24]. Eq. (1) shows the response expected for a one-site model when looking at the change in the apparent moles of a bound analyte (A) or drug on an affinity column as a function of the applied molar concentration for the analyte, [A] [24–26].

$$m_{Lapp} = \frac{m_L K_{a1} [A]}{(1 + K_{a1} [A])} \quad (1)$$

In this equation, m_{Lapp} is the apparent moles of drug/analyte needed to reach the mean point of the breakthrough curve at a given applied concentration for the analyte, m_L is the moles of active sites that can bind to this analyte, and K_a is the association equilibrium constant for the interaction [8–13,24–26]. Eq. (2) can be similarly used to fit frontal analysis data to a two-site binding model,

$$m_{Lapp} = \frac{m_{L1} K_{a1} [A]}{(1 + K_{a1} [A])} + \frac{m_{L2} K_{a2} [A]}{(1 + K_{a2} [A])} \quad (2)$$

where K_{a1} and K_{a2} are the association equilibrium constants for the drug or analyte at the two groups of sites (e.g., with K_{a1} representing moderate-to-high affinity regions, such as Sudlow sites 1 and 2, and K_{a2} representing weak or non-specific sites on the immobilized binding agent) [25,26]. The terms m_{L1} and m_{L2} are the moles of these two groups of binding sites that are present in the column.

Another factor to consider in such studies is the presence of any secondary binding and interactions the analyte or drug may have with the support [9–11,24]. If a relatively large amount of binding agent is present, as was the case in this study, then it may also be necessary to consider the extent to which the presence of the immobilized binding agent covers and reduces the availability of these secondary sites [13]. The binding of tolazamide with a control support (i.e., with no immobilized HSA present) followed a one-site model, such as described by Eq. (1) (see Supplemental Material). This data made it possible to estimate the total moles of sites for tolazamide on the original support (as described here by the term m_S) and the association equilibrium constant for this binding (K_{aS}). These values were then used in fits to the data for the HSA columns by using expanded versions of Eqs. (1–2), as shown in Eqs. (3–4), that included an additional term for binding by tolazamide to the support.

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

$$m_{Lapp} = \frac{m_{L1}K_{a1}[D]}{(1 + K_{a1}[D])} + \frac{m_{L2}K_{a2}[D]}{(1 + K_{a2}[D])} + \frac{\alpha m_S K_{aS}[D]}{(1 + K_{aS}[D])} \quad (4)$$

The values of m_S and K_{aS} were determined in separate experiments for the control column and the only additional fitted parameter that was added to Eqs. (3–4), when compared to Eqs. (1–2), was the term α . This term was used to represent the fraction of binding sites on the original support that was still accessible to tolazamide in the presence of immobilized HSA, and which had an expected value between zero and one.

The results of these fits are shown in Table 1. These results were obtained using a best-fit value of $1.34 (\pm 0.01) \times 10^3 \text{ M}^{-1}$ for K_{aS} (i.e., representing binding by tolazamide to the support) and a value of $8.3 (\pm 0.1) \times 10^{-7} \text{ mol}$ for m_S (i.e., the moles of binding sites for tolazamide on the support in the absence of any HSA). Based on the results shown in Table 1, the presence of HSA led to a decrease in secondary binding between tolazamide and the support. For example, the data for the fit of the two-site drug/protein binding model in Table 1 indicated that about half (51%) of the original binding sites for tolazamide on the support were still available to tolazamide once normal or glycosylated HSA had been immobilized. Use of a one-site drug/protein model similarly indicated that 58–60% of the original sites on the support were accessible for binding to tolazamide after protein immobilization.

Figure 2 shows some typical frontal analysis data for tolazamide on a column containing HSA and when fit to Eqs. (3) and (4). For each type of HSA, it was found that the two-site drug/protein binding model, as described by Eq. (4), gave a much better fit to the data than a one-site drug/protein model based on Eq. (3). These two types of fits had correlation coefficients ranging from 0.9998–0.9999 and 0.9829–0.9972 ($n = 12$), respectively, for all the types of HSA samples that were examined. The fits to the two-site model based on Eq. (4) also gave more random variations of the data about the best-fit lines (see insets in Figure 2) and smaller values for the sums of the squares of the residuals. In the latter case, values of $0.1\text{--}2.0 \times 10^{-18}$ were obtained for the two-site drug/protein binding model in (Eq. 4) vs. values of $0.3\text{--}1.4 \times 10^{-16}$ for the one-site drug/protein model in (Eq. 3), with these differences being significant at the 95% confidence level. These results, and the general shape of the frontal analysis curves, indicated that the two-site drug/protein model was better than the one-site model for describing interactions between tolazamide and normal HSA or glycosylated HSA. This conclusion was consistent with the two-step frontal analysis curves that are shown in Figure 1(a) for normal HSA at moderate-to-high tolazamide concentrations. This two-site model also agrees with previous findings for tolazamide and several other first-generation sulfonylureas with normal HSA or BSA [6,7], as well as for alternative first-generation sulfonylurea drugs with similar preparations of glycosylated HSA to those used in this study [8–13].

The association equilibrium constants and moles of binding sites that were obtained with the two-site drug/protein binding model are provided in Table 1 (Note: the results for the one-site model are also provided for reference). The association equilibrium constants for the higher affinity sites that were found for tolazamide with normal HSA, gHSA1, and gHSA2

were $5.3 (\pm 0.6) \times 10^4 \text{ M}^{-1}$, $4.3 (\pm 0.5) \times 10^4 \text{ M}^{-1}$, and $6.0 (\pm 0.2) \times 10^4 \text{ M}^{-1}$, respectively. The precisions of these values varied from ± 3.3 – 11.6% (average, $\pm 8.8\%$). This range of binding constants was similar to a value of $8.67 \times 10^4 \text{ M}^{-1}$ that has been previously measured for the higher affinity sites of tolazamide at pH 7.4 and 37°C when using dynamic dialysis and a 1% solution of normal HSA [7]. In addition, these binding constants were consistent with binding strengths of 12 – $22 \times 10^4 \text{ M}^{-1}$, 8.4 – $12 \times 10^4 \text{ M}^{-1}$, and 4.5 – $9.9 \times 10^4 \text{ M}^{-1}$ that have been reported for three other first-generation sulfonylurea drugs (i.e., acetohexamide, tolbutamide, and chlorpropamide, respectively) with normal HSA and glycated HSA [7–11,13].

The values obtained from the same fits for the binding strengths of the weak or non-specific sites (as represented by K_{a2}) are also listed in Table 1. The value of this term for normal HSA, gHSA1, and gHSA2 was $0.49 (\pm 0.01) \times 10^4 \text{ M}^{-1}$, $0.85 (\pm 0.27) \times 10^4 \text{ M}^{-1}$, or $0.91 (\pm 0.01) \times 10^4 \text{ M}^{-1}$, respectively (i.e., binding constants that were 5.1- to 10.8-fold lower than K_{a1} for the higher affinity sites). The precisions of these K_{a2} values ranged from ± 1.1 – 32% (average, $\pm 11.6\%$). These binding constants were similar to those for weak/non-specific sites that have been reported for three other first-generation sulfonylurea drugs with normal HSA and glycated HSA [7–11,13].

The amount of binding sites that had a moderate-to-high affinity for tolazamide was found from the data in Table 1 to be 0.14 – 0.19 mol per mol HSA (average, 0.16) for the three types of normal or glycated HSA that were examined in this study. These results were based on the content of HSA in each type of microcolumn (i.e., 41 – 45 nmol, as calculated by using the measured amount of HSA on the support and the support's known packing density) and the values for m_{L1} that are listed in Table 1, as obtained for the same preparations of HSA when using a two-site binding model. These values suggested that only a portion of the immobilized HSA was active [9–12] and that only one or a few binding regions on HSA made up tolazamide's higher affinity sites. The number of lower affinity binding sites for tolazamide was found through the same approach to range from 3.9 – 19.6 (average, 14.0) mol per mol HSA.

The data in Table 1 indicate that some changes occurred in the value of K_{a1} at the moderate-to-high affinity sites for tolazamide when going from normal HSA to glycated HSA and using a two-site binding model. For instance, there was a 19% decrease in the value of K_{a1} for tolazamide when going from normal HSA to the gHSA1 and a 13% increase between normal HSA and gHSA2. Each of these differences was significant at 95% confidence level. Both increased binding and decreased binding strength has been seen for other sulfonylurea drugs with HSA that had similar or identical levels of glycation to that present in this study [8–11,13]. These changes in affinity have been proposed to be due to glycation or related modifications that take place at or near Sudlow sites I and II [8–13,27–29]. In addition, an increase in K_{a2} of 1.7- to 1.9-fold and an increase of 4.4- to 4.6-fold in m_{L2} for the weak/non-specific sites were seen for tolazamide in going from normal to glycated HSA. This latter set of increases explains why two fronts were sometimes seen in the breakthrough curves for tolazamide with normal HSA (i.e., which had the largest difference between K_{a1} vs. K_{a2} and m_{L1} vs. m_{L2}), while only one front was seen for the microcolumns that contained glycated HSA.

3.2 Zonal competition studies

3.2.1 Zonal elution studies with tolazamide at Sudlow site I—Zonal elution competition studies were used to investigate any site-specific changes in the binding of tolazamide with normal or glycosylated HSA and to identify the regions on this protein which made up the moderate-to-high sites that were represented by K_{aI} in the frontal analysis studies. In this method, a small amount of a site-specific probe (e.g., *R*-warfarin or L-tryptophan) was injected onto an HSA microcolumn in the presence of a mobile phase that contained a known and fixed concentration of tolazamide [24]. Figure 3 shows typical chromatograms for these competition experiments for normal HSA and gHSA1; similar behavior was seen for gHSA2. In these studies, the probe eluted in under 1.5–2.0 min (for L-tryptophan) or under 7–8 min (for *R*-warfarin). The retention factor (k) for the probe was determined at each concentration of tolazamide in the mobile phase. The precision of the retention factors for the injected probes in these studies ranged from ± 0.1 –8.4% (average, $\pm 1.3\%$).

Previous studies have shown that direct interaction of a probe with a drug at a single site during a zonal elution experiment yields a linear relationship when plotted according to Eq. (5) [24,30].

$$\frac{1}{k} = \frac{K_{aA}V_M[A]}{K_{aP}m_L} + \frac{V_M}{K_{aP}m_L} \quad (5)$$

In (Eq. 5), K_{aP} and K_{aA} are the association equilibrium constants for the probe and analyte at the competition site, V_M is the void volume, and m_L is the moles of common binding sites. The ratio of the slope to intercept for a plot of $1/k$ versus $[A]$ provides the value of K_{aA} , which is the local association equilibrium constant for the drug at the common binding region [24]. Examples of this type of plot are shown in Figure 4.

Interactions of tolazamide at Sudlow site I were investigated by employing *R*-warfarin as the site-specific probe [21,23]. These experiments provided linear plots according to Eq. (5) for the columns that contained normal or glycosylated HSA. The correlation coefficients of these plots spanned from 0.8962 to 0.9502 ($n = 6$ –8). These fits were consistent with a system in which tolazamide had direct interactions with *R*-warfarin at Sudlow site I on normal and glycosylated HSA. This result agreed with observations made in previous studies with other first-generation sulfonylurea drugs [9–12].

Table 2 shows the association equilibrium constants that were determined for tolazamide at Sudlow site I on each HSA microcolumn. These values were $1.8 (\pm 0.3) \times 10^4 \text{ M}^{-1}$, $1.4 (\pm 0.2) \times 10^4 \text{ M}^{-1}$, and $1.4 (\pm 0.3) \times 10^4 \text{ M}^{-1}$ for tolazamide on normal HSA, gHSA1 and gHSA2, respectively, and had precisions of ± 14 –21% (average, $\pm 17\%$). These binding constants were within the range of 10^4 – 10^5 M^{-1} that was measured by frontal analysis for K_{aI} at the moderate-to-high affinity sites of tolazamide on HSA, indicating that Sudlow site I was one of these regions. There was a 22% decrease in the binding strength for tolazamide at Sudlow site I in going from normal HSA to both gHSA1 and gHSA2. In each case, this decrease in local affinity was significant at the 95% confidence level. These changes in

binding strength are again probably related to glycation-related modifications that occur at or near Sudlow site I, as have been observed for residues K199, K276, and K281 on HSA [16].

The results for tolazamide in the zonal elution studies were compared to those that have been measured by the same method with other first-generation sulfonylurea drugs (see Table 2). In the case of acetohexamide, a non-significant decrease in binding strength of 3–10% has been noted at Sudlow site I with HSA that had similar or identical levels of glycation to that utilized in this study; however, lower levels of glycation gave a 40% increase in the binding strength of acetohexamide at this same site [9,11]. Small-to-moderate increases in binding strength have been noted for chlorpropamide and tolbutamide (i.e., increases of 10–21% and 18–20%, respectively) with samples of glycated HSA that were similar to those used in this report [8–11,13].

The difference in the relative direction and sizes of these changes in binding with glycated HSA and with the various sulfonylureas in Table 2 provides some clues as to the influence of their two side chains (i.e., R1 and R2) on these interactions. As an example, tolazamide and tolbutamide both have a methyl group at R1, while tolazamide has a large azepane group at R2 and tolbutamide has a much smaller butyl group. This comparison suggests that the 22% decrease in binding strength for the glycated HSA with tolazamide vs. the 18–20% increase with tolbutamide, and the 3.1- to 4.7-fold higher affinity seen for tolbutamide with both normal and glycated HSA, is due to a better fit of the smaller group at R2 on tolbutamide in Sudlow site I. This observation is supported by the fact that chlorpropamide (i.e., which has only a propyl group at R2 and a chlorine at R1) has a similar binding strength and change in affinity to that seen with tolbutamide in going from normal HSA to glycated HSA. The binding strength and change in binding for acetohexamide at Sudlow site I are closest to the results for tolazamide. This similarity may be due to the relatively large cyclic rings that are present at R2 in both compounds.

3.2.2 Zonal elution studies with tolazamide at Sudlow site II—Similar zonal elution studies were used to examine the binding of tolazamide at Sudlow site II, for which L-tryptophan was used as a site-specific probe [18,23]. The average precision obtained for the retention factors in these studies was $\pm 1.5\%$ (range, ± 0.1 – 8.5%). Plots of the resulting data were made according to (Eq. 5), as illustrated in Figure 4(b). These plots gave linear responses with correlation coefficients for all the tested protein samples that ranged from 0.8967 to 0.9653 ($n=7$ – 8). This type of response indicated that tolazamide was binding at Sudlow site II in both normal and glycated HSA.

Table 2 lists the site-selective binding constants that were measured for tolazamide at Sudlow site II for each type of HSA. These values ranged from 1.9 to $3.0 \times 10^4 \text{ M}^{-1}$ and had precisions of ± 14 – 23% (average, $\pm 18\%$). These results were slightly larger than the binding constants measured for tolazamide at Sudlow site I and were again consistent with the values for K_{dI} that were determined by frontal analysis for the moderate-to-high affinity sites of tolazamide on HSA. The values in Table 2 for tolazamide at Sudlow site II were also the same order of magnitude but lower than binding constants that have been reported for acetohexamide and tolbutamide under comparable conditions with similar preparations of

HSA [10–13]. There was a small, non-significant increase of 11% in the local affinity for tolazamide at Sudlow site II when comparing gHSA1 to normal HSA; however, there was also a 58% increase, which was significant at the 95% confidence level, in going from normal HSA to gHSA2. These changes were again thought to reflect modifications that were occurring at or near this site due to glycation, as have been observed at K378 and K439 [16,27–29].

The results obtained for tolazamide at Sudlow site II were compared with those noted for other first-generation sulfonylurea drugs with similar samples of modified HSA [10–12]. In this case, chlorpropamide gave the closest behavior to that seen for tolazamide at Sudlow site II. Chlorpropamide had local affinities that were similar to those seen for tolazamide and had an increase in affinity of up to 55% being seen in going from normal HSA to samples of glycated HSA [13]. These similarities indicated that Sudlow site II was mostly interacting with the R1 region of these compounds (i.e., a change from only a methyl group to a chlorine between tolazamide and chlorpropamide). However, some interactions with R2 were probably present as well, as suggested by the differences in affinities at Sudlow site II that were seen between tolazamide and tolbutamide (i.e., both compounds having methyl groups at R1). These conclusions agree with recent observations that have been made when comparing the interactions of chlorpropamide with those of acetohexamide and tolbutamide at Sudlow site II on normal or glycated HSA [13].

4 Conclusion

In this study, HPAC was used to investigate the binding of tolazamide to normal HSA and glycated HSA. These results were then compared to those obtained in prior work with other first-generation sulfonylurea drugs. Frontal analysis was first used to examine the overall interactions of tolazamide with the various preparations of HSA. A two-site model yielded binding constants for tolazamide that included a set of moderate-to-high affinity regions ($4.3\text{--}6.0 \times 10^4 \text{ M}^{-1}$) and a set of lower affinity regions ($4.9\text{--}9.1 \times 10^3 \text{ M}^{-1}$). Both a slight decrease (19%) and small increase (13%) in binding strength at the higher affinity regions on HSA were seen for tolazamide at the two levels of glycation that were considered, which represented typical conditions seen in controlled or advanced diabetes. Both these trends and binding model agreed with previous observations that have been made for other sulfonylurea drugs [8–11,13].

Zonal elution studies were used to examine the interactions of tolazamide at specific sites on normal and glycated HSA. This drug was found to bind to both Sudlow sites I and II, as has been reported for other first-generation sulfonylurea drugs [8–13]. A 22% decrease in affinity was observed for tolazamide at Sudlow site I for the glycated samples of HSA when compared to normal HSA, while up to a 58% increase in affinity was observed at Sudlow site II. These results were also consistent with prior observations made for other sulfonylurea drugs, which have typically been found to both Sudlow sites I and II and which can have increased or decreased affinities at these sites as a result of glycation [8–11,13].

The results for tolazamide at Sudlow sites I and II were compared in more detail to prior data obtained for related first-generation sulfonylurea drugs to learn more about the portions

of these drugs that contributed to binding at each of these sites. It was found that changes in the side group on the urea portion of these drugs (i.e., R2 in Table 2) were important in determining their affinities at Sudlow site I. Changes at the side group on the phenylsulfonyl region of these compounds (i.e., R1 in Table 2) were important in affecting binding at Sudlow site II; however, some contributions from R2 were also probably present at this site.

It was illustrated in this report how HPAC can be employed with affinity microcolumns to look at changes in both the overall binding and site-selective interactions that take place as a result of protein modification. This approach was shown to be relatively fast, reproducible, and able to provide precise estimates of binding constants for such work. It was these features, plus the ability to use a small amount of protein for many experiments, which made it possible to examine even relatively minor changes in the affinity of tolazamide for normal and glycated HSA. These properties also made it possible to compare the binding behavior noted for tolazamide with that seen for related drugs and similar or equivalent preparations of normal or glycated HSA. These results provided a better understanding of how HSA glycation can affect the transport and protein binding of sulfonylurea drugs in serum, as well as information that can be used in future studies aimed at personalized medicine based on these agents [8].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The binding of tolazamide with albumin was examined using affinity microcolumns.
- Normal human serum albumin and glycated forms of this protein were considered.
- Frontal analysis was used to examine the overall changes in binding with glycation.
- Zonal elution was used to examine binding of this drug at Sudlow sites I and II.
- The changes in binding were compared with those seen for other sulfonylurea drugs.

Figure 1.

Structure of tolazamide and typical chromatograms obtained by frontal analysis for the application of various concentrations of tolazamide at 0.50 mL/min to affinity microcolumns containing immobilized samples of (a) normal HSA or (b) glycated HSA1 (gHSA1).

Figure 2.

Fit of (a) one-site or (b) two-site drug/protein binding models to frontal analysis data for tolazamide with an immobilized sample of gHSA1, as obtained by using Eq. (3) or (4), respectively, and including the use of a term to correct for secondary binding by tolazamide to the support (Note: see Supplemental Materials for more details). The insets show the corresponding residual plots. The correlation coefficients for these fits were (a) 0.9965 and (b) 0.9999 ($n = 12$).

Figure 3.

Typical chromatograms obtained by zonal elution competition studies and in the presence of 0 or 20 μM tolazamide in the mobile phase for (a) the injection of L-tryptophan as a probe for Sudlow site II on a normal HSA microcolumn or (b) the injection of *R*-warfarin as a probe for Sudlow site I on a microcolumn containing gHSA1. These chromatograms were acquired at a flow rate of 0.50 mL/min.

Figure 4.

Examples of zonal elution competition studies and plots made according to Eq. (5) when using tolazamide as a competing agent and (a) *R*-warfarin as the injected probe on a column containing gHSA2 or (b) L-tryptophan as the injected probe on a normal HSA column. The correlation coefficients for these plots were (a) 0.9287 ($n = 8$) and (b) 0.9653 ($n = 8$).

Table 1.

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

Type of HSA & binding model	α	K_{a1} ($M^{-1} \times 10^4$)	m_{L1} ($mol \times 10^{-9}$)	K_{a2} ($M^{-1} \times 10^4$)	m_{L2} ($mol \times 10^{-7}$)
<i>One-site model</i>					
Normal HSA	0.58 (± 0.04)	3.4 (± 0.4)	48.4 (± 3.3)	-----	-----
gHSA1	0.60 (± 0.02)	1.7 (± 0.1)	66.9 (± 2.2)	-----	-----
gHSA2	0.60 (± 0.06)	3.9 (± 0.5)	40.4 (± 3.0)	-----	-----
<i>Two-site model</i>					
Normal HSA	0.51 (± 0.01)	5.3 (± 0.6)	6.2 (± 0.3)	0.49 (± 0.01)	1.8 (± 0.1)
gHSA1	0.51 (± 0.01)	4.3 (± 0.5)	7.6 (± 0.4)	0.85 (± 0.27)	8.0 (± 2.4)
gHSA2	0.51 (± 0.01)	6.0 (± 0.2)	6.1 (± 0.2)	0.91 (± 0.01)	8.2 (± 1.1)

^aThese results were measured at pH 7.4 and 37 °C. The ranges given in parentheses are ± 1 S.D. Eqs. (3) and (4) were used to obtain the parameters for the one-site and two-site binding models of tolazamide with HSA by employing independent estimates for K_{aS} and m_{LS} of $1.34 (\pm 0.01) \times 10^3 M^{-1}$ and $8.3 (\pm 0.1) \times 10^{-7}$ mol.

Site-specific association equilibrium constants (K_d) determined by zonal elution for the binding of tolazamide and other first-generation sulfonylureas at Sudlow sites I and II of HSA^a

Table 2.

Sulfonylurea [Refs.]	R1	R2	K_d , Sudlow site I ($M^{-1} \times 10^4$)			K_d , Sudlow site II ($M^{-1} \times 10^4$)		
			Normal HSA	gHSA1	gHSA2	Normal HSA	gHSA1	gHSA2
Tolazamide	-CH ₃		1.8 (± 0.3)	1.4 (± 0.2)	1.4 (± 0.3)	1.9 (± 0.3)	2.1 (± 0.3)	3.0 (± 0.7)
Acetohexamide [9,11]			4.2 (± 0.4)	3.8 (± 0.3)	4.1 (± 0.6)	13 (± 1)	11 (± 1)	12 (± 1)
Chlorpropamide [13]	-Cl		3.9 (± 0.2)	4.3 (± 0.4)	4.7 (± 0.5)	2.0 (± 0.4)	2.8 (± 0.4)	3.1 (± 0.6)
Tolbutamide [10,11]	-CH ₃		5.5 (± 0.5)	6.6 (± 0.5)	6.5 (± 0.2)	5.3 (± 0.2)	7.2 (± 0.3)	6.4 (± 0.3)

^aThese results were measured at pH 7.4 and 37 °C. The ranges in parentheses are ± 1 S. D., as based on error propagation and the precisions of the best-fit slopes and intercepts obtained when using Eq. (5). The protein samples referred to here as "gHSA1" and "gHSA2" were prepared under the same conditions as used in this report for these preparations of HSA.