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Binding of tolbutamide to glycated human serum albumin

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ABSTRACT

The presence of elevated levels of glucose in blood during diabetes can lead to the non-enzymatic glycation of serum proteins such as human serum albumin (HSA). This study examined the changes that occur in binding of the sulfonylurea drug tolbutamide to HSA as the level of glycation for this protein was increased. High-performance affinity chromatography was used in this work along with columns containing various preparations of *in vitro* glycated HSA. It was found in frontal analysis experiments that the binding of tolbutamide with all of the tested preparations of glycated HSA could be described by a two-site model involving both strong and weak affinity interactions. The association equilibrium constants (K_a) for tolbutamide at its high affinity sites on glycated HSA were in the range of 0.8–1.2 $\times 10^5$ M^{-1} and increased by 1.4-fold in going from normal HSA to mildly glycated HSA. It was found through competition studies that tolbutamide was binding at both Sudlow sites I and II on the glycated HSA, in agreement with previous studies. The K_a for tolbutamide at Sudlow site II increased by 1.1- to 1.4-fold in going from normal HSA to glycated HSA. At Sudlow site I, the K_a for tolbutamide increased by 1.2- to 1.3-fold in going from normal HSA to the glycated HSA samples. This information demonstrates the effects that glycation can have on drug interactions on HSA and should provide a better quantitative understanding of how the protein binding of tolbutamide in serum may be affected for individuals with diabetes.

1. Introduction

It has been estimated that approximately 151 million people worldwide suffer from diabetes [1] and that 23.6 million children and adults have diabetes in the U.S. [2]. Diabetes is a group of disorders that pertain to insulin deficiency, insulin ineffectiveness, or a combination of the two, resulting in glucose intolerance or hypoglycemia [1]. Type I diabetes (i.e., juvenile-onset diabetes) occurs when the immune system destroys the cells that make insulin and control blood glucose levels. Type II diabetes (i.e., non-insulin dependent diabetes) begins with insulin resistance and leads to an eventual decrease in insulin production. Type I and II diabetes occur in 5–10% and 90–95% of patients with diabetes, respectively [2]. Diabetes can lead to complications such as cardiovascular disease, nerve damage, renal failure, and blindness. However, with proper treatment many of these complications can be delayed or prevented. Roughly 57% of people with diabetes take oral medication alone to treat this disease and 13% take both insulin and oral medication for treatment [2].

Sulfonylurea drugs are often used to treat type II diabetes, either alone or in combination with other drugs [3]. Tolbutamide (see Fig. 1) was the first drug in this class to be used to treat diabetes and has remained an important agent for this use since its discovery in 1956 [3]. At therapeutic levels, tolbutamide is 90% protein-bound in the circulation. This binding is of interest for tolbutamide and other first-generation sulfonylureas (e.g., chlorpropamide, acetohexamide, and tolazamide) because these agents are thought to be more easily displaced from their carrier proteins than their second-generation counterparts [4]. In addition, even a small change in the free (non-protein bound) fraction of tolbutamide can lead to severe hypoglycemia [5,6].

Human serum albumin (HSA) is the most abundant protein in human plasma [6–12] and is the main carrier protein for tolbutamide in serum [4]. This protein has a serum concentration of ~40 g/L and is known to have two major binding sites for drugs: Sudlow sites I and II [13]. Sudlow site I, which is found in subdomain IIA of HSA, binds to bulky heterocyclic compounds such as coumarins, sulfonamides, and salicylate [6,8,9,14–16]. Sudlow site II, found in subdomain IIIA, binds to aromatic carboxylic acids and profens [6,8,9,15]. HSA is able to bind and transport both endogenous solutes (e.g., unesterified fatty acids) as well as a wide range of drugs [6]. This binding can have a large affect on the pharmacologic and pharmacokinetic properties of a drug such as tolbutamide by affecting its metabolism, excretion, and bioavailability [6,17].

One process that is believed to affect the binding of drugs to HSA is glycation. Glycation refers to the modification of a protein by a process that begins with the reaction between a reducing sugar and a free amine group on a protein [10,18]. This process can occur for

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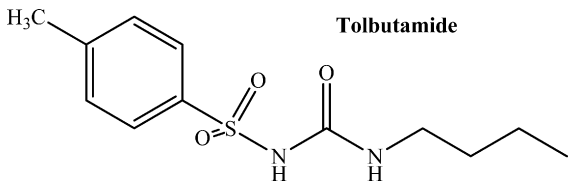

Fig. 1. Structure of tolbutamide.

HSA and becomes more pronounced in diabetes when an elevated amount of glucose is present in the bloodstream. While an average individual has 6–13% of HSA in a glycated form, a person with diabetes may have 20–30% or more glycated HSA in the circulation [6,10,11]. It has recently been shown that some of the primary modification sites for glycated HSA are at or near Sudlow sites I and II [11,18,19]. This information, in turn, has created interest in how these modifications may affect the binding of drugs such as tolbutamide to HSA [20].

This study will use high-performance affinity chromatography (HPAC) to examine the binding of tolbutamide to HSA with various levels of glycation. HPAC is a type of HPLC that uses a biological ligand as the stationary phase. HPAC has been used for many years as a selective separation and analysis technique for chemical isolation and analysis, but this approach can also be used as a tool to study various biological interactions (e.g., see recent review in Ref. [21]). This current study will use columns that contain immobilized glycated HSA to examine the binding of tolbutamide with this protein under various experimental conditions. Some benefits of using HPAC over more traditional methods for drug binding studies (e.g., equilibrium dialysis or ultrafiltration) include the good precision and reproducibility of HPAC plus its small sample requirements and ease of automation. The ability to reuse the same protein preparation for many experiments is also an attractive feature of HPAC, as well as its good correlation versus reference methods (e.g., as has been shown in numerous studies for normal HSA; see review in Refs. [21,22]).

This current report will first use frontal analysis to determine how the association equilibrium constants and binding capacities change for columns containing *in vitro* glycated HSA with increasing levels of glycation [21,22]. Zonal elution studies will then be performed to specifically examine the binding of tolbutamide at Sudlow sites I and II of HSA as the levels of glycation are increased [21,22]. The knowledge gained from these studies should give a better description of how glycation changes the binding of tolbutamide with HSA. This information, in turn, may lead to the development of more effective drug treatment regimes based on personalized medicine for patients with diabetes.

2. Theory

2.1. Frontal analysis

Frontal analysis (or frontal affinity chromatography) was used to determine the association equilibrium constant(s), K_a, and moles of binding sites, m_L, for tolbutamide with glycated HSA. An example of this type of experiment is given in Fig. 2(a). If a system has single-site binding, Eqs. (1) and (2) in Table 1 can be used with frontal analysis data to provide information on the binding of an applied drug to the immobilized protein [21,22]. In these equations, m_{Lapp} is the apparent moles of drug required to reach the central position of the breakthrough curve at a given concentration of the applied drug. According to Eq. (1), a plot of $1/m_{Lapp}$ versus $1/[A]$ should give a linear relationship if the drug binds to only one type of binding site on the protein. In addition, deviations from linearity at high drug concentrations (i.e., low $1/[A]$ values) can be used to detect multi-site interactions between the drug and protein [22].

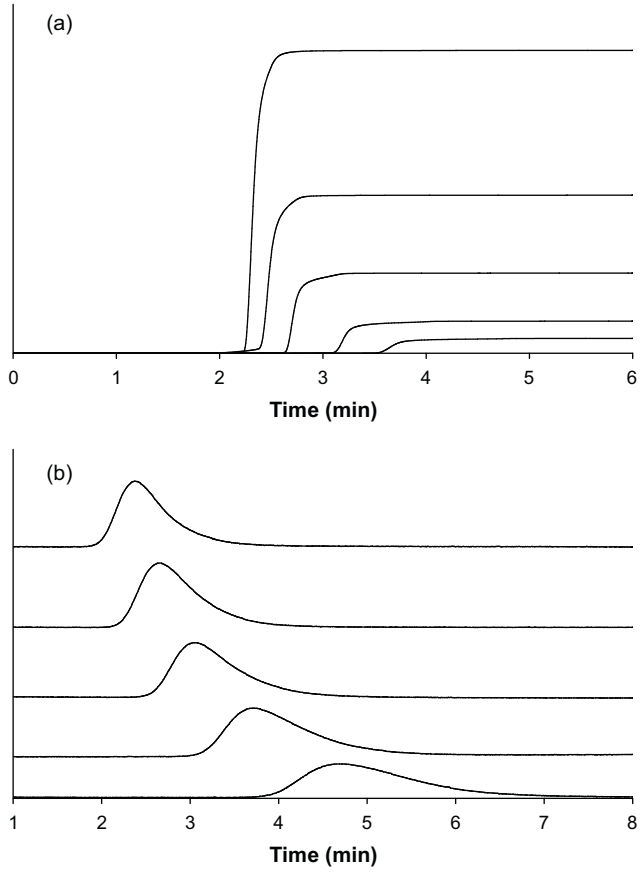

Fig. 2. (a) Frontal analysis and (b) zonal elution studies for tolbutamide on the gHSA1 column. The tolbutamide concentrations in (a) were (top-to-bottom) 200, 100, 50, 20, and 10 μM. The results in (b) were obtained using *R*-warfarin as a probe for Sudlow site I, along with tolbutamide concentrations in the mobile phase (top-to-bottom) of 20, 15, 10, 5, and 1 μM.

Similar expressions to Eqs. (1)–(2) can be written for a drug–protein system with more than one type of interaction site, as shown in Eqs. (3) and (4) for a two-site binding model [21,22]. In these equations, K_{a1} and K_{a2} are the association equilibrium constants of the applied analyte *A* at sites 1 and 2, and m_{L1} or m_{L2} are the binding capacities for *A* at these sites. The term α_1 represents the fraction of all binding regions that make up the high affinity binding sites (i.e., $\alpha_1 = m_{L1,tot}/m_{Ltot}$). The term β_2 is the ratio of the association equilibrium constants for the lower affinity site (K_{a2}) versus the highest affinity site, where $\beta_2 = K_{a2}/K_{a1}$ and $0 < K_{a2} < K_{a1}$. According to Eq. (3), a non-linear response for a plot of $1/m_{Lapp}$ versus $[A]$ would be expected throughout a broad range of concentrations for a system with multi-site binding. However, at low concentrations a linear response can still be observed. In this case, Eq. (3) approaches the linear form shown in Eq. (5) that can be used to estimate the association equilibrium constant for the highest affinity sites by using data obtained at low analyte concentrations [23].

2.2. Zonal elution

HPAC and the method of zonal elution were used to examine the binding of tolbutamide to glycated HSA at specific binding sites (see Fig. 2(b)). In this approach, the retention time (t_R) of an injected probe A (i.e., *R*-warfarin or L-tryptophan in this study) is measured in the presence of various mobile phase concentrations of competing agent I (e.g., tolbutamide) and is used to calculate the retention

Table 1
Equations used in frontal analysis and zonal elution experiments to obtain binding parameters for a drug with an immobilized protein such as HSA[a].

	Frontal analysis	
Single-site binding	$\frac{1}{m_{Lapp}} = \frac{1}{(K_a m_L[A])} + \frac{1}{m_L}$	(1)
	$m_{Lapp} = \frac{m_L K_a[A]}{(1 + K_a[A])}$	(2)
Two-site binding	$\frac{1}{m_{Lapp}} = \frac{1 + K_{a1}[A] + \beta_2 K_{a1}[A] + \beta_2 K_{a1}^2[A]^2}{m_{Ltot}\{(\alpha_1 + \beta_2 - \alpha_1\beta_2)K_{a1}[A] + \beta_2 K_{a1}^2[A]^2\}}$	(3)
	$m_{Lapp} = \frac{m_{L1} K_{a1}[A]}{(1 + K_{a1}[A])} + \frac{m_{L2} K_{a2}[A]}{(1 + K_{a2}[A])}$	(4)
	$\lim_{[A]\to 0} \frac{1}{m_{Lapp}} = \frac{1}{m_{Ltot}(\alpha_1 + \beta_2 - \alpha_1\beta_2)K_{a1}[A]} + \frac{\alpha_1 + {\beta_2}^2 - \alpha_1{\beta_2}^2}{m_{Ltot}(\alpha_1 + \beta_2 - \alpha_1\beta_2)^2}$	(5)
	Zonal elution	
Single-site competition	$\frac{1}{k} = \frac{K_{a1} V_M[I]}{K_{aA} m_L} + \frac{V_M}{K_{aA} m_L}$	(6)

[a] A more detailed discussion of these equations and their use can be found in Refs. [21,22]. The terms used in these equations are defined in the text.

factor (k) for the injected probe, where $k = (t_R - t_M)/t_M$ and t_M is the elution time of a non-retained solute (e.g., sodium nitrate).

If the injected probe and competing agent have direct competition at a single type of site on an immobilized protein, Eq. (6) in Table 1 can be used to describe the relationship between the measured retention factor for A and the concentration of I [21,22]. In this equation, K_{aI} and K_{aA} are the association equilibrium constants for the competing agent and site-selective probe, respectively, at their site of competition and V_M is the void volume. A plot of $1/k$ versus $[I]$ in this case should yield a linear response if A and I follow a model based on direct competition at a single type of binding site. The best-fit line for such a system can then be used to find the association equilibrium constant for I at the site of competition by dividing the slope by the intercept. If multi-site competition or allosteric effects are present, a non-linear response would instead be observed [22].

3. Experimental

3.1. Reagents

The tolbutamide (≥99.9% pure), *R*-warfarin (≥97%), L-tryptophan (98%), HSA (essentially fatty acid free, ≥96%), and commercial glycated HSA (Lot 058K6087) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The Nucleosil Si-300 (7 μm particle diameter, 300 Å pore size) was from Macherey–Nagel (Düren, Germany). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). The assay kit for fructosamine (used for measuring glycation levels) was from Diazyme Laboratories (San Diego, CA, USA). All aqueous solutions were made using water from a Nanopure system (Barnstead, Dubuque, IA, USA) and filtered with a 0.20 μm GNWP nylon membrane from Millipore (Billerica, MA, USA).

3.2. Apparatus

The HPLC system consisted of a Jasco DG-2080-53 three-solvent degasser (Tokyo, Japan), two Jasco PU-2080 isocratic pumps, a Rheodyne Advantage PF six-port valve (Cotati, CA, USA), a Jasco AS-2055 autosampler, a Jasco CO-2060 column oven, and a Jasco UV-2075 UV/vis detector. The HPLC system hardware was controlled by EZChrom Elite software v3.2.1 (Scientific Software, Pleasanton, CA, USA) via Jasco LC Net hardware. An in-house version of Labview 5.1 software (National Instruments, Austin, TX, USA) was used to analyze the frontal analysis curves while PeakFit 4.12 (Jandel Scientific Software, San Rafael, CA, USA) was used to determine the central moments of peaks obtained from zonal elution experiments. Linear regression was performed using Excel 2003 (Microsoft Corporation, Redmond, WA, USA) and non-linear regression was performed using DataFit (Oakdale Engineering, PA, USA).

3.3. Methods

3.3.1. Column and sample preparation

Diol silica was made using Nucleosil Si-300 silica, and HSA was immobilized to the diol silica by the Schiff base method, both according to previously published procedures [16]. Control supports were prepared in the same manner but with no HSA being added during the immobilization step. Although both the Schiff base immobilization method and glycation involve the use of free amine groups on a protein, it has been found that these two processes mainly involve different residues on HSA; it has also been shown that the prior glycation of HSA (e.g., under the same conditions as used in this report) does not change the specific activities at Sudlow sites I and II when using warfarin or L-tryptophan as probes for these sites [24].

A BCA assay was performed in triplicate to determine the protein content of each glycated HSA support, using soluble glycated HSA as the standard and the control support as the blank. This report used three batches of HSA for immobilization, with each batch containing a different level of glycation. The first HSA sample was purchased from Sigma (referred to here as "gHSA1"). The second and the third samples (i.e., "gHSA2" and "gHSA3", respectively) were made *in vitro* by incubating a 42 g/L HSA solution for one month in sterile pH 7.4 phosphate buffer with 15 mM or 30 mM glucose at 37 °C (*Note*: Sodium azide was also added to prevent bacterial growth during incubation). The protein was then purified, lyophilized, and stored at −80 °C until further use, according to methods described in Ref. [24]. An enzymatic fructosamine assay from Diazyme Laboratories was used to determine the level of glycation for each sample, as described previously [24], with the results being expressed as mol hexose/mol HSA.

The glycated HSA supports were downward slurry-packed into separate 2.0 cm × 2.1 mm I.D. columns at 3500 psi (24 MPa) using pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. The control supports for each gHSA sample were packed into separate columns under the same conditions. These columns were stored at 4 °C in the packing solution and used over a period of one year and fewer than 500 sample applications per column. The routine washing of these columns with fresh portions of sterile phosphate buffer during the binding studies made it unnecessary to include a preservative in this buffer; this approach also helped

maintain conditions that closely corresponded to those seen *in vivo* in serum during the binding studies. Columns containing normal HSA that have been used in this manner have been found in previous studies to retain good stability for drug–protein binding studies under such conditions [25].

The tolbutamide, *R*-warfarin, and L-tryptophan solutions were all made in pH 7.4, 0.067 M potassium phosphate buffer. This same buffer was used as the application and elution buffer in the chromatographic studies. All mobile phases were filtered through a 0.2 μM nylon filter and degassed for 10–15 min prior to use. A flow rate of 0.5 ml/min was used for both the frontal analysis and zonal elution studies, with all experiments being carried out at 37 °C. This flow rate has been shown in previous studies to give reproducible retention factors and binding capacities for drug binding studies conducted on similar columns containing normal HSA [26,27].

3.3.2. Chromatographic studies

In the frontal analysis studies, the column was first equilibrated with pH 7.4, 0.067 M potassium phosphate buffer. A switch was then made between the pH 7.4 buffer and a drug solution that was prepared in the same buffer. Once the drug had saturated the column and produced a breakthrough curve, the pH 7.4 buffer was again passed through the column to elute the retained drug. The drug solutions contained 1–200 μM tolbutamide and the elution of tolbutamide was monitored at 250 nm. Although the pK_a of sulfonylurea drugs like tolbutamide range from 5.2 to 6.2, even the solutions containing the highest concentration of tolbutamide in this study gave less than a 0.05 change in pH for the pH 7.4 buffer, and the pH of this solution was found to vary by less than 0.05 units over the course of this study. The frontal analysis experiments were performed in triplicate at each drug concentration. The breakthrough curves were analyzed by using Labview 5.1 and the equal areas method [22]. A correction for non-specific binding to the system was made by subtracting the results for the control column from the data obtained for each glycated HSA column.

Zonal elution competition studies were performed in triplicate using *R*-warfarin as a probe for Sudlow site I and L-tryptophan as a probe for Sudlow site II [13]. Tolbutamide concentrations of 1–20 μM were placed in the mobile phase while 20 μL injections of 5 μM *R*-warfarin or L-tryptophan were made. These sample concentrations have been shown to provide linear elution conditions for similar HSA columns [14,28]. The elution of *R*-warfarin and L-tryptophan was monitored at 308 or 280 nm, respectively. Injections containing 20 μL of 20 μM sodium nitrate were made under the same conditions using only the pH 7.4 buffer as the mobile phase. Sodium nitrate was used as a non-retained solute to determine the column void time, with the elution of sodium nitrate being monitored at 205 nm. The resulting peaks were fit to an exponentially modified Gaussian curve and analyzed using PeakFit v4.12.

4. Results and discussion

4.1. Preparation of glycated HSA

Earlier reports examining the binding of tolbutamide with HSA and glycated HSA have examined either changes in the binding capacities of these proteins or the differences in their apparent binding constants [5,20]. These same reports, however, did not consider how the tolbutamide–HSA interactions might change with various levels of glycation for HSA. In addition, these reports measured binding constants at lower temperatures than are present in the body (e.g., 20 °C for the fluorescence quenching assay in Ref. [5]) or used higher glucose concentrations for HSA glycation than are found in the body during diabetes [20]. These issues and limitations were overcome in this current investigation by carrying out all binding studies at 37 °C and by using HSA samples that had undergone various known levels of glycation and that were prepared under conditions similar to those found in serum during diabetes.

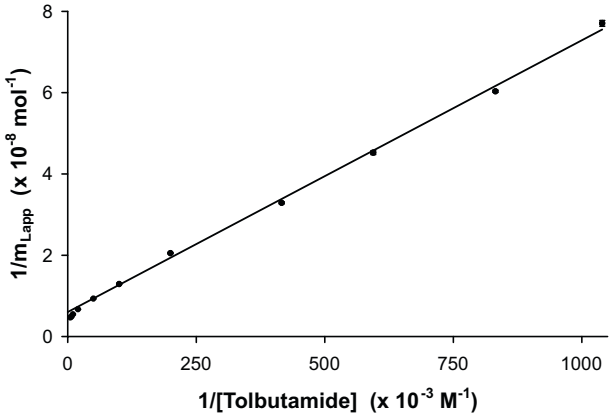

Fig. 3. Double-reciprocal plot of frontal analysis data obtained for tolbutamide on the gHSA1 column. The best-fit line for the upper linear region when analyzed according to Eq. (5) is $y = 670\ (\pm 20)\ x + [6.1\ (\pm 1.0) \times 10^7]$, $r = 0.999$, $n = 6$. The error bars represent a range of ±1 S.D. and are of a comparable size to the data markers in this plot.

The glycated HSA samples that were used in this study were the same as employed in recent work examining the changes in the HSA interactions with warfarin and L-tryptophan during glycation [24]. The first sample of glycated HSA (gHSA1) contained 1.31 (±0.05) mol hexose/mol HSA (*Note:* The value in parentheses represents ±1 S.D. for the population of results). This sample was used to represent mildly glycated HSA, as might be found during prediabetes or early state diabetes. The second glycated HSA sample (gHSA2) had a glycation level of 2.34 (±0.13) mol hexose/mol HSA; this sample was made using a glucose concentration and conditions typical of those seen in many diabetic patients [29]. The third sample (gHSA3) had a glycation level of 3.35 (±0.14) mol hexose/mol HSA, as might be present in uncontrolled or advanced diabetes. After these various preparations of glycated HSA had been immobilized, the resulting supports were determined to have protein contents of 29 (±4), 47 (±8), and 40 (±3) mg protein/g silica, respectively, or ~440–710 nmol HSA/g silica. These protein contents were comparable to those noted in earlier work with normal HSA and comparable supports prepared by the same methods [16].

4.2. Frontal analysis studies of tolbutamide binding to glycated HSA

The method of frontal analysis was used to gather information about the overall binding of tolbutamide to glycated HSA. The data that were obtained for each type of glycated HSA column were first plotted using a double-reciprocal plot (see example in Fig. 3). When using this type of model and plot, each glycated HSA column gave deviations at high drug concentrations (i.e., low values of 1/[Tolbutamide]) from the expected linear response for a single-site binding model, as described by Eq. (1). This result indicated that tolbutamide was binding to more than one binding site on each type of glycated HSA. This behavior was consistent with similar studies using normal HSA, which has also been found to have multi-site interactions for tolbutamide [30].

Eq. (5) was next used with the upper linear region of plots like those in Fig. 3 to estimate the association equilibrium constant at the high-affinity sites for tolbutamide, according to a method from Refs. [23,24]. This fit was obtained using data at tolbutamide concentrations of 1–10 μM for each of the columns. The resulting K_a values were between 0.9 and 1.1 × 10^5 M^{-1} at 37 °C and pH 7.4, with

Table 2
Binding constants obtained for tolbutamide with HSA or glycated HSA at pH 7.4 and 37 °C when using frontal analysis and a two-site binding model[a].

Column	K_{a1} ($\times 10^5$ M^{-1})	m_{L1} ($\times 0^{-8}$ mol)	Specific activity	K_{a2} ($\times 10^3$ M^{-1})	m_{L2} ($\times 10^{-8}$ mol)	Specific activity[b]
Normal HSA[c]	0.87 (±0.06)	2.0(±0.1)	1.1 (±0.1)	8.1(±1.7)	1.8(±0.1)	1.0(±0.1)
gHSA1	1.2(±0.2)	1.1(±0.2)	0.82(±0.18)	9.5(±3.2)	1.7(±0.1)	1.2(±0.2)
gHSA2	0.84(±0.16)	2.2(±0.4)	1.0(±0.3)	7.8(±5.1)	1.9(±0.2)	0.87(±0.18)
gHSA3	0.89(±0.06)	1.9(±0.1)	1.1(±0.1)	1.7(±1.1)	3.6(±1.4)	1.9(±0.8)

[a] The values in parenthesis represent a range of ±1 S.D. for the population of results (n = 9–11). These values were determined by using error propagation and the standard deviations of the parameters that were obtained from best-fit lines generated according to Eq. (4).
[b] The specific activity refers to the moles of tolbutamide that were bound per mole of immobilized HSA or glycated HSA.
[c] The results provided for normal HSA are based on previous work described in Ref. [30] and were acquired using experimental conditions identical to those used in this current report for glycated HSA.

correlation coefficients of at least 0.999 (n = 5 or 6) being noted for a linear fit to the given region of the double-reciprocal plots for all glycated HSA columns. These results were similar but slightly higher than a value of 0.82 (±0.04) × 10^5 M^{-1} that was obtained by the sample approach when examining the high affinity sites of tolbutamide on normal HSA [30].

A two-site model was next employed to examine the binding of tolbutamide with the glycated HSA columns (see Fig. 4). This was accomplished by analyzing the frontal analysis data through the use of Eq. (4) and non-linear regression. A two-site model has been shown in earlier work to give the best fit for frontal analysis data acquired for tolbutamide with normal HSA [30]. This model was also found in this current report to give a good fit for the results obtained with tolbutamide on each glycated HSA column, giving correlation coefficients of at least 0.999 (n = 9–11). Only random variations in the data about the best-fit lines were observed in these plots, along with a small sum of the square of the residuals (range 0.8 × 10^{-19} to 3.8 × 10^{-19}). This overall fit gave much better agreement with the data than a single-site model, which instead exhibited non-random variations of the residuals about the best-fit lines along with correlation coefficients that ranged from 0.965 to 0.998, and larger values for the sum of the square of the residuals (range 4.3 × 10^{-18} to 7.4 × 10^{-18}).

A summary is provided in Table 2 of the association equilibrium constants and amount of each class of binding sites that were noted when using the two-site model for tolbutamide on the glycated HSA columns. In this table, the association equilibrium constants found for the high affinity regions (K_{a1}) were in good general agreement with the values of 0.9–1.1 × 10^5 M^{-1} that were estimated from the linear regions of plots prepared according to Eq. (5), as described previously in this section. When these results were compared to data that were obtained by the same approach for tolbutamide on a normal HSA column (see entry from Ref. [30] for normal HSA in Table 2), a 1.4-fold increase in affinity was present in going from normal HSA (K_{a1} = 0.87 (±0.06) × 10^5 M^{-1}) to the gHSA1 sample (K_{a1} = 1.2 (±0.2) × 10^5 M^{-1}), a change which was significant at the 95% confidence level. The apparent association equilibrium constant for the high affinity regions returned to comparable values versus normal HSA when using the gHSA2 and gHSA3 samples, which had K_{a1} values of 0.84 (±0.16) × 10^5 M^{-1} and 0.89 (±0.06) × 10^5 M^{-1}, respectively. The corresponding m_{L1} values for these high-affinity regions were 1.1–2.2 × 10^{-8} mol and corresponded to specific activities of approximately 0.82–1.0 for all of these high affinity regions, with no apparent change in these values being seen as the level of glycation was varied.

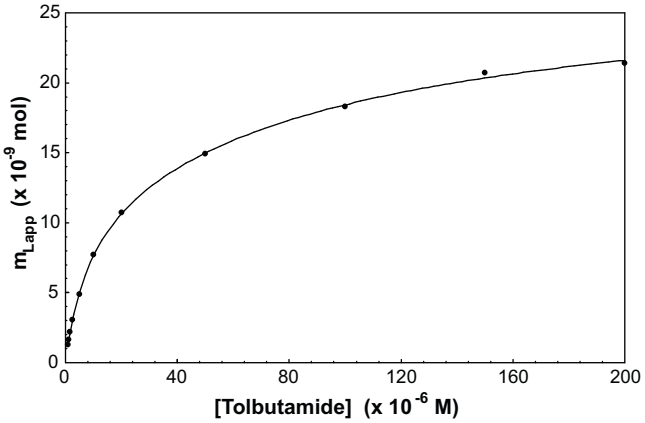

Fig. 4. Plot of m_{Lapp} versus [Tolbutamide] for the gHSA1 column, as analyzed according to Eq. (4) and a two-site model. The best-fit values obtained for the association equilibrium constants and binding capacities are summarized in Table 2. The data used in this plot were the same as in Fig. 3. The error bars for the data points (not shown) are comparable in size to the data markers in this plot.

The association equilibrium constants calculated for the lower affinity sites were comparable to those obtained for tolbutamide when using similar affinity columns containing normal HSA (for the latter value, see entry for normal HSA in Table 2 from Ref. [30]). The strength of these weak affinity interactions was similar for normal HSA (K_{a2} = 8.1 (±1.7) × 10^3 M^{-1}) [30] versus gHSA1 or gHSA2, in which these last two samples gave K_{a2} values of 9.5 (±3.2) × 10^3 M^{-1} and 7.8 (±5.1) × 10^3 M^{-1}, respectively. However, there was an apparent decrease in K_{a2} in going from normal HSA to gHSA3, in which this last sample had a K_{a2} value of 1.7 (±1.1) × 10^3 M^{-1}. The corresponding specific activities for tolbutamide at these low affinity sites ranged from 0.87 to 1.9 for the various glycated samples, which were comparable to a value of 1.0 (±0.1) that has been noted for tolbutamide with similar columns containing normal HSA [30].

4.3. Site-specific interactions of tolbutamide at Sudlow site II

Zonal elution competition studies were used to further examine the interactions of tolbutamide with glycated HSA. L-Tryptophan was first used in such work as a site-selective probe to examine the binding of tolbutamide at Sudlow site II [30]. When the data from these experiments were plotted according to Eq. (6), a linear fit for all the glycated HSA columns was observed (see example of such a plot in Fig. 5). Similar linear behavior and correlation coefficients between 0.998 and 0.999 (n = 5) were obtained for each glycated HSA column examined in this study. In each case, only random residuals were noted about the best-fit line. Based on this fit, it was determined that tolbutamide and L-tryptophan had direct competition at a single site. In addition, it was possible to determine that this competition was taking place at Sudlow site II because L-tryptophan is known to bind selectively at this site on both normal HSA and the types of glycated HSA that were examined in this study [24,28]. This result agrees with previous competition studies in which tolbutamide has been found to be displaced by probes for Sudlow site II from both normal HSA and glycated HSA [30].

By using the best-fit lines from these plots and Eq. (6), it was possible to find the association equilibrium constant for tolbutamide at Sudlow site II by taking the ratio of the slope to the intercept for each plot. This method has been used in a previous study with

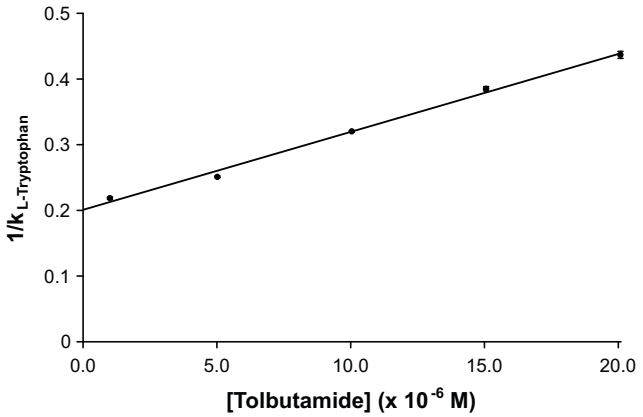

Fig. 5. Zonal elution studies on the gHSA1 column for the injection of L-tryptophan as a site-selective probe for Sudlow site II in the presence of tolbutamide as a competing agent. The best-fit line obtained when using Eq. (6) is $y = [1.2\ (\pm 0.1) \times 10^4]\ x + 0.20\ (\pm 0.01)$, $r = 0.998$, $n = 5$. The error bars represent a range ±1 S.D. and are comparable in size to the data markers in this plot.

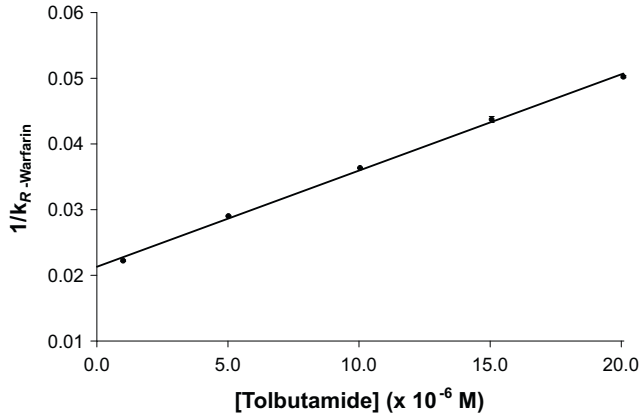

Fig. 6. Zonal elution studies on the gHSA1 column for the injection of *R*-warfarin as a site-selective probe for Sudlow site I in the presence of tolbutamide as a competing agent. The best-fit line obtained when using Eq. (6) is $y = 1500\ (\pm 100)\ x + 0.021\ (\pm 0.001)$, $r = 0.999$, $n = 5$. The error bars represent a range of ±1 S.D. and are comparable in size to the data markers in this plot.

normal HSA to obtain a site-specific association equilibrium constant of 5.3 (±0.2) × 10^4 M^{-1} for tolbutamide at Sudlow site II [30]. The association equilibrium constants values that were obtained at the same site in this study are summarized in Table 3. In going from normal HSA to the mild level of glycation that was present in gHSA1, the size of K_a for tolbutamide at Sudlow site II was found to increase by 1.1-fold to 5.9 (±0.3) × 10^4 M^{-1} (i.e., an increase that was significant at the 90% confidence level). This was followed by higher values of 7.2 (±0.3) × 10^4 M^{-1} for the gHSA2 sample and 6.4 (±0.3) × 10^4 M^{-1} for the gHSA3 sample, or 1.4- to 1.2-fold increases versus normal HSA that were significant at the 95% confidence level. It was also possible from the intercepts of these plots to determine how the specific activity for tolbutamide at Sudlow site II was affected as the level of glycation for HSA was increased. When this was done, no significant change in specific activity was seen at the 95% confidence level for tolbutamide at Sudlow site II in going from normal HSA to any of the glycated HSA columns.

4.4. Site-specific interactions of tolbutamide at Sudlow site I

Site-specific studies where also carried out using competition studies and *R*-warfarin as a site-specific probe for Sudlow site I [14,16]. These results were again plotted according to Eq. (6), with an example being given in Fig. 6. All of the glycated HSA columns gave linear behavior for this type of plot, with correlation coefficients that ranged from 0.994 to 0.999 ($n = 5$) and with only random variations in the residuals being observed about the best-fit line. This type of behavior indicated that tolbutamide and *R*-warfarin were competing directly at Sudlow site I on HSA. Similar results have been noted in prior competition studies for tolbutamide with probes for Sudlow site I when using both normal HSA and glycated HSA [30].

The association equilibrium constant for tolbutamide at Sudlow site I on each of the glycated HSA samples was found by using the best-fit lines of plots prepared according to Eq. (6). The results are summarized in Table 3. The K_a value for tolbutamide at Sudlow site I on the gHSA1 column was roughly 1.3-fold higher than the value of 5.5 (±0.2) × 10^4 M^{-1} that has previously been measured for tolbutamide at this site on normal HSA [30]. A comparable, roughly 1.2-fold increase in affinity versus normal HSA was measured at the same site for the gHSA2 and gHSA3 samples. All of the association equilibrium constants measured at Sudlow site I for tolbutamide with the glycated HSA samples where significantly different at the 95% confidence level when compared to the K_a value reported for tolbutamide at the same site on normal HSA. No significant change at the 95% confidence level was seen in the specific activity for tolbutamide at Sudlow site I in going from normal HSA to the glycated HSA columns.

Table 3
Association equilibrium constants measured for tolbutamide at Sudlow sites I and II at pH 7.4 and 37 °C[a].

Column	Sudlow site I (*R*-warfarin) (× 10^4 M^{-1})	Sudlow site II (L-tryptophan) (× 10^4 M^{-1})
Normal HSA[b]	5.5 (±0.2)	5.3 (±0.2)
gHSA1	6.9 (±0.2)	5.9 (±0.3)
gHSA2	6.6 (±0.5)	7.2 (±0.3)
gHSA3	6.5 (±0.2)	6.4 (±0.3)

[a] The values in parenthesis represent a range of ±1 S.D. for the population of results. These values were determined by using error propagation and the standard deviations of the slopes and intercepts that were obtained from best-fit lines generated according to Eq. (6).

[b] The results provided for normal HSA are based on previous work described in Ref. [30] and were acquired using experimental conditions identical to those used in this current report for glycated HSA.

5. Conclusion

This report looked at how the binding of tolbutamide with HSA changes as the level of glycation for HSA is increased. Experiments based on the use of HPAC and frontal analysis indicated that the binding of tolbutamide with glycated HSA followed a two-site model that included a group of high affinity sites and a set of lower affinity interactions. This behavior was in agreement with a previous report that has studied the binding of tolbutamide with normal HSA [30]. The association equilibrium constants for the high affinity interactions were in the general range of 0.8–1.2 × 10^5 M^{-1} and increased by 1.4-fold in going from normal HSA to mildly glycated HSA (i.e., the gHSA1 sample), with the overall affinity returning to more normal levels at higher levels of glycation (as noted for the gHSA2 and gHSA3 samples). Competition studies based on zonal elution experiments found that the association equilibrium constant of tolbutamide at Sudlow site I increased by 1.2- to 1.3-fold in going from normal HSA to all of the tested samples of glycated HSA. At Sudlow site II, the association equilibrium constant for tolbutamide increased by 1.1- to 1.4-fold in going from normal HSA to all of the tested samples of glycated HSA. Such an increase in binding affinity to HSA upon glycation could potentially lower the amount of non-bound drug that is available for binding to receptors and may

affect the rate of metabolism or extraction and the overall half-life of tolbutamide in the circulation.

As demonstrated in this report, the binding data that can be obtained through this type of research can result in a better understanding of how glycation may change the interactions of drugs such as tolbutamide to HSA during diabetes. This information should aid in the future development of better patient treatments for personalized medicine by allowing physicians to better understand how glycation may affect the activity of such a drug in the circulation and to make appropriate adjustments in the prescribed dose when using tolbutamide and related drugs. Similar binding studies are now being carried out with *in vivo* glycated HSA. Work with additional pharmaceutical agents, including other sulfonylurea compounds, is also in progress to determine the extent to which the interactions of these drugs may vary during glycation of HSA.

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