

High-performance affinity chromatography and the analysis of drug interactions with modified proteins: binding of gliclazide with glycosylated human serum albumin

Ryan Matsuda · Jeanethe Anguizola · K. S. Joseph · David S. Hage

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Abstract This study used high-performance affinity chromatography (HPAC) to examine the binding of gliclazide (i. e., a sulfonylurea drug used to treat diabetes) with the protein human serum albumin (HSA) at various stages of modification due to glycation. Frontal analysis conducted with small HPAC columns was first used to estimate the number of binding sites and association equilibrium constants (K_a) for gliclazide with normal HSA and glycosylated HSA. Both normal and glycosylated HSA interacted with gliclazide according to a two-site model, with a class of high-affinity sites (average K_a , $7.1\text{--}10 \times 10^4 \text{ M}^{-1}$) and a group of lower-affinity sites (average K_a , $5.7\text{--}8.9 \times 10^3 \text{ M}^{-1}$) at pH 7.4 and 37 °C. Competition experiments indicated that Sudlow sites I and II of HSA were both involved in these interactions, with the K_a values for gliclazide at these sites being 1.9×10^4 and $6.0 \times 10^4 \text{ M}^{-1}$, respectively, for normal HSA. Two samples of glycosylated HSA had similar affinities to normal HSA for gliclazide at Sudlow site I, but one sample had a 1.9-fold increase in affinity at this site. All three glycosylated HSA samples differed from normal HSA in their affinity for gliclazide at Sudlow site II. This work illustrated how HPAC can be used to examine both the overall binding of a drug with normal or modified proteins and the site-specific changes that can occur in these interactions as a result of protein modification.

Keywords Drug–protein interactions · Gliclazide · Human serum albumin · Glycation · High-performance affinity chromatography

Introduction

The American Diabetes Association reports that an estimated 25.8 million children and adults in the USA have diabetes, representing almost 8.3% of the population [1]. Diabetes is a health condition that is related to insulin deficiency or a resistance to insulin. This disorder results in an increased level of glucose in blood. There are two main types of diabetes. Type I diabetes (i. e., juvenile or insulin-dependent diabetes) is caused when the immune system attacks pancreatic beta cells and results in little or no production of insulin. These patients require insulin for treatment. Type II diabetes (i. e., non-insulin-dependent or adult onset diabetes) is the most common type of diabetes and is created by insulin resistance [1].

Type II diabetes is frequently treated by using sulfonylurea drugs [2]. Sulfonylurea drugs increase the amount of insulin that is released from beta cells in the pancreas, which helps control the buildup of glucose in blood. Gliclazide (see Fig. 1) is a second-generation sulfonylurea drug that is taken orally. Second-generation sulfonylurea drugs like gliclazide have a better effectiveness than first-generation sulfonylurea drugs (e. g., acetohexamide and tolbutamide) in the treatment of diabetes and are more easily excreted by the body [3].

Sulfonylureas such as gliclazide are known to be tightly bound to serum proteins when these drugs are in blood. The most abundant serum protein is human serum albumin (HSA) [4–10], which is also the main carrier protein for sulfonylurea drugs in the circulation [4]. HSA has a mass of

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R. Matsuda · J. Anguizola · K. S. Joseph · D. S. Hage (✉)
Department of Chemistry, University of Nebraska-Lincoln,
Lincoln, NE 68588-0304, USA
e-mail: dhage@unlserve.unl.edu

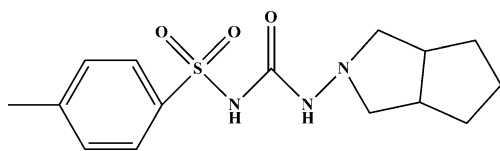


Fig. 1 Structure of gliclazide

66.5 kDa and has two major binding sites for drugs: Sudlow sites I and II [4]. Sudlow site I is located in subdomain IIA of HSA and is known to bind a variety of drugs, such as warfarin, azapropazone, phenylbutazone, and salicylate [5]. Sudlow site II is in subdomain IIIA of HSA and has been shown to bind to ibuprofen, fenoprofen, ketoprofen, benzodiazepines, and L-tryptophan [5]. Both Sudlow sites I and II have also been found to bind to the first-generation sulfonylurea drugs acetohexamide and tolbutamide [6, 7].

Glycation is a type of protein modification that is believed to alter the interactions of some drugs with HSA. Glycation occurs when the presence of glucose in blood leads to the non-enzymatic addition of glucose with proteins. This process initially occurs through a reaction between glucose or a reducing sugar and a free amine group on a protein (see Fig. 2) [8–13]. Normal individuals have 6–13% of their HSA in a form that is glycated in blood [8, 9, 11]. A person with diabetes has approximately 20–30% or more of their HSA in a glycated form [8, 9, 11]. In addition, it is known that both Sudlow sites I and II can be modified as a result of glycation [8–13] and that this modification can affect the binding of first-generation sulfonylurea drugs at these sites [6, 7]. Although the pharmacokinetics and overall serum protein binding of gliclazide has been previously examined for healthy and diabetic subjects [14, 15], no detailed information was provided in this prior work on the strength of this binding with HSA or on the effects of glycation on gliclazide–HSA interactions.

The purpose of this study will be to use high-performance affinity chromatography (HPAC) to examine the binding of

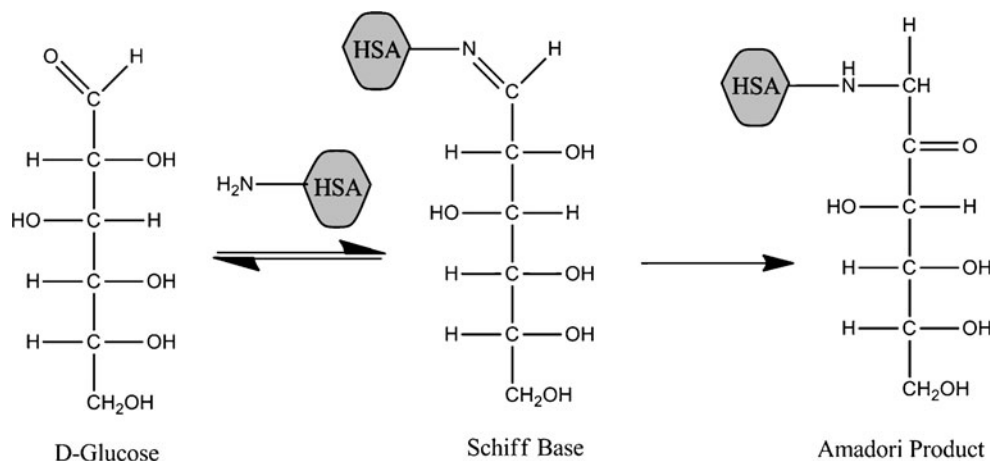
gliclazide to normal HSA and HSA that has been modified in vitro to contain various stages of glycation [16–18]. HPAC is a type of HPLC that uses an immobilized biological molecule (e.g., HSA) as the stationary phase [16]. It is known from prior work that HPAC can be used with HSA columns to provide precise and fast measurements of drug–protein interactions with results that give good agreement with those obtained for soluble HSA [17]. Other advantages of using HPAC for this type of research are its ease of automation and its ability to use the same preparation of a protein for hundreds of experiments [16–18]. Recent work with HPAC as a screening method has indicated that significant changes can occur in the binding of gliclazide with HSA during glycation, resulting in trends similar to those seen for acetohexamide and tolbutamide [19]. This current study will examine these interactions in a quantitative manner by first using the method of frontal analysis (or frontal affinity chromatography) to determine the overall equilibrium constants and binding capacities for gliclazide with normal HSA and glycated HSA. Competition studies will then be conducted to examine the specific binding of gliclazide at Sudlow sites I and II on HSA at various stages of glycation. The results should be useful in determining how glycation can affect the binding of gliclazide, and related drugs, to HSA during diabetes. This report will also illustrate how HPAC can be used as a tool to examine the overall binding and site-selective interactions of drugs or other solutes with modified proteins.

Experimental

Chemicals

The gliclazide ($\geq 99.9\%$ pure), *R*-warfarin ($\geq 97\%$), L-tryptophan ($\geq 98\%$), D-(+)-glucose (99.5%), sodium azide ($>95\%$), HSA (essentially fatty acid free, $\geq 96\%$), and commercial sample of in vitro glycated HSA (Lot 058K6087) were from

Fig. 2 General reactions involved in the glycation of HSA



Sigma-Aldrich (St. Louis, MO, USA). Nucleosil Si-300 (7- μ m particle diameter, 300 Å pore size) was obtained from Macherey-Nagel (Düren, Germany). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). A fructosamine assay kit, which was used for measuring glycation levels, was purchased from Diazyme Laboratories (San Diego, CA, USA). All aqueous solutions were prepared using water from a Nanopure system (Barnstead, Dubuque, IA, USA) and were filtered through 0.20 μ m GNWP nylon membranes from Millipore (Billerica, MA, USA).

Instrumentation

The HPLC system consisted of a DG-2080-53 degasser, two PU-2080 pumps, an AS-2057 autosampler, a CO-2060 column oven, and a UV-2075 absorbance detector from Jasco (Tokyo, Japan), plus a Rheodyne Advantage PF six-port valve (Cotati, CA, USA). Chromatograms were collected using EZChrom Elite v3.2.1 (Scientific Software, Pleasanton, CA, USA) and Jasco LC Net. Non-linear regression was carried out by using Data Fit 8.1.69 (Oakdale, PA, USA).

Methods

Nucleosil Si-300 silica was converted into a diol-bonded form, and HSA was immobilized onto the diol-bonded silica through the Schiff base method, as described previously [20–24]. A control support was prepared in the same manner but with no HSA being added during the immobilization step (note: although free amine groups are involved in both the Schiff base immobilization method and glycation, these processes tend to involve different residues on HSA [6, 7, 21]). All supports were downward slurry packed into separate 2.0 cm \times 2.1 mm I.D. columns at 3,500 psi (24 MPa). A pH 7.4, 0.067 M potassium phosphate buffer was used as the packing solution. The columns were stored at 4 °C, and all experiments were performed over the course of less than 500 sample applications, with the columns being routinely washed and used with sterile pH 7.4, 0.067 M phosphate buffer. No significant changes in binding properties were noted under these conditions during the course of this study [22].

A BCA assay was used to directly determine the immobilized protein content for each support, using HSA or glycosylated HSA as the standard and the control support as the blank. The amount of protein in the normal HSA support was 38 (\pm 3) mg HSA/g silica. Three glycosylated HSA supports, each having different levels of modification, were used. The first glycosylated HSA sample (gHSA1) was purchased from Sigma and was prepared under proprietary conditions. The second and third samples (gHSA2 and gHSA3) were prepared in vitro as described previously [6,

7, 21] and using conditions similar to those found in the serum of patients with controlled or advanced diabetes [25, 26]. The amount of protein on these glycosylated HSA supports was 29 (\pm 4), 47 (\pm 8), or 40 (\pm 3) mg HSA/g silica, respectively; this amount corresponded to 10.9–17.6 nmol protein within a 2.0 cm \times 2.1 mm I.D. column.

The level of glycation for each HSA sample was determined in replicate through the use of a fructosamine assay [6, 7]. The gHSA1 sample was found by this assay to contain 1.31 (\pm 0.05) mol hexose/mol HSA and represented mildly glycosylated HSA, as might be present in pre-diabetes or early stage diabetes. The gHSA2 sample contained 2.34 (\pm 0.13) mol hexose/mol HSA and was representative of many patients with controlled diabetes [25]. The gHSA3 sample had 3.35 (\pm 0.14) mol hexose/mol HSA and represented a situation found in patients with uncontrolled or advanced diabetes [26].

Solutions of gliclazide, *R*-warfarin, and *L*-tryptophan were prepared in pH 7.4, 0.067 M potassium phosphate buffer. The same buffer was used as the application and elution buffer in the chromatographic studies. The mobile phases were filtered using a 0.2 μ m nylon filter and degassed for 10–15 min before use. All experiments were carried out at 37 °C and 0.5 mL/min, which has been shown in prior work to allow for the measurement of reproducible retention factors, binding capacities, and association equilibrium constants during frontal analysis and zonal elution studies for the types of columns that were used in this report [23, 24].

The columns were first equilibrated with pH 7.4, 0.067 M potassium phosphate buffer. In the frontal analysis experiments, a switch was then made to the same buffer that contained a known concentration of gliclazide. Once a breakthrough curve had formed, pH 7.4, 0.067 M potassium phosphate buffer was then passed again through the column to elute the retained drug. Concentrations of 1–200 μ M gliclazide were used in these experiments and the elution of gliclazide was monitored at 250 nm. Although gliclazide is a weak acid with a pK_a of 5.8 [27], less than a 0.05 unit change in pH occurred when this drug was added to the pH 7.4 phosphate buffer over the entire tested range of gliclazide concentrations. Each frontal analysis experiment was performed in quadruplicate and the central location of each breakthrough curve was determined using the equal area method and PeakFit 4.12 (Jandel Scientific Software, San Rafael, CA, USA) [16]. Based on the results that were obtained for the control column, a correction was made for non-specific binding of gliclazide to the support by subtracting the control results from the data for a column containing normal HSA or glycosylated HSA. Non-specific binding to the support made up approximately 39% of the total binding for 1 μ M gliclazide on an HSA column and was easily

corrected by this approach, as noted previously for related drugs on similar columns [6, 7].

The zonal elution studies were carried out in quadruplicate using *R*-warfarin as a site-specific probe for Sudlow site I and *L*-tryptophan as a probe for Sudlow site II [5]. During these experiments, 1–20 μM gliclazide was placed into the mobile phase as 20- μL injections of 5 μM *R*-warfarin and *L*-tryptophan were made (i.e., sample conditions found earlier to represent linear elution conditions on the types of columns that were examined in this study) [23]. The elution of *R*-warfarin or *L*-tryptophan was monitored at 308 or 280 nm, respectively. Sodium nitrate was injected and monitored at 205 nm as a non-retained solute; this solute has been found in numerous studies to be a good index of the void volume and void time for similar HSA columns (e.g., see Refs. [17–24]). Data from the competition studies were fit to exponentially modified Gaussian curves and analyzed using PeakFit v4.12.

Results and discussion

Frontal analysis studies

The first set of experiments used frontal analysis to examine the overall binding of gliclazide to samples of either normal or glycosylated HSA within HPLC columns. This work was used to provide initial estimates of the association equilibrium constants and moles of binding sites for gliclazide with these protein preparations. Some typical chromatograms that were generated for normal HSA during these experiments are given in Fig. 3(a). The resulting data were first analyzed by using a one-site binding model, as represented by Eqs. 1 and 2 [16–18].

One-site model:

$$m_{\text{Lapp}} = \frac{m_{\text{Ltot}}K_a[A]}{(1 + K_a[A])} \quad (1)$$

$$\frac{1}{m_{\text{Lapp}}} = \frac{1}{(K_a m_{\text{Ltot}}[A])} + \frac{1}{m_{\text{Ltot}}} \quad (2)$$

The term m_{Lapp} in Eqs. 1 and 2 represents the apparent moles of the applied analyte (i.e., gliclazide) that were required to reach the central point of a breakthrough curve at a given concentration of analyte in the mobile phase, $[A]$ [17]. The association equilibrium constant and total moles of binding sites for the analyte in the column are described in Eqs. 1 and 2 by K_a and m_{Ltot} .

Similar equations can be created for systems with multiple binding sites, as shown for a two-site model in Eqs. 3 and 4 [16–18].

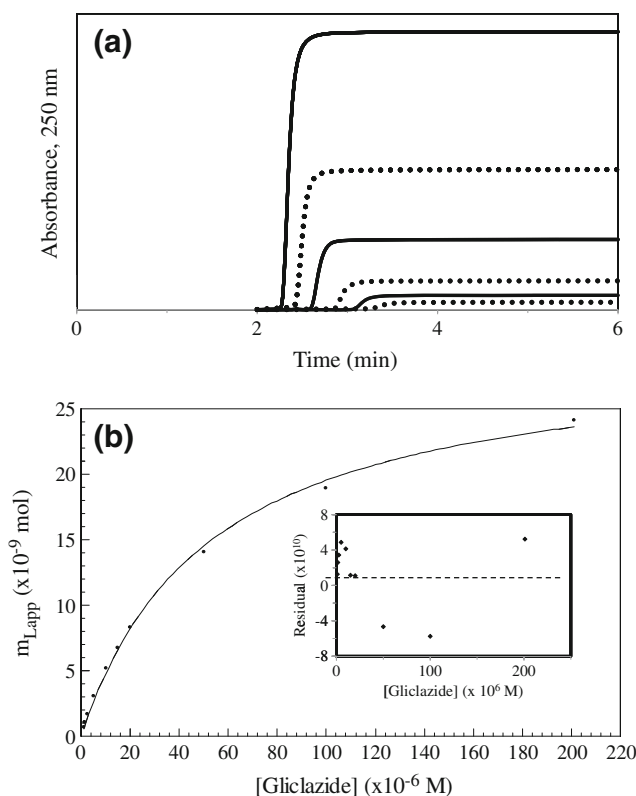


Fig. 3 (a) Example of frontal analysis studies for gliclazide on a normal HSA column and (b) a binding isotherm that was generated from such a study. The results in (a) were obtained at gliclazide concentrations of 200, 100, 50, 20, 10, and 5 μM (top to bottom). The best-fit line in (b) was generated by using Eq. 1 and a one-site model; further details on this fit are given in the text. The inset in (b) shows the corresponding residual plot

Two-site model:

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

$$\frac{1}{m_{\text{Lapp}}} = \frac{1 + K_{a1}[A] + \beta_2 K_{a1}[A] + \beta_2 K_{a1}^2[A]^2}{m_{\text{Ltot}}\{\alpha_1 + \beta_2 - \alpha_1\beta_2\}K_{a1}[A] + \beta_2 K_{a1}^2[A]^2} \quad (4)$$

These equations now include two association equilibrium constants (K_{a1} and K_{a2}), which represent the high- and lower-affinity sites in the column. The amounts of these two types of sites, in moles, are described by m_{L1} and m_{L2} . Equation 4 includes the term α_1 , which represents the fraction all binding sites for an analyte that consist of the high-affinity regions (i.e., $\alpha_1 = m_{\text{L1}}/m_{\text{Ltot}}$). The term β_2 in Eq. 4 is the ratio of the association equilibrium constants for the low versus high-affinity sites, or $\beta_2 = K_{a2}/K_{a1}$.

Figure 3(b) shows a typical binding isotherm that was obtained when the frontal analysis data for the normal

HSA column were examined according to Eq. 1. The use of non-linear regression gave a best-fit line for Eq. 1 that had a correlation coefficient of 0.998 ($n=10$). The best-fit parameters for this line provided a K_a value of $1.9 (\pm 0.1) \times 10^4 \text{ M}^{-1}$ and a value for $m_{L\text{tot}}$ of $3.0 (\pm 0.1) \times 10^{-8} \text{ mol}$. The binding data were also examined by using double-reciprocal plots of $1/m_{L\text{app}}$ vs. $1/[A]$, as illustrated in Fig. 4(a) for the normal HSA column. According to Eq. 2, this type of plot should result in a linear relationship if one-site binding is present between the applied analyte and immobilized binding agent [17]. The plot that was obtained in this case gave a linear response (correlation coefficient, 0.999 for $n=7$) at the lowest analyte concentrations, or highest values of $1/[A]$. However, a small amount of curvature at lower values of $1/[A]$ was observed. According to Eq. 4, this curvature indicates that some multi-site interactions were present.

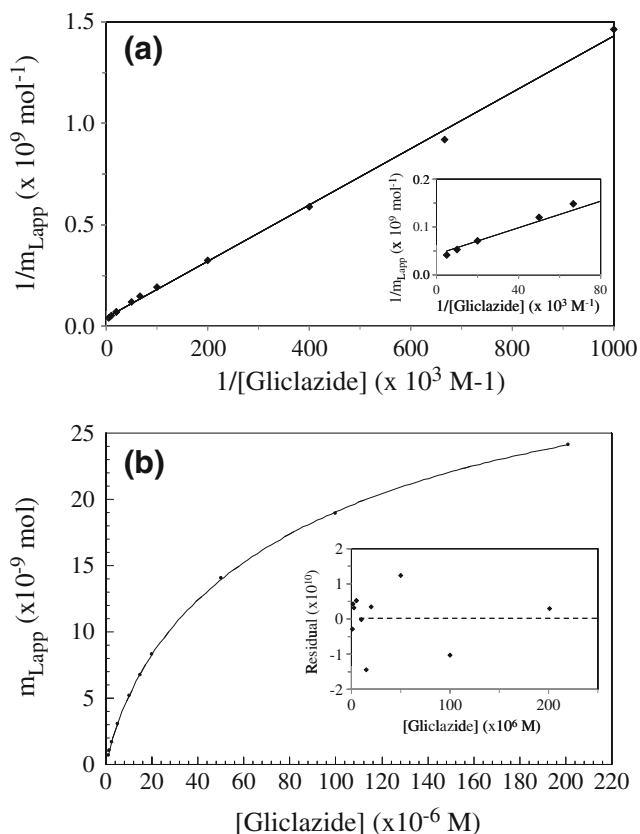


Fig. 4 (a) A double-reciprocal plot for data obtained from frontal analysis experiments that examined the binding of gliclazide with normal HSA, and (b) analysis of the binding isotherm for gliclazide and normal HSA when using a two-site model. The best-fit line in (a) was generated by using the data at 10–200 μM gliclazide to the right of this plot. The inset in (a) shows the small deviations from linearity that occurred at low values of $1/[\text{Gliclazide}]$. The inset in (b) provides the residual plot for the fit of a two-site model to the frontal analysis data

The linear behavior seen in Fig. 4(a) at high values of $1/[A]$ (or low values of $[A]$) is predicted by Eq. 5 [16].

$$\lim[A] \rightarrow 0 \frac{1}{m_{L\text{app}}} = \frac{1}{m_{L\text{tot}} \{(\alpha_1 + \beta_2 - \alpha_1\beta_2)K_{a1}[A]\}} + \frac{\alpha_1 + \beta_2^2 - \alpha_1\beta_2^2}{m_{L\text{tot}}(\alpha_1 + \beta_2 - \alpha_1\beta_2)^2} \quad (5)$$

It is known from a previous work that this linear range will be seen for any system with multiple and independent binding sites and can be used to estimate the association equilibrium constant for the highest affinity sites in the system [16]. From this linear range, an estimate of $3.4 (\pm 0.1) \times 10^4 \text{ M}^{-1}$ was obtained for the average K_a of gliclazide at its high-affinity sites on HSA.

Because the plots in Figs. 3 and 4 suggested that multi-site binding was present for gliclazide with HSA, the frontal analysis data were next examined by using a two-site model. The resulting fit that was obtained for gliclazide with normal HSA is shown in Fig. 4(b). The two-site model provided a slightly better fit than the one-site model, giving a correlation coefficient of 0.999 ($n=10$). The better agreement of this fit with the data was more clearly indicated in the residual plots for Figs. 3(b) and 4(b) (see insets), in which the two-site model gave a more random distribution of data points about the best-fit line. The better fit of the two-site model was also apparent when comparing the sum of the squares of the residuals for Figs 3(b) and 4(b), in which the two-site model gave a much smaller value than the one-site model (i.e., 3.77×10^{-12} vs. 1.34×10^{-9}). Similar results were obtained when examining the binding of gliclazide with a column containing glycated HSA (e.g., using a sample of gHSA3). No further improvement in the fit was noted when using a higher-order model, so a two-site model was utilized in all further binding studies with gliclazide and normal HSA or glycated HSA. Similar results and conclusions were obtained based on ultrafiltration experiments that used HSA or glycated HSA in a soluble form for equivalent binding studies (see the [Electronic supplementary material](#)). However, the HPAC method required much less protein than the ultrafiltration studies (e.g., 11–18 nmol per column vs. 260–280 nmol for one ultrafiltration measurement) and was capable of reusing the same columns and proteins for hundreds of experiments [6, 7, 16–18]. HPAC was also a much faster method than ultrafiltration for these measurements, providing results within 4–5 min per drug sample compared to 80–90 min per sample for ultrafiltration (e.g., see Fig. 3(a) and the [Electronic supplementary material](#)).

The association equilibrium constants that were obtained by frontal analysis for the two-site model with gliclazide and normal HSA were $7.1 (\pm 1.9) \times 10^4$ and $8.9 (\pm 1.5) \times 10^3 \text{ M}^{-1}$. The corresponding values for glycated HSA, using the gHSA3 column as an example, were $1.0 (\pm 0.8) \times 10^5$ and $5.7 (\pm 3.9) \times 10^3 \text{ M}^{-1}$. A summary of the results that

were obtained at pH 7.4 and 37 °C is provided in Table 1. These values were similar to those that have been reported when using a two-site model to describe the interactions of tolbutamide and acetohexamide with normal HSA or glycosylated HSA [6, 7]. The relative amounts of the two groups of binding sites were estimated to be $7.1 (\pm 2.2) \times 10^{-9}$ and $2.7 (\pm 0.1) \times 10^{-8}$ mol for normal HSA, with values of $5.6 (\pm 3.8) \times 10^{-9}$ and $2.6 (\pm 0.4) \times 10^{-8}$ mol being obtained for gHSA3. Based on the protein content of the HPAC columns, these results corresponded to specific activities of $0.50 (\pm 0.16)$ and $1.90 (\pm 0.16)$ mol/mol normal HSA or $0.38 (\pm 0.25)$ and $1.71 (\pm 0.29)$ mol/mol gHSA3. Given the fact that HSA which has been immobilized by the Schiff base method is roughly 50–60% active, these results and those obtained by ultrafiltration (see the [Electronic supplementary material](#)) indicated that one to two major binding sites and two to three or more weaker binding regions were involved in the interactions of gliclazide with normal HSA or glycosylated HSA. Similar conclusions have been reached when examining the interactions of acetohexamide and tolbutamide by this approach with normal HSA or glycosylated HSA [6, 7].

Zonal elution studies with gliclazide at Sudlow site I

Competition studies based on zonal elution experiments were next used with the HPAC columns to identify specific sites for gliclazide on normal HSA and glycosylated HSA. These experiments were first conducted by using *R*-warfarin as a site-selective probe for Sudlow site I. This region was of interest because it has recently been proposed to be one of high-affinity sites on HSA for sulfonylurea drugs such as acetohexamide and tolbutamide, as determined through zonal elution studies [6, 7]. A typical set of chromatograms for this type of experiment is shown in Fig. 5, in which a small amount of *R*-warfarin was injected into the presence of mobile phases that contained various concentrations of gliclazide as a competing agent. Sodium nitrate was also injected as a non-retained solute to determine the void time, which was then used to calculate the retention factor (k) for *R*-warfarin.

Equation 6 can be used in this type of experiment to describe a system in which direct competition at a single-site is involved in the binding of the injected probe A and

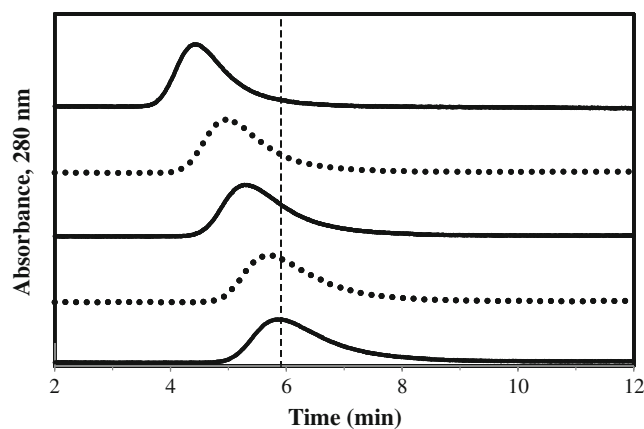


Fig. 5 Typical zonal elution competition studies on a normal HSA column using *R*-warfarin as an injected site-specific probe and gliclazide as a mobile phase additive. These results are for gliclazide concentrations of 20, 10, 5, 1, or 0 μM (top to bottom). The vertical dashed line is shown for reference and demonstrates how the retention time for the injected probe changed as the concentration of gliclazide was varied in the mobile phase

the competing agent I that has been added to the mobile phase [7].

$$\frac{1}{k} = \frac{K_{aI}V_M[I]}{K_{aA}m_L} + \frac{V_M}{K_{aA}m_L} \quad (6)$$

The terms K_{aA} and K_{aI} in Eq. 6 represent the association equilibrium constants for the probe and competing agent, respectively, and V_M is the void volume. According to Eq. 6, a plot of $1/k$ versus the competing agent concentration $[I]$ should produce a linear relationship for a system with single-site competition [7]. The value of K_{aI} can be obtained from this plot by determining the ratio of the slope to the intercept. In this way, it is possible to specifically examine the binding of the mobile phase additive I at its site of competition with the injected probe.

As shown in Fig. 6(a), a linear fit to Eq. 6 was obtained for gliclazide on each column that contained normal HSA or glycosylated HSA when *R*-warfarin was used as an injected probe for Sudlow site I. The best-fit lines for these plots had correlation coefficients in the range of 0.960 to 0.998 ($n=5-6$). The corresponding residual plots gave only random variations in the data about the best-fit lines, and the sums of the squares for the residuals were between $1.0 \times$

Table 1 Best-fit parameters obtained for a two-site model for the binding of gliclazide with normal HSA and gHSA3 at pH 7.4 and 37°C

Type of HSA	$K_{a1} (\times 10^4 \text{ M}^{-1})$	$m_{L1} (\times 10^{-9} \text{ mol})$	$K_{a2} (\times 10^4 \text{ M}^{-1})$	$m_{L2} (\times 10^{-9} \text{ mol})$
Normal HSA	7.1 (± 1.9)	7.1 (± 2.2)	0.89 (± 0.15)	27 (± 1)
gHSA3	10.0 (± 0.8)	5.6 (± 3.8)	0.57 (± 0.39)	26 (± 4)

The values in parentheses represent a range of ± 1 SD, as based on error propagation and the precisions of the best-fit slopes and intercepts obtained when using Eq. 3 for $n=10$

10^{-5} and 1.7×10^{-3} . All of these results confirmed that gliclazide and *R*-warfarin had direct competition at Sudlow site I on both normal HSA and glycosylated HSA. Binding at Sudlow site I has also been noted for acetohexamide and tolbutamide on normal HSA and glycosylated HSA [6, 7].

The association equilibrium constants for gliclazide at Sudlow site I, as represented in this case by K_{aI} in Eq. 6, were determined on the various HSA columns from the best-fit lines in Fig. 6(a). The results that were obtained at pH 7.4 and 37 °C are summarized in Table 2. An association equilibrium constant of $1.9 (\pm 0.1) \times 10^4 \text{ M}^{-1}$ was measured for gliclazide with normal HSA. This result is slightly lower than the K_a values of $4.2\text{--}5.5 \times 10^4 \text{ M}^{-1}$ that have been reported for tolbutamide and acetohexamide at the same site on normal HSA [6, 7].

A comparison was next made in the association equilibrium constants that were determined for gliclazide at Sudlow site I in going from normal HSA to the glycosylated HSA samples. There was no significant difference in the K_a values for normal HSA and gHSA1 or gHSA3. However, there was a 1.9- to 2.0-fold increase in the K_a for gliclazide

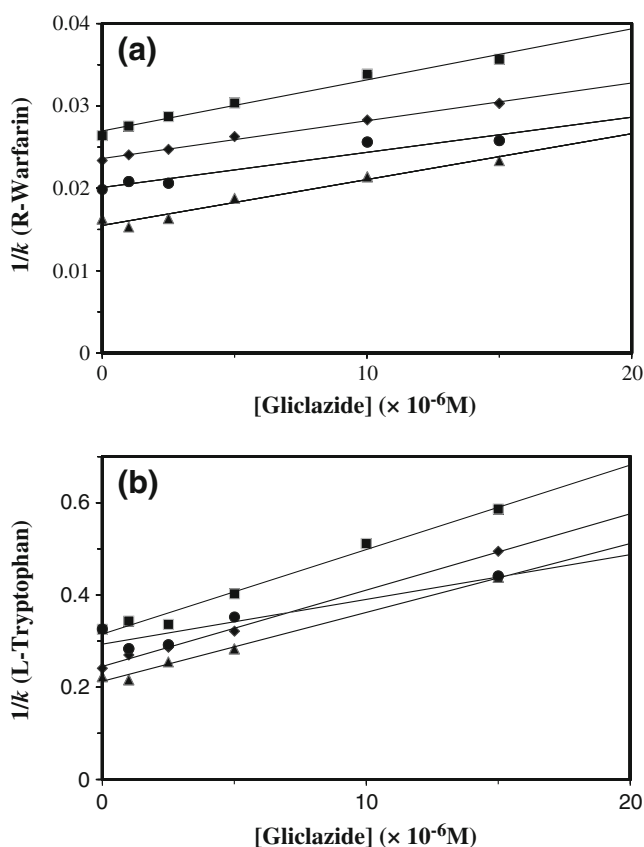


Fig. 6 Plots prepared according to Eq. 6 that show how the reciprocal of the retention factor for (a) *R*-warfarin or (b) *L*-tryptophan changed on HSA or glycosylated HSA columns as the concentration of gliclazide was varied in the mobile phase. These results are for normal HSA (diamonds), gHSA1 (squares), gHSA2 (triangles), and gHSA3 (circles)

Table 2 Association equilibrium constants measured at pH 7.4 and 37 °C for gliclazide at Sudlow sites I and II for normal HSA and in vitro glycosylated HSA

Type of HSA	Sudlow site I $K_a (\times 10^4 \text{ M}^{-1})$	Sudlow site II $K_a (\times 10^4 \text{ M}^{-1})$
Normal HSA	1.9 (± 0.1)	6.0 (± 0.5)
gHSA1	1.8 (± 0.2)	4.6 (± 0.4)
gHSA2	3.6 (± 0.3)	7.6 (± 0.6)
gHSA3	2.1 (± 0.2)	3.8 (± 0.4)

The values in parentheses represent a range of ± 1 SD, as based on error propagation and the precisions of the best-fit slopes and intercepts obtained when using Eq. 6 for $n=5\text{--}6$

at Sudlow site I in going from normal HSA or gHSA1 to gHSA2 and a similar decrease in affinity in going from gHSA2 to gHSA3. These differences were all significant at the 95% confidence level. This change in affinity with the level of glycation for HSA has also been noted for acetohexamide and tolbutamide, although the degree and direction of this change does vary from one type of sulfonylurea drug to the next [6, 7]. It has been proposed in earlier work that these alterations in affinity are related to the extent and types of glycation products that form at or near Sudlow site I as the level of HSA glycation is altered [6, 7, 28, 29].

Zonal elution studies with gliclazide at Sudlow site II

Competition studies on the HPLC columns were also carried out using *L*-tryptophan as a site-selective probe for Sudlow site II. This site was of interest because it also has been demonstrated to be one of high-affinity sites on HSA for acetohexamide and tolbutamide [6, 7]. The results of these experiments were again plotted and analyzed through the use of Eq. 6. All of the normal HSA or glycosylated HSA columns gave a linear response to this equation, as illustrated in Fig. 6(b), with correlation coefficients that ranged from 0.967 to 0.996 ($n=5\text{--}6$). The corresponding residual plots all gave a random distribution of the data points about the best-fit lines and the sum of the squares of the residuals for these best-fit lines ranged from 4.3×10^{-4} to 1.7×10^{-4} . The agreement of these plots with the behavior predicted by Eq. 6 indicated that gliclazide and *L*-tryptophan had direct competition at Sudlow site II. The same conclusion has been reached when the same approach was used to examine the binding of acetohexamide and tolbutamide at Sudlow site II [6, 7].

The results from the plots in Fig. 6(b) were used to determine the association equilibrium constants for gliclazide at Sudlow site II on each normal HSA or glycosylated HSA column. These results are included in Table 2. The K_a value of $6.0 (\pm 0.5) \times 10^4 \text{ M}^{-1}$ that was determined for

gliclazide at Sudlow site II on normal HSA at pH 7.4 and 37 °C agreed with the average association equilibrium constant that was estimated by frontal analysis for the high-affinity sites of gliclazide on this protein. In addition, the K_a determined for gliclazide at Sudlow site II on normal HSA was slightly lower than the affinities of $5.3\text{--}13 \times 10^4 \text{ M}^{-1}$ that have been measured for tolbutamide and acetohexamide at the same site [6, 7].

All of the glycosylated HSA samples had affinities at Sudlow site II that were in the range of $10^4\text{--}10^5 \text{ M}^{-1}$ for gliclazide. However, the size of these values varied with the extent of glycation. For instance, there was a decrease of 1.3-fold in K_a for gliclazide at Sudlow site II in going from normal HSA to gHSA1. This change was similar to what has been seen for acetohexamide with the same samples of normal and glycosylated HSA, in which a 1.6-fold decrease in affinity was observed [6]. There was a 1.6-fold increase in K_a between gHSA1 and gHSA2, or a 1.3-fold increase between normal HSA and gHSA2. This was followed by a 2-fold decrease in K_a in going from gHSA2 to gHSA3, or a 1.6-fold decrease between normal HSA and gHSA3. These differences were all significant at the 95% confidence level. As stated in the previous section, these changes in affinity with glycation are thought to be due to differences in the glycation products that are formed at or near specific regions on HSA as the overall level of glycation for this protein is increased [6, 7, 28, 29].

Concluding remarks

This report used HPAC as a tool to examine the binding of gliclazide to normal HSA and HSA with various levels of glycation. Frontal analysis experiments indicated that gliclazide was binding with normal HSA and glycosylated HSA through a two-site model that involved both high- and lower-affinity sites. There were one or two high-affinity regions with an average association equilibrium constant of approximately $7.1\text{--}10 \times 10^4 \text{ M}^{-1}$ and two or more low affinity sites with an average association equilibrium constant of $5.7\text{--}8.9 \times 10^3 \text{ M}^{-1}$ at pH 7.4 and 37 °C.

Zonal elution studies demonstrated that gliclazide was binding to both Sudlow sites I and II in normal HSA and glycosylated HSA. The association equilibrium constants for these sites were in the range of $10^4\text{--}10^5 \text{ M}^{-1}$. The binding of gliclazide at Sudlow sites I and II for normal HSA gave association equilibrium constants of 1.9×10^4 and $6.0 \times 10^4 \text{ M}^{-1}$, respectively. Two of the glycosylated HSA samples (i. e., gHSA1 and gHSA3) had similar affinities to normal HSA for gliclazide at Sudlow site I; however, one of the protein samples (gHSA2) had a 1.9-fold increase in affinity for gliclazide at this site. All of the glycosylated HSA samples differed from normal HSA in their affinity for gliclazide at

Sudlow site II. These data indicate that modifications due to glycation can have different effects on the interactions of gliclazide with HSA at separate binding sites on this protein. These results are in agreement with previous data that have been obtained with acetohexamide and tolbutamide [6, 7] and with structural studies that have examined the glycation products that can form at or near Sudlow sites I and II [28, 29]. Similar studies with *in vivo* glycosylated HSA are now in progress to further characterize these effects and to determine their possible clinical significance.

The experiments in this report illustrated how HPAC could be used to provide detailed information on the binding of a drug or solute with a modified protein. This included data on the overall model, equilibrium constants and amount of binding sites for a drug–protein interaction. It was also demonstrated how HPAC can be used to examine the changes in interactions that occur at specific regions on a protein (e.g., Sudlow sites I and II of HSA). The methods used in this study were relatively fast (i.e., taking only minutes per sample) and were easily automated. The small amounts of protein in the HPAC columns and the ability to reuse these columns for hundreds of binding experiments made this approach more attractive than ultrafiltration for work with valuable or limited samples of modified proteins. The techniques used in this study are not limited to gliclazide and normal HSA or glycosylated HSA but could be used with many other types of biological interactions. These features should result in the further use of HPAC for biointeraction analysis.

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