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Studies of drug interactions with glycated human serum albumin by high-performance affinity chromatography

Abstract: Diabetes is a health condition associated with the elevated levels of glucose in the bloodstream and affects 366 million people worldwide. Type II diabetes is often treated with sulfonylurea drugs, which are known to bind tightly in the blood to the transport protein human serum albumin (HSA). One consequence of the elevated levels of glucose in diabetes is the nonenzymatic glycation of proteins such as HSA. Several areas of HSA are now known to be affected by glycation-related modifications, which may in turn affect the binding of sulfonylurea drugs and other solutes to this protein. This review discusses some recent studies that have examined these changes in drug-protein binding by employing high-performance affinity chromatography (HPAC). A description of the theoretical and experimental techniques that were used in these studies is given. The information on drug interactions with glycated HSA, as obtained through this method, is also summarized. In addition, the potential advantages of this approach in the areas of biointeraction analysis and personalized medicine are considered.

Keywords: binding studies; diabetes; drug-protein binding; human serum albumin; glycation; high-performance affinity chromatography; sulfonylurea drugs.

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Introduction

Diabetes is a health condition that is associated with hyperglycemia or the elevated levels of glucose in the bloodstream (Nelson and Cox 2005). This disease affects 366 million people worldwide, and in the United States, diabetes affects 25.8 million people (International Diabetes Federation 2011). Type I diabetes (i.e., juvenile or insulin-dependent diabetes) is associated with the insufficient production of insulin and occurs when the immune system attacks insulin-producing pancreatic β cells (International Diabetes Federation 2011, U.S. Centers for Disease Control 2011). This type of diabetes accounts for 5–10% of the diagnosed cases of diabetes and requires insulin for treatment. Type II diabetes (i.e., non-insulin-dependent or adult-onset diabetes) makes up the remaining 90–95% of the patients diagnosed with this disease and is caused by insulin deficiency or insulin resistance (International Diabetes Federation 2011, U.S. Centers for Disease Control 2011).

Type II diabetes is often treated with sulfonylurea drugs, which are oral medications that are responsible for stimulating the release of insulin to lower glucose levels in the blood (Skillman and Feldman 1981). The basic structure of a sulfonylurea drug is shown in Figure 1. The structures of several first- and second-generation sulfonylurea drugs are also included in the figure. These sulfonylurea drugs contain phenysulfonyl and urea groups with various nonpolar functional groups on either side of the core structure (Foster 1998, Zavod et al. 2008). The first-generation sulfonylureas (e.g., acetohexamide and tolbutamide) were the drugs in this class that were used the earliest. However, the second-generation drugs (e.g., glibenclamide and gliclazide) tend to be more effective and are more easily excreted from the body (Jakoby et al. 1995).

Sulfonylurea drugs are known to bind tightly to serum transport proteins and especially to human serum albumin (HSA) (Crooks and Brown 1974, Anguizola et al. 2013a). HSA is the most abundant serum protein and is found in concentrations ranging from 30 to 50 g/L in the blood, accounting for approximately 60% of the total serum protein concentration (Tietz 1986, Peters 1996). Figure 2 shows the structure of HSA. HSA has a molecular weight of 66.7 kDa and contains 585 amino acids, 59 lysines, and 24 arginines. This protein is involved in many physiological processes, which include the regulation of osmotic pressure, the control of pH in the bloodstream, and the transportation of numerous low mass substances within the blood (e.g., hormones, fatty acids, and drugs) (Peters 1996).

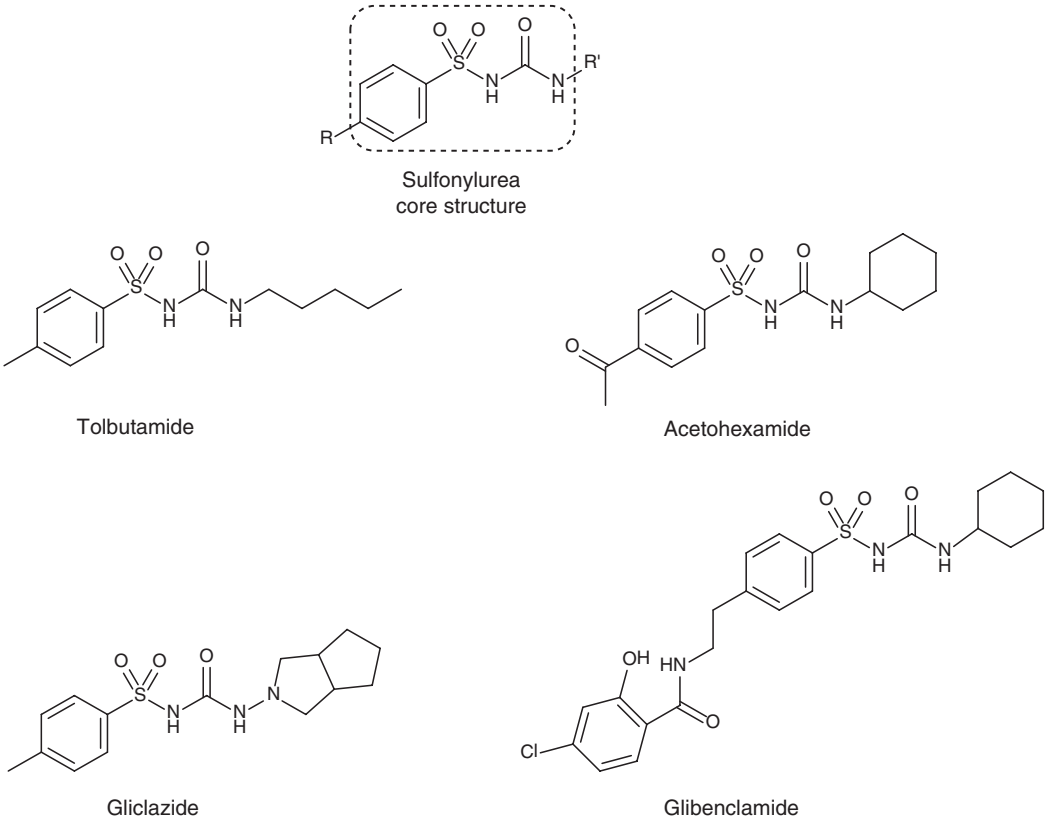

Figure 1 Core structure of a sulfonylurea drug and examples of first-generation sulfonylurea drugs (e.g., tolbutamide and acetohexamide) or second-generation sulfonylurea drugs (e.g., gliclazide and glibenclamide).

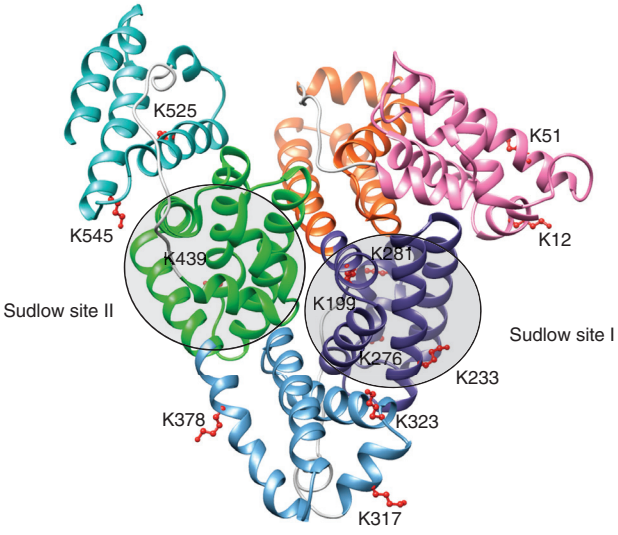

Figure 2 Structure of HSA, including the location of several lysines that often take part in glycation and the location of the major drug-binding sites of this protein (i.e., Sudlow sites I and II).
Each subdomain of HSA is shown in a different color. This structure was generated using Protein Data Bank (PDB) file ID: 1A06 (Anguizola et al. 2013a).

There are many regions on HSA that can take part in its binding to low mass solutes (Figure 2). For instance, HSA contains several hydrophobic binding sites for fatty acids (Curry et al. 1998, Simard et al. 2005). This protein also has two major binding sites for drugs, known as Sudlow sites I and II (Sudlow et al. 1975, Peters 1996, Otagiri 2005). Sudlow site I (i.e., the warfarin-azapropazone site) is located in subdomain IIA of HSA and tends to bind bulky heterocyclic compounds, anticoagulants, or nonsteroid anti-inflammatory drugs, such as warfarin, salicylate, azapropazone, and phenylbutazone (Sudlow et al. 1975, Loun and Hage 1994, Peters 1996, Otagiri 2005). Sudlow site II (i.e., the indole-benzodiazepine site) is located in subdomain IIIA and is the primary binding site for aromatic compounds such as ibuprofen, fenoprofen, ketoprofen, benzodiazepines, and L-tryptophan (Sudlow et al. 1975, Yang and Hage 1993, Peters 1996, Otagiri 2005). Another minor binding site for drugs on HSA is the digitoxin site (Brock 1975, Sjoholm et al. 1979, Brors et al. 1993, Hage and Sengupta 1999, Chen et al. 2004).

Elevated levels of glucose can result in the nonenzymatic glycation of proteins (Iberg and Fluckiger 1986, Koyama et al. 1997, Colmenarejo 2003, Nakajou et al. 2003,

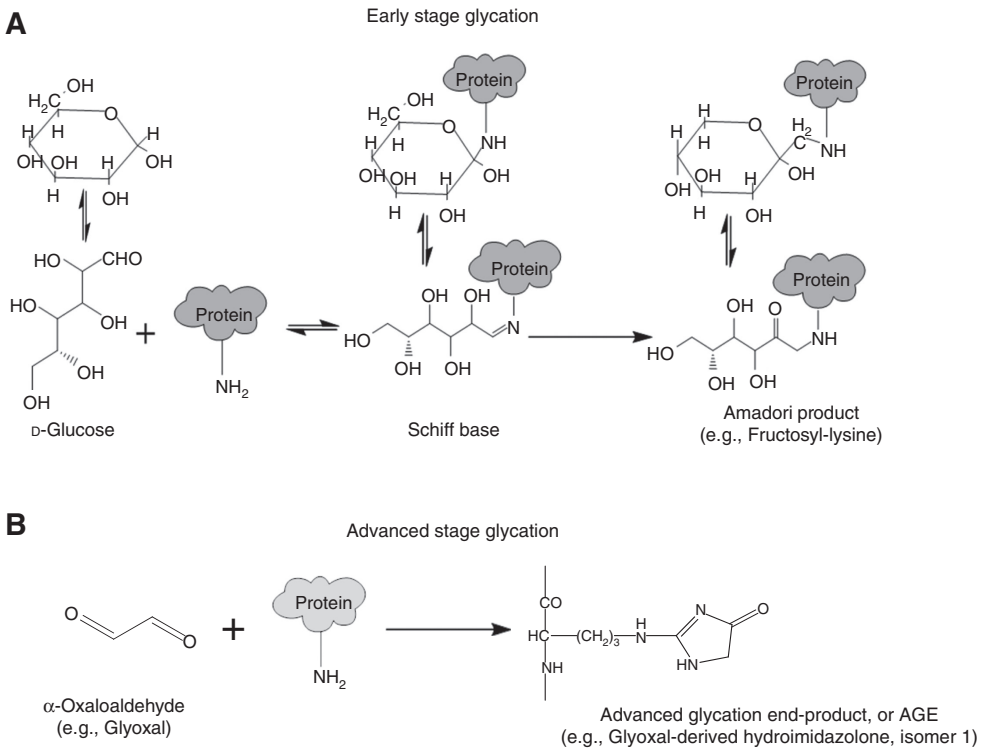

Figure 3 General reactions involved in the glycation of HSA, including both early-stage glycation (e.g., as illustrated through the formation of fructosyl-lysine in the presence of glucose) and advanced-stage glycation (e.g., as illustrated in the formation of an AGE through the reaction of α-oxaloaldehyde with HSA) (Anguizola et al. 2013a).

Mendez et al. 2005). For instance, patients with diabetes can have a 2- to 5-fold increase in the amount of HSA that is glycated when compared to normal individuals (Roohk and Zaidi 2008). Early-stage glycation, as illustrated in Figure 3A, is characterized by the nucleophilic addition of reducing sugars to free amine groups on a protein (Lapolla et al. 2005, 2006). This type of reaction can occur at the N-terminus or with free amine groups on lysine residues. The intermediate of this reaction is a Schiff base, which is unstable but can undergo rearrangement to form a more stable Amadori product or a keto amine derivative (Zhang et al. 2009). When this reaction involves glucose, such a process will form a product known as fructosyl-lysine. Advanced glycation end-products (AGEs) can also form through further dehydration, oxidation, and/or cross-linking steps, such as those that result in the production of reactive dicarbonyls such as α-oxaloaldehyde (or glyoxal). These reactive dicarbonyls can then combine with lysine or arginine residues on proteins to form AGEs, as illustrated in Figure 3B (Anguizola et al. 2013a). Several areas of HSA at or near Sudlow sites I and II are now known to be affected by glycation-related modifications (Figure 2), which have also been shown to affect the binding of various sulfonylurea drugs to this protein (Iberg and Fluckiger 1986, Koyama et al. 1997, Colmenarejo 2003, Nakajou et al. 2003, Mendez et al. 2005, Joseph et al. 2010, 2011, Matsuda et al. 2011, 2012).

One way in which these changes in drug-protein binding can be examined is by employing high-performance affinity chromatography (HPAC). This method is a liquid chromatographic technique that makes use of an immobilized biological agent (e.g., HSA) as a stationary phase (Hage 2002, Schiel et al. 2010, Hage et al. 2011a). The basic operation of this method is shown in Figure 4, in which an immobilized binding agent is used for the selective recognition and retention of a given set of target compounds in a sample. This method has often been used for the separation, purification, and analysis of specific targets through the use of appropriate binding agents that are immobilized in the HPAC column (Hage et al. 2011b). However, HPAC can also be used to examine solute- and drug-protein interactions by using HSA or some other protein as the immobilized binding agent. Some advantages of HPAC over other methods for analyzing solute-protein interactions are that it is a high-throughput technique that is relatively fast and easy to perform. In addition, this

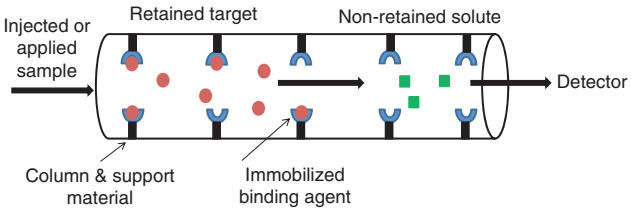

Figure 4 General scheme for using an immobilized binding agent in an HPAC column system to recognize and separate a target from other nonretained sample components.

method can have high levels of precision and can often use the same binding agent and column over hundreds of experiments (Hage et al. 2011b).

This review will describe recent studies that have used HPAC to examine the binding of sulfonylureas to normal HSA and samples of *in vitro* or *in vivo* glycated HSA. An overview of the theoretical and experimental techniques that were used in these studies will be provided along with a summary of the information that was obtained through HPAC on drug interactions with glycated HSA. These results will be used to illustrate how HPAC can be employed as a tool to examine the complex interactions that involve heterogeneous binding agents. The possible advantages of this method as a tool in the areas of biointeraction analysis and personalized medicine will also be considered as well as the extension of this approach to other systems of biomedical interest that involve solute-protein binding or heterogeneous binding agents.

Preparation of normal HSA and glycated HSA columns

The first item to consider in HPAC-based binding studies is the approach used to prepare the immobilized binding agent. In most HPAC studies of solute-protein interactions, high-performance liquid chromatography (HPLC)-grade porous silica has been used as the starting support material. This material is usually converted into a diol-bonded form to provide low nonspecific binding for most biological compounds (Kim and Hage 2006, Hage et al. 2011a). Diol-bonded silica can then be used for the covalent immobilization of a protein (e.g., HSA or glycated HSA) through an approach such as the Schiff base method, as illustrated in Figure 5A. One way the Schiff base method can be carried out is by oxidizing diol-bonded silica to form aldehyde groups on the surface of this support. This activated support is then reacted with free amine groups on the protein or binding agent of interest, which results in the formation of a Schiff base. This Schiff base

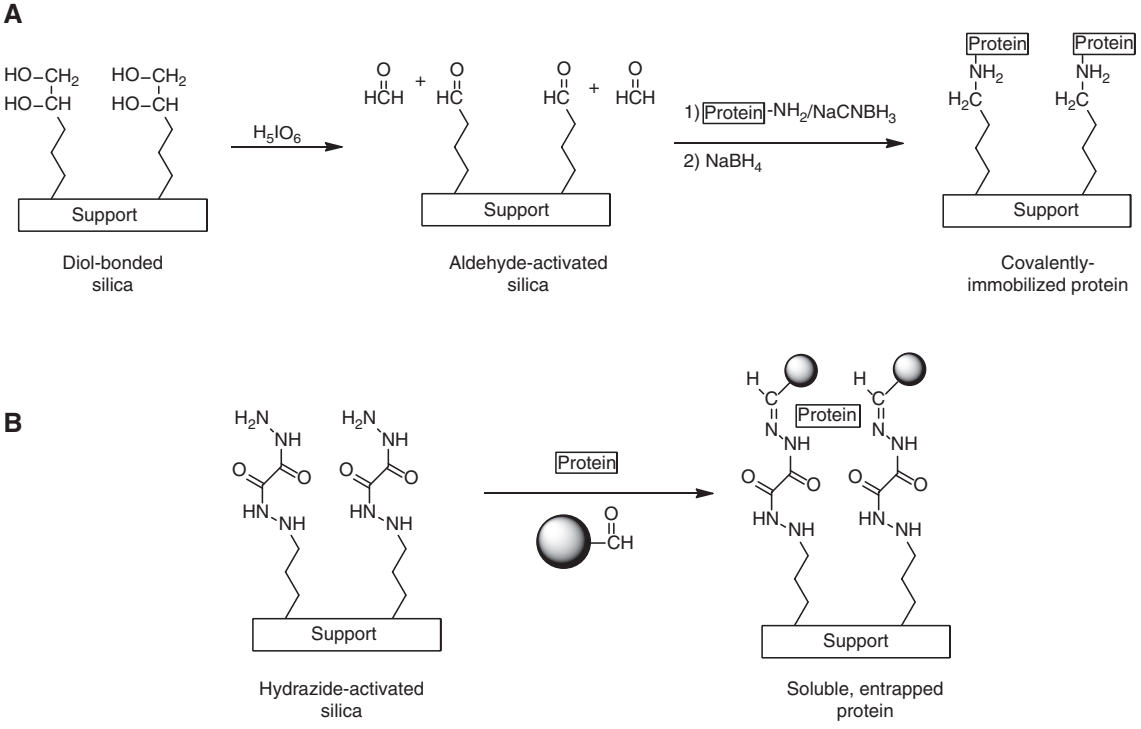

Figure 5 General reactions involved in the immobilization of a protein onto silica by (A) the Schiff base method or (B) entrapment based on the reaction of mildly oxidized glycogen with a hydrazide-activated support.

is reduced upon its formation by using sodium cyanoborohydride to form a stable secondary amine linkage. Any aldehyde groups that remain after the immobilization process can be removed by later adding a strong reducing agent, such as sodium borohydride (Kim and Hage 2006). The immobilization of HSA by this method tends to occur through the N-terminus or lysines that are not located at Sudlow site I or II. These particular lysines also tend to differ from the residues that usually take part in glycation (Wa et al. 2006, 2007, Barnaby et al. 2010). In addition, prior studies that have used the Schiff base method with HSA have found that this approach gives an immobilized protein that has good qualitative and quantitative agreement with the binding behavior seen for the same protein in its soluble form (Hage et al. 2011a).

Several reports have used the Schiff base method to prepare HPAC columns that contained various types of glycated HSA. In one of these studies, samples of *in vitro* glycated HSA were prepared or obtained that had levels of glycation similar to those found in individuals with pre-diabetes, controlled diabetes, and advanced or poorly controlled diabetes (Joseph and Hage 2010a). The pre-diabetes sample was acquired from a commercial source and was prepared under proprietary conditions that involved mixing a fixed concentration of glucose with HSA at 37°C and for a period of time that was no longer than a week (Joseph and Hage 2010a; Barnaby et al. 2011a). The other samples were prepared in a similar manner by using glucose concentrations of 15–30 mM and physiological levels of HSA at 37°C and an incubation time of 4 weeks (Joseph et al. 2010). The glycated HSA samples were then dialyzed and lyophilized, immobilized by the Schiff base method, and used to prepare HPAC columns for various drug-binding studies (Joseph and Hage 2010a), as will be described later in this review. These columns were stable for up to 500 sample injections or applications, making it possible to conduct many binding experiments with different drugs and conditions on the same HPAC columns (Joseph et al. 2010, 2011, Matsuda et al. 2011, 2012).

The Schiff base method has also been used to prepare HPAC columns that contained *in vivo* glycated HSA obtained from patients with diabetes (Anguizola et al. 2013b). Figure 6 shows the general procedure that was employed to isolate *in vivo* glycated HSA from serum or plasma. In this procedure, the glycated HSA was extracted from a sample by using an anti-HSA polyclonal antibody column. The samples were then dialyzed, lyophilized, and immobilized by the Schiff base method. The resulting columns were found to be stable for over 250 sample injections or application cycles when they were used in drug-binding studies (Anguizola et al. 2013b).

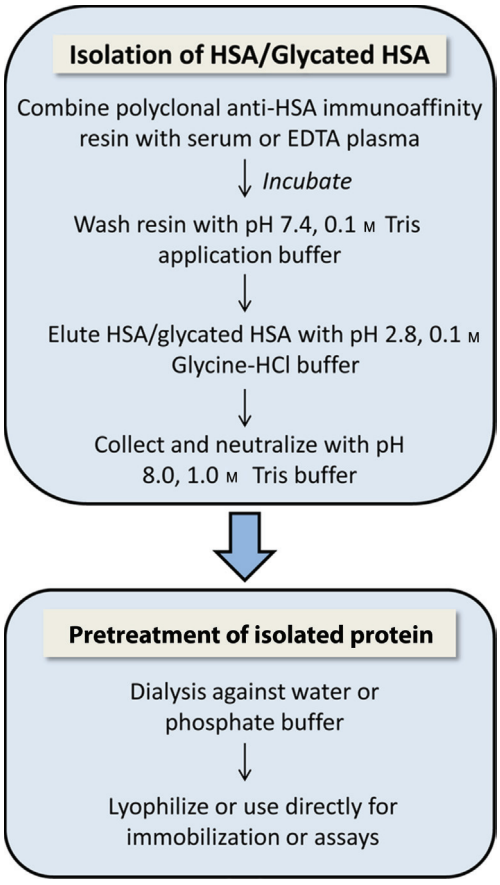

Figure 6 General scheme for the purification and isolation of *in vivo* glycated HSA from the serum or plasma of patients with diabetes (Anguizola et al. 2013b).

A possible disadvantage of covalent immobilization is that it can lead to a loss of activity for the binding agent, resulting from effects such as multisite attachment, improper orientation of the binding agent, or steric effects (Kim and Hage 2006). Another immobilization approach that can overcome many of these effects is entrapment. This method involves the physical entrapment of the binding agent in a support such as HPLC-grade silica (Jackson et al. 2010, 2013). One reported scheme for entrapment is shown in Figure 5B, in which large particles of mildly oxidized glycogen are allowed to covalently attach to a hydrazide-activated support and used to entrap a protein at the surface or within the pores of this support (Jackson et al. 2010). The advantages of this method are that the binding agent remains fully active and in a soluble form once it is entrapped.

Entrapment has been shown with normal HSA to produce an immobilized protein with binding properties that have good agreement with the results expected for the

same protein in solution (Jackson et al. 2010). In a recent study, entrapment has also been used to develop affinity supports that contained glycated HSA for examining drug interactions with this protein (Jackson et al. 2013). This work used a commercially available sample of *in vitro* glycated HSA and a zonal elution format to examine the overall binding by site-selective probes and sulfonylurea drugs to normal HSA and glycated HSA. These columns were found to be stable over at least 60 sample injection or application cycles and 4 months of operation (Jackson et al. 2013).

Frontal analysis studies of drug interactions with glycated HSA

General principles of frontal analysis

Frontal analysis, or frontal affinity chromatography (FAC), is a common technique used in HPAC to examine solute-protein interactions (Schriemer 2004, Hage et al. 2011b). In this technique, a solution of the solute (or target) is continuously applied to a column that contains an immobilized biological agent, allowing the binding sites in the column to eventually become saturated. This process forms a characteristic breakthrough curve as the nonbound target elutes from the column, as shown in Figure 7A. When generated by using HPAC, this type of curve can often be obtained in a matter of minutes and with a variation of only a few percent. In addition, the shape and position of this curve can be used to provide information on the interaction that is taking place between the applied target and the immobilized binding agent (Hage 2002).

The mean position of the breakthrough curve, and the way in which this position changes as the concentration of the target is varied, can be used to simultaneously estimate both the binding capacity of the column for the target and strength with which the target is binding to the column. For instance, if relatively fast association and dissociation kinetics between the target and the binding agent are present on the timescale of the experiment, the mean positions of the breakthrough curves can be used to determine the association equilibrium constants (K_a) and the total moles of binding sites (m_{Ltot}) for the target on the column (Hage 2002, Schiel et al. 2010). If a single-site interaction occurs between the target (or analyte, A) and the binding agent (or ligand, L), the frontal analysis data can be described by using Equations (1) and (2):

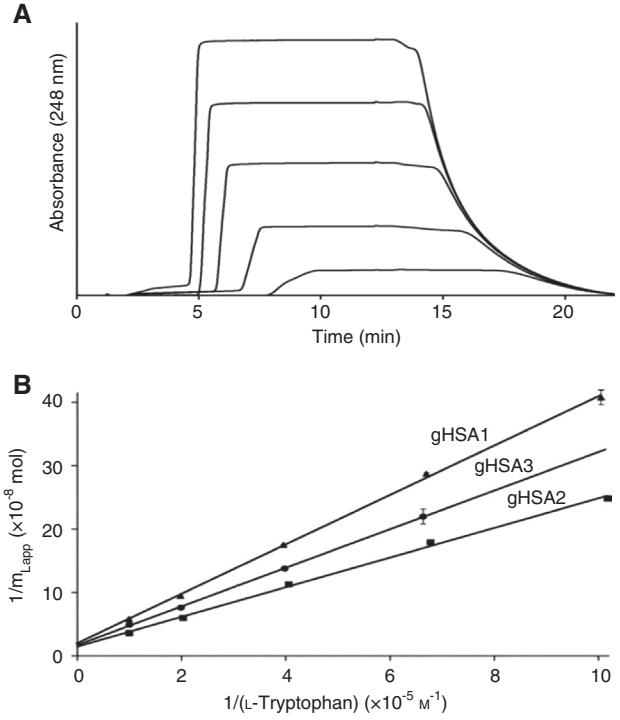

Figure 7 (A) Typical results for a frontal analysis experiment involving the application of acetohexamide to an HPAC column containing normal HSA using a 2.0 cm×2.1 mm i.d. column at 0.5 mL/min and (B) examples of double-reciprocal plots that were generated according to Equation (2) for the binding of L-tryptophan to HPAC columns that contained three different types of *in vitro* glycated HSA.
The concentrations of the acetohexamide in (A) were 10, 7.5, 5, 2.5, and 1 μM (from top to bottom); this plot was obtained from Joseph and Hage (2010b). Copyright 2010 Elsevier and reproduced with permission. The error bars in (B) represent a range of ±1 SD; this figure is from Joseph and Hage (2010a). Copyright 2010 Elsevier and reproduced with permission.

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

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

In these equations, m_{Lapp} represents the moles of applied target that are required to reach the mean point of the breakthrough curve at a given molar concentration of the target or analyte, [A]. Equation (1) describes the nonlinear binding isotherm that is expected for this system, and Equation (2) is a double-reciprocal transform of Equation (1) to produce a linear relationship between $1/m_{Lapp}$ and 1/[A]. An example of this latter type of plot is shown in Figure 7B. The values of K_a and m_{Ltot} can be determined for a single-site system by either employing nonlinear regression with Equation (1) or by using linear regression with Equation (2) (Hage 2002).

In a system that involves two types of binding sites, Equations (1) and (2) can be expanded to the forms that are given in Equations (3) and (4) (Tweed et al. 1997, Hage 2002).

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

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

The association equilibrium constants K_{a1} and K_{a2} in Equation (3) represent the highest- and lowest-affinity sites in the column, respectively. The moles of active binding regions for these two groups of sites are represented by m_{L1} and m_{L2}. In Equation (4), α_1 is the fraction of all binding regions for the target that are represented by the highest-affinity sites, where $\alpha_1=m_{L1}/m_{Ltot}$. In a similar manner, β_2 is the ratio of the association equilibrium constants for the lowest-affinity versus highest-affinity sites, where $\beta_2=K_{a2}/K_{a1}$. For cases involving multisite binding, Equation (4) would be expected to show some deviations from the linear behavior that is predicted by Equation (2) for a single-site system. However, Equation (5) demonstrates that, at low concentrations of the target, or high values of 1/[A], the relationship of $1/m_{Lapp}$ versus 1/[A] does approach a linear form even for a multisite system (Tweed et al. 1997).

$$\lim_{[\mathrm{A}]\to 0}\frac{1}{m_{Lapp}}=\frac{1}{m_{Ltot}(\alpha_1+\beta_2-\alpha_1\beta_2)K_{a1}[\mathrm{A}]}+\frac{\alpha_1+\beta_2^2-\alpha_1\beta_2^2}{m_{Ltot}(\alpha_1+\beta_2-\alpha_1\beta_2)^2} \quad (5)$$

Under these conditions, in which [A] approaches zero, the slope and intercept of the linear region in a plot made according to Equation (4) or (5) can be used to provide a reasonable estimate for the association equilibrium constant of the highest-affinity site in the multisite system (Tweed et al. 1997, Tong and Hage 2011).

Frontal analysis studies of site-specific probes with normal or glycated HSA

In the analysis of glycated HSA, frontal analysis has been used to characterize the binding by common site-specific probes for Sudlow sites I and II (Joseph and Hage 2010a). These studies were conducted on *in vitro* glycated HSA samples with various levels of glycation that might be found in pre-diabetes or diabetes. Racemic warfarin was employed as a site-specific probe for Sudlow site I and gave a good fit with a single-site binding model for each of the tested samples of glycated HSA. As shown in Table 1, the association equilibrium constants that were measured for these samples ranged from 2.3×10^5 to 2.7×10^5 M^{-1} at pH 7.4 and 37°C, which were similar to an average literature value of $2.4\pm0.4\times10^5$ M^{-1} for warfarin and normal HSA. It was also found that the specific activities for warfarin with these glycated HSA samples showed no significant variations versus normal HSA at the 95% confidence level. These data indicated that the extent of *in vitro* glycation did not have any appreciable effect on the strength of this interaction or on the number of binding sites of glycated HSA for warfarin under the conditions used in this study. In addition, these results showed that warfarin could be used as a site-specific probe for Sudlow site I not only on normal HSA but also on these samples of glycated HSA (Joseph and Hage 2010a).

Table 1 Association equilibrium constants (K_a) for warfarin and L-tryptophan with normal HSA and various samples of *in vitro* glycated HSA.[a]

Drug or solute	Type of HSA and value of K_a ($\times10^5$)			
	Normal HSA[b]	gHSA1	gHSA2	gHSA3
Warfarin	2.4±0.4	2.3±0.2	2.3±0.2	2.7±0.3
L-tryptophan	0.11±0.03	0.52±0.09	0.64±0.17	0.57±0.05

[a]Data for glycated HSA are based on results from Joseph and Hage (2010a). These results were measured at 37°C in pH 7.4, 0.067 M potassium phosphate buffer. The values of K_a have units of M^{-1}, and the numbers in parentheses represent a range of ±1 SD. The levels of glycation for the HSA samples were as follows: gHSA1, 1.31±0.05; gHSA2, 2.34±0.13; and gHSA3, 3.35±0.14 mol hexose/mol HSA.
[b]K_a values for racemic warfarin and L-tryptophan with normal HSA are based on data from Loun and Hage (1994) and Yang and Hage (1993), respectively.

Similar frontal analysis studies have been conducted with L-tryptophan, which is a solute known to interact with Sudlow site II of HSA (Joseph and Hage 2010a). The results that were obtained with the same *in vitro* glycated HSA samples and columns that were used to examine racemic warfarin again gave good agreement with a single-site binding model for L-tryptophan. The association equilibrium constants that were measured for L-tryptophan with these samples (Table 1) ranged from 5.2×10^4 to 6.4×10^4 M^{-1} at pH 7.4 and 37°C. These values were approximately 4.7–5.8 times higher than the reported association equilibrium constant for normal HSA with L-tryptophan (Yang and Hage 1993). Thus, glycation did affect the interaction between L-tryptophan and HSA in this study. Although there was an observed increase in the affinity

of L-tryptophan for HSA as a result of glycation, the specific activity in the glycated HSA samples was comparable to that seen for normal HSA. It was concluded from these data that L-tryptophan could still be used as a site-specific probe for Sudlow site II of glycated HSA, but the change in the affinity of this interaction due to glycation did have to be considered when using this probe in binding studies (Joseph and Hage 2010a).

Frontal analysis studies of sulfonylurea drugs with normal or glycated HSA

Frontal analysis was next used to characterize the overall binding of various first- and second-generation sulfonylurea drugs with normal HSA (Joseph and Hage 2010b; Matsuda et al. 2011, 2012). The results from these experiments were analyzed according to a number of binding models, such as those represented in Equations (1)–(4) (Figure 8). A comparison of these fits, their correlation coefficients, and the corresponding residual plots indicated that each of the tested sulfonylurea drugs followed a two-site model in their binding with normal HSA. Table 2 summarizes the association equilibrium constants and moles of binding sites that were determined for the various sulfonylurea drugs with normal HSA when using a two-site model. These sites were composed of approximately one or two high-affinity regions and a larger number of weaker- or moderate-affinity sites. The K_a values for the highest-affinity sites were in the range of 10^5–10^6 M^{-1}, while the weak-to-moderate affinity sites had K_a values in the range of 10^3–10^4 M^{-1}. Glibenclamide had the highest value for K_{a1} followed by acetohexamide, tolbutamide, and gliclazide (Joseph and Hage 2010b; Matsuda et al. 2011, 2012).

Similar frontal analysis experiments were conducted with these sulfonylurea drugs and using several preparations of *in vitro* glycated HSA (Joseph et al. 2010, 2011, Matsuda et al. 2011, 2012). The sulfonylurea drugs were again found to interact with the glycated HSA through a two-site model. The corresponding association equilibrium constants and moles of active binding sites that were determined by HPAC are provided in Table 2 for one of the glycated HSA samples. The high-affinity regions in the two-site model for acetohexamide, tolbutamide, and gliclazide were composed of one or two binding sites (Joseph et al. 2010, 2011, Matsuda et al. 2011), while glibenclamide had one high-affinity site and several weaker- or

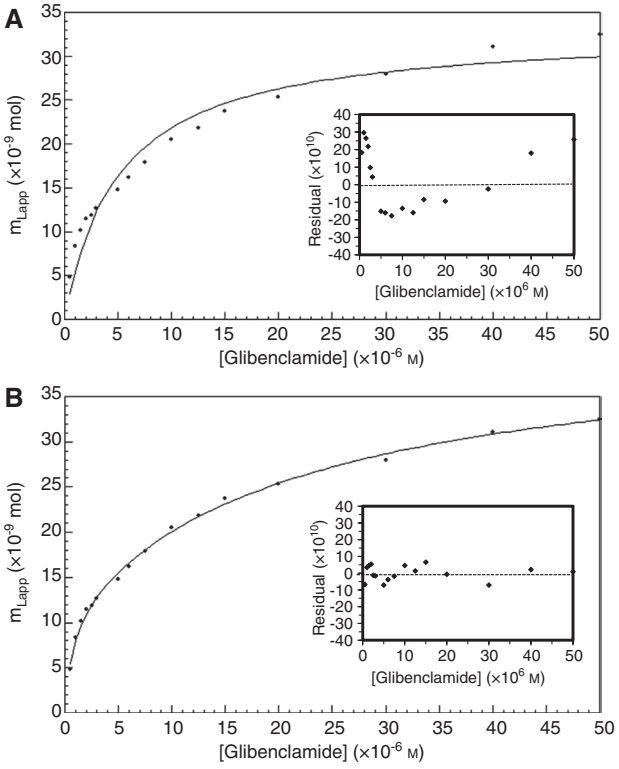

Figure 8 Analysis of data obtained for the frontal analysis of glibenclamide on an HPAC column containing normal HSA when plotted according to (A) a single-site binding model based on Equation (1) or (B) a two-site binding model based on Equation (3).
The insets show the corresponding residual plots, where each point represents the average of four experiments (Matsuda et al. 2012).

Table 2 Association equilibrium constants (K_a) and binding capacities (m_L) for various sulfonylurea drugs with normal HSA or *in vitro* glycated HSA when using a two-site binding model.[a]

Type of HSA and drug	K_{a1} (×10^5)	m_{L1} (×10^{-8})	K_{a2} (×10^3)	m_{L2} (×10^{-8})
Normal HSA				
Acetohexamide	1.3±0.2	2.4±0.1	0.4±0.1	9.3±5.5
Tolbutamide	0.9±0.1	2.0±0.1	8.1±1.7	1.8±0.1
Gliclazide	0.7±0.2	0.7±0.2	8.9±0.2	2.7±0.1
Glibenclamide	14±5	1.1±0.2	44±10	3.1±0.1
Glycated HSA[b]				
Acetohexamide	2.0±0.3	1.5±0.1	4.1±0.7	3.0±0.1
Tolbutamide	0.9±0.2	1.9±0.1	7.8±5.1	1.9±0.2
Gliclazide	1.0±0.1	0.6±0.4	5.7±3.9	2.6±0.4
Glibenclamide	19±15	0.9±0.3	72±28	2.4±0.2

[a]Based on data from Joseph and Hage (2010b), Joseph et al. (2010, 2011), and Matsuda et al. (2011, 2012). The results for each drug were measured at 37°C in pH 7.4, 0.067 M potassium phosphate buffer. The values of K_a have units of M^{-1}, and m_L has units of mol. The numbers in parentheses represent a range of ±1 SD based on error propagation and the precisions of the best-fit slopes and intercepts when using Equation (3) for n=9–16.
[b]The level of modification for the glycated HSA sample was 3.35±0.14 mol hexose/mol HSA (Joseph and Hage 2010a).

moderate-strength binding sites (Matsuda et al. 2012). The ranges of K_a values for the high and weak-to-moderate affinity sites were similar to those found for normal HSA. However, some variation in these values versus normal HSA were noted for at least some of the drugs and samples of glycated HSA (Joseph et al. 2010, 2011, Matsuda et al. 2011, 2012).

Frontal analysis was further used to examine the binding by sulfonylurea drugs with *in vivo* glycated HSA from diabetic patients (Anguizola et al. 2013b). These experiments confirmed that the sulfonylurea drugs interacted with glycated HSA through a two-site model. Analysis of the relative activities for these sites gave similar results to those noted for *in vitro* glycated HSA. The interactions of these samples with acetohexamide, tolbutamide, and gliclazide had a set of high-affinity sites that were composed of one or two binding regions and a set of lower-affinity sites that were made up of a larger number of weaker binding regions. The association equilibrium constants for these sites showed good agreement with previous results that had been obtained with *in vitro* glycated HSA having similar or slightly lower levels of modification (Anguizola et al. 2013b).

Zonal elution studies of the overall binding of drugs to glycated HSA

General principles of zonal elution

Zonal elution is another technique that can be utilized in HPAC to examine solute-protein interactions (Hage 2002). In a typical zonal elution experiment, a small amount of a target is injected onto a column containing the binding agent or protein of interest. The retention factor (k) of the target, as defined in Equation (6), is then determined and employed as a measure of the target's interaction with the immobilized binding agent.

$$k=\frac{t_R-t_M}{t_M}=\frac{V_R-V_M}{V_M} \tag{6}$$

In this equation, t_R and V_R are the retention time or retention volume for the target, respectively, and t_M or V_M are the void time or void volume for a nonretained solute. Equations (7) and (8) show that it is possible to relate the retention factor directly to the overall strength of interaction between the target and binding agent as well as to the moles of binding sites for the target in the column (Schiel et al. 2010).

$$k=\frac{(nK'_a m_{Ltot})}{V_M} \tag{7}$$

$$nK'_a=\sum(n_i K_{ai}) \tag{8}$$

In Equations (7) and (8), the global affinity constant for the target with the immobilized binding agent is represented by the term nK'_a, and m_{Ltot} is the total moles of binding sites for the target in the column. In this situation, the retention factor is a measure of the overall strength with which the injected target is binding to all of the available sites on the immobilized binding agent.

Equation (8) indicates that the global affinity constant can be written as the weighted sum of the individual association equilibrium constants for each type of binding site for the target with the immobilized binding agent (Schiel et al. 2010). The moles of binding site i per mole of binding agent are represented in Equation (8) by n_i, and the association equilibrium constant for this site is given by K_{ai}. In the special case where only one type of binding site is present for the target, Equation (7) can be simplified to produce Equation (9) (Schiel et al. 2010, Jackson et al. 2013).

$$k=\frac{(K_a m_{Ltot})}{V_M} \tag{9}$$

In this specific case, the global affinity constant is now equal to K_a, because n is equal to 1. It is under these conditions that the retention factor can be used as a direct measure of the affinity of the injected solute at a single type of site on the immobilized binding agent.

Zonal elution studies of sulfonylurea drugs using normal or glycated HSA

One study used zonal elution and retention measurements to examine the combined effects that glycation and fatty acids can have on the interactions between sulfonylurea drugs and HSA (Basiaga and Hage 2010). The effects of fatty acids, such as those shown in Figure 9, were of interest because diabetes can lead to changes in fatty acid concentrations in the serum. In addition, HSA can bind many of these fatty acids, and this type of interaction can result in either direct or allosteric competition in the binding of drugs to this protein (Noctor et al. 1992, Anguizola et al. 2013c). This study first measured the retention of acetohexamide, tolbutamide, gliclazide, and glibenclamide on columns containing covalently immobilized normal

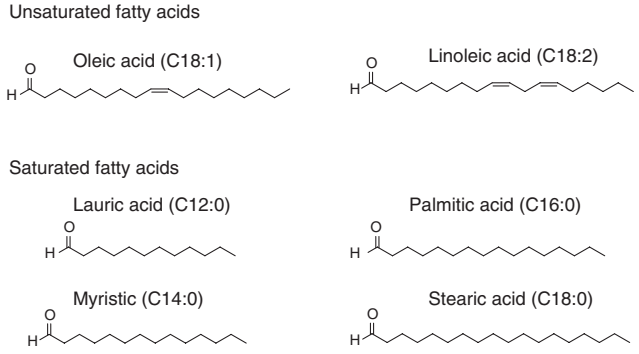

Figure 9 Examples of fatty acids that have been examined by HPAC for their effects on the binding of sulfonylurea drugs with normal HSA or glycated HSA.

HSA or *in vitro* glycated HSA. The HPAC columns containing glycated HSA had a 2.7- to 3.6-fold increase in retention versus the columns that contained normal HSA, as demonstrated in Figure 10. However, a decrease in retention was seen for these drugs as the concentration of fatty acids was increased in the mobile phase. Similar trends were observed for the sulfonylurea drugs in the presence of the same fatty acids and glycated HSA (Basiaga and Hage 2010).

Another report used zonal elution and HPAC to investigate the binding of various sulfonylurea drugs and other solutes with entrapped samples of normal HSA or *in vitro* glycated HSA (Jackson et al. 2013). With this approach, it was possible to obtain retention data in 3 min or less and with precisions of only a few percent. Because the sulfonylurea drugs were previously found to interact

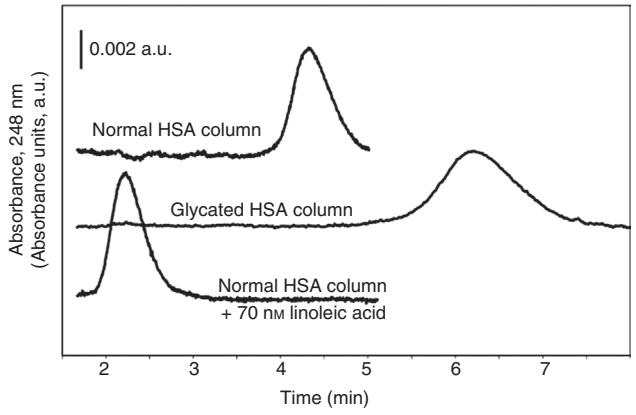

Figure 10 Zonal elution experiments examining the retention of acetohexamide on 2.0 cm×2.1 mm i.d. HPAC columns containing normal HSA or glycated HSA at 0.5 mL/min and in the presence of pH 7.4 buffer or pH 7.4 buffer containing 70 nM linoleic acid (Basiaga and Hage 2010).

Table 3 Specific retention factors and global affinity constants for sulfonylurea drugs on columns containing normal HSA or *in vitro* glycated HSA.[a]

Type of HSA and drug	Retention factor, k	Global affinity constant, nK'_a (M^{-1})
Normal HAS		
Acetohexamide	46.5±0.1	$1.6\pm0.1\times10^5$
Gliclazide	17.1±0.2	$5.8\pm0.2\times10^4$
Tolbutamide	30.8±0.1	$1.0\pm0.1\times10^5$
Glycated HSA[b]		
Acetohexamide	62.7±0.1	$2.5\pm0.1\times10^5$
Gliclazide	23.4±0.1	$9.1\pm0.3\times10^4$
Tolbutamide	47.0±0.2	$1.9\pm0.1\times10^5$

[a]The information in this table was obtained from Jackson et al. (2013). The results for each drug were measured at 37°C in pH 7.4, 0.067 M potassium phosphate buffer. The value of each retention factor in this table was obtained by taking the difference between the overall retention factor on an entrapped protein column and the retention factor due to nonspecific interactions for the same solute on a control column. The values for nK'_a were estimated by using Equation (7). The numbers in parentheses represent a range of ±1 SD.
[b]The level of modification for the glycated HSA sample was 1.8 mol hexose/mol HSA.

with HSA through a two-site model, Equations (7) and (8) were employed to estimate the global affinity constants for these drugs from their measured retention factors (Table 3). These values were comparable to those estimated from frontal analysis and zonal elution studies utilizing similar preparations of covalently immobilized normal HSA or glycated HSA (Joseph and Hage 2010a,b, Joseph et al. 2011, Matsuda et al. 2011, 2012). Similar experiments were conducted by using Equation (9) and injections of *R*-warfarin and L-tryptophan to estimate the binding constants for these drugs or solutes at Sudlow sites I and II of normal HSA or glycated HSA. The results also showed good agreement with values that had been determined by methods such as frontal analysis (Jackson et al. 2013).

Competition studies examining drug interactions with glycated HSA

General principles of competition studies based on zonal elution

The most popular use of zonal elution and HPAC for characterizing biological interactions is a competition study. This type of study can be used to identify the sites that are

taking part in an interaction of a drug with a protein and can provide data on the local equilibrium constant that describes binding at this specific site (Hage et al. 2011b). In a typical competition experiment (Figure 11A), a small amount of a probe compound is injected in the presence of a known concentration of a competing agent in the mobile phase. If direct or indirect competition occurs between these compounds, there will be a shift in the observed retention for the probe as the concentration of the competing agent is varied. If the probe has a known interaction site with the immobilized binding agent, this same experiment can be used to determine whether the competing agent also has some interactions at this site (Hage 2002).

It is possible to further determine the nature of the interaction between the probe and competing agent by fitting the measured retention factors for the probe to a given binding model. For instance, if direct competition at a single site is occurring between the competing agent and the probe, a linear relationship should be present between $1/k$ for the probe and the concentration of the competing agent, [I], as predicted by Equation (10).

$$\frac{1}{k}=\frac{K_{aI}V_M[\mathrm{I}]}{K_{aA}m_L}+\frac{V_M}{K_{aA}m_L} \tag{10}$$

In this equation, K_{aA} and K_{aI} are the association equilibrium constants for the probe and the competing agent, respectively, at their site of competition, and all the other terms are as defined previously. An example of a plot that has been made according to Equation (10) is provided in Figure 11B. The value of K_{aI} in this type of linear relationship can be calculated by using the ratio of the slope and the intercept. The deviations from linearity in such a plot can occur if allosteric or multisite interactions are present. Thus, a plot made according to Equation (10) can be used to determine if direct competition at a single type of site is present between the probe and competing agent or if more complex interactions are present between these solutes. In this latter situation, alternative models and equations can be employed to describe such interactions (Hage 2002, Chen and Hage 2004, Schiel et al. 2010).

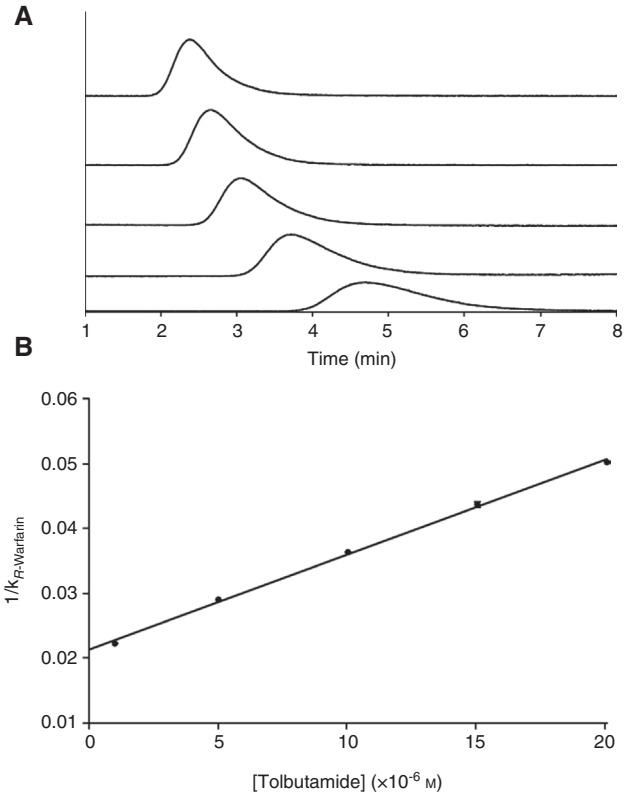

Figure 11 Examples of (A) a competition zonal elution study for injections of *R*-warfarin in the presence of tolbutamide in the mobile phase on an HPAC column containing glycated HSA and (B) the fit of the resulting shift in retention to Equation (10).
The results in (A) are for tolbutamide concentrations of 20, 15, 10, 5, and 1 μM (from top to bottom) using a 2.0 cm×2.1 mm i.d. column at 0.5 mL/min (Joseph et al. 2011). Copyright 2011 Elsevier and reproduced with permission.

Competition studies for sulfonylurea drugs on normal and glycated HSA

Zonal elution and competition studies have been used in several reports to profile the site-specific interactions that occur between sulfonylurea drugs and normal HSA or *in vitro* glycated HSA with various levels of modification (Joseph and Hage 2010b; Joseph et al. 2010, 2011, Matsuda et al. 2011, 2012). It was found that all of the sulfonylurea drugs that were examined had strong- or moderate-strength interactions at both Sudlow sites I and II when using *R*-warfarin and L-tryptophan as probes for these sites. A direct competition of the drugs with these probes was indicated by the linear relationships that were obtained for plots made according to Equation (10). Table 4 summarizes the association equilibrium constants that were measured for these drugs with normal HSA (Joseph and Hage 2010b; Matsuda et al. 2011, 2012). A summary of how the affinity at Sudlow site I or II changed for these drugs as the level of glycation for HSA was varied is provided in Table 5 (Joseph et al. 2010, 2011, Matsuda et al. 2011, 2012).

The results in Table 5 demonstrate that the level of *in vitro* glycation did have an effect on binding by these sulfonylurea drugs at Sudlow sites I and II. This change in affinity ranged from 0.6- to 6-fold when the binding by these drugs with *in vitro* glycated HSA was compared to the binding seen with normal HSA (Anguizola et al.

Table 4 Association equilibrium constants (K_a) for various sulfonylurea drugs at specific binding regions on normal HSA.[a]

Drug	Sudlow site I	Sudlow site II	Digitoxin site
	K_a ($\times 10^4$ M^{-1})	K_a ($\times 10^4$ M^{-1})	K_a ($\times 10^6$ M^{-1})
Acetohexamide	2.4±0.3	4.7±0.3	–
Tolbutamide	4.2±0.4	1.3±0.1	–
Gliclazide	1.9±0.1	6.0±0.5	–
Glibenclamide	2.4±0.3	3.9±0.2	2.1±0.8

[a]The results in this table were obtained from Joseph and Hage (2010b) and Matsuda et al. (2011, 2012). These values were measured at 37°C in pH 7.4, 0.067 M potassium phosphate buffer. The numbers in parentheses represent a range of ±1 SD.

2013a). The extent of this change varied from one drug to the next and between these interaction sites. The differences in affinity between these sites and drugs with a change in the level of glycation have been proposed to be due to variations in the extent and types of modifications that have been observed to occur at Sudlow sites I and II during the glycation process (Barnaby et al. 2011a,b; Anguizola et al. 2013a).

In the works with glibenclamide, competition studies with digitoxin as an alternative probe were also carried out to help locate the highest-affinity sites for this drug on HSA (Matsuda et al. 2012). Linear plots made according to Equation (10) revealed that the digitoxin site of HSA was heavily involved in the interactions of glibenclamide with both normal HSA and *in vitro* glycated HSA. This observed behavior was reasonable for glibenclamide because the digitoxin site is known to bind other large hydrophobic drugs (Brock 1975, Sjoholm et al. 1979, Brors et al. 1993, Hage and Sengupta 1999, Chen et al. 2004, Ohnmacht et al. 2006). The association equilibrium constants that were determined for glibenclamide at this site on normal HSA are included in Table 4. The binding of glibenclamide at this site may have had an overall decrease in affinity of up to 1.9-fold as the level of glycation was increased to values seen in patients with advanced diabetes. However, the change in affinity at this site was not statistically significant for any individual sample of glycated HSA (Matsuda et al. 2012).

Additional competition studies have been conducted using *in vivo* glycated HSA (Anguizola et al. 2013b). When *R*-warfarin and L-tryptophan were employed as probes, the drugs acetohexamide, tolbutamide, or gliclazide were again observed to have direct competition and binding at both Sudlow sites I and II. Table 6 shows how the affinities

Table 5 Changes in the binding of site-selective probes and various sulfonylurea drugs at Sudlow sites I and II when comparing *in vitro* glycated HSA with normal HSA.[a]

	Relative change in association equilibrium constant (↑ or ↓)		
Drug and binding site	**gHSA1**	**gHSA2**	**gHSA3**
Sudlow site I			
Acetohexamide	↑ 1.4-fold	NS (↓ 10%)[b]	NS (<5%)
Tobutamide	↑ 1.3-fold	↑ 1.2-fold	↑ 1.2-fold
Gliclazide	NS (<10%)	↑ 1.9-fold	NS (↑ 11%)
Glibenclamide[c]	NS (<5%)	↑ 1.7-fold	↑ 1.9-fold
Sudlow site II			
Acetohexamide	↓ 0.6-fold	↓ 0.8-fold	NS (<10%)
Tobutamide	↑ 1.1-fold	↑ 1.4-fold	↑ 1.2-fold
Gliclazide	↓ 0.8-fold	↑ 1.3-fold	↓ 0.6-fold
Glibenclamide[c]	↑ 4.3-fold	↑ 6.0-fold	↑ 4.6-fold

[a]Adapted and reproduced with permission from Anguizola et al. (2013c). The results for each drug were obtained using the same preparations of in vitro glycated HSA and were measured at 37°C in pH 7.4, 0.067 M potassium phosphate buffer. The levels of glycation for the in vitro glycated HSA samples were as follows: gHSA1, 1.31±0.05; gHSA2, 2.34±0.13; and gHSA3, 3.35±0.14 mol hexose/mol HSA. All of these values are being compared to the association equilibrium constants that were measured for normal HSA, as listed in Table 4. The term NS stands for "not significant" and indicates that the association equilibrium constant was not significantly different from the value for normal HSA at the 95% confidence level.
[b]This association equilibrium constant was significantly different at the 90% confidence level from the reference value for normal HSA but was not significantly different at the 95% confidence level.
[c]Glibenclamide also binds to the digitoxin site of HSA, which may have displayed a slight decrease in binding in going from normal HSA to the samples of in vitro glycated HSA.

Table 6 Changes in the binding of site-selective probes and various sulfonylurea drugs at Sudlow sites I and II when comparing *in vivo* glycated HSA with normal HAS.

	Relative change in association equilibrium constant (↑ or ↓)[a]	
Drug and binding site	**Clinical sample 1**	**Clinical sample 2**
Sudlow site I		
Acetohexamide	↑ 1.1-fold	NS (<5%)
Tobutamide	↑ 1.2-fold	↑ 1.4-fold
Gliclazide	↑ 1.2-fold	↓ 0.7-fold
Sudlow site II		
Acetohexamide	NS (<5%)	↑ 1.3-fold
Tobutamide	↑ 1.4-fold	↑ 1.5-fold
Gliclazide	↑ 1.8-fold	↑ 1.5-fold

[a]Based on the data obtained from Anguizola et al. (2013b). The results for each drug were obtained using the same preparations of in vivo glycated HSA and were measured at 37°C in pH 7.4, 0.067 M potassium phosphate buffer. The levels of glycation for the in vivo glycated HSA samples were as follows: Clinical sample 1, 1.19±0.15 mol hexose/mol HSA; Clinical sample 2, 1.51±0.20 mol hexose/mol HSA. The results for these samples were compared to the association equilibrium constants measured for normal HSA, as provided in Table 4. The term NS stands for "not significant" and indicates that the association equilibrium constant was not significantly different from the value for normal HSA at the 95% confidence level.

of these drugs changed for *in vivo* glycated HSA versus normal HSA at these sites. This change was as large as 1.8-fold in some cases. The observed trends were similar to those seen for *in vitro* glycated HSA with comparable levels of modification, with the variations in affinity at Sudlow sites I and II being dependent on both the type of sulfonylurea drug and the extent of glycation for HSA (Anguizola et al. 2013b).

Another report used zonal elution and competition studies to provide more details on the effects of glycation and fatty acids on the interactions of sulfonylurea drugs with normal HSA and *in vitro* glycated HSA (Anguizola et al. 2013c). Fatty acids at low concentrations were used as competing agents to examine the highest-affinity sites for these solutes, while the sulfonylurea drugs were used as the injected probes. The resulting shifts in retention were analyzed by using Equation (10) to see if any of these changes could be described by direct competition between the sulfonylurea drugs and fatty acids. It was found through this approach that some sulfonylurea drugs showed direct competition with the fatty acids, while others had a mixed-mode interaction. There were some situations in which a change was seen in the type of interaction between glycated HSA and normal HSA, such as what was observed for the sulfonylurea drugs in the presence of linoleic acid. This result indicated that glycation could affect the types of interactions that occurred between these fatty acids and sulfonylureas on HSA. Based on a fit to Equation (10) and a direct competition model, myristic acid and stearic acid showed an increase in affinity in going from normal HSA to glycated HSA, which indicated that glycation could also affect the association equilibrium constants for some fatty acids with this protein (Anguizola et al. 2013c).

Summary

This review examined several recent reports that have used HPAC to examine the binding by sulfonylurea drugs and other solutes with normal HSA and glycated HSA. Various ways of preparing columns for this type of work were described. These methods included covalent immobilization techniques such as the Schiff base method as well as an approach based on noncovalent entrapment. A number of approaches for using these columns in binding studies were then considered. One such method was frontal analysis, which has been used to determine both the equilibrium constants and the amount of binding sites that are present for sulfonylurea drugs and other solutes on normal and glycated HSA. In addition, zonal elution methods were discussed. This set of techniques has been employed to compare the global affinities of sulfonylurea drugs with glycated HSA and normal HSA and to examine the effects of fatty acids on these interactions. The use of zonal elution in competition studies to investigate the site-specific interactions of sulfonylurea drugs with normal or glycated HSA was also discussed.

Several important pieces of information have already been obtained through the use of HPAC with normal HSA and glycated HSA. For example, it has been shown that warfarin and L-tryptophan can be used as probes for Sudlow sites I and II on HSA that has been glycated under conditions similar to those found in diabetes. It has been further shown that warfarin does not have any appreciable change in affinity when comparing normal HSA with these samples of glycated HSA, but that L-tryptophan does have an appreciable increase in the binding strength for the glycated HSA. A more complete model of the binding of sulfonylurea drugs with HSA and glycated HSA has also been developed through this work. In this model, all of the sulfonylurea drugs that have been examined have a set of high-affinity sites and a group of lower-affinity interactions. These drugs each bind to both Sudlow sites I and II, with some drugs in this class (i.e., glibenclamide) also binding to the digitoxin site. It has been further demonstrated that the strength of these interactions can be altered by glycation, with this change being dependent on the type of drug and level of modification. Finally, some of these changes in affinity can be quite large, ranging from 0.6- to 6.0-fold. This last item is of clinical importance in that it could affect the nonbound fraction and effective dose of a drug in the circulation. This change, in turn, could lead to conditions such as hypoglycemia or hyperglycemia if the effective dose of a sulfonylurea drug varies significantly from the level expected in the presence of only normal HSA (Matsuda et al. 2012, Anguizola et al. 2013a).

There are various properties of HPAC that make it attractive for these studies. For instance, most of the HPAC methods described in this review can be carried out in a matter of minutes. In addition, the columns used in this work can often be used in hundreds of experiments, resulting in good precision and allowing the same protein preparation to be used in examining the effects of many drugs. This last feature, especially when combined with the small columns that were used in these HPAC studies, reduces the amount of binding agent that is needed for a drug-protein binding study and makes it possible to work with proteins that have been modified under various conditions or that have been isolated for individual patients. Both of these last two situations were illustrated in this review through HPAC studies that were conducted using either *in vitro* or *in vivo* glycated HSA (Joseph et al. 2010, Anguizola et al. 2013b).

Although this review examined interactions that mainly involved sulfonylurea drugs and normal or glycated HSA, the same approaches and types of experiments could be adapted for use with other drugs and modified proteins. These methods could also be employed with alternative systems that involve heterogeneous interactions. The range of methods that are available for HPAC, the advantages of this technique, and the large amount of information that can be obtained through HPAC should continue to make this technique a powerful tool for characterizing drug-protein interactions or other systems of clinical or pharmaceutical interest. These same features make this method of potential interest for future work in personalized medicine, by allowing data to be obtained on drug interactions with proteins that have been obtained from individual patients (Anguizola et al. 2013b).

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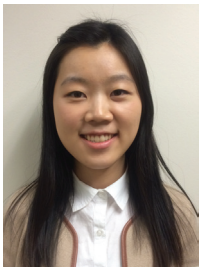

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