Spring 2019

ANALYSIS OF DRUG-PROTEIN INTERACTIONS DURING DIABETES BY HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

Pingyang Tao
University of Nebraska - Lincoln, pingyang.tao@huskers.unl.edu

Follow this and additional works at: https://digitalcommons.unl.edu/chemistrydiss

Part of the Analytical Chemistry Commons, and the Medicinal-Pharmaceutical Chemistry Commons


This Article is brought to you for free and open access by the Chemistry, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Student Research Projects, Dissertations, and Theses - Chemistry Department by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
ANALYSIS OF DRUG-PROTEIN INTERACTIONS DURING DIABETES BY HIGH-
PERFORMANCE AFFINITY CHROMATOGRAPHY

By
Pingyang Tao

A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science
Major: Chemistry

Under the Supervision of Professor David S. Hage

Lincoln, Nebraska

May, 2019
ANALYSIS OF DRUG-PROTEIN INTERACTIONS DURING DIABETES BY HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

Pingyang Tao, M.S.

University of Nebraska, 2019

Advisor: David S. Hage

High-performance affinity chromatography (HPAC) is a type of liquid chromatography in which solutes are separated based on their binding to a stationary phase that is a biologically-related agent. Sulfonylurea drugs have significant binding to proteins in blood, with most of this binding is believed to occur with human serum albumin (HSA). HSA is known to be modified as a result of the high serum levels of glucose that is present during diabetes, which may also alter the function of HSA as a binding agent for many drugs in circulation. Some modifications of HSA that can occur during diabetes involves early stage glycation and advanced glycation. Drugs that may be affected by these changes include various sulfonylurea drugs, as are commonly used to treat type II diabetes. This thesis describes the development of tools and techniques based on HPAC to examine the effects of glycation on the binding of HSA. A major portion of this research describes the use of HPAC to examine the effect of early stage glycation on the binding of two first-generation sulfonylurea drugs. Frontal analysis and zonal elution studies were used to provide information on association equilibrium constants and the number of binding sites for these interactions. Significant levels of both increases and decreases in affinity were observed for these drugs at the levels of glycation that were examined. A second portion of this research describes the utilization of HPAC to investigate the effect of advance glycation on the binding of two first-generation
sulfonylurea drugs and one second-generation sulfonylurea drug. Two major advanced
glycation end-products (AGEs) of HSA, glyoxal- and methylglyoxal-modified HSA,
were studied in this work. Immunoextraction and zonal elution were used to estimate the
global affinity of these drugs with normal or in vitro modified HSA, while competition
studies based on zonal elution were used to measure the binding strengths of these drugs
at specific sites on these preparations of HSA.
ACKNOWLEDGEMENTS

I would first like to thank my advisor Prof. David Hage for his continuous support of my M.S. studies and research, as well as for his patience, motivation and immense knowledge. The door to Prof. Hage office was always open whenever I ran into a trouble spot or had a question about my research or writing. I would also like to acknowledge Prof. Robert Powers and Prof. Eric Dodds as the readers of this thesis, and I am gratefully indebted for their very valuable comments.

I would also like to thank the rest of my supervisory committee: Prof. Liangcheng Du and Prof. Randy Wehling. I thank them for their insightful comments and encouragement. I would also like to thank Prof. Stephen Morin, who accepted me into his laboratory for my first rotation when I came to Nebraska. He gave me a lot of help and encouragement during the earlier period of my graduate studies. I also thank my fellow lab mates for all the fun we have had over the last three years.

Finally, I must express my very profound gratitude to my parents and to my wife for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.
# TABLE OF CONTENTS

## CHAPTER 1

**OVERVIEW OF THESIS**

1.1 Overview of thesis........................................................................................................1

## CHAPTER 2

**HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY: APPLICATIONS IN DRUG-PROTEIN BINDING STUDIES**

2.1 Introduction..................................................................................................................3

2.2 Zonal elution studies ..................................................................................................5

2.3 Frontal analysis studies ..............................................................................................8

2.4 References..................................................................................................................19

## CHAPTER 3

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

3.1 Introduction................................................................................................................26

3.2 Experimental.............................................................................................................29

3.2.1 Reagents...............................................................................................................29

3.2.2 Instrumentation......................................................................................................29

3.2.3 Glycation and modification of HSA........................................................................30

3.2.4 Column preparation..............................................................................................30

3.2.5 Chromatographic studies.....................................................................................31

3.3 Results and discussion..............................................................................................33

3.3.1 Overall binding of chlorpropamide with HSA.....................................................33
3.3.2 Site-selective binding of chlorpropamide with HSA .........................41

3.4 Conclusion .............................................................................................................47

3.5 References .............................................................................................................64

CHAPTER 4

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

4.1 Introduction ............................................................................................................71

4.2 Experimental .........................................................................................................72
  4.2.1 Reagents ...........................................................................................................72
  4.2.2 Instrumentation ................................................................................................72
  4.2.3 Glycation and modification of HSA ................................................................72
  4.2.4 Column preparation ..........................................................................................72
  4.2.5 Chromatographic studies ................................................................................73

4.3 Results and discussion ........................................................................................74
  4.3.1 Overall binding of tolazamide with HSA .......................................................74
  4.3.2 Site-selective binding of tolazamide with HSA .............................................79

4.4 Conclusion .............................................................................................................83

4.5 References .............................................................................................................98

CHAPTER 5

CHROMATOGRAPHIC CHARACTERIZATION OF INTERACTIONS BY FIRST- AND SECOND-SULFONYLUREA DRUGS WITH GLYOXAL-MODIFIED ALBUMIN MICROCOLUMNS
CHAPTER 6

CHROMATOGRAPHIC CHARACTERIZATION OF INTERACTIONS BY FIRST- AND SECOND-SULFONYLUREA DRUGS WITH METHYLGLYOXAL-MODIFIED ALBUMIN MICROCOLUMNS

6.1 Introduction.......................................................................................130

6.2 Experimental....................................................................................131

6.2.1 Reagents..........................................................................................131

6.2.2 Instrumentation.................................................................................132

6.2.3 Modification of HSA.........................................................................132
6.2.4 Column preparation........................................................................132
6.2.5 Chromatographic studies.................................................................133
6.3 Results and discussion........................................................................136
  6.3.1 Characterization of MGo-modified HSA and related supports ........136
  6.3.2 Estimation of global binding for sulfonylureas with MGo-modified
       HSA........................................................................................................136
  6.3.3 Site-selective binding of sulfonylureas with HSA.........................137
6.4 Conclusion............................................................................................141
6.5 References............................................................................................151

CHAPTER 7

SUMMARY AND FUTURE WORK

7.1 Summary of work..................................................................................156
7.2 Future studies........................................................................................158
7.3 References............................................................................................159
CHAPTER 1

OVERVIEW OF THESIS

1.1 Overview of thesis

High-performance affinity chromatography (HPAC) is a type of liquid chromatography in which solutes are separated based on their binding to a stationary phase that is a biologically-related agent. Sulfonylurea drugs have significant binding to proteins in blood, with most of this binding is believed to occur with human serum albumin (HSA). HSA is known to be modified as a result of the high serum levels of glucose that is present during diabetes, which may also alter the function of HSA as a binding agent for many drugs in circulation. Some modifications of HSA that can occur during diabetes involves early stage glycation and advanced glycation. Drugs that may be affected by these changes include various sulfonylurea drugs, as are commonly used to treat type II diabetes. This thesis describes the development of tools and techniques based on HPAC to examine the effects of glycation on the binding of HSA.

The Chapter 2 provides a general introduction to HPAC and explained how HPAC can be used to examine interactions between drugs and proteins. The method of frontal analysis is introduced and described as a method for determining the global affinity binding of a drug with a protein. Zonal elution will also be introduced as a method that can be utilized to investigate the association equilibrium constants at specific sites on a protein with drugs.

Chapters 3 and 4 describe the use of HPAC to profile the binding of two first-generation sulfonylurea drugs (i.e., chlorpropamide and tolazamide) to normal HSA and samples of HSA with levels of glycation corresponding to controlled or advanced
diabetes. Frontal analysis and zonal elution competition studies will be applied and provided information on binding of drug with normal and glycated HSA. The results obtained from these experiments will indicate how much changes in affinity of binding between these sulfonylureas and HSA due to early stage glycation.

Chapter 5 and 6 describe the utilization of HPAC to profile the binding of two first-generation sulfonylurea drugs (chlorpropamide and tolazamide) and second-generation sulfonylurea drugs (gliclazide) to samples of HSA with levels of advanced stage glycation (i.e., glyoxal and methylglyoxal modification) corresponding to healthy individuals or those with controlled diabetes. Immunoextraction zonal elution and competition studies will be used to examine the effects of advanced stage glycation on the binding of HSA. The results obtained from these experiments will indicate how much changes in affinity of binding between these sulfonylureas and HSA due to advanced stage glycation.
CHAPTER 2

HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY: APPLICATIONS IN DRUG-PROTEIN BINDING STUDIES

Portions of this material have previously appeared in the following publication and are reproduced with permission: P. Tao, S. Poddar, Z. Sun, D.S. Hage, J. Chen, Analysis of solute-protein interactions and solute-solute competition by zonal elution affinity chromatography, Methods 146 (2018) 3–11.

2.1 Introduction

High performance affinity chromatography (HPAC) is a chromatographic technique that uses an immobilized binding agent (e.g., a protein) as the stationary phase [1-3]. In previous work, HPAC has been used to isolate and analyze various specific targets; due to its versatility, this approach can also be applied to studying the interactions between biological molecules in methods that provide fast analysis times and have good precisions. In this thesis, HPAC’s ability to determine binding regions, equilibrium constants, and the amounts of binding sites for drug-protein interactions will be utilized [1-8].

Human serum albumin (HSA) is most abundant serum protein in humans. HSA makes up more than 50% of an individual’s plasma proteins, with a normal concentration between 30–50 mg/ml. This plasma protein is a single chain globular protein with a molecular weight of 66.5 kDa [9]. It contains 585 amino acids, including 35 cysteines, 1 tryptophan, 59 lysines, and 17 disulfide chains [9-15]. HSA has two major binding sites for drugs: Sudlow sites I and II [9]. Sudlow site I is located in subdomain IIA of HSA.
and is known to bind many types of hydrophobic drugs, such as warfarin and salicylate [12]. Sudlow site II is located in subdomain IIIA of HSA and is known to bind to L-tryptophan, ibuprofen, and ketoprofen, as well as many other drugs and solutes [12]. In previous reports it has been found that both of these binding sites can bind to many drugs that are used to treat type II diabetes, such as acetohexamide and tolbutamide [13-15].

High levels of glucose during diabetes can result in the modification of serum proteins, such as through the glycation of HSA. Glycation is a non-enzymatic modification in which reducing sugars bind to proteins [16]. The glycation of proteins has been linked to many of the long-term complications of diabetes [16]. Glycation can lead to both early stage products (e.g., fructosamines) and advanced glycation end-products (AGEs) such as argpyrimidine, and imidazolone [16]. In addition, it is known that both Sudlow sites I and II can be modified as a result of glycation and that these modifications can affect the binding by HSA to various anti-diabetic drugs [17-21].

Sulfonylurea drugs are commonly used to treat type II diabetes by oral administration. The core structure of sulfonylurea drugs is shown in Fig. 2-1 and is composed of two main groups: a phenylsulfonyl group and a urea group. The first-generation sulfonylurea drugs chlorpropamide and tolazamide have been found to bind tightly with HSA; these drugs can lower elevated glucose levels in human blood by binding to ATP K\(^+\) channels in pancreatic beta cells and inducing closure of these channels [22-26]. Overall, these sulfonylurea drugs can lower high glucose levels in blood by closing the K\(^+\) channel in pancreatic cells and opening the Ca\(^{2+}\) channel, which can stimulate insulin secretion from the pancreas [22-26].
This work will use HPAC to investigate the binding of sulfonylurea drugs to normal HSA, glycated HSA and HSA that has been modified to contain advanced glycation end-products (AGEs). The method of frontal analysis will be used to determine the overall equilibrium constants and the moles of binding sites for sulfonylurea drugs with HSA. Zonal competition studies will also be employed to examine the interactions of sulfonylurea drugs with Sudlow sites I and II on HSA. The results obtained from this work should provide a better understanding of the effects of glycation and AGE formation on HSA and of how these processes alter the binding of sulfonylurea drugs to HSA at different stages of diabetes.

2.2 Zonal elution studies of drug-protein interactions

Zonal elution is the most prevalent form of affinity chromatography that is used for the analysis of solute-protein interactions and solute-solute competition for binding sites on proteins [27-31]. In this method, a small amount of an analyte or solute (A) is injected into a chromatographic system that contains an affinity column with an immobilized biological ligand or binding agent (L). This injection is often made in the presence of a mobile phase that contains a known concentration of a competing agent or interacting agent (I). The time or mobile phase volume that is required to elute the solute from the column is then monitored and used to provide information on the interactions between the injected solute, the agent in the mobile phase, and/or the immobilized binding agent [32]. This section 2.2 will examine the theory, principles and applications of zonal elution affinity chromatography method in the study of solute-protein binding and solute-solute competition for proteins.
A typical HPLC system for performing a zonal elution experiment is shown in Figure 2-2 [32]. This particular system design includes an online detector; however, a fraction collector and an offline detector can also be employed, particularly when the experiment involves a low-performance affinity column [30,32-33]. Temperature control is ideally needed for this type of system to obtain optimum precision and accuracy [32]. Linear elution conditions are also often required, in which the amount of the injected solute is negligible compared to the amount of the binding agent in the column [28,32,34]. However, nonlinear elution conditions can be employed in some situations for zonal elution experiments [35-37]. There are several advantages to using zonal elution and affinity chromatography for the analysis of solute-protein binding and solute-solute competition. First, only a small amount of solute is needed per injection and the same preparation of an immobilized binding agent can often be used for many experiments [30,32]. In addition, it is possible in some situations (e.g., with chiral compounds) to examine the binding by more than one solute per run, as long as sufficient resolution is present to differentiate between the corresponding peaks for these solutes [30]. There are several other advantages when using this type of method as part of an HPLC system, including the good precision and speed of HPLC as well as the variety of detectors that can be used to monitor solute elution [38]. Zonal elution has several additional advantages when compared to frontal analysis, which is the other major form of affinity chromatography that is used for binding studies [32]. These advantages include the ability of zonal elution to work with a smaller amount of a solute than frontal analysis and the longer time that is often needed for frontal analysis experiments. Zonal elution can also be used in work with binding agents that may contain multiple sites for a solute
and can allow binding strengths to be measured at individual sites [39-40]. The main
disadvantages of zonal elution are that frontal analysis tends to give more precise binding
constants and can be used to independently determine both the number of binding sites
that are present for a solute and the equilibrium constants for each group of sites [32].

Zonal elution has been employed in many reports for examining competition and
displacement in solute-ligand systems [41]. In this type of study, an analyte or solute is
injected into an affinity column in the presence of a mobile phase that contains a fixed
concentration of a possible competing agent. An example of such a study was given
earlier in Fig. 2-3 [42]. From the change in retention that is observed for the solute as the
concentration of the competing agent is varied, it can be determined whether the two
solutes are interacting as they bind to the same affinity ligand [32].

The nature of this interaction can be determined by comparing the change in the
measured retention with the response that is expected for various models [32]. Examples
of interactions that may be present include direct competition between the injected
analyte and agent in the mobile phase, allosteric interactions, or the absence of any
interactions between these two chemicals with the immobilized binding agent [41]. One
way these interactions can be examined is by making a plot of the retention data
according to Eq. (2-1).

\[ \frac{1}{k_A} = \frac{K_I[D]}{K_A CL} + \frac{1}{K_A CL} \]

(2-1)

In this equation, \( k_A \) is the retention factor for the injected analyte; \( K_A \) and \( K_I \) are
the association equilibrium constants for the analyte and the competing agent,
respectively, at their site of competition; \([I]\) is the concentration of the competing agent;
and \( C_L \) is the concentration of the affinity ligand in the column. A corrected retention
factor that is equal to the term $k_A - X$ is also sometimes used instead of $k_A$ in Eq. (2-1), where $X$ represents the retention factor for the injected analyte due to binding sites in the column at which competing agent has no interactions [39].

Fig. 2-4 shows the results that are obtained for various types of interactions when a plot of $1/k_A$ versus the competing agent concentration is made according to Eq. (2-1) [40]. In the absence of any competition between the analyte and mobile phase additive, a plot that is prepared in this manner shows only random variations about a horizontal line, as illustrated in Fig. 2-4(a) for digitoxin as the injected analyte and when using glimepiride as a mobile phase additive. When direct competition is present at the same binding site for the analyte and competing agent, a linear plot with a positive slope is generated, as shown in Fig.2-4(b) for L-tryptophan as the analyte and in the presence of mobile phases containing glimepiride. Deviations from a positive linear response, as demonstrated in Fig. 2-4(c) for injections of $R$-warfarin as the analyte and glimepiride as the mobile phase additive, can occur if a negative allosteric effect or multisite competition is present. A positive allosteric interaction results in a plot like the one shown in Fig. 2-4(d) for tamoxifen as the analyte tamoxifen and glimepiride as the mobile phase additive, in which the value $1/k_A$ decreases with an increase in the concentration of the agent that has been placed into the mobile phase [32].

2.3 Frontal analysis of drug-protein interactions

Frontal analysis is one approach that has been employed in HPAC for drug-protein interaction studies and work related to personalized medicine [21,43-51]. In this method, solutions containing known concentrations of a drug were continuously applied onto a given column. As the solution of the target passes through the column, some or
most of the binding sites for this target in the column will become occupied by the drug. This process will lead to an increase in the amount of the target that elutes from the column over time. The mean position of each breakthrough curve for a given drug solution was then determined. The mean position of this curve was then fit to various models to determine the association equilibrium constants and the moles of binding sites for the drug on the column.

For a system in which there is relatively fast association and dissociation between a drug (A) and an immobilized protein or binding agent (L), the following equations can be used to describe the response that would be expected for a system with a single type of binding site [52].

\[
m_{L_{\text{app}}} = \frac{m_{L1}K_a[A]}{(1+K_a[A])} \quad (2-2)
\]

\[
\frac{1}{m_{L\text{app}}} = \frac{1}{K_a m_L[A]} + \frac{1}{m_L} \quad (2-3)
\]

In Eqs. (2-2) and (2-3), \(m_{L\text{app}}\) refers to the moles of the applied drug that are required to reach the mean point of the breakthrough curve at a given concentration of the drug, [A]. The association equilibrium constant and total moles of active binding sites for this interaction are described by the terms \(K_a\) and \(m_L\). In Eq. (2-3) predicts that a single system should give a linear plot of \(1/m_{L\text{app}}\) versus \(1/[A]\), in which the value of \(m_L\) can be obtained from the inverse of the intercept and \(K_a\) can be obtained from the ratio of the intercept over the slope.

Similar models can be created for systems with two binding sites, as shown for a two-site model in Eqs. (2-4) and (2-5).

\[
m_{L_{\text{app}}} = \frac{m_{L1}K_a[A]}{(1+K_a[A])} + \frac{m_{L2}K_a[A]}{(1+K_a[A])} \quad (2-4)
\]
\[
\frac{1}{m_{L,\text{app}}} = \frac{1+K_{a1}[A]+\beta_2 K_{a1}[A]+\beta_2 K_{a1}^2[A]^2}{m_L((\alpha_1+\beta_2-\alpha_1\beta_2)K_{a1}[A]+\beta_2 K_{a1}^2[A]^2)}
\]  

These equations include two association equilibrium constants (\(K_{a1}\) and \(K_{a2}\)), which show the higher and lower affinity in the column. The moles of these two types of sites are represented by \(m_{l,1}\) and \(m_{l,2}\). The \(\alpha_1\) and \(\beta_2\) are the ratio of the moles of active binding sites high affinity site to all the active binding sites and the ratio of association equilibrium constants for the low versus high affinity sites.

One advantage of this method is that the equilibrium constant for the target with the binding agent can be determined at the same time as an estimate is obtained for the moles of binding sites that are present [21,43,53]. This makes this approach useful in examining the stoichiometry of a reaction and in comparing binding constants that are obtained from columns that may contain different amounts of the binding agent. However, this method can require large amounts of the target and, because of the number of experiments involved, does tend to take longer to carry out than some of the alternative methods that are described later in this thesis [43,54-55].
Figure 2-1. Structure of chlorpropamide, a typical sulfonylurea drug. The section in the dashed box shows the core structure of all sulfonylurea drugs.
**Figure 2-2.** A typical HPLC system for use in zonal elution affinity chromatography and studies of solute-protein binding or solute-solute competition for proteins.
Figure 2-3. Typical chromatograms obtained in zonal elution studies using injections of \( R \)-warfarin as a site-selective probe for HSA and mobile phases that contained various concentrations of chlorpropamide as a competing agent or interacting agent. This figure was adapted and reproduced with permission from Ref. [42].
Figure 2-4. Plots made according to Eq. (1-3) for various injected solutes – (a) digitoxin, (b) L-tryptophan, (c) R-warfarin and (d) tamoxifen – and mobile phases containing glimepiride that were applied to HSA columns. These plots represent systems in which there was (a) no interaction between the injected solute and agent in the mobile phase for binding sites in the column, (b) direct competition of these agents at a single type of common binding site, (c) negative allosteric effects between these agents during their binding to the column, or (d) positive allosteric effects as these agents were binding to the column. Behavior similar to that in (c) can also be produced by direct competition at multiple binding sites. This figure was adapted and reproduced with permission from Ref. [40].
2.4 REFERENCES


47. R. Matsuda, S. Kye, J. Anguizola, D.S. Hage, Studies of drug interactions with
glycated human serum albumin by high-performance affinity chromatography,

or glycated human serum albumin by high-performance affinity chromatography,

interactions by high-performance affinity chromatography: binding by
glimepiride to normal or glycated human serum albumin, J. Chromatogr. A 1408
(2015) 133-144.


66.

52. S.A. Tweed, B. Loun, D.S. Hage, Effects of ligand heterogeneity in the
characterization of affinity columns by frontal analysis, Anal. Chem. 69 (1997)
4790-4798.

53. H.S. Kim, I.W. Wainer, Rapid analysis of the interactions between drugs and
human serum albumin (HSA) using high-performance affinity chromatography

CHAPTER 3

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

Portions of this material have previously appeared in the following publication and are reproduced with permission: P. Tao, Z. Li, R. Mastuda, D.S. Hage, Chromatographic studies of chlorpropamide interactions with normal and glycated human serum albumin based on affinity microcolumns, J. Chromatogr. B 1097-1098 (2018) 64-73.

3.1 Introduction

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

Sulfonylurea drugs are often used in the treatment of type II diabetes [4,5]. The basic structure of a sulfonylurea drug is composed of a phenylsulfonyl group and a urea group. Chlorpropamide is an oral first-generation sulfonylurea drug that has been used in the past to lower glucose levels in blood by stimulating insulin secretion [4-7]. The structure of this drug is given in Figure 3-1(a). Like many other sulfonylurea drugs [8-11], chlorpropamide is known to bind with more than one site on HSA or the related
protein bovine serum albumin (BSA); however, only a limited amount of previous work has been carried out in characterizing these interactions [5,12,14,15].

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

The high levels of glucose that are present in blood during diabetes can result in the modification of proteins, including HSA [23-27]. This modification occurs through a process that is known as glycation [8,16,23-25], which is illustrated in Figure 3-1(b). Glycation is a non-enzymatic reaction during which glucose combines with an amine group on a protein to create a Schiff base; the Schiff base can then later rearrange to form a stable ketoamine, or Amadori product [8,16,25]. The glycation of proteins is linked to many of the long-term complications of diabetes [16]. This process has also been shown to alter the binding of some sulfonylurea drugs to HSA [8-11,25-27]. However, no previous studies have examined the effects of glycation on the interactions of chlorpropamide with HSA.
Work in this chapter will combine the use of high-performance affinity chromatography (HPAC, also known as high-performance liquid affinity chromatography) with affinity microcolumns to examine the binding of chlorpropamide to normal HSA and glycated HSA. HPAC is a type of liquid chromatography that uses an immobilized binding agent (e.g., HSA) as the stationary phase [28-30]. Advantages of HPAC with regards to the study of biological interactions include its ability to be automated and to provide relatively fast and precise results, while also allowing the same binding agent to often be used for hundreds of experiments [8-11,28-32]. HPAC has been used in prior work to examine the effects of HSA glycation on the binding of this protein to sulfonylurea drugs such as tolbutamide and acetohexamide [8-11]. It has been further shown in recent studies how affinity microcolumns with volumes in the low-to-mid microliter range can be employed with small amounts of a protein such as glycated HSA to rapidly provide a detailed characterization of how a target solute or drug interacts with this binding agent [8,33,34].

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

3.2.1. Chemicals

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

3.2.2. Instrumentation

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

Two samples of glycated HSA (i.e., gHSA1 and gHSA2) were prepared in vitro as described previously under sterile conditions and using 1 mM sodium azide as a buffer additive to prevent bacterial growth [9,10,35]. This was done by using reaction mixtures made in pH 7.4, 0.067 M potassium phosphate buffer that contained a typical physiological concentration of normal HSA (42 g/L, using the same type of commercial preparation as employed in Section 2.4 when making the normal HSA microcolumns) and either 15 or 30 mM of D-glucose (i.e., as used to make gHSA1 or gHSA2 samples, which represented glucose levels that might be seen during controlled or advanced diabetes, respectively) [9,35]. These mixtures were incubated for about 4 weeks at 37 °C to allow glycation to occur, followed by use of a desalting column to remove the excess and unreacted glucose. The glycated HSA samples were lyophilized and stored at -80 °C.

A fructosamine assay was carried out on each HSA sample, in duplicate, to determine the level of glycation that was present [35]. The measured levels of glycation for the normal HSA, gHSA1, and gHSA2 samples were < 0.1, 1.40 (± 0.06), and 3.24 (± 0.07) mol hexose/mol HSA (Note: the values in parenthesis represent ± 1 S.D.).

3.2.4. Column preparation

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

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

3.2.5. Chromatographic studies

The solutions of chlorpropamide, L-tryptophan, R-warfarin and sodium nitrate were prepared in pH 7.4, 0.067 M phosphate buffer, which was also used as the mobile phase for the chromatographic studies (Note: this buffer was selected because it has been routinely used in prior studies to examine binding by other sulfonylurea drugs with HSA) [9-11,34,38]. This buffer was passed through a 0.2 µm filter and degassed for 10-20 min prior to its use. The solutions of R-warfarin were used within two weeks of preparation,
and the L-tryptophan solutions were used within 1-2 days, as based on previous studies examining the stability of such solutions [38-40]. All of the chromatographic experiments were performed at 0.30-0.50 mL/min and 37 °C. The back pressure for the microcolumns under these conditions was 2.0 MPa (290 psi) or less. As noted previously, no significant changes in the binding parameters that were measured in this work were seen when using other flow rates as long as sufficient time was still allowed for a local equilibrium to be established between the immobilized binding agent and the injected/applied drugs and probe compounds [8-11].

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

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

3.3. Results and discussion

3.3.1. Overall binding of chlorpropamide with HSA

3.3.1.1. General approach
The overall interactions of chlorpropamide with normal and glycated HSA were examined by using frontal analysis [34,38]. These studies were carried out to provide an initial estimate for the number and types of sites (e.g., high vs. low affinity) that took part in these interactions; the higher affinity sites were of particular interest, as examined in more detail later through zonal elution (Section 3.3.2). In the frontal analysis experiments, several solutions containing known concentrations of chlorpropamide were applied onto an HSA microcolumn or control microcolumn and used to obtain a series of breakthrough curves, as shown in Figure 3-2. The mean breakthrough times for these curves occurred within 4-15 min of solution application at 0.30 mL/min (i.e., applied solution volumes of 1.2-4.5 mL) and when using a 1.0 cm × 2.1 mm i.d. column containing normal HSA. The glycated HSA microcolumns exhibited similar behavior. Even shorter breakthrough times could be obtained with these microcolumns by using higher flow rates for sample application, but some loss in the precision of this method is known to occur as the flow rate is increased [41].

The frontal analysis results were analyzed by looking at how the moles of chlorpropamide that were needed to reach the mean breakthrough time of each curve changed as the applied concentration of chlorpropamide was varied. For instance, Eqs. (3-1) and (3-2) were used to represent a system with a single set of saturable sites and relatively fast association and dissociation between a drug (D) and an immobilized protein (or affinity ligand, L) [41-44].

\[
\frac{1}{m_{L,app}} = \frac{1}{K_a m_L[D]} + \frac{1}{m_L} \tag{3-1}
\]

\[
m_{L,app} = \frac{m_L K_a[D]}{(1+K_a[D])} \tag{3-2}
\]
In Eqs. (3-1), \( m_{Lapp} \) is the apparent binding capacity (i.e., moles of drug needed to reach the mean point of the breakthrough curve) of an immobilized protein at a given applied concentration for the drug [41]. Other terms in these equations include the association equilibrium constant (\( K_a \)) and moles of active sites for this interaction (\( m_L \)).

Eq. (3-1) indicates that a drug and protein with a one-site interaction should give a linear response for a plot of \( 1/m_{Lapp} \) versus \( 1/[D] \); the value \( m_L \) can be acquired from the reciprocal of the intercept for this plot, and \( K_a \) can be found by using the ratio of the intercept over the slope [41]. A non-linear fit to Eq. (3-2) can also be used to obtain these best-fit parameters [9-11,41-43]. An advantage of using frontal analysis and expression like Eqs. (3-2) is it allows separate estimates to be obtained for both the binding constants and moles of binding sites for an applied solute with an immobilized protein or binding agent [28,29,41]. However, no information is provided on the location of these binding regions [10,11,28,41].

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

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

(3-3)

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

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

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

$$m_{Lapp,tot} = \frac{m_L K_{a1}[D]}{(1+K_{a1}[D])} + \frac{\alpha m_S K_{aS}[D]}{(1+K_{aS}[D])}$$  \hspace{1cm} (3-4)

$$m_{Lapp,tot} = \frac{m_L K_{a1}[D]}{(1+K_{a1}[D])} + \frac{m_L K_{a2}[D]}{(1+K_{a2}[D])} + \frac{\alpha m_S K_{aS}[D]}{(1+K_{aS}[D])}$$  \hspace{1cm} (3-5)

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

Both of these approaches for considering secondary interactions were employed at various stages in this study. Similar trends and binding constants for chlorpropamide with HSA were obtained by each approach (see Section 3.3.1.2). However, the final results given in Section 3.3.1.2 were obtained by using Eqs. (3-4, 3-5). It was determined in these equations that the presence of HSA did lead to a decrease in the amount of secondary binding between chlorpropamide and the support. For instance, Table 3-1 shows that the term $\alpha$ was equal to 0.40-0.42 when using Eq. (3-5) to fit the frontal analysis data for the HSA microcolumns to a two-site drug/protein binding model. This indicated that up to 58-60% of the support’s secondary sites were no longer accessible to chlorpropamide once HSA had been immobilized to this material. The average precision for the overall moles of bound drug that were measured and used in these fits was $\pm 2.2\%$ (range, $\pm 0.2-6.7\%$) under the concentration, column size, and flow rate conditions that were employed.
3.3.1.2. Interactions of chlorpropamide with normal and glycated HSA

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

The linear fits of double-reciprocal plots to both the entire data set and data obtained at lower drug concentrations, with the latter emphasizing the strongest interaction sites, were used to estimate the value of \( K_{a1} \) for the higher affinity sites of chlorpropamide on the various types of HSA microcolumns. The resulting estimates that were obtained for chlorpropamide with normal HSA from the entire data set and the data at lower drug concentrations were 4.3 (± 0.3) × 10^4 M^-1 and 3.5 (± 0.6) × 10^4 M^-1, respectively; this was equivalent to dissociation equilibrium constants (\( K_d \), where \( K_d = 1/K_{a} \)) of 2.3 (± 0.2) × 10^-5 M and 2.9 (± 0.5) × 10^-5 M. The corresponding estimates of \( K_{a1} \) that were obtained for chlorpropamide with microcolumns containing gHSA1 were
4.9 (± 0.2) × 10^4 M^{-1} and 4.9 (± 0.4) × 10^4 M^{-1}, or $K_d$ values of 2.0 (± 0.1) × 10^{-5} M and 2.0 (± 0.2) × 10^{-5} M. The $K_{d1}$ values for the microcolumn containing gHSA2 were 5.6 (± 0.4) × 10^4 M^{-1} and 6.8 (± 0.3) × 10^4 M^{-1}, giving values for $K_d$ of 1.8 (± 0.1) × 10^{-5} M and 1.5 (± 0.1) × 10^{-5} M. The best estimates for the highest affinity sites for gHSA1 and gHSA2, as obtained from the lower drug concentration data, were 1.40-fold to 1.94-fold higher than the estimate of $K_{d1}$ that was obtained for normal HSA, with each of these changes being significant at the 95% confidence level. These changes indicated that the glycation of HSA did have an effect on the binding of chlorpropamide with at least some regions on this protein.

The same data were analyzed in more detail by using non-linear regression and one-site or two-site models, with a simultaneous correction for binding the support as based on Eqs. (3-4) or Eq. (3-5). Figure 3-4 shows how these two types of models fit with the experimental data, as demonstrated by using the results obtained for chlorpropamide on a normal HSA microcolumn. Similar trends were seen for the glycated HSA microcolumns. The one-site model gave lower correlation coefficients than the two-site model, with values that ranged from 0.9489-0.9856 and 0.9993-0.9997, respectively ($n = 11$). In addition, the two-site model gave a more random distribution of the data about the best-fit lines. The use of higher-order models did not improve the fit of these data sets any further. These results, along with the deviations from linearity seen for some of the plots made according to Eq. (3-1), suggested that the two-site model gave a better description than the one-site model for the interactions between chlorpropamide and normal HSA or glycated HSA. The same observation has been made for other first-generation sulfonylurea drugs [8-11] and in previous binding studies between
chlorpropamide and normal HSA or BSA [14,15]. The best-fit parameters that were obtained with both the one- and two-site models for HSA, including the parameters used to correct for binding by chlorpropamide to the support, are summarized in Table 3-1. The overall association equilibrium constants that were obtained for chlorpropamide at pH 7.4 and 37ºC with normal HSA and glycated HSA varied from 3.5 to 4.7 × 10^4 M\(^{-1}\) when using a one-site model for HSA and, for the higher affinity sites, ranged from 6.2 to 9.9 × 10^4 M\(^{-1}\) when using a two-site model for HSA. The corresponding values for \(K_d\) ranged from 2.9 to 2.1 × 10\(^{-5}\) M for the one-site model and from 1.6 to 1.0 × 10\(^{-5}\) M for the higher affinity sites in the two-site model. The overall \(K_a\) values that were obtained were consistent with a value of 0.99 × 10\(^{-5}\) M for \(n_1K_{a1}\) (where \(n\) is the number of a given type of site per protein), as calculated based on prior data reported at pH 7.4 and 37ºC for the high affinity site of chlorpropamide with normal HSA in solution [14]. The range of these values for chlorpropamide also agreed with the overall or high affinities of 12 to 20 × 10^4 M\(^{-1}\) and 8.4 to 12 × 10^4 M\(^{-1}\) that have been observed for acetohexamide and tolbutamide with normal HSA and similar samples of glycated HSA [9-11]. The precisions of these values varied from ± 8.5-17% (average, ± 13.0%) for \(K_{a1}\) in the one-site model and from ± 6.5-9.1% (average, ± 7.8%) for \(K_{a1}\) in the two-site model. The number of the high affinity sites per HSA molecule (i.e., as based on the measured content of HSA in the microcolumns and the values for \(m_{L1}\) when using the two-site model) was 0.12 to 0.18 (average, 0.15). Given that not all of the immobilized HSA was active [9-11,37,38], these results indicated that only one or a few binding regions on HSA probably made up the higher affinity sites for chlorpropamide, as suggested in a prior solution-phase studies [14]. The number of lower affinity sites (e.g., with \(K_{a2}\) values of
1.8 to $5.7 \times 10^3$ M$^{-1}$ and representing weak or non-specific binding regions on HSA) was less well-defined but was determined in the same manner to range from at least 2.1 to 7.0 (average, 5.0). This latter result was also consistent with previous solution-phase binding studies that have been conducted between chlorpropamide and normal HSA or BSA [14,15].

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

3.3.2. Site-selective binding of chlorpropamide with HSA

3.3.2.1. General approach

Zonal elution was next used to provide a more detailed examination of how the binding of chlorpropamide changed at Sudlow sites I or II of HSA as a result of glycation. This was done by using a competition study, in which a site-specific probe (e.g., $R$-warfarin or $L$-tryptophan) was injected onto an HSA or glycated HSA microcolumn as a solution of chlorpropamide with a known and constant concentration was passed through the same microcolumn. Figure 3-5 shows some typical chromatograms from these experiments. The elution of the probe compounds occurred within 6 min or less when
using an injection flow rate of 0.50 mL/min. The retention time for each injected probe was measured as the concentration of the chlorpropamide was varied, and the corresponding value of the probe’s retention factor ($k$) was calculated for each mobile phase that was employed.

It is known from prior work with this type of experiment that if the injected probe and drug in the mobile phase have a single site of competition, a plot made of $1/k$ versus the molar drug concentration, $[D]$, should follow the linear relationship that is given by Eq. (3-6) [28,34,41]. Examples of such plots are provided in Figure 3-6.

$$\frac{1}{k} = \frac{K_{aD}V_M[D]}{K_{aP}m_L} + \frac{V_M}{K_{aP}m_L}$$ (3-6)

The association equilibrium constants for the probe and drug at their site of competition are represented in Eq. (3-6) by $K_{aP}$ and $K_{aD}$, while $V_M$ is the void volume, and $m_L$ is the moles of the common binding sites the probe and drug have in the column. If competition occurs between the drug and probe at a single common site, the ratio of the slope versus intercept for a plot made according to this equation should provide the local association equilibrium constant for the drug at this common site [28,34,41]. One advantage of this approach is it provides a value for $K_{aD}$ that is independent of the moles of active protein or binding sites in the column [41]. This value is also independent of any other interaction regions the applied drug may have with the immobilized binding agent or with the support as long as the injected probe does not interact with these other regions [28,41], as has been noted to be the case in prior work with the site-selective probes that were employed in this study (i.e., R-warfarin and L-tryptophan) [28,35,38,40,44].

3.3.2.2. Competition studies at Sudlow site I
Competition studies were first carried out by using zonal elution experiments and R-warfarin as a probe for Sudlow site I [35,38,44,49]. This site was of interest because it has been suggested through prior competition studies to be a binding region for chlorpropamide on HSA [12]. Furthermore, this region is known to bind other first-generation sulfonylurea drugs [8-11]. The data from zonal elution competition studies that used R-warfarin as a probe gave a linear plot for each type of HSA microcolumn when the data were examined by using Eq. (3-6), as illustrated in Figure 3-6(a). The correlation coefficients for these lines spanned from 0.9758 to 0.9930 (n = 8). This behavior confirmed that chlorpropamide had direct interactions with R-warfarin and binding at Sudlow site I on normal HSA and glycated HSA. The average precision of the retention factors that were obtained in these measurements was ± 1.2% (range, ± 0.2-4.1%). Table 3-2 lists the association equilibrium constants that were acquired by this method at Sudlow site I for chlorpropamide on the normal HSA and glycated HSA microcolumns. These values were in the general range of 3.9-4.7 × 10^4 M\(^{-1}\) at pH 7.4 and 37ºC (i.e., K\(_d\) values of 2.6 to 2.1 × 10\(^{-5}\) M), with precisions of ± 5.1-10.6% (average, ± 8.4%).

These binding constants were consistent with the general estimates of 3.4-9.6 × 10^4 M\(^{-1}\) that had been obtained by frontal analysis in Section 3.3.1 for the higher affinity regions of chlorpropamide with normal or glycated HSA. Previous competition studies that were conducted at pH 7.0 and a lower temperature of 25ºC gave an estimated binding constant of 2.9 × 10^5 M\(^{-1}\) for chlorpropamide at Sudlow site I of normal HSA [12]; however, it is known that the binding strength of drugs with HSA tends to decrease with an increase in temperature [28]. For instance, a decrease in affinity of 20-30% has being
observed at pH 7.4 for R/S-warfarin at Sudlow site I when going from 25 to 37°C [44], and a decrease of 21% in affinity for the strongest binding sites has been reported for chlorpropamide with BSA in going from 15 to 30°C [15]. The values shown in Table 3-2 for chlorpropamide were in the same general range as binding constants of $3.8-5.9 \times 10^4$ M$^{-1}$ and $5.5-6.9 \times 10^4$ M$^{-1}$ that have been measured at Sudlow site I at pH 7.4 and 37°C for acetohexamide and tolbutamide, respectively, with normal HSA and samples of glycated HSA like those used in this report [9-11]. Chlorpropamide gave a binding constant at Sudlow site I that was 29% weaker than seen for tolbutamide with normal HSA, with similar decreases of 28-35% being seen in the binding strength for glycated forms of HSA [9,11]. The two differences in the structures of tolbutamide and chlorpropamide, as shown in Table 3-1(a), are the change from a methyl group to a chlorine on the phenyl ring ($R_1$) and a change from a butyl group to a propyl group at the other end of the structure ($R_2$). Given that Sudlow site I is known to prefer compounds with bulky heterocyclic structures [13,17,18], it is the change on the phenyl group that is probably responsible for most of the decrease in binding strength for chlorpropamide vs. tolbutamide at this site for both normal and glycated HSA. This observation is consistent with the much smaller change noted in binding strength between chlorpropamide and acetohexamide, with a 7% decrease for chlorpropamide for normal HSA and a 13-15% increase for glycated HSA [10,11]. For these latter two compounds, there is a change from a chlorine to an acetyl group on the phenyl ring at $R_1$ and a change from a propyl group to a cyclohexyl group at $R_2$, neither of which appears to have led to any major differences in the binding of these two drugs to Sudlow site I.
The effect of glycation on the site-specific affinities measured at Sudlow site I were consistent with the patterns seen in Section 3.1 for the overall affinities or higher affinity constants for chlorpropamide with HSA. For instance, there was an apparent 1.10-fold increase in the binding strength for chlorpropamide at Sudlow site I in going from normal HSA to gHSA1 and an increase of 1.21-fold in going from normal HSA to gHSA2. Although these differences were relatively small and approached the level of precision for the zonal elution measurements, the results for gHSA2 were significant at the 95% confidence level. As stated in Section 3.1, such changes in binding strength with glycation are believed to reflect the different amounts and types of modification that occur on HSA as its level of glycation is increased [8-11,46-48,50]. Examples of specific modification sites due to glycation that have been found to occur at or near Sudlow site I include K199, K281, and K276 [25].

2.3.2.3. Competition studies at Sudlow site II

Zonal elution and competition studies were further used to examine the interactions of chlorpropamide at Sudlow site II by using L-tryptophan as a site-selective probe for this region [28,38,40,51]. This site was of interest because it has been found to bind to other first-generation sulfonylurea drugs such as acetohexamide and tolbutamide [8-11]. The results for these experiments when chlorpropamide was added to the mobile phase gave a linear plot for both the normal and glycated HSA microcolumns when the data were plotted according to Eq. (3-6), as shown by the example in Figure 3-6(b). These plots had correlation coefficients that ranged from 0.8877 to 0.9546 (n =7-8). These results indicated that chlorpropamide had direct interactions at Sudlow site II on normal or glycated HSA, in addition to the binding that occurred at Sudlow site I. The
retention factors used to generate these plots had an average precision of ± 3.8% (range, ± 0.2-18%).

Table 3-2 includes the association equilibrium constants measured at Sudlow site II during the zonal elution experiments for chlorpropamide with normal HSA or the samples of glycated HSA. These values were in the range of 2.0-3.1 × 10^4 M⁻¹ at pH 7.4 and 37°C (i.e., K_d values of 5.0 to 3.2 × 10⁻⁵ M) and had precisions of ± 14-20% (average, ± 18%). The results were also only slightly lower than the estimated binding constants of 3.9-4.7 × 10^4 M⁻¹ that were acquired in the previous section for chlorpropamide at Sudlow site I on the same microcolumns. In addition, these values were of the same order of magnitude, but lower than, binding constants for Sudlow site II of 7.9-13 × 10^4 M⁻¹ and 5.3-7.2 × 10^4 M⁻¹ that have been reported at pH 7.4 and 37°C for acetohexamide and tolbutamide with normal HSA and comparable preparations of glycated HSA [9-11]. The binding constant for chlorpropamide at Sudlow site II was 63% lower than seen for tolbutamide with normal HSA; similar decreases of 52-61% were observed in the binding constants for glycated HSA [9,11]. Sudlow site II is known to interact with aromatic carboxylic acids and related compounds [13,17,18], so these changes in binding were again mostly likely due to the change from a methyl group to a chlorine group at R₁. It is interesting to note that this alteration in structure produced a much larger change than was seen at Sudlow site I, with normal and glycated HSA having similar patterns in their relative binding strengths for chlorpropamide vs. tolbutamide. A large decrease in relative binding strength was further seen when comparing chlorpropamide with acetohexamide, with chlorpropamide giving an 85% lower affinity at Sudlow site II for normal HSA and 74-75% lower values for glycated HSA [10,11]. These differences
were also thought to be primarily the result of changes in structure at R₁, although some additional contributions from the changes at R₂ may have been present. As was seen for Sudlow site I, glycation again resulted in an increase in the affinity of chlorpropamide at Sudlow site II and at the levels of modification employed in this report. In this case, there was a 1.40-fold increase in the affinity for chlorpropamide at Sudlow site II in going from normal HSA to gHSA1 and an increase of 1.55-fold in going from normal HSA to gHSA2, with all of these differences being significant at the 95% confidence level and larger than the relative uncertainty expected due to only random errors. These changes in binding were again probably a result of the different amounts and types of glycation-related modifications that occurred on these HSA samples [8-11,47,48,50]. For instance, K439 is one major glycation site that occurs at Sudlow site II of HSA [25].

3.4 Conclusion

HPAC was used in this work to investigate the binding of chlorpropamide, a first-generation sulfonylurea drug, to normal HSA and HSA with levels of glycation corresponding to controlled or advanced diabetes. Frontal analysis was first used to examine the overall binding of chlorpropamide with normal and glycated HSA. Although a one-site model gave a reasonable fit to the binding data for these interactions, a slightly better fit was obtained when using a two-site model based on a set of moderate-to-high affinity sites (i.e., the equivalent of two sites with overall binding constants around 6.2-9.9 × 10⁴ M⁻¹ at pH 7.4 and 37°C) and a set of lower affinity binding sites (binding constants, 1.8-5.7 × 10³ M⁻¹). This type of behavior and observed range of affinities were consistent with interaction models that have been reported for tolbutamide and acetohexamide, two other first-generation sulfonylurea drugs, with normal HSA and
similar preparations of glycated HSA [8-11]. A net increase in the overall binding strength of chlorpropamide for the higher affinity sites of up to 1.6-fold was noted when comparing the glycated HSA samples to normal HSA.

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

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

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

**Acknowledgements**

This work was supported by the National Institutes of Health under grant R01 DK069629.
Figure 3-1. (a) Structures of the first-generation sulfonylurea drugs chlorpropamide (i.e., 1-[(p-chlorophenyl)sulfonyl]-3-propylurea), tolbutamide and acetohexamide; (b) the addition of glucose to an amine group on a protein through the process of glycation. The dashed box in (a) shows the core structure of a sulfonylurea drug. The reactions in (b) represent the formation of an early stage glycation product; further reactions can occur to form advanced glycation end-products (AGEs) [23].
(a) \[ \text{Chlorpropamide} \]
\[ \text{Tolbutamide} \]
\[ \text{Acetohexamide} \]

(b) \[ \text{D-Glucose} \quad \text{Schiff base} \quad \text{Amadori product} \]
Figure 3-2. Typical chromatograms obtained by frontal analysis for the application of chlorpropamide to a 1.0 cm × 2.1 mm i.d. column containing normal HSA column. The concentrations of the applied drug are shown on the right. The flow rate was 0.30 mL/min. These studies were performed at 37°C and pH 7.4.
Figure 3.3. Double-reciprocal plots made according to Eq. (3-1) to examine data obtained for the binding of chlorpropamide with (a) normal HSA and (b) glycated HSA2 during frontal analysis studies. The best-fit lines shown are for the lower concentration data (i.e., 1.0 to 7.5 µM) and had correlation coefficients of 0.9998-0.9999 ($n = 5$). The error bars represent a range of ± 1 S.D.
(a) Normal HSA

\[
\frac{1}{m_{\text{app}}} \ (\text{mol}^{-1} \times 10^9)
\]

\[
\frac{1}{[\text{Chlorpropamide}]} \ (\text{M}^{-1} \times 10^3)
\]

(b) Glycated HSA (gHSA2)

\[
\frac{1}{m_{\text{app}}} \ (\text{mol}^{-1} \times 10^9)
\]

\[
\frac{1}{[\text{Chlorpropamide}]} \ (\text{M}^{-1} \times 10^3)
\]
Figure 3-4. Comparison of the fits for (a) one-site and (b) two-site models to frontal analysis data for the overall binding of chlorpropamide to a column containing normal HSA. These fits were obtained by using Eqs. (3-4) and (3-5), respectively. The insets show the corresponding residual plots. The relative standard deviations for the values of $m_{Lapp,tot}$ that are shown in these plots ranged from ± 0.17% to ± 4.7% (average, ± 2.0%).
**Figure 3-5.** Typical chromatograms obtained by zonal elution competition studies on a 1.0 cm × 2.1 mm i.d. column containing normal HSA and for the injection of *R*-warfarin in the presence of application of mobile phases that contained 20 or 0 µM chlorpropamide. The flow rate was 0.50 mL/min. These studies were performed at 37°C and pH 7.4.
Figure 3-6. Example of plots made according to Eq. (3-6) when using chlorpropamide as a competing agent in the mobile phase and (a) R-warfarin or (b) L-tryptophan as the injected probe on a column containing glycated HSA1. The best-fit lines in (a) and (b) had correlation coefficients of 0.9813 and 0.9546, respectively. The error bars represent ± 1 S.D.
Table 3-1. Best-fit parameters based on one- or two-site models for the overall binding of chlorpropamide with normal HSA or glycated HSA.

<table>
<thead>
<tr>
<th>Type of HSA &amp; binding model</th>
<th>( K_a^1 ) (M(^{-1} \times 10^4 ))</th>
<th>( m_{L1} ) (mol ( \times 10^{-9} ))</th>
<th>( K_a^2 ) (M(^{-1} \times 10^4 ))</th>
<th>( m_{L2} ) (mol ( \times 10^{-8} ))</th>
<th>( \alpha )</th>
</tr>
</thead>
<tbody>
<tr>
<td>One-site model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal HSA</td>
<td>3.5 (± 0.3)</td>
<td>16.5 (± 0.7)</td>
<td>-----</td>
<td>-----</td>
<td>0.48 (± 0.12)</td>
</tr>
<tr>
<td>gHSA1</td>
<td>4.5 (± 0.6)</td>
<td>15.9 (± 0.9)</td>
<td>-----</td>
<td>-----</td>
<td>0.49 (± 0.19)</td>
</tr>
<tr>
<td>gHSA2</td>
<td>4.7 (± 0.8)</td>
<td>15.7 (± 1.3)</td>
<td>-----</td>
<td>-----</td>
<td>0.48 (± 0.31)</td>
</tr>
<tr>
<td>Two-site model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal HSA</td>
<td>6.2 (± 0.4)</td>
<td>3.4 (± 0.3)</td>
<td>0.57 (± 0.10)</td>
<td>4.3 (± 0.6)</td>
<td>0.42 (± 0.02)</td>
</tr>
<tr>
<td>gHSA1</td>
<td>8.9 (± 0.7)</td>
<td>3.7 (± 0.3)</td>
<td>0.18 (± 0.01)</td>
<td>12.4 (± 7.1)</td>
<td>0.41 (± 0.03)</td>
</tr>
<tr>
<td>gHSA2</td>
<td>9.9 (± 0.9)</td>
<td>2.4 (± 0.2)</td>
<td>0.19 (± 0.06)</td>
<td>14.8 (± 0.3)</td>
<td>0.40 (± 0.01)</td>
</tr>
</tbody>
</table>

\( \alpha \)These results were measured at 37 °C in the presence of pH 7.4, 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ± 1 S.D. The parameters shown for the one-site and two-site binding of chlorpropamide with HSA were obtained by using Eqs. (4) and (5), respectively, along with independently-estimated values for \( K_{aS} \) and \( m_{LS} \) of \( 1.64 \times 10^4 \) M\(^{-1} \) and \( 8.6 \times 10^{-9} \) mol.
Table 3-2. Site-specific association equilibrium constants ($K_a$) for the interactions of chlorpropamide at Sudlow sites I and II

<table>
<thead>
<tr>
<th>Type of HSA</th>
<th>Sudlow site I</th>
<th>Change vs.</th>
<th>Sudlow site II</th>
<th>Change vs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal HSA</td>
<td>3.9 (± 0.2) × 10⁴</td>
<td>------</td>
<td>2.0 (± 0.4) × 10⁴</td>
<td>------</td>
</tr>
<tr>
<td>gHSA1</td>
<td>4.3 (± 0.4) × 10⁴</td>
<td>↑ 1.10-fold (N.S.)</td>
<td>2.8 (± 0.4) × 10⁴</td>
<td>↑ 1.40-fold</td>
</tr>
<tr>
<td>gHSA2</td>
<td>4.7 (± 0.5) × 10⁴</td>
<td>↑ 1.21-fold</td>
<td>3.1 (± 0.6) × 10⁴</td>
<td>↑ 1.55-fold</td>
</tr>
</tbody>
</table>

These results were measured at 37 °C in the presence of pH 7.4, 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ± 1 S. D.

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


7. J. Judis, Binding of sulfonylureas to serum proteins, J. Pharm. Sci. 61 (1972) 89-93.


CHAPTER 4

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

 Portions of this material have previously appeared in the following publication and are reproduced with permission: P. Tao, Z. Li, A. Woolfork, D.S. Hage, Chromatographic studies of tolazamide binding with glycated and normal human serum albumin by using high-performance liquid chromatography, J. Pharm. Biomed. Anal. 166 (2019) 273-280.

4.1 Introduction

As described in Section 3.1, sulfonylurea drugs are frequently used to treat type II diabetes. Like other compounds in this group, tolazamide is known to have a moderately strong interaction with the transport protein human serum albumin (HSA) and the closely-related protein bovine serum albumin (BSA) [1-9]. Prior work has suggested that either one or two groups of sites are involved in the binding of tolazamide to serum albumin [2,3]. High-performance affinity chromatography (HPAC) will be utilized in this study to examine the interactions between tolazamide and HSA at various levels of glycation. Frontal analysis and zonal elution will be utilized in this chapter with HPAC to investigate the overall binding and site-specific interactions of normal and in vitro glycated forms of HSA with tolazamide. This study will also include a comparison between these results and prior observations made for alternative first-generation sulfonylurea drugs to see how alterations in the structure of these compounds affect their binding to HSA and glycated HSA [2-9]. The final results should provide a better
understanding of the effects of HSA glycation on the transport and protein binding by sulfonylurea drugs in serum. These data should also provide useful information for future applications in the field of personalized medicine, as pertaining to the ability to adjust a drug dosage for a given patient in a disease such as diabetes [4].

4.2 Experimental

4.2.1 Chemicals

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

4.2.2 Instrumentation

The same Jasco HPAC system was used for this work as described in Section 3.2.2.

4.2.3 Preparation of glycated HSA

Glycated samples of HSA (i.e., gHSA1 and gHSA2) were made in vitro by using a prior method, as described in Section 3.2.3 [5-7].
4.2.4 Preparation of HSA microcolumns

In this study, the affinity microcolumns were prepared by following same procedures as described in Section 3.2.4.

4.2.5 Chromatographic studies

All drug solutions were prepared in phosphate buffer (pH 7.4, 0.067 M) and filtered through a 0.2 µm membrane, followed by degassing for 10-20 min. The L-tryptophan solutions were prepared fresh daily, while the R-warfarin solutions where prepared and used within two weeks [10-12]. The chromatographic experiments were conducted at 37 °C and 0.50 mL/min, as employed in previous drug-binding studies using similar protein supports or columns [4-9].

Frontal analysis studies were conducted by applying a potassium phosphate buffer (pH 7.4, 0.067 M) onto a microcolumn until a steady baseline was obtained. A step change was then made for the application of a known concentration of tolazamide, as prepared in the same buffer and passed continuously through the microcolumn. A total of 12 solutions containing 0.5 to 50 μM tolazamide were applied in triplicate to each HSA microcolumn and control microcolumn. After reaching a stable plateau at a given concentration of tolazamide, the original buffer was then reapplied to the microcolumn to elute the bound tolazamide and regenerate the system. The elution of tolazamide was detected at a wavelength of 254 nm, and all concentrations of this drug that were examined were within the linear range of the detector at this wavelength. The first derivative function in PeakFit 4.12 was used to find the mean position of each frontal analysis (or breakthrough) curve. Identical studies were conducted on a control column to correct for the system void time and non-specific binding of tolazamide to the support,
as has been used in previous work with similar support materials and columns [4-10]. The relative extent of non-specific binding ranged from 27-36% of the total measured binding on the HSA microcolumns over the range of tolazamide concentrations that were examined; these results are in line with previous data that have been acquired with another first-generation sulfonylurea drugs (i.e., chlorpropamide) on similar columns [9].

Zonal elution competition studies were conducted by employing R-warfarin and L-tryptophan as site specific probes for HSA [10]. Eight mobile phase concentrations prepared in phosphate buffer (pH 7.4, 0.067 M) and ranging from 0 to 20 μM tolazamide were used in these experiments. Each sample contained 5 μM of the given probe, as prepared in the mobile phase. A 20 μL portion of each sample was injected (four times) under each set of mobile phases and microcolumn conditions. The wavelengths that were utilized for detection were 308 and 280 nm for R-warfarin and L-tryptophan, respectively. A 20 μM solution of sodium nitrate in phosphate buffer (pH 7.4, 0.067 M) was used as a void volume marker and was monitored at 205 nm. Zonal elution experiments were performed in replicate (four times), and an exponentially-modified Gaussian curve fit was used to find the mean retention time of each peak.

4.3 Results and discussion

4.3.1 Frontal analysis studies

Frontal analysis was used to examine the overall interactions of tolazamide with normal or glycated HSA, as described in prior work in section 3.3.1 [10]. In this method, a drug solution with a known concentration was continuously applied onto a microcolumn containing one of these protein preparations. During application of the drug solution, the amount of drug that eluted was monitored and formed a breakthrough
curve. These curves reached stable plateaus within 2.5-4.0 min under the given conditions. The precision for the moles of bound tolazamide that were measured during these experiments ranged from ± 0.1-5.5% (average, ± 1.8%). Each frontal analysis chromatogram for tolazamide showed a small initial plateau (i.e., at about 2.2-2.5 min) which represented the void volume and which was created by a slight difference in the background absorbance of the tolazamide solution when compared to the application buffer. The frontal analysis results after the void volume and with normal HSA gave a set of two curves at the higher concentrations of applied tolazamide, indicating that multiple types of binding sites were present for this drug. The glycated HSA samples exhibited only one overall breakthrough curve beyond the void volume, demonstrating that some changes in the binding of tolazamide had taken place in going from normal HSA to glycated HSA. The overall breakthrough time for the combined curves in each plot, beyond the void volume, were used to determine the binding capacity for tolazamide to the immobilized HSA; this approach for data analysis made it possible to directly compare the results obtained at different concentrations of tolazamide with the same preparation of HSA or on different types of HSA microcolumns. The overall binding capacities that were measured at different applied concentrations of tolazamide were fit to various binding models to obtain the equilibrium constants and amounts of binding sites for these interactions [13]. Eq. 3-3 was used to examine the higher or lower affinity binding sites on HSA for tolazamide, as described in Section 3.3.1. The presence of any secondary binding and interactions the analyte or drug may have with the support was also considered.
The results of these fits are shown in Table 4-1. These results were obtained using a best-fit value of $1.34 \pm 0.01 \times 10^3 \text{ M}^{-1}$ for $K_{aS}$ (i.e., representing binding by tolazamide to the support) and a value of $8.3 \pm 0.1 \times 10^7 \text{ mol}$ for $m_S$ (i.e., the moles of binding sites for tolazamide on the support in the absence of any HSA). Based on the results shown in Table 4-1, the presence of HSA led to a decrease in secondary binding between tolazamide and the support. For example, the data for the fit of the two-site drug/protein binding model in Table 4-1 indicated that about half (51%) of the original binding sites for tolazamide on the support were still available to tolazamide once normal or glycated HSA had been immobilized. Use of a one-site drug/protein model similarly indicated that 58-60% of the original sites on the support were accessible for binding to tolazamide after protein immobilization. Figure 4-1 shows some typical frontal analysis data for tolazamide on a column containing HSA and when fit to Eqs. (3-3) and (3-4). For each type of HSA, it was found that the two-site drug/protein binding model, as described by Eq. (3-4), gave a much better fit to the data than a one-site drug/protein model based on Eq. (3-3). These two types of fits had correlation coefficients ranging from 0.9998-0.9999 and 0.9829-0.9972 ($n = 12$), respectively, for all the types of HSA samples that were examined. The fits to the two-site model based on Eq. (3-4) also gave more random variations of the data about the best-fit lines (see insets in Figure 4-1) and smaller values for the sums of the squares of the residuals. In the latter case, values of $0.1-2.0 \times 10^{-18}$ were obtained for the two-site drug/protein binding model in Eq. (3-4) vs. values of $0.3-1.4 \times 10^{-16}$ for the one-site drug/protein model in Eq. (3-3), with these differences being significant at the 95% confidence level. These results, and the general shape of the frontal analysis curves, indicated that the two-site drug/protein model was
better than the one-site model for describing interactions between tolazamide and normal HSA or glycated HSA. This conclusion was consistent with the two-step frontal analysis curves that are shown in Figure 4-1(a) for normal HSA at moderate-to-high tolazamide concentrations. This two-site model also agrees with previous findings for tolazamide and several other first-generation sulfonylureas with normal HSA or BSA [8,9], as well as for alternative first-generation sulfonylurea drugs with similar preparations of glycated HSA to those used in this study [4-9].

The association equilibrium constants and moles of binding sites that were obtained with the two-site drug/protein binding model are provided in Table 4-1 (Note: the results for the one-site model are also provided for reference). The association equilibrium constants for the higher affinity sites that were found for tolazamide with normal HSA, gHSA1, and gHSA2 were 5.3 (± 0.6) × 10^4 M⁻¹, 4.3 (± 0.5) × 10^4 M⁻¹, and 6.0 (± 0.2) × 10^4 M⁻¹, respectively. The precisions of these values varied from ± 3.3-11.6% (average, ± 8.8%). This range of binding constants was similar to a value of 8.67 × 10^4 M⁻¹ that has been previously measured for the higher affinity sites of tolazamide at pH 7.4 and 37ºC when using dynamic dialysis and a 1% solution of normal HSA [9]. In addition, these binding constants were consistent with binding strengths of 12-22 × 10^4 M⁻¹, 8.4-12 × 10^4 M⁻¹, and 4.5-9.9 × 10^4 M⁻¹ that have been reported for three other first-generation sulfonylurea drugs (i.e., acetohexamide, tolbutamide, and chlorpropamide, respectively) with normal HSA and glycated HSA [3-7, 9].

The values obtained from the same fits for the binding strengths of the weak or non-specific sites (as represented by \( K_a2 \)) are also listed in Table 4-1. The value of this term for normal HSA, gHSA1, and gHSA2 was 0.49 (± 0.01) × 10^4 M⁻¹, 0.85 (± 0.27) ×
$10^4 \text{ M}^{-1}$, or $0.91 \pm 0.01 \times 10^4 \text{ M}^{-1}$, respectively (i.e., binding constants that were 5.1- to 10.8-fold lower than $K_{a1}$ for the higher affinity sites). The precisions of these $K_{a2}$ values ranged from $\pm 1.1-32\%$ (average, $\pm 11.6\%$). These binding constants were similar to those for weak/non-specific sites that have been reported for three other first-generation sulfonylurea drugs with normal HSA and glycated HSA [3-7, 9].

The amounts of binding sites that had a moderate-to-high affinity for tolazamide was found from the data in Table 4-1 to be 0.14-0.19 mol per mol HSA (average, 0.16) for the three types of normal or glycated HSA that were examined in this study. These results were based on the content of HSA in each type of microcolumn (i.e., 41-45 nmol, as calculated by using the measured amount of HSA on the support and the support’s known packing density) and the values for $m_{L1}$ that are listed in Table 4-1, as obtained for the same preparations of HSA when using a two-site binding model. These values suggested that only a portion of the immobilized HSA was active [5-8] and that only one or a few binding regions on HSA made up tolazamide’s higher affinity sites. The number of lower affinity binding sites for tolazamide was found through the same approach to range from 3.9-19.6 (average, 14.0) mol per mol HSA.

The data in Table 4-1 indicate that some changes occurred in the value of $K_{a1}$ at the moderate-to-high affinity sites for tolazamide when going from normal HSA to glycated HSA and using a two-site binding model. For instance, there was a 19% decrease in the value of $K_{a1}$ for tolazamide when going from normal HSA to the gHSA1 and a 13% increase between normal HSA and gHSA2. Each of these differences was significant at 95% confidence level. Both increased binding and decreased binding strength has been seen for other sulfonylurea drugs with HSA that had similar or identical
levels of glycation to that present in this study [3-7, 9]. These changes in affinity have been proposed to be due to glycation or related modifications that take place at or near Sudlow sites I and II [4-9,15-17]. In addition, an increase in $K_{a2}$ of 4.4- to 4.6-fold was seen for tolazamide in going from normal to glycated HSA. This increase, along with the increase in the value of $m_{L2}$ for these weak/non-specific sites, explains why two fronts were seen in the breakthrough curves for tolazamide with normal HSA (i.e., which had the largest difference between $K_{a1}$ vs. $K_{a2}$ and $m_{L1}$ vs. $m_{L2}$), while only one broad front was seen for the microcolumns that contained glycated HSA.

4.3.2 Zonal competition studies

4.3.2.1 Zonal elution studies with tolazamide at Sudlow site I

Zonal elution competition studies were used to investigate any site-specific changes in the binding of tolazamide with normal or glycated HSA and to identify the regions on this protein which made up the moderate-to-high sites that were represented by $K_{a1}$ in the frontal analysis studies. In this method, a small amount of a site-specific probe (e.g., R-warfarin or L-tryptophan) was injected onto an HSA microcolumn in the presence of a mobile phase that contained a known and fixed concentration of tolazamide [18]. Figure 4-2 shows typical chromatograms for these competition experiments for normal HSA and gHSA1; similar behavior was seen for gHSA2. In these studies, the probe eluted in under 1.5-2.0 min (for L-tryptophan) or under 7-8 min (for R-warfarin). The retention factor ($k$) for the probe was determined at each concentration of tolazamide in the mobile phase. The precision of the retention factors for the injected probes in these studies ranged from ± 0.1-8.4% (average, ± 1.3%).
Previous studies have shown that direct interaction of a probe with a drug at a single site during a zonal elution experiment yields a linear relationship when plotted according to Eq. (3-5), as described in section 3.3.2. Examples of this type of plot are shown in Figure 4-3. Interactions of tolazamide at Sudlow site I were investigated by employing R-warfarin as the site-specific probe [10]. These experiments provided linear plots according to Eq. (3-5) for the columns that contained normal or glycated HSA. The correlation coefficients of these plots spanned from 0.8962 to 0.9502 (n = 6-8). These fits were consistent with a system in which tolazamide had direct interactions with R-warfarin at Sudlow site I on normal and glycated HSA. This result agreed with observations made in previous studies with other first-generation sulfonylurea drugs [5-8].

Table 4-2 shows the association equilibrium constants that were determined for tolazamide at Sudlow site I on each HSA microcolumn. These values were 1.8 (± 0.3) × 10⁴ M⁻¹, 1.4 (± 0.2) × 10⁴ M⁻¹, and 1.4 (± 0.3) × 10⁴ M⁻¹ for tolazamide on normal HSA, gHSA1 and gHSA2, respectively, and had precisions of ± 14-21% (average, ± 17%). These binding constants were within the range of 10⁴-10⁵ M⁻¹ that was measured by frontal analysis for K_a1 at the moderate-to-high affinity sites of tolazamide on HSA, indicating that Sudlow site I was one of these regions. There was a 22% decrease in the binding strength for tolazamide at Sudlow site I in going from normal HSA to both gHSA1 and gHSA2. In each case, this decrease in local affinity was significant at the 95% confidence level. These changes in binding strength are again probably related to glycation-related modifications that occur at or near Sudlow site I, as have been observed for residues K199, K276, and K281 on HSA [17].
The results for tolazamide in the zonal elution studies were compared to those that have been measured by the same method with other first-generation sulfonylurea drugs (see Table 4-2). In the case of acetohexamide, a non-significant decrease in binding strength of 3-10% has been noted at Sudlow site I with HSA that had similar or identical levels of glycation to that utilized in this study; however, lower levels of glycation gave a 40% increase in the binding strength of acetohexamide at this same site [5,7]. Small-to-moderate increases in binding strength have been noted for chlorpropamide and tolbutamide (i.e., increases of 10-21% and 18-20%, respectively) with samples of glycated HSA that were similar to those used in this report [4-7,9].

The difference in the relative direction and sizes of these changes in binding with glycated HSA and with the various sulfonylureas in Table 4-2 provides some clues as to the influence of their two side chains (i.e., R1 and R2) on these interactions. As an example, tolazamide and tolbutamide both have a methyl group at R1, while tolazamide has a large azepane group at R2 and tolbutamide has a much smaller butyl group. This comparison suggests that the 22% decrease in binding strength for the glycated HSA with tolazamide vs. the 18-20% increase with tolbutamide, and the 3.1- to 4.7-fold higher affinity seen for tolbutamide with both normal and glycated HSA, is due to a better fit of the smaller group at R2 on tolbutamide in Sudlow site I. This observation is supported by the fact that chlorpropamide (i.e., which has only a propyl group at R2 and a chlorine at R1) has a similar binding strength and change in affinity to that seen with tolbutamide in going from normal HSA to glycated HSA. The binding strength and change in binding for acetohexamide at Sudlow site I are closest to the results for tolazamide. This
similarity may be due to the relatively large cyclic rings that are present at R2 in both compounds.

3.3.2.2 Zonal elution studies with tolazamide at Sudlow site II

Similar zonal elution studies were used to examine the binding of tolazamide at Sudlow site II, for which L-tryptophan was used as a site-specific probe [10-11]. The average precision obtained for the retention factors in these studies was ± 1.5% (range, ± 0.1-8.5%). Plots of the resulting data were made according to Eq. (3-5), as illustrated in Figure 4-3(b). These plots gave linear responses with correlation coefficients for all the tested protein samples that ranged from 0.8967 to 0.9653 (n =7-8). This type of response indicated that tolazamide was binding at Sudlow site II in both normal and glycated HSA.

Table 4-2 lists the site-selective binding constants that were measured for tolazamide at Sudlow site II for each type of HSA. These values ranged from 1.9 to 3.0 × 10⁴ M⁻¹ and had precisions of ± 14-23% (average, ± 18%). These results were slightly larger than the binding constants measured for tolazamide at Sudlow site I and were again consistent with the values for Kₐ₁ that were determined by frontal analysis for the moderate-to-high affinity sites of tolazamide on HSA. These values given in Table 4-2 for tolazamide at Sudlow site II were also the same order of magnitude but lower than binding constants that have been reported for acetohexamide and tolbutamide under comparable conditions with similar preparations of HSA [12-15]. There was a small, non-significant increase of 11% in the local affinity for tolazamide at Sudlow site II when comparing gHSA1 to normal HSA; however, there was also a 58% increase, which was significant at the 95% confidence level, in going from normal HSA to gHSA2. These
changes were again thought to reflect modifications that were occurring at or near this site due to glycation, as have been observed at K378 and K439 [19-21].

The results obtained for tolazamide at Sudlow site II were compared with those noted for other first-generation sulfonylurea drugs with similar samples of modified HSA [6-8]. In this case, chlorpropamide gave the closest behavior to that seen for tolazamide at Sudlow site II. Chlorpropamide had local affinities that were similar to those seen for tolazamide and had an increase in affinity of up to 55% being seen in going from normal HSA to samples of glycated HSA [9]. These similarities indicated that Sudlow site II was mostly interacting with the R1 region of these compounds (i.e., a change from only a methyl group to a chlorine between tolazamide and chlorpropamide). However, some interactions with R2 were probably present as well, as suggested by the differences in affinities at Sudlow site II that were seen between tolazamide and tolbutamide (i.e., both compounds having methyl groups at R1). These conclusions agree with recent observations that have been made when comparing the interactions of chlorpropamide with those of acetohexamide and tolbutamide at Sudlow site II on normal or glycated HSA [9].

4.4 Conclusion

In this study, HPAC was used to investigate the binding of tolazamide to normal HSA and glycated HSA. These results were then compared to those obtained in prior work with other first-generation sulfonylurea drugs. Frontal analysis was first used to examine the overall interactions of tolazamide with the various preparations of HSA. A two-site model yielded binding constants for tolazamide that included a set of moderate-
to-high affinity regions (4.3-6.0 \times 10^4 \text{ M}^{-1}) and a set of lower affinity regions (4.9-9.1 \times 10^3 \text{ M}^{-1}). Both a slight decrease (19\%) and small increase (13\%) in binding strength at the higher affinity regions on HSA were seen for tolazamide at the two levels of glycation that were considered, which represented typical conditions seen in controlled or advanced diabetes. Both these trends and binding model agreed with previous observations that have been made for other sulfonylurea drugs [4-7,9].

Zonal elution studies were used to examine the interactions of tolazamide at specific sites on normal and glycated HSA. This drug was found to bind to both Sudlow sites I and II, as has been reported for other first-generation sulfonylurea drugs [4-9]. A 22\% decrease in affinity was observed for tolazamide at Sudlow site I for the glycated samples of HSA when compared to normal HSA, while up to a 58\% increase in affinity was observed at Sudlow site II. These results were also consistent with prior observations made for other sulfonylurea drugs, which have typically been found to both Sudlow sites I and II and which can have increased or decreased affinities at these sites as a result of glycation [4-7,9].

The results for tolazamide at Sudlow sites I and II were compared in more detail to prior data obtained for related first-generation sulfonylurea drugs to learn more about the portions of these drugs that contributed to binding at each of these sites. It was found that changes in the side group on the urea portion of these drugs (i.e., R2 in Table 4-2) were important in determining their affinities at Sudlow site I. Changes at the side group on the phenylsulfonyl region of these compounds (i.e., R1 in Table 4-2) were important in affecting binding at Sudlow site II; however, some contributions from R2 were also probably present at this site.
It was illustrated in this report how HPAC can be employed with affinity microcolumns to look at changes in both the overall binding and site-selective interactions that take place as a result of protein modification. This approach was shown to be relatively fast, reproducible, and able to provide precise estimates of binding constants for such work. It was these features, plus the ability to use a small amount of protein for many experiments, which made it possible to examine even relatively minor changes in the affinity of tolazamide for normal and glycated HSA. These properties also made it possible to compare the binding behavior noted for tolazamide with that seen for related drugs and similar or equivalent preparations of normal or glycated HSA. These results provided a better understanding of how HSA glycation can affect the transport and protein binding of sulfonylurea drugs in serum, as well as information that can be used in future studies aimed at personalized medicine based on these agents [4].

Acknowledgements

This work was funded by the National Institutes of Health under grant R01 DK069629.
Figure 4-1. Fit of (a) one-site or (b) two-site drug/protein binding models to frontal analysis data for tolazamide with an immobilized sample of gHSA1, as obtained by using Eqs. (3-3) or (3-4), respectively, and including the use of a term to correct for secondary binding by tolazamide to the support. The insets show the corresponding residual plots. The correlation coefficients for these fits were (a) 0.9965 and (b) 0.9999 ($n = 12$).
Figure 4-2. Typical chromatograms obtained by zonal elution competition studies and in the presence of 0 or 20 µM tolazamide in the mobile phase for (a) the injection of L-tryptophan as a probe for Sudlow site II on a normal HSA microcolumn or (b) the injection of R-warfarin as a probe for Sudlow site I on a microcolumn containing gHSA1. These chromatograms were acquired at a flow rate of 0.50 mL/min.
Figure 4-3. Examples of zonal elution competition studies and plots made according to Eq. (3-5) when using tolazamide as a competing agent and (a) R-warfarin as the injected probe on a column containing gHSA2 or (b) L-tryptophan as the injected probe on a normal HSA column. The correlation coefficients for these plots were (a) 0.9287 ($n = 8$) and (b) 0.9653 ($n = 8$).
**Table 4-1.** Best-fit parameters for the overall binding of tolazamide with normal HSA or glycated HSA based on one-site and two-site models

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

*These results were measured at pH 7.4 and 37 °C. The ranges given in parentheses are ± 1 S.D. Eqs. (3-4) and (3-5) were used to obtain the parameters for the one-site and two-site binding models of tolazamide with HSA by employing independent estimates for $K_{aS}$ and $m_{LS}$ of 1.34 (± 0.01) × 10$^3$ M$^{-1}$ and 8.3 (± 0.1) × 10$^{-7}$ mol.*
Table 4-2. Site-specific association equilibrium constants ($K_a$) determined by zonal elution for the binding of tolazamide and other first-generation sulfonylureas at Sudlow sites I and II of HSA\textsuperscript{(a)} (see following page)

\textsuperscript{a}These results were measured at pH 7.4 and 37 °C. The ranges in parentheses are ± 1 S. D., as based on error propagation and the precisions of the best-fit slopes and intercepts obtained when using Eq. (3-4).
<table>
<thead>
<tr>
<th>Sulfonhydrae</th>
<th></th>
<th></th>
<th>$K_a$, Sudlow site I</th>
<th>$K_a$, Sudlow site II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1</td>
<td>R2</td>
<td>Normal HSA (M$^{-1} \times 10^4$)</td>
<td>gHSA1 (M$^{-1} \times 10^4$)</td>
</tr>
<tr>
<td>Tolazamide</td>
<td>-CH$_3$</td>
<td></td>
<td>1.8 (±0.3)</td>
<td>1.4 (±0.2)</td>
</tr>
<tr>
<td>Acetohexamide [7]</td>
<td></td>
<td></td>
<td>4.2 (±0.4)</td>
<td>3.8 (±0.3)</td>
</tr>
<tr>
<td>Chlorpropamide [9]</td>
<td>-Cl</td>
<td>-(CH$_2$)$_2$CH$_3$</td>
<td>3.9 (±0.2)</td>
<td>4.3 (±0.4)</td>
</tr>
<tr>
<td>Tolbutamide [7]</td>
<td>-CH$_3$</td>
<td>-(CH$_2$)$_3$CH$_3$</td>
<td>5.5 (±0.5)</td>
<td>6.6 (±0.5)</td>
</tr>
</tbody>
</table>
4.5 References
1. J. Judis, Binding of sulfonylureas to serum proteins, J. Pharm. Sci. 61 (1972) 89-93.


CHAPTER 5
CHROMATOGRAPHIC CHARACTERIZATION OF INTERACTIONS BY 
FIRST- AND SECOND-SULFONYLUREA DRUGS WITH GLYOXAL-
MODIFIED ALBUMIN MICROCOLUMN

5.1 Introduction

Sulfonylurea drugs like chlorpropamide, gliclazide, and tolazamide are known to have significant binding with human serum albumin (HSA), as described in Section 3.1 [1-6]. HSA is known to be modified as a result of the presence of glucose and related products in blood [7-12]. For instance, glucose can react with free amino groups on HSA through a non-enzymatic process known as glycation to form a Schiff base that can then be converted into a stable fructosamine, as described in Section 3.1 [7]. Glyoxal, a type of α-oxoaldehyde, can be created from the oxidation of these glycated protein adducts, as well as through the auto-oxidation of glucose [13]. Glyoxal can then react with arginine or lysine residues on a protein such as HSA to form advanced glycation end-products (AGEs), as illustrated in Figure 5-1 [13-16]. Examples of these products are Nε-carboxymethyl-lysine and glyoxal-derived hydroimidazolone isomer 1, as shown in Fig. 5-2. The formation of these AGEs in proteins is related to many of the complications that originate from high glucose levels, including atherosclerosis, retinopathy, nephropathy, and neuropathy [17-18]. This study will utilize high-performance affinity chromatography (HPAC) to examine the binding of chlorpropamide, gliclazide, and tolazamide to HSA that has been modified with various physiological levels of glyoxal.

In this chapter, both the global and site-specific changes in the interactions of chlorpropamide, gliclazide, and tolazamide with glyoxal-modified HSA will be
investigated by using HPAC. A comparison will be made between these results and prior measurements made with normal or glycated HSA [4-6] to see how alterations in the modifications of HSA or in the structure of these compounds affect these interactions. This information, in turn, should be useful in future work aimed at creating customized treatments based on these and other sulfonylurea drugs for personalized medicine [1].

5.2 Experimental

5.2.1 Chemicals

The gliclazide (≥ 99.9% pure), R-warfarin (≥ 97%), L-tryptophan (≥ 98%), HSA (product A1887, from human serum, essentially fatty acid free, ≥ 96%), sodium dodecyl sulfate (≥ 99%), sodium tetraborate (≥ 99%), and glyoxal were from Sigma-Aldrich (St. Louis, MO, USA). The chlorpropamide (≥ 99%) and tolazamide (≥ 99%) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The o-phthalaldehyde (OPA) (≥ 99%), 9,10-phenanthrenequinone (≥ 98%) were from TCI America (Portland, OR, USA). The β-mercaptoethanol (≥ 98%) was from Alfa Aesar (Ward Hill, MA, USA). The Nucleosil Si-300 (pore size, 300 Å; particle size, 7 µm) was acquired from Macherey-Nagel (Duren, Germany). The bicinchoninic acid (BCA) protein assay reagents were from Pierce (Rockford, IL, USA). Water that had been purified by a Milli-Q-Advantage A 10 system (EMD Millipore, Billerica, MA, USA) was used to make all the aqueous solutions and mobile phases that were utilized in this research. These solutions were filtered by passing them through 0.20 µm GNWP nylon membranes from Fisher Scientific (Pittsburgh, PA, USA).

5.2.2 Instrumentation
A Jasco (Tokyo, Japan) HPLC system was used in this report, as described in Section 3.2.2.

5.2.3 Preparation of glyoxal-modified HSA

HSA was modified with glyoxal by adapting previous conditions that have been used to prepare glycated HSA in vitro [19]. This modification was carried out under sterile conditions and using 1 mM sodium azide as a buffer additive to prevent bacterial growth [1-6]. All reaction mixtures were made in pH 7.4, 0.067 M potassium phosphate buffer that contained a typical physiological concentration of normal HSA (42 g/L) and either 130 nM or 210 nM of glyoxal (i.e., representing glyoxal levels in serum seen in healthy individuals or those with controlled diabetes, respectively) [20-21]. These conditions gave modified forms of HSA that are referred to in this paper as GoHSA1 and GoHSA2. Each of these mixtures was incubated for about 4 weeks at 37 °C to allow the modification reaction to occur. A desalting column was then used to remove the excess and unreacted glyoxal [36], followed by dialysis against water using Slide-A-Lyzer dialysis cassettes from Pierce. The resulting samples of modified HSA were then lyophilized and stored at -80 °C. The level of modification for the HSA samples was determined by assays for free lysine or arginine residues, as described previously [22-23]. In the assay for free arginine residues, a 1 mg/mL solution of the modified HSA was prepared in pH 7.4, 0.067 M potassium phosphate buffer, with a solution of normal HSA being prepared in the same manner as a reference. A 1.0 mL portion of each protein solution was then combined with 3.0 mL of a 0.1 mg/mL solution of 9,10-phenanthrenequinone in absolute ethanol, with 0.5 mL of 2 N sodium hydroxide then being added to this mixture and incubated in the dark for 3 h at 60°C. The 9,10-
phenanthrenequinone solution was prepared immediately before each assay because it is light sensitive and unstable at room temperature [22]. After this incubation step, 0.5 mL of 1.2 N HCl was added to the solution in the dark and gently mixed by shaking. The new combined solution was transferred to a cuvette and analyzed by making fluorescence measurements at excitation wavelength of 312 nm and an emission wavelength of 395 nm [22]. Each sample measurement was carried out in quadruplicate.

In the assay for free lysine residues [23], a 0.25 mM solution of normal HSA or modified HSA was prepared in pH 7.4, 0.067 M potassium phosphate buffer that also contained 2.5 mM sodium azide as an antibacterial reagent. OPA (density, 1.13 g/mL) was dissolved in 1 mL of 95% ethanol at a concentration of 40 mg/mL; this solution was then mixed with 25 ml of 0.10 M sodium tetraborate in water and 2.5 mL of 20% (w/w) sodium dodecyl sulfate (SDS) in water. A 0.10 mL aliquot of β-mercaptoethanol was then added to this mixture. This reagent was stored in an amber bottle (i.e., to protect it from light) and with minimal air space, giving a working solution that was stable for several days [23]. A 50 µL portion of the 0.25 mM protein solution was then combined with 3.0 mL of the OPA reagent and allowed to react for 10 min in the dark at room temperature. Fluorescence measurements were carried out at an excitation wavelength of 340 nm and an emission wavelength of 455 nm [23] using normal HSA in water as the blank. The lysine modification levels of the protein samples were determined indirectly by frustosamine assay results of glycated HSA samples based on a comparison of absorbance values between the AGE samples, glycated HSA and normal HSA.

5.2.4 Column preparation
The affinity microcolumns were prepared in the same manner as described in Section 3.2.3.

5.2.5 Chromatographic studies

The solutions of gliclazide, L-tryptophan, R-warfarin and sodium nitrate were prepared in pH 7.4, 0.067 M phosphate buffer, which was also used as the mobile phase for the chromatographic studies (Note: this buffer was selected because it has been routinely used in prior studies to examine binding by other sulfonylurea drugs with HSA) [2-6,24]. This buffer was passed through a 0.2 µm filter and degassed for 10-20 min prior to its use. The solutions of R-warfarin were used within two weeks of preparation, and the L-tryptophan solutions were used within 1-2 days, based on previous studies examining the stability of such solutions [24-26]. All the chromatographic experiments were performed at 0.50 mL/min and 37 °C. The back pressure for the microcolumns under these conditions was 2.0 MPa (290 psi) or less. As noted previously, no significant changes in the binding parameters that were measured in this work were seen when using other flow rates as long as sufficient time was applied for a local equilibrium to be established between the immobilized binding agent and the injected/applied drugs and probe compounds [1-5].

The overall binding between sulfonylurea drugs and HSA were investigated by using zonal elution which was carried out by saturating an anti-HSA immunoaffinity microcolumn with 5.0 µM of controlled Go-modified HSA or diabetic Go-modified HSA. In this saturation process, any non-bound protein was removed by applying pH 7.4, 0.067 M potassium phosphate buffer alone for 30.0 min at 0.10 mL/min. After equilibrating the column, 20.0 µL of each sulfonylurea drug was injected onto the retained HSA in the
antibody column using a concentration of 10.0 µM at a flow rate of 0.10 mL/min at 37oC, this was performed in triplicates. Detection of each sulfonylurea drug was performed at its highest absorbance wavelength. The detection wavelengths were as follows: tolzamide, gliclazide, 226 nm; tolbutamide, 228 nm; chlorpropamide, 231 nm; and warfarin, 308 nm. All injections were made in triplicate. The microcolumn was regenerated after every 20 injection of these drugs samples by applying pH 2.5 0.10 M potassium phosphate buffer at 0.10 mL/min for 30 min. Control experiments were conducted by injecting the sulfonylurea drugs onto the anti-HSA column in the absence of HSA to measure possible interactions between the drugs and the immobilized antibodies. The retention factor (k) of each drug was then tabulated by subtracting any possible extra-column interactions (i.e. antibody-drug and support-drug interactions) from HSA-drug interactions.

The competition studies based on zonal elution were carried out by using R-warfarin (i.e., a probe for Sudlow site I) and L-tryptophan (i.e., a probe for Sudlow site II) [24]. These studies were performed with eight different concentrations of gliclazide in the mobile phase, spanning from 0 to 20 µM. The same solutions of gliclazide were used to make samples of the desired probe at a probe concentration of 5 µM (Note: the sulfonylurea drugs were added to minimize changes in the local concentration of this drug in the mobile phase, and the corresponding changes in the background response during sample injection, as has been used in prior studies with other sulfonylurea drugs) [2-6,24]. The injection volume for all samples was 20 µL. The probe samples were injected onto each type of microcolumn while the probe was monitored at 308 nm for R-warfarin or 280 nm for L-tryptophan. A 20 µM solution of sodium nitrate, which was
used as a non-retained solute for the HSA and glycated HSA supports [24], was also injected onto each microcolumn, as well as onto the chromatographic system with no microcolumn present; this solute was monitored at 205 nm. Each zonal elution experiment was carried out in quadruplicate, and the mean retention time of each peak was determined by using PeakFit 4.12.

5.3 Results and discussion

5.3.1 Characterization of Go-modified HSA and related supports

It is known that glyoxal can react with both lysine and arginine residues on proteins such as HSA [16]. These levels of modification were determined by using assays based on OPA and 9,10-phenanthrenequinone, respectively [22,23]. The modification levels of lysine in the samples of GoHSA1 and GoHSA2 were 2.6 (± 0.1) % and 4.2 (± 0.1) %. A 1.62-fold increase can be found in the relative levels change when comparing GoHSA2 with GoHSA1. The levels of arginine modification for GoHSA1 and GoHSA2 samples were 2.9 (± 0.4) % and 5.7 (± 0.3) %, respectively. A 1.97-fold increase relative change can be observed in the modification levels on GoHSA2 when compared to GoHSA1, which is consistent with levels of AGE modification on arginine that have been found on bovine serum albumin (BSA) under similar reaction conditions [27]. Examples of residues where the highest levels of these and other AGE modifications have been reported to occur on HSA include R521, R197, R117 and R428, as well as K525, K414, K51 and K439 [48]. The protein content of the supports that were prepared with these modified forms of HSA were also evaluated. These supports were found to contain 89 (± 3), 55 (± 2) or 69 (± 4) mg HSA/g silica for the samples of NHSA, GoHSA1 and GoHSA2, respectively. When these supports were placed into 1.0 cm × 2.1
mm i.d. microcolumns, they contained 0.86-1.08 mg (13-16 nmol) of GoHSA1 or GoHSA2. Each of these columns was found to be stable when used over the course of about 300 injections and had typical backpressures of 1.5-2.0 MPa (220-290 psi) at a flow rate of 0.5 mL/min.

5.3.2 Estimation of global binding for sulfonylureas with Go-modified HSA

The global affinities of each sulfonylurea drug with GoHSA1 and GoHSA2 were performed by using zonal elution on samples of these proteins that were adsorbed to an anti-HSA microcolumn. In this method, the retention factor ($k$) of each drug were obtained. These values were then used to calculate each sulfonylurea global affinity binding constant ($nK_a'$) using Eq. (5-1), where $m_L$ is the moles of the ligand and $V_m$ is the void volume of the column.

$$k = \frac{nK_a' m_L}{V_m}$$  \hspace{1cm} (5-1)

Based on Eq. (5-1), the values of $nK_a'$ values that were found for gliclazide, chlorpropamide and tolazamide on normal HSA and the samples Go-modified HSA are summarized in Table 5-1. The results that were obtained for chlorpropamide at pH 7.4 and 37°C with NHSA, GoHSA1 and GoHSA2 were 5.9 (± 0.5) × 10⁴ M⁻¹, 7.5 (± 0.9) × 10⁴ M⁻¹ and 7.8 (± 0.9) × 10⁴ M⁻¹, respectively. The values for these same protein samples with gliclazide were 8.0 (± 1.2) × 10⁴ M⁻¹, 5.2 (± 0.8) × 10⁴ M⁻¹ and 6.4 (± 0.5) × 10⁴ M⁻¹, while the values obtained with tolazamide were 2.8 (± 0.6) × 10⁴ M⁻¹, 3.4 (± 0.8) × 10⁴ M⁻¹ and 6.6 (± 0.8) × 10⁴ M⁻¹. It can be observed that there is a 1.3-fold increase in global affinity for chlorpropamide when comparing GoHSA1 to normal HSA and GoHSA2 to normal HSA, respectively. A similar increase of 1.2-fold and 2.4-fold were found for tolazamide in going from normal HSA to GoHSA1 and GoHSA2, respectively.
However, a decrease of 0.7-fold and 0.8-fold was observed in global affinity for gliclazide when comparing GoHSA1 to normal HSA and GoHSA2 to normal HSA, respectively. The changes that observed for chlorpropamide and tolazamide were consistent with the results obtained from sulfonylurea drugs when comparing normal HSA to glycated HSA at various levels (i.e., 1.6-fold increase for chlorpropamide, 1.1-fold increase for tolazamide) [2-6].

5.3.3. Site-selective binding of sulfonylureas with HSA

5.3.3.1 General Approach

Zonal elution is the most prevalent form of affinity chromatography that is used to examine the binding of solute-protein interactions at a specific region [22]. In this study, competition study based on zonal elution experiments provides a more detailed examination of how the binding of gliclazide changed at Sudlow sites I or II of HSA due to advanced glycation. These experiments were conducted by injecting a site-specific probe (e.g., R-warfarin or L-tryptophan) onto an HSA or glycated HSA microcolumn as a solution of gliclazide with a known and constant concentration was passed through the same microcolumn. Some typical chromatograms from these experiments as shown in Fig. 5-3. The elution peaks of the probe compounds came out within 5 min or less under a flow rate of 0.50 mL/min. Sodium nitrate, as a non-retained solute, was also injected to determine the void time. The retention time and retention factor (k) for each probe was then calculated as the concentration of gliclazide was varied.

Eq. (5-2) can be used to describe this type of zonal elution system in which direct competition occurs at a single-site between an injected probe A and competing agent I. A
plot made of 1/k versus the molar drug concentration, [I], should follow the linear relationship that is given by Eq. (5-2) [49].

\[
\frac{1}{k} = \frac{K_a V_M [I]}{K_{aA} m_L} + \frac{V_M}{K_{aA} m_L}
\] (5-2)

The term \(K_{aA}\) and \(K_{aI}\) in Eq. (5-2) represent the association equilibrium constants for the probe and drug at their site of competition, while \(V_M\) is the void volume, and \(m_L\) is the apparent moles of the common binding sites the probe and drug have in the column. If competition occurs between the drug and probe at a single common site, the \(K_{aI}\) can be obtained from the ratio of the slope versus intercept for a plot made [28-30]. One advantage of this approach is it provides a value in the column for \(K_{aI}\) that is independent of the moles of active protein or binding sites as well as any other interaction regions that probes does not interact with, as has been noted to be the case in prior work with the site-selective probes that were employed in this study (i.e., \(R\)-warfarin and \(L\)-tryptophan) [28-30].

5.3.3.2 Competition studies at Sudlow site I

Competition studies based on zonal elution experiments were first carried out for Sudlow site I by using \(R\)-warfarin as site-selective probe [28-30]. This site was of interest because it has been found to bind to other second-generation and third-generation sulfonylurea drugs (i.e., glipizide, glibenclamide and glimepiride) on both normal HSA and glycated HSA [2-4]. In this study, competition study between \(R\)-warfarin with each drug was carried out on columns containing glyoxal modified HSA at various levels. The data were analyzed by use of Eq. (5-2), as illustrated in Figure 5-4(a). Linear plots were observed on both the GoHSA1 and GoHSA2 microcolumns, with correlation coefficients \((n = 7-8)\) that spanned from 0.9947-0.9989 for gliclazide, 0.9866-0.9988 for
chlorpropamide, and 0.9554-0.9689 for tolazamide. This behavior confirmed that the direct interactions between these drugs and R-warfarin at Sudlow site I on glyoxal-modified HSA. The average precision of the retention factors that were obtained in these measurements was ± 0.7% (range, ± 0.1-1.5%) for gliclazide, ± 0.3% (range, ± 0.1-0.5%) for chlorpropamide, and ± 0.5% (range, ± 0.1-1.3%) for tolazamide. Table 5-2 shows the association equilibrium constants for gliclazide that were measured at Sudlow site I on the GoHSA1 and GoHSA2 microcolumns. These values for gliclazide spanned from $2.0 \times 10^4$ M$^{-1}$ to $2.4 \times 10^4$ M$^{-1}$ at pH 7.4 and 37ºC, with precisions of ± 4.2-5.0% (average, ± 4.6%). The binding constants for chlorpropamide and tolazamide were in a general range of $5.1-5.4 \times 10^4$ M$^{-1}$, $2.7-8.8 \times 10^4$ M$^{-1}$, with precisions of ± 3.7-7.8% (average, ± 5.8%) and ± 14.8-15.9% (average, ± 15.4%), respectively, as shown in Table 5-2. Previous competition studies that were conducted in same manner gave an estimated binding constant of $1.9 (\pm 0.1) \times 10^4$ M$^{-1}$ for gliclazide at Sudlow site I of normal HSA, which is also listed in Table 5-1 for comparison [4]. It was found that there was no significant change in the binding affinity for gliclazide when comparing GoHSA1 to normal HSA. However, a 1.26-fold increase in binding strength was found for gliclazide at Sudlow site I in going from normal HSA to GoHSA2, which was significant at the 95% confidence level. Table 5-2 also shows the results for chlorpropamide and tolazamide at Sudlow site I on normal HSA column, as determined previously [12-13]. It was found that there is an increase in affinity for chlorpropamide of 1.38- or 1.31-fold when going from normal HSA to GoHSA1 or GoHSA2. A similar increase of 1.50-fold was observed in the affinity for tolazamide when comparing GoHSA1 to normal HSA, while a 4.9-fold increase in the affinity was seen for tolazamide in going from normal HSA to GoHSA2.
All these differences were significant at the 95% confidence level. Such changes in binding strength are believed to reflect the different amounts and types of modification that occur on HSA as its level of AGE formation is increased [1-7,31-34]. Look back to our previous work on glycated HSA, chlorpropamide had a similar tendency (i.e. 1.2-fold increase). However, the changes for tolazamide and gliclazide are reversed when comparing the results that obtained from glycated HSA (i.e. 0.8-fold decrease for tolazamide, 1.9-fold increase for gliclazide) [9-13]. Examples of specific modification sites for AGE formation that have been found to occur at or near Sudlow site I include K199, K281, K276 and R197 [31-34].

4.3.3.3. Competition studies at Sudlow site II

Competition studies were also used to examine the interactions of each drug at Sudlow site II by using L-tryptophan as a site-selective probe for this region [1-5, 30]. Sudlow site II was of interest in this work because previous studies with gliclazide have suggested that this region has a relatively high affinity for this drug on both normal and glycated HSA [11]. This site is also known to bind other second-sulfonylurea drugs [1-3]. The results for each of drug that was examined in this study gave a linear plot for both GoHSA1 and GoHSA2 when the data were plotted according to Eq. (5-2). An example of such a plot is provided in Figure 5-4(b). The correlation coefficients (n = 7-8) for these plots ranged from 0.9606-0.9977 for gliclazide, 0.9837-0.9956 for chlorpropamide, and 0.9730-0.9879 for tolazamide. This behavior indicated that all of these drugs had direct interactions at Sudlow site II on both GoHSA1 and GoHSA2. The retention factors used to generate these plots had an average precision of ± 0.7% (range, ± 0.1-2.0%) for
gliclazide, ± 0.9% (range, ± 0.1-2.0%) for chlorpropamide, and ± 0.4% (range, ± 0.1-0.7%) for tolazamide.

Table 5-1 shows the association equilibrium constants that were measured at Sudlow site II during these experiments for gliclazide with the samples of glyoxal-modified HSA. These values were in the range of 3.6-4.3 × 10^4 M^-1 at pH 7.4 and 37°C and had precisions of ± 2.3-13.8% (average, ± 8.1%). The binding constant for gliclazide at Sudlow site II of normal HSA is also listed in Table 5-1 [11]. It was found through this comparison that modification with glyoxal gave a decrease in the affinity of gliclazide at Sudlow site II. In this case, there was a 1.40-fold decrease in the affinity for gliclazide at Sudlow site II in going from normal HSA to GoHSA1 and a decrease of 1.67-fold in going from normal HSA to GoHSA2, with all of these differences being significant at the 95% confidence level.

Table 5-2 provides the measured binding constants at Sudlow site II for chlorpropamide and tolazamide on the Go-modified samples of HSA. The values obtained for $K_a$ were in a range of 4.4-6.2 × 10^4 M^-1 for chlorpropamide and 2.8-3.2 × 10^4 M^-1 for tolazamide, with precisions of ± 4.8-9.1% (average, ± 7.0%) and 10.7-15.6% (average, ± 13.2%), respectively. The results that have been obtained for the same drugs with normal HSA column are also listed in Table 5-2 [12-13]. When comparing these results, there was a 2.20-fold increase in the affinity for chlorpropamide at Sudlow site II for GoHSA1 vs. normal HSA and an increase of 3.10-fold in going from normal HSA to Go-HSA2. Tolazamide gave 1.47-fold and 1.68-fold increases when going from normal HSA to GoHSA1 or GoHSA2, respectively. All of these differences were significant at the 95% confidence level. These changes in binding were again probably a result of the
different amounts and types of modifications that occurred on these HSA samples [1-5,31-34]. For instance, K439 and R428 are major sites for AGE formation that occur at Sudlow site II of HSA [31-34].

A closer examination of overall binding affinity for each sulfonylurea drug on each type of HSA was performed by combining the results at both Sudlow sites I and II. The combined association equilibrium constants for gliclazide, chlorpropamide and tolazamide with GoHSA1 were $6.3 \pm 0.1 \times 10^4 \text{ M}^{-1}$, $9.8 \pm 0.4 \times 10^4 \text{ M}^{-1}$ and $5.5 \pm 0.5 \times 10^4 \text{ M}^{-1}$. The corresponding values with GoHSA2 were $6.0 \pm 0.5 \times 10^4 \text{ M}^{-1}$, $11.3 \pm 0.5 \times 10^4 \text{ M}^{-1}$ and $12.0 \pm 1.5 \times 10^4 \text{ M}^{-1}$, respectively. When compared with normal HSA, there was a 1.7-fold or 2.0-fold increase in the combined association equilibrium constants for chlorpropamide and tolazamide on GoHSA1; however, there was a decrease of 0.8-fold for gliclazide with the same preparation of Go-modified HSA. There were larger increases of 1.9-fold and 4.8-fold in these combined association equilibrium constants for chlorpropamide and tolazamide in going from normal HSA to GoHSA2, respectively; gliclazide again gave a 0.75-fold decrease for the Go-modified HSA. All of these changes were significant at the 95% confidence level. These changes gave similar trends with the results obtained from zonal elution studies based on the immunoaffinity microcolumn in Section 4.3.2 (i.e., 1.27-fold to 1.32-fold increase for chlorpropamide, 1.2-fold to 2.4-fold increase for tolazamide and 0.7-fold to 0.8-fold decrease for gliclazide in going from normal HSA to GoHSA1 and GoHSA2, respectively).

4.4. Conclusion

HPAC was used in this work to investigate the binding of first- and second-generation sulfonylurea drugs, to HSA at levels of advanced glycation corresponding to
health adults or patients with diabetes. Zonal elution-based competition studies were used to provide more site-specific information on the interactions between these drugs at Sudlow sites I and II of normal HSA or AGE-modified HSA. The drugs in this study were found to bind to both Sudlow sites I and II, as has been noted for other sulfonylurea drugs [1-3]. Advanced glycation levels like those found in patients with diabetes gave a slight increase in the binding strength of gliclazide at Sudlow site I by up to 1.26-fold and an apparent decrease in binding strength at Sudlow site II of 1.67-fold when compared to the binding observed for normal HSA. There was no significant difference in the binding of gliclazide at Sudlow site I but a 1.4-fold decrease at Sudlow site II in going from normal HSA to some of the Go-modified HSA. Similar results were obtained for chlorpropamide and tolazamide.

Affinity microcolumns were used for examining drug-protein interactions in this report. The small size of the affinity microcolumns made it possible to carry out detailed binding studies in a short time (minutes) and with systems up to affinities of $10^5 \text{ M}^{-1}$ under isocratic conditions. The small amount of HSA in the microcolumns and the ability to reuse the columns for hundreds of binding experiments made it practical to use this approach with modified forms of HSA. For instance, a single affinity microcolumn was used in this study for over 300 sample injections or application cycles, which was the equivalent of using less than 5 µg HSA per experiment. The approach used in this report is not limited to AGEs but could be employed in binding studies with other modified proteins or even proteins that have been isolated from individual clinical samples [1]. These features should result in further use of HPAC in bioanalysis and personalize medicine.
Acknowledgements

This work was supported by the National Institutes of Health under grant R01 DK069629.
Figure 5-1. The processes of early stage glycation and advanced stage glycation.
a. Process of Early Stage Glycation

D-Glucose + Protein ⇌ Schiff base → Amadori product

b. Process of Advanced Stage Glycation

α-oxoaldehydes (e.g. glyoxal) + Protein → advanced glycation end-product, or AGE glyoxal-derived hydroimidazolone, isomer 1
**Figure 5-2.** AGEs modification of (a) lysine and (b) arginine residue for the formation of glyoxal-modified HSA.
\[
\text{glyoxal-derived hydroidimidazole, isomer 1 (G-H1)}
\]

\[
\text{N}_{\text{c}}\text{-carboxymethyl-L-lysine (CML)}
\]
Figure 5-3. Typical chromatograms obtained by zonal elution competition studies on a 1.0 cm × 2.1 mm i.d. column containing diabetic Go-modified HSA and for the injection of R-warfarin in the presence of application of mobile phases that contained 20 or 0 µM gliclazide. The flow rate was 0.50 mL/min. These studies were performed at 37°C and pH 7.4.
Figure 5-4. Example of plots made according to Eq. (5-2) when using gliclazide as a competing agent in the mobile phase and (a) R-warfarin or (b) L-tryptophan as the injected probe on a column containing controlled Go-modified HSA. The best-fit lines in (a) and (b) had correlation coefficients of 0.9947 and 0.9977, respectively. The error bars represent ± 1 S.D.
**Table 5-1.** Overall association equilibrium constants ($nK_a'$) for the interactions of several sulfonylurea drugs with normal or Go-modified HSA.

Global affinity, $nK_a'$ (M$^{-1} \times 10^4$)$^a$

<table>
<thead>
<tr>
<th>Drug</th>
<th>Normal HSA</th>
<th>GoHSA1</th>
<th>GoHSA2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chlorpropamide</strong></td>
<td>5.9 (± 0.5) [5]</td>
<td>7.5 (± 0.9)</td>
<td>7.8 (± 0.9)</td>
</tr>
<tr>
<td><strong>Gliclazide</strong></td>
<td>8.0 (± 1.2) [4]</td>
<td>5.2 (± 0.8)</td>
<td>6.4 (± 0.5)</td>
</tr>
<tr>
<td><strong>Tolazamide</strong></td>
<td>2.8 (± 0.6) [6]</td>
<td>3.4 (± 0.8)</td>
<td>6.6 (± 0.8)</td>
</tr>
</tbody>
</table>

These results were measured at 37 °C in the presence of pH 7.4, 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ± 1 S. D ($n = 3$).
Table 5-2. Site-specific association equilibrium constants \((K_a)\) for the interactions of chlorpropamide, gliclazide, and tolazamide at Sudlow site I and II

<table>
<thead>
<tr>
<th>Type of drug &amp; HSA [Ref.]</th>
<th>Sudlow site I</th>
<th>Change vs. Normal HSA</th>
<th>Sudlow site II</th>
<th>Change vs. Normal HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpropamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal HSA [5]</td>
<td>3.9 (± 0.2)</td>
<td>-----</td>
<td>2.0 (± 0.4)</td>
<td>-----</td>
</tr>
<tr>
<td>GoHSA1</td>
<td>5.4 (± 0.2)</td>
<td>↑ 1.38-fold</td>
<td>4.4 (± 0.4)</td>
<td>↑ 2.20-fold</td>
</tr>
<tr>
<td>GoHSA2</td>
<td>5.1 (± 0.4)</td>
<td>↑ 1.31-fold</td>
<td>6.2 (± 0.3)</td>
<td>↑ 3.10-fold</td>
</tr>
<tr>
<td>Gliclazide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal HSA [4]</td>
<td>1.9 (± 0.1)</td>
<td>-----</td>
<td>6.0 (± 0.5)</td>
<td>-----</td>
</tr>
<tr>
<td>GoHSA1</td>
<td>2.0 (± 0.1)</td>
<td>↑ 1.05-fold</td>
<td>4.3 (± 0.1)</td>
<td>↓ 1.40-fold</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(N.S.)</td>
<td></td>
</tr>
<tr>
<td>GoHSA2</td>
<td>2.4 (± 0.1)</td>
<td>↑ 1.26-fold</td>
<td>3.6 (± 0.5)</td>
<td>↓ 1.67-fold</td>
</tr>
<tr>
<td>Tolazamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal HSA [6]</td>
<td>1.8 (± 0.3)</td>
<td>-----</td>
<td>1.9 (± 0.3)</td>
<td>-----</td>
</tr>
<tr>
<td>GoHSA1</td>
<td>2.7 (± 0.4)</td>
<td>↑ 1.50-fold</td>
<td>2.8 (± 0.3)</td>
<td>↑ 1.47-fold</td>
</tr>
<tr>
<td>GoHSA2</td>
<td>8.8 (± 1.4)</td>
<td>↑ 4.89-fold</td>
<td>3.2 (± 0.5)</td>
<td>↑ 1.68-fold</td>
</tr>
</tbody>
</table>

These results were measured at 37 °C in the presence of pH 7.4, 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ± 1 S. D., as based on error propagation and the precisions of the best-fit slopes and intercepts obtained when using Eq. (5-2) \((n = 7-8)\).

This value was not significantly different (N.S.) from that for normal HSA at the 95% confidence level.
5.5 REFERENCES


CHAPTER 6

CHROMATOGRAPHIC CHARACTERIZATION OF INTERACTIONS BY FIRST- AND SECOND-SULFONYLUREA DRUGS WITH METHYLGLYOXAL-MODIFIED ALBUMIN MICROCOLUMNS

6.1 Introduction

The binding between first- and second-generation sulfonylurea drugs with HSA and glycated HSA have been examined, as described in Chapters 3-5 [1-4]. The high levels of glucose in blood during diabetes can lead to the non-enzymatic modification of HSA through a process known as glycation. In this process, the free amino groups on HSA initially react with the aldehyde group of glucose to form a reversible Schiff base. This Schiff base can then convert to a stable Amadori product through rearrangement [5], as shown in Figure 5-1(a). Additional reactions can also occur to form advanced glycation end-products (AGEs) through pathways such as hydration, oxidation and cross-linking steps [6-7]. Reactive aldehydes are produced due to the degradation of Amadori products, and these molecules can directly react with arginine and lysine residues on a protein; this results in the formation of AGEs [6-8], as illustrated in Fig. 5-1(b).

Methylglyoxal (MGo) is a highly reactive aldehyde that is formed during these processes and which can combine with arginine and lysine residues on proteins to form AGEs [9]. One example is shown in Figure 6-1, in which MGo reacts with lysine and arginine to $N_c$-carboxyethyl-lysine (CEL) and methylglyoxal-derived hydroimidazolone isomer 1 (MG-H1), respectively. These types of AGEs have been found to be involved in
many of the complications that arise from hyperglycemia, including nephropathy, atherosclerosis, neuropathy and retinopathy [10, 11]. Recently, HSA has been applied in some research to examine how early stage glycation affects the structure of this protein and its interactions with drugs [1-4]. However, the effect of modifying HSA with MGo on these processes has not been examined in prior studies.

The purpose of this study is to see how the binding of sulfonylurea drugs such as gliclazide, chlorpropamide and tolazamide change with HSA that has undergone modification with MGo. These changes were examined by using high-performance affinity chromatography (HPAC), as described in Section 5.1. In this work, zonal elution will be employed with HPAC to examine the site-specific interactions of sulfonylureas with MGo-modified HSA. A comparison will then be made between the results and previous data obtained for the same drugs with normal, unmodified HSA. This comparison will make it possible to see how changes in the structure of proteins such as HSA can affect their binding processes [3]. The results should also provide a more complete picture on the effects of binding by sulfonylurea drugs to HSA during diabetes [3].

6.2. Experimental

6.2.1 Chemicals

The chlorpropamide (≥ 96% pure), tolazamide (≥ 96% pure), gliclazide (≥ 96% pure), and R-warfarin (≥ 97% pure) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). L-Tryptophan (≥ 98%), HSA (lyophilized powder, essentially fatty acid free, ≥ 96% pure), methylglyoxal (~40 wt% in water), sodium azide (> 95%) and D-(+)-glucose (99.5%) were obtained from Sigma Aldrich (St. Louis, MO, USA). The
bicinchoninic acid (BCA) assay kit was purchased from Pierce (Rockford, IL, USA) and used to measure the protein content of each support. All buffers and drug solutions were prepared using purified water from a Milli-Q-Advantage A 10 system, and all the aqueous solutions were filtrated through 0.20 µm GNWP nylon membranes (both of which were purchased from EMD Millipore, Billerica, MA, USA).

6.2.2. Instrumentation

The HPLC system utilized in this study was from Jasco (Tokyo, Japan), as described in Section 3.2.2.

6.2.3. Modification of HSA

Two samples of MGo-modified HSA were prepared for this study: MGoHSA1 and MGoHSA2. These protein samples were prepared in vitro by using 40 nM or 120 nM MGo and reaction conditions similar to those used in prior work to prepare glycated HSA [13]. The levels of MGo that were used to make these samples represented typical serum levels that are seen for this reactive aldehyde in healthy adults and patients with controlled-to-advanced diabetes [14]. Incubation of these solutions with a physiological level of HSA was carried out for about 4 weeks at pH 7.4 and 37 °C. This was followed by use of a desalting column to remove any excess and unreacted MGo. The MGo-modified HSA samples were then lyophilized and stored at -80 °C.

6.2.4. Column preparation

The columns which contained MGo-modified HSA were prepared in the same manner as stated in a previous work with normal or glycated HSA, as described in section 5.2.4 [4]. A control column containing no HSA was also made. The protein content of
each support was determined by using a BCA protein assay. In this assay, all the measurements were performed in triplicate; normal HSA was used as the standard, and the control support was used as the blank. The protein contents were found to be 51 (± 1) and 65 (± 5) mg HSA/g silica for the MGoHSA1 and MGoHSA2 columns, respectively. The extent of modification for the arginine and lysine residues in the MGoHSA1 and MGoHSA2 samples were determined by means of fluorescent assays using 9,10-phenanthrenequinone and o-phthalaldehyde (OPA) [4]. The protein supports were downward slurry packed into individual 1.0 cm × 2.1 mm i.d. stainless steel columns at a packing pressure of 3500 psi (24 MPa). In this work, potassium phosphate buffer (pH 7.4, 0.067 M) was used as the packing buffer. These microcolumns were found to contain 0.79-1.01 mg of modified HSA, as calculated by using the known packing density of the support (i.e., 0.45 g silica/cm³) and the support’s measured protein content. After packing, the columns and remaining supports (each kept the pH 7.4 phosphate buffer) were stored at 4 °C until use. As has been reported in prior work [1-4], these microcolumns were stable during the course of this entire study (i.e., over 300 sample injections) without any significant changes in their binding properties or drug-protein interactions.

6.2.5. Chromatographic studies

The chlorpropamide, tolazamide, gliclazide, L-tryptophan, R-warfarin and sodium nitrate solutions were prepared in pH 7.4, 0.067 M phosphate buffer, which acted as the mobile phase for the chromatographic studies (Note: this buffer resembles physiological conditions and has routinely been used to examine binding of other sulfonylurea drugs with HSA) [1-4, 14]. Before use, this buffer was passed through a 0.2 µm filter and
degassed for 10-20 min. All solutions containing \( R \)-warfarin were used within two weeks of preparation, and the \( L \)-tryptophan solutions were prepared and used within 1-2 days [15-16]. All the chromatographic experiments were performed at 0.50 mL/min and 37 °C. The back pressure for the microcolumns under these conditions was 2.0 MPa (290 psi) or less. No significant changes in the binding properties were observed when compared with similar experiments performed at other flow rates, provided that sufficient time was allowed for an equilibrium to be established between the immobilized binding agent and the injected/applied drugs and probe compounds (\( R \)-warfarin and \( L \)-tryptophan) [1-4].

Zonal elution study was conducted to evaluate the overall affinity between sulfonylurea drugs and HSA by utilizing an immunoaffinity microcolumn. Anti-HSA antibodies were covalently immobilized onto silica for this purpose. This immunoextraction microcolumn was initially equilibrated with pH 7.4, 0.067 M potassium phosphate buffer at 0.10 mL/min and 37ºC for 60 min. Application of MGoHSA1 or MGoHSA2 to this column was done by using 5.0 µM solutions that were passed through the anti-HSA microcolumn at 0.10 mL/min until a breakthrough curve was obtained. A syringe pump was employed for protein application in this step. Any non-bound protein was then removed by applying the application buffer at the same flow rate for 30 min. For zonal elution studies, a small plug of the desired drug in the same pH 7.4 buffer was injected. In this study, a 20.0 µL sample containing a 10.0 µM drug solution was injected onto chromatographic system; equivalent retention was seen for the injection of 2.5 µM drug samples, indicating that linear elution conditions were present. The detection wavelengths used in this work were 231 nm for chlorpropamide, and 226 nm for gliclazide or tolamazide. All injections were made in a triplicate. The
microcolumn was regenerated by using pH 2.5, 0.10 M potassium phosphate buffer at 0.10 mL/min for 30 min. Column regeneration was carried out after 20 drug sample injections in order to release the captured HSA. This process was repeated by passing a fresh solution of modified HSA to the immunoextraction column. Control experiments were conducted in the sample manner but with no MGoHSA1 or MGoHSA2 being passed through the immunoextraction microcolumn. These later experiments were used to measure any interactions that were present between the drugs and immobilized antibodies. The corrected retention factor (k) for each drug was found by subtracting the results obtained in the presence and absence of modified HSA on the immunoextraction microcolumn.

A competition method based on zonal elution was performed by using R-warfarin (i.e., a probe for Sudlow site I) and L-tryptophan (a probe for Sudlow site II) [15]. Eight concentrations of each sulfonylurea drug, ranging from 0 to 20 μM, were applied in the mobile phase to the column. The same drug solutions were used to make 5 μM solutions of the probe compounds. (Note: This was done to minimize variations in the background signal due to difference in the local concentrations of the drug in the mobile phase versus the sample) [1-4, 15]. The sample injection volume was 20 μL. The probe samples were injected onto each microcolumn while monitoring their absorbance at 308 nm for R-warfarin and 280 nm for L-tryptophan. A sample containing 20 μM of sodium nitrate was used as a non-retained solute, which was monitored at 205 nm [15]. Sodium nitrate was also injected onto the chromatographic system with no microcolumn present to obtain the system void time. All zonal elution experiments were carried out in quadruplicate, and the retention time of each peak was determined by using PeakFit 4.12.
6.3. Results and discussion

6.3.1. Determination of AGEs levels of MGo-modified HSA

The measured levels of arginine modification for the MGoHSA1 and MGoHSA2 samples were 5.6 (± 0.3) % and 13.1 (± 0.2) %, respectively. It is found that there is a 2.33-fold increase in relative change of modification levels when comparing MGoHSA2 with MGoHSA1, which was consistent with levels of modification for arginine that have been seen with BSA in prior studies [17-19]. The lysine modification levels for the MGoHSA1 and MGoHSA2 samples were 1.6 (± 0.1) % and 3.4 (± 0.1) %. A 2.11-fold increase can be observed in relative change of modification levels on MGoHSA2 when compared to MGoHSA1. Arginines R410, R114, R186 and R428 and lysines K525, K414, K12 and K233 have been reported to be the regions on HSA that undergo the highest levels of modification with agents such as MGo [20].

As evaluated through a BCA protein assay, the supports contained 51 (± 1) and 65 (± 1) mg protein/g silica for MGoHSA1 and MGoHSA2, respectively. When placed in microcolumns with dimensions of 1.0 cm × 2.1 mm i.d., about 0.80-1.02 mg (12-15 nmol) of these modified forms of HSA were present. These columns experienced backpressures of 1.5-2.0 MPa (220-290 psi) at a flow rate of 0.5 mL/min and were found to be stable over the course of 300 injections.

6.3.2 Estimation of global binding for sulfonylureas with MGo-modified HSA

The global affinities of sulfonylurea drugs used in this study were measured for MGoHSA1 and MGoHSA2 by using zonal elution, as described in section 5.3.2. Table 6-1 summarizes the \( nK_a' \) values that were obtained for gliclazide, chlorpropamide and tolvazamide on the MGo-modified HSA samples and compares these results with those for
normal HSA from the literature. All these values were obtained at 37 °C and using pH 7.4, 0.067 potassium phosphate buffer as the mobile phase. In the case of chlorpropamide, the values obtained were \(5.9 (\pm 0.5) \times 10^4\) M\(^{-1}\), \(7.6 (\pm 0.5) \times 10^4\) M\(^{-1}\) and \(10.3 (\pm 0.5) \times 10^4\) M\(^{-1}\), for normal HSA, GoHSA1 and GoHSA2 respectively. The values obtained with gliclazide were \(8.0 (\pm 1.2) \times 10^4\) M\(^{-1}\), \(12.6 (\pm 0.8) \times 10^4\) M\(^{-1}\) and \(8.4 (\pm 0.4) \times 10^4\) M\(^{-1}\) for these same protein samples, whereas tolazamide gave global affinity constants of \(2.8 (\pm 0.6) \times 10^4\) M\(^{-1}\), \(6.4 (\pm 0.7) \times 10^4\) M\(^{-1}\) and \(4.7 (\pm 0.5) \times 10^4\) M\(^{-1}\). About 1.3- to 1.8-fold increase in global affinity was obtained for chlorpropamide when comparing the values for MGoHSA1 and MGoHSA2 with that of normal HSA. In going from normal HSA to MGoHSA1, tolazamide showed a 2.3-fold increase in affinity, however this increase was around 1.7-fold for MGoHSA2. No significant increase was been observed for gliclazide on MGoHSA2 when compared to normal HSA. Gliclazide showed a similar trend to chlorpropamide and tolazamide, with an increase in global affinity of 1.6-fold between MGoHSA1 and normal HSA. Similar consistency was found for these sulfonylurea drugs when their changes in global affinities were compared to those seen previously for glycated HSA [1-4].

6.3.3 Site-selective binding of sulfonylureas with MGo-modified HSA

6.3.3.1 Theory

Zonal elution can also be used for the analysis of solute-ligand interactions at a specific region [19]. In this work, zonal competition elution was used which to provide detailed information about how the binding of the sulfonylurea drugs changed at the major binding regions on HSA (i.e., Sudlow sites I and II) due to modification with MGo,
as described in section 5.3.1. A typical chromatogram of zonal elution in this study, as shown in Figure 6.2.

6.3.3.2 Interactions at Sudlow site I

*R*-Warfarin was used as a site-selective probe for Sudlow site I during the competition studies and zonal elution experiments [21-24]. This site was of interest because other second-generation sulfonylurea drugs (i.e., glipizide and glibenclamide) have been found to bind here on both normal HSA and glycated HSA [1-2]. In this study, *R*-warfarin was used as a probe with mobile phases containing gliclazide, chlorpropamide and tolazamide and using columns containing MGoHSA1 or MGoHSA2. The data were analyzed by using Eq. (5-2), as illustrated in Figure 6-3(a). Linear relationships were observed for gliclazide on the MGoHSA1 and MGoHSA2 columns during these experiments, with correlation coefficients ranging from 0.9949 to 0.9952 (*n* =7-8). Similar linearities were found on these columns for chlorpropamide, with correlation coefficients of 0.9824 to 0.9927 (*n* =7-8), and for tolazamide, with correlation coefficients of 0.9035 to 0.9671 (*n* = 6-8). This behavior indicated that there was direct competition between these drugs and *R*-warfarin at Sudlow site I on the methylglyoxal-modified HSA. The average precision of the retention factors obtained in these measurements was ± 2.6% (range, ± 0.6-6.1%) for gliclazide, ± 1.8% (range, ± 0.2-4.2%) for chlorpropamide and ± 3.0% (range, ± 0.1-7.2%) for tolazamide. The association equilibrium constants that were obtained in these studies at Sudlow site I are provided in Table 6-1. The equilibrium constants for gliclazide on the MGoHSA1 and MGoHSA2 columns were $2.3 \times 10^4$ M$^{-1}$ and $4.9 \times 10^4$ M$^{-1}$ at pH 7.4 and 37 ºC, with precisions of ± 4.3-6.1% (average, ± 5.2%). Prior work that was conducted in same manner gave an
estimated binding constant of $1.9 \times 10^4 \text{ M}^{-1}$ for gliclazide at Sudlow site I of normal HSA, is also listed in Table 6-1 [1-4]. It was found that there was a 1.2-fold increase in the binding strength for gliclazide at Sudlow site I in going from normal HSA to MGoHSA1. A larger increase of about 2.6-fold occurred for gliclazide on the MGoHSA2 column vs. normal HSA. All of these differences were significant at the 95% confidence level. As shown in a previous study, the differences in binding is believed to reflect changes in the amounts and types of modification on HSA that were present [21-24]. K199, K281, K276 and R197 are prone to be specific modification sites due to glycation at or near Sudlow site I [25-28].

The results for chlorpropamide and tolazamide are also shown in Table 6-2. The $K_a$ values between chlorpropamide and MGoHSA1 or MGoHSA2 were $3.8 \times 10^4 \text{ M}^{-1}$ and $4.1 \times 10^4 \text{ M}^{-1}$, with precisions of $\pm 4.9-7.9\%$ (average, $\pm 8.4\%$). There was no significant change in affinity for chlorpropamide at Sudlow site I when comparing both samples of MGo-modified HSA to normal HSA (i.e., $3.9 \times 10^4 \text{ M}^{-1}$), as describe in section 3.3.2 [4]. Similar patterns were seen for tolazamide, with no significant change in binding for MGoHSA1 but a decrease in binding strength of 0.83-fold when comparing MGoHSA2 with normal HSA.

6.3.3.3 Interactions at Sudlow site II

L-Tryptophan was used as a site-selective probe for competition studies to examine the interactions by the sulfonylurea drugs at Sudlow site II of HSA [1-4]. Linear curves were obtained by plotting the data according to Eq. (5-2) from these sulfonylurea drugs with both samples of MGo-modified HSA. An example of such a plot is shown in Figure 6-3 (b). The correlation coefficients of these plots for gliclazide ranged from
0.9848 to 0.9975 (n = 7-8), which indicated that gliclazide had direct interactions at Sudlow site II with both MGoHSA1 and MGoHSA2. The retention factors for the probe I this case had an average precision of ± 5.4% (range, ± 1.0-11.2%). Direct competition at Sudlow site II was also found to occur through these plots for the first-generation drugs chlorpropamide and tolazamide on the MGoHSA1 and MGoHSA2 columns. The correlation coefficients for chlorpropamide ranged from 0.9474 to 0.9597 (n = 7-8) and the retention factors had an average precision of ± 6.2% (range, ± 1.3-16.2%). For tolazamide, the correlation coefficients ranged from 0.9568 to 0.9798 (n = 7-8) and the retention factors had an average precision of ± 6.6% (range, ± 0.9-16.1%). Table 6-2 includes the association equilibrium constants at Sudlow site II for gliclazide with the MGo-modified HSA samples. The constants ranged from 10.4-14.8 × 10^4 M^-1 at pH 7.4 and 37ºC. The precision of these values was ± 4.8-8.8% (average, ± 6.8%). The results for normal HSA, as based on prior work, is also listed in Table 6-2 for comparison [1-4]. A 1.2- to 2.6-fold increase was seen for gliclazide at Sudlow site II was observed when going from normal HSA to MGoHSA1 or MGoHSA, with all of these differences being significant at the 95% confidence level.

Information for the affinity of chlorpropamide and tolazamide at Sudlow site II on both the MGo-modified HSA columns are also shown in Table 6-2. The values for chlorpropamide ranged from 2.0-5.3 × 10^4 M^-1, with an average precision of ± 16% (range, 15-17%). For tolazamide, the affinity ranged from 1.8-2.5 × 10^4 M^-1 with an average precision of ± 11.5% (range, 11.1-12.0%). There was no significant change in affinity for either of these drugs when going from normal HSA to MGoHSA1. However, for MGoHSA2 there was an increase of 2.7-fold for chlorpropamide and an increase of
1.3-fold for tolazamide. These changes in binding were again probably due to the variations in the amount and types of modification on the HSA samples [3-4, 21-24]. For instance, K439 and R428 are known to be two major sites for AGE-based modifications that are at or near Sudlow site II of HSA [25-28]. By combining association equilibrium constants that were measured for Sudlow site I and II, it was possible to obtain a second estimate of the overall binding affinity for each drug with HSA. For gliclazide, chlorpropamide and tolazamide, these values were $17.1 \pm 1.3 \times 10^4$ M$^{-1}$, $5.8 \pm 0.4 \times 10^4$ M$^{-1}$ and $3.7 \pm 0.3 \times 10^4$ M$^{-1}$ for MGoHSA1. The corresponding combined equilibrium constants for MGoHSA2 were $15.3 \pm 0.6 \times 10^4$ M$^{-1}$, $9.4 \pm 0.9 \times 10^4$ M$^{-1}$ and $4.0 \pm 0.5 \times 10^4$ M$^{-1}$, respectively. These values were consistent with the estimated given in Table 6-1, indicating that Sudlow sites I and II were the major binding regions for these drugs with the MGo-modified forms of HSA.

6.4 Conclusion

This study examined the binding of several sulfonylurea drugs with HSA that had been modified with MGo at levels seen in healthy adults or in patients with diabetes. All of these drugs were found to bind to both Sudlow sites I and II, as has been noted for other sulfonylurea drugs with normal HSA [1-4]. Zonal elution-based competition studies were used to provide more site-specific information about the interactions between these drugs and MGo-modified HSA at Sudlow sites I and II. Significant changes were generally seen for these drugs with MGo-modified HSA that had been prepared under conditions to mimic those seen in controlled-to-advanced diabetes, with these changes ranging from a 0.83-fold decrease to a 2.7-fold increase in affinity. The methods used in this study are not limited to MGo-modified HSA but could be employed in binding
studies with other types of modified proteins or even proteins isolated from clinical samples [29].

**Acknowledgements**

This work was supported by the National Institutes of Health under grant R01 DK069629.
Figure 6-1. Examples of modifications of (a) lysine and (b) arginine residues that may occur during the formation of MGo-modified HSA.
advanced glycation end-product, or AGE methylglyoxal-derived hydroimidazolone, isomer 1

\[ \text{N}^\omega\text{-carboxyethyl-lysine (CEL)} \]
Figure 6-2. Typical chromatograms obtained by zonal elution competition studies on a 1.0 cm × 2.1 mm i.d. column containing diabetic MGo-modified HSA and for the injection of R-warfarin in the presence of application of mobile phases that contained 20 or 0 μM gliclazide. The flow rate was 0.50 mL/min. These studies were performed at 37°C and pH 7.4.
**Figure 6-3.** Example of plots made according to Eq. (5-2) when using gliclazide as a competing agent in the mobile phase and (a) R-warfarin or (b) L-tryptophan as the injected probe on a column containing controlled MGomodified HSA. The best-fit lines in (a) and (b) had correlation coefficients of 0.9952 and 0.9948, respectively. The error bars represent ± 1 S.D.
Table 6-1. Global affinity constants ($nK_a$') for the interactions of sulfonylurea drugs with normal or MGo-modified HSA.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Normal HSA</th>
<th>MGoHSA1</th>
<th>MGoHSA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpropamide</td>
<td>5.9 (± 0.5) [32]</td>
<td>7.6 (± 0.5)</td>
<td>10.3 (± 0.5)</td>
</tr>
<tr>
<td>Gliclazide</td>
<td>8.0 (± 1.2) [29, 30]</td>
<td>12.6 (± 0.8)</td>
<td>8.6 (± 0.4)</td>
</tr>
<tr>
<td>Tolazamide</td>
<td>2.8 (± 0.6) [31]</td>
<td>6.4 (± 0.7)</td>
<td>4.7 (± 0.5)</td>
</tr>
</tbody>
</table>

*aThese results were measured at 37 °C in the presence of pH 7.4, 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ± 1 S. D (n = 3).*
Table 6-2. Site-specific association equilibrium constants ($K_a$) for the interactions of chlorpropamide, gliclazide, and tolazamide at Sudlow site I and II

<table>
<thead>
<tr>
<th>Type of drug &amp; HSA [Ref.]</th>
<th>Sudlow site I $K_a$ (M$^{-1}$× 10$^4$)</th>
<th>Change vs. Normal HSA</th>
<th>Sudlow site II $K_a$ (M$^{-1}$× 10$^4$)</th>
<th>Change vs. Normal HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chlorpropamide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal HSA [32]</td>
<td>3.9 (± 0.2)</td>
<td>-----</td>
<td>2.0 (± 0.4)</td>
<td>-----</td>
</tr>
<tr>
<td>MGoHSA1</td>
<td>3.8 (± 0.3)</td>
<td>N.S.</td>
<td>2.0 (± 0.3)</td>
<td>N.S.</td>
</tr>
<tr>
<td>MGoHSA2</td>
<td>4.1 (± 0.2)</td>
<td>↑ 1.05-fold N.S.</td>
<td>5.3 (± 0.9)</td>
<td>↑ 2.7-fold</td>
</tr>
<tr>
<td><strong>Gliclazide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal HSA [29, 30]</td>
<td>1.9 (± 0.1)</td>
<td>-----</td>
<td>6.0 (± 0.5)</td>
<td>-----</td>
</tr>
<tr>
<td>MGoHSA1</td>
<td>2.3 (± 0.1)</td>
<td>↑ 1.2-fold</td>
<td>14.8 (± 1.3)</td>
<td>↑ 2.5-fold</td>
</tr>
<tr>
<td>MGoHSA2</td>
<td>4.9 (± 0.3)</td>
<td>↑ 2.6-fold</td>
<td>10.4 (± 0.5)</td>
<td>↑ 1.7-fold</td>
</tr>
<tr>
<td><strong>Tolazamide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal HSA [32]</td>
<td>1.8 (± 0.3)</td>
<td>-----</td>
<td>1.9 (± 0.3)</td>
<td>-----</td>
</tr>
<tr>
<td>MGoHSA1</td>
<td>1.9 (± 0.2)</td>
<td>↑ 1.05-fold N.S.</td>
<td>1.8 (± 0.2)</td>
<td>N.S.</td>
</tr>
<tr>
<td>MGoHSA2</td>
<td>1.5 (± 0.4)</td>
<td>↓ 0.83-fold</td>
<td>2.5 (± 0.3)</td>
<td>↑ 1.3-fold</td>
</tr>
</tbody>
</table>

*a*These results were measured at 37 °C in the presence of pH 7.4, 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ± 1 S. D., as based on error propagation and the precisions of the best-fit slopes and intercepts obtained when using Eq. (5-2) ($n = 7-8$).

*b*This value was not significantly different (N.S.) from that for normal HSA at the 95% confidence level.
6.5 References


CHAPTER 7

SUMMARY AND FUTURE WORK

7.1 Summary

HPAC was used in this thesis to investigate the binding of various sulfonylurea drugs to normal HSA, glycated HSA and AGE-modified HSA. The results from frontal analysis experiments and using a two-site binding model indicated that most of sulfonylurea drugs that were examined had relatively strong binding with normal HSA and glycated HSA. Zonal elution studies were used to provide information on the interactions between sulfonylurea drugs at Sudlow sites I and II on normal HSA, glycated HSA and AGE-modified HSA. These drugs were found to bind to both Sudlow site I and II, and it was also found that glycation or AGE modification could either increase or decrease the binding strength of these drugs to these sites. This data should provide a better insight in determining how effective the dosage of a sulfonylurea drug may change as the glucose concentration in blood and protein glycation levels vary in diabetic patients.

The first chapter provided a general introduction to HPAC and use of HPAC for investigating the effects of interactions between drug and protein due to glycation. The processes of protein glycation (i.e. early stage glycation and advanced stage glycation) was discussed. The change of structure and binding of HSA during glycation were also considered. Frontal analysis and zonal elution, as common chromatographic approach, were next introduced and utilized to demonstrate how HPAC could be used as a tool to provide information about interactions between drug and protein.
 Chapters 3-4 contained two studies which utilized HPAC to examine how glycation can affect the binding of two first-generation sulfonylurea drugs (i.e., chlorpropamide and tolazamide) to normal HSA and HSA with levels of glycation corresponding to controlled or advanced diabetes. Frontal analysis and zonal elution competition studies were applied and provided information about interactions between each drug with normal and glycated HSA. It was found that a 0.8- to 1.6-fold change in affinity can be observed for the interactions of the sulfonylureas at each Suldow sites on glycated HSA when comparing with HSA. These data should provide a better insight of glycation and provide useful information in altering the effective dose or free concentrations of these drugs and help in the use of these drugs for personalized medicine during the treatment of diabetes.

 Chapters 5-6 included several studies that involved the use of HPAC to examine the effects of advanced glycation end-product formation (i.e., glyoxal and methylglyoxal modification) on the binding of two first-generation sulfonylurea drugs (i.e., chlorpropamide and tolazamide) and a second-generation sulfonylurea drug (i.e., gliclazide) to HSA and samples of HSA with levels of advanced stage glycation (i.e., glyoxal and methylglyoxal modification) corresponding to healthy adults or patients with controlled diabetes. Immunoextraction zonal elution and zonal elution competition studies were applied and provided information about interactions between each drug with normal and glycated HSA. A 0.1- to 5.0-fold change in affinity was observed for the interactions of the sulfonylurea drugs at specific sites on AGE-modified HSA when compared to HSA. The results from immunoextraction zonal elution and competition studies indicated that modifications with methylglyoxal and glyoxal could significantly
affect the binding of these drugs to HSA. These results provided a better understanding of how HSA advanced stage glycation can affect the transport and protein binding of sulfonylurea drugs in serum, as well as information that can be used in future studies aimed at personalized medicine based on these agents. The approach used in this report could be employed in binding studies with other modified proteins or proteins that have been isolated from individual clinical samples [1].

In this study, frontal analysis and zonal elution competition method were both used to examine the interaction between sulfonylurea drugs and HSA with various levels of glycation. Frontal analysis could provide higher accuracy in the results for overall binding (i.e. the correlation coefficient value can reach to 0.9995). However, this chromatographic method required longer experimental times and larger amounts of targets. Zonal elution competition was of interest due to its faster analysis time and ability to work with smaller amounts of an analyte. The zonal elution method could also provide more binding information on the specific location of protein binding sites. Thus, zonal elution could be used to help us understand more about how glycation affects each major binding site of HSA in this research.

7.2 Future work

The work in this thesis described the development and employment of HPAC and microcolumns for examining binding of various sulfonylurea drugs with proteins. The use of HPAC is not limited to determine the binding of chlorpropamide, tolazamide and gliclazide but could be used with other second-generation or third-generation sulfonylurea drugs (i.e. glipizide, glibenclamide and glimepiride) with glyoxal-modified HSA and methylglyoxal-modified HSA. The binding of other anti-diabetic drugs (i.e.,
nateglinide, repaglinide, rosiglitazone and pioglitazone) to AGE-modified forms of HSA could also be examined by using HPAC and affinity microcolumns. Another approach may include the development of new immobilization methods to immobilize drug analogs and use these instead of proteins in HPAC microcolumns for binding studies. Through this approach, drugs with mild to strong binding affinities could be tested in their ability to extract transport proteins from serum. The immobilized drugs could also be tested to see if they are capable of separating different types of modified proteins. This approach could then be used to identify and separate modified proteins that are present in clinical samples, which might then be used with mass spectrometry to identify key changes in structure that may underlie the observed changes in binding strength.

7.3 References