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# DEVELOPMENT OF ENTRAPMENT COLUMNS FOR THE STUDY OF AFFINITY BASED ANALYSIS OF DRUG-PROTEIN INTERACTIONS

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# DEVELOPMENT OF ENTRAPMENT COLUMNS FOR THE STUDY OF AFFINITY BASED ANALYSIS OF DRUG-PROTEIN INTERACTIONS

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# DEVELOPMENT OF ENTRAPMENT COLUMNS FOR THE STUDY OF AFFINITY BASED ANALYSIS OF DRUG-PROTEIN INTERACTIONS

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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. Because of the strong and selective nature of many biological interactions, this method has already become a powerful technique for the purification and analysis of solutes that are complementary to the immobilized binding agent. Human serum albumin (HSA), the most abundant protein in the blood with concentrations of 35-50 mg/mL in serum, has interactions with many drugs, which can affect the absorption, distribution, metabolism and excretion of such agents.

The overall goal of this thesis is to examine the use of on-column entrapment methods based on hydrazide-activated silica and oxidized glycogen as a capping agent for the immobilization of proteins as affinity ligands in HPAC. Although this general type of entrapment method has been previously examined reported by our group, this method still needs further optimization for its use in an on-column format and in new applications based on HPAC. For example, it is necessary to conduct studies to further increase the amount of the entrapped affinity ligand that can be obtained by using alternative types of supports.

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## TABLE OF CONTENTS

**CHAPTER 3 MONOLITH AS A SUPPORT FOR THE ENTRAPMENT OF HSA**

## CHAPTER FOUR: SUMMARY AND FUTURE WORK

# CHAPTER 1

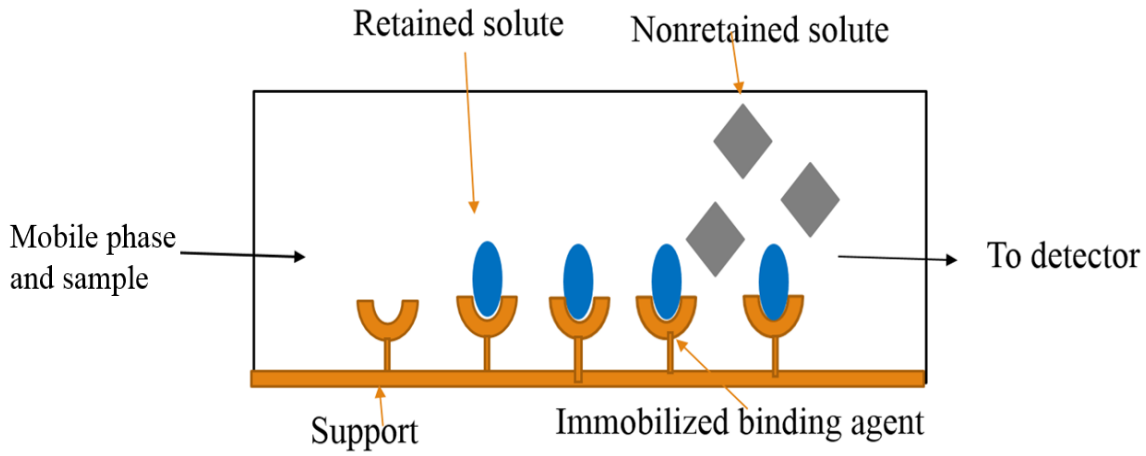
# GENERAL INTRODUCTION

## 1.1. High-performance Affinity chromatography

Affinity chromatography is a liquid chromatographic technique that utilizes biologically-related interactions [1]. This method uses the selective, reversible interactions that are characteristic of most biological systems as shown in Fig. 1.1. These biological interactions are used in affinity chromatography to separate or analyze different sample components [1,2]. The stationary phase in this method is a biological agent, or "affinity ligand", that can placed within a support by methods such as covalent coupling, entrapping, or absorbing this agent within or on the support [1,2]. The solid support and stationary phase are then packed within a column which can be used for the separation or analysis of target molecules that can bind to the affinity ligand [3-5].

The retention and separation of the sample components in affinity chromatography is based on the specific and reversible interactions that are characteristic of many biological interactions [1]. Some typical examples of these interactions include the binding of an antibody with an antigen, an enzyme's binding with a substrate or inhibitor, the binding of a hormone with a receptor, or the binding of lectin with a polysaccharide [1,8]. The ineractions that occur between the affinity ligand and target molecules can be due to electrostatic interactions, van der Waals' forces, dipole-dipole interactions, or hydrogen bonding, among others, and may also involve steric effects [8]. Traditional affinity chromatography uses inexpensive supports with low-to-moderate efficiencies, like agarose gels or carbohydrate-based substances [3,5]. To have

**Figure 1.1.** A typical system utilized in affinity chromatography or high-performance affinity chromatography. In this approach an analyte is applied to the affinity column in an appropriate application buffer. Molecules that are complementary to the immobilized affinity ligand are allowed to bind to the column, and the non-retained sample components are eluted from the column. Next, an elution buffer is applied that will elute the bound target for collection, measurement or characterization. The application buffer is then re-applied to regenerate the column.


Retained solute
Nonretained solute
Mobile phase
and sample
To detector
Support
Immobilized binding agent

sufficient mechanical stability and efficiency that is needed for use in the high pressure in HPLC, the support must be selected and optimized acordingly [3,5]. The use of an HPLC- type of support, results in a method known as high-performance affinity chromatography (HPAC), which has better precision and a much faster speed than are usually present in traditional affinity chromatography [3,5]. Automation with the use of HPLC systems also is relatively easy to accomplish in HPAC [1,4,5].

Modified silica or glass and hydroxylated polystyrene media are some of the materials used as a supports for HPAC [1]. Several types of monolithic supports have also been used for use in HPAC, such as those based on organic polymers, silica monoliths, cryogels and modified forms of agarose [6,7]. Some of the useful features of these supports include their rapid mass transfer, low back pressures, and ability to be made in many shapes and sizes for use in affinity-based separations [8].

## 1.2. QUANTITATIVE AFFINITY CHROMATOGRAPHY

In addition to the use of affinity chromatography and HPAC for the isolation of substances or their analysis, these methods can also be used to study the interactions between a ligand and chemicals [9,10]. The use of HPAC or affinity chromatography for this purpose is referred to as analytical affinity chromatography, quantitative affinity chromatography, or biointeraction chromatography [1-3]. By collecting data on the retention time or retention volume of an applied solute, it is possible to the determine such factors as the equilibrium constants and binding capacity for an analyte with an immobilied ligand. Using additional solutes in the mobile phase as competetive binding agents can also provide information on how

the inject and applied solutes may compete for the affinity ligand as they both undergo solute-ligand interactions [1-3,9,10].

The main convenience of using HPAC for the analysis of solute-ligand binding is the ability to reuse a single affinity column for multiple experiments. This reduces the amount of affinity ligand that is needed and minimizes the cost required per analysis. Using the same affinity ligand preparation for many experiments also improves the reproducibility and precision by decreasing batch-to-batch and run-to-run variations [11].

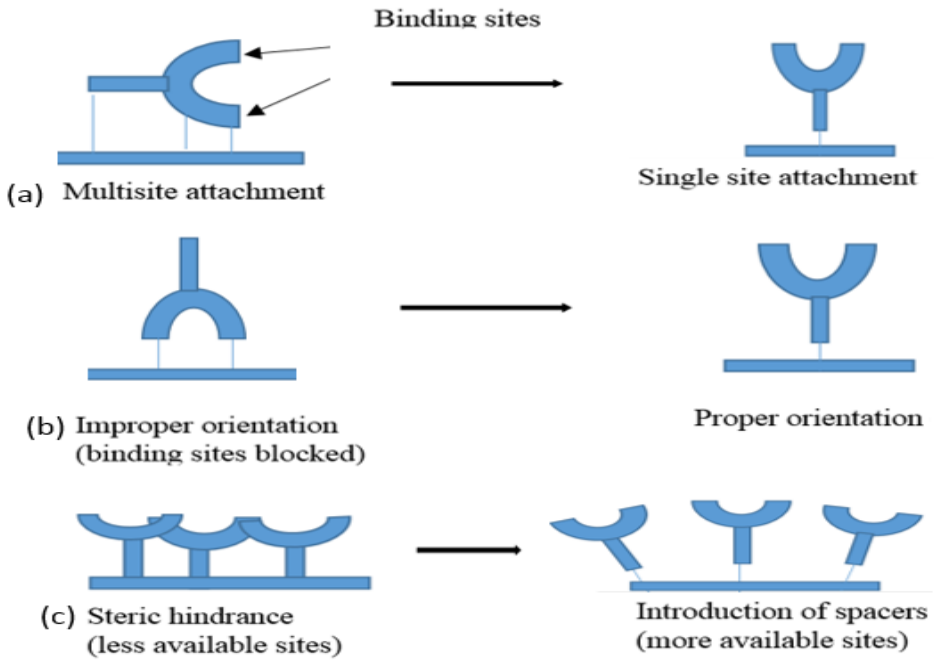
One experimental technique that is commonly used in HPAC for studying biological interactions is zonal elution. This method involves making small injections of an analyte into an affinity column that contains an immobilized ligand. The outcome of this experiment is a peak for the injected compound that can be monitored by on-line detection. The most common parameter that is obtained from this experiment and chromatogram is the retention time of the injected analyte, which is then used to help determine the strength of this binding or the identify the type of binding that is occuring for this analyte to the affinity ligand [1,2].

## 1.3. IMMOBILIZATION TECHNIQUES

Immobilization refers to the means by which an affinity ligand is attached to the chromatographic support. This is another important factor to consider in HPAC. The ideal immobilization method should not alter or denature the binding agent and should not adversely affect this agent's activity [6]. Examples of some undesirable effects that can occur are improper orientation or multisite attachment of the binding agent and steric hindrance for the target as it attempts to interact with this immobilized affinity ligand (see Figure 1.2) [14]. There are various

approaches that can be used for the immobilization of binding agents in affinity chromatography. These include covalent immobilization, biospecific adsorption, and entrapment [6].

**Figure 1.2.** Non-ideal effects that can occur during ligand immobilization. Covalent immobilization techniques can result (a) multisite attachment, where the ligand is immobilized through multiple sites; (b) improper orientation, where binding sites may be obstructed and not available for binding; or (c) steric hindrance, where active sites on the affinity ligand are blocked by the support or by neighboring ligands.


Binding sites
(a) Multisite attachment
Single site attachment
(b) Improper orientation
(binding sites blocked)
Proper orientation
(c) Steric hindrance
(less available sites)
Introduction of spacers
(more available sites)

The support that holds the ligand within the column must be in a form that can be easily modified for ligand attachment and should have minimal nonspecific binding. The amount of ligand that can be immobilized will determine binding capacity and degree of analyte retention by the support material, and the type of both the support and ligand will determine the mobile phase conditions that can be used with the column. The pore size is also important to consider. As the pore size increases, the surface area of the support material will decrease and this will a give smaller surface area for the ligand to be attached to the surface of the support. However, a pore size that is too small may also be inaccessible to the affinity ligand for immobilization [1].

## 1.4. ENTRAPMENT

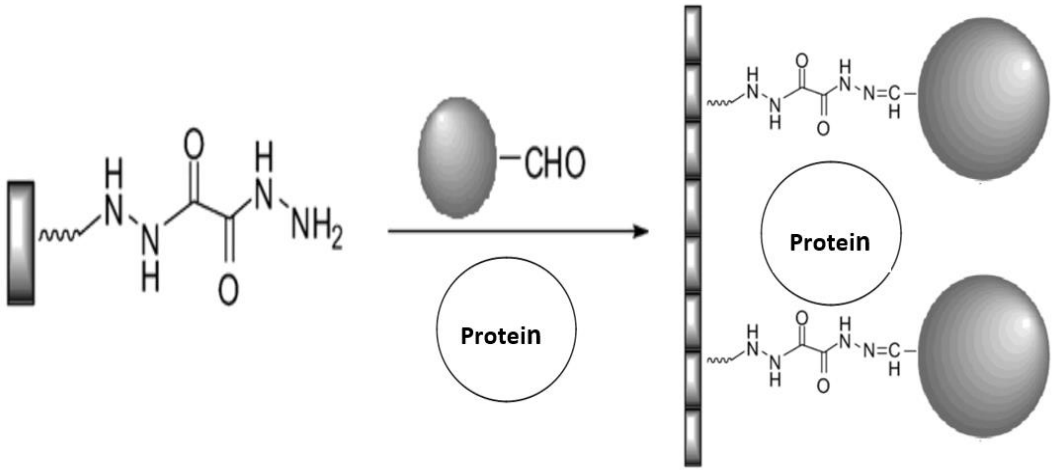
Entrapment refers to the physical containment or encapsulation of an affinity ligand in a support that contains small pores or a highly cross-linked polymer network [5]. This immobilization method overcomes such effects as steric hindrance and improper orientation that can occur during covalent immobilization techniques and can give high activities for immobilized proteins [15-17].

### 1.4.1. Hydrazide silica as a support

Recent work has been conducted in using entrapment with HPLC-grade silica and has been tested by using human serum albumin (HSA) as a model affinity ligand [18]. This method has been employed for examining the interactions of HSA with sulfonylurea drugs by HPAC [19]. One entrapment procedure that was used in these studies involved circulating solutions

containing HSA and mildly-oxidized glycogen into a packed column that contained hydrazide-activated porous silica. The glycogen had been previously oxidized by treatment with periodic acid to form aldehyde groups within its structure. The aldehyde groups on the glycogen were able to react with the hydrazide groups on the silica

**Figure 1.3.** General scheme for entrapment of a protein using by a glycogen-capped and hydrazide-activated support.


CHO
Protein
Protein

to form stable covalent bonds, while the HSA remained free solution as it was trapped within the pores or on the surface of the support [19].

As is illustrated in Figure 1.3, a protein such as HSA gets entrapped during this process in a soluble form because glycogen is a large molecule that blocks the protein from leaving the support. However, the entrapped protein can still be reached by small molecules that can pass into and out of the pores of the support or reach its surface [15, 19]. This method has been shown to provide good activity for entrapped proteins and avoids many of the undesirable effects of covalent immobilization [15].

Prior methods using entrapment have often done this by incorporating the binding agent during the formation of the support. This has most often been done by using sol-gels based on silica [20]. This method has been used for various biological agents but care must be used in selecting the proper reagents and conditions for support formation to allow entrapment to occur without significant denaturation of the entrapped agent [21-23].

### 1.4.2. Monolith as a support

A monolith is a single piece of polymer that is prepared within a column. Monolithic supports have a higher external porosity than particle-based supports, which can also give monoliths higher permeability, better efficiency, and lower back pressure than particle-based supports [24-26]. These features are useful with affinity-based separations in allowing work at high flow rates and in providing high efficiencies for applications such as high-throughput drug screening and rapid antibody-based assays [27, 28]. The combination of monoliths with affinity ligands is known as affinity monolith chromatography (AMC) [28].

Monoliths generally have two types of pores: “flow-through pores”, or macropores, and smaller “diffusion pores”, or mesopores [28]. In a chromatographic system flow occurs mostly through the large macropores, while most of the solute interactions with the stationary phase occur at or near the surfaces of the macropores. In affinity-based separations these structures are important in permitting high flow rates and in giving high efficiencies for applications such as chiral separations, antibody-based assays, and high-throughput screening [29-31].

Several types of monoliths can be used in affinity columns. These supports have been based on agarose, organic polymers, cryogels, and silica [30-36]. Organic polymer-based monoliths and inorganic-based silica monoliths are the two most common types of monoliths that have been used in affinity monolith chromatography. The organic polymer-based monoliths were first developed in the 1980s, while silica monoliths appeared in the 1990s [37-39].

## 1.5. Overall Goal and Summary of Work

The overall goal of this thesis is to examine the use of on-column entrapment methods based on hydrazide-activated silica and oxidized glycogen as a capping agent for the immobilization of proteins as affinity ligands in HPAC. Although this general type of entrapment method has been previously examined reported by our group, this method still needs further optimization for its use in an on-column format and in new applications based on HPAC. For example, it is necessary to conduct studies to further increase the amount of the entrapped affinity ligand that can be obtained by using alternative types of supports.

**Chapter 2** will mainly concentrate on a fast method for studying drug-protein binding on HPAC columns that contain entrapped proteins. This study will examine the development

and application of a new and rapid approach for performing drug-protein binding studies by using preparations of entrapped proteins and zonal elution experiments. **Chapter 3** will focus on the adaptation and optimization of current methods for the entrapment of proteins on silica for use in the immobilization on monoliths based on co-polymers of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA). These monoliths will be used to entrap HSA and then will be examined by using zonal elution analysis experiments that are conducted with probe compounds for the major binding sites on HSA. **Chapter 4** will present an overview of this work and discuss future projects that might be done with entrapment method on both dihydrazide-activated silica and monolith supports. For example, this future work could include automation of the on-column entrapment method and the application of this approach to a wider variety of biomolecules, such as alpha$_1$-acid glycoprotein (AGP) and lectins.

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# CHAPTER 2:

# RAPID ANALYSIS OF DRUG PROTEIN BINDING BASED ON COLUMNS PREPARED BY ENTRAPMENT

## 2.1. Introduction

It is important to know the activity and fate of pharmaceutical agents in the body. When a drug enters the circulatory system, it may undergo interactions with proteins found in blood, such as human serum albumin (HSA), $\alpha_1$-acid glycoprotein (AGP) and lipoproteins. These interactions can be described by using a parameter such as the association equilibrium constant ($K_a$) for the drug-protein complex. These drug-protein interactions are often strong and significant, with approximately 43% of the 1500 most common drugs having at least 90% binding to serum proteins. As a result, these interactions can have a large impact on the absorption, distribution, metabolism and excretion (ADME) of a drug in the human body [1].

Because of the potential impact of these interactions, it is important to conduct drug-protein binding studies on pharmaceutical agents to help determine their dosages and the frequency at which the drugs should be taken [2]. Binding studies that are conducted *in vitro* may give general information for a particular drug and its interactions, while *in vivo* studies can potentially provide specific information that is more tailored to an individual patient's needs for use in personalized medicine [3].

Human serum albumin (HSA) is a transport protein in the circulatory system that can bind to many endogenous and exogenous compounds. HSA is the most abundant

protein in human plasma, where it is found at concentrations ranging from 30–50 g/L (0.53–0.75 mM). The high concentration of HSA accounts for approximately 60% of the total protein content in serum and gives this protein the ability to greatly influence the pharmacokinetics and activity of many common drugs [4-6]. HSA plays a key role in the reversible binding and transport of drugs, metabolites, and various endogenous ligands, such as fatty acids. In addition, HSA can increase the solubility of lipophilic drugs, sequester toxins, and act as an important antioxidant in plasma [7,8].

There are two major binding sites for drugs on HSA, which are located in subdomains IIA and IIIA of this protein. These sites are often referred to as Sudlow sites I and II after Gillian Sudlow, who proposed their existence in 1975 [9,10]. Sudlow site I, which is also known as the warfarin-azapropazone site, binds to bulky heterocyclic anions such as warfarin and salicylate. Sudlow site II, or the indole-benzodiazepine site, binds primarily to aromatic carboxylic acids like ibuprofen and L-tryptophan. At least two minor binding sites for drugs on HSA have also been proposed for compounds such as digitoxin and tamoxifen (i.e., the digitoxin site and tamoxifen site) [11,12].

In recent years, it has been found that the glycation of HSA may affect its binding to solutes such as many drugs. Sulfonylureas, which are often used to treat type 2 diabetes, are one group of drugs that have been found to be affected by this glycation process. These drugs are highly bound to serum proteins, and especially to HSA [14,19-24]. Binding studies based on high-performance affinity chromatography (HPAC) have found that glycation can affect the equilibrium constants of sulfonylurea drugs with HSA

and that the extent of this change is affected by both the degree of protein glycation and the specific drug that is being examined [3,14,19-23,25].

## 2.2. Experimental section

### 2.2.1. Materials

The HSA (essentially fatty acid free, purity ≥ 96%), glycogen (bovine liver, type IX; total glucose ≥ 85%, dry basis), racemic warfarin (purity ≥ 98%), *R*-warfarin (purity ≥ 97%), L-tryptophan (purity ≥ 98%) and periodic acid reagent ($H_5IO_6$, purity 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nucleosil Si-300 silica (7 μm particle diameter, 300 Å pore size, 100 $m^2/g$ surface area) was obtained from Macherey-Nagel (Düren, Germany). The *in vitro* samples of glycated HSA were purified through the use of Econo-Pac 10DG desalting columns from Bio-Rad Laboratories (Hercules, CA, USA) and Slide-A-Lyzer digest 7K dialysis cassettes (7 kDa MW cutoff; 0.5-3, 3-12 or 12-30 mL sample volumes) from ThermoScientific (Rockford, IL, USA). All other chemicals were of the purest grades available.

### 2.2.2. Apparatus

The chromatographic system that was used in the zonal elution studies consisted of a DG-2080 degasser, two PU-2080 pumps, an AS-2057 autosampler, a CO-2060 column oven, and a UV-2075 absorbance detector from Jasco (Tokyo, Japan), plus a Rheodyne LabPro six-port valve (Cotati, CA, USA). LC Net and ChromNav from Jasco (Tokyo, Japan) were used to control the chromatographic system and to collect the data.

For the frontal analysis studies, two 1200 isocratic pumps and a 1200 autosampler from Agilent (Santa Clara, CA, USA) were used, along with a Series 200 UV-Vis detector and a vacuum degasser from Perkin Elmer (Waltham, MA, USA). A Rheodyne LabPro valve and an Isotemp 9100 circulating water bath (Fisher Scientific, Pittsburgh, PA, USA) with 0.067 M potassium phosphate buffer as the packing solution. The columns that were packed with the hydrazide-activated silica were used for the on-column entrapment of normal HSA or HSA with various levels of glycation.

In the entrapment process, a solution containing 50 mg/mL of the normal HSA or glycated HSA in pH 5.0, 0.10 M potassium phosphate buffer was first circulated through the column at 20 µL/min for 4 h. This was followed by the application of a solution over 16 h that contained a mixture of the normal HSA or glycated HSA at 50 mg/mL and oxidized glycogen at 4.2 mg/mL in the same phosphate buffer. Finally, 200 µL of 1 mg/mL of oxalic dihydrazide in the same buffer was added to the reaction mixture, and the circulation of this solution through the column was continued for two more hours. A control column was made under the same conditions but with the solutions of HSA being replaced with an equal volume of pH 5.0, 0.10 M potassium phosphate buffer. All of the columns were washed by flowing pH 7.4, 0.067 M potassium phosphate buffer through them at 0.5 mL/min for 1 h or until a stable baseline response was reached for these columns.

### 2.2.3. Chromatographic studies

Stock solutions of the sulfonylurea drugs (i.e., acetohexamide, tolbutamide and gliclazide) were prepared in pH 7.4, 0.067 M potassium phosphate buffer at a typical concentration of 100 µM. The stock solutions of the site-specific probes were also prepared in this buffer and had typical concentrations of 100 µM for warfarin and 1.3 mM for L-tryptophan. The acetohexamide, tolbutamide and gliclazide solutions were used within one week of preparation. The warfarin solutions were used within one week, and L-tryptophan solutions were used within two days of preparation. It has been demonstrated in previous studies that similar solutions of these drugs and solutes are stable over for these given periods of time [24].

The mobile phases that were used in the competition studies with the site-specific probes were prepared at concentrations that were typically 1 mM for L-tryptophan and 40 µM for warfarin, as made by diluting the stock solutions for these probes with pH 7.4, 0.067 M potassium phosphate buffer. The injected samples for these experiments were prepared by using the stock solutions for the sulfonylurea drugs, warfarin or L-tryptophan and the pH 7.4, 0.067 M potassium phosphate buffer. The concentrations of the sulfonylurea drugs ranged from 20 µM to 40 µM. Additional experiments were performed by using 10 µM L-tryptophan or 20 µM warfarin in the same pH 7.4 buffer, as used for determining the retention factors and protein content of the normal HSA and glycated HSA columns. It has been shown in previous work that the sample concentrations that were used in this study provide linear elution conditions for the given drugs on HSA columns [23-24]. The chromatographic experiments were carried out at a

typical flow rate of 0.50 mL/min and at a temperature of 37 °C. All of the injected solutions and mobile phases that were used in the experiments were passed through 0.2 μm filters and degassed for 10-15 min prior to use.

## 2.3. Results and Discussion

### 2.3.1. Zonal elution studies

The global affinity of a target compound for a binding agent with several independent sites (e.g., as often occurs for the binding of drugs with HSA) can be described as the addition of the affinities for all the sites on the binding agent. For a column that is made by entrapment, essentially all the binding agent will be active and the overall retention factor (*k*) of a drug or solute due to binding at these sites can be described by the relationship in Equation 2.1 [19],

$$k = (n_1 K_{a1} + n_2 K_{a2} + \cdots n_n K_{an}) m_{Ltot} / V_m \quad (2.1)$$

where $n_i$ is the relative moles of binding site *i* for a given drug or solute per mole of protein, $K_{ai}$ is the association equilibrium constant for the same site and drug/solute, $m_{Ltot}$ is the total moles of all binding sites for the solute in the column, and $V_m$ is the column void volume. The global affinity in this equation is the term in parentheses and can also be represented by the sum of these terms, $nK_a'$. This is a number-weighted sum of the equilibrium constants for all the binding sites for the drug or solute on the immobilized binding agent [19].

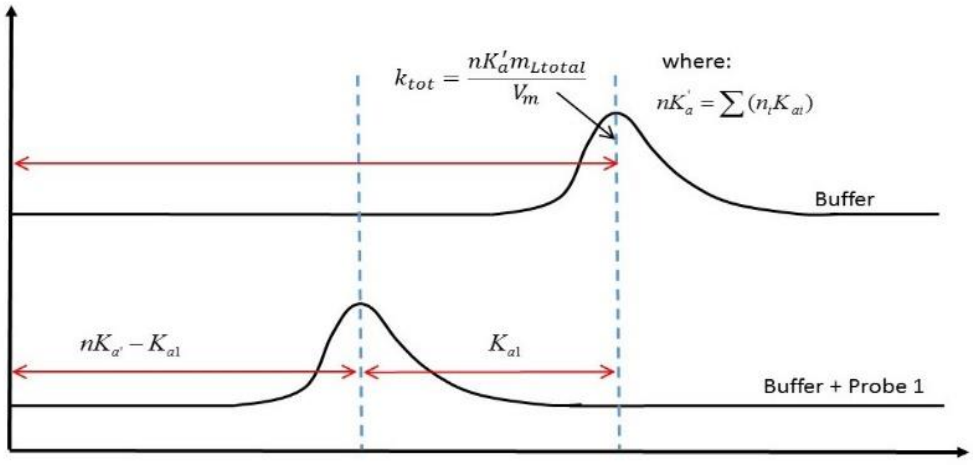
When a probe interacts with a single type of site on an entrapped binding agent, Equation 2.1 can be reduced to the following expression,

$$k = K_a \frac{m_{Ltot}}{V_m} \quad (2.2)$$

where $K_a$ is the association equilibrium constant for the probe with the specific binding site [19].

Equations 2.1 and 2.2 indicate that the measured retention factor for a solute on a column that contains an entrapped binding agent can allow a direct determination of the global binding constant or site-specific binding constant for this solute. This can be accomplished by using experiments like those shown in Figure 2.1. First, an injection of a drug or solute that is dissolved in the buffer alone can be made onto a column containing the entrapped binding agent, making it possible to determine the total retention factor, $k_{tot}$. This parameter, along with a previous measurement of the column void volume and $m_{Ltot}$, makes it possible to obtain the global affinity, as is shown in the following reduced form of Equation 1.1 [19],

**Figure 2.1.** Use of zonal elution experiments for estimating the global affinity ($nK_a'$) or site-selective affinity ($K_{a1}$) for a solute on a column that contains an entrapped protein or binding agent. Other terms in this figure are described in the text.


$k_{tot} = \frac{nK_a' m_{Ltotal}}{V_m}$
where:
$nK_a' = \sum (n_i K_{ai})$
Buffer
$nK_{a'} - K_{a1}$
$K_{a1}$
Buffer + Probe 1

$$k_{tot} = \frac{nK'_a m_{Ltot}}{V_m} \quad (1.3)$$

where $nK'_a = \sum(n_i K_{ai})$.

The same drug or solute can then be injected in the presence of a mobile phase that contains a site-selective probe (e.g., warfarin for Sudlow site I or L-tryptophan for Sudlow site II of HSA). This agent added to the mobile phase is present at a level that saturates a known fraction of the given site in the column. During this second injection, the retention for the solute will be lower because the probe in the mobile phase is binding to one of the sites on the entrapped agent. The shift in retention can make it possible to find the association equilibrium constant for this specific binding site ($K_{a1}$), as is indicated in Equation 2.4.

$$k = \frac{(nK'_a - K_{a1})m_{Ltot}}{V_m} \quad (2.4)$$

The value of $m_{Ltot}$ in Equations 2.3 and 2.4 can be obtained through independent frontal analysis measurements or by making a retention factor measurement with a probe that has a known value for its association equilibrium constant with the same binding agent. Repeating the experiment in Figure 2.1 with probes for other binding sites should make it possible to also find the solute's affinity for each of the other binding regions [19].

The concentration of a site-specific probe (e.g., warfarin and L-tryptophan for HSA) that is needed in the mobile phase to obtain a given level of saturation for the entrapped binding agent can be found by using the following relationship,

$$\frac{k - k_{min}}{k_{max} - k_{min}} = \frac{1}{1 + K_I [I]} \quad (2.5)$$

where $I$ represents the probe, $K_I$ is the association equilibrium constant for this probe at its binding site, and [$I$] is the concentration of the probe in the mobile phase [33]. The terms $k$, $k_{min}$ and $k_{max}$ are the retention factor that should result for the injected solute at a given concentration of the probe, the minimum possible retention factor for this solute (i.e., when all the given binding sites are saturated), and the maximum possible retention factor for the solute (i.e., when all these sites are available for binding).

### 2.3.2. Estimation of the amount of an entrapped protein in a column

The amount of HSA that was entrapped in a given column was determined by using both frontal analysis and zonal elution experiments with warfarin and L-tryptophan. The zonal elution experiments that were carried out with the same column and using the same analyte or probes as were used in the frontal analysis experiments. The values for the moles of entrapped protein that were obtained by both of these methods are provided in Table 2.1. There was good agreement between the two methods, with no significant difference being present at the 95% confidence level. This indicated that it was possible to measure the total moles of binding sites and protein by either method, with the zonal elution technique being preferred in later studies because it required much less time and reagents than the frontal analysis experiments.

The value of $K_a$ that was obtained from the double-reciprocal plot for warfarin in the frontal analysis studies was 2.6 (± 0.3) × $10^5$ $M^{-1}$, which was in good agreement with an average value of 2.4 (± 0.4) × $10^5$ $M^{-1}$ that has been previously determined for the *R*-

and *S*-enantiomers of warfarin with HSA. The value of $K_a$ that was obtained from the double-reciprocal plot for L-tryptophan was 0.93 (± 0.23) × $10^4$ $M^{-1}$, which was also in

**Table 2.1.** Estimates of the amount of entrapped normal HSA, as determined by zonal elution and frontal analysis.[1] These values were measured at 37 °C in pH 7.4, 0.067 M phosphate buffer. The values in parentheses represent a range of ± 1 S.D.

| **Probe or analyte** | **Column void volume, $V_m$ (L × $10^{-5}$)** | **Moles of entrapped protein[1] (mol × $10^{-8}$)** | |
|---|---|---|---|
| | | **Zonal elution** | **Frontal analysis** |
| Warfarin | 3.35 ( ± 0.07) | 1.83 (± 0.31) | 1.46 (± 0.17) |
| L-Tryptophan | | 1.26 (± 0.35) | 1.09 (± 0.27) |

Good agreement with a value of 1.1 (± 0.3) × $10^4$ $M^{-1}$ that has been reported in previous studies with HSA *(26,33).*

### 2.3.3. Estimating the global affinity constant and site-specific binding constants for acetohexamide with entrapped normal HSA

Once the total moles of entrapped normal HSA had been determined, the global affinity constant for acetohexamide with the entrapped HSA was determined by making injections of acetohexamide on both the entrapped HSA column and a control column. A second set of injections were then made for acetohexamide in the presence of a site-selective probe for HSA (e.g., warfarin for Sudlow site I or L-tryptophan for Sudlow site II) to determine the binding constants of acetohexamide at specific sites on HSA.

Equation 2.5 was used to determine the concentrations of the site-specific probes that were needed in these experiments to achieve near complete saturation of the binding sites. This was done by setting the left side of Equation 2.9 equal to a relative retention of 0.05 (representing 95% site saturation) for calculating the concentration that would produce a 95% shift in retention from its maximum possible value. The known association equilibrium constants for HSA with warfarin and L-tryptophan (i.e., the probes that were used as site-selective additives in this work) were also used in these calculations. The conditions needed for 95% site saturation were estimated to occur when the mobile phase contained 79 μM warfarin or 1.7 mM L-tryptophan. Experiments that were also done at 90% site saturation, which corresponded to the use of 40 μM warfarin or 850 μM L-tryptophan in the mobile phase.

Table 2.2 shows the association equilibrium constants that were measured by zonal elution for acetohexamide in the presence of various amounts of the mobile phase additives.

**Table 2.2.** Site-specific association equilibrium constants measured for acetohexamiden zonal elution experiments and using various concentrations of site-selective probes in the mobile phase.

| Probe[1] | Mobile phase concentration | Site-specific association equilibrium constant, $K_a$ ($M^{-1} \times 10^4$)[2] | |
|---|---|---|---|
| | | Sudlow site I | Sudlow site II |
| Warfarin/L-tryptophan | 101.6 µM/1.7 mM | 10.9 (± 1.9) | 6.9 (± 2.0) |
| Warfarin/L-tryptophan | 40 µM/850 µM | 9.9 (± 0.18) | 6.8 (± 2.0) |
| Warfarin (racemate) | 40 µM | 8.3 (± 1.4) | N.A. |
| *R*-Warfarin | 40 µM | 8.9 (± 0.9) | N.A. |

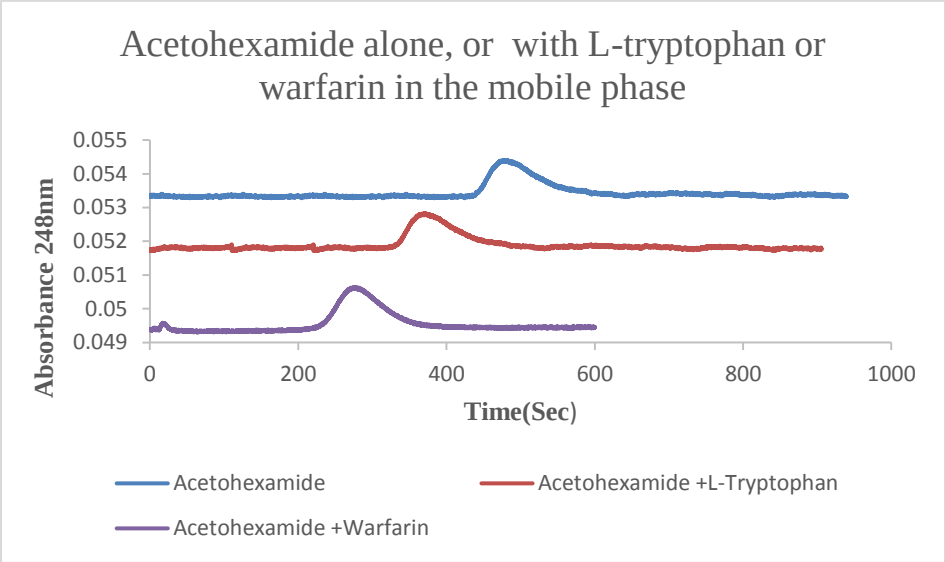
Warfarin was used as a probe for measuring the binding constant for Sudlow site I and L-tryptophan was used for Sudlow site II. These probes were used in separate sample solutions for each experiment. Physiological conditions were also used in these measurements (i.e., pH 7.4, 0.067 M potassium phosphate buffer and a column temperature of 37 °C). [2]The values in parentheses represent a range of ± 1 S.D.

One observation made was that there was no significant difference at the 95% confidence level in the association equilibrium constants that were obtained when using mobile phase additives that corresponded to 95% or 90% site saturation. Given the fact that lower concentrations of these additives also resulted in a decrease in reagent costs and a lower background signal for the detector, mobile phase concentrations of 40 μM warfarin or 1000 μM L-tryptophan were selected for use in all subsequent zonal elution studies. The global affinity constant that was obtained in these experiments and from the retention factor for acetohexamide was 22.8 (± 2.0) × $10^4$ $M^{-1}$, which was is in good

agreement with values of 1.2-2.0 × $10^5$ $M^{-1}$ that have been previously obtained for this drug with HSA [19-21].

The impact of using racemic warfarin versus a single warfarin enantiomer as a mobile phase additive for probing Sudlow site I was also considered in this study. The association equilibrium constants that were obtained for acetohexamide at Sudlow site I when using either type of probe were statistically equivalent at the 95% confidence level. From this result, it was decided that racemic warfarin would be used as the probe for Sudlow site I in all later experiments with other sulfonylurea drugs. Examples of some chromatograms that were obtained in these experiments are shown in Figure 2.2 and the results are included in Table 2.2.

**Figure 2.2.** Chromatograms obtained for the injection of acetohexamide onto a column containing entrapped HSA and in the absence or presence of *R*-warfarin or racemic warfarin in the mobile phase.


Acetohexamide alone, or with L-tryptophan or warfarin in the mobile phase
Absorbance 248nm
0.055
0.054
0.053
0.052
0.051
0.05
0.049
0
200
400
600
800
1000
Time(Sec)
Acetohexamide
Acetohexamide +L-Tryptophan
Acetohexamide +Warfarin

### 2.3.4. Estimation of global affinity constants and site-specific binding constants for sulfonylurea drugs with entrapped samples of normal or glycated HSA

The use of on-column entrapment and zonal elution was next used to examine the global affinities and site-specific binding constants for various sulfonylurea drugs with either normal HSA or glycated HSA. As was demonstrated previously, the total moles of HSA in such a column could be obtained from retention measurements that were made using a well-characterized probe for such a protein. For these experiments, warfarin was selected as the probe for measuring $m_{Ltot}$ because this solute has been shown in previous studies to have no significant change in its affinity in the presence of low-to-moderate levels of glycation for HSA [19,26].

In this section, injections of racemic warfarin were first made onto columns containing entrapped samples of normal HSA or glycated HSA, and onto a control column, to provide an estimate of the total protein content of each column. The same columns were then used for the injection of samples that contained the sulfonylurea drugs acetohexamide, tolbutamide or gliclazide. These injections were initially made in the presence of only buffer to obtain the global affinity constants for each drug with normal HSA or the samples of glycated HSA. The same drugs were then injected in the presence of a mobile phase that contained a site-selective probe for HSA. To examine the binding of these drugs at Sudlow site I, 40 μM warfarin was added to the mobile phase. To examine the binding of these drugs at Sudlow site II, 1000 μM L-tryptophan was added. The differences in the retention times that were seen for each drug in the presence of only buffer or in the presence of a known concentration of a site-specific probe were then used

to calculate the site-specific association equilibrium constants for each drug at Sudlow sites I and II. The global affinity constants and site-specific association equilibrium constants that were measured for these drugs at Sudlow sites I and II are summarized in Tables 2.3 through 2.5.

Table 2.3 shows the global affinity constants that were measured for the sulfonylurea drugs with normal HSA or HSA with three levels of glycation: gHSA1, which had a modification level that was typical of that seen in prediabetes; gHSA2, which had a modification level corresponding to controlled diabetes; and gHSA3, which corresponded to advanced/uncontrolled diabetes. For all sulfonylurea drugs, there was an increase in the global affinity constant when going from normal HSA to gHSA1 and a further increase when going to gHSA2. However, the global affinity constant decreased when going to gHSA3. The highest increase that was observed was 1.3-fold, as noted for acetohexamide and gliclazide with the gHSA2 sample. However, none of these apparent differences were statistically significant when the standard deviations (in parentheses) were considered. The global affinity constants for these drugs with normal HSA were in good agreement with those reported in previous studies: 22.8 (± 2.0) × $10^4$ $M^{-1}$ for acetohexamide, 12.6 (± 1.1) × $10^4$ $M^{-1}$ for tolbutamide, and 6.17 (± 0.53) × $10^4$ $M^{-1}$ for gliclazide [14,19,22,24].

**Table 2.3.** Global affinity constants obtained for various sulfonylurea drugs with columns containing entrapped samples of normal HSA or HSA with variouslevels of glycation. [1]These values were measured at 37 °C in pH 7.4, 0.067 M phosphate buffer. The values in parentheses represent a range of ± 1 S.D.

| Drug | Global affinity constant ($M^{-1} \times 10^4$) and type of HSA[1] | | | |
|---|---|---|---|---|
| | Normal has | gHSA1 | gHSA2 | gHSA3 |
| Acetohexamide | 17.0 (± 1.9) | 17.6 (± 1.9) | 24.0 (± 3.1) | 21.2 (± 2.6) |
| Tolbutamide | 10.2 (± 1.1) | 10.7 (± 1.2) | 12.8(± 1.6) | 12.2(± 1.5) |
| Gliclazide | 4.9 (± 0.5) | 5.4(± 0.6) | 5.8(± 0.7) | 5.6(± 0.6) |

**Table 2.4.** Association equilibrium affinity constants obtained at Sudlow site I for various sulfonylurea drugs with columns containing entrapped samples of normal HSA or HSA with various levels of glycation. [1]These values were corrected for the fact that only 90% site saturation was used. These results were measured at 37 °C in pH 7.4, 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ± 1 S.D.

| Drug | Association equilibrium constant ($M^{-1} \times 10^4$) and type of HSA[1] | | | |
|---|---|---|---|---|
| | Normal HSA | gHSA1 | gHSA2 | gHSA3 |
| Acetohexamide | 7.7 (± 1.4 ) | 8.7 (± 0.9) | 10.6 (± 1.4) | 8.8 (± 1.1) |
| Tolbutamide | 4.9 (± 0.9) | 5.8 (± 0.6) | 6.2 (± 0.8) | 5.5 (± 0.6) |
| Gliclazide | 2.2(± 0.4) | 2.7(± 0.3) | 3.5(± 0.4) | 3.4(± 0.3) |

**Table 2.5.** Association equilibrium affinity constants obtained at Sudlow site II for various sulfonylurea drugs with columns containing entrapped samples of normal HSA or HSA with various levels of glycation. [1]These values were corrected for the fact that only 90% site saturation was used. These results were measured at 37 °C in pH 7.4, 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ± 1 S.D.

| Drug | Association equilibrium constant ($M^{-1} \times 10^4$) and type of HSA[1] | | | |
|---|---|---|---|---|
| | Normal HSA | gHSA1 | gHSA2 | gHSA3 |
| Acetohexamide | 4.9 (± 0.9) | 5.3 (± 0.6) | 7.8(± 1.1) | 6.2 (± 0.7) |
| Tolbutamide | 2.4 (± 0.4) | 2.6 (± 0.3) | 3.5(± 0.4) | 3.9(± 0.4) |
| Gliclazide | 1.6 (± 0.3) | 1.7(± 0.2) | 2.7(± 0.3) | 2.3(± 0.2) |

Tables 2.4 and 2.5 compare the sulfonylurea drugs in terms of their association equilibrium constants for the two main drug binding sites on HSA: Sudlow sites I and II. In general, an increase in these site-specific binding site constants was observed for the three drugs when going from normal HSA to gHSA2. The affinity then tended to decrease with further glycation, as was typical of uncontrolled diabetes (gHSA3). The binding constant for Sudlow site I increased by 1.4-fold for acetohexamide, 1.3-fold for tolbutamide and 1.6-fold for gliclazide for gHSA2 relative to normal HSA. The binding constant for Sudlow site II increased by 1.6-fold for acetohexamide, 1.5-fold for tolbutamide and 1.7-fold for gliclazide for gHSA2 relative to normal HSA.

The association equilibrium constants at Sudlow site I and II were also compared between the various drugs. For instance, the binding constant at Sudlow site I was higher than the value at Sudlow site II for acetohexamide in normal HSA as well as in all of the samples of glycated HSA. The same trend was true for tolbutamide. For gliclazide, there was no statistically significant difference between the values that were measured at Sudlow sites I and II.

## 2.4. CONCLUSIONS

In this study, a novel approach was developed and tested for measuring global affinity constants and site-specific binding constants for drug-protein interactions. This method made use of columns containing entrapped proteins. With these columns, it was possible to estimate drug-protein binding constants by using relatively simple retention measurements for a drug in the presence of only a buffer or in the presence of a probe for each specific binding site that was to be studied.

The system was applied to the sulfonylurea drugs acetohexamide, tolbutamide and gliclazide, which are known to bind tightly to HSA and to have interactions at both Sudlow sites I and II of this protein [14,19-24]. A sample of normal HSA and three samples of HSA with various levels of glycation were immobilized by on-column entrapment using the conditions that were optimized previously.

In order to calculate the binding constants for these drugs with normal HSA or glycated HSA, it was necessary to also have an estimate of the total moles of active protein that were in each column. This estimate was made by using both zonal elution and frontal analysis experiments with a probe compound (i.e., warfarin) that had known interactions with these proteins. It was found that the values that were obtained by zonal elution were statistically equivalent to these obtained with the more time-consuming method of frontal analysis. Racemic warfarin was used for this type of measurement in the later work with the normal HSA and glycated HSA, because it has been shown in previous studies that the affinity of this drug for HSA is not affected significantly by the glycation of HSA at the modification levels that are normally seen in diabetes [19,26].

The global affinity constants for several sulfonylurea drugs were measured by this new approach. Good agreement was seen between these values that those that have been previously estimated for these drugs with HSA. It was also shown how site-specific association equilibrium constants for these drugs could be measured with either normal HSA or glycated HSA. Good agreement was seen between the global affinity constants that were found in this study and values that have been calculated previously for the same drugs with HSA.

The rapid approach addressed in this chapter for studying protein-drug interactions by using entrapped proteins in HPAC columns can be applied for screening the binding of drugs with proteins isolated from serum of patients with some pathological condition in order to tailor the treatment to each individual.

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# CHAPTER THREE: MONOLITH AS A SUPPORT FOR THE ENTRAPMENT OF HSA

## 3.1. INTRODUCTION

Monolithic columns have been of great recent interest for use in high performance affinity chromatography (HPAC) because of their low back pressures, ease of preparation and good mass transfer properties [1-4]. Monoliths generally contain two types of pores. Macropores are the flow-through pores which allow movement of the mobile phase through the column and are usually in the size range of micrometers for typical chromatographic applications. Mesopores are smaller in size and are also known as diffusion pores, since analytes enter these pores primarily by means of diffusion [3].

The combination of affinity ligands with monolith columns is known as affinity monolith chromatography (AMC) [1,5-10]. Various types of monoliths have been used in AMC, with many such reports using co-polymers of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) [1-4]. The co-solvents utilized to prepare these monoliths are usually cyclohexanol and 1-dodecanol. These "porogens" are used to generate the pores within the monolith column. These monolith columns are typically prepared through the use of an initiator and heat [3-4]. GMA/EDMA monoliths are advantageous because they have been shown to be effective in immobilizing proteins such as human serum albumin (HSA), antibodies such as IgG, and other binding agents of importance (e.g., protein A) [4].

HPAC is an effective tool for the separation and analysis of many compounds that can bind to various biologically-related ligands [3-5, 8, 12, 13]. HPAC is a type of high performance liquid chromatography in which a biological binding agent (i.e., the affinity

ligand) is immobilized within a column and used as the stationary phase. This binding agent usually has the ability to bind with moderate-to-strong affinity and with good specificity to the analyte of interest, such as occurs between the drug warfarin and the protein HSA [14, 15]. HPAC can be useful for studying biological interactions, for protein purification, and for chiral separations [1]. This method also has many advantages, such as its ease of automation, high specificity, speed, and good reproducibility [1]. In many of the applications of HPAC (e.g., binding studies) the total amount of ligand that is immobilized in the column is vital to the success of this method by providing good retention and high resolution between retained and non-retained sample components[1].

The work in this chapter will focus on primarily optimizing and determining the total amount of protein that can be entrapped in monolithic supports based on co-polymers of GMA/EDMA. The optimization of protein content in monolithic columns for use with HPAC has been shown to be important in allowing for these columns to be used for studying drug interactions involving proteins such as HSA [4]. In this chapter, the amount of monomers GMA and EDMA will be held constant and the polymerization temperature will be kept at 80º C. The relative amounts of cyclohexanol to 1-dodecanol will be varied to generate a library of monoliths. The hydrazide activation technique will be utilized for the entrapment of HSA and to study the relative change in the total protein content of the different monoliths under the various porogenic ratios. HPLC will be used to study the binding and elution of model analytes to the entrapped HSA within each monolith. The results should make it possible to determine the optimum ratio of 1-dodecanol to cyclohexanol for monolith synthesis and the optimum conditions for

monolith preparation with respect to achieving the highest total amount of entrapped protein. These results, in turn, should make it possible to generate more effective monolithic columns for use in future HPAC experiments that involve HSA or similar proteins.

## 3.2. EXPERIMENTAL SECTION

### *3.2.1. Reagents*

The GMA (97% pure), EDMA (98%), cyclohexanol (> 99%), 1-dodecanol (98%), 2,2'-azobisisobutryonitrile (AIBN, 98% pure), HSA (essentially fatty acid free, >96%), sodium borohydride (98%, a strong reducing agent), periodic acid (> 99%, an oxidizing agent), racemic warfarin (purity ≥ 98%), and L-tryptophan (purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). All aqueous reagents, solutions and buffers were prepared using water from a Nanopure system (Barnstead, Dubuque, IA, USA) and were filtered using 0.2 µm GNWP nylon filters from Millipore (Billerica, MA, USA).

### *3.2.2. Apparatus*

The monoliths were prepared in 4.6 mm i.d. × 0.5 cm columns with PEEK inner liners from Alltech (Deerfield, IL, USA). These columns included a special frit that could be used to compress the monoliths and to avoid the formation of gaps within the columns during and after their preparation. The monoliths were placed into 4.6 mm i.d. × 0.5 mm PEEK disks. The chromatographic system that was used in the zonal elution

studies consisted of a DG-2080 degasser, two PU-2080 pumps, an AS-2057 autosampler, a CO-2060 column oven, and a UV-2075 absorbance detector from Jasco (Tokyo, Japan), plus a Rheodyne LabPro six-port valve (Cotati, CA, USA). LCNet and ChromNav from Jasco (Tokyo, Japan) were used to control the chromatographic system and to collect the data.

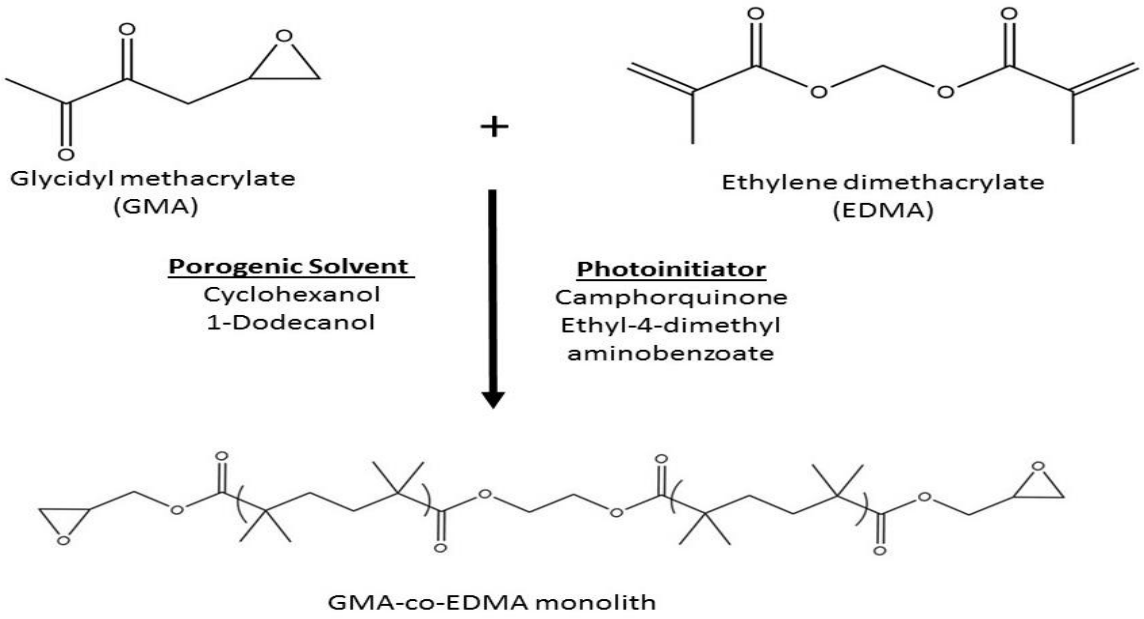
The results of the zonal elution experiments were analyzed by using PeakFit 4.12 (Systat Software, San Jose, CA, USA). Calculation of the retention factors and binding constants was performed by using Excel 2013 (Microsoft, Redmond, WA, USA). Two PHD Ultra syringe pumps (Harvard Apparatus, Holliston, MA, USA) were used for the on-column entrapment method. A Jasco V-630 UV/VIS spectrophotometer (Jasco, Kyoto, Japan) equipped with temperature control was used for the protein assay.

### *3.2.3. Preparation of Monolithic Columns*

Figure 3-1 shows the general procedure that was used for the preparation of the monolith columns and for the immobilization of HSA in these columns[16]. This procedure began with mixing of the monomers, cross-linkers and initiator, followed by polymerization. After the completion of monolith formation, this support was washed for 1.5 h at 0.5 mL/min with 50 mL of water using an HPLC pump. Activation of this support was accomplished by first converting the monolith into a diol form through treatment with 0.5 M sulfuric acid. An HPLC pump was used to pass, without recycling, 5 mL of a 0.5 M solution of sulfuric acid in water through the monolithic column at 0.5 mL/min and room temperature for 10 min. The two ends of the monolithic column were then sealed using plugs and placed into a water bath set at 60 $^{o}$C for 4 h. The monolithic

column was removed from the water bath and was washed with 100 mL of water at 0.5 mL/min using an HPLC pump at room temperature for 3 h. Next, 40 mL of a solution containing 2 g periodic acid in a 90:10(v/v) mixture of acetic acid and water was prepared. This solution was cycled through the column at room temperature and 0.5 mL/min for 4hours. During this procedure, the container holding the periodic acid solution was wrapped in aluminum foil to protect it from light. The monolithic column was then washed with 100 mL of water that was applied without recycling at 0.5 mL/min using an HPLC pump at room temperature for 3 h. This resulted in an aldehyde-activated monolith.

**Figure 3.1.** General scheme for the preparation of a GMA/ EDMA monolith [16].




The aldehyde-activated support was next reacted with 30 mL of 0.5 M oxalic dihydrazide in pH 5.0, 0.10 M phosphate buffer by passing this solution through the column at 0.5 mL/min for 1 h at room temperature and PU-2080 Jasco pump was used. Another 20 mL of the same solution was circulated through the column for another 2 h at 0.5 mL/min and room temperature. Unreacted aldehyde groups on the monolith were reduced to alcohols by using 2.5 mg/mL sodium borohydride in pH 8. 0, 0.1 M potassium phosphate buffer. A 20 mL portion of this solution was applied to the monolith at 0.5

mL/min for 4 h at room temperature and PU-2080 Jasco pump was used. Finally, the column was washed by using an HPLC pump and by passing 120 mL of pH 7.4, 0.067 M potassium phosphate buffer for 4 h at 0.5 mL/min at room temperature It was used immediately and if it is going to be stored for the next day it was stored in the fridge at 4 °C.

HSA was entrapped by the reaction of mildly-oxidized glycogen with hydrazide-activated silica, as described in Chapter 2. For this entrapment process, a solution containing 100 mg/mL of normal HSA in pH 5.0, 0.10 M potassium phosphate buffer was circulated through the hydrazide-activated column at 20 µL/min for 4 h, at room temperature and two PHD Ultra syringe pumps (Harvard Apparatus, Holliston, MA, USA) were used for pumping the solution. This was followed by the application of a solution over 16 h at room temperature, that contained a mixture of 100 mg/mL normal HSA and 4.2 mg/mL oxidized glycogen in the same phosphate buffer. Finally, 200 µL of 1 mg/mL of oxalic dihydrazide in the same buffer was added to the reaction mixture, and circulation of this solution through the column was carried out for two more hours [5] at room temperature. A control column was made under the same conditions but with the solutions of HSA being replaced with an equal volume of pH 5.0, 0.10 M potassium phosphate buffer. All of the columns were washed by flowing pH 7.4, 0.067 M potassium phosphate buffer through them at 0.5 mL/min for 1 h, at room temperature or until a stable baseline response was reached for these columns.

The relative ratio of dodecanol to cyclohexanol was varied during the preparation of the GMA/EDMA supports to create a library of monoliths at a polymerization temperature of 80ºC. The total amount of entrapped protein was determined for each

monolith by using a BCA assay [16]. For this assay, each monolith was prepared in triplicate during the optimization studies and washed with 100 mL of water at 0.5 mL/min for 3 h at room temperature to remove any non-entrapped components.

### *3.2.4. Chromatographic Studies*

The chromatographic studies were performed at room temperature using pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase. The mobile phase buffer was degassed and sonicated for approximately 30 min prior to use. A sample containing a 20 μM solution of racemic warfarin was prepared in pH 7.4, 0.067 M potassium phosphate buffer. A 20 μM solution of L-tryptophan was also prepared in pH 7.4, 0.067 M potassium phosphate buffer. All samples were used within one day of preparation, and a 20 μL injection of each analyte or a void marker (i.e., sodium nitrate) was made in triplicate at flow rate of 0.5 mL/min. The elution of warfarin was monitored at 308 nm. The L-tryptophan were monitored at 280 nm. A 20 μL injection of 0.2 mM sodium nitrate was monitored at 205 nm. The extra-column void time was determined by injecting sodium nitrate onto a zero dead volume connector and monitoring the elution at 205 nm.

## 3.3 RESULTS AND DISCUSSION

The general procedure for the polymerization of monoliths was similar to a previously reported method [16], and the entrapment step was also similar to the entrapment method done on hydrazide-activated silica that was optimized in the lab [17]. In these studies several parameters were held constant while the relative amounts of the porogenic solvents were varied.

The polymerization conditions that were optimized were the volume ratios of the 1-dedecanol and cyclohexane in the porogen solvent mixture. HSA was used as a model protein to compare the amount of entrapped protein that could be placed within such supports.

As shown in Table 3.1, the retention factor obtained for the injection of warfarin and L-tryptophan were used to determine the retention factor of these analytes on the column. Figure 3.1 also shows the overall trends noted in the retention factors that were obtained for warfarin and L-tryptophan on these monoliths. As was found that a 20:40 porogenic ratio gave a monolith column that had a retention factor of 17.4 (± 0.1) for warfarin and 0.5 (± 0.1) for L-tryptophan. This was the highest set of retention factors that were seen and thus represented the highest protein content. A 10:50 porogenic ratio gave a monolith column with a retention factor of 13.8 (± 0.1) for warfarin and 3.2 (± 0.1) for L-tryptophan, which was the second highest set of retention factors. The precisions of the retention factors measured for each of the probes warfarin and L-Tryptophan ranged from ± 0.1 to ± 0.4 % and ± 0.1 to ± 0.5% respectively. As it can be seen in the table there was proportional variation in the retention factor of the probe drugs used warfarin and L-tryptophan, which have different binding sites.

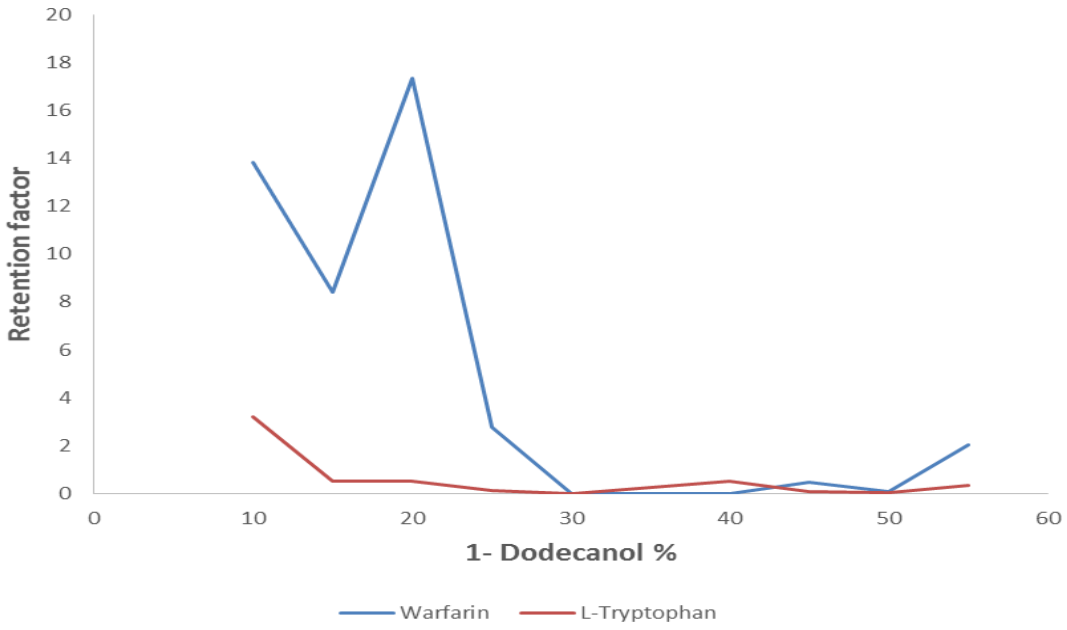
**Table 3.1.** Retention factors measured for various monoliths containing entrapped ha

[a]The numbers in parentheses represent a range of ± 1 S.D.

| **Porogen content** | **Retention factor (*k*) measured for probes for Sudlow sites I (warfarin) and II (L-tryptophan)** | | |
|---|---|---|---|
| 1-Dodecanol% (v/v) | Cyclohexanol% (v/v) | Warfarin | L-Tryptophan |
| 55 | 5 | 2.0 (± 0.4) | 0.5 (± 0.1) |
| 50 | 10 | 0.1 (± 0.2) | 0.1 (± 0.5) |
| 45 | 15 | 0.5 (± 0.4) | 0.1 (± 0.5) |
| 40 | 20 | (± 0.1) | 0.5 (± 0.1) |
| 30 | 30 | 0.0(± 0.2) | 0.0 (± 0.1) |
| 25 | 35 | 2.8 (± 0.1) | 0.1 (± 0.5) |
| 20 | 40 | 17.4 (± 0.1) | 0.5 (± 0.1) |
| 15 | 45 | 8.4 (± 0.1) | 0.5 (± 0.1) |
| 10 | 50 | 13.8 (± 0.1) | 3.2 (± 0.1) |

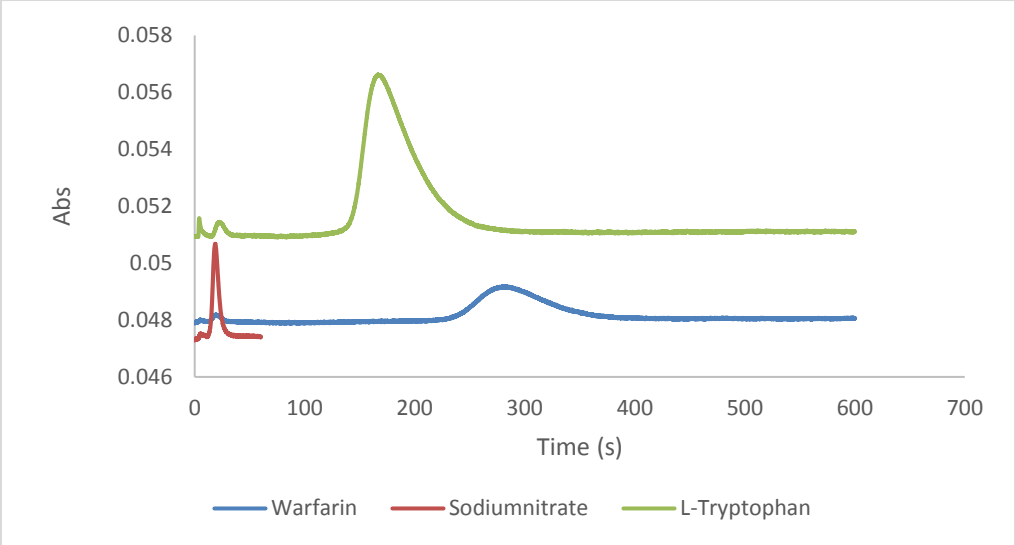
As can be seen in the Figures 3.2 the overall trends noted in the total amount of protein that could be immobilized as the amount of 1-dodecanol to cyclohexanol was varied in the monoliths. It was found that lower levels of 1-dodecanol compared to cyclohexanol for the GMA/EDMA monolith gave a slightly higher total protein content. This higher value at the lower 1-dodecanol amount is similar to the trend observed on monolith column made by epoxy method and Schiff base method for immobilization [16].

**Figure 3.2.** Comparison of the retention factors for warfarin and L-tryptophan on GMA/EDMA monoliths containing entrapped HSA as the ratio of the porogenic solvents was varied.


20
18
16
14
12
10
8
6
4
2
0
Retention factor
0
10
20
30
40
50
60
1- Dodecanol %
Warfarin
L-Tryptophan

Some typical chromatograms obtained in these studies are shown in Fig. 3.3. As noted earlier with warfarin and L-tryptophan, each of the drugs exhibited a large difference in retention between the entrapped-protein columns and the control column. Warfarin, which has an association equilibrium constant of roughly $2 \times 10^5$–$3 \times 10^5$ $M^{-1}$ for normal HSA [18], was eluted within 3.5–4.5min at 0.5 mL/min. L-Tryptophan, which has a tenfold lower association equilibrium constant of approximately $1 \times 10^4$–$2 \times 10^4$ $M^{-1}$ for normal HSA [19], was eluted in 2–3 min at 0.5 mL/min from the entrapped-protein columns 0.5mL/min. As it can be seen in fig. 3.3 the chromatograms were symmetrical without not much tailing. The chromatographic studies were performed at room temperature using pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase. The mobile phase buffer was degassed and sonicated for approximately 30 min prior to use. A sample containing a 20μM solution of warfarin was prepared in pH 7.4, 0.067 M potassium phosphate buffer. Sodium nitrate was used as a void marker as it doesn't have any affinity to the protein HSA. The elution of warfarin was monitored at 308 nm. The L-tryptophan were monitored at 280 nm. A 20 μL injection of 0.2 mM sodium nitrate was monitored at 205 nm. The extra-column void time was determined by injecting sodium nitrate onto a zero dead volume connector and monitoring the elution at 205 nm.

**Figure 3.3.** Examples of chromatograms obtained for the injection of sodium nitrate, warfarin, and L-tryptophan on monoliths containing entrapped HSA.

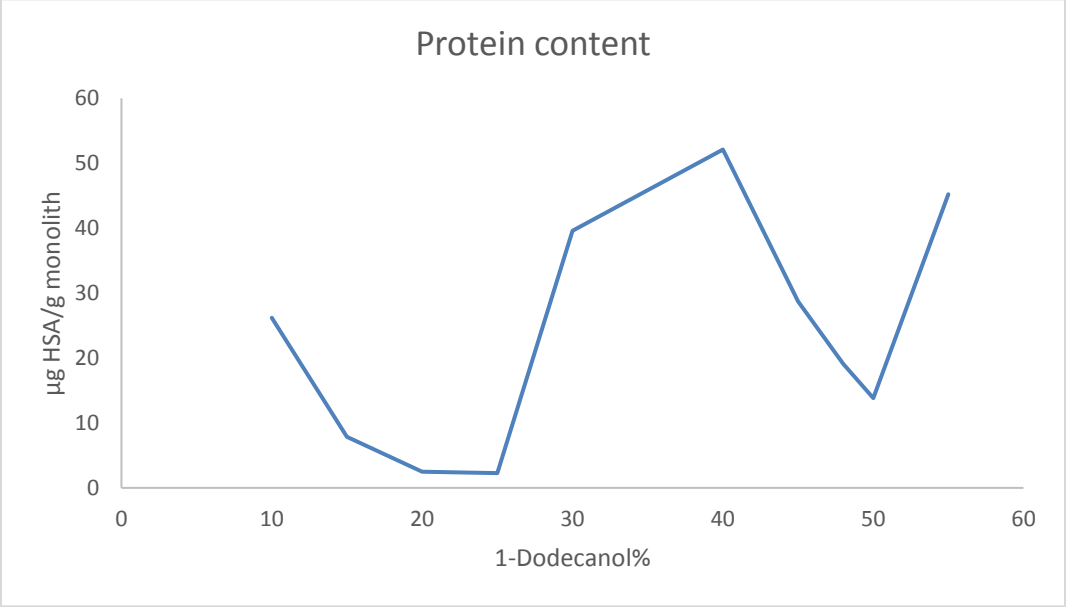

0.058
0.056
0.054
0.052
0.05
0.048
0.046
Abs
0
100
200
300
400
500
600
700
Time (s)
Warfarin
Sodiumnitrate
L-Tryptophan

### 3.4. Assessment of Monoliths

The total amount of protein was determined for each monolith via a BCA assay [16]. For this assay, each monolith was prepared in triplicate during the optimization studies and washed with 100 mL of water at 0.5 mL/min for 3 h at room temperature. The monolith column was then removed from the housing and ground to a fine powder through the use of a mortar and pestle. The powder was placed on a watch glass and dried in a vacuum oven overnight at room temperature. This same procedure was repeated for a control monolith that did not contain any immobilized protein. All samples were analyzed and prepared in triplicate, with HSA being utilized as the standard in the BCA assay.

**Figure 3.4** shows the overall trend in the total amount of protein that could be entrapped in µg of HSA/g of monolith as the amount of 1-dodecanol to cyclohexanol was varied in the monolith. It was found that higher levels of protein content was found between 30-40% 1-dodecanol. This result of amount of protein corresponds with previously made columns by epoxy method and Schiff base method of immobilization [16].

**Figure 3.4**. Effects of varying the porogen composition on the amount of HSA that could be entrapped to GMA/EDMA monoliths prepared at 80ºC.


Protein content
µg HSA/g monolith
60
50
40
30
20
10
0
0
10
20
30
40
50
60
1-Dodecanol%

## 3.5 Conclusion

In this study the goal was to optimize polymerization ratio of 1-dodecanol to cyclohexanol and maximize the entrapment of HSA protein on methacrylate based monoliths. Two columns, one entrapment and one control column were made and evaluated using retention factor value and BCA assay for their total protein content.

The retention factor measurement showed that the 10-20% 1- dodecanol ratio gave the highest entrapped protein content. The BCA assay method showed that the highest protein content was between 30-40 % 1-dodecanol. Although the two methods for estimating the protein content didn't point to the same porogonic ratio colulmns, they gave a good estimation on which porogenic ratio is a potential for further research and optimization. The direct measurement of the retention factor method to estimate the protein content seems more plausible method, since the BCA method gave high response to the control column and the HSA support. It can be said that the presence of the hydrazide groups on the support could reduce the $Cu^{2+}$ to $Cu^{1+}$ in the BCA. This large response for the control support creates small difference in the final absorbance that was measured. This will produce high variability in the final result of protein content which can lead to an inconsistency.

This chapter has considered and studied the method of entrapment on monolith supports, which was previously developed in the lab on hydrazide-activated supports was considered on monolith supports. It has showed a promising results which opens a novel way and experiments to examine the activities for many of these entrapped agents. These features should make this approach valuable for the entrapment of other proteins and biomacromolecules for HPLC and affinity separation methods.

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# CHAPTER FOUR: SUMMARY AND FUTURE WORK

## 4.1. SUMMARY OF WORK

This thesis has been mainly on the study of on-column method of entrapment on hydrazide-activated silica or monolith supports using oxidized glycogen as a capping agent. This method of entrapment can make supports that can withstand the high pressures and flow rates in HPAC. The entrapment method is based on the physical containment of a ligand in a polysaccharide-capped dihydrazide support or monolith. Careful attention needs to be given to the nature in which the affinity ligand is incorporated into the stationary phase, since it is for the study of biologically-related molecules. Ideally, the behavior of the immobilized ligand in the use of high performance affinity chromatography (HPAC) should mimic the behavior of the ligand in its natural environment. One effective way to maintain the activity of the ligand is to maintain the soluble form of the ligand by avoiding covalent immobilization.

**Chapter 1** presented a general introduction to HPAC, the different immobilization methods that are available, the use of monoliths as supports, and quantitative techniques of affinity chromatography. Background information on the entrapment method was also provided. The potential advantages of the entrapment method was also discussed.

**Chapter 2** mainly concerned a method for measuring the global affinity constants and site specific binding constants for drug-protein interactions. Samples of normal HSA and HSA with different glycation levels characteristic of patients with pre-diabetes, controlled diabetes or advanced diabetes were placed into supports made by entrapment and the on-

column method. The high protein contents that were achieved by the on-column method, and the fact that the activity of this protein was maintained in these supports, allowed the use of this method in a simplified approach for measuring drug-protein interactions at specific sites on normal HSA or glycated HSA. Using these columns it was possible to estimate binding constants by using simple retention measurements for a drug in the presence of only a buffer or a probe for each specific binding site that was to be studied.

In **Chapter 3** the work focused primarily on surveying the total amount of protein that can be entrapped in monolithic supports based on co-polymers of GMA/EDMA. This was done by preparing monolith HPAC columns that contained an entrapped HSA support. Zonal elution analysis experiments were then conducted with probe compounds for the major binding sites on HSA. This makes the immobilization technique a novel method from the traditional covalent monolith immobilization methods.

A variety of experiments were then conducted to optimize the protein content of these supports. These experiments were accomplished by varying the pore size of the support, by changing relative amounts of cyclohexanol to 1-dodecanol ratios. A library of monolith columns with different porogen ratios was made. The resulting protein content was estimated for each study, and optimum conditions were selected. It was also determined that high capacity supports could be produced.

## 4.2. FUTURE WORK

The optimization of the entrapment method has created many options for further implementation of this method. The work in this thesis involved the development of high capacity supports from hydrazide-activated silica and monolith columns containing entrapped

proteins for use in HPAC. For example, this approach may allow the possibility to study analytes with low affinities by using columns with high protein coverage.

The entrapment of wide range of ligands with various molecular weights is also another potential area of study. The ability to entrap a large variety of ligands will allow the production of new affinity supports. One example is the entrapment of streptavidin. To obtain binding information specific to each variant of the serum proteins, the different genetic variants of AGP could also be entrapped. Lectins are also another possibility (e.g., jacalin or *Sambucus nigra* agglutinin). These lectin columns could be used in studies for glycoproteomics, in which the binding of the entrapped lectins might be used for the isolation of glycopeptides or glycans prior to the analysis of these analytes by methods such as mass spectrometry.

Automation of the on-column entrapment method should be possible due to the intact nature of the entrapped protein. This could be done by using a system of pumps for applying the reagents through a hydrazide-activated silica column, and a system of valves that can switch between the various reagent solutions. One further step in automation would be to make a complete on-line entrapment system where the protein that is to be entrapped (e.g., HSA) could also be isolated from a biological sample (e.g., human serum) by using an antibody column for the protein, followed by elution of the captured protein directly into the hydrazide-activated silica support, where entrapment is then performed. Such a system would minimize loss of the protein during sample handling steps and would provide a convenient way of entrapping proteins or other biologically-related agents that are obtained from *in vivo* samples.

The protein remains fully active when it is immobilized by entrapment therefore this should make it possible the method to create fast screening methods that can be used to obtain the binding affinities for solutes with the entrapped proteins. As shown in Chapter 2, this can be accomplished by simply measuring retention time shifts when this solute is injected in the presence or absence of the entrapped protein.

## 4.3 ACKNOWLEDGMENTS

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**References**

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