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Optimization and Implementation of Entrapment: A Novel Immobilization Technique for High-performance Affinity Chromatography

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OPTIMIZATION AND IMPLEMENTATION OF ENTRAPMENT: A NOVEL IMMOBILIZATION TECHNIQUE FOR HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY

by

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OPTIMIZATION AND IMPLEMENTATION OF ENTRAPMENT: A NOVEL IMMOBILIZATION TECHNIQUE FOR HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY

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The proper use of high performance affinity chromatography (HPAC) in the study of biologically-related systems requires that careful attention be paid to the nature in which the affinity ligand is incorporated into the stationary phase. Ideally, the behavior of the immobilized ligand should mimic the behavior of the ligand in its natural environment. Retaining the soluble form of the ligand, by avoiding covalent immobilization completely, is one effective way to retain the activity of the ligand. Previously, noncovalent immobilization techniques that do not modify the ligand of interest have included physical entrapment onto low performance supports.

This dissertation introduces an alternative method of entrapment onto supports that can withstand the high pressures and flow rates associated with HPAC. The entrapment method is based on the physical containment of a ligand in a polysaccharide-capped dihydrazide support. This method was optimized for maximum ligand content, and employed for the immobilization of several different ligands onto HPAC supports, all of which retained essentially 100% of their binding activity. These supports were used to

study the binding affinities of a variety of drugs and the results were compared with previously established values obtained from studies using covalently immobilized ligands.

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CHAPTER 1:

GENERAL INTRODUCTION

Affinity Chromatography

Affinity chromatography is a type of liquid chromatography that employs biologically related interactions for the separation and analysis of specific analytes within a sample.[1,2] This process is accomplished through the use of a stationary phase containing a biologically-related agent, known as the "affinity ligand".[1,3] The specific, reversible interactions between this ligand and its target provide the basis for selective separations. In this method, one of the pair of interacting molecules is immobilized onto the support composing the stationary phase, while the other molecule is contained within the mobile phase or injected sample. Initially, affinity chromatography separations were conducted using low-performance carbohydrate-based supports, such as agarose, sepharose or polyacrylamide. Although easy and inexpensive to use, these support materials are only structurally stable at low pressures.[1-3]

High-performance affinity chromatography (HPAC) is an adaptation of affinity chromatography, combining the selective nature of affinity chromatography with the small, rigid support particles of HPLC systems, that are capable of withstanding high pressures. This increased pressure stability provides an efficient and rapid means of selective separation and analysis of complex biological systems. When compared to its low-performance counterpart, HPAC tends to be more costly to perform, but the resultant advantages of this method outweigh these drawbacks.[3-5]

One of the central uses of HPAC, which will be discussed in this dissertation, is its ability to quantitatively characterize biological interactions. There are many advantages to using HPAC for these types of studies, the main advantage being the stability of the immobilized ligand. Because the ligand is incorporated directly into the stationary phase, the resulting column can often be used for a large number of experiments. This feature not only reduces the amount of total affinity ligand needed for the experiments, but also leads to an increase in the overall precision by using the same stationary phase for multiple studies.[3, 6, 7]

Zonal Elution.

Zonal elution is the most common HPAC method used to analyze analyte-ligand binding. This method is preformed by injecting a small plug of analyte onto a column in the presence of a mobile phase, often a buffer at a physiological pH, that is applied at a constant flow-rate. The retention time or volume of the analyte is monitored either on- or off-line using an appropriate detector. Analyte retention is dependent upon the interaction between the analyte and the immobilized ligand contained in the column and can be represented by the analyte's overall retention factor (k) as determined by **Equation 1-1**.

$$k = \frac{(t_R - t_M)}{t_M} \quad \text{or} \quad k = \frac{(V_R\text{-}V_M)}{V_M} \tag{1-1}$$

In this equation, t_R is the retention time of the analyte, V_R is the corresponding retention volume, t_M is the void time of the column, and V_M is the void volume. A shift in analyte retention will be caused by a change in binding strength or the number of binding sites within the column.

When comparing the retention of an analyte on two separate columns, the retention factor can be normalized by dividing the calculated retention factor by the total protein content of the support. The retention factor can be related to the number of binding sites in the column and to the equilibrium constant or the analyte according to **Equation 1-2**.

$$k = \frac{(K_{a1}n_1 + \cdots K_{an}n_n)m_L}{V_m} \quad (1\text{-}2)$$

Here, K_{a1} through K_{an} are the association equilibrium constants for binding sites 1 through n, n_1 through n_n represent the number of each type of site in the column, m_L is the moles of total binding sites in the column, and V_M is again the column void volume[6].

Frontal Analysis.

Frontal analysis, while originally used as a purification technique, has become a popular tool for obtaining information about the interactions between various ligand-analyte systems. Frontal analysis is preformed when an analyte is continuously applied to a column containing an immobilized affinity ligand. As the ligand interacts with the analyte, binding sites on the ligand gradually become saturated. Once all of the available binding sites on the ligand are bound to the analyte, excess analyte passes through the column to the detector. This sequence of events creates what is known as a breakthrough curve. If fast association/dissociation kinetics are present in the system, the breakthrough curves obtained through frontal analysis measurements can be related to both the association equilibrium constant (K_a), the moles of active binding sites in the column (m_L) and the concentration of the applied analyte, [A]. **Equation 1-3** can be used to

describe the frontal analysis response for an analyte that binds to an immobilized ligand at a single site.

$$\frac{1}{m_{Lapp}} = \frac{1}{(m_L K_a [\mathrm{A}])} + \frac{1}{m_L} \qquad (1\text{-}3)$$

In this equation, m_{Lapp} is the apparent moles of analyte required to saturate the column at a given concentration of applied analyte. Using **Equation 1-3**, a plot of $1/m_{\mathrm{Lapp}}$ vs. 1/[A] should yield a linear relationship if there is single-site binding between the ligand and analyte. The inverse of the intercept will give m_{L}, while dividing the intercept by the slope of the best-fit line will give K_{a}.[6]

Affinity Ligands

Human Serum Albumin (HSA).

HSA is the most abundant protein in human serum, with typical concentrations of approximately 40-45 g/L. This protein makes up about 60% of the total protein in blood serum.[8, 9] HSA is composed of a single chain of 585 amino acid residues, stabilized by 17 internal disulfide bonds. This globular, heart-shaped protein has a molecular weight of 66.5 kDa and a size of approximately 80 x 80 x 30 Å.[10] HSA has a variety of functions in the body; however, this dissertation will focus on the role of HSA as a carrier protein in serum and will investigate the binding of HSA to several target analytes. This protein has two major binding sites for drugs, Sudlow sites I and II. Sudlow site I, which is also known as the warfarin-apapropazone site, binds to bulky heterocyclic compounds. Sudlow site II, the indole-benzodiazapine site, binds to aromatic carboxylic acids.[10-12] HSA is commonly used as an immobilized ligand in HPAC to study drug binding.[13] This

binding can be affected by many factors,[14] and portions of this dissertation will investigate the effects of protein modification due to glycation on drug binding.

In adults, an average of 6-15% of serum HSA is glycated, with that value increasing to 20-30% in adults with type II diabetes.[15, 16] Glycation, or the modification of a protein through the reaction between a reducing sugar and a free amine group on the protein, can take place when HSA is in the presence of glucose. This process initially forms a reversible Schiff base that can rearrange to form an irreversible ketoamine, also known as an Amadori product.[15] This process is especially prevalent in diabetes, resulting in the increased percentage of glycated HSA. Glycation is thought to alter the secondary and tertiary structure of HSA, potentially affecting the binding of analytes to HSA,[17] as some of the glycation sites are near Sudlow sites I and II.[18] Studies have been conducted to investigate the effect of glycation on the binding of certain drugs to HSA, including the use of covalently immobilized HSA in HPAC.[19-22] This dissertation will present data on this effect, as obtained through the use of an entrapment method for immobilization and HPAC zonal elution binding studies.

α_1-Acid Glycoprotein (AGP).

Human AGP, also known as orosomucoid, is a negatively charged, acidic glycoprotein, consisting of 59% protein and 41% carbohydrate. This 41 – 43 kDa glycoprotein has a serum concentration of approximately 1 g/L; however, different conditions affect his concentration, including acute inflammation, cancer, rheumatoid arthritis, and pregnancy. AGP is among the major binding proteins for drugs in serum. It is a major transport protein for basic drugs, but it has also been shown to be a carrier for

steroids and acidic drugs.[23, 24] The binding of drugs to AGP has been extensively studied and reviewed.[23-28]

AGP contains seven total binding sites: five low affinity sites for the binding of endogenous substances, and two drug-binding sites (i.e., one with low affinity and one with high affinity). For practical purposes, one drug binding site (i.e., the high affinity site) is the only site considered of clinical relevance.[23, 24] The binding affinity of drugs to AGP is also dependent upon the relative concentrations of the three genetic variants of AGP. These variants can exhibit different drug specificity.[23, 24, 29] For the studies in this dissertation, the same sample of commercial (unfractionated) AGP was used for all drug-binding studies, eliminating binding discrepancies based on variant concentration.

The binding of drugs to AGP has been studied using ultra-filtration and equilibrium dialysis.[23,25, 29] Recently an immobilization technique was developed making it possible to study AGP-drug interactions via HPAC. This immobilization was achieved through the mild oxidation of carbohydrate residues on the surface of AGP.[26, 28] This dissertation will investigate the use of the entrapment method as a means for AGP immobilization in the preparation of HPAC supports. Data obtained from AGP-drug binding studies, with this approach is presented in **Chapter 3** and **Chapter 5**.

Protein G.

Protein G is a bacterial cell surface protein that is commonly used as a binding agent in affinity chromatography and other methods for the selective adsorption of immunoglobulins and antibodies.[30-33] This protein is produced by groups C and G streptococci and binds to the F_c regions of many types of immunoglobulins, including all

subclasses of mouse immunoglobulin G (IgG), rabbit IgG, goat IgG, and human IgG[31]. Protein G has strong binding to these targets at a neutral or slightly acidic pH (e.g., pH 5-7.5) and yet can be made to release any retained immunoglobulins at pH 2.5-3.0.[31] These properties have made protein G popular as a binding agent for the purification of immunoglobulins and as a secondary binding agent that can be used to adsorb antibodies for use in immunoaffinity chromatography or immunoassays.[30-33]

Over the last decade there have been several examples in which immobilized protein G has been used in small affinity columns or in affinity capture systems for the adsorption of immunoglobulins.[34-37] The small size of many of these columns and affinity sorbents ideally requires that a relatively large amount of protein G be present in a small volume for the effective capture of the desired target.[37] However, no previous studies have determined the maximum amount of protein G that can be placed on common porous HPAC supports. An estimate of the maximum amount of protein G that can be covalently immobilized to silica with various pore sizes and an examination of the binding of these supports to immunoglobulins, using rabbit IgG as a model, is presented in **Chapter 6**.

Traditional Immobilization Techniques for HPAC

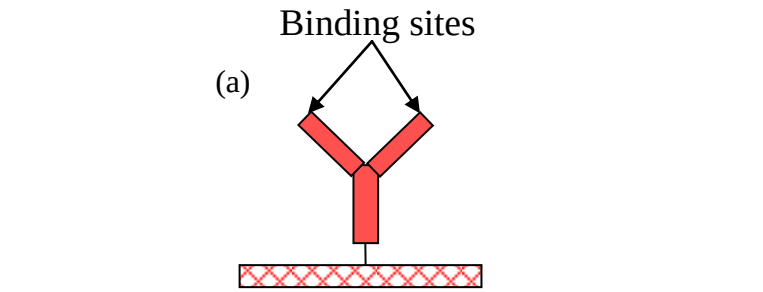
The proper use of HPAC in the study of biologically-related systems requires that careful attention be paid to the nature in which the affinity ligand is incorporated to the stationary phase. The type of immobilization method is extremely important because it can alter the actual or apparent activity of the ligand within the support.[3, 13, 38] In order to obtain accurate protein binding data, consideration of the type of ligand immobilization

technique is essential. Ideally, the behavior of the immobilized ligand should mimic the behavior of the ligand in its natural environment. In this dissertation, the term "immobilization" will not be limited to a covalent linkage of the ligand to the support; rather it will be used to refer to the way the ligand is incorporated within the support.

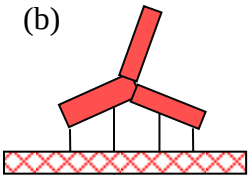
Traditionally, a ligand, such as a protein, is covalently immobilized onto the surface of the HPAC support. This immobilization can be instituted through several different functional groups on the ligand. During protein immobilization, free amine groups are often used in techniques such as the cyanogen bromide method, reductive amination (i.e. the Schiff base method), the *N*-hydroxysuccinimide technique, and the carbonyldiimidazole method.[39-44] Protein immobilization is not limited to free amine groups alone; other functional groups that may be employed include sulfhydryl groups, carboxyl groups, and carbonyl groups.[28, 45]

Complications can arise during covalent immobilization because there is often more than one functional group present on a ligand such as a protein. This can not only lead to attachment of the ligand to the support through multiple sites but can also lead to an orientation of the ligand that interferes with the ligand's binding sites, as shown in **Figure 1-1**. These effects can lead to a decrease or complete loss in the activity of the ligand.[2,28,38,46,47] However, multi-site attachment and random orientation can be minimized by coupling the ligand to the support through functional groups that occur in a select number of places in the structure of the ligand. One example is the use of carbohydrate chains in glycoproteins like AGP for site-selective attachment to hydrazide-activated supports.[28]

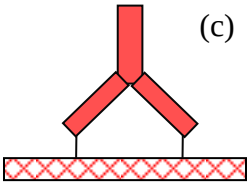
Figure 1-1. Covalent immobilization techniques can result in several orientations of the immobilized ligand: (a) proper orientation; all binding sites are unobstructed and available for analyte binding, (b) multisite attachment; the ligand is immobilized through multiple sites, (c) improper orientation; the ligand is immobilized through residues at or near the binding sites.



Proper orientation



Multi-site attachment



Improper orientation

Noncovalent immobilization techniques can involve the simple adsorption of a ligand to a surface, the binding of one ligand to a secondary ligand, or the formation of a coordination complex between the ligand the support. An alternative noncovalent immobilization method is that of entrapment or encapsulation. Physical entrapment of a ligand within a support is a technique that has been widely employed in the field of chromatography for many years. The long-standing definition of entrapment focuses on the physical containment of a ligand within a support that contains small pores or a highly cross-linked polymer network.[48-50] The attraction to this type of method stems from the lack of modification to the ligand itself; therefore any conformational changes to the ligand are avoided, and all of the ligand's activity is retained.

Physical containment of a ligand has been popularized through the wide usage of sol-gel materials. Most recently, bioencapsulation of ligands in sol-gels, including but not limited to proteins, enzymes, phospholipids, and nucleic acids, has been used for the development of biosensors and bioreactors. Developments in whole cell encapsulation has lead to advancements in cell-based biosensing and bioproduction, allowing the development of artificial organs.[48-52] Liposomal entrapment has been used for the encapsulation of a wide variety of ligands, including anti-infective agents, vaccine adjuvants, peptides and DNA.[53,54] These previous examples all employ physical containment of the ligand of interest, allowing for increased ligand lifetime and prolonged ligand activity through degradation prevention. Traditionally, entrapment has been limited to low-performance supports and is therefore restricted to low pressures and flow rates.[48-57]

Entrapment for Ligand Immobilization in HPAC

This dissertation introduces an alternative approach to entrapment that is capable of withstanding conditions used in HPAC. This entrapment method is based on the physical containment of a ligand in a polysaccharide-capped dihydrazide support. This support is produced by first activating a porous or nonporous silica with hydrazide groups.[58] This hydrazide-activated support is then incubated with a solution containing the ligand of interest and an oxidized form of a large polysaccharide. The oxidized polysaccharide will be prepared according to methods adopted from previous work in the oxidation of glycoproteins by periodate.[28, 59, 60]

When these oxidized polysaccharides are incubated with the hydrazide-activated support, aldehyde groups on the oxidized polysaccharide should form a stable covalent bond with the hydrazide groups. This process should entrap a portion of the ligand in the support. The initial examination and optimization of the entrapment method is presented in **Chapter 2**.

Overall Goal and Summary of Work

The overall goal of this dissertation is to examine the use of entrapment and other methods as ligand immobilization techniques for HPAC. **Chapter 2** will first examine the viability of the entrapment method as an alternative to the traditional covalent immobilization of ligands for use with HPAC. The rest of **Chapter 2** will focus on the optimization of the entrapment method in an effort to maximize the protein content of immobilized ligands on such supports. **Chapter 3** will examine the optimized entrapment parameters through the zonal elution analysis of drugs with known binding to

HSA. These results will be compared with data obtained from a support made with the Schiff base method under identical conditions. **Chapter 4** will assess the ability of the entrapment method to immobilize glycated HSA, through zonal elution studies of site-specific probe compounds and a series of sulfonylureas. The resulting data for the entrapped glycated HSA will be compared not only to the values obtained for the entrapped HSA but also to established literature values. **Chapter 5** will test the ability of the method to entrap AGP and examine the binding of a series of drugs to entrapped HSA and entrapped AGP. Association equilibrium values will be calculated based on the measured retention factors and column efficiency will be compared based on plate height calculations. **Chapter 6** will describe work done to create high-capacity protein G supports through traditional covalent immobilization techniques. Finally, **Chapter 7** will discuss the future prospects of the entrapment method, including plans for further optimization and application to a wider variety of chromatographic situations.

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CHAPTER 2:

ENTRAPMENT – AN ALTERNATIVE IMMOBILIZATION METHOD FOR USE IN HPAC BINDING STUDIES: METHOD DEVELOPMENT AND OPTIMIZATION

Introduction

Affinity chromatography employs the interactions between a ligand and a particular analyte to determine the manner in which ligand-analyte binding occurs. Traditionally, the ligand is covalently immobilized onto the surface of the support. This immobilization can be instituted through several different functional groups on the ligand. Free amine groups on protein-based ligands are often used in immobilization techniques such as the cyanogen bromide method, reductive aminiation (i.e., Schiff base method), the *N*-hydroxysuccinimide technique, and the carbonyldiimidazole method.[1-6] However, protein immobilization is not limited to free amine groups alone; other functional groups that may be employed include sulfhydryl groups, carboxyl groups, and carbonyl groups.[7,8]

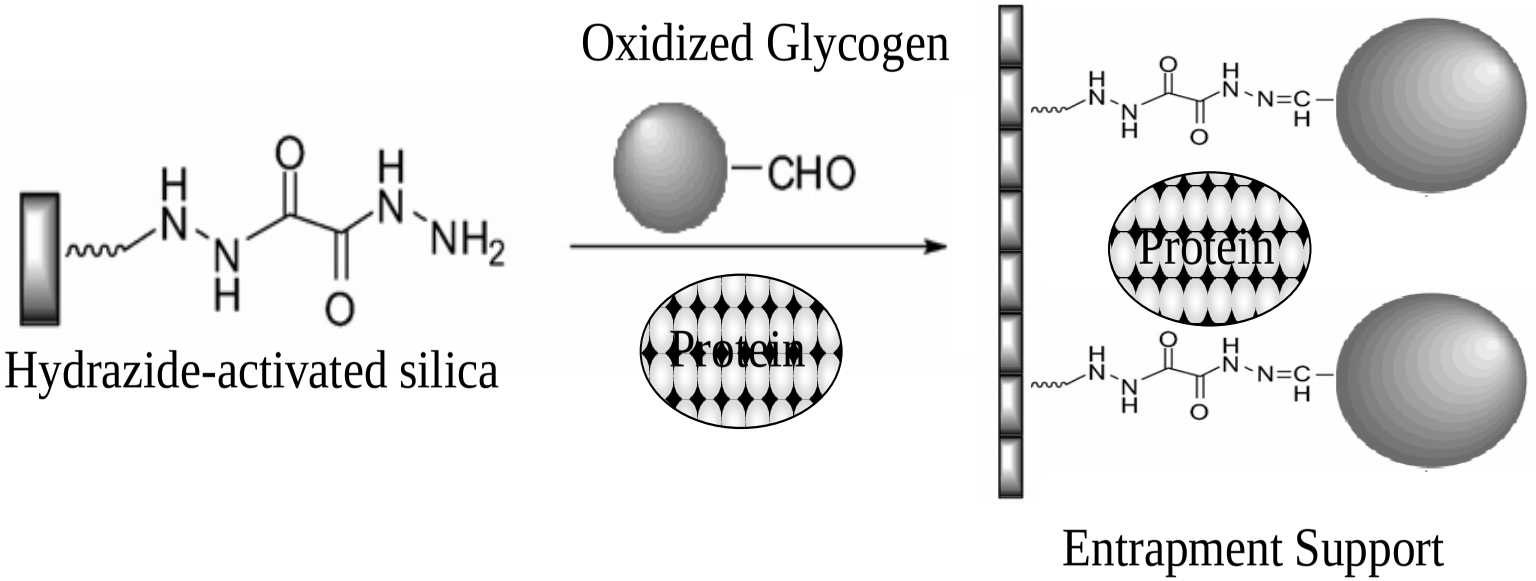
Complications can arise during covalent immobilization because there is often more than one functional group present on a ligand such as a protein. This can not only lead to multi-site attachment but can also lead to random orientation which can lead to a decrease or complete loss in the activity of the ligand.[7, 9-11] Noncovalent immobilization techniques can involve the simple adsorption of a ligand to a surface, the binding of one ligand to a secondary ligand, or physical entrapment or encapsulation.[12] These techniques avoid undesirable immobilization effects because the ligand itself is not

modified, therefore retaining all ligand activity.[13] Traditionally, entrapment has been limited to low-performance supports and has therefore been restricted by pressure and flow rate constraints.[14]

The work in this chapter introduces an alternative approach to entrapment that is capable of withstanding conditions used in high performance ligand chromatography (HPAC). This entrapment method involves the physical containment of a ligand in a polysaccharide-capped dihydrazide support. This method is performed by first activating a porous or nonporous silica support with hydrazide groups, as prepared according to a previously-published technique.[15] This hydrazide-activated support is then incubated under slightly acidic pH conditions with a solution containing the ligand of interest and an oxidized form of a large polysaccharide. The oxidized polysaccharide is prepared according to methods adopted from previous work in the oxidation of glycoproteins by periodate.[7]

When these oxidized polysaccharides are incubated with the hydrazide-activated support, aldehyde groups on the oxidized polysaccharide should form a stable covalent bond with the hydrazide groups, a reaction known to occur even in a neutral aqueous buffer[15, 16]. This process should entrap the ligand in the support, as shown in **Figure 2-1**. The performance of this method will be examined and optimized through the use of human serum albumin (HSA) as a model ligand. The binding capacity and affinity of HSA will be determined and compared with data obtained from covalently immobilized HSA supports.

Figure 2-1. General scheme for entrapment of a biomolecule such as a protein using a glycogen-capped and hydrazide-activated support.


Oxidized Glycogen
CHO
Protein
Protein
Hydrazide-activated silica
Entrapment Support

Frontal Analysis.

The method of frontal analysis was used to determine the association equilibrium constants (K_a) and the moles of active binding sites (m_L) for *S*-warfarin and L-tryptophan (see structures in **Figure 2-2**). If fast association/dissociation kinetics are present in a drug-protein system, the breakthrough curves obtained through frontal analysis measurements can be related to both K_a, m_L and the concentration of the applied analyte, [A]. The **Equation 2-1** can be used to describe the frontal analysis response for an analyte that binds to an immobilized protein through a single site.[17]

$$\frac{1}{m_{Lapp}} = \frac{1}{(m_{Lapp}K_a[\mathrm{A}])} + \frac{1}{m_L} \qquad (2\text{-}1)$$

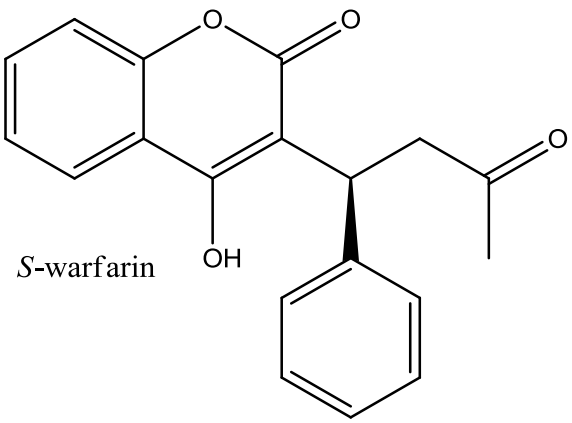
In this equation, m_{Lapp} is the apparent moles of analyte required to saturate the column at a given concentration of applied analyte. Using **Equation 2-1**, a plot of $1/m_{Lapp}$ vs. 1/[A] should yield a linear relationship is there is single-site binding between the ligand and analyte. The inverse of the intercept will give m_L, while dividing the intercept by the slope of the best-fit line will give K_a.[17]

Experimental

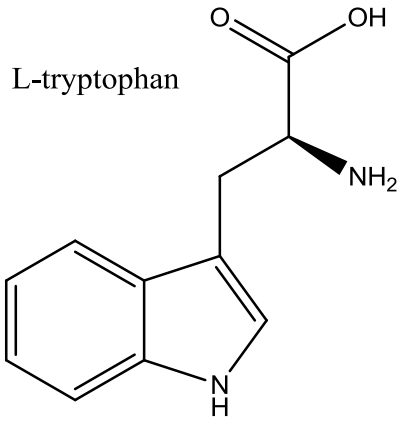
Reagents.

The *p*-periodic acid (periodic acid reagent, or H_5IO_6), glycogen (from bovine liver)**,** HSA (Cohn fraction V, 99% globulin free, 99% fatty acid free), human alpha 1-acid glycoprotein (AGP, 99%), glycated HSA (95% lyophilized, lot no. 115K6108), goat immunoglobulin G (IgG), *S*-warfarin, L-tryptophan, insulin, myoglobin, streptavidin, avidin, ferritin, monobasic and dibasic potassium phosphate salts, sodium chloride and

Figure 2-2. Structures of *S*-warfarin and L-tryptophan.



S-warfarin



L-tryptophan

sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO**).** The Nucleosil Si-50, Si-100, Si-300, Si-500 and Si-1000 silica (all 7 μm diameter, with nominal pore sizes of 50 Å, 100 Å, 300 Å, 500 Å, and 1000 Å, respectively) were purchased from Macherey-Nagel (Düren, Germany). The nonporous glass beads (3-10 μm particle size) were purchased from Polysciences (Warrington, PA). Reagents for the micro bicinchoninic acid (BCA) protein assay were purchased from Pierce (Rockford, IL). Other chemicals were of the purest grades available. The Econo-Pac 10 DG disposable 30 x 10 mL desalting columns were purchased from Bio-Rad Laboratories (Hercules, CA). All solutions were prepared using water from a NANOpure purification system (Barnstead, Dubuque, IA) and filtered with a 0.20 μm GNWP nylon membrane from Millipore (Billerica, MA).

Apparatus.

The chromatographic system consisted of two Jasco PU-980i intelligent HPLC isocratic pumps (Tokyo, Japan), a Rheodyne Advantage PF ten-port valve (Cotati, CA), and a Jasco UV-975 UV/Vis detector**.** Data were collected using an interface and software from National Instruments (Austin, TX). The temperature of the columns and mobile phases was controlled using a PolyScience circulating VWR circulating water bath (Buffalo Grove, IL) and a water jacket from Alltech. All columns were packed using an HPLC column slurry packer from Alltech.

Methods.

Preparation of Oxidized Glycogen.

The procedure for oxidizing glycogen was adapted from previously-published work and optimized for use with the entrapment method.[7, 18, 19] The oxidation of

glycogen was done in a pH 5.0 buffer containing 20 mM sodium acetate and 15 mM sodium chloride. A 10.0 mL solution was prepared by dissolving 200 mg of periodic acid and 50 mg of glycogen from bovine liver in the aforementioned buffer. This solution was covered in aluminum foil – due to the light sensitivity of periodic acid and allowed to shake at room temperature for 12-24 h. After oxidization, the glycogen was purified using three Econo-Pac 10DG disposable desalting columns and pH 5.0, 0.10 M potassium phosphate buffer. This process was done by first removing the upper cap of each column and decanting the excess storage buffer. The next step was a buffer exchange via the addition of 10.0 mL of pH 5.0, 0.10 M potassium phosphate buffer to each column. Next, 3.3 mL of the oxidized glycogen sample were allowed to pass through each column causing the elution of the newly introduced potassium phosphate buffer. To each column, 4.0 mL of potassium phosphate buffer was then added to elute the oxidized glycogen. The larger glycogen molecules eluted from the column during this step, while the smaller periodic acid molecules took much longer to pass through the column matrix. The three fractions of purified oxidized glycogen were collected and combined to produce a 4.2 mg/mL solution with a final volume of 12.0 mL. This solution was stored at 4 °C.

Preparation of Entrapped Supports.

Diol silica was prepared from nonporous glass beads and Nucleosil Si-50, Si-100, Si-300, Si-500 or Si-1000, and was then converted into a dihydrazide-activated form according to previously-established procedures.[15] A 50 mg/mL stock solution of HSA was prepared with pH 5.0, 0.10 M potassium phosphate buffer. To create the entrapped support, 30 mg of dihydrazide activated silica was combined with 500 µL of the HSA

solution. This mixture was sonicated under vacuum for 15 min to ensure the proper mixing of the silica and protein. A 100 µL portion of the purified oxidized glycogen was added, and the mixture was shaken at room temperature for 24 h. During the final hour of the reaction, 200 µL of a 2 mg/mL oxalic dihydrazide solution in the same buffer was added to the reaction mixture to cover any remaining aldehyde groups on the silica support. After immobilization, the support was washed several times with pH 7.4, 67 mM potassium phosphate buffer A control support was also prepared through the addition of a 500 µL aliquot of potassium phosphate buffer in place of the HSA solution. Binding of an analyte to the prepared control support was used to monitor non-specific interactions in the frontal analysis studies.

After immobilization, all supports were downward slurry-packed into separate stainless steel columns at 4000 psi using pH 7.4, 67 mM potassium phosphate buffer as the packing solution**.** Columns used in the S-warfarin frontal analysis studies were packed into 5.0 cm × 2.1 mm I.D. stainless steel columns, while those used in the L-tryptophan frontal analysis studies had dimensions of 1.0 cm × 2.1 mm I.D. All columns were stored at 4 °C in the packing solution.

Initial Chromatographic Studies.

All samples for the chromatographic studies were prepared in pH 7.4, 67 mM potassium phosphate buffer. The warfarin solution was prepared through the dilution of a 30 µM stock solution of *S*-warfarin. The L-tryptophan solution was prepared through the dilution of a 100 µM of L-tryptophan. This pH 7.4 buffer was also used as the mobile phase. All solutions were stored at 4 °C. Prior to use, all mobile phases were filtered through a 0.20 µm GNWP nylon filter and were degassed for a minimum of 20 min. The

column temperature was maintained at 37.0 (±0.1) °C during all experiments through the use of a temperature-controlled water jacket.

Frontal analysis studies were performed using two well-characterized probe compounds for HSA: *S*-warfarin, known to bind to Sudlow site I, and L-tryptophan, known to bind to Sudlow site II.[20, 21] In the frontal analysis studies, the column was first equilibrated with pH 7.4, 67 mM potassium phosphate buffer, with the equilibration being completed between each successive run. A switch was made between the pH 7.4 buffer and the sample solution using an automated ten-port valve at the initiation of data collection. The sample solution was continuously applied to the entrapped HSA column until all binding sites were saturated, producing a breakthrough curve. The valve was then switched to return the flowing mobile phase to the pH 7.4 buffer, which was used to elute any remaining bound analyte from the column. All runs were performed in triplicate on both the entrapped HSA column and the control column. These studies were conducted to not only determine the activity of the entrapped HSA but also to determine an association equilibrium constant for comparison with an established immobilization method. Both *S*-warfarin (304 nm) and L-tryptophan (280 nm) were analyzed, with concentration ranging from 5 to 25 µM and 1 to 25 µM, respectively. Breakthrough times for each analyte on both the entrapped HSA column and control column were determined by using the equal area method. The amount of analyte needed to saturate these columns was determined by integration of the resulting breakthrough curves, after correcting for the void time of the system and non-specific binding to the support, as measured on the control column.[22] The non-specific binding of *S*-warfarin and L-

tryptophan was found to be less than 5% and 9%, respectively, of the total binding measured on columns containing entrapped HSA.

Protein Content Determination Using the Micro BCA Assay.

A sample of each support was washed several times with water and dried at 50 °C. These samples were used to determine the overall protein content of support materials used in entrapped HSA columns. This determination was done through use of a micro bicinchoninic acid (BCA) protein assay.[23, 24] This assay was used not only for analysis of the HPAC supports but also for the smaller samples that were prepared during all subsequent optimization experiments.

Entrapment Optimization.

After initial chromatographic measurements were made, studies were done to determine the optimum conditions for the entrapment method in an effort to obtain maximum protein content. These studies investigated the effect of porosity, the concentration of both the glycogen and protein in the initial reaction mixture, the rate of immobilization, and the ratio of periodic acid to glycogen with regard to the overall final protein content. All studies were done with small samples of silica and analyzed for protein content using a micro BCA assay.

Results and Discussion

Initial Studies of the Entrapment Method.

The viability of the entrapment method as a means to create HPAC supports containing a biologically active ligand was initially tested through the injection of *S*-warfarin onto three separate HPAC columns. These columns included an entrapped HSA

support, a control support prepared in the same manner as the entrapped HSA support but without the addition of HSA to the reaction mixture, and a support containing only dihydrazide-activated silica. Injections on the entrapped HSA support indicated not only that HSA was entrapped, but also that it was accessible for binding with small molecules such as *S*-warfarin. Injections on the two subsequent supports indicated that *S*-warfarin had low nonspecific binding to both the dihydrazide activated silica and the oxidized glycogen on the control support.[18] These studies indicated that the entrapped method could be a useful technique for the immobilization of a protein for drug-protein binding studies in HPAC. In the remainder of this chapter, the entrapment method was examined using frontal affinity chromatography and optimized through variations of individual initial reaction conditions.

Frontal Analysis Studies of Entrapped HSA.

Initial frontal analysis experiments employed *S*-warfarin as the model analyte for the study of drug binding to Sudlow site I. For this study, supports from several pore sizes of silica were prepared. Silica with pore sizes of 50 Å, 100 Å, 300 Å, 500 Å, or 1000 Å was converted to dihydrazide-activated silica and used for the preparation of both control supports and supports containing entrapped HSA.[15] These supports were slurry packed into separate 5.0 cm × 2.1 mm I.D. columns. A concentration range of 5-20 μM *S*-warfarin was used for this frontal analysis study. Breakthrough times were calculated from the resultant curves and m_{Lapp} values were determined at each analyte concentration. Breakthrough curves from the frontal analysis of *S*-warfarin on the 300 Å entrapped HSA column can be seen in **Figure 2-3**.

Double-reciprocal plots of $1/m_{Lapp}$ vs. 1/[*S*-warfarin] were obtained for each set of columns. **Figure 2-4** shows a double-reciprocal plot made according to **Equation 2-1** for frontal analysis data obtained for the binding of *S*-warfarin to an entrapped HSA support with the following pore sizes of 50 Å, 100 Å, and 300 Å. The correlation coefficients were 0.998 (n = 5) for the 50 Å pore size silica, 0.997 (n = 5) for the 100 Å pore size silica and 0.994 (n = 5) for the 300 Å pore size silica. No noticeable deviations from linearity were noted over the concentration range studied for any of these pore sizes. This indicated that there was single-site binding for *S*-warfarin to the entrapped HSA under the conditions of this experiment (pH 7.4 and 37 ºC), agreeing with previous work using covalently immobilized HSA.[25] Similar binding behavior was observed in frontal analysis experiments performed using entrapped HSA columns prepared with other pore size silica supports. The double-reciprocal plots for these studies had correlation coefficients ranging from 0.994-0.998 (n = 5) over the same concentration range as shown in **Figure 2-4**.

By using the intercepts and slopes from these double reciprocal plots along with **Equation 2-1**, it was possible to determine the association equilibrium constants for *S*-warfarin with the entrapped HSA columns. The five pore sizes of silica supports gave an average association equilibrium constant of 1.7 (± 0.2) × 10^5 M^{-1} at pH 7.4 and 37 ^oC. These results are in good agreement with values reported for soluble HSA (e.g., 2.0 × 10^5 M^{-1}) as well as those measured for HSA that had been immobilized by the Schiff base method (e.g., 2.0 × 10^5 M^{-1}).[25]

Figure 2-3. Breakthrough curves for *S*-warfarin on the 300 Å pore size entrapped HSA column. Concentrations from left to right: 25, 20, 15, 10, and 5 µM *S*-warfarin.

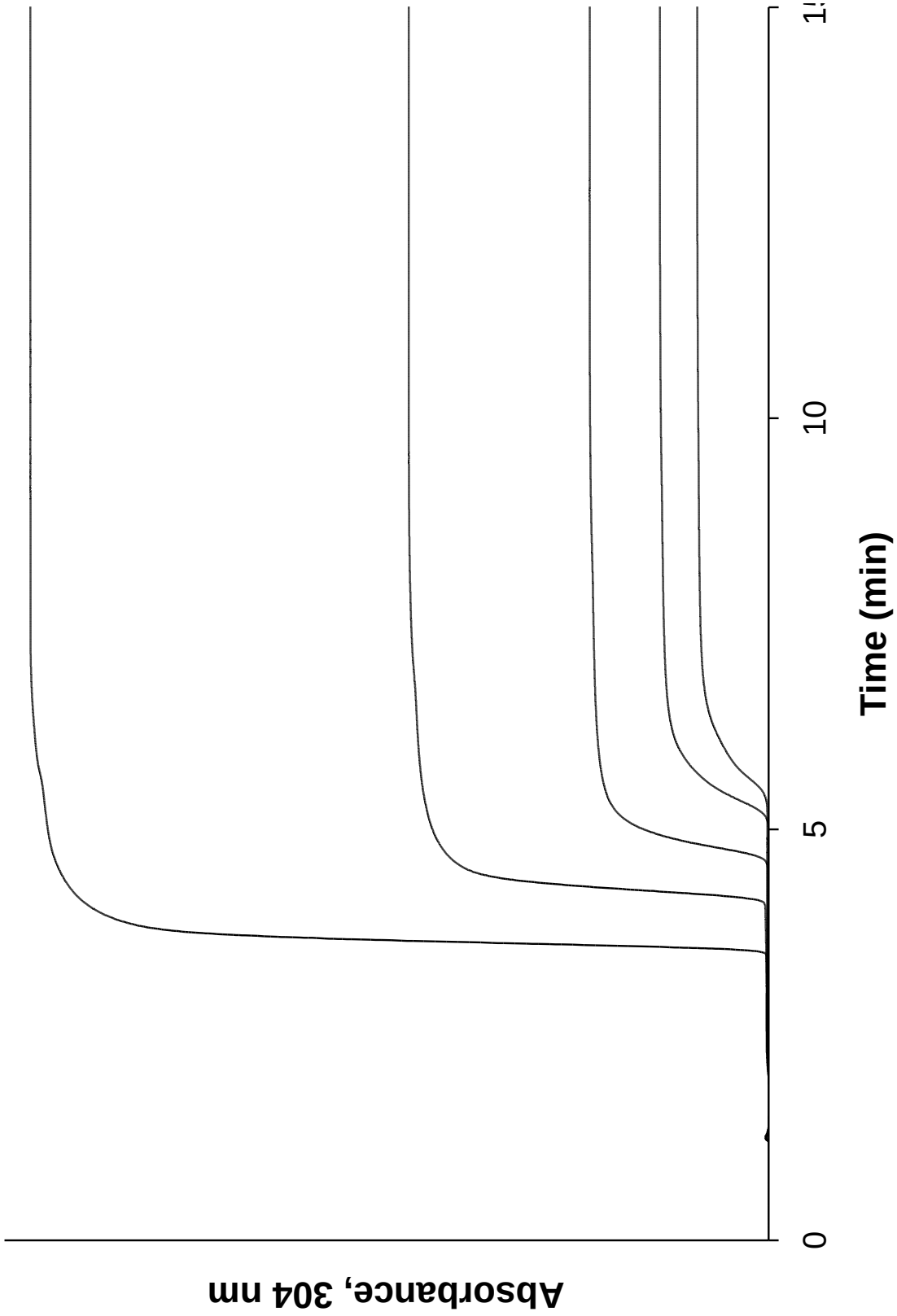
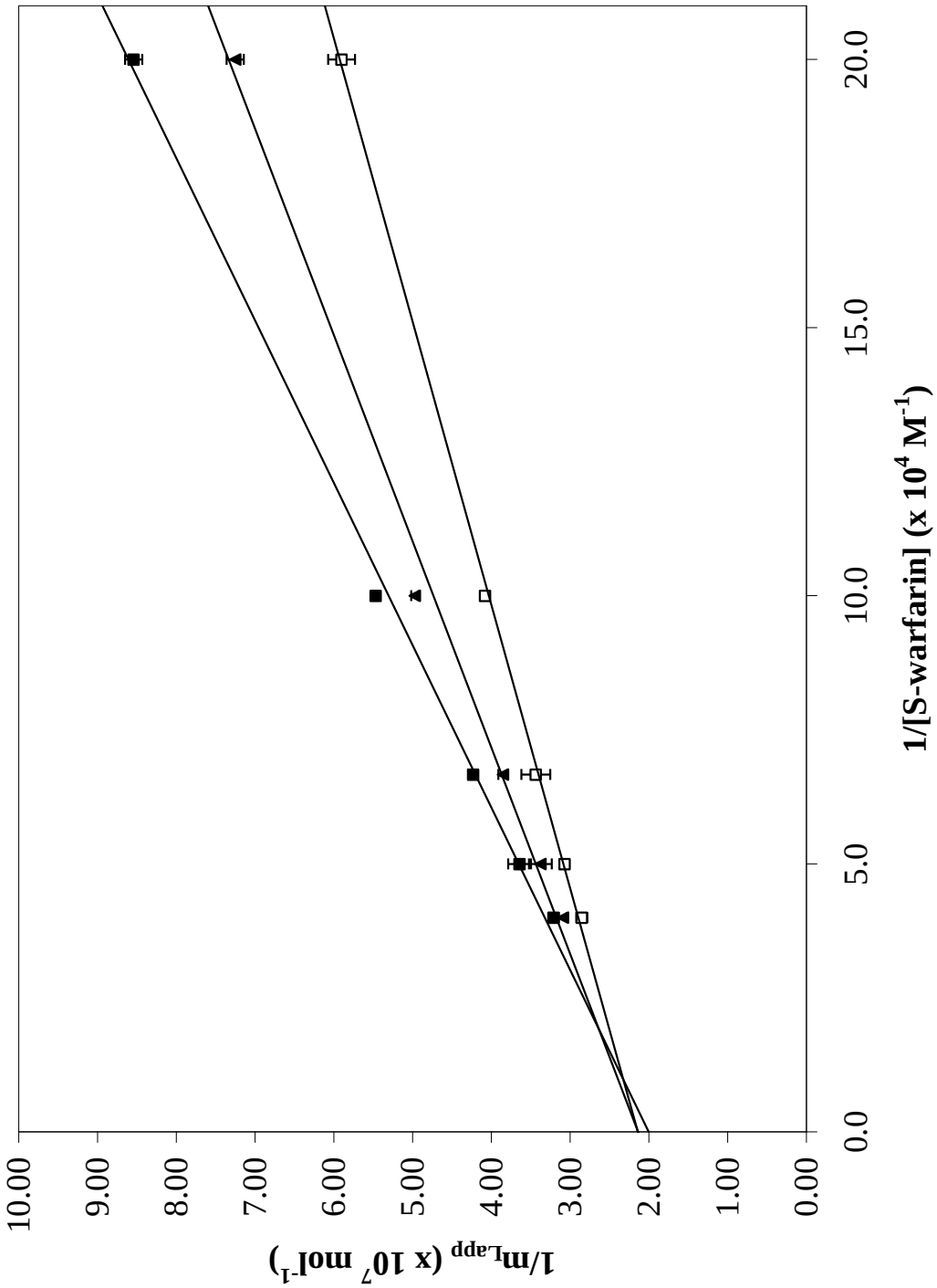

Absorbance, 304 nm
0
5
10
Time (min)

Figure 2-4. Double-reciprocal plot made according to **Equation 2-1** for frontal analysis data obtained for the binding of *S*-warfarin to HSA that had been entrapped on silica with the following pore sizes: 50 Å (□), 100 Å (■), and 300 Å (▲). The best-fit slopes and intercepts for these plots were as follows: 50 Å pore size silica, slope = 0.189 (±0.003) x 10^4, intercept = 2.14 (±0.04) x 10^7; 100 Å pore size silica, slope = 0.330 (±0.009) x 10^4, intercept = 2.0 (±0.1) x 10^7; 300 Å pore size silica, slope = 0.26 (±0.01) x 10^4, intercept = 2.1 (±0.1) x 10^7. The correlation coefficients were 0.998 (n = 5) for the 50 Å pore size silica, 0.997 (n = 5) for the 100 Å pore size silica and 0.994 (n = 5) for the 300 Å pore size silica. The conditions are given in the text. The error bars represent a range of ± 1 S.D.


10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
1/mL_app (x 10^7 mol^-1)
0.0
5.0
10.0
15.0
20.0
1/[S-warfarin] (x 10^4 M^-1)

A frontal analysis study was also conducted to examine the binding of HSA to L-tryptophan, a compound with known single site binding to Sudlow Site II. For this study, both control and entrapped HSA supports were prepared from 300 Å pore size silica and were slurry packed into separate 1 cm × 2.1 mm I.D. columns. A concentration range of 1-25 μM L-tryptophan was used for this frontal analysis study. The double-reciprocal plot of $1/m_{Lapp}$ vs 1/[L-tryptophan] had a correlation coefficient of 0.991 (n = 7), indicating agreement with a system that had single site binding.[26] Using **Equation 2-1** a value of K_a for L-tryptophan on this support was calculated to be 2.0 (± 0.5) × 10^4 M^{-1}. This value was in good agreement with a value of 2.4 (± 0.3) × 10^4 M^{-1} that has been previously reported for HSA immobilized by the Schiff base method. The association equilibrium values that were determined for both *S*-warfarin and L-tryptophan are shown in **Table 2-1**.

Activity of Entrapped HSA.

One of main advantages of noncovalent immobilization is the ability to avoid undesirable effects such as multisite attachment and improper orientation that can lead to the loss of activity for a covalently immobilized protein. The specific activity of a support can be obtained using **Equation 2-1** and the data from the double reciprocal plots created from the frontal analysis studies. Recall that the term m_L in **Equation 2-1** represents the total binding capacity of the column, or the moles of ligand available for binding. These results paralleled those for the amount of entrapped HSA that was determined by the BCA assay. Using the known HSA content of these supports and the binding capacities that were measured via frontal analysis, it was possible to determine the specific activity for the entrapped HSA in each column.

Table 2-1. Results obtained from frontal analysis studies of *S*-warfarin and L-tryptophan on entrapped HSA.

Analyte	Column Length	Protein Content (moles)	Specific Activity	Association Equilibrium Constant (M^{-1})
*S-warfarin	5 cm	1.3 (± 0.2) × 10^{-8}	0.91 (± 0.29)	2.0 (± 0.3) × 10^{5}
L-tryptophan	1 cm	6.9 (± 0.1) × 10^{-9}	0.88 (± 0.14)	2.0 (± 0.5) × 10^{4}

***** Average values for all pore sizes.

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

The average specific activity for the five columns used in the frontal analysis studies of *S*-warfarin was 0.92 (± 0.29). This value is significantly higher than the typical specific activity of 0.67 that has been reported for *S*-warfarin with HSA that has been covalently immobilized by the Schiff base method.[25] Similar work with sulfhydryl-reactive immobilization methods for HSA has given specific activities of 0.81-0.87.[8] The specific activity determined for entrapped HSA with L-tryptophan was 0.88 (± 0.14). This value was also significantly higher than the specific activity of 0.34 that has been reported for L-tryptophan with HSA which has been covalently immobilized by the Schiff base method.[26] Both of the specific activities determined for entrapped HSA were statistically equal to 100%, which indicated that Sudlow sites I and II of the entrapped HSA were completely available for binding. This increased column activity translates into more efficient supports for use in HPAC drug-protein binding studies.

Preliminary evaluations of the entrapment method revealed that it was a viable alternative to covalent immobilization. These studies indicated that small molecules were able to access both Sudlow Sites I and II, and that statistically, the entrapped HSA did not suffer any loss of activity during the immobilization process. The association equilibrium constants obtained using the entrapment method were also in good agreement with those established for covalent immobilization. The next step was to isolate and modify specific parameters of the entrapment method in an attempt to optimize the binding capacity of the entrapped support.

Effect of Pore Size on Protein Entrapment.

The first parameter investigated in the optimization of the entrapment method was the porosity of the support. This study looked at nonporous silica in addition to silica

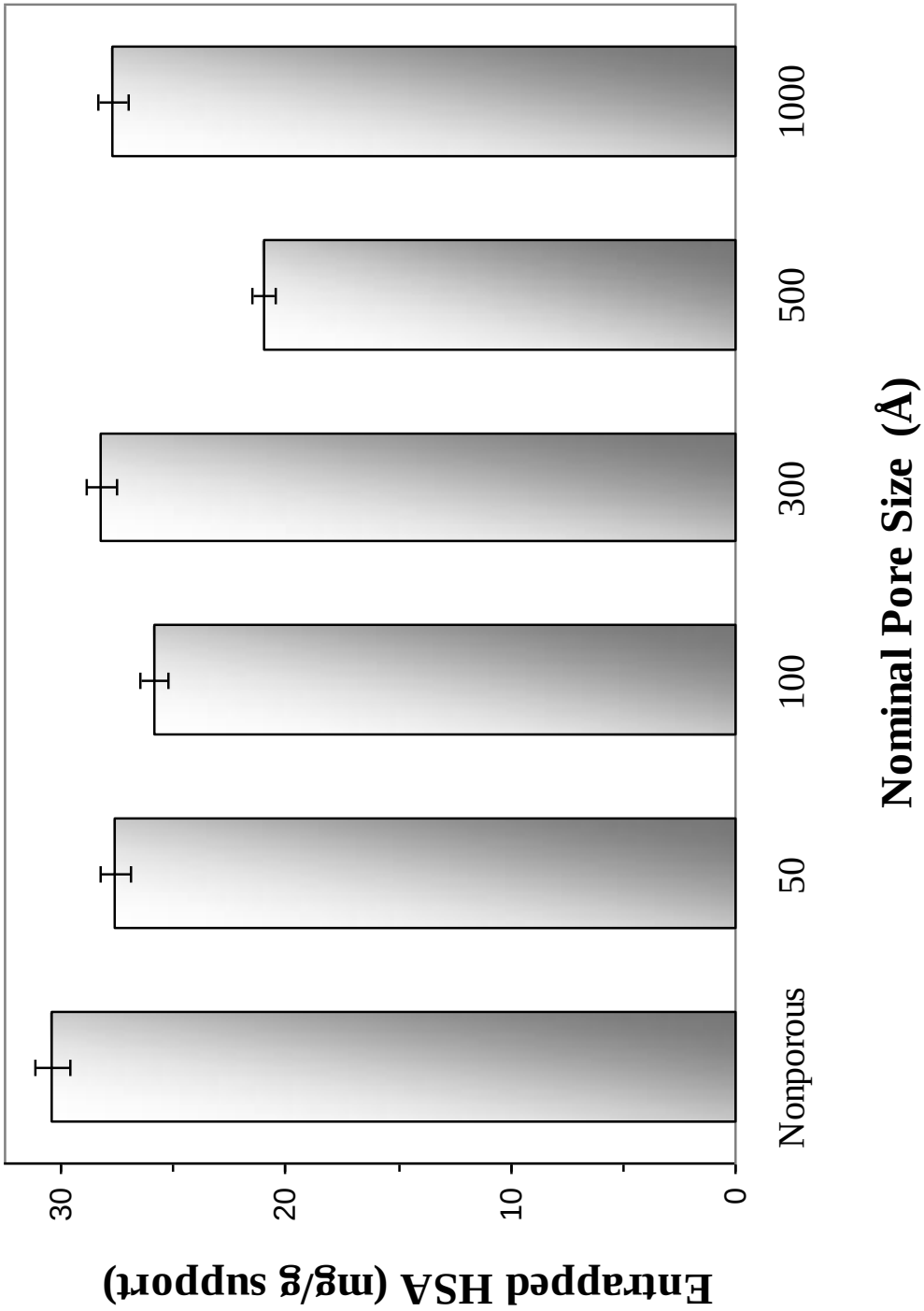
with pore sizes ranging from 50 Å to 1000 Å. In this work, 10 mg of dihydrazide activated silica was combined with 125 µL of 8 mg/mL HSA and 500 µL of oxidized glycogen. The reaction mixture was brought to a total volume of 1 mL with the addition of 365 µL of pH 5.0, 0.10 M potassium phosphate buffer. This mixture was shaken at 4 ^{o}C for 12 h. After immobilization was complete, the samples were washed several times with water and dried for several days at 50 °C. The protein content was then determined using a micro BCA assay. The protein content in all supports was similar, averaging 26.8 (± 3.2) mg HSA per gram silica, as seen in **Figure 2-5**. Entrapment of the nonporous support resulted in a protein content of 30.3 (± 0.8).

When taking into account the surface areas of the supports, the total surface area could be dissected into two regions: the interior of the pore and the outermost surface of the silica. By comparison, the nonporous support acquires 100% of its surface area from the outer surface, while the majority of the surface area for the porous supports would be contributed by the interior of the pores. The results obtained in this study suggested that the mechanism for protein entrapment under the given preparation conditions involved HSA being held by the oxidized glycogen within pockets at or near the outer surface of the supports, where similar surface areas would have been present for each of the supports examined. Comparison of the entrapment results with those for covalent immobilization techniques showed similar results for all pore sizes in terms of the protein content per unit of surface area of the support.[8]

Effect of Amount of Glycogen on HSA Entrapment.

The amount of HSA that could be entrapped was examined as a function of the amount of glycogen that was used during the entrapment process. This was studied by

Figure 2-5. Amount of HSA that was entrapped on various types of glycogen-capped and hydrazide-activated porous silica or nonporous glass beads. The pore size or type of support was varied while all other entrapment conditions (e.g., the amount of added protein and oxidized glycogen) were held constant. The conditions are given in the text. The error bars represent a range of ± 1 S.D.


Entrapped HSA (mg/g support)
0
10
20
30
Nonporous
50
100
300
500
1000
Nominal Pore Size (Å)

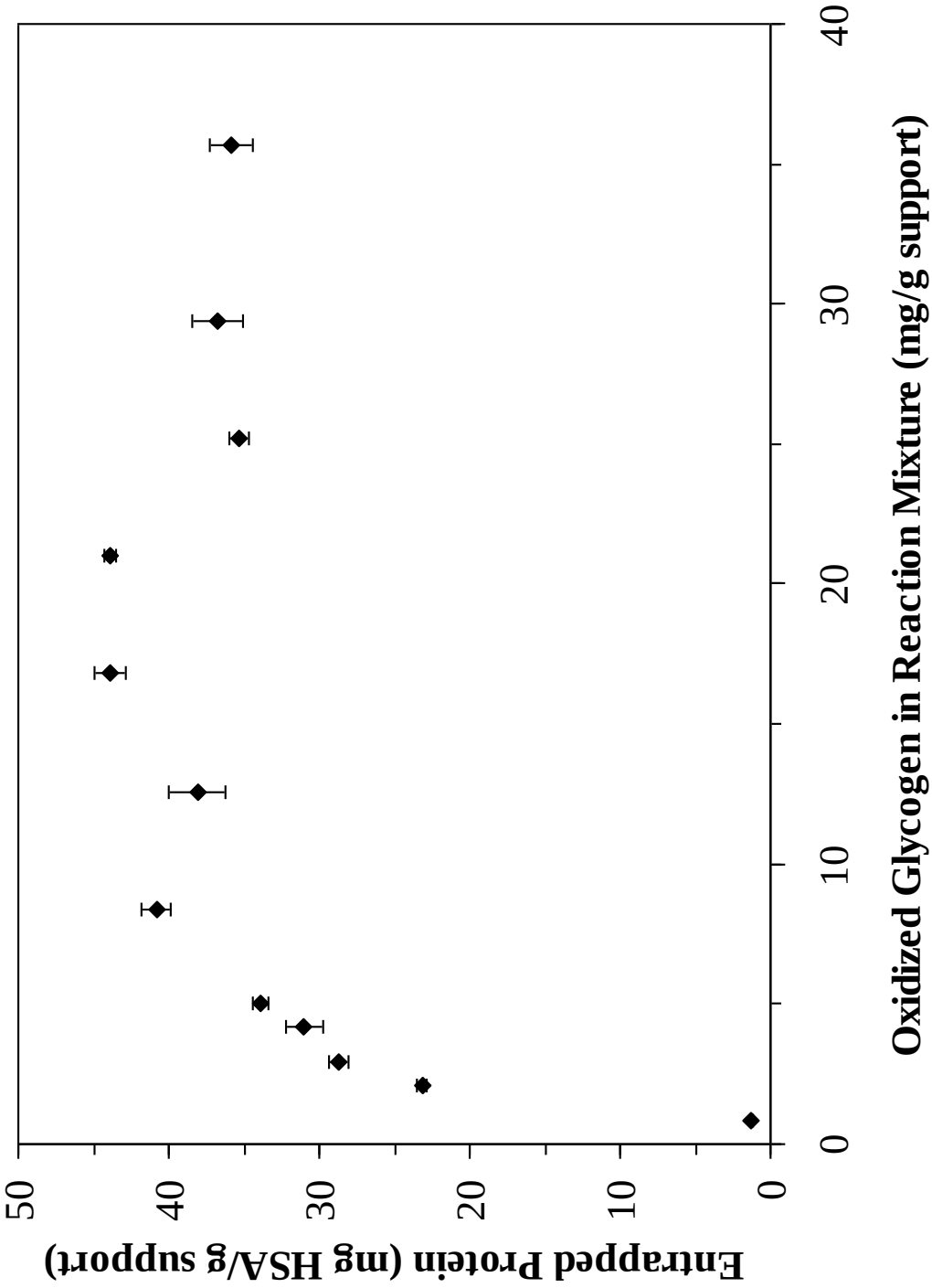
incubating 10 mg of 300 Å hydrazide-activated silica with various amounts of oxidized glycogen and a solution containing 8 mg/mL HSA. The amount of glycogen in this study ranged from 0.84 to 35.70 mg oxidized glycogen per gram of silica in the reaction mixture. Other than the change in the amount of glycogen in the reaction mixture, all conditions used in the previous porosity study were kept constant. The protein content was again determined using a micro BCA assay. The results are summarized in **Figure 2-6**. In this plot, the ratio of glycogen per silica (in mg/g) represents the relative amount of glycogen that was added, while the ratio of entrapped HSA per support (also in mg/g) represents the amount of entrapped HSA that was obtained with the final supports.

It can be seen from **Figure 2-6** that as amount of oxidized glycogen increased from 0.84 mg to 17 mg per gram of silica, the amount of immobilized HSA increased from 1.2 to 44 mg/g. A further increase in the amount of added glycogen to levels of 21 mg per gram silica gave a relatively consistent amount of entrapped HSA. However, the use of larger amounts of glycogen (up to 36 mg/g silica) may have given a slight decrease in the amount of entrapped HSA. The exact reason for this possible small decrease is not known, but it may be due to the smaller amount of time that was allowed for the entrapment of HSA to occur as a larger excess of glycogen was available to bind to the hydrazide-activated support. From this point on, the amount of glycogen in the reaction mixture was set at an optimized value of 20 mg per gram silica.

Rate of Protein Immobilization.

All studies until this point have illustrated that the entrapment method is a viable alternative to covalent immobilization techniques for use in HPAC binding studies. The main covalent immobilization technique for such work is the Schiff base method. The

Figure 2-6. Amount of entrapped HSA as a function of the amount of oxidized glycogen that was used for the entrapment process. The conditions are given in the text. The error bars represent a range of ± 1 S.D.

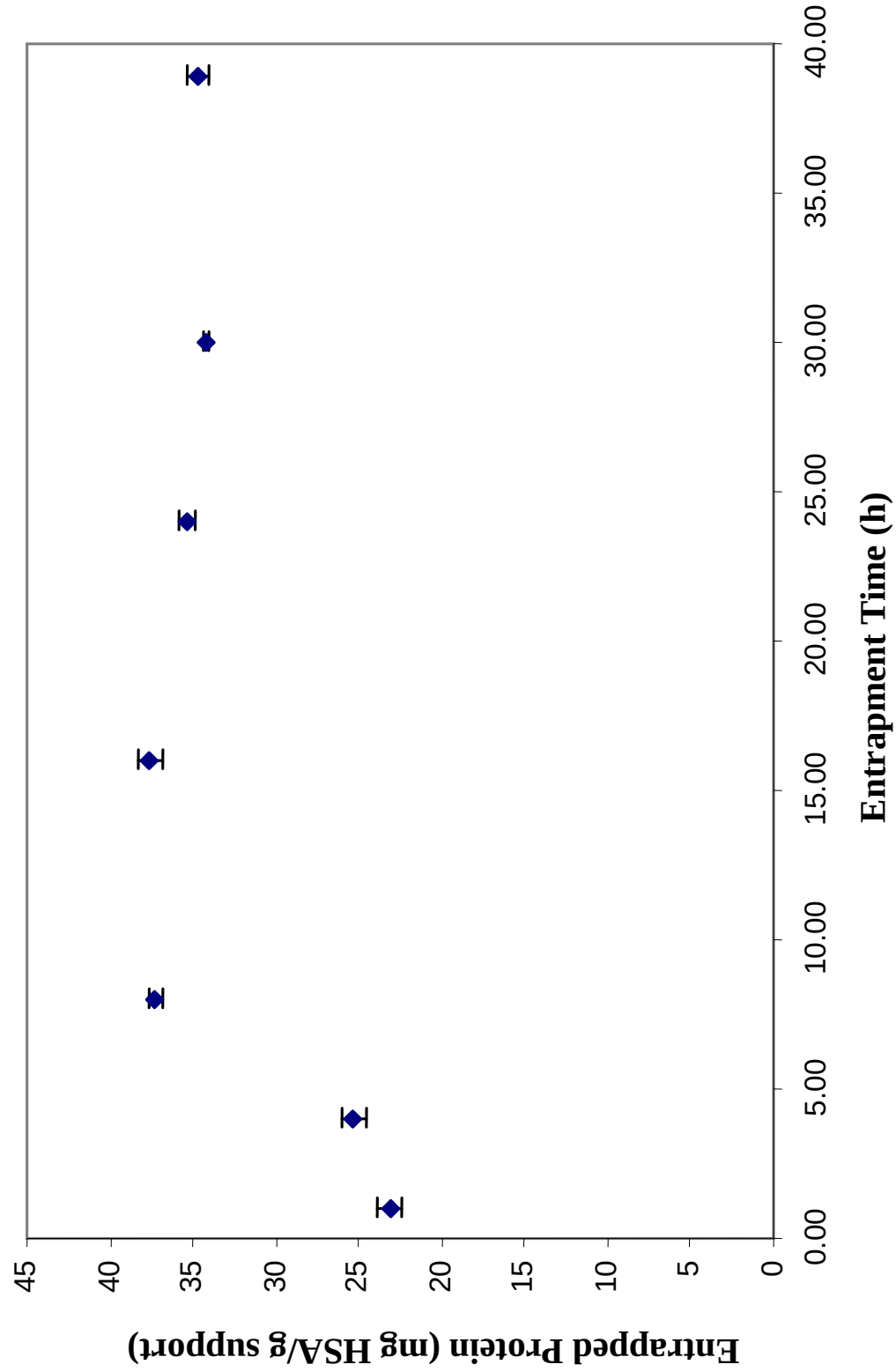

Entrapped Protein (mg HSA/g support)
0
10
20
30
40
50
0
10
20
30
40
Oxidized Glycogen in Reaction Mixture (mg/g support)

association equilibrium constants for both *S*-warfarin and L-tryptophan were in good agreement between the two methods, and similar protein content was achieved. The largest variation between the two methods up to this point was the specific activity of the immobilized protein, with little loss of activity when using the entrapment method as compared to an approximately 44% loss of activity when using the Schiff base method. The next step in the optimization of the entrapment method was to determine and compare the rate at which immobilization occurs.

In this study, 300 Å pore size silica was used as the support and all concentrations of the reaction components were held constant. The time allowed for entrapment to occur was varied from 1 to 40 hours. Seven samples were taken over this time period and a micro BCA assay was used to determine the amount of immobilized protein. The results of this study can be seen in **Figure 2-7**.

A maximum amount of entrapped HSA or 37.6 (±0.6) mg HSA/g support was achieved within the first 8 h of immobilization. Between 8 and 16 h, the amount of entrapped HSA remained constant, with only a small change in protein content of 2.5 (±0.5) mg HSA per gram support follow taking place between 16 and 40 hours. Therefore, the procedure for entrapment was modified to limit the time for immobilization to between 8 and 16 h. A standard procedure for the immobilization of HSA and other proteins through the Schiff base method calls for an immobilization time of 6 days. This rate study indicated that HSA immobilization via the entrapment method is markedly faster than Schiff base immobilization. This represents another advantageous aspect of the entrapment method.

Figure 2-7. Amount of entrapped HSA immobilized over a period of 40 h. The conditions are given in the text. The error bars represent a range of ± 1 S.D.


Entrapped Protein (mg HSA/g support)
0
5
10
15
20
25
30
35
40
45
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
Entrapment Time (h)

Effect of Protein Concentration on HSA Entrapment.

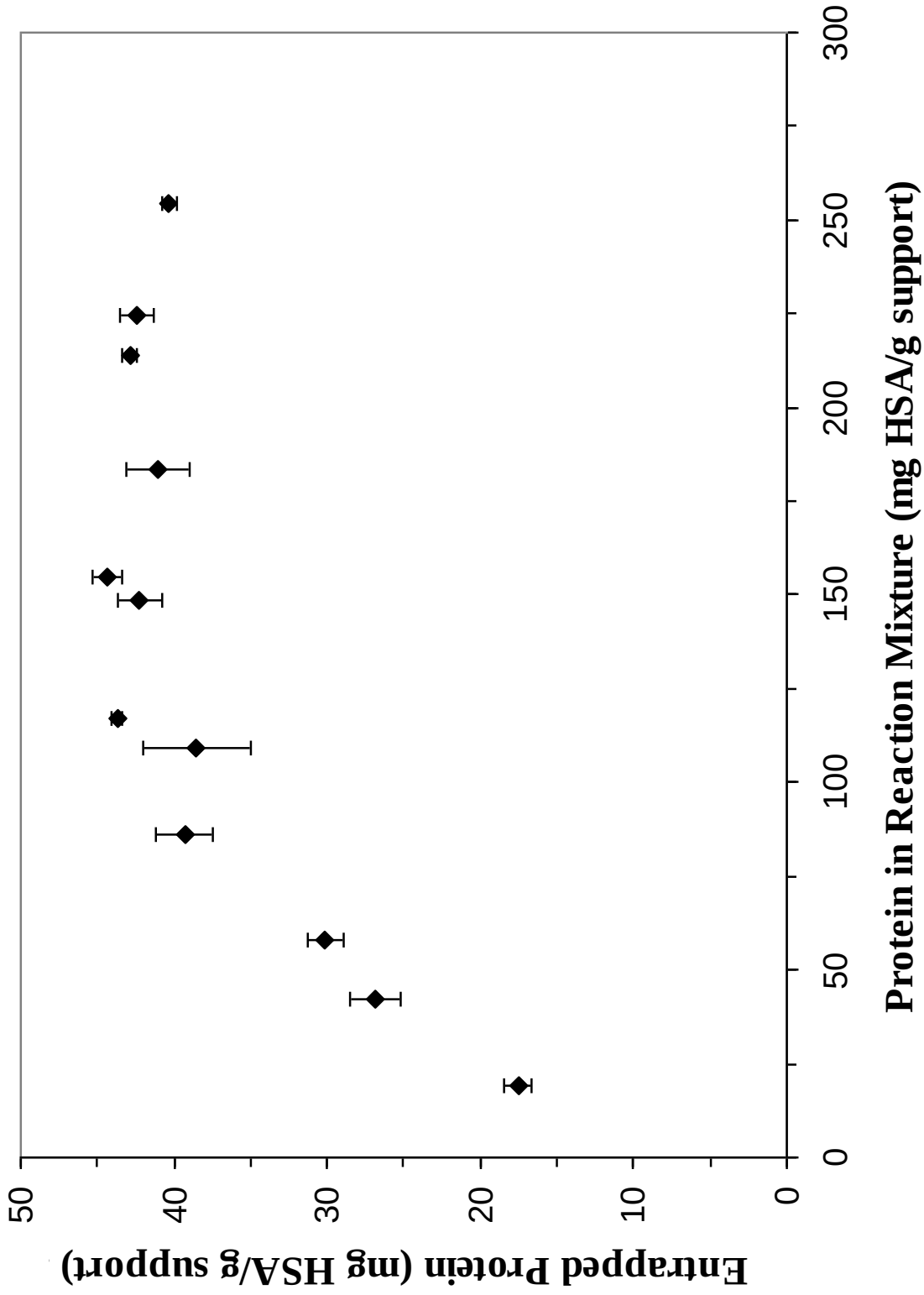
Another factor of interest was the relationship between the amount of protein added into the reaction mixture and the resulting amount of entrapped protein. Because the protein of interest was sterically entrapped between the surface of the silica and the glycogen, the amount of protein entrapped should have been affected by the concentration of protein in the reaction solution. By using different concentrations for this protein solution, the amount of protein entrapped into hydrazide-activated silica was examined and related to the initial protein concentration.

This relationship was examined by using 10 mg of 300Å pore size hydrazide-activated silica that was reacted with a solution containing various concentrations of HSA and 0.20 mg of oxidized glycogen. The mixture was reacted for 12 h at 4 °C. The results are summarized in **Figure 2-8**. As the concentration of HSA in the reaction mixture was increased from 19 to 118 mg HSA per gram silica, there was a subsequent increase in the amount of HSA that was entrapped onto the support. As larger amounts of HSA were added, the amount of entrapped protein reached a plateau at approximately 42.9 (±0.5) mg HSA per gram support. For all subsequent optimization studies a concentration of 100 mg HSA per gram silica was used in the reaction mixture.

Alternative Ligand Entrapment.

The method of entrapment that is described in this report is not limited to HSA but can also be extended to other proteins and biomacromolecules. **Table 2-2** shows the results that were obtained for a series of other proteins and ligands that were entrapped in silica with a 300 Å pore size, using the final conditions developed for work with HSA. The ligands that were examined ranged in size from insulin (MW, 5.8 kDa) to ferritin

Figure 2-8. Amount of entrapped HSA immobilized as a function of the amount of HSA that was used for the entrapment process. The amount of added protein was varied, while the amount of added oxidized glycogen, the degree of oxidation and the pore size of the support (300 Å) remained constant. The conditions are given in the text. The error bars represent a range of ± 1 S.D.


Entrapped Protein (mg HSA/g support)
0
10
20
30
40
50
0
50
100
150
200
250
300
Protein in Reaction Mixture (mg HSA/g support)

(MW, 450 kDa). It was found that ligands up to a size of at least 150 kDa (as represented by IgG) could be successfully entrapped by this method, where the amount of entrapped ligand was inversely related to the mass of the ligand. All of these ligands were much lower in molecular weight than the glycogen that was used for entrapment (i.e., approximate MW, 10^4 kDa). The results fit with the previously proposed mechanism, in which these ligands were physically contained within pockets created between the surface of the support and the oxidized glycogen. Although there was a small amount of ferritin immobilized by this approach, there was also a large decrease in the relative amount of this ligand that was entrapped versus the amount that was obtained for IgG or lower mass ligands. Thus, it was found that this approach could be used with ligands and proteins with masses in the general range of 5.8 to 150 kDa, with some entrapment also being noted at higher masses (up to 450 kDa).

Glycogen Oxidation Levels.

Initially the oxidation of glycogen for use in the entrapment method called for a concentration of 88 mM periodic acid in the reaction mixture, corresponding to a 4:1 ratio of periodic acid to glycogen. These conditions lead to the oxidation of only 0.5% of the glucose units in the glycogen molecule.[18] This was within the range suggested for the oxidation of antibody carbohydrate residues for site-specific immobilization.[19]

However, in this study the entrapment method used glycogen only as a means to entrap ligands, so activity of the oxidized glycogen was not of importance. The following study investigated how increasing the concentrations of oxidizing agent would affect the ability of the system to entrap HSA.

Table 2-2. Alternative ligands immobilized by the entrapment method.

Identity of ligand	Ligand mass (kDa)	Amount of immobilized ligand (mg ligand/g support)	Amount of immobilized ligand (mol ligand/g support)[a]
Insulin	**5.8**	**93 (± 2)**	**160 (± 3) x 10^{-7}**
Myoglobin	**16.7**	**21 (± 3)**	**12 (± 2) x 10^{-7}**
Streptavidin	**52.8**	**12.6 (± 0.5)**	**2.38 (± 0.01) x 10^{-7}**
Avidin	**69.0**	**10.7 (± 0.5)**	**1.55 (± 0.01) x 10^{-7}**
gHSA	**71.4**	**14.9 (± 1.4)**	**2.09 (± 0.19) x 10^{-7}**
IgG	**150.0**	**15.7 (± 0.2)**	**1.05 (± 0.01) x 10^{-7}**
Ferritin	**450.0**	**9.0 (± 0.8)**	**0.20 (± 0.01) x 10^{-7}**

[a]Abbreviations: gHSA, glycated human serum albumin; IgG, immunoglobulin G (rabbit).

The values in parentheses represent ± 1 S.D.

The concentration of periodic acid was expressed as a mass-to-mass ratio of periodic acid to glycogen. This study looked at unoxidized glycogen up to a ratio of 12.5 mg periodic acid per mg glycogen, which was an approximate three-fold increase versus the original entrapment conditions. All supports used in the previous porosity study were also used in this comparison. The oxidation level of glycogen was the single variable; all other reaction conditions were held constant. All porosities examined in this study used 3 mg samples of dihydrazide activated silica, a concentration of 20 mg oxidized glycogen per gram silica, and 100 mg HSA per gram silica. The reaction mixture was mixed at 4 °C for 12 h.

The first support examined was prepared using nonporous silica. Initially, as the oxidation level of glycogen increased the amount of entrapped HSA also increased, reaching a maximum of 33 (± 1) mg HSA per gram support at a mass ratio of approximately 6.5 mg periodic acid per mg of glycogen. Higher levels of oxidation resulted in a decrease in entrapped HSA. The 1000 Å pore size support showed an increase in entrapped HSA, which reached a maximum of 28 (± 1) mg HSA per gram support at a mass ratio approximately 6.5 mg periodic acid per gram silica. This trend was repeated for the 500 Å pore size support, where the amount of entrapped protein increased to a maximum of 33 (± 2) at a mass ratio of approximately 6.5 mg periodic acid per mg glycogen.

These three supports resulted in data that shared a similar trend. This trend could be attributed to the complete exclusion of glycogen from pore sizes of 300 Å and smaller.[18] In supports with pore sizes of 500 Å and 1000 Å, both the glycogen and protein have access to the pores. It can therefore be assumed that a portion of the pores

are occupied with glycogen causing the results to look similar to that of the nonporous support.

When the entrapment samples using 300 Å porous silica were analyzed, the amount of entrapped HSA increased steadily over the entire analysis range. The highest three levels of glycogen oxidation gave protein content in the range of 34.5 (± 1.5) mg HSA per gram of support. Analysis of the 50 Å porous silica samples gave a much different set of results. In this study, a maximum protein content of 37 (± 1) mg HSA per gram of support was achieved early, at a mass ratio of approximately 3 mg periodic acid per mg glycogen, followed by a decrease in protein content at higher levels of oxidation to a protein content of 13 (± 1) mg HSA per gram of support, at 8 – 12 mg periodic acid per mg glycogen. Results from these four porosities were combined into one plot presented in **Figure 2-9**.

The support with a porosity of 100 Å was examined further in **Figure 2-10** due to the fact that higher levels of protein were entrapped on this support compared to the other materials. The amount of entrapped HSA increased to a maximum of 174 (± 2) mg HSA per gram of support at a mass ratio of approximately 6.75 mg periodic acid per mg glycogen. Higher levels of oxidation resulted in only a slight decrease in protein content, with the highest level of oxidation entrapping 158 (± 2) mg HSA per gram of support. These levels of protein are roughly four times the protein content of traditional HPAC supports containing HSA that has been immobilized via covalent techniques.[17]

Both the 300 Å and 100 Å supports showed a steady amount of entrapped protein after the maximum, this may be due to the ability of higher levels of oxidation to force the HSA down into the pores and therefore increase the protein content.

Figure 2-9. Amount of entrapped HSA as a function of the mass ratio of periodic acid to glycogen that was used for glycogen oxidation in the initial step of the entrapment process. The porosity of the supports ranged from a pore size of 50Å to nonporous (NP) glass beads. The conditions are given within the text. The error bars represent a range of ± 1 S.D.

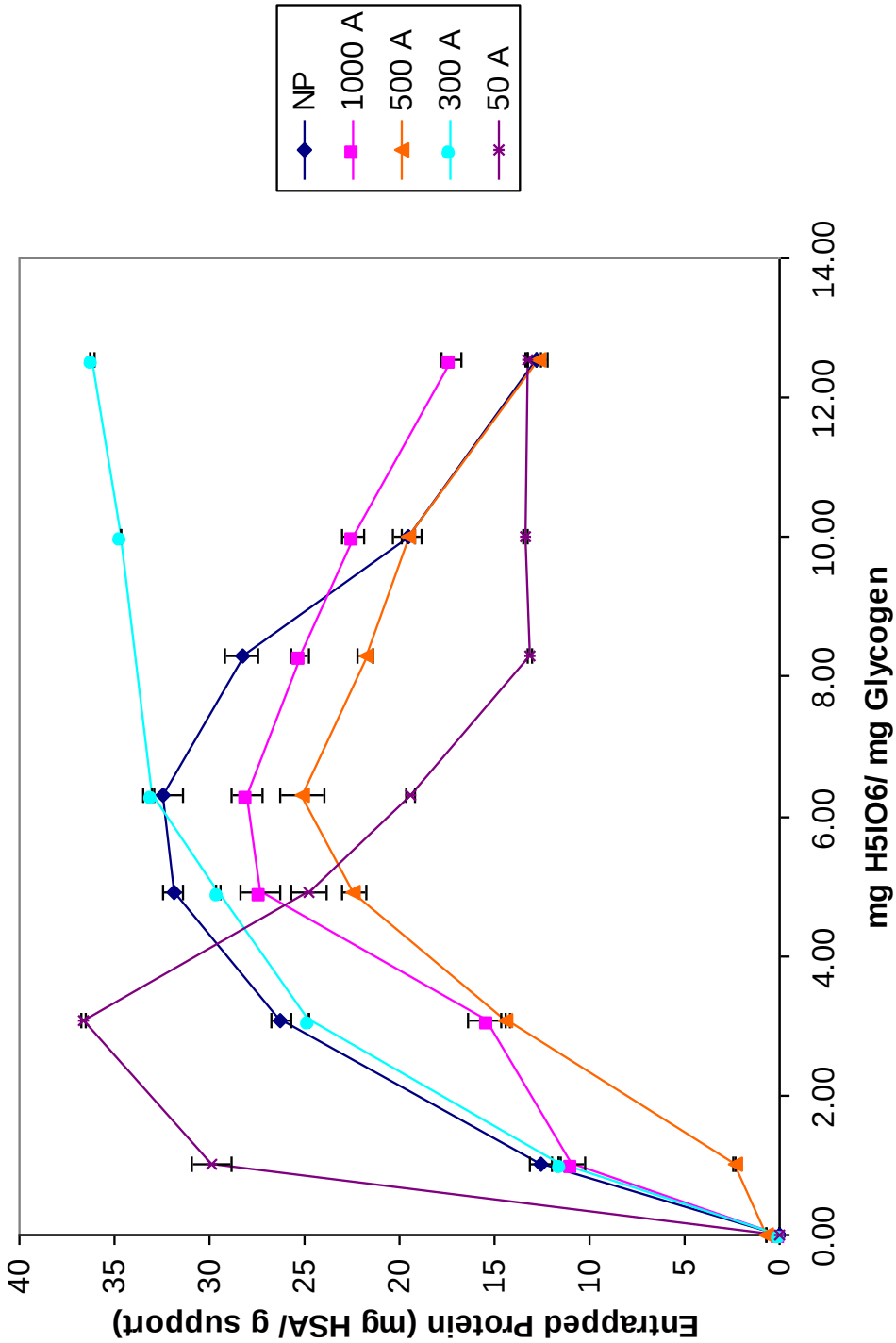
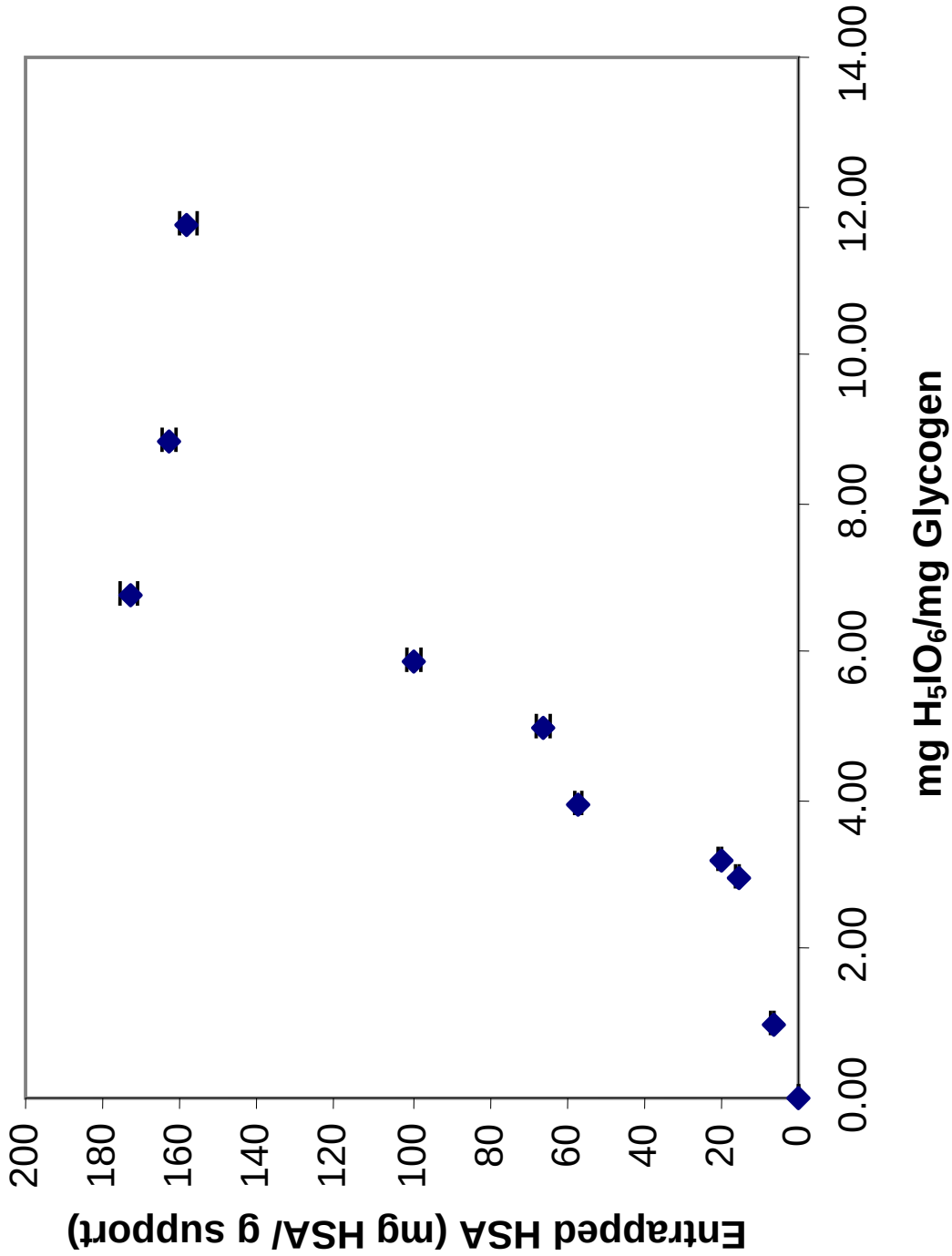

NP
1000 A
500 A
300 A
50 A
Entrapped Protein (mg HSA/ g support)
0
5
10
15
20
25
30
35
40
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
mg H5IO6/ mg Glycogen

Figure 2-10. Amount of entrapped HSA as a function of the mass ratio of periodic acid to glycogen that was used for glycogen oxidation in the initial step of the entrapment process on a pore size of 100 Å silica support. The conditions are given within the text. The error bars represent a range of ± 1 S.D.


Entrapped HSA (mg HSA/ g support)
0
20
40
60
80
100
120
140
160
180
200
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
mg H5IO6/mg Glycogen

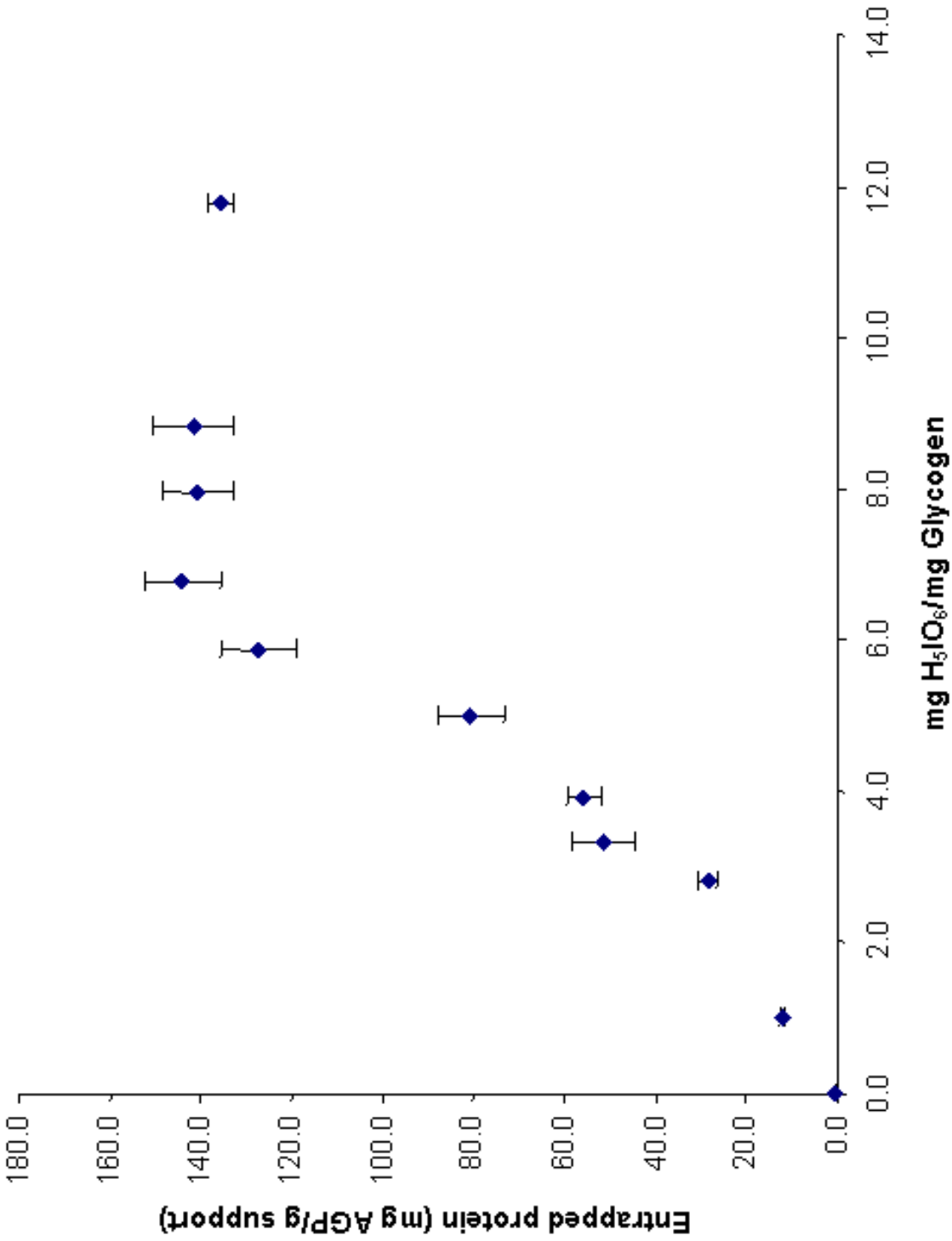
Results for the 50 Å support do not follow this trend, possibly because the pores are too close in size to the HSA, causing steric interference.

A study using 100 Å pore size silica and AGP as the model ligand was also conducted. All parameters were consistent with those used for the entrapment of HSA in 100 Å porous supports. The results for this study can be seen in **Figure 2-11**. The amount of entrapped AGP increased to a maximum of 144 (± 8) mg AGP per gram of support at a mass ratio of approximately 6.75 mg periodic acid per mg glycogen. Higher levels of oxidation resulted in only a slight change in protein content, with the highest level of oxidation entrapping 135 (± 3) mg AGP per gram of support. This high protein content combined with the retention of all protein activity when immobilizing with the entrapment method would produce supports with extremely high binding capacities.

High Capacity Supports.

The high protein content achieved in the previous study indicated that it could be possible to make high capacity HPAC supports using the entrapment method. This optimization study looked at both HSA and IgG as model ligands and employed 100 Å porous silica as the support material. The glycogen was oxidized using a mass ratio of 7.95 mg periodic acid per mg glycogen and was added to the reaction mixture at a concentration of 20 mg glycogen per gram silica. In order to determine the maximum amount of ligand that could be entrapped, the concentration of HSA was varied from 25 to 400 mg initial HSA per gram silica. **Figure 2-12** shows the results for the entrapped HSA under these reaction conditions. The amount of entrapped HSA increased directly with the amount of initial HSA in the reaction mixture up to a value of 200 mg of initial HSA per gram silica, which indicated that efficient HSA immobilization was present.

Figure 2-11. Amount of entrapped AGP as a function of the mass ratio of periodic acid to glycogen that was used for glycogen oxidation in the initial step of the entrapment process on a100 Å pore size silica support. The conditions are given within the text. The error bars represent a range of ± 1 S.D.


Entrapped protein (mg AGP/g support)
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
mg H5IO6/mg Glycogen

A maximum of 211 (± 9) mg entrapped HSA per gram support, was reached at 400 mg initial HSA per gram silica, but was similar to values obtained at 200 and 300 mg initial HSA per gram silica.

Similar results and trends to those for HSA were also seen in the entrapment of IgG under the same reaction conditions (**Figure 2-13**). The amount of entrapped IgG increased steadily up to 200 mg IgG per gram silica. Concentrations above this value produced relatively similar amounts of immobilized IgG, hovering around 80 mg entrapped IgG per gram of support.

Although the upper concentrations of both ligands achieved a high ligand content, a considerable portion of the ligand in the reaction mixture was not immobilized – less than 70% for HSA entrapment and less than 40% for the entrapment of IgG. This will be something to take into consideration when this method is adapted for use with the larger quantities of silica required to pack HPAC columns.

Conclusions

This chapter described the preparation of glycogen-capped hydrazide supports for the entrapment of proteins and other ligands. In this approach, the silica support was first activated to contain hydrazide groups. The hydrazide-activated support was then incubated under slightly acidic pH conditions with a solution containing the protein or binding agent of interest and an oxidized form of glycogen. While aldehyde groups on the oxidized glycogen formed a stable covalent bond with the hydrazide groups, the soluble binding agent was entrapped in the support.

Figure 2-12. Amount of entrapped HSA as a function of the concentration of HSA in the reaction mixture. This study was done with 100 Å pore size silica and a periodic acid ratio of 7.95 mg H_5IO_6/g glycogen. The conditions are given within the text. The error bars represent a range of ± 1 S.D.

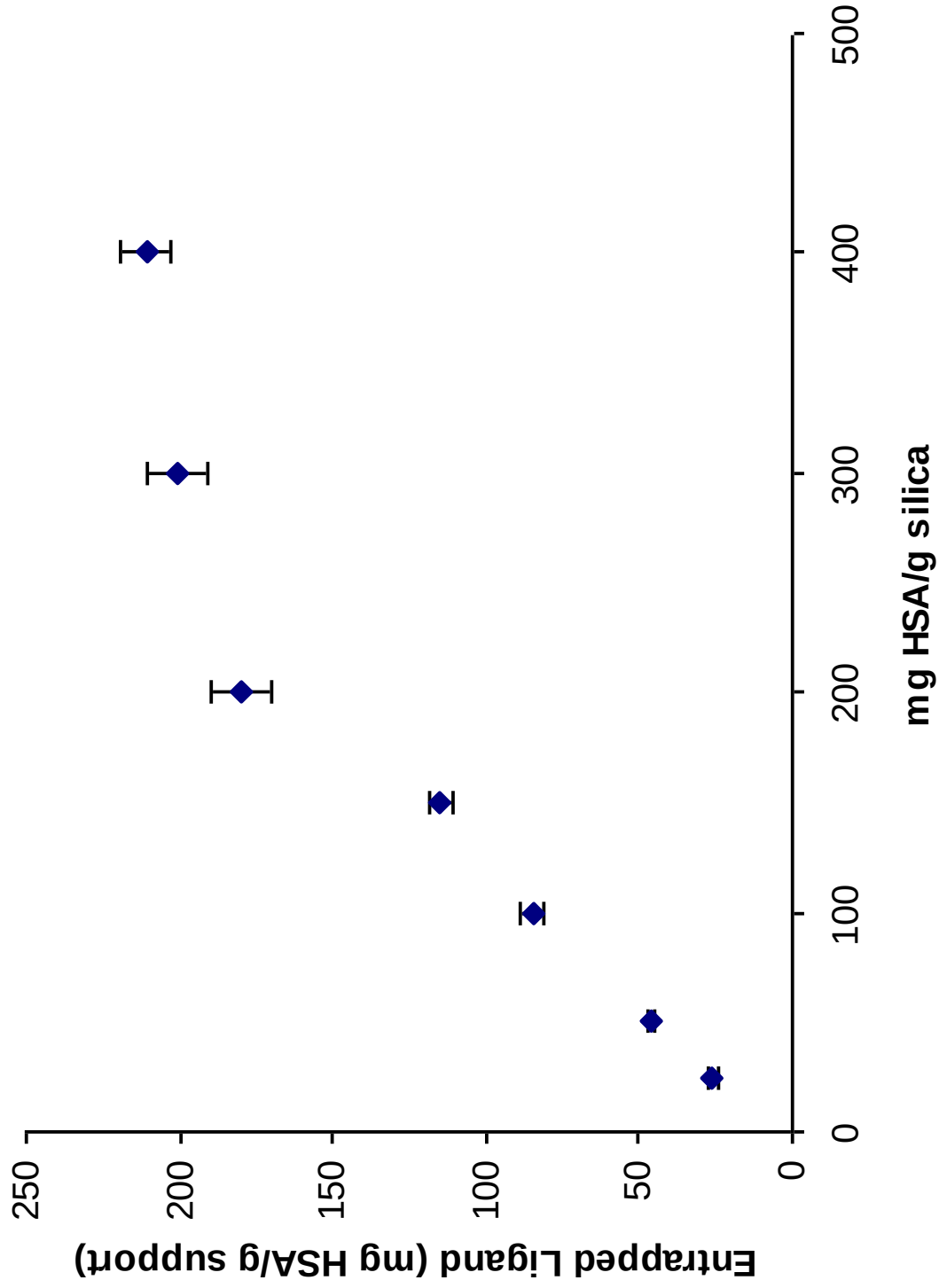
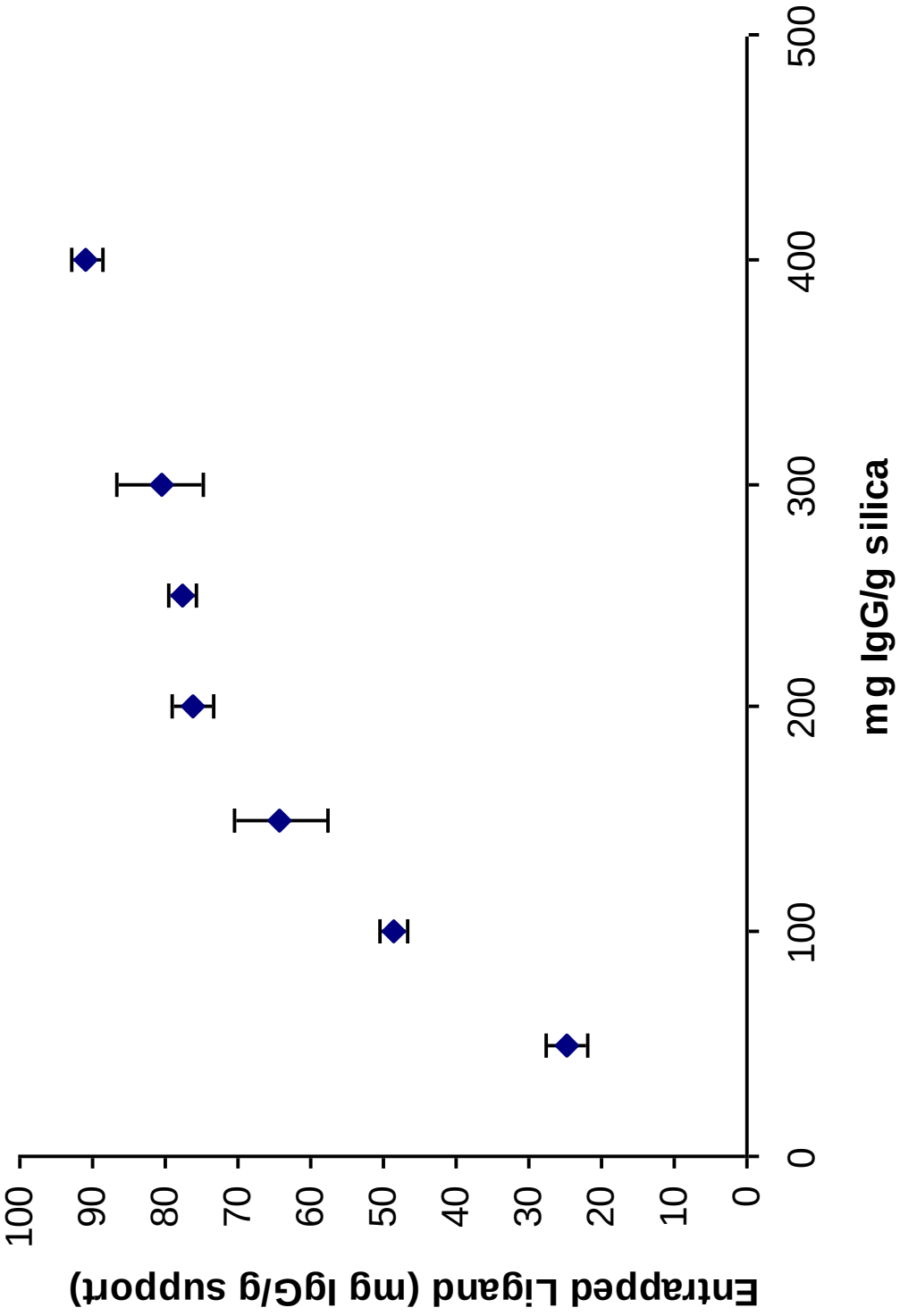

Entrapped Ligand (mg HSA/g support)
250
200
150
100
50
0
0
100
200
300
400
500
mg HSA/g silica

Figure 2-13. Amount of entrapped IgG as a function of the concentration of IgG in the reaction mixture. This study was done with 100 Å pore size silica and a periodic acid ratio of 7.95 mg H_5IO_6/g glycogen. The conditions are given within the text. The error bars represent a range of ± 1 S.D.


Entrapped Ligand (mg IgG/g support)
0
10
20
30
40
50
60
70
80
90
100
0
100
200
300
400
500
mg IgG/g silica

Frontal analysis was used to determine K_a values for both *S*-warfarin (a probe for Sudlow site I) and L-tryptophan (a probe for Sudlow site II) with the entrapped HSA. The binding capacity of the support was also determined by using frontal analysis. The binding capacity was closely related to the overall protein content of the supports as determined by a micro BCA protein assay, and translated to a specific activity of essentially 100% for both tested analytes. This study revealed that both major binding sites of the entrapped HSA were active and available for binding.

The entrapment method was then optimized to achieve maximum protein content through the variation of several different reaction components and procedures. The method was tested on five silica supports with varying porosities, as the concentrations of oxidized glycogen and HSA in the reaction mixtures were varied, as well as the time allowed for the reaction to take place. Other components tested were the ability of the entrapment method to work with ligands other than HSA, and the effect of increasing the oxidation level of the glycogen on the amount of immobilized ligand.

This alternative method of entrapment allows for physical containment of a ligand in a high performance support material. The resulting entrapped ligand is not only accessible to small analytes, but also retains essentially 100% of its binding activity. Association equilibrium constants for the chosen system were in good agreement with previously reported values. After optimization of the reaction conditions, a greater than five-fold increase in immobilized protein content was achieved versus traditional covalent immobilization techniques, indicating the possibility of the production of high capacity columns for HPAC. This novel immobilization technique provides the ability to examine ligands that mimic native behavior using the speed and precision of HPAC.

Further studies can be done to examine how the optimized parameters affect the ability of drugs to access and bind to the entrapped ligand.

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CHAPTER 3:

THE EFFECT OF VARYING THE OXIDATION LEVEL OF GLYCOGEN IN THE PREPARATION OF ENTRAPPED HSA SUPPORTS

Introduction

Drug interactions with serum proteins are important in determining the activity, transport, excretion and metabolism of many pharmaceutical agents in the body. Human serum albumin (HSA), the most abundant protein in serum, is commonly involved in this reversible drug-protein binding. HSA is known to bind to a large number of drugs, hormones and other small analytes in the body.[1-3] Due to the widespread occurrence of these interactions, numerous analytical techniques, including high performance affinity chromatography (HPAC), have been employed to study the interaction of drugs with HSA.[3-6] It has been previously shown that HPAC supports containing immobilized HSA can effectively represent drug-binding involving soluble HSA.[7]

In **Chapter 2** a new noncovalent immobilization technique was introduced as an alternative to traditional covalent immobilization. Part of this optimization examined the effect of glycogen oxidation levels on the amount of entrapped HSA. This study was conducted by using various amounts of periodic acid for the oxidation of glycogen during the initial steps of the entrapment method. This glycogen was then used to make entrapped HSA supports with several levels of oxidized glycogen as the capping agent.

This chapter will examine the binding of several analytes as a function of the level of glycogen oxidation. These analytes will also include *R*-warfarin and L-tryptophan,

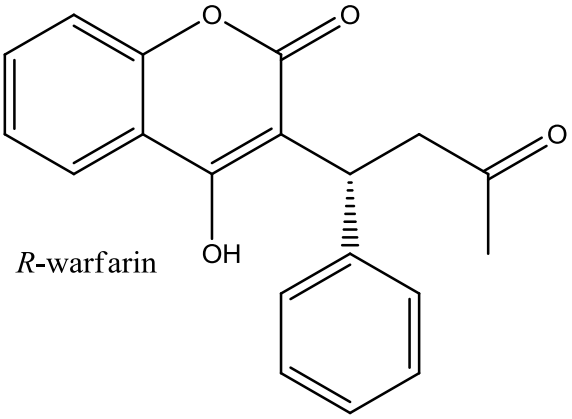
which are common site-selective probes for HSA. *R*-warfarin, an anticoagulant, binds to Sudlow site I, while L-tryptophan, an essential amino acid, binds to Sudlow site II.[8,9] The structures of these compounds can be seen in **Figure 3-1**. These probe compounds will be injected at various flow rates on to each column to determine if the ability of the drug to reach the entrapped protein is effected by changes in the flow rate. Measurements of the protein content and retention factor will be used to examine the interactions of drugs to the immobilized HSA and to determine the effect of the level of glycogen oxidation on the binding capabilities of the column. The efficiency for each column will also be investigated through the use of band broadening measurements to determine plate heights. Finally, the entrapment method will be compared to the Schiff base immobilization method through the examination of the retention of a variety of injected analytes on columns that are prepared by these two methods.

Experimental

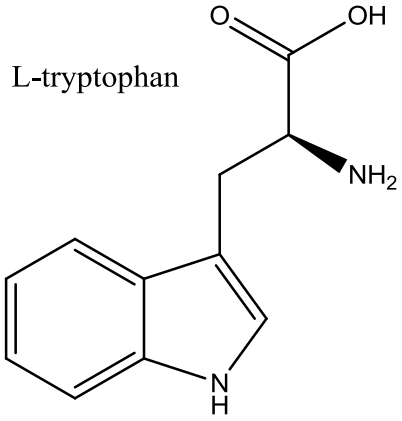
Reagents.

The *p*-periodic acid (periodic acid reagent, or H_5IO_6), glycogen (from bovine liver)**,** HSA (Cohn fraction V, 99% globulin free, 99% fatty acid free), *R*-warfarin, *S*-warfarin, L-tryptophan, acetohexamide, gliclazide, tolbutamide, monobasic and dibasic potassium phosphate salts, sodium chloride and sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO**).** The Nucleosil Si-300 silica (300 Å nominal pore size, 7 μm diameter) was purchased from Macherey-Nagel (Düren, Germany). Reagents for the micro bicinchoninic acid (BCA) protein assay were purchased from Pierce (Rockford, IL). Other chemicals were of the purest grades available. The Econo-Pac 10 DG

Figure 3-1. Structures of *R*-warfarin and L-tryptophan.



R-warfarin



L-tryptophan

disposable 30 x 10 mL desalting columns were purchased from Bio-Rad Laboratories (Hercules, CA). All solutions were prepared using water from a NANOpure purification system (Barnstead, Dubuque, IA) and filtered with a 0.20 μm GNWP nylon membrane from Millipore (Billerica, MA).

Apparatus.

The chromatographic system consisted of a Jasco PU-980i intelligent HPLC isocratic pump (Tokyo, Japan), a Rheodyne Advantage PF ten-port valve (Cotati, CA), and a Jasco UV-975 UV/Vis detector**.** Data were collected using an interface and software from National Instruments (Austin, TX). The temperature of the columns and mobile phases was controlled using a PolyScience circulating VWR circulating water bath (Buffalo Grove, IL) and a water jacket from Alltech. All columns were packed using an HPLC column slurry packer from Alltech. Peakfit v.4.12 and an exponentially-modified Gaussian curve fit were used to determine the central moments of all eluting peaks.

Methods.

Nucleosil Si-300 was converted into a dihydrazide-activated form according to a previously-established procedure.[10] Optimized entrapment conditions established in **Chapter 2** were used to prepare columns containing entrapped HSA and control supports. Three separate oxidized glycogen solutions were prepared in a pH 5.0 buffer containing 20 mM sodium acetate and 15 mM sodium chloride. Each 4.0 mL solution was prepared by dissolving various amounts of periodic acid and 17 mg of glycogen from bovine liver in the aforementioned buffer. The different samples will be referred to by the mass ratio of periodic acid to oxidized glycogen used in the initial step of the

oxidation. The three samples contained the following amounts of periodic acid: HSA 3.95, 67 mg; HSA 7.95, 135 mg; and HSA 12.05, 205 mg – where the number designations of 3.95, 7.95 and 12.05 refer to the ratio of mg periodic acid to mg glycogen.

Each solution was covered in aluminum foil – due to the light sensitivity of periodic acid – and allowed to shake at room temperature for 12-24 h. After oxidization, each glycogen solution was purified using a separate Econo-Pac 10DG disposable desalting column and pH 5.0, 0.10 M potassium phosphate buffer. This process was done by first removing the upper cap of the column and decanting the excess storage buffer. The next step was a buffer exchange via the addition of 10.0 mL of pH 5.0, 0.10 M potassium phosphate buffer to the column. Next, 3.3 mL of the oxidized glycogen sample were allowed to pass through the column, followed by an addition of 4.0 mL of potassium phosphate buffer to elute the oxidized glycogen. The larger glycogen molecules eluted from the column during this step, while the smaller periodic acid molecules took much longer to pass through the column matrix. Each final purified oxidized glycogen solution had a concentration of 4.2 mg/mL in a volume of approximately 4.0 mL. These solutions were stored at 4 °C.

Three entrapped HSA supports were prepared under the same immobilization conditions. To create the HSA-3.95 entrapped support, 80 mg of dihydrazide-activated silica was combined with 160 µL of a 50 mg/mL stock solution of protein prepared in pH 5.0, 0.10 M potassium phosphate buffer. This mixture was sonicated under vacuum for 15 min to ensure the proper mixing of the silica and protein. A 380 µL portion of the specified oxidized glycogen was added, and the mixture was shaken at room temperature

for 12 h. During the final hour of the reaction, 200 µL of a 2 mg/mL oxalic dihydrazide solution in the same buffer was added to the reaction mixture to cover any remaining aldehyde groups on the silica support. After immobilization, the support was washed several times with pH 7.4, 67 mM potassium phosphate buffer. A control support was also prepared using the specified oxidized glycogen solution, with the addition of a 160 µL aliquot of potassium phosphate buffer in place of the protein solution. Binding of an analyte to the prepared control support was used to monitor non-specific interactions. This procedure was repeated for the preparation of the HSA-7.95 support and the HSA-12.05 support.

Another 80 mg portion of Nucleosil Si-300 was converted to diol-bonded silica, and HSA was immobilized to this support by the Schiff base method according to previously-published procedures.[11,12] A control support was also made using this method, without the addition of HSA.

The protein content of the entrapped HSA and Schiff base HSA supports was determined by using a micro BCA assay, with soluble HSA being used to create a standard curve and the control support being used as the blank. The protein content, determined in mg HSA per gram support for all supports were as follows: HSA-3.95 support, 25.2 (± 0.5); HSA-7.95 support, 37 (± 1); HSA-12.05 support, 36 (± 1); and 39 (± 2) for the Schiff base HSA support. All supports were downward slurry-packed into separate 1.0 cm × 2.1 mm I.D stainless steel columns at 4000 psi using pH 7.4, 67 mM potassium phosphate buffer as the packing solution**.** All columns were stored at 4°C in the packing solution.

Zonal elution studies were performed on all four sets of columns using two well-characterized probe compounds for HSA: *R*-warfarin, which is known to bind to Sudlow site I, and L-tryptophan, which is known to bind to Sudlow site II. Binding of drugs to the entrapped HSA-7.95 column and the Schiff base HSA column were then examined in further detail using four additional analytes: *S*-warfarin, acetohexamide, gliclazide and tolbutamide. All sample solutions for the chromatographic studies were prepared in pH 7.4, 67 mM potassium phosphate buffer. Stock sample solutions with a concentration of 30 µM *R*-warfarin or *S*-warfarin and a concentration of 100 µM L-tryptophan, acetohexamide, gliclazide or tolbutamide were prepared in pH 7.4, 67 mM potassium phosphate buffer. The stock solutions were diluted with this pH 7.4 buffer to give 10 µM working samples of all analytes.

A 20 µL volume of each sample was injected onto the HSA columns and control columns in triplicate at 37 °C in the presence of pH 7.4, 67 mM potassium phosphate buffer. Flow rates of 0.1-1.0 mL/min were used to examine the binding of each analyte to the entrapped HSA columns or Schiff base HSA column. The void time of each column was determined by making triplicate 20 µL injections of 25 µM sodium nitrate in the presence of pH 7.4, 67 mM potassium phosphate buffer at each sampled flow rate. The void time of the system was determined by repeating these injections of sodium nitrate on a zero-volume union. Peakfit v.4.12 and an exponentially-modified Gaussian curve fit were used to determine the central moments of all eluting peaks.

Results and Discussion

Zonal elution studies of analyte retention on entrapped HSA columns

Initial zonal elution studies using the probe compounds *R*-warfarin and L-tryptophan were performed on the HSA-3.95, HSA-7.95, HSA-12.05 columns and the Schiff base HSA column. The retention factor (*k*) was measured for each analyte at flow rates of 0.10, 0.25, 0.50, 0.75 and 1.00 mL/min on each column. The value of *k* was calculated according to **Equation 3-1**, where t_R is the retention time of the analyte and t_M is the void time of the column.

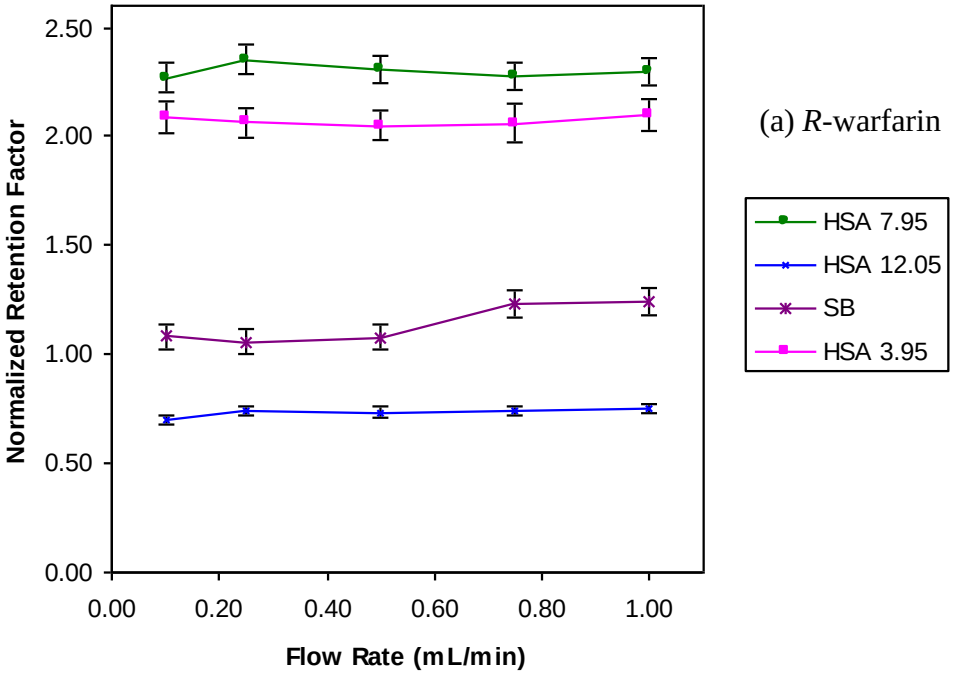
$$k = \frac{(t_R - t_M)}{t_M} \qquad (3\text{-}1)$$

Figure 3-2 shows how varying the flow rate affected the retention factors measured for *R*-warfarin and L-tryptophan on each column. These retention factors were normalized by dividing *k* by the measured total protein content for each support. This allowed for a more accurate comparison of retention factors between columns. Each column showed good consistency in retention factor across all flow rates, indicating that access to either Sudlow site I or Sudlow site II on the immobilized HSA was not hindered by an increase in flow rate on these columns.

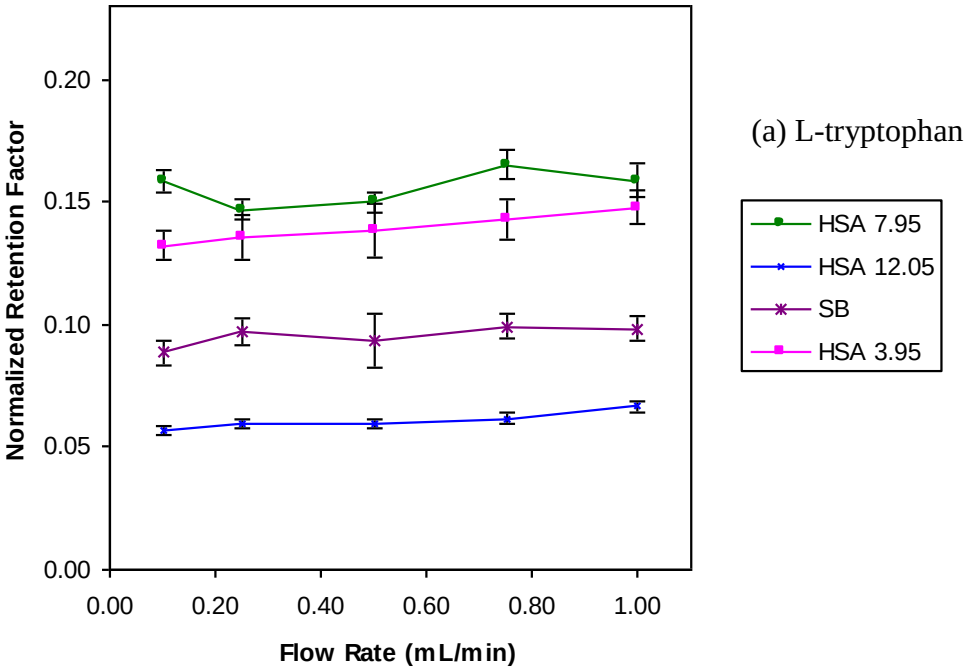
As the oxidation level of the glycogen was increased, an initial increase in protein content was seen in going from column HSA-3.95 to column HSA-7.95, followed by a leveling off with column HSA-12.05. This trend agrees with data collected for the optimization of glycogen oxidation for 300 Å pore size silica in **Chapter 2**. The direct relationship between retention factor and protein content can been seen in **Equation 3-2**,

$$k = \frac{(K_a' m_{Ltotal})}{V_m} \qquad (3\text{-}2)$$

Figure 3-2. Normalized retention factors determined at various flow rates for the HSA-3.95, HSA-7.95, HSA-12.05, and Schiff base immobilized HSA supports for (a) *R*-warfarin and (b) L-tryptophan. All measurements were adjusted for nonspecific binding through the use of a control support. Other experimental conditions are given in the text. The error bars represent ± 1 S.D.



(a) *R*-warfarin



(a) L-tryptophan

Table 3-1. Measured binding capacities and retention factors for *R*-warfarin and L-tryptophan on the HSA-3.95, HSA-7.95, HSA-12.05 and the Schiff base HSA supports. All measurements were adjusted for nonspecific binding through the use of a control support.

***R*-warfarin**	**Protein content (nmoles)**	**Retention factor (k)**	**Normalized retention factor k/(protein content)[a]**
HSA-3.95	5.6 (± 0.4)	52 (± 1)	2.07 (± 0.07)
HSA-7.95	8.2 (± 0.2)	84.7 (± 0.5)	2.30 (± 0.07)
HSA-12.05	8.1 (± 0.2)	27.7 (± 0.2)	0.73 (± 0.2)
Schiff Base	8.7 (± 0.4)	41.4 (± 0.3)	1.14 (± 0.06)

L-tryptophan	**Protein content (nmoles)**	**Retention factor (k)**	**Normalized retention factor k/(protein content)[a]**
HSA-3.95	5.6 (± 0.4)	4 (± 1)	0.14 (± 0.07)
HSA-7.95	8.2 (± 0.2)	5.7 (± 0.3)	0.16 (± 0.04)
HSA-12.05	8.1 (± 0.2)	2.2 (± 0.1)	0.06 (± 0.02)
Schiff Base	8.7 (± 0.4)	3.5 (± 0.4)	0.10 (± 0.06)

[a] These normalized retention factors were calculated by dividing the listed retention factors by a measured total protein content for the given support in units of mg HSA/g support. The values in parentheses represent a range of ± 1 S.D.

where V_m is the void volume of the column and K_a' and m_{Ltotal} are the global association constant (the average of K_{a1} and K_{a2}) and the moles of total active protein in the column. **Table 3-1** presents the measured protein contents and retention factors for all supports that were analyzed. The data for columns HSA-3.95 and HSA-7.95 were in agreement with **Equation 3-2**, but a significant deviation was seen in the data for column HSA-12.05. The protein content was statistically equal to the value for HSA-7.95, but a decrease of approximately three-fold in retention was seen between the two columns for both *R*-warfarin and L-tryptophan. Because the only varied parameter between the two supports was the amount of periodic acid used in the oxidation of glycogen, this decrease in retention factor was most likely due to overoxidation of the glycogen, blocking access to some of the entrapped protein.

Overoxidation has been seen with the use of high periodic acid concentrations during antibody oxidation. This can occur when diol groups are oxidized past aldehyde groups to carboxylic acids, or when cross-linking occurs between oxidized glycoproteins.[13, 14] Another effect of overoxidation is decreased solubility. After glycogen oxidation, any remaining solution was stored at 4 °C Samples HSA-3.95 and HSA-7.95 remained in solution, while sample HSA-12.05 produced a white precipitate within a few days following preparation. This observation is consistent with previously-reported observations of antibody precipitation after oxidization with high levels of periodic acid[13]. In this study the overoxidation of glycogen appeared to inhibit the accessibility of an analyte for the entrapped HSA.

Although the protein content of the HSA-7.95 column and the Schiff base HSA column were similar, the entrapment column had an increased value for the retention

factor. As seen in **Equation 3-2**, the retention factor is directly related to the protein content, so it would follow that similar protein content would result in similar values for retention factor. However, differences in specific activity between the two types of immobilization can account for the discrepancy in retention factor. This concept was examined further through the use of a wider variety of injected analytes, and will be discussed later in this chapter.

Band broadening for analytes on entrapped HSA columns

Band broadening is another parameter that can be used in zonal elution studies with HPAC to obtain information about the interaction between an analyte and an immobilized ligand.[5,7] In this type of study, an affinity column is viewed as being comprised of a series of equal-sized regions as represented by (*N*), the number of theoretical plates. Each theoretical plate represents a single interaction between the analyte and the stationary phase. The distance along the column that makes up a single theoretical plate is represented by the plate height (*H*). These two terms are related to one another through the total length of the column (*L*), where $H = L/N$.[7, 15] Plate height can be used as an indication of column efficiency, where *H* decreases with increasing efficiency. The value of *H* is related to the width, or the second moment (σ^2) of a peak. In this type of study, measurements are taken for injections at several different flow-rates. Plate height values can then be calculated according to **Equation 3-3**, where H_R

$$H_R = \frac{L\sigma^2{}_R}{t_R{}^2} \qquad (3\text{-}3)$$

is the total plate height measured for the column, *L* is the column length (in cm), $\sigma^2{}_R$ is the second moment of the retained analyte peak, and t_R is the analyte's retention time. The plate height contribution of the stationary phase is found by taking the difference

between the total plate height measured for the analyte on the HSA column and the plate height measured for a control column.[7]

Figure 3-3 shows the values for plate height that were obtained for the injection of 10 µM *R*-warfarin for all supports studied. These values were used as a means for comparison of column efficiency. Over the range of linear velocities 0.1 – 0.8 cm/s the plate height values for the individual entrapment-based columns stayed consistent, indicating that there was no loss in efficiency as the flow rate was increased up to 0.8 cm/s. When the entrapped HSA columns were compared to each other, the efficiency decreased as the oxidation level of glycogen was increased. The HSA-3.95 and HSA-7.95 supports had similar values for the plate height, ranging from 0.010 to 0.018 (± 0.001). The HSA-12.05 support had a much higher values for plate height, with an average value of 0.057 (± 0.001). Similar results were noted for of L-tryptophan. This reduction in column efficiency at high levels of oxidation can most likely be attributed to the overoxidation of glycogen and slower mass transfer for the injected analytes on the entrapped HSA support. This analysis further supports the hypothesis that overoxidation of glycogen can limit the accessibility of an analyte to reach the entrapped HSA.

Correlation study

For a comparison between the entrapment and Schiff base immobilization methods, injections of four additional analytes were made: *S*-warfarin, acetohexamide, gliclazide and tolbutamide. The supports used for this comparison were the Schiff base HSA column and the HSA-7.95 entrapped column. The HSA-7.95 column was chosen for this study because it had good efficiency and similar protein content to the Schiff base HSA column. Correlation charts for the injection of six analytes on these columns are

Figure 3-3. Plate height plots obtained for 20 μL injections of *R*-warfarin on the entrapped HSA columns. Other experimental conditions are given in the text. The error bars represent ± 1 S.D.

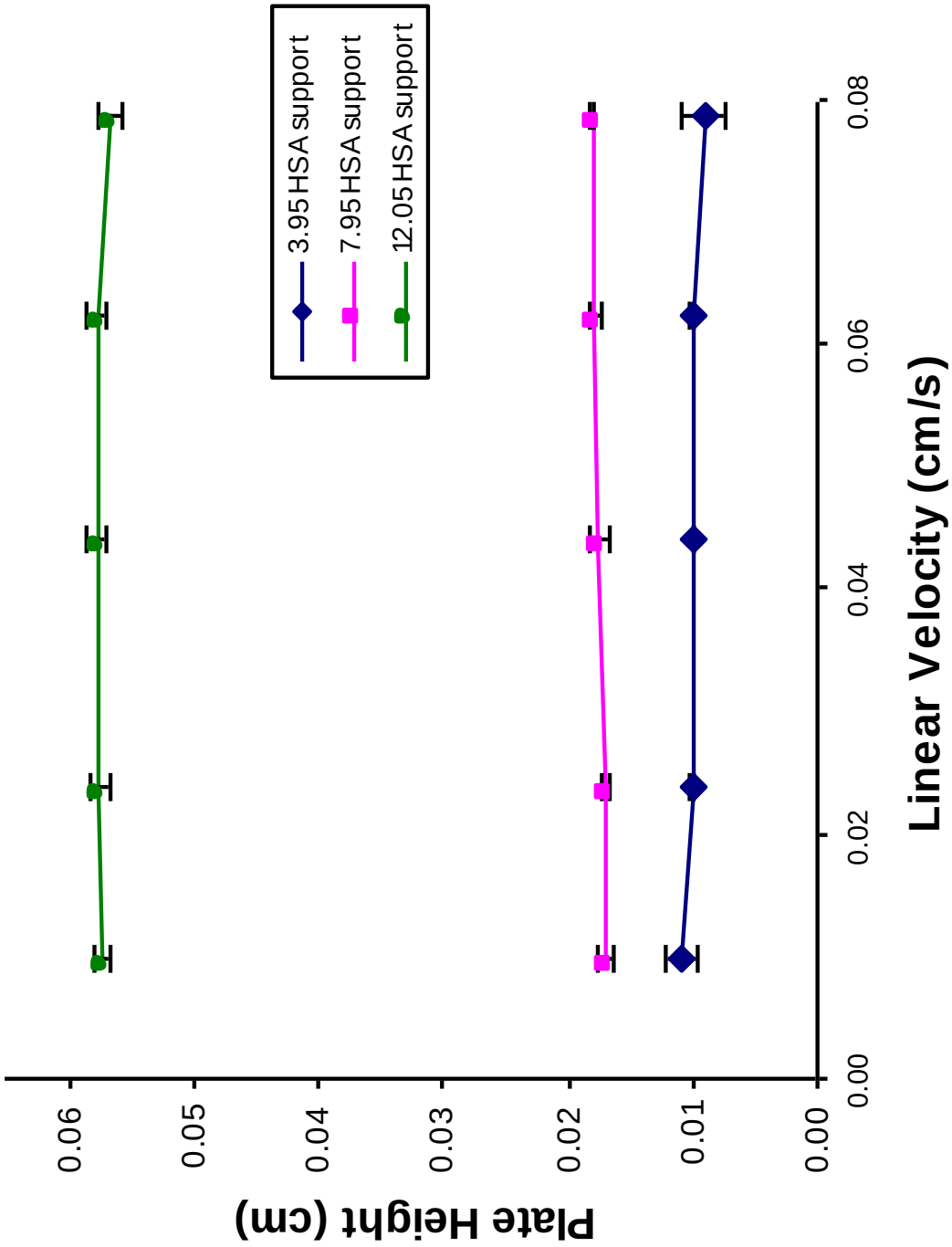
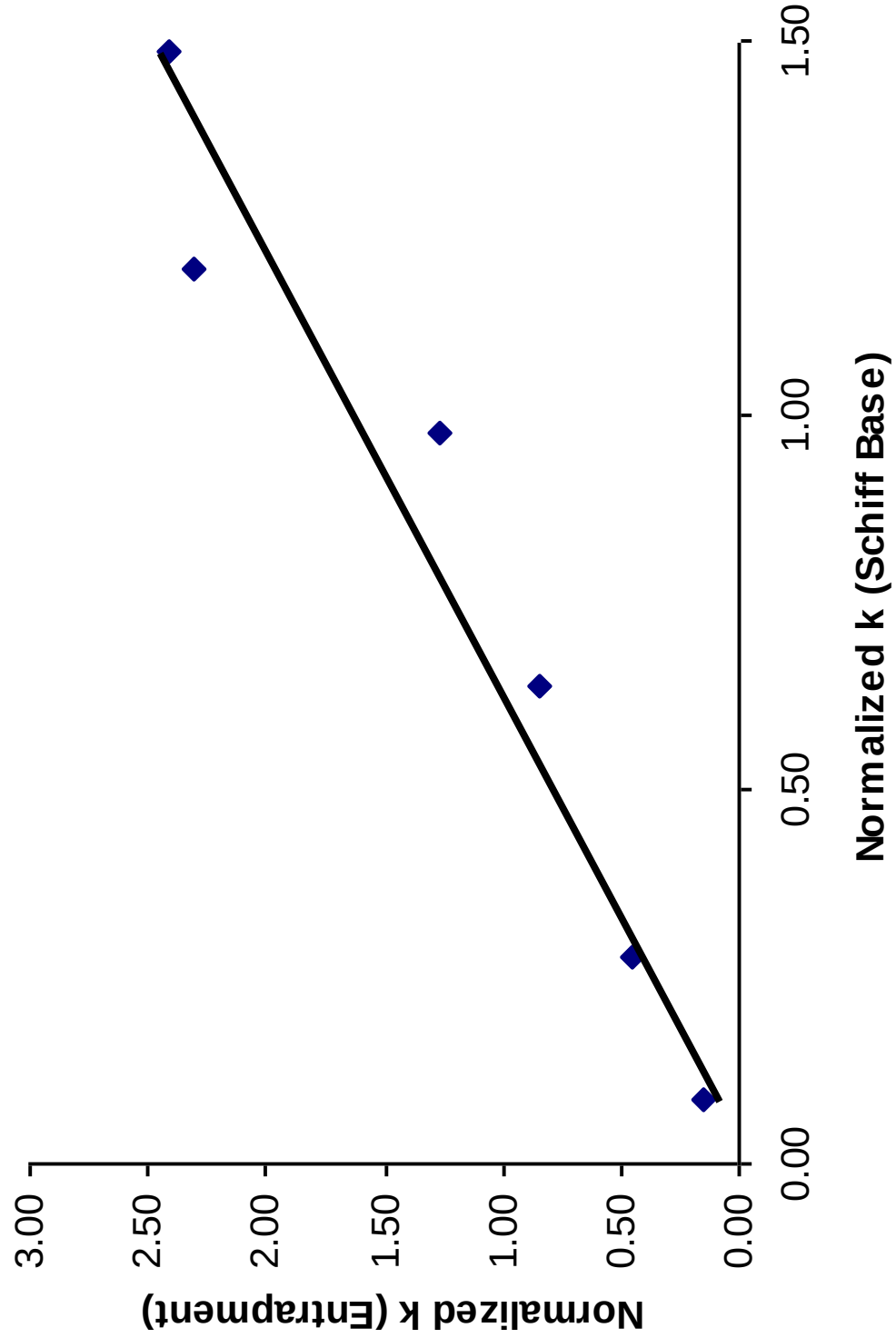


Plate Height (cm)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.00
0.02
0.04
0.06
0.08
Linear Velocity (cm/s)
3.95 HSA support
7.95 HSA support
12.05 HSA support

Figure 3-4. Correlation plots between the normalized retention factors for various drugs on the entrapped HSA-7.95 column and the Schiff base immobilized HSA column. Conditions can be found in the text. The correlation coefficient for this plots was 0.940 (n = 6).


Normalized k (Entrapment)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.00
0.50
1.00
1.50
Normalized k (Schiff Base)

shown in **Figure 3-4**. Each value shown is the average normalized retention factor for triplicate injections at five different flow rates. The plot of entrapment vs. Schiff base results had a slope of 1.6946. This indicated that the entrapment method results in an approximately a 70% higher retention factor when compared to the Schiff base method, further illustrating the effect of specific activity on the retention factor.

Conclusions

This chapter used HPAC zonal elution studies to examine the changes in analyte binding as a function of the level of initial glycogen oxidation for entrapped HSA. Three levels of glycogen oxidation were used to prepare entrapped HSA supports. Zonal elution studies with *R*-warfarin and L-tryptophan showed that retention factors were consistent over a range of flow rates, but the value of the retention factor was effected by the level of oxidized glycogen used to prepare the supports. The entrapped HSA-12.05 support did not have retention factors consistent with its measured protein content. This indicated that the analyte was not fully able to access the entrapped protein, due to possible glycogen overoxidation. Normalized retention factors for the other two entrapped columns were higher than the values reported for the Schiff base immobilized HSA support. The relationship between retention and protein content suggests that a larger portion of the entrapped HSA was active compared to the HSA in the Schiff base column. This fits with previously-established data for the specific activity of each method.

This work also investigated the efficiency of each column as a function of glycogen oxidation through a determination of plate heights. The values for the plate

height remained consistent throughout the span of sampled flow rates but increased as the oxidation level of glycogen increased. The entrapped HSA-12.05 column showed a significant decrease in efficiency compared to the other entrapped supports, reinforcing the idea that the analyte's ability to interact with entrapped protein was inhibited by the highly oxidized glycogen in this support.

The results of this work indicate that entrapment supports made with glycogen that has undergone a moderate level of oxidation can be used in place of covalently immobilized supports for the analysis of drug-protein interactions. A relatively small 1.0 cm column contains a similar protein content to a comparable Schiff base column, but higher values of retention can be achieved, due to the presence of essentially 100% protein activity. The entrapped HSA column also shows consistent efficiency at common HPLC flow rates.

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CHAPTER 4:

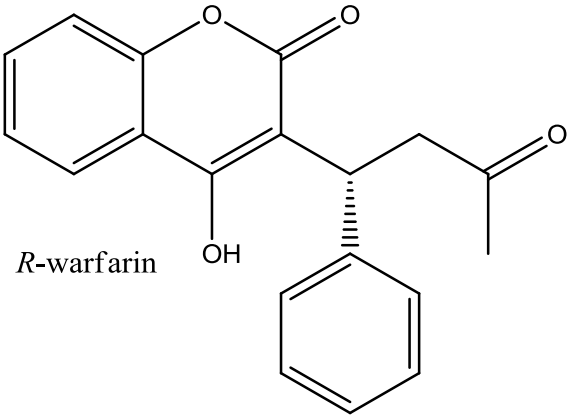
THE USE OF ENTRAPMENT TO EXAMINE THE EFFECT OF GLYCATION ON THE BINDING OF SEVERAL DRUGS TO HUMAN SERUM ALBUMIN

Introduction

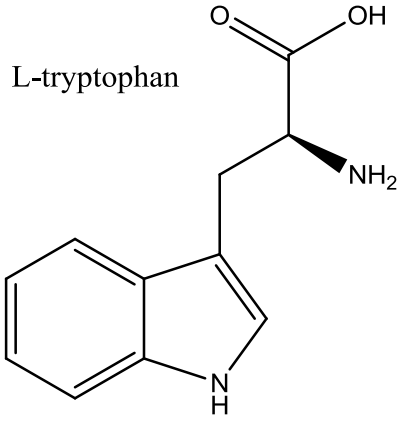
Human serum albumin (HSA), serves as an important carrier protein, is the most abundant protein in human plasma.[1, 2] This protein has two major binding sites, Sudlow site I and Sudlow site II.[3, 4] The binding of drugs to HSA can be affected by many factors.[2] This chapter will focus on the effects of glycation on analyte binding, a process especially prevalent in individuals with diabetes.[2, 5] The glycation process is thought to alter the secondary and tertiary structure of HSA. Because some of the glycation sites are near Sudlow sites I and II, the glycation process can potentially alter the overall binding affinity for analytes to HSA.[5-7]

The first portion of this study will compare the binding of *R*-warfarin and L-tryptophan (structures seen in **Figure 4-1**) to HSA versus glycated HSA (gHSA) using the entrapment immobilization method. Warfarin and L-tryptophan are often used as site-selective analytes for Sudlow sites I and II, respectively.[8] This study will then be extended to examine how the binding of three sulfonylureas were affected by the glycation of HSA. Sulfonylureas, including acetohexamine, gliclazide and tolbutamide, are a class of drugs commonly used to treat type II, or non-insulin dependent diabetes.[9, 10] The structures for these analytes can be seen in **Figure 4-2**. It has been shown that the three sulfonylureas examined in this study bind to both major sites of HSA, as characterized by HPAC and covalently immobilized HSA.[11, 12]

Figure 4-1. Structures of *R*-warfarin and L-tryptophan.

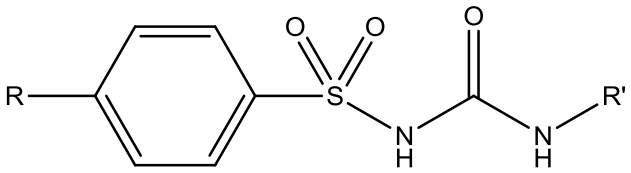


R-warfarin

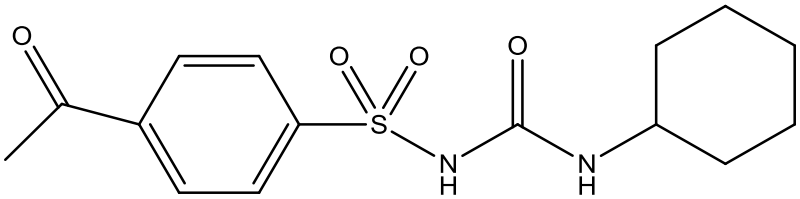


L-tryptophan

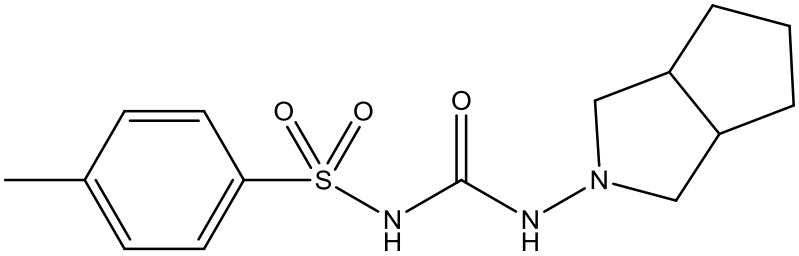
Figure 4-2. Structures of the sulfonylurea drugs used in this study.



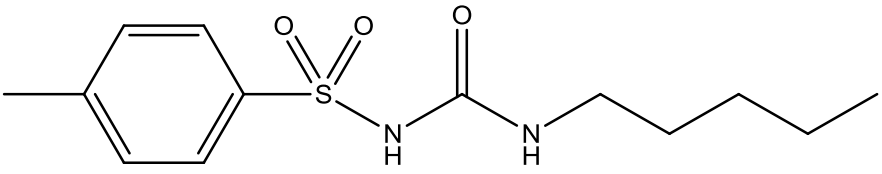
Basic sulfonylurea structure



Acetohexamide



Gliclazide



Tolbutamide

This chapter will use the entrapment method for the immobilization of both HSA and gHSA. These entrapped supports will be used in zonal elution to explore the effects of glycation on the binding of the aforementioned target analytes to entrapped HSA and entrapped gHSA.

Experimental

Reagents.

The *p*-periodic acid (periodic acid reagent, or H_5IO_6), glycogen (from bovine liver)**,** HSA (Cohn fraction V, 99% globulin free, 99% fatty acid free), glycated HSA (95% lyophilized, lot no. 115K6108), *R*-warfarin, L-tryptophan, acetohexamide, gliclazide, tolbutamide, monobasic and dibasic potassium phosphate salts, sodium chloride and sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO**).** The Nucleosil Si-300 silica (300 Å pore size, 7 micron particle diameter) was purchased from Macherey-Nagel (Düren, Germany). Reagents for the micro bicinchoninic acid (BCA) protein assay were purchased from Pierce (Rockford, IL). Other chemicals were of the purest grades available. The Econo-Pac 10 DG disposable 30 x 10 mL desalting columns were purchased from Bio-Rad Laboratories (Hercules, CA). All solutions were prepared using water from a NANOpure purification system (Barnstead, Dubuque, IA) and filtered with a 0.20 µm GNWP nylon membrane from Millipore (Billerica, MA).

Apparatus.

The chromatographic system consisted of a Jasco PU-980i intelligent HPLC isocratic pump (Tokyo, Japan), a Rheodyne Advantage PF ten-port valve (Cotati, CA), and a Jasco UV-975 UV/Vis detector**.** The detection wavelengths were as follows: 205

nm, sodium nitrate; 226 nm, gliclazide; 248 nm, acetohexamide, 250 nm, tolbutamide; 280 nm, L-tryptophan, and 304 nm, *R*-warfarin. Data were collected using an interface and software from National Instruments (Austin, TX). The temperature of the columns and mobile phases was controlled using a PolyScience circulating VWR circulating water bath (Buffalo Grove, IL) and a water jacket from Alltech. All columns were packed using an HPLC column slurry packer from Alltech. The chromatographic data were analyzed using Peakfit 4.12 (Jandel Scientific Software, San Rafael, CA).

Methods.

Nucleosil Si-300 was converted into a dihydrazide-activated form according to previously-established procedures.[13] Optimized entrapment conditions established in **Chapter 2** were used to prepare columns containing entrapped HSA, entrapped glycated HSA, and control supports. The oxidation of glycogen was carried out in a pH 5.0 buffer containing 20 mM sodium acetate and 15 mM sodium chloride. A 4.0 mL solution was prepared by dissolving 135 mg of periodic acid and 17 mg of glycogen from bovine liver in the aforementioned buffer. This solution was covered in aluminum foil – due to the light sensitivity of periodic acid – and allowed to shake at room temperature for 12 – 24 h. After oxidization, the glycogen was purified using an Econo-Pac 10DG disposable desalting column and pH 5.0, 0.10 M potassium phosphate buffer. This process was done by first removing the upper cap of the column and decanting the excess storage buffer. The next step was a buffer exchange via the addition of 10.0 mL of pH 5.0, 0.10 M potassium phosphate buffer to the column. Next, 3.3 mL of the oxidized glycogen sample were allowed to pass through the column, followed by a volume of 4.0 mL of potassium phosphate buffer to elute the oxidized glycogen. The larger glycogen

molecules eluted from the column during this step, while the smaller periodic acid molecules took much longer to pass through the column matrix. The resulting purified oxidized glycogen solution had a concentration of 4.2 mg/mL with a final volume of approximately 4.0 mL. This solution was stored at 4 °C.

Both HSA and gHSA supports were prepared under the same immobilization conditions. A 50 mg/mL stock solution of protein was prepared with pH 5.0, 0.10 M potassium phosphate buffer. To create the entrapped support, 80 mg of dihydrazide activated silica was combined with 160 µL of the protein solution. This mixture was sonicated under vacuum for 15 min to ensure the proper mixing of the silica and protein. A 380 µL portion of the purified oxidized glycogen was added, and the mixture was shaken at room temperature for 12 h. During the final hour of the reaction, 200 µL of a 2 mg/mL oxalic dihydrazide solution in the same buffer was added to the reaction mixture to cover any remaining aldehyde groups on the silica support. After immobilization, the support was washed several times with pH 7.4, 67 mM potassium phosphate buffer. A control support was also prepared through the addition of a 160 µL aliquot of potassium phosphate buffer in place of the protein solution. Binding of an analyte to this support was used to monitor non-specific interactions. The protein content of the entrapped HSA and gHSA supports was determined using a micro BCA assay, with soluble HSA or gHSA being used to create a standard curve and the control support being used as a blank. The HSA support was found to contain 37 (± 1) mg HSA per gram support, and the gHSA support contained 32 (± 2) mg gHSA per gram support.

All supports were downward slurry-packed into separate 1.0 cm × 2.1 mm I.D stainless steel columns at 4000 psi using pH 7.4, 67 mM potassium phosphate buffer as

the packing solution**.** All columns were stored at 4 °C in the packing solution. All sample solutions for the chromatographic studies were prepared in pH 7.4, 67 mM potassium phosphate buffer. Stock sample solutions with a concentration of 30 µM *R*-warfarin and a concentration of 100 µM L-tryptophan, acetohexamide, gliclazide or tolbutamide, were prepared in pH 7.4, 67 mM potassium phosphate buffer. The stock solutions were diluted with this pH 7.4 buffer to give 10 µM working samples of all analytes.

A 20 µL volume of all samples was injected onto the entrapped HSA column, entrapped gHSA column and control column in triplicate at 37 °C in the presence of pH 7.4, 67 mM potassium phosphate buffer. Flow rates of 0.1-1.0 mL/min were used to examine the binding of each analyte to the protein of interest. The void time of each column was determined by making triplicate 20 µL injections of 25 µM sodium nitrate in the presence of pH 7.4, 67 mM potassium phosphate buffer at all sampled flow rates. The void time of the system was determined by repeating these injections of sodium nitrate on a zero-volume union. Peakfit v.4.12 and an exponentially-modified Gaussian curve fit were used to determine the central moments of all eluting peaks.

Results and Discussion

Initial Zonal Elution Studies of Entrapped HSA and Entrapped gHSA.

Affinity chromatography has been shown to be a useful tool to examine the overall affinity of drugs to immobilized proteins such as HSA.[14] Zonal elution studies can be used to determine the retention factor (*k*), which can then be used to calculate the global association equilibrium constant (K_a') of the analyte. K_a' is an average of the

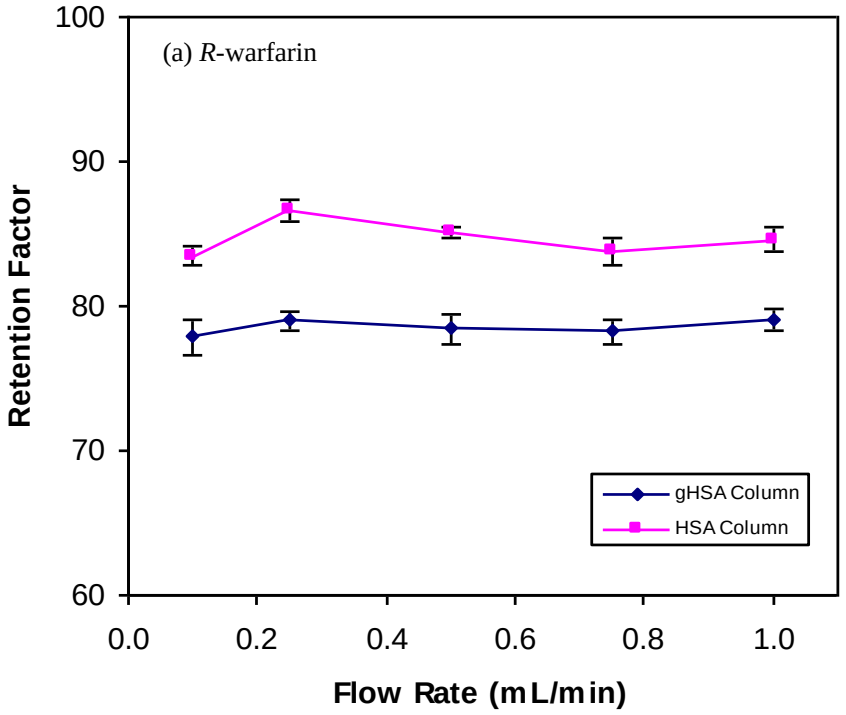
association equilibrium constants for both the high and low affinity binding sites. **Equation 4-1** shows how the retention factor is related to the K_a' for an injected analyte with an immobilized protein, where m_L is the moles of total moles of active binding sites in the column and V_M is the column void volume.[15, 16]

$$k = \frac{(K_a' m_{Ltotal})}{V_m} \tag{4-1}$$

Initially, the retention of *R*-warfarin and L-tryptophan on entrapped HSA and entrapped gHSA columns was studied by performing zonal elution. These analytes were chosen for this optimization, as they are often used as site-selective probes for Sudlow sites I and II, respectively. **Figure 4-3** shows how varying the flow rate affected the retention factors measured for these two analytes. The consistency of the retention factors across all flow rates examined indicated that altering the flow rate did not hinder access to either Sudlow sites I and II by the injected analytes. The ability to use higher flow rates made it possible to decrease the analysis time needed to obtain retention factors on the 1.0 cm × 2.1 mm I.D. entrapped protein columns.

Table 4-1 summarizes the average retention factors that were measured for each of the tested drugs over the range of sampled flow rates on both the entrapped HSA and entrapped gHSA columns. For a more accurate comparison, the retention factors were normalized by dividing these values by the measured total protein content in mg protein per gram support. Normalized retention factors for *R*-warfarin were relatively consistent between the entrapped HSA and the entrapped gHSA column. The calculated K_a values for *R*-warfarin ranged from 2.9 x 10^5 M^{-1} to 3.1 x 10^5 M^{-1}, with a relative precision of

Figure 4-3. Retention factors measured at various flow rates on the entrapped HSA and the entrapped gHSA columns for (a) *R*-warfarin and (b) L-tryptophan. Other experimental conditions are given in the text. The error bars represent ± 1 S.D.


(a) R-warfarin
Retention Factor
100
90
80
70
60
0.0
0.2
0.4
0.6
0.8
1.0
Flow Rate (mL/min)
gHSA Column
HSA Column

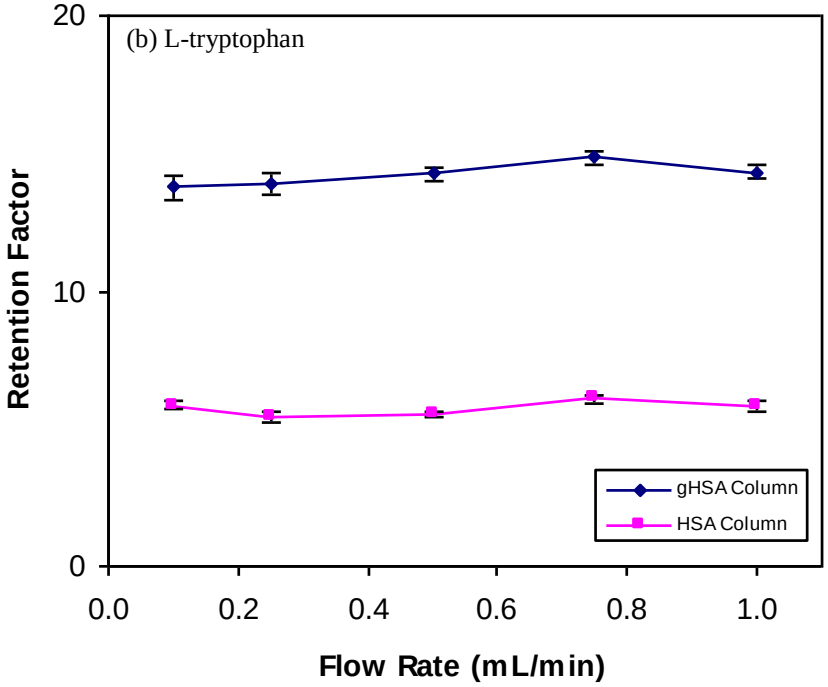

(b) L-tryptophan
Retention Factor
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
Flow Rate (mL/min)
gHSA Column
HSA Column

Table 4-1. Retention and calculated association equilibrium constants for *R*-warfarin and L-tryptophan on entrapped HSA and glycated HSA (gHSA) columns.

HSA	Retention factor (k)	Normalized retention factor k/(protein content)[a]	K_a (M^{-1})
R-warfarin	84.7 (± 0.5)	2.30 (± 0.03)	2.9 (± 0.2) x 10^5
L-tryptophan	5.7 (± 0.2)	0.16 (± 0.04)	1.9 (± 0.3) x 10^4

gHSA	Retention factor (k)	Normalized retention factor k/(protein content)[a]	K_a (M^{-1})
R-warfarin	78.5 (± 0.8)	2.46 (± 0.06)	3.1 (± 0.3) x 10^5
L-tryptophan	14.22 (± 0.03)	0.45 (± 0.07)	5.6 (± 0.2) x 10^4

The values in parentheses represent a range of ± 1 S.D.

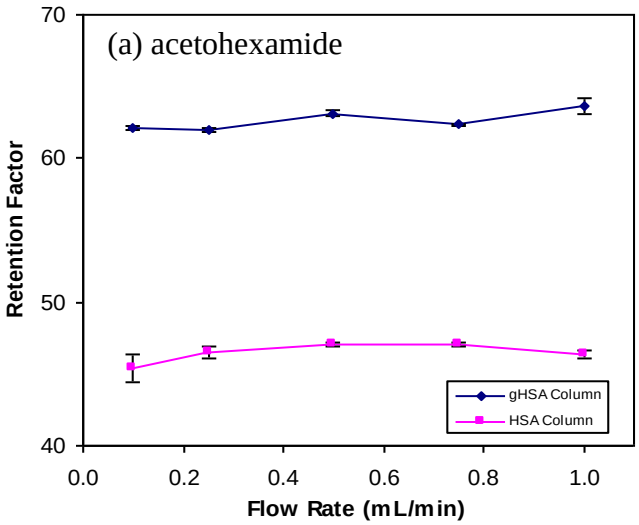
[a] These normalized retention factors were calculated by dividing the listed retention factors by a measured total protein content for the given supports of 37 (± 1) mg HSA per gram support or 32 (± 2) mg glycated HSA per gram support.

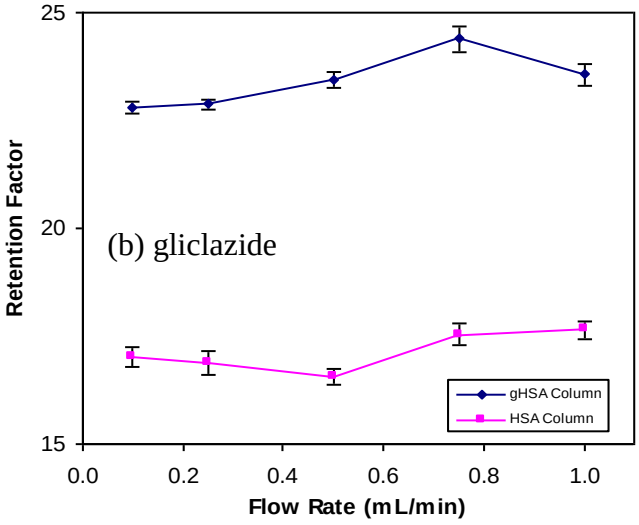
± 7-10%. These results did not show any significant variation between the entrapped HSA and the entrapped gHSA columns, with K_a values for *R*-warfarin within ± 1 S.D. of one another. These results indicated that the glycation of HSA did not have an observable effect on the binding of *R*-warfarin. These findings are consistent with previously- reported results for the effect of glycation on Sudlow site I.[8] The range of K_a values for *R*-warfarin was slightly higher than the previously-reported value of 2.5 – 3.0 x 10^5 M^{-1} for Schiff base immobilized HSA.[8, 17]

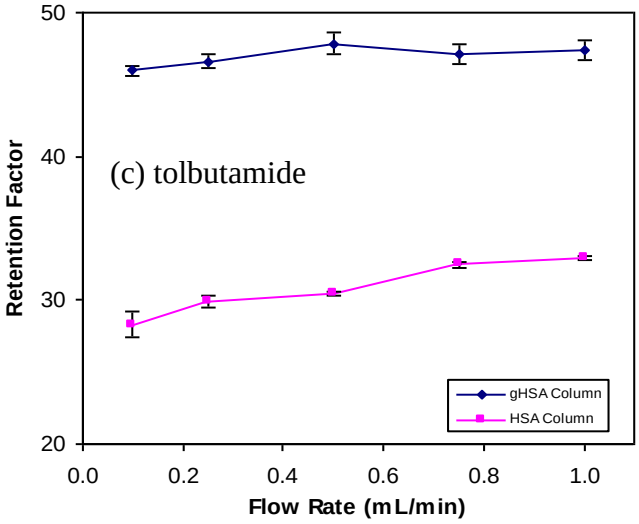
Unlike the retention of *R*-warfarin, the retention of L-tryptophan was affected by the glycation of HSA. The normalized retention factor for L-tryptophan on the entrapped gHSA column was approximately three times higher than the analyte's normalized retention factor for entrapped HSA. The value of K_a increased by the same factor, from 1.9 x 10^4 M^{-1} for entrapped HSA to 5.6 x 10^4 M^{-1} for entrapped gHSA. These results indicated that the glycation of HSA affected the binding of L-tryptophan to Sudlow site II, consistent with recently-published results.[17] While no major glycation sites occur in close proximity to Sudlow site II, it has been suggested that small amounts of glycation alter the tertiary structure of HSA, causing local unfolding near the binding site.[7] The value of K_a for L-tryptophan on the entrapped HSA column was slightly higher than the previously-reported value of 1.1 (± 0.3) x 10^4 M^{-1} for Schiff base immobilized HSA.[18]

The discrepancies in K_a values can possibly be attributed to the increased specific activity of the entrapment method as compared to the Schiff base method. When calculating the association equilibrium constant from retention factor data, the value of K_a is directly related to the value of m_L. Essentially 100% of the total protein content (m_L) is

Figure 4-4. Retention factors measured at various flow rates on the entrapped HSA and the entrapped gHSA columns for (a) acetohexamide, (b) gliclazide and (c) tolbutamide. Other experimental conditions are given in the text. The error bars represent ± 1 S.D.


(a) acetohexamide
Retention Factor
70
60
50
40
0.0
0.2
0.4
0.6
0.8
1.0
Flow Rate (mL/min)
gHSA Column
HSA Column


(b) gliclazide
Retention Factor
25
20
15
0.0
0.2
0.4
0.6
0.8
1.0
Flow Rate (mL/min)
gHSA Column
HSA Column


(c) tolbutamide
Retention Factor
50
40
30
20
0.0
0.2
0.4
0.6
0.8
1.0
Flow Rate (mL/min)
gHSA Column
HSA Column

active when using the entrapment method, while immobilization through the Schiff base method results in a typical specific activity of up to 67%.[17]

Zonal Elution Studies of Additional Analytes.

This study was extended to include acetohexamide, gliclazide and tolbutamide; three sulfonylureas commonly used to treat type II, or non-insulin dependent, diabetes. **Figure 4-4** shows how varying the flow rate affected the retention factors measured for these three analytes. Similar to the results seen for *R*-warfarin and L-tryptophan, the relative consistency of the retention factors across all sampled flow rates indicated that altering the flow rate did not hinder the binding ability of these drugs to the immobilized protein.

Table 4-2 summarizes the average retention factors measured for each of the tested drugs over the range of flow rates on both the entrapped HSA and entrapped gHSA columns. For a more accurate comparison, the retention factors were normalized, by dividing these values by the measured total protein content (in mg protein per gram support). All three sulfonylurea drugs showed an increase of approximately 50-60% for the normalized retention factors on the entrapped gHSA column versus the value obtained for the entrapped HSA column. This increase in normalized retention factor is in agreement with previously-reported values for these three sulfonylureas, using the same sample of gHSA for immobilization.[19] All three of these analytes are known to have two-site binding to HSA, with the majority of the binding attributed to the high affinity site.[11, 12] If the binding affinity to each individual site is known, a global K_a can be determined by calculating an averaging of the K_a values for each site. This value can be used for comparison to the K_a values obtained using zonal elution experiments in this

Table 4-2. Retention and calculated association equilibrium constants for acetohexamide, gliclazide and tolbutamide on entrapped HSA and glycated HSA (gHSA) columns.

HSA	Retention factor (k)	Normalized retention factor k/(protein content)[a]	K_a (M^{-1})
Acetohexamide	46.5 (± 0.1)	1.26 (± 0.03)	1.6 (± 0.1) x 10^5
Gliclazide	17.1 (± 0.2)	0.46 (± 0.03)	5.8 (± 0.2) x 10^4
Tolbutamide	30.8 (± 0.1)	0.84 (± 0.03)	1.0 (± 0.1) x 10^5

gHSA	Retention factor (k)	Normalized retention factor k/(protein content)[a]	K_a (M^{-1})
Acetohexamide	62.7 (± 0.1)	1.97 (± 0.06)	2.5 (± 0.3) x 10^5
Gliclazide	23.4 (± 0.1)	0.74 (± 0.06)	9.3 (± 0.3) x 10^4
Tolbutamide	47.0 (± 0.2)	1.47 (± 0.06)	1.9 (± 0.1) x 10^5

The values in parentheses represent a range of ± 1 S.D.

[a] These normalized retention factors were calculated by dividing the listed retention factors by a measured total protein content for the given supports of 37 (± 1) mg HSA per gram support or 32 (± 2) mg glycated HSA per gram support.

study. These values are presented in **Table 4-3**. The K_a values determined using entrapped HSA were approximately two times larger than previously reported reference values. This increase in K_a, although more significant than seen in *R*-warfarin and L-tryptophan, may also be attributed to the discrepancy in specific activity of the immobilized protein content used to determine K_a from retention factor.

Conclusions

This chapter used high-performance affinity chromatography zonal elution studies to examine the changes in binding and overall affinity for various analytes that occur with the glycation of HSA. Retention factors were calculated using a series of increasing flow rates. These changes in flow rate had little effect on the binding of all examined drugs to either immobilized protein. Initial studies examined the binding of two probe compounds for the two main binding sites on HSA, Sudlow sites I and II. An average retention factor for the examine flow rates was used to calculated a global K_a value for each analyte on both the entrapped HSA and entrapped gHSA columns. The normalized retention and association equilibrium constant for *R*-warfarin remained constant for both entrapped HSA and entrapped gHSA. L-tryptophan, however, experienced a nearly three-fold increase in normalized retention factor, which translated into an increase of K_a from 1.9 ($\pm$ 0.3) x 10^4 M^{-1} to 5.6 ($\pm$ 0.2) x 10^4 M^{-1}. This study was extended for the examination of three sulfonylaurea drugs used for the treatment of type II diabetes. Acetohexamide, gliclazide and tolbutamide all showed increased binding and association constants with the glycation of HSA. All K_a values determined on the entrapped HSA column were slightly higher than previously established values for the same analytes binding to Schiff

Table 4-3. Comparison of K_a values calculated using zonal elution experiments to reference global K_a values on HSA and gHSA with similar levels of glycation.

HSA	K_a (M^{-1})	K_a (M^{-1})[a]	Reference
Acetohexamide	1.6 (± 0.1) x 10^5	8.7 (± 0.7) x 10^4	[11]
Gliclazide	5.8 (± 0.2) x 10^4	3.1 (± 0.6) x 10^4	[20]
Tolbutamide	1.0 (± 0.1) x 10^5	5.4 (± 0.2) x 10^4	[11]

gHSA	K_a (M^{-1})	K_a (M^{-1})[a]	Reference
Acetohexamide	2.5 (± 0.3) x 10^5	6.1 (± 0.2) x 10^4	[21]
Gliclazide	9.3 (± 0.3) x 10^4	N/A[b]	
Tolbutamide	1.9 (± 0.1) x 10^5	6.5 (± 0.2) x 10^4	[22]

The values in parentheses represent a range of ± 1 S.D.

[a] These K_a values were determined by calculating the average value of both the low and high affinity binding sites of each analyte to the protein of interest.

[b] Studies are currently being conducted to determine a K_a value for gliclazide on covalently immobilized gHSA.

base immobilized HSA.[11, 12] This discrepancy could be attributed to the reported differences in specific activity determine for the two methods.

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CHAPTER 5:

BINDING OF DRUGS TO ENTRAPPED HSA AND ENTRAPPED AGP SUPPORTS IN HPAC

Introduction

High performance affinity chromatography (HPAC) makes use of the selective and reversible interactions between an analyte and biologically relevant ligand. This ligand is immobilized onto a support with sufficient stability to withstand high pressure conditions. A major advantage of using HPAC for drug-binding studies is the ability for its supports to be used for multiple experiments. This feature is of great interest when comparing the binding of a large number of analytes to a specific ligand. Using the same support for all studies eliminates the variation between immobilized supports.[1-4]

One desirable feature in the immobilization of a protein for drug-binding studies is to have the protein in a final form that closely mimics that behavior of the protein's native form. The work in previous chapters examined the ability of entrapped human serum albumin (HSA) to bind with the site specific probes *R*-warfarin and L-tryptophan. This chapter will expand on the use of an entrapped HSA support for the analysis of a variety of other drugs. This chapter will also introduce the use of an entrapped α_1-acid glycoprotein (AGP) support.

Traditionally, AGP has been immobilized through cross-linking and thiol coupling. The former has had poor correlation with soluble AGP binding behavior, while the latter has shown good correlation with soluble AGP for binding with some drugs but not others.[1, 2] Recently, a new immobilization technique has made use of the

carbohydrate residues on this glycoprotein. This method immobilizes AGP through controlled oxidation of these carbohydrate residues, followed by a coupling of this oxidized protein to hydrazide-activated silica. This immobilization technique has been shown to give results with better agreement to soluble AGP-binding values than previous methods.[1] This chapter will investigate the use of the entrapment method to immobilize AGP and compare the measured binding constants for propranolol, a beta-blocker used for the treatment of hypertension and a drug with well-categorized binding to soluble AGP with established values.[1, 3, 4] The structure of propranolol is given in **Figure 5-1**.

Both the entrapped HSA and entrapped AGP supports will be used to investigate the binding of a series of analytes. Several of the analytes are known to bind to these ligands with a certain affinity, but this study will also investigate the binding of other analytes where the interactions are unknown. This list of drugs includes amitriptyline and notriptyline, both antidepressants; chloramphenicol, an antibiotic; chlorpromazine, an antipsychotic; phenobarbital, an anticonvulsant; quinidine, an antiarrhythmic; and theophylline, a bronchiodialator. The structures for these seven compounds are shown in **Figure 5-2**.

These studies will be preformed using zonal elution, the most popular method used in chromatographic biointeraction studies.[1,4,9] This method was performed by injecting a small plug of analyte onto the column and measuring the retention time of the injected analyte. This retention time can be correlated to the association equilibrium constant, when the void volume and the binding capacity are known. The bulk of this chapter will be designated to the study the interactions between both AGP and HSA to the seven compounds listed in the previous paragraph.

Figure 5-1. Structure of propranolol.

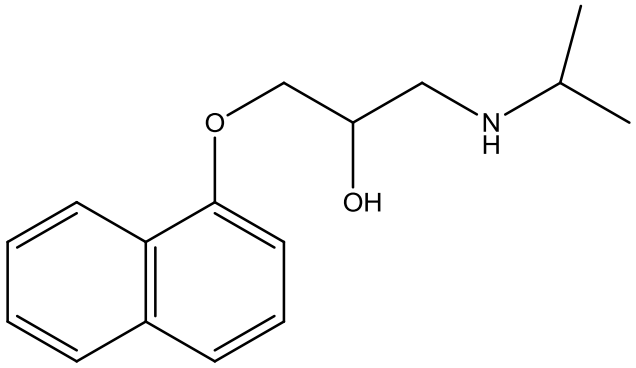
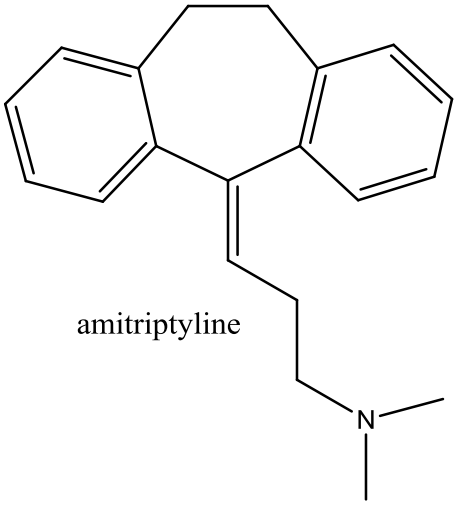
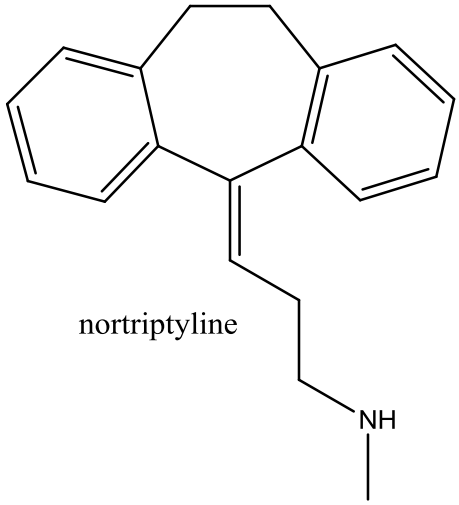

O
N
H
OH

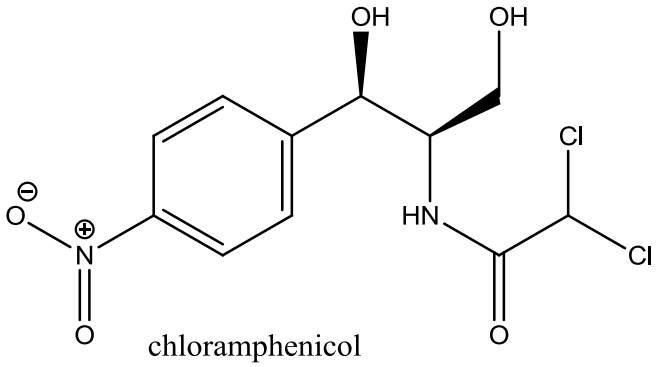
Figure 5-2. Structures for analytes injected onto both the entrapped HSA and the entrapped AGP columns.



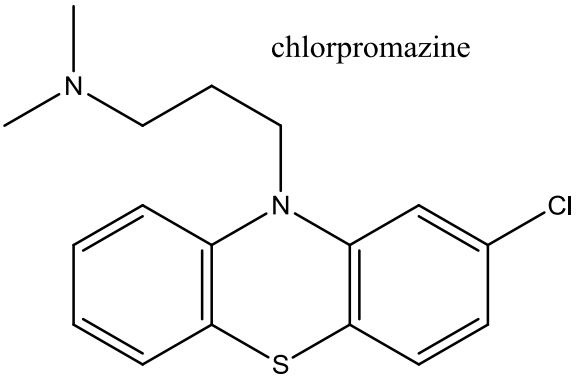
amitriptyline



nortriptyline

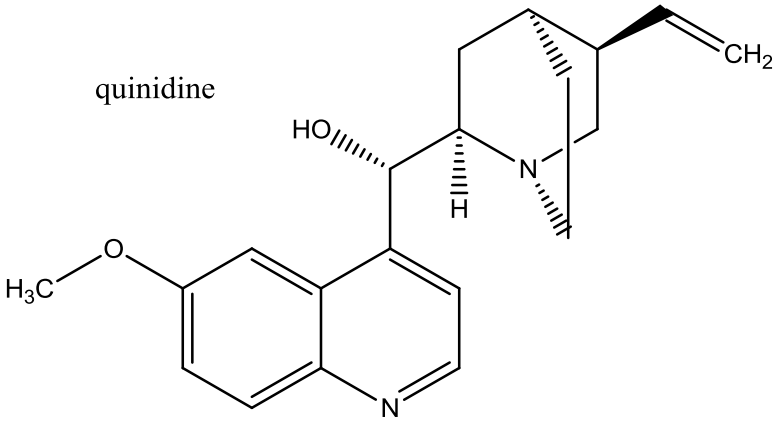


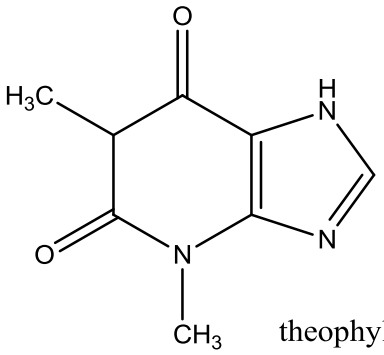
chloramphenicol



chlorpromazine

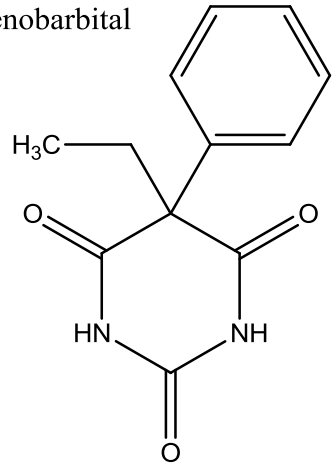
quinidine





theophylline

phenobarbital



Experimental

Reagents.

The *p*-periodic acid (periodic acid reagent, or H_5IO_6), glycogen (bovine liver)**,** HSA (Cohn fraction V, 99% globulin free, 99% fatty acid free), AGP (99%), racemic propranolol, amitryptiline, chloramphenicol, chlorpromazine, nortriptyline, phenobarbital, quinidine, theophylline, monobasic and dibasic potassium phosphate salts, sodium chloride and sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO**).** The Nucleosil Si-300 silica (300 Å pore size, 7 micron particle diameter) was purchased from Macherey-Nagel (Düren, Germany). Reagents for the micro bicinchoninic acid (BCA) protein assay were purchased from Pierce (Rockford, IL). Other chemicals were of the purest grades available. The Econo-Pac 10 DG disposable 30 x 10 mL desalting columns were purchased from Bio-Rad Laboratories (Hercules, CA). All solutions were prepared using water from a NANOpure purification system (Barnstead, Dubuque, IA) and filtered with a 0.20 µm GNWP nylon membrane from Millipore (Billerica, MA).

Apparatus.

The chromatographic system consisted of a Jasco PU-980i intelligent HPLC isocratic pump (Tokyo, Japan), a Rheodyne Advantage PF ten-port valve (Cotati, CA), and a Jasco UV-975 UV/Vis detector**.** The detection wavelengths were as follows: 205 nm, sodium nitrate; 225 nm, propranolol; 209 nm, amitriptyline; 204 nm, chloramphenicol; 253 nm, chlorpromazine, 209 nm, nortriptyline; 206 nm, phenobarbital; 234 nm, quinidine; and 209 nm, theophyline. Data were collected using an interface and software from National Instruments (Austin, TX). The temperature of the columns and

mobile phases was controlled using a PolyScience circulating VWR circulating water bath (Buffalo Grove, IL) and a water jacket from Alltech. All columns were packed using an HPLC column slurry packer from Alltech. The chromatographic data were analyzed using Peakfit 4.12 (Jandel Scientific Software, San Rafael, CA).

Methods.

Nucleosil Si-300 was converted into a dihydrazide-activated form according to previously-established procedures.[5] Optimized entrapment conditions determined in **Chapter 2** were used to prepare columns containing entrapped HSA, entrapped AGP, and a control support. The oxidation of glycogen was done in a pH 5.0 buffer containing 20 mM sodium acetate and 15 mM sodium chloride. A 4.0 mL solution was prepared by dissolving 135 mg of periodic acid and 17 mg of glycogen from bovine liver in the aforementioned buffer. This solution was covered in aluminum foil – due to the light sensitivity of periodic acid – and allowed to shake at room temperature for 12-24 h. After oxidization, the glycogen was purified using an Econo-Pac 10DG disposable desalting column and pH 5.0, 0.10 M potassium phosphate buffer. This process was done by first removing the upper cap of the column and decanting the excess storage buffer. The next step was a buffer exchange via the addition of 10.0 mL of pH 5.0, 0.10 M potassium phosphate buffer to each column. Next, 3.3 mL of the oxidized glycogen sample were allowed to pass through the column, causing the elution of the newly introduced potassium phosphate buffer. A volume of 4.0 mL of potassium phosphate buffer was then added to the column to elute the oxidized glycogen. The larger glycogen molecules eluted from the column during this step, while the smaller periodic acid molecules took much longer to pass through the column matrix. The resulting purified

oxidized glycogen solution had a concentration of 4.2 mg/mL solution with a final volume of approximately 4.0 mL. This solution was stored at 4 °C.

Both the HSA and AGP supports were prepared under the same immobilization conditions. A 50 mg/mL stock solution of each protein was prepared with pH 5.0, 0.10 M potassium phosphate buffer. To create the entrapped support, 80 mg of dihydrazide activated silica was combined with 160 µL of the protein solution. This mixture was sonicated under vacuum for 15 min to ensure the proper mixing of the silica and protein. A 380 µL portion of the purified oxidized glycogen was added, and the mixture was shaken at room temperature for 12 h. During the final hour of the reaction, 200 µL of a 2 mg/mL oxalic dihydrazide solution in the same buffer was added to the reaction mixture to cover any remaining aldehyde groups on the silica support. After immobilization, the support was washed several times with pH 7.4, 67 mM potassium phosphate buffer. A control support was also prepared through the addition of a 160 µL aliquot of potassium phosphate buffer in place of the protein solution. Binding of an analyte to this support was used to monitor non-specific interactions. This control support was used in the chromatographic studies.

Another portion of Nucleosil Si-300 was converted to diol-bonded silica, and HSA was immobilized to this support by the Schiff base method according to previously-published procedures.[5, 6] A control support was also made using this method, without the addition of HSA.

The protein content of the Schiff base HSA, entrapped HSA and AGP supports was determined using a micro BCA assay, with soluble HSA or AGP being used to create a standard curve, and the control support being used as the blank. The entrapped HSA

support was found to contain 37 (± 1) mg HSA per gram support, the Schiff base HSA support was found to contain 39 (± 2) mg HSA per gram support, and the AGP support contained 21 (± 1) mg AGP per gram support.

All supports were downward slurry-packed into separate 1.0 cm × 2.1 mm I.D stainless steel columns at 4000 psi using pH 7.4, 67 mM potassium phosphate buffer as the packing solution**.** All columns were stored at 4 °C in the packing solution. All sample solutions for the chromatographic studies were prepared in pH 7.4, 67 mM potassium phosphate buffer. Stock sample solutions with a concentration of 100 µM propanolol and a concentration of 70 µM for all other analytes examined were prepared in pH 7.4, 67 mM potassium phosphate buffer. The stock solutions were diluted with this pH 7.4 buffer to give 20 µM working samples of all analytes.

A 20 µL volume of all samples was injected onto the entrapped HSA, entrapped AGP and control columns in triplicate at 37 °C in the presence of pH 7.4, 67 mM potassium phosphate buffer. For a majority of the studies, a flow rate of 0.5 mL/min was used, for those samples with weaker retention, slower flow rates of 0.10 or 0.30 mL/min were employed to examine the binding of each analyte to the protein of interest. The void time of each column was determined by making triplicate 20 µL injections of 25 µM sodium nitrate in the presence of pH 7.4, 67 mM potassium phosphate buffer. The void time of the system was determined by repeating these injections of sodium nitrate on a zero-volume union. Peakfit v.4.12 and an exponentially-modified Gaussian curve fit were used to determine the central moments of all eluting peaks.

Results and Discussion

Initial binding studies of racemic propranolol to AGP.

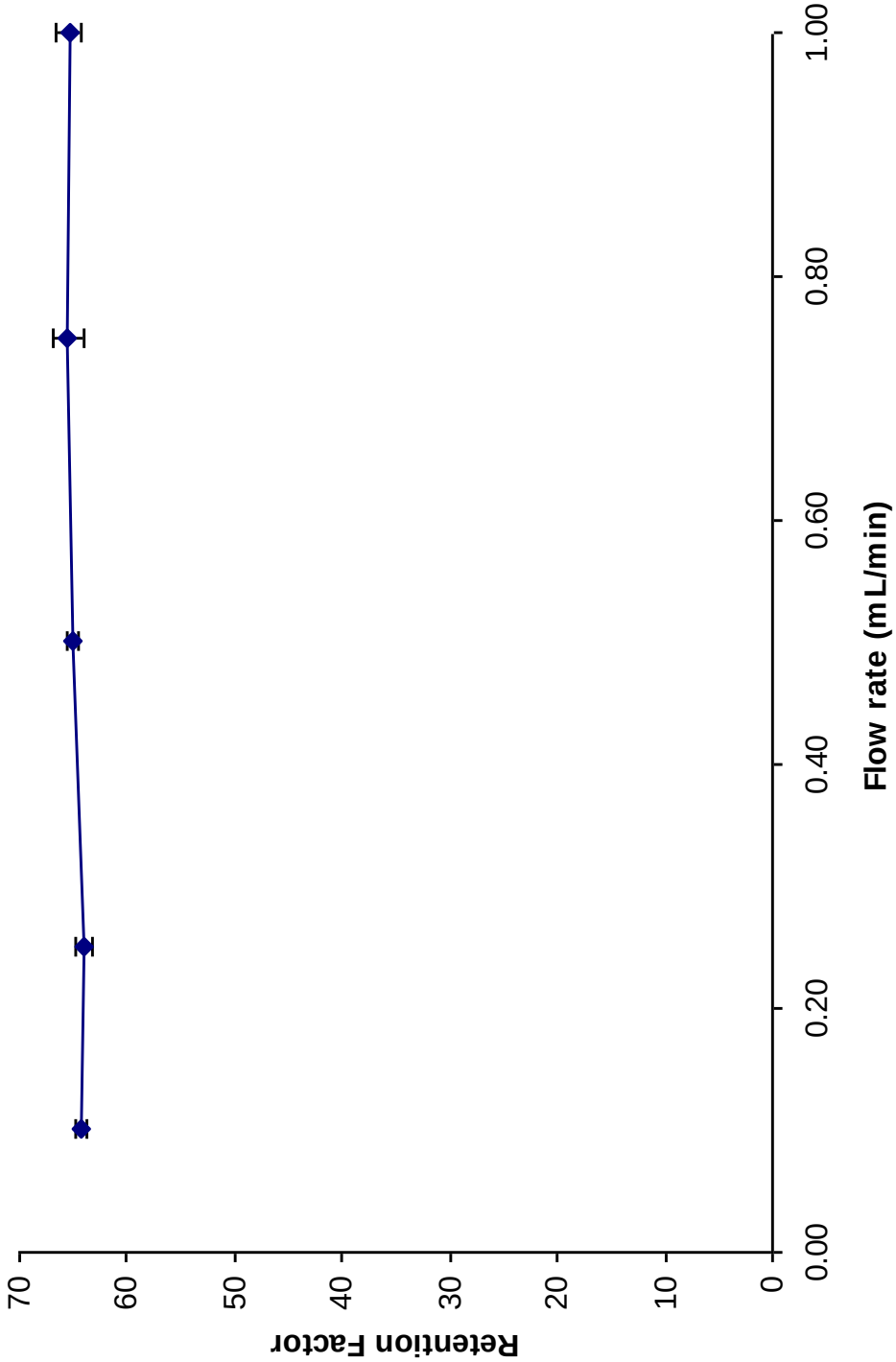
Affinity chromatography has been shown to be a useful tool to examine the overall affinity of drugs to immobilized proteins such as HSA.[7] Zonal elution studies can be used to determine the retention factor (k), which can then be used to calculate the association equilibrium constant (K_a) of the analyte. **Equation 5-1** shows how the retention factor is related to the K_a for an injected analyte with an immobilized protein, where m_L is the moles of total binding sites in the column and V_M is the column void volume.

$$k = \frac{K_a m_L}{V_M} \qquad (5\text{-}1)$$

The retention of propranolol on the entrapped AGP column was determined through zonal elution experiments at flow rates ranging from 0.10-1.00 mL/min. The retention factor remained relatively constant throughout this range of flow rates, as indicated in **Figure 5-3**. This shows that flow rates up to 1.00 mL/min could be used without causing any significant change in the retention factor. This result is consistent with data seen for entrapped HSA columns over the same range of flow rates. An average of these retention values was used to calculate a K_a value for propranolol and AGP.

The K_a value for propranolol and AGP, calculated from the average measured retention factor, was 3.1 (± 0.3) x 10^5 M^{-1}. A variety of association equilibrium constants have been reported for propranolol and AGP, ranging from 1.0 – 8.4 x 10^5.[1,3,8] The results obtained in this study fall within this range of values, and are most similar to the values obtained using equilibrium dialysis under similar buffer and temperature

Figure 5-3. Retention factors measured at various flow rates for propranolol on an entrapped AGP column. The conditions are given in the text. The error bars represent ± 1 S.D.


Retention Factor
70
60
50
40
30
20
10
0
0.00
0.20
0.40
0.60
0.80
1.00
Flow rate (mL/min)

conditions.[3] This indicates that the entrapped AGP is accessible for binding by small molecules like propranolol. The average value for non-specific binding of propranolol to this column was approximately 28%.

Binding of various analytes to entrapped AGP.

The second phase of this study used entrapped HSA and entrapped AGP columns to calculate association equilibrium constants for a series of analytes based on their measured retention factors. Injections were made on a control column to correct for nonspecific binding to the entrapment support. The interactions between these drugs and AGP were examined first. Out of the seven drugs studied, both phenobarbital and theophylline eluted at the void time, showing no apparent binding to the entrapped AGP. The measured association equilibrium constants for all seven drugs are listed in **Table 5-1**, along with published values of K_a for these drugs with soluble AGP. The data collected on the entrapped AGP was in good agreement for three of the four drugs with published data. The measured value for chlorpromazine on the entrapped AGP column was nearly two-fold higher than the previously reported value. This discrepancy will be discussed later in this section. No established literature K_a values could be located for theophylline, chloramphenicol or phenobarbital.

Binding of Various Analytes to Entrapped HSA.

The zonal elution injections were repeated with the same drugs on the entrapped HSA column, (see **Table 5-2**). The binding of these drugs to HSA was also analyzed on a Schiff base HSA column of the same dimensions. This was done to determine if nonspecific interactions between the injected drugs and the entrapment support had a significant impact on the resulting association equilibrium constant. These values are

Table 5-1. Measured association equilibrium constants (K_a) on the entrapped AGP support and previously reported values for soluble AGP with various drugs.

Drug	K_a (M^{-1})	K_a (M^{-1})*	Reference
Amitryptyline	7.1 (± 0.3) x 10^5	> 10^5	8
Nortriptyline	7.4 (± 0.2) x 10^5	1.0 – 6.7 x 10^5	8
Theophylline	[a]No Retention		
Chloramphenicol	1.9 (± 0.5) x 10^3		
Phenobarbitol	[a]No Retention		8
Quinidine	3.0 (± 0.3) x 10^5	2.0 – 3.0 x 10^5	8, 9
Chlorpromazine	1.5 (± 0.4) x 10^6	0.3 -3.9 x 10^6	8

*For drugs that have a range of association equilibrium constants reported, the average value of the constants is listed in the table.

The numbers in parentheses represent ± 1 S.D.

[a] No retention on the entrapped AGP support.

Table 5-2. Measured association equilibrium constants (K_a) on the entrapped HSA support, the Schiff base immobilized HSA support and previously reported values for HSA and various drugs.

Drug	K_a (M^{-1}) *Entrapment*	K_a (M^{-1}) *Schiff Base*	K_a (M^{-1})*	Ref
Amitryptyline	5.6 (± 0.2) x 10^4	6.3 (± 0.2) x 10^4	4.35 x 10^4	10
Nortriptyline	6.2 (± 0.3) x 10^4	5.8 (± 0.1) x 10^4	1.41 x 10^2	10
Theophylline	4.0 (± 0.6) x 10^2	9.6 (± 0.1) x 10^2		
Chloramphenicol	1.1 (± 0.5) x 10^3	1.1 (± 0.1) x 10^3	2.54 x 10^4	11
Phenobarbitol	[a]N/A	[a]N/A		
Quinidine	2.1 (± 0.4) x 10^4	1.2 (± 0.4) x 10^4		
Chlorpromazine	1.2 (± 0.2) x 10^5	5.9 (± 0.2) x 10^4	6.4 – 8.9 x 10^4	12

*For drugs that have a range of association equilibrium constants reported, the average value of the constants is listed in the table.

The numbers in parentheses represent ± 1 S.D.

[a] No retention on the entrapped AGP support.

also shown in **Table 5-2**. The results of the binding studies on the Schiff base HSA column were similar to the results for the entrapped HSA column. Both supports showed no retention for phenobarbital and minimal retention for theophylline. A significant discrepancy was noted for the K_a values of chlorpromazine, which were approximately two-fold higher for the entrapment column. Reference values for the binding affinities of these drugs on both immobilized and soluble forms of HSA are also listed in **Table 5-2**. Values for amitriptyline were consistent for the entrapped HSA, the Schiff base HSA and the reference value. Values for nortriptyline were in good agreement between the Schiff base and the entrapment columns, but these values were two-orders of magnitude larger than the reference value for soluble HSA. While the Schiff base HSA had a K_a value fairly consistent with the reported literature value for chlorpromazine, the K_a for the same drug on the entrapped HSA support was 150 – 200% higher. The lack of retention of phenobarbital and the measured K_a values for theophylline and quinidine were consistent between immobilization techniques, however no reference values were available for further comparison in these cases.

The entrapment support and the Schiff base support each had an average of approximately 30% nonspecific binding for all of the bound drugs, with the exception of chlorpromazine on the entrapped support. This drug had larger nonspecific interactions of 60%. All calculated K_a values were adjusted for nonspecific interactions through the use of a control column. Due to the high value of nonspecific binding obtained for chlorpromazine, additional injections of all drugs were performed on a column containing only dihydrazide-activate silica for comparison with the control column to further investigate the nature of these nonspecific interactions. The retention of the drugs on the

bare dihydrazide column accounted for an average of 55% of the retention noted for the control column, indicating that nonspecific binding was taking place not only with the dihydrazide base of the support, but also with the oxidized glycogen present in the control column. Further studies are needed to minimize the amount of nonspecific binding experienced by some drugs on the entrapped support.

Conclusions

This work implemented the use of biologically-relevant ligand entrapment as an immobilization method for HPAC. Initially, the binding of a well-characterized drug – propranolol – was investigated through the use of an entrapped AGP column. The results indicated that this small molecule was able to access and bind to the entrapped AGP with similar affinity to that seen with soluble AGP. This column, along with an entrapped HSA column as initially tested through experiments in **Chapter 3**, were then used to screen a series of drugs in a pursuit of the determination of association equilibrium constants for these interactions. The binding of this series of drugs was examined on both the entrapped AGP and entrapped HSA supports. The use of a Schiff base HSA support was also used as a means of comparison and to determine if nonspecific interactions between the analytes and the entrapment support had an appreciable effect on analyte retention. All measured K_a values were compared to established literature values, when available. Generally, there was good agreement between the entrapment results and the established values for these drugs with the soluble form of the examined proteins.

This study indicates that these entrapment columns can be used for the initial screening of a variety of drugs to determine binding affinity with proteins. Further

examination of drugs with established binding could then be done using more time-consuming frontal analysis studies, in order to achieve more detailed binding data. This method could also be expanded for the use with other affinity ligands, such as immunoglobulins, to create a series of columns containing additional serum agents to obtain a more complete profile of the binding of drugs in serum.

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CHAPTER 6:

PREPARATION OF HIGH-CAPACITY SUPPORTS CONTAINING PROTEIN G IMMOBILIZED TO POROUS SILICA

Introduction

Protein G is a bacterial cell surface protein that is commonly used as a binding agent for immunoglobulins in affinity chromatography and other analytical methods.[1-4] Protein G has strong binding to these targets at pH 5.0-7.5 and yet can be made to release any retained immunoglobulins at pH 2.5-3.0.[2] These properties have made protein G popular not only as a binding agent for the purification of immunoglobulins but also as a secondary binding agent that can be used to adsorb antibodies for applications such as immunoaffinity chromatography or immunoassays.[1-4]

Over the last decade there have been several examples in which immobilized protein G has been used in small affinity columns or in affinity capture systems for the adsorption of immunoglobulins.[5-8] The small size of many of these columns and affinity sorbents requires that a relatively large amount of protein G be present in a small volume for the effective capture of the desired target.[8] However, no previous studies have determined the maximum amount of protein G that can be placed on common porous HPAC supports. The goal of this chapter was to estimate the maximum amount of protein G that could be covalently immobilized to silica with various pore sizes and to examine the binding of these supports to immunoglobulins.

Experimental

Reagents.

The Nucleosil Si-50, Si-100, Si-300, Si-500, Si-1000 or Si-4000 (all 7 μm diameter with nominal pore sizes of 50 Å, 100 Å, 300 Å, 500 Å, 1000 Å, 4000 Å, respectively) were purchase from Alltech (Deerfield, IL). The protein G (recombinant, albumin binding domains removed), rabbit immunoglobulin G (IgG), monobasic and dibasic potassium phosphate salts, sodium cyanoborohydride, and sodium borohydride was from Sigma-Aldrich (St. Louis, MO) or Pierce (Rockford, IL). Reagents for the bicinchoninic acid (BCA) protein assay were also from Pierce. All solutions were prepared using water from a NANOpure purification system (Barnstead, Dubuque, IA) and filtered with a 0.20 μm GNWP nylon membrane from Millipore (Billerica, MA).

Apparatus.

Samples for a micro BCA protein assay were analyzed using a Shimadzu UV 160U absorbance spectrophotometer (Kyoto, Japan). The protein and supports were mixed using an Aliquot 4651 Mixer from Ames (Elkhart, IN). Columns were packed using a CM3200 pump from Thermoseparations (Riviera Beach, FL) with a modified Valco N60 six-port valve (Houston, TX, USA). Chromatographic studies were conducted using a Thermoseparations CM3200 pump, two Rheodyne six-port injection valves (Cotati, CA), two Jasco PU980 pumps (Tokyo, Japan) and a Thermoseparations SM3100 variable wavelength detector.

Methods.

Protein G was immobilized onto silica for use in sandwich microcolumns. Supports with six different pore sizes were prepared to determine total immobilized

protein G content as a function of porosity. All of the supports used in this study were HPLC-grade porous silica with an average particle size of 7 μm and nominal pore sizes of 50 Å, 100 Å, 300 Å, 500 Å, 1000 Å or 4000 Å. According to the manufacturer, these supports had surface areas of 420, 350, 100, 35, 25 and 10 m^2 per gram, respectively. All silica samples were converted into a diol-bonded form, which was then used to immobilize protein G by the Schiff base method, according to methods adapted from the literature for use with other proteins.[9, 10]

> Each reaction mixture contained 100 mg of silica and approximately 3 mg of protein G in pH 6.0, 0.10 M potassium phosphate buffer. The samples were degassed under vacuum for 5 min and then allowed to shake for seven days at 4 °C in the presence of 100 mg per ml sodium cyanoborohydride. The immobilization reaction took place at pH 6.0 because this promotes immobilization at the N-terminus of the protein rather than at its lysine residues.[11] Immobilization at the lysine residues decreases the activity of the protein because of incorrect orientation for binding of the analyte.

At the end of the immobilization period, the supports were washed four times with water, once with pH 8.0, 0.10 M potassium phosphate buffer, and allowed to shake for 90 min with 2 mg per ml sodium borohydride to remove excess aldehyde groups on the silica. The supports were again washed with water and pH 7.4, 0.10 M potassium phosphate buffer, and stored at 4 °C in the pH 7.4 buffer. A portion of each support was set aside for an analysis of protein content using a micro BCA assay.

Each slurry was diluted to the desired concentration and used to pack a sandwich microcolumn.[4] Six 1.0 cm x 2.05 mm I.D. microcolumns were packed, each containing a support of a different pore size. A thin layer of inert diol-bonded silica was first packed into the column, followed by a series of 64 injections of protein G silica, each with a volume of 90 μL. The remainder of the column was filled with diol-bonded silica until the pressure was over 3000 psi, indicating that the column was full. Next, the flow rate was decreased until the backpressure on the column reached 3000 psi (the desired

packing pressure) and the column was then allowed to equilibrate for 20 min or until the pressure stabilized. Columns of the same length and inner diameter were packed with diol-bonded silica to find the column void time and to examine non-specific binding of analytes to such columns.

A micro BCA assay was used to determine the protein content of each microcolumn. The set aside portions of the immobilized supports were washed with water and dried under vacuum without heat or organic solvents. The samples were examined in triplicate, with bovine serum albumin (BSA) as the standard. An assay was run to compare the responses of protein G and BSA in the BCA assay. There were no notable differences between the responses of the two proteins, indicating that BSA could serve as a suitable substitute for preparation of the standard curve. The concentration of protein per gram of silica was calculated for each sample.

Frontal analysis studies were performed to examine the binding of IgG to the immobilized protein G supports in all six microcolumns. Solutions of varying concentrations of IgG were made in pH 7.4, 0.10 M potassium phosphate buffer. The IgG solution was continuously applied to the protein G column until all binding sites were saturated, producing a breakthrough curve. After each run, the columns were washed for approximately 10 min at flow rates of 1 to 2 ml per min with pH 2.5, 0.10 M potassium phosphate buffer, to elute the IgG remaining on the column. Finally, the column was regenerated through the application of pH 7.4, 0.10 M potassium phosphate buffer for 10 min at 1.00 mL per min. The flow rate was then decreased slowly to 0.10 mL per min and the next run began. All runs were performed in triplicate on each

immobilized protein G column and corresponding inert diol-bonded silica column to serve as the control.

Breakthrough times for column and control column were determined by using the equal area method. The amount of analyte needed to saturate these columns was determined by integration of the resulting breakthrough curves, after correcting for the void time of the system and non-specific binding to the support, as measured on the control column. The nonspecific binding for these supports accounted for less than 5% of total binding.

These studies were conducted to determine not only the amount of bound IgG, but also the specific activity of each support. The moles of IgG bound to the column were found by multiplying the time required to reach the breakthrough point by the flow rate and IgG solution concentration. The specific activity of each column was determined by dividing the amount of IgG bound to the column by the total protein G content, as determined by a micro BCA assay.

Results and Discussion

Effect of Porosity on the Amount of Immobilized Protein G.

Recombinant protein G with the albumin binding domain removed was covalently immobilized onto supports with six different porosities, using the Schiff base method.[2] This method has been shown in previous work to give higher activities for protein G and protein A (i.e., a related bacterial cell wall protein) than other amine-based coupling methods.[2,3,9] The concentration of protein G used for immobilization was two-fold larger than the maximum amount that was later found to be immobilized to any of the materials that were examined in this study. A micro BCA assay was used to determine the protein content of all six protein G supports.[12]

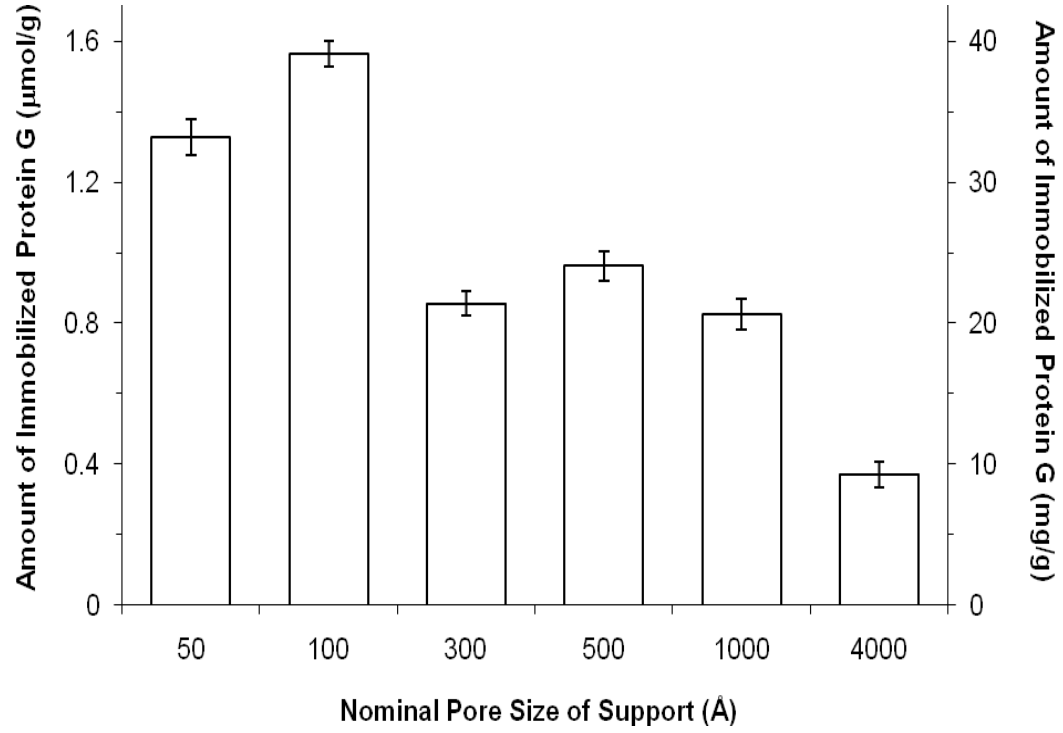
Figure 1A shows the maximum amount of protein G that was immobilized using the Schiff base method onto silica with various pore sizes. Each sample was measured in

triplicate using a mirco BCA assay. The highest protein content was obtained for the 100 Å support, which contained 1.56 (± 0.04) μmol protein G per gram support or 39 (± 1) mg protein G per gram support (note: the numbers in parentheses represent ± 1 standard deviation of the mean). The second highest amount was obtained for the 50 Å support, which gave 33 (± 1) mg protein G per gram support. A decrease in the amount of immobilized protein G per gram of support was seen as the pore size increased to 300 through 4000 Å, with roughly 21-24 mg protein G per gram support being obtained for the 300-1000 Å pore size supports and 9.3 mg protein G per gram support being measured for 4000 Å pore size supprt.

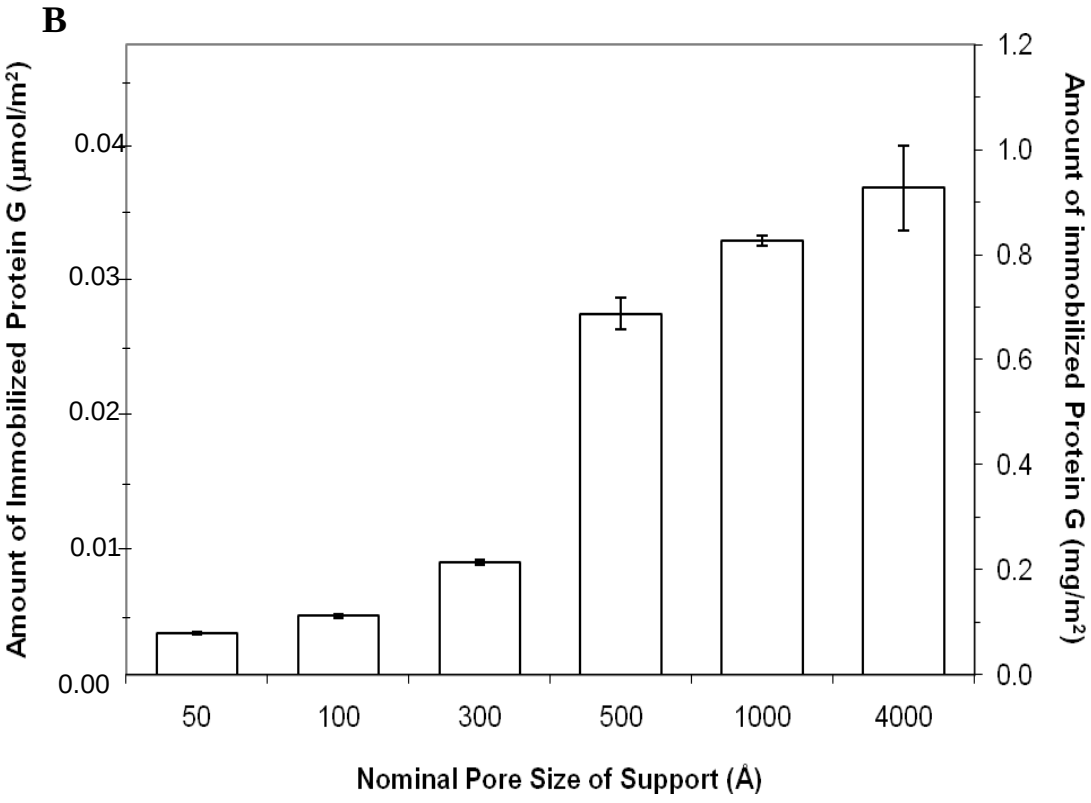
It has previously been suggested in work with the immobilization of other proteins, and in theoretical calculations of effective diffusivity, that the optimum pore size for protein immobilization is roughly 3-5 times the diameter of the protein.[13, 14] Based on a previous crystal structure and hydrodynamic studies,[15, 16] the estimated diameter of recombinant protein G with the albumin binding removed was less than 30-35 Å. This size range agreed with the data shown in **Figure 1A**, which confirmed that a maximum protein content was obtained when using a support with pores that were

Figure 1. ***Amount of protein G that was immobilized by the Schiff base method to silica with various pore sizes. The results are shown in terms of (A) the moles or mass of protein G that was immobilized per gram of support and (B) the moles or mass that was immobilized per unit surface area of the support. The error bars represent a range of ± 1 S.D. of the mean (n = 3).***

A


Amount of Immobilized Protein G (µmol/g)
Amount of Immobilized Protein G (mg/g)
1.6
1.2
0.8
0.4
0
40
30
20
10
0
50
100
300
500
1000
4000
Nominal Pore Size of Support (Å)

B


Amount of Immobilized Protein G (µmol/m²)
Amount of immobilized Protein G (mg/m²)
0.04
0.03
0.02
0.01
0.00
1.2
1.0
0.8
0.6
0.4
0.2
0.0
50
100
300
500
1000
4000
Nominal Pore Size of Support (Å)

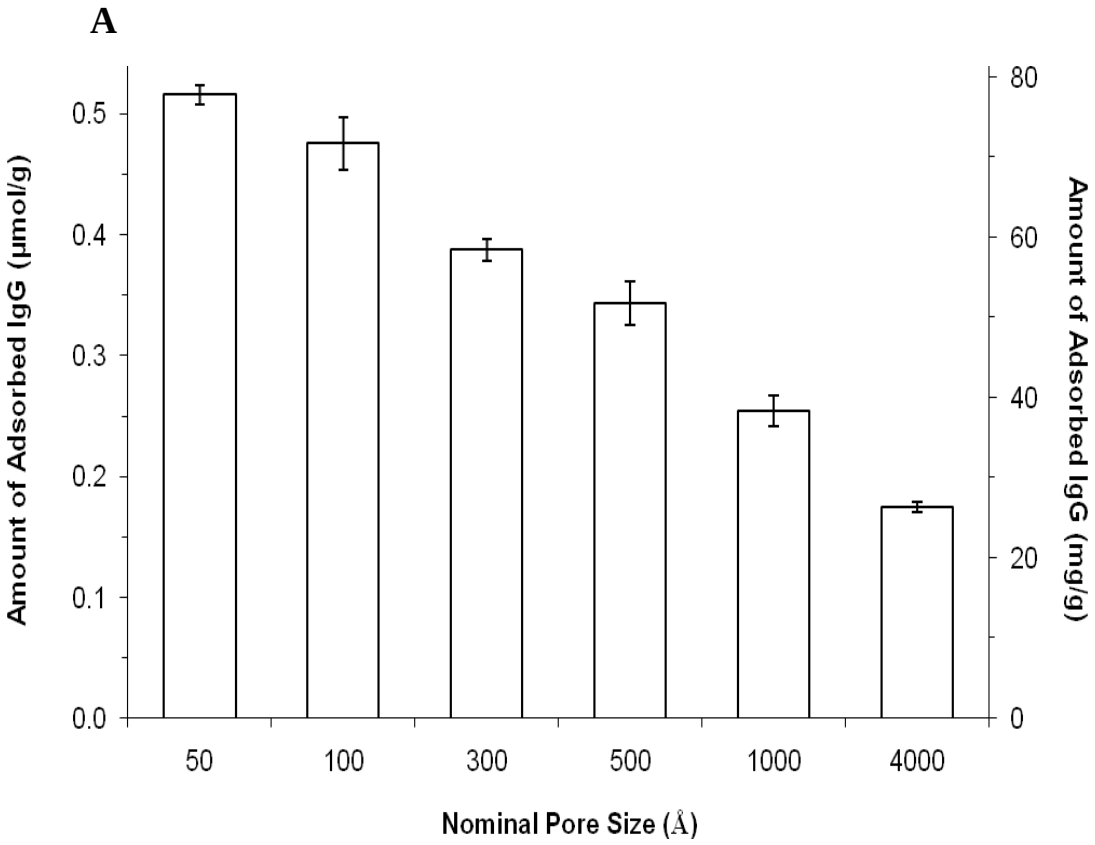
roughly three times the diameter or protein G (i.e., as noted when using 100 Å pore size silica).

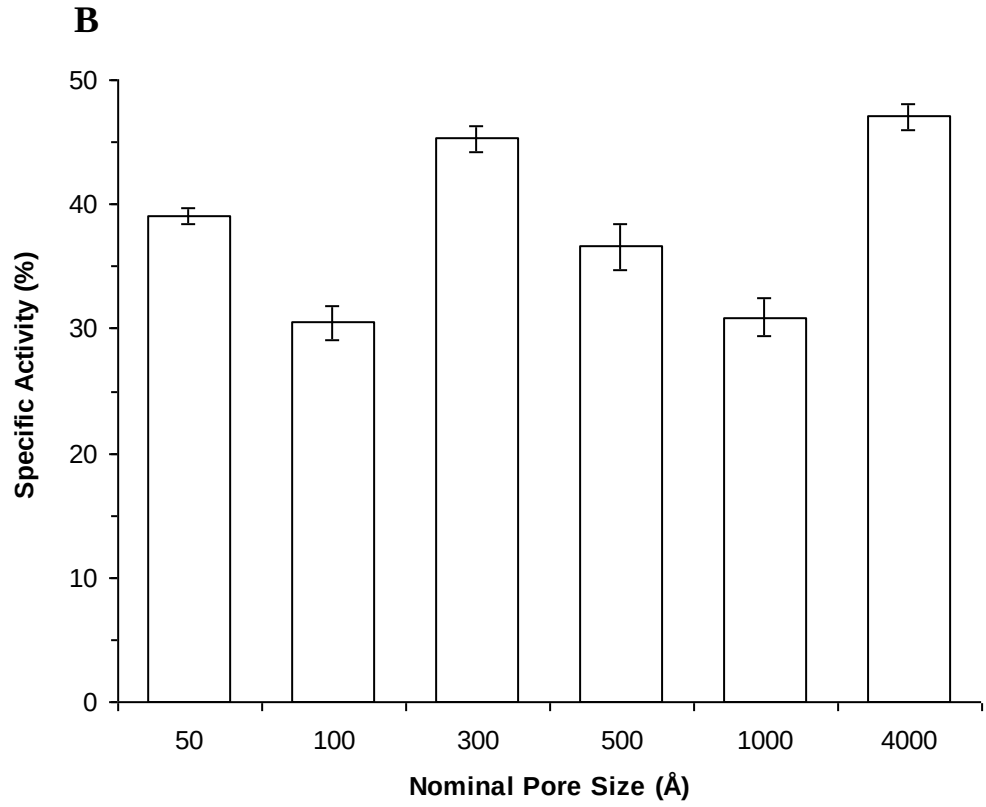
The amount of immobilized protein G was also examined in terms of the quantity that was coupled per unit area for each type of silica. These results, shown in **Figure 1B**, indicate that there was an increase in amount of immobilized protein G per unit area as the pore size of the support was increased. This effect has been observed in previous studies for other proteins (e.g., IgG and F_{ab} fragments)[13] and is a result of the protein being able to access more surface area on the support as the pores increase in size. The use of a support with a pore size of 300 Å or smaller resulted in a large fraction of the total surface area being inaccessible to protein G, thus resulting in a relatively low surface coverage. Silica with a pore size of 500 Å or larger gave a dramatic increase in surface coverage, with a maximum value of 0.037 (± 0.003) μmol per m^2 or 0.93 (± 0.08) mg per m^2 being measured for 4000 Å pore size silica. This trend fits with previous observations that have been made with other proteins on porous silica.[13]

Rabbit IgG Frontal Analysis Studies.

The binding capacity and activity for each protein G support were examined by placing these materials into affinity columns and conducting frontal analysis. Rabbit IgG was used as the model analyte for this work. The results are summarized in **Figure 2**. In **Figure 2A**, the 50 Å and 100 Å pore size supports gave the largest overall binding capacities for rabbit IgG, with values in the range of 71 – 77 mg protein per g support. As the pore size of the support was increased to larger values, there was a decrease in binding capacity that followed the general trend noted for the total amount of immobilized protein G in **Figure 1A**. The maximum binding capacities obtained in this

Figure 2. ***Amount of rabbit IgG that was adsorbed to protein G supports prepared by using silica with various pore sizes. The protein G supports that were used in this figure were the same supports examined in Figure 1. The specific activities were calculated by assuming a maximum of one IgG could bind to each protein G molecule. The error bars represent a range of ± 1 S.D. of the mean (n = 3).***


A
Amount of Adsorbed IgG (µmol/g)
0.0
0.1
0.2
0.3
0.4
0.5
Amount of Adsorbed IgG (mg/g)
0
20
40
60
80
50
100
300
500
1000
4000
Nominal Pore Size (Å)


B
Specific Activity (%)
0
10
20
30
40
50
50
100
300
500
1000
4000
Nominal Pore Size (Å)

study

were approximately 1.6 – to 1.7 – fold higher than values listed for commercial supports that contain protein G immobilized to comparable materials.

The apparent activity of the immobilized protein G in each type of column was found by dividing the measured binding capacity by the total amount of immobilized protein G. These results are given in **Figure 2B**. In this case, all of the supports had similar apparent activities in the range of 30-47%. These values indicated that about one out of every three protein G molecules could bind to rabbit IgG when the support was saturated with this target. Some of this protein G may have lost its activity during immobilization, a possibility that has been seen in work with protine A.[9] Steric hindrance between neighboring protein G molecules in their binding to IgG would also be expected to account for part of the drop in apparent activity below 100%. The similarity of the apparent activities in **Figure 2A** indicates that steric hindrance arising from pore size effects was not an issue for the adsorption of IgG to protein G that had been immobilized to the 50 to 4000 Å porous silica.

Previous work has examined the amount of rabbit IgG that can be covalently immobilized to the same types of supports that were used in this present study[13]. Both general amine-based coupling methods (e.g., the Schiff base and 1,1'-carbonyldiimidazole techniques) and a site-specific immobilization technique (e.g., the coupling of IgG through oxidized carbohydrate chains in its F_c region to a dihydrazide-activated support) were studied in the previous report. The results for these various methods were fairly comparable, with contents for rabbit IgG of 212 (± 20) mg per pgram support being obtained for 100 Å pore size silica and 255 (± 12) mg per gram support being reported for 300 Å pore size silica when using site-selective coupling by the

dihydrazide method.[13] A comparison of these values with those in **Figure 2A** indicates that the covalent coupling of immunoglobulins/antibodies to these supports can allow for about a three-fold higher maximum coverage of these agents than when using secondary adsorption of the same agents to immobilized protein G.

Conclusions

In summary, this report investigated the immobilization of protein G to silica supports with regards to the maximum protein content that could be obtained for these materials. A maximum content of 39 mg protein G per gram support was obtained when using 100 Å pore size silica, with a slightly lower level being obtained for 50 Å silica and even lower levels for 300 Å to 4000 Å silica. These data agreed with previous observations that maximum content for an immobilized protein is generally obtained when using a pore size that is three to five times the diameter of the protein. The surface coverage of protein G was found to increase with pore size, with a maximum level of 0.037 μmol/m^2 being obtained for 4000 Å silica.

When the binding of these supports for immunoglobulins was determined using frontal analysis, all gave apparent activities in the range of 30-47% for rabbit IgG, with the highest binding capacities (i.e., 71 – 77 mg IgG/g silica) being obtained for the 50 and 100 Å silica supports. Although these values are lower than those that can be obtained for the covalent immobilization of antibodies, they should still be appropriate for the use of protein G in affinity microcolumns or miniaturized analytical systems.[5-8] In addition, using protein G as a secondary binding agent can allow adsorbed immunoglobulins/antibodies to be released at a mildly acidic pH for column regeneration,

and this binding agent can allow a variety of immunoglobulins/antibodies to be adsorbed to the same support.[2-8] The ability of protein G to attach to the F_c region of immunoglobulins is another attractive feature that can aid in obtaining site-selective attachment and high activity for adsorbed antibodies. These properties should continue to make protein G supports, like those developed in this report, valuable tools in immunoaffinity separations, immunoassays and related bioanalytical applications.

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CHAPTER 7:

SUMMARY AND FUTURE WORK

SUMMARY OF WORK

Optimization of the Entrapment Method.

The proper use of high performance affinity chromatography (HPAC) in the study of biologically-related systems requires that careful attention be paid to the nature in which the affinity ligand is incorporated into the stationary phase. Ideally, the behavior of the immobilized ligand should mimic the behavior of the ligand in its natural environment. Retaining the soluble form of the ligand, by avoiding covalent immobilization completely, is one effective way to retain the activity of the ligand. Previously, noncovalent immobilization techniques that do not modify the ligand of interest have included physical entrapment onto low performance supports.

This dissertation has introduced an alternative method of entrapment onto supports that can withstand the high pressures and flow rates associated with HPAC. The entrapment method is based on the physical containment of a ligand in a polysaccharide-capped dihydrazide support. In **Chapter 2**, initial chromatographic studies were performed in order to determine the activity of proteins in the entrapped support. This was done by preparing HPAC columns that contained an entrapped HSA support, and conducting frontal analysis experiments with probe compounds for the major binding sites on HSA. It was determined that the entrapped HSA retained essentially 100% of its binding activity, making this immobilization technique a viable alternative to traditional covalent immobilization methods.

A variety of experiments were then conducted to optimize the protein content of these supports. These experiments included varying the pore size of the support, the amount of glycogen in the reaction mixture, the amount of ligand in the reaction mixture, the time allowed for the entrapment process to occur, and the level of periodic acid used in the oxidation of glycogen. The resulting protein content was determined for each study, and optimum conditions were selected. The entrapment method was also expanded for use with other immobilized ligands. A range of different molecular weight ligands were tested in the method, and it was determined that a range of 5.8-150 kDa moleculare weights could be successfully entrapped, with smaller amounts of ligand entrapment occurring for molecular weights up to 450 kDa. It was also determined that high capacity supports could be produced, with protein contents up to four times higher than seen with traditional immobilization techniques.

Implementation of Entrapment Supports for Use in HPAC Binding Studies.

It was found in **Chapter 2** that as the level of periodic acid used in the initial oxidation of glycogen increased, the binding capacity of the entrapped support also increased. The work in **Chapter 3** involved the use of several columns, each prepared with a different amount of periodic acid in the reaction mixture. Zonal elution studies were performed at multiple flow rates on all supports to determine how the retention of the aforementioned probe compounds was affected. The measured retention factors for each individual column remained consistent over flow rates ranging from 0.10 – 1.00 mL/min, indicating that higher flow rates could be used with these 1.0 cm columns without impacting the retention of these analytes. When the columns were compared

with each other, there was not only a notable decrease in retention factor for higher oxidation levels but also a notable decrease in column efficiency. These results indicated that higher levels of oxidation inhibit the ability of the analyte to bind to the entrapped support. Several drugs were injected onto both sets of columns, and the resulting correlation chart showed a 70% higher normalized retention factor for analytes injected on the entrapped HSA column versus the Schiff base column, reinforcing previous data for indicating the higher activity of the entrapped ligand.

Chapter 4 examined the binding of three sulfonylurea drugs to both entrapped HSA and entrapped glycated HSA (gHSA). The binding ability of the entrapped gHSA column was initially tested using probe compounds with known binding to HSA. These results indicated that the gHSA was accessible for binding. The zonal elution data obtained for the injections of sulfonylurea drugs were in agreement with previously reported values for the binding of these drugs to gHSA. An increase in normalized retention factor was seen in these experiments for the entrapped supports when compared to Schiff base supports.

The studies in **Chapter 5** focused on the use of the entrapment columns as a screening tool to examine a wide range of analyte binding affinities. These experiments were conducted using both entrapped HSA and entrapped AGP supports. The entrapped AGP support was initially tested for viability through the injection of a compound with known retention to this glycoprotein. Resulting data showed that the entrapped AGP was accessible to small analytes for binding, with measured association equilibrium constants that were in agreement with previously-established literature values. A series of zonal elution injections for seven drugs was performed on the entrapped HSA, the entrapped

AGP, and the prepared control column. Injections were made on the control column to account for nonspecific binding between the analyte and the support. Measured K_a values were compared with established literature values when available.

Preparation of High Capacity Protein G Supports.

The final study in this dissertation focused on the covalent immobilization of protein G using the Schiff base method – a method that was often used for comparison in the entrapment studies. These supports were optimized to achieve maximum immobilized protein content through the variation of pore size. The ability of this immobilized protein G to selectively attach to the F_c region of immunoglobulins was examined. This type of adsorption creates a stationary phase containing highly active antibodies, which is useful for the immunoaffinity separations.

FUTURE WORK

Entrapment.

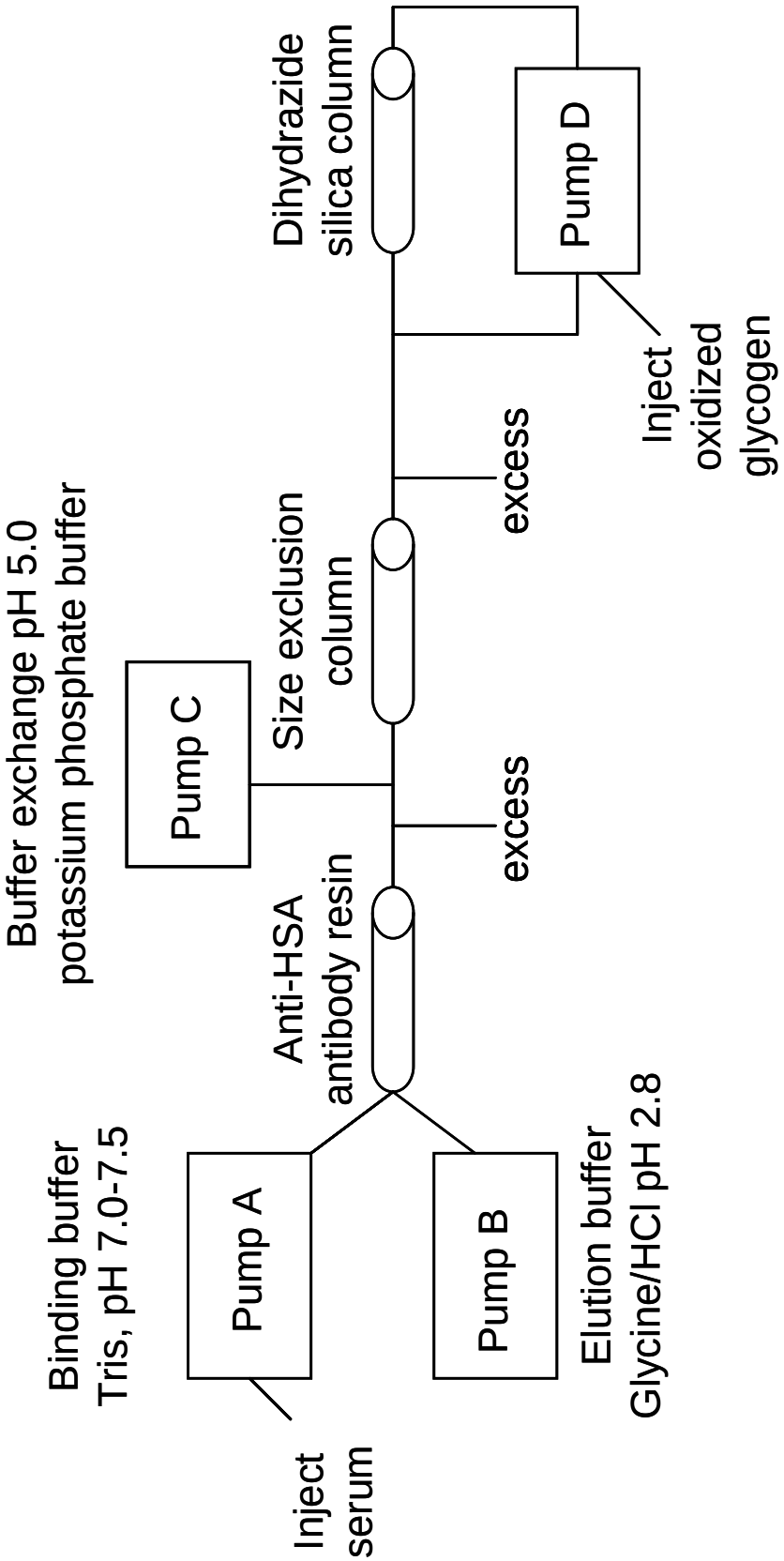
The optimization of the entrapment method has created many options for further implementation of this method. The high protein content supports, prepared using 100 Å silica and high levels of ligand in the reaction mixture, will be used to make HPAC columns for future binding studies. At the optimum periodic acid ratio determined in the preliminary studies, the amount of glycogen oxidation should allow for complete access of the analyte to the immobilized ligand. A support with such high protein content would make it possible to study analytes with relatively low affinities and to avoid experimental problems such as sample overloading. The work done in this dissertation showed that

analyte retention on these supports was not affected at flow rates up to 1.00 mL/min. Using this moderately high flow rate, a high protein content support would make it possible to create an efficient screening method that could obtain binding affinities for analytes that have not yet been studied by HPAC.

The entrapment method has been shown to be viable with a wide range of ligands at various molecule weights. The ability to entrap a large variety of ligands will allow production of new affinity supports. For example, work has been done to prepare an entrapped streptavidin support with a relatively high measured protein content of 56 (± 1) mg streptavidin per gram support. The activity of this support still needs to be tested. If sufficient activity is present, this support could be combined with biotinylated compounds of interest, which could be bound to the streptavidin stationary phase and used for affinity binding studies. This would make it possible to extend the entrapment method for the use of ligands with small molecular weights outside the optimized range. The different genetic variants of AGP could also be entrapped, to obtain binding information specific to each variant.

The work in **Chapter 4** with commercially gHSA will next be extended for work with gHSA from human serum samples obtained from diabetic patients. The nature of the entrapment method makes it viable for automation. **Figure 7-1** shows a proposed scheme to carry out the entrapment immobilization in an online fashion extending from serum injection to finished HPAC column. The first step would be to purify the HSA (or the protein of interest) from human serum using the binding specificity between albumin and its immobilized antibodies. An anti-albumin antibody agarose resin will be prepared with a size large enough to accommodate the quantity of HSA needed for later

Figure 7-1. Schematic for online HSA entrapment from serum.


Binding buffer
Tris, pH 7.0-7.5
Pump A
Inject
serum
Anti-HSA
antibody resin
Pump B
Elution buffer
Glycine/HCl pH 2.8
excess
Buffer exchange pH 5.0
potassium phosphate buffer
Pump C
Size exclusion
column
excess
Dihydrazide
silica column
Pump D
Inject
oxidized
glycogen

entrapment. Current resins available from Vivapure® have a binding capacity of 2 mg/mL and 5 mL of a 50% resin slurry could purify the HSA in 20 µL of serum or up to 800vµg of HSA. Larger columns could be produced to process larger volumes of serum. This purification step will be done by first flowing binding buffer through the column, a Tris-based buffer of low salt concentration between pH 7.0-7.5. Next, a known volume of human serum, with a size compatible with the amount of resin, would be injected onto the column. All non-bound proteins will be eluted from the column during the application of binding buffer and collected in an excess container. This washing step will ensure that only purified HSA is present on the resin. The next step would be to elute the HSA from the resin. This will be done by the application of an elution buffer, 0.10 M glycine/HCl at pH 2.8. The bound HSA would be eluted from the resin into a size exclusion column where the pH 2.8 buffer will be exchanged for 0.10 M potassium phosphate buffer at pH 5.0 for compatibility with the entrapment method. The HSA will now be cycled through an HPAC column containing dihydrazide-activated silica. Next, oxidized glycogen will be added to the HSA mixture and cycled through the column. This step will create the entrapped support. The final step will be to make a small injection of oxalic dihydrazide to cover any remaining aldehyde groups on the support to minimized non-specific binding. The column will then be washed with pH 7.4 potassium phosphate buffer prior to use in HPAC.

Studies conducted during the optimization of the entrapment rate employed initial silica samples of only 3 mg for the successful entrapment of HSA. These results indicated that small columns with relatively high binding capacities could be made using these human serum samples. Current studies are being done to test the ability for

entrapment to occur in pH 2.8 buffer. This would eliminate the buffer exchange step in the proposed online procedure allowing for the entrapment HPAC columns to be prepared from serum to support with minimal exposure and human interference.