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Affinity Chromatography in Environmental Analysis and Drug-Protein Interaction Studies

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AFFINITY CHROMATOGRAPHY IN ENVIRONMENTAL ANALYSIS AND DRUG-PROTEIN INTERACTION STUDIES

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This dissertation will examine the use of novel affinity sorbents to extract emerging contaminants from water. These contaminants include carbamazepine, an anti-epileptic drug which is resistant to natural degradation in the environmental and to drinking water treatment procedures. This drug has been found in fish, drinking water, estuarine and coastal waters, and river sediment and has been used as a general marker of contaminants in wastewater. Carbamazepine was one of the most commonly detected compounds in surface-water and groundwater samples in a recent reconnaissance study of untreated drinking water sources in the U.S. Besides using this drug as a representative contaminant for testing albumin-based extraction methods, other sections of this dissertation will include a discussion of the combination of on-line immunoextraction using anti-carbamazepine antibodies with RPLC/MS. Research will be presented involving the use of this method with molecularly imprinted polymers (MIPs) to extract emerging contaminants from water.

Other studies in this dissertation will include the use of serum protein columns to not only retain drugs but to provide chiral separations. This approach will be used to examine the retention of some chiral drugs by the serum protein α_1-acid glycoprotein. Another part of this dissertation will include a discussion of how chromatographic theory

can be used to describe the binding and extraction behavior of albumin columns when used to retain emerging contaminants. In addition, it will be shown how the same types of protein columns can be used to examine the kinetics of drug-protein interactions. Possible future directions for this work will also be discussed.

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TABLE OF CONTENTS

CHAPTER 1 GENERAL INTRODUCTION..... 1

BIOLOGICALLY ACTIVE ENVIRONMENTAL CONTAMINANTS..... 1

ANALYSIS OF ENVIRONMENTAL CONTAMINANTS.....7

ALTERNATIVE ANALYSIS STRATEGIES FOR EMERGING CONTAMINANTS..... 9

REFERENCES..... 11

CHAPTER 2 HIGH-PERFORMANCE IMMUNOSORBENTS FOR THE SELECTIVE TRACE ANALYSIS OF EMERGING CONTAMINANTS IN WATER..... 15

INTRODUCTION..... 15

MATERIALS AND METHODS.....18

RESULTS..... 25

DISCUSSION.....35

CONCLUSIONS..... 37

ACKNOWLEDGEMENTS.....38

REFERENCES..... 39

CHAPTER 3 PREPARATION, CHARACTERIZATION AND CHROMATOGRAPHIC EVALUATION OF MOLECULARLY IMPRINTED POLYMERS FOR USE IN THE ONLINE EXTRACTION OF TETRACYCLINE FROM WATER 43

INTRODUCTION..... 43

EXPERIMENTAL..... 52

Reagents..... 52

Apparatus..... 52

Polymer Preparation..... 53

Bulk polymer synthesis..... 53

Grafting of MIPs on silica particles..... 54

Chromatographic Conditions..... 54

Particle Size Distribution Evaluation..... 54

RESULTS AND DISCUSSION..... 56

CONCLUSIONS..... 68

REFERENCES..... 69

CHAPTER 4 COMPARISON OF VARIOUS α_1-ACID GLYCOPROTEIN IMMOBILIZATION METHODS FOR USE IN THE SEPARATION OF RACEMIC MIXTURES OF PHARMACEUTICAL AGENTS..... 71

INTRODUCTION..... 71

EXPERIMENTAL..... 81

Reagents..... 81

Apparatus..... 81

AGP Immobilization Method 1..... 82

Oxidation of AGP..... 82

Immobilization of AGP.....82

AGP Immobilization Method 2..... 83

AGP Immobilization Method 3..... 84

AGP Immobilization Method 4..... 85

AGP Immobilization Method 5..... 85

Chromatographic Conditions.....86

RESULTS AND DISCUSSION.....87

Method 1.....87

Method 2.....88

Method 3.....106

Method 4.....106

Method 5.....112

CONCLUSIONS..... 112

ACKNOWLEDGEMENTS.....113

REFERENCES..... 113

CHAPTER 5 DEVELOPMENT OF AFFINITY SORBENTS FOR ON-LINE EXTRACTION AND CONCENTRATION OF BIOLOGICALLY-ACTIVE CONTAMINANTS FROM WATER..... 115

INTRODUCTION..... 115

EXPERIMENTAL.....129

Reagents.....129

Apparatus.....129

Column Preparation.....130

Chromatographic Conditions.....130

Computer Model.....131

RESULTS AND DISCUSSION.....132

CONCLUSIONS.....156

ACKNOWLEDGEMENTS.....157

REFERENCES.....158

CHAPTER 6 THE USE OF CHROMATOGRAPHIC PEAK PROFILING FOR THE STUDY OF DRUG-PROTEIN BINDING KINETICS.....161

INTRODUCTION.....161

THEORY.....166

EXPERIMENTAL.....176

Reagents.....176

Apparatus.....176

Column Preparation.....177

Chromatographic Studies.....177

RESULTS AND DISCUSSION.....179

Peak Profiling on Control Column.....179

Effects of Stagnant Mobile Phase Mass Transfer on Control Column.....182

Peak Profiling on Protein Columns..... 185

CONCLUSIONS..... 192

REFERENCES..... 193

CHAPTER 7 SUMMARY AND FUTURE WORK..... 197

SUMMARY..... 197

FUTURE WORK..... 198

APPENDIX..... 199

Representative partial spreadsheet for 2,4-D simulation..... 199

CHAPTER 1

GENERAL INTRODUCTION

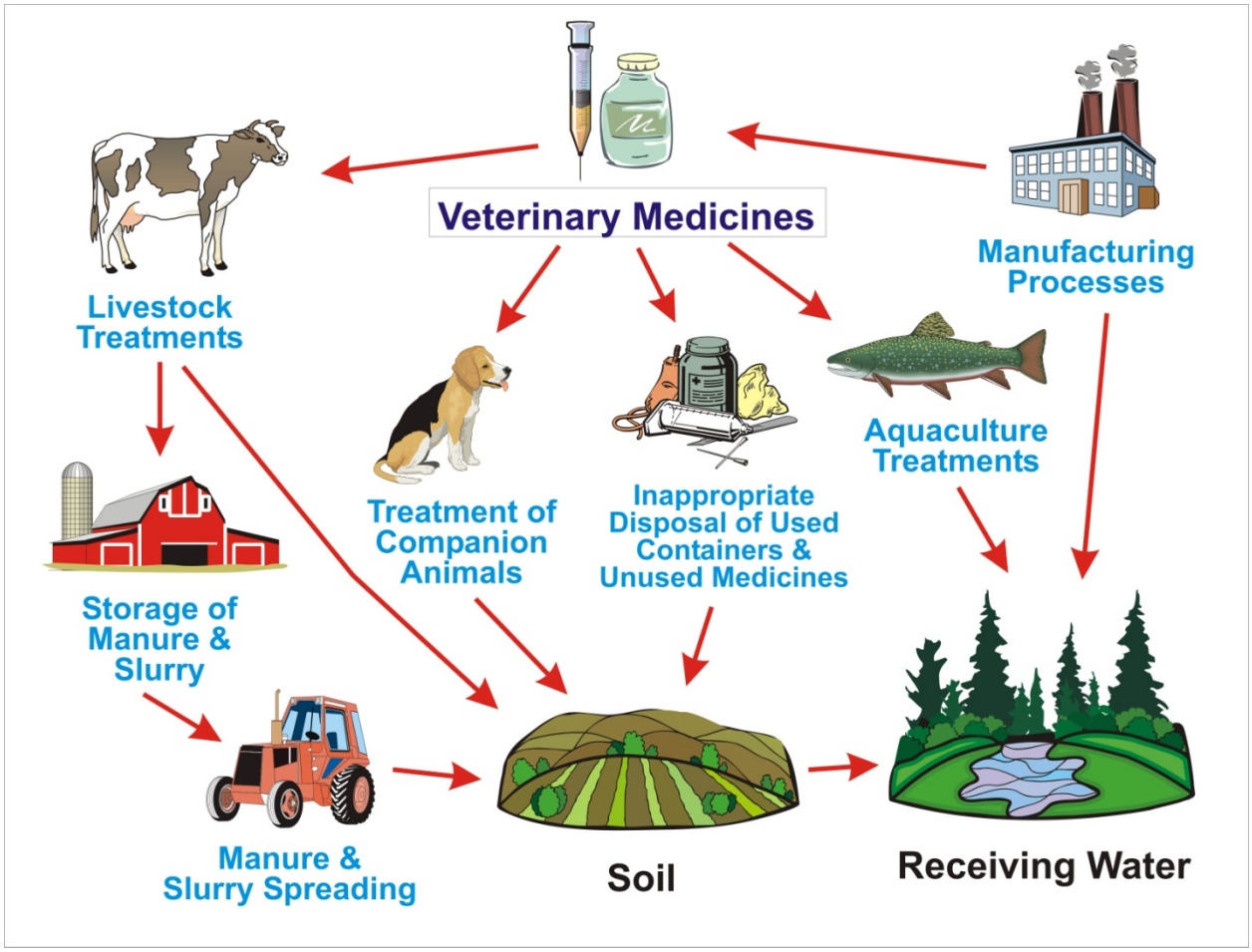
BIOLOGICALLY-ACTIVE ENVIRONMENTAL CONTAMINANTS

Over the last decade there has been increasing research in the area of biologically-active "emerging contaminants". These environmental pollutants are organic compounds that have both industrial and agricultural sources and include substances whose environmental effects are not fully understood and for which regulations are not yet in place.[1] These contaminants are typically present in trace amounts and are of concern in surface water, soil, sediment and groundwater. Included in this class of compounds are human and veterinary pharmaceuticals and their metabolites, personal care products, natural and synthetic hormones, endocrine disrupting compounds, disinfection byproducts, flame retardants, surfactants, plasticizers, pesticide degradation products and new pesticides, as well as sucralose, antimony, nanomaterials, antibiotic resistance genes and prion proteins.[2-4]

There are many pathways that biologically active contaminants can follow when entering the environment. Some examples include pesticide application, manure application, feedlot runoff, wastewater treatment plant effluent, land application of treated municipal wastewater and biosolids, stormwater runoff, leaching into groundwater, uptake into food crops, discharge of wastewater into water bodies, sewer overflows, septic tank effluent, industrial waste discharge, release into surface waters of

antibacterials used in aquaculture, and improper disposal of unused medicines.[3, 5,6, 7] Figure 1-1 shows some examples of pathways for veterinary medicines. Knowing the sources and pathways of contaminants is not only helpful in reducing the input of these compounds into the environment but also aids in assessing the risk they pose to human health and to the ecosystem.[6]

Figure 1-1. Pathways for the entry of biologically-active contaminants into the environment (Reproduced with permission from Ref. 5).


Veterinary Medicines
Livestock Treatments
Manufacturing Processes
Treatment of Companion Animals
Inappropriate Disposal of Used Containers & Unused Medicines
Aquaculture Treatments
Storage of Manure & Slurry
Manure & Slurry Spreading
Soil
Receiving Water

There have been many studies of the occurrence of biologically-active contaminants in various settings. These contaminants have been found in plants and animals, including human tissue.[8] These contaminants have also been detected in waters throughout the world,[6, 9] including sources of public drinking water.[8] It is difficult to set drinking water standards because information on the large-scale occurrence of biologically-active contaminants is only beginning to become available and there are few standardized methods for analyzing these agents.[8] There is also evidence that drought caused by climate change can lead to increased concentrations of pharmaceuticals and endocrine-disrupting compounds in surface water and drinking water sources.[10] Population growth and urbanization have led to the use of treated, partially treated and even untreated wastewater for agricultural irrigation in many parts of the world. There is the potential for pharmaceuticals in such water to be leached into groundwater.[3]

These contaminants are of concern because their fate in the environment and therefore, their potential impact on the environment and on human health are not well known.[3, 11, 12] However, many of these contaminants are suspected of causing diseases or developmental abnormalities in wildlife and humans and some confirmed to do so .[3, 13] Biologically-active contaminants can also interact with each other in the environment.[6] For example, the degradation of naproxen has been shown to be diminished by the presence of antibiotics in soil.[14] Sorption of these compounds by soil creates the potential for extensive and long-term water contamination. Soil also acts as a reaction catalyst for some contaminants.[3]

Personal care products are the source of the antibacterial compounds triclosan and triclocarban, which are added to soaps, deodorants, skin creams, mouthwashes,

toothpastes, cosmetics, fabrics and plastics.[15,16] Triclosan and triclocarban easily pass through wastewater treatment systems because they are hydrophobic and may end up in the environment through biosolids application to agricultural fields, which is an increasingly common practice in the United States.[3] Not much is known about the fate of these compounds after they enter the soil.[17] The concern about triclosan is that it may be an endocrine disruptor[18] and cause resistance to antibiotics in bacteria.[19] Although triclocarban is not an endocrine disruptor on its own, it is thought to enhance testosterone action.[20]

Other biologically-active compounds (e.g., pharmaceuticals, hormones and endocrine disrupting compounds) are also difficult to remove completely during wastewater treatment. The concern over the environmental release of pharmaceuticals such as antibiotics is their contribution to the development of antibiotic-resistant bacteria as well as the reduced biodegradation of plant material. This latter issue is a concern because plants are an important source of food for aquatic organisms. Natural hormones and synthetic chemicals that mimic hormones have possible estrogenic effects and other effects on animals and humans. For example, the contraceptive α-ethinylestradiol (EE2) affects sexual characteristics and decreases egg fertilization in fish at low and subnanogram per liter levels.[21]

ANALYSIS OF ENVIROMENTAL CONTAMINANTS

Detection levels for manufactured and natural organic compounds are often in the sub-parts per billion to sub-parts per trillion levels.[8] Liquid chromatography-mass spectrometry (LC/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS) are commonly used for the analysis of these and other compounds in environmental samples.[8, 9, 21, 22] These techniques combine compound-specific fragments that are produced by the mass spectrometer with the use of chromatographic retention to clearly separate and identify compounds. Another advantage of using mass spectrometry as part of these methods is the ability of this approach to consult data files even months after an analysis for the identification of unknown compounds.[21] In order to overcome matrix effects and ion suppression in wastewater samples, isotopically labeled standards are often used in these measurement methods. These internal standards are usually deuterated or contain ^{13}C and are often quite costly.[2, 23] More efficient and selective extraction or pretreatment methods for the desired analytes can be useful in these situations.

In order to provide relevant detection limits for trace analysis in environmental samples, extraction and preconcentration are typically required prior to sample introduction to an HPLC system. Solid-phase extraction (SPE) is the most common method of extraction and concentration for environmental samples; in some instances, online SPE has even been used.[21, 24] To provide more efficient and selective methods of extraction, affinity based sorbents have been used in some research for the analysis of pesticides.[25-27] Affinity ligands are usually of biological origin and include antibodies, enzymes, serum proteins, lectins, carbohydrates and avidin/streptavidin. These binding

agents, or "affinity ligands", make use of selective interactions of many biological systems to efficiently extract sample components for analysis or purification. These ligands may be immobilized to high-performance chromatographic supports to give a technique known as high-performance affinity chromatography (HPAC); this method is also known more specifically as high-performance immunoaffinity chromatography (HPIAC) when antibodies are used as the ligands.[28, 29]

An example of this latter type of work is a previous study in which HPIAC was coupled to reversed-phase liquid chromatography (RPLC) to analyze atrazine and its major degradation products in water.[25] The compounds of interest in this particular example were extracted by the anti-atrazine monoclonal antibodies immobilized to silica in the immunoaffinity column and then separated using an on-line reversed-phase column. Compared to the reference methods of gas chromatography-mass spectrometry (GC/MS) and gas chromatography with nitrogen-phosphorus detection (GC/NPD), the HPIAC/RPLC method had similar accuracy and precision. However, since there was no need for extraction or sample derivatization before analysis, the HPIAC/RPLC method was quicker, less labor intensive and required smaller amounts of solvents.[25] It was also possible to use this system to measure atrazine degradation products at environmentally significant concentrations in the parts-per-trillion range (i.e., nanograms per liter concentrations).[26] A similar, portable immunoextraction/RPLC system was developed to analyze atrazine and other triazine herbicides in groundwater and surface water.[27] In another example of immunoextraction, monoclonal antibodies to 17β-estradiol and estrone were used to selectively extract these steroid estrogens from wastewater samples.

The extracts were then analyzed using HPLC-electrospray MS with a C_{18} column for separation.[30]

ALTERNATIVE ANALYSIS STRATEGIES FOR EMERGING CONTAMINANTS

Another, less expensive option to antibodies that will be explored in this dissertation is the use of serum transport proteins as the basis for affinity sorbents for steroid hormones, drugs and other emerging contaminants. Like antibodies, proteins such as human serum albumin (HSA) and bovine serum albumin (BSA) have relatively high selectivity and strong binding for their target compounds.[29, 31] Albumin is the most abundant plasma protein in vertebrates. HSA has a molecular weight of 66.5 kDa, and has a single polypeptide chain that contains 585 amino acids. HSA also contains 17 disulfide bridges, which stabilize its structure by folding and looping together α-helices. BSA has a similar mass and structure to HSA but contains only 583 amino acids.[29]

Albumin is produced by the liver and performs a variety of functions in the body. These functions include the ability of albumin to bind and deliver organic anions, long-chain fatty acids, drugs, vitamins and other substances through the blood stream. Other functions of albumin involve its role in regulating osmotic pressure, protecting low-density lipoproteins from peroxidative effects, and acting as a buffering system for extravascular fluids.[29] Chapter 5 will present the development of affinity sorbents for use of albumins in the on-line extraction of biologically-active environmental contaminants in water analysis using LC/MS/MS.

One compound that will be used to test this approach is carbamazepine. Carbamazepine is an anti-epileptic drug which is resistant to natural degradation in the environment and to drinking water treatment procedures.[32-34] This drug has been found in fish, drinking water, estuarine and coastal waters, river sediments[21, 35, 36] and has been used as a general marker of contaminants in wastewater.[37] Carbamazepine was one of the most commonly detected compounds in surface-water and groundwater samples in a recent reconnaissance study of untreated drinking water sources in the U.S.[8] Also, the chronic toxicity lowest observed effect concentration (LOEC) of carbamazepine is close to its levels that are observed in wastewater effluents.[21] Chapter 5 will illustrate using this drug as a representative contaminant for testing albumin-based extraction methods, and Chapter 2 will include discussion of the combination of on-line immunoextraction using anti-carbamazepine antibodies with RPLC/MS. In Chapter 3 research will be presented involving the use of this method with molecularly imprinted polymers (MIPs) to extract emerging contaminants from water.

Another sampling and concentration option for environmental contaminants that will be considered in this dissertation is the use of a polar organic chemical integrative sampler (POCIS) extraction membrane. This method has been shown to be useful when it is not feasible, convenient or helpful to collect grab samples. Under some conditions, POCIS provides a time-weighted average concentration of the compound of interest. It also has the advantage of being equivalent to the respiratory exposure of aquatic organisms[38].

Other studies in this dissertation will include the use of serum protein columns to not only retain drugs but to provide chiral separations. This approach will be used in

Chapter 4 to examine the retention of some chiral drugs by the serum protein α1-acid glycoprotein. Chapter 5 will include a discussion of how chromatographic theory can be used to describe the binding and extraction behavior of albumin columns when used to retain emerging contaminants. In addition, Chapter 6 will show how the same types of protein columns can be used to examine the kinetics of drug-protein interactions. Chapter 7 will summarize the results of this dissertation and discuss some possibilities for future studies.

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CHAPTER 2

HIGH-PERFORMANCE IMMUNOSORBENTS FOR THE SELECTIVE TRACE ANALYSIS OF EMERGING CONTAMINANTS IN WATER

Note: The following is adapted from *High-Performance Immunosorbents for the Selective Trace Analysis of Emerging Contaminants in Water*, D. S. Hage, E. Papastavros, D. D. Snow, Proceedings WEFTEC, 2010.

INTRODUCTION

A wide variety of polar organic chemicals such as pharmaceuticals, steroids, and personal care products are released into the environment through municipal wastewater and the application of biosolids. Studies of surface and ground water quality are now considering the impact of these difficult-to-analyze contaminants on aquatic organisms and human health. Low concentration chemicals such as X-ray contrast agents, steroids, antibiotics and other pharmaceuticals have been detected in water bodies impacted by wastewater.[1-5]Because of their persistence, a few refractory organics, ranging from health care products and human steroid hormones to caffeine and its metabolites and even artificial sweeteners, are now considered potential wastewater markers in surface and ground water systems.

Exceedingly low, parts-per-trillion, levels of these individual chemicals in water may not in itself raise serious health concerns. However, the persistence and occurrence of these chemicals in water supplies may have implications for both human water-borne diseases and risks to environmental health.[3, 6-9] Since drinking water treatment

technologies do not typically remove these contaminants, some may even be found in treated water supplies.[10] Many questions remain as to the relative importance of different sources of these agents and the environmental factors that control the occurrence and fate of these chemicals. Because of these concerns and uncertainty, there is an increasing need to assess the sources, occurrence and effects of these biologically-active environmental contaminants in water. Many of these chemicals are water soluble, making their separation more challenging. They are typically found in very complex matrices, such as bio-solids or sludge, sediments, manures, and waste-impacted natural waters, further adding to the difficulty of analysis. These factors have created a pressing need for improved sampling and detection technologies for these micro-constituents.

Highly efficient extraction and purification methods are needed to extract these compounds from complex matrices and to separate them from interferences, i.e. species other than the analyte of interest that can affect the response of the analytical method. . Existing methods for such work typically use either bonded silica or polymeric sorbents for concentrating contaminants from water. Although recoveries can be quite good using these nonselective sorbents, large quantities of potential interferences are often co-extracted leading to reduction in the sensitivity and accuracy of the analysis. Immunosorbents (i.e., supports containing immobilized antibodies as selective binding agents) show great promise as an alternative approach for this type of work.[11, 12] Immunosorbents have previously been employed for both off- and on-line extraction with LC and LC/MS, as well as in the automation of immunoassays.[13] Advantages of using immunosorbents for the extraction and detection of micro-contaminants such as steroids include their high selectivity and strong binding for a target, as illustrated in recent work

performed with such supports in the liquid chromatography-tandem mass spectrometry analysis of estrogens (17β-estradiol and estrone) in raw sewage samples.[14] Immunosorbents and related sorbent technologies are only beginning to see applications in environmental analysis.[15] These materials provide a high degree of selectivity and retention needed for concentrating polar organic contaminants such as pharmaceuticals form large volumes of water.[16]

Development of a selective sorbent first requires identification of a contaminant or contaminant class to be concentrated and separated from water. Though many organics have been reported in wastewater effluent and in water impacted by wastewater, a few are frequently observed, probably reflecting both widespread use and resistance to biological degradation.

For example, traces of the anti-seizure medications primidone and carbamazepine, as well as the stimulant caffeine, were detected downstream from wastewater treatment plants in California, Pennsylvania, Colorado, Oklahoma and New Jersey.[17] In another study,[18] researchers detected traces of carbamazepine in groundwater near a municipal wastewater treatment lagoon by Tel Aviv, Israel. A recent national reconnaissance by the U.S. Geological Survey found traces of 63 organic contaminants in untreated drinking water supplies across the U.S.[6] The five most frequently detected of the targeted chemicals in surface water used as a drinking water source included cholesterol, metolachlor (a herbicide), cotinine (a nicotine metabolite), β-sitosterol (a plant sterol), and 1,7-dimethylxanthine (a caffeine metabolite). The same study also investigated ground water supplies, finding traces of carbamazepine along with tetrachloroethylene (a chlorinated solvent), bisphenol-A (a plasticizer), 1,7-

dimethylxanthine and tri(2-chloroethyl) phosphate (a flame retardant). Based on these and other reports, carbamazepine frequently appears in municipal wastewater[19] and is resistant to biodegradation with little removal during wastewater treatment.[20]

The objective of this chapter is to illustrate the use of immunosorbents for development of an improved method for concentration and detection of pharmaceuticals such as carbamazepine in order to help understand and measure impacts of wastewater on water quality. Other contaminants reported here to illustrate the use and selectivity of immunosorbents are triazine herbicides, such as atrazine, and chlorophenoxyacetic acid herbicides, such as 2,4-D. Atrazine is particularly ubiquitous in the environment and has been detected at low levels in drinking water supplies from across the U.S. In a recent study of pharmaceuticals and endocrine disruptors in U.S. drinking water[5], the most frequently detected compounds included atenolol, atrazine, carbamazepine, estrone, gemfibrozil, meprobamate, naproxen, phenytoin, sulfamethoxazole, TCEP, and trimethoprim. Atrazine, together with its degradation products deethyl- and deisopropylatrazine, are among the most widely detected herbicide residues in surface water and ground water.[21]

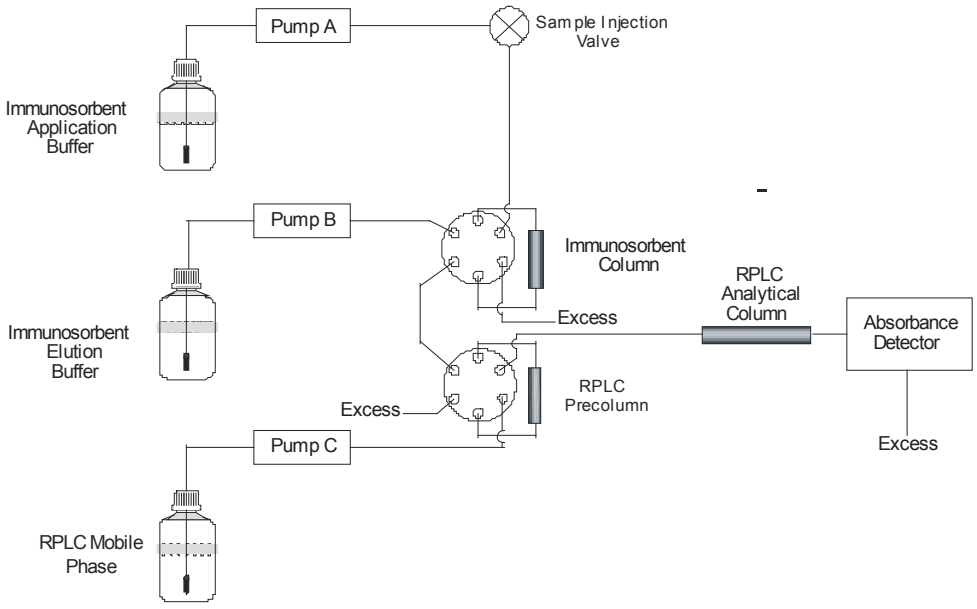
MATERIALS AND METHODS

The immunosorbents used in these examples were prepared using HPLC-grade silica modified with a preparation of antibodies immobilized using the Schiff base method or comparable technique.[13] In the examples presented here, these immunosorbents were tested and coupled on-line with either reversed-phase liquid chromatography (RPLC) with ultraviolet (UV) detection or liquid chromatography-mass

spectrometry (LC/MS) for detection and analysis of micro-constituents in water and other aqueous matrices.

Figure 2-1 gives a schematic of the on-line immunosorbent column coupled to RPLC pre-column configuration discussed in this chapter.[11, 16, 22, 23] The system uses three pumps and two switching valves to control flow between the immunosorbent column and two reversed phase columns. A similar system has been used to study the interfacing of immunosorbents with RPLC[22] and in studies of the binding strengths and kinetics of environmental contaminants as they bind to and elute from immunosorbents in HPLC systems.[24] In this type of system, a small column containing the immunosorbent is first placed on-line with an appropriate application buffer as sample is injected onto the system. The application buffer is typically a neutral pH aqueous buffer (e.g., pH 7-7.4 phosphate) that will allow strong binding to occur between the analytes in the injected samples and immobilized antibodies in the immunosorbent column. After non-retained sample components have been washed from the column, a valve is switched and the immunosorbent is placed on-line with a small RPLC precolumn. An elution buffer is pumped through the immunosorbent, releasing target compounds which are then retained by the RPLC column. After the desired solutes have been recaptured by the RPLC precolumn, the valve is then switched again and the RPLC precolumn is placed in series with a longer analytical RPLC column. Mobile phase containing organic solvent is passed through these two columns, which causes analytes to be eluted from the precolumn and separated on the analytical column based on polarity. These solutes are then detected as they elute from the analytical column and enter an appropriate detector. While this separation is occurring, the application buffer is reapplied to the

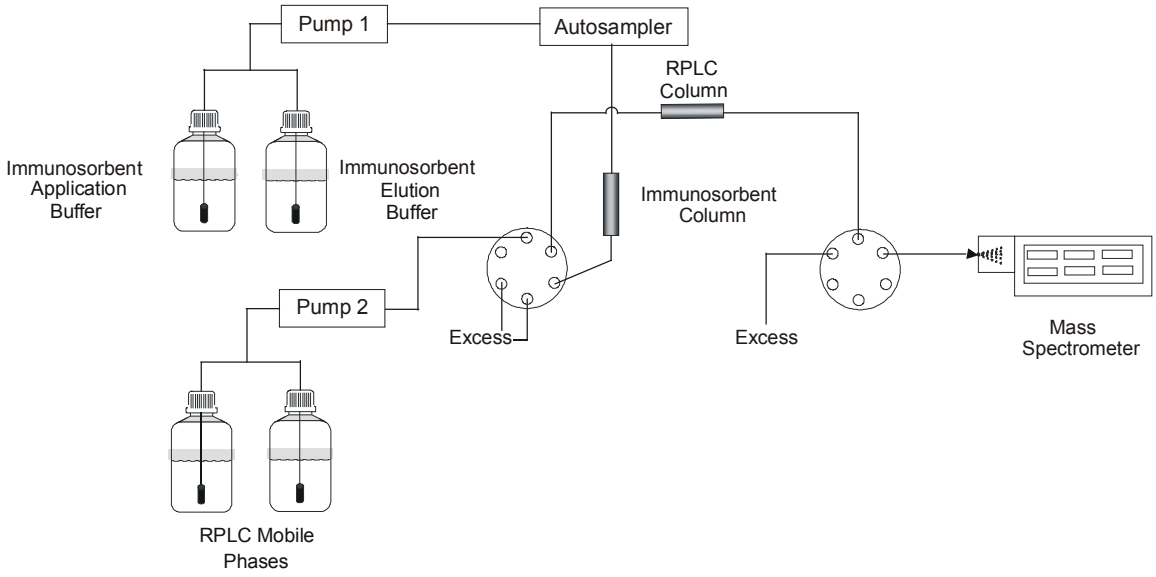
Figure 2-1. A typical system for coupling an HPLC immunosorbent column on-line with reversed-phase liquid chromatography and absorbance detection (Reproduced with permission from Ref. 25).


Pump A
Sample Injection Valve
Immunosorbent Application Buffer
Pump B
Immunosorbent Column
RPLC Analytical Column
Absorbance Detector
Immunosorbent Elution Buffer
Excess
Excess
RPLC Precolumn
Pump C
Excess
RPLC Mobile Phase

immunosorbent as this material is allowed to regenerate. The entire process is then repeated for the next sample injection. This type of analysis can be carried out using a standard benchtop HPLC system.[16, 22, 23] The same approach can also be modified as part of a smaller field-portable system.[25]

A similar scheme to that shown in Figure 2-1 can be utilized for the on-line coupling of LC/MS with immunosorbents. An example of one such system, which has been used in the detection of carbamazepine in aqueous samples,[26] is shown in Figure 2-2. The initial steps for operation in the system shown in Figure 2-2 are similar to those already described in Figure 2-1. However, in Figure 2-2 the RPLC column is also used for an intermediate step in which a switch is made from one type of aqueous buffer, which is used for the release of solutes that have been retained by the immunosorbent (e.g., pH 2.5 potassium phosphate buffer) to a more volatile buffer that is compatible with the LC/MS system (e.g., acetate buffer). In this particular case, the RPLC precolumn is used alone for the separation of solutes based on polarity once the second, more volatile, buffer is combined with some organic modifier (e.g., acetonitrile). The eluting analyte is then detected as it leaves the RPLC precolumn using an on-line mass spectrometer as the detector.[26] If desired, a second, longer RPLC column can also be placed after the RPLC precolumn to aid in the separation of sample components based on their polarity.[12]

Figure 2-2. System for detecting an analyte by using an HPLC immunosorbent and LC/MS (Based on results presented in Ref. 12).

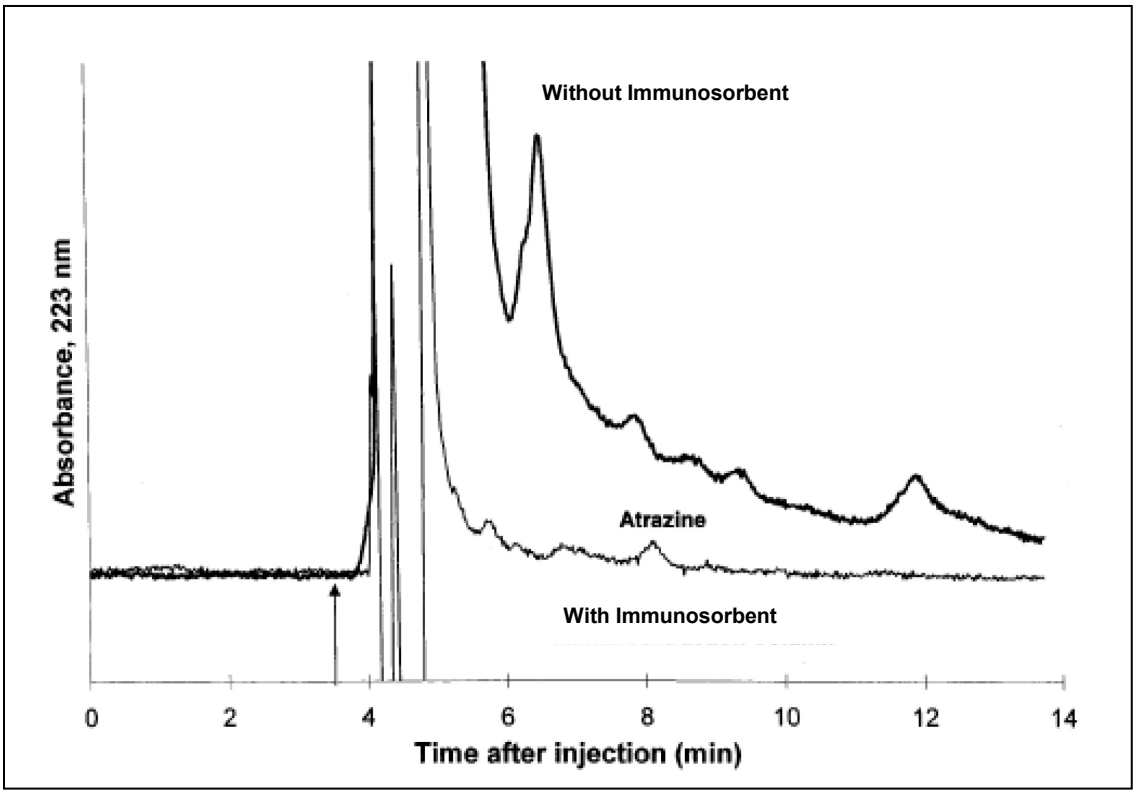

Pump 1
Autosampler
RPLC
Column
Immunosorbent
Application
Buffer
Immunosorbent
Elution
Buffer
Immunosorbent
Column
Pump 2
Excess
Excess
Mass
Spectrometer
RPLC Mobile
Phases

RESULTS

There are various ways for combining immunosorbents with other methods for the analysis of micro-constituents. One method that can easily be coupled with the on-line use of immunosorbents is RPLC. The combination of immunosorbents with this approach produces a method that has been referred to as immunoaffinity/RPLC, or IA-RPLC. There are several examples of the use of IA-RPLC for the analysis of micro-constituents in the environment. This method has been employed as a tool in a number of studies to measure atrazine and related degradation products in various types of water samples.[16, 23, 25] An example of how an immunosorbent can aid in such an analysis is shown in Figure 2-3. The upper chromatogram in this figure shows the result that is obtained when a groundwater sample is injected directly onto only a RPLC column. The result is a chromatogram with a large number of peaks and a high background signal, making atrazine undetectable because of other sample components. This illustrates the difficulty in detecting trace levels of solutes such as atrazine at low parts-per-billion levels in a complex matrix.

When the same sample is first allowed to pass through an immunosorbent column selective for atrazine, the retained fraction that was then allowed to go on to the RPLC column gave a much simpler chromatogram. In this second case the number of background peaks is greatly reduced and it is now relatively easy to identify and quantify 2 ppb atrazine present in the original sample even when using a relatively nonspecific UV-absorbance detector.

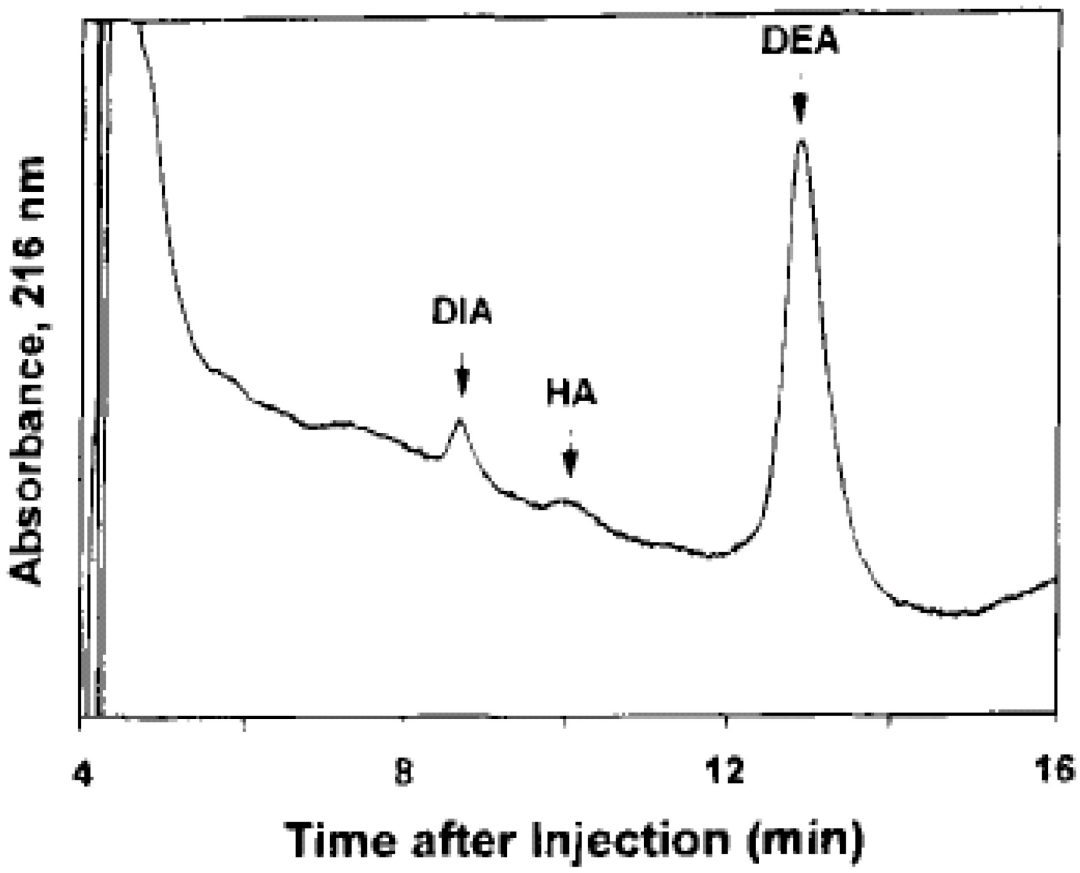
Figure 2-3. The analysis of atrazine in a groundwater sample by using only a RPLC column (top) or an anti-atrazine immunosorbent column followed by the same RPLC column (bottom). The detected atrazine was present in the groundwater sample at a level of 2 parts-per-billion (Adapted with permission from Ref. 25).


Without Immunosorbent
Atrazine
With Immunosorbent
Absorbance, 223 nm
0
2
4
6
8
10
12
14
Time after injection (min)

In addition for use in measuring triazine herbicides,[16, 23, 25] IA-RPLC has been used with carbofuran,[27] carbendazim,[28] and 2,4-D and related compounds.[22, 24] An analysis of carbendazim in soil and lake water employed a protein G column that was coupled to a reversed-phase analytical column by using a restricted access media trapping column. The limit of detection for carbendazim was 0.025 ppb and the throughput was on the order of three samples per hour.[28] In some of these studies, limits of detection in the parts-per-billion and parts-per-trillion range have been reported even when using UV absorbance detection after the immunoextraction and RPLC separation.[16]

The cross-reactivity of antibodies for structurally-similar compounds is often a problem in immunoassays and related methods. However, this ability can be used in work with immunosorbents and IA-RPLC to allow for the simultaneous analysis of closely-related micro-constituents, such as a contaminant and its degradation products. This approach has been used to measure the occurrence of atrazine and its degradation products such as hydroxyatrazine, deethylatrazine and deisopropylatrazine using a single IA-RPLC system. An example of one such system that was optimized for the analysis of atrazine degradation products at low parts-per-trillion levels is shown in Figure 2-4. In this method, the immunosorbent is used to selectively extract chemically-related compounds, as opposed to a single compound, from a sample. The extracted compounds are then separated based on their polarity by later releasing these chemicals from the immunosorbent and passing them through one or more RPLC columns (e.g., a RPLC precolumn and a large analytical column). This latter type of separation is particularly useful in discriminating between a parent compound and its metabolites or degradation

Figure 2-4. Separation and analysis of deisopropylatrazine (DIA), hydroxyatrazine (HA), and deethylatrazine (DEA) by IA-RPLC. This result was obtained for a 45 mL groundwater sample containing 60, 10 and 210 parts-per-trillion DIA, HA and DEA, respectively. (Reproduced with permission from Ref. 16).


DEA
DIA
HA
Absorbance, 216 nm
4
8
12
16
Time after Injection (min)

products, which do tend to have large differences in polarity and which are relatively easy to examine simultaneously by such an approach.[16, 23]

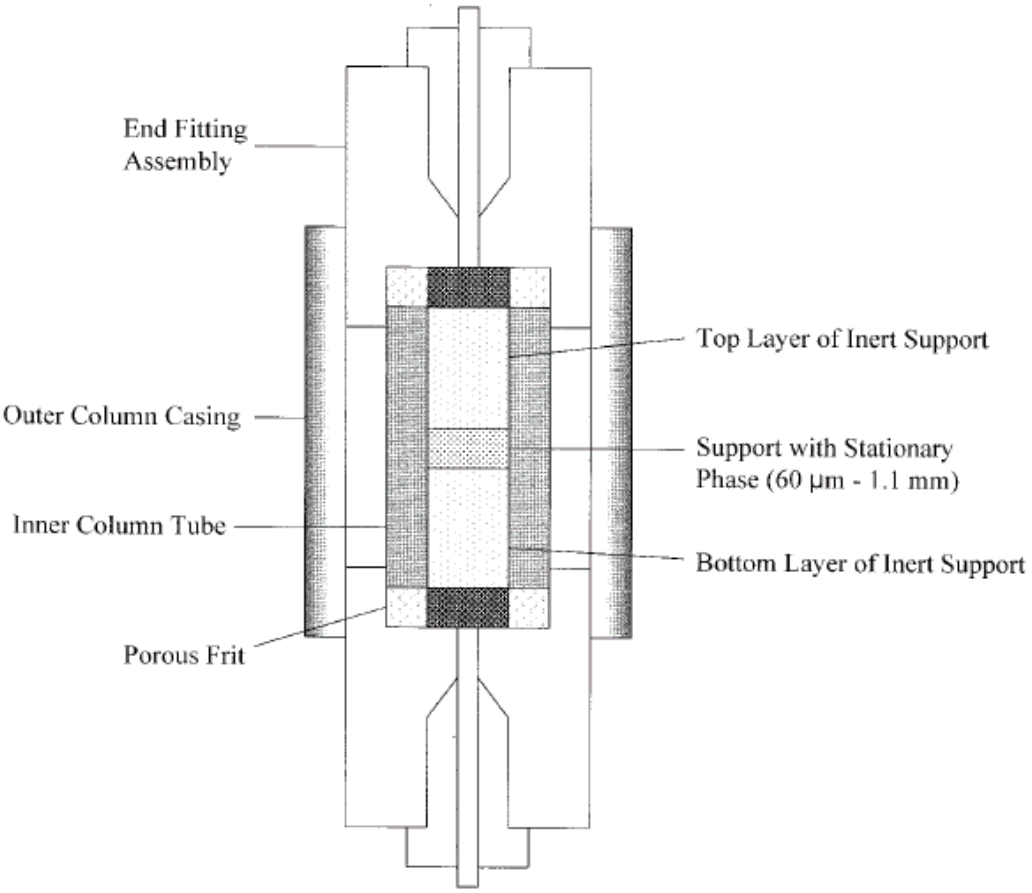
A field-portable system for the analysis of triazine herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D) and related compounds in groundwater and surface water has also been developed.[25] No sample pretreatment besides filtering through a 0.2 μm syringe filter was necessary in this work before sample injection. The sample analysis took 10 minutes or less and the detection limit for atrazine was near 0.3 ppb. Other analytes with which IA-RPLC has been used include work that has been reported with estrogens,[29] polycyclic aromatic hydrocarbons,[30, 31] isoproturon,[32] phenylurea pesticides,[32-35] aflatoxins,[36] *E. coli*[37] and compounds related to azo dyes.[38]

Varying the position and function of the immunosorbent column, along with adjustment of buffering conditions, are other options to consider when optimizing IA-RPLC methods as illustrated in the analysis of the herbicide 2,4-D.[22] In this case, an immunosorbent column for 2,4-D and related herbicides was followed by an RPLC precolumn and an RPLC analytical column. Dissociation rates for these compounds as they were eluted from the immunosorbent were first determined in the presence of various buffers. The retention of these analytes on the RPLC precolumn was also examined and this information was combined with that collected from the immunosorbent for use in computer modeling of the IA-RPLC interface. A modified countercurrent distribution model was used and found to give good agreement with the experimental results. It was shown through this work that both the immunosorbent column conditions and RPLC precolumn conditions could be adjusted in an IA-RPLC

system to adjust the selectivity of this method for a given analyte (e.g., 2,4-D) or for a group of analytes (e.g, 2,4-D and related compounds).

Although IA-RPLC can be used with absorbance detectors (e.g., see results in Figures 2-3 and 2-4), immunosorbents have also been applied in a number of studies with more sensitive instrumentation such as liquid chromatography mass spectrometry, or LC/MS (e.g., see reviews in refs 12 and 13). An example of a general scheme that can be used for on-line immunoextraction coupled with LC/MS was described in Figure 2-2. This particular scheme involved the use of small immunosorbent columns to extract carbamazepine prior to detection using LC/MS.[26] This method used a small immunosorbent column with the design that is shown in Figure 2-5. This column consisted of two layers of an inert support that served as a mechanical support for an immunosorbent active layer that was only 500 μm long with an inner diameter of 2.1 mm. The small size of this column made it inexpensive to prepare and practical for use with even small amounts of immobilized antibodies. The same design could easily be used to prepare other types of immunosorbents and can be used with either RPLC or LC/MS systems.

Figure 2-5. Design of a housing for construction of microaffinity columns containing an immunsorbent layer.[39] (Reproduced with permission from Ref. 39).


End Fitting
Assembly
Outer Column Casing
Inner Column Tube
Porous Frit
Top Layer of Inert Support
Support with Stationary
Phase (60 µm - 1.1 mm)
Bottom Layer of Inert Support

DISCUSSION

The previous section gave several examples that illustrated the on-line use of immunosorbents with RPLC or LC/MS for analysis of environmental contaminants. The selectivity of antibodies has allowed these methods to be used with a wide variety of samples that have included groundwater, surface water, drinking water, soil extracts, food samples, and biological samples.[12, 13] As shown in Figure 2-3, this selectivity allows matrix components that are not similar to the analyte to be effectively removed before the analyte is detected. This essentially lowers the background signal and helps to remove potential interferences from the sample prior to analyte detection.

Although some cross-reactivity may still be present between the analyte and structurally-related compounds in the sample, this effect can be used to an advantage. This idea was illustrated in Figure 2-4, in which the combined use of an immunosorbent and RPLC was used to first isolate a group of atrazine degradation products from a sample and then to separate and simultaneously measure the degradation products as they passed through the RPLC column.[16] The same effect can be used in a single IA-RPLC method to look at several closely-related herbicides, such as atrazine and simazine or 2,4-D and related agents.[22, 23]

The strong binding of immunosorbents under physiological conditions allows many of these materials to retain and concentrate their targets prior to their analysis. In many cases, the antibodies in an immunosorbent will bind irreversibly to their target compounds at a neutral pH and hold onto these until an appropriate elution buffer is applied. The overall effect is that the size of the captured analyte fraction is more directly related to the mass or moles of analyte that have been applied to the

immunosorbent rather than to the concentration of this analyte.[40] As a result, the use of a large sample volume can be used to obtain lower concentration limits of detection for RPLC and LC/MS systems that first use immunosorbents for sample pretreatment. The result of such an approach was illustrated in Figure 2-4, in which the use of a 45 mL sample volume made it possible to modify an IA-RPLC method for atrazine to allow for low parts-per-trillion detection limits of its degradation products.[16]

There are several reasons why immunosorbents are commonly used with RPLC columns in these methods. First, the aqueous elution buffers that are often used with immunosorbents will act as weak mobile phases for RPLC columns. This property means the retained chemicals that are released from an immunosorbent in the presence of such a buffer will tend to bind strongly and be concentrated at the top of the RPLC column. This effect tends to take the peak for the dissociating solutes that are leaving the immubosorbent and focus the eluting mix into a narrow band, thus making it possible to more easily later separate these chemicals on the RPLC column in the presence of an appropriate mobile phase.[22] The fact that a RPLC column provides a separation based on the general property of chemical polarity is also useful in providing a complementary scheme for isolating and resolving the chemicals that have been retained and concentrated by the immunosorbent.[12, 13]

CONCLUSIONS

This chapter has examined the use of immunosorbents with RPLC and LC/MS and has considered several aspects of such methods. A number of examples from the literature were provided to illustrate some of the useful features of immunosorbents in work with environmental contaminants. It has been demonstrated in the literature that immunosorbents can be effective tools in concentrating trace analytes from large sample volumes, making it possible to obtain limits of detection at the parts-per-billion level or even in the parts-per-trillion range. These methods have been used with a variety of samples, such as groundwater, surface water, food, and biological samples. This approach has promise for both laboratory and field-portable analytical methods for organic micro-contaminants. A number of factors have also been considered in the literature in the design and development of such systems, such as the conditions needed for interfacing the immunosorbent with RPLC or LC/MS. The selective binding of immunosorbents, their ability to be interfaced with LC or LC/MS/MS, and the ability to obtain antibodies against a wide range of micro-constituents are all properties that should continue to make this approach attractive as an alternative to other current analysis and sample pretreatment methods for the capture and integrated sampling of micro-constituents in water samples.

ACKNOWLEDGMENTS

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CHAPTER 3

PREPARATION, CHARACTERIZATION AND CHROMATOGRAPHIC EVALUATION OF MOLECULARLY IMPRINTED POLYMERS FOR USE IN THE ONLINE EXTRACTION OF TETRACYCLINE FROM WATER

INTRODUCTION

Emerging contaminants, as discussed in Chapter 1, include several classes of veterinary pharmaceuticals. Antibiotics are often used in agriculture to treat and prevent diseases, as well as to promote growth and feed efficiency in livestock operations.[1-3] At large animal-feeding operations many animals are within close proximity to each other, enabling diseases to spread quickly and making the use of veterinary pharmaceuticals necessary.[2] There has been concern that the sub-therapeutic doses of antibiotics that are used regularly for animals during food production are causing an increase in antibiotic-resistant bacteria.[2] Antibiotic-resistant *E. coli* has been found in the waste of feedlot animals that have been administered sub-therapeutic doses of antibiotics.[4] In addition, even though some antimicrobials that are used for animals are not always the same as those used to treat disease and infection in humans, there is still the possibility of bacterial resistance occurring to both types of antibiotics even given exposure of bacteria to only one group of these drugs. The structures of the antimicrobials that are usually employed for animals are similar enough to those used for humans to cause resistance to more than one of these compounds.[3]

Antibiotics are often poorly absorbed by an animal's body and, depending on the compound, most of the drug may be excreted unchanged from its original form. Furthermore, antibiotic metabolites can also be bioactive. This situation poses an environmental risk because it is common in many parts of the world to supplement fertilizer with animal waste.[2] Pharmaceuticals may also be present in wastewater from feeding operations and make their way into groundwater and surface water.[3]

The focus of this research is on the tetracycline group because these are widely used in cattle, pork and poultry production.[3, 5] Tetracycline and macrolide antibiotics make up more than 16% of all veterinary antibiotics in the U.S.[1] The general structure of tetracycline is shown in Figure 3-1.

In order to successfully detect antibiotics and other emerging contaminants in environmental samples, effective pretreatment, extraction and concentration of these agents from complex matrices is necessary. The required detection limits are low for these analytes and these compounds must often be separated from interferences in the sample.[1, 3] Antibiotic analysis is most commonly performed by using liquid chromatography-tandem mass spectrometry (LC/MS/MS). This method has the combined advantages of good sensitivity, reproducibility and specificity.[1] A common method of extraction and concentration that is used prior to chromatographic analysis and LC/MS/MS is solid phase extraction (SPE).[3, 5] However, this method is not efficient for samples with low concentrations of tetracyclines and requires multiple sample-preparation steps, often with the use of the standard addition method .[3]

Affinity extraction is an alternative to SPE, with the advantages of higher selectivity, potentially less interference and faster sample processing than SPE.[6]

Immunoaffinity extraction, based on the use of antibodies, has been successfully used for the extraction of analytes from various types of complex matrices both in off-line[7-11] and on-line[12-15] configurations, including on-line extraction in a field-portable system.[16] Detection limits have been obtained with this approach in the parts-per-trillion range for off-line methods and in the parts-per-billion and parts-per-trillion range for on-line methods. These low limits of detection can be reached because the affinity column is mass sensitive and responds to the amount of analyte (i.e. moles, rather than the concentration of the analyte).[6] On-line immunoaffinity extraction has been combined with reversed-phase liquid chromatography (RPLC) in the analysis of herbicides, carbofuran and carbendazim and has been shown to give excellent reproducibility and short analysis times in such an approach. On-line immunoextraction allows the transfer of the analyte to occur more quickly and inexpensively than off-line methods, with greater precision and recovery and with the use of fewer reagents.[6]

Molecularly imprinted polymers (MIPs) have been employed as alternatives to antibodies in affinity supports. MIPs are easy to prepare, less expensive than antibodies and can be used for a wider variety of analytes. For example, MIPs can be used with nonaqueous samples and with analytes for which antibodies cannot easily be generated (e.g., due to toxicity).[6, 17] During the synthesis of MIPs, a binding site is created which allows recognition and binding of a target molecule to take place later. The preparation of MIPs, as illustrated in Figure 3-2, involves the polymerization of functional monomers in the presence of a cross-linker and template, or imprint molecule. One or more functional groups are chosen for the process based on the interactions that will take place between these groups and the template molecule. A complex is formed between the imprint

molecule and the functional monomers. Polymerization then is allowed to take place and the cross-linked structure holds the functional groups in place. The template molecule, which has been chosen based on the final desired target, is next extracted after formation of the polymer. This leaves behind a binding site in the polymer that is capable of recognizing and binding the target molecule. This type of recognition can occur because the binding site has a size and shape that are complementary to those of the template and target molecule.[17]

Another, recently developed option for affinity ligands is the use of aptamers. These are oligonucleotides that are designed for binding to a specific target. They are chosen from random single-stranded DNA and then enriched through the use of a technique known as systematic evolution of ligands by exponential enrichment (SELEX). They can be created for target proteins that do not bind to DNA and for a wide variety of compounds.[18]

Although the most common applications of MIPs in separations has been the use of these materials in SPE for processing food, environmental and medical samples; MIPs have also been used in membrane extractions.[17] In another example, MIPs were used in affinity membranes to remove tetracycline from water.[19] However, the use of MIPs as affinity ligands in HPLC has been more limited because of the binding site heterogeneity of these agents.[17] However, the need for improved on-line extraction at a lower cost than that possible with immunoaffinity columns makes MIPs an attractive alternative for such an application.

This research investigated the use of various forms of MIPs as stationary phases in HPLC. Bulk polymers were prepared and used in HPLC columns. Polymerization

was also attempted on silica particles that are used as chromatographic supports. Tetracycline was used as the template molecule and methacrylic acid (MAA) was used as the functional monomer because it can form hydrogen bonds with tetracycline (see structure in Figure 3-1).

Figure 3-1. The structure of tetracycline.[19]

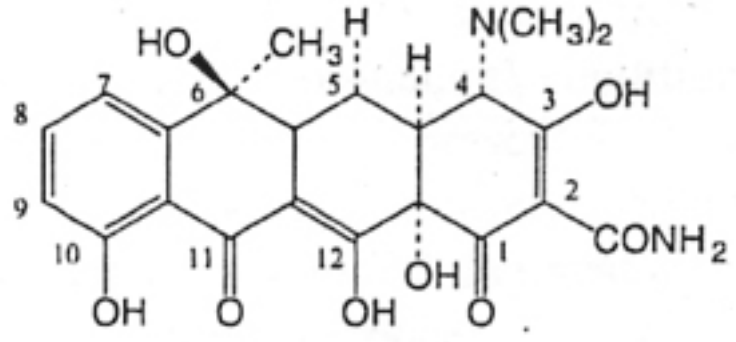
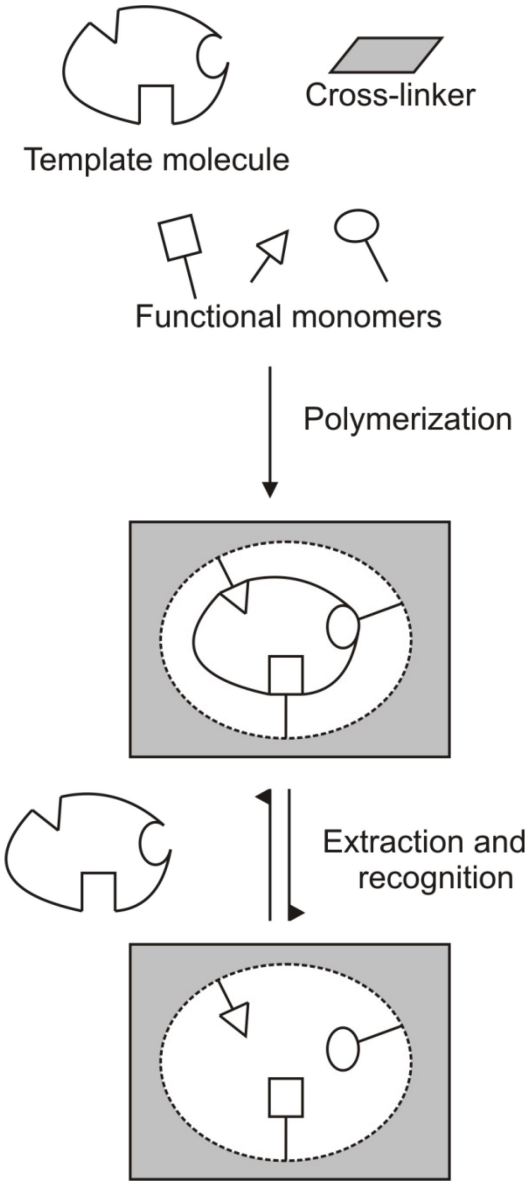

HO
CH3
H
H
N(CH3)2
OH
CONH2
OH
O
OH
OH
O
1
2
3
4
5
6
7
8
9
10
11
12

Figure 3-2. The preparation of a molecularly imprinted polymer (MIP).


Cross-linker
Template molecule
Functional monomers
Polymerization
Extraction and
recognition

EXPERIMENTAL

Reagents

Tetracycline, methacrylic acid (MAA) and (3-aminopropyl)-triethoxysilane (APS) were obtained from Sigma-Aldrich (St. Louis, MO). Ethylene glycol dimethacrylate (EDMA) was obtained from Aldrich (Milwaukee, WI). 2-2‘-Azodi (2,4-dimethylvaleronitrile) was obtained from Wako Pure Chemical Industries (Osaka, Japan). HPLC-grade Nucleosil 1000-5 and 1000-7 silica (1000 Å pore size, 5 µm and 7 µm diameter) were obtained from Macherey-Nagel (Düren, Germany). All other chemicals were of the purest grades available. Solutions were prepared using water from a Nanopure purification system (Barnstead, Dubuque, IA).

Apparatus

The chromatographic system used for the testing of MIP columns consisted of a LC-10AT pump, two Advantage PF Valves, and aSPD-10AV UV-Vis detector from Shimadzu (Kyoto, Japan). A Jasco (Easton, MD) PU-980i pump, a Thermo Separation Products (Waltham, MA) Consta Metric 4100 pump and a Hitachi (Schaumburg, IL) L-6000 pump were also used in testing the backpressure of columns. A Thermo Separation Products Spectra Series (Fremont, CA) UV100 UV-Vis detector was also used.

Data were collected using software and an interface from National Instruments (Austin, TX). All supports were downward-slurry packed using an HPLC column packer from Alltech (Deerfield, IL).

Polymer Preparation

Bulk polymer synthesis

A method previously used to prepare MIPs for tetracycline recognition in affinity membranes[19] was followed with some modifications. For instance, a different initiator was used: 2-2‘-azodi (2,4-dimethylvaleronitrile) instead of azobis(isobutyronitrile) (AIBN). The reaction mixture consisted of 9.95 g EDMA, 1.05 g MAA, 0.12 g initiator, 15 ml acetonitrile, 10 ml benzyl alcohol and 1.24 g tetracycline. The polymerization reaction was carried out at 50 °C for 24 h. A control, or non-imprinted polymer (NIP) was prepared following the same procedure, with the exception of the addition of tetracycline. After grinding the material by using a mortar and pestle, the polymers were dried and sieved to sort them with regard to size.

For the MIP that contained tetracycline, the fraction that was obtained by using a 25 μm sieve (≥ 25 μm and < 53 μm) was placed into a cellulose thimble and extraction was carried out with this MIP at 100 °C for 11.5 days. The extraction solution was changed to 10% acetic acid in acetonitrile twice and then replaced twice with 100% acetonitrile. The extraction solvent was light orange and still appeared to be extracting tetracycline after 11.5 days. The polymer was washed with water and packed into a 50 mm x 3 mm I.D. column and two 15 mm x 2.1 mm I.D. stainless steel columns. Because the backpressure of the 50 mm x 3 mm I.D. column was too high for practical use, acetonitrile was pumped through the 15 mm x 2.1 mm I.D. column at 0.1 ml/min for 20 hr to flush out any remaining tetracycline and to use this column. The NIP was similarly packed into a 15 mm x 2.1 mm I.D. stainless steel columns after sieving.

Grafting of MIPs on silica particles

A previously reported method was followed to prepare porous silica particles with polymerization precursors attached to their surface.[20] Nucleosil silica was reacted with APS in various ways: under ambient conditions, under an inert atmosphere and using a pyridine catalyst. IR spectra were obtained after reaction of the silica with APS and after a second reaction between the silica-APS with azobiscyanovaleric acid (ACVA).

Chromatographic Conditions

Each polymer support was downward-slurry packed at 1500-3500 psi for 1-3 h into separate 50 mm x 3 mm I.D. or 15 x 2.1 mm I.D. stainless steel columns. The packing solvent was water. A Rheodyne (Oak Harbor, WA) Lab Pro valve was also used for column packing. The flow rates during the HPLC experiments were 0.1, 0.2, 0.5, 1 and 5 ml/min. Samples of 5 μM , 20 μM and 50 μM tetracycline in water were injected using a 5 μL sample loop onto the 15 mm x 2.1 mm I.D. column containing the tetracycline imprinted polymer while using water as the mobile phase. The flow rates were 0.2 and 0.5 ml/min for these injections and detection was carried out at a wavelength of 275 nm.

Particle Size Distribution Evaluation

The NIPs were ground for 10 min or 20 min using a mortar and pestle, dried in a vacuum oven overnight at room temperature, and sieved for 20 min to separate according to particle size using a Tyler (Mentor, OH) sieve shaker. Alternatively, the polymers were ground for 10 min using an Angstrom (Belleville, MI) pulverizer. Pictures were

taken of the NIP under a light microscope to determine the range of particle sizes that were included in a given size range after processing by using the sieves and sieve shaker.

RESULTS AND DISCUSSION

As noted earlier the extraction solvent used to treat the final MIPs became light orange and appeared to be extracting tetracycline from these polymers even after 11.5 days. The difficulty of extracting the template molecule is a common problem during the preparation of MIPs. This problem often occurs because some of the template is buried deep in the polymer.[17] Unfortunately, even with successful extraction, the low accessibility for many of the remaining binding sites can be problematic in HPLC applications of the resulting MIPs.[17] After further flushing the 15 mm x 2.1 mm I.D. MIP column with acetonitrile using an HPLC pump, the solvent was then colorless, indicating that no further tetracycline was being removed from the polymer after this treatment. This size of column was used in all further studies because the back pressure of a similar 50 mm x 3 mm I.D. tetracycline MIP column was too high for practical use.

In initial testing of the 15 mm x 2.1 mm I.D. column, a large peak was observed almost immediately following each injection of tetracycline, which may have been due to overloading of the column. However, there was also a broad peak with a retention time of 40 min which was believed to be due to the retention and later elution of tetracycline. Further injections of tetracycline samples produced no observable peaks when water, acetonitrile and combinations of both solvents were used as the mobile phase. Acetonitrile works well as a mobile phase for MIPs based on methacrylic acid. These polymers are also resistant to this solvent.[21] When acetonitrile was used to regenerate the column, the solvent was clear, indicating that no appreciable amount of retained tetracycline was eluting under these conditions. A second 15 mm x 2.1 mm I.D. column

was then packed with the same tetracycline MIP, but the back pressure of this second column quickly reached the pump's limit during testing.

The effect of particle size on column performance was examined by preparing 15 mm x 2.1 mm I.D. columns that contained NIPs of various sizes. The 25-45 μm fraction of the NIPs appeared to have a back pressure that was lower than typical column back pressures. This may have been due to inefficient packing of that particular column since all of the other fractions (<25 μm, ≥25 μm and <45 μm, ≥45 μm and <53 μm, ≥53 μm and <106 μm, ≥ 106 μm) gave backpressures that were too high. The limit of the HPLC pump was either reached or the pump was stopped in order to prevent reaching the upper pressure limit in each of these cases. A possible explanation for the high back pressures seen with these supports was that the frits at the column ends became clogged. This may have been the case if small particles were attached to larger ones and stayed in the larger size fraction during the sieving process. This hypothesis was possibly confirmed when the packing of a NIP column was attempted by using a valve and HPLC pump instead of a slurry packer. Injections of the NIP slurry were made and the back pressure quickly reached the upper limit. When the column was removed, there was no packing visible inside except for a small amount of slurry that was found on the frit at the bottom. If fine particles were attached to larger ones and stayed in the sieved fraction for larger particles, they would have clogged the frit during the packing process and led to this type of behavior.

When examined under a microscope, the NIPs from the >106 μm and 53-106 μm size fractions contained a significant amount of fine powder. These small particles were believed to be the source of the clogged frits and high back pressures that were seen

during the column packing process. As shown in Figure 3-3, longer and higher intensity grinding of the NIPs gave smaller particle sizes. Although grinding ensured more uniform particle sizes were present, as would be desirable for HPLC supports, this approach also introduced unwanted fine particles. For instance, Figure 3-4 shows a picture of the NIPs in the 53-106 μm size fraction sieve after it was allowed to settle in water for 1 day.

Wet-sieving was done in an effort to remove fine particles. It appeared successful in this respect but the particle sizes seemed irregular under the microscope. Grinding afterwards seemed to reintroduce some unwanted fine powder. However, dry sieve shaking for 60 min appeared to remove fine particles, even though the particle size distribution seemed fairly large for the >106 μm fraction NIP. This distribution appeared more uniform for the 53-106 μm fraction. Wet sieving followed by grinding and extended dry sieving appeared to give the best particle size distribution while also excluding fine powder from the final preparation.

Because it can be difficult to obtain MIPs that are suitable for chromatography through the use of bulk polymerization techniques, alternative methods of preparation were sought in this study. Crushing and sieving procedures can result in a large loss of material and destruction of binding sites and, although resulting MIPs can have high affinity and selectivity, they often suffer from low capacity and poor site accessibility. Ideally, the preparation method should give a high yield of particles with a uniform pore size and particle size distribution that does not depend on the monomers, template molecules, or solvents that are used.[22, 23]

Figure 3-3. Particle size distribution of tetracycline molecularly imprinted polymer. Typical precision for these measurements was 0.003-0.14%.

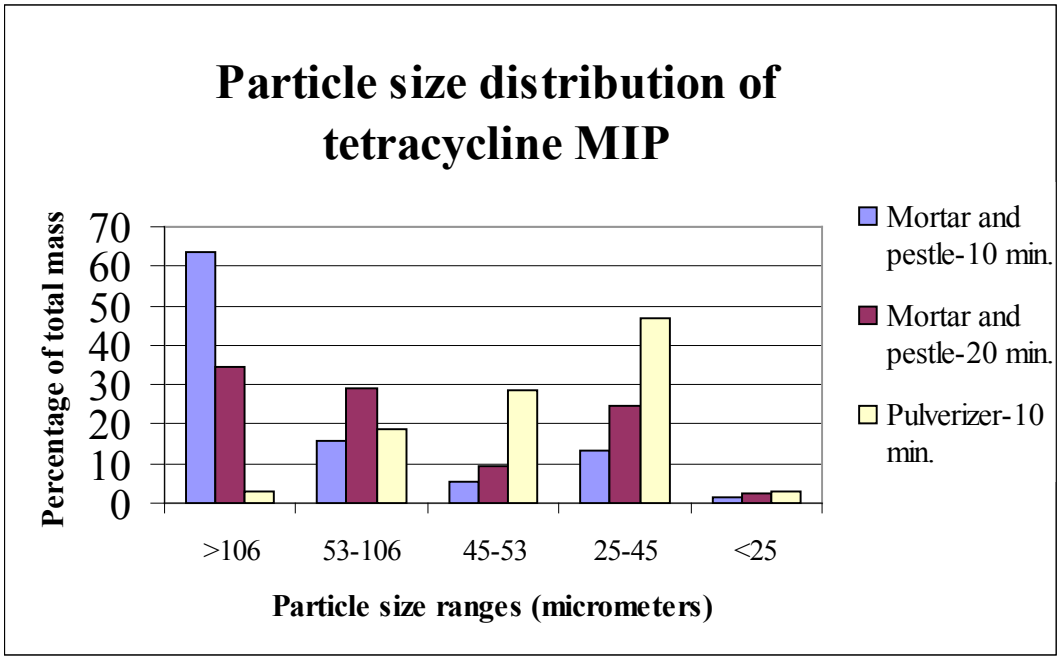
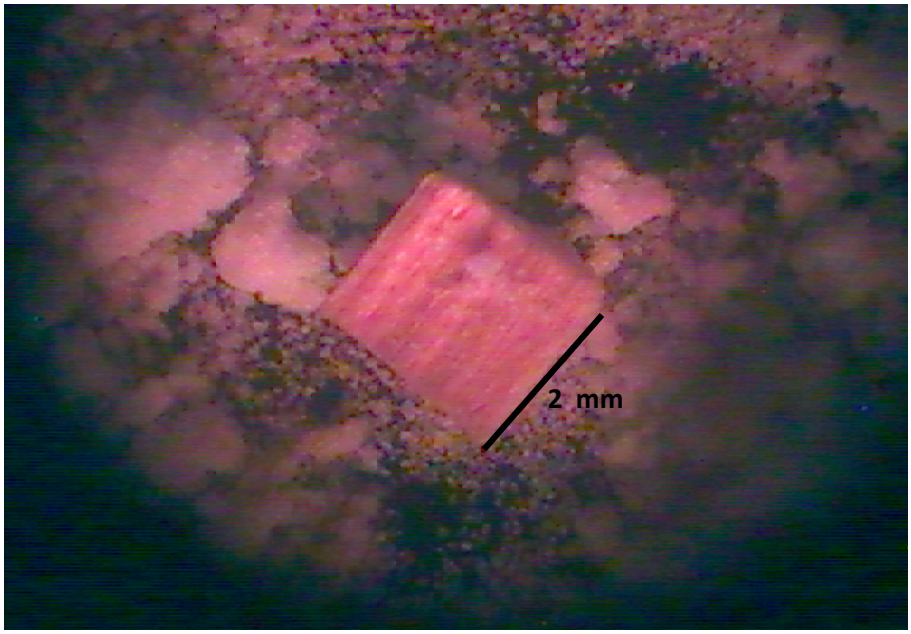

Particle size distribution of tetracycline MIP
Percentage of total mass
70
60
50
40
30
20
10
0
>106
53-106
45-53
25-45
<25
Particle size ranges (micrometers)
Mortar and pestle-10 min.
Mortar and pestle-20 min.
Pulverizer-10 min.

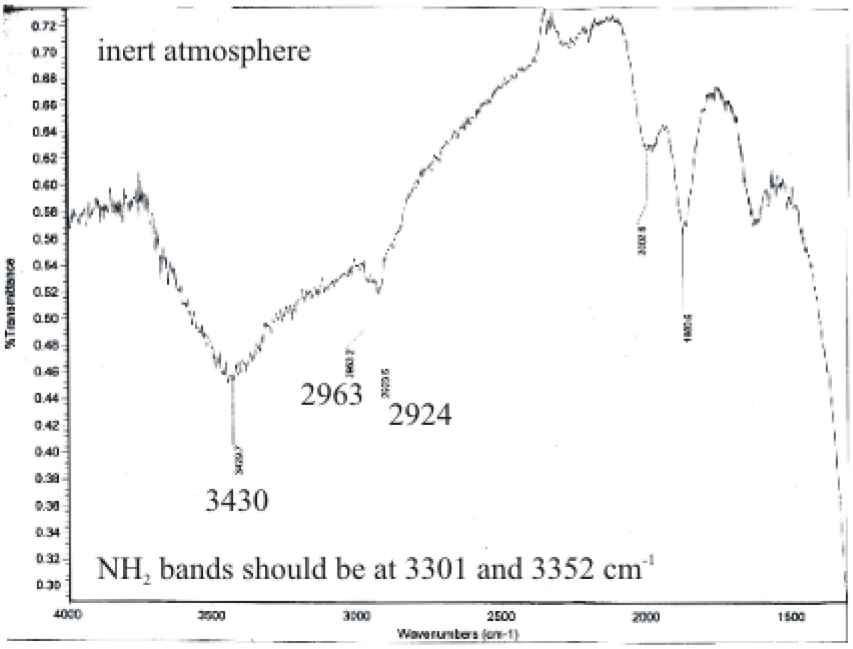
Figure 3-4. Image seen under a light microscope for the 53-106 μm fraction of a NIP.


2 mm

In a previously reported method, the grafting of polymerization azo initiators onto silica was carried out, followed by the synthesis of MIP films on the silica particles.[22] In an attempt to create MIPs that were suitable for the on-line extraction of tetracycline, the grafting reaction of (3-aminopropyl)-triethoxysilane (APS) to silica support particles was next carried out in this study. This reaction was conducted under ambient conditions, under an inert atmosphere, and with a pyridine catalyst. A representative IR spectrum of the silica-APS that was prepared under inert atmosphere is shown in Figure 3-5. The two small NH_2 bands, interpreted as being the result of hydrogen bonding between the amine group and unreacted silanol groups,[20] were absent at 3301 and 3352 cm^{-1}. It is possible that the yield in this case was higher than that obtained in Ref. 19 and that there were few remaining silanol groups; it is also possible that condensation of the APS had taken place because of a trace amount of moisture that may have been present,[20] thus giving a low yield of silica APS.

The silica APS was further reacted with the initiator azobiscyanovaleric acid (ACVA) in order to anchor this agent to the particle's surface for use in future polymerization reactions. The IR spectrum obtained after this reaction is shown in Figure 3-6 and is compared to the spectrum for this product that was obtained in Ref. 19. The stretching vibrations of the amide groups are absent in this new IR spectrum.

Figure 3-5. IR spectrum of silica APS synthesized under an inert atmosphere (top), as compared with a spectrum from Ref. 19 (reproduced with permission, bottom).


inert atmosphere
2963
2924
3430
NH2 bands should be at 3301 and 3352 cm-1
%Transmittance
Wavenumbers (cm-1)

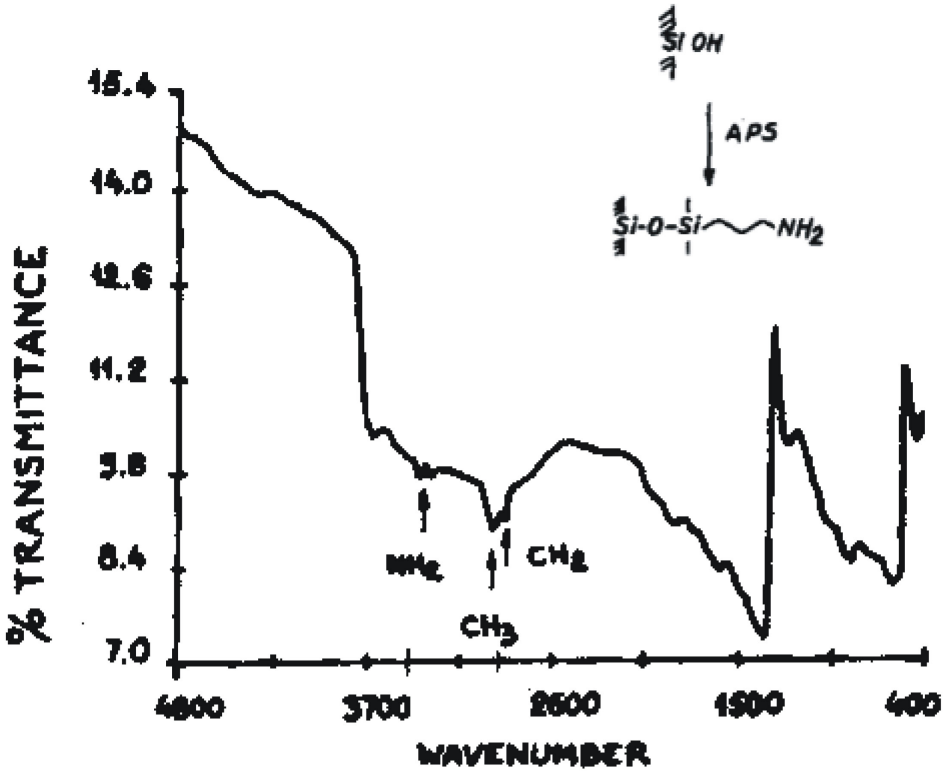
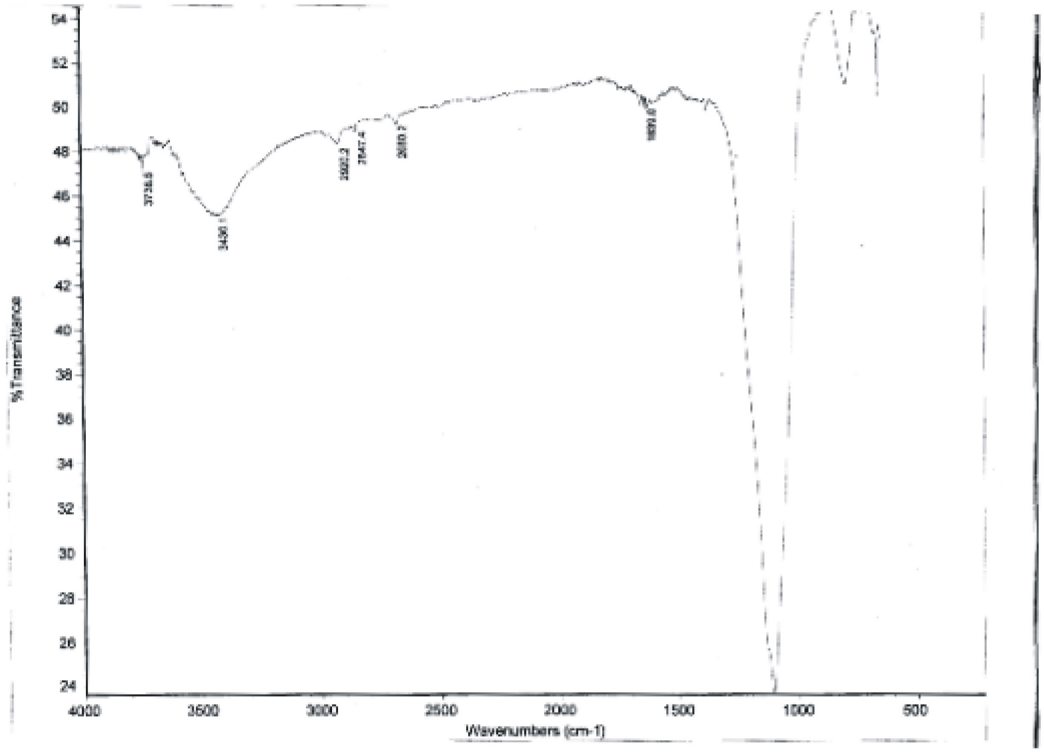
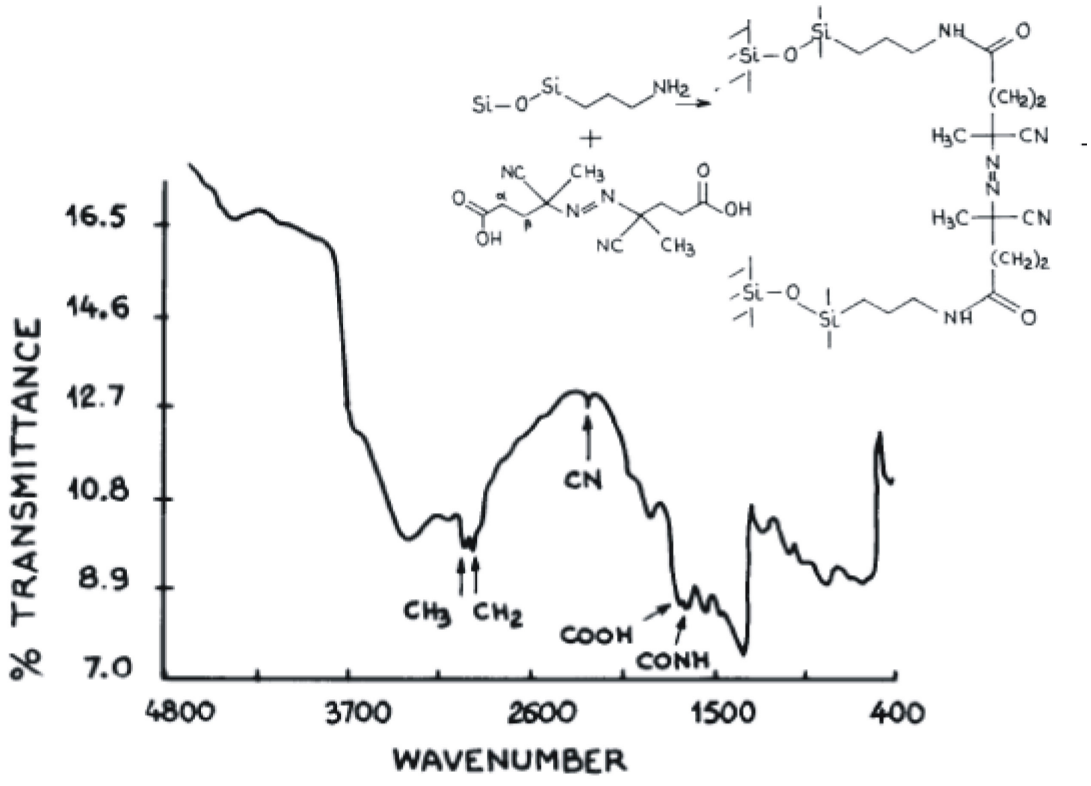

SI OH
APS
Si-O-Si
NH2
% TRANSMITTANCE
15.4
14.0
12.6
11.2
9.8
8.4
7.0
NH2
CH2
CH3
4000
3700
2600
1500
400
WAVENUMBER

Figure 3-6. IR spectrum of silica APS ACVA synthesized under an inert atmosphere (top), as compared with a spectrum from Ref. 19 (reproduced with permission, bottom).


%Transmittance
Wavenumbers (cm-1)
4000
3500
3000
2500
2000
1500
1000
500


% TRANSMITTANCE
16.5
14.6
12.7
10.8
8.9
7.0
4800
3700
2600
1500
400
WAVENUMBER
CH3
CH2
CN
COOH
CONH

CONCLUSIONS

This study explored MIP preparation for use in the on-line extraction of tetracycline for HPLC. Although some results indicated that an MIP for this analyte could be produced and that it had reasonably high retention for tetracycline, the resulting support often gave high back pressures and was difficult to use in making reproducible columns. The high backpressures appeared to be a result of bulk polymerization methods and the generation of fine particles that seemed to attach to larger particles and follow with the larger particles though a sieving process. Wet sieving followed by extended dry sieve shaking may have removed most of these fine particles and allowed a more uniform particle size of the MIPs to be maintained.

An alternative approach based on the synthesis of MIPs on silica particles was also pursued. A comparison of IR spectra for the intermediates and products from this synthesis differed with reference spectra from the literature. However, this may have been due to low recoveries of the product or the presence of trace amounts of water in the reagents or starting materials. Future optimization of this approach is also needed.

Other possible strategies for MIP synthesis include multi-step swelling polymerization, suspension polymerization and precipitation polymerization. These techniques have produced spherical MIPs with uniform sizes that are well suited for chromatographic applications.[23]

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CHAPTER 4

COMPARISON OF VARIOUS α_1-ACID GLYCOPROTEIN IMMOBILIZATION METHODS FOR USE IN THE SEPARATION OF RACEMIC MIXTURES OF PHARMACEUTICAL AGENTS

INTRODUCTION

The chiral separation of pharmaceutical agents is important in the development and production of drugs because often only one enantiomer is active while the other can have unwanted side effects and no beneficial activity.[1] Although there are regulations requiring production of the desired enantiomer or separation of enantiomers, no process is completely efficient. It is necessary to monitor industrial production of pharmaceuticals to ensure enantiomeric purity.[2] In addition, some drugs are used as racemic mixtures and their stereochemistry affects their pharmacological action and toxicity.[3] Also, the ability to measure the relative amounts of the chiral forms of drugs in plasma and urine samples can provide useful pharmacokinetic information and can indicate whether one drug form is more effective than another.[1]

α_1-Acid glycoprotein (i.e., orosomucoid or AGP) immobilized to chromatographic supports has been used effectively in chiral separations for a wide variety of compounds, such as benzodiazepines,[4] non-steroidal anti-inflammatory drugs (NSAIDs),[5] calcium antagonist drugs[6] and β-receptor blocking agents.[7] Separations in AGP columns can be adjusted by adding low concentrations of alcohol to the mobile phase and by varying the

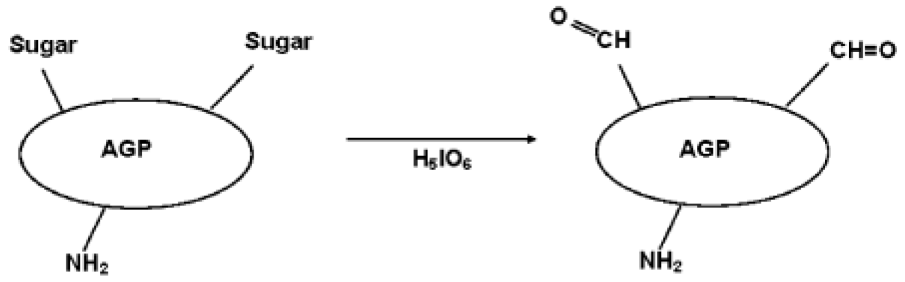
pH.[1, 8] AGP provides better retention and resolution for many chiral drugs than other proteins because of AGP's higher affinity for the compounds to which it binds.[8]

AGP is an important component of blood plasma and has a molecular mass of approximately 41,000 g/mol.[1, 8, 9] It is produced by the liver and is involved in transporting various substances in the body. It is thought to be involved in the immune response because its levels rise during many disease states.[8] The structure of AGP consists of a single polypeptide chain made up of 181 amino acids and five carbohydrate groups. Of all the potential ways the carbohydrate groups can be attached to AGP, only 12 to 20 combinations have been detected. Disease states also affect the extent of AGP glycosylation as well as the arrangement of the carbohydrate groups, which may influence binding by this glycoprotein to drugs.[8]

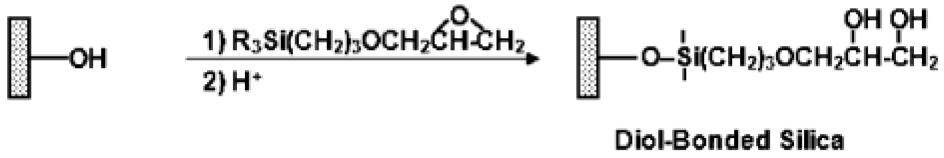
There is interest in the chromatographic resolution of racemic mepivacaine and other drugs by companies such as Regis Technologies, Inc. (Morton Grove, IL). Mepivacaine is a local anesthetic that is used as a racemic mixture.[3] To meet this need, several methods for the immobilization of AGP onto silica were tested in this chapter for preparing columns for these types of separations. One method that has been reported for the immobilization of AGP was developed by Xuan and Hage;[10] this method involves the mild oxidation of the carbohydrate regions of AGP to generate aldehyde groups, which are then used for the immobilization of AGP to hydrazide-activated silica. Figure 4-1 shows the reaction scheme for this approach. This immobilization method for AGP was developed to create protein supports that show good agreement with the drug binding behavior of AGP in solution.[10]

Figure 4-1. (A) Oxidation of AGP by periodic acid, (B) preparation of hydrazide-activated silica, and (C) immobilization of oxidized AGP to hydrazide-activated silica. Reproduced with permission from Ref. 10.

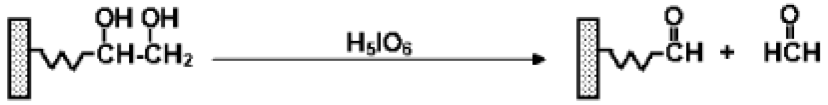
A Oxidation of AGP



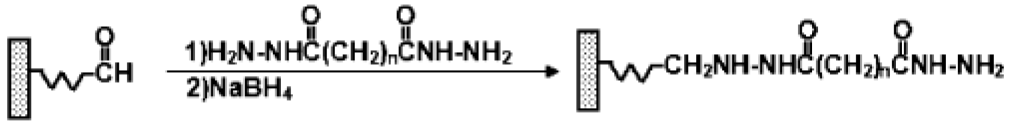
B Preparation of Hydrazide-Activated Silica



Diol-Bonded Silica



Aldehyde-Activated Silica



Hydrazide-Activated Silica

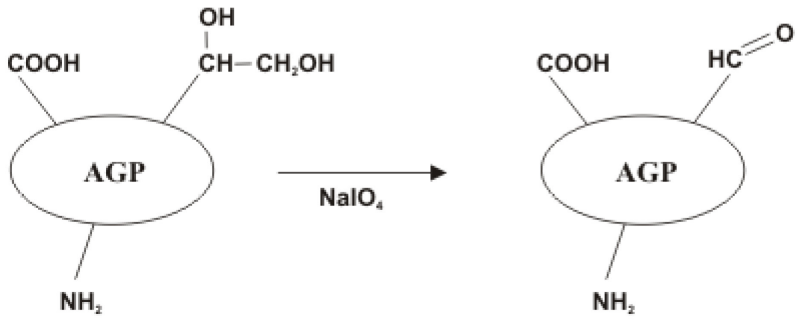
C Immobilization of Oxidized AGP

Immobilized AGP

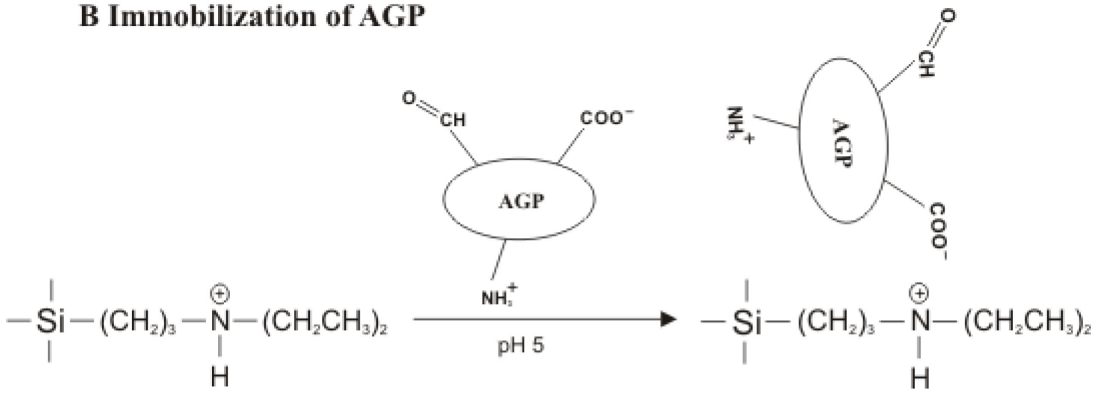
A European patent by Hermansson[1] describes two other methods which have also been employed for the immobilization of AGP to supports such as silica. These two methods, also based on the oxidation of AGP, were investigated in the research described in this chapter. Stationary phases resulting from use of these methods have been used for chiral separations involving the drugs disopyramide, mepensolate bromide, RAC 109, bupivacaine, mepivacaine, propiomazine and oxyphencyclimine.[1] The first of these methods involves oxidation of the alcoholic hydroxyl groups of AGP, followed by cross-linking of this glycoprotein to itself and immobilization to a support that contains a tertiary amine or quaternary ammonium groups. The steps involved are illustrated in Figure 4-2. In this approach, the positive groups on an amine-containing support attract the protein's negative acidic groups that are present at the pH employed for immobilization. The second approach by Hermansson involves covalent coupling of amine groups on AGP to epoxide-activated silica,[1] as shown in Figure 4-3. The first method by Hermansson has been reported to produce a greater final protein content than the second method, but both techniques were evaluated in this work for use in chiral separations for the drugs of interest.

Figure 4-2. (A) Oxidation of AGP by periodic acid, (B) adsorption of AGP to *N,N'*-diethylaminopropyl silica, and (C) cross-linking of AGP on the support.

A Oxidation of AGP



B Immobilization of AGP



C Cross-linking of AGP

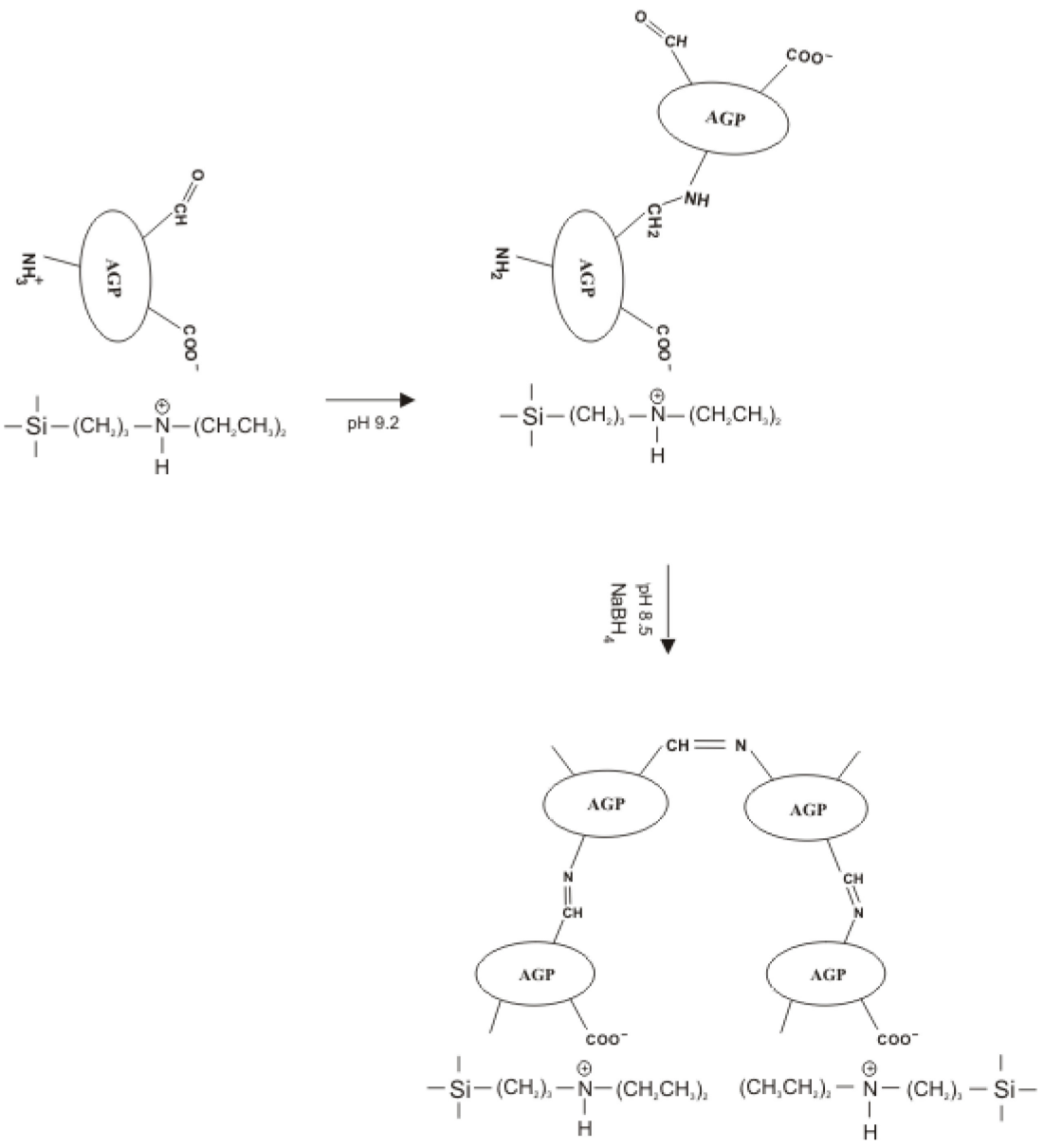
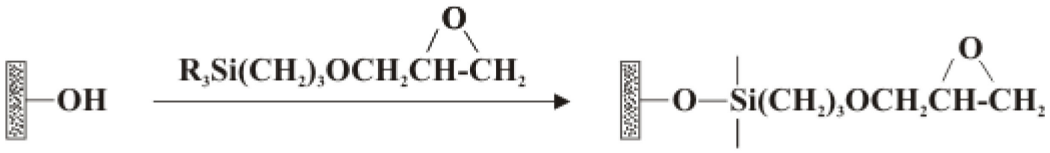
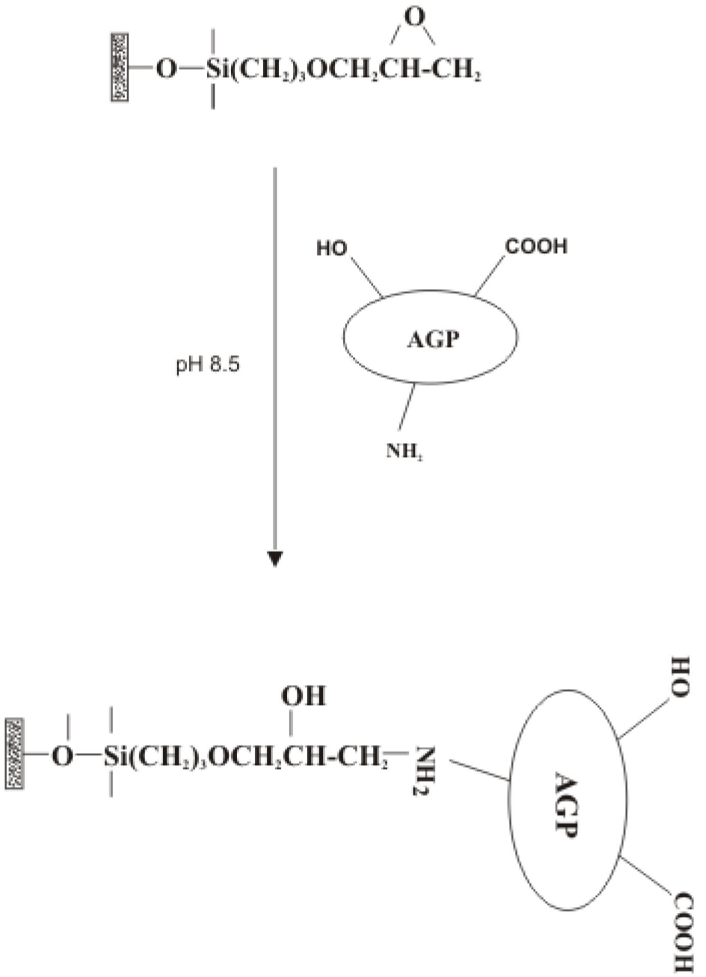


Figure 4-3. (A) Preparation of epoxide-activated silica and (B) immobilization of AGP to this type of support.

A Preparation of epoxide activated silica



B Immobilization of AGP



EXPERIMENTAL

Reagents

Lucifer yellow CH (LyCH), ethylene glycol, periodic acid, sodium metaperiodate, oxalic dihydrazide, glycerol, sodium borohydride, glycidylpropyltrimethoxysilane (GPS), racemic propranolol and mepivacaine were obtained from Sigma-Aldrich (St. Louis, MO). Nucleosil 300-5 silica (300 Å pore size, 5 µm diameter) was obtained from Macherey-Nagel (Düren, Germany). *N,N*-Diethylaminopropyl silica was obtained from ES Industries Chromega Columns (West Berlin, NJ). All other chemicals were of the purest grades available. All solutions were prepared using water from a Nanopure purification system (Barnstead, Dubuque, IA). Human AGP was obtained from Sigma (St. Louis, MO) and had a purity of at least 99%.

Apparatus

Slide-A-Lyzer 7K dialysis cassettes (7,000 MW cutoff) were purchased from Pierce (Rockford, IL). Econo-Pac 10DG disposable, prepacked desalting gravity flow columns were obtained from BioRad (Hercules, CA). The chromatographic system consisted of a LC-10AD solvent delivery system from Shimadzu (Kyoto, Japan), a LabPro injection valve from Rheodyne (Oak Harbor, WA) and a UV-2075 Plus Intelligent absorbance detector from Jasco (Easton, MD). Data were collected using software and an interface from National Instruments (Austin, TX). Data analysis was performed using Peak Fit (SeaSolve Software). All supports were downward-slurry packed using an HPLC column packer from Alltech (Deerfield, IL).

AGP Immobilization Method 1

Oxidation of AGP

The first procedure for immobilizing AGP was based on that described by Xuan and Hage (Figure 4-1).[10] To oxidize AGP for this immobilization method (see Figure 4-1A), 14 mL of a 5 mg/ml solution of AGP were prepared in pH 7.0, 20 mM sodium acetate buffer containing 0.15 M sodium chloride. This solution was combined in a 1:1 (v/v) ratio with 20 mM periodic acid in the same buffer, and the AGP was allowed to react with the periodic acid at room temperature in the dark. After 15 min, the oxidation of AGP was quenched by adding 7 mL of ethylene glycol (i.e., 0.25 mL ethylene glycol per milliliter sample). After 2 min, dialysis was carried out on this mixture for 2 h at 4 °C against 2 L of pH 7.0, 20 mM sodium acetate buffer containing 0.15 M sodium chloride. Three more dialysis cycles were carried out against fresh 2 L portions of pH 7.0, 0.10 M potassium phosphate buffer, with each dialysis cycle being conducted for 2 h at 4 °C. AGP that was prepared using this procedure has been previously reported to have an average of five aldehyde groups generated per AGP molecule.[10]

Immobilization of AGP

Hydrazide-activated silica was prepared as described by Ruhn et al.[11], as illustrated in Figure 4-1B. Oxidized AGP was immobilized to the hydrazide-activated silica by using the procedure that is summarized in Figure 4-1C. The hydrazide-activated silica (roughly 3.6 g) was placed in 12 mL of pH 7.0, 0.10 M potassium phosphate buffer and sonicated under vacuum for 15 min to remove any air bubbles. A 40 mL portion of

approximately 2 mg/mL oxidized AGP in pH 7.0, 0.10 M potassium phosphate buffer was added to the silica slurry, and the resulting suspension was split into two equal portions. Each portion was sonicated under vacuum for 5 min. The test tubes containing these suspensions were placed onto a shaker and the immobilization reaction was allowed to take place at 4 °C. After 3 days, shaking was stopped, the reaction mixture was centrifuged, and the supernatant was removed. The silica was washed four times with pH 7.0, 0.10 M potassium phosphate buffer and four times with deionized water. Any remaining hydrazide groups were neutralized by adding 3.4 g of glyceraldehyde, which represented a 670-fold excess of glyceraldehydes versus the support's original hydrazide content.[11] The resulting mixture was shaken for 6 h at 4 °C and then washed four times with pH 7.0, 0.10 M potassium phosphate buffer and stored in this buffer at 4 °C until use. The final amount of immobilized AGP was estimated by a protein assay to be 15.8 mg protein/g silica.[10]

AGP Immobilization Method 2

The procedure for this approach was a modified version of Method 1 by Xuan and Hage.[10] One change made was that only 4.52 mL of a 5 mg/ml AGP solution was prepared for oxidation, with the amounts of 20 mM periodic acid solution and ethylene glycol being adjusted accordingly. In addition, instead of using four dialysis cycles to purify the AGP following the oxidation step, a desalting step was performed followed by two dialysis cycles. The buffer used for desalting was pH 7.0, 20 mM sodium acetate buffer that contained 0.15 M sodium chloride. Dialysis was performed for 2 h against pH

7.0, 0.10 M potassium phosphate buffer at 4 °C and then overnight under the same conditions. In the remainder of this method, 1.13 g of hydrazide-activated silica were used for immobilization, along with 10.5 ml of the oxidized AGP solution. The immobilization reaction was allowed to take place for 3.5 days and the amount of glyceraldehyde that was added made up a 200-fold excess versus the support's original hydrazide content.[11]

AGP Immobilization Method 3

The procedures followed in this method have previously been described in the patent by Hermansson.[1] In this method, 50.65 mg of AGP were placed into 10 mL of pH 5.05, 0.01 M sodium acetate buffer and kept at 4 °C. A 0.03 g portion of sodium metaperiodate was then added, giving a 120-fold mol excess of the metaperiodate versus AGP. This mixture was allowed to react for 1 h in the dark at 4 °C. Glycerol was then added in a 19-fold mol excess versus metaperiodate to quench the oxidation reaction and was allowed to react with the mixture at room temperature for 10 min. The AGP was purified using disposable desalting gravity flow columns containing pH 5.0, 0.01 M sodium acetate buffer. The purified AGP in the pH 5.0 buffer was then mixed with 2.5 g of *N,N'*-diethylaminopropyl silica, as supplied by Regis Technologies. According to Hermansson, this step should have resulted in the adsorption of AGP to the surface of the *N,N'*-diethylaminopropyl silica through ionic interactions. [1] After allowing this adsorption to occur for approximately 3 h, the silica was centrifuged and washed three times with 0.03 M, pH 9.2 borate buffer. The pH was raised in this step so that the amino

groups in the peptide chain of AGP would be mainly present in a neutral form that could undergo a cross-linking reaction with aldehyde groups on the oxidized carbohydrate regions of adjacent AGP molecules. [1] This cross-linking reaction and immobilization step was allowed to continue at 4 °C in the dark and with continuous shaking for approximately 17 h. The silica was then washed with pH 8.5, 0.1 M borate buffer and reacted with excess sodium borohydride at room temperature to reduce the remaining aldehyde groups and convert the Schiff bases that formed during cross-linking into stable secondary amine linkages. The final support was washed three times with pH 7.0, 0.10 M potassium phosphate buffer and stored in this buffer at 4° C until use.

AGP Immobilization Method 4

The procedure followed in this approach was the same as in Method 3 by Hermansson[1] but used twice the amount of AGP for the immobilization process.

AGP Immobilization Method 5

This approach was also one of the methods that has been previously described by Hermansson and involved the covalent coupling of AGP to silica particles.[1] In this method, 2.5 g of Nucleosil 300-5 silica were first reacted with 3-glycidoxypropyltri-methoxysilane in pH 5.5, 0.01 M sodium acetate buffer at 90 °C for 5 h. The resulting epoxide-activated silica was reacted with 103 mg AGP in a pH 8.5 buffer; immobilization in this buffer was allowed to take place for 41 h at 4 °C. The amounts of reagents that

were used in these steps were all selected based on similar and previously-described methods for other proteins.[10] The final support was washed several times with pH 7.0, 0.1 M potassium phosphate buffer and stored in this buffer at 4 °C prior to use.

Chromatographic Conditions

Each AGP silica support was downward-slurry packed at 4000-4500 psi for 1-5 h into separate 100 mm x 4.0 mm I.D. or 36 mm x 2.1 mm I.D. stainless steel columns. The packing solution was pH 7.0, 0.10 M potassium phosphate buffer. The injected samples had a volume of 5 µL. The flow rate was 1 ml/min, unless otherwise indicated. The mepivacaine and propranolol samples each had a concentration of 100 µM and were prepared in corresponding mobile phase. The mobile phase was pH 7.0, 0.10 M potassium phosphate buffer; pH 7.0, 0.01 M sodium phosphate buffer; or pH 7.0, 0.01 M sodium phosphate buffer containing 9% isopropyl alcohol. The detection wavelength for mepivacaine was 210 nm and the detection wavelength for propranolol was 225 nm. Each sample was injected in triplicate, unless otherwise indicated, and an average was taken for retention times used in the calculation of retention factors and other parameters. All experiments were performed at room temperature.

RESULTS AND DISCUSSION

The following section summarizes the results that were obtained for each of the given immobilization methods when the corresponding AGP columns were tested for use in the chiral separation of mepivacaine or propranolol.

Method 1

A 100 mm x 4.0 mm I.D. AGP column that was prepared using Method 1 was evaluated using one sample that contained 100 μM racemic propranolol and that was injected onto the column in the presence of pH 7.0, 0.10 M potassium phosphate buffer at 1.0 ml/min and room temperature. When these conditions were used along with a detection wavelength of 225 nm, a separation of the enantiomers was observed for propranolol. The peaks were quite broad, as is common for such columns under aqueous conditions; however, close to baseline resolution was obtained, with retention times of approximately 68 and 99 min and retention factors of 62 and 91 for the two enantiomers. This behavior indicated that the AGP column prepared by Method 1 had both strong retention and good stereoselectivity for these two enantiomers in the presence of only a pH 7.0 phosphate buffer.

The results for this method were in general agreement with those seen in a previous study for the same type of AGP column in which separation of propranolol was carried out under similar conditions with some organic modifier also being present in the mobile phase. In that study, a 50 mm x 4.1 mm I.D. column was used along with a mobile phase that consisted of pH 7.4, 0.067 M potassium phosphate buffer containing 2% (v/v) 2-propanol, operated at a temperature of 37°C and a flow rate of 2.0 ml/min.[12]

It was interesting to note in comparing these conditions that the addition of 2-propanol to the mobile phase acted to decrease the degree of retention while also sharpening the peaks for the two enantiomers of propranolol. However, the addition of 5% (or more) propanol gave rise to a sufficiently low retention for propranolol that no separation was then observed for the separate enantiomers of this drug.[12]

When the same column was used by Regis Technologies for separation of the enantiomers for mepivacaine, no resolution of these chiral forms was initially noted (the retention time was 6.4 min and the retention factor was 4.8). Mepivacaine was chosen by Regis for this work because it has been noted in work with other types of protein columns that is more difficult to achieve chiral separation for this compound than for other drugs. The mobile phase used for this separation was 9% 2-propanol in pH 7.0, 0.01 M sodium phosphate buffer, which is typically used by this company for chiral separations on AGP columns. The mepivacaine sample was prepared in this mobile phase and was injected onto the column at a flow rate of 0.9 ml/min at room temperature. The lack of a chiral separation in this case was not surprising, given the lack of stereoselectivity that was previously noted for propranolol when using a mobile phase that contained more than 5% 2-propanol.

Method 2

A second group of columns were prepared using Method 2, in which the same general immobilization scheme was employed as in Method 1 but now modifying this approach to yield a higher protein on the final support. Two columns were prepared using Method 2; a 100 mm x 4.0 mm I.D. AGP column and a 36 mm x 2.1 mm I.D. AGP

column. For the 100 mm x 4.0 mm I.D. column, the mepivacaine and propranolol samples were analyzed under three different conditions. First, 100 μM samples of each drug were prepared in pH 7.0, 0.10 M potassium phosphate buffer, which was also used as the mobile phase. Two samples of propranolol and three samples of mepivacaine were injected. Under these conditions, a separation was achieved for both compounds, as shown in Figures 4-4 and 4-5 for propranolol and mepivacaine, respectively. The selectivity factor for propranolol was 1.11 and the resolution between the peaks for its enantiomers was 0.85 (the retention times were 82 and 90 min and the retention factors were 74 and 82). The corresponding selectivity factor and resolution for the mepivacaine peaks were 1.43 and 1.11, respectively, along with retention times of 10 and 14 as well as retention factors of 8.1 and 12. These results indicated that a slightly modified form of the method of Xuan and Hage[10] could be used to prepare an AGP column that allowed the chiral separation of either propranolol or mepivacaine in the presence of a pH 7.0 buffer alone and at room temperature.

The samples were next injected onto the 100 mm x 4.0 mm I.D. column in the presence of pH 7.0 phosphate buffer after rinsing this column with water. This rinsing step was done to determine whether water could be used as a storage solution for such a column without affecting the ability to later use the column for a chiral separation. It was found that both of the tested drugs again gave a chiral separation on the tested column (e.g., see Figure 4-6). The selectivity factors and the resolution improved slightly for both drugs (the selectivity factors increased by 0.7 and 0.1 for propranolol and mepivacaine, respectively and the resolution increased by 0.3 and 0.2 for propranolol and

mepivacaine, respectively) after the rinsing step, which may have reflected the removal of some remaining reagents after the immobilization step.

Figure 4-4. Chiral separation of propranolol using a 100 mm x 4.0 mm I.D. AGP column prepared using Method 2. The sample was prepared in pH 7.0, 0.10 M potassium phosphate buffer, which was also used as the mobile phase. Other conditions are given in the text.

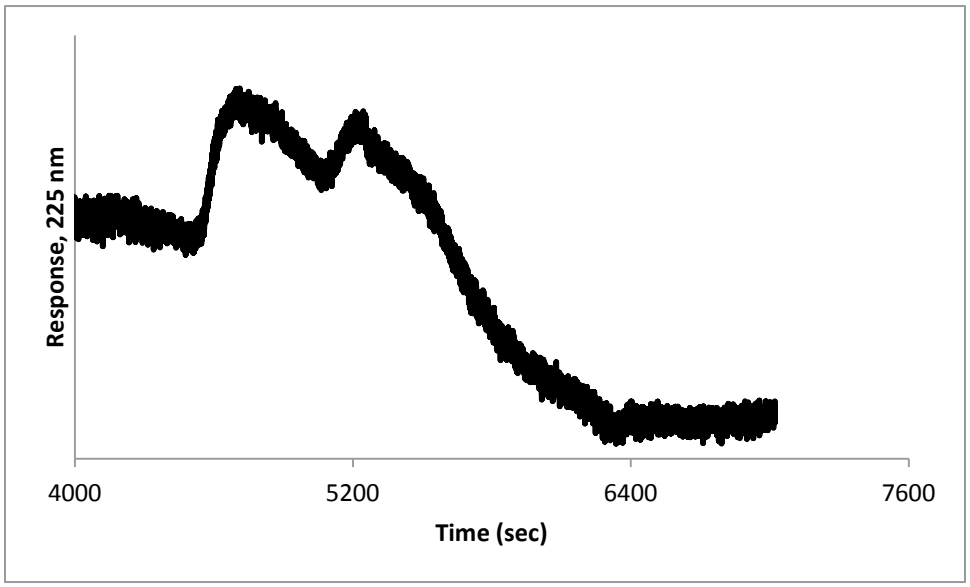

Response, 225 nm
4000
5200
6400
7600
Time (sec)

Figure 4-5. Chiral separation of mepivacaine using a 100 mm x 4.0 mm I.D. AGP column prepared using Method 2. The sample was prepared in pH 7.0, 0.10 M potassium phosphate buffer, which was also used as the mobile phase. Other conditions are given in the text.

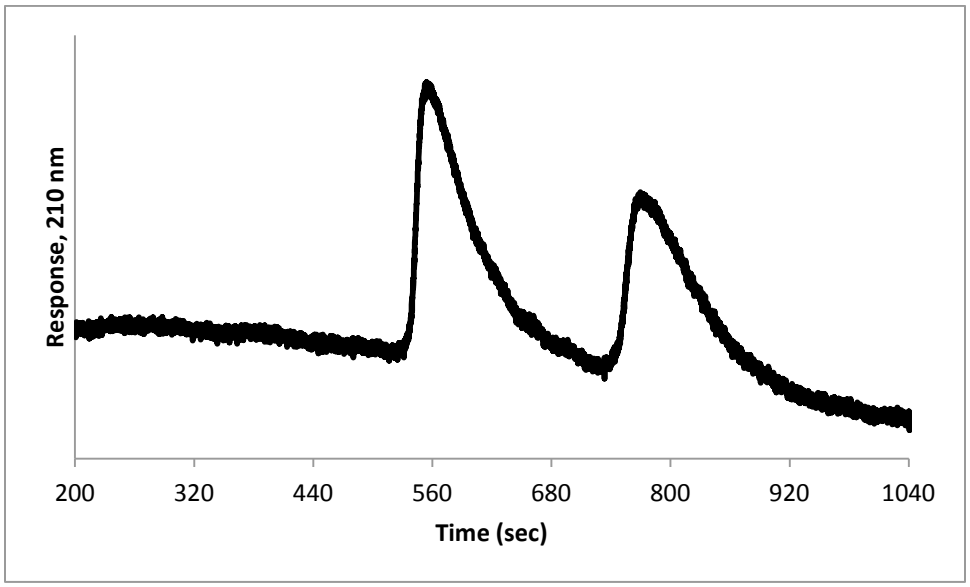
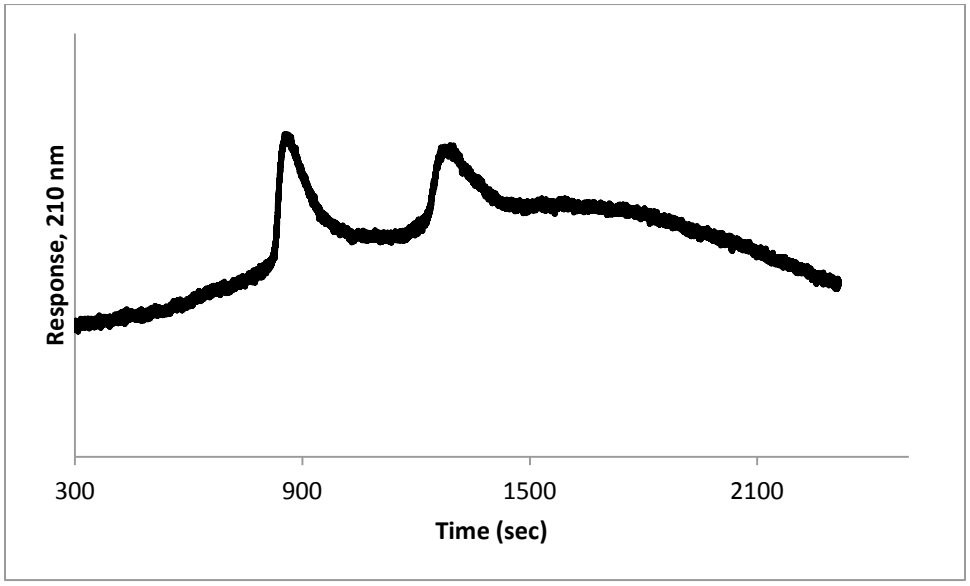

Response, 210 nm
200
320
440
560
680
800
920
1040
Time (sec)

Figure 4-6. Chiral separation of mepivacaine on a 100 x 4.0 mm AGP column prepared by using Method 2 and after rinsing with water. The sample was prepared in pH 7.0, 0.10 M potassium phosphate buffer, which was also used as the mobile phase. Other conditions are given in the text.


Response, 210 nm
300
900
1500
2100
Time (sec)

After another rinsing step of the 100 x 4.0 mm AGP column with water, samples of propranolol and mepivacaine were prepared and injected (one mepivacaine sample was injected) in the presence of pH 7.0, 0.010 M sodium phosphate buffer that contained 9% isopropyl alcohol. This solvent composition was again chosen because of its routine use by Regis in the evaluation of chiral columns. As mentioned previously, it is known that chiral separations using proteins such as AGP are affected by the addition of small amounts of organic modifiers in the mobile phase.[2, 13] For instance, placing organic modifiers such as 2-propanol in the mobile phase has been shown to affect the binding of propranolol to AGP to a greater extent than changes in pH, ionic strength or temperature.[10] This can be explained by the fact that hydrophobic interactions are important in the binding of propranolol to AGP, which involves nonpolar residues on the protein.[12]

Figure 4-7 shows that propranolol was not resolved in the presence of pH 7.0 phosphate buffer and 9% 2-propanol for the 100 x 4.0 mm AGP column that was prepared using Method 2 (the retention time was 37 min and the retention factor was 33). This result was expected based on the results that were obtained with Method 1.[10] However, a partial resolution of the mepivacaine enantiomers was observed under these conditions (Figure 4-8) with retention times of 4.9 and 5.2 min and retention factors of 3.5 and 3.8. Uncharged modifiers added to the mobile phase usually result in reduced retention and increased efficiency but also in loss of chiral separation.[14] In this case, reduced retention was observed along with an increase in efficiency for mepivacaine. For both drugs, the separation factor decreased with increasing 2-propanol content, which

resulted in a loss of chiral selectivity. Such an effect can be explained by a possible change in conformation for AGP.[12]

Figure 4-7. Injection of racemic propranolol on a 100 x 4.0 mm AGP column prepared using Method 2 and in the presence of pH 7.0, 0.010 M sodium phosphate buffer that contained 9% isopropyl alcohol. Other conditions are given in the text.

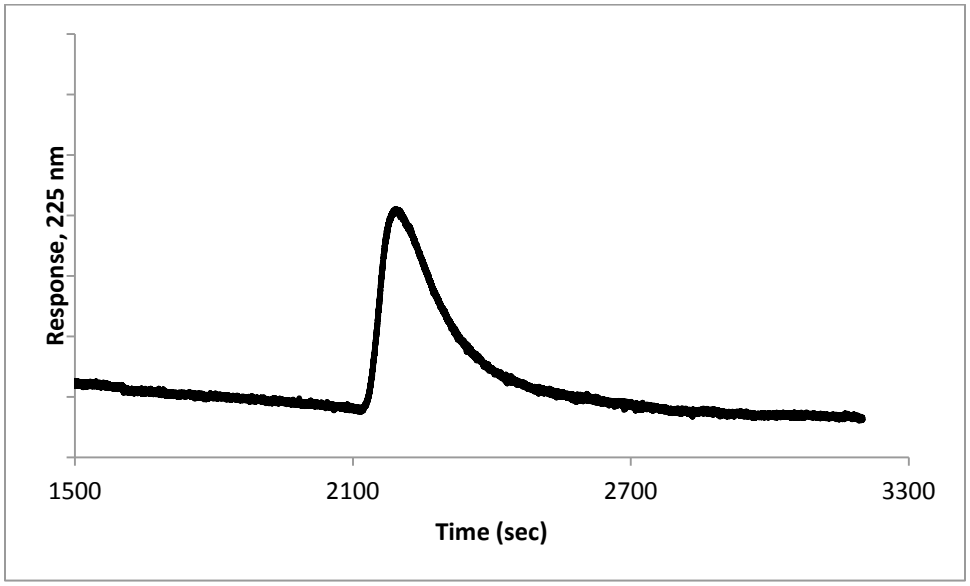
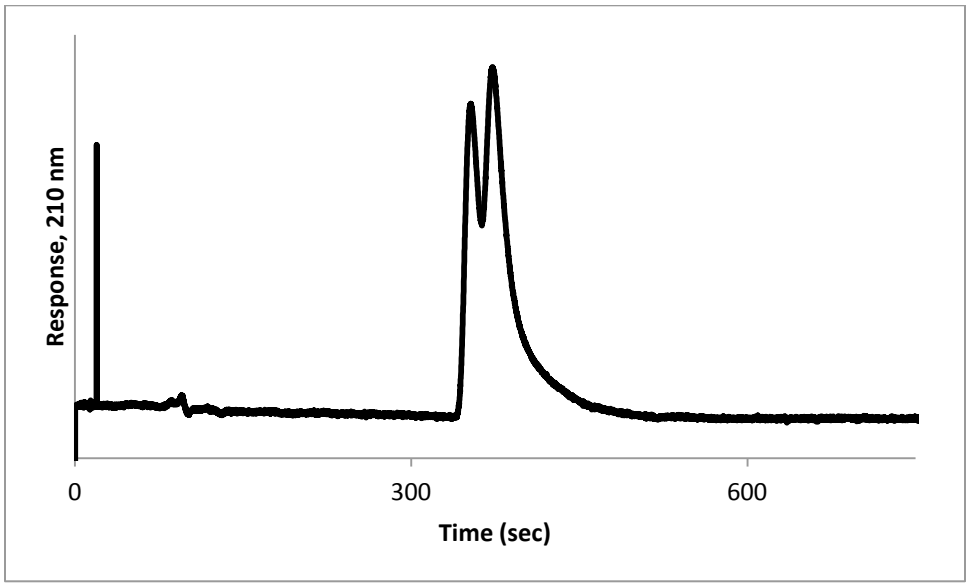
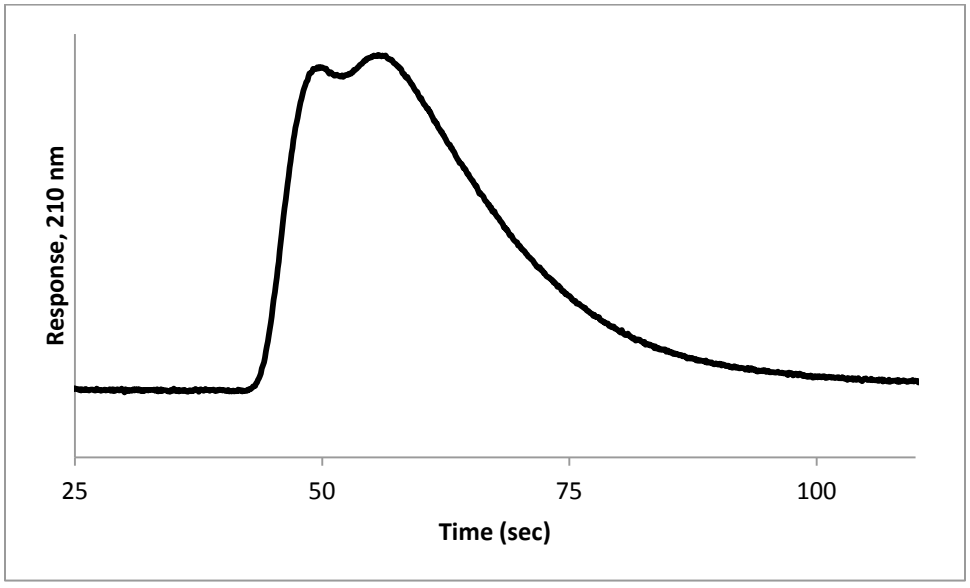

Response, 225 nm
1500
2100
2700
3300
Time (sec)

Figure 4-8. Injection of racemic mepivacaine on a 100 x 4.0 mm AGP column prepared using Method 2 and in the presence of pH 7.0, 0.010 M sodium phosphate buffer that contained 9% isopropyl alcohol. Other conditions are given in the text.


Response, 210 nm
0
300
600
Time (sec)

A short 36 mm x 2.1 mm I.D. AGP column was also prepared using Method 2 for the initial screening of binding by drugs to the AGP column. This column was evaluated by using 100 μM samples of mepivacaine or propranolol that were injected into the presence of pH 7.0, 0.10 M potassium phosphate buffer. On this short column, there was retention for both drugs but little or no resolution was observed. The chromatogram for mepivacaine (Figure 4-9) gave only a slight separation of enantiomers (the selectivity factor was 1.23, the retention times were 0.8 and 0.9 and the retention factors were 1.1 and 1.3). An injection of racemic propranolol gave only a single peak for its enantiomers, with a retention time of 7.1 and a retention factor of 17.1. The lower resolution on this column was expected because it had a much smaller number of theoretical plates than the 100 mm long AGP column. However, the short column was found to be useful to quickly screen the effects of mobile phase composition on the binding of drugs such as mepivacaine or propranolol to immobilized AGP.

Figure 4-9. Injection of mepivacaine on a 36 mm x 2.1 mm I.D. AGP column that was prepared using Method 2. The sample was prepared in pH 7.0, 0.10 M potassium phosphate buffer, which was also used as the mobile phase. Other conditions are given in the text.


Response, 210 nm
25
50
75
100
Time (sec)

Method 3

For the evaluation of the 100 mm x 4.0 mm I.D. AGP column that was prepared using Method 3, samples were made using both pH 7.0, 0.010 M sodium phosphate buffer and 9% isopropyl alcohol in pH 7.0, 0.010 M sodium phosphate buffer. The mobile phase for all of these samples was 9% 2-propanol in pH 7.0, 0.010 M sodium phosphate buffer. Under these conditions, there was no separation observed for propranolol. There did not appear to be any separation for the mepivacaine sample (Figure 4-10). There was a decrease in retention for both compounds when 9% 2-propanol was used in the mobile phase. For propranolol, retention times decreased from 2.7 to 2.0 min and retention factors decreased from 1.5 to 0.8. For mepivacaine, retention times decreased from 2.3 min to 1.7 min while retention factors decreased from 1.1 to 0.5.

Method 4

The column prepared using Method 4 contained twice as much AGP as the support prepared using Method 3. Although there was no separation for the propranolol sample on this column (retention time was 4.2 min and retention factor was 2.9), it did give some separation for the mepivacaine sample, with a retention time of approximately 2.5 min and a retention factor of 1.3. The latter separation was better than the results for the column prepared using Method 3. The chromatogram for mepivacaine on the column that was prepared by Method 4 is shown in Figure 4-11. The samples were again

prepared in pH 7.0, 0.01 M sodium phosphate containing 9% 2-propanol, which was also used as the mobile phase.

Figure 4-10. Injection of mepivacaine on a 100 mm x 4.0 mm I.D. column prepared using Method 3. The sample was prepared in pH 7.0, 0.010 M sodium phosphate buffer containing 9% isopropyl alcohol, which was also used as the mobile phase. Other conditions are given in the text.

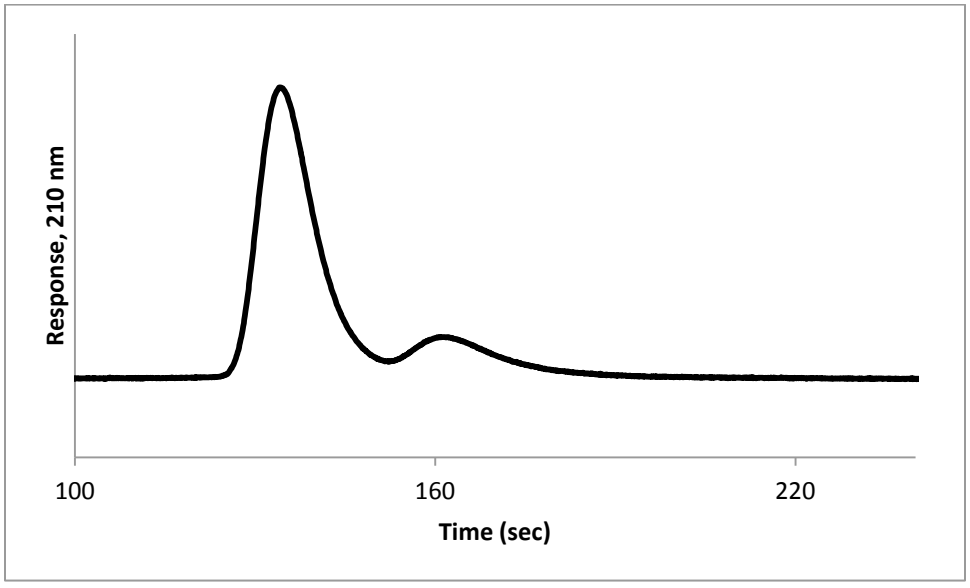
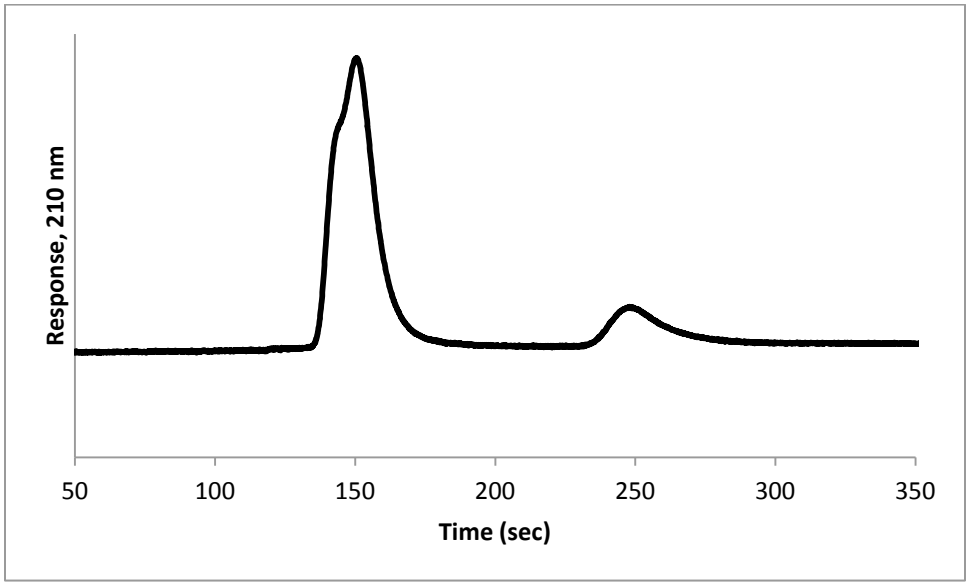

Response, 210 nm
100
160
220
Time (sec)

Figure 4-11. Injection of mepivacaine on a 100 mm x 4.0 mm I.D. column prepared using Method 4. The sample was prepared in pH 7.0, 0.010 M sodium phosphate buffer containing 9% isopropyl alcohol, which was also used as the mobile phase. Other conditions are given in the text.


Response, 210 nm
50
100
150
200
250
300
350
Time (sec)

Method 5

A 100 mm x 4.0 mm I.D. AGP column was also prepared by Method 5. This column was evaluated using the same separation conditions as already described for Methods 3 and 4. However, in this case no separation was noted for either the enantiomers of propranolol (retention time of 10 min, retention factor of 8.6) or mepivacaine (retention time of 3.4 min, retention factor of 2.1).

CONCLUSIONS

Method 2, based on a modified procedure adapted by Xuan and Hage[10] gave the best results for the separation of the tested drug enantiomers. When pH 7.0 phosphate buffer was used as the mobile phase in the absence of other additives, the separation of enantiomers for both mepivacaine and propranolol was accomplished. However, the addition of 9% 2-propanol resulted in a loss of chiral separation for propranolol and a large decrease in chiral selectivity for mepivacaine on such a column. Method 1 gave the next best results, with separation of propranolol but not mepivacaine. Method 4 gave partial separation of the enantiomers of mepivacaine. Method 3 also gave partial separation for mepivacaine.

In previous work, an increase in temperature has been observed to decrease affinity.[10] It is more difficult to predict the effects of temperature on chiral separations, although there is evidence that affinity decreases for both enantiomers of propranolol with temperature and that the affinity of AGP for S-propranolol is greater than that for R-propranolol.[10]

ACKNOWLEDGEMENTS

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CHAPTER 5

DEVELOPMENT OF AFFINITY SORBENTS FOR ON-LINE EXTRACTION AND CONCENTRATION OF BIOLOGICALLY-ACTIVE CONTAMINANTS FROM WATER

INTRODUCTION

Chapter 1 introduced the problem of emerging contaminants and outlined the need to develop selective and efficient methods for their extraction from environmental samples. These non-traditional pollutants include pharmaceuticals, steroid hormones and many other organic compounds. Steroid hormones and other natural and synthetic compounds that can mimic these hormones are considered endocrine disrupting compounds because they interfere with hormonal systems in humans and animals. Some examples are natural estrogens, natural androgens, phytosteroids, isoflavenoids, synthetic estrogens, pesticides, phthalates, bisphenol A, dioxins and organotins.[1] Some of these agents are known to disturb the reproductive systems of aquatic organisms when present at ng/L concentrations.[2]

Biologically active environmental pollutants are often present at quite low concentrations (i.e., ng/L or even pg/L levels) and are often found incorporated within complex matrices such as bio-solids or sludge, sediments, manures, waste-impacted surface and groundwater, as well as drinking water.[3-5] LC/MS/MS has successfully been used to detect steroid hormones at low levels but the extraction efficiency and selectivity for these compounds in current sample pretreatment methods limit the sensitivity of this approach.[6]

Antibodies have been frequently used for the extraction and concentration of environmental contaminants. Previous work has used antibodies to create immunosorbents to obtain low limits of detection for various target compounds in analytical methods, as outlined in Chapters 1 and 2.[6-9] Antibodies have many advantages for this type of work, such as their high selectivity for a class of compounds, the flexibility with which they can be developed for use with many analytes and types of samples, and their ability to be functionalized with a variety of labels and reporting agents to help achieve low limits of detection. However, the cost of antibodies is quite high and their use in immunosorbents requires a step gradient to elute analytes. In addition, there is the possibility that there will be interferences with immunosorbents from compounds that are structurally similar to the analyte.

Serum transport proteins such as human serum albumin (HSA) and bovine serum albumin (BSA) are possible alternatives to antibodies for use in the extraction of emerging contaminants. These proteins bind many drugs and hormones with relatively high affinity,[10, 11] they are readily available, and they are much lower in cost than antibodies. This chapter will examine the development of new affinity sorbents based on BSA for the extraction and concentration of several compounds of environmental concern from water samples.

Carbamazepine, estradiol, and estrone, were chosen as initial targets for this study. Carbamazepine (Figure 5-1) is a widely used drug that is effective in treating epilepsy as well as trigeminal neuralgia and bipolar depression[12, 13] It is one of the most commonly occurring pharmaceuticals in the environment and is found in municipal sewage and surface water samples.[14] Less than 10% of carbamazepine is removed during

sewage treatment and this drug has been detected at levels up to 1.075 μg/L in surface water, up to 1.1 μg/L in groundwater, and up to 0.030 μg/L in drinking water.[14] Carbamazepine's wide occurrence and relatively large concentrations in water samples make it ideal for use as an anthropogenic marker for wastewater.[13] In addition, it is one of the few drugs for which the lowest observed effect concentration (LOEC) for chronic toxicity to aquatic organisms is close to levels observed in wastewater effluents.[1]

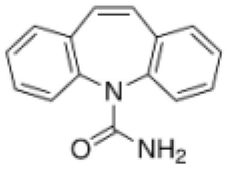
The naturally-occurring female hormone 17β-estradiol and its oxidation product estrone (Figure 5-1) have also been reported in sewage treatment plant effluents.[2] These steroid hormones are of concern because they disrupt the endocrine function of aquatic organisms at environmentally relevant levels.[2]

The remaining compounds used in this study were chosen based on their wide occurrence in natural and treated waters and their inclusion in the current lists of priority emerging contaminants.[15-18] These other targets are listed in Table 5-1, which also shows the uses and properties of these agents. The structures of these other targets are provided in Figure 5-1.

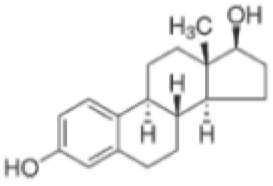
Table 5-1. Compounds used in this study and their properties and uses.

Compound	Use
2,4-Dichlorophenoxyacetic acid	Herbicide
Atrazine	Herbicide
Caffeine	Central nervous system stimulant
Carbamazepine	Anticonvulsant
17β-Estradiol	Natural female hormone
Estrone	Oxidation product of 17β-estradiol
Ibuprofen	Anti-inflammatory agent with analgesic properties
Testosterone	Natural male hormone

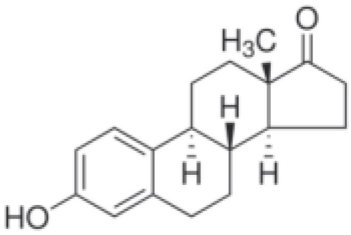
Figure 5-1. Structures used as model target analytes in this study.



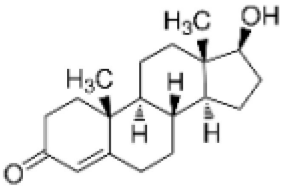
Carbamazepine



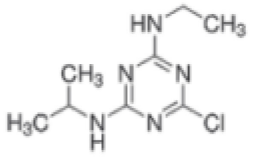
Estradiol



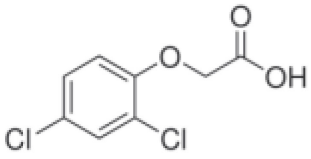
Estrone



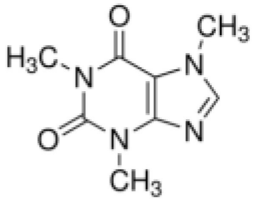
Testosterone



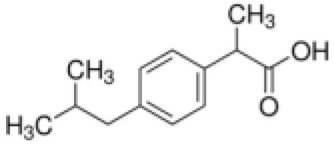
Atrazine



2,4-Dichlorophenoxyacetic acid



Caffeine



Ibuprofen

Because the ultimate goal of this research was to provide a means for the on-line extraction and concentration of emerging contaminants prior to LC/MS/MS analysis, the performance of a BSA column was tested in such an experimental set-up using carbamazepine as the analyte. The system that was used for this type of study is illustrated in Figure 5-2, with an enlarged view of the valve configuration also being provided. In this system, the sample was applied to the BSA column in water, and eluted and passed onto a reversed-phase liquid chromatography (RPLC)precolumn using a mobile phase that was also suitable for use with the BSA (e.g., 2% 1-propanol in water containing 0.5 g/L ammonium formate). The mobile phase passing through the RPLC precolumn was then switched to 75% methanol/25% water and the sample was applied as a narrow plug to a larger RPLC analytical column. Tandem mass spectrometry was used for detection as compounds eluted from the analytical column.

Figure 5-2a. Experimental set-up used for testing an affinity column containing BSA on-line with detection based on tandem mass spectrometry.

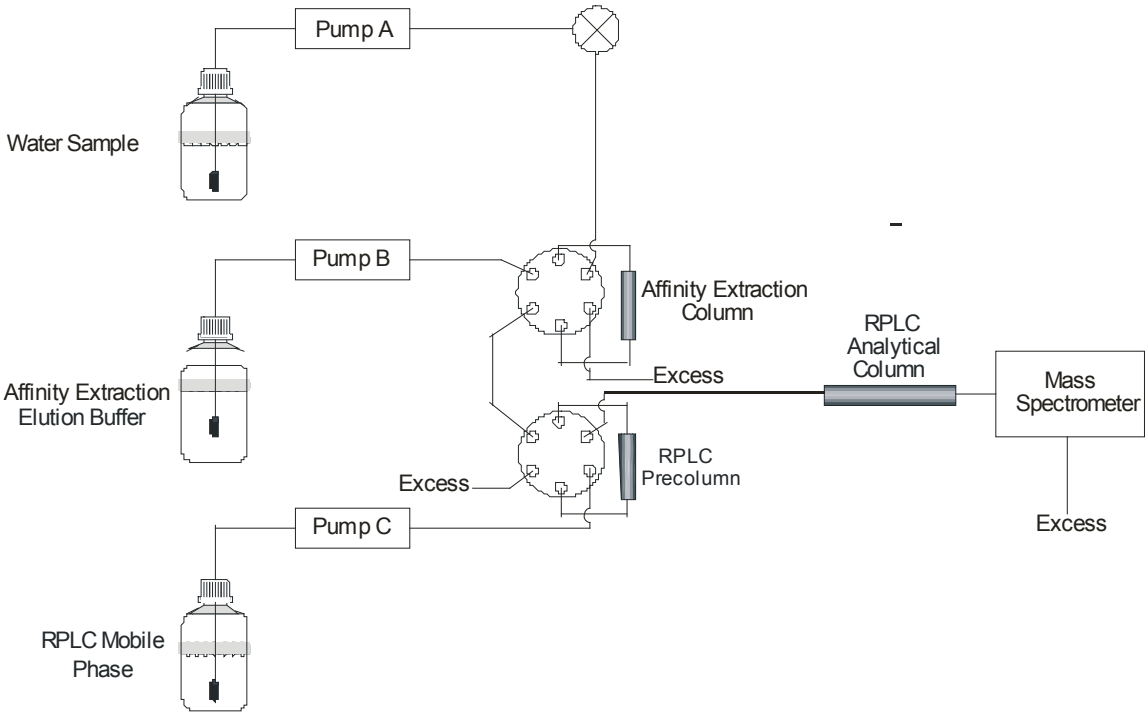
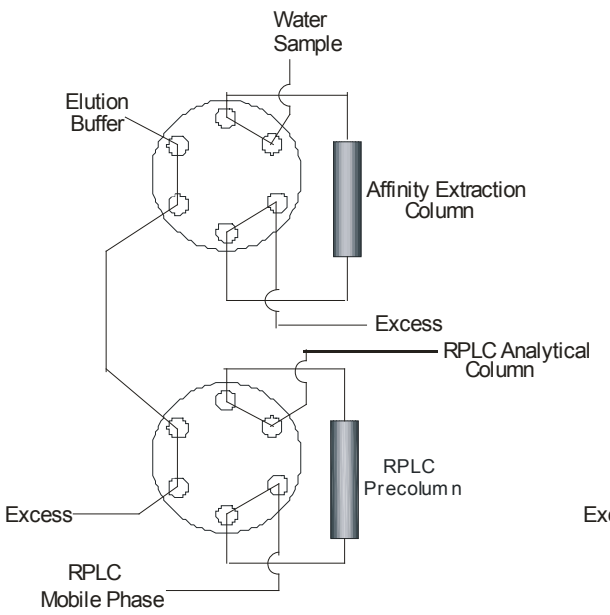
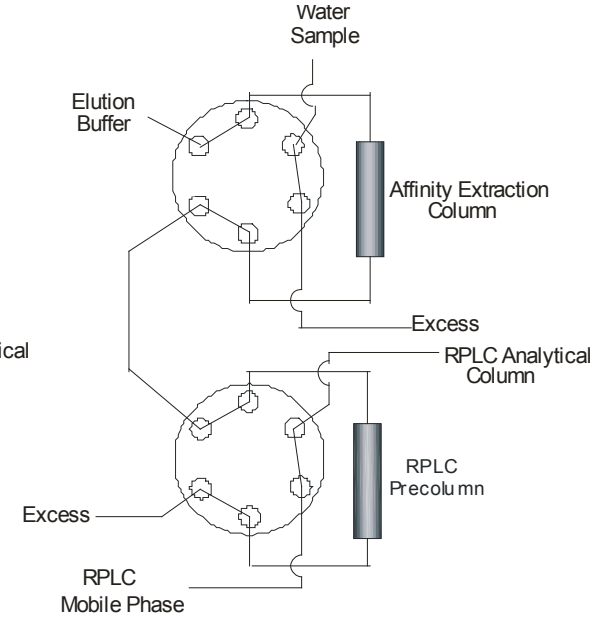

Pump A
Water Sample
Pump B
Affinity Extraction Column
RPLC Analytical Column
Mass Spectrometer
Affinity Extraction Elution Buffer
Excess
RPLC Precolumn
Excess
Pump C
Excess
RPLC Mobile Phase

Figure 5-2b. An expanded view of the valves used in the chromatographic system for affinity extraction coupled with tandem mass spectrometry.



Valve Position 1



Valve Position 2

C_{18} RPLC columns or cartridges are usually used to extract and concentrate analytes from environmental samples. In order to compare the binding capacity of columns containing BSA sorbents to those containing C_{18}, frontal analysis experiments were conducted as part of this study. In this type of experiment, a sample containing an analyte of known concentration is continuously applied to the column until the binding sites in the column are saturated. As this occurs, the amount of analyte being detected in the eluent increases and a breakthrough curve is obtained. If several different concentrations of the analyte are applied and the association/dissociation kinetics for analyte binding are relatively fast, the binding capacity of the analyte on the column can be calculated by using the following equation, which relates the time at which the center of the breakthrough curve occurs to the concentration and moles of applied analyte.[19]

$$\frac{1}{m_{Lapp}} = \frac{1}{K_A m_L [A]} + \frac{1}{m_L} \qquad (1)$$

In Equation 1, m_{Lapp} is the apparent moles of analyte required to saturate the column at a given concentration of analyte, K_a is the association equilibrium constant for the binding between the analyte and its ligand binding sites in the column, m_L is the total binding capacity of the ligand binding sites in the column, and [A] is the concentration of analyte.[19]

In this study, to determine and compare the relative affinity of each type of column that was used for the given target analytes, the retention times and retention factors for each target on each column were determined. The samples were prepared and injected into various buffers and solvents to compare various conditions for sample

application and elution on such columns. A pH 7.4, 0.067 M potassium phosphate buffer was used as the main reference application buffer because this buffer is commonly employed with columns containing BSA or other proteins to study their binding under physiological conditions. A 2% solution of 1-propanol in water was also tested as a possible mobile phase for the BSA column. Low concentrations of polarity-reducing agents such as 1-propanol or other alcohols are often used in elution buffers for immunoaffinity columns because they reduce the hydrophobic forces between antigens and antibodies.[20] A mobile phase containing a 75% methanol/25% water mixture was also tested because this solution is a typical mobile phase that could be used with a C_{18} column. Ammonium acetate buffer was included in this system design because this buffer is compatible with mass spectrometric detection.

The retention factor (*k'*) for each eluting target was determined by using Equation 2, where t_R is the retention time of the analyte and t_M is the column void time, as determined by measuring the retention time of a non-retained compound such as sodium nitrate.[21]

$$k' = \frac{t_R - t_M}{t_M} \qquad (2)$$

To compare the band-broadening for each peak of the injected targets, the plate height (*H*) was calculated for each analyte on each column by using Equation 3,

$$H = L\frac{\sigma_t^2}{t_R^2} \qquad (3)$$

where σ_t^2 is the variance of the analyte's peak with respect to time, *L* is the length of the column. and t_R is the retention time of the analyte.[21] To understand how retention of each

analyte changed with different elution and column conditions, computer simulations were carried out. These simulations were based on a countercurrent distribution model,[22-25] with the results then being compared to the experimental results and to the results for other target analytes.

EXPERIMENTAL

Reagents

BSA was obtained from Sigma-Aldrich (St. Louis, MO) and had a purity of 98%. HPLC-grade Nucleosil 300-5 silica (300 Å pore size, 5 μm diameter) was obtained from Macherey-Nagel (Düren, Germany). Carbamazepine, β-estradiol, estrone, ibuprofen and testosterone were obtained from Sigma-Aldrich. Atrazine and 2,4-D were obtained from Riedel-de Haën/Sigma-Aldrich (Seelze, Germany). Caffeine was obtained from Alfa Aesar (Ward Hill, MA). All other chemicals were of the purest grades available. All solutions were prepared using water from a Nanopure purification system (Barnstead, Dubuque, IA).

Apparatus

The chromatographic system used for the determination of binding capacity and retention consisted of a LC-10AD solvent delivery system from Shimadzu (Kyoto, Japan), a 515 HPLC pump from Waters (Milford, MA), a LabPro injection valve from Rheodyne (Oak Harbor, WA), and a UV-2075 Plus Intelligent absorbance detector from Jasco (Easton, MD). Data were collected using software and an interface from National Instruments (Austin, TX). Data analysis was performed using Peak Fit (SeaSolve Software) and Excel (Microsoft, Redmond, WA). The on-line extraction experiments were performed on a ThermoFinnigan (San Jose, CA) LCQ ion trap liquid chromatograph/mass spectrometer system (LC/MS/MS). Simulations were performed using spreadsheet calculations prepared in Excel. All supports were downward-slurry packed using an HPLC column packer from Alltech (Deerfield, IL). The C_{18} guard

cartridges (10 x 2.1 mm, 5 μm particle size) were obtained from Thermo Hypersil (Waltham, MA).

Column Preparation

The diol-bonded silica used for BSA immobilization was prepared from Nucleosil 300-5 silica according to a previous method.[26] The BSA was immobilized to this silica support by the reductive amination method.[27]

Chromatographic Conditions

Each BSA silica support was downward-slurry packed at 4000-4500 psi for 1 h into separate 50 x 4.6 mm I.D. or 10 x 2.1 mm I.D. stainless steel columns. The packing solution was pH 7.4, 0.067 M potassium phosphate buffer. Injected samples had a volume of 5 μL. The flow rate used in all chromatographic experiments was 1 ml/min.

For the determination of retention, all samples had a concentration of 2 ppm and were prepared in pH 7.4, 0.067 M potassium phosphate buffer, pH 7.4 0.010 M ammonium acetate buffer, 2% 1-propanol in water or 75% methanol/25% water. The detection wavelengths used in this study were 230 nm for carbamazepine, 225 nm for estrone, 225 nm for estradiol, 249 nm for testosterone, 275 nm for caffeine, 220 nm for ibuprofen, 223 nm for atrazine, 223 nm for 2,4-D and 205 nm for sodium nitrate. Each sample was injected in triplicate and the average of the resulting peak parameters were used for the calculation of retention factors or plate heights. All experiments were performed at room temperature. For the determination of binding capacity, the carbamazepine samples were prepared in water at concentrations of 1×10^{-5} M, 2×10^{-5}

M and 5 x 10^{-5} M. For the on-line extraction experiments, the carbamazepine samples were prepared in water and had concentrations ranging from 10 ng/L to 10 μg/L.

Computer Model

Simulations were performed using spreadsheet calculations prepared in Excel and a countercurrent distribution model. [22-25, 28, 29] The input parameters included the analyte's retention factor and plate height, sample volume and concentration, flow rate and column size. An example of this type of spreadsheet is provided in the Appendix. Further details on the simulation method are provided in the Results and Discussion.

RESULTS AND DISCUSSION

Initial studies demonstrated that a 50 x 4.6 mm i.d. BSA column could be used to successfully trap a 24 ng sample of carbamazepine from an injected 10 ng/L sample. The carbamazepine was eluted in this case onto a RPLC precolumn using 2% 1-propanol as the mobile phase. This precolumn was used to refocus the eluted carbamazepine and to avoid later injecting phosphate buffer into the LC/MS/MS system. The retained carbamazepine was then eluted from the RPLC precolumn and applied onto a longer analytical RPLC column by using 75% methanol/25% water as the mobile phase. The resulting mass spectrum that was obtained when using this approach is shown in Figure 5-3. The peaks seen at *m/z* values of 194 and 237 are characteristic of carbamazepine. The corresponding chromatographic peak, as shown in Figure 5-4, gave an easy to detect signal for carbamazepine but was approximately 8 min wide.

In an effort to obtain a narrower peak, smaller 10 x 2.1 mm i.d. BSA columns were prepared. The binding capacity of this type of column was then estimated and compared to that for a C_{18} precolumn with the same dimensions. The latter is commonly used for the pretreatment of environmental samples. It was found in this comparison that the C_{18} column had a binding capacity of approximately 1.0×10^{-8} mol for carbamazepine, as determined by frontal analysis. The BSA column had only a slightly lower binding capacity of 0.8×10^{-8} mol, which indicated that both types of supports could be used to bind to roughly equivalent amounts of this drug in the presence of an aqueous mobile phase.

Figure 5-3. Mass spectrum obtained after on-line extraction of carbamazepine from water using a BSA column in an LC/MS/MS experiment.

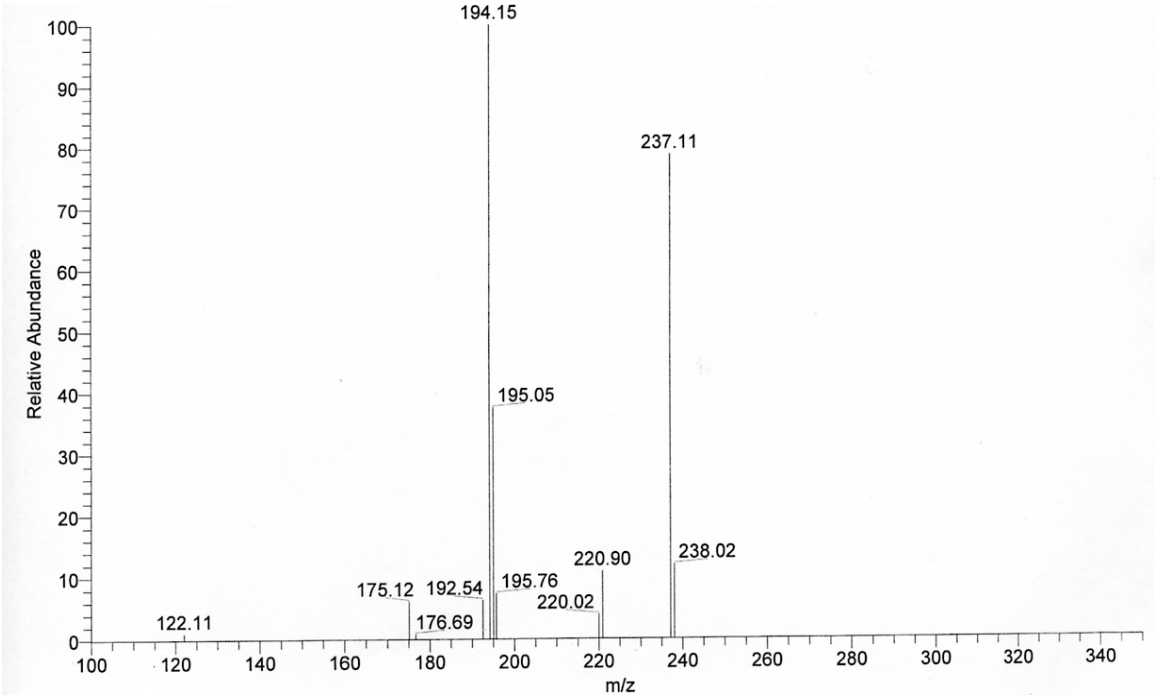
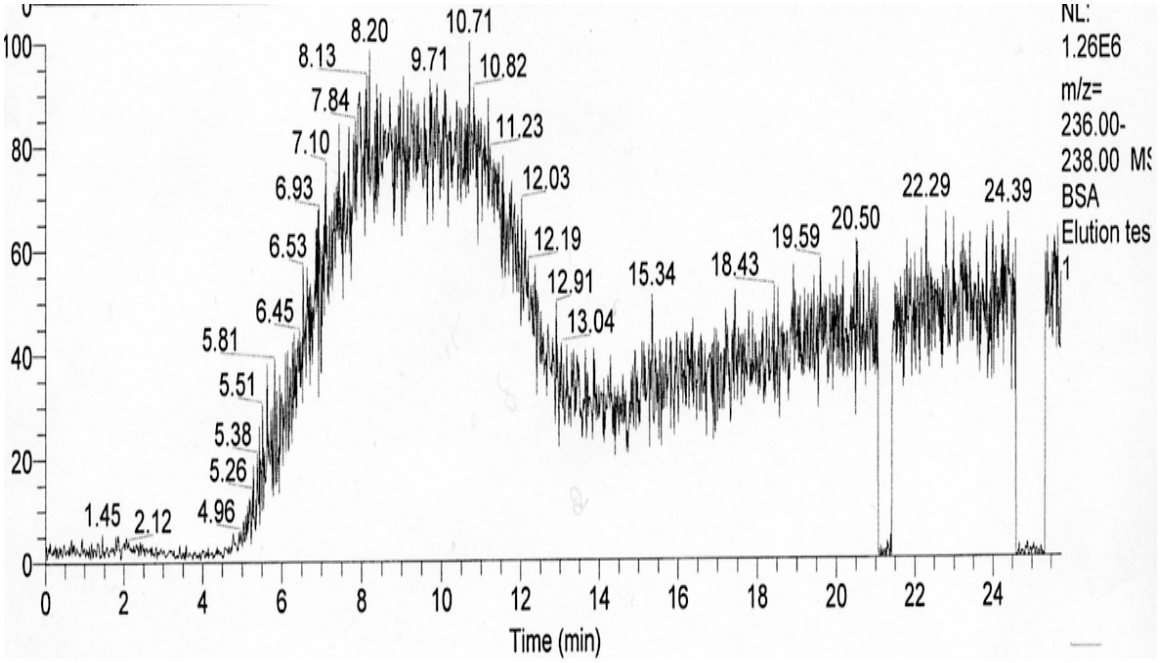

194.15
237.11
195.05
238.02
220.90
175.12
192.54
195.76
220.02
176.69
122.11
Relative Abundance
m/z
100
90
80
70
60
50
40
30
20
10
0
100
120
140
160
180
200
220
240
260
280
300
320
340

Figure 5-4. Chromatogram obtained after on-line extraction of carbamazepine from water using a BSA column in an LC/MS/MS experiment.


NL:
1.26E6
m/z=
236.00-
238.00 MS
BSA
Elution tes
1
1.45
2.12
4.96
5.26
5.38
5.51
5.81
6.45
6.53
6.93
7.10
7.84
8.13
8.20
9.71
10.71
10.82
11.23
12.03
12.19
12.91
13.04
15.34
18.43
19.59
20.50
22.29
24.39
Time (min)

Tables 5-2 and 5-3 list the average retention factors and plate heights that were next measured on 10 x 2.1 mm i.d. columns containing BSA and C_{18} and using pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase. For some of the compounds that had low retention (e.g., caffeine), the plate heights were difficult to measure accurately because of the effects of extra-column band-broadening at the short retention times observed for such compounds. Carbamazepine was found to have a retention factor of 1.0 on the BSA column in the presence of the pH 7.4, 0.067 M phosphate buffer, which made this a useful reference point for comparison with the retention of the other compounds, columns and mobile phases that were also examined in this study. Several of the tested compounds (i.e., ibuprofen, testosterone, β-estradiol, estrone and 2,4-D) had much greater retention factors than carbamazepine on the BSA column. Also, the majority of the retention factors on the BSA column were significantly greater than the corresponding retention factors for the same analytes on the C_{18} column. These results indicated that BSA could have significant binding to a range of different compounds (i.e., drugs, steroid hormones, and pesticides). The greater affinity of this material for these compounds of interest in the presence of an aqueous solvent makes this type of support an attractive alternative to C_{18} for on-line extraction and the concentration of emerging contaminants from water.

Excluding the compound with the lowest retention (i.e., for which the efficiencies were artificially high due to extra-column effects), the plate heights measured for the BSA column ranged from 0.02 to 0.35 cm, with an average of 0.13 cm. Because most of the tested compounds had low retention on the C_{18} column under aqueous conditions, the plate heights were difficult to measure accurately for all analytes except ibuprofen. The

plate height for this drug was 0.4 cm on the C_{18} column while it was 0.02 cm on the BSA column. This greater efficiency for the BSA column under aqueous conditions is another potential advantage for this material as an alternative to C_{18} for sample extraction.

Table 5-4 lists the retention factors that were measured with other mobile phases on the same two types of columns. In general, the retention factors decreased significantly in going from a pH 7.4, 0.067 M potassium phosphate buffer to a mobile phase that contained 2% 1-propanol on the BSA column. This difference would make the latter mobile phase a good elution buffer when transferring the extracted analytes from the BSA column to a RPLC column. In contrast, going from the pH 7.4, phosphate buffer to a 75% methanol/25% water mobile phase on the C_{18} column resulted in a slight increase in the retention factor. However, this was to be expected since this new mobile phase is a typical RPLC solvent and the retention times with the phosphate buffer on the C_{18} column were quite low, as can occur in the presence of a highly aqueous solution for this type of support. These results confirmed that an aqueous buffer was a better mobile phase for application to the BSA column than for the C_{18} column. This difference indicated that a BSA column would be more suitable than a C_{18} column for use in extracting emerging contaminants from water. An ammonium acetate buffer was also tested for use on these columns because of its compatibility with mass spectrometric detection. While the retention factors on the BSA column were similar for some of the compounds, they decreased drastically for β-estradiol, estrone and ibuprofen, making this mobile phase a poor choice for a sample application buffer but a possible candidate as a mild elution buffer for this column. In contrast, the ammonium acetate buffer gave greater retention for some of the compounds on the C_{18} column.

Table 5-2. Retention factors measured for the model analytes on BSA and C_{18} column in the presence of pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase.

Analyte	**Retention factor, *k*'[a]**	
	BSA	**C_{18}**
Caffeine	0.04	0.04
Atrazine	0.64	0.06
Carbamazepine	1.0	0.08
Testosterone	2.4	0.18
Estrone	11.0	0.09
Ibuprofen	11.0	0.62
2,4-D	12.0	0.13
β-Estradiol	19.8	0.07

[a]These results are the averages for triplicate injections. The typical precision for these measurements was ± 2-3%.

Table 5-3. Plate height values measured for the model analytes on BSA and C_{18} column in the presence of pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase.

Analyte	**Plate height, *H* (cm)[a]**	
	BSA	**C_{18}**
Caffeine	>1.0	>1.0
Atrazine	0.35	>1.0
Carbamazepine	0.14	>1.0
Testosterone	0.21	>1.0
Estrone	0.02	>1.0
Ibuprofen	0.02	0.4
2,4-D	0.03	>1.0
β-Estradiol	0.13	>1.0

[a]These results are the averages for triplicate injections. The typical precision for these measurements was ± 7-11%%.

Table 5-4. Retention factors measured for the model analytes on BSA and C_{18} column in the presence of mobile phases containing 2% 1-propanol, 75% methanol/25% water or pH 7.4, 0.010 M ammonium acetate buffer.

Analyte	**Retention factor, *k*'[a]**			
	BSA ***2% 1-propanol***	C_{18} ***75% methanol/25% water***	BSA ***pH 7.4, 0.010 M ammonium acetate***	C_{18} ***pH 7.4, 0.010 M ammonium acetate***
Caffeine	1.18	0.17	0.10	0.22
Atrazine	0.32	0.29	0.50	8.32
Carbamazepine	0.63	0.23	0.95	14.2
Testosterone	1.03	0.42	1.75	-
Estrone	0.24	0.31	0.03	-
Ibuprofen	0.31	0.19	0.04	1.63
2,4-D	7.78	0.14	-	0.13
β-Estradiol	0.17	0.33	1.03	0.06

[a]These results are the averages for triplicate injections. The typical precision for these measurements was ± 3-5%.

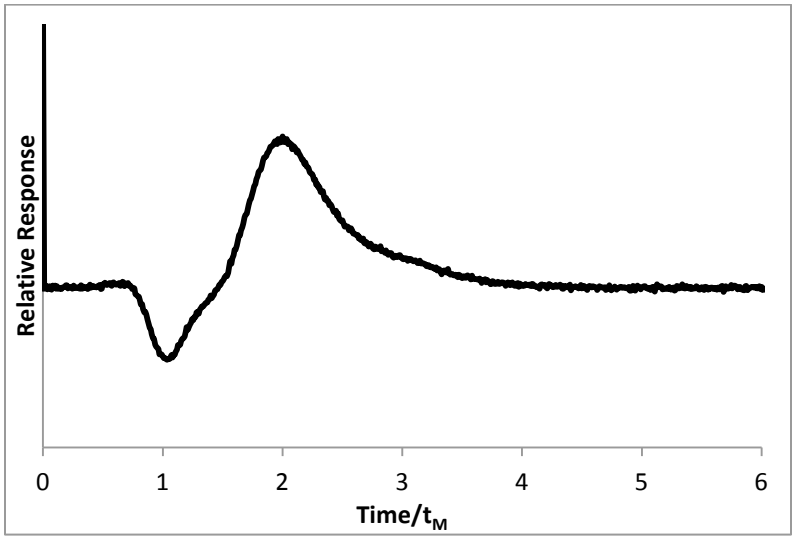
Computer modeling was next used to simulate the elution profiles for the target analytes under various retention conditions. In this approach, the number of theoretical plates for the column was represented by equal segments in an Excel spreadsheet. The movement of an analyte through this column was simulated by using a countercurrent distribution model.[22-25, 28, 29] In this model, a given amount of analyte was applied to the first segment of the column, allowed to distribute between the mobile and stationary phases in that segment, and then moved to the next segment while additional analyte was applied to the first segment. The process was repeated throughout the length of the column. The amount of analyte eluting in the mobile phase from the last segment was then recorded and plotted as a function of time to produce the simulated chromatogram. A representative spreadsheet (e.g., as used in modeling the elution profile of 2,4-D) is provided in the Appendix.

Examples of the actual chromatograms and simulated chromatograms that were obtained for some of the target analytes in this study are provided in Figures 5-5 through 5-9 for representative compounds from each category of analyte (e.g., drugs, pesticides and hormones). The simulated chromatograms gave good agreement with the experimental chromatograms in each case. For each analyte, the retention factor, plate height, flow rate and column dimensions were input in order to calculate the void volume, void time and number of segments required. A response value was calculated using an arbitrary relative response factor and the number of segments calculated.

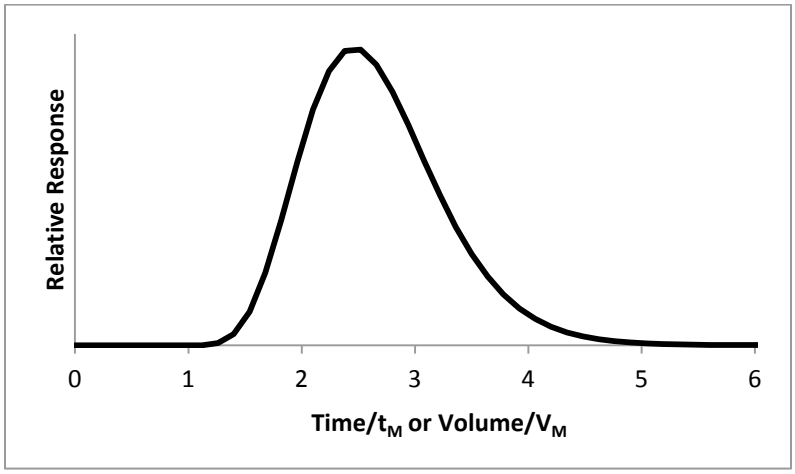
Figure 5-10 compares the simulated chromatograms for the compounds in Figure 5-5 through 5-9. It was possible from these plots to determine the sample application conditions that were needed to collect or elute each compound in the presence of pH 7.4

phosphate buffer. For instance, the compounds that were observed to have relatively mild retention on the BSA column (e.g., atrazine, carbamazepine, testosterone) did not begin to exit this column until after 1.2-1.5 times the column void volume (or void time) but were almost completely eluted after 7-8 column volumes (e.g., carbamazepine and atrazine) or 10 column volumes (testosterone). 2,4-D had a higher amount of retention, with its elution from the column beginning around 7 column volumes and nearing completion after 20 column volumes. β-Estradiol had the highest retention, with elution beginning at 7 column volumes and continuing up to 50 column volumes. From this information and these types of plots it is thus possible to estimate the conditions that are needed for effective capture for each of these analytes and to adjust the selectively of the BSA column to provide information on some groups of compounds versus others with different levels of retention.

Figure 5-5. Experimental and simulated chromatograms for carbamazepine, using a normalized value of time on the x-axis. The BSA column was used with a mobile phase consisting of pH 7.4, 0.067 M potassium phosphate buffer.

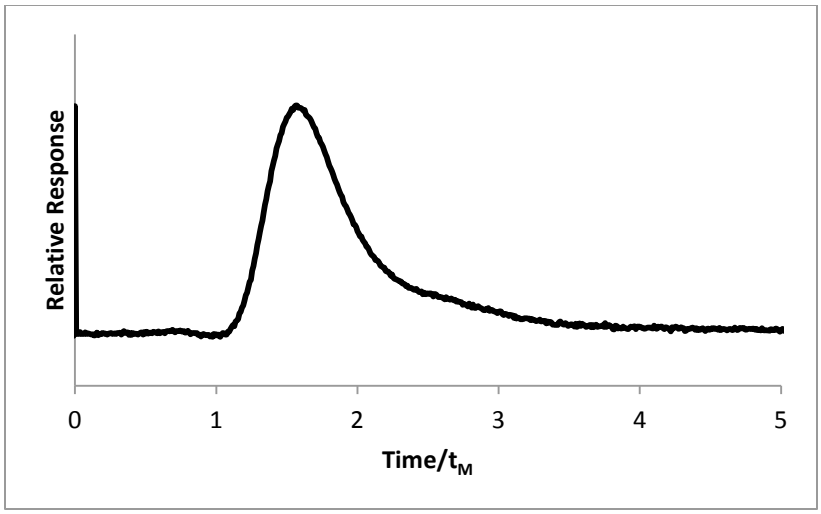


Carbamazepine – experimental result

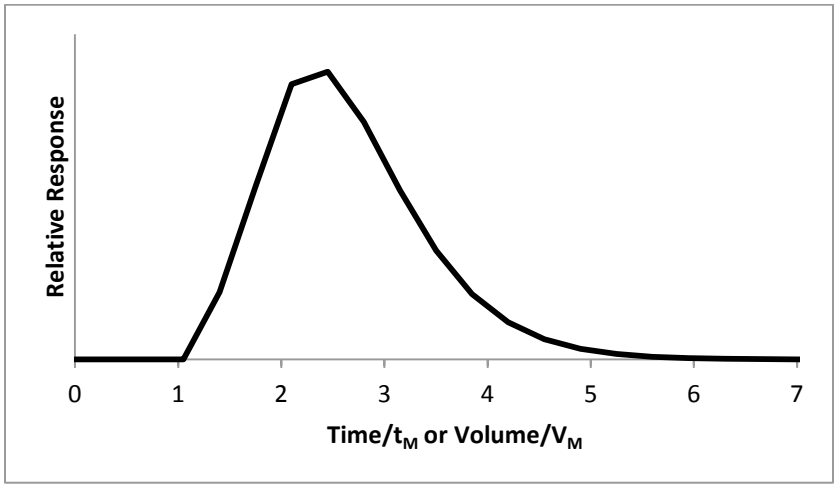


Carbamazepine – simulated result

Figure 5-6. Experimental and simulated chromatograms for atrazine, using a normalized value of time on the x-axis. The BSA column was used with a mobile phase consisting of pH 7.4, 0.067 M potassium phosphate buffer.

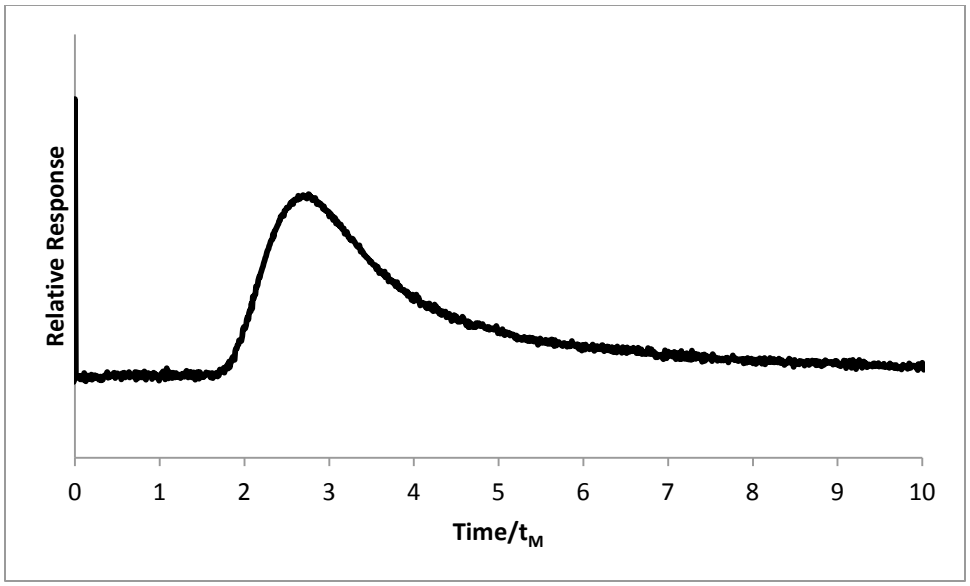


Atrazine – experimental result

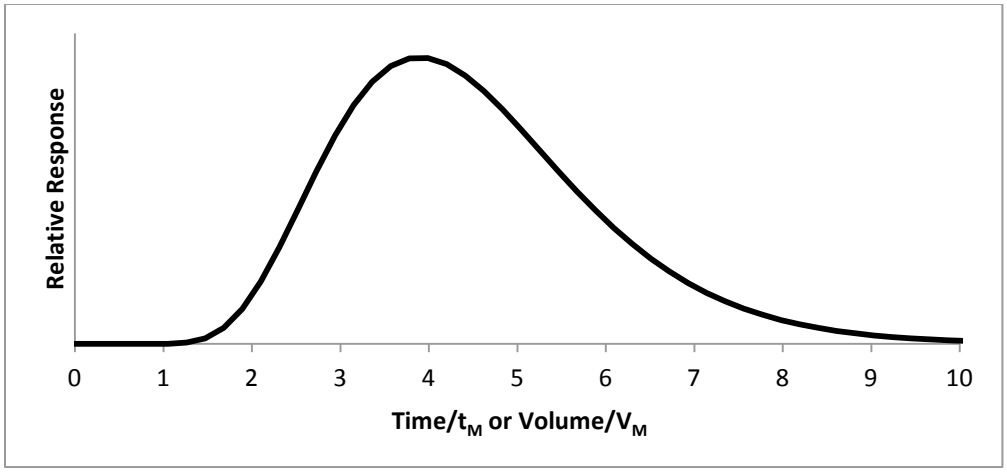


Atrazine – simulated result

Figure 5-7. Experimental and simulated chromatograms for testosterone, using a normalized value of time on the x-axis. The BSA column was used with a mobile phase consisting of pH 7.4, 0.067 M potassium phosphate buffer.

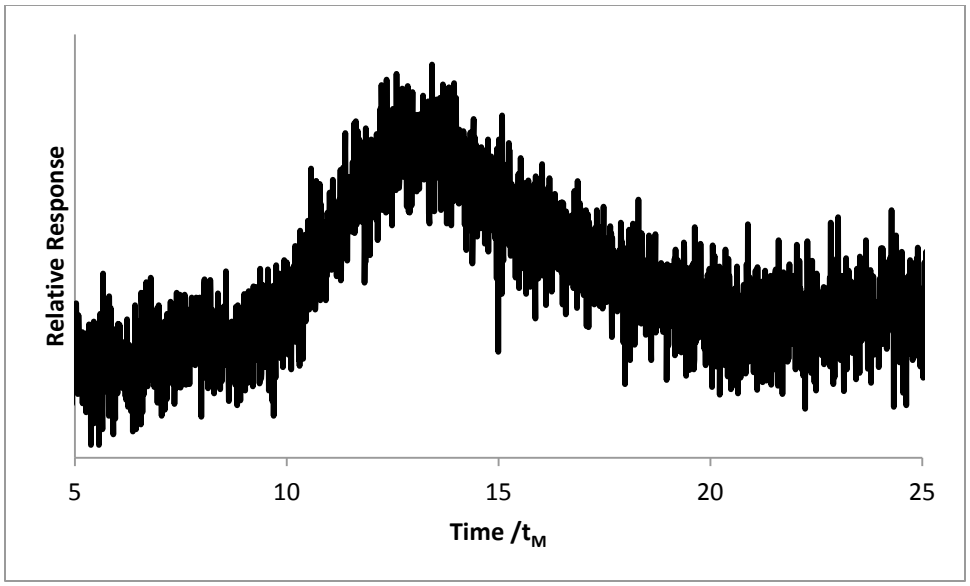


Testosterone – experimental result

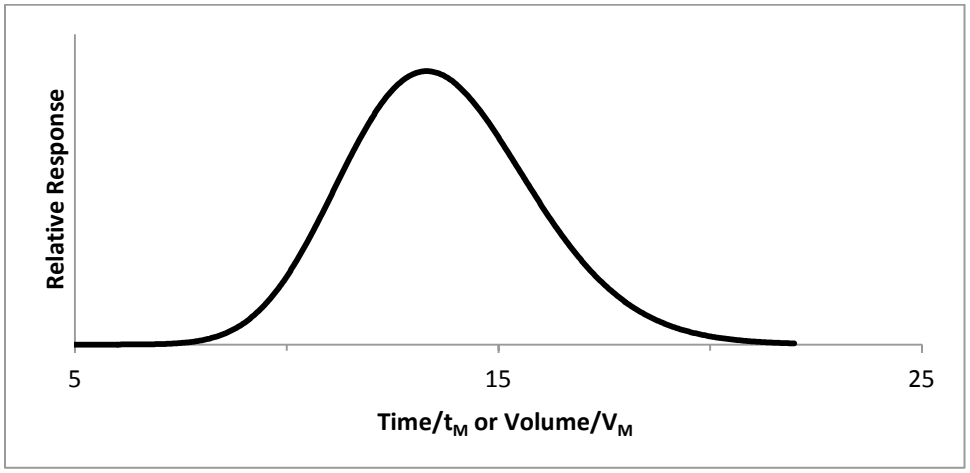


Testosterone – simulated result

Figure 5-8. Experimental and simulated chromatograms for 2,4-D, using a normalized value of time on the x-axis. The BSA column was used with a mobile phase consisting of pH 7.4, 0.067 M potassium phosphate buffer.

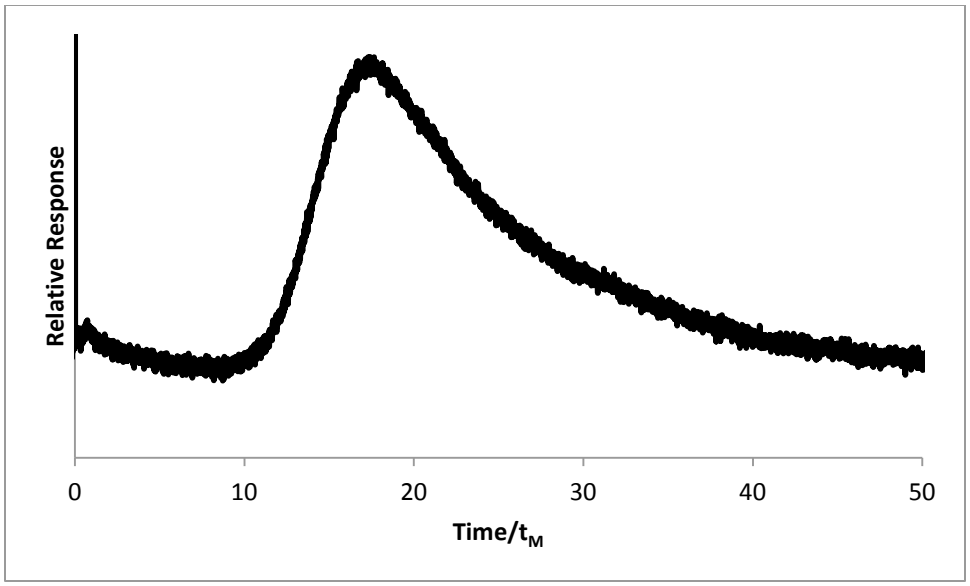


2,4-D – experimental result

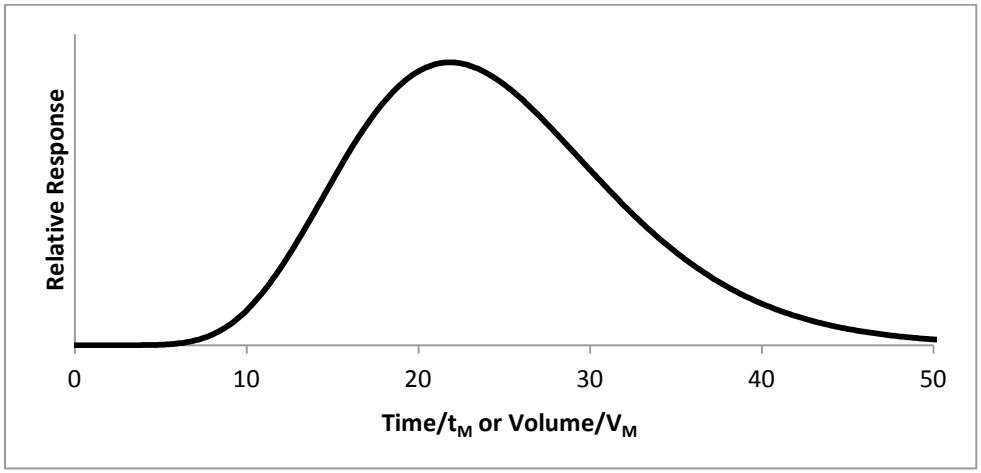


2,4-D – simulated result

Figure 5-9. Experimental and simulated chromatograms for β-estradiol, using a normalized value of time on the x-axis. The BSA column was used with a mobile phase consisting of pH 7.4, 0.067 M potassium phosphate buffer.

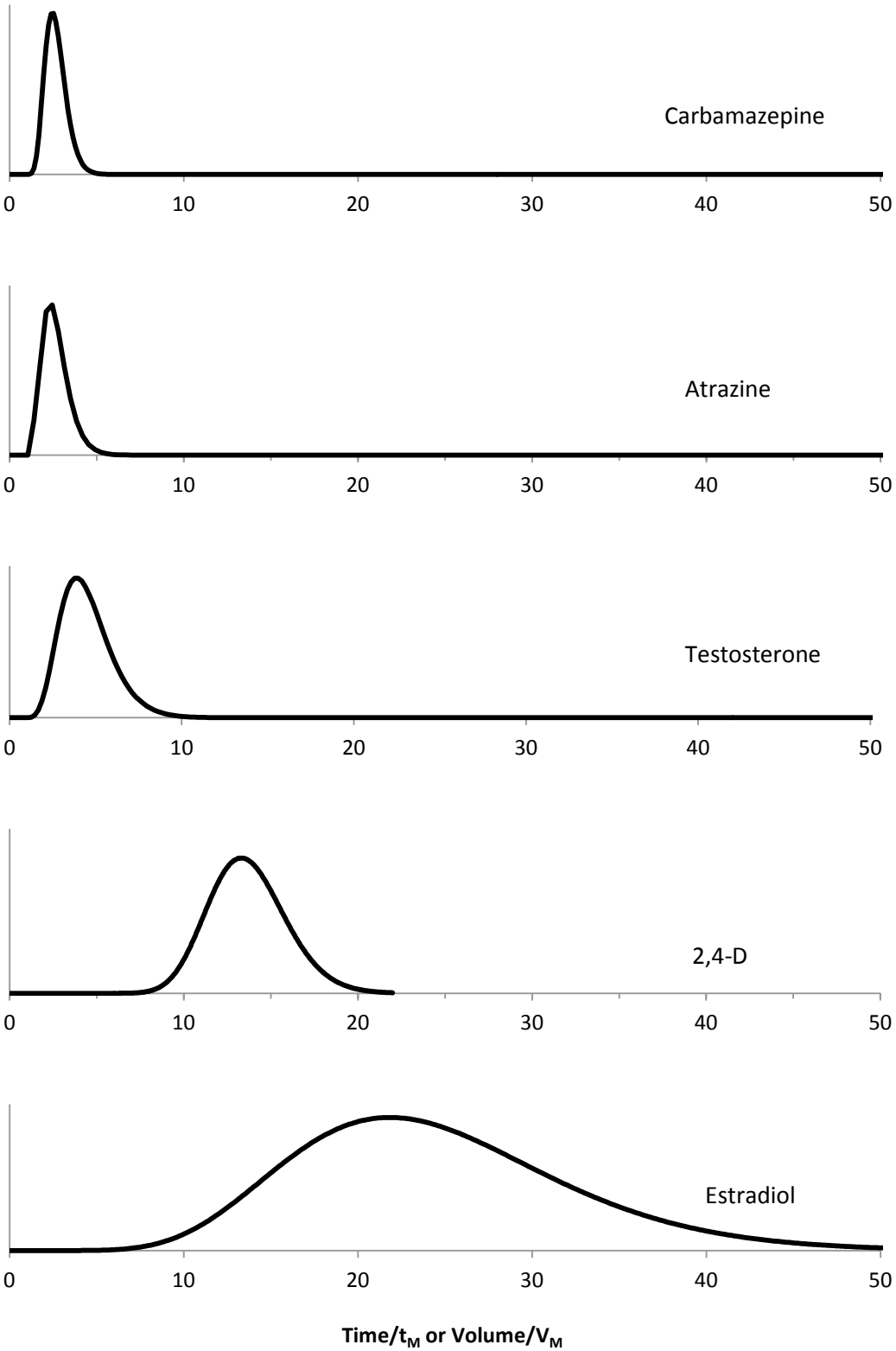


Estradiol – experimental result



Estradiol – simulated result

Figure 5-10. Comparison of the simulated response for the compounds in Figures 5-5 through 5-9, using a normalized value of time on the x-axis. The BSA column was used with a mobile phase consisting of pH 7.4, 0.067 M potassium phosphate buffer.


Carbamazepine
0
10
20
30
40
50
Atrazine
0
10
20
30
40
50
Testosterone
0
10
20
30
40
50
2,4-D
0
10
20
30
40
50
Estradiol
0
10
20
30
40
50
Time/t_M or Volume/V_M

CONCLUSIONS

A column containing immobilized BSA was successfully used for the on-line extraction of carbamazepine from water in a LC/MS/MS experiment. Although BSA columns were found to have a slightly lower binding capacity than C_{18} columns of the same size, they also had greater affinity for the target compounds examined in this study. One advantage of BSA is that it is a general ligand for such compounds and can be used to trap and elute these agents under isocratic elution conditions. In comparison, antibodies tend to be specific for one compound or a few structurally similar compounds. In addition, antibodies typically require a step gradient to release the captured analytes. BSA is also easy to obtain and is inexpensive (i.e., less than 1 cent per milligram, compared to hundreds of dollars per milligram for many antibodies). BSA also offers the ability to provide more selective and stronger retention than C_{18} supports for the emerging contaminants that were tested in this study. In addition, BSA is more suitable for the application of aqueous samples.

The binding and elution of the target compounds from the BSA columns were successfully simulated by using an Excel spreadsheet and a countercurrent distribution model. The same approach could be used to examine other emerging contaminants or elution conditions by changing the retention factor (i.e., to represent a change in the analyte, mobile or stationary phase) and the plate height (i.e., to represent a change in efficiency). As indicated in this study, these simulations should be useful for the future optimization of on-line extraction based on BSA or other binding agents for other emerging contaminants or compounds of interest in environmental samples.

ACKNOWLEDGEMENTS

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CHAPTER 6

THE USE OF CHROMATOGRAPHIC PEAK PROFILING FOR THE STUDY OF DRUG-PROTEIN BINDING KINETICS

INTRODUCTION

Drugs in the human body are often bound to plasma proteins, such as human serum albumin (HSA) and α_1-acid glycoprotein (AGP). HSA is the most important plasma binding protein for many drugs and endogenous substances.[1] It has a molecular weight of 66,500 g/mol and is made up of a single polypeptide chain that contains 585 amino acids.[2] The concentration of HSA in plasma ranges between 30 and 50 g/l, or roughly 500 to 750 μM.[3, 4] HSA is able to bind compounds with widely varying structures. Two of the most important binding sites on HSA are Sudlow sites I and II.[2] Sudlow site I binds to drugs such as warfarin that contain a cationic center. Sudlow site II tends to binds drugs with arylpropionic acid, fenamanate or benzodiazepine groups. Binding at both sites involves mostly hydrophobic interactions, but ionic interactions or dipole-dipole interactions can also be involved.[1] AGP binds mostly to basic drugs[4] and appears to have one, high-affinity site that involves mainly hydrophobic binding forces.[5] The concentration of AGP in plasma is only 1/50th that of HSA,[3] but the levels of AGP do rise significantly during many disease states.[4]

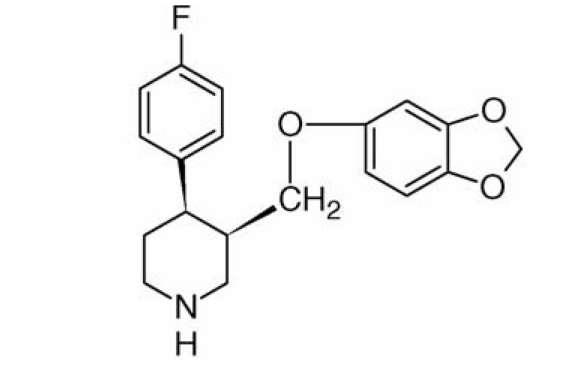
Drug-protein binding in blood has been widely studied for many years because it frequently affects the transport, distribution, metabolism and excretion of pharmaceutical agents.[3] It is for this reason that drug-protein binding with serum proteins is a routine

part of drug development.[6] In addition, a number of disease states, especially those involving the liver or kidneys, are known to cause changes in drug binding in blood, thus affecting the distribution and elimination of many pharmaceutical agents.[1]

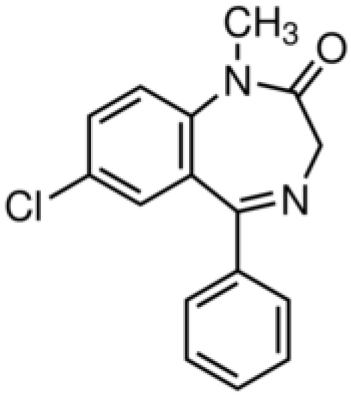
Equilibrium constants are commonly used as a measure of the binding strength for drug-protein interactions. However, only when kinetics are taken into account can a complete description of this binding be obtained.[4] Examples of processes in which the rates of association and dissociation between drugs and proteins are important include hepatic clearance and passage of a drug across the blood-brain barrier.[4] This chapter will use the methods of high-performance affinity chromatography and peak profiling to examine the interaction rates of various drugs with HSA and AGP.

The peak profiling method allows calculation of the dissociation rate constant, k_d, between an analyte and a ligand by measuring the plate heights on a column containing the ligand when injections are made for the analyte and a non-retained species at various flow rates.[4, 7, 8] This approach was used in this chapter to study binding by the drugs paroxetine and diazepam to HSA and binding by the drug propranolol to AGP. Figure 6-1 shows the structures of these drugs. Paroxetine is a selective serotonin reuptake inhibitor (SSRI) that is used to treat major depression, obsessive-compulsive disorder, panic disorder, generalized anxiety disorder, post-traumatic stress disorder and social phobia in adults.[9] Previous studies have demonstrated relatively strong binding of paroxetine to HSA (e.g., previously giving a retention factor greater than 5.7 on an HSA column).[10] Diazepam is one of the most often used benzodiazepine drugs. This group of drugs has sedative, hypnotic, anti-anxiety, muscle relaxant and antiepileptic properties

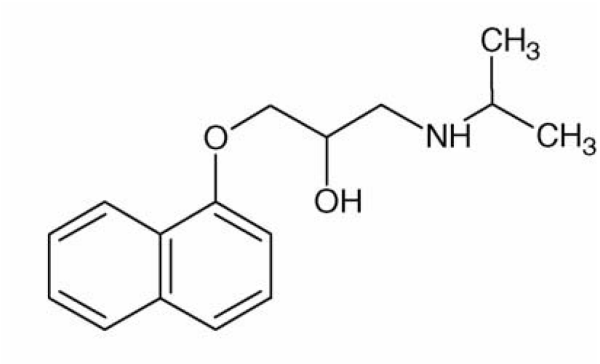
Figure 6-1. Structures of the drugs examined in this study.



Paroxetine



Diazepam



Propranolol

and is among the most commonly prescribed families of medications.[11, 12] Diazepam is also known to bind fairly strongly to HSA. The association equilibrium constants that have been reported in the literature for this interaction have ranged from 1.2 x 10^5 to 1.7 x 10^6 M^{-1}.[13-15] Propranolol is a non-selective beta-adrenoceptor blocking agent that is used to treat hypertension, coronary heart disease, cardiac arrhythmias and heart failure.[16] The association equilibrium constants for the binding of propranolol to AGP have been reported to be in the range of 10^5-10^6 M^{-1}.[17-22] The binding of propranolol to HSA has been reported to have an association equilibrium constant in the range of 10^3-10^4 M^{-1}.[23, 24]

The binding of a small drug with serum proteins is important because the drug-protein complex is not able to diffuse across membranes because of its high molecular weight; only the free, or non-complexed, form of the drug is able to cross membranes.[25] For many drugs, this makes the free drug form the biologically-active fraction in the circulation.[26] However, there are some cases in which the interactions of the binding proteins with components in the microcirculation (e.g., arterioles, venules and capillaries) can cause dissociation of the drug from a serum protein and enable transport of the drug into tissues.[27, 28] For example, it has been suggested that a fraction of warfarin that is bound to serum proteins is available for tissue extraction as a result of enhanced dissociation of the drug-protein complex in the tissue microcirculation, as noted in a study of brain and salivary gland extraction of this drug in rats.[29] Enhanced dissociation from albumin of valproic acid in the brain microcirculation has also been observed.[30]

These effects make information on the kinetics of drug interactions with serum proteins of great potential interest in helping to determine the relative importance of these processes for drugs such as paroxetine, diazepam and propranolol.

THEORY

The binding of a drug (*D)* to a serum protein (*P*) to form a reversible complex (*DP*) can be described by the following equation,[31]

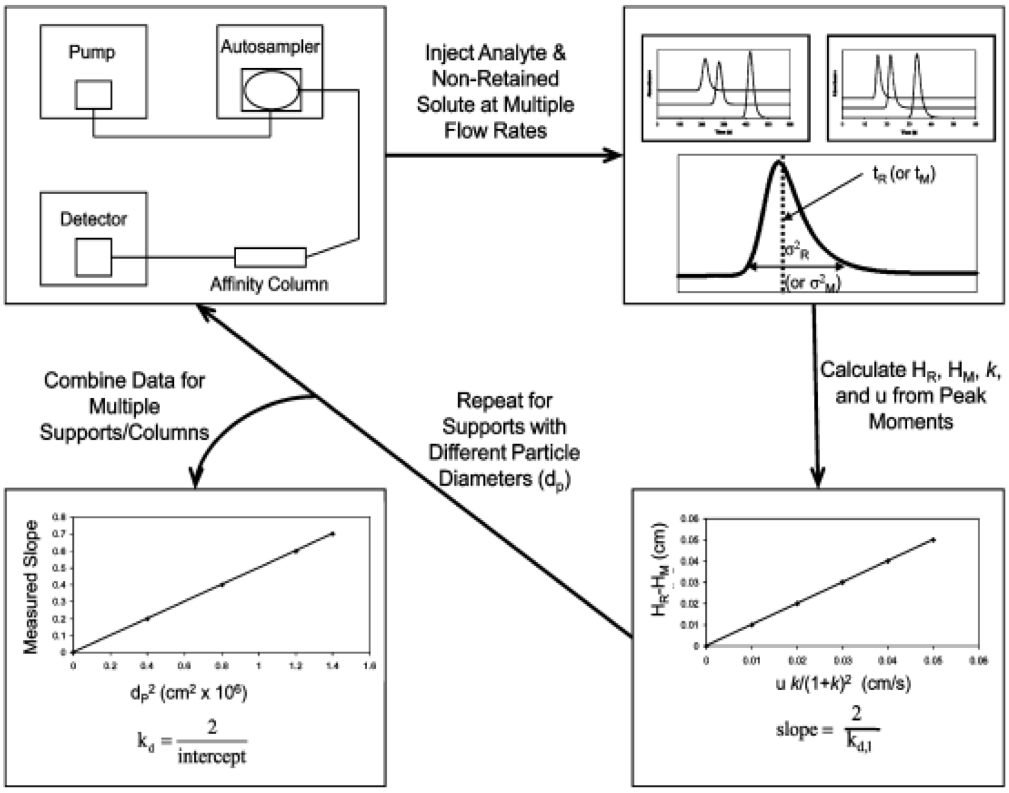
$$D + P \underset{k_d}{\overset{k_a}{\rightleftharpoons}} DP \qquad (1)$$

where k_a and k_d are the association and dissociation rate constants for the binding process, respectively. The association equilibrium constant for this process, K_a, is related to these rate constants through the following expression.[31]

$$K_a = k_a/k_d \qquad (2)$$

In this study, the peak profiling method was used to determine the dissociation rate constants for several drugs with serum proteins. In this method, an immobilized binding agent such a serum protein is employed while injections of a drug and of a non-retained solute are made at one or more flow rates.[4, 8] An example of such a study is shown in Figure 6-2. The chromatographic profiles (i.e., the positions and widths of the peaks) for the retained and non-retained compounds are then measured at each flow rate.

Figure 6-2. Experimental set-up and procedure for a peak profiling experiment.[8]


Pump
Autosampler
Detector
Affinity Column
Inject Analyte & Non-Retained Solute at Multiple Flow Rates
t_R (or t_M)
σ^2_R
(or σ^2_M)
Calculate H_R, H_M, k, and u from Peak Moments
Repeat for Supports with Different Particle Diameters (d_p)
Combine Data for Multiple Supports/Columns
Measured Slope
d_P^2 (cm^2 x 10^6)
$k_d = \frac{2}{\text{intercept}}$
$H_R - H_M$ (cm)
u $k/(1+k)^2$ (cm/s)
$\text{slope} = \frac{2}{k_d}$

The retention time for a retained drug can be related to the association equilibrium constant for the drug-protein interaction and the width of the peak can be related to the rates of association and dissociation for this interaction. The data that is obtained for the non-retained compound is used to account for peak broadening due to sources other than drug-protein binding, such as mass transfer and diffusion of the drug within the mobile phase.[4]

The peak profiling method is a result of the work that was initially done by Giddings and Eyring in 1955, when they developed a statistical description of molecular migration in chromatography. This description depicted chromatographic peaks as probability distribution functions.[32] This theory was further developed, made more general and applied to affinity chromatography by Denizot and Delaage in 1975.[33] Based on this previous work, the dissociation rate constant for the interaction of an injected analyte with an immobilized binding agent can be described by the following equation,[4]

$$k_d = \frac{2(E[t_o])^2(E[t_R] - E[t_0])}{\sigma_R{}^2(E[t_o])^2 - \sigma_0{}^2(E[t_R])^2} \qquad (3)$$

in which $E[t_o]$ and $E[t_R]$ are the mean retention times of the chromatographic peaks for the analyte and for a non-retained compound, respectively, and σ_R^2 and σ_0^2 are the corresponding peak variances (i.e., measures of peak width). In order for this equation to be valid, all sources of band-broadening other than analyte interactions with the stationary phase must be similar for the analyte and non-retained compound or negligible compared to analyte-stationary phase interaction under the conditions of the experiment. This requirement typically means that relatively high flow rates are used for such studies to maximize the contribution of analyte-stationary phase interactions to band-broadening versus other band-broadening processes.[4, 8]

An affinity column, like other types of chromatographic columns, can be thought of as being made up of a series of equally sized regions, or theoretical plates. Each theoretical plate can be thought of as representing a single interaction between the analyte and the stationary phase. The length of the column that is taken up by a single theoretical plate is referred to as the "plate height" and is represented by the term *H*. The value of the plate height is related to the column length *L* by the term *N*, the number of theoretical plates, where $H = L/N$. The number of theoretical plates, in turn, can be related to the square of the retention time for an analyte, t_R^2, and the variance of the analyte's peak, σ^2, as given by the relationship $N = t_R^2 / \sigma^2$.[34, 35]

As a small sample of an injected analyte travels through a column, there are several processes that cause broadening of the analye's peak and contribute to the total observed plate height, H_{total}. The contributions to H_{total} can be represented by the summation of several separate plate height terms, as shown in Equation 4.

$$H_{total} = \frac{L\sigma_R^2}{t_R^2} = H_m + H_L + H_{sm} + H_k \qquad (4)$$

In this equation, H_m represents the plate height contribution due to mobile phase mass transfer and eddy diffusion, H_L represents the contribution due to longitudinal diffusion, H_{sm} results from stagnant mobile phase mass transfer, and H_k is the contribution from stationary phase mass transfer. Another term representing extra-column band broadening, H_{ec}, is sometimes included with these other terms but can be eliminated if a correction is made for such effects by subtracting from H_{total} the band broadening results for the analyte that are measured when no column is present in the system.

In the peak profiling method, a simplification in Equation 4 is made which assumes that H_L produces only a negligible contribution to H_{total}; this assumption is valid for most types of liquid chromatography. It is also assumed that H_m is constant at the flow rates that are typically used during the peak profiling measurements. If these assumptions hold, the following equation can be used to determine the rate constant k_{-1} that describes stagnant mobile phase mass transfer on a control column that has little or no retention for the analyte (i.e., the retention factor, k, is equal to zero).[34, 36]

$$H_{sm} = \frac{2uV_P}{k_{-1}V_M} \qquad for\ k = 0 \tag{5}$$

In this equation, u is the linear velocity of the mobile phase (i.e., as measured by using a non-retained molecule), V_P is the pore volume of the stationary phase, V_M is the column void volume, and k is the retention factor. To use this relationship, sample injections are made at various flow rates to obtain plate height values on a control support that contains no immobilized binding agent or stationary phase. Alternatively, a non-retained molecule can be injected onto a column that does contain a stationary phase. If linear regression is performed on a plot of H_{total} versus u, the slope of this plot can be used to obtain a value for k_{-1} by also using the known values of V_P and V_M for the column and support. The intercept of this plot, which may be a positive non-zero value, can be used to estimate the value of H_m.[34, 37, 38]

It is also possible to estimate the plate height contribution due to stagnant mobile phase mass transfer by making injections of the analyte onto the affinity column at several flow rates and measuring H_{total} along with k. In this approach, the value obtained

for k_{-1} from the previous set of experiments conducted on a control column or with a non-retained solute is used in the following equation to estimated H_{sm}.

$$H_{sm} = \frac{2uV_P(1+\frac{V_M}{V_P}k)^2}{k_{-1}V_M(1+k)^2} \quad (6)$$

With this approach, estimates for H_{sm} can be made at each flow rate and subtracted from H_{total} to give H_k. The expression for H_k, as given by Equation 7, can then be used to calculate k_d, the dissociation rate constant for the analyte/ligand interaction.[34, 37]

$$H_k = \frac{2uk}{k_d(1+k)^2} \quad (7)$$

If a plot is made of H_k versus $uk/(1+k)^2$, Equation 7 indicated that k_d can be determined from the slope of this plot.[34, 37] To use this relationship, the ligands being studied must have weak-to-moderate binding so that the association and dissociation rates of the analyte-ligand interaction are fast enough to allow multiple binding and dissociation steps to take place as the analyte passes through the column. This generally means that the analyte-ligand interaction will have an association equilibrium constant in the range of 10^6 M^{-1} or lower. Another important consideration in this type of experiment is that the amount of analyte that is injected should be small enough to ensure that linear elution conditions are present (i.e., the retention factor should not change with the concentration of the injected analyte).[36-39]

Band broadening measurements have been used in the past to examine a few types of analyte interactions with affinity columns. For instance, an early example of the use of this method to study reaction rates was in studies that examined the interactions of

sugars with the lectin concanavalin A (Con A).[40] Rate constants for the interactions of *R/S*-warfarin and D/L-tryptophan with HSA have also been measured at various temperatures by using this method.[37, 38] Such information, along with studies on the effects on pH, ionic strength and solvent polarity on kinetics, has been further obtained and used to optimize chiral separations for *R/S*-warfarin and D/L-tryptophan on HSA columns.[37, 38, 41] Other examples of the usefulness of such studies can be found in the use of rate constants of drug-protein interactions to examine the pharmacokinetics of drug binding to HSA[8, 42, 43] and to develop new assays for measuring free drug or hormone fractions in serum.[44]

One assumption that was made in Equation 7 is that the stagnant mobile phase mass transfer is the same for the retained and the non-retained species or that the plate height contribution due to stagnant mobile phase mass transfer is significantly smaller than that due to stationary phase mass transfer. However, since the retention factor k affects the plate height due to stagnant mobile phase mass transfer, different contributions to this term can occur for retained and non-retained solutes. In order to examine such effects, the difference in each plate height contribution may be plotted versus the term $u\,k/(1+k)^2$ to compare the relative contributions due to stagnant mobile phase mass transfer (Equation 8), stationary phase mass transfer from analyte interactions with the support (Equation 9) and stationary phase mass transfer from analyte interactions with an immobilized protein or other ligand (Equation 10).[43]

$$(H_R - H_M)_{sm} = \frac{2uk}{(1+k)^2}\left[\frac{(1+{}^3/_2 k)}{k_{-1}}\right] \qquad (8)$$

$$(H_R - H_M)_{k,n} = \frac{2uk}{(1+k)^2}\left[\frac{\propto_n}{k_{d,n}}\right] \quad (9)$$

$$(H_R - H_M)_{k,P} = \frac{2uk}{(1+k)^2}\left[\frac{\propto_p}{k_{d,P}}\right] \quad (10)$$

In Equation 8, k_{-1} is given by the following equation,

$$k_{-1} = \frac{60\gamma D}{d_p^2} \quad (11)$$

where γ is the tortuosity factor, D is the diffusion coefficient for the retained and non-retained solutes, and d_p is the particle diameter of the support (i.e., 7 µm in this study).[43] Typical values[34] for γ and D of 0.5 and 1 x 10^{-5} cm^2/s, respectively, were used in this study. In Equation 9, $k_{d,n}$ is the dissociation rate constant due to binding of the analyte to the support and α_n is given by Equation 12,

$$\alpha_n = \frac{(1-f_p)k_{control}}{k} \quad (12)$$

where $k_{control}$ is the retention factor for the analyte on the control column and f_p is the fraction of the support surface covered by immobilized ligand,[26, 34, 45] as calculated based on the surface area and packing density of the support, the protein content of the column, and the area of the protein.[43] In Equation 10, $k_{d,P}$ is the dissociation rate constant due to binding of the analyte with the protein or immobilized ligand, and α_p is given by Equation 13.

$$\alpha_p = 1 - \alpha_n \quad (13)$$

Another situation of interest is when there is more than one site of interaction (e.g., a specific interaction of the drug with the immobilized protein as well as a non-

specific interaction of the drug with the support). In this case, Equation 7 can be written as shown below.[43]

$$H_R - H_M = H_{k,P} + H_{k,n}$$

$$= \frac{2uk_p}{k_{d,P}(1+k)^2} + \frac{2uk_n}{k_{d,n}(1+k)^2}$$

$$= \frac{2uk}{(1+k)^2}\left[\frac{\propto_P}{k_{d,P}} + \frac{\propto_n}{k_{d,n}}\right] \quad (14)$$

EXPERIMENTAL

Reagents. The HSA (essentially fatty acid free, > 96%) and AGP (99 % pure) were from Sigma-Aldrich (St. Louis, MO). The paroxetine (≥ 98%pure) and diazepam were from Sigma-Aldrich. Racemic propranolol (99% pure) was from Aldrich (Milwaukee, WI). The Nucleosil Si-300 (7 μm particle diameter, 300 Å pore size) was from Macherey-Nagel (Duren, Germany). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL). All other chemicals were of reagent grade or better. All aqueous solutions were prepared with water obtained from a Nanopure water system (Barnstead, Dubuque, IA).

Apparatus. The chromatographic system consisted of an LC-10AD solvent delivery system from Shimadzu (Kyoto, Japan), a LabPro injection valve from Rheodyne (Oak Harbor, WA) and a UV-2075 Plus Intelligent UV/Vis absorbance detector from Jasco (Easton, MD). The sample loop had a volume of 5 μL. The column temperature was maintained at 37.0^{o}C by using a water jacket from Alltech (Deerfield, IL) and a Fisher Scientific 9100 circulating bath (Westbury, NY). Data were collected using LabView 5.0 software (National Instruments, Austin, TX) and an interface from National Instruments (Austin, TX). Data analysis was performed using PeakFit 4.12 (Systat Software, San Jose, CA), in which moment analysis was performed using an exponentially-modified Gaussian fit (EMG) with a linear progressive baseline. The residual option in PeakFit was used to determine the best fit for the chromatographic peaks. All supports were downward-slurry packed using an HPLC column packer from Alltech (Deerfield, IL).

Column Preparation. The columns containing immobilized HSA or the control support were prepared using Nucleosil Si-300 silica. The silica was first converted into a diol form according to a previously reported method.[46] The diol content of this material was estimated to be 230 (± 20) μmol diol/g silica (± 1 S.D.), based on previous determinations by an iodometric capillary electrophoresis assay.[47] Part of the diol silica was used to immobilize HSA by the Schiff base method[41] and part was used to prepare a control support in which no protein was added during the immobilization step. AGP was immobilized to hydrazide-activated silica, following a previous method.[17] The control support for the AGP column was the hydrazide-activated silica with no protein immobilized to it. The HSA support was found to have 40 (± 2) mg HSA/g silica, as determined in triplicate by a BCA protein assay using HSA as the standard and the control support as the blank. The supports were downward slurry-packed at 24-28 MPa (3500-4000 psi) into 5 cm × 4.6 mm i.d. and 1.9 cm x 2.1 mm i.d. stainless steel columns using pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. The columns were stored at 4^{o}C in pH 7.4, 0.067 M phosphate buffer.

Chromatographic Studies. Experiments were performed at 37°C with a mobile phase of pH 7.4, 0.067 mM potassium phosphate buffer in order to simulate physiological conditions. All samples were also prepared in this buffer. The flow rate used ranged from 0.5 to 4.0 ml/min and was increased in 0.5 ml/min increments. The detection wavelengths were 205 nm, 209 nm and 225 nm for sodium nitrate, paroxetine and both propranolol and diazepam, respectively. Sodium nitrate was used as the non-retained species. Retention times were corrected by subtracting the time for analytes to elute from a zero-volume connector. The injected samples contained 25 μM paroxetine; 100 μM

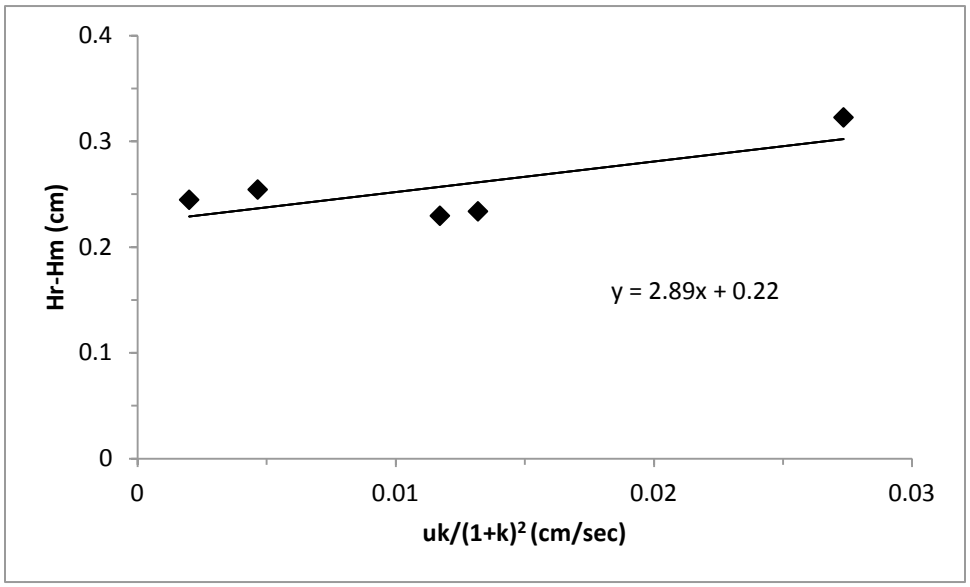
diazepam; 25 or 100 μM propranolol; and 25 or 100 μM sodium nitrate (i.e., used as a non-retained solute), as based on conditions identified previously as being suitable for peak profiling.[8] Injections for all analytes were typically made in triplicate. Similar injections were made using a zero volume union to correct for the contributions to the elution time or band-broadening by extra-column components of the system. The elution times and variances for the eluting peaks (after correcting for extra-column effects) were used to determine the retention factors and total plate heights for the analytes on the HSA column, AGP column and control columns.

RESULTS AND DISCUSSION

Peak Profiling on Control Column. The types of HSA and AGP supports used in this study have been shown previously to be good models for drug-protein interactions and to have low non-specific binding for many pharmaceutical agents.[17, 48-53] However, it was found that the tested drugs did have some non-specific binding to these supports. The retention factors on the control column for the HSA support were 0.03 (± 0.02) and 4.95 (± 0.59) for paroxetine and diazepam, respectively, and made up roughly 0.18% and 7.0% of the total retention seen for the same drugs on the HSA columns. The retention factor for propranolol on the control column for the AGP support was 44.6 (± 4.0) and made up 55.1% of the total retention seen for the propranolol on the AGP columns.

The dissociation rates for these non-specific interactions were studied by first carrying out peak profiling experiments for each drug on its corresponding control column. These data were used to prepare plots of (H_R - H_M) vs. $uk/(1+k)^2$ and analyzed according to a single-site model by using Equation 7. In the case of diazepam and propranolol, the dissociation rate for the binding of these drugs with the control support was negligible compared to the dissociation rates that were later measured for these drugs with immobilized serum proteins. In the case of paroxetine, the dissociation rate constant from the support was estimated to be 0.7 (± 0.1) s^{-1}, as determined from the plot shown in Figure 6-3.

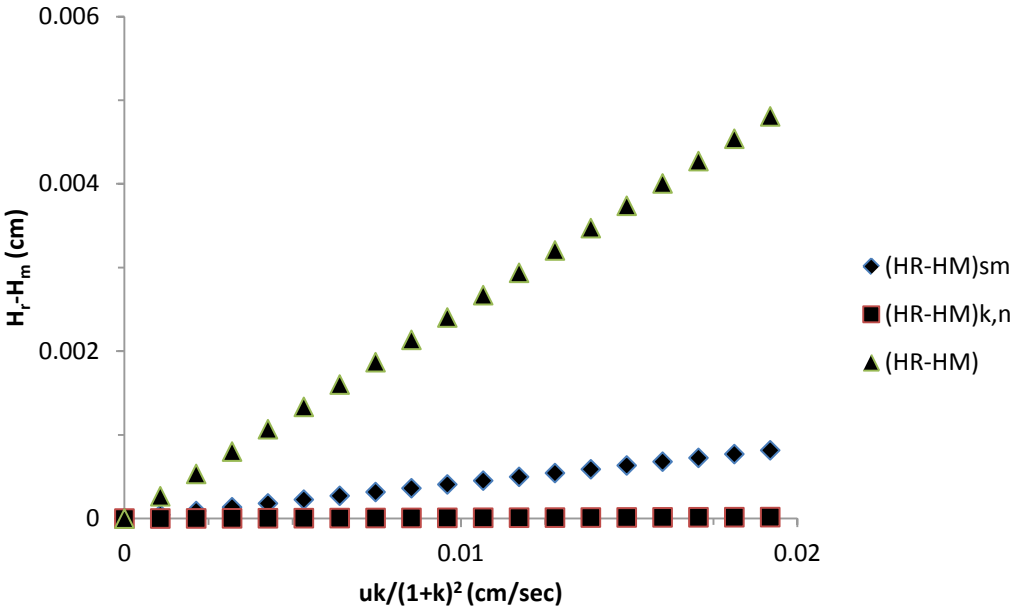
Figure 6-3. Use of the multiple flow rate peak profiling method to estimate the dissociation rate constant for non-specific binding of paroxetine to the control support. The equation of the best-fit line is shown. The standard error of the slope is 0.65.


0.4
0.3
0.2
0.1
0
Hr-Hm (cm)
y = 2.89x + 0.22
0
0.01
0.02
0.03
uk/(1+k)² (cm/sec)

Effects of Stagnant Mobile Phase Mass Transfer on Control Column. As stated earlier, Equation 7 assumes stagnant mobile phase mass transfer is approximately the same for the retained solute and non-retained solute or that its plate height contribution is small compared to that for stationary phase mass transfer. However, the retention factor (*k)* can affect the plate height due to stagnant mobile phase mass transfer and lead to differences in this term when comparing retained and non-retained solutes. The importance of this effect was examined by using a series of calculations based on chromatographic theory along with the known experimental conditions and estimated values of $k_{d,n}$ and $k_{control}$ for each drug on the control column, as described in the Theory. These calculations were carried out for paroxetine but not for diazepam or propranolol, given the negligible values of $k_{d,n}$ for these drugs on the control support.

Figure 6-4 shows a theoretical plot of $(H_R - H_M)$ vs. u $k/(1+k)^2$ for paroxetine on the control column. These results confirmed that the overall change in $(H_R - H_M)$ for this system would have been only about 0.005 cm over the flow rate range that was examined in this study, which was on the same order of magnitude as the precision of the experimental measurements made on the HSA and AGP columns.

Figure 6-4. Predicted contributions from various band-broadening processes for paroxetine on a control column.

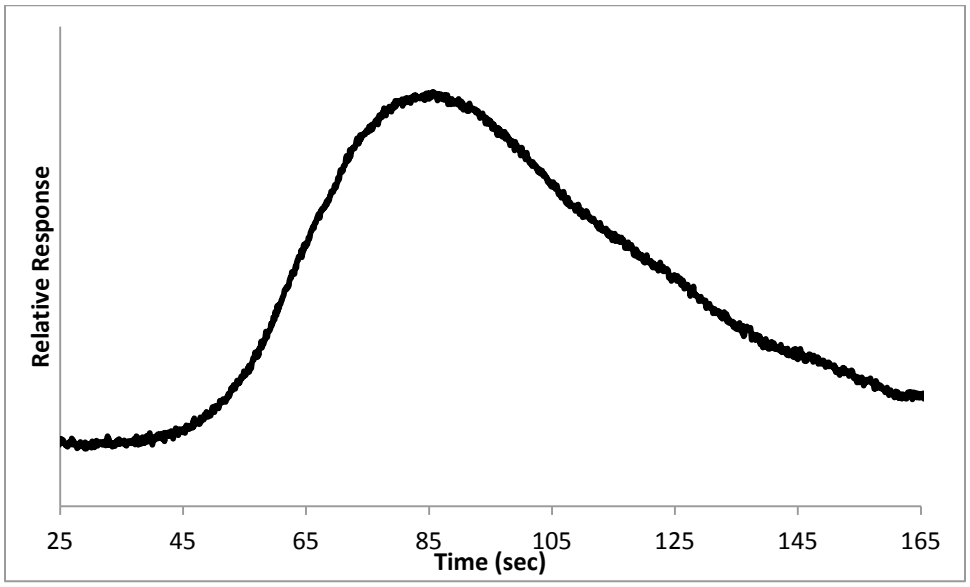

0.006
0.004
0.002
0
0
0.01
0.02
H_r-H_m (cm)
uk/(1+k)^2 (cm/sec)
(HR-HM)sm
(HR-HM)k,n
(HR-HM)

Peak Profiling on Protein Columns. The next set of experiments used peak profiling to determine the dissociation rate constants for diazepam and paroxetine on an HSA column. At 2.5 mL/min, the elution of diazepam occurred in less than 1.5 min (see Figure 6-5), and the elution of paroxetine required less than 5 min. Plots of (H_R - H_M) vs. u $k/(1+k)^2$ gave an overall positive slope that could be used to estimate the k_d values for paroxetine interacting with HSA and propranolol with AGP (e.g., see example in Figure 6-6). However, the single flow rate method of peak profiling was used to examine the interactions of diazepam interacting with HSA because of the strong binding, slow dissociation, and small changes in plate height that were present in the latter case.

Table 6-1 summarizes the results that were obtained. The apparent dissociation rate constant that was estimated for paroxetine on the HSA column, and without any correction for non-specific binding to the support, was 3.96 (± 0.54) s^{-1} at pH 7.4 and 37 ºC. For propranolol on the AGP column, the dissociation rate constant was estimated to be 0.45 (± 0.25) s^{-1}. The interaction of diazepam with HSA was found to be 0.11 (± 0.06) s^{-1}, as based on Equation 3. When a correction was made for non-specific binding, the corresponding values for these same rate constants were 3.95, 0.20 and 0.10 s^{-1}, respectively. During this process, it was found that it was not necessary to correct for the protein coverage of the support, which had only a small or insignificant effect on the amount of support surface that was available for non-specific binding. For instance, the values of α_P that were calculated by using the measured protein content of the supports and a cross sectional areas for the given proteins were only 0.02 (± 0.01), 0.0006 (± 0.0004), and 0.13 (± 0.01) for diazepam, paroxetine and propranolol, respectively.[5,28,54]

Figure 6-5. Chromatograms for (a) diazepam and (b) paroxetine on an HSA column. The broad peaks result from H_k making the main contribution to broadening and mainly reflect dissociation, which is slow.

(a)


Relative Response
25
45
65
85
105
125
145
165
Time (sec)

(b)

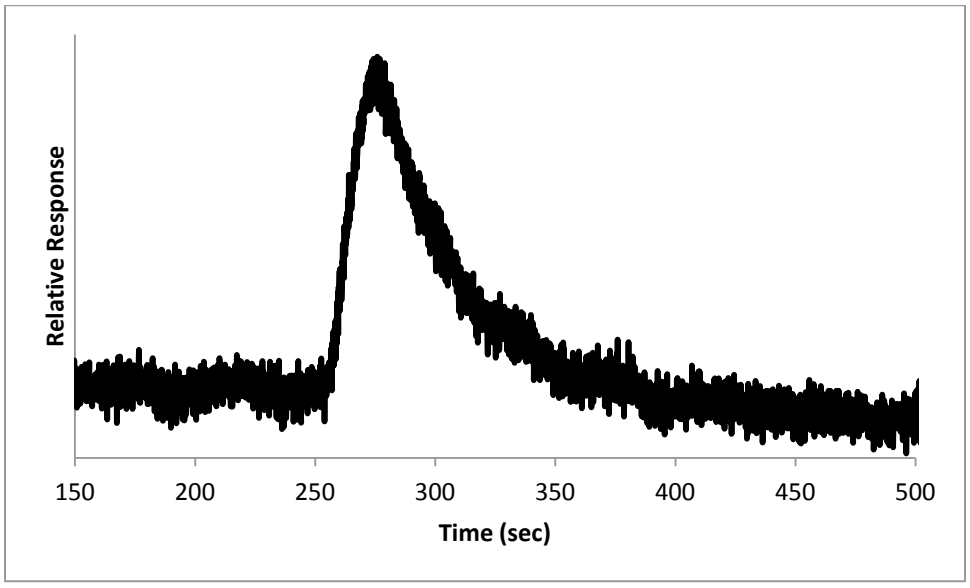
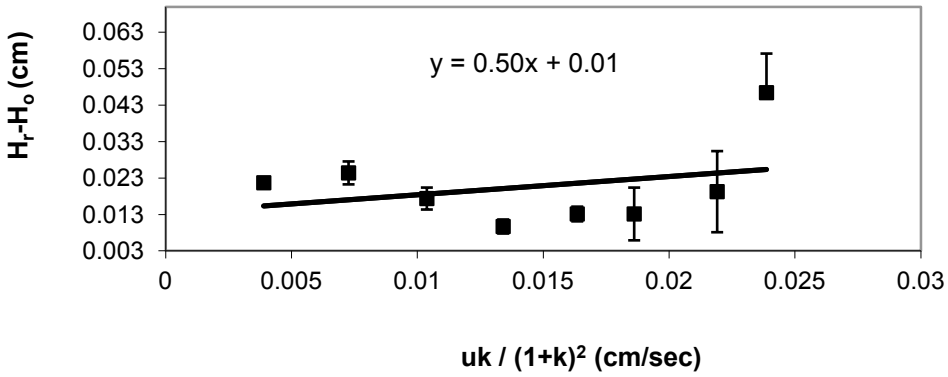

Relative Response
150
200
250
300
350
400
450
500
Time (sec)

Figure 6-6. Plot of $(H_R - H_M)$ vs. u $k/(1+k)^2$ obtained for paroxetine on an HSA column. The error bars represent the standard deviation in $(H_R - H_M)$ values, taken as an average at each flow rate. The equation of the best-fit line is shown. The standard error of the slope is 0.22.


y = 0.50x + 0.01
0.063
0.053
0.043
0.033
0.023
0.013
0.003
0
0.005
0.01
0.015
0.02
0.025
0.03
H_r-H_o (cm)
uk / (1+k)^2 (cm/sec)

No previous studies have reported the dissociation rate constants for these interactions, although a k_d value for diazepam interacting with HSA was measured using another method and found to be 0.44 (± 0.02).[55] However, these results do agree with the range of k_d values that have been measured for other drugs and solutes with HSA, such as warfarin[37, 56] and L-tryptophan.[4, 8, 38]

Table 6-1. Dissociation rate constants estimated by the peak profiling method.[a]

Drug-Protein	$\mathbf{k_d}$ (s^{-1})
Without correction for non-specific binding	
Paroxetine – HSA	3.96 (± 0.54)
Diazepam – HSA	0.11 (± 0.06)[b]
Propranolol- AGP	0.45 (± 0.25)
With correction for non-specific binding	
Paroxetine – HSA	3.95 (± 0.54)
Diazepam – HSA	0.10 (± 0.06)[b]
Propranolol- AGP	0.20 (± 0.25)

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

[b]The single flow rate method gave better results for this drug.

CONCLUSIONS

The method of peak profiling was used with HPAC to examine the binding kinetics of the drugs diazepam and paroxetine with HSA and propranolol with AGP. The estimated dissociation rate constants for diazepam and paroxetine with HSA were 0.10-0.11 s^{-1} and 3.95-3.96 s^{-1}, respectively, at pH 7.4 and 37 °C. The dissociation rate constant measured for propranolol with AGP was 0.20-0.45 s^{-1} at pH 7.4 and 37 °C. Although these drugs, with the possible exception of paroxetine, had relatively fast dissociation during their non-specific interactions with the support, these interactions did indirectly affect the peak profiling results obtained on HSA and AGP columns through the contribution of non-specific binding to the overall retention of the drugs. A correction was made for these retention effects. The approach described in this report is not limited to the particular drugs and supports that were used in this study. An advantage of this peak profiling approach is its use of chromatographic data that is relatively simple to obtain (i.e., retention times and peak widths) and the ability to acquire such data in a matter of minutes. These features should make this method attractive for use in solute-protein binding studies and in the high-throughput determination of dissociation rate constants for drug/protein interactions for biomedical and pharmaceutical research.

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CHAPTER 7

SUMMARY AND FUTURE WORK

SUMMARY

This dissertation presented research involving various applications of affinity chromatography. A brief introduction to emerging contaminants and related research needs was given in Chapter 1. Chapter 2 provided an overview of immunosorbent use for extraction of pesticides and carbamazepine from samples combined with RPLC and mass spectrometric detection.

Chapters 3 and 5 centered on the development of novel affinity extraction sorbents for environmental contaminants. Chapter 3 dealt with the synthesis of MIPs for this purpose and associated difficulties, while Chapter 5 examined the successful use of BSA for affinity extraction of emerging contaminants form water.

The use of immobilized serum proteins, HSA and AGP, to study their interactions with pharmaceutical agents was the focus of Chapters 4 and 6. Various methods of immobilization of AGP to silica were employed and optimized for use of this support in the chiral resolution of mepivacaine and propranolol. These experiments were described in Chapter 4 and it was found that a method of mild oxidation of AGP, followed by immobilization to hydrazide-activated silica worked best for this application. Chapter 6 presented the use of chromatographic peak profiling to determine dissociate rate constant values for the interactions of paroxetine and diazepam with HSA and propranolol with AGP. Results were similar to literature values obtained using other methods. Non-

specific binding of these drugs to the stationary phase was taken into account and corrections to the values were made as a result.

FUTURE WORK

In order to produce MIPs suitable for chromatographic applications, other methods of synthesis can be employed. These include multi-step swelling polymerization, suspension polymerization and precipitation polymerization. These methods are known to produce spherical MIPs with uniform sizes.

Future experiments dealing with emerging contaminants include the use of antibody extraction columns for estrone, β-estradiol and possibly other compounds in water samples. BSA and antibody columns can also be tested with spiked wastewater samples and compared to C_{18} extraction cartridges. Ultimately, these sorbents can be tested in field sampling, along with the use of POCIS extraction membranes.

Chapter 6 presented the first known use of AGP in the chromatographic profiling method. These types of experiments with AGP will be continued with other drugs and the interactions of chiral compounds with this protein will be examined in the context of peak profiling.

APPENDIX - Representative partial spreadsheet for 2,4-D simulation

Linear Elution Model - Simulated column - Results in terms of analyte in mobile phase																		
Input Column Parameters										**Calculated parameters**								
k:			1.20E+01			**Flowrate (ml/min):**	1.00E+00			**VM (mL):**		0.027709		**mL/slice (mol):**				
mL (mol):						**Col. length (cm):**	1.00E+00			**tM (min):**		0.027709		**tM/slice (min):**	0.000831257			
Conc. A (M):			9.05E-06			**Col. ID (mm):**	2.10E+00			**Ka:**				**VM/slice (mL):**	0.000831257			
						No. Slices:	3.33E+01			**Ka/VM (mol):**				**mol A/cycle (mol):**	7.52287E-12			
H (cm):			3.00E-02			**Detector Cell:**	3.43E+01			**tR (min):**		0.360211						
Sample vol (mL):			5.00E-03			**Slices for Sample Inj:**	6.014989			**Total A (mol):**		4.53E-11						
Rel Response A:			1.00E+12															
tM/cycle (min):			Adjust to			mol A/cycle:												
0.000831			Detector Cell		Normalize	7.52287E-12												
		Time/tM or		Cumulat	Cumulat					Column Slice No. - A in mobile phase								
Time(min)	Vol (mL)	Vol/VM	Response	Response	Response	Next A input (mol)				1	2	3	4	5	6	7	8	9
0	0	0	0.00E+00	0.00E+00	0.00E+00	7.52287E-12				0	0	0	0	0	0	0	0	0
0.000831	0.000831	0.03	0.00E+00	0.00E+00	0.00E+00	7.52287E-12				5.79E-13	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
0.001663	0.001663	0.06	0.00E+00	0.00E+00	0.00E+00	7.52287E-12				1.11E-12	4.45E-14	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
0.002494	0.002494	0.09	0.00E+00	0.00E+00	0.00E+00	0				1.61E-12	1.27E-13	3.42E-15	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
0.003325	0.003325	0.12	0.00E+00	0.00E+00	0.00E+00	0				1.48E-12	2.40E-13	1.29E-14	2.63E-16	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
0.004156	0.004156	0.15	0.00E+00	0.00E+00	0.00E+00	0				1.37E-12	3.36E-13	3.04E-14	1.24E-15	2.03E-17	0.00E+00	0.00E+00	0.00E+00	0.00E+00
0.004988	0.004988	0.18	0.00E+00	0.00E+00	0.00E+00	0				1.26E-12	4.15E-13	5.39E-14	3.48E-15	1.14E-16	1.56E-18	0.00E+00	0.00E+00	0.00E+00
0.005819	0.005819	0.21	0.00E+00	0.00E+00	0.00E+00	0				1.17E-12	4.81E-13	8.17E-14	7.36E-15	3.73E-16	1.02E-17	1.20E-19	0.00E+00	0.00E+00
0.00665	0.00665	0.24	0.00E+00	0.00E+00	0.00E+00	0				1.08E-12	5.33E-13	1.12E-13	1.31E-14	9.10E-16	3.81E-17	8.95E-19	9.22E-21	0.00E+00
0.007481	0.007481	0.27	0.00E+00	0.00E+00	0.00E+00	0				9.93E-13	5.75E-13	1.45E-13	2.07E-14	1.85E-15	1.05E-16	3.75E-18	7.73E-20	7.09E-22
0.008313	0.008313	0.3	0.00E+00	0.00E+00	0.00E+00	0				9.17E-13	6.07E-13	1.78E-13	3.03E-14	3.30E-15	2.39E-16	1.16E-17	3.60E-19	6.60E-21
0.009144	0.009144	0.33	0.00E+00	0.00E+00	0.00E+00	0				8.47E-13	6.31E-13	2.11E-13	4.16E-14	5.37E-15	4.74E-16	2.91E-17	1.22E-18	3.38E-20
0.009975	0.009975	0.36	0.00E+00	0.00E+00	0.00E+00	0				7.81E-13	6.48E-13	2.43E-13	5.46E-14	8.16E-15	8.51E-16	6.33E-17	3.36E-18	1.25E-19
0.010806	0.010806	0.39	0.00E+00	0.00E+00	0.00E+00	0				7.21E-13	6.58E-13	2.74E-13	6.92E-14	1.17E-14	1.41E-15	1.24E-16	7.97E-18	3.74E-19
0.011638	0.011638	0.42	0.00E+00	0.00E+00	0.00E+00	0				6.66E-13	6.63E-13	3.04E-13	8.49E-14	1.62E-14	2.21E-15	2.23E-16	1.69E-17	9.59E-19
0.012469	0.012469	0.45	0.00E+00	0.00E+00	0.00E+00	0				6.15E-13	6.63E-13	3.31E-13	1.02E-13	2.14E-14	3.28E-15	3.76E-16	3.28E-17	2.18E-18
0.0133	0.0133	0.48	0.00E+00	0.00E+00	0.00E+00	0				5.67E-13	6.59E-13	3.57E-13	1.19E-13	2.76E-14	4.68E-15	5.99E-16	5.91E-17	4.54E-18
0.014131	0.014131	0.51	0.00E+00	0.00E+00	0.00E+00	0				5.24E-13	6.52E-13	3.80E-13	1.38E-13	3.47E-14	6.44E-15	9.13E-16	1.01E-16	8.74E-18
0.014963	0.014963	0.54	0.00E+00	0.00E+00	0.00E+00	0				4.83E-13	6.42E-13	4.01E-13	1.56E-13	4.26E-14	8.62E-15	1.34E-15	1.63E-16	1.58E-17
0.015794	0.015794	0.57	0.00E+00	0.00E+00	0.00E+00	0				4.46E-13	6.30E-13	4.20E-13	1.75E-13	5.14E-14	1.12E-14	1.90E-15	2.54E-16	2.71E-17
0.016625	0.016625	0.6	0.00E+00	0.00E+00	0.00E+00	0				4.12E-13	6.16E-13	4.36E-13	1.94E-13	6.09E-14	1.43E-14	2.62E-15	3.80E-16	4.46E-17
0.017456	0.017456	0.63	0.00E+00	0.00E+00	0.00E+00	0				3.80E-13	6.00E-13	4.50E-13	2.13E-13	7.11E-14	1.79E-14	3.52E-15	5.52E-16	7.04E-17
0.018288	0.018288	0.66	0.00E+00	0.00E+00	0.00E+00	0				3.51E-13	5.83E-13	4.61E-13	2.31E-13	8.20E-14	2.20E-14	4.62E-15	7.80E-16	1.07E-16
0.019119	0.019119	0.69	0.00E+00	0.00E+00	0.00E+00	0				3.24E-13	5.65E-13	4.71E-13	2.49E-13	9.35E-14	2.66E-14	5.96E-15	1.08E-15	1.59E-16
0.01995	0.01995	0.72	0.00E+00	0.00E+00	0.00E+00	0				2.99E-13	5.47E-13	4.78E-13	2.66E-13	1.05E-13	3.18E-14	7.55E-15	1.45E-15	2.30E-16
0.020781	0.020781	0.75	0.00E+00	0.00E+00	0.00E+00	0				2.76E-13	5.28E-13	4.83E-13	2.82E-13	1.18E-13	3.74E-14	9.41E-15	1.92E-15	3.24E-16