Order Number 9510983

Development of high-performance immunoaffinity chromatography

Thomas, David H., Ph.D.

The University of Nebraska - Lincoln, 1994

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

DEVELOPMENT OF HIGH-PERFORMANCE IMMUNOAFFINITY CHROMATOGRAPHY

by

David H. Thomas

A DISSERTATION

Presented to the Faculty of

The Graduate College at The University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Chemistry

Under the Supervision of Professor David S. Hage

Lincoln, Nebraska

May, 1994

DISSERTATION TITLE

Development of High-Performance Immunoaffinity Chromatography

BY

David H. Thomas

SUPERVISORY COMMITTEE:

	APPROVED	DATE
Signature		4/26/94
Typed Name	David Hage	
Signature		4/26/94
Typed Name	Carolyn Price	
Signature		4/26/94
Typed Name	Michael L. Gross	
Signature		4/26/94
Typed Name	T. A. George	
Signature		4/26/94
Typed Name	James D. Carr	
Signature		4/26/94
Typed Name	Roy F. Spalding	

GRADUATE COLLEGE
UNIVERSITY OF NEBRASKA

DEVELOPMENT OF HIGH-PERFORMANCE IMMUNOAFFINITY CHROMATOGRAPHY

David H. Thomas, Ph. D.

University of Nebraska, 1994

Advisor: David S. Hage

The theory and behavior of a chromatographic competitive binding immunoassay with sequential injection of sample and labeled analyte were examined. An equation was derived relating assay response (B/B_0) to the column's binding capacity, the moles of analyte and labeled analyte injected, and the flow rate/adsorption kinetics of the system. There was good agreement between this equation and experimental data for the binding of human serum albumin (HSA) to an immobilized anti-HSA antibody column. It was found that the amount of labeled analyte injected, when applied in excess *versus* binding sites in the column, had little effect on the calibration curve. The position of the calibration curve was determined mainly by the number of binding sites on the column, but could be shifted over several orders of magnitude by varying the flow rate used for analyte injection.

A fully automated HPLC method was developed for the analysis of atrazine in water. This method used a high-performance immunoaffinity column for the extraction of atrazine and other triazines from samples, followed by separation of the retained compounds with an on-line reversed-phase column. This technique used only 250 μL of

sample, required minimal sample pretreatment and showed no significant interferences from the sample matrix or several common pesticides tested. Atrazine plus all of its major degradation products could be determined in 20 min with this system. The calibration curve for atrazine was linear over two orders of magnitude and had a lower limit of detection of 0.1 μg/L. The within-day precision was $\pm$ 5.4% for samples containing 1.1 μg/L atrazine. The results of this method showed good correlation with those obtained by GC/MS or GC/NPD. By using different immunoaffinity columns and elution conditions, this method could be adapted for use in the determination of other compounds of environmental interest.

The feasibility of coupling Flow Injection Analysis to the affinity column waste stream was demonstrated using the determination of urinary catecholamines and creatinine as an example. This effectively doubled the throughput of the high-performance affinity chromatographic technique. Accuracy and precision of this method compared favorably with standard methods.

These studies lay the foundation for the rapid, fully automated determination of analytes in complex matrices by the use of High-performance immunoaffinity chromatography.

To my Father

and

In Memory of my Mother

With gratitude to David Hage, Yiren Gu, Bounthon Loun, Brother John, and many other long persevering friends.

Table of Contents

CHAPTER 1

INTRODUCTION

High-performance immunoaffinity chromatography (HPIAC) exploits the strong, highly specific, yet reversible binding that occurs between certain molecules and the immunoglobulins that recognize them. One of these compounds, immobilized onto a porous microparticulate silica support, is used as an affinity ligand to cleanly extract the other compounds from a complex mixture. After a sample is injected onto an HPIAC system, the non-specifically bound sample components pass through unretained. The specifically bound analytes remain bound to the affinity ligand until the mobile phase conditions are changed in order to disrupt the analyte-ligand interactions.

There are many ways in which the antibody-antigen interaction can be manipulated to perform a chromatographic separation or to make an analytical measurement. Two easily recognized approaches differ in the amount of immobilized antibody used relative to the amount of analyte. In the first approach, called a chromatographic competitive binding immunoassay, an HPLC column containing a limited amount of immobilized antibody is used. In the second approach, which can be denoted an immunoaffinity extraction, an HPLC column containing a large excess of immobilized antibody is used to selectively extract the analyte from a sample. The work presented in this dissertation advances the theory and practical application of both of these approaches.

In Chapter 2, the theory and behavior of one type of chromatographic competitive binding immunoassay is examined. In this approach, called the sequential injection

mode, sample is first injected onto a column containing a small amount of immobilized antibodies (Step 1). After non-retained sample components have been washed away, a fixed amount of an analyte conjugated to a highly detectable label is injected (Step 2). Since some of the antibody binding sites have been occupied by sample analyte, fewer are available for the labeled analyte. This results in slower adsorption and less retention of the labeled analyte then would occur if no sample analyte had been injected. The amount of labeled analyte bound to the column can be determined indirectly by monitoring the non-retained fraction in Step 2 or by later applying an elution buffer that dissociates the retained analyte and labeled analyte from the column (Step 3). After eluting these retained components, the column is brought back to its initial mobile phase conditions, allowed to regenerate, and the next sample is injected. By determining the fraction of labeled analyte retained or non-retained at different loads of sample analyte, a standard curve can be generated for quantitating the amount of analyte in unknown samples.

Chapter 2 presents the derivation of an equation that describes this calibration curve in terms of fundamental parameters of the system, namely flowrate, adsorption kinetics and relative amounts of immobilized antibody, analyte and label. This equation is then used to predict the behavior of the assay under a variety of chromatographic conditions, and corroborated by comparison with experimental results.

In Chapter 3, the second approach is explored. A combined HPIAC/RPLC system using a column containing a large excess of immobilized antibodies is used to perform an automated immunoaffinity extraction and reversed-phase HPLC separation of

triazine compounds. Practical issues considered in the design of this system include the application and elution conditions for the HPIAC and RPLC columns and ways of minimizing chromatographic interferences. The system is then characterized with respect to speed, response precision and accuracy. We find that this combined system allows rapid, sensitive assays with minimal interference from the sample matrix. The crossreactivity that limits the specificity of some manual immunoasays is eliminated by the subsequent reversed-phase separation, thus allowing quantitation of atrazine in the presence of similar triazine herbicides and their degradation products.

The exquisite specificity of immunochemical methods results in the disadvantage that analytically useful information may be wasted in the nonretained fraction that passes through the column. Chapter 3 presents a novel way to recover some of this information, by coupling a flow injection analysis system to the HPIAC waste stream. The system is designed to allow the simultaneous determination of urinary catecholamines along with creatinine. This is necessary so that the level of catecholamines can be excretion-volume normalized and reported in a clinically useful form.

CHAPTER 2

CHARACTERIZATION OF A SEQUENTIAL ADDITION COMPETITIVE BINDING IMMUNOASSAY BASED ON HIGH-PERFORMANCE IMMUNOAFFINITY CHROMATOGRAPHY

INTRODUCTION

The competitive binding immunoassay is a powerful method for the analysis of biological samples. An immunoassay may be defined as an analytical method based on the use of biochemical reagents known as antibodies for the selective determination of the compound of interest. Antibodies, or immunoglobulins, are a diverse group of glycoproteins that make up part of the body's immune system. There are approximately 10^6-10^8 types of antibodies in the body, each of which binds to a unique foreign agent (1). Compounds to which antibodies may be produced include drugs, hormones, enzymes, nucleic acids, whole cells and viruses (2). The selectivity and diversity of available antibodies have made the immunoassay a popular analytical tool in the areas of clinical chemistry, biochemistry, and pharmaceutical testing.

In a competitive binding immunoassay, a small amount of antibody is mixed with sample and a fixed amount of an analyte analog (i.e., the label) that is conjugated with an easily detectable chemical compound, such as a radioisotope, fluorescent tag or enzyme. This mixture is then incubated, allowing the analyte in the sample and the label to compete for the limited number of antibody binding sites. As the concentration of analyte in the sample increases, less of the antibody binding sites are available for the

label. After the incubation, the analyte and label that are bound to the antibody are separated from that remaining free in solution. The amount of analyte in the original sample is then determined indirectly by measuring the relative amount of label in the bound and/or free fractions (2).

Competitive binding immunoassays are popular in bioanalysis because they are both selective, as a result of the specific nature of the antibody-analyte interaction, and sensitive, owing to the use of radio or other labels. Immunoassays can be used for a wide range of analytes and require little or no sample preparation. However, current competitive binding immunoassays suffer from a number of disadvantages. One disadvantage is that these assays are usually manual techniques because multiple reagent additions, washing steps and/or separation steps are involved in their use. A second disadvantage is that the long incubations often required lead to analysis times of hours or days. High sample throughput can be obtained by performing a batch analysis, but this makes quality control difficult because it is not known whether an assay has worked until all of the samples, standards and controls have been processed. The long analysis times also make troubleshooting difficult because several days may be required to isolate and fix assay problems.

One method being investigated for overcoming these disadvantages is to automate competitive binding immunoassays by using high-performance immunoaffinity chromatography (HPIAC). In HPIAC, a high-performance column containing immobilized antibodies is used for the rapid separation and determination of a given sample component (3). Like immunoassays, the use of antibodies makes this technique

very selective for a given analyte. Furthermore, HPIAC has the additional advantages of being fast, typically requiring only minutes per analysis, and easily automated (4-6). Because HPIAC analyzes samples in serial rather than a batch mode, it is also easier to maintain quality control than in standard immunoassay methods (7).

A number of recent papers have reported the use of competitive binding immunoassays based on HPIAC and other flow-based systems (8-15). Most of these papers have used schemes in which the sample and label are injected simultaneously onto an immobilized antibody column (8-13). An alternative method proposed by Nilsson et al. (14) and Cassidy et al. (15) is to inject sequentially the sample and label, as illustrated in Figure 1. In this approach, sample is first applied to a column containing a small amount of immobilized antibodies (Step 1). After washing away non-retained sample components, a fixed amount of label is also injected (Step 2). Because some of the antibody binding sites are already occupied by sample analyte, fewer of these sites are now available for the label. This results in slower adsorption and less retention of the label than if no sample analyte had been injected previously. The amount of label bound to the column is then determined either indirectly, by measuring the non-retained fraction in Step 2, or directly, by eluting the retained analyte and label from the column and measuring the amount of label released (Step 3). After these retained components have been eluted, the column is brought back to its initial mobile-phase conditions and allowed to regenerate before the next sample is injected. A standard curve can be generated for quantitating the amount of analyte in unknown samples by determining the fraction of

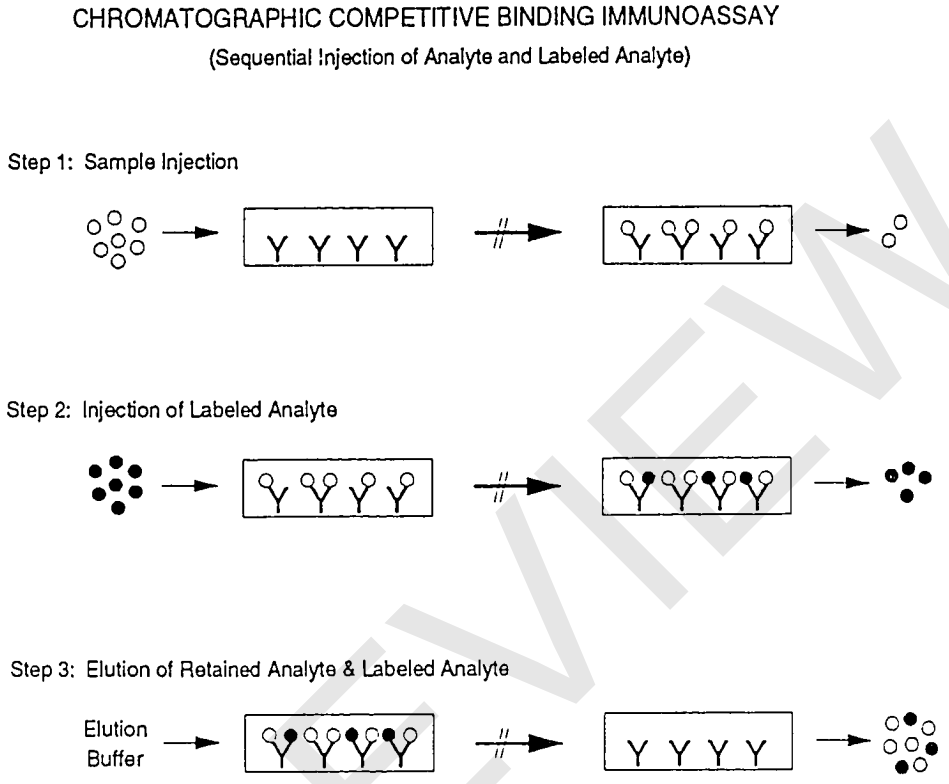

Figure 1. Chromatographic competitive binding immunoassay with sequential addition of sample analyte (○) and label (●).

label bound by the column at different loads of sample analyte (14,15).

The sequential addition method offers several advantages over assays based on the simultaneous injection of sample and label. One advantage is that sample pretreatment, such as the addition of label, is minimized prior to injection. A second advantage is that no contact is ever made between the label and sample components. This is important if the label undergoes degradation in the presence of sample (e.g., cleavage of an enzymatic label by proteolytic factors) or if the label has secondary binding to the sample matrix, affecting its later retention on the immobilized antibody column. A third advantage of the sequential injection method is that the amount of free (and indirectly the amount of bound) label can be determined with no interference from the analyte or other sample components. This means that even a nonderivatized "labeled" analyte and relatively non-specific detection methods, such as UV/Vis absorbance, can be used as long as they provide a sufficient limit of detection for the analyte range to be determined (15).

Although it has been shown that a sequential-addition, competitive-binding immunoassay can be performed by HPIAC, little or no work has examined the theoretical basis of this method. In this chapter, the theory and behavior of a sequential addition assay will be examined by using non-linear chromatographic theory and a model antibody system. From non-linear chromatographic theory, an equation will be developed to describe the response of this assay in a system with adsorption-limited kinetics and homogeneous binding sites. The results predicted by this equation will be compared to experimental data obtained for the binding of human serum albumin (HSA) to an immobilized anti-HSA antibody column. The role of various assay parameters, such as

the amount of label applied and the injection flow rate, will be studied. From this work a series of general guidelines will be developed for use in the design and optimization of this type of assay.

THEORY

Predicted Assay Response. The response of a standard competitive binding immunoassay is commonly expressed in terms of the ratio B/B_o, where B_o is the amount of label bound to antibody when sample analyte is absent and B is the amount of label bound when sample analyte is present. B/B_o has a maximum value of one when there is no sample analyte present in the system. This ratio decreases as increasing amounts of sample analyte are added. A plot of B/B_o *versus* log[Analyte] produces a sigmoidal-shaped curve with a dynamic range covering several orders of magnitude in analyte concentration. The shape and position of this curve are affected by the equilibrium constants for the binding of analyte (A) and label (A*) to the antibody (Ab), and the concentrations of A, A* and Ab used in the assay (16).

The chromatographic model shown in Figure 2 was used to describe the binding of A and A* to an immobilized antibody column. In this Figure, F is the flow rate, k_1 and k_{-1} are the forward and reverse mass transfer rate constants for the movement of A between the flowing mobile phase and stagnant mobile phase, and k_3 is the second-order adsorption rate constant describing the binding of A to the immobilized antibody (17,18). A similar set of reactions can be written for the label (A*). Previous work with this model has shown that there is a finite chance that some analyte will pass through the column without interacting with any of the immobilized binding sites (18,19). This phenomenon, known as the "split-peak" effect, leads to the formation of two peaks from a single sample injection: a non-retained (or free) fraction and a strongly retained (or bound) fraction.

CHROMATOGRAPHIC MODEL

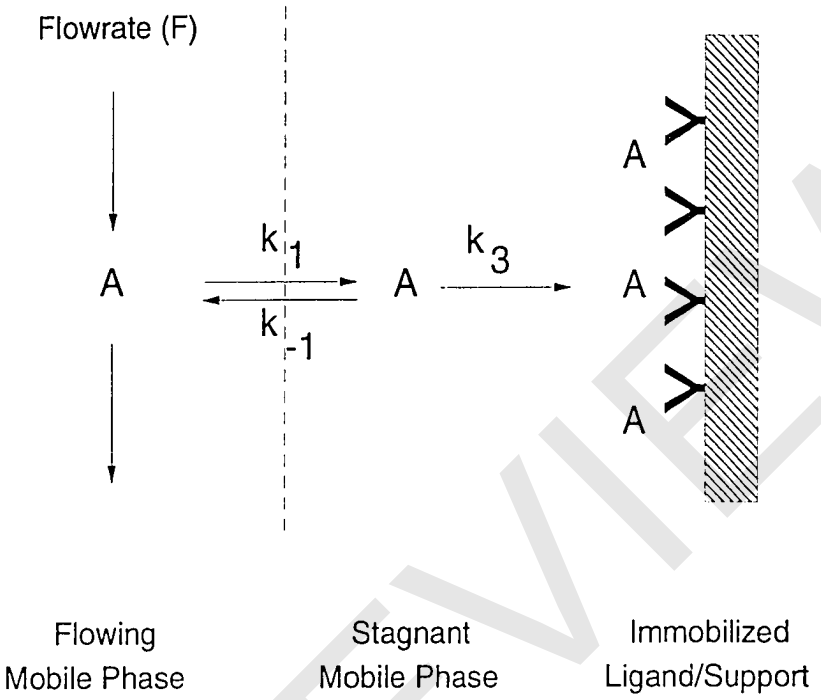

Figure 2. Model used in describing a chromatographic competitive binding immunoassay. A is the compound injected onto the column and Y represents the immobilized antibody. All other terms are described in the text.

This effect has been shown to increase with fast flow rates, slow kinetics, or a small number of binding sites (18,19). These are the same conditions expected to occur with a column used in a rapid, competitive binding assay format.

If it is assumed that adsorption is the rate-limiting step in analyte retention, then the following equations predict the fraction of A that elutes in the free fraction (f) when the amount of analyte injected is relatively small (linear conditions) or approaching the number of binding sites on the column (non-linear chromatographic conditions) (18-20):

Linear Conditions:

$$f = e^{-(k_3 m_L)/F} = e^{-1/S_o} \qquad (1)$$

Non-Linear Conditions:

$$f = \{S_o/Load\ A\} \ln \left[1 + \left\{e^{(Load\ A)/S_o} - 1\right\} e^{-1/S_o}\right] \qquad (2)$$

In these equations, m_L is the moles of antibody (or ligand) sites originally present in the column, and Load A is the relative amount of analyte injected (Load A $\equiv$ moles A/m_L). Under linear chromatographic conditions (eq 1), the free fraction f is determined by the flow rate, the number of binding sites, and the adsorption kinetics of the chromatographic system (18). For convenience, these factors are described by the split-peak constant S_o, where $S_o = F/(k_3 m_L)$ for adsorption-limited kinetics. Under non-linear chromatographic conditions (eq. 2), f is a function of both S_o and the relative amount of A applied to the column (19,20).