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DEVELOPMENT OF SANDWICH MICROCOLUMNS FOR USE IN RAPID CHROMATOGRAPHIC IMMUNOASSAYS

by

William A. Clarke

A DISSERTATION

Presented to the Faculty of

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In Partial Fulfillment of Requirements

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Major: Chemistry

Under the Supervision of Professor David S. Hage

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DISSERTATION TITLE

Development of Sandwich Microcolumns for Use in Rapid Chromatographic Immunoassays

BY

William A. Clarke

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GRADUATE COLLEGE
UNIVERSITY OF NEBRASKA

DEVELOPMENT OF SANDWICH MICROCOLUMNS FOR USE IN RAPID CHROMATOGRAPHIC IMMUNOASSAYS

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University of Nebraska, 2000

Adviser: David S. Hage

The work in my dissertation involves the development of microcolumns for performing rapid chromatographic immunoassays. The areas of study involved the development of microcolumns with lengths of less than 1 mm, optimization of antibody immobilization to chromatographic supports, and the separation of free warfarin from serum protein bound warfarin. In addition, sandwich microcolumns were used in the development of a rapid chromatographic immunoassay for free thyroxine (T_4) in serum.

Part one deals with optimizing the amount of antibody that can be immobilized to a chromatographic support. This study examined several factors, including immobilization of Fab fragments versus entire antibodies, the use of general or site-specific attachment, and differences between the immobilization of antibodies to silica or other commercially available supports.

Part two explores the development of microcolumns with sub-millimeter lengths and the reproducibility of making these columns. Reproducibility of the method was determined by packing hemoglobin microcolumns and analyzing each microcolumn for total protein content. Studies were performed to determine the feasibility of using these columns for rapid

chromatographic immunoextractions under both diffusion-limited and adsorption-limited conditions.

In part three, the use of microcolumns to separate the free and protein-bound fractions of warfarin is explored. The extraction of warfarin in a sub-second time frame was optimized in order to minimize the interference from protein bound warfarin released during this extraction. Microcolumn immunoextraction coupled with restricted-access media chromatography was used to separate the free and bound warfarin and determine the amount of free warfarin in the sample. The experimental results were then compared with mathematically predicted values as well as the results of a computer simulation.

The last part of this dissertation involves the development of a chromatographic immunoassay for free T_4 in serum. The analysis was performed using a displacement immunoassay format with chemiluminescent detection and a labeled analog. The reproducibility of the method was established and a standard curve was obtained using various T_4 concentrations. Finally, the amount of free T_4 in a serum sample was determined.

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CHAPTER 1

GENERAL INTRODUCTION

This dissertation discusses the development of a rapid chromatographic immunoassays for measuring, drugs, hormones, and other analytes. Areas of study in this work include: optimization of antibody immobilization to chromatographic supports for immunoaffinity chromatography, development of sandwich microcolumns for rapid extractions, determination of free warfarin in solutions containing warfarin and human serum albumin, and development of a chromatographic immunoassay for the determination of free thyroxine in serum.

Antibody Structure:

Antibodies were discovered in 1890 when Emil von Behring and Shibasaburo Kitasato discovered proteins that bound to the relevant pathogen in the serum of vaccinated individuals [1]. An antibody is defined as a plasma protein produced by B-lymphocytes that specifically binds a substance which is then referred to as an antigen. The site on the antigen to which the antibody molecule binds is called an epitope. Antibodies have two functions; the first is to bind foreign agents, or antigens, in the body and the second is an effector function. The effector function is responsible for eliminating the antigen after the antibody-antigen complex has been formed. An immune response results in the production of antibodies to a particular pathogen that has been introduced into the system. Each antibody molecule has unique structural components that allow it to bind to a specific antigen, but all antibodies have the same general structure and are known as a family of proteins called

immunoglobulins (Ig). The immunoglobulin family can be divided into five classes, or isotypes: IgM, IgA, IgE, IgD and IgG. Each of these isotypes can be distinguished from one another both by structure and by their function.

IgM has an average serum concentration of 1.5 mg/mL in humans and accounts for 5%-10% of the total serum immunoglobulin. IgM is the first immunoglobulin produced in the primary response to an antigen and is the first to be produced in neonates. Monomeric IgM is expressed on the surface of B-cells; when the B-cell is activated, giving rise to a plasma cell, IgM is secreted as a pentamer.

IgA constitutes 10%-15% of the total immunoglobulins in serum in humans. It is the predominant immunoglobulin class found in external secretions such as tears, saliva, breast milk and mucus of the bronchial and digestive tracts. The IgA in secretions, called secretory IgA, consists of either a dimer or tetramer of the protein. In serum, IgA exists primarily as a monomer, although it can also be found as dimers, trimers or tetramers.

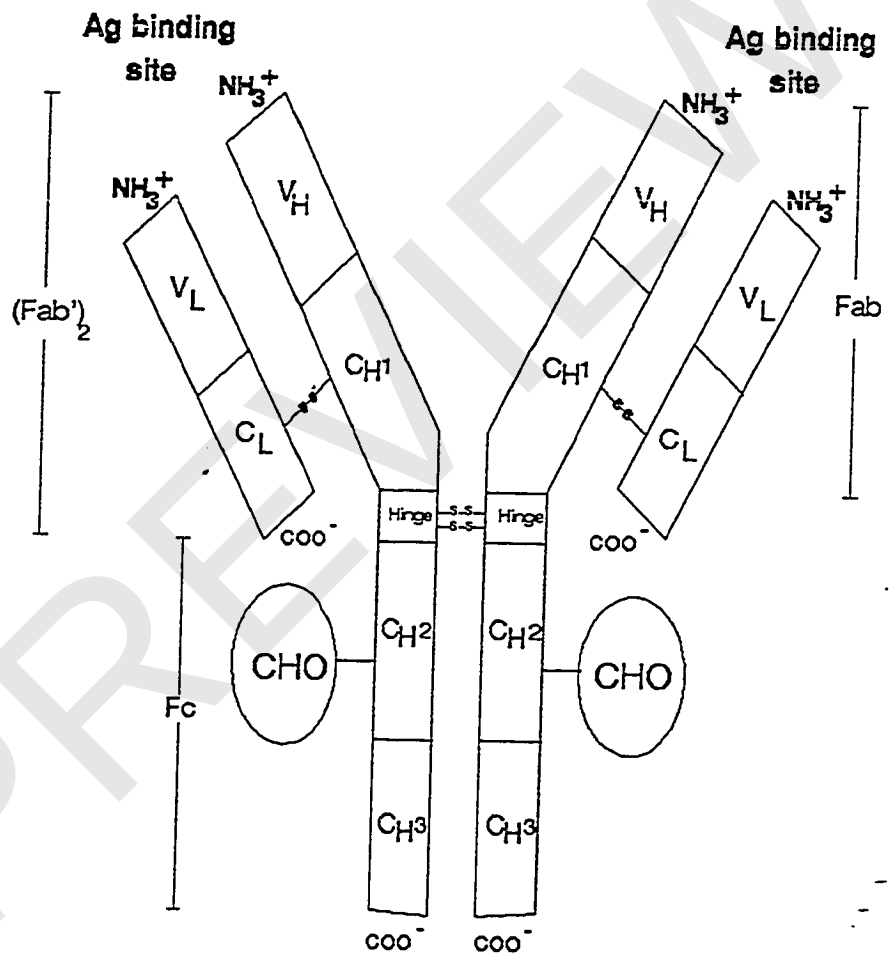
IgE has a low serum concentration (0.3 μg/mL) in humans and accounts for a small percentage of the total Ig concentration in serum, but it is easy to detect due to its potent biological activity. IgE antibodies control the immediate hypersensitivity reactions that are responsible for the symptoms of hay fever, asthma, hives and anaphylactic shock. When IgE molecules are cross-linked by antigen (also known as an allergen) allergic reactions occur.

IgD has a serum concentration of 30 μg/mL in humans and constitutes about 0.2% of the total immunoglobulin in serum. Along with IgM, IgD is the major membrane-bound immunoglobulin. It is thought to function in the activation of B-cells by antigen; however, no biological effector function has yet been identified for IgD.

IgG is the most abundant type of immunoglobulin in serum, constituting approximately 80% of the total serum immunoglobulins. Human IgG is divided into four subclasses that are numbered with respect to their decreasing serum concentrations: IgG_1, IgG_2, IgG_3 and IgG_4. IgG is the only immunoglobulin that can cross the placental barrier and provide protection to an unborn child. The subclasses of IgG contain differences in structure that give rise to variance in their effector functions. Rabbit IgG, which only has one subclass, was the main immunoglobulin isotype that was used during our immobilization studies.

The basic structure of an antibody can be seen in Figure 1-1, using IgG as an example of a typical antibody molecule. The region of the antibody that binds antigen is highly variable in nature and is known as the variable region, or V region. This is located at the amino terminus of the protein. The region of the antibody that engages the effector functions of the immune system is essentially constant in structure and is known as the constant region, or C region. The antibody is composed of four polypeptide chains: two identical heavy chains linked together by disulfide bonds, and two identical light chains which are each linked to the heavy chains by disulfide bonds. These four chains are held together by a combination of disulfide bonds and noncovalent interactions to form the classic "Y"-shaped immunoglobulin molecule. Experiments by Edelman and Porter [2,3] revealed that each heavy chain is composed of a polypeptide chain having a molecular weight of about 50 kilodaltons (kDa), and each light chain is composed of a polypeptide chain with a molecular weight of 25 kDa. The heavy chains have amino acid sequences that fall into one of five categories; mu (μ), alpha (α), epsilon (ε), delta (δ) and gamma (γ). These five categories

Figure 1-1. Basic Structure of an IgG Molecule



correspond to the five isotypes of immunoglobulins.

The heavy chain (H) of immunoglobulins is composed of approximately 440 amino acids, which are divided into four domains of 110 amino acids each. The domain whose sequence begins at the amino terminus shows considerable variation from antibody to antibody. This region is referred to as the variable region (V_H) of the heavy chain. The remaining three domains remain fairly constant within each isotype and these domains comprise the constant region (C_H) of the antibody. These three domains are numbered with respect to their position in relation to the amino terminus: C_H1, C_H2 and C_H3. An oligosaccharide side chain, containing as many as seven sugars, is attached to the C_H2 region and is very heterogenous within each isotype.

The light chain (L) of immunoglobulins consists of two domains, also containing 110 amino acids each. The sequence of amino acids in the domain that begins at the amino terminus is highly variable and is known as the variable region (V_L) of the light chain. The second domain of each light chain does not show much amino acid variation and is known as the constant region (C_L). When the heavy and light chains are associated to form the immunoglobulin molecule, the variable regions are responsible for antigen binding. Differences in amino acid sequence found in the variable region of the antibody account for the specific nature of antibody-antigen binding.

The hinge region of the antibody is located between the C_H1 and C_H2 domains in the immunoglobulin structure. This region consists of a sequence of 10-15 amino acids that connect the two domains. Each isotype has a different hinge region that plays a role in the flexibility of the antibody arms containing the light chains and, thus, the binding sites. This

area of the antibody is susceptible to attack by proteolytic enzymes.

Papain is a proteolytic enzyme that cleaves the antibody on the amino side of the hinge region that holds the heavy chains together. Three protein fragments are obtained from cleavage by papain. The first is a 50 kDa fragment that contains the C_H2 and C_H3 domains of both heavy chains. This fragment is referred to as the Fc fragment because it contains most of the constant region of the antibody, giving an almost identical amino acid sequence within an isotype and making it easy to crystallize from solution. The Fc abbreviation stands for fragment crystallizable. Two other identical 50 kDa fragments are obtained from the cleavage, each containing the V_L, C_L, V_H and C_H1 domains from the immunoglobulin. These fragments retain their antigen binding capabilities and are named the Fab fragments, which stands for fragment antigen binding.

A different enzyme, pepsin, also cleaves the immunoglobulin within the hinge region. Pepsin cleaves the heavy chains on the carboxyl side of the hinge region, leaving the entire hinge region untouched. This process produces a 100 kDa fragment with its binding region still intact. This fragment is different from an Fab fragment in that it contains both binding sites from the antibody and is thus capable of binding two antigen molecules rather than one; therefore, this fragment is known as the $(Fab')_2$ fragment. This is the only fragment obtained from digestion with pepsin, because the Fc fragment is destroyed during the digestion.

Affinity Chromatography:

Affinity chromatography is a separation technique that is based on the same types of specific, reversible interactions that are found in biological systems, such as the binding of

an enzyme with a substrate or an antibody with an antigen. Affinity chromatography was first demonstrated by Starkenstein in 1910 when insoluble starch was used to purify α-amylase [4]. The technique of affinity chromatography was further developed in 1951 when Campbell et. al immobilized antigens onto a solid matrix in order to purify antibodies [5]. In 1967, Porath et. al developed a method to attach ligands to agarose by activating the support with cyanogen bromide, giving rise to modern affinity chromatography [6].

Affinity chromatography is a valuable tool in areas such as biochemistry, pharmaceutical science, clinical chemistry and environmental testing, where it has been used for both the purification and analysis of compounds in complex sample mixtures. The strong and relatively specific binding that characterizes many affinity ligands allows solutes that are quantitated or purified by these ligands to be separated with little or no interferences from other sample components. Often the solute of interest can be isolated in one or two steps, with purification yields of one hundred to several thousand-fold being common. Similar selectivity has been observed when using affinity chromatography for compound quantitation in such samples as serum, plasma, urine, food, cell cultures, water and soil extracts.

The most common way of performing affinity chromatography is by using a step gradient for elution, as shown in Figure 1-2. This involves injecting a sample onto the affinity column in the presence of a mobile phase that has the right pH and solvent composition for solute-ligand binding. This solvent, which represents the weak mobile phase of the affinity column, is called the application buffer. During the application phase of the separation, compounds which are complimentary to the affinity ligand will bind as the sample is carried through the column by the application buffer. However, due to the high

Figure 1-2. Protocol for Affinity Chromatography Using Step-Gradient Elution