CHARACTERIZATION OF SOLUTE-PROTEIN INTERACTIONS USING HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY AND AFFINITY CAPILLARY ELECTROPHORESIS

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High-performance affinity chromatography and affinity capillary electrophoresis is used for the study of interactions between drugs and human serum albumin (HSA). In chapter 2 and 3, frontal analysis was used to determine the association equilibrium constant and binding capacity for carbamazepine on immobilized HSA column at various temperatures. The results indicated that carbamazepine had a single binding site on HSA with an association constant of 5.3 x 10^3 M^{-1} at pH 7.4 and 37°C. This was confirmed through the use of zonal elution self-competition studies. The value of ΔG for this reaction was -5.35 kcal/mol at 37°C, with an associated change in enthalpy (ΔH) of -6.45 kcal/mol and a change in entropy (ΔS) of -3.56 cal/mol·K. The location of this binding region was examined by competitive zonal elution experiments using probe compounds with known sites on HSA. It was found that carbamazepine had direct competition with L-tryptophan, a probe for the indole-benzodiazepine site of HSA, but allosteric interactions with probes for the warfarin, tamoxifen and digitoxin sites. Changes in the pH, ionic strength, and organic modifier content of the mobile phase were

used to identify the predominant forces in the carbamazepine-HSA interaction.

In chapter 4, a method was developed and evaluated for preparing *N*-hydroxysuccinimide (NHS)-activated silica for use in immobilizing human serum albumin (HSA) in HPLC columns. HSA column prepared in this manner showed excellent chrial separation ability and long term stability on separation of racemic warfarin and tryptophan.

A technique based on affinity capillary electrophoresis (ACE) and chemically-modified proteins was used to screen the binding sites of various drugs on human serum albumin (HSA) in chapter 5. This involved using HSA as a buffer additive, following the site-selective modification of this protein at two residues (tryptophan 214 or tyrosine 411) located in its major binding regions. The results of this method showed good agreement with those of previous reports.

In chapter 6. capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) and electrospray ionization mass spectrometry (ESI-MS) were used to study the preparation of singly labeled protein from conjugation of myoglobin with fluorescent molecules and the kinetics of this conjugation reaction.

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CHAPTER 1.

BINDING OF DRUGS BY HUMAN SERUM ALBUMIN

INTRODUCTION

The development of new approaches for studying solute-protein interactions is an area of ongoing and active research. The interaction of solutes with proteins is important in many biological processes. Examples include the binding of hormones with their receptors or antigens with antibodies. The binding of endogenous compounds or pharmaceutically important molecules with proteins in blood is yet another example of such a process. These interactions can be important in determining the overall distribution, elimination, therapeutic activity and toxicity of a drug (1-3).

In some cases, the binding of these solutes will occur with a general ligand, such as the blood transport proteins human serum albumin (HSA) and α_1-acid glycoprotein (AGP) (1,2). In other situations these interactions may occur in a specific manner, as is the case for interactions of the hormone L-thyroxine with thyroxine-binding globulin or the binding of corticosteroids and sex hormones to various steroid-binding globulins (3).

The direct or indirect competition of two solutes for the same binding protein can be an important source of displacement, which can alter the availability of one solute relative to the other. An example of this is the displacement of phenytoin from HSA by valproic acid or the displacement of disopyramide from AGP by mono-*N*-dealkyldisopyramide (4,5). Competition between drugs and endogenous compounds, such as the displacement of various drugs from HSA by fatty acids or bilirubin, is another

source that can alter the availability of a drug (6).

The degree of drug-protein binding in plasma can have a significant effect on the pharmacokinetic and pharmacodynamic properties of a drug. This is due to the fact that the unbound drug can penetrate the wall of the blood vessel and become therapeutically effective once it finds a target receptor. The protein bound form of a drug is small enough to penetrate the blood vessel and is typically eliminated in the urine or feces. Furthermore, drug-plasma protein binding is often stereoselective due to the inherent chirality of plasma proteins like HSA and AGP. This topic has been of particular interest in recent years as agencies such as the U.S. Food and Drug Administration have begun to increase rules and regulations involving the marketing and use of chiral drugs (7). This has also resulted in increased research aimed at resolving such agents, some work of which has involved the use of proteins as chiral ligands in chromatographic and electrophoretic methods (8,9).

HUMAN SERUM ALBUMIN

Human serum albumin (HSA) is the most abundant protein in serum, with typical blood concentration of 50 g/L. It is probably the most extensively studied of all proteins (1). It has a number of important physiological properties in blood (10). Although HSA was first studied more than a century ago, the elucidation of its three-dimensional structure was achieved only recently (1989) by means of crystals grown in the space shuttle and through the use of recombinant technologies. In addition, the characterization of the significance of HSA as a protective agent, as a factor in lipid metabolism, as a carrier for solutes and their metabolites, and in other functions is still

being recognized. Table 1 shows some of the important functions of HSA in the body.

HSA has a molecular mass of 66,438 Da and consists of a single polypeptide chain of 585 amino acids that is held together by 17 disulfide bonds (1). Minghetti *et al.* reported the amino acid sequence for HSA based on complementary DNA data, and the three-dimensional structure of HSA was determined by He *et al.* at a resolution of 6.0 Å (17,18). The crystal structure of HSA indicates that this protein is composed of three homologous domains (referred to as I, II and III), with each domain being divided into two subdomains (referred to as A and B). Figure 1 shows the three-dimensional structure of HSA.

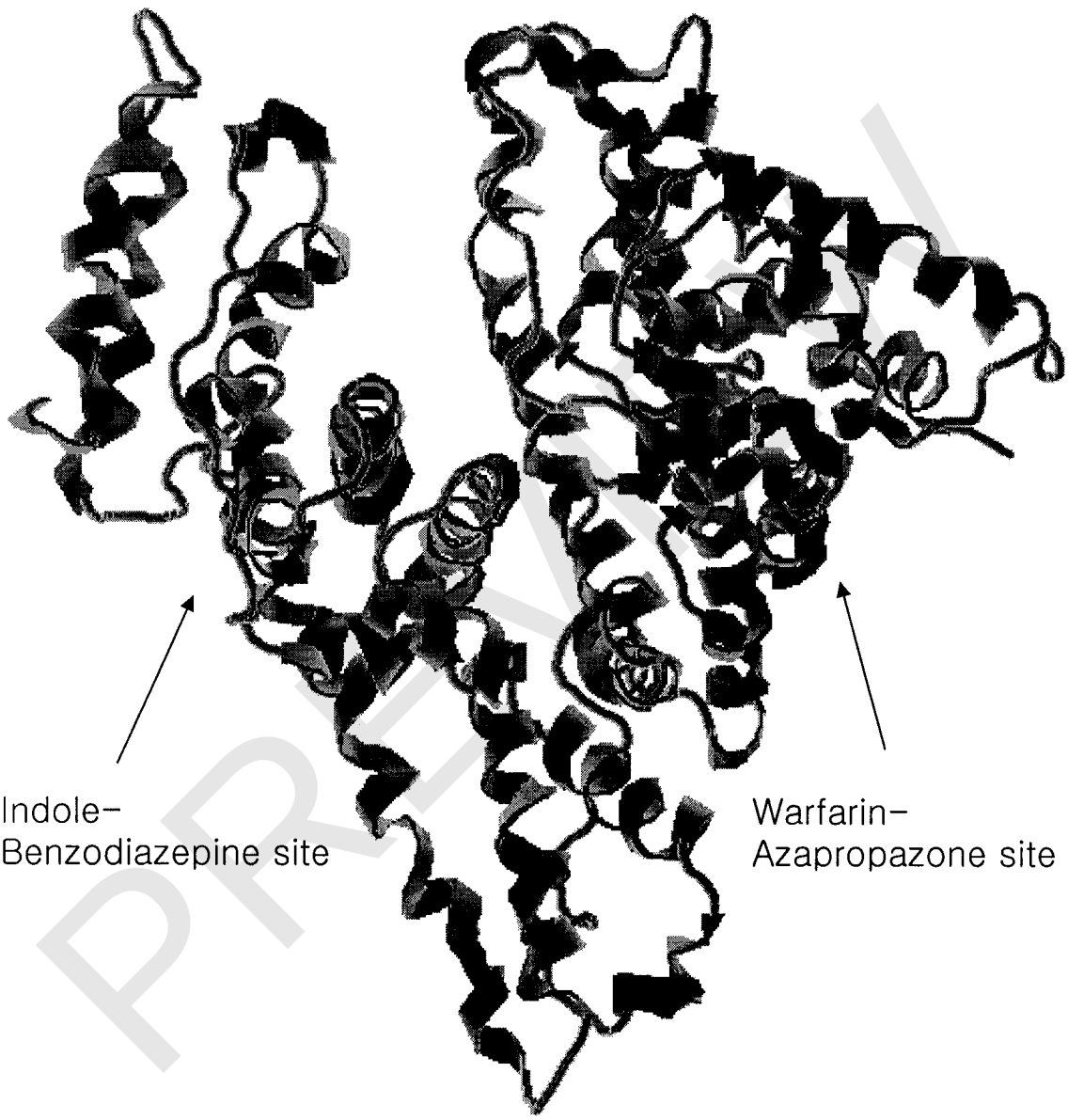
Many low mass solutes show reversible binding to HSA, including both endogenous agents (e.g., long chain fatty acids, steroids, and bilirubin) and exogenous compounds (e.g., salicylate, diazepam, and warfarin). The binding of HSA with these substances occurs at relatively well-defined regions on this protein. For drugs, the two most important binding regions on HSA are Sudlow sites I and II. These are also referred to as the warfarin-azapropazone and indole-benzodiazepine sites, respectively, due to their affinities for solutes belonging to these groups of compounds (see Fig. 1). Sudlow site I is located in the IIA subdomain of HSA and specifically binds to warfarin and coumarins, along with salicylate and many other drugs. Sudlow site II is located in the IIIA subdomain of HSA and interacts with such agents as L-tryptophan, diazepam, and naproxen (1,3-5). The locations of sites I and II on HSA have been identified by X-ray crystallography (19).

Characterization of the binding sites on a protein is important for understanding solute-protein interactions as well as for drug discovery. The binding sites of HSA and

Table 1. General functions of HSA in the body.

Function	Comment	Ref.
Circulatory roles	Accounts for 80% of colloidal osmotic blood pressure	11
	Supplies most of the acid/base buffering	12
Protective agent	Acts as an antioxidant	13
	Sequesters exogenous toxins	14
Metabolic effect	Stimulates lipoprotein lipase activity and inhibits pancreatic lipase	15,16
Carrier	Transports endogenous and exogenous compound	1

Figure 1. Three dimensional structure of HSA and its warfarin-azapropazone and indole-benzodiazpine site (www.rcsb.org/pdb).



the composition of these sites have been examined by various techniques, including UV difference spectroscopy, fluorescence spectroscopy, and circular dichroism measurements (20-22). Modification of amino acids in these sites, with changes in the binding affinities or chiral selectivities, have been reported (23,24). These studies have shown that the change of binding observed between solutes and the modified HSA derivatives is one way to determine whether or not a given amino acid participates in these binding sites.

The binding of a protein to a small solute is often described by the reaction model shown in Eqs. (1) and (2), where S is the solute of interest, L is the binding site on the protein, and S-L is the resulting solute-protein complex.

$$S + L \leftrightarrow S\text{-}L \quad (1)$$

$$K_a = k_a/k_d = [S\text{-}L]\,/[S][L] \quad (2)$$

In eq 2, [] represents the molar concentration of S and L in solution, K_a is the association equilibrium constant for the reaction, k_a is the second-order association rate constant for solute-protein binding, and k_d is the first-order dissociation rate constant.

Many methods are available for the study of drug-protein binding. Equilibrium dialysis and ultrafiltration have been most widely used for this purpose (25). Equilibrium dialysis has been the reference method for such measurements. One reason for this is its simplicity as well as its relatively low cost. It, however, suffers from several disadvantages. One disadvantage is the long period of time typically required for establishing an equilibrium during the dialysis process (hours or even days). This not only makes this method inconvenient for routine analysis, but it creates problems if the analyte is unstable or if its binding is susceptible to changes with temperature or pH.

Ultrafiltration is another technique used in evaluating solute-protein binding.

Its operation is similar to that of equilibrium dialysis but requires less time to establish an equilibrium. This method, as well as dialysis, requires a labeled solute and is often performed using a second method for analysis, such as an immunoassay, GC or HPLC. In addition, the effects of analyte adsorption to the membrane or filter must be considered.

The problems associated with these traditional methods have accelerated the effort to find better, faster, more efficient and convenient approaches for the analysis of drug-protein binding. Two such efforts involve the use of high-performance affinity chromatography (HPAC) and affinity capillary electrophoresis (ACE).

HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

High-performance affinity chromatography (HPAC) is a type of HPLC in which the separation or purification is based on specific and reversible interactions between the analyte and an immobilized ligand. The retention is based on the interactions commonly seen in biological systems. Examples include the binding of a hormone with its receptor, an enzyme with its substrate, or an antibody with an antigen. In affinity chromatography, a sample containing the analyte is applied to a column containing an immobilized affinity ligand. The analyte is then retained by this ligand due to their specific interactions while other components are quickly washed from the column. The analyte is later eluted off the column by altering the mobile phase, pH, or ionic strength, or by adding competing agents. The column is then restored to the original mobile phase conditions, allowed to regenerate, and the next analysis is performed.

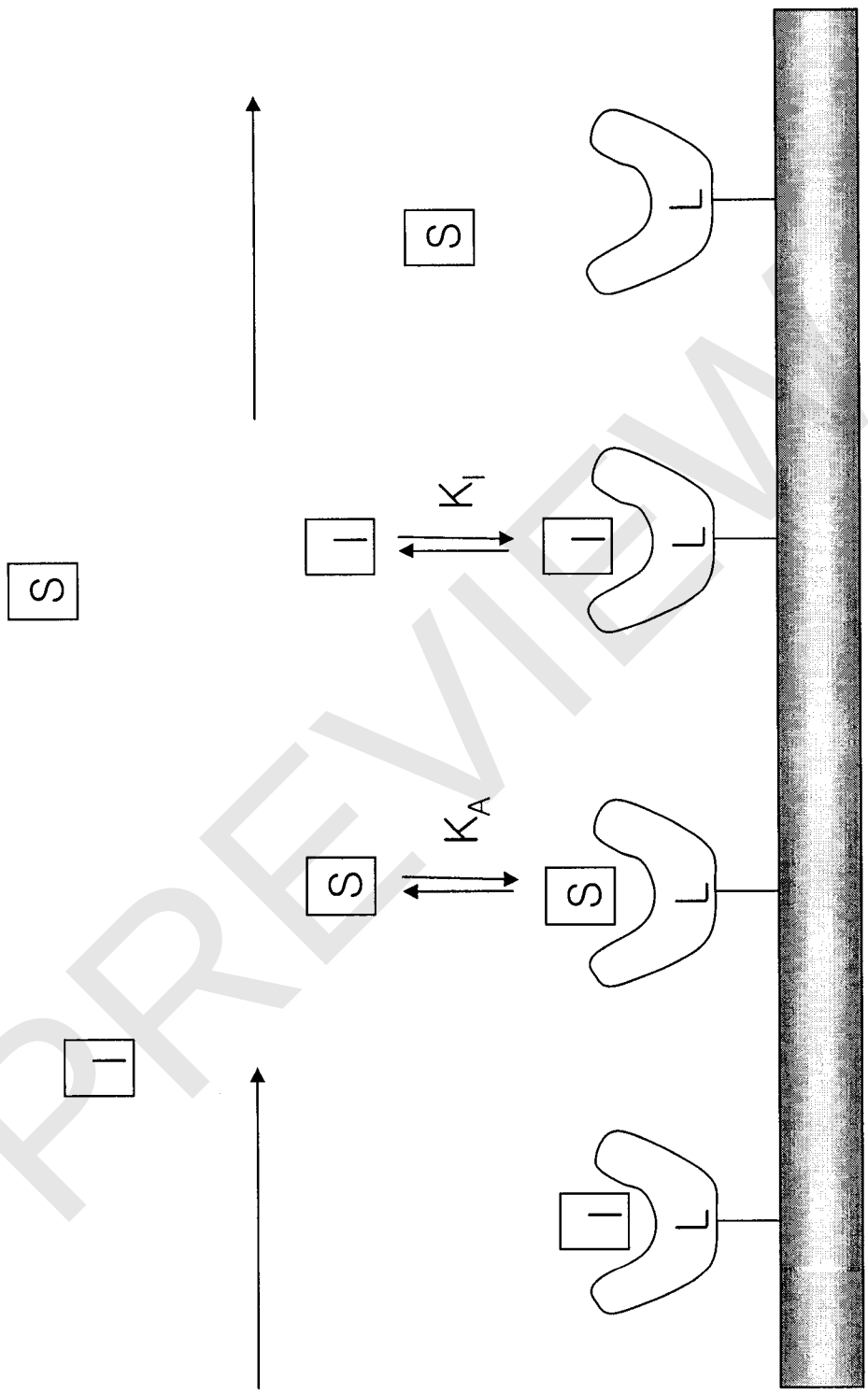
Traditional affinity chromatography exploits these interactions by immobilizing one of a pair of interacting molecules onto a solid support and packing this into a column.

The support material typically used in low-performance affinity chromatography is a large diameter, non-rigid material such as agarose or dextran. These materials not only show poor mass transfer properties, but they also suffer from limited stabilities at high flow rates and pressures. The low back pressure produced from these materials means that they can be operated under gravity flow, making them relatively simple and inexpensive to use for the purification or pretreatment of samples.

HPAC combines the speed and efficiency of high performance liquid chromatography (HPLC) with the specificity of affinity chromatography. These properties are produced by using support materials which consist of small, rigid particles capable of withstanding high flow rates or pressures and that possess good mass transfer properties. Examples include silica, glass, and hydroxylated polystyrene media. Compared to low performance affinity chromatography, the separation in HPAC requires standard HPLC equipment such as high precision pumps and valves. Although these make HPAC more expensive than low performance affinity chromatography, the better speed and precision of HPAC make it preferable for routine work and analytical applications. .

Affinity chromatography is sometimes used to evaluate equilibrium or kinetic parameters of biological systems. The use of affinity chromatography for this purpose is referred to as analytical affinity chromatography, quantitative affinity chromatography, or biochromatography (26,27). This is typically performed by using the approach illustrated in Figure 2. The ligand of interest is immobilized onto a suitable support and an injection of analyte is made onto the column. Information regarding equilibrium or kinetic parameters can later be obtained by carefully observing the elution time or

Figure 2. Reaction model for the study of solute-protein binding by HPAC, where *S* is the injected or applied analyte and *I* is a competing agent which has been added at a known concentration to the mobile phase. The terms K_A and K_I represent the association equilibrium constants for the binding of *S* and *I* to *L*, respectively



volume of analyte as it passes through the column. As will be seen later, this approach will be used to evaluate the binding of HSA with the drug carbamazepine.

AFFINITY CAPILLARY ELECTROPHORESIS

Affinity capillary electrophoresis (ACE) is a relatively new technique that can be used for the study of molecular interactions. This method uses shifts in electrophoretic mobilities of a solute as it binds with an affinity ligand. ACE can provide both qualitative and quantitative information on solute-protein binding. This information includes the detection of complex formation, the identification of an active component for binding in a multi-component mixture, the identification of structural requirements for recognition, the determination of the equilibrium constant and stoichiometry for a binding reaction, and concentration measurements based on immunochemical recognition (28). ACE is especially useful for evaluating solute-protein binding because it requires only small quantities of proteins (i.e., usually less than several nanoliters). Typical analysis times are short and ACE can be easily automated. Protein adsorption inside the capillary surface, however, can be a drawback and cause non-reproducible results. This can be corrected by employing a surfaced modified capillary to prevent protein adsorption.

There are a number of ways to evaluate solute-protein interactions in ACE. One particular method used in this work is mobility shift assay. Figure 3 illustrates the general format of a mobility shift assay in ACE. In a mobility shift assay, the evaluation of solute-protein binding is performed by injecting a small amount of solute (S) into a capillary that contains a soluble protein (P) in the running buffer. This is represented by

Figure 3. Principle of the mobility shift assay in ACE. In (a) the solute migrates with its native electrophorectic mobility, and in (b) the migration of solute is enhanced or retarded when it interacts with soluble ligands in the running buffer.