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CHARACTERIZATION OF DRUG-PROTEIN INTERACTIONS IN BLOOD USING HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

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Abstract

The binding of drugs with proteins in blood, serum or plasma is an important process in determining the activity, distribution, rate of excretion, and toxicity of drugs in the body. High-performance affinity chromatography (HPAC) has received a great deal of interest as a means for studying these interactions. This review examines the various techniques that have been used in HPAC to examine drug-protein binding and discusses the types of information that can be obtained through this approach. A comparison of these techniques with traditional methods for binding studies (e.g., equilibrium dialysis and ultrafiltration) will also be presented. The use of HPAC with specific serum proteins and binding agents will then be discussed, including human serum albumin and α_1 -acid glycoprotein. Several examples from the literature are provided to illustrate the applications of such research. Recent developments in this field are also described, such as the use of improved immobilization techniques, new data analysis methods, techniques for working directly with complex biological samples, and work with immobilized lipoproteins. The relative advantages and limitations of the methods that are described will be considered and the possible use of these techniques in the high-throughput screening or characterization of drug-protein binding will be discussed.

Keywords

drug-protein binding; high-performance affinity chromatography; human serum albumin; α_1 -acid glycoprotein; lipoproteins

1 Introduction

1.1 Drug-protein binding in blood

The binding of drugs with proteins in the blood stream is an important process in determining the eventual activity and fate of such drugs once they have entered the circulation. These interactions, in turn, help control the distribution, rate of excretion, and toxicity of drugs in the body. Furthermore, the presence of direct or indirect competition between two drugs or between a drug and an endogenous compound (e.g., a fatty acid) for the same binding proteins can be an important source of drug-drug interactions or drug displacement effects [1–7]. This binding also helps improve the solubility of hydrophobic agents [8]. These features make it important for pharmaceutical and clinical chemists to have a good understanding of how pharmaceutical agents bind to proteins in blood and of how these interactions may be affected by the presence of other substances [1–10].

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Figure 1 shows a simple model that is used to describe these interactions and their effect on the biological effects and distribution of a drug in the body. The central box in this model represents blood and the circulatory system. From this central component, a drug is able to reach its receptor and target tissue, to be metabolized by the liver, or to be excreted by the kidneys. The binding of drugs with agents in blood is a reversible process that usually involves proteins like human serum albumin (HSA) and α_1 -acid glycoprotein (AGP); however, more complex agents such as lipoproteins, red blood cells or platelets can be involved as well [2,3,11–23]. These interactions are often significant, with 43% of the 1500 most frequently prescribed drugs having 90% or greater binding to serum proteins [11]. The importance and widespread occurrence of these interactions makes the assessment of drug-protein binding in blood an important part of the adsorption/distribution/metabolism/excretion (ADME) data this is needed prior to the approval of any new pharmaceutical agent.

Many techniques have been utilized to study drug interactions with serum proteins and related binding agents. These techniques have included X-ray crystallography [24,25], fluorescence spectroscopy [26], absorption spectroscopy [26], ultrafiltration or equilibrium dialysis [27–29], capillary electrophoresis [30–37], surface plasmon resonance [38] and nuclear magnetic resonance (NMR) spectroscopy [39,40]. Another method which has seen a great deal of use in such studies is high-performance affinity chromatography (HPAC). This review will examine the various experimental formats through which HPAC can be utilized to study drug-protein binding in blood. These formats will be illustrated by using specific examples from the literature. The general information that can be obtained from these studies will be discussed and the relative advantages or limitations of each format will be considered, particularly with respect to traditional methods for drug-protein binding work (e.g., equilibrium dialysis and ultrafiltration). New directions in the use of HPAC to study drug-protein binding in complex systems and in the high-throughput characterization of these interactions will also be considered.

1.2 General principles of HPAC

The method of HPAC is a subset of a broader technique known as affinity chromatography. Affinity chromatography is a liquid chromatographic technique that uses a biologically-related agent as a stationary phase for the purification or analysis of sample components [41–43]. The retention of solutes in this method 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. These interactions are exploited in affinity chromatography by immobilizing (or adsorbing) one of a pair of interacting molecules onto a solid support and using this agent as a stationary phase. This immobilized agent is called the affinity ligand.

In the past, affinity chromatography has often been carried out by using carbohydrate-based materials (e.g., agarose) as the support material to which the affinity ligand is immobilized. These supports typically have low non-specific binding for the components of biological samples and yet can be easily modified for ligand attachment. Columns for affinity chromatography that contain these materials are commonly used in the purification of biological agents. However, these same materials tend to have limited stability at the flow rates and pressures that are often required or desired for analytical applications in HPLC [41,42].

HPAC overcomes these limitations and allows the use of affinity columns in HPLC systems by employing a support that consists of small, rigid particles capable of withstanding high flow-rates and/or pressures [41–44]. Examples of affinity supports that are suitable for work under these conditions include modified silica or glass, azalactone beads, and hydroxylated

polystyrene media [41,44]. Several monolithic supports and sol gel materials have also been recently adapted for use in HPAC [45–50]. Although the need for HPLC instrumentation does make HPAC more expensive to perform than traditional affinity chromatography, the better speed and precision of HPAC make it preferable for analytical applications. These same features make HPAC attractive as a tool for the characterization and high-throughput screening of drug-protein binding in blood or serum.

The use of affinity chromatography to study solute-ligand interactions has been referred to by various names, including analytical affinity chromatography, quantitative affinity chromatography, and biointeraction chromatography [9,10,51,52]. Figure 2 shows two general ways in which HPAC can be used for such experiments: zonal elution and frontal analysis (see Sections 2.1 and 2.2 for more details). In both of these formats, the protein of interest is used as the immobilized ligand and an injection or application of analyte is made onto the affinity column in the presence of only buffer or buffer plus a mobile phase modifier/competing agent. By examining the elution time or volume of the analyte after it has passed through the column, it is possible to obtain information on the equilibrium constants that describe binding by the analyte to the affinity ligand. If additional agents are present in the mobile phase, data can be obtained on how these agents affect the analyte-ligand interactions. Furthermore, information on the rates of these binding processes can be acquired by examining the elution profile for the analyte. All of these approaches have been used to examine the binding of drugs to various proteins and transport agents in blood. Specific examples that will be discussed later in this review will include the use of HPAC to investigate the binding of drugs to HSA, AGP and lipoproteins.

One advantage of utilizing HPAC for drug-protein studies is the ability of this method to reuse the same ligand preparation for multiple experiments. For instance, columns containing HSA immobilized to silica have been used for 500–1000 injections [7,9]. This creates a situation in which only a relatively small amount of protein is needed for a large number of studies. This helps to give good precision by minimizing run-to-run variations. Other advantages include the ease with which HPAC methods can be automated and the relatively short periods of time that are required in HPAC for most binding studies (i.e., often a total of 5–10 min per analysis, which is much shorter than the time usually needed for sample equilibrium and analysis in ultrafiltration or equilibrium dialysis). The fact that the immobilized protein is continuously washed with an applied solvent is yet another advantage of HPAC because this eliminates the effects produced by any organic contaminants (e.g., fatty acids) that might have been present in the initial protein preparation [32,52].

An important factor to consider in the initial development of an HPAC column for these types of studies is the degree to which the immobilized protein acts as a model for the same protein in a soluble form in blood or serum. In the case of serum albumins, there is now a large body of evidence indicating that immobilized HSA and BSA can provide good qualitative and quantitative agreement with the behavior seen for these proteins when they are in solution. As an example, it has been demonstrated in many reports that the displacement effects and allosteric interactions seen on HSA columns are similar to those observed for soluble HSA [32,53–61]. Good agreement with values that have been determined by equilibrium dialysis and other solution-based methods has also been found in the equilibrium constants measured by HPAC for immobilized albumins and in the temperature dependence of these equilibrium constants, the associated changes in entropy and enthalpy, and the rate constants for solute-albumin systems that have been measured by HPAC [32,53,62–65].

There are some cases in which differences in the behavior of immobilized and non-immobilized serum proteins have been noted. These types of differences were noted in the past for columns that were prepared using cross-linked albumin [66] or cross-linked AGP [67,68]. These differences illustrate the need to use model compounds with known binding properties to assess any new immobilized protein before it is used to examine other substances [32,69]. This approach has worked well in the past with HSA and serum albumins, and has led to the development of immobilization schemes that are now used routinely to make HPAC columns to examine the binding of these proteins to drugs (see Section 3). Recent work in the creation and optimization of a new immobilization approach for AGP has similarly been found to produce columns for this protein that closely follow the behavior expected for AGP in its soluble form in blood (see Section 4) [70–72].

2 Approaches for studying of drug-protein binding by HPAC

2.1 Zonal elution

The most common method that is used in HPAC to study drug interactions with serum proteins is zonal elution. A typical example of this type of experiment is shown in Figure 2(a), as illustrated by the competition between verapamil and S-warfarin on an immobilized HSA column [60]. A typical zonal elution experiment involves the application of a small amount of analyte (in the absence or presence of a competing agent) to a column that contains an immobilized ligand. The retention of the analyte in this case will depend on how strongly the analyte and competing agent bind to the ligand and on the amount of active ligand that is present in the column. This type of experiment makes it possible to measure the equilibrium constants for these binding processes by examining the change in analyte retention as the competing agent's concentration is varied.

Eq. (1) represents one specific type of zonal elution study, in which the injected analyte (A) and competing agent (I) bind to a single common site on an immobilized ligand.

$$\frac{1}{k} = \frac{K_I V_M [I]}{K_A m_L} + \frac{V_M}{K_A m_L} \quad (1)$$

Similar equations can be derived for other systems, such as those involving multiple binding sites or that involve both soluble and immobilized forms of the ligand [9,51,52]. In Eq. (1), K_A and K_I are the association equilibrium constants for the binding of the ligand to the analyte and competing agent at their site of competition. The term $[I]$ is the concentration of I that is being applied to the column in the mobile phase, m_L is the moles of common binding sites on the immobilized ligand for A and I, and V_M is the void volume of column. The term k in Eq. (1) is the retention factor that is measured for A, as given by the relationship $k = (t_R/t_M) - 1$, where t_R is the retention time for A and t_M is the column void time. In this case, the values of the association constants K_A and/or K_I can be obtained by examining how the retention factor for A changes with $[I]$.

There are a variety of ways in which zonal elution has been used to obtain information on the binding of solutes to a ligand. These methods include not only measurements of the degree and affinity of solute-ligand binding but also studies examining changes in binding with variations in the mobile phase composition or temperature and experiments that consider how alterations in solute or ligand structure affect these interactions. Each of these applications relies on the fact that the retention that is observed for an injected analyte is a direct measure of the strength with which the analyte is binding to a ligand within the column. This idea is described by Eq. (2), which shows how the overall retention factor for

an analyte is related to the number of binding sites it has in the column and to the association equilibrium constants for the analyte at each of these sites [7].

$$k = \frac{(K_{A1}n_1 + \dots + K_{An}n_n)m_L}{V_M} \quad (2)$$

In Eq. (2), the association equilibrium constants for the analyte at the individual sites are given by the terms K_{A1} through K_{An} , while the fraction of each type of site in the column is given by the terms n_1 through n_n (Note: this summation of terms is also known as the global association equilibrium constant, $n K_A$). It can be seen from Eq. (2) that a change in either the strength of binding, the number of binding sites, or the relative distribution of these sites can result in a shift in analyte retention.

One way zonal elution has been employed in quantitative studies of solute-ligand interactions is as a means to measure the average extent of binding that is occurring between a solute and immobilized ligand [7]. This approach is based on the fact that the retention factor, when measured at true equilibrium, is equal to the fraction of an injected solute that is bound to the ligand (b) divided by the fraction of solute which remains free in the mobile phase (f), or $k = b/f$. The result obtained through this experiment is comparable to the free or bound drug fractions that are provided by equilibrium dialysis or ultrafiltration for drug-protein mixtures and is a simple measure of the overall degree of drug binding that is taking place in the system. The relative binding of two solutes can also be compared by using zonal elution and by taking the ratio of their retention factors on the same affinity column. According to Eq. (2), if both solutes have a single, common binding site on the ligand, the ratio of their retention factors should equal the ratio of their association constants at this site. However, caution must be exercised when using this approach with solutes that have multisite binding or slightly different binding regions on a ligand, because these regions and sites may have different susceptibilities to a loss of activity during ligand immobilization [7,32].

The second and most common use for zonal elution and affinity chromatography in studies of solute-ligand interactions has been in competition and displacement studies. This work is performed by injecting the analyte while a fixed concentration of a potential competing agent is passed through the column in the mobile phase. It is relatively easy from such an experiment to determine whether two compounds interact as they bind to the same immobilized ligand. However, to obtain further information on this interaction (e.g., the nature of this competition and the number of sites that are involved) it is necessary to compare the zonal elution data to the response expected for various models. Figure 3 illustrates the use of Eq. (1) in such an analysis to see if the system being studied fits a model with 1:1 competition between the injected analyte and a competing agent. The data obtained is equivalent to that of a displacement study between a probe compound and drug that is carried out by using equilibrium dialysis (or ultrafiltration); however, with HPAC this type of study can be conducted in much less time and with only trace amounts of the probe being required. These features make HPAC appealing for the high-throughput screening of drug-drug interactions [9,10].

A third way zonal elution and affinity chromatography can be used is to consider how changes in the reaction conditions affect solute-ligand binding. For instance, these factors can be examined by varying the temperature, pH, ionic strength, or general content of the mobile phase. This information can be valuable in determining the relative contributions of various forces to the formation and stabilization of a solute-ligand. As an example, changing the pH can affect the interactions between a ligand and solute by altering their

conformations, net charges, or coulombic interactions. An increase in ionic strength tends to decrease coulombic interactions through a shielding effect, but may also cause an increase in non-polar solute adsorption. Adjusting the solvent polarity by adding a small amount of organic modifier can alter solute-ligand binding by disrupting non-polar interactions or by causing a change in solute and ligand structure. Changing the temperature can be used to provide information on the change in enthalpy and entropy for the interaction between the solute and ligand [9,32]. Although the same types of experiments could be conducted by techniques like equilibrium dialysis and ultrafiltration, the greater precision and reproducibility of HPAC makes it a better tool for detecting small changes in binding properties. In addition, the use of the same protein preparation for many experiments in HPAC significantly reduces the cost for such work and minimizes the effects of batch-to-batch variations.

Another application of zonal elution in affinity chromatography has been its use in determining the location and structure of a binding region on a ligand. If it is known that one agent interacts with a specific site on a ligand, competition studies with this agent can be used to determine if other compounds bind at the same site. Information on binding sites can also be obtained by looking at how a change in the structure of a solute or ligand will affect their interactions. This is the principle behind the use of zonal elution to develop a quantitative structure-retention relationship (i.e., a QSRR). This method involves measuring the retention factors on an affinity column for a large set of structurally-related compounds under constant temperature and mobile phase conditions. The resulting data are then compared to factors that describe various structural features of the solutes [73–75]. A complementary approach is to use zonal elution to investigate how solute retention changes as alterations are made to binding sites on a ligand, as has been performed in work with modified proteins and protein fragments [9,76,77]. These experiments could again be performed by equilibrium dialysis or traditional solution-based methods, but the good precision, speed, and ability to reuse the same protein preparation make HPAC more attractive for such work.

While zonal elution is a relatively easy method to use, there are several practical factors to consider if this technique is to be properly performed [9,32]. As an example, when a new column is made with unknown binding properties, this column should first be tested with an analyte that has known binding properties to the ligand. This type of work will ensure that the column, support, and immobilized ligand have all been chosen correctly for later studies with other analytes. It is also important when using zonal elution for binding studies to measure the true center of the peak when determining the retention factor. This is necessary because it is the center of this peak that is generally assumed to represent a point of local equilibrium between the analyte and immobilized ligand. It can be verified that a local equilibrium is present by determining if the measured retention factor is independent of the flow rate under the conditions used in the study. In addition, a control column made according to the same procedure as utilized to make the affinity column (but with no ligand being added) should be used to account for any non-specific binding that might occur.

Zonal elution studies are usually performed under linear elution conditions, so this method should use samples that provide a retention factor that is essentially independent of analyte concentration. Other factors to consider are the solubility and detector response of the analyte. Solubility will place an upper limit on the concentration range that can be used for the analyte, while the detector response will place a lower limit on this range. Solubilizing agents such as cyclodextrins can be used to increase the solubility but do require the use of more complex models to describe how the analyte retention will depend on the concentrations of both the competing agent and solubilizing agent [32].

2.2 Frontal analysis

An alternative approach for equilibrium constant measurements in affinity chromatography is to use frontal analysis. This method is sometimes known as frontal affinity chromatography (FAC). In this technique, a solution containing a known concentration of the analyte is continuously applied to an affinity column at a fixed flow rate, as shown in Figure 2(b). As the analyte binds to the immobilized ligand, the ligand becomes saturated and the amount of analyte eluting from the column gradually increases. This forms a characteristic breakthrough curve. The volume of analyte solution or moles of applied analyte that is required to reach the mean position of this breakthrough curve is then measured. If the association and the dissociation kinetics are fast, the mean position of this curve can be related to the concentration of the applied solute, the amount of ligand in the column and the association equilibrium constants for solute-ligand binding [7,9].

A simple example of a frontal analysis system is one where an applied analyte binds to a single type of immobilized ligand site. In this situation, the following equation can be used to relate the total moles of active binding sites in the column (m_L) to the apparent moles of analyte ($m_{L,app}$) that are required to reach the mean position of the breakthrough curve at a given concentration of applied analyte, $[A]$.

$$m_{L,app} = \frac{m_L K_A [A]}{1 + K_A [A]} \quad \text{or} \quad \frac{1}{m_{L,app}} = \frac{1}{K_A m_L [A]} + \frac{1}{m_L} \quad (3)$$

As defined earlier, K_A is the association equilibrium constant for the binding of the analyte to the ligand. In this case, the value of K_A can be determined by calculating the ratio of the intercept to the slope in the second form of Eq. (3), and m_L can be obtained from the inverse of the intercept. Similar relationships can be derived for cases in which there is more than one type of binding site or in which both a competing agent and solute are applied simultaneously to the column. Like zonal elution, frontal analysis can provide a variety of information regarding a solute-ligand system, including the affinity and number of binding sites for a solute, the nature of this binding (e.g., single site or multisite), the effects of temperature or solvent on this binding, and the changes that occur in the presence of a competing agent [7,9]. The advantages of using frontal analysis for this work compared to equilibrium dialysis or other traditional solution-based methods are comparable to those discussed in the last section for zonal elution. The speed and relatively large amount of information that can be obtained by this method make it attractive for use both in detailed studies of drug-protein binding or in the high-throughput screening of drug-protein interactions [9,10].

Many quantitative applications of frontal analysis have involved its use in providing quantitative data on the affinity and amount of ligand in a column. This is accomplished by measuring the breakthrough times for a solute at several concentrations and fitting the results to expressions like Eq. (3) based on a given reaction model. The main advantage of frontal analysis over traditional zonal elution is it can simultaneously provide information on both the association constant for a solute and its number of binding sites. This makes frontal analysis the method of choice when information is needed on the binding capacity. Frontal analysis is also preferred for accurate association constant measurements, because the values it provides for K_A can be determined independent from the binding capacity.

A second application of frontal analysis has been as a tool to examine the competition between solutes for an immobilized ligand [78]. This is performed in a similar manner to that described for zonal elution, in which the change in analyte retention is measured as a function of the competing agent's concentration in the mobile phase. In frontal analysis,

direct competition between the analyte and competing agent leads to a smaller breakthrough time for the analyte as the level of competing agent is increased. Positive or negative allosteric effects can also be observed, which lead to a shift to higher or lower breakthrough times, respectively, with an increase in the competing agent's concentration. The same technique can be used to examine how temperature, pH, ionic strength or solvent polarity might affect solute-ligand binding [7,9]. Like zonal elution, frontal analysis has been used to examine the binding of solutes to modified proteins to provide information on the nature of solute-ligand binding sites [76,77].

One disadvantage of frontal analysis is it requires a relatively large amount of analyte for study. However, frontal analysis does provide information on both the association constant for a solute and its total number of binding sites in a column. This feature makes frontal analysis the method of choice for high accuracy in equilibrium measurements, because the resulting association constants are essentially independent of the number of binding sites in the column [7,9].

Many of the practical factors that should be considered in the use of frontal analysis are the same as those that have already been described for zonal elution. The key differences in these methods are the approaches they use for sample application and data analysis. In the case of binding studies that are performed by frontal analysis, it is necessary to have an observable shift in the breakthrough curve as the concentration of analyte is varied. For a 1:1 binding system, the greatest change in this response occurs when $[A] = 1/K_A$; however, a range of analyte concentrations that extends both well above and below this optimum should be used for binding studies [9,32]. The larger this range, the greater the final degree of certainty will be that a particular model does or does not fit the binding data. Analyte solubility will often set the upper limit to this range, while analyte detectability will usually determine the lowest analyte concentrations that can be examined [9].

It is relatively easy to analyze and determine the breakthrough time of a simple, symmetrical frontal analysis curve. In this situation, the point that is halfway between the baseline and plateau would be the breakthrough time. Unfortunately, many breakthrough curves are not perfectly symmetrical in shape and, therefore, the analysis approach has to be slightly altered. One approach is to find the point at which the areas below the front portion of the curve and above the latter half of the curve are equal. An equivalent approach for analyzing a breakthrough curve is to take the first derivative of the curve and then determine the central moment of this derivative [9].

2.3 Other methods

Several methods have been developed or modified for use with quantitative affinity chromatography to examine drug interactions with serum proteins. Band-broadening measurements represent one such approach. This approach is a modification of the zonal elution method, in which the widths of the eluting peaks are measured along with their retention times. This approach has been used to study the interactions of drugs and amino acids with serum albumin, and the kinetics of protein-based chiral stationary phases [51,62,63]. This type of experiment involves injecting a small amount of analyte onto an affinity column while carefully monitoring the retention time and width of the eluting peak. These injections are performed at several flow-rates on both the affinity column and on a column of the same size which contains an identical support but with no immobilized ligand present. This control column is needed to correct for any band-broadening that occurs due to processes other than the binding and dissociation of analyte from the immobilized ligand. By comparing plots of the peak widths (or plate heights) for the affinity and control columns, it is possible to determine the value of the dissociation rate constant for the analyte-ligand interaction. An example of such a study is given in Figure 4. A variation of

this approach, known as peak profiling, involves simultaneously determining the band-broadening for both a retained and non-retained solute on an affinity column to examine the contributions of stationary phase mass transfer versus other band-broadening processes [79–81].

Once the plate height contribution due to the stationary phase interaction has been determined, this can be related to the kinetics of analyte dissociation from the ligand in the HPAC column. This is shown by Eq. (4),

$$H_k = \frac{2 \cdot u \cdot k}{k_d \cdot (1+k)^2} \quad (4)$$

where u is the linear velocity of mobile phase in the column, k is the retention factor of the injected solute, H_k is the plate height due to the stationary phase interaction, and k_d is the dissociation rate constant between the analyte and immobilized ligand. Based on Eq. (4), a plot of H_k versus $u/(1+k)^2$ should give a slope of $2/k_d$ and an intercept of zero, as depicted in Figure 4. Thus, the slope of this plot can be used to determine the dissociation rate constant for the solute-protein system. By using the k_d values obtained from these plots along with independent estimates of the equilibrium constants for the system, the association rate constants for the solute and protein can also be obtained [62,63].

It is also possible to use HPAC to examine the binding of drugs and other solutes with soluble proteins or with complex mixtures and samples such as serum. This has recently been accomplished by using HPAC columns that are designed for ultrafast immunoextraction [82–84]. The basis of this technique is the use of microcolumns that contain antibodies which bind the drug or solute of interest and are capable of extracting this solute in very short periods of time (e.g., 80–120 ms). By rapidly passing a drug-protein mixture through this type of immunoaffinity microcolumn, it should be possible to quickly isolate the portion of this drug which is free in solution while allowing its protein-bound fraction to elute non-retained. The result is a tool that can be used to measure the amount of free, as well as bound, solute that existed in the original sample (see example in Figure 5). This approach has been found to give good correlation with traditional methods like ultrafiltration for the analysis of free drug and hormone levels in clinical samples and drug-protein mixtures, but this new approach provides results in considerably less time (typically 20–30 s after sample injection) [82–84]. These properties should make this method valuable in the future for the high-throughput determination of free drug fractions and drug-protein binding in various biological samples.

Another approach in HPAC that has recently been adapted for use with serum proteins is the peak decay method [85,86]. This technique can be used to measure a dissociation rate constant for a drug from an immobilized protein. This method is performed by first equilibrating and saturating a small affinity column with a solution that contains the analyte of interest or an easily detected analog of this analyte. The column is then quickly switched to a mobile phase in which the analyte is not present. The release of the bound analyte is then monitored over time, resulting in a decay curve. This decay is related to the dissociation rate of the analyte and the mass transfer kinetics within the column. If the mass transfer rate is known or is fast compared to analyte dissociation, then the decay curve can be used to provide the dissociation rate constant for the analyte from the immobilized ligand [85,86].

3 HPAC studies of HSA and other serum albumins

3.1 General properties of serum albumins

HPAC has already been used to study a variety of serum proteins. However, most of this work has focused on human serum albumin (HSA) and related proteins such as bovine serum albumin (BSA). Albumin is the most abundant protein in the plasma of vertebrates. HSA is the type of albumin found in humans and has a typical concentration in plasma of 35–50 g/L. The structure of HSA is shown in Figure 6 [87]. HSA is a 66.5-kDa single-chain protein with 585 amino acids that is produced by the liver. BSA has a similar mass and structure but contains only 583 amino acids. The structure of HSA is stabilized by 17 disulfide bridges. The close coupling of adjacent half-cysteine residues that is associated with these disulfide bridges gives albumin both flexibility and resistance to extreme conditions. These bridges also form the pseudo-nine (eight and one-half) double loops that are usually grouped together as three homologous domains (I, II, and III) [4].

Albumin serves many functions in the body. For example, it is involved in the regulation of osmotic pressure and plays a large role in providing almost all of the anions in plasma. In addition, it acts as the primary buffering system for extravascular fluids and protects low-density lipoproteins from peroxidative effects [4]. Another function of albumin is to bind and deliver a number of substances within the body. Besides organic anions and long-chain fatty acids, albumin also binds to vitamins and acidic drugs. HSA, BSA and related proteins have also been employed in HPAC as chiral stationary phases for drugs and other solutes [45,46,66,88–93].

HSA or BSA supports for HPAC can be prepared by covalently attaching albumin to diol-bonded silica activated with 1,1'-carbonyldiimidazole [54,94], by attaching albumin to silica through a two-step [53] or three-step Schiff base method (i.e., reductive amination) [94–96], or by using modified silica that has been activated with an *N*-hydroxysuccinimide ester [97–100]. In addition, some studies have used albumin which is non-covalently adsorbed to ion-exchange columns [100] and HPLC-grade silica [96], or which is immobilized to HPLC supports based on agarose [101] or hydroxyethylmethacrylate (HEMA) [102,103]. Commercial albumin columns for chiral separations, sold under the trade name Resolvosil, are prepared by cross linking albumin in the presence of silica with such agents as glutaraldehyde or *N,N'*-disuccinimidyl carbonate [66]. New developments include the creation of a sulfhydryl-based coupling method for the site selective immobilization of HSA through its lone free cysteine [104]. HSA has also been recently immobilized in monolith columns based on organic polymers or silica for use in drug binding studies and chiral separations [46,47]. In addition, the entrapment of soluble BSA in sol gels has been reported [105] and a method has been presented for the entrapment of soluble HSA in silica particles [106,107].

3.2 Examination of strength and degree of binding

Zonal elution has been used in several studies to determine the average extent of binding by drugs and other solutes to HSA and other types of immobilized serum albumin. One way this is accomplished is by relating the retention factor to the ratio of the moles of a drug that is free versus bound to the immobilized albumin; this can be carried out according to methods described in Section 2.1 [7,32]. This technique has been used to compare the percent binding measured by HPAC with that determined by ultrafiltration for various coumarin compounds mixed with HSA, giving the best correlation for compounds with medium-to-strong binding [108]. This approach has also been used with liquid chromatography/mass spectrometry (LC/MS) to allow the simultaneous determination of

binding for several drugs in a mixture [109]. Similar studies have been conducted with other classes of compounds [110].

A related approach has appeared in recent years in which the ratio of the retention factor to the protein content of a support is used to follow changes in the binding activity of HSA columns that are prepared by different methods. This technique makes it possible to correct for any variations in total protein content that occur as the immobilization method is varied. Instead, this ratio $k/(\text{amount protein})$ provides a value that is directly proportional to the global association equilibrium constant for a drug with the immobilized protein ($n K_A$), as suggested by Eq. (2). This approach has been used to compare the effect of the immobilization method on the binding of carbamazepine to HSA [98].

A new technique that has been used to measure the free versus bound fraction of drugs in the presence of HSA is ultrafast immunoextraction [82–84]. An example of one way in which this approach can be performed was given earlier in Figure 5. This method has been used to measure the binding of R- and S-warfarin to soluble HSA [82], the free fraction of L-thyroxine in serum samples [83], and the free fraction of phenytoin in either serum samples or in the presence of soluble HSA [84]. The results in each case have been shown to give good correlation with reference methods but typically provide results in only 25–30 s after sample injection [83,84].

The most accurate approach for measuring association equilibrium constants for HSA and other serum albumins by HPAC is to use frontal analysis. Illustrations of such an experiment are shown in Figure 7 for the binding of coumarin and 4-hydroxycoumarin to HSA. The top graph shows results obtained for a solute that gave a good fit to the response predicted by Eq. (3) for a single-site binding model. However, the solute examined in the lower graph gave a curved response for a similar plot. Such deviations indicate that multi-site interactions were present in this latter case, as confirmed by the fact that these results gave a good fit to a two-site model when using equations that have been derived for multi-site systems (e.g., see Refs. [9,32,111]). Equivalent expressions that make use of Scatchard plots have also been used for this type of data analysis [9,52,112–115]. Examples of drugs or solutes that have been studied by frontal analysis for their binding to HSA include carbamazepine, L-thyroxine, L-tryptophan, R/S-warfarin and various indole or coumarin compounds [53,58,98,116–118].

3.3 Competition studies

A large number of binding studies using HPAC have examined the competition and displacement of drugs by other solutes from HSA and related proteins. This type of experiment is often conducted by using zonal elution. An example of such an experiment was given earlier in Figure 2, in which S-warfarin was found to have competition with racemic verapamil on HSA, as indicated by the shift in warfarin retention as the mobile phase concentration of verapamil is varied. The extent of this shift as a function of the concentration of competing agent is then analyzed according to various equations to determine the type of interaction that is taking place between these agents and to measure the corresponding equilibrium constants or coupling constants for this interaction. Figure 3 shows a plot that was made according to Eq. (1) of $1/k$ versus verapamil concentration, which indicated that the competition between verapamil and S-warfarin was occurring at a specific site on HSA (i.e., the warfarin-azapropazone site or Sudlow site I) [60]. The same type of plot for a compound that has no competition with verapamil (e.g., digitoxin) results in only random variations in the measured values of $1/k$ [9,32,60].

More complex behavior occurs when there is an allosteric interaction between two solutes as they bind to HSA. An alternative way of analyzing zonal elution data was recently described

in which the results are plotted according to the following expression, in which k_0 is the retention factor for the analyte A in the presence of any competing agent and k is the retention factor measured when a competing agent concentration of $[I]$ is present in the mobile phase.

$$\frac{k_0}{k - k_0} = \frac{1}{\beta_{I \rightarrow A} - 1} \cdot \left(\frac{1}{K_{IL}[I]} + 1 \right) \quad (5)$$

In this equation, the ligand is viewed as having at least two binding sites, one for the injected analyte and one for the competing agent. The interaction of A with the ligand is altered as I also binds to the ligand, which causes the association equilibrium constant for A with the ligand to change from K_{AL} to K_{AL}' . This change is represented in the above equation by the coupling constant $\beta_{I \rightarrow A}$, which is equal to the ratio K_{AL}'/K_{AL} . Eq. (5) predicts that a plot of $k_0/(k - k_0)$ versus $1/[I]$ will give a linear relationship for a simple allosteric interaction and that, through this relationship, the values of $\beta_{I \rightarrow A}$ and K_{IL} can be obtained [119]. An example of such a plot is shown in Figure 8 for the interaction between verapamil and tamoxifen on HSA [60]. Similar work with Eq. (5) and related expressions has been conducted to examine the allosteric effects that occur on HSA during the interactions of *R*- or *S*-ibuprofen with various benzodiazepine enantiomers, the interactions between L-tryptophan and phenytoin, and the interactions between warfarin and tamoxifen [119–121]. A particularly useful feature of this approach is it can be used to look independently at both directions of an allosteric effect, thus allowing independent measurements to be made for the two directions of such an interaction [119].

3.4 Effects of solvent conditions and temperature on binding

The effect of various solvent and temperature conditions on drug-albumin binding has also been examined by HPAC. An example of such a study is shown in Figure 9, in which the effect on HSA binding to carbamazepine as a result of changes in the pH, organic modifier content (i.e., 1-propanol) and ionic strength (as represented by a change in phosphate concentration) of the mobile phase. The lack of any appreciable changes in retention with pH and absence of a decrease in retention with an increase in ionic strength suggest coulombic interactions do not play a major role in this particular system. However, the decrease in retention that occurs with an increase in the content of 1-propanol and the increase in retention with ionic strength both indicate that non-polar interactions are important in the binding of carbamazepine to HSA [122]. Similar studies have been conducted with many other drugs to examine their binding to HSA or other serum albumins (e.g., see reviews in Refs. [32] and [93]). Along with changes in pH, ionic strength and solvent polarity, the use of different buffer salts, chaotropic agents and other additives have also been employed in such work [32].

Temperature is another experimental factor that is often varied during zonal elution studies to study drug-protein interactions. If used correctly, this type of experiment can provide not only qualitative data on the effects of temperature on binding but can also be used to determine some thermodynamic constants. For instance, the following relationship shows how the retention factor for a drug would be expected to vary on an albumin column if this system has single site binding and the analyte has no other significant source of retention on the column [32].

$$\ln k = -(\Delta H/R T) + \Delta S/R + \ln(m_L/V_M) \quad (6)$$

In Eq. (6), T is absolute temperature at which the retention factor is measured, R is the ideal gas law constant, ΔH is the change in enthalpy for the reaction, ΔS is the change in entropy, and the other terms (m_L and V_M) are the same as defined in previous sections. The resulting plot of $\ln k$ versus $1/T$ has been often employed in thermodynamic studies [64,122–129] and in reports that consider how changes in temperature affect the selectivity of albumin columns [130,131]. However, caution needs to be exercised in using Eq. (5) for thermodynamic studies in that it requires that the value of m_L does not change with temperature. This assumption is not always valid for the binding of drugs to albumin, as has been shown for *R/S*-warfarin and carbamazepine [58,122]. A better approach is to use frontal analysis to obtain independent estimates of the association equilibrium constant (K_A) and the binding capacity (m_L) for the drug-protein interaction. For a system with single site binding, the resulting K_A values that are obtained from the frontal analysis data can be plotted as a function of temperature according to Eq. (7).

$$\ln K_A = -(\Delta H/R T) + \Delta S/R \quad (7)$$

If the resulting plot is found to be linear, the slope and intercept can then be used to obtain the values of ΔH and ΔS [9,32]. The frontal analysis data can also be used to see how the apparent activity of the immobilized protein (m_L) changes with temperature [58,122].

3.5 Determining the location and structure of binding sites

Many reports have used HPAC to determine the location and structure of binding regions for a drug or solute on serum albumins. This is often done with zonal elution and competition studies through the use of an injected probe that has a known binding site on a protein such as HSA. This type of experiment, which was illustrated earlier in Figure 2 and Figure 3(a), has been used to investigate the binding of HSA and other albumins with non-steroidal anti-inflammatory drugs [132,133], *R*- and *S*-ibuprofen [134], *cis*- and *trans*-clomiphene [135], digitoxin or acetyldigitoxin [136], benzodiazepines [137], phenytoin [138], carbamazepine [122], L-thyroxine [116] and verapamil [60]. Probe compounds that have been employed in such work include *R/S*-warfarin, L-tryptophan, phenylbutazone, *R/S*-ibuprofen, 2,3,5-triiodobenzoic acid, *cis/trans*-clomiphene, acetyldigitoxin, digitoxin, and phenol red [60,116,122,132–138]. Other agents that have been explored for such work included 4-hydroxycoumarin as a probe for Sudlow site I [117] and 3-acetylindole as a probe for Sudlow site II [118]. It is possible through the use of multiple probes to generate maps that show the relationship between the various binding regions on serum albumin for a drug [137,138].

An additional approach for learning about binding sites on a serum albumin is to use a set of structurally-related compounds to see how changes in the structure of a drug or solute will affect interactions with this protein. This has been done in examining the binding of HSA to L-thyroxine and related thyronines, warfarin and coumarins, and indole compounds that are similar to L-tryptophan in structure [116–118]. If a large set of test solutes are sampled, the data can also be used to develop a quantitative structure-retention relationship [75,139,140]. This approach has been utilized to investigate the binding of HSA to benzodiazepines, which was used to develop binding sites for both the *M*- and *P*-conformations of these agents [141]. Other compounds whose binding with HSA has been examined by this approach include 2,3-substituted-3-hydroxy-propionic acids and related compounds [142,143].

Another technique that has been used in HPAC to characterize solute binding to serum albumins is to use forms of the protein that have been modified at specific sites. For instance, one report used HSA that has been treated with *p*-nitrophenyl acetate, a reagent

thought to mainly modify the Tyr-411 residue of HSA (i.e., a residue located at the indole-benzodiazepine site of this protein). This modification was shown to change the retention of a variety of solutes injected onto normal versus modified HSA columns [144]. A similar study used *o*-nitrophenylsulfenyl chloride to modify the lone tryptophan residue on HSA, Trp-214, which is located within the warfarin-azapropazone site of HSA [145]. This latter modification did not change the moles of binding sites but did result in a complete loss of HSA's stereoselectivity for *R*- and *S*-warfarin. As a result, it was concluded that Trp-214 and/or its neighboring residues played an important role in determining the chiral recognition of these compounds by HSA. Similar studies have involved modification of the lone free cysteine residue on HSA with ethacrynic acid [146] and the use of BSA fragments in the chiral separation of benzoin and other drugs [147,148].

3.6 Kinetic studies of drug binding to HSA

Although analysis of the rates of drug interactions with HSA is much less common than binding studies, there have been several reports that have used HPAC for such work. For instance, band-broadening has been employed as a tool to examine the rate of interaction between *R*- or *S*-warfarin and *D*- or *L*-tryptophan with HSA over a variety of temperatures. The work with *D*- and *L*-tryptophan and HSA also looked at how the association and dissociation rates for this system varied with pH, ionic strength and organic modifier content in the mobile phase. This information has been shown to be important in optimizing chiral separations that use HSA [62,63]. This kinetic data has also been shown to be useful in describing the pharmacokinetics of drugs that bind to HSA and in developing new assays for measurement of free drug or hormone fractions in serum [82].

Several other approaches have recently been developed for examining the dissociation rates for drugs from HSA by HPAC. One such method is peak profiling. This approach has recently been used at both single flow rates [79] and with multi-flow rate measurements to examine the dissociation rate of *L*-tryptophan from HSA [80], giving good agreement with literature values. Data analysis methods in peak profiling have also been modified for work with drugs that have significant binding to both HSA and the support with an HSA column. This expanded approach has been successfully used to examine the kinetics of binding between immobilized HSA and the drugs imipramine and propranolol, which both had significant non-specific binding to the chromatographic support employed in these experiments [81]. Yet another technique that has been used to examine drug dissociation from HSA is the peak decay method. This approach has been used to examine the release of *R*- and *S*-warfarin from HSA and gave good correlation with previous results that have been reported for this system [86].

4 HPAC studies of AGP

4.1 General properties of AGP

Another important plasma protein is α_1 -acid glycoprotein (AGP) [149]. Like albumin, AGP is produced by the liver. It is a fairly heterogeneous protein with an approximate molecular weight of 41 kDa. AGP contains a single polypeptide chain with 181 amino acids and five carbohydrate groups [149–151]. There are many ways in which these carbohydrate groups can potentially be attached to AGP, but only 12 to 20 combinations have been detected. The extent of this glycosylation and the arrangement of these carbohydrate groups are dependent on the disease state of the body. This, in turn, affects the structure of AGP and its binding properties. For instance, it has been proposed for some solutes that binding occurs through sialic acid residues in the carbohydrate chains rather than through interactions with the protein core of AGP [152]. While it is generally accepted that the binding of most solutes

occurs at a single hydrophobic core of this protein, the presence of more than one site on this ligand has also been postulated.

AGP has many biological functions in serum. Many of these functions are shared with albumin, such as the ability of AGP to bind and transport many classes of drugs throughout the body [151,153,154]. Normal plasma levels of AGP are around 0.50 to 1.0 g/l, but its levels increase during disease. This has led investigators to predict that there is a direct involvement of AGP in the immune response [151]. Although serum albumin is present in plasma at a greater concentration and has greater binding capacity than AGP, this latter protein is more important as a binding agent for basic and neutral drugs. Like HSA, AGP has also been placed into chromatographic columns for use as a chiral stationary phase [66,72,155–165]. One main advantage of using AGP over serum albumins in chiral separations is the higher affinity that AGP typically has for its target solutes, giving it better retention and resolution.

AGP columns for chiral separations can be purchased from several companies (e.g., ChromTech, Astec, and Regis Technologies) and can be prepared by several immobilization techniques [91]. One commercial AGP chiral stationary phase is made by ionically binding a monolayer of AGP to diethylaminoethyl silica. This step is followed by oxidizing the protein with periodate to form aldehyde groups that can cross-link AGP through Schiff base formation. Alternatively, AGP can be immobilized directly through its carbohydrate residues by oxidizing these groups with periodate and reacting the resulting aldehyde groups with a support containing free amine or hydrazide groups [70,132]. The use of hydrazide groups of this purpose along with mild oxidation conditions (see Figure 10) has recently been shown to be successful in producing immobilized AGP that is a good model for soluble AGP in terms of its ability to bind to various drugs under physiological conditions [70,159].

4.2 HPAC Studies of drug interactions with AGP

The majority of reports that have used AGP columns to examine drug binding have examined the effects of various reaction conditions on these binding processes. These studies have often varied such conditions as the pH, ionic strength and organic content of the mobile phase. The resulting changes in retention were then determined to see how altering these conditions affected the binding of a given drug to AGP or a chiral separation that was being performed on the AGP column [91]. These experiments have been conducted with propranolol, kynurenine, methylphenobarbital, dihydropyranimidazopyridines, *N*-substituted amino acid derivatives, quinazolone derivatives, and bupropion hydrochloride [66,71,160,163–166]. Altering the solvent conditions has been used in another study to examine the separation of the biologically active components in *Rhizomachuanxiong* when using an AGP column [167].

Temperature is another parameter that has been frequently examined in terms of its role in the chiral retention and binding of various compounds to AGP [91,70,159,167–169]. Temperature is one factor that has been used to control the elution order of mosapride enantiomers on an AGP column [168,170] and has been studied with regards to its effect on chiral separations for naproxen, ibuprofen and propranolol [159,169].

The use of improved immobilization conditions has made it possible to use some AGP columns to directly model the binding of drugs to soluble AGP. Frontal analysis has been used with such columns to measure the association equilibrium constants for R- and S-propranolol with AGP under physiological conditions (see Figure 11) [70,159]. The same columns have also been used to examine the correlation between the retention factors found on such media and the reported association equilibrium constants of AGP for solutes that include epinephrine, isoproterenol, phenylbutazone, carbamazepine, pindolol, lidocaine,

perphenazine, quinidine, imipramine, bupivacaine, and trifluoperazine [70]. In addition, the effect of temperature on the binding of R- and S-propranolol to AGP has been examined with such columns [70,71,159].

Competing or displacing agents have both been used in HPAC to study the processes by which drugs and other solutes bind to AGP columns (see Figure 11 for an example) [159]. One report used dimethyloctylamine as a competing agent to examine the binding of AGP to 4-aryl-1,4-dihydropyridine calcium-channel blockers, making it possible to identify two distinct binding regions for these drugs [171]. In another study, propranolol was used as a competing agent to identify the high affinity site for carbamazepine on AGP [172].

Similar approaches to those used for HSA have been described for structural requirements for the binding and stereoselective interactions of drugs with AGP columns. Comparisons of the retention for structurally-related compounds have been reported for tetracyclic and pentacyclic vinca alkaloid analogs [161], quinolones [162], quinazoline derivatives [166], amino alcohols [173], and 4-aryl-1,4-dihydropyridine calcium-channel blockers [171]. These types of experiments have been further employed with AGP columns to create quantitative structure-retention relationships that describe the binding of beta-adrenolytic drugs & antihistamines (see in Figure 12) [174–176].

5 Lipoproteins

Lipoproteins are a third class of drug binding agents in blood that have recently been examined by HPAC. The general structure of a lipoprotein is given in Figure 13(a). A lipoprotein is a soluble macromolecular complex of proteins and lipids that can transport hydrophobic compounds such as cholesterol, triacylglycerols (triglycerides) and other lipids in the circulatory system of humans [177–180]. Based on their densities, lipoproteins in humans are traditionally classified into five main categories: chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate low density lipoproteins (IDL) and high density lipoproteins (HDL) [178–185]. The composition and relative amount of each lipoprotein in any individual will depend on their sex, age, race, metabolic condition and disease state [177,178,182]. A typical distribution of lipoproteins in a healthy fasting adult male is about 280 mg/dL HDL, 410 mg/dL LDL, 150 mg/dL VLDL, and 0 mg/dL CM, with chylomicrons normally appearing only immediately after a meal [12]. These serum concentrations are much lower than those for HSA but are comparable to those for AGP [177–180]. Of these, HDL and LDL are of greatest interest in drug binding [180,186].

Recent experiments with HDL have found that this type of lipoprotein can be immobilized to silica by the Schiff base method and used in HPAC for drug binding studies. The resulting column was found to be stable for over 3000 h of continuous operation in the presence of pH 7.4, 0.067 M phosphate buffer [187]. This column was then used to examine the binding of R- and S-propranolol to HDL through frontal analysis studies, as illustrated in Figure 13(b). The binding isotherms that were obtained indicated that a mixed mode interaction was occurring between propranolol and HDL, which is believed to represent both interactions at a saturable site on the lipoprotein (e.g., as part of the apolipoprotein component) and partitioning of the drug within the core of HDL [187]. Similar behavior has also been noted for the binding of propranolol to LDL columns [188].

6 Concluding remarks

It can be seen from this review that there is indeed a growing interest in using HPAC as a tool for study drug interactions with serum proteins by HPAC. The tools that are most commonly used in these studies are zonal elution and frontal analysis. With these techniques

it is possible to obtain information on the extent of drug-protein binding, the equilibrium constants for these processes, the ability of a drug to compete with other compounds, the effects of temperature or solvent composition on these reactions, and the structure and location of the drug binding sites on the immobilized protein. Several newer approaches for using HPAC in this type of research were also described, including methods based on band-broadening measurements, peak profiling, ultrafast immunoextraction, and peak decay analysis.

These tools have already been used in a large number of reports to examine the interactions of drugs with HSA and related albumins. A considerable amount of work has also been carried out in the use of HPAC to characterize the interactions of drugs with AGP. Recent studies have further indicated that drug interactions with lipoproteins can be examined through the use of HPAC. In addition, new techniques based on ultrafast immunoextraction have allowed HPAC to directly examine the binding of drugs with soluble proteins or in complex mixtures such as serum. The many ways in which HPAC can be used in these studies and the wealth of information this approach can provide make it a powerful means for characterizing drug-protein binding in blood. The speed, precision and reproducibility of HPAC, along with its ability to often reuse the same protein preparation for a large number of studies, also make this approach appealing for the high-throughput characterization or screening of drug-protein interactions. It is expected that the applications of HPAC will continue to grow in clinical and pharmaceutical research as a means to examine the interactions of drugs and other solutes with proteins and binding agents in blood or serum.

Abbreviations

AGP	α_1 -acid glycoprotein
BSA	bovine serum albumin
HDL	high-density lipoprotein
HPAC	high-performance affinity chromatography
HSA	human serum albumin
LDL	low-density lipoprotein
QSRR	quantitative structure-retention relationship

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Figure 1.

General model for drug-protein binding in blood and the relationship of this binding to the ability of a drug to reach its target or be acted on by the liver and kidneys.

Figure 2. Typical chromatograms obtained on an HSA column during (a) zonal elution competitive binding experiments in which *S*-warfarin was the injected analyte and racemic verapamil was a mobile phase additive, and (b) frontal analysis experiments with *S*-verapamil being applied as the analyte. The concentrations that are shown represent the concentration of (a) racemic verapamil or (b) *S*-verapamil that was applied to the HSA column. (Reproduced with permission from Ref. [60])

Figure 3.

Analysis of data from a zonal elution competitive binding experiment in which S-warfarin was the injected analyte and racemic verapamil was the mobile phase additive. This plot was obtained using the data from Figure 2(a), as analyzed according to Eq. (1). The linear response in this case indicates that the injected analyte and mobile phase additive are competing at a single common binding site on the immobilized ligand (i.e., HSA). (Reproduced with permission from Ref. [60])

Figure 4.

A typical band-broadening study examining the effect of a change in pH on the plate height contribution due to stationary phase mass transfer (H_k) for D-tryptophan on an HSA column. These plots were analyzed according to Eq. (4) to provide the dissociation rate constant (k_d) under each of the reaction conditions. (Reproduced with permission from Ref. 63).

Figure 5.

(a) General scheme for the use of affinity microcolumns in an ultrafast immunoextraction/displacement assay and (b) a typical chromatogram obtained by this approach for determining the free fraction of phenytoin in a sample containing binding proteins. In (a), the closed circles represent a labeled analog of the drug of interest, the open circles represent the drug, the U-shaped symbols represent binding proteins in the sample and the Y-shaped symbols represent immobilized antibodies in the HPAC column. In (b), an excess of a labeled analog of phenytoin was injected onto an anti-phenytoin antibody column at 0 min, followed by the injection of a phenytoin sample at 6 min. The size of the displacement peak

in this chromatogram is then used to measure the free fraction of drug in the injected sample. (Reproduced with permission from Ref. [84])

Figure 6. Structure of HSA and location of its two major binding sites for drugs, Sudlow sites I and II. The lysine residues that are highlighted in this figure have been found to be important coupling sites when this protein is immobilized through the Schiff base method. (Reproduced with permission from Ref. [87])

Figure 7.

Frontal analysis results for (a) coumarin and (b) 4-hydroxycoumarin on an HSA column with the data being analyzed according to a double-reciprocal plot. The results in (a) are linear over the entire sampled range of concentrations and indicate that binding at a single major site is occurring for coumarin on HSA. The deviations from linearity noted in (b) indicate that multi-site binding is present for 4-hydroxycoumarin on HSA. (Reproduced with permission from Ref. [116])

Figure 8.

Allosteric effect of verapamil on the binding of tamoxifen to an HSA column. The plot (a) shows how the results deviate from the linear response that would be expected for a 1:1 direct competition model when such data are that are obtained according to Eq. (1). The plot in (b) shows the graph that was obtained for the same data when these results were analyzed according to Eq. (5). The slope and intercept of this second plot can then be used to find the coupling constant that describes the allosteric interaction. (Reproduced with permission from Ref. [60])

Figure 9. Effect of (a) pH, (b) ionic strength, and (c) organic modifier content of the mobile phase on the binding of carbamazepine to an HSA column. (Reproduced with permission from Ref. [122].)

(a) Oxidation of AGP

(b) Preparation of Hydrazide-Activated Silica

(c) Immobilization of Oxidized AGP

Figure 10.

Immobilization of AGP through its oxidation under mild conditions with periodate and coupling of the oxidized AGP with a hydrazide-activated support. (Reproduced with permission from Ref. [70])

Figure 11.

(a) A zonal elution self-competition experiment on an AGP column using *R*-propranolol as both the analyte and mobile phase additive, and (b) results of frontal analysis studies conducted on the same type of AGP column using *R*-propranolol as the applied analyte. (Reproduced with permission from Refs. [70] and [159])

Figure 12. Models developed through the use of quantitative structure-retention relationships to describe the binding of basic drugs to AGP. (Reproduced with permission from Ref. 174).

Figure 13.

(a) General structure of a lipoprotein and (b) frontal analysis data obtained for the binding of R-propranolol to an HDL column at (○) 4°C, (●) 15°C, (▲) 27°C, or (■) 37°C.