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Review

High-performance affinity chromatography and immobilized serum albumin as probes for drug- and hormone-protein binding

David S. Hage*, John Austin

Department of Chemistry, University of Nebraska – Lincoln, 738 Hamilton Hall, Lincoln, NE 68588-0304, USA

Abstract

The binding of drugs and hormones to proteins within the blood is an important process in determining the transport, excretion, metabolism and activity of such agents. This paper discusses the combined use of immobilized serum albumin and high-performance affinity chromatography (HPAC) as tools for the study of such binding processes. The general approaches that are used in such work and are illustrated by several examples taken from previous work in the author's laboratory. The type of qualitative and quantitative information that can be obtained by such work is described, including the comparison of relative binding affinities, competitive displacement by other agents or the measurement of equilibrium and rate constants based on immobilized albumin columns. A comparison is also provided between the results that are obtained by these methods and those that are provided by solution-phase albumin. Some newer advances that are highlighted include use of HPAC to examine the binding of non-polar compounds to albumin, the effects of binding site heterogeneity on HPAC measurements and the use of chemically-modified albumin as a tool to examined the site-specific interactions of solutes with albumin.

Keywords: Reviews; Proteins; Drugs; Hormones

Contents

*Corresponding author. Tel.: +1-402-4722-744; fax: +1-402-4729-402.

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1. Introduction

Protein binding is important in many processes that determine the eventual activity and fate of a drug once it has entered the body. An example of one such interaction is the binding of many pharmaceutical agents with blood proteins [1]. These interactions help determine the overall distribution, excretion, activity and toxicity of a drug within the body. In addition, the direct or indirect competition of two drugs for the same binding proteins can be an important source of drug–drug interactions [1,2]. Competition can also occur between drugs and endogenous compounds, such as the displacement of various drugs from human serum albumin by fatty acids or bilirubin [2–5]. Since the binding of some drugs to proteins can be stereoselective in nature [1,6–8], it is possible that these interactions may play a further role in determining the fate of the different forms of a chiral drug within the body.

One group of proteins that are important in these binding processes are the serum albumins. Examples include human serum albumin (HSA) and bovine serum albumin (BSA). These proteins have the ability to carry a wide variety of endogenous and exogenous compounds throughout the body, such as drugs, fatty acids, metal ions, bilirubin and several low-molecular-mass hormones. HSA (M_r 66 500) is a 585 amino acid monomeric protein that is stabilized by the presence of 17 internal disulfide bonds. It is believed to have several relatively well-defined binding regions for solutes, including fatty acids, metal ions and many small organic compounds. Most drugs and hormones interact with two major binding sites on HSA, known as the warfarin–azapropazone and indole–benzodiazepine sites (see protein structure shown in Fig. 1). Other minor binding regions for organic solutes have also been proposed, including those that bind to bilirubin, digitoxin and tamoxifen [9,10].

The interaction of a drug or hormone with albumin is usually described by the model shown in Eqs. (1) and (2), where D is the solute of interest, L is the region or binding site on the protein, and D–L is the resulting solute–protein complex:

$$D + L \underset{k_d}{\overset{k_a}{\rightleftharpoons}} D\text{–}L \quad (1)$$

$$K_a = \frac{k_a}{k_d} = \frac{[D\text{–}L]}{[D][L]} \quad (2)$$

In the above expressions, [] represents the molar concentration of each species in solution, k_a is the second-order association rate constant for solute–protein binding, k_d is the first-order dissociation rate constant, and K_a is the association equilibrium constant for the interaction.

Two common methods that have traditionally been used in evaluating the binding of drugs and hormones to albumin include equilibrium dialysis and ultrafiltration [1,2,4]. Equilibrium dialysis is considered by many to be the reference method for such analyses; however, it does suffer from several disadvantages. Perhaps its greatest disadvantage is the long periods of time that are required to establish an equilibrium during the dialysis process (i.e., hours or even days) [1]. Furthermore, it is necessary to correct for the alterations in free and bound analyte concentrations that occur during the dialysis procedure; the possible effects of analyte adsorption onto the dialysis membrane must also be considered [1,2,4]. Ultrafiltration is similar in its operation to equilibrium dialysis but requires much less time to perform (i.e., typically less than 30 min) [1,2]. Like dialysis, it requires the use of a labeled drug and/or an additional analysis step for the actual measurement of the final free drug concentration (e.g., by using an immunoassay, gas chromatographic or high-performance liquid chromatographic method). In addition, the effects of analyte adsorption to the ultrafiltration membrane must be considered [1]. Other problems that may be associated with ultrafiltration include

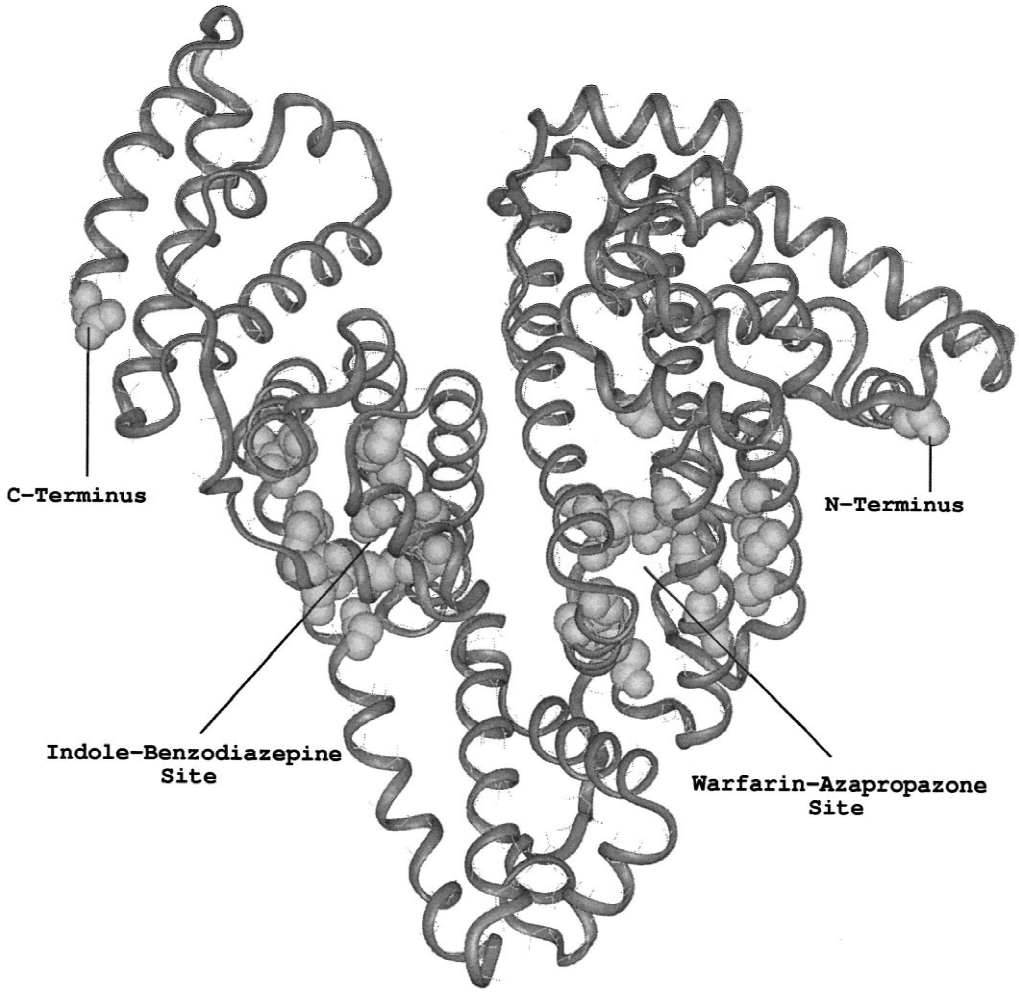

Fig. 1. The structure of human serum albumin. This image was generated by using Image II software (Molecular Simulations, San Diego, CA, USA) based on coordinates obtained from Ref. [10] as file PDB ID 1AO6 in the protein data bank of the Research Collaboratory for Structure Bioinformatics (http://www.rcbs.org/pdb/).

difficulties with temperature changes during the separation (requiring the use of a centrifuge with temperature-control) and problems when working with highly bound drugs, which will produce only small amounts of measurable analyte in the final filtrate [1].

Because of these limitations, there has been continuing research to find better, faster and more convenient approaches for the analysis of drug–protein binding. One such approach involves the use of immobilized albumin supports and high-performance affinity chromatography (HPAC) [11]. HPAC is a high-performance liquid chromatography (HPLC)-based method in which the stationary phase consists of an immobilized biologically-related ligand. In the case of solute–albumin studies, this ligand consists of serum albumin which has been adsorbed or covalently linked to a support like silica. For in-

stance, this is usually done by covalently attaching albumin to diol-bonded silica activated with 1,1′-carbonyldiimidazole [12,13], by attaching albumin to silica through a two-step [14] or three-step Schiff base method (reductive amination) [13,15,16], or by using modified silica that has been activated with *N*-hydroxysuccinimide ester [17]. In addition, some studies have used albumin which is non-covalently adsorbed to ion-exchange columns [18] or HPLC-grade silica [16] or which is immobilized to HPLC supports based on agarose [19] or hydroxyethylmethacrylate (HEMA) [20,21]. Purified albumin is usually used in preparing these columns to avoid interferences from other proteins in the later binding studies. However, in one report albumin and another protein (α_1-acid glycoprotein) were immobilized on the same support for use in HPLC studies [22].

One advantage of using HPAC and immobilized albumin columns for binding studies is the ability to reuse the same ligand preparation for multiple experiments. For instance, columns containing HSA immobilized to silica have been used for up to 500–1000 injections in some cases [23–25]. This creates a situation in which only a relatively small amount of protein is needed for a large number of studies and 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 solute binding studies (i.e., often 5–15 min per analysis). The fact that the immobilized protein is continuously washed with an applied solvent is yet another advantage of HPAC since this eliminates the effects produced by any organic contaminants (e.g., fatty acids) that might have been present in the initial protein preparation [14].

2. General HPAC methods for the study of solute–albumin interactions

There are several different approaches in HPAC that can be used to examine the binding of small solutes with immobilized albumin. These methods are: (1) zonal elution and (2) frontal analysis. The general principles behind each of these techniques will now be discussed, along with their advantages, disadvantages and common assumptions made during their use.

2.1. Zonal elution

This is the most common method used in HPAC to study solute–protein interactions. This technique requires that relatively fast association/dissociation kinetics be present in the solute–protein system so that multiple contacts are made between these two species as the solute travels through an immobilized protein column. Zonal elution is usually performed by injecting a small amount of the drug or solute of interest (i.e., linear elution conditions); however, there have been studies in which larger amounts of sample have also been employed [26]. This sample is injected either in the presence of only buffer or in the presence of a fixed concentration of a competing agent. Analysis of the results is performed by determining how the retention factor (k') for the injected solute changes as a function of the mobile phase conditions or the competing agent's concentration. The value of k' in this case is calculated by using the equation $k' = (t_R - t_M)/t_M$, where t_R is the mean retention time for the injected solute and t_M is the column void time.

Fig. 2 shows an example of a typical zonal elution study. For this particular example the injected drug or solute (D) has competition with a mobile phase additive (A) at a single class of common binding sites on HSA. If no other types of binding sites for D are present in the column, then Eq. (3) shows the relationship that would be expected between the retention factor that is measured for D and the concentration of the mobile phase additive,

$$\frac{1}{k'} = \frac{K_{aA} V_M [A]}{K_{aD} m_L} + \frac{V_M}{K_{aD} m_L} \quad (3)$$

where K_{aA} and K_{aD} are the association equilibrium constants for the binding of A and D at the site of competition, [A] is the molar concentration of the mobile phase additive, V_M is the column void volume, and m_L is the moles of common binding sites for D and A [14]. Eq. (3) predicts that a system with single-site competition will give a linear plot for $1/k'$ versus [A], as shown in Fig. 2. By determining the ratio of the slope to the intercept for this plot, the

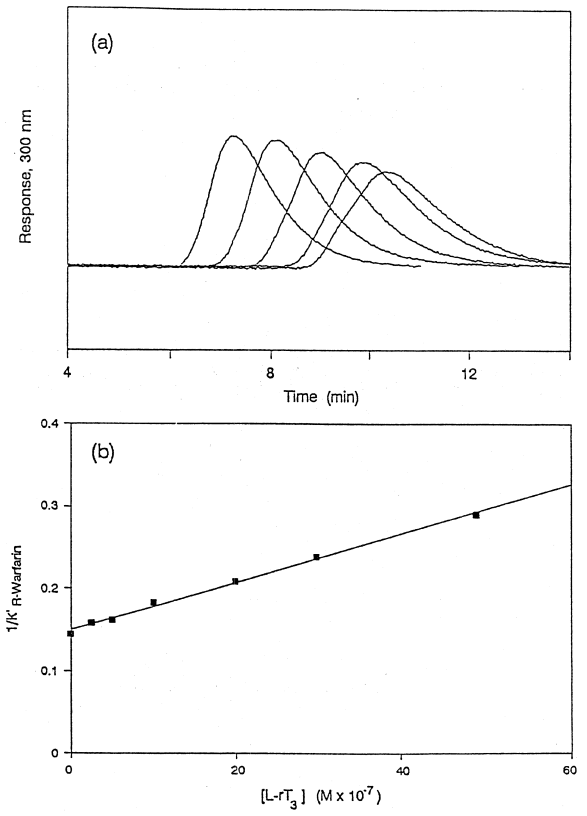

Fig. 2. (a) Chromatograms and (b) relationship between k' and ligand concentration for zonal elution experiments examining the competition of *R*-warfarin with L-reversed triiodothyronine (L-rT_3) for binding sites on immobilized HSA. In (a) 20-μl samples of 6.5 μ*M* *R*-warfarin in the appropriate mobile phase were injected into the presence of mobile phases containing (left to right) 1.90, 0.97, 0.49, 0.24 or 0 μ*M* L-rT_3. The resulting data were then plotted according to Eq. (3), as shown in (b). Adapted with permission from Ref. [23].

value of K_{aA} can be obtained. If a separate estimate of m_L/V_M is made (e.g., by frontal analysis, as described in the next section), then the value of K_{aD} can also be determined from the intercept.

Similar expressions to Eq. (3) can be derived for other situations, such as for drugs and additives with multiple sites of competition or injected solutes that have other binding sites which do not interact with the mobile phase additive [27,28]. This second case (as illustrated in Fig. 3) is particularly common [27–31] and is performed by simply replacing the left-hand term of Eq. (3) by the expression $1/(k'-X)$, where X is a constant for the injected solute that

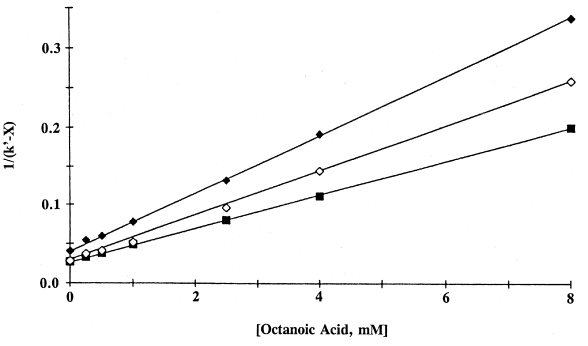

Fig. 3. Zonal elution plots using the term $1/(k'-X)$ to correct for retention of the injected analyte at binding sites on the column other than those involved in competition with the mobile phase additive (octanoic acid). The data shown are for *R*-warfarin (♦), *S*-warfarin (◇) and phenylbutazone (■). Reproduced with permission from Ref. [27].

represents its k' value due to all regions that are not involved in the competitive binding processes [27]. Finally, zonal elution has also been utilized as a means for examining the kinetics of solute interactions with immobilized albumin. Further details on this last topic will be provided in Section 3.

2.2. Frontal analysis

Frontal analysis is the second technique that is commonly used in HPAC studies of drug or hormone interactions with albumin. In this approach, a solution containing a known concentration of the solute to be studied is continuously applied to the affinity column. As the solute binds to the immobilized ligand, the ligand becomes saturated and the amount of solute eluting from the column gradually increases, forming a characteristic breakthrough curve (see examples shown in Fig. 4). If fast association and dissociation kinetics are present in the system, then the mean positions of the breakthrough curves can be related to the concentration of applied solute, the amount of ligand in the column, and the association equilibrium constants for solute–ligand binding.

The example shown in Fig. 4 was obtained for a solute that has 1:1 binding with immobilized albumin. The results that would be expected in this situation are given by Eq. (4), where the applied drug or solute (D) is viewed as binding to only a single type of immobilized ligand or binding site (L).

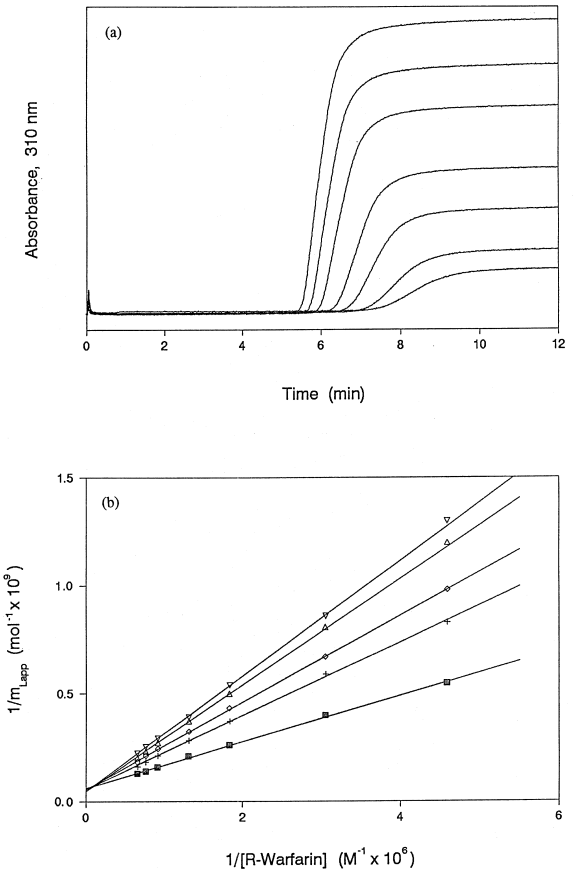

Fig. 4. Typical chromatograms obtained for the frontal analysis of *R*-warfarin on the immobilized HSA column at 4°C (a) and plots made according to Eq. (4) for data obtained at temperatures of 4 (■), 15 (+), 25 (◇), 37 (△) and 45°C (▽). In (a) the *R*-warfarin concentrations (left to right) were 1.50, 1.30, 1.10, 0.76, 0.55, 0.33 and 0.22 μ*M*. Adapted with permission from Ref. [39].

Eq. (4) shows how the true number of active binding sites in the column (m_L) are related to the apparent moles of drug (m_{Lapp}) that are required to reach the mean position of the breakthrough curve in the absence of any competing agent [32].

$$\frac{1}{m_{Lapp}} = \frac{1}{K_a m_L[D]} + \frac{1}{m_L} \quad (4)$$

In the above relationship, K_a is the association constant for the binding of D to L, and [D] is the molar concentration of drug applied to the column. This equation predicts that a plot of $1/m_{Lapp}$ versus $1/[D]$ for a system with single-site binding will give a straight line with a slope of $(1/K_a m_L)$ and an intercept of $1/m_L$ (see Fig. 4). In this case, K_a can be determined by calculating the ratio of the intercept to the slope, and m_L is obtained from the inverse of the intercept. Similar relationships can be derived for more complex systems, such as those with multisite interactions [33] or competitive binding between the applied solute and a known concentration of a mobile phase additive [34]. If desired, expressions are also available where the results of frontal analysis experiments can be examined by Scatchard plots [35–38].

One disadvantage of frontal analysis is the relatively large amount of solute that is required for each study. However, it also has a distinct advantage over normal zonal elution in that it can simultaneously 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 valuable in monitoring the stability of affinity columns during their use in the long-term studies [39]. In addition, the same feature makes frontal analysis the method of choice for accurate association constant measurements between a solute and an immobilized protein, since the resulting K_a values are essentially independent of the number of binding sites present in the column [38,39].

3. Information obtained through HPAC studies of solute–albumin binding

3.1. Relative binding strengths

The simplest type of experiment for solute–protein binding in HPAC involves simply injecting the drug or hormone of interest onto an immobilized protein column and measuring the solute's retention in the presence of only buffer or simple buffer additives. This relies on the fact that the resulting retention time or retention factor (k') for the injected solute is related to the various equilibrium constants for the solute's interactions in the column. For instance, in the case of a solute that is injected onto a column that contains a protein with a series of binding sites L_1 through L_n, the following relationship has been

shown to relate k' to the solute–ligand global association constant (K'_a):

$$k' = (K_{a1}n_1 + \ldots K_{an}n_n)m_{L,tot}/V_M \quad (5)$$

$$= K'_a(m_{L,tot}/V_M) \quad (6)$$

where V_M again represents the column void volume, $m_{L,tot}$ is the total moles of immobilized protein in the column, K_{a1} through K_{an} are the association equilibrium constants for each individual type of binding site, and n_1 through n_n are the relative number of each type of binding site within the column [11].

Based on the relationship shown in Eq. (6), retention factor measurements have been used in a number of studies with HSA, BSA or albumin from other sources to examine how their mechanisms of retention change when varying solute structure, temperature or mobile phase composition (e.g., pH, ionic strength or organic solvent content) [13,15,18–21,40–49]. An example of such a study is shown in Fig. 5. However, as also shown in Fig. 5, some caution must be exercised when using k' as a direct measure of binding affinity, since changes in this parameter can be caused by alterations in either K_{ai} or n_i. This creates more than one factor that can produce changes in the measured k' values [32].

The retention time for a drug or injected solute can also be used in other ways to study solute–protein interactions. For instance, the value of k' can be used as indicator of the average bound fraction of a solute on a column, since the retention factor is simply a measure of the relative moles of solute that is bound at equilibrium to the stationary phase. This approach has been used to compare the percent binding of HSA with various benzodiazepines, coumarins and triazole derivatives [50]. The same approach has been combined with liquid chromatography–mass spectrometry (LC–MS) to allow the simultaneous determination of binding properties for a mixture of drugs [51]. A related use for k' values has been in the development of quantitative structure–retention relationships (QSRRs) for the binding of drugs to immobilized protein columns [29,52]. This involves collecting k' values, or other types of retention information, under constant temperature and mobile phase conditions for a large set of drugs representing a range of structural variations. These data are then compared to various parameters that can be used to describe the structure of these solutes, and regression is performed to determine which of these factors are most important in controlling retention [53]. Such an approach has been used to examine the binding immobilized HSA to 1,4-benzodiazepines [29,52], indolocarbazole derivatives [54], and acyclovir esters [55] (see Fig. 6).

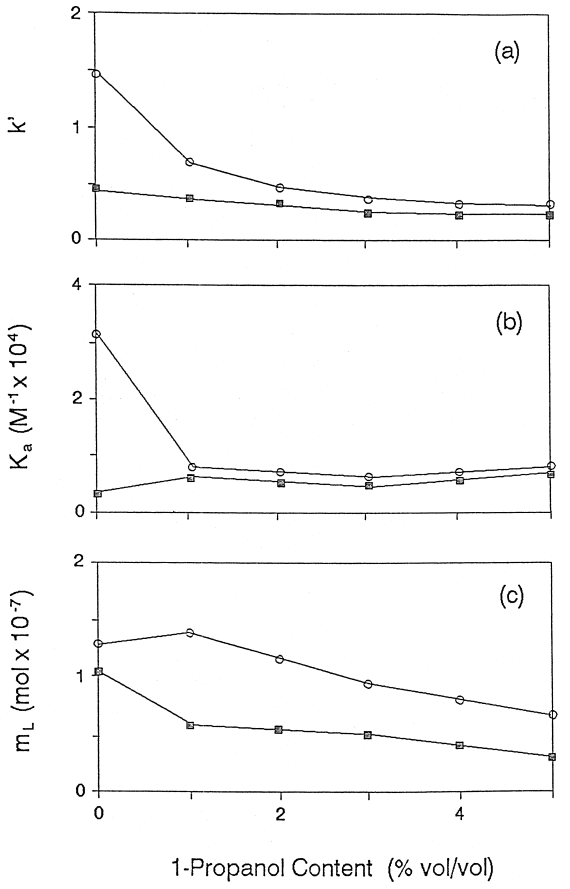

Fig. 5. Effect of adding 1-propanol to the mobile phase on the retention factor (a), association equilibrium constant (b) and total moles of active binding sites (c) for L-tryptophan (○) and D-tryptophan (■) on an immobilized HSA column. Reproduced with permission from Ref. [32].

3.2. Displacement by other solutes

Another common use of HPAC and zonal elution has been as a tool for examining the displacement of drugs from proteins by other solutes, as illustrated in

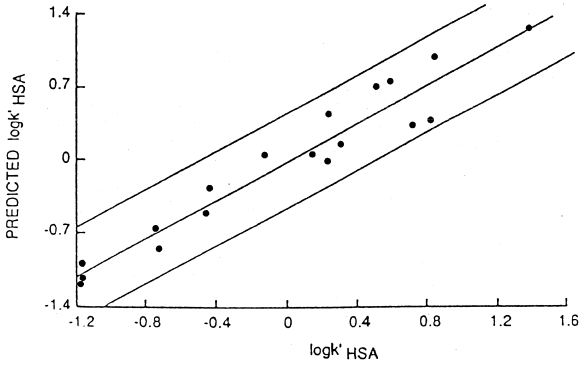

Fig. 6. Predicted versus actual values of log k' for a series of acyclovir esters injected onto an immobilized HSA column. The upper and lower lines in the graph represent the 95% confidence interval of the best-fit response. Adapted with permission from Ref. [55].

Figs. 2 and 3. Examples based on HPLC columns have included the use of zonal elution to examine the displacement of D/L-thyronine and D/L-tryptophan from immobilized HSA by bilirubin or caprylate [56]; the competition of *R*/*S*-warfarin with racemic oxazepam, lorazepam and their hemisuccinate derivatives on an HSA column [57]; the direct or allosteric competition of octanoic acid on immobilized HSA for the binding sites of *R*/*S*-warfarin, phenylbutazone, tolbutamide, *R*/*S*-oxazepam hemisuccinate, ketoprofen A/B, suprofen A/B [27] and ketorolac [58]; and the competitive binding of *R*/*S*-ibuprofen, salicylate, *R*/*S*-oxazepam hemisuccinate, fluorouracil, diazepam, phenylbutazone and *R*/*S*-warfarin on HSA [59]. Other studies have used this to examine the competition of *R*-warfarin and L-tryptophan with D-tryptophan [40] or L-thyroxine and related thyronine compounds on immobilized HSA [14,23], and the competitive binding of diazepam with itself or diclofenac [60], phenylbutazone [61] and *R*- or *S*-ketoprofen [62] on HSA columns. In addition, this method has been employed in investigations of the displacement of *R*- and *S*-ibuprofen [28], *cis*- and *trans*-clomiphene [63], and digitoxin or acetyldigitoxin [64] by one another or various probe compounds [65] at their binding regions on HSA. The same technique has been used to characterize the binding sites of non-steroidal anti-inflammatory drugs on HSA [30], and the displacement of non-steroidal anti-inflammatory drugs and benzodiazepines by phenylbutazone, *R*/*S*-ibuprofen or 2,3,5-triiodobenzoic acid from human, rat and rabbit serum albumin columns [31].

3.3. Equilibrium constant and binding capacity measurements

HPAC studies can also be used to provide quantitative information on the strength or degree of solute–protein interactions. For instance, Eq. (3) and related expressions have now been used in several studies to determine the equilibrium constants for solute–albumin interactions [14,23,27–31,63,64]; examples of such experiments were provided earlier in Figs. 2 and 3. One particular advantage of zonal elution experiments is that they can be designed for determining association constants at specific binding regions for solutes that have multisite interactions to the immobilized protein. As indicated by Eq. (3), this can be done by applying the solute of interest as the mobile phase additive while making injections of probe compounds that are known to have single-site interactions at the binding sites to be tested. Such an approach has been used to investigate the mechanisms involved in the binding of thyroid hormones to the warfarin and indole sites of HSA (e.g., see Fig. 2) [14,23]. Similar studies have been used to examine the interactions of *cis*- and *trans*-clomiphene or digitoxin and acetyldigitoxin at their binding regions on HSA [63–65].

As discussed earlier, frontal analysis can also be employed in quantitative studies of solute–albumin interactions (see Fig. 4). For instance, this approach has been used to investigate the binding of HSA to *R*- or *S*-warfarin [14,39] and D- or L-tryptophan [14,27,32,40], and the binding of salicylate to BSA [36]. In addition, it has been used to determine the binding capacities of monomeric versus dimeric HSA for salicylic acid, warfarin, phenylbutazone, mefenamic acid, sulfamethizole and sulfonylureas [37], as well as the changes in binding capacity that occur with different temperatures or mobile phases for *R*- or *S*-warfarin [39], and D- or L-tryptophan [32] on immobilized HSA columns (see Fig. 5). Furthermore, frontal analysis has been utilized to examine the competition of sulfamethizole with salicylic acid for HSA binding regions [38], or salicylate with

clofibric acid, octanoic acid or oestradiol for sites on BSA [35].

3.4. Rate constant measurements

Other information that can be obtained from HPAC involves the kinetics of solute–albumin interactions. One way in which this can be done is to perform zonal elution studies at several different flow-rates and prepare van Deemter-type plots showing how the measured column efficiency changes with the solute's linear velocity. The plate height contribution due to stationary phase mass transfer (H_s) is of particular interest in such studies since it is directly related to the dissociation rate constant between the injected solute and the immobilized ligand (k_d), as shown by Eq. (7),

$$H_s = \frac{2uk'}{k_d(1+k')^2} \tag{7}$$

where u is the linear velocity of mobile phase in the column and k' is the retention factor of the injected solute. Based on Eq. (7), a plot of H_s versus $uk'/(1+k')^2$ should give a slope of $2/k_d$ and an intercept of zero. Some typical graphs made according to Eq. (7) are shown in Fig. 7 [25]. By using the k_d values obtained from these plots along with independent estimates for the equilibrium constants of the system, the association rate constants for the drug and protein can also be obtained. If these studies are done at several temperatures, then it is possible to generate a reaction profile for the solute–protein system (see Fig. 7b). Such experiments have now been performed for *R*- or *S*-warfarin [24] and D- or L-tryptophan [25] on immobilized HSA columns and provide useful insights into how the energetics of these binding processes change under typical chromatographic operating conditions.

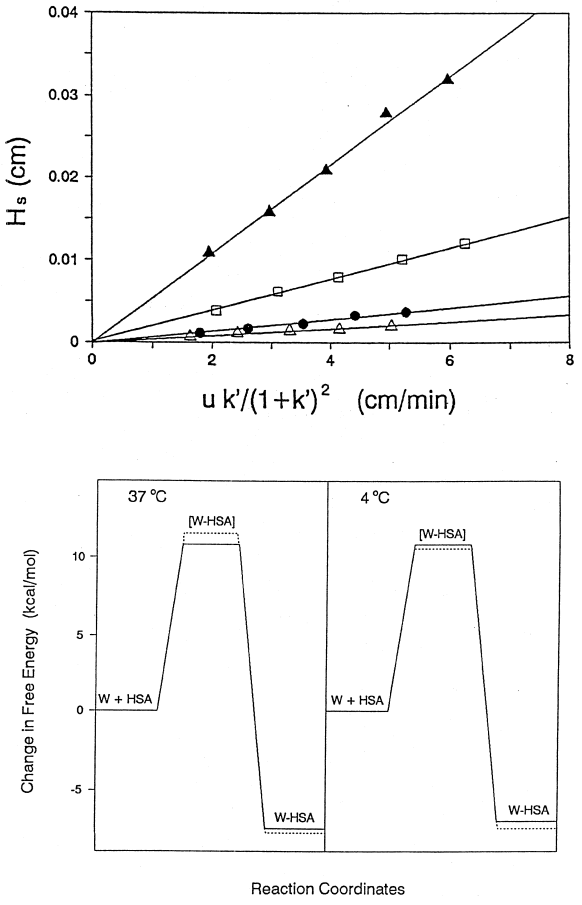

Fig. 7. (Top, a) Plots of the plate height contribution due to stationary phase mass transfer (H_s) versus $[uk'/(1+k')^2]$ for D-tryptophan injected onto an immobilized HSA column at 25°C and mobile phase pH values of 4.0 (△), 5.0 (●), 6.0 (□) or 7.0 (▲). (Bottom, b) Reaction profiles developed for *R*-warfarin (solid lines) and *S*-warfarin (dashed lines) based on such plate height studies. Reproduced with permission from Refs. [24,25].

4. Comparison of results for soluble albumin and immobilized albumin in HPAC

A logical question to ask is this: how well does immobilized albumin work as a model for the behavior of soluble albumin in the body? There are several different levels at which this question can be addressed. First, there is the evidence that comes from a comparison of the relative binding and displacement properties that have been noted for both types of proteins. For instance, it has now been shown in numerous studies that displacement phenomena and allosteric interactions seen on HSA columns are similar to those observed for HSA in solution [12,14,27,29,57,66]. Although such comparisons are qualitative in nature, they do lend support to the validity of using immobilized albumin as a model for albumin in solution.

The next level of evidence comes from a comparison of the actual equilibrium constants that have been measured for immobilized versus soluble albumin. Table 1 give some examples for representative solutes that have been used in such comparisons [14,39,61,67–71]. Again, the results obtained by HPAC and methods such as frontal analysis or zonal elution show good agreement with solution-phase binding constants that have been measured under equivalent conditions by methods like equilibrium dialysis or ultrafiltration. In addition, a comparison of the temperature dependence of these equilibrium constants, or the changes in entropy and enthalpy, that have reported by HPAC have shown similar agreement with values reported for solution-phase albumin [14,32,39,69].

A third piece of evidence that supports the use of immobilized albumin columns as tools for solute binding studies comes from the kinetics that have been observed for such columns. For instance, the range of association rate constants that have been measured for D- or L-tryptophan, and *R*- or *S*-warfarin on immobilized HSA columns have been found to be typical of the range of rate constants that have been reported for many small solutes with soluble HSA [24,25]. A possible explanation for this is the fact that solute–albumin binding, like many other biological interactions, is believed to involve a two-step process: (1) initial diffusion of the solute and protein together to form an intermediate complex and (2) changes in the binding or conformation of the solute and/or protein to form the final solute–protein complex. It has long been suspected from solution-phase studies that the association of albumin with small solutes is limited by the second step in this process. If this is true, then changes in the rate of albumin diffusion (as would occur due to immobilization) would be expected to have only a minimal effect on the net association rate of albumin with solutes, as indicated by the HPAC kinetic studies [24,25].

5. Recent developments in HPAC studies of drug and hormone binding to albumin

5.1. HPAC studies of low solubility compounds

One recent development in the use of HPAC in solute-binding studies has been in the modification of this method for dealing with compounds that have low solubility in aqueous solvents [63–65]. This is an important problem since many of these substances also bind to albumin and other proteins in blood. The

Table 1
Comparison of association constants measured for soluble HSA and HSA immobilized in HPLC columns[a]

Solute	Type of HSA	Association constant, K_a (M^{-1})	Method of measurement	Ref.
L-Thyroxine	Immobilized	$1.4\ (\pm 0.1)\cdot 10^5$ (site I)	Zonal elution	[14]
	Immobilized	$5.7\ (\pm 0.8)\cdot 10^5$ (site II)	Zonal elution	[14]
	In solution	$3\cdot 10^5$ (two sites)	Equilibrium dialysis	[67]
L-Tryptophan	Immobilized	$1.1\ (\pm 0.3)\cdot 10^4$	Frontal analysis	[14]
	In solution	$1.3\cdot 10^4$	Equilibrium dialysis	[68]
R-Warfarin	Immobilized	$2.1\ (\pm 0.2)\cdot 10^5$	Frontal analysis	[39]
	In solution	$2.5\cdot 10^5$	Equilibrium dialysis	[69]
	In solution	$2.06\ (\pm 0.02)\cdot 10^5$	Equilibrium dialysis	[70]
S-Warfarin	Immobilized	$2.6\ (\pm 0.4)\cdot 10^5$	Frontal analysis	[39]
	In solution	$5.69\cdot 10^5$	Equilibrium dialysis	[69]
	In solution	$2.44\ (\pm 0.04)\cdot 10^5$	Equilibrium dialysis	[70]
Phenylbutazone	Immobilized	$1.84\ (\pm 0.13)\cdot 10^5$ (site I)	Zonal elution	[61]
	In solution	$1.17\cdot 10^5$	Ultrafiltration	[71]

[a] Values in parentheses represent ±1 SD.

difficulty in studying these solutes directly by HPAC is that it must be possible to get enough of these substances into the mobile phase to either detect or to use as competing agents. Although improved monitoring schemes may allow the detection of smaller concentrations of such species, obtaining a high enough concentration to use these compounds as competing agents is more difficult to address since the levels needed for this are dictated by the nature of the experiment and the strength of the binding process that is being studied.

There are several approaches that might be used to increase the concentration of a solute for HPAC; however, most of these approaches produce undesirable side effects. For example, the temperature might be increased to improve a compound's solubility, but this will also change the equilibrium constants and kinetics of a solute–protein interaction. Similar changes can occur when the pH or ionic strength of the mobile phase is varied or when an organic modifier is added. In some cases a supersaturated solution of the solute can be prepared, but such solutions are stable for only limited periods of time and need to be carefully monitored during their use. An alternative approach is to place a solubilizing agent into the mobile phase that helps to increase the effective concentration of the solute of interest. β-Cyclodextrin is one solubilizing agent that has been used for this purpose. This agent was selected since it is known to bind to many small, non-polar compounds and yet it has no measurable binding to HSA [63,72]. This means that the presence of β-cyclodextrin in the mobile phase should not alter the nature or strength of the binding between the immobilized albumin and injected or applied solutes. Instead, β-cyclodextrin and albumin act as independent ligands for these drugs, with the β-cyclodextrin being used to merely help keep the solute in the mobile phase through the formation of soluble host:guest complexes (see Fig. 8) [63,64]. Another advantage of using β-cyclodextrin as a complexing agent is that it has no appreciable UV–Vis absorbance under the conditions that are commonly employed in monitoring solutes during HPAC studies of drug– or hormone–protein interactions.

Two cases in which β-cyclodextrin has been utilized in HPAC studies include experiments that have examined the binding of immobilized HSA to

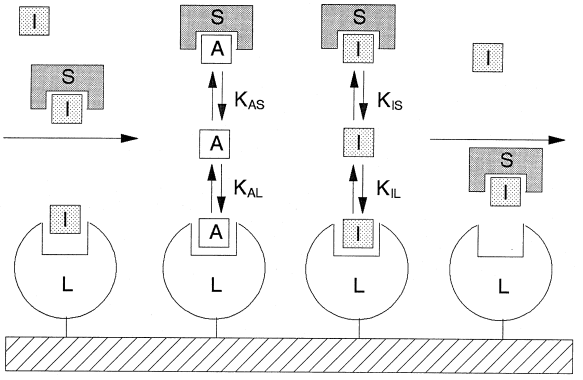

Fig. 8. General model for the binding of an injected analyte (A) and competing agent (I) with an immobilized ligand (L) and solubilizing agent (S) in an HPAC column. The terms K_{AL}, K_{IL}, K_{AS} and K_{IS} represent the association equilibrium constants for the binding of A and I to L and S.

cis- or *trans*-clomiphene [63] and digitoxin or acetyldigitoxin [64]. This was performed by using zonal elution studies similar to those described previously, but with theory of this method now being adapted to consider how the elution of the injected solute changes with the concentrations of both the solubilizing agent and competing agent in the mobile phase. The equations and experiments that are used for this purpose are described in detail in Ref. [63]. This approach first involves using the solubilizing agent in sufficient excess so that a linear relationship is obtained between $1/k'$ for the injected analyte and the concentration of competing agent. The slope and intercept of this linear region are then measured at several different concentrations of the solubilizing agent. The intercept/slope ratio from these graphs is next plotted as a function of the solubilizing agent's concentration; if the system being studied has 1:1 interactions between the injected solute and ligand, then this second plot should result in a linear relationship. The association equilibrium constant between the immobilized ligand and the competing agent is then obtained from the reciprocal of the intercept for this second plot [63,64].

Based on this approach, it has been possible to study the competitive binding of *cis*- and *trans*-clomiphene or acetyldigitoxin and digitoxin with each other and with other probe compounds. For instance, it was found that *cis*- and *trans*-clomiphene each have a single, common binding region on HSA

that is separate from either the warfarin–azapropazone site or indole–benzodiazepine region of this protein. It was further found that this same binding region gave 1:1 interactions with tamoxifen, confirming the existence of a separate ''tamoxifen site'' on HSA for such compounds [63]. Similar studies performed with digitoxin and acetyldigitoxin demonstrated that these solutes have a single common binding area on HSA that is distinct from HSA's warfarin or indole binding sites, thus confirming the alleged presence of a ''digitoxin site'' on this protein [64]. These results have recently been used to compare the specificity of such probes in characterizing the interactions of other solutes at the minor binding regions of HSA [65]. The same methods should prove valuable in future work involving other compounds that are non-polar in nature and/or that have low solubility in water.

5.2. HPAC binding studies based on chemically-modified albumin

A second area of on-going research has been in the construction and use of columns which contain albumin that has been chemically-modified at specific residues in its structure. One example is a report in which *o*-nitrophenylsulfenyl chloride was employed as a reagent for modification of the lone tryptophan residue on HSA, Trp-214 [73]. The reaction that was involved in this process is shown in Fig. 9a. This type of modification was of interest since Trp-214 is known to be located within the warfarin–azapropazone site of HSA.

It was found in frontal analysis studies that HSA which had been chemically-modified at Trp-214 gave the same number of binding sites as normal HSA for *R*-warfarin but with a lower association equilibrium constant for this solute. This result was interpreted as being due to the direct blocking of HSA's warfarin binding region by the modified tryptophan residue. Further studies based on zonal elution and the injection of racemic warfarin showed that Trp-214 and/or its neighboring residues played an important role in determining both the affinity and stereoselectivity of HSA for the *R*- and *S*-enantiomers of warfarin (see Fig. 9b).

Albumin columns that have been modified at specific binding regions offer an attractive alternative

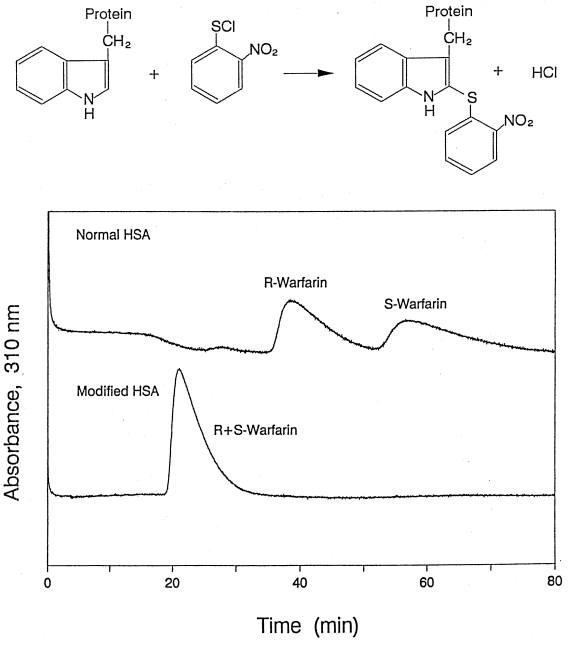

Fig. 9. (Top, a) Modification of tryptophan residues on a protein with the reagent *o*-nitrophenylsulfenyl chloride and (bottom, b) use of normal HSA and tryptophan-modified HSA columns in the separation of *R*- and *S*-warfarin at 25°C. Reproduced with permission from Ref. [73].

to displacement studies in determining the binding regions of compounds on albumin. Such columns may also eventually play a role in adjusting and optimizing albumin-based chiral separations. However, more work is still needed in improving the selectivity of the modification reactions that are used for this purpose. For example, frontal analysis experiments performed with L-tryptophan indicated that this solute also had a decrease in binding affinity following the modification of Trp-214 by *o*-nitrophenylsulfenyl chloride. This was believed to be due to an allosteric interaction between the warfarin and indole sites of HSA that was created by modification of the tryptophan residue [73]. Current work is examining the use of alternative reagents that may avoid the presence of such long-range interactions, thus allowing the warfarin and/or indole sites to be blocked without producing noticeable changes in the affinities or reactivities of the other binding regions on HSA. Similar experiments have recently been reported in which the lone free cysteine residue on

HSA (Cys-34) was modified with ethacrynic acid [74].

A second way in which modified albumin columns can be prepared is by digesting this protein and immobilizing specific fragments. For instance, this was recently illustrated with BSA, in which a peptic digest was made of BSA [75,76]. Some of the resulting fragments were then isolated by size-exclusion and anion-exchange chromatography and characterized by mass spectrometry. Columns were then prepared using either the intact BSA or one of two major fragments which consisted of only amino acid residues 1–307 or the N-terminal half of BSA. The chiral recognition of these supports were then compared for a variety of different drugs. Some drugs, like benzoin, gave better separations on the immobilized fragment supports while others (e.g., warfarin) were better resolved on the intact HSA column (see Fig. 10). Such differences may be useful in future studies in providing clues on the general regions of albumin that are involved in the binding and enantioselective recognition of a particular analyte.

5.3. Effects of binding site heterogeneity on HPAC studies of solute–protein binding

A third area of recent work has been in determining the possible effects of binding site heterogeneity on HPAC measurements of drug– and hormone–protein interactions [32,60–62]. This issue is of concern because such heterogeneity can be produced in several different ways. For instance, this might be the result of natural heterogeneity in the ligand, such as caused by the presence of a mixture of binding proteins, chemically-modified proteins or genetic variations within the same group of ligands. In addition, heterogeneity might be caused by the presence of multiple binding regions on a single ligand or, when using an immobilized ligand, by the creation of heterogeneity due to the immobilization process (e.g., random ligand orientation or multi-site attachment).

Fig. 11a shows the effects observed when performing frontal analysis with an analyte (L-thyroxine) that has several different binding sites on HSA [32]. For a system with only one solute binding site on HSA, a linear relationship should be seen for a double-reciprocal plot of $1/m_{\mathrm{Lapp}}$ versus $1/[\mathrm{Ana}$-

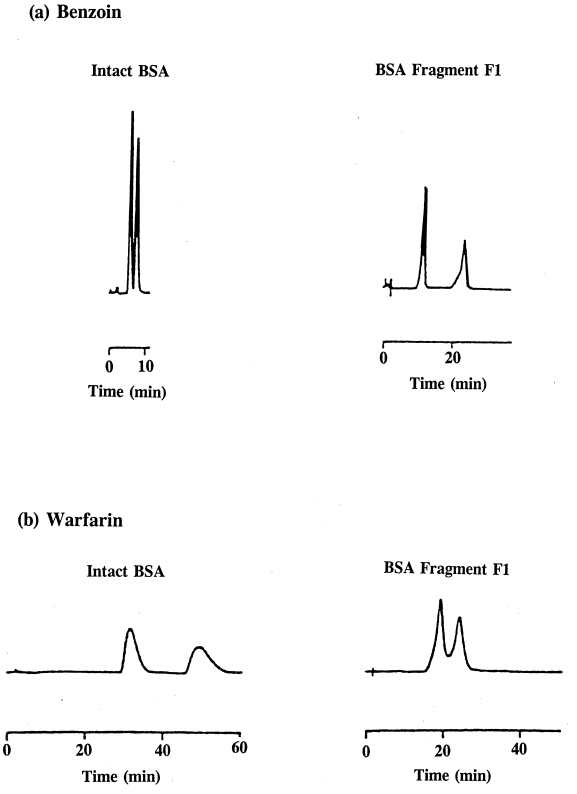

Fig. 10. Chromatograms for the chiral separation of (a) benzoin and (b) warfarin on HPLC columns that contained immobilized intact BSA or BSA fragment F1 (amino acids 1–307). Adapted with permission from Ref. [75].

lyte]. However, in a heterogeneous system, such as the binding of L-thyroxine to HSA, the response is linear only at low analyte concentrations and becomes curved at higher solute levels. This behavior can be explained by the fact that at low solute concentrations only the highest affinity sites on the column will tend to bind to solute, making this system appear to be homogeneous in nature. But as higher solute concentrations are used, a significant amount of the lower affinity sites will also begin to take part in solute retention, now giving the ligand heterogeneous binding properties.

Equations have recently been developed for frontal analysis studies that indicate how such double-reciprocal plots will change when varying either the relative amount of each binding site or the relative size of the association constants for these sites. The same theory and equations have been used to ex-

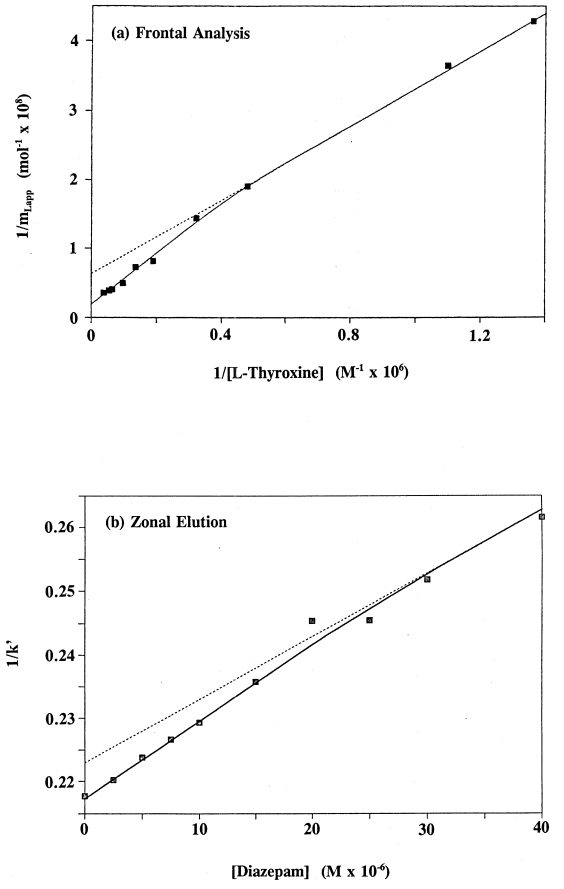

Fig. 11. Heterogeneity effects in (a) a double-reciprocal frontal analysis plot for the application of L-thyroxine to an immobilized HSA column and (b) a zonal elution plot for the self-competition of diazepam on an HSA column. Adapted with permission from Refs. [32,60].

amine the accuracy of binding capacity or association constants measured from the linear region of double-reciprocal plots when heterogeneous binding sites are present in the column. It has been found that a large proportion of two-site systems give a good estimate (i.e., less than 10–20% error) of the true total column capacity and the association constant for the highest affinity ligand in the column. A smaller, but still appreciable, fraction of all three- and four-site cases also produce good estimates of these values. These results are of great practical value because of they again indicate that HPAC methods, particularly those based on frontal analysis, can provide an accurate measure of solute–protein interactions even if some degree of ligand heterogeneity is present in the column.

Similar heterogeneity effects have been reported in competitive binding zonal elution studies [60–62]. An example of such behavior is shown in Fig. 11b for the competitive binding of diazepam present in both the mobile phase and in an injected sample [60]. The same general trends have been observed for the self-competition of phenylbutazone [61] and the competition of diazepam as a mobile phase additive with injections of diclofenac [60], phenylbutazone [61], or *R*- and *S*-ketoprofen [62]. These data have been used to determine the binding constants for these various agents at their low and high affinity sites on HSA. Part of this approach involves the use of a linear fit to the high concentration region of plots like Fig. 11b. From this fit, parameters for the low affinity site are then estimated. These parameters are then used with the rest of the graph to further determine the binding constants for the high affinity site.

6. Conclusions

In recent years there has been growing interest in the development of new approaches for the study of solute–albumin interactions. HPAC techniques that use immobilized albumin columns are one group of tools that have been explored for this purpose. This paper has discussed how these items can be used in solute binding studies and has examined several recent applications of these techniques. Approaches based on both zonal elution and frontal analysis were described, as well as the types of information that can be obtained by these methods. This information includes the relative binding strengths and displacement properties of drugs or other small solutes, and the equilibrium constants and rate constants for their interactions with albumin. A comparison was then provided between the results obtained with immobilized albumin and those that have been reported for solution-phase albumin. Some newer advances in the use of immobilized albumin were then considered, such as techniques for measuring the binding of non-polar compounds to albumin, and studies on the effects of binding site heterogeneity or chemically-modified albumin in HPAC. Based on past and

current work, it is expected that HPAC and immobilized albumin columns will become increasingly important in clinical and pharmaceutical research for the detection or study of drugs, hormones or other small solutes that bind to albumin.

Acknowledgements

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