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Review

# High-performance affinity chromatography: a powerful tool for studying serum protein binding

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**Abstract**

High-performance affinity chromatography (HPAC) is a method in which a biologically-related ligand is used as a stationary phase in an HPLC system. This approach is a powerful means for selectively isolating or quantitating agents in complex samples, but it can also be employed to study the interactions of biological systems. In recent years there have been numerous reports in which HPAC has been used to examine the interactions of drugs, hormones and other substances with serum proteins. This review discusses how HPAC has been used in such work. Particular attention is given to the techniques of zonal elution and frontal analysis. Various applications are provided for these techniques, along with a list of factors that need to be considered in their optimization and use. New approaches based on band-broadening studies and rapid immunoextraction are also discussed. 

*Keywords:* Reviews; Protein binding

## Contents



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## 1. Introduction

The interaction of small solutes with proteins is important in many biological processes. Examples include the action of enzymes upon substrates and the binding of hormones with their receptors. The binding of drugs and other compounds with serum proteins is yet another example of such a process. Protein binding in blood is important in determining the eventual activity and fate of 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. In addition, the presence of direct or indirect competition between two drugs or a drug and 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].

For all of these reasons, it is important to have a good understanding of how pharmaceutical agents bind to serum proteins and of how these interactions are affected by other substances. This review will discuss one powerful technique that can be used in these studies, that of high-performance affinity chromatography (HPAC). The underlying principles of HPAC will be discussed, along with a description of how it can be used with serum proteins. A comparison of this approach versus other techniques will also be made. A detailed description will then be given of two specific methods (zonal elution and frontal analysis) that have been widely employed in HPAC for the study of serum proteins. Newer methods that use band-broadening measurements or rapid immunoextraction will be described as well. The basis of each approach will be discussed and several examples from the literature will be provided. In addition, a number of practical considerations will be examined that should be considered in the optimization and use of these methods for the investigation of solute–protein binding.

### *1.1. Definition and principles of HPAC*

A common definition of affinity chromatography is that of a liquid chromatographic technique that uses a ‘‘biologically related’’ agent as a stationary phase for the purification or analysis of sample components [8–10]. 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 as a stationary phase. This immobilized molecule is known as the affinity ligand and is what gives an affinity column the ability to bind to a particular compound in a sample.

Along with the affinity ligand, another important factor in affinity chromatography is the material used to hold this ligand within the column. Ideally this

support should have low non-specific binding for sample components, it should be easy to modify for ligand attachment, and it should be stable under the flow-rate, pressure and solvent conditions that will be employed in the analysis or purification of samples. In low-performance (or column) affinity chromatography, the support is usually a large diameter, non-rigid material (e.g. a carbohydrate-based gel or one of several synthetic organic-based polymers). The low back-pressures that are produced by these supports means that these materials can be operated under gravity flow or with a peristaltic pump, making them relatively simple and inexpensive to use for the purification or pretreatment of samples. However, the poor mass transfer properties and limited stabilities of these materials at high flow-rates and pressures limit their use in analytical applications [8].

High-performance affinity chromatography (HPAC) is characterized by a support which consists of small, rigid particles capable of withstanding high flow-rates and/or pressures [8–11]. Examples of affinity supports that are suitable for work under these conditions include modified silica or glass, azalactone beads, and hydroxylated polystyrene media. The stability and efficiency of these supports allow them to be used with standard HPLC equipment. Although the need for HPLC instrumentation does make HPAC more expensive to perform than low-performance affinity chromatography, the better speed and precision of HPAC make it preferable for analytical applications. An example of one such application is the use of HPAC to study drug–protein binding.

When affinity columns are utilized as tools to study solute–ligand interactions, this approach is referred to as analytical affinity chromatography, quantitative affinity chromatography, or biochromatography [7,9,12]. One way this might be performed is by using the format given in Fig. 1, in which the protein of interest is used as the immobilized ligand and an injection of analyte is made onto the column in the presence of only buffer or buffer plus a mobile phase modifier or 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 the analyte's binding to the affinity ligand. If additional agents are also 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 shape of the analyte's elution profile. As will be seen later, all of these approaches have been used to examine serum proteins and to provide measures of the extent and nature of their interactions with drugs and other small solutes.

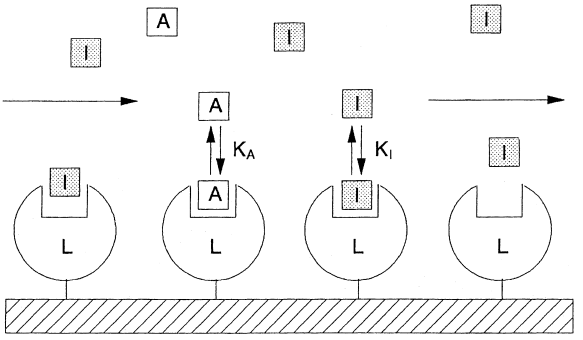



Fig. 1. Reaction model for the study of solute–protein binding by HPAC, where "*A*" is the injected or applied analyte, "*I*" is a competing agent which has been added in a known concentration to the mobile phase, and "*L*" is the immobilized ligand in the column. The terms $K_A$ and $K_I$ represent the association equilibrium constants for the binding of *A* and *I* to *L*, respectively.

### *1.2. HPAC and the study of serum proteins*

HPAC has already been used to study a variety of serum proteins. However, most of this work has focused on human serum albumin (HSA), bovine serum albumin (BSA), and $\alpha_1$-acid glycoprotein (AGP). HSA or BSA supports for HPAC can be prepared by covalently attaching albumin to diol-bonded silica activated with 1,1′-carbonyldiimidazole [13,14], by attaching albumin to silica through a two-step [15] or three-step Schiff base method (i.e. reductive amination) [14,16,17], or by using modified silica that has been activated with *N*-hydroxysuccinimide ester [18]. An example of one such technique (the Schiff base method) is shown in Fig. 2a. In addition, some studies have used albumin which is non-covalently adsorbed to ion-exchange columns [19] and HPLC-grade silica [17], or which is immobilized to HPLC supports based on agarose

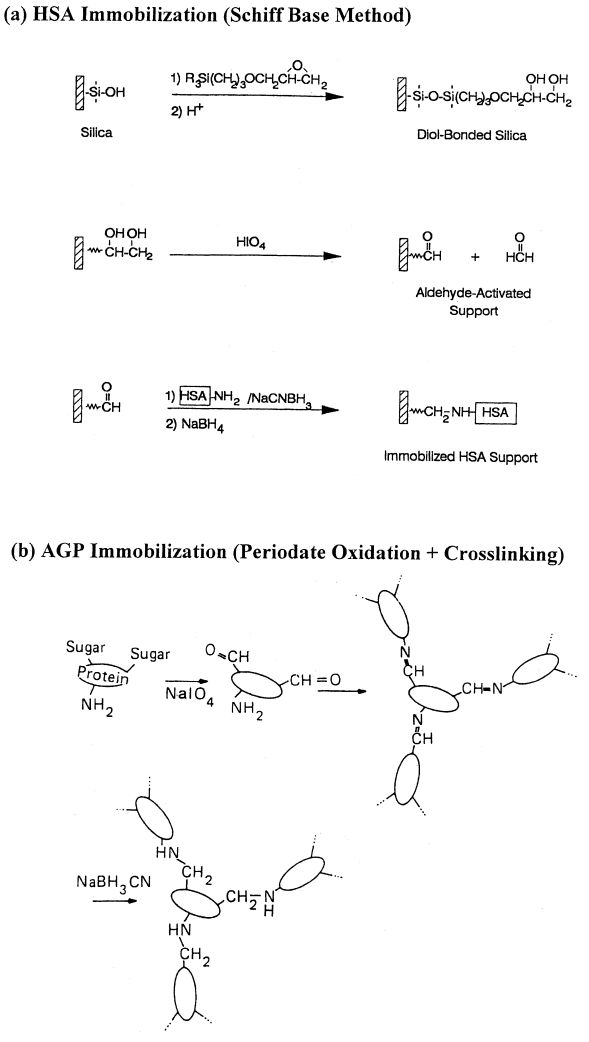



Fig. 2. (a) The Schiff base method for the immobilization of proteins to silica, and (b) the use of mild oxidation plus crosslinking and reductive amination for the preparation of AGP columns. The lower figure is reproduced with permission from Ref. [23].

[20] or hydroxyethylmethacrylate (HEMA) [21,22]. Commercial albumin columns, sold under the tradename Resolvosil, are prepared by crosslinking albumin in the presence of silica with such agents as glutardialdehyde or *N*,*N*′-disuccinimidyl carbonate [23].

HPLC supports that contain immobilized AGP, which are available commercially as chiral stationary phases under the tradenames Enantiopac and Chiral AGP, are made by first oxidizing the carbohydrate residues of AGP with periodate, causing the formation of aldehyde groups in these residues (see Fig. 2b). The pH is then raised and these aldehydes are allowed to react with free amine groups on other AGP molecules in the presence of silica. The resulting imine bonds are then stabilized by reduction with sodium cyanoborohydride to form secondary amine groups. This provides an HPLC support coated with crosslinked AGP [23]. In one study, both albumin and AGP were immobilized onto the same support for use in HPAC [24].

One advantage of utilizing HPAC for solute–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 [25–27]. 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 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 [15].

Whenever HPAC and immobilized proteins are used to study solute–ligand interactions, an important question to consider is ‘‘How well does the protein in the column work as a model for the same protein in the body?’’ The answer will depend on the protein that is being considered and the way in which it has been immobilized. In the case of serum albumins, there is a large body of evidence indicating that immobilized HSA and BSA can indeed provide good qualitative and quantitative agreement with the behavior seen for these proteins when they are in solution. For instance, it has been shown in numerous studies that displacement phenomena and allosteric interactions seen on HSA columns are similar to those observed for soluble HSA [13,15,28–32]. Also, the equilibrium constants measured by HPAC for immobilized albumins have close

Table 1
Comparison of binding constants measured by HPAC for immobilized HSA versus soluble HSA[a]

| Solute | Association constant $K_A$ ($M^{-1}$) | |
|---|---|---|
| | Immobilized HSA | Soluble HSA |
| Digitoxin | 5.2 ($\pm$0.2)$\times 10^4$ | 4–7$\times 10^4$ |
| Phenylbutazone | 1.84 ($\pm$0.13)$\times 10^5$ (Site 1) | 1.17$\times 10^5$ |
| L-Thyroxine | 1.4 ($\pm$0.1)$\times 10^5$ (Site 1) | 3$\times 10^5$ (Two sites) |
| | 5.7 ($\pm$0.8)$\times 10^5$ (Site 2) | |
| L-Tryptophan | 1.1 ($\pm$0.3)$\times 10^4$ | 1.3$\times 10^4$ |
| *R*-Warfarin | 2.1 ($\pm$0.2)$\times 10^5$ | 2.1–2.5$\times 10^5$ |
| *S*-Warfarin | 2.6 ($\pm$0.4)$\times 10^5$ | 2.4–5.7$\times 10^5$ |

[a] The values in this table are from Refs. [6,7,33]. The digitoxin, phenylbutazone and L-thyroxine results for immobilized HSA were obtained by zonal elution; the L-tryptophan, *R*-warfarin, and *S*-warfarin results for immobilized HSA were determined by frontal analysis.

agreement with those reported in solution using methods like equilibrium dialysis or ultrafiltration under comparable temperature and buffer conditions (see Table 1). Similar agreement has been noted in the temperature dependence of these equilibrium constants, the changes in entropy and enthalpy, and the rate constants that have been determined for solute–albumin systems by HPAC and liquid-phase methods [15,26,27,34–36].

However, there are some cases in which differences in the behavior of immobilized and non-immobilized serum proteins have been noted. For instance, it is known that columns with crosslinked albumin prepared by different methods can have variations in their chromatographic properties [23]. Also, crosslinked AGP columns have been found to have different displacement behavior from that seen for the same protein in solution [37,38]. Although the reasons for such differences are not fully understood, they do indicate the importance of using model compounds with known binding properties to assess any new immobilized protein before it is used to examine other substances.

## 2. Zonal elution

For all of the serum proteins that have been studied so far, the most popular HPAC method that has been used to examine solute binding has been zonal elution. This is performed in the same mode used for most analytical applications of chromatography, in which a narrow plug of solute is injected onto a column while the solute's elution time or volume is monitored [12].

The first use of zonal elution for the study of solute–ligand binding was in 1974 by Dunn and Chaiken [39], who examined the retention of staphylococcal nuclease on a low-performance affinity column containing immobilized thymidine-5′-phosphate-3′-aminophenylphosphate. The earliest application involving a drug–protein system was by Lagercrantz et al. [40] in 1979, who used low-performance Sepharose columns to measure the interactions of fatty acids, steroids and various drugs with immobilized BSA. In the mid-1980s, zonal elution was used as part of HPLC systems to examine the properties of proteins as chiral stationary phases [41–43]. And by the late 1980s and early 1990s, reports began to appear in which HPAC and zonal elution were used in quantitative studies of drug–protein interactions [15,20,28–30,44,45].

### 2.1. General basis of method

Zonal elution is generally performed by injecting a small amount of an analyte through a column (i.e. linear elution conditions) while elution of the analyte is monitored by an on-line detector. Non-linear conditions can also be employed for injection [46,47], and fraction collection plus off-line detection may be used in some cases, with the latter frequently being encountered in work with low-performance affinity systems [12]. The mobile phase used in these studies has a known composition (usually a buffer with a physiological pH) and often contains a fixed concentration of an additive or

competing agent. It is by looking at how analyte retention changes with the mobile phase composition or other conditions (such as changes in temperature or solute structure) that information is obtained on the interactions between an analyte and affinity ligand.

A typical zonal elution experiment is shown in Fig. 3. Factors which can be altered in this work include: (1) the pH, ionic strength and polarity of the mobile phase; (2) temperature; (3) the type of solute or ligand in the column; and (4) the presence of displacing agents in the mobile phase. In Fig. 3, the change in retention of *cis*-clomiphene (the injected solute) is being examined on an immobilized HSA column as various amounts of *R*/*S*-warfarin are added to the mobile phase. In this case, the retention of clomiphene shifts to higher values as the warfarin concentration is increased, indicating that positive allosteric effects occur during the binding of these two solutes to HSA [32].

An important advantage of zonal elution is that it requires only a small amount of solute per injection (often in the pmol–nmol range). It is also possible to examine more than one compound per injection (e.g. a mixture of drug enantiomers), provided that adequate resolution can be obtained between the corresponding peaks. Another advantage of zonal elution is that it can easily be performed with standard HPLC equipment. The only modification required is the addition of temperature control for the column and mobile phase. If performed properly, changes in retention of only a few seconds can easily be detected by this approach [48].

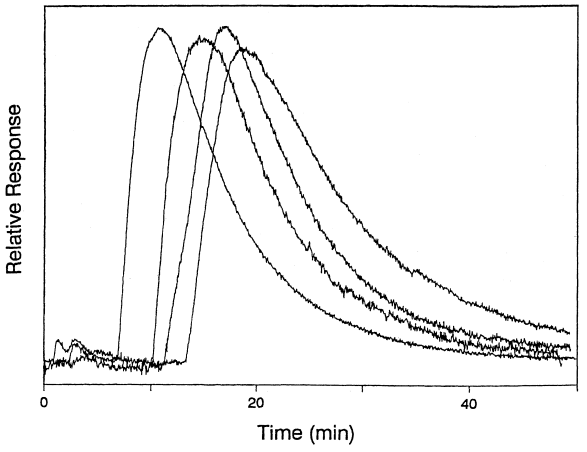



Fig. 3. Chromatograms obtained in a zonal elution study for the injection of *cis*-clomiphene into the presence of racemic warfarin as a mobile phase additive. The mobile phase concentrations of warfarin (from left-to-right) were 0, 0.5, 1.0 and 7.5 μ*M*. All of these studies were performed at 37°C in a pH 7.4, 0.067 *M* phosphate buffer. Reproduced with permission from Ref. [32].

### *2.2. Applications*

There are a variety of ways in which zonal elution has been used to obtain information on the binding of solutes to serum proteins. A list of these applications and examples from the literature can be found in Table 2. These include measurements of the degree and affinity of solute–protein binding, studies examining changes in binding with mobile phase composition or temperature, and experiments that consider how variations in solute or protein structure affect the interactions of these species. More details on each application will be provided later in this section.

Each of the applications in Table 2 relies on the fact that the retention observed for an injected analyte is a direct measure of that analyte's interactions within the column. This is described by Eq. (1), which shows how the analyte's overall retention factor ($k$) is related to the number of binding sites it has in the column and to the equilibrium constants for the analyte at these sites [7]:

$$k = (K_{A1}\, n_1 + \cdots K_{An}\, n_n)\, m_L / V_M \quad (1)$$

In this equation, the retention factor is calculated by using $k = (t_R - t_M)/t_M$ or $k = (V_R - V_M)/V_M$, where $t_R$ is the retention time of the injected compound, $V_R$ is the corresponding retention volume, $t_M$ is the column void time and $V_M$ is the void volume. Other terms in Eq. (1) include the total moles of analyte binding sites in the column ($m_L$), the association equilibrium constants for the analyte at the individual sites in this population ($K_{A1}$ through $K_{AN}$), and the fraction of each type of site in the column ($n_1$ through $n_n$). From this equation, it can be seen that a change in the strength of binding, the number of binding sites, or the relative distribution of these sites can result in a shift in analyte retention. It will be shown later how this shift can then be used in HPAC to provide both qualitative and quantitative

Table 2
Applications of zonal elution and HPAC in binding studies with serum proteins

| Application | Protein | Solutes [References] |
|---|---|---|
| Measurement of degree of binding | HSA | Benzodiazepines, coumarins, triazole derivatives [49]; mixtures of drugs [50]; various pharmaceuticals [51] |
| Competition and displacement studies | HSA | D/L-Thyronine and D/L-tryptophan (additive, bilirubin or caprylate) [44]; oxazepam, lorazepam and hemisuccinate derivatives (additive, *R*/*S*-warfarin) [30]; *R*/*S*-warfarin, phenylbutazone, tolbutamide, *R*/*S*-oxazepam hemisuccinate, ketoprofen A/B, suprofen A/B (additive, octanoic acid) [28]; ketorolac (additive, octanoic acid) [52]; *R*/*S*-ibuprofen, salicylate, *R*/*S*-oxazepam hemisuccinate, fluorouracil, diazepam, phenylbutazone and *R*/*S*-warfarin [53]; *R*-warfarin and L-tryptophan (additives, D-tryptophan [54], or L-thyroxine [15] and thyronine compounds [25]); diazepam (additives, diazepam and diclofenac [55], phenylbutazone [56], or *R*- and *S*-ketoprofen [57]); non-steroidal anti-inflammatory drugs (additives, warfarin, phenylbutazone, *R*-ibuprofen) [58]; *R*- and *S*-ibuprofen (additives, *R*- and *S*-ibuprofen) [59]; *cis*- and *trans*-clomiphene (additives, *cis*- and *trans*-clomiphene) [32,60]; digitoxin and acetyldigitoxin (additives, digitoxin and acetyldigitoxin) [32,48]; non-steroidal anti-inflammatory drugs and benzodiazepines (additives, phenylbutazone, *R*/*S*-ibuprofen and 2,3,5-triiodobenzoic acid) [61]; piroxicam (additives, phenylbutazone and diazepam) [62]; indomethacin, sulindak and diclofenac (additives, phenylbutazone and diazepam) [63] |
| | Miscellaneous albumins | Non-steroidal anti-inflammatory drugs and benzodiazepines (additives, phenylbutazone, *R*/*S*-ibuprofen and 2,3,5-triiodobenzoic acid) [61]. |
| Effects of reaction conditions on binding | HSA | D- and L-Tryptophan [27,34,54]; *R*- and *S*-warfarin [35]; D/L-dansyl amino acids [64–69]; calcium and magnesium [70,71]; active components in *Rhizoma chuanxiong* [72] |
| | BSA | *N*-benzoyl D/L-amino acids [42]; *N*-aroyl D/L-amino acids [42]; barbiturates [43]; nonaromatic carboxylic acids [73] |
| | AGP | Kynurenine, methylphenobarbital [23]; dihydropyranoimidazopyridines [74] |
| Creation of quantitative structure–retention relationships | HSA | 1,4-Benzodiazepines [29,45,77]; acyclovir esters [78,79]; indolocarbazole derivatives [80]; 2,3-substituted 3-hydroxypropionic acids [81] |
| | AGP | Basic drugs and antihistamines [82,83] |
| | Miscellaneous proteins | Various compounds [84,85] |
| Studies with modified proteins | HSA | *R*/*S*-Warfarin, D/L-tryptophan (reagent, *o*-nitrophenylsulfenyl chloride) [86,87]; *R*/*S*-oxazepam hemisuccinate (reagent, *p*-nitrophenyl acetate) [88]; (reagent, ethacyrnic acid) [89] |
| | BSA | Benzoin, warfarin (BSA fragments) [90,91] |

information on the interactions between a solute and an immobilized serum protein.

### 2.2.1. Estimation of relative binding

One way in which zonal elution has been employed in HPAC is to use this as a means to measure the average extent of binding of a drug to an immobilized protein. This is based on the fact that the retention factor, when measured at true equilibrium, is equal to the moles or fraction of an injected solute that is bound to the ligand ($b$) divided by the moles or fraction of solute which remains free in the surrounding mobile phase ($f$):

$$k = b/f \quad (2)$$

By using the fact that the bound plus free fractions must be equal to one, it is possible to rearrange Eq. (2) into the following form, which allows the relative

bound fraction of the solute to be calculated directly from its retention factor [49].

$$b = k/(1 + k) \quad (3)$$

An example of the results obtained with this approach are shown in Fig. 4, which compares the percent binding measured by HPAC with that determined by ultrafiltration for various coumarin compounds mixed with HSA. The same approach has been used with other classes of drugs, as shown in Table 2, and has been used with liquid chromatography–mass spectrometry (LC–MS) to allow the simultaneous determination of binding for several drugs in a mixture [50]. This technique has been shown to give a good correlation versus reference methods for compounds with medium-to-strong binding to HSA [49].

An item that must be kept in mind during these studies is that they are generally performed with only a small amount of solute. This creates a situation in which a large excess of protein is present and the relative degree of solute binding is independent of both the solute and protein concentration. The same type of measurement could, in theory, be used for drugs at concentrations that approach or exceed those of their binding proteins; however, in this situation the results will be dependent on both the solute and protein levels, making it essential to know or determine the amount of active protein in the column. Also, care must be exercised when dealing with drugs that have multisite binding on a protein, especially when these sites have different susceptibilities to loss of activity during immobilization. For instance, it has been reported that the warfarin–azapropazone and indole–benzodiazepine sites on HSA are not affected to the same degree by the immobilization of this protein [15,54]. Unless it can be ensured that such sites have equal levels of activities, the relative binding determined by this type of experiment should be viewed as only an approximation of the behavior that would be expected for the same protein in solution.

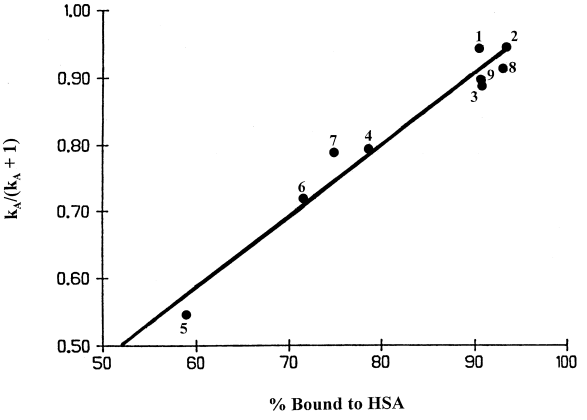



Fig. 4. Relationship between the binding predicted by retention measurements on an immobilized HSA column, $k/(k+1)$, and the binding measured by ultrafiltration for a series of coumarins with soluble HSA. The compounds studied in this experiment were as follows: (1) *R*-warfarin, (2) *S*-warfarin, (3) 7-hydroxycoumarin-4-acetic acid, (4) coumarin, (5) coumarin, (6) 4-hydroxycoumarin, (7) umbelliferone, (8) 4-trifluoromethylumbelliferone, and (9) 7-amino-4-methylcoumarin. Reproduced with permission from Ref. [49].

### *2.2.2. Competition and displacement studies*

The most common use for HPAC in the study of solute–protein systems has been as a tool to examine the competition and displacement of drugs from proteins by other solutes. This is performed by injecting one or more compounds as the analyte while a fixed concentration of a possible competing agent is passed through the column in the mobile phase. Several examples of such studies are given in Table 2. This is also the type of experiment illustrated in Fig. 3, in which *cis*-clomiphene was shown to have competition with racemic warfarin on HSA by looking at the shift in clomiphene retention as the mobile phase concentration of warfarin was varied.

It is relatively easy from such work to see whether or not two compounds interact as they bind to the same immobilized protein. But to obtain further information on this competition, such as the nature of the competition and the number of sites that are involved, it is necessary to compare the zonal elution data to the responses that would be expected for various types of interaction models. Various equations and models that have been developed for this are shown in Table 3.

Making a reciprocal plot of $1/k$ versus competing agent concentration is a useful place to begin in this analysis, since these plots provide only random variation in the case of non-competition (see Fig. 5a) and give a linear relationship with a positive slope for systems with direct competition at a single

Table 3
Relationships used to describe the retention of injected solutes during zonal elution

| Type of system [Reference] | Model | Predicted response |
|---|---|---|
| Self-competition of an injected analyte, *A*, with itself as a competing agent at a single type of binding site, *L* [46,59] | $A + L \overset{K_A}{\rightleftharpoons} A - L$ | $k_A = \frac{K_A m_L}{V_M(1 + K_A[\mathrm{A}])}$ or $1/k_A = \frac{V_M[\mathrm{A}]}{m_L} + \frac{V_M}{K_A m_L}$ |
| Self-competition of an injected analyte, *A*, with itself as a competing agent at two types of binding sites, $L_1$ and $L_2$ [46,55,59] | $A + L_1 \overset{K_{A1}}{\rightleftharpoons} A - L_1$<br>$A + L_2 \overset{K_{A2}}{\rightleftharpoons} A - L_2$ | $k_A = \frac{K_{A1} m_{L1}}{V_M(1 + K_{A1}[\mathrm{A}])} + \frac{K_{A2} m_{L2}}{V_M(1 + K_{A2}[\mathrm{A}])} = k_{\mathrm{Site1}} + k_{\mathrm{Site2}}$<br>or $1/(k_A - k_{\mathrm{Site2}}) = \frac{V_M[\mathrm{A}]}{m_{LI}} + \frac{V_M}{K_{A1} m_{L1}}$ |
| Direct competition of an injected analyte, *A* and competing agent, *I*, at a single common binding site, *L*, where *A* has no other binding sites and is present in a small amount versus *L* [105] | $A + L \overset{K_A}{\rightleftharpoons} A - L$<br>$I + L \overset{K_I}{\rightleftharpoons} A - L$ | $k_A = \frac{K_A m_L}{V_M(1 + K_I[\mathrm{I}])}$ or $1/k_A = \frac{V_M K_I[\mathrm{I}]}{K_A m_L} + \frac{V_M}{K_A m_L}$ |
| Direct competition of an injected analyte, *A*, and competing agent, *I*, at a single common binding site, *L*; *A* has one or more additional sites, $L_{N1} \ldots L_{Nn}$, that produce no competition, and *A* is present in a small amount versus *L* [28] | $A + L \overset{K_A}{\rightleftharpoons} A - L$<br>$I + L \overset{K_I}{\rightleftharpoons} I - L$<br>$A + L_{N1} \overset{K_{AN1}}{\rightleftharpoons} A - L_{N1}$<br>$A + L_{Nn} \overset{K_{ANn}}{\rightleftharpoons} A - L_{Nn}$ | $k_A = \frac{K_A m_L}{V_M(1 + K_I[\mathrm{I}])} + \frac{(K_{AN1} m_{LN1} + \cdots K_{ANn} m_{LNn})}{V_M} = k_{AL} + X$<br>or<br>$1/(k_A - X) = 1/k_{AL} = \frac{V_M K_I[\mathrm{I}]}{K_A m_L} + \frac{V_M}{K_A m_L}$ |

common binding site (Fig. 5b). In contrast to this, deviations from a linear response occur if allosteric competition or multisite interactions are present (Fig. 5c and d). If the injected analyte has some binding sites that are not being affected by the presence of the competing agent, then a related plot that can be used is one in which $1/(k - X)$ is plotted versus [I], where $X$ is a fitted value that represents the analyte retention due to sites of non-competition [28]. Other equations and models are used in specialized applications, such as when the same solute acts as both the analyte and mobile phase additive [46,55,59] or when a solubilizing agent is in the mobile phase [60].

### *2.2.3. Measurement of affinity and number of binding sites*

The next level of information that can be obtained by HPAC and zonal elution is quantitative data on the strength and number of solute–protein interactions (see Table 2 for examples). This is done by fitting the equations and models in Table 3 to the experimental data and then using the best-fit parameters to extract the binding constants for the system. As one example, the equations shown in Table 3 predict that a linear relationship should be seen when plotting $1/k$ versus [I] for a drug and competing agent with single-site competition on an immobilized protein. For instance, such behavior is seen when examining the competition of *trans*-clomiphene with phenol red on an HSA column, as shown in Fig. 5b. By determining the best-fit slope and intercept for this plot and taking ratio of the slope to intercept, the result should be the value of the association constant for the competing agent at the site of competition ($K_I$), as demonstrated in Eq. (4):

$$\mathrm{Slope/Intercept} = \frac{(V_M K_I / K_A m_L)}{(V_M / K_A m_L)} = K_I \tag{4}$$

In this particular situation, the value of $K_A$ can also be obtained by using the intercept and a separate measurement of $m_L/V_M$. A similar approach can be used with the other equations in Table 3 to examine cases where the analytes and additives have multiple sites of competition or the injected solutes have other binding sites which do not interact with the mobile phase additive.

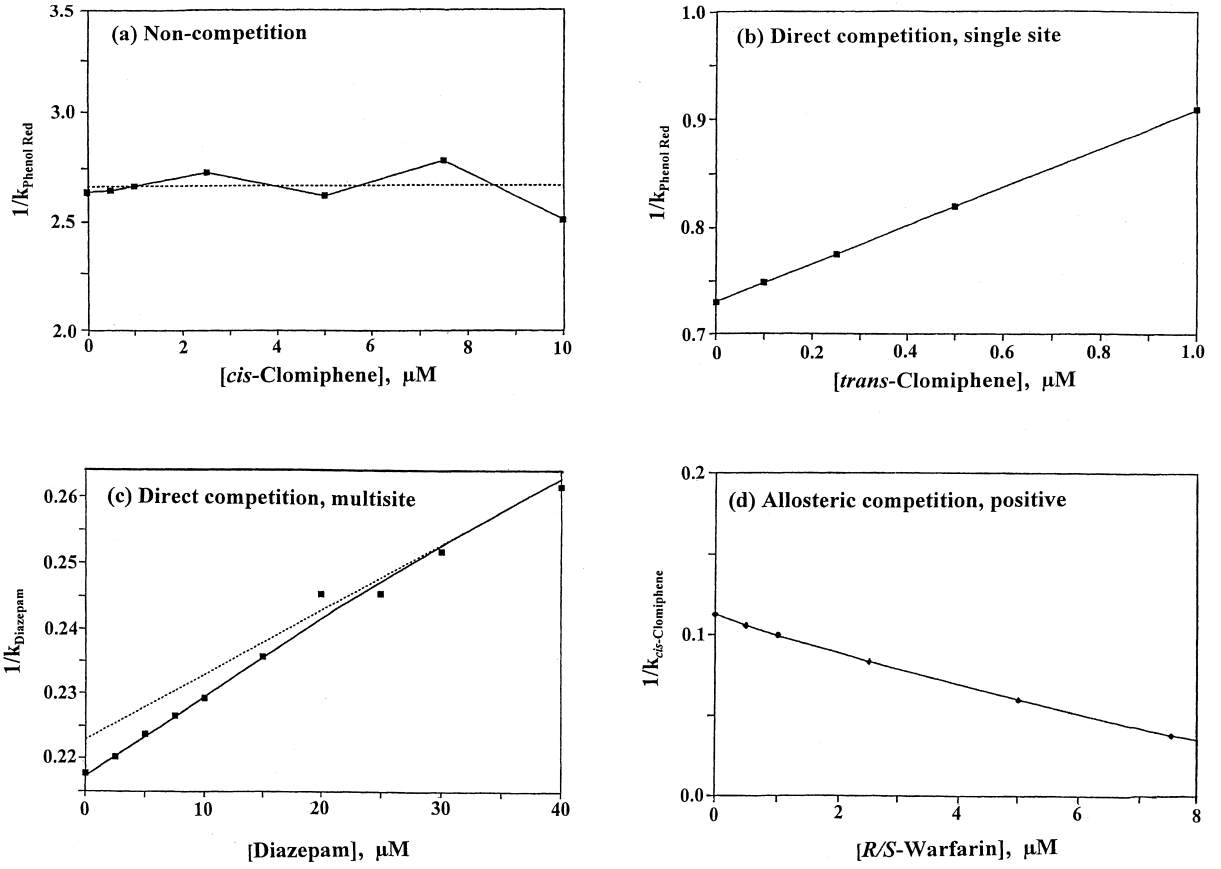



Fig. 5. Examples of reciprocal plots prepared for analyte and competing agents with various types of competition on immobilized HSA columns. These figures are based on results reported in Refs. [32] and [55].

Some advantages of using zonal elution for this type of work is that it is a relatively rapid approach, it requires only a small amount of analyte per run, and it allows for the possible study of multiple analytes per injection. In addition, the binding constants measured by this approach have good precision (typically 5–10%) and agree well with solution-phase values. Some association constants determined for HSA by zonal elution include the values shown for digitoxin, phenylbutazone and L-thyroxine in Table 1. The use of slope/intercept ratios in generating such numbers (as demonstrated in Eq. (4)) has the added benefit of giving binding constants that show little variation with changes in the protein content of an HPAC column. For instance, it has been shown that a long-term change in activity of 20% for an immobilized HSA support gave a random change of only ±2% in equilibrium constants that were measured by this strategy [15]. This provides a big advantage over the method described in Section 2.2.1 for measuring the relative degree of binding, where a consistent and high degree of protein activity was required to obtain accurate and reproducible results.

### *2.2.4. Solvent and temperature studies*

A third way in which zonal elution can be used to examine solute–protein interactions is to consider how changes in the reaction conditions affect these binding processes. Solvent effects can be studied by varying the pH, ionic strength or general content of the mobile phase. This is valuable in helping estimate the relative contributions made by various forces to the formation and stabilization of a solute–

protein complex. For instance, changing the pH can affect the interactions between a protein and solute by changing their net charges and coulombic interactions, or by changing the conformation of the protein at its binding regions. Serum proteins tend to have their most specific and strongest binding at or near pH 7.4; however, a small change in pH above or below this value can actually enhance their binding to charged solutes (e.g. as might be seen for a cationic compound as the pH is increased). An increase in ionic strength tends to decrease coulombic interactions through a shielding effect, but at the same time may cause an increase in non-polar solute adsorption. Adjusting the solvent's polarity by adding a small amount of organic modifier (e.g. a few percent of 1-propanol, 2-propanol or methanol) can alter solute–protein binding by disrupting non-polar interactions or causing a change in the protein's structure. This usually decreases the protein's binding to solutes, along with decreasing the width and tailing of the injected solute peaks [23,92].

An example of a solvent study is shown in Fig. 6, in which the effect of an increasing amount of organic modifier was used to examine the interactions of *R*- and *S*-warfarin with HSA. It was concluded in this case that even though *R*- and *S*-warfarin are known to have a common binding site on HSA, they were interacting at different locations within this site. *R*-Warfarin, which had an initial decrease in binding with the addition of a small amount of organic solvent, was proposed to interact within the hydrophobic interior, while *S*-warfarin, which had an initial increase in binding with the addition of modifier, was thought to bind more to the site's polar exterior [35]. Similar comparisons have been made to learn about the geometry and relative location of binding regions on HSA for D- and L-tryptophan and dansyl amino acids (see Table 2). 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.

Temperature is another factor that can be varied during zonal elution studies. An example is given in Fig. 7 for experiments examining the binding of D- and L-tryptophan to immobilized BSA. Besides providing qualitative data on the effects of temperature on binding, zonal elution can be used to determine some thermodynamic constants. For instance, the following equation can be used for a solute–ligand system with 1:1 binding:

$$\ln k = -(\Delta H/RT) + \Delta S/R + \ln(m_L/V_M) \quad (5)$$

where $T$ is the absolute temperature at which the retention factor is measured, $R$ is the ideal gas law constant, $\Delta H$ is the change in enthalpy for the reaction, $\Delta S$ is the change in entropy, and the other terms are as defined previously. Eq. (5) has frequently been employed in thermodynamic studies that involve zonal elution experiments [54,75,76,93–96] and in studies that consider how changes in tempera-

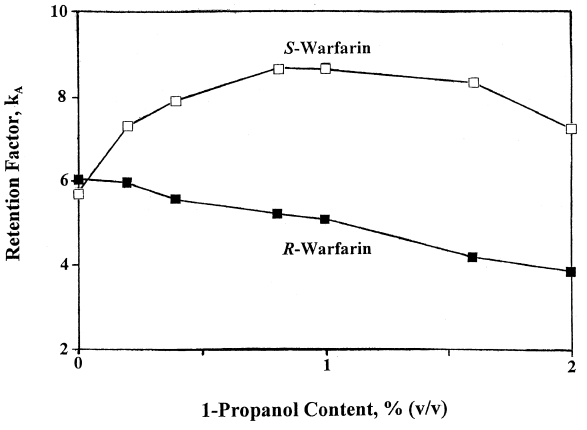



Fig. 6. Change in retention with the addition of 1-propanol to the mobile phase for the binding of *R*- and *S*-warfarin to immobilized HSA. Reproduced with permission from Ref. [35].

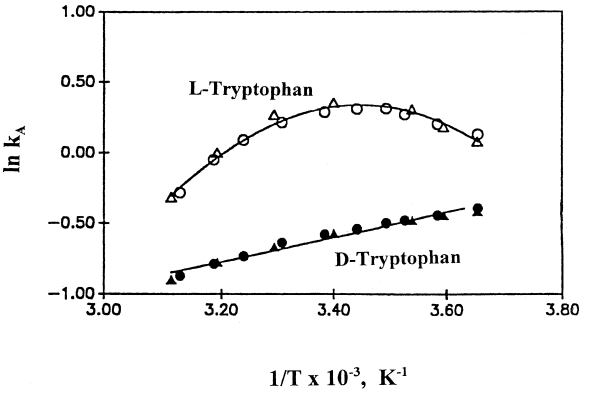



Fig. 7. Effects of temperature on the binding of D- and L-tryptophan to BSA. Reproduced with permission from Ref. [93].

ture affect the selectivity of protein columns [97,98]. For instance, the data shown in Fig. 7 for D-tryptophan supports a model in which this solute has a 1:1 binding mechanism with BSA over the temperature range shown, while the same conditions cause a change in the way that L-tryptophan binds to this protein [93]. If it is known that there is no temperature dependence in the number of binding sites ($m_L$), then the slope of a linear plot of $\ln k$ versus $1/T$ can also be used to determine the value of $\Delta H$ for a solute–protein system [34,54], as indicated by Eq. (5).

One precaution that needs to be exercised in interpreting the results of temperature and solvent studies is that the observed changes in retention may be due to variations in either the number of active sites on the protein or in the degree of solute binding at these sites. For instance, it has been shown that changing temperature, pH, or organic modifier content of the mobile phase can cause changes in the retention factors for L-tryptophan on immobilized HSA that are related to alterations in both the association constants and number of sites for this analyte on the column. However, for D-tryptophan the change in retention seen under identical conditions is related only to a change in its association constant; a similar observation has been made for both D- and L-tryptophan when varying the concentration of phosphate buffer in the mobile phase [27].

### *2.2.5. Determining the location and structure of binding sites*

Yet another application of zonal elution is to use this to determine the location and structure of binding regions on a protein. One way this can be done is through competition and displacement studies. For instance, if it is known in advance where one particular agent interacts with a protein, then competition studies with this agent can be used to determine whether other compounds bind to this same region. This has been used in many of the competitive binding studies listed in Table 2, including work reported with HSA and other albumins in their binding to non-steroidal anti-inflammatory drugs [58,61], *R*- and *S*-ibuprofen [59], *cis*- and *trans*-clomiphene [60], digitoxin or acetyldigitoxin [48], and benzodiazepines [61]. 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 [32,48,58–61]. It is even possible to generate maps that show the relationship between the binding regions on a ligand. An example is shown in Fig. 8 based on competition studies performed between various probes for the major and minor binding regions of HSA [32].

Another approach for learning about binding sites is to study how changes in the structure of a solute or protein affects their interactions. This is the principle behind the use of zonal elution to develop quantita-

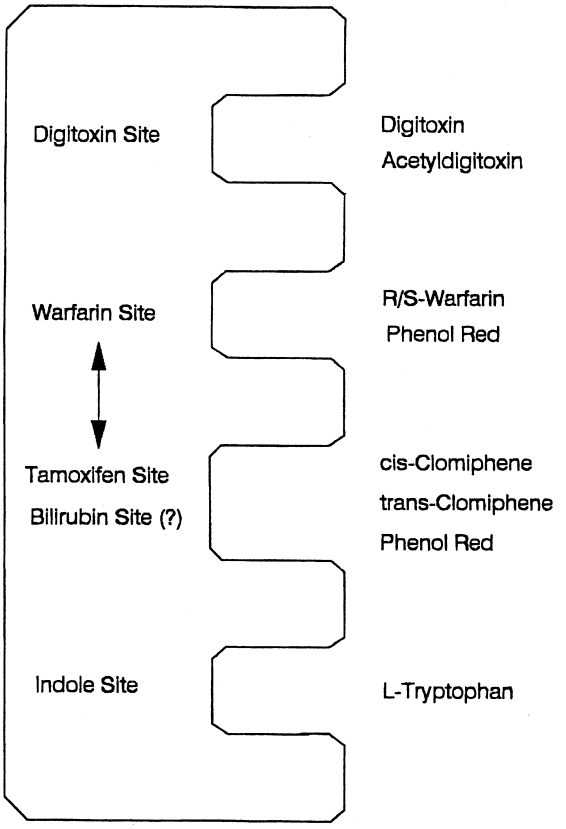



Fig. 8. Relative arrangement of the major and minor binding sites of HSA based on competition studies between various probe compounds. The compounds which were examined in this work are shown on the right and their binding regions are shown to the left. The arrow between the warfarin and tamoxifen sites indicates the presence of allosteric effects between these two regions. Reproduced with permission from Ref. [32].

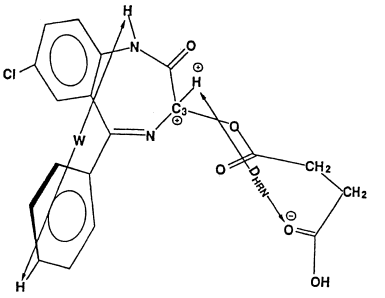
tive structure–retention relationships (QSRRs) that describe the binding of drugs and their analogs to protein columns. This involves measuring retention factors for a large set of structurally related compounds under constant temperature and mobile phase conditions. The resulting data are then compared to several factors that describe various structural features of the solutes. This is done to determine which of these factors are most important in the retention and binding of the tested compounds. From this data, information can be obtained on the forces involved in the drug–protein binding and an approximate description can be developed for the sites that are involved in these interactions [99].

An example of such a study is shown in Fig. 9, which examined the binding of immobilized HSA to benzodiazepines [45]. A general structure for this group of compounds is shown at the top of Fig. 9 along with some parameters that were used to help describe the retention of these substances. The structural parameters used in this study included the width of the molecule along the length of its phenyl group ($W$) and the excess charge on the $C_3$ carbon ($C(3)$). Other terms that were employed included $f_{X+Y}$, which is the sum of the hydrophobic constants for the two substituents on the phenyl rings (represented here by the hydrogen and chlorine to the left of Fig. 9a), and a submolecular polarity parameter ($P_{SM}$), which was determined by using the distance across the compound ($D_{HRN}$) and the excess charge on the hydrogen attached to $C_3$ ($\Delta$), where $P_{SM} = \Delta \cdot D_{HRN}$. By testing different weighting factors for each of these parameters, the following best-fit relationships were generated for a group of benzodiazepines with two chiral forms, $P$ and $M$:

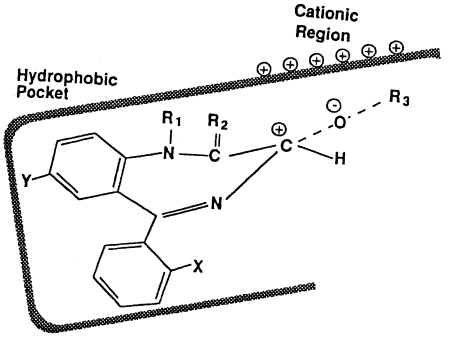
$$\log k_P = 2.4790 + 1.834 f_{X+Y} - 0.2779W \quad (6)$$

$$\log k_M = 0.5558 + 0.8354 P_{SM} + 0.3645 f_{X+Y} - 2.6904 C(3) \quad (7)$$

Based on the different parameters and weighting factors used in Eqs. (6) and (7), it was possible to determine some features of the binding sites for these agents on HSA. An example of this binding site model is given in Fig. 9b for the $M$-conformation. A similar model, but lacking the cationic region in the binding sites, was developed for the $P$-conformation [45].




(a) Structural Descriptors used for Benzodiazepines




(b) Binding Pocket Model ($M$-Conformation of Benzodiazepines)

Fig. 9. Models used in QSRR studies to describe (a) benzodiazepine structure and (b) interactions of the $M$-conformation of such compounds with HSA. The structural descriptor $W$ represents the width of the molecule between the two indicated hydrogens, and $D_{HRN}$ is the distance between the indicated hydrogen and oxygen atoms. Reproduced with permission from Ref. [45].

A complementary approach to QSRRs is to utilize zonal elution to investigate how solute retention changes as alterations are made to its binding sites on a protein. An example is work that involved the acetylation of HSA with *p*-nitrophenyl acetate, a reagent thought to mainly modify the Tyr-411 residue of HSA, which is 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 [88]. 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 [87]. 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 [89] and the use of BSA fragments in the chiral separation of benzoin and other drugs [90,91].

### *2.3. Practical considerations*

Although zonal elution is relatively easy to perform, the proper use of this method does require that several variables and experimental factors be considered. Examples include the need for an accurate measurement of analyte retention, the correct choice of mobile phase additive concentrations, and the use of an appropriate sample size. These and other items will be considered in more detail in the following section.

#### *2.3.1. Determination of analyte retention*

A requirement for any type of zonal elution experiment is that the analyte must have reproducible and observable retention on the column. As pointed out earlier, this retention will be related to the equilibrium constants for solute–ligand binding and the moles of active binding sites within the column. Although the amount of protein in a standard HPAC column may only be in the nmol–μmol range, the relatively strong binding that occurs in many solute–protein systems can result in quite high retention factors during zonal elution experiments. Having large retention factors makes it easy to detect small shifts in retention, but it also can lead to long analysis times. One way to reduce the retention time for strongly retained compounds is to simply increase the flow-rate; however, care must be taken to ensure that this does not affect the solute's retention factor (see discussion in next paragraph). Another option is to change the protein content or size of the column, with a reduction in either of these items leading to a proportional decrease in the retention time. The use of mobile phase additives can also be used to adjust retention, but caution must again be taken with this option to avoid altering the nature of the solute–protein interaction that is being studied.

One assumption made in most zonal elution studies is that the center of an analyte's peak represents a point of local equilibrium within the column. This assumption should be tested for each new analyte by performing a few zonal elution studies at several flow-rates. If the assumption is not valid, then a shift in retention factor should result, indicating that a slower flow-rate and/or a longer column should be used in the experiment.

To obtain an accurate measure of retention, it is recommended that the true center of a solute's peak be employed. It is important to note that this is not the same as the peak maximum, since many solutes on immobilized protein columns produce tailing peaks under physiological conditions (e.g. see the chromatograms in Fig. 3). This tailing occurs even under linear elution conditions and is a result of relatively slow association and dissociation kinetics. For chromatograms that are collected by a computer, the position of an analyte's true retention time can be determined by calculating the central moment of its peak ($t_R$):

$$t_R = \frac{(\Delta t)^2 \cdot \sum(i \cdot h_i)}{\Delta t \cdot \sum(h_i)} \quad (8)$$

where $\Delta t$ is the difference in time between each data point in the peak, and $h_i$ is the signal for data point $i$ after correcting for the background response [12]. An alternative approach is to use one of several empirical expressions that allow $t_R$ to be calculated by using the elution time at the peak maximum, the width of the peak at a specific height (e.g. the width at half the peak maximum), and the peak's asymmetry factor (see Refs. [100] and [101]).

#### *2.3.2. Choice of additive concentrations*

When examining binding constants or changes in retention due to a mobile phase additive, it is necessary to have a shift in analyte retention that can easily be measured. The conditions needed for this can be selected by considering the change in the retention factor $k$ as it moves between its maximum and minimum values, $k_{max}$ and $k_{min}$. In a system

where the injected analyte and additive have direct competition at a single type of site, the following relationship describes the relative shift in retention, $(k - k_{min})/(k_{max} - k_{min})$, that should be observed at various additive concentrations:

$$\frac{(k - k_{min})}{(k_{max} - k_{min})} = \frac{1}{(1 + K_I[I])} \quad (9)$$

Note that the size of this shift depends only on the association constant for the competing agent ($K_I$) and this agent's concentration in the mobile phase ([I]). Another way of viewing this ratio is to look at it as the average fraction of binding sites which remain unoccupied and able to bind to the analyte as it contacts the stationary phase. Based on this model, the analyte's retention factor should be equal to $k_{max}$ when the concentration of competing agent is zero, but will approach $k_{min}$ as [I] approaches infinity.

Fig. 10 shows how Eq. (9) can be used to determine the optimum range of competing agent concentrations for zonal elution. For a minimum detectable shift in retention of 10%, the competing agent concentrations which can be used are those that provide values for $K_I[I]$ between 0.1 and 9.0 (i.e. conditions in which [I] is equal to 0.1–9 times $1/K_I$). For a minimum detectable shift of 5%, competing agents which give $K_I[I]$ values of approximately 0.05 to 19 can be used. For more precise retention measurements an even greater range of competing agent levels may be employed.

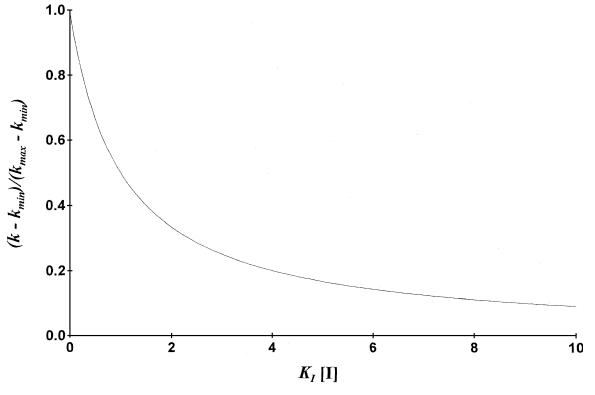



Fig. 10. Relative shift in analyte mobility, $(k - k_{min})/(k_{max} - k_{min})$, as a function of competing agent concentration for a zonal elution experiment in which there is direct competition between *A* and *I* at a single site on an immobilized ligand.

Although Fig. 10 shows the range of competing agent concentrations which could ideally be used, other factors may prevent this full range from being tested. For instance, the competing agent may have a limited solubility in the mobile phase or it may have a high background signal which prevents its use at high concentrations. In some cases it is possible to switch to a different competing agent that has a lower signal or to one with better solubility. Another means of increasing solubility is to add an organic modifier or change the pH of the mobile phase, but this can also affect the binding between the immobilized protein and the injected analyte or competing agent. A third alternative is to employ a solubilizing agent [48,60]. β-Cyclodextrin is especially useful for this purpose since it is known to bind to many small, non-polar compounds, and yet it has no measurable binding to HSA. This agent also gives little or no background signal with UV–Vis absorbance detection. One disadvantage of using a solubilizing agent is that it does complicate the data analysis during zonal elution studies, since both the concentration of the solubilizing and competing agents must be varied to obtain binding constant information. Also, the binding of an analyte to a solubilizing agent produces lower retention, which will decrease the range of retention factors that can be used during the study [48].

Since many zonal elution studies involve the use of several different mobile phases, it is crucial to ensure that the chromatographic system has been equilibrated with each new solvent before any final measurements are made of retention. For this reason, it is always recommended that several analyte injections be made under each set of conditions to ensure that reproducible results are being obtained. When changing the pH, buffer composition or organic modifier content of the mobile phase, washing the column with 10–20 void volumes of a new solvent is usually sufficient to establish a new equilibrium in the system. However, an even larger volume may be needed when altering a competing agent's concentration. As will be discussed in Section 3.3., the volume required for this latter case will be determined by the amount of binding sites in the column, the affinity of the competing agent for these sites, and the concentration of the applied competing agent.

*2.3.3. Selection of sample size*

Even though it is possible to perform work under non-linear conditions through the use of computer modeling and chromatographic theory [46,47], all of the equations shown in Table 3 assume that the amount of applied analyte is small compared to the amount of active ligand in the column. This creates a small problem with immobilized protein columns because these usually have a much lower sample capacity than more traditional columns, like those used for reversed-phase chromatography. Fortunately, it is easy to test for linear elution conditions by injecting the analyte at several concentrations and seeing if the measured retention factor is a consistent value. An example of such an experiment is shown in Fig. 11 for the injection of D- and L-tryptophan on an HSA column [34]. As can be seen in this figure, the sample size dependence of the retention factor can vary significantly between two different solutes on the same protein column. Thus, this needs to be examined on a case-by-case basis. However, even solutes with large sample size effects tend to give satisfactory results with sample loads of 0.01–0.2% versus the total column binding capacity (in Fig. 11, $10^{-10}$–$10^{-11}$ mol of L-tryptophan for a column with 50–100 nmol of sites).

Two other factors to consider when selecting sample concentrations for zonal elution studies are the solubility and detectability of the analyte. Solubility places an upper limit on the practical amount of analyte which can be applied with each injection. For most compounds, work in the micromolar range, as is often needed to obtain linear elution conditions, provides sufficient solubility for injection. However, this can be difficult to accomplish when working with non-polar compounds that are only sparingly soluble in aqueous buffer. One possibility is to use a small amount of organic modifier in the sample solvent. But caution must be followed in doing this since this may create background peaks that make it difficult to examine solute retention, or it may cause shifts in retention due to differences between the mobile phase and injection solvent. Another possibility (as discussed previously) is to add a solubilizing agent like a cyclodextrin to the injection solvent and/or mobile phase. Although this greatly expands the types of compounds that can be examined in zonal elution experiments, it does add an extra variable to the study (i.e. the solubilizing agent's concentration) that must be considered in obtaining binding constant information.

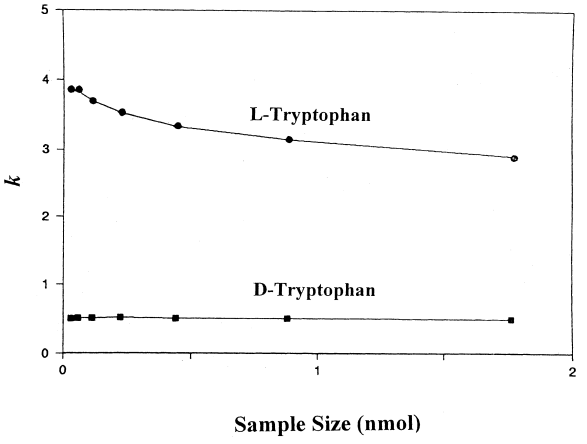



Fig. 11. Effect of sample size on the retention factors measured for D- and L-tryptophan on an immobilized HSA column. The column size was 10 cm×4.1 mm I.D. and contained 500 nmol HSA/g silica. Reproduced with permission from Ref. [54].

The detectability of the analyte will depend on the type of detection scheme which is being employed, the properties of the analyte, and the background signal that is present due to the mobile phase. Detection in zonal elution studies is most often accomplished by using UV–Vis absorbance detection, but other approaches like LC–MS have also been employed [33]. Using sample concentrations in the micromolar range is compatible with most of these methods. If an additive is in the mobile phase which also gives a response to the detector, then an increase in background signal will be seen as greater amounts of this additive are used. This may result in greater noise and give a lower signal-to-noise ratio for the analyte, requiring the use of higher sample concentrations for detection. Also, a change in the mobile phase's composition (e.g. its pH or organic modifier content) may increase or decrease the detector response of the analyte by changing its physiochemical properties. Thus, detectability of the analyte should be evaluated in each zonal elution study by using the full range of mobile phases that are to be examined.

If the analyte cannot be placed onto the column at a level suitable for detection, it might be possible to use a labeled analog of the analyte which can be

monitored at lower concentrations than the analyte itself. An example is work by Lagercrantz et al. [40], in which radiolabels were used in early zonal elution studies to examine the binding of various solutes to low-performance albumin columns. Alternative labels, such as those based on fluorescent tags, might also be used. But precautions must be taken in this approach to ensure that the labeled analogs are adequately mimicking the binding behavior that would be expected for the analyte of interest.

#### *2.3.4. Other considerations*

There are a number of additional items which need to be considered during zonal elution studies. For instance, it is essential to control and report the pH and composition of the mobile phase and samples (including buffer type and concentration, as well as the use of organic modifiers or other additives), since even small changes in the content of these solvents can alter the degree of solute–ligand binding which is observed. In addition, the temperature should be reported and controlled by using a column heater or a column jacket attached to a circulating water bath. For high accuracy work, the temperature of the mobile phases and samples should be adjusted so that they are at or near the desired temperature in the column; this helps avoid the presence of local temperature gradients across the column during the zonal elution studies.

Other items which should be reported for zonal elution studies are the size and type of column which was used. This includes the length and diameter of the column, the type of support within the column, the type of ligand present, and how this ligand was immobilized. Ideally, the total protein content of the column should also be provided, along with the amount of this protein that was actually active. Such information is useful in comparing the results of different studies and in selecting the conditions for a zonal elution experiment or in troubleshooting such a system.

Yet another experimental factor which needs to be reported and monitored is the back-pressure of the chromatographic system. Although very high pressures are needed to affect protein structure, recent studies have shown that at least some solute–protein systems do have slight changes in binding near the upper pressure limits of HPLC systems [102]. To test for such effects, some initial zonal elution studies should be performed at various flow-rates and back-pressures to identify conditions in which minimal variations in retention are present. The acceptable operating range for analytes which have been tested so far (i.e. D- and L-tryptophan) appears to be a back-pressure of less than a few hundred bar. However, this range is dependent on the solute/protein system and should be considered separately for each new zonal elution experiment [102].

## 3. Frontal analysis

The second most common method in HPAC for studying solute–protein binding is frontal analysis. This differs from zonal elution in that it involves the continuous, rather than plug-type, application of an analyte to a column. The result is essentially a titration of the number of active binding sites within the column [7,12].

Frontal analysis was first employed in the investigation of solute–ligand binding in 1975 by Kasai and Ishiii [103], who used this method with low-performance affinity columns. In 1978 low-performance columns were used by Nakano et al. [104] to study the binding of immobilized BSA with salicylate, and in 1979 Lagercrantz et al. [40] employed a similar method to examine the binding of salicylate with HSA. Finally, Loun and Hage [15] reported the use of frontal analysis and HPAC as a means to characterize the binding of immobilized HSA for various solutes.

The following section discusses the underlying principles of frontal analysis and various applications that have been reported for this method in the area of drug–protein interactions. As was done for zonal elution, a number of practical factors will also be discussed that need to be considered in obtaining the best results from this method.

### *3.1. General basis of method*

In frontal analysis, a solution containing a known concentration of solute is continuously applied to a column that contains an immobilized ligand. As the solute binds to this ligand, the column becomes

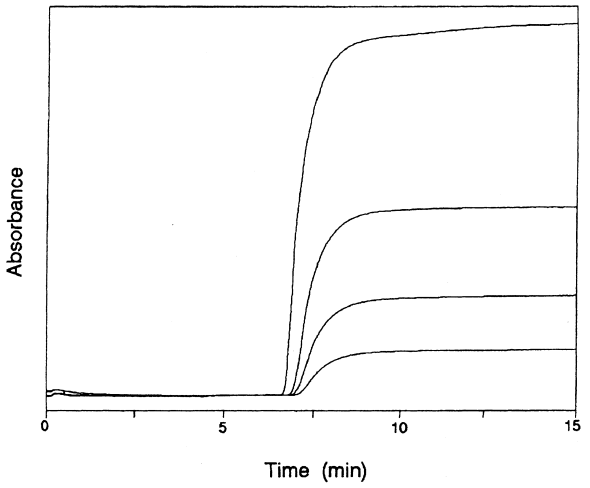



Fig. 12. Typical frontal analysis experiment performed with L-tryptophan on an immobilized HSA column. The concentrations of applied L-tryptophan (from left-to-right) were 100, 50, 25 and 12.5 μ*M*. The flow-rate was 0.25 ml/min and the column void time was 3.6 min. Reproduced with permission from Ref. [34].

saturated and the amount of solute eluting from the column gradually increases, forming a characteristic breakthrough curve (see Fig. 12). If fast association and dissociation kinetics are present in the system, the mean position of the breakthrough curve 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. As will be shown later, this provides a means for quantitating the active ligand in a column and the affinity of this ligand for the applied solute.

An example of frontal analysis experiment is shown in Fig. 12, where the binding of L-tryptophan (the applied analyte) is being examined on an immobilized HSA column as various concentrations of L-tryptophan are passed through this column. As can be seen in this example, the use of a higher concentration of L-tryptophan results in a faster saturation of HSA and produces a breakthrough curve that appears at faster elution times. The position of this curve is then examined as the analyte's applied concentration is changed, allowing the affinity and number of binding sites in the column to be determined [34]. By varying the temperature, type of immobilized ligand, and solvent that is passing through the column, this same type of study can be used to help characterize the nature and types of interactions that are occurring between the analyte and ligand in the column.

Like zonal elution, frontal analysis can easily be performed with standard HPLC equipment, with the addition of temperature control for the column and mobile phases and data handling routines for measuring the elution time of breakthrough curves. Although frontal analysis does require more analyte than zonal elution studies, it also provides more information per study. Its main advantage, as will be seen in the next section, is the ability to separately measure both the equilibrium constants and number of binding sites within a column. This makes this approach valuable in characterizing the properties of a column and in obtaining careful measurements of binding affinity and activity.

### *3.2. Applications*

Like zonal elution, frontal analysis can be used to provide a variety of information regarding a solute–protein system. As shown in Table 4, applications that have been reported include the use of this method to determine the affinity and number of binding sites a solute has in a column, the type of binding that is taking present in this column (e.g. single site or multisite), the effects of temperature or solvent on this binding, and the effect of using a competing agent or different type of ligand for the study. Each of these applications is described in this section.

#### *3.2.1. Measurement of affinity and number of binding sites*

The main application of frontal analysis has been to provide quantitative data on the affinity and moles of active ligand in a column. This is done by measuring the breakthrough times for a solute at several solute concentrations and then fitting the resulting data to equations that are based on various reaction models. Some examples are shown in Fig. 13. Double-reciprocal plots are particularly useful for this purpose, where $1/m_{Lapp}$ (i.e. the apparent moles of analyte that are required to saturate the column) is plotted versus 1/[A] (i.e. the inverse of the applied analyte's concentration). According to the equations given in Table 5, the result of this type of plot should

Table 4
Applications of frontal analysis and HPAC in binding studies with serum proteins

| Application | Protein | Solutes [References] |
|---|---|---|
| Measurement of binding constants | HSA | *R*- or *S*-Warfarin [15,35,108]; D- and L-tryptophan [15,27,34,54]; salicylic acid, warfarin, phenylbutazone, mefenamic acid, sulphamethizole and sulphonylureas [105] |
| | BSA | Salicylate [104]; mandelic acid, tryptophan, 2-phenylbutyric acid and *N*-benzoylalanine [109] |
| | Miscellaneous albumins | *R*-warfarin [108] |
| Effects of reaction conditions on binding | HSA | *R*- and *S*-Warfarin [35]; D- and L-tryptophan [34] |
| Competition and displacement studies | HSA | Sulphamethizole (additive, salicylic acid) [106] |
| | BSA | Salicylate (additives, clofibric acid, octanoic acid and oestradiol) [107] |
| Studies with modified proteins | HSA | *R*/*S*-Warfarin, D/L-tryptophan (reagent, *o*-nitrophenylsulfenyl chloride) [87]; salicylic acid, warfarin, phenylbutazone, mefenamic acid, sulphamethizole, sulphonylureas (dimeric HSA) [105] |

be a linear relationship if the analyte has a single type of binding site on the immobilized ligand. If more than one type of binding site is present, then negative deviations will be seen in this plot at high analyte levels (i.e. low values for $1/[A]$) [108].

Once it has been determined which reaction model best describes a solute–protein system, the affinity and number of binding sites in the column for the solute can be determined from the best-fit parameters for the experimental data. For instance, the equations given in Table 5 for a system with single site binding predict that a plot of $1/m_{Lapp}$ versus $1/[A]$ will give a linear response with a slope equal to $1/(K_A\, m_L)$ and an intercept of $1/m_L$. The total binding capacity of a column that fits this model can then be obtained from the inverse of the intercept, while the association constant for binding can be found by dividing the intercept by the slope. A similar approach, but using a combination of both non-linear and linear fits, can be used for more complex systems that involve multisite interactions [108]. If desired, equivalent information can also be obtained by analyzing frontal analysis data with Scatchard plots [104–107].

The main advantage of frontal analysis over zonal elution is 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 makes it the method of choice when information is needed on the binding capacity of a column. Frontal analysis is also the preferred technique for highly accurate association constant measurements, since the values that it provides for $K_A$ can be determined independently from the column binding capacity [35]. Examples of systems in Table 1 which have been studied by frontal analysis include the binding of L-tryptophan, *R*-warfarin and *S*-warfarin to HSA [15,35]. As shown in this table, the results obtained by this approach have good precision and show excellent agreement with solution-phase values.

### *3.2.2. Solvent and temperature studies*

It was mentioned for zonal elution that caution must always be used in interpreting solvent and temperature studies performed by this approach, since the retention shifts that are observed may be due to alterations in either the affinity or number of binding sites. But this is not an issue in frontal analysis, since data on both affinity and binding site activity are provided in the same experiment. For instance, Fig. 14 shows how frontal analysis was used to determine the change in $m_L$ and $K_A$ for *R*- and *S*-warfarin at various temperatures on an immobilized HSA column. Based on this information, it was possible to predict how these analytes would behave during zonal elution. Frontal analysis has also been used to examine the binding and separation of D- and L-tryptophan on immobilized HSA under a variety of temperatures and mobile phase conditions, many of which affect both the strength and number of binding sites for these agents [34].

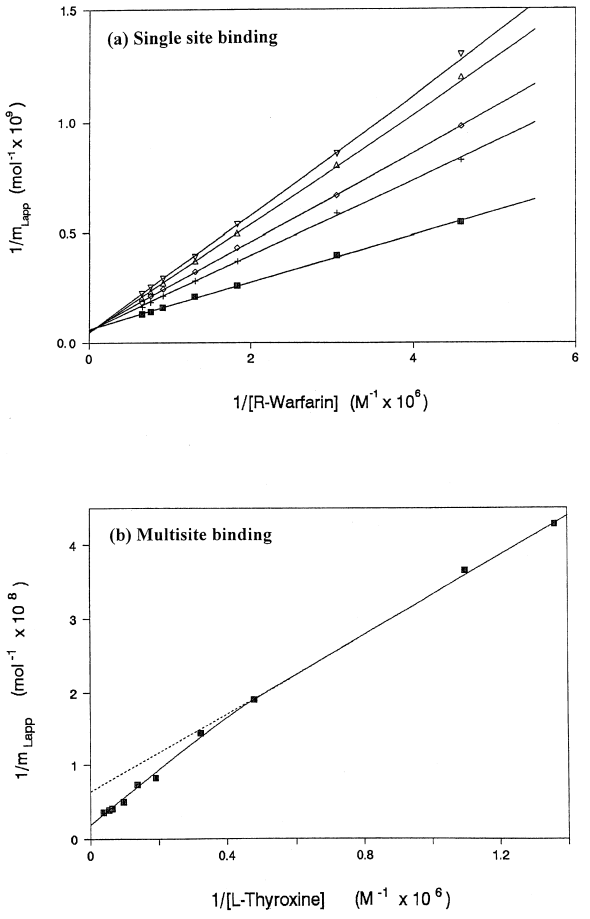



Fig. 13. Examples of double-reciprocal frontal analysis plots for systems with (a) single site binding and (b) multisite binding. Reproduced with permission from Refs. [35] and [108].

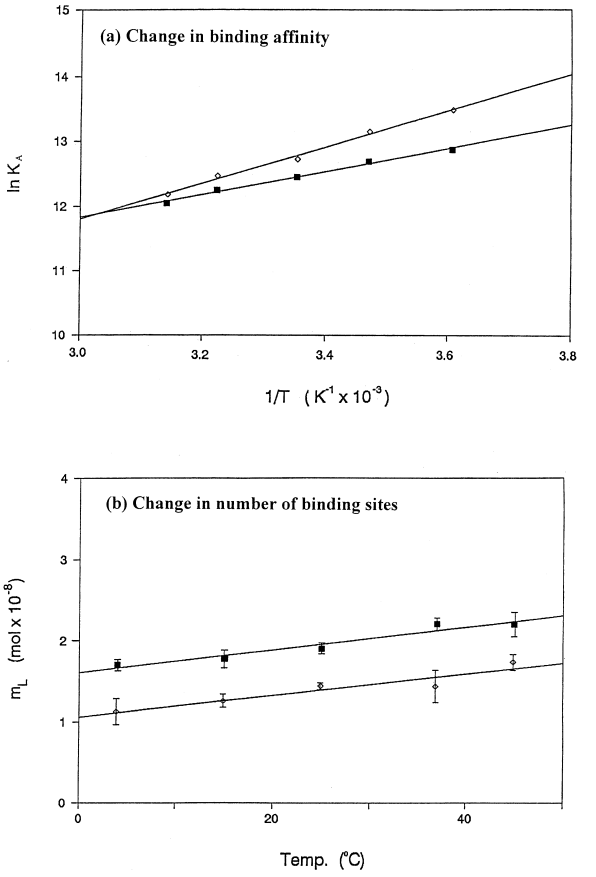



Fig. 14. Use of frontal analysis to examine the change in binding affinity and number of binding sites for *R*-warfarin (■) and *S*-warfarin (◇) on an immobilized HSA column as a function of temperature. Reproduced with permission from Ref. [35].

Table 5
Examples of relationships used to fit frontal analysis data

| Type of system [Reference] | Model | Predicted response |
|---|---|---|
| Binding of analyte, *A*, at a single type of site, *L* [15] | $A + L \overset{K_A}{\rightleftharpoons} A - L$ | $m_{Lapp} = \dfrac{m_L K_A[\mathrm{A}]}{(1 + K_A[\mathrm{A}])}$ or $1/m_{Lapp} = \dfrac{1}{K_A m_L[\mathrm{A}]} + \dfrac{1}{m_L}$ |
| Binding of analyte, *A*, at two independent sites, $L_1$ and $L_2$ [108] | $A + L_1 \overset{K_{A1}}{\rightleftharpoons} A - L_1$ | $k_A = \dfrac{m_{L1} K_{A1}[\mathrm{A}]}{(1 + K_{A1}[\mathrm{A}])} + \dfrac{m_{L2} K_{A2}[\mathrm{A}]}{(1 + K_{A2}[A])}$ |
| | $A + L_2 \overset{K_{A2}}{\rightleftharpoons} A - L_2$ | $1/m_{Lapp} = \dfrac{1 + K_{A1}[\mathrm{A}] + \beta_2 K_{A1}[\mathrm{A}] + \beta_2 K_{A1}^2[\mathrm{A}]^2}{m_L\{(\alpha_1 + \beta_2 - \alpha_1\beta_2) K_{A1}[\mathrm{A}] + \beta_2 K_{A1}^2[\mathrm{A}]^2\}}$ |
| | | where: $\alpha_1 = m_{L1}/m_L$ $\beta_2 = K_{A2}/K_{A1}$ |

### 3.2.3. Competition and displacement studies

A third application of frontal analysis has been as a tool to examine the competition between solutes for sites on an immobilized protein. This has been utilized to examine the competition of sulphamethizole with salicylic acid for HSA [105], and salicylate with clofibric acid, octanoic acid or oestradiol for sites on BSA [106]. This type of experiment 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. Direct competition between the analyte and competing agent leads to a smaller breakthrough time for the analyte as the competing agent's level 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.

### 3.2.4. Studies with modified proteins

Like zonal elution, frontal analysis has been used to examine the binding of solutes to modified proteins. In one case, frontal analysis was used to compare the binding capacities for monomeric versus dimeric HSA in their interactions with various solutes [105]. In addition, this approach has been used to examine HSA that had been reacted with *o*-nitrophenylsulfenyl chloride at Trp-214. In these latter studies, it was determined that this modification did not change the moles of warfarin–azapropazone sites on HSA, but it did lower the association constant of this protein for *R*-warfarin (see Fig. 15). Similar studies with L-tryptophan indicated that the moles of indole–benzodiazepine sites were not affected; however, an allosteric decrease in affinity was detected on the modified HSA [87].

## 3.3. Practical considerations

As was shown for zonal elution, frontal analysis has a number of factors that need to be considered and optimized for the proper use of this method. Some of these items include the correct determination of breakthrough times and the selection of appropriate analyte concentrations. These and other factors will be examined in this section.

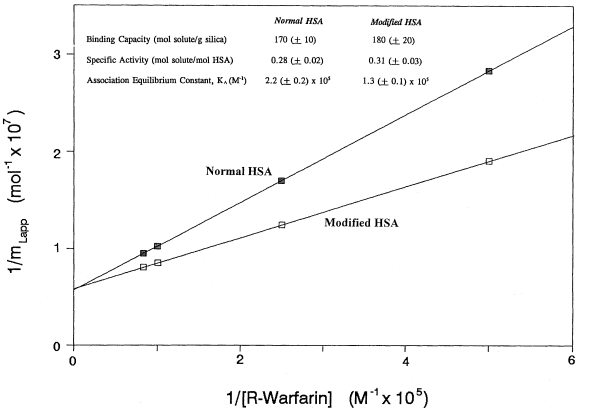



Fig. 15. Use of frontal analysis to examine the change in binding affinity and number of binding sites for *R*-warfarin on normal and modified HSA. Reproduced with permission from Ref. [87].

### 3.3.1. Determination of breakthrough times

The determination of binding capacities and association constants by frontal analysis requires that careful measurements be made of the analyte's average breakthrough time or volume through the column. For a symmetric breakthrough curve, this will be equal to the point which is half-way between the baseline and upper plateau. However, most such curves are not symmetric, so an alternative way for determining this position is needed. One approach is to integrate below the front portion of the curve and above the latter part until a point is reached at which these two areas are equal, as shown in Fig. 16. This is the equivalent of converting the frontal analysis

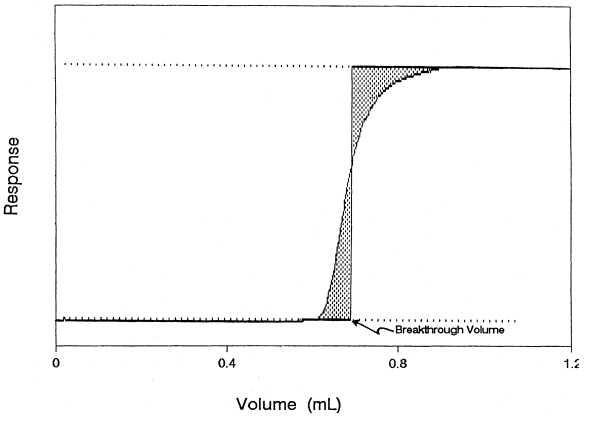



Fig. 16. Determination of the breakthrough volume for L-tryptophan applied to an immobilized HSA column.

data into a step function, where the mean breakthrough time corresponds to the point at which the step function changes its value. Another method is to take the first-derivative of the breakthrough curve and determine the central moment of this derivative; the central moment in this second approach can be obtained by using the same equations and techniques that were discussed in Section 2.3.1 for zonal elution.

In order to use the equations in Table 5 for binding constant measurements, it is assumed that the mean point of a breakthrough curve represents a point of equilibrium between the analyte and ligand in the column. As was seen for zonal elution, the validity of this assumption can be tested by performing frontal analysis studies at several flow-rates and seeing if consistent breakthrough volumes are obtained. If a shift in this volume is noted, then a longer column or a slower flow-rate is needed to provide more time for the analyte to bind within the column. The same type of study can be employed to determine whether or not the breakthrough volume is independent of the back-pressure across the column (see Section 2.3.4).

When acquiring data for a frontal analysis experiment, it is best to have a well-defined difference between the void time of the column and beginning of the breakthrough curve. This allows a stable baseline to be obtained at the beginning of the curve that can then be used to determine this curve's true center (see Fig. 16). It is also necessary to make sure that the analyte is applied for a sufficient amount of time to reach and form the upper plateau of the breakthrough curve, which is often used as a second reference point in determining the curve's central position. This generally involves applying an analyte solution 1.5–2 times the mean breakthrough time, although for some systems smaller or larger amounts of this solution may be required.

### *3.3.2. Choice of analyte concentrations*

The main variable in frontal analysis studies of solute–protein interactions is the concentration of analyte that is applied to the column. A key factor in choosing this concentration is the size of the analyte's equilibrium constant for its binding to the column. This is demonstrated in Eq. (10), where the fraction of active column sites that are bound to the analyte ($m_{Lapp}/m_L$) is shown to be dependent only on the applied analyte's concentration and the association constant for the analyte at these sites:

$$m_{Lapp}/m_L = K_A[\mathrm{A}]/(1 + K_A[\mathrm{A}]) \quad (10)$$

As indicated in Fig. 17, the value of $m_{Lapp}/m_L$ that is obtained with Eq. (10) will be equal to zero when no analyte is applied and will approach one as the analyte concentration approaches infinity. Between these two extremes is a range of intermediate concentrations in which the ratio of $m_{Lapp}/m_L$ has a detectable change in value, which translates into a shift in the mean position of the resulting breakthrough curve. For a minimum shift of $\pm 10\%$ (i.e. 10–90% of the binding sites are occupied), the analyte concentrations needed to produce this must provide a value for the product $K_A[\mathrm{A}]$ that is equal to roughly 0.1–10 (i.e. [A] is equal to 0.1–10 times $1/K_A$). Similarly, for at least a 5% shift in the breakthrough curve's mean position (i.e. a binding range of 5–95%), the value of $K_A[\mathrm{A}]$ should be in the range of 0.05–20.

Detectability and solubility are other issues that must be considered when choosing analyte conditions for frontal analysis. In this case, the applied solutions of the analyte should all provide a measurable increase in signal versus the baseline, and the maximum change in signal should have a linear dependence on analyte concentration. Whether this is

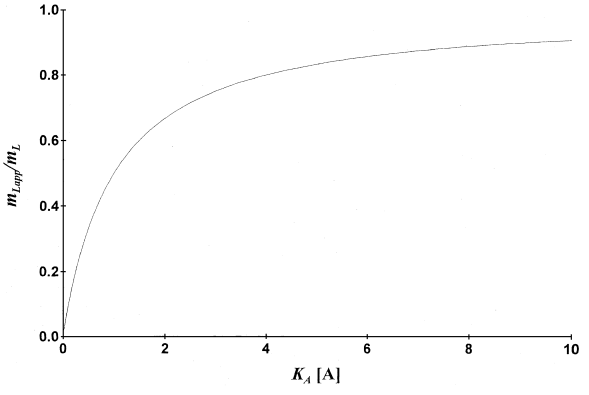



Fig. 17. Change in the degree of column saturation, $m_{Lapp}/m_{L,}$ versus the product of applied analyte concentration and the analyte's association constant, $K_A[\mathrm{A}]$, for frontal analysis performed on a system that has single site binding.

the actual case for a given solute can be determined by passing the selected analyte solutions through the chromatographic system when no column is present, with the resulting signal then being examined to see if it is both measurable and proportional to the analyte's concentration. If analyte solubility is an issue, it is possible that an additive could be placed into the mobile phase to overcome this problem (see previous discussion in Section 2.3.3). However, additional studies will then be needed to see if the presence of the additive changes the binding between the analyte and ligand.

Once an appropriate range of analyte concentrations has been identified, it is necessary to select other conditions that will help make any shifts in binding easier to observe. Factors that can be adjusted for this are the size of the column, the amount of ligand in the column, and the application flow-rate. This can be demonstrated with a modified version of Eq. (10) (as shown below), which indicates how the difference in time between the mean position of the breakthrough curve ($t_{\text{Mean}}$) and the void time will vary with flow-rate ($F$), column size and protein content ($m_L$):

$$(t_{\text{Mean}} - t_M) = \frac{m_L K_A[\text{A}]}{F[\text{A}](1 + K_A[\text{A}])} \tag{11}$$

As can be seen from Eq. (11), the difference ($t_{\text{Mean}} - t_M$) will increase by using a column with more immobilized protein or by using a slower flow-rate for analyte application. The downside to this is that using more protein will require the use of more moles of analyte per study, and an increase in column size or a decrease in flow-rate will increase the time needed for each experiment.

#### *3.3.3. Other considerations*

Along with analyte concentrations, other factors that should be reported and considered in frontal analysis include the pH and composition of the mobile phase and temperature that was used during the study. The flow-rates that were employed and the back-pressures that were generated across the column should also be given. And finally, the size of the column, the support it contained, the column's total protein content, and the means by which this protein was immobilized should be described. As mentioned previously, this information is useful in comparing data from different studies and in troubleshooting HPAC experiments.

## 4. Other methods

The main goal in the approaches discussed up to this point has been to examine solute–ligand interactions under equilibrium conditions. But HPAC can also be employed as a means to study the kinetics of these processes. Two examples of methods which allow this information to be obtained are band-broadening studies and free fraction measurements.

### *4.1. Band-broadening studies*

This approach makes use of peak width or band-broadening measurements that are made during zonal elution experiments. This is accomplished by injecting substances at several different flow-rates while determining their corresponding widths and plate height values. This information is then used to prepare van Deemter-type plots of plate height versus flow-rate or linear velocity. These studies are performed both on the HPAC column of interest and on an identical inert control column that contains the same type of support but no immobilized protein. Work on this second column is used to determine the plate height contributions due to processes in the column other than analyte–stationary interactions. Finally, the plate height contribution made by the stationary phase interactions are then calculated by taking the difference between the total plate height measured for the HPAC column and the plate height measured on the control column for the other non-stationary phase processes.

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. (12):

$$H_s = \frac{2uk}{k_d(1 + k)^2} \tag{12}$$

where $u$ is the linear velocity of mobile phase in the column, $k$ is the retention factor of the injected

solute, $H_s$ 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. (12), a plot of $H_s$ versus $uk/(1+k)^2$ should give a slope of $2/k_d$ and an intercept of zero. 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 [26,27].

An example of a band-broadening study is shown in Fig. 18 for injections of *R*-warfarin onto immobilized HSA [26]. As can be seen from Fig. 18b, this system gave good agreement with the behavior predicted by Eq. (12). The same types of studies have been performed with *S*-warfarin [26], D-tryptophan and L-tryptophan [27] on immobilized HSA columns. From these experiments it has been possible to obtain measurements of the association and dissociation rates for these solutes with HPAC columns. The resulting data have provided useful insights into the energetics of these binding processes and on how they are affected by changes in temperature or solvent. For instance, Fig. 19 shows the combined results of frontal analysis and band-broadening measurements for the interactions of D- and L-tryptophan with HSA. Based on these data, it was possible to show that the change in binding affinity with pH for these solutes was the result of alterations in both their association and dissociation rates. The same types of experiments have been used to examine how these rates are affected by the changing buffer composition, temperature, and organic modifier content of the mobile phase [26,27].

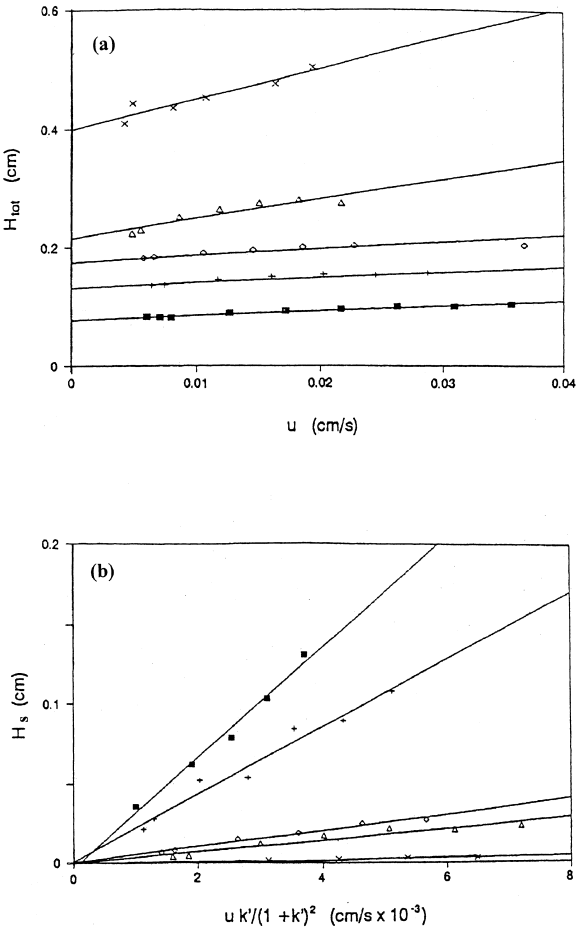



Fig. 18. (a) Total plate height ($H_{tot}$) versus linear velocity ($u$) for the injection of *R*-warfarin onto an immobilized HSA column, and (b) the plate height contribution due to stationary phase mass transfer ($H_s$) versus $[uk/(1+k)^2]$ for the same system. The individual lines in each plot represent data obtained at 4 (■), 15 (+), 25 (◇), 37 (△) and 45°C (×). Reproduced with permission from Ref. [26].

### *4.2. Free fraction analysis*

All of the HPAC methods that have been discussed up to this point have involved the use of immobilized serum proteins. But it is also possible to use HPAC to examine the binding of drugs and other solutes with serum proteins that are still in solution. This has recently been accomplished by using HPAC columns that are designed for rapid immunoextraction. 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. An example of a column that has been used in this work is shown in Fig. 20a, in which a small layer of an immobilized antibody support is placed between two layers of another inert material, such as diol-bonded silica [110]. These columns have been prepared with a thickness for the affinity layer as small as 60 μm and can provide residence times in the millisecond range at standard HPLC flow-rates. In addition, these columns have been used in immunoextraction to quantitatively bind

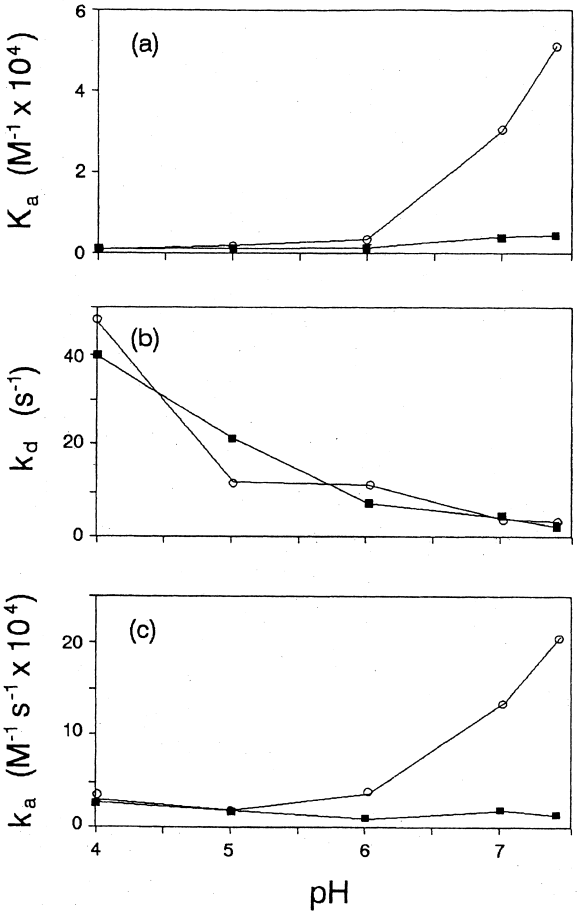



Fig. 19. Change in (a) the association equilibrium constant, $K_a$; (b) the first-order dissociation rate constant, $k_d$; and (c) the second-order association rate constant, $k_a$, for the interactions of D-tryptophan (■) and L-tryptophan (○) with immobilized HSA at various pH values. Reproduced with permission from Ref. [27].

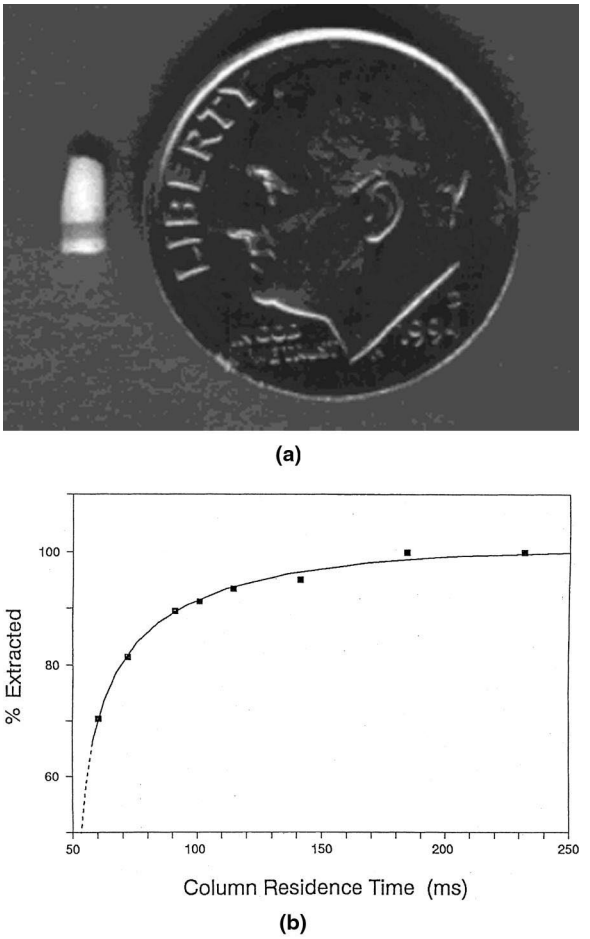



Fig. 20. (Top) Example of a sandwich microcolumn used for free drug and hormone analysis and (bottom) the use of such a column with immobilized antibodies for the extraction of fluorescein on the millisecond time scale. The active layer of the sandwich microcolumn is represented by the dark layer located in the middle of this column. Reproduced with permission from Ref. [110].

several small solutes in times as low as 80–120 ms (see Fig. 20b).

Based on previous kinetic studies of drug–protein systems, it is known that many of these systems have dissociation rates that occur on the order of a few seconds. Thus, by rapidly passing a drug–protein mixture through an immunoaffinity microcolumn for a particular drug, it should be possible to quickly isolate the portion of 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.

This approach has already been demonstrated by using the binding of *R*- and *S*-warfarin to HSA as a model system [111]. In this case, anti-warfarin antibodies were used in an immunoaffinity microcolumn and warfarin/HSA samples were injected onto this column with only 180 ms being allowed for extraction. Under these conditions, it was predicted that the amount of extracted warfarin should have very little interference from any warfarin that was initially bound to HSA and later released as the

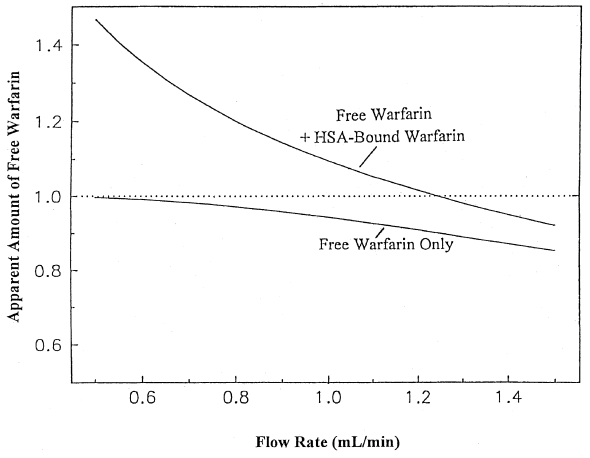



Fig. 21. Calculated results for the extraction of free *R*-warfarin from warfarin–HSA mixtures by a sandwich microcolumn that contains anti-warfarin antibodies. The upper line shows the total amount of warfarin which would be expected to bind to the column, including both initial free fraction plus HSA-bound warfarin which is released while passing through column. The bottom lines shown the amount of warfarin which is extracted when it is injected in the absence of any binding proteins in the sample. Reproduced with permission from Ref. [111].

sample passed through the immunoaffinity support (see Fig. 21). The amount of free warfarin in each sample was then determined by using fluorescence measurements to examine the amount of warfarin which eluted non-retained and comparing this to the total amount of warfarin in the original sample. The free fractions measured by this approach showed good agreement with values predicted from the known composition of the sample and the equilibrium constants for HSA in its binding to *R*- and *S*-warfarin. The advantage of this approach is that it is extremely fast and uses serum proteins which are in solution instead of immobilized to a solid support. Studies are now being performed to expand the use of this method to alternative systems for the analysis of other free drug fractions.

## 5. Conclusions

In this review a variety of methods have been discussed that can be used to study solute interactions with serum proteins by HPAC. Particular attention was given to the techniques of zonal elution and frontal analysis, which are the most popular formats for this type of work. Various applications for these methods were described, including their use to determine the extent of solute–protein binding, the number of sites involved in these interactions, the equilibrium constants for these processes, the ability of a solute to be displaced by other compounds, the effects of temperature or solvent composition on these reactions, and the structure and location of the binding sites on an immobilized ligand. Numerous practical issues were discussed for the design and use of these methods, such as the concentration of analyte or competing agents that should be used, and factors that affect the accurate measurement of analyte retention. Some newer approaches to the study of reaction rates in solute–protein systems were described as well, including methods based on band-broadening measurements and rapid immunoextraction. The variety of ways in which HPAC can be used and the wealth of information it can provide have resulted in this being utilized in a rapidly increasing number of applications. It is expected that this trend will continue as HPAC becomes even more common in clinical and pharmaceutical research as a means for studying the interactions of drugs, hormones and other solutes with serum proteins.

## 6. Nomenclature

| | |
|---|---|
| AGP | $\alpha_1$-acid glycoprotein |
| BSA | bovine serum albumin |
| HPAC | high-performance affinity chromatography |
| HSA | human serum albumin |
| QSRRs | quantitative structure–retention relationships |

## Acknowledgements

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