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Review

Chromatographic and electrophoretic studies of protein binding to chiral solutes

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Abstract

Protein interactions are important in determining the transport, metabolism and/or activity of many chiral compounds within the body. This review examines data that have been obtained on these interactions by various chromatographic and electrophoretic methods, especially those based on either high-performance liquid chromatography or capillary electrophoresis. Zonal elution, frontal analysis and vacancy methods are each considered, as are approaches that employ either soluble or immobilized proteins. There are a variety of different items that can be learned about a solute–protein system through these techniques. This includes information on the binding constants and number of binding sites for a solute–protein system, as well as the thermodynamic parameters, rate constants, interaction forces and binding site structure for the protein and solute. Numerous examples are provided throughout this review, as taken from the literature and from work performed within the author's laboratory. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

1.1. Significance of protein interactions with chiral solutes

The interactions of small molecules with proteins is an important process in determining the activity and eventual fate of many compounds within the body. Examples include the binding of a hormone or drug with a target receptor to produce a given biological effect. These interactions are also important in determining the ability of enzymes to metabolize drugs and the ability of blood proteins to adsorb and transport small solutes throughout the body. In some cases this binding occurs with general ligands, such as the interaction of many drugs with blood transport proteins like human serum albumin (HSA) or α_1-acid glycoprotein (AGP). In other situations these interactions are highly specific, such as the binding of substrates to enzymes or of a hormone to its receptor [1–7].

Since all proteins are inherently chiral, it should come as no surprise that their interactions with chiral solutes often show stereoselectivity [7]. This topic has been of particular interest in recent years as agencies such as the US Food and Drug Administration have begun to increase rules and regulations involving the marketing and use of chiral drugs [8]. This has also resulted in increased research aimed at resolving such agents, some work of which has involved the use of proteins as chiral ligands in chromatographic or electrophoretic methods [9–13].

Along with the increased need for better separations of chiral compounds has arisen the necessity for obtaining more fundamental information on how such agents bind to proteins. This review will examine various chromatographic and electrophoretic methods that have been developed for such studies. A survey of the various approaches that can be used for solute–protein binding studies will be presented, with an emphasis being placed on techniques that employ high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE). The types of information that can be obtained from such methods will be examined, and some typical results of these methods will be given to illustrate how these approaches have been used to study protein interactions with chiral solutes.

1.2. General description of solute–protein binding

The binding of a protein to a small solute, regardless of whether or not this substance is chiral, is usually described by the reactions shown in Eqs.

(1)–(4). In this model, S represents the solute of interest, L_1 through L_n are the binding regions on the protein for this solute, and $S–L_1$, $S–L_2$, etc., are the resulting solute–protein complexes:

$$S + L_1 \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} S–L_1 \quad (1)$$

$$K_{a1} = \frac{k_{a1}}{k_{d1}} = \frac{[S–L_1]}{[S][L_1]} \quad (2)$$

$$S + L_n \underset{k_{dn}}{\overset{k_{an}}{\rightleftharpoons}} S–L_n \quad (3)$$

$$K_{an} = \frac{k_{an}}{k_{dn}} = \frac{[S–L_n]}{[S][L_n]} \quad (4)$$

In these reactions [] represents the molar concentration of each species in solution, k_{a1} through k_{an} are the second-order association rate constants for solute–protein binding, k_{d1} through k_{dn} are the first-order dissociation rate constants, and K_{a1} through K_{an} are the association equilibrium constants for the solute at each of its binding regions on the protein.

The above reaction scheme assumes that the individual binding regions on the protein have constant and independent affinities for the solute. Such an assumption is only true when allosteric interactions are not present; if this is not the case, then more a complex reaction model must be used. Another assumption in these equations is that the binding of the solute to each region on a protein can be described by a single-step, reversible process. In actuality, this process probably involves multiple steps, such as the diffusion of solute to the protein and changes in the protein's conformation as a result of its binding to the solute [14–16]. However, even in a multi-step process the model in Eqs. (1)–(4) can still provide a useful approximation of the net reaction which takes place between a small solute and a protein.

The variety of functional groups that are available on proteins allows many different types of processes to play a role in stabilizing a solute–protein complex. Examples of these processes include non-polar, dipole or coulombic interactions, as well as hydrogen bonding and steric effects. The combination of these various factors can lead to either the specific or general binding of solutes to a protein. These same forces, combined with the well-defined three dimensional structure of proteins, makes it possible for proteins to discriminate between the different chiral forms of a solute.

2. Separation techniques for the study of solute–protein interactions

Liquid chromatography and electrophoresis are two approaches that have received particular attention in recent years as techniques for examining solute–protein interactions. In some of these methods both soluble proteins and compounds are used, while in others the protein or solute is immobilized onto a solid support. The following section summarizes various methods for binding studies that use HPLC or CE (see Refs. [14] and [17–35] for general reviews). Although some of these methods have not yet been employed with chiral solutes, all could potentially be used to examine the interactions of such substances with proteins or other ligands.

2.1. Chromatographic methods based on soluble proteins

There are several chromatographic methods that can be used to directly analyze the binding of solutes and proteins in solution. These have been discussed in several past reviews dealing with both low-performance [17,18] and high-performance chromatographic supports [14,18,19]. Most of these are based on columns that contain a size-exclusion or internal surface reversed-phase (ISRP) support; both types of columns provide a means for resolving low- to intermediate-molecular-mass solutes from proteins or solute–protein complexes.

2.1.1. Zonal elution

The traditional chromatographic method of zonal elution (i.e., the injection of a small sample band onto a column) is the first approach that can be used to study solute–protein interactions in solution. One way of performing this is to mix the protein and drug or solute of interest in solution, allow this mixture to equilibrate, and then inject it onto a column for separation of the free and protein-bound fractions of the solute. This *direct separation method* requires

little or no dissociation of the protein-bound fraction during the separation process, so most drug–protein systems (which generally have reasonably fast association and dissociation kinetics) are not amenable to this approach. However, it has been used to study the binding of a few steroids, such as prednisolone and cortisol, to corticosteroid-binding globulin [36–38].

For solutes that have intermediate dissociation rates from proteins, a related method that can be used is that of *peak-splitting measurements* performed on an ISRP column. In this approach, the protein and solute–protein complex are excluded from the reversed-phase sites located only within the pores of this column. However, the non-bound fraction of a small solute will be able to enter the pores and access these reversed-phase sites, leading to retention. If the rate of solute–protein dissociation is comparable to the time of this separation, it is possible to get two solute peaks from a single injection. The solute peak which elutes last from the column represents the solute that was originally free in the sample or that was quickly released from low affinity proteins. The other peak corresponds to solute which was initially bound tightly to proteins but later dissociated as the sample traveled through the column. This technique has been employed with several systems that have intermediate binding affinities, such as mixtures of bovine serum albumin (BSA) with warfarin [39,40], and human serum incubated with phenytoin [41,42] or imirestat [41].

Another way in which zonal elution can be used to study solute–protein systems in solution is to employ the protein as a mobile phase additive while injecting small samples of solute into the column. When this is done for a low- or intermediate-molecular-mass solute on a size-exclusion column, the binding of the protein to the solute should result in a shift in the solute's retention time to lower values. This is performed both in the absence of protein and in presence of two more protein concentrations to determine the net binding constant for the solute–protein system (also known as the global association constant, K'_a). Such a technique has been used to examine the binding of HSA to warfarin, phenylbutazone, furosemide [43], tryptophan [44,45] and omeprazole [45]. One of its advantages it that it can be employed to study the individual chiral forms of a racemic solute mixture if these forms have significantly different protein binding properties [44,45].

2.1.2. Frontal analysis

Frontal analysis is another chromatographic technique that can be used to examine solute–protein binding in solution [14,17–19]. This is performed by applying to the column a large volume sample that contains both the solute and protein of interest. If the column has different retention times for the solute and the solute–protein complex, and the solute–protein mixture is at a local equilibrium within the column, then the resulting chromatogram should consist of several plateau regions (see Fig. 1). These regions represent the non-complexed protein, the solute–protein complex and the free solute fraction. By using the height of this last region and the known total concentration of the solute, the relative amount of solute that is bound per protein can be calculated. If this process is repeated at several solute concentrations, it is possible to determine the binding constants for the solute and protein. Applications of this technique in HPLC-based methods (a method known as high-performance frontal analysis, or

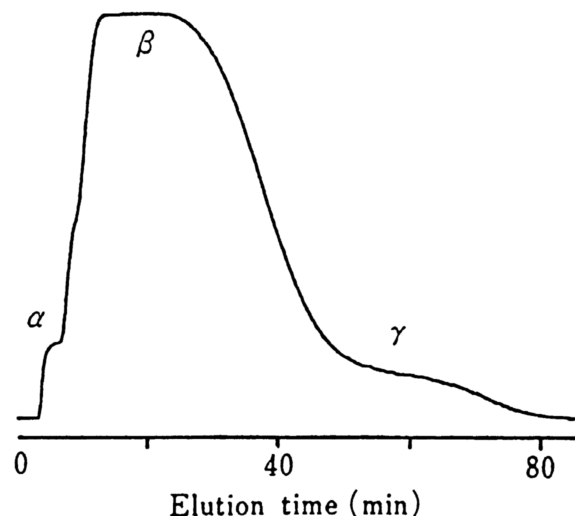

Fig. 1. Frontal analysis experiment in HPLC for examining the binding of BSA to racemic warfarin. The three bands shown in this profile (from left-to-right) represent non-complexed protein, the solute–protein complex and the non-complexed solute, respectively. Reproduced with permission from Ref. [46].

HPFA) have included its use to examine the binding of BSA to warfarin and indometacin [46], and the interactions of HSA with warfarin [47–49], diazepam [50], carbamazepine [51], troglitazone [52] and fenoprofen [53]. This method has also been used in combination with chiral HPLC columns for the separation and quantitation of individual chiral species in a given plateau region, as has been demonstrated in work examining the stereoselective binding of proteins to fenoprofen [53], warfarin [54], ketoprofen [55], nilvadipine [56], BOF-4272 [57,58] and semotiadil/levosemotiadil [59,60].

2.1.3. Vacancy techniques

A third group of chromatographic methods for solute–protein studies are based on the constant application of a mobile phase containing the protein and solute, or only the solute, of interest. As this mobile phase elutes from the column, the concentrations of these additives are monitored with an appropriate detection scheme. An injection is then made of a sample that lacks one or more of these additives. As this sample passes through the column, the local equilibrium between the solute and protein is perturbed, resulting in the formation of one or more troughs, or vacancy peaks. These troughs represent a depletion of some mobile phase component in the region of the peak and, under the correct circumstances, can be used to help determine the amount of free versus protein-bound solute that is present in the system at equilibrium.

The most common vacancy technique is the *Hummel–Dreyer method* [14,19,61]. This approach employs a size-exclusion, ISRP or ion-exchange column in the presence of a mobile phase that contains a fixed, known concentration of the solute of interest. An injection of a small amount of protein is made into the presence of this mobile phase (see Fig. 2). If the protein and solute have rapid association/dissociation kinetics and the column gives different retention times for the solute–protein complex versus free solute, then a chromatogram should result in which the first, positive peak represents the eluting protein and its associated bound solute fraction. The second negative peak appears at the retention time expected for the free solute. Since this peak is produced by binding of sample protein with the solute in the mobile phase, the area of this peak

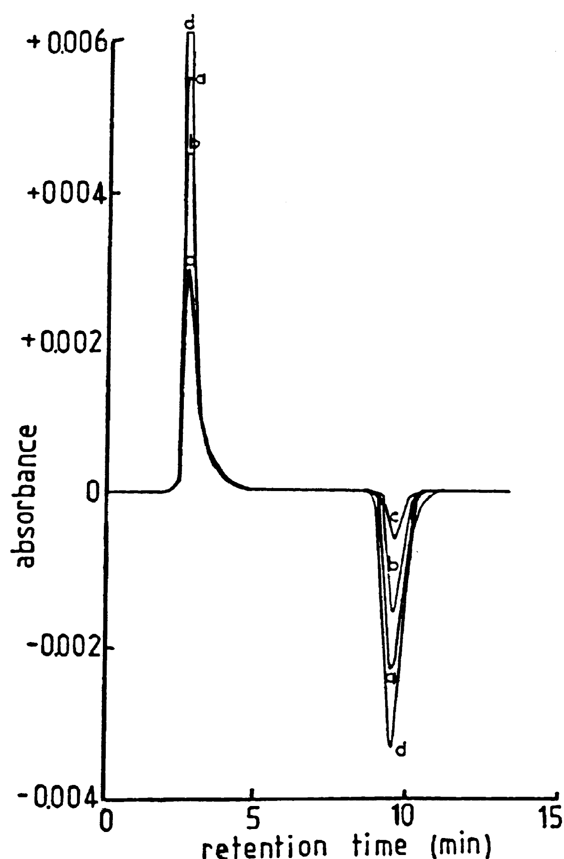

Fig. 2. Hummel–Dreyer experiment examining the binding of racemic warfarin to HSA in the presence of several different competing agents (a–d). The first peak represents protein-bound warfarin, while the second peak represents the non-complexed form of warfarin. Reproduced with permission from Ref. [63].

can be used to help quantitate the amount of bound solute. This information can then be used to obtain the binding constants and number of binding sites for the solute–protein interaction. The Hummel–Dreyer method has been employed in the study of a large number of solute–protein systems [14,17,19]. Some examples are the binding of HSA with warfarin [48,49,62,63], furosemide [48], diazepam [50], *R*/*S*-isradipine [64] or propanolol [65]; the binding of BSA with warfarin [62] or L-tryptophan [66]; the interactions of AGP with *R*/*S*-isradipine [64] or propanolol [65,67]; the binding of tubulin with colchicine [68]; and the binding of low-density lipoproteins to propanolol [65].

A technique that is closely related to the Hummel–

Dreyer method is the *equilibrium saturation method*, or vacancy peak method. This is again performed with a size-exclusion column or related support that can resolve the solute and solute–protein complex of interest. However, in this method both the solute and protein are used as mobile phase additives and injections are made of a sample containing only the mobile phase buffer. A chromatogram generated by this approach contains a series of two vacancy peaks that correspond to the retention times of the solute–protein complex and free solute, respectively. As in the Hummel–Dreyer method, the size of these peaks can be used with calibration to determine the fractions of the free and bound solute at equilibrium. If this is conducted in the presence of several different solute–protein mixtures, the binding parameters of this system can then be obtained [61]. Such an approach has been used to examine the interactions of HSA with diazepam [50], and the effect of fatty acids [63] or sodium dodecyl sulfate [69] on the binding of HSA to warfarin.

2.2. Chromatographic methods based on immobilized proteins

The use of an immobilized ligand in a chromatographic system for the study of biomolecular interactions is a method known as *analytical affinity chromatography* or *biochromatography*. This may be performed on either a low-performance or high-performance support. A general overview of the experimental approaches used in this field has been discussed in a previous book [21], and its used in solute–protein interactions has been the subject of several previous reviews [14,18,19,22,23]. To study solute–protein interactions by affinity chromatography, either the protein or solute (or some related analog) can be used as the immobilized ligand [14,21–24]. However, in most cases the protein is the species which is attached to the column.

An important factor to consider when using an immobilized protein column is the degree to which this support will model the behavior of the same protein in its soluble form. Ideally, work with any new type of protein column should begin with experiments that compare the binding properties of the immobilized and soluble protein in order to determine whether or not such effects are important. Fortunately, there is growing evidence that at least some immobilized proteins can be successfully used as models for the study of solute–protein interactions in solution. For example, it has been shown in several studies that the binding constants, displacement phenomena and allosteric interactions of immobilized HSA closely match those observed for the same protein in solution (see Refs. [70–81] and the review provided in Ref. [23]). On the other hand, silica-based supports containing immobilized AGP have been reported in some studies to exhibit different displacement properties than AGP in solution [14].

2.2.1. Zonal elution

As was true for soluble protein methods, zonal elution is also an approach that is frequently used to study the binding of solutes on immobilized protein columns [14,19,22,23]. This is generally performed using a sample that contains only a small amount of the drug or solute of interest (i.e., linear elution conditions), but the effects of using larger amounts of sample have also been studied. The sample may be injected either in the presence of only buffer or in the presence of a fixed concentration of a competing agent in the mobile phase. Analysis of the results is usually performed by determining how the retention factor (k) for the injected solute changes as a function of competing agent. Since the retention factor is directly related to the global association constant for the solute and protein, this parameter can then be used as a direct index of solute–protein binding and of the overall interactions that are taking place within the column. For instance, the retention factor has been used as a tool to compare the percent binding of immobilized HSA with various benzodiazepines, coumarins and triazole derivatives [82]. An alternative approach, as discussed later in Section 3.1, involves the use of a mobile phase additive that competes with solutes for binding sites on the protein. If a solute's retention varies as the competing agent's concentration is changed (as shown in Fig. 3a), then the degree of this shift in retention can be used to determine the binding constants for these agents at their site of competition on the protein.

2.2.2. Frontal analysis

Frontal analysis is a second format that can be

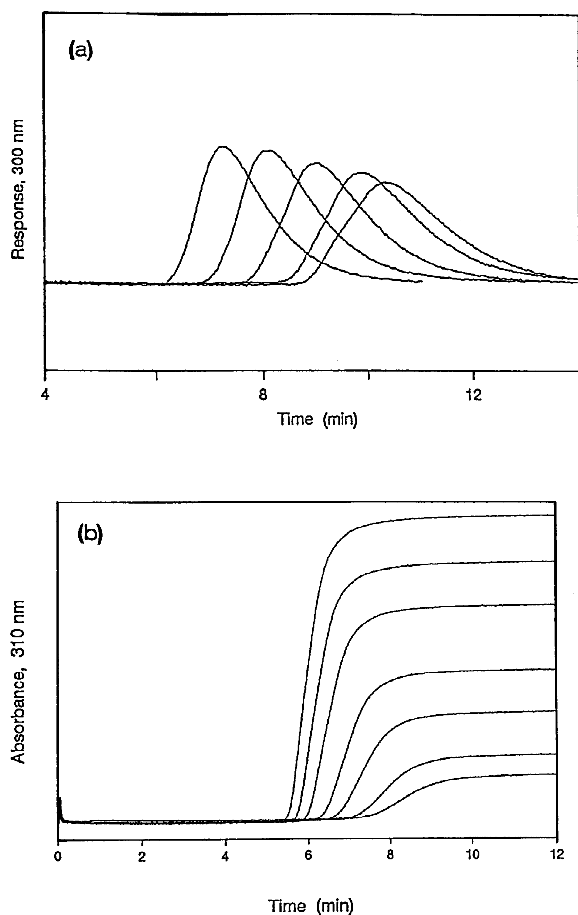

Fig. 3. (a) Typical zonal elution chromatograms for the injection of *R*-warfarin onto an immobilized HSA column in the presence of various concentrations (increasing from right-to-left) of a competing agent and (b) chromatograms for the frontal analysis of various concentrations of *R*-warfarin (increasing from right-to-left) applied to an immobilized HSA column. Reproduced with permission from Refs. [77] and [152].

used for solute–protein binding studies in affinity chromatography. This differs from the methods described in Section 2.1.2 for soluble proteins in that now an immobilized protein is used along with a solution containing a known concentration of solute that is continuously applied to an affinity column. As the solute binds to the immobilized ligand, the ligand becomes saturated and the amount of solute eluting from the column gradually increases, forming a characteristic breakthrough curve (see Fig. 3b). If fast association and dissociation kinetics are present in the system, the mean positions of the breakthrough curves can be related to the concentration of applied solute, the amount of ligand in the column, and the association equilibrium constants for solute–ligand binding. Frontal analysis affinity chromatography has been used to investigate the binding of HSA to *R*- or *S*-warfarin [71,77,83] and D- or L-tryptophan [71,72,78,83,84], and the binding of salicylate to BSA [85]. It has also been used to determine the binding capacities of monomeric versus dimeric HSA for salicylic acid, warfarin, phenylbutazone, mefenamic acid, sulfamethizole and sulfonylureas [86] and to examine the competition of sulfamethizole with salicylic acid for HSA binding regions [87], or salicylate with clofibric acid, octanoic acid or oestradiol for sites on BSA [88].

2.2.3. Vacancy techniques

Like size-exclusion or ISRP supports, affinity columns can be employed in vacancy techniques such as the Hummel–Dreyer method [89]. For example, a HPLC column containing an immobilized BSA silica support has been used along with a mobile phase that contained a mixture of D- and L-tryptophan at a fixed concentration to determine the different affinities and retention times for these enantiomers to BSA or other ligands. For instance, when a BSA sample was injected into the column the result was a broad positive peak corresponding to the retention time for the protein and solute–protein complexes, followed by a series of negative peaks that were related to the depletion of D- and L-tryptophan from the mobile phase. The areas of these vacancy peaks were then compared to those obtained for the injection of only buffer and from this data an area ratio was calculated to describe the stereoselective binding of D- and L-tryptophan to BSA. The same general system was then used to examine the interactions of these solutes with other injected ligands, such as HSA, garden pea lectin and various cyclodextrins [89].

2.3. Electrophoretic methods based on soluble proteins

Like chromatography, electrophoresis can be used as a tool to study solute–protein interactions. Many of the same approaches can be used in electro-

phoresis as have already been discussed for chromatographic methods (i.e., zonal elution, frontal analysis or vacancy techniques). Past work with gel-based electrophoresis systems has used both soluble and immobilized proteins or ligands for the study of biomolecular interactions [25–27]. In more recent years, capillary electrophoresis has also been used in such work, giving rise to a technique known as affinity capillary electrophoresis (ACE) [28–35]. As its name implies, this method is performed as part of a CE system, with the ligand of interest being placed within the capillary as a running buffer additive. Advantages of ACE versus traditional electrophoresis include its speed, resolving power, and ability to work with small amounts of ligand or analyte. Since the CE system also acts to separate the analyte from other sample components, this method can often be used with impure samples or it can be used to simultaneously study the binding of several different compounds with the ligand of interest.

2.3.1. Zonal elution

This method makes up the largest category of modern electrophoretic approaches for the study of solute–protein interactions. The general assumptions and formats are similar to those already discussed in Section 2.1 for solution-phase measurements by chromatography. However electrophoresis, and ACE in particular, is usually more amenable than chromatographic methods to direct solution-phase studies since this technique generally does not require any type of stationary phase for analyte separation.

As is the case for the chromatographic methods, zonal elution in electrophoresis is only seldom used for the direct separation of the free and protein-bound fractions of a solute. The reason for this is again that the dissociation kinetics for many solute–protein complexes are too rapid to allow a separation of free and bound solute on the time scale of a typical electrophoretic run [25]. Some exceptions include work in which CE was used to investigate the binding and stoichiometry between antisense peptide nucleic acids and their complementary oligonucleotide sequences [90], the binding of anionic carbohydrates [91] or oligonucleotides [92] to synthetic peptides derived from human serum amyloid P component, the binding of procainamide and related compounds to hemoglobin and histone proteins [93], the interactions of BSA with anti-BSA antibodies [94], the binding of cyclophilin with cyclosporin A [95] and association of the protein HLA-DR4 with a viral peptide fragment [96].

Mobility shift assays are the format in which zonal elution is usually used to study solute–protein interactions in ACE. This is done by injecting a small amount of the analyte of interest into the presence of a soluble ligand in the CE running buffer. If the solute and ligand have fast association–dissociation kinetics and there are different mobilities for the injected solute versus the solute–ligand complex, then there will be a shift observed in the position of the solute peak as the ligand concentration in the running buffer is varied, as shown in Fig. 4. This shift in peak position is then used to determine the number and strength of binding sites by using the change in migration time, velocity or mobility of the solute observed at each ligand concentration.

Mobility shift assays have been used to examine a number of solute/protein systems, including the binding of leucovorin with BSA [97], D/L-tryptophan with HSA [98], human or bovine carbonic anhydrase with arylsulfonamides [99–104], vancomycin to small peptides [104], deoxyspergualin to heat shock proteins [105], Src Homology III domains with peptides from receptor proteins [106], phosphorylcholine with human C-reactive protein [107], serum amyloid P component with heparin or chrondrotin sulfate [108], and HLA protein with viral peptides [96]. This has been used to investigate the binding of antibodies with phosphotyrosine [109], *N*-2,4-dinitrophenyl compounds [110], phosvitin [111], or a diphosphotyrosine peptide [112] as the antigen. This format has also been used to examine the binding of benzoin enantiomers or phenothiazine derivatives to soluble HSA in the presence of various displacing agents [113].

2.3.2. Frontal analysis

A few reports have examined the use of frontal analysis in CE for the study of solute–protein binding [30,31,114,115]. The basic principle of this method and the appearance of its results are similar to those described in Section 2.1 for solution-phase chromatographic methods; the only difference in CE is that the free solute, solute–protein complex and non-complexed protein bands are now separated

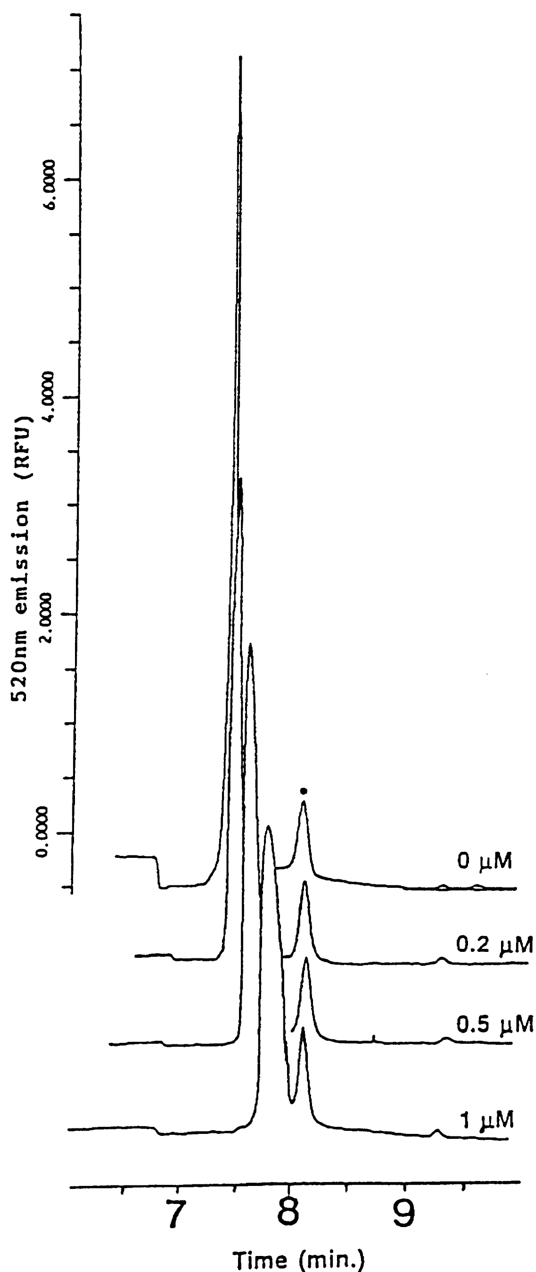

Fig. 4. Mobility shift assay in affinity capillary electrophoresis for injections of a peptide into the presence of a running buffer containing various concentrations of the protein HLA-DR4 as a binding agent. The peak under the circle is an impurity in the samples that was used as an internal marker. Reproduced with permission from Ref. [96].

based on their different electrophoretic mobilities. This method was initially used in work with warfarin and BSA, in which such an approach was compared to the Hummel–Dreyer and equilibrium saturation methods, as performed on the same CE system [114]. One variation on this approach that has been reported involved the use of a coated capillary and a physiological pH to separate the free and protein-bound fractions of verapamil from HSA, which provided data that were then used to determine the binding parameters for this system [116]. Alternatively, hydrodynamic or electrokinetic injection has been tested for isolation of the free fractions of verapamil, followed by the solute's injection onto a capillary and separation into its individual enantiomers by using a second ligand (i.e., trimethyl-β-cyclodextrin) as a chiral binding agent [115].

2.3.3. Vacancy techniques

It has been shown that CE can used to perform both the Hummel–Dreyer and equilibrium saturation methods. This was demonstrated in work in which these methods were used in the analysis of warfarin–BSA interactions [114]. As in the solution-phase chromatographic technique described in Section 2.1.3, the CE-based Hummel–Dreyer method is performed by injecting a sample that contains only protein into the presence of a buffer that has a fixed and known concentration of the solute to be studied. As the sample passes through the CE capillary, the different electrophoretic mobilities of the solute and solute–protein complex will give a positive peak for the injected protein and a negative, vacancy peak that represents the depleted free solute concentration in the running buffer. From the size of this vacancy peak, the concentration of bound versus free solute can be quantitated and used to determine the binding affinity and number of binding sites for the solute on the protein. The equilibrium saturation method can be performed on a CE system in a similar fashion but now using a running buffer that contains both a protein–solute mixture and a sample that contains only buffer. This latter situation results in two vacancy peaks, one for the protein and the other for the solute, and can again be used with Scatchard plots to provide the binding parameters for the solute–protein system [114].

2.4. Electrophoretic techniques based on immobilized proteins

There have been many past reports on the study of biomolecular interactions based on ligands immobilized to traditional gel electrophoresis supports [27]. The overall approach is similar to what is used for immobilized proteins in liquid chromatography, as described in Section 2.2, but now uses an electric field instead of a pressure gradient to elute the applied sample. The ligands in these methods can be immobilized directly onto the electrophoresis support, entrapped in a soluble form within an electrophoresis gel during the gel's formation, or attached to other supports and then entrapped within the gel. Common ligands used in these techniques include immobilized lectins and antibodies, but a variety of other agents have been employed, such as dyes, sugars and enzyme inhibitors or co-factors [27]. Although gel-based electrophoretic systems with immobilized ligands have been used for many years, relatively little work has been performed in using immobilized ligands in CE for solute–protein studies. Instead, research in this area has focused on the use of proteins in CE for chiral separations. A review of this topic can be found in Ref. [12].

3. Information from separation methods on protein binding to chiral solutes

There are variety of ways in which the techniques discussed in Section 2 have been used to study protein interactions with chiral solutes. Examples of such work that will now be considered include the use of these approaches to examine the strength and number of solute–protein binding sites, the thermodynamics and kinetics of these processes, the effects of solvent changes on these interactions, and the nature of the binding regions that are involved in the recognition of chiral agents by proteins.

3.1. Strength and number of protein binding sites

By far the most common type of solute–protein study that is performed by chromatographic or electrophoretic methods is an investigation of the extent of solute binding to a protein. This may involve just determining the relative fraction of a solute that is bound at a given protein concentration, or it might involve a more detailed examination of the equilibrium constants and number of binding regions that are involved in this interaction.

3.1.1. Data analysis methods

One common way of using chromatographic or electrophoretic data for this purpose is to measure the bound and/or non-bound fractions of the solute under various concentration conditions and to plot the results by using Scatchard analysis [117]. This is based on measurements of the total fraction of solute bound per protein (r, or B/P) as a function of the concentration of solute that remains free in solution [S], as described by the following relationship:

$$r = \sum \{n_i K_{ai}[\mathrm{S}]/(1 + K_{ai}[\mathrm{S}])\} \qquad (5)$$

where n_i is the moles of binding site i per mole of protein and K_{ai} is the association equilibrium constant for solute binding at this site. Since Eq. (5) is based on the reaction scheme given earlier in Eqs. (1)–(4), it assumes that the values for n and K_a for each site are independent and constant. This relationship is convenient for the study of solute–ligand binding since it can be adapted for use with any number of binding sites. However, in practice only one or two types of sites are usually used with this expression to describe solute–protein interactions. For example, in the case of a single-site interaction, Eq. (5) reduces to form shown below:

$$r/[\mathrm{S}] = nK_a - K_a r \qquad (6)$$

which predicts that a plot of $r/[\mathrm{S}]$ versus r for a system with 1:1 binding will yield a straight with a slope of $-K_a$ and intercept of nK_a, thus providing the binding parameters for the solute–protein system.

Eqs. (5) and (6) are often used directly with the data obtained with zonal elution, frontal analysis and vacancy methods in both liquid chromatography and electrophoresis. It is also possible to use this approach in mobility shift assays. This is demonstrated for a 1:1 binding system in Eq. (7), which relates the observed change in the migration time or net mobility of an injected protein to the concentration of solute in the running buffer [29]:

$$\Delta\mu_{net,L}/[S] = K_a\Delta\mu_{net,L}^{max} - K_a\Delta\mu_{net,L} \quad (7)$$

In this expression, [S] is the molar concentration of solute in the running buffer, $\Delta\mu_{net,L}$ is the observed shift in the net mobility of the protein in the presence of [S] as compared to the net mobility observed when no solute is present, and $\Delta\mu_{net,L}^{max}$ is the maximum possible shift in mobility for the protein peak, as occurs when all binding sites on the protein are saturated with the solute. According to Eq. (7), a plot of $\Delta\mu_{net,L}/[S]$ versus $\Delta\mu_{net,L}$ for a 1:1 binding system should provide a linear relationship with the slope of this line giving the value of the association equilibrium constant K_a.

One problem associated with Scatchard plots is that the terms on the right and left-hand sides both depend on the same parameter (*r*), thus invalidating the use of normal linear regression techniques for such graphs. This also produces a non-uniform variance throughout these plots which must be considered when finding their best-fit parameters. Because of these limitations, other methods of data analysis have also been developed for chromatographic and electrophoretic studies of solute–protein interactions. For instance, in zonal elution studies it is common to use reciprocal graphs that do not have the co-dependence problems of Scatchard plots. One example is shown below for zonal elution studies with an immobilized protein, in which a small amount of probe solute is injected in the presence of a known concentration of a soluble competing agent (A) [71]:

$$\frac{1}{k} = \frac{K_{aA}V_m[A]}{K_{aS}m_L} + \frac{V_m}{K_{aS}m_L} \quad (8)$$

In this relationship, k is the measured retention factor, K_{aA} and K_{aS} are the association equilibrium constants for the binding of A and S at a common site of competition on the protein, [A] is the molar concentration of the mobile phase additive and m_L is the moles of common binding sites on the protein for S and A. This equation predicts that a system with single-site competition will give a linear plot for $1/k$ versus [A], and by determining the ratio of the slope to the intercept for this plot, the value of K_{aA} can be obtained. Similar expressions have been derived for other situations, such as for solutes and additives with multiple sites of competition or injected solutes that have other binding sites which do not interact with the mobile phase additive [72].

Related reciprocal plots are commonly used in frontal analysis studies with immobilized proteins [77,78,83] and have employed in CE studies of solute–protein interactions in solution. For instance, the following equation shows how the observed migration of an injected solute in CE would be expected to change as the concentration of its binding protein is varied in the running buffer [98,118]:

$$1/(\mu_{net,S} - \mu_S) = 1/\{(K_a[L])(\mu_{SL} - \mu_S)\} + 1/(\mu_{SL} - \mu_S) \quad (9)$$

In this expression, $\mu_{net,S}$ is the net mobility observed for the solute in the presence of a given protein or ligand concentration [L], μ_S is the net solute mobility in the absence of protein, and μ_{SL} is the mobility for the resulting solute–protein complex. This equation, which is derived for a 1:1 binding system, predicts that a plot of $1/(\mu_{net,S} - \mu_S)$ versus $1/[L]$ should allow the value of K_a to be determined from the ratio of the intercept to the slope. The same basic approach can be used in CE studies that involve the injection of proteins into the presence of solute additives in the running buffer (e.g., by using [S] in place of [L], $\mu_{net,L}$ in place of $\mu_{net,S}$, etc.) [107,109].

3.1.2. Typical binding parameters for proteins and chiral solutes

The measurement of binding parameters is the most common way in which separation methods have been used to examine solute/protein interactions. Numerous examples of this can be found in Section 2 of this review. Some specific examples are shown in Table 1. These particular cases were selected for this table since they each involved the measurement of protein binding for the separate chiral forms of the given solutes under otherwise identical measurement conditions. These examples were also chosen because the competition of the separate isomeric forms have been studied and found to show several ways in which chiral compounds can differ in their protein binding properties. For example, *R*- and *S*-warfarin are known to both bind to the

Table 1
Binding parameters measured for proteins and chiral solutes by chromatography or electrophoresis

Method	Protein	Solute	Binding parameters	Conditions	Ref.
Hummel–Dreyer	HSA (in solution)	*R*-Isradipine	$3.6 \cdot 10^4$ M^{-1} ($n=1$)[a]	pH 7.4, 37°C	[64]
		S-Isradipine	$1.8 \cdot 10^5$ M^{-1} ($n=1$)		
	AGP (in solution)	*R*-Isradipine	$1.2 \cdot 10^7$ M^{-1} ($n=1$)	pH 7.4, 37°C	[64]
		S-Isradipine	$1.3 \cdot 10^6$ M^{-1} ($n=1$)		
HFPA	HSA (in solution)	Semotiadil	$2.2 \cdot 10^5$ M^{-1} ($n=1$)	pH 7.4, 37°C	[59]
		Levosemotiadil	$6.6 \cdot 10^5$ M^{-1} ($n=1$)		
	AGP (in solution)	Semotiadil	$3.2 \cdot 10^7$ M^{-1} ($n=1$)	pH 7.4, 37°C	[60]
		Levosemotiadil	$2.6 \cdot 10^7$ M^{-1} ($n=1$)		
Frontal analysis	HSA (immobilized)	*R*-Warfarin	$2.1 \cdot 10^5$ M^{-1} ($n=1$)	pH 7.4, 37°C	[77]
		S-Warfarin	$2.6 \cdot 10^5$ M^{-1} ($n=1$)		
		D-Tryptophan	$3.6 \cdot 10^3$ M^{-1} ($n=1$)	pH 7.0, 37°C	[78]
		L-Tryptophan	$2.4 \cdot 10^4$ M^{-1} ($n=1$)		
Zonal elution	HSA (immobilized)	*R*-Ibuprofen	$5.3 \cdot 10^5$ M^{-1} ($n=1$)	pH 6.9, 25°C[b]	[119]
		S-Ibuprofen	$1.1 \cdot 10^5$ M^{-1} ($n=2$)		
			$1.2 \cdot 10^5$ M^{-1}		

[a] The K_a value given here represents (nK_a) for this solute. Multiple bonding sites may actually have been present.
[b] 15% Acetonitrile was also present in the mobile phase during the ibuprofen study.

same general region on HSA, but they have different association constants at this site [77]; similar behavior has been reported for the binding of semotiadil and levosemotiadil to HSA and AGP [59,60]. D-Tryptophan and L-tryptophan each have 1:1 binding with HSA but have a much larger difference in their binding constants and may even interact with different regions on this protein [78]. *R*-Ibuprofen and *S*-ibuprofen have fairly similar binding constants; however, the *R*-enantiomer binds at only one major region on HSA, while the *S*-enantiomer has at least two major binding sites [119]. Thus, the strength, number and even location of major binding sites on a protein can differ between the various forms of a chiral solute.

In some cases it is possible to use HPLC or CE to examine interactions at specific protein regions even when a solute has multiple binding sites on the protein. One way this can be done is by using zonal elution or mobility shift experiments in which probe compounds are injected that have known, specific sites on a protein at which they interact. As shown earlier in Eq. (8) for zonal elution studies, this allows the binding of a second solute, used here as a competing agent, to be examined at that same particular binding region. An example of such an experiment is shown in Fig. 5, which illustrates how the different number of binding sites were detected for *R*- and *S*-ibuprofen [119]. This same approach has been used in numerous other studies, as will be discussed further in Section 3.5.

3.2. Thermodynamic parameters for protein binding to chiral solutes

3.2.1. Data analysis methods

Along with estimating binding constants for solute–protein interactions, chromatography and electrophoresis can be used as tools to provide even further thermodynamic information on these interactions. A simple example of this is the overall change in free energy that is associated with the binding process (ΔG), which can be calculated directly from K_a through the following equation:

$$\Delta G = -RT \ln K_a \qquad (10)$$

where R is the ideal gas law constant and T is the temperature (in Kelvin) at which the association constant was measured.

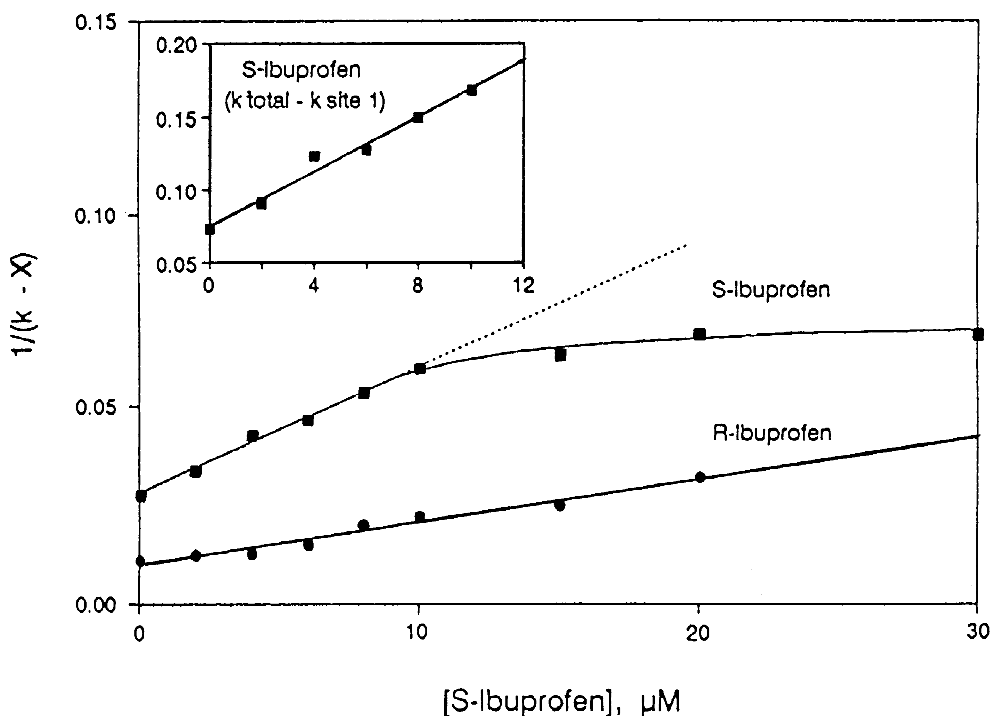

Fig. 5. Zonal elution studies examining the competitive binding of *R*- and *S*-ibuprofen for their binding sites on an immobilized HSA column. The linear relationship shown for *R*-ibuprofen as an injected analyte (●) indicates that this only has one site of competition with *S*-ibuprofen, the mobile phase additive. The non-linear results obtained for injections of *S*-ibuprofen in competition with itself (■) means that this enantiomer has at least two binding sites on HSA. Adapted with permission from Ref. [119].

If the association constants for a system are measured over a range of temperatures, with all other conditions being constant, then it is also possible to estimate the changes in enthalpy and entropy that are associated with these binding processes. This can be done by using the van't Hoff equation:

$$\ln K_a = -(\Delta H/RT) + \Delta S/R \quad (11)$$

In this relationship, ΔH is the change in enthalpy for the reaction and ΔS is the change in entropy. By making a plot of ln K_a versus $1/T$, it is possible to use the slope and intercept of this graph to obtain the values of ΔH and ΔS for a solute–protein interaction. Since this particular equation assumes a 1:1 binding process, the presence of multiple bindings sites or secondary interactions can give rise to curvature in plots prepared according to Eq. (11). This equation further requires that the values of ΔH and ΔS be independent of temperature, which is a reasonably good assumption for solute–protein systems that are examined over a moderate temperature range.

Since the retention factor is directly related to the association constant, it is sometimes possible to make van't Hoff plots by using values for k in place of K_a, as indicated below:

$$\ln k = -(\Delta H/RT) + \Delta S/R + \ln (m_L/V_m) \quad (12)$$

Eq. (12) is obtained directly from Eq. (11) by using the fact that $k = K_a m_L/V_m$ for an immobilized protein that has 1:1 interactions with an injected solute. Eq. (12) is convenient for use in thermodynamic studies involving zonal elution experiments and has frequently been used for such work [84,120–125]. Closely-related expressions that relate changes in temperature to the selectivity (or ratio of retention factors) for two chiral compounds have also been employed in this type of research [126,127]. How-

ever, some caution needs to be followed when using Eq. (12) and related equations since these generally assume that only changes in K_a, and not in m_L, are what give rise to the observed changes in retention with temperature. This is of concern since such an assumption has been shown to not be valid for at least some solute–protein systems [77,78].

3.2.2. Results for proteins and chiral solutes

Although much less common than binding constant measurements, HPLC has been used to estimate the changes in free energy and changes in enthalpy or entropy for several solute–protein systems. Some examples of the data that have been obtained from such studies are given in Table 2. As can be seen from this table, both the enthalpy and entropy terms are usually significant factors in these interactions. A positive value for ΔS (or a negative value for $-T\Delta S$), as shown in the table, is often present for these reactions. This is due to the change in the local environment about the solute and protein binding site during the binding process, which generally results in the net release of solvent. In the examples shown here, the contribution due to ΔH is also favorable, which reflects the net gain in bond formation that occurs as the solute and protein interact.

3.3. Solvent effects in chiral recognition by proteins

Another important application of chromatographic and electrophoretic methods has been their use as tools to investigate how changing the composition of the solvent affects the nature of a solute–protein interaction. This is valuable in helping estimate the relative contributions that are made by non-polar interactions, dipole forces, coulombic interactions and hydrogen bonding to the formation and stabilization of a solute–protein complex. Such information not only provides a better picture of solute–protein binding, but also results in data that can be used for the optimization and design of better chiral separation systems.

3.3.1. Data analysis methods

There a variety of approaches in chromatography and electrophoresis that allow solvent effects to be examined in solute–protein interactions. The simplest of these schemes makes use of the changes in analyte retention or migration that are observed as the mobile phase or running buffer composition is changed in the separation system. In the case of chromatography, this is generally accomplished by using the retention factor for an injected solute as a direct measure of that solute's interactions with an immobilized protein. For instance, a compound that is injected onto a column that contains a protein with a series of binding sites for that substance (L_1 through L_n) will give a retention factor that is determined by the following relationship:

$$k = (K_{a1}n_1 + ...K_{an}n_n)m_L/V_m \tag{13}$$

$$k = K'_a(m_L/V_m) \tag{14}$$

where K'_a is the global association constant for the solute and protein. This means that k can be used as an index of how solvent effects alter the overall extent of solute–protein binding. However, some care is needed when using this approach since, as mentioned in Section 3.2, the value of k is determined by both the association constants and amount of each binding site within the system, either of which might be affected by a change in solvent

Table 2
Thermodynamic constants measured by HPLC for the interactions of immobilized HSA with chiral solutes[a]

Protein	Solute	ΔG at 37°C (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol K)
HSA	*R*-Warfarin	−7.5 (±0.3)	−3.5 (±0.2)	13 (±1)
	S-Warfarin	−7.7 (±0.4)	−5.6 (±0.3)	7 (±1)
HSA	D-Tryptophan	−4.8 (±0.3)	−1.7 (±0.2)	10.5 (±0.6)
	L-Tryptophan	−6.2 (±0.6)	−5.7 (±0.4)	1.7 (±1.3)

[a] Data taken from Refs. [77] and [78]. The warfarin values were determined at pH 7.4; the tryptophan values were measured at pH 7.0.

[78]. A similar problem arises when using shifts in mobility in electrophoresis to describe solvent effects in solute–protein interactions. In addition, solvent studies performed in CE need to consider the fact that the electroosmotic flow and solute or protein mobilities will probably change as the solvent composition is altered.

A more selective way of examining solvent effects is to independently measure the changes that occur in the association constants and number of binding sites. This can potentially be done by any one of the methods that were described earlier for the estimation of binding parameters. An example of a technique which has been employed for this purpose is frontal analysis with immobilized protein columns. As described in Section 2.2.2, this involves the saturation of an immobilized protein with a known concentration of an applied solute, [S]. If this experiment is repeated at several solute concentrations, the apparent moles of solute that are required to reach the center of the saturation curve (m_{Lapp}) can be related to the true number of binding sites for that solute (m_{L}) through the following equation:

$$1/m_{\mathrm{Lapp}} = 1/(K_{\mathrm{a}} m_{\mathrm{L}}[\mathrm{S}]) + 1/m_{\mathrm{L}} \tag{15}$$

This predicts that a plot of $1/m_{\mathrm{Lapp}}$ versus $1/[\mathrm{S}]$ for a system with 1:1 binding will give an intercept of $1/m_{\mathrm{L}}$ and an intercept/slope ratio of K_{a}. In this way it is possible to study changes in both the association constant and number of binding sites as the solvent is changed during a solute–protein binding study (see Fig. 6).

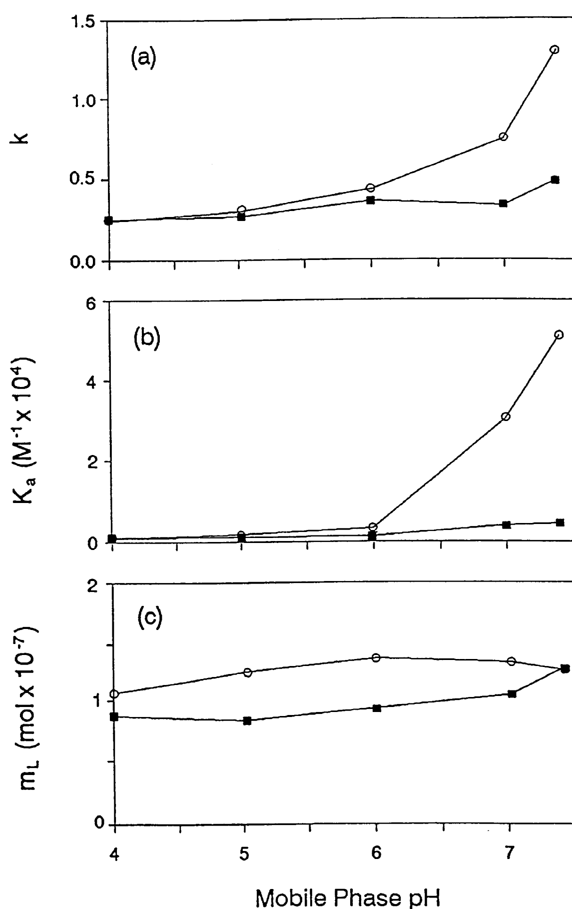

Fig. 6. Effect of mobile phase pH on the retention factor (a), association equilibrium constant (b) and total moles of active binding sites (c) for L-tryptophan (○) and D-tryptophan (■) on an immobilized HSA column. Reproduced with permission from Ref. [78].

3.3.2. Results of solvent studies

Most work in this area has used zonal elution to examine the effects of different solvent conditions on the retention of compounds on immobilized protein columns [9,14,84,120–145]. However, more recent studies have also used frontal analysis on immobilized protein columns [78,143,144] and ACE [12,146]. Proteins that have been examined in these reports include HSA, BSA, and AGP, among others. Solvent effects which are generally considered include variations in pH, ionic strength and solvent polarity. In addition, temperature changes, which were discussed in the previous section, are often included in this type of research.

Solvent pH is of interest in these studies since it 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 [9,12]. Altering the pH can have a large effect on the binding and separation of chiral solutes by proteins, but the exact extent of these effects is hard to predict and usually must be determined on a case-by-case basis. In general, studies have shown that proteins tend to have their most specific and strongest binding when working at

a pH mimicking that of their native environments. Working at a much higher or lower pH will often lead to protein denaturation and a loss of specific binding; however, a small pH change above or below physiological levels can actually enhance the binding of charged solutes (e.g., an increase in binding for cationic species as the pH is increased).

Ionic strength and solvent polarity are two other factors that have been shown in chromatographic and electrophoretic studies to have an effect on the interactions of solutes with proteins. An increase in ionic strength tends to decrease coulombic interactions through a shielding effect [9,12], but at the same time may cause an increase in non-polar solute adsorption. Adding a small amount of organic modifier to change the polarity of the solvent is another means for controlling solute–protein interactions. In this case, a few percent of an agent such as 1-propanol, 2-propanol or methanol can be used to alter solute–protein binding by disrupting non-polar interactions. This usually decreases the binding of solutes to a protein, along with decreasing the width and degree of tailing in solute peaks. The net result can be an improvement in the resolution of chiral solutes, provided that the peak width and tailing decrease faster than the strength of the solute–protein interaction. But caution must be used to avoid adding too much organic modifier to the system since this can cause denaturation of the protein, with a corresponding loss in chiral selectivity.

3.4. Kinetic studies of protein interactions with chiral solutes

Another set of information that can be obtained from separation-based method concerns the kinetics of solute–protein interactions. When combined with thermodynamic information, this provides a relatively complete picture of the dynamics and energetics that are involved in solute–protein binding.

3.4.1. Data analysis methods

Zonal elution and band-broadening measurements have been used in previous work to examine solute–protein kinetics in HPLC columns [147,148]; however, the same approach could be adapted for use in CE [32]. This type of study is done by performing zonal elution studies on immobilized protein columns and inert control columns of the same size that contain the same type of packing material. Injections of solute are then made under well-defined retention conditions and at several flow-rates, with the plate number for the solute's peak being determined under each set of conditions. A van Deemter-type plot is next prepared for both the protein column and control column in order to determine the amount of band-broadening that was associated with each of the various mass transfer processes that were occurring within the column. Of particular interest here is the plate height contribution due to stationary phase mass transfer (H_s), which is directly related to the dissociation rate constant (k_d) between the injected solute and the immobilized protein, as shown in Eq. (16):

$$H_s = \frac{2uk}{k_d(1+k)^2} \qquad (16)$$

where u is the linear velocity of solute and k is its measured retention factor. According to Eq. (16), a plot of H_s versus $uk/(1+k)^2$ should give a slope of $2/k_d$ and an intercept of zero (see Fig. 7). By using the k_d values obtained from these plots along with independent estimates for the equilibrium constants of the system, the association rate constants for the drug and protein can be obtained.

3.4.2. Rates of protein interactions with chiral solutes

Two groups of chiral solutes have previously been studied by using band-broadening measurements. The first group, *R*- and *S*-warfarin, were examined to see how their binding to HSA was affected by changes in temperature at pH 7.4 [147]. In a second study, the association and dissociation rate constants were determined between immobilized HSA and D- or L-tryptophan under a variety of temperatures and solvent conditions [148]. Some representative results that were obtained in these reports are shown in Table 3. In both systems, the solutes and elution conditions which produced the smallest dissociation rate constants also produced the most band-broadening on the immobilized HSA columns. This is simply a reflection of the fact that the stationary phase mass transfer term (H_s) is inversely related to k_d, as shown in Eq. (16). In addition, the difference in k_d values

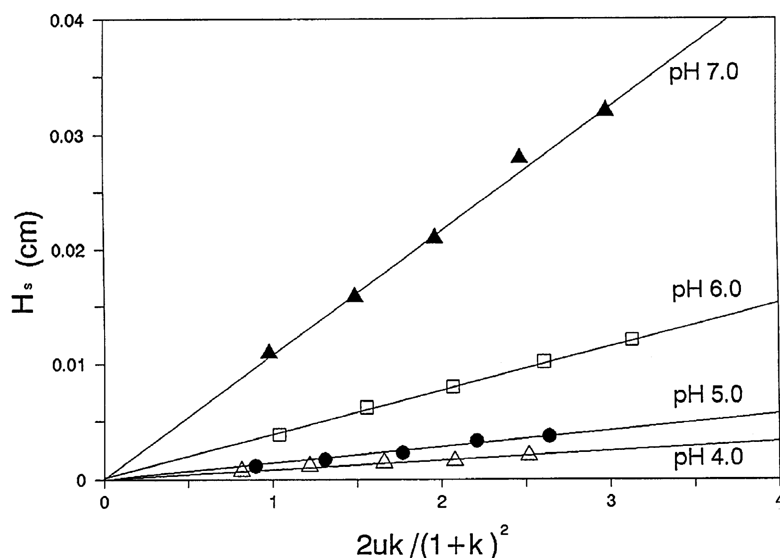

Fig. 7. Plate height contribution to stationary phase mass transfer (H_s) versus [$uk/(1+k)^2$] for D-tryptophan injected onto an immobilized HSA column at 25°C and at several different mobile phase pH values. Modified with permission from Ref. [148].

Table 3
Rate constants for the interactions of immobilized HSA with chiral solutes, as determined by HPLC[a]

Solute	Temperature (°C)	K_a (M^{-1})	k_a (M^{-1} s^{-1})	k_d (s^{-1})
R-Warfarin	45	$1.7\cdot10^5$	$32\cdot10^4$	1.9
	37	$2.1\cdot10^5$	$12\cdot10^4$	0.56
	25	$2.6\cdot10^5$	$10\cdot10^4$	0.40
	4	$4.0\cdot10^5$	$2.4\cdot10^4$	0.06
S-Warfarin	45	$2.0\cdot10^5$	$7.2\cdot10^4$	0.36
	37	$2.6\cdot10^5$	$6.2\cdot10^4$	0.24
	25	$3.4\cdot10^5$	$4.8\cdot10^4$	0.14
	4	$7.3\cdot10^5$	$4.4\cdot10^4$	0.06
L-Tryptophan	45	$2.0\cdot10^4$	$1.3\cdot10^5$	6.4
	37	$2.4\cdot10^4$	$1.4\cdot10^5$	6.0
	25	$3.2\cdot10^4$	$1.5\cdot10^5$	4.7
	4	$8.2\cdot10^4$	$2.7\cdot10^5$	3.3
D-Tryptophan	45	$3.0\cdot10^3$	$2.3\cdot10^4$	7.7
	37	$3.6\cdot10^3$	$2.5\cdot10^4$	7.0
	25	$3.7\cdot10^3$	$1.9\cdot10^4$	5.1
	4	$3.8\cdot10^4$	$0.7\cdot10^4$	1.8

[a] Data taken from Refs. [147] and [148]. The warfarin values were obtained at pH 7.4; the tryptophan values were measured at pH 7.0.

between each pair of enantiomers explains why such different peak widths are obtained in chiral separations for these agents. For instance, the separation of D- and L-tryptophan at neutral pH generally results in a chromatogram where the L-tryptophan peak shows much greater broadening than the D-tryptophan peak. According to these kinetic measurements, a key factor in producing this difference is the much slower dissociation rate of L-tryptophan from HSA under such conditions. Analogous differences in the association and dissociation kinetics of HSA columns have been observed for D- and L-tryptophan when altering the pH, ionic strength and organic content of the eluting solvent [148].

3.5. Characterization of protein binding sites

Obtaining information on solute–protein binding also provides clues on the nature of the binding sites that are involved in these interactions. Such clues can be obtained from any of the various methods that have already been described for the measurement of binding parameters, thermodynamic constants and rate constants, or for studying solvent effects. This helps indicate how a specific solute is binding to a protein, and allows some predictions to be made on

how other compounds may interact with the same protein.

3.5.1. Competitive binding studies

One common way in which chromatography and electrophoresis can be used to study and identify protein binding sites is by examining the competition and displacement of some model solutes with other substances that are placed in contact with the protein. An example of such a study was given earlier in Fig. 5, which illustrated the competition of *R*- and *S*-ibuprofen for their binding sites on HSA. Other examples of these studies have included work with HPLC columns to examine the displacement of D/L-thyronine and D/L-tryptophan from immobilized HSA by bilirubin or caprylate [149]; the competition of *R*/*S*-warfarin with racemic oxazepam, lorazepam and their hemisuccinate derivatives on an HSA column [74]; the direct or allosteric competition of octanoic acid on immobilized HSA for the binding sites of *R*/*S*-warfarin, phenylbutazone, tolbutamide, *R*/*S*-oxazepam hemisuccinate, ketoprofen A/B, suprofen A/B [72] and ketorolac [150]; and the competitive binding of *R*/*S*-ibuprofen, salicylate, *R*/*S*-oxazepam hemisuccinate, fluorouracil, diazepam, phenylbutazone and *R*/*S*-warfarin on HSA [151]. Other studies have used this to examine the competition of *R*-warfarin and L-tryptophan with D-tryptophan [84] or L-thyroxine and related thyronine compounds on immobilized HSA [71,152], and the competitive binding of diazepam with itself or diclofenac [153], phenylbutazone [76] and *R*- or *S*-ketoprofen [154] on HSA columns. In addition, this method has been employed in investigations of the displacement of *R*- and *S*-ibuprofen [119], *cis*- and *trans*-clomiphene [155], and digitoxin or acetyldigitoxin [156] by one another or various probe compounds [157] at their binding regions on HSA. The same technique has been used to characterize the binding sites of non-steroidal anti-inflammatory drugs on HSA [158], and the displacement of non-steroidal anti-inflammatory drugs and benzodiazepines by phenylbutazone, *R*/*S*-ibuprofen or 2,3,5-triiodobenzoic acid from human, rat and rabbit serum albumin columns [159]. Related studies have used competing agents to alter the extent of solute–protein binding in CE as a screening method for determining the binding sites of HSA and BSA for various solutes [160,161].

3.5.2. Structure–retention relationships

A related use for zonal elution studies has been in the development of quantitative structure–retention relationships (QSRRs) for the binding of drugs to immobilized protein columns [162,163]. This involves collecting retention factors, or other types of retention information, under constant temperature and mobile phase conditions for a large set of drugs representing a range of structural variations. This data is then compared to various parameters that can be used to describe the structure of these solutes, and regression is performed to determine which of these factors are most important in controlling retention. Such an approach has been used to examine the binding of immobilized HSA to 1,4-benzodiazepines [73,164,165], indolocarbazole derivatives [166], and acyclovir esters [167]. This method has also been employed in studies of the binding of basic drugs and antihistamines to AGP [168,169] and in the binding of melanin to phenothiazine and dibenzazepine drugs [170]. Based on these relationships, it has been possible in some instances to develop models that describe the protein binding sites for these solutes. A specific example of this is shown in Fig. 8 for the binding of basic drugs to AGP.

3.5.3. Solvent and temperature studies

A third way in which information can be gained about a protein binding site is through the effects that different temperatures or solvent conditions have on a solute–protein interaction at that site. This might include the use of kinetic and thermodynamic data as well as information on how various solvent compositions change the binding of a solute to a protein. Two model systems which have been studied in some detail by this approach include the adsorption of HSA to *R*- and *S*-warfarin [77,147] and to D- and L-tryptophan [78,84,148]. This has led to the development of models in which *R*- and *S*-warfarin are believed to bind to slightly different sections of the same binding site on HSA, while the sites for D- and L-tryptophan appear to be much more distinct and perhaps even separate regions on HSA. Other studies in this category include investigations of the roles played by surface tension, pH, temperature cha-

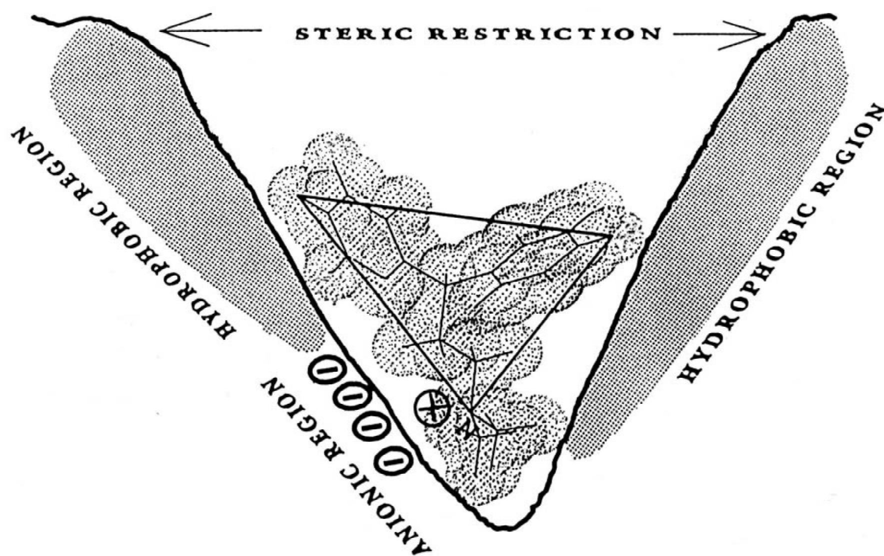

Fig. 8. Important structural features and interaction forces identified by quantitative structure–retention relationships for the binding of basic drugs to AGP. Reproduced with permission from Ref. [168].

otropic agents, solvent polarity, and solute structure in the binding of dansyl amino acids to HSA [120,122,124,126,127,135–137]. Such data have recently being used to describe the geometry of HSA's binding site for these amino acid derivatives, as illustrated by the diagram shown in Fig. 9 [135–137].

3.5.4. Work with modified proteins

A fourth approach to studying protein binding regions involves the use of a protein that has been modified at or near its binding sites. An early example of such work was the use of *p*-nitrophenyl acetate for the acetylation of HSA [171]. This reagent is 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, as demonstrated in Fig. 10 for *R*- and *S*-oxazepam hemisuccinate. A similar example is a report in which *o*-nitrophenylsulfenyl chloride was employed as a reagent for modifying the lone tryptophan residue on HSA, Trp-214, which is located within the warfarin-azapropazone site of HSA [83]. This modification did not change the moles of binding sites for *R*-warfarin, but it produce a lower association constant for this solute. Furthermore, the modification of Trp-214 resulted in a complete loss of stereoselectivity of HSA for *R*- and *S*-warfarin, indicating that Trp-214 and/or its neigh-

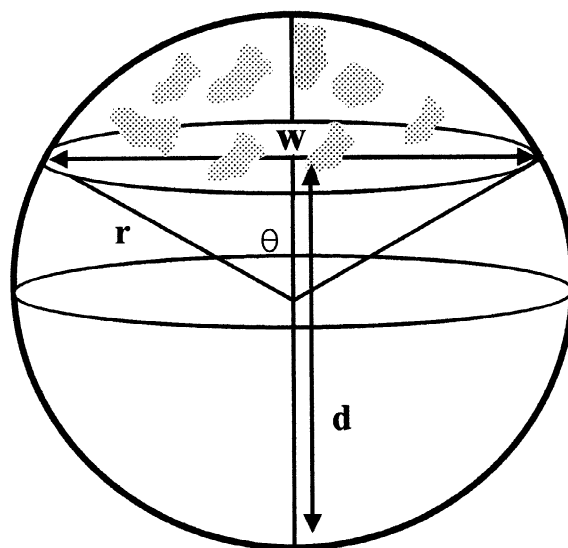

Fig. 9. Proposed binding site geometry on HSA for dansyl amino acids based on solvent and temperature studies. This site is estimated to have a depth (d) of roughly 16 Å, a width (w) of 8 Å and a radius of curvature (r) of 8.5 Å. Reproduced with permission from Ref. [137].

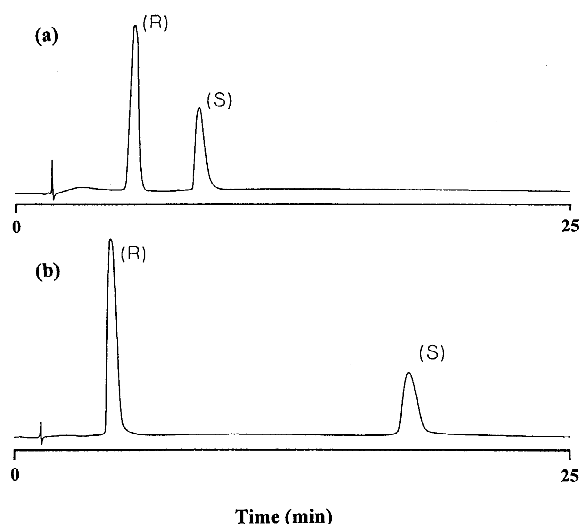

Fig. 10. Chromatograms for a racemic mixture of *R*- and *S*-oxazepam hemisuccinate injected onto a column that contained (a) unmodified HSA or (b) HSA which had been acetylated with *p*-nitrophenyl acetate. All conditions, other than the type of protein that was used, were the same in both separations. Reproduced with permission from Ref. [171].

boring residues played an important role in determining the chiral recognition of these compounds by HSA. Similar work examining changes in chiral selectivity with protein modification have been reported involving the modification of the lone free cysteine residue on HSA with ethacrynic acid [172] and in the use of BSA fragments in the chiral separation of benzoin and other drugs [173,174].

4. Conclusions

This review has examined several new developments and uses of separation-based methods for the study of protein interactions with chiral solutes. Several different formats for these types of experiments were discussed, including methods which used either HPLC or CE and soluble or immobilized proteins. The variety of techniques that can be employed in such work is useful since this provides a means for studying a wide array of solute–protein systems. There are also several types of information that can be gained through these methods. This might involve determining the association constants and number of sites involved in solute–protein binding, a measurement of thermodynamic values or rate constants for this process, a consideration of the role played by various forces within this interaction, or an investigation of the locations and properties of the binding sites that are involved in this interaction. Such methods have already been used to examine many solute–protein systems of common interest. However, given the great potential of these tools, it is expected that they will play an even greater role in the future in the investigation of protein binding to chiral solutes.

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References

[1] W.E. Lindup, in: J.W. Bridges, L.F. Chasseaud, G.G. Gibson (Eds.), Progress in Drug Metabolism, Vol. 10, Taylor & Francis, New York, 1987, Chapter 4.
[2] T.C. Kwong, Clin. Chim. Acta 151 (1985) 193.
[3] C.K. Svensson, M.N. Woodruff, J.G. Baxter, D. Lalka, Clin. Pharmacokin. 11 (1986) 450.
[4] J. Barre, F. Didey, F. Delion, J.-P. Tillement, Ther. Drug Monit. 10 (1988) 133.
[5] S. Refetoff, P.R. Larsen, in: L.J. DeGroot et al. (Ed.), Endocrinology, Vol. 1, Saunders, Philadelphia, PA, 1989, Chapter 38.
[6] U. Westphal, Steroid–Protein Interactions, Springer-Verlag, New York, 1971.
[7] I.W. Wainer, J. Ducharme, C.P. Granvil, H. Parenteau, S. Abdullah, J. Chromatogr. A 694 (1995) 169.
[8] Chem. Eng. News 71 (Sept. 27) (1993) 38.
[9] S. Allenmark, Chromatographic Enantioseparation – Methods and Applications, 2nd ed., Ellis Horwood, New York, 1991, Chapter 7.
[10] D.W. Armstrong, Anal. Chem. 59 (1987) 84A.
[11] I.W. Wainer, Trends Anal. Chem. 12 (1993) 153.
[12] D.S. Hage, Electrophoresis 18 (1997) 2311.
[13] J. Haginaka, J. Chromatogr. A 906 (2001) 253.
[14] D.S. Hage, S.A. Tweed, J. Chromatogr. B 699 (1997) 499.
[15] N. Reitbrock, A. Lassmann, Naunyn-Schmiedeberg's Arch. Pharmacol. 313 (1980) 269.
[16] G. Menke, W. Worner, W. Kratzer, N. Rietbrock, Naunyn-Schmiedeberg's Arch. Pharmacol. 339 (1989) 42.
[17] C.C. Wood, P.F. Cooper, Chromatogr. Rev. 12 (1970) 88.

[18] T. Cserhati, K. Valko, Chromatographic Determination of Molecular Interactions, CRC Press, Boca Raton, FL, 1994, Chapter 2.
[19] B. Sebille, R. Zini, C.-V. Madjar, N. Thuaud, J.-P. Tillement, J. Chromatogr. 531 (1990) 51.
[20] J. Oravcova, B. Bohs, W. Lindner, J. Chromatogr. B 677 (1996) 1.
[21] I.M. Chaiken (Ed.), Analytical Affinity Chromatography, CRC Press, Boca Raton, FL, 1987.
[22] I.W. Wainer, J. Chromatogr. A 666 (1994) 221.
[23] D.S. Hage, J. Austin, J. Chromatogr. B 739 (2000) 39.
[24] C. Rochette-Egly, E. Boschetti, P. Basset, J.-M. Egly, J. Chromatogr. 241 (1982) 333.
[25] J.R. Cann, Anal. Biochem. 237 (1996) 1.
[26] K. Takeo, J. Chromatogr. A 698 (1995) 89.
[27] V. Hořejší, M. Tichá, J. Chromatogr. 376 (1986) 49.
[28] D.M. Goodall, Biochem. Soc. Trans. 21 (1993) 125.
[29] Y.-H. Chu, L.Z. Avila, J. Gao, G.M. Whitesides, Acc. Chem. Res. 28 (1995) 461.
[30] M.H.A. Busch, J.C. Kraak, H. Poppe, J. Chromatogr. A 777 (1997) 329.
[31] M.H.A. Busch, L.B. Carels, H. Poppe, J. Chromatogr. A 777 (1997) 311.
[32] K. Shimura, K.-I. Kasai, Anal. Biochem. 251 (1997) 1.
[33] G. Rippel, H. Corstjens, J. Frank, Electrophoresis 18 (1997) 2175.
[34] I.J. Colton, J.D. Carbeck, G.M. Whitesides, Electrophoresis 19 (1998) 367.
[35] N.H.H. Neegaard, S. Nilsson, N.A. Guzman, J. Chromatogr. B 715 (1998) 29.
[36] J.C.K. Loo, N. Jordan, A.H. Ngoc, J. Chromatogr. 305 (1984) 194.
[37] P.F. Dixon, J. Endocrinol. 40 (1968) 457.
[38] W. Hoffman, U. Westphal, Anal. Biochem. 32 (1969) 48.
[39] A. Shibukawa, T. Nakagawa, M. Miyake, H. Tanaka, Chem. Pharm. Bull. 36 (1988) 1930.
[40] A. Shibukawa, T. Nakagawa, M. Miyake, N. Nishimura, H. Tanaka, Chem. Pharm. Bull. 37 (1989) 1311.
[41] T.C. Pinkerton, T.D. Miller, L.J. Janis, Anal. Chem. 61 (1989) 1171.
[42] B.J. Gurley, M. Marx, K. Olsen, J. Chromatogr. B 670 (1995) 358.
[43] B. Sebille, N. Thuaud, J.P. Tillement, J. Chromatogr. 204 (1981) 285.
[44] B. Sebille, N. Thuaud, J. Liq. Chromatogr. 3 (1980) 299.
[45] I. Marle, C. Pettersson, T. Arvidsson, J. Chromatogr. 456 (1988) 323.
[46] A. Shibukawa, T. Nakagawa, N. Nishimura, M. Miyake, H. Tanaka, Chem. Pharm. Bull. 37 (1989) 702.
[47] B. Sebille, N. Thuaud, J.-P. Tillement, C. R. Acad. Sci., Ser. C 285 (1977) 535.
[48] B. Sebille, N. Thuaud, J.-P. Tillement, J. Chromatogr. 167 (1978) 159.
[49] T.C. Pinkerton, K.A. Koeplinger, Anal. Chem. 62 (1990) 2114.
[50] N. Thuaud, B. Sebille, M.H. Livertoux, J. Bessiere, J. Chromatogr. 282 (1983) 509.
[51] A. Shibukawa, N. Nishimura, K. Nomura, Y. Kuroda, T. Nakagawa, Chem. Pharm. Bull. 38 (1990) 443.
[52] A. Shibukawa, T. Sawada, C. Nakao, T. Izumi, T. Nakagawa, J. Chromatogr. A 697 (1995) 337.
[53] A. Shibukawa, M. Nagao, A. Terakita, J. He, T. Nakagawa, J. Liq. Chromatogr. 16 (1993) 903.
[54] A. Shibukawa, M. Nagao, Y. Kuroda, T. Nakagawa, Anal. Chem. 62 (1990) 712.
[55] A. Shibukawa, A. Terakita, J.-Y. He, T. Nakagawa, J. Pharm. Sci. 81 (1992) 710.
[56] A. Shibukawa, C. Nakao, T. Sawada, A. Terakita, N. Morokoshi, T. Nakagawa, J. Pharm. Sci. 83 (1994) 868.
[57] A. Shibukawa, M. Kadohara, J.-Y. He, M. Nishimura, S. Naito, T. Nakagawa, J. Chromatogr. A 694 (1995) 81.
[58] A. Shibukawa, M. Kadohara, J.-Y. He, M. Nishimura, S. Naito, T. Nakagawa, J. Chromatogr. A 708 (1995) 364, (Erratum).
[59] M.E. Rodriquez Rosas, A. Shibukawa, Y. Yoshikawa, Y. Kuroda, T. Nakagawa, Anal. Sci. 15 (1999) 217.
[60] M.E. Rodriquez Rosas, A. Shibukawa, Y. Yoshikawa, Y. Kuroda, T. Nakagawa, Anal. Biochem. 274 (1999) 27.
[61] J.P. Hummel, W.J. Dreyer, Biochim. Biophys. Acta 63 (1962) 530.
[62] S.F. Sun, S.W. Kuo, R.A. Nash, J. Chromatogr. 288 (1984) 377.
[63] B. Sebille, N. Thuaud, J.P. Tillement, J. Chromatogr. 180 (1979) 103.
[64] J. Oravcova, D. Sojkova, E. Bencsikova, P. Bohov, T. Trnovec, Chirality 7 (1995) 167.
[65] F. Bree, J.-P. Tillement, B. Sebille, J. Chromatogr. 375 (1986) 416.
[66] S.F. Sun, C.L. Hsiao, J. Chromatogr. 648 (1993) 325.
[67] J. Oravcova, S. Bystricky, T. Trnovec, Biochem. Pharmacol. 38 (1989) 2575.
[68] R.F. Williams, M.J. Aivaliotis, L.D. Barnes, A.K. Robinson, J. Chromatogr. 266 (1983) 141.
[69] B. Sebille, N. Thuaud, J.P. Tillement, Farad. Disc. Royal Chem. Soc., Faraday Symp. 15 (1980) 139.
[70] E. Domenici, C. Bertucci, P. Salvadori, G. Felix, I. Cahagne, S. Motellier, I.W. Wainer, Chromatographia 29 (1990) 170.
[71] B. Loun, D.S. Hage, J. Chromatogr. 579 (1992) 225.
[72] T.A.G. Noctor, I.W. Wainer, D.S. Hage, J. Chromatogr. 577 (1992) 305.
[73] T.A.G. Noctor, C.D. Pham, R. Kaliszan, I.W. Wainer, Mol. Pharmacol. 42 (1992) 506.
[74] E. Domenici, C. Bertucci, P. Salvadori, I.W. Wainer, J. Pharm. Sci. 80 (1991) 164.
[75] E. Domenici, C. Bertucci, P. Salvadori, S. Motellier, I.W. Wainer, Chirality 2 (1992) 263.
[76] V.N. Russeva, Z.D. Zhivkova, Int. J. Pharm. 168 (1998) 23.
[77] B. Loun, D.S. Hage, Anal. Chem. 66 (1994) 3814.
[78] J. Yang, D.S. Hage, J. Chromatogr. A 725 (1996) 273.
[79] M. Tabachnick, J. Biol. Chem. 239 (1964) 1242.
[80] H.M. Solomon, J.J. Schrogie, D. Williams, Biochem. Pharmacol. 17 (1968) 143.
[81] J.H.M. Miller, G.A. Smail, J. Pharm. Pharmacol. 29 (1977) 33.

[82] T.A.G. Noctor, M.J. Diaz-Perez, I.W. Wainer, J. Pharm. Sci. 82 (1993) 675.
[83] A. Chattopadhyay, T. Tian, L. Kortum, D.S. Hage, J. Chromatogr. B 715 (1998) 183.
[84] J. Yang, D.S. Hage, J. Chromatogr. 645 (1993) 241.
[85] N.I. Nakano, T. Oshio, Y. Fujimoto, T. Amiya, J. Pharm. Sci. 67 (1978) 1005.
[86] N.I. Nakano, Y. Shimamori, S. Yamaguchi, J. Chromatogr. 237 (1982) 225.
[87] N.I. Nakano, Y. Shimamori, S. Yamaguchi, J. Chromatogr. 188 (1980) 347.
[88] C. Lagercrantz, T. Larsson, H. Karlsson, Anal. Biochem. 99 (1979) 352.
[89] L. Soltes, B. Sebille, N. Thuaud, Chromatographia 38 (1994) 761.
[90] D.J. Rose, Anal. Chem. 65 (1993) 3545.
[91] N.H.H. Heegaard, F.A. Robey, Anal. Chem. 64 (1992) 2479.
[92] N.H.H. Heegaard, F.A. Robey, J. Liq. Chromatogr. 16 (1993) 1923.
[93] C.V. Thomas, A.C. Cater, J.J. Wheeler, J. Liq. Chromatogr. 16 (1993) 1903.
[94] N.H. Chiem, D.J. Harrison, Electrophoresis 19 (1998) 3040.
[95] S. Kiessig, H. Bang, F. Thunecke, J. Chromatogr. A 853 (1999) 469.
[96] N.H.H. Heegaard, B.E. Hansen, L.H. Fugger, J. Chromatogr. A 781 (1997) 91.
[97] G.E. Barker, P. Russo, R.A. Hartwick, Anal. Chem. 64 (1992) 3024.
[98] S. Bose, J. Yang, D.S. Hage, J. Chromatogr. B 697 (1997) 77.
[99] F.A. Gomez, J.N. Mirkovich, V.M. Dominguez, K.W. Liu, D.M. Macias, J. Chromatogr. A 727 (1996) 291.
[100] Y.-H. Chu, L.Z. Avila, H.A. Biebuyck, G.M. Whitesides, J. Med. Chem. 35 (1992) 2915.
[101] L.Z. Avila, Y.-H. Chu, E.C. Blossey, G.M. Whitesides, J. Med. Chem. 36 (1993) 126.
[102] F.A. Gomez, L.Z. Avila, Y.-H. Chu, G.M. Whitesides, Anal. Chem. 66 (1994) 1785.
[103] Y.-H. Chu, W.J. Lees, A. Stassinopoulos, C.T. Walsh, Biochemistry 33 (1994) 10616.
[104] J. Kawaoka, F.A. Gomez, J. Chromatogr. B 715 (1998) 203.
[105] J. Liu, K.J. Volk, M.S. Lee, E.H. Kerns, I.E. Rosenberg, J. Chromatogr. A 680 (1994) 395.
[106] F.A. Gomez, J.K. Chen, A. Tanaka, S.L. Schreiber, G.M. Whitesides, J. Org. Chem. 59 (1994) 2885.
[107] N.H.H. Heegaard, F.A. Robey, J. Immunol. Methods 166 (1993) 103.
[108] N.H.H. Heegaard, H.D. Mortensen, P. Roepstorff, J. Chromatogr. A 717 (1995) 83.
[109] N.H.H. Heegaard, J. Chromatogr. A 680 (1994) 405.
[110] M. Mammen, F.A. Gomez, G.M. Whitesides, Anal. Chem. 67 (1995) 3526.
[111] S. Lin, I.-Y. Hsiao, S.-M. Hsu, Anal. Biochem. 254 (1997) 9.
[112] S. Lin, P. Tang, S.-M. Hsu, Anal. Biochem. 20 (1999) 3388.
[113] D.K. Lloyd, S. Li, P. Ryan, Chirality 6 (1994) 230.
[114] J.C. Kraak, S. Busch, H. Poppe, J. Chromatogr. 608 (1992) 257.
[115] T. Ohara, A. Shibukawa, T. Nakagawa, Anal. Chem. 67 (1995) 3520.
[116] A. Shibukawa, Y. Yoshimoto, T. Ohara, T. Nakagawa, J. Pharm. Sci. 83 (1994) 616.
[117] G. Scatchard, Ann. NY Acad. Sci. 51 (1949) 660.
[118] R. Kuhn, R. Frei, M. Christen, Anal. Biochem. 218 (1994) 131.
[119] D.S. Hage, T.A.G. Noctor, I.W. Wainer, J. Chromatogr. A 693 (1995) 23.
[120] E. Peyrin, Y.C. Guillaume, N. Morin, C. Guinchard, J. Chromatogr. A 808 (1988) 113.
[121] R.K. Gilpin, S.E. Ehtesham, R.B. Gregory, Anal. Chem. 63 (1991) 2825.
[122] E. Peyrin, Y.C. Guillaume, C. Guinchard, Anal. Chem. 69 (1997) 4979.
[123] R.K. Gilpin, S.B. Ehtesham, C.S. Gilpin, S.T. Liao, J. Liq. Chromatogr. Rel. Technol. 19 (1996) 3023.
[124] E. Peyrin, Y.C. Guillaume, N. Morin, C. Guinchard, Anal. Chem. 70 (1998) 2812.
[125] W. Su, R.B. Gregory, R.K. Gilpin, J. Chromatogr. Sci. 31 (1993) 285.
[126] E. Peyrin, Y.C. Guillaume, C. Guinchard, J. Chromatogr. Sci. 36 (1998) 97.
[127] E. Peyrin, Y.C. Guillaume, Chromatographia 48 (1998) 431.
[128] G. Schill, I.W. Wainer, S.A. Barkan, J. Chromatogr. 365 (1986) 73.
[129] S. Allenmark, B. Bomgren, H. Boren, J. Chromatogr. 316 (1984) 617.
[130] S. Allenmark, B. Bomgren, H. Boren, J. Chromatogr. 264 (1983) 63.
[131] J. Hermansson, J. Chromatogr. 269 (1983) 71.
[132] T. Miwa, T. Miyakawa, M. Kayano, Y. Miyake, J. Chromatogr. 408 (1987) 316.
[133] S. Allenmark, S. Andersson, J. Bojarski, J. Chromatogr. 436 (1988) 479.
[134] T. Fornstedt, G. Zhong, Z. Bensetiti, G. Guiochon, Anal. Chem. 68 (1996) 2370.
[135] E. Peyrin, Y.C. Guillaume, C. Guinchard, Anal. Chem. 70 (1998) 4235.
[136] E. Peyrin, Y.C. Guillaume, Anal. Chem. 71 (1999) 1496.
[137] E. Peyrin, Y.C. Guillaume, C. Guinchard, Biophys. J. 77 (1999) 1206.
[138] R.K. Gilpin, H. Gao, M. Jaroniec, J. Liq. Chromatogr. 15 (1992) 2503.
[139] M.C. Millot, B. Sebille, C. Mangin, J. Chromatogr. A 776 (1997) 37.
[140] M. Enquist, J. Chromatogr. 519 (1990) 271.
[141] S. Allenmark, Chirality 5 (1993) 295.
[142] J. Iredale, A.F. Aubry, I. Wainer, Chromatographia 31 (1991) 329.
[143] N.L. Taleb, M.-C. Millot, B. Sebille, J. Chromatogr. A 776 (1997) 45.
[144] I.W. Wainer, Y. Zhang, K.J. Kellar, J. Chromatogr. B 725 (1999) 65.

[145] M.C. Banks, A.F. Fell, R.D. McDowall, Anal. Proc. 30 (1993) 98.
[146] D.K. Lloyd, A. Ahmed, F. Pastore, Electrophoresis 18 (1997) 958.
[147] B. Loun, D.S. Hage, Anal. Chem. 68 (1996) 1218.
[148] J. Yang, D.S. Hage, J. Chromatogr. A 766 (1997) 15.
[149] L. Dalgaard, J.J. Hansen, J.L. Pedersen, J. Pharm. Biomed. Anal. 7 (1989) 361.
[150] P.J. Hayball, J.W. Holman, R.L. Nation, J. Chromatogr. B 662 (1994) 128.
[151] G.A. Ascoli, C. Bertucci, P. Salvadori, Biomed. Chromatogr. 12 (1998) 248.
[152] B. Loun, D.S. Hage, J. Chromatogr. B 665 (1995) 303.
[153] Z. Zhivkova, V. Russeva, J. Chromatogr. B 707 (1998) 143.
[154] Z.D. Zhivkova, V.N. Russeva, J. Chromatogr. B 714 (1998) 277.
[155] D.S. Hage, A. Sengupta, Anal. Chem. 70 (1998) 4602.
[156] D.S. Hage, A. Sengupta, J. Chromatogr. B 724 (1999) 91.
[157] A. Sengupta, D.S. Hage, Anal. Chem. 71 (1999) 3821.
[158] S. Rahim, A.-F. Aubry, J. Pharm. Sci. 84 (1995) 949.
[159] A.-F. Aubry, N. Markoglou, A. McGann, Comp. Biochem. Physiol. 112C (1995) 257.
[160] D.K. Lloyd, S. Li, P. Ryan, Chirality 6 (1994) 230.
[161] T. Arai, N. Nimura, T. Kinoshita, Biomed. Chromatogr. 9 (1995) 68.
[162] R. Kaliszan, J. Chromatogr. B 715 (1998) 229.
[163] R. Kaliszan, Trends Anal. Chem. 18 (1999) 400.
[164] R. Kaliszan, T.A.G. Noctor, I.W. Wainer, Mol. Pharmacol. 42 (1992) 512.
[165] R. Kaliszan, T.A.G. Noctor, I.W. Wainer, Chromatographia 33 (1992) 546.
[166] D.S. Ashton, C.R. Beddell, G.S. Cockerill, K. Gohil, C. Gowrie, J.E. Robinson, M.J. Slater, K. Valko, J. Chromatogr. B 677 (1996) 194.
[167] D.S. Ashton, C. Beddell, A.D. Ray, K. Valko, J. Chromatogr. A 707 (1995) 367.
[168] R. Kaliszan, A. Nasal, M. Turowski, Biomed. Chromatogr. 9 (1995) 211.
[169] R. Kaliszan, A. Nasal, M. Turowski, J. Chromatogr. A 722 (1996) 25.
[170] A. Radwanska, T. Frackowiak, H. Ibrahim, A.-F. Aubry, R. Kaliszan, Biomed. Chromatogr. 9 (1995) 233.
[171] T.A.G. Noctor, I.W. Wainer, Pharm. Res. 9 (1992) 480.
[172] C. Bertucci, I.W. Wainer, Chirality 9 (1997) 335.
[173] J. Haginaka, N. Kanasugi, J. Chromatogr. A 769 (1997) 215.
[174] J. Haginaka, N. Kanasugi, J. Chromatogr. A 694 (1995) 71.