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Review

Recent advances in chromatographic and electrophoretic methods for the study of drug–protein interactions

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Abstract

Drug–protein binding is an important process in determining the activity and fate of a pharmaceutical agent once it has entered the body. This review examines various chromatographic and electrophoretic methods that have been developed to study such interactions. An overview of each technique is presented along with a discussion of its strengths, weaknesses and potential applications. Formats that are discussed include the use of both soluble and immobilized drugs or proteins, and approaches based on zonal elution, frontal analysis or vacancy peak measurements. Furthermore, examples are provided that illustrate the use of these methods in determining the overall extent of drug–protein binding, in examining the displacement of a drug by other agents and in measuring the equilibrium or rate constants for drug–protein interactions. Examples are also given demonstrating how the same methods, particularly when used in high-performance liquid chromatography or capillary electrophoresis systems, can be employed as rapid screening tools for investigating the binding of different forms of a chiral drug to a protein or the binding of different proteins and peptides to a given pharmaceutical agent. © 1997 Elsevier Science B.V.

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1. Introduction

1.1. Importance of drug–protein interactions

Protein binding is important in many processes that determine the eventual activity and fate of a drug once it has entered the body. Some examples of such binding include the interactions of a drug with a target enzyme or receptor. Another type of interaction, and the one that will be emphasized in this report, is the binding of pharmaceutical agents with blood proteins [1]. These interactions can be important in determining the overall distribution, excretion, activity and toxicity of a drug. In some instances this protein binding occurs with general ligands, such as the interaction of many drugs with human serum albumin (HSA) or α_1-acid glycoprotein (AAG) [1–4]. In other cases this binding is highly specific in nature (e.g., interactions of the hormone L-thyroxine to thyroxine-binding globulin or the binding of corticosteroids and sex hormones to various steroid-binding globulins) [5,6].

Besides affecting the distribution and elimination of drugs, it is known that the direct or indirect competition of two drugs for the same binding proteins can be an important source of drug–drug interactions. An example of this is the displacement of phenytoin from HSA by valproic acid [1,2]. Competition can also occur between drugs and endogenous compounds, such as the displacement of various drugs from HSA by fatty acids or bilirubin [2–4,7]. Since the binding of some drugs to proteins can be stereoselective in nature [1,8–10], it is possible that these interactions may also play a role in determining the fate of the different forms of a chiral drug within the body.

Two common methods that are used in evaluating the binding of drugs to proteins include equilibrium dialysis and ultrafiltration [1,2,4]. Equilibrium dialysis is considered by many to be the reference method for such analyses; however, it does suffer from several disadvantages. Perhaps its greatest disadvantage is the long periods of time that are typically required to establish an equilibrium during the dialysis process (i.e., hours or even days) [1]. This not only makes this method inconvenient for routine testing [1], but it also creates problems if the analyte of interest is unstable or if its binding is susceptible to any changes that might occur in the pH or fatty acid levels of the sample during the dialysis process [2,4]. Furthermore, it is necessary with this method to correct for the alterations in free and bound analyte concentrations that occur during the dialysis procedure. The possible effects of analyte adsorption onto the dialysis membrane must also be considered [1,2,4].

Ultrafiltration is similar in its operation to equilibrium dialysis but requires much less time to perform (i.e., typically less than 30 min) [1,2]. However, like dialysis, it still requires the use of a labeled drug and/or an additional analysis step for the actual measurement of the final free drug concentration

[e.g., by using an immunoassay, gas chromatography (GC) or high-performance liquid chromatography (HPLC) method]. In addition, the effects of analyte adsorption to the ultrafiltration membrane must also be considered [1]. Other problems associated with this method include difficulties with temperature control during the separation (particularly when a centrifuge is used) and complications when working with highly bound drugs, which will produce only small amounts of measurable analyte in the final filtrate [1].

Because of these limitations, there has been continuing research to find better, faster and more convenient approaches for the analysis of drug–protein binding. Many of the newer techniques developed for this purpose are based on chromatographic or electrophoretic systems. The main purpose of this review is to examine the various formats that can be employed in these systems for the study of drug–protein interactions. An overview of each technique will be presented along with a discussion of its strengths, weaknesses and potential applications.

1.2. Description of drug–protein binding

The interaction of a drug with a protein is often described by the reaction model shown in Eqs. (1)–(4), where D is the drug of interest, L_1 through L_n are individual regions or binding sites on the protein and D–L_1 through D–L_n are the resulting drug–protein complexes:

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

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

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

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

In the above expressions, [ ] represents the molar concentration of each species in solution, k_{a1} through k_{an} are the second-order association rate constants for drug–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 individual binding sites.

The model in Eqs. (1)–(4) assumes that the individual binding regions on the protein have constant and independent affinities for the drug (i.e., the values of $K_{a1}...K_{an}$ are not affected by binding of the drug at other regions on the protein); this assumption does not hold when allosteric interactions are present, in which case more complex reaction models must be employed [1]. Another assumption in the above reaction scheme is that the binding of drug to given region can be described by a single-step, reversible process. In actuality, the binding process probably involves multiple steps (e.g., diffusion of drug to the protein and changes in the protein's conformation as a result of drug binding) [11,12]. However, even in these multi-step schemes the model in Eqs. (1)–(4) can still provide a useful approximation of the net reaction which takes place between the drug and protein.

Scatchard analysis is commonly used to determine the binding parameters for drug–protein systems [13]. This is based on measurements of the total fraction of drug bound per protein (r, or B/P) as a function of the concentration of drug that remains free in solution [D]:

$$r = \sum \{n_i K_{ai}[D]/(1 + K_{ai}[D])\} \quad (5)$$

In Eq. (5), n_i represents the moles of binding site i per mol of protein and all other terms are as defined earlier. Eq. (5) again assumes that the values for n and K_a at each site are independent and constant. This relationship is convenient for the study of solute–ligand binding in general 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 in the description of drug–protein interactions.

In the case of a single-site interaction, Eq. (5) reduces to following form [13]:

$$r/[D] = nK_a - K_a r \quad (6)$$

This equation predicts that a plot of $r/[D]$ vs. 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 drug–protein system. One problem associated with the use of Eq. (6) is

that the terms on the right and left-hand sides both depend to the same parameter (r), thus invalidating the use of normal linear regression techniques. This co-dependence also produces a non-uniform variance throughout plots made according to Eq. (6), a factor that must be considered when determining the reliability of binding parameters estimated from such plots.

When the concentration of a drug is much lower than that of the protein being tested, the following equation can be employed to describe the drug–protein binding process [14]:

$$K'_a = \sum n_i K_{ai} \quad (7)$$

In the above expression K'_a is a factor known as the global association constant, which represents a number-average association equilibrium constant for the system being studied. This term is typically used to describe drug–protein binding in methods that employ only small amounts of drug, such as the zonal elution methods described later in Section 2.1.3 Section 3.1.1.

In order to properly use the reaction model in Eqs. (1)–(4) and the related expressions given in Eqs. (5)–(7), it is necessary to consider the binding of each major form of the drug that exists under the conditions being tested. This is particularly important when working with chiral drugs, since the binding processes for many drug–protein systems can be stereoselective in nature [8–10]. One way in which this stereoselectivity can be produced is by the chiral forms of a compound having different binding affinities for the protein (e.g., the binding *R*- and *S*-warfarin to HSA at the same region) [15]. Stereoselectivity can also be produced by the chiral forms having different binding regions, such as the interactions of HSA with D- and L-tryptophan [16] or *R*- and *S*-oxazepam hemisuccinate [17].

2. Chromatographic techniques based on soluble proteins and drugs

There are several chromatographic methods that can be used to directly analyze the binding of drugs and proteins in solution. A previous review by Wood and Cooper [18] concentrated on formats based on low-performance supports, while a more recent survey by Sebille et al. [14] emphasized HPLC methods. Examples of both high- and low-performance methods were discussed in a text by Cserhati and Valko [19]. Many chromatographic methods for solution-phase studies 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 drugs from proteins or drug–protein complexes. Such supports can be used in three general formats to investigate the binding of soluble drugs and proteins. The first of these formats is zonal elution, which includes the techniques of direct drug and protein separation, peak-splitting measurements and the use of proteins as mobile phase additives. The second format is frontal analysis, and the last format is that of the vacancy techniques, which includes both the Hummel–Dreyer method and the equilibrium saturation (or vacancy peak) method.

2.1. Zonal elution techniques

2.1.1. Direct separation methods

The traditional chromatographic method of zonal elution (i.e., the injection of a small sample band onto a column) is one approach that can be used to study drug–protein interactions in solution. The easiest scheme to envision for this is an experiment in which the drug and protein are mixed together, allowed to equilibrate and injected as a sample onto a column for separation of the free drug and protein-bound fraction. Although this type of direct separation would seem at first glance to be straightforward, it is actually limited in terms of applications for drug–protein studies. This is the case because such a separation requires that there be little or no dissociation of the drug–protein complex during the time scale of the separation, a situation that is not present for many common drug–protein systems [14]. The kinetic requirements for this type of analysis have been previously examined from a theoretical viewpoint by Nimmo and Bauermeister regarding the dissociation of any ligand–protein complex during a size-exclusion separation [20].

There have been a few reported cases in which direct separation techniques have been successfully used to study drug–protein interactions. One exam-

ple is work by Loo et al. [21], who found that direct injection and zonal elution separations by HPLC gave good results for the interactions between prednisolone and corticosteroid-binding globulin (CBG), a system which has slow dissociation and a high binding affinity ($K_a = 3 \cdot 10^7$ M^{-1}). However, the same method produced significant dissociation for a system with weaker interactions (i.e., the binding of prednisolone to HSA, where $K_a = 2 \cdot 10^3$ M^{-1}). In work by Dixon [22] or Hoffman and Westphal [23] these differences in behavior were used as a means for examining the high affinity interactions between CBG and cortisol without interferences from weaker binding processes between cortisol and other plasma proteins.

2.1.2. Peak-splitting measurements

An interesting variation on the use of zonal elution for drug–protein binding studies concerns the analysis of drug and protein mixtures with intermediate dissociation rates on ISRP columns. On an ISRP support, the protein (and drug–protein complex) is excluded from the reversed-phase sites located only within the pores of the matrix; however, the non-bound fraction of the drug is able to enter the pores and access these sites, thus leading to its retention. In the case where drug–protein dissociation occurs at a rate comparable to the time scale of the separation, it is possible to get two drug peaks from a single sample injection. One of these peaks (i.e., that which elutes last from the column) corresponds to the amount of drug that was originally free in the sample or quickly released from low affinity proteins [24,25]. The other peak, which elutes earlier and is generally broader in appearance, corresponds to drug which was initially bound tightly to sample proteins but later dissociated as the sample traveled through the column (see Fig. 1 for an example).

Peak-splitting has been reported for several drug–protein systems with intermediate binding affinities. These systems include mixtures of bovine serum albumin (BSA) with warfarin [24,25], and human serum incubated with phenytoin [26,27] or imirestat [26]. As demonstrated in Fig. 1, the generation of peak-splitting behavior usually requires a relatively large sample volume, an item important to control in such studies [24–27]. The advantage of peak-splitting is that it can sometimes be used to simultaneously determine both the free and bound fractions of a drug in a single run, giving results for high or moderate affinity interactions that show good agreement with data obtained by reference methods [24–27]. However, applications for peak-splitting may be limited in scope, since this method does

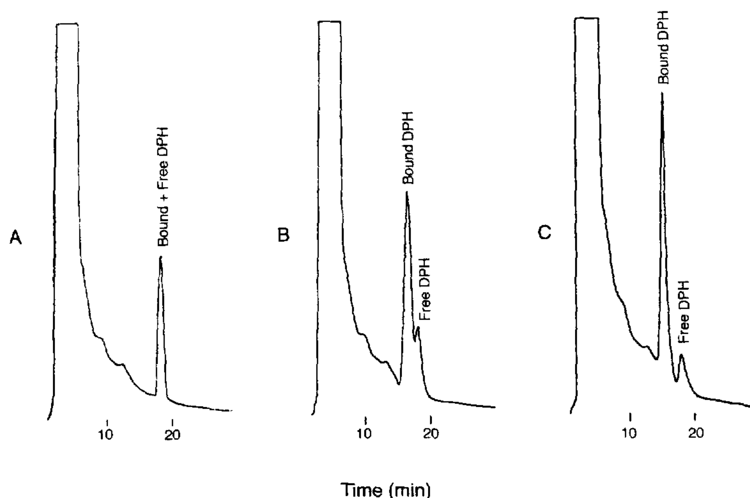

Fig. 1. Peak-splitting for phenytoin (DPH) in human serum. These results were obtained at 37°C on a 15 cm×4.6 mm I.D. ISRP silica column for 20 μg/ml phenytoin in human serum injected in sample volumes of (A) 100, (B) 200 or (C) 400 μl. The flow-rate in each run was 1.0 ml/min and the mobile phase was pH 7.4, 0.0125 M potassium phosphate buffer containing 1% tetrahydrofuran. This figure was reproduced with permission from Ref. [27].

require a test system with a specific set of kinetic properties. For instance, the drug–protein system must have dissociation that is fast enough to give quantitative release of the bound drug as it passes through the column and yet must be slow enough to allow the production of separate peaks for the free and bound drug fractions. An illustration of this problem is given by the work of Shibukawa et al. [25], who found that an ISRP column capable of separating the bound and free fractions of warfarin in the presence of BSA could not resolve these fractions for a mixture of antipyrine and BSA, a system with much weaker binding and a faster dissociation rate.

2.1.3. Use of proteins as mobile phase additives

One way that zonal elution can be modified for the study of drug–protein systems with fast dissociation is by using the protein as a mobile phase additive and injecting small samples of the drug into this mobile phase. When this is done for a low or intermediate molecular mass drug on a size-exclusion column, the binding of the protein to the drug should result in a shift in the drug's retention time (or elution volume) to lower values. By comparing the retention observed for the drug in the absence of protein and in presence of two more protein concentrations, it is possible to use the resulting shifts in retention to determine the global association constant (K'_a, as defined in Eq. (7)) for the drug–protein interaction [28,29].

Protein additives have been used to examine a number of drug–protein systems, including the binding of HSA to warfarin, phenylbutazone, furosemide [28], tryptophan [29,30] and omeprazole [30]. This approach can be employed to study the individual chiral forms of a racemic drug mixture (e.g., D- and L-tryptophan or the enantiomers of omeprazole) if these forms have significantly different protein binding properties [29,30]. Such methods have been performed with a variety of column supports, including both size-exclusion [28–30] and reversed-phase materials [30], but have the limitation of requiring a relatively large amount of protein per analysis.

One assumption made in this type of experiment is that the association and dissociation rates for the drug–protein interaction are rapid enough to allow the establishment of a local equilibrium between these two agents during the time spent by the sample within the column. This can be tested by comparing the results obtained at several different flow-rates to see if consistent binding parameters are produced. Another assumption often made in this technique is that the amount of injected drug is small vs. the amount of active protein in the mobile phase; to verify this, several concentrations of the sample can be injected to see whether or not uniform retention times and peak symmetries are being generated [28,29]. A practical consideration is the need to fully equilibrate the column with the protein additive before making sample injections. This is particularly important to evaluate when dealing with supports that may adsorb the protein (e.g., reversed-phase materials), since failure to reach full equilibration can lead to variable column behavior and non-reproducible sample retention times [30].

2.2. Frontal analysis

Frontal analysis is another chromatographic technique that can be use to examine drug–protein interactions in solution. This approach was first described by Nichol and Winzor for the analysis of protein–protein interactions [31] and was soon employed by Scholtan for the study of drug–protein binding [32]. Unlike zonal elution, frontal analysis is performed by applying to the column a large volume sample containing both the drug and protein of interest. If the column has different retention times for the drug vs. drug–protein complex and the drug–protein mixture is at a local equilibrium within the column, then the chromatogram that results should consist of several plateau regions (see Fig. 2a). When using a column that has longer retention for the drug than the protein (e.g., when working with a low molecular mass drug and a size-exclusion column), the first plateau region (or α-band) is produced by the non-complexed protein in the sample; this region may or may not be seen depending on the detection scheme being used to monitor drug elution. The second plateau region (the β-band) corresponds to the drug–protein complex, and the third region (the γ-band) represents the equilibrium concentration of the free drug fraction [33]. From the height of the γ-band and the known total concentration of the drug, the relative amount of drug bound per protein

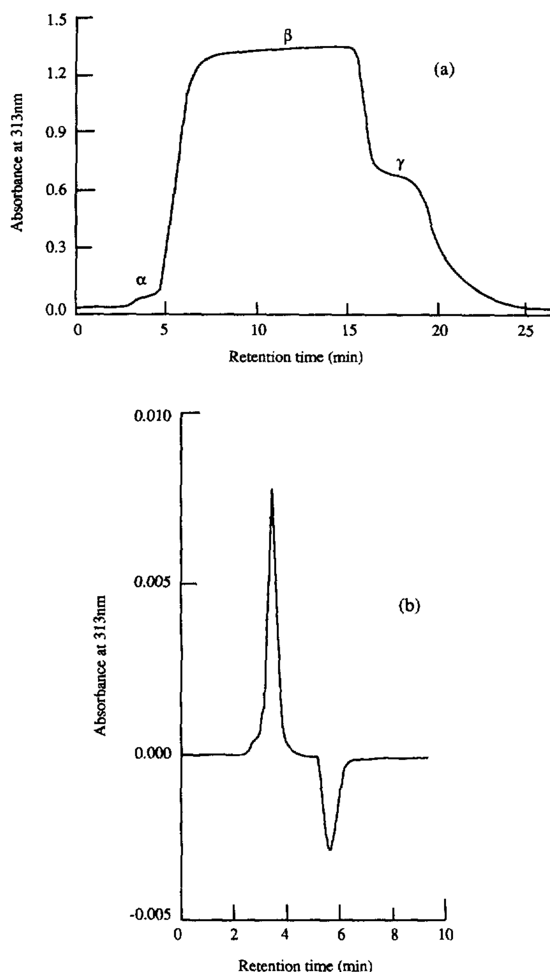

Fig. 2. Typical chromatograms for the binding of warfarin to HSA in (a) frontal analysis and (b) the Hummel–Dreyer method. The profile in (a) was obtained for an 18 ml mixture of 100 μ*M* warfarin and 2 g/l HSA applied at 37°C and 1.5 ml/min in pH 7.4, 0.067 *M* phosphate buffer to a 30 cm×3.9 mm I.D. μBondagel column. The results in (b) were generated using a 12.5 μl injection of 2 g/l HSA into a mobile phase containing 0.5 μ*M* warfarin in pH 7.4 phosphate buffer flowing at 0.5 ml/min through a 15 cm×4.2 mm I.D. Glycophase G column held at 37°C. This figure was reproduced with permission from Ref. [35].

(r) can be calculated. If this process is repeated at several drug concentrations, Scatchard analysis can be used to determine the binding parameters for the drug–protein system.

There have been many reported applications for frontal analysis in drug–protein studies [14,18,19], but most recent work has focussed on HPLC-based methods (i.e., high-performance frontal analysis, or HPFA). Applications of HPFA have included its use in investigations concerning the binding of BSA to warfarin and indometacin [33], and the interactions of HSA with warfarin [34–36], diazepam [37], carbamazepine [38], troglitazone [39] and fenoprofen [40]. 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. This latter approach has been employed to study the stereoselective protein binding of fenoprofen [40], warfarin [41], ketoprofen [42], nilvadipine [43] and BOF-4272 [44,45].

One disadvantage of frontal analysis is its need for large volume samples of the drug and protein to be studied. The typical sample volumes needed in past work with HPFA have often been in the range of 10–20 ml [33,35]; however, the required volume will depend on the degree of separation that can be obtained between the free drug and drug–protein complex. This has recently been examined by Shibukawa and Nakagawa, who used computer simulations to study the role of sample volume, degree of drug retention, column efficiency and drug–protein binding parameters on the appearance observed for HPFA chromatograms [46]. In practice, it has been shown that consideration of these parameters can allow the use of sample volumes as low as 80–400 μl when working with ISRP columns, which tend to give better resolution between the drug and drug–protein bands than ordinary size-exclusion supports [33,40].

One possible difficulty in working with frontal analysis is that sometimes it can be difficult to obtain a stable plateau region for the γ-band. This can occur if there is any retention of the drug or protein with the stationary phase and this retained species dissociates during the elution of the γ-band, thus distorting the appearance of this region. Another potential difficulty can occur when applying a large amount of protein to a column to which the protein is strongly retained. This can slowly change the column properties over time and give non-reproducible frontal analysis curves. However, frontal analysis also has a major advantage in that both the drug and protein in this technique start out at equilibrium and at known total concentrations. This helps to avoid any problems due to protein dilution or self-association that

may be seen in other approaches, such as the Hummel–Dreyer method (see Section 2.3.1) [14].

2.3. Vacancy techniques

This group of methods is based on the constant application of a mobile phase containing the protein and drug, or only drug, 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 drug 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 vs. protein-bound drug that is present in the system at equilibrium.

2.3.1. Hummel–Dreyer method

The most common vacancy technique, and perhaps the most frequently-used chromatographic approach for examining drug–protein interactions, is the Hummel–Dreyer method. This technique was originally described in 1962 by Hummel and Dreyer [47] and usually employs a column that contains a size-exclusion support. This method is performed by continuously applying to the column a mobile phase that contains a fixed, known concentration of the drug of interest. An injection of a small amount of protein is then made into the presence of this mobile phase. If the protein and drug have rapid association/dissociation kinetics and the column gives different retention times for the drug–protein complex vs. free drug, then a chromatogram similar to that shown in Fig. 2b should result. In this chromatogram, the first positive peak represents the eluting protein and its associated bound drug fraction. The second negative peak appears at the retention time expected for the free drug. Since this peak is produced by binding of sample protein with the drug in the mobile phase, the area of this peak can be used to help quantitate the amount of bound drug. This information can then be used with Scatchard analysis to obtain the binding constants and number of binding sites for the drug–protein interaction.

The Hummel–Dreyer method has been employed in the study of a large number of drug–protein systems [14,18]. Some examples are shown in Table 1. Hummel–Dreyer measurements of such systems are usually conducted on size-exclusion columns, but other types of columns have also been used. For example, Sebille et al. have described the use of an ion-exchange column to perform the Hummel–Dreyer method [34], and an ISRP column has been used by Pinkerton and Koeplinger to carry out this technique [36].

The amount of protein-bound drug in the Hummel–Dreyer method can be estimated from the drug vacancy peak by using an internal calibration method, in which the same protein sample is applied in the presence of several different drug concentrations [35,47]. External calibration can also be used, in which the absolute size of the vacancy peak is directly compared to the peak area measured for the same drug when injected onto the column in the presence of only the mobile phase buffer [48]. Both calibration methods have been shown to give similar results for certain model systems (e.g., the binding of warfarin with HSA) [49], but detector linearity should be examined in either case to ensure that accurate and consistent results are being obtained. The amount of bound drug can also sometimes be determined by examining the peak that corresponds to the drug–protein complex, especially when the drug or test solute contains a readily detectable label [50].

One requirement of the Hummel–Dreyer method is that the drug–protein interaction must have sufficiently fast association and dissociation kinetics to allow the establishment of a local equilibrium within the column. A second requirement is that there must be good resolution between the peaks that correspond to the drug–protein complex and free drug. The limited retention range of size-exclusion columns and the presence of any peak tailing can create problems with this last requirement. Peak tailing can be caused by interactions of the drug or protein with the support, by slow drug–protein interaction kinetics or by self-association of the injected protein. The last of these situations has been the subject of particular attention, as discussed in Refs. [51,52].

Table 1
Drug–protein systems examined by HPLC and the Hummel–Dreyer method

Protein	Drug/solute[a]	Conditions[b]	Reference
HSA	Warfarin	pH 7.4, 37°C	[35]
		pH 7.4, 23–25°C	[36]
		pH 7.4, 25°C	[49]
		pH 7.4, 37°C	[53]
	Furosemide	pH 7.4, 37°C	[35]
	Diazepam	pH 7.4, 37°C	[37]
	R/*S*-Isradipine	pH 7.4, 37°C	[153]
	Propranolol	pH 7.4, 37°C	[154]
BSA	Warfarin	pH 7.4, 25°C	[49]
	L-Tryptophan	pH 7.4, 25°C	[48]
AAG	*R*/*S*-Isradipine	pH 7.4, 37°C	[153]
	Propranolol	pH 7.4, 37°C	[154]
	(+)/(−)Propranolol	pH 7.4, 37°C	[155]
Tubulin	Colchicine	pH 7.2, 25°C	[156]
Low-density lipoproteins	Propranolol	pH 7.4, 37°C	[154]

[a] Unless otherwise indicated, racemic solute mixtures were used in the above studies.
[b] All studies were performed in 0.067 *M* phosphate buffer except for those in Refs. [48,156], which instead used a 0.05 *M* phosphate buffer.

The importance of these effects in creating peak overlap can be minimized by using an alternative type of column, such as one containing an ISRP support, that may provide better resolution between the peaks for the free drug and drug–protein complex.

2.3.2. Equilibrium saturation method

A technique that is closely related to the Hummel–Dreyer method is the equilibrium saturation, or vacancy peak, method. This approach was first reported by Sebille et al. in 1979 [53] and is performed with a size-exclusion column or related support that can resolve the drug and drug–protein complex of interest. However, in this method both the drug and protein are now 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 drug–protein complex and free drug, respectively. As in the Hummel–Dreyer method, the size of these peaks can be used along with internal or external calibration to determine the fraction of the free vs. bound drug at equilibrium. The experiment is conducted in the presence of several different drug–protein mixtures and the binding parameters are again obtained through Scatchard analysis [53].

The equilibrium saturation method has been used to examine the interactions of HSA with diazepam [37], and the effect of fatty acids [53] or sodium dodecyl sulfate [54] on the binding of HSA to warfarin. Many of the requirements for this approach are the same as in the Hummel–Dreyer method, including the need for fast drug–protein interaction kinetics and good resolution between the peaks for free drug and drug–protein complex. Although the equilibrium saturation method does require more protein than the Hummel–Dreyer method, it also has a number of advantages vs. this other technique. For example, the fact that the protein and drug are applied at fixed concentrations to the column avoids the problems with protein dilution that can occur in the Hummel–Dreyer method. The presence of protein in the mobile phase as a binding agent is also appealing since this helps keep drugs in solution that may have low solubility in aqueous buffers [14].

3. Chromatographic techniques based on immobilized proteins or drugs

The use of an immobilized ligand in a chromatographic system for the study of biomolecular interactions is a method known as analytical or quantitative affinity chromatography. This may be performed on either a low-performance or high-performance support; when using silica or another HPLC-type matrix, the method is called high-performance affinity chromatography (HPAC). A book edited by Chaiken gives a general overview of the experimental approaches used in analytical affinity chromatography [55], while recent reviews by Wainer [56] or Cserhati and Valko [19] have focused on some applications concerning drug–protein binding. The two main categories of techniques in such studies are zonal elution and frontal analysis, but some work with vacancy methods has also been reported. Each of these categories will be examined in detail in the following section.

Early work in the area of analytical affinity chromatography concentrated on the use of various low-performance gels (e.g., agarose), but HPLC-grade supports (e.g., silica) are now common in such work [14,55,56]. To study drug–protein interactions by affinity chromatography, either the protein or drug (or some related analog) can be used as the immobilized ligand, but most previous reports have employed protein-based columns [14]. Several manufacturers now supply activated matrixes that can be used for protein immobilization [57] and a number of proteins of interest in drug binding studies (e.g., HSA, BSA and AAG) can be obtained already attached to silica or agarose [14,55,58]. Alternatively, one of various literature methods can be used for protein immobilization [57]. Many of these same procedures can also be used for attaching a small ligand (e.g., a drug or drug analog) to a support for the preparation of an affinity column [57].

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. This is of potential concern since the immobilization process can affect protein activity through denaturation, improper orientation or steric hindrance of the protein at the binding sites to be studied [59]. 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 [16,60,61]. Fortunately, there is growing evidence that at least some immobilized proteins can be successfully used for the study of drug–protein interactions. For example, it has been shown that K_a values measured by equilibrium dialysis for soluble HSA with *R*- and *S*-warfarin or L-tryptophan (i.e., solutes that interact with one of the two major binding regions of HSA) are in close agreement with K_a values determined using immobilized HSA columns [15,16,60] (see Table 2). It has also been found that displacement phenomena and allosteric interactions observed for HSA columns are representative of the behavior noted for HSA in solution [17,60,62–65]. On the other hand, silica-based supports containing immobilized AAG, which are useful in performing HPLC-based chiral separations [58], have been reported in at least one study to exhibit different displacement properties than AAG in solution [66], a feature that may be due to ionic interactions between the silica and sialic groups on AAG [67].

One advantage of using an immobilized protein column for binding studies is the ability to reuse the same ligand preparation for multiple experiments. For instance, columns containing HSA immobilized to silica have been used for up to 500–1000 injections in some cases [61,68,69]. This creates a situation in which only a relatively small amount of protein is needed for a large number of studies and helps to give good precision by minimizing run-to-run variations. However, if the protein can not be immobilized in a suitably active form, then a ligand based on an immobilized analog of the drug of interest can instead be used. Although this approach avoids the problems of denaturation or inactivation that can be associated with an immobilized protein, steric hindrance can still be an important factor. Particular care must be taken to use a spacer arm between the immobilized drug or ligand and the surface of the support in order to allow sufficient access of this compound to the protein for binding [59]. The point of attachment between a small ligand and the support is also important in that it can affect the types of interactions which take place with the

Table 2
Comparson of association constants measured at 37°C for *R*- and *S*-warfarin to soluble and immobilized HSA[a]

Type of warfarin	Type of HSA	Association constant, K_a ($M^{-1} \cdot 10^5$)[b]	Method of measurement	Reference
R-Warfarin	Immobilized	2.1 (±0.2)	Frontal analysis	[15]
	Immobilized	3.3	Zonal elution	[8]
	In solution	2.5	Equilibrium dialysis	[157]
	In solution	2.06 (±0.02)	Equilibrium dialysis	[158]
S-Warfarin	Immobilized	2.6 (±0.4)	Frontal analysis	[15]
	Immobilized	4.4	Zonal elution	[8]
	In solution	5.69	Equilibrium dialysis	[157]
	In solution	2.44 (±0.04)	Equilibrium dialysis	[158]
Racemate	Immobilized	2.03	Frontal analysis	[159]
	In solution	2.31	Equilibrium dialysis	[160]
	In solution	2.3 (±0.02)	Equilibrium dialysis	[161]

[a] This table was adapted with permission from Ref. [15].
[b] Values in parentheses represent ±1 S.D. All association constants were measured at pH 7.4 except those from Ref. [158], which were determined at pH 10.0.

protein. One way around this last problem is to also apply the drug as a mobile phase additive and examine how protein retention changes with the additive's concentration, thus allowing the affinity column to be used as a direct probe for solute–protein binding in solution [14,55].

3.1. Zonal elution techniques

Zonal elution methods have frequently been used to study the binding and competition of drugs and other solutes on immobilized protein columns [14,56]. This approach was first reported for the study of biological interactions by Dunn and Chaiken [70] and was later adapted by Lagercrantz and co-workers for use in drug–protein studies [8,71]. This technique assumes that relatively fast association/dissociation kinetics are present for the test system. It 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 [72]. 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 capacity factor (k') for the injected solute changes as a function of competing agent concentration, with k' being given by the term $(t_r/t_m - 1)$, where t_r is the mean measured retention time for the injected solute and t_m is the column void time. Fig. 3 shows an example of a typical zonal elution study and the relationship seen between k' and competing agent concentration for a system with 1:1 competition between the sample solute and competing agent.

3.1.1. Binding and displacement studies

A number of reports have used zonal elution to study drug–protein interactions by simply injecting the drug of interest onto an immobilized protein column in the presence of only buffer. This type of experiment can be used to provide information on the bound fraction for a drug, since the capacity factor itself is a direct measure of the relative mol of drug at equilibrium that is bound to the immobilized protein vs. free in the mobile phase. Based on the definition of k', it has been shown that a relatively strong correlation exists between the term $k'/(k'+1)$, which is a measure of the bound fraction of drug in the column, and the percent of drug–protein binding that is observed in solution-phase studies (see Fig. 4). This approach has been used as a tool to compare the percent binding of HSA with various benzodiazepines, coumarins and triazole derivatives [73].

Other reports have used k' as a direct measure of binding affinity to examine how various solvent conditions affect drug–protein interactions. In the

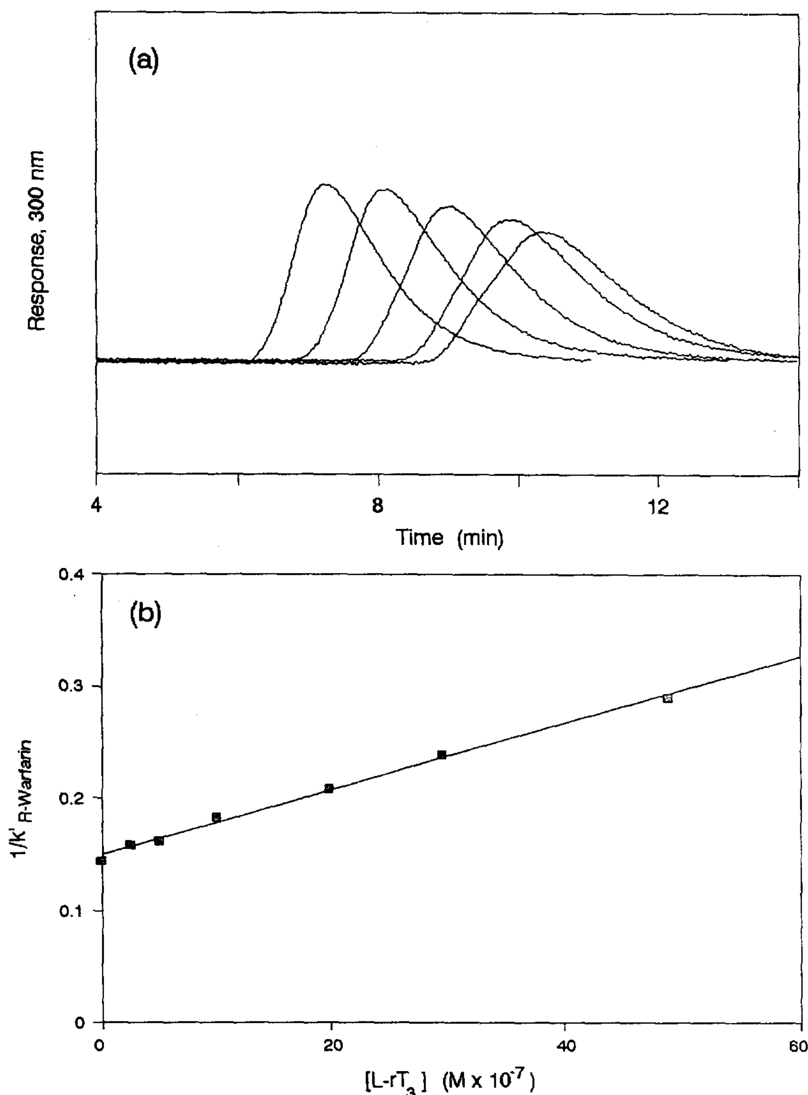

Fig. 3. (a) Chromatograms and (b) relationship between k' and ligand concentration for zonal elution experiments examining the competition of *R*-warfarin with L-reversed triiodothyronine (L-rT$_3$) for binding sites on immobilized HSA. These studies were conducted at 37°C in pH 7.4, 0.067 *M* phosphate buffer. In (a) 20 μl samples of 6.5 μ*M* *R*-warfarin in the appropriate mobile phases were injected at 0.5 ml/min into the presence of mobile phases containing (left to right) 1.90, 0.97, 0.49, 0.24 or 0 μ*M* L-rT$_3$. The resulting data were then plotted according to Eq. (10), as shown in (b). The column was 4.5 cm×4.1 mm I.D. and contained HSA immobilized to diol-bonded Nucleosil Si-1000. This figure was adapted with permission from Ref. [61].

case of a drug that is injected onto a column that contains a protein with a series of binding sites L_1 through L_n, the following relationship shows that k' is a direct measure of the global association constant (K'_a):

$$k' = (K_{a1}n_1 + \dots K_{an}n_n)m_{L,tot}/V_m \quad (8)$$

$$= K'_a(m_{L,tot}/V_m) \quad (9)$$

where V_m is the column void volume, $m_{L,tot}$ is the

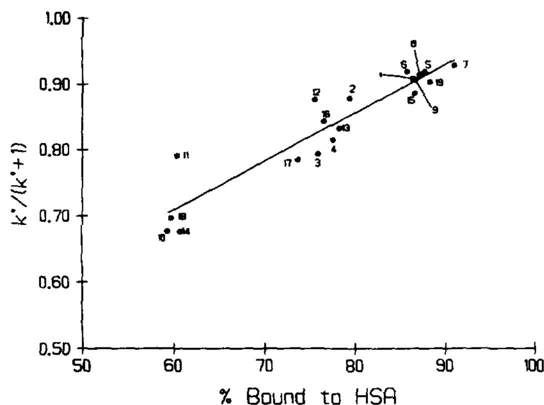

Fig. 4. Relationship between $k'/(k'+1)$ measured on an immobilized HSA column and observed degree of benzodiazepine binding to HSA in solution. The benzodiazepines tested were as follows: 1, chlordiazepoxide; 2, clorazepate; 3, flunitrazepam; 4, clonazepam; 5, desmethyldiazepam; 6, delorazepam; 7, diazepam; 8, lormetazepam; 9, oxazepam; 10, alprazolam; 11, triazolam; 12, clobazam; 13, norfludiazepam; 14, oxazolam; 15, flurazepam; 16, prazepam; 17, estazolam; 18, bromazepam and 19, temazepam. This figure was reproduced with permission from Ref. [73].

total mol of immobilized protein in the column, and all other terms are the same as defined earlier. Based on this general relationship, k' measurements have been used in a number of studies with HSA, BSA, AAG and other immobilized proteins to examine how their mechanisms of retention change when varying solute structure or mobile phase composition (e.g., pH, ionic strength or organic solvent content). Examples of such experiments are provided in Refs. [16,58,67,74–79]. An alternative approach is to monitor the elution of the protein when injected on a series of columns containing different drug analogs, as used by Rochette-Egly et al. to investigate the binding of calmodulin to various phenothiazines [80]. In either format, some caution must be exercised when using k' as a direct measure of binding affinity under non-physiological conditions, since such circumstances may cause variations in either K_{ai} or n_i, thus creating more than one factor that can create changes in the measured k' values [81].

A related use for k' values has been in the development of quantitative structure-retention relationships (QSRRs) for the binding of drugs to immobilized protein columns [82,83]. This involves collecting k' values, or other types of retention information, under constant temperature and mobile phase conditions for a large set of drugs representing a range of structural variations. 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 [56]. This approach has been used by Kaliszan and co-workers to examine the binding of various 1,4-benzodiazepines to immobilized HSA [82,83]. Based on the QSRR equations that were developed, it was hypothesized that two different types of binding regions were involved in retaining the *M*- vs. *P*-conformation of benzodiazepines [83].

The most common application of zonal elution affinity chromatography in drug–protein studies has been as a tool for examining the displacement of drugs from proteins by other solutes, as illustrated by Fig. 3. This topic was discussed in a recent mini-review by Noctor and Wainer [84]. Examples based on HPLC columns have included the use of zonal elution to examine the displacement of D/L-thyronine and D/L-tryptophan from immobilized HSA by bilirubin or caprylate [85], the competition of *R*/*S*-warfarin with racemic oxazepam, lorazepam and their hemisuccinate derivatives on an HSA column [63], 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 and suprofen A/B [65], the competition of *R*-warfarin and L-tryptophan with D-tryptophan [16] or L-thyroxine and related thyronine compounds on immobilized HSA [60,61], and the displacement of *R*- and *S*-ibuprofen by one another at their binding regions on HSA [86]. The same technique has been used to characterize the binding sites of non-steroidal anti-inflammatory drugs on HSA [87], 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 [88].

3.1.2. Equilibrium constant measurements using immobilized proteins

Zonal elution experiments with immobilized protein columns can be used to provide not only qualitative information on binding and displacement

but also quantitative information on the strength or degree of these processes. For instance, the data in Fig. 3 represent a case in which an injected drug (D) has competition with a mobile phase additive (A) at a single class of sites on the protein. If no other types of binding sites for D are present in the column, then Eq. (10) shows the relationship that would be expected between the capacity factor measured for D and the concentration of the mobile phase additive [60]:

$$\frac{1}{k'} = \frac{K_{aA} V_m [A]}{K_{aD} m_L} + \frac{V_m}{K_{aD} m_L} \qquad (10)$$

In Eq. (10), K_{aA} and K_{aD} are the association equilibrium constants for the binding of A and D at the site of competition, [A] is the molar concentration of the mobile phase additive and m_L is the mol of common binding sites for D and A. This equation predicts that a system with single-site competition will give a linear plot for $1/k'$ vs. [A], as shown in Fig. 3b. By determining the ratio of the slope to the intercept for this plot, the value of K_{aA} can be obtained. If a separate estimate of m_L/V_m is made (e.g., by frontal analysis affinity chromatography, as described in Section 3.2), then the value of K_{aD} can also be determined from the intercept. Similar expressions can be derived for other situations, such as for drugs and additives with multiple sites of competition or injected solutes that have other binding sites which do not interact with the mobile phase additive [65,86]. The latter case is particularly common [65,82,86–88] and is performed by simply replacing the left-hand term of Eq. (10) by the expression $1/(k' - X)$, where X is a constant for the injected solute that represents its k' value due to all regions that are not involved in the competitive binding processes [65].

There are now several reports in which Eq. (10) and related expressions have been used to quantitate the equilibrium constants for drug–protein interactions. Some examples include those provided in Refs. [60,61,65,82,86–88], as described in Section 3.1.1. One particular advantage of zonal elution experiments is that they can be designed for determining association constants at specific binding regions for solutes that have multi-site interactions to the immobilized protein. As indicated by Eq. (10), this can be done by applying the solute of interest as the mobile phase additive while making injections of probe compounds that are known to have single-site interactions at the binding sites to be tested. Such an approach has been used to investigate the mechanisms involved in the binding of thyroid hormones and related compounds to the warfarin and indole sites of HSA (e.g., see Fig. 3) [60,61].

3.1.3. Equilibrium constant measurements using immobilized drugs

Several papers have used small ligands to investigate the binding of enzymes with immobilized inhibitors or co-factors (e.g., see review provided in Ref. [55]), but only a few reports have extended this same approach to the study of drug–protein interactions. Two specific examples include work by Rochette-Egly et al., who studied the binding of immobilized phenothiazines to calmodulin [80], and work by Veronese et al., who used immobilized perphenazine to examine the binding of soluble phenothiazines and butyrophenones to bovine glutamate dehydrogenase [89].

Veronese and co-workers were able to measure association constants in their work by using the following relationship [89]:

$$\frac{1}{V - V_0} = \frac{K_{aID}}{(V_0 - V_m)[ID]} + \frac{K_{aID}[D]}{K_{aD}(V_0 - V_m)[ID]} \qquad (11)$$

In Eq. (11), V represents the elution volume for the protein in the presence of soluble drug at a concentration of [D], V_0 is the protein elution volume in the absence of the immobilized ligand, V_m is the column void volume and [ID] is the effective concentration of the immobilized drug in the column. The term K_{aID} is the association equilibrium constant for the binding of protein to the immobilized drug and K_{aD} is the association constant for the interactions of the protein and soluble drug. A plot made according to Eq. (11) should give a linear relationship for a system with single-site competition. Note that by taking the ratio of the intercept to slope, the value of K_{aD} is obtained, thus allowing the affinity column to be used in the direct measurement of solution-phase binding constants.

3.1.4. Rate constant measurements

Zonal elution experiments can also be adapted to examine the kinetics of drug–protein interactions.

This is illustrated in recent reports by Hage and co-workers, in which plate height measurements were used to study the association and dissociation kinetics of *R*/*S*-warfarin [68] and D/L-tryptophan [69] on immobilized HSA columns. In this approach, Van Deemter-type plots were used to estimate the various plate height contributions for these solutes on the immobilized protein supports. The plate height contribution due to stationary phase mass transfer (H_s) is of particular interest in such studies since it is directly related to the dissociation rate constant between the injected solute and the immobilized ligand (k_d), as shown by Eq. (12),

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

where u is the linear velocity of mobile phase in the column and k' is the capacity factor of the injected solute. Based on Eq. (12), a plot of H_s vs. $uk'/(1+k')^2$ should give a slope of $2/k_d$ and an intercept of zero. Some typical Van Deemter plots and graphs made according to Eq. (12) are provided in Fig. 5 for D-tryptophan and HSA [69]. By using the k_d values obtained from these plots along with independent estimates for the equilibrium constants of the system, the association rate constants for the drug and protein can also be obtained. Such kinetic experiments require the presence of relatively fast association and dissociation between the injected drug and immobilized protein so that multiple binding steps can occur as the drug passes through the column. In addition, careful control and monitoring of several experimental conditions (e.g., solute retention, temperature and flow-rate) must be used in these studies in order to allow accurate and precise plate height measurements [68].

For the studies involving *R*- and *S*-warfarin, the dissociation and association rate constants were evaluated at pH 7.4 and at several temperatures over the range of 4 to 45°C. From this data it was possible to estimate the activation energies and changes in enthalpy or entropy that occurred during formation of the warfarin-HSA activated complex [68]. In the work with D- and L-tryptophan, the changes in association and dissociation rate constants were examined as a function of temperature as well as pH, ionic strength and solvent polarity (i.e., organic modifier content). From the results of this study, it was possible to determine the role that various forces play in forming the activated complex between HSA and each solute. The results of the tryptophan study were also used to illustrate the importance of considering kinetics and band-broadening in the design and optimization of protein-based chiral separations [69].

3.2. Frontal analysis

Frontal analysis affinity chromatography was first used in 1975 by Kasai and Ishii to study biological interactions [90] and was soon employed by Lagercrantz et al. to the investigate the binding of fatty acids and drugs to BSA [71]. In this technique, a solution containing a known concentration of the solute to be studied 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 examples shown in Fig. 6). If fast association and dissociation kinetics are present in the system, then the mean positions of the breakthrough curves can be related to the concentration of applied solute, the amount of ligand in the column and the association equilibrium constants for solute–ligand binding.

In the area of drug protein interactions, frontal analysis affinity chromatography has been used to investigate the binding of HSA to *R*- or *S*-warfarin [15,60] and D- or L-tryptophan [16,60,65,81], and the binding of salicylate to BSA [91]. It has also been used to determine the binding capacities of monomeric vs. dimeric HSA for salicylic acid, warfarin, phenylbutazone, mefenamic acid, sulphamethizole and sulphonylureas [92] and to examine the competition of sulphamethizole with salicylic acid for HSA binding regions [93], or salicylate with clofibric acid, octanoic acid or oestradiol for sites on BSA [71].

The results obtained in a frontal analysis experiments can be examined by Scatchard plots [71,91–93], but alternative methods for data treatment can also be used [15,16,60,65,81]. For example, in the case where an applied drug (D) binds to a single type of immobilized ligand site (L), the following equation can be used to relate the true number of active binding sites in the column (m_L) to the apparent mol of drug ($m_{L,app}$) required to reach the mean position

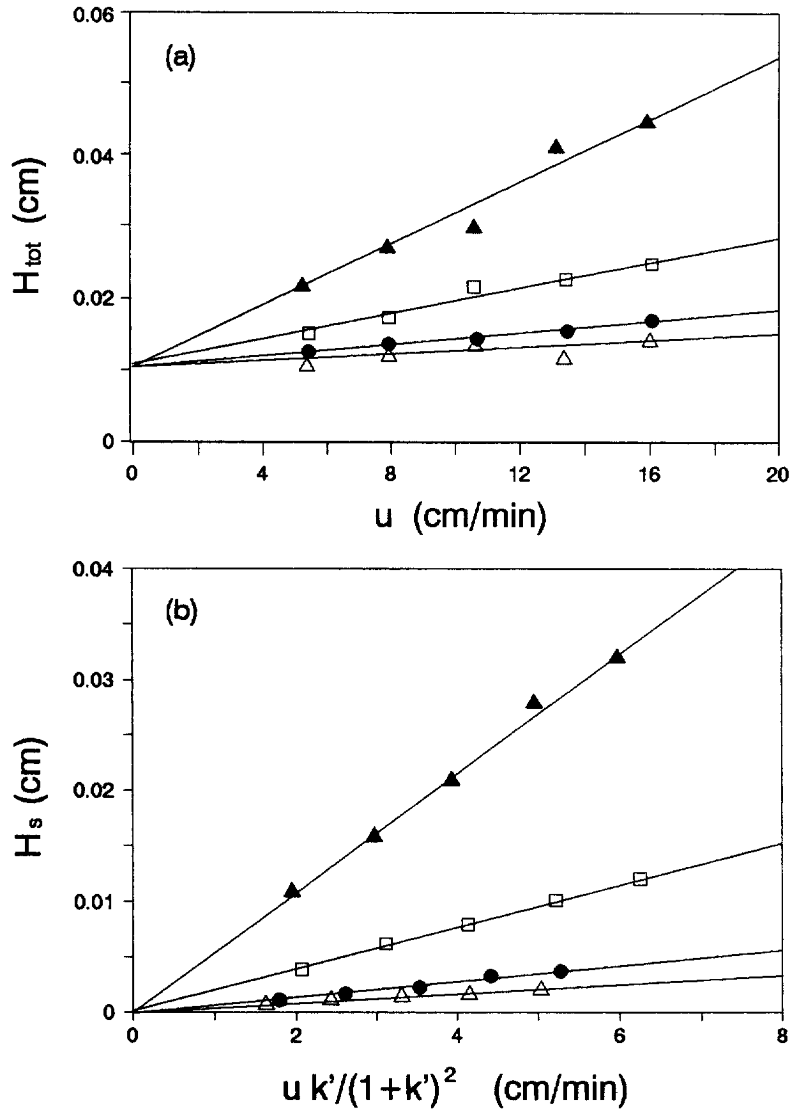

Fig. 5. (a) Van Deemter plots of total plate height (H_{tot}) vs. linear velocity u and (b) plots of the plate height contribution due to stationary phase mass transfer (H_s) vs. $[uk'/(1+k')^2]$ for D-tryptophan injected onto an immobilized HSA column at 25°C and mobile phase pH values of 4.0 (△), 5.0 (●), 6.0 (□) or 7.0 (▲). These data were generated with 20 μl samples of 10 μM D-tryptophan injected into pH 7.4, 0.067 M phosphate buffer as the mobile phase. A 10 cm×4.1 mm I.D. column was used that contained HSA immobilized onto diol-bonded Nucleosil Si-300. This figure was reproduced with permission from Ref. [69].

of the breakthrough curve in the absence of any competing agent [81]:

$$\frac{1}{m_{\mathrm{L,app}}} = \frac{1}{K_a m_{\mathrm{L}}[\mathrm{D}]} + \frac{1}{m_{\mathrm{L}}} \quad (13)$$

In Eq. (13), K_a is the association constant for the binding of D to L, and [D] is the molar concentration of drug applied to the column. Eq. (13) predicts that a plot of $1/m_{\mathrm{L,app}}$ vs. $1/[\mathrm{D}]$ for a system with single-site binding will give a straight line with a slope of $(1/K_a m_{\mathrm{L}})$ and an intercept of $1/m_{\mathrm{L}}$ (see Fig. 6b). In this case, K_a can be determined by calculating the ratio of the intercept to the slope, and m_{L} is

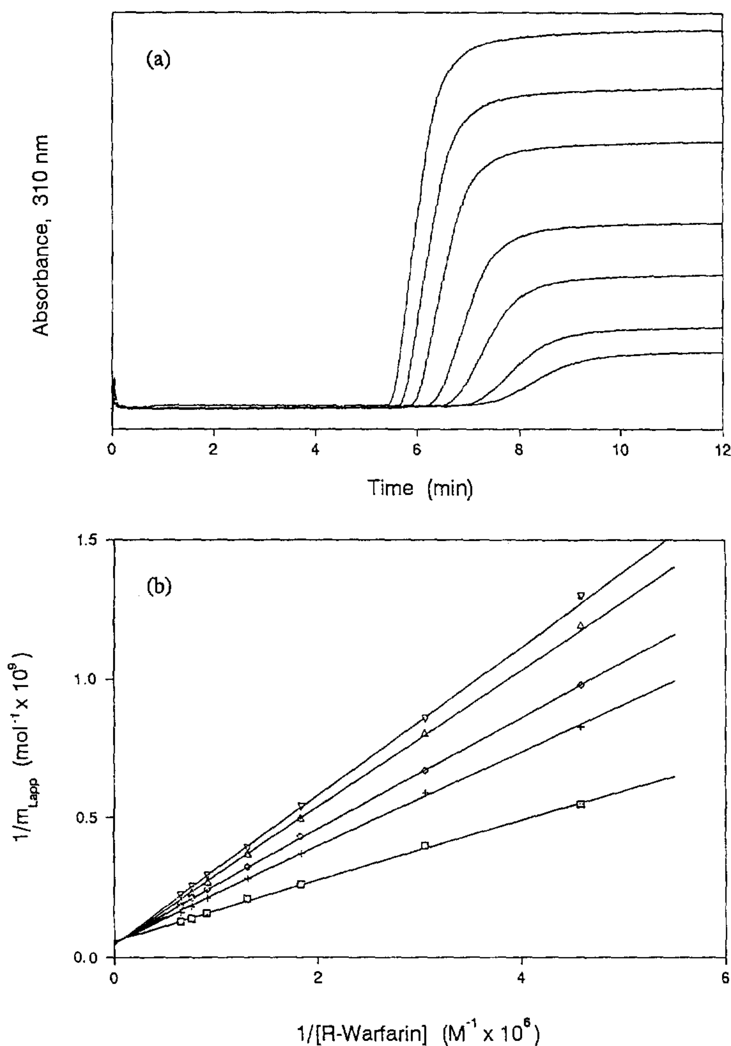

Fig. 6. (a) Typical chromatograms obtained for the frontal analysis of *R*-warfarin on an immobilized HSA column at 4°C and (b) plots made according to Eq. (13) for data obtained at temperatures of 4 (■), 15 (+), 25 (◊) 37 (△) and 45°C (▽). In (a) the *R*-warfarin concentrations (left to right) were 1.50, 1.30, 1.10, 0.76, 0.55, 0.33 and 0.22 μ*M*. The mobile phase buffer was pH 7.4, 0.067 *M* phosphate buffer applied to a 4.5 cm×4.1 mm I.D. column packed with HSA immobilized onto diol-bonded Nucleosil Si-1000. This figure was adapted with permission from Ref. [15].

obtained from the inverse of the intercept. Similar relationships can be derived for more complex systems, such as those with multi-site interactions or competitive binding between the applied solute and a known concentration of a mobile phase additive [55].

One disadvantage of frontal analysis is the relatively large amount of solute that is required for each study. However, it also has a distinct advantage versus zonal elution affinity chromatography in that frontal analysis can simultaneously provide information on both the association constant for a solute and its total number of binding sites in a column. This feature makes frontal analysis valuable in monitoring the stability of affinity columns during their use in the long-term studies [15]. In addition, the same feature makes frontal analysis the method of choice for accurate association constant measurements between a solute and an immobilized protein,

since the resulting K_a values are essentially independent of the number of binding sites present in the column [15,81]. For example, this approach has recently been used to examine changes in both the association constants and number of binding sites for D- and L-tryptophan on immobilized HSA under various pH, ionic strength and solvent polarity conditions [81], and to quantitate the changes in binding affinity and capacity that accompany the interactions of *R*- and *S*-warfarin with immobilized HSA at a variety of temperatures [15].

3.3. Vacancy techniques

Like size-exclusion or ISRP supports, affinity columns can be employed in vacancy techniques such as the Hummel–Dreyer method. This was recently demonstrated in work by Soltes et al. [94]. In this study, a HPLC column containing an immobilized BSA silica support was placed in the presence of a mobile phase that contained a mixture of D- and L-tryptophan at a fixed concentration. The different affinities and retention times for these enantiomers were then used to simultaneously study the binding of each solute to BSA or other ligands. For example, 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 [94].

4. Electrophoretic techniques based on soluble proteins and drugs

Like chromatography, electrophoresis can be used as a tool to study drug–protein interactions. Many of the same approaches can be used in electrophoresis 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. Both Cann [95] and Takeo [96] have written recent reviews concentrating on the use of traditional gel electrophoresis methods for the study of biological interactions. A past review by Hořejší and Tichá [97] has also appeared on this topic, with an emphasis on methods that use immobilized ligands.

A relatively new development in the study of biomolecular interactions by electrophoresis has been in the development of the technique known as affinity capillary electrophoresis (ACE). As its name implies, this method is performed as part of a capillary electrophoresis (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. A fairly large number of papers have appeared in the last few years describing the use of ACE for drug-binding studies. Recent reviews by Goodall [98] and Chu et al. [99] have discussed the use of CE for such work.

4.1. Zonal elution techniques

This group of methods makes up the largest category of modern electrophoretic approaches used for the study of drug–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.

4.1.1. Direct separation methods

As was the case for the chromatographic methods in Section 2.1, zonal elution in electrophoresis is only seldom used for the direct separation of the free and protein-bound fractions of a drug. The reason for this is the same as discussed earlier in that the dissociation kinetics for many drug–protein complexes are too rapid to allow a separation of free and bound drug on the time scale of a typical electrophoretic run [95]. However, there are a few exceptions to this. For example, many protein–protein or protein–DNA interactions have relatively slow dissociation and can be analyzed by such an approach [95]. One example is a report by Rose in which capillary gel electrophoresis (CGE) was used to investigate the binding kinetics and stoichiometry between antisense peptide nucleic acids and their complementary oligonucleotide sequences (see Fig. 7) [100]. Direct separations have also been used to study the binding of anionic carbohydrates [101] or oligonucleotides [102] to synthetic peptides derived from human serum amyloid P component, as well as the binding of procainamide and related compounds to hemoglobin and histone proteins [103]. In addition, direct separations are employed in immunoassay methods based on CE, where the CE system is used to separate the bound and free fractions of an analyte in the presence of antibodies or antibody-related fragments. Applications for this last approach in the area of pharmaceutical or clinical testing include assays developed for human growth hormone [104,105], insulin [106–108], cortisol [109–111], digoxin [112,113], opiates [114] and chloramphenicol [115].

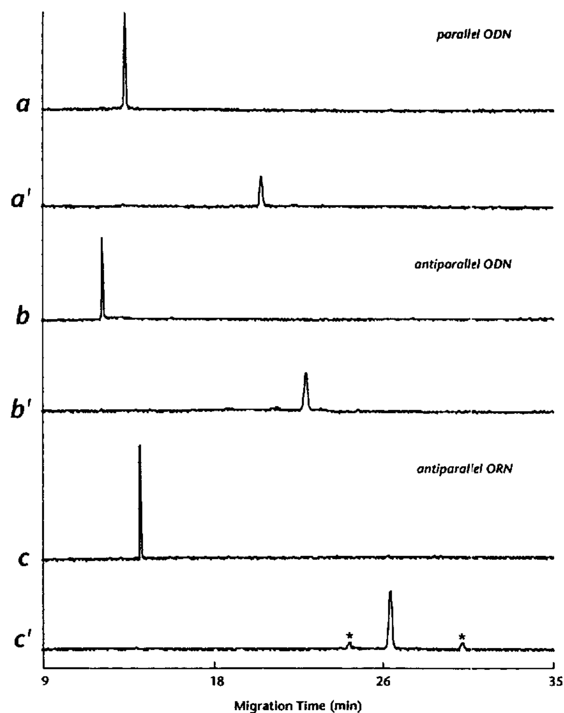

Fig. 7. Electropherograms obtained in capillary gel electrophoresis studies on the binding of peptide nucleic acids (PNAs) with complementary strands of oligodeoxynucleotide (ODN) or oligoribonucleotide (ORN). The results in a, b and c show runs obtained for parallel ODN (a), antiparallel ODN (b) and antiparallel ORN (c) in the absence of PNA. The data in a′–c′ were obtained for the same oligonucleotides after reaction with PNA and show the peaks that were produced by the resulting oligonucleotide–PNA complexes. The running buffer was pH 7.0, 75 m*M* Tris–phosphate containing 10% methanol and the capillary was a 50 cm×50 μm I.D. Micro-Gel 100 packed column operated at a voltage of −15 kV. This figure was reproduced with permission from Ref. [100].

4.1.2. Mobility shift assays

Mobility shift assays are the way in which drug–protein interactions are usually studied by 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 vs. 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 (e.g., see Fig. 8). This shift in peak position can be described by using the change in migration time or velocity of the solute observed at each ligand concentration. Another parameter often used to describe the solute migration in these assays is the solute's net electrophoretic mobility (μ_{Net}). The value of this term can be calculated by using the expression $\mu_{Net} = (L_{eff}L_{tot})/(tV)$, where t is the solute's measured migration time, L_{eff} is the effective capillary length from the injection end to the detector, L_{tot} is the total length of the capillary and V is the applied voltage in the CE system. Note that the value of μ_{Net} actually represents the combined effect of the inherent mobility of the solute (μ) and the mobility due to

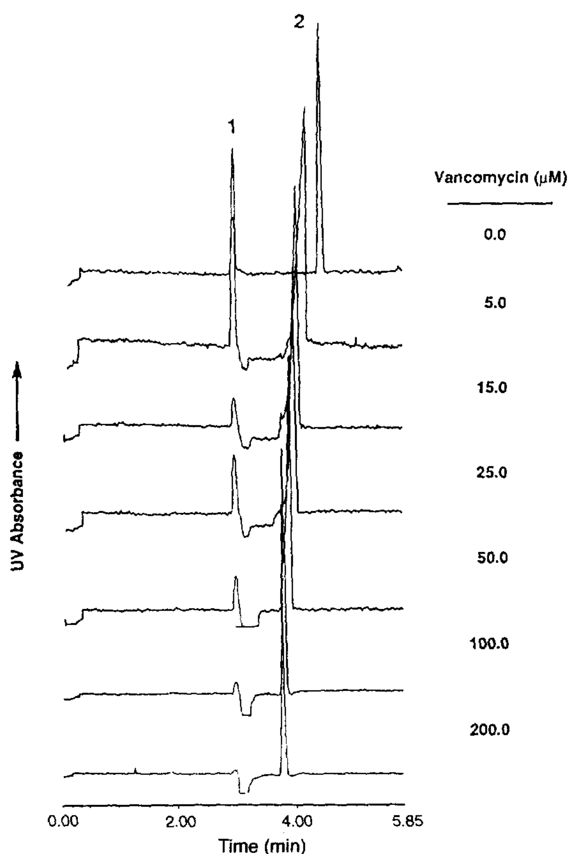

Fig. 8. Mobility shift assay for the binding of vancomycin to injected samples of a pentapeptide percursor from *S. aureus* 209P. Peak 1 represents a neutral marker (mesityl oxide) and peak 2 is the pentapeptide precursor. The running buffer contained vancomycin in pH 8.3, 0.2 *M* glycine–0.03 *M* Tris. An untreated 57.3 cm×50 μm I.D. fused-silica capillary was used along with an operating voltage of 25 kV. This figure was reproduced with permission from Ref. [122].

electroosmotic flow (μ_{eo}) in determining the actual migration seen for the injected solute.

In the last five years there have been a number of applications reported for ACE in mobility shift assays involving drugs and proteins or related systems. Examples include work using ACE to examine the binding of leucovorin with BSA [116], D/L-tryptophan with HSA [117], vancomycin with peptides [98,118–123], human or bovine carbonic anhydrase with arylsulfonamides [121,124–127] or zinc ions [128], deoxyspergualin to heat shock proteins [129], Src Homology III domains with peptides from receptor proteins [130], calcium ions and phosphorylcholine with human C-reactive protein [131], calcium ions with calmodulin or parvalbumin [128], serum amyloid P component with heparin or chondroitin sulfate [132] and antibodies with phosphotyrosine [133] or *N*-2,4-dinitrophenyl compounds [134] as the antigen.

Another group of studies based on the mobility shift assay are those that have examined the interactions of drug enantiomers with protein buffer additives as a means for performing chiral separations in CE. For example, Busch et al. explored the use of orosomucoid, ovomucoid, fungal cellulase and BSA for separating the enantiomers of warfarin, benzoin, tryptophan, promethazine and pindolol [135]. Cellulase was similarly employed as a running buffer additive by Valtcheva et al. for the stereoselective separation of various β-blockers [136]. Vespalec and co-workers studied the use of HSA in the analysis of chiral amino, mono- or dicarboxylic acids [137], while Arai et al. used BSA as a chiral selector for quinolone bactericidal reagents [138]. The mechanisms of protein-based chiral separations based on HSA were investigated by Yang and Hage for the separation of D/L-tryptophan and *R*/*S*-warfarin [139]. Lloyd et al. used CE to examine the binding of benzoin enantiomers or phenothiazine derivatives to soluble HSA in the presence of various displacing agents [140]. In addition, this group has compared the chiral separation conditions required for benzoin in HPLC and CE methods based on HSA as a stereoselective binding agent [141]. A similar comparison has been made by other workers for the separation of β-blocker enantiomers using cellulase as a chiral ligand [136].

One assumption made in zonal ACE studies of drug–protein binding is that the drug and protein are only interacting in solution. This can be problem when working with a physiological buffer since many proteins have a tendency to adsorb to normal fused-silica CE capillaries under such conditions [102,116,120,124,139,140,142,143]. It is possible to avoid or at least minimize such adsorption by carefully selecting the ionic strength and pH of the running buffer or to use capillaries that have been treated to minimize their interactions with proteins [102,116,142,143]. However caution must be used when varying the running buffer in order to avoid affecting the nature of the drug–protein interaction

[122,139]. In the same manner, the applied voltage used in the mobility assay must be low enough to avoid significant Joule heating within the electrophoretic system, since this would give rise to an increase in temperature and a change in the binding constants for the drug–protein interaction.

Another practical problem that must be dealt with in ACE binding studies is the change in viscosity that occurs as different concentrations of ligand are placed into the CE running buffer. This in turn causes a change in the observed mobility for the injected analyte, regardless of whether or not it has any interactions with the ligand [117,126,144,145]. This is an undesirable situation since it means that mobilities measured during the study are affected by the ligand through more than one mechanism, thus hindering the use of these values for binding measurements. One means of overcoming this problem is to determine the running buffer viscosity under each set of test conditions [144], but a more common approach is to use one or more non-binding solutes as reference markers to measure the viscosity-induced shifts in mobility [117,124,126,133,134,145]. These results can then be used to normalize or correct the mobilities measured for the test analyte to eliminate viscosity-related effects.

4.1.3. Equilibrium constant measurements using drugs as buffer additives

Quantitation of the binding parameters for a drug–protein interaction can be obtained through ACE. Currently the format used most for this purpose is one in which a sample of a protein (plus appropriate marker compounds) is injected into the presence of a CE running buffer that contains various known concentrations of the drug or solute of interest. The migration time, velocity or mobility observed for the protein peak is then determined as several different drug concentrations. An example of such an experiment is shown in Fig. 8. As mentioned earlier, this method requires that the drug and protein have fast association and dissociation on the time scale of the experiment (note: the importance of this assumption and methods for testing it are provided in Ref. [125]). This type of procedure also requires that the binding of drug to the protein cause an observable change in the protein's apparent net electrophoretic mobility. Whether or not this is the case will depend on such items as the charge and hydrodynamic radius (i.e., size) of the protein vs. drug–protein complex [99] and the precision of the migration time or mobility measurements [117].

There are several examples in which drug additives in CE systems have been used to evaluate the binding constants for drug–protein interactions. These include the work in Refs. [118–122,124–127,129–131,133,134], as discussed in Section 4.1.2. Although reciprocal plots have been occasionally used for data analysis in such studies (e.g., see Eq. (15) as provided in Section 3.1.3) [131,133], Scatchard analysis has been employed in most cases. This latter approach may be performed using either the observed changes in the migration time or net mobility of the protein as a function of solute concentration, as shown in Eq. (14) for a system with 1:1 binding [99]:

$$\Delta\mu_{\mathrm{net,L}}/[\mathrm{D}] = K_{\mathrm{a}}\Delta\mu_{\mathrm{net,L}}^{\max} - K_{\mathrm{a}}\Delta\mu_{\mathrm{net,L}} \quad (14)$$

In Eq. (14), [D] is the molar concentration of drug in the running buffer, $\Delta\mu_{\mathrm{net,L}}$ is the observed shift in the net mobility of the ligand or protein in the presence of [D] as compared to the net mobility observed when no drug is present, and $\Delta\mu_{\mathrm{net,L}}^{\max}$ is the maximum possible shift in mobility for the protein peak, as occurs when all binding sites on the protein are saturated with the drug. According to this expression, a plot of $\Delta\mu_{\mathrm{net,L}}/[\mathrm{D}]$ vs. $\Delta\mu_{\mathrm{net,L}}$ should provide a linear relationship for a 1:1 drug–protein interaction, with the slope of this line giving the value of the association equilibrium constant K_{a}. If non-linear behavior is observed, then multisite relationships analogous to Eq. (5) could also be used for data treatment.

One advantage of having drugs as running buffer additives is that these can be used in situations in which only small amounts of proteins (or peptides) are available or in which multiple proteins and peptides are present in the test sample. Such a method has been used by Chu et al. to simultaneously measure the binding of several peptides to vancomycin [119], and the interactions of carbonic anhydrase A and B to 4-alkylbenzyne sulfonamides [124]. An interesting modification of this approach involved its use to determine the peptide with the highest binding affinity for vancomycin in a peptide library [120]. In this latter case, some theoretical studies have been performed to determine the maxi-

mum number of binding agents that can be simultaneously screened by ACE in such an experiment [120].

4.1.4. Equilibrium constant measurements using proteins as buffer additives

Proteins can also be used as running buffer additives in quantitative ACE studies. Examples include studies on the binding of BSA to the 6*R*- and 6*S*-stereoisomers of leucovorin [116], the interactions of vancomycin to various peptides [119], and the binding of HSA to D- or L-tryptophan [117,139] and *R*- or *S*-benzoin [140]. An example of this type of study is shown in Fig. 9. This particular technique is complementary to the one discussed in Section 4.1.3 in that now the shift in mobility for the drug, rather than protein, is monitored. The advantages of this approach are that it can be used to simultaneously examine the binding of multiple forms of the drug (e.g., different stereoisomers, as shown in Fig. 9) [116,117,139,140]. This approach is also appealing for use when working with a large or highly-charged protein that may not exhibit a detectable shift in mobility upon binding to the drug of interest, thus preventing studies such as those described in Section 4.1.3.

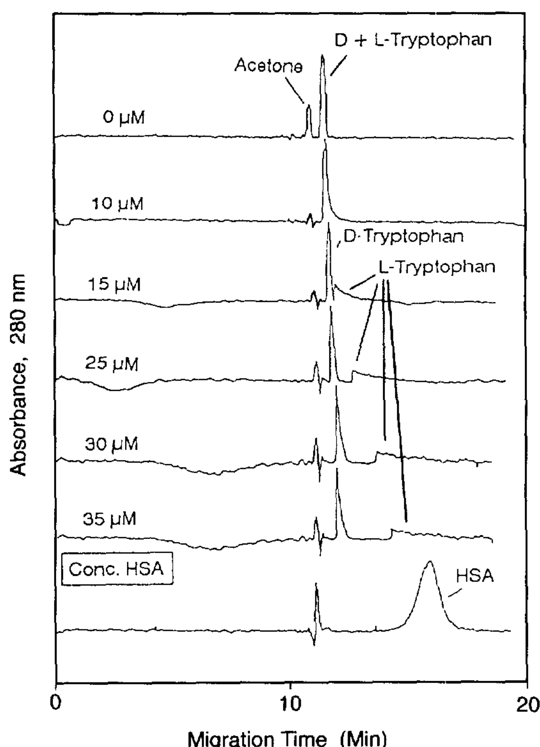

Fig. 9. CE Mobility shift assay for D- and L-tryptophan in the presence of HSA as a running buffer additive. The samples contained 50 μ*M* D/L-tryptophan and 0.5% (v/v) acetone as a neutral marker. The applied voltage was 18 kV and the capillary was a 50 cm×50 μm I.D. CE 200/glycerol coated fused-silica column filled with a pH 7.4, 0.0125 *M* potassium phosphate running buffer. This figure was adapted with permission from Ref. [117].

Binding data obtained when using protein additives can be performed by Scatchard analysis, using expressions similar to that given in Eq. (14) [119]. But alternative methods based on reciprocal plots can also be employed. This second approach is illustrated by Eq. (15), as derived for a drug–protein system with 1:1 interactions [117,146]:

$$1/(\mu_{\mathrm{net,D}} - \mu_{\mathrm{D}}) = 1/[(K_{\mathrm{a}}[\mathrm{L}])(\mu_{\mathrm{DL}} - \mu_{\mathrm{D}})] + 1/(\mu_{\mathrm{DL}} - \mu_{\mathrm{D}}) \quad (15)$$

In this relationship, $\mu_{\mathrm{net,D}}$ is the net mobility observed for the drug in the presence of a given protein or ligand concentration [L], μ_{D} is the net drug mobility in the absence of protein and μ_{DL} is the mobility for the resulting drug–protein complex. To use this equation, a plot of $1/(\mu_{\mathrm{net,D}} - \mu_{\mathrm{D}})$ is made vs. 1/[L] and the association constant K_{a} is determined from the ratio of the intercept to the slope. The same type of equation can be employed in ACE studies that involve drug additives by reversing the role of the drug and protein (e.g., using [D] in place of [L], $\mu_{\mathrm{net,L}}$ in place of $\mu_{\mathrm{net,D}}$, etc.) [131,133]. One advantage of this type of data treatment is that it avoids the co-dependence of the left- and right-hand terms that is present during Scatchard analysis; in addition, the associated problems concerning non-uniform variance are also reduced.

4.2. Frontal analysis

A few reports have examined the use of frontal analysis in CE for the study of drug-protein binding [147–149]. The basic principle of this method and the appearance of its results are similar to those described in Section 2.2 for solution-phase chromatographic methods; the only difference in CE is that the free drug, drug–protein complex and non-

complexed protein bands are now separated based on their different electrophoretic mobilities. Fig. 10c shows an example of a CE frontal analysis run. This approach assumes that relatively fast association and dissociation kinetics are present for the test system. Ideally, little or no protein adsorption to the capillary or support is also desired so that minimal bleeding into the γ-band, or free drug region, occurs during the analysis. Although CE-based frontal analysis has not received as much use or attention as the chromatographic-based approach, the CE method does have the distinct advantage of requiring much smaller sample volumes than are needed in even HPFA [148,149].

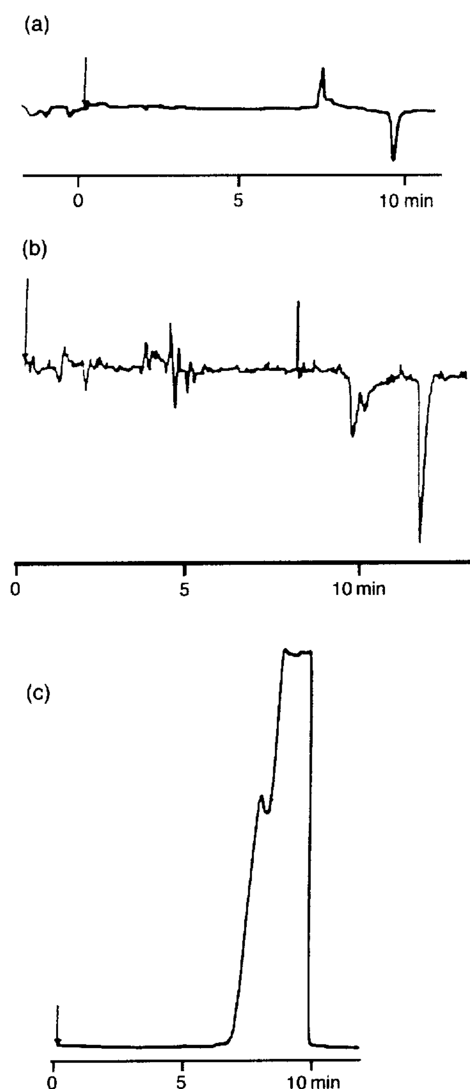

Fig. 10. Electropherograms obtained for warfarin and BSA in CE using the (a) Hummel–Dreyer method, (b) the equilibrium saturation method and (c) frontal analysis. This figure was adapted with permission from Ref. [147].

The use of frontal analysis in CE was first reported by Kraak et al. in work with warfarin and BSA as a model. They compared the results of this approach to the Hummel–Dreyer and equilibrium saturation methods, as performed on the same CE system, and concluded that frontal analysis was the method of choice among these techniques [147]. An interesting variation on the combination of frontal analysis and CE was later described by Shibukawa and co-workers in experiments using *R*/*S*-verapamil and HSA [148,149]. In their work, they first used a polyacrylamide-coated capillary and a physiological pH to separate the free and protein-bound fractions of verapamil from HSA [149]. This approach was then used along with Scatchard analysis to determine the binding parameters between verapamil and AAG, with the results showing good agreement vs. those obtained by equilibrium dialysis [149]. The same group later explored the use of hydrodynamic vs. electrokinetic injection to isolate the free fractions of verapamil. The verapamil applied to the capillary was next separated into its individual enantiomers by using a secondary ligand (i.e., trimethyl-β-cyclodextrin) as a chiral binding agent. The resulting peaks were then used to estimate the free concentration of each enantiomer in the initial sample [148].

4.3. Vacancy techniques

Both the Hummel–Dreyer and equilibrium saturation methods can be adapted for use in CE systems. This was demonstrated by Kraak et al. [147], who examined the use of these methods in the analysis of warfarin–BSA interactions. As in the solution-phase chromatographic technique described in Section 2.3.1, 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 drug to be studied. As the sample passes through the CE capillary, the different electrophoretic mobilities of the drug and

drug–protein complex will give a positive peak for the injected protein and a negative, vacancy peak that represents the depleted free drug concentration in the running buffer. From the size of this vacancy peak, the concentration of bound vs. free drug can be quantitated and used along with Scatchard analysis to determine the binding affinity and number of binding sites for the drug 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 a protein–drug 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 drug, and can again be used with Scatchard plots to provide the binding parameters for the drug–protein system [147].

Examples of CE runs based on the Hummel–Dreyer and equilibrium saturation methods are provided in Fig. 10. The overall appearance of these separations is similar to that seen in the corresponding chromatographic methods (see Section 2.3.1 Section 2.3.2). The underlying assumptions behind these methods (e.g., the presence of fast association/dissociation kinetics) are also the same as in the chromatographic techniques. However, the CE-based methods have the advantage of requiring much less sample to perform. An initial evaluation of these methods was conducted by Kraak et al. in their work with warfarin and BSA. Some difficulties were encountered early in their studies with both techniques, such as the presence of noisy baselines and run-to-run variations in the electropherograms, but it was found that these problems could be avoided or minimized by using stringent protocols for sample pretreatment and analysis. Scattering and imprecision of the data at low r values in the Scatchard plots was another difficulty encountered. This was caused by the relatively small peaks that had to be analyzed under these experimental conditions, thus requiring the use of a large number of sample replicates [147].

5. 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, as reviewed by Hořejší and Tichá [97]. The overall approach is similar to that of analytical affinity chromatography, as described in Section 3, but now uses an electric field to elute the applied sample components. 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 also been employed such as dyes, sugars and enzyme inhibitors or co-factors [97].

Although gel-based electrophoretic systems with immobilized ligands have been used for many years, much less work has been performed in the use of immobilized ligands in CE for drug-protein studies. Furthermore, the work that has been done has focussed more on the use of CE for chiral drug separations than on the direct quantitation of drug–protein binding. For example, Sun et al. studied the use of BSA coupled to high-molecular-mass dextran for the separation of leucovorin enantiomers [150]. Similarly, Birnbaum and Nilsson used a gel-filled CE capillary and glutaraldehyde cross-linked BSA for the resolution of D- and L-tryptophan [151]. A HPLC silica-based AAG support was placed into a CE capillary and used by Li and Lloyd for separating the enantiomers of benzoin, hexobarbital, pentobarbital, ifosfamide, cyclophosphamide, disopyramide, metoprolol, oxprenolol, alprenolol and propanolol. In the same study the effects of pH, organic solvent content and ionic strength on the retention and drug–protein stereoselectivity were also investigated [152]. In a report by Yang and Hage, the resolution of D/L-tryptophan and *R*/*S*-warfarin was investigated in CE capillaries that contained either HSA within the running buffer or HSA adsorbed to capillary wall. *R*/*S*-Warfarin could be resolved by the HSA-coated capillary but not D/L-tryptophan, an effect that was confirmed by using protein binding data to compare the behavior expected for these solutes in the presence of soluble vs. immobilized HSA [139].

6. Conclusions

In recent years there has been several new developments or improvements in separation-based methods for the study of drug–protein interactions.

This review has examined various formats for such methods, including the use of both soluble and immobilized drugs or proteins. Approaches based on zonal elution, frontal analysis or vacancy peak measurements were also presented. These approaches are attractive alternatives to traditional methods, such as equilibrium dialysis and ultrafiltration, that are commonly used for drug–protein binding studies. The variety of chromatographic and electrophoretic techniques that can be employed in such work provides the means for studying drug–protein systems with either fast or slow kinetics. In addition, these methods can be designed to provide information on a wide range of items of interest in the analysis of drug–protein interactions. For instance, examples were provided regarding the use of such techniques for determining the overall extent of drug–protein binding, examining the displacement of a drug by other agents from binding sites on a protein and measuring the equilibrium or rate constants for drug–protein interactions. In many cases the same methods, particularly when used in HPLC or CE systems, can be employed as rapid screening tools for investigating the binding of different forms of a chiral drug to a protein or the binding of different proteins and peptides to a given pharmaceutical agent. Together, these capabilities should make these methods increasingly useful in clinical and pharmaceutical research for the detection of drugs, the development of new pharmaceutical agents and in the design of improved treatment regimes for these agents.

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