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CRITICAL REVIEW

Chromatographic analysis of drug interactions in the serum proteome

David S. Hage,* Jeanethe A. Anguizola, Abby J. Jackson, Ryan Matsuda, Efthimia Papastavros, Erika Pfaunmiller, Zenghan Tong, John Vargas-Badilla, Michelle J. Yoo and Xiwei Zheng

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The binding of drugs with serum proteins and binding agents such as human serum albumin, α_1-acid glycoprotein, and lipoproteins is an important process in determining the activity and fate of many pharmaceuticals in the body. A variety of techniques have been used to study drug interactions with serum proteins, but there is still a need for faster or better methods for such work. High-performance liquid chromatography (HPLC) is one tool that has been utilized in many formats for these types of measurements. Advantages of using HPLC for this application include its speed and precision, its ability to be automated, its good limits of detection, and its compatibility with a wide range of assay formats and detectors. This review will discuss various approaches in which HPLC can be employed for the study of drug-protein interactions. These techniques include the use of soluble proteins in zonal elution and frontal analysis methods or vacancy techniques such as the Hummel-Dreyer method. Zonal elution and frontal analysis methods that make use of immobilized proteins and high-performance affinity chromatography will also be presented. A variety of applications will be examined, ranging from the determination of free drug fractions to the measurement of the strength or rate of a drug-protein interaction. Newer developments that will be discussed include recent work in the creation of novel mathematical approaches for HPLC studies of drug-protein binding, the use of HPLC methods for the high-throughput screening of drug-protein binding, and the creation and use of affinity monoliths or affinity microcolumns for examining drug-protein systems.

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1. Introduction

The binding of drugs with proteins in serum (*i.e.*, the serum proteome) is an important process in determining the eventual activity and fate of many pharmaceutical agents in the body.[1–13] Fig. 1 shows a general model for this type of interaction, as illustrated for the formation of a 1 : 1 reversible complex between a drug and a serum protein. In this model, K_a is the association equilibrium constant for the formation of a drug-protein complex, while k_a and k_d are the corresponding association and dissociation rate constants for this process.[1,7] This type of interaction frequently involves common serum transport proteins and binding agents such as human serum albumin (HSA), α_1-acid glycoprotein (AGP), and lipoproteins (*e.g.*, high-density lipoprotein, or HDL, and low-density lipoprotein, or LDL).[1–8] In some cases more specific binding agents can take part in these interactions. An example of this latter situation is the binding of steroids with corticosteroid-binding globulin (CBG).[7,14]

These drug-protein interactions are often significant, with approximately 43% of the 1500 most common drugs having at least 90% binding to proteins in serum.[13] For instance, these interactions can affect the amount of the free, or non-complexed, form of a drug in the circulation and can have a large effect on the distribution, effective activity, excretion, and metabolism of a drug.[1–4] The direct or indirect competition between drugs and other solutes for binding sites on serum proteins can not only be

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an important source of drug-drug interactions but can lead to interactions between drugs and endogenous agents such as fatty acids, which also bind to some serum proteins.[1–8] In addition, the binding of drugs with serum proteins can improve the overall solubility of a hydrophobic drug within the circulation.[9] Because the binding of some chiral drugs with proteins can be stereoselective in nature, it is further possible that these interactions in serum may affect the apparent activities for different forms of a chiral drug in the body.[5,9,11,12]

A variety of techniques have been used in the past to study drug interactions with serum proteins. Ultrafiltration and equilibrium dialysis are the most common methods utilized for this type of work.[15,16] In addition, fluorescence spectroscopy,[18] absorption spectroscopy,[18] X-ray crystallography,[6,19,20] nuclear magnetic resonance (NMR) spectroscopy,[21,22] capillary electrophoresis,[23–25] and surface plasmon resonance[26] have been employed. However, there is still a growing need for general methods that can provide faster and more convenient approaches for the analysis and characterization of drug-protein binding in serum and related samples.

High-performance liquid chromatography (HPLC) is one tool that has been used in many formats for such work. Some attractive features of HPLC for this application include its speed and precision, its ability to be automated and conducted with common equipment, its good limits of detection and efficiency, and its compatibility with a wide range of assay formats and detectors.[7–10] The purpose of this review is to examine the various approaches in which HPLC can be employed for the study of drug-protein interactions with both soluble drugs and proteins or with immobilized forms of these agents. An overview of these techniques will be presented and various applications will be described for these methods.

2. Zonal elution methods using soluble proteins

One approach that can be employed in HPLC to study drug-protein interactions is to use zonal elution to separate the free and protein-bound fractions of a drug in a sample. In this case, a small sample of a drug-protein mixture is injected onto a column which has different retention for the drug and drug-protein complex. If little to no dissociation occurs for the drug-protein complex during the experiment, the measured amount of drug in the free or protein-bound fraction that elutes from the column can be used to determine the extent of drug-protein binding in the sample and the equilibrium constant for this interaction.[7]

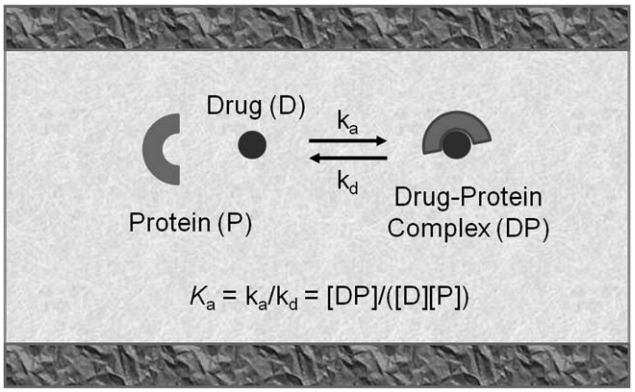

Fig. 1 General model for the reversible binding of a drug with a protein in blood or serum. The model shown here is for the formation of a 1 : 1 complex between the protein and drug, as described by the association equilibrium constant (K_a) and corresponding association rate constant (k_a) and dissociation rate constant (k_d). Similar reactions can be written for multi-site drug-protein interactions or mixed-mode interactions.

The earliest use of this approach involved the injection of small samples onto columns that contained size-exclusion supports.[27] In this type of experiment, the drug-protein complex eluted first, followed later by the free drug fraction. This approach has been reported to require an association equilibrium constant for a drug-protein complex that is greater than 10^7 M^{-1}, which would generally provide only a small level of dissociation as the complex passes through the size-exclusion column.[7,28,29] For instance, this method has been used to study the interaction between prednisolone and CBG, which has a relatively large association equilibrium constant ($K_a = 3 \times 10^7$ M^{-1}), but was not successful when used to examine the binding of prednisolone

The following graduate students in the Hage laboratory contributed to this review, shown in the photo going from the front (left-to-right) to back (left-to-right) and then to the right column (top-to-bottom). Ryan Matsuda is using high-performance affinity chromatography (HPAC) to examine drug interactions with modified serum proteins. Jeanethe A. Anguizola is using HPAC and mass spectrometry in proteomics to see how the activity and structure of human serum albumin change during diabetes. Efthimia Papastavros is developing approaches for the selective detection and extraction of biologically-active contaminants in water. Erika Pfaunmiller is creating novel monolithic supports for rapid affinity-based separations. John Vargas-Badilla is investigating new approaches for the immobilization of biological agents in chromatographic media. Zenghan Tong is using chromatographic theory to develop novel approaches for the characterization of binding site heterogeneity and solute-protein interactions by HPAC. Abby J. Jackson is creating improved methods for the entrapment of proteins in porous supports for HPAC. Michelle J. Yoo is combining the use of monolith supports with affinity ligands for the creation of high-throughput methods for drug-protein binding studies. Xiwei Zheng is also examining new methods based on affinity monoliths for rapid assays of drug-protein binding.

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with HSA ($K_a = 2 \times 10^3$ M^{-1}).[29] HPLC size-exclusion columns have been used to examine the binding of ATX-S10(Na) to high-density lipoprotein (HDL) and to serum albumin, as well as other serum proteins.[30] In a similar manner, gel fitration has been utilized to study the binding of cosalane to HSA and AGP.[31] In this latter study, gel filtration was further used to examine the competition between cosalane and various site-selective probes for known binding sites on HSA.[31]

It is also possible to examine drug-protein interactions by injecting a small sample of a drug onto a size-exclusion column in the presence of a known concentration of protein in the mobile phase. As the drug binds to the protein, a drug-protein complex will form and there will be a decrease in the drug's observed retention. The global association equilibrium constant for the drug-protein interaction can then be determined by comparing the analyte's retention times in the presence and absence of protein in the mobile phase.[7,10] This method has been used to examine the binding of HSA to warfarin, furosemide, phenylbutazone, tryptophan, and omeprazole.[32–34] In order to use this approach to study drug-protein binding, it is necessary to have association and dissociation rates for the interaction that are fast enough to allow a local equilibrium to be established between the drug and protein as these components pass through the column. It is also necessary for the amount of injected drug to be small compared to the amount of protein in the mobile phase.[32,33]

Zonal elution methods can further be employed with internal surface reversed-phase (ISRP) columns to perform peak-splitting measurements of drug-protein interactions.[7,35–39] The ISRP columns utilized in this approach consist of a reversed-phase stationary phase that is located only within the pores of the support. When a drug-protein mixture is injected onto this type of support, the drug-protein complex is excluded from the pores while the free drug fraction is allowed to enter the pores and interact with the reversed-phase sites. A peak-splitting effect can then arise when dissociation of the drug-protein complex occurs at a rate similar to the timescale of the separation. This effect results in a situation where two drug peaks elute from the column. The first, broad peak corresponds to the drug that was originally bound to the protein and the second, sharper peak corresponds to the original free drug fraction. The result is a chromatogram in which both the free and protein-bound fractions of a drug can be determined within a single run. Systems examined by this approach have included mixtures of HSA with phenytoin,[35,36] imirestat,[36] or warfarin[37] and mixtures of warfarin with bovine serum albumin (BSA).[38,39]

Several recent reports have used immobilized antibodies and high-performance immunoaffinity chromatography to measure free drug fractions.[40–44] In one of these studies, ultrafast immunoextraction was used to examine the free drug fractions in warfarin/HSA mixtures.[40] In this study, sandwich microcolumns containing a 1.1 mm layer of a support with anti-warfarin antibodies were used to extract the free warfarin fraction in about 180 ms. This column was then used in series with ISRP columns to determine the remaining fraction of warfarin that was bound to HSA. The results gave good agreement with those predicted by solution-phase measurements.[40]

Other studies have used ultrafast immunoextraction with HPLC-based displacement immunoassays to measure the free fractions of thyroxine or phenytoin in serum.[41,42] In this work, a sample containing the free and protein-bound forms of the analyte was injected onto a small immobilized antibody column that contained a reversibly-bound and labeled analog of the analyte, as illustrated in Fig. 2. As the sample passed through the column, the free fraction of the analyte displaced some of the labeled analog. This displacement produced a peak for the analog that was proportional to the amount of free analyte in the original sample. Using this approach, the free fractions of thyroxine and phenytoin were extracted from serum samples within 90–100 ms, and the free concentrations of these analytes were determined within 0.5–3.0 min of sample injection. Detection was achieved by using a chemiluminescent label and a post-column reactor in the case of thyroxine[41] and a near-infrared fluorescent label in the case of phenytoin.[42] When used in the analysis of serum, both methods gave good correlation *versus* reference methods. The same approach has been used to examine interactions between phenytoin and HSA.[42] Similar work using ultrafast immunoextraction and LC/MS detection has been described in the analysis of free carbamazepine fractions.[43,44]

3. Frontal analysis using soluble proteins

Another common technique for examining drug-protein interactions in solution is frontal analysis.[7,45–51] This approach was first employed in 1964–1965 with soluble agents to examine protein-protein binding[45] and drug-protein interactions[46] and is still an important method for such measurements. Frontal analysis using soluble proteins is different from the zonal elution methods described in the previous section because a large volume of a drug/protein mixture is used instead of a small plug of sample.

A typical elution pattern that is seen during this type of frontal analysis experiment is shown in Fig. 3, in which a mixture of a low molecular weight drug and a protein is applied to a size-exclusion column. In this situation, the drug will be able to enter the pores of the support and will have a later elution time than

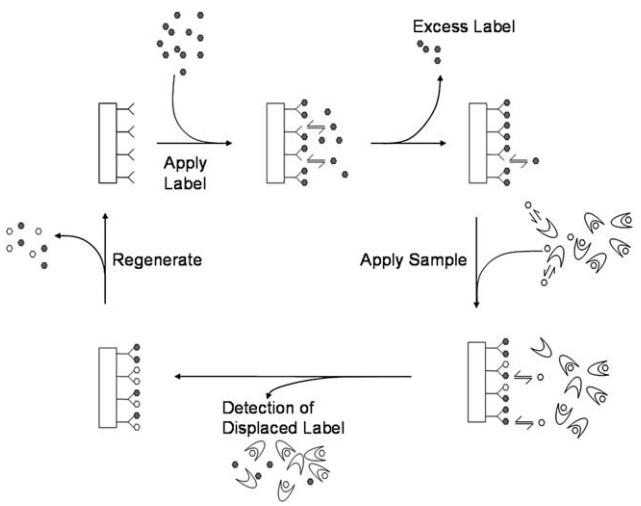

Fig. 2 Scheme for an immunoextraction/displacement assay for the detection of a free drug fraction in a serum sample. Symbols: (●), labeled analog of analyte; (○), unlabeled analyte from sample; (⊃), binding agent in a sample for the analyte; (–<), immobilized antibody. Reproduced with permission from ref. 42.

the protein or drug-protein complex.[48,49] Because a relatively large sample volume has been applied, the column will produce only a partial separation of these fractions. The elution profile that is generated will ideally have several plateau regions.[7,50] In Fig. 3, the first plateau represents the non-complexed protein in the original sample. This region in the chromatogram may or may not be seen, depending on the detection method that is used to monitor elution of the sample. The second plateau, or the "β band", represents the drug-protein complex. The final plateau (*i.e.*, the "γ band") represents the free drug fraction at its equilibrium concentration.[7,51] Information about the total and free concentrations of the drug can be obtained from this profile. If various concentrations of the drug are used in such an experiment, it is also possible to determine the equilibrium constants and number of binding sites that are involved in the drug-protein interaction.[52–55]

The use of HPLC with frontal analysis and soluble proteins produces a method known as high-performance frontal analysis (HPFA). This approach has been used in many reports to examine drug-protein binding.[7,28,51,56,57] For instance, HPFA has been used to study the interactions of HSA with carbamazepine, diazepam, fenoprofen, troglitazone, or warfarin.[58–64] HPFA has also been coupled with chiral HPLC columns for the separation and measurement of the individual chiral forms of a drug within the frontal analysis profile. This approach has been used to examine the stereoselective binding of BOF-4272, fenoprofen, ketoprofen, nilvadipine, and warfarin to proteins.[37,64–68]

The typical sample size in HPFA when using a size-exclusion column is about 10–20 mL;[7,51,59] however, the amount of sample that is selected will be dependent on the degree of separation that can be obtained between the free drug and drug-protein complex.[69–71] HPFA can also be conducted with ISRP columns, which provides better resolution between the drug and drug-protein complex bands than size-exclusion columns and requires only 80–400 μL of sample.[7,51,64] In addition, HFPA can be adopted for use with capillary electrophoresis, which needs even less sample and which separates the drug and drug-protein bands based on their differences in electrophoretic mobility.[7,65,72–76]

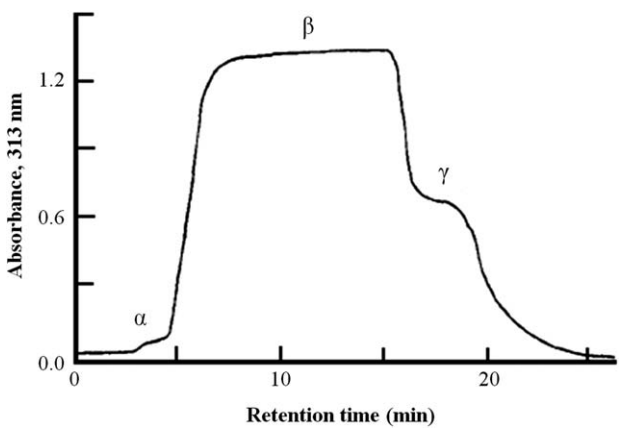

Fig. 3 Chromatogram obtained by high-performance frontal analysis (HPFA) for a mixture of warfarin and HSA. The sample was a mixture of 100 μM warfarin plus 2 g L^{-1} HSA that was injected onto a 30 cm × 3.9 mm I.D. size-exclusion column. Reproduced with permission from ref. 60.

In all of these methods, obtaining stable plateau regions, particularly for the free drug band, can be difficult if strong retention occurs between any of the sample components and the stationary phase or walls of the capillary.[7,28,65]

4. Vacancy methods

Vacancy methods are a group of techniques that are based upon the continual application of a mobile phase that contains either the drug of interest or a combination of the drug and a protein.[7] An injection is made of a sample that lacks one or more of these components. As the sample plug passes through the column, the local equilibrium between the drug and protein in the mobile phase is perturbed. This effect produces a trough or vacancy peak that occurs in response to a depletion of one of the mobile phase components. The size of this peak is then used to determine the amount of free drug or protein-bound drug that was present in the mobile phase at equilibrium.[7]

The most common example of a vacancy technique is the Hummel-Dreyer method.[77] In this method, a solution containing a known concentration of the drug of interest is passed through a size-exclusion support. A sample of a protein is then injected onto this system, causing one of two situations to occur. If the protein does not bind to the drug, a single peak corresponding to the protein will result. If reversible binding of the drug with the protein is present, a portion of the free drug in the mobile phase will be depleted and will become bound to the protein in the sample. As a result, two peaks will appear as the sample passes through a size-exclusion column. The first, positive peak will represent the protein and drug-protein complex that is formed as the protein passes through the column. The second, negative peak will appear at the expected retention time for the drug and will represent the depleted free fraction of the drug in the mobile phase (see Fig. 4).[7,77–79] The area of the negative peak can then be used to measure the amount of drug that is bound by the protein in the mobile phase. If this experiment is carried out at several drug concentrations, the data can be used to obtain the binding constants and the number of binding sites for the drug-protein interaction.[7,77–79]

The Hummel-Dreyer method was originally developed in 1962 for use with low-performance size-exclusion columns.[77] This method was modified for work with HPLC in 1978 and was validated by examining the binding that takes place between HSA and warfarin.[78] The HPLC version of the Hummel-Dreyer method has since been used for the analysis of many drug-protein systems. Examples of these systems have included the interactions of HSA with buspirone, ceftrixone, diazepam, isradipine, phenobarbitol, phenytoin, propranolol, warfarin, carvedilol, furosemide, phenylbutazone and pirprofen; the binding of AGP with isradipine, propranolol, propanfenone, and carvedilol; and the binding of HDL or LDL with amlodipine isradipine, or propranolol.[7,77–79] This method has typically been conducted by using size-exclusion columns, but ion-exchange columns and ISRP columns have also been used.[7] In addition, this approach has been modified for use in CE.[7,71] The Hummel-Dreyer method has been utilized in both binding studies[78] and competition studies, as has been shown in work with ISRP columns.[7,79] In addition, the Hummel-Dreyer technique has been employed with both serum proteins and other proteins or binding agents

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(*e.g.*, enzymes, polysaccharides, and cyclodextrins).[79] Two recent examples that have illustrated the use of the Hummel-Dreyer method with other binding agents have involved studies of steroid-cyclodextrin inclusion complexes[80] and the interactions between tubulin and dolastatin 15.[81]

To relate the amount of a free drug and protein-bound drug to the size of the vacancy peak in the Hummel-Dreyer method, the system must be calibrated either internally or externally. When using internal calibration, the protein is injected in the presence of increasing concentrations of the drug while the total sample volume is held constant.[7,77,79] For external calibration, the size of the vacancy peak is directly compared with the peak area that is measured when the drug is injected onto the column in the absence of any protein.[7] One requirement in either calibration method is the need to have a local equilibrium present between the drug and protein, which requires the presence of sufficiently fast association and dissociation kinetics on the time scale of the experiment. Good resolution between the peaks for the protein-bound drug and the free drug fractions is also required.[7,79]

The equilibrium saturation method is another example of a vacancy technique.[82] This approach is similar to the Hummel-Dreyer method in that a column containing a material such as a size-exclusion support is utilized to resolve a drug and drug-protein complex from a mixture of these components.[7] However, in the equilibrium saturation method both the protein and drug are used as mobile phase additives, and the sample contains only the mobile phase buffer. The chromatogram that is produced during sample injection will ideally contain two negative, vacancy peaks. These two vacancy peaks appear at the retention times for the drug-protein complex and the free form of the drug. The size of these peaks can be used along with internal or external calibration, as described in the previous paragraph, to determine the free and protein-bound concentrations of the drug in the mobile phase at equilibrium. If this experiment is carried out in the presence of mobile phases that contain several different drug-protein mixtures, it is possible to determine the association equilibrium constants and number of binding sites that are involved in the drug-protein interaction.[82]

The equilibrium saturation method has been used to study the interactions of HSA with diazepam[62] and to study the effects of fatty acids[82] and sodium dodecyl sulfate[83] on the binding of HSA with warfarin. Like the Hummel-Dreyer method, this approach has been adapted for use in CE.[7] The equilibrium saturation method has also been used to study the competitive binding of solutes to a protein for systems that have good resolution between the peaks for the individual sample components.[79]

It is necessary in any application of the equilibrium saturation method to have good resolution between the peaks for the free drug and drug-protein complex and to have reasonably fast kinetics for the drug-protein interaction on the time scale of the study. Although the equilibrium saturation method requires a larger amount of protein than the Hummel-Dreyer method, drugs with lower solubility can be analyzed by the former method because these drugs can bind to the protein in the mobile phase, which will aid drug solubility.[7]

5. Zonal elution in HPAC

Various methods based on affinity chromatography can also be used to study drug-protein interactions. Affinity chromatography is a type of liquid chromatography in which a biologically-related agent (*e.g.*, a protein or drug) is used as the stationary phase.[84,85] When HPLC media such as silica or monolithic supports are used with an immobilized binding agent, the resulting technique is known as high-performance affinity chromatography (HPAC).[86–89] Affinity chromatography has long been an important tool for the separation, purification and analysis of compounds that can bind to immobilized agents such as proteins, enzymes, dyes, nucleic acids and immobilized metal ions.[85] However, affinity chromatography and HPAC can also be used to study biological interactions. The use of HPAC or affinity chromatography for this purpose is referred to as

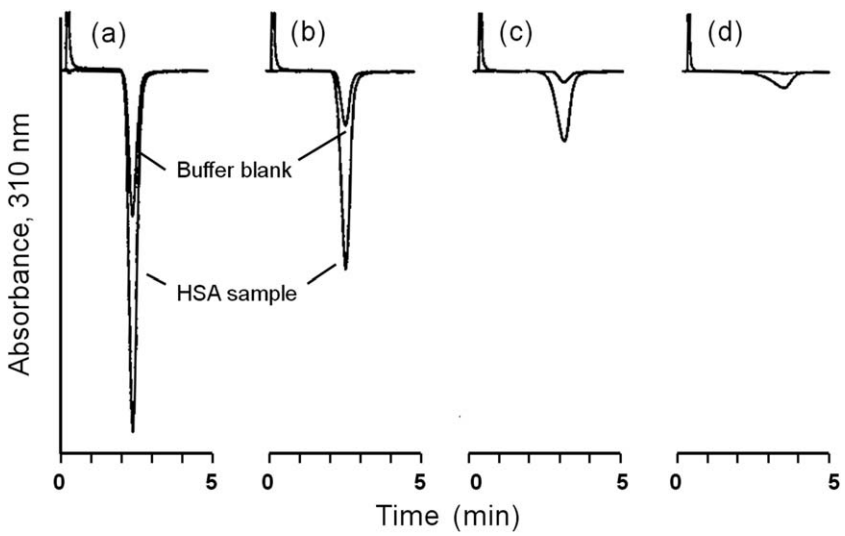

Fig. 4 Use of the Hummel-Dreyer method for examining the binding of warfarin with HSA. These experiments were conducted on a 5 cm long internal surface reversed-phase (ISRP) column by injecting samples that contained either a buffer blank (top trace) or a sample of HSA (bottom trace) in the presence of mobile phases that contained various, known concentrations of warfarin. The mobile phase concentrations of warfarin were as follows: (a) 81.1 μM, (b) 32.5 μM, (c) 8.1 μM, and (d) 1.6 μM. Reproduced with permission from ref. 61.

analytical affinity chromatography, biointeraction affinity chromatography or quantitative affinity chromatography.[86–92]

One advantage of using affinity chromatography to study biological interactions is that the same immobilized binding agent can often be used for a large number of sample injections. This feature helps minimize the effects of batch-to-batch variations in the binding agent, resulting in good run-to-run precision, and decreases the total amount of binding agent that is needed for such studies. In addition, biointeraction studies carried out by using HPAC can be automated and used in high-throughput methods for drug-protein binding measurements.[10]

The proper use of affinity chromatography and HPAC for binding studies requires that careful attention be given to the preparation and treatment of the immobilized binding agent. In work with HSA and AGP or closely-related proteins, it has been shown that conditions for covalent attachment to a support can often be found in which the immobilized form of the protein is a good model for its soluble form.[86,92–103] Similar results have been noted for the covalent immobilization of lipoproteins within HPAC columns.[104] An alternative approach based on non-covalent physical entrapment has also been used in some studies to place soluble proteins within a support for binding studies.[105,106]

Zonal elution is one method that has frequently been used with HPAC columns to obtain information on the binding of solutes to proteins or other agents.[10,11,86] For instance, this combination of techniques can be utilized for measuring the average affinity of a drug or solute with an immobilized protein. In this type of experiment, a small amount of a drug or solute is injected onto a column that contains the protein of interest. The retention factor (k) of the drug or solute is then calculated by using the observed retention time and the column void time. This retention factor can then be related to the bound fraction (b) and the free fraction (f) of the drug through the expression $k = b/f$, provided a local equilibrium and linear elution conditions are present.[7,10] It is possible from this relationship to determine either the value of b or f given the fact that the sum of the bound plus free fractions for the drug must always be equal to one.[107]

This zonal elution approach has been used with various coumarin compounds and HSA to compare the overall extent of solute-protein binding that is measured by HPAC *versus* ultrafiltration.[108] The same method has been used with other pharmaceuticals,[109] including anti-HIV chemotherapy drugs,[110] and has been combined with LC/MS for the simultaneous examination of the protein interactions of several drugs in a mixture.[107] Related reports have used k as a direct measure of the global affinity of injected solutes for an immobilized protein. This approach has been utilized to compare immobilization methods and columns containing AGP or HSA for applications such as binding studies or chiral separations involving *R*/*S*-warfarin,[102,103] *R*/*S*-propanolol[102] and D/L-tryptophan.[103] This technique has also been used for screening bioactive compounds from plants by employing immobilized β_2-adrenoceptors.[111]

A number of other HPAC studies have used measurements of k to see how the mechanism of analyte retention changes on an immobilized serum protein when factors such as the pH, ionic strength or organic content of the mobile phase are varied.[10,112–120] A related set of applications have involved the use of retention factor measurements for a series of structurally-related compounds on the same column to learn about binding sites and to see how changes in the structure of a drug can affect its interaction with a protein. This approach has been employed to examine the binding of L-thyroxine, warfarin, coumarins, sulfonylurea drugs and several indole compounds to HSA and glycated HSA.[121–126] Several studies involving a large set of samples have used this method to develop quantitative structure-retention (or reactivity) relationships (QSRRs) to describe drug-protein interactions.[127–129] As an example, QSRR models have been created with this technique to describe the binding of HSA to benzodiazepines and 2,3-substituted-3-hydroxy-propionic acid[130,131] and to study the stereoselective interactions of AGP with amino alcohols, antihistamines, beta-adrenolytic drugs, cyclic vinca alkaloid analogues, and quinazolone derivatives.[132–136]

The most common application of zonal elution and HPAC in the study of drug-protein interactions is the use of these methods to investigate the competition and displacement of drugs from proteins by other drugs or solutes.[10] In this type of experiment, a small pulse of one analyte is injected on an HPAC column containing the immobilized protein while a fixed concentration of a potential competing agent is passed through the column in the mobile phase. Fig. 5 illustrates such an experiment, in which the drug tolbutamide is used as a mobile phase additive on a glycated HSA column and *R*-warfarin is injected as a site-selective probe for Sudlow site I on the immobilized protein.[126] The shift in the chromatograms in Fig. 5(a) for *R*-warfarin as the concentration of tolbutamide is increased indicates that these two drugs interact on the immobilized protein. When the measured retention data are plotted and analyzed according to the plot shown in Fig. 5(b), it is possible to show that this interaction involves direct competition at a common site. It is also possible from this graph to determine the association equilibrium constant for tolbutamide at Sudlow site I. Other examples of this approach include reports that have examined the displacement of D/L-thyronine and D/L-tryptophan by bilirubin or caprylate from HSA;[137] the competition of warfarin with oxazepam, larazepam and hemisuccinate derivatives on a HSA column;[138] the competition of octanoic acid with oxazepam hemisuccinate, ketoprofen A/B and suprofen A/B on HSA;[139] the competition of β-estradiol with progesterone on HSA;[140] and the site-specific interactions of sulfonylurea drugs with both normal HSA and glycated HSA.[125,126,141]

6. Frontal analysis in HPAC

Another common technique that has been used for studying drug-protein interactions by affinity chromatography and HPAC is frontal analysis.[7,10,142] Frontal analysis was first utilized to study biological interactions by means of low-performance affinity chromatography in 1975.[143] This technique was then used to examine the binding of salicylate with HSA[144] and the binding of fatty acids and drugs to BSA.[145] The combined use of frontal analysis with HPAC and HSA was later reported in the early 1990s.[94,95,99,139]

Frontal analysis in affinity chromatography and HPAC is performed by continuously applying a known concentration of a drug or analyte to a column that contains an immobilized binding agent.[7,10] As the immobilized agent becomes saturated by the applied substance, the remaining drug or analyte in the

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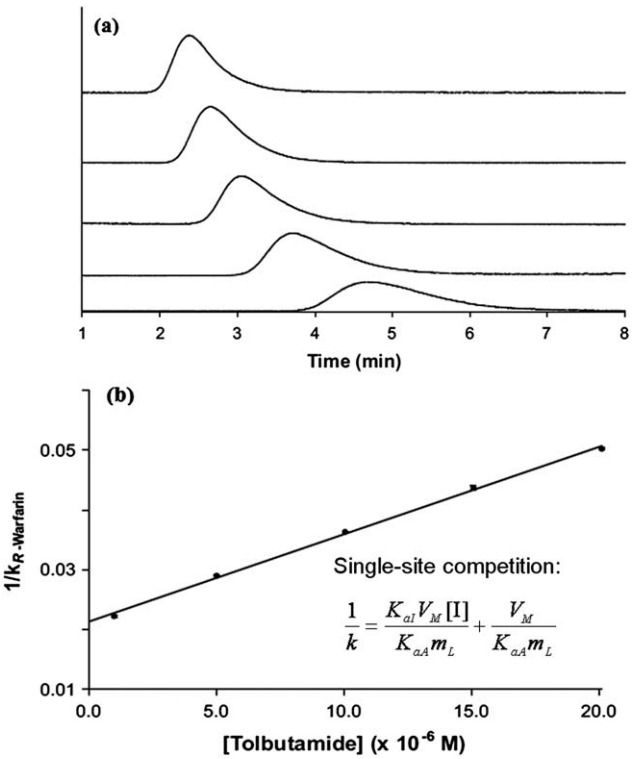

Fig. 5 (a) Typical chromatograms obtained in a zonal elution study on a column containing glycated HSA and examining the competition between injected samples of *R*-warfarin (*i.e.*, a probe for Sudlow site I of HSA) and tolbutamide, which was used here as a mobile phase additive. The mobile phase concentrations of tolbutamide in (a) were (from top-to-bottom) 20, 15, 10, 5 and 1 μM. The plot in (b) shows how the reciprocal of the measured retention factor for *R*-warfarin ($1/k_{R\text{-Warfarin}}$) changed as the mobile phase concentration of tolbutamide was varied. The inset equation shows the result that would be expected for 1 : 1 competition of the injected analyte (A) and the mobile phase additive (I), in which K_{aA} and K_{aI} are the association equilibrium constants for the analyte and mobile phase additive at their site of competition, [I] is the mobile phase concentration of I, m_L represents the total moles of these binding sites, and V_M is the void volume of the column. Adapted with permission from ref. 126.

mobile phase is allowed to pass through the column. This process results in a breakthrough curve, as shown in Fig. 6(a). If relatively fast association and dissociation kinetics are present for this system, the mean position of the breakthrough curve can be related to the concentration of the applied analyte and used to determine the affinity and the number of binding sites for the analyte on the immobilized agent.[7,10,142] A major advantage of using frontal analysis and affinity chromatography or HPAC to study drug-protein binding is that both the equilibrium constants and binding capacities for these interactions can be determined simultaneously.

Frontal analysis in HPAC has been utilized to investigate the binding of HSA to *R*- or *S*-warfarin[94,95] and D- and L-tryptophan.[94,99,100,139] Another application of frontal analysis has been the use of this method to determine the binding capacities of monomeric *vs.* dimeric HSA for salicylic acid, warfarin, phenylbutazone, mefenamic acid, sulphamethizole and sulfonylurea drugs.[146] In addition, frontal analysis has been used to examine the binding of various sulfonylurea drugs and site-specific probes to HSA and glycated HSA.[124,126,141] Frontal analysis has also been used to study the binding of *R*/*S*-propranolol and carbamazepine with AGP, as well as the binding of *R*/*S*-propranolol and imipramine with HDL.[147,148]

When used in binding studies, the data from a frontal analysis experiment are typically analyzed according to various models. An example of such a comparison is given in Fig. 6(b) for the binding of tolbutamide to HSA. Similar plots can be employed to examine the fit of single-site or multi-site models to frontal analysis data.[10,149] These fits can often be used to determine both the equilibrium constants and number of binding sites for a drug-protein interaction.[7,10,149] For example, this approach has been used to examine the changes in both the association equilibrium constants and number of binding sites for D- and L- tryptophan on immobilized HSA under various pH conditions.[100] Similar work has been carried out to examine the changes in binding affinity and capacity that accompany the interactions of *R*/*S*-warfarin, D/L-tryptophan and carbamazepine with immobilized HSA at a variety of temperatures.[95,100,150] In such studies it has been shown that shifts in retention with temperature can correspond to a change in either the number of available binding sites or to changes in the affinity of an applied analyte for an immobilized binding agent.[95,100,150–152]

7. Methods for kinetic analysis

There are several ways in which affinity chromatography, and particularly HPAC, can be adapted for use in examining the kinetics of a drug-protein interaction.[7,10,90,154] One approach for this type of study is to use band-broadening measurements, as occurs in the plate height method. In this approach, the total plate height for an analyte is measured on both an affinity column that contains the immobilized binding agent and on an otherwise identical control column that contains no binding agent. These plate heights are then plotted as a function of flow rate or linear velocity and used to find the plate height contribution due to stationary phase mass transfer (H_k). This value, in turn, can then be plotted as a function of the linear velocity and used to determine the dissociation rate constant between the analyte and the immobilized binding agent.[154]

The plate height method has been utilized to examine the association and dissociation kinetics of *R*/*S*-warfarin and D/L-tryptophan with HSA.[96,153] This approach has further been used to examine the effects of changes in various reaction conditions (*e.g.*, pH, temperature and organic modifier content of the mobile phase) on the chiral separation of *R*/*S*-warfarin or D/L-tryptophan on HSA columns.[96,153] It has been shown that this technique can be used for systems with weak-to-moderate binding (*i.e.*, $K_a \leq 10^6$ M^{-1}) in the presence of linear elution conditions.[10,154]

One variation on band-broadening measurements is the peak profiling method.[154] In this technique, which is illustrated in Fig. 7, retention times and peak widths are measured on an affinity column for both the analyte and for a non-retained species; these measured values are then used to determine the kinetics of analyte dissociation from the immobilized binding agent by using the data for the non-retained species to correct for other band-broadening processes.[155,156] The peak profiling

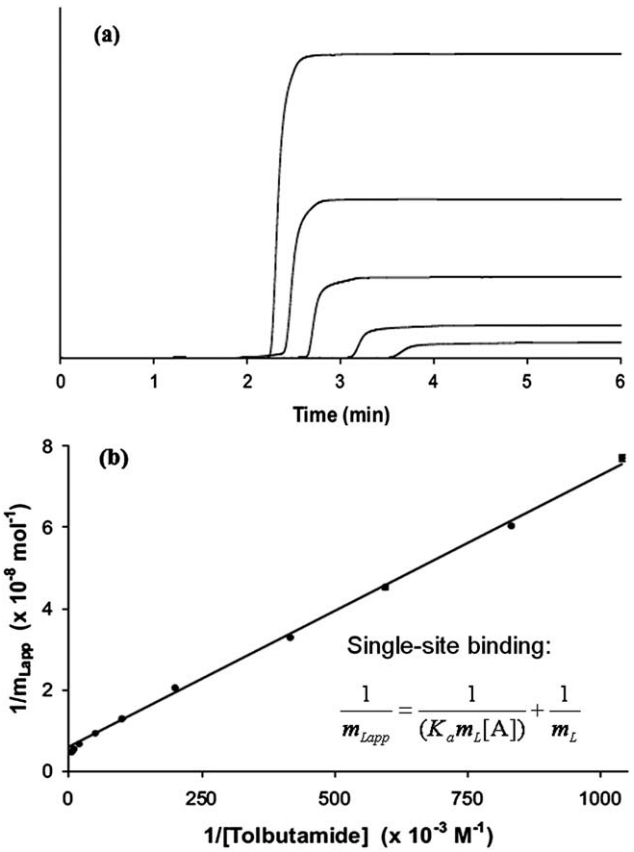

Fig. 6 (a) Breakthrough curves obtained during frontal analysis studies for tolbutamide on an HPAC column containing glycated HSA. The applied concentrations of tolbutamide were (from left-to-right) 200, 100, 50, 20, and 10 μM. The graph in (b) shows a double-reciprocal plot that was used to examine the frontal analysis results for tolbutamide with the glycated HSA column. The inset equation shows the result that would be expected for single-site binding between an applied analyte and an immobilized binding agent, in which [A] is the concentration of the applied analyte, K_a is the association equilibrium constant for the analyte with the binding agent, m_L is the total moles of binding sites involved in these interactions, and m_{Lapp} represents the moles of analyte needed to reach the mean position of the breakthrough curve. In this particular example, deviations from a linear response occur at low values of 1/[Tolbutamide], indicating that multi-site interactions are present for this system. Adapted with permission from ref. 126.

method can be carried out by using data collected at a single, high flow rate;[155] however, work at multiple flow rates tends to result in more robust and accurate estimates of dissociation rate constants.[156]

The peak profiling method has been utilized to examine the dissociation of L-tryptophan from HSA, as well as the dissociation of carbamazepine and imipramine.[156,157] In addition, this technique has been modified for work with multi-site systems, such as those in which a drug has significant binding to both an immobilized protein and a support.[157]

Another approach that has been used to obtain information on the kinetics of drug-protein interactions is the peak decay method.[158–160] This technique is conducted by first injecting a pulse of the analyte onto a small affinity column. A mobile phase is then applied to the column under conditions that prevent re-association of the analyte as it dissociates from the immobilized ligand. As the analyte dissociates under these conditions, it is eluted from the column and produces a decay curve. If the rate of stagnant mobile phase mass transfer rate is fast compared to the rate of analyte dissociation, the slope of the decay curve can be used to provide the dissociation rate constant for the analyte from the immobilized binding agent.[158–160]

The peak decay method has been used in recent studies to determine the dissociation rates of various drugs from HSA.[158,159] It has been found that this method works best at high flow rates, which has made it of interest as a tool for the high-throughput screening of drug-protein dissociation rates.[159,160]

8. Recent trends and developments

Given the wealth of information that HPLC can provide on drug-protein binding, it is not surprising that these methods have recently been explored as a means for the high-throughput screening and analysis of these interactions. Examples were provided in the previous section during the discussion of kinetic measurements by HPAC.[159,160] There have also been several studies that have examined the use of chromatographic methods in estimating or determining the affinities of drug-protein systems. For instance, frontal analysis-mass spectrometry using HPAC columns has been described as an approach for screening drug candidates for a given target.[161] An HSA column operated under gradient elution conditions has been described for rapidly examining the binding of various drugs with this serum protein.[162] In addition, frontal analysis and zonal elution have been used to quickly examine the interaction of various drugs with HSA by using a standard plot of retention factors and association equilibrium constants for reference compounds to determine the association equilibrium constants for other drugs with HSA.[163]

The search for faster HPLC methods has, in turn, led to the use of alternative supports such as monolithic columns for drug-binding studies.[160,164,165] This type of application typically makes use of the good mass transfer properties of monolithic supports and their low back pressures, which allows such materials to provide efficient separations at high flow rates.[89] It has been shown in one study that affinity monoliths based on a copolymer of glycidyl methacrylate and ethylene dimethacrylate (also known as GMA/EDMA monoliths) can be used for the ultrafast immunoextraction of fluorescein. In this case, a 4.5 mm i.d. × 0.95 mm monolith disk containing anti-fluorescein antibodies provided up to 95% extraction of fluorescein within 100 ms.[164] The speed of this approach has made this type of support useful in the direct analysis of free drug fractions in clinical samples, as described in Section 2.

Related work has examined the use of several immobilization methods for the preparation of GMA/EDMA monolithic columns containing HSA. It has been found that the Schiff base method provides for the greatest amount of immobilized HSA and results in the best stereoselectivity and retention for *R*/*S*-warfarin and D/L-tryptophan.[165] Affinity silica monoliths containing HSA or AGP have also been used for chiral separations and in drug-protein binding studies.[166,167] Affinity silica monoliths containing immobilized AGP have been used for the chiral separation of *R*/*S*-warfarin and *R*/*S*-propranolol, while the chiral

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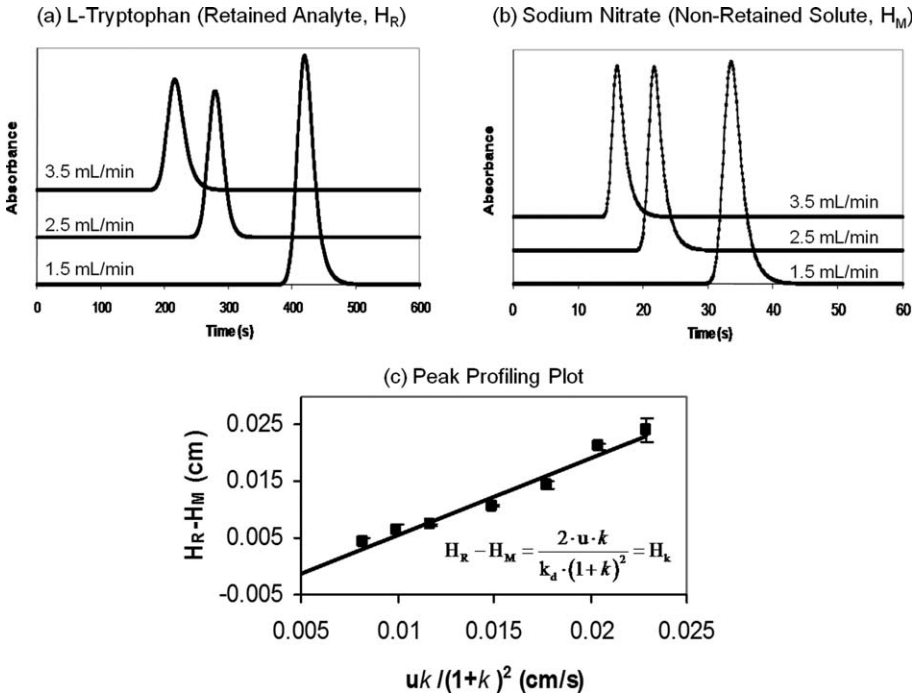

Fig. 7 Use of the peak profiling method to examine the dissociation rate of L-tryptophan from HSA. The chromatograms in (a) and (b) were used to provide the total plate heights H_R and H_M at several flow rates for L-tryptophan and sodium nitrate (*i.e.*, a non-retained solute), respectively, on an HSA column. These results were then examined by using a plot like the one shown in (c) to determine the dissociation rate constant (k_d) for L-tryptophan from HSA. The equation shown in the inset indicates that this type of plot should have a linear response with a slope that is inversely related to k_d. Other terms in this equation include the retention factor (k) for the retained analyte and the linear velocity (u). The value of H_k represents the plate height contribution due to stationary phase mass transfer in the column. Adapted with permission from ref. 156.

separation of *R*/*S*-warfarin, D/L-tryptophan, and *R*/*S*-ibuprofen have been carried out by using a HSA silica monolith column. In each of these situations, silica monolith columns have been found to give better resolution, stereoselectivity, and efficiency when compared to columns that contain the same serum proteins on silica particles or within GMA/EDMA monoliths.[102,167]

Other reports have examined the use of affinity microcolumns that contain either silica monoliths or silica particles in drug-binding studies. The initial goal of this work has been to determine if these columns can be used in place of more traditional HPAC columns for the examination of drug-protein binding by methods such as frontal analysis or zonal elution. One study used immobilized HSA in silica monolith microcolumns with lengths of 1–5 mm for drug-protein binding studies. These microcolumns were tested by using them to measure the retention factors and plate height measurements for warfarin and carbamazepine in the presence of the immobilized HSA. It was found that these silica monolith affinity microcolumns could be used to produce reliable estimates of retention factors and plate heights while decreasing the analysis times that are needed for such measurements down to 10 s or less when using a 1 mm long microcolumn.[102] In addition, it has been shown that affinity silica monoliths as small as 1 mm × 4.6 mm i.d. can be used in the peak decay method to estimate dissociation rate constants for drugs from immobilized HSA.[160]

Affinity microcolumns based on silica particles have also been used with immobilized HSA to perform zonal elution and frontal analysis studies.[168] In this work, warfarin and L-tryptophan were used as model analytes because these two solutes are commonly used as site-specific probes for HSA. It was found that affinity microcolumns as short as 1 mm that contained silica particles could provide comparable results to those obtained with much longer HPAC columns, while significantly decreasing the analysis times and amount of protein needed for such experiments. It was concluded that these features make affinity microcolumns based on either silica particles or silica monoliths attractive for the rapid analysis of drug-protein interactions.[168]

The use of smaller columns may not only make it possible to increase the speed of existing HPLC methods for drug-protein binding studies, but these microcolumns create new opportunities for this type of work. For instance, Section 2 described the use of small immunoextraction columns for the isolation and measurement of free drug fractions.[40–44] Another recent report examined the use of affinity microcolumns containing immobilized HSA as tools for measuring free drug fractions in samples that contained soluble HSA.[169] In this method, a mixture containing a drug and the protein of interest was injected onto an HSA affinity microcolumn. This injection was carried out at a sufficiently high flow rate to minimize dissociation of the drug from the protein as the sample passed through the HSA microcolumn. Under these conditions, a non-retained peak for the drug-protein complex first elutes from the column, followed later by a second peak that represents the free drug fraction that was extracted and retained by the column (see Fig. 8). It was found with this method that greater than 95% extraction could be obtained within 250 ms for the free drug fractions, with this fraction then being separated from the drug-protein complex in the sample. This fraction was then eluted as a separate peak

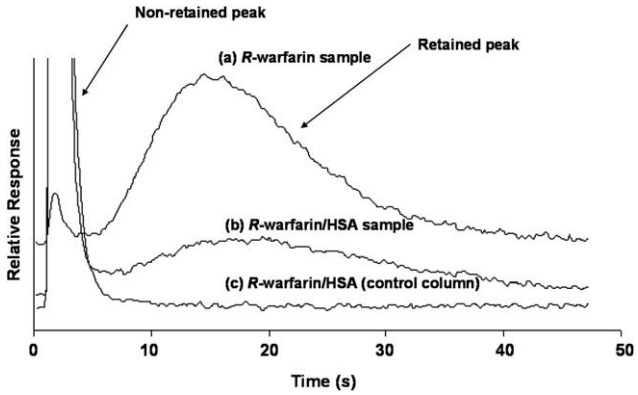

Fig. 8 Chromatograms obtained using ultrafast affinity extraction and a 4.5 mm × 2.1 mm I.D. HSA microcolumn. These chromatograms were obtained by injecting (a) 10 μM *R*-warfarin onto an HSA microcolumn, (b) 20 μM HSA plus 10 μM *R*-warfarin onto an HSA microcolumn, or (c) 20 μM HSA plus 10 μM *R*-warfarin onto a control column at 4.5 mL/min. The non-retained peaks represent the HSA and complex between *R*-warfarin and HSA in the injected sample. The second, retained peak represents the free fraction of *R*-warfarin in the injected sample. Reproduced with permission from ref. 169.

under isocratic conditions within 40 s. This method was used with soluble HSA and several drugs, including *R*-warfarin, *S*-warfarin, *S*-ibuprofen, and imipramine, with association equilibrium constants that ranged from 10^3–10^5 M^{-1} for HSA. This approach was found to give binding parameters that gave good agreement with values obtained by reference methods such as ultrafiltration and equilibrium dialysis.[169]

Another area of ongoing research has been the creation of new mathematical tools and methods for data analysis that have made it possible to expand the types of information on drug-protein binding that can be obtained by HPLC. A good example is a recent method that has been described for the examination of allosteric binding.[170–172] This method can be used with zonal elution and competition or displacement studies that are carried out by HPAC. In this method, the retention factors for an analyte are measured in the presence of an immobilized binding agent and in the presence (k) or absence (k_0) of known concentrations of a competing agent (I) that may have either direct competition or allosteric competition with the analyte. A plot is then made of the term $k_0/(k\text{-}k_0)$ *versus* 1/[I], which can be used to determine if direct competition or allosteric interactions are present. If allosteric interactions are found to occur, the same plot can be used to measure the coupling constant for the effect of the competing agent on the analyte.[170–172] Other examples of recent developments in the use of chromatography to study drug-protein binding are the extension of this general approach to examine systems with multi-mode interactions (*e.g.*, the binding of a drug with a lipoprotein or multi-site binding of a drug with a protein)[104,124–126] and experiments that consider the effects of multiple factors (*e.g.*, protein modification and the presence of fatty acids) on this type of binding.[173]

Acknowledgements

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