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CHARACTERIZATION OF DRUG INTERACTIONS WITH SERUM PROTEINS BY USING HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

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Abstract

The binding of drugs with serum proteins can affect the activity, distribution, rate of excretion, and toxicity of pharmaceutical agents in the body. One tool that can be used to quickly analyze and characterize these interactions is high-performance affinity chromatography (HPAC). This review shows how HPAC can be used to study drug-protein binding and describes the various applications of this approach when examining drug interactions with serum proteins. Methods for determining binding constants, characterizing binding sites, examining drug-drug interactions, and studying drug-protein dissociation rates will be discussed. Applications that illustrate the use of HPAC with serum binding agents such as human serum albumin, α_1-acid glycoprotein, and lipoproteins will be presented. Recent developments will also be examined, such as new methods for immobilizing serum proteins in HPAC columns, the utilization of HPAC as a tool in personalized medicine, and HPAC methods for the high-throughput screening and characterization of drug-protein binding.

Keywords

α_1-acid glycoprotein; drug-protein binding; high-performance affinity chromatography; high-throughput screening; human serum albumin; lipoproteins; personalized medicine

Introduction

Drug interactions with transport proteins and binding agents in serum can play an important role in determining the activity, distribution, rate of excretion or metabolism, and toxicity of many pharmaceutical agents in the body. The binding of drugs with agents in blood is a reversible process that usually involves proteins like human serum albumin (HSA) and α_1-acid glycoprotein (AGP); however, more complex agents such as lipoproteins, red blood cells or platelets can be involved as well [1-7]. The widespread occurrence of these interactions has made the assessment of drug-protein binding in blood an important part of the adsorption/distribution/metabolism/excretion (ADME) data needed prior to the approval of new pharmaceutical agents. In addition, the binding of drugs with serum agents can help to improve the solubility of hydrophobic agents and can be an important source of direct or indirect competition between drugs or drugs and endogenous compounds such as fatty acids [1,2,8-13]. These effects make it essential for pharmaceutical chemists to determine and

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understand how drugs may interact with proteins or other binding agents in blood and how this binding may be affected by the presence of other compounds in serum [1,2,8-15].

Ultrafiltration and equilibrium dialysis are often used to study the binding of drugs to serum agents [1,2,4,5,16-18], but many other techniques can be employed for such work. These other methods have included structural studies based on X-ray crystallography [11] and binding assays based on fluorescence or absorption spectroscopy [19], capillary electrophoresis [20-26], surface plasmon resonance (SPR) [27] and nuclear magnetic resonance (NMR) spectroscopy [28,29]. It is also possible to study these interactions by using chromatographic systems. One chromatographic approach that has been of great recent interest for such work is high-performance affinity chromatography (HPAC) [10,12,14,15,30]. This review will look at how HPAC can be used in these studies and will explore the various types of information that can be gained by this method when investigating drug interactions with serum proteins and other binding agents. Methods for determining binding constants, characterizing binding sites, examining drug-drug interactions, and studying drug-protein dissociation rates will be presented. Applications will be given that illustrate the use of HPAC with serum binding agents such as human serum albumin, α_1-acid glycoprotein, and lipoproteins. Recent developments will also be examined, such as new methods for immobilizing serum agents in HPAC columns, the utilization of HPAC as a tool in personalized medicine, and HPAC methods for the high-throughput screening of drug-protein binding.

Overview of HPAC and HPAC-Based Binding Studies

The method of HPAC is a subset of affinity chromatography, in which a biologically-related agent is used as the stationary phase [31-33]. In HPAC, this stationary phase is placed in a column that is suitable for use in high-performance liquid chromatography (HPLC). The retention of solutes in both HPAC and affinity chromatography is based on the specific, reversible interactions that are often found in biological systems, such as the binding of an enzyme with a substrate or a drug with a serum protein. These interactions can be examined in HPAC by immobilizing one of the pair of interacting species onto a solid support and using this agent as a stationary phase in an HPLC column (see Figure 1). The other species is then injected onto the column or applied in the mobile phase to examine its interactions with the immobilized molecule. By examining the elution profile for this retained species, information can be obtained on the strength of binding between the two agents, the rate of this interaction, and the binding sites [14,15,30,34-36].

The use of HPAC, or affinity chromatography in general, for studying a biological interaction is referred to as quantitative affinity chromatography, analytical affinity chromatography, or biointeraction affinity chromatography. The theory and various applications of this approach have been discussed in previous reviews [14,15,30,34-36]. As will be demonstrated in this review, HPAC can be used to provide a variety of information on a drug-protein interaction. This information can involve data on the degree of binding, equilibrium constants, number of binding sites, rates of association or dissociation, drug-drug competition, and even the location of binding sites for a drug on a protein.

One advantage of utilizing HPAC over methods such as ultrafiltration or equilibrium dialysis for drug binding studies is the ability of HPAC to reuse the same ligand preparation for multiple experiments. It has now been shown in many studies that columns containing HSA attached to silica can often be used for 500-1000 injections or sample applications under conditions that are typically used in drug binding studies (e.g., pH 7-7.4 phosphate buffer and 4-45°C) [14,36]. Similar long term stability under the same conditions has been noted with AGP columns [37-39]. This good stability creates a situation in which only a relatively small amount of protein is needed for a large number of studies (i.e., μg to mg

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quantities for hundreds of experiments), which helps to provide good precision by minimizing run-to-run variations [14]. HPAC methods are also attractive for use in studies of drug interactions with serum agents because these methods can be automated with standard HPLC equipment. Furthermore, these methods are relatively fast and require only short periods of time for most binding studies, with run times of 5-15 min being common and some methods even allowing results to be obtained in less than a minute (see later discussion of high-throughput methods) [14,15]. Another useful feature of HPAC is the column containing the immobilized binding agent is continuously washed with an applied solvent between experiments; this feature is useful with immobilized proteins such as HSA in eliminating the effects produced by common contaminants (e.g., fatty acids) that may have been present in the original protein sample [30,36].

Serum Agents Examined by HPAC

Many common binding agents for drugs in serum have been investigated by HPAC. Most of these studies have examined the binding of drugs to HSA and related serum albumins [10,12,14,15,30,36,41]. HSA is the most abundant serum protein in humans and has a typical concentration in plasma of 35-50 g/L. This protein has a molecular mass of 66.5 kDa and contains a single chain of 585 amino acids that is stabilized by 17 disulfide bridges. One of the functions of HSA is to take part in the binding and delivery of various substances throughout the body, such as acidic drugs, organic anions, long-chain fatty acids, and some vitamins [9]. In addition to its use in drug binding studies, HSA and related proteins have been utilized in HPAC for chiral separations of drugs and other solutes [42-50].

AGP is another serum binding agent that has been examined by HPAC [15,36-41]. This protein has an approximate molecular mass of 41 kDa. AGP has a heterogeneous composition that consists of a single polypeptide chain of 181 amino acids and five carbohydrate groups [51-53]. Normal serum levels of AGP are around 0.5-1.0 g/L, but the concentration of this protein can increase during disease [53]. Although AGP has a much lower concentration than HSA in serum, AGP can be a major binding protein for basic or neutral drugs. Like HSA, AGP has been used in HPAC columns to carry out chiral separations of drugs and small solutes [39,44,54-64].

Recent work has also explored the use of HPAC in studying the binding of drugs with lipoproteins [41,65]. A lipoprotein is a soluble complex of lipids and proteins that transports various non-polar substances in blood, including triglycerides, cholesterol esters, and related lipids [66-69]. Lipoproteins are often classified based on their density, resulting in five general categories: chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL), immediate low density lipoprotein (ILDL) and high density lipoprotein (HDL) [67-74]. These lipoproteins have the following typical concentrations in a healthy fasting adult male: 280 mg/dL HDL, 410 mg/dL LDL, and 150 mg/dL VLDL (Note: Chylomicrons generally appear only after a meal) [66-68]. Of these various forms, HDL and LDL are of the most interest in drug binding studies; however, some binding by drugs can also occur with VLDL [41,65,69,75].

Preparation of HPAC Columns for Drug-Protein Binding Studies

Traditional affinity chromatography is often carried out by using a carbohydrate-based material as the support (e.g., agarose), while HPAC instead makes use of a more rigid and efficient support that is suitable for HPLC columns [31-33,41,76]. Examples of supports that have been utilized in HPAC include modified silica or glass, azalactone beads, hydroxylated polystyrene media, and some sol gel materials [31,76,77]. Silica has been the main support used in HPAC [78]; this material has good long-term stability under the pH and buffer conditions that are typically employed in drug binding studies (e.g., pH 7-7.4), but this type

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of material should not be used at a pH below 2 or above 8. A number of recent studies have examined the creation and use of monolithic columns that contain proteins such HSA or AGP [39,42,43,49]. Monolithic supports in chromatography consist of a single block of a porous polymer or porous silica [42,76,79]. When these supports are combined with the immobilization of a binding agent, the resulting method is called affinity monolith chromatography (AMC) [42,79]. Advantages of monolithic supports include their low back pressures and better mass transfer properties than silica particles, allowing for more efficient separations or the use of higher flow rates for binding studies in HPAC [39,42,43,49,76,79]. The ability of either silica particles or monoliths to be used at relatively high flow rates and as part of HPLC systems has made HPAC an attractive tool for the characterization and high-throughput screening of drug interactions with serum proteins and other binding agents [42,79,81-87].

An important factor to consider in the development of an HPAC column for drug binding studies is the degree to which the immobilized binding agent acts as a model for the same agent in its soluble form in blood or serum. In the case of HSA, many studies have found that immobilized HSA can provide good qualitative and quantitative agreement with the drug binding behavior seen for soluble HSA (Note: Similar agreement has been seen with new types of AGP columns, as discussed later in this section). This work has included a comparison of HSA columns and soluble HSA in terms of their displacement effects and allosteric interactions [30,88-96] and their equilibrium constants and rate constants for drugs [30,88,97-99]. However, there are some cases in which differences in the behavior of HPAC columns and non-immobilized serum proteins has been noted, such as in work using cross-linked albumin [44] or cross-linked AGP [100,101]. It is because of these potential differences that the development of a new type of column for HPAC should be followed by validation using model compounds with known binding properties before the column is then utilized to examine other substances [30,102].

There are some commercial albumin columns used for chiral separations, which are prepared by cross-linking albumin in the presence of glutardialdehyde or *N*,*N*'-disuccinimidyl carbonate on silica [44]. HSA supports that are used in drug binding studies in HPAC have typically been prepared by covalently immobilizing HSA through its amine groups. This type of immobilization can be easily carried out by using silica that has been activated with 1,1'-carbonyldiimidazole [89,103], that has used in the Schiff base method (i.e., reductive amination) [88,103-105], or that has been activated with an *N*-hydroxysuccinimide ester [106-109]. Of these amine-based approaches, the Schiff base and *N*-hydroxysuccinimide methods tend to give the best recovery of activity for the final immobilized protein [107,108]. Some reports have also used albumin that has been adsorbed to ion-exchange columns and silica [105,109], but some desorption of this protein from these materials may occur over time. Alternative supports based on agarose [110] or hydroxyethylmethacrylate [111,112] have been employed in some studies, although silica or monoliths have been used in most work with HPAC binding studies because of their better mechanical stability and flow properties [78,79]. It is also possible to immobilize HSA through its lone free cysteine group by using silica and a sulfhydryl-based coupling method, which has been shown to give site-selective immobilization for this protein [113]. The use of sol gels has been reported for the entrapment of soluble BSA [114] and a recent method has been described by which soluble HSA can be entrapped in dihydrazide-activated silica by using oxidized glycogen as a capping agent (see Figure 2) [115]. This latter approach can be used with standard HPLCsupports and has been shown with model systems (e.g., the binding of HSA with warfarin) to provide essentially 100% activity for the entrapped protein along with association equilibrium constants that agree with values for the same protein in its soluble form [115].

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Several types of AGP columns are available commercially for chiral separations [48]. For instance, a chiral stationary phase can be made by adsorbing AGP to an ion-exchange support such as diethylaminoethyl silica and then oxidizing this protein with periodate to form aldehyde groups that can cross-link the AGP through Schiff base formation. Another reported approach involves the mild oxidation of AGP and the immobilization of this oxidized protein onto a support that contains hydrazide groups [37-40]. This second approach has been used with silica particles or various monoliths and has been demonstrated to give immobilized AGP that correlates well with the behavior of soluble AGP in drug binding studies [37-40,116]. The entrapment of soluble AGP in dihydrazide-activated silica by using oxidized glycogen as a capping agent, as described in the previous paragraph for HSA, has also been reported [115].

Lipoproteins have been immobilized to silica by the Schiff base method for use in HPAC and drug binding studies. This approach has been utilized with HDL particles and found to give a column that is stable for over 3000 h of continuous operation in the presence of pH 7.4, 0.067 M phosphate buffer [65]; the same approach has been employed with LDL particles (see example in next section). It has been shown that these columns can be used in studies of drug binding with lipoproteins and can provide detailed information on the processes that are occurring during these interactions [65].

In some cases, modified forms of serum binding agents have been studied by HPAC. For instance, HPAC has been used to compare the binding capacities of monomeric versus dimeric HSA for various solutes [117]. Several recent reports have used HPAC to determine how the non-enzymatic glycation of HSA during diabetes might affect the binding of this protein with various sulfonylurea drugs (e.g., acetohexamide and tolbutamide) or common site-selective probes for HSA (i.e., L-tryptophan and warfarin) [118-120]. In addition, HPAC columns have been developed that contain HSA that has been acetylated [121] or chemically altered at Sudlow site I by using *o*-nitrophenylsulfenyl chloride to modify Trp-214 on HSA [122].

General Approaches for Studying of Drug-Protein binding by HPAC

There are many approaches that can be used in HPAC to examine drug interactions with binding agents from serum. Zonal elution is the most popular method when information is desired on drug-drug competition, the overall extent of drug interactions with a binding agent, or the effect of temperature, pH or solvent polarity on this binding. A zonal elution experiment typically involves the injection of a narrow plug of analyte onto a column that contains an immobilized binding agent. The elution time or profile of the analyte is then monitored. The retention of the analyte will be directly dependent upon how strongly this analyte is bound by the immobilized agent, while the width and shape of the peak will be related to the kinetics of these interactions. This method can be used to examine how the retention of the analyte changes with temperature or as the contents of the mobile phase are varied, such as occurs during the inclusion of a competing drug or solute. An example of such a study is shown in Figure 3, in which zonal elution is used to examine the competition of tolbutamide in the mobile phase with small pulses of L-tryptophan that are injected onto a column that contains HSA [123]. An important advantage of zonal elution is that it requires only a small amount of analyte per injection. This method also has the ability to examine more than one compound per injection, provided that there is adequate resolution between the peaks for these compounds [30,36].

Frontal analysis is a second technique that can be utilized in HPAC to study the binding of a drug with a protein or serum binding agent. In this technique, a solution containing a known concentration of the drug or analyte is continuously applied to a column that contains the immobilized binding agent. As the immobilized agent binds to the analyte, the column will

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eventually become saturated and the amount of analyte that elutes from the column will increase. The result is the formation of a breakthrough curve, as illustrated in Figure 4. This example shows a series of breakthrough curves obtained during the application of *R*-propranolol to a column that contains the lipoprotein LDL. If fast association and dissociation kinetics are present for the interaction being examined in this type of experiment, the amount of applied analyte that is required to reach the mean position of the breakthrough curve can be used to obtain information on both the moles of active binding agent in the column and the equilibrium constant(s) for the analyte as it interacts with this binding agent. This feature makes frontal analysis attractive for use in detailed studies of drug-protein binding and in the high-throughput screening of drug-protein interactions [30,36].

A number of techniques will be described later in this review that can be used in special situations for drug binding studies [124]. For example, the plate height method and peak profiling methods are variations on the zonal elution technique that are used to study the kinetics of drug-protein interactions [34,36,97,98,124-126]. The peak decay method is a special type of zonal elution that uses fast flow rates and small HPAC columns to examine the dissociation rates of drugs from immobilized binding agents [127-129]. Finally, ultrafast affinity extraction is an approach used to rapidly isolate the free fraction of drugs from samples; this method can be used to either measure the overall binding of a drug in serum or to study drug-protein binding in aqueous solutions [130-133].

Measurement of the Degree of Drug Binding

An important item to measure when describing drug interactions in serum is the overall degree of binding that a drug may have with proteins or binding agents in such a sample. Zonal elution can be used to obtain such information based on the retention factor (k) that is measured for a small amount of drug that is injected onto an HPAC column containing the binding agent of interest. The value of k can be determined experimentally by using the relationship $k = (t_R - t_M)/t_M$, in which t_R is the observed retention time for the drug and t_M is the column void time. If a local equilibrium and linear elution conditions are present during this measurement (i.e., the value of k is not affected by the amount of injected sample and fast association/dissociation kinetics are present), Eqn. (1) can be used to relate the value of k to the fraction of the drug that is bound to the immobilized binding agent (*b)* versus the fraction of drug that remains free in solution in the mobile phase (*f*) [12,30].

$$k = b/f \tag{1}$$

It is then possible from this data to calculate the value of either *b* or *f* and the percent binding by using the fact that $b + f = 1$. This method for determining the extent of binding for a drug has been found to give good correlation with ultrafiltration for the interactions of HSA with various coumarin compounds that have medium-to-strong binding to this protein [134]. Similar work has been conducted with other classes of compounds [135] and has been used with liquid chromatography/mass spectrometry (LC/MS) for the simultaneous examination of several drugs in a mixture [136].

In more recent work, HPAC methods have been created to directly measure the free fraction or bound fraction of drugs and endogenous solutes in clinical samples for use in personalized medicine. These methods have made use of small HPAC columns for the ultrafast extraction of free drug fractions [130-133,137]. In one study, ultrafast immunoaffinity chromatography was used to analyze the bound drug fractions of warfarin/HSA mixtures [130]. This report used small HPAC columns that could selectively extract the free fraction of warfarin in less than 180 ms. The HSA and HSA-bound warfarin which

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eluted non-retained from this column were then separated by using internal surface reversed-phase columns to measure the HSA-bound fraction of warfarin in the sample [130]. This approach was then used with other antibodies for other targets and combined with a displacement immunoassay format to measure the free fractions of thyroxine [131] and phenytoin in clinical samples [132]. These methods have made use of labels that are detected through on-line measurements of chemiluminescence or near-infrared fluorescence and have been found to provide good correlation versus reference methods [131,132]. A similar approach has been developed for the measurement of free carbamazepine fractions by using HPAC columns for ultrafast immunoextraction followed by detection using LC/MS [137].

Another recent study demonstrated that the free and bound fractions of a drug in a sample can also be measured by using immobilized HSA in affinity microcolumns [133]. This approach is illustrated in Figure 5. First, the drug/protein mixture of interest is injected onto the HSA microcolumn at a high flow rate. These conditions are chosen so that the free fraction of the drug in the sample can be extracted by the immobilized HSA while the bound fraction of the drug in the sample elutes non-retained with the sample proteins. When this method was tested, an HSA microcolumn was noted to give greater than 95% extraction within 250 ms for all of the tested drugs. The captured, free fraction of each drug was then eluted from the HSA microcolumn within 40 s under the same mobile phase conditions, making it possible to directly determine the free fraction of drug that was present in the original sample. The degree of binding that was measured by this method for HSA gave good agreement with values that were obtained by reference methods, such as ultrafiltration and equilibrium dialysis [133].

Measurement of the Strength of Drug Binding

A more specific measure of the strength of drug binding to a given agent can be obtained by determining the equilibrium constant for this interaction. One simple means for estimating this binding strength is through the use of a zonal elution experiment in which a small amount of drug is injected onto an HPAC column that contains the desired binding agent. The retention factor k for the injected drug that is measured can then be related through Eqn. (2) to the number of binding sites the drug has in the column and to the association equilibrium constants for the drug at these sites [12,30].

$$k = \frac{(K_{A1} n_1 + \ldots + K_{An} n_n)\, m_L}{V_M} \tag{2}$$

The term m_L in this equation is the total moles of all binding sites for the drug in the column and V_M is the column void volume. The association equilibrium constant for the drug at each of its binding sites on the column is given by the terms K_{A1} through K_{An}. The fraction of each type of site in the column is given by the terms n_1 through n_n. The summation of the terms $K_{A1}\, n_1$ through $K_{An}\, n_n$ is also known as the global association equilibrium constant ($n\, K_A$), which is directly related to the value of k.

It can be seen from the relationship in Eqn. (2) that the overall retention factor will be affected by changes in both the number of binding sites available to a drug in the column and the association equilibrium values for the drug at these sites. This relationship has been used to compare the activities and properties for HSA columns that have been prepared by different methods, in which the value of k is first divided by the protein content to correct for any variations in the total protein content of a support as the immobilization method is varied [107]. The relative binding of two solutes can also be compared by using Eqn. (2) and calculating the ratio of their retention factors on the same HPAC column. This technique has

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been used in recent work with the high-throughput screening of drug binding to HSA [138]. However, care must be used when employing Eqn. (2) with drugs that may have multisite binding to a column because these sites may have different susceptibilities to a loss of activity during the immobilization process [12,30].

More detailed information on the number of binding sites and their equilibrium constants can be obtained by using frontal analysis to examine the interactions of a drug with an immobilized binding agent over an appropriately large range of analyte concentrations. This method involves first measuring the apparent moles of analyte (m_{Lapp}) that are required to reach the mean position of the breakthrough curve at each applied concentration of the analyte ([A]). A plot of the data is then examined according to several models to determine what type of binding is present. For instance, the presence of a 1:1 interaction between the analyte and the immobilized binding agent should result in a response that follows Eqns. (3) or (4) [14].

$$\frac{1}{m_{Lapp}} = \frac{1}{K_A m_L [A]} + \frac{1}{m_L} \tag{3}$$

$$m_{Lapp} = \frac{m_L K_A [A]}{1 + K_A [A]} \tag{4}$$

According to Eqn. (3), a plot of $1/m_{Lapp}$ versus 1/[A] for a system with 1:1 binding should provide a linear response with an intercept and slope that can be used to obtain the total moles of active binding sites in the column (m_L, from the inverse of the intercept) and the association equilibrium constant for this interaction (K_A, from the intercept/slope ratio). Eqn. (4) shows how the same data could be used with non-linear regression to obtain such information. Similar equations to those shown in Eqn. (4) can be used with non-linear regression to examine systems with multi-site binding or mixed-mode interactions [14]. This approach has been used in a number of studies to examine the binding of drugs and other solutes with HSA and AGP columns [14,30,37,40]. An example of one such study is shown in Figure 6, in which frontal analysis data for the binding of *R*-warfarin to HSA was examined by using Eqn. (3) and a 1:1 binding model [88].

It is possible using HPAC to obtain further information on binding strength by using site-specific probes and competition studies to examine the interactions of a drug at particular regions on an immobilized binding agent. This type of information can be acquired through zonal elution experiments by injecting a small amount of a site-specific probe in the presence of the immobilized binding agent and several solutions that contain known concentrations of the drug. Eqn. (5) shows the response that is expected if the injected probe (A) and drug (I) bind to a single common site on the binding agent [14].

$$\frac{1}{k} = \frac{K_I V_M [I]}{K_A m_L} + \frac{V_M}{K_A m_L} \tag{5}$$

In this equation, K_A and K_I are the association equilibrium constants for the interactions of the immobilized binding agent with the probe and the drug at their site of competition. If direct competition is occurring between these compounds, then a plot of 1/k versus [I] should result in a linear relationship that can be used to find the association equilibrium constant for the drug at its site of competition with the probe. An example of such a study is shown in Figure 7, in which carbamazepine and propranolol were found to share a common

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binding site on AGP based on the linear behavior of a plot that was prepared for these drugs according to Eqn. (5) [40].

Competition and Displacement Studies

HPAC is also a powerful tool for examining the effects of one solute on another as both interact with an immobilized binding agent. This feature makes HPAC valuable in studying drug-drug interactions or drug-solute competition and displacement effects involving HSA, AGP or related serum binding agents. Zonal elution is the mode that is most frequently used for competition and displacement studies [14,30]. This work is performed by injecting a small pulse of the analyte while a fixed concentration of a potential competing agent is passed through the column in the mobile phase. It is relatively easy from such an experiment to determine whether two compounds interact as they bind to the same immobilized agent because the presence of one compound in the mobile phase will cause a shift in the retention of the other compound as it passes through the column. To obtain further information on this interaction (e.g., the nature of this competition and the number of sites that are involved) it is necessary to compare the zonal elution data to the response that is expected when using various binding models.

Plots prepared according to Eqn. (5) can be used in a zonal elution competition study to see if the system fits a model with 1:1 direct competition between the injected analyte and competing agent. If no interactions are present between these two agents, the measured value of 1/k should show no significant change as the concentration of the competing agent is increased. If linear behavior is seen for a plot that is prepared according to Eqn. (5), the results can be said to show good agreement with a 1:1 model involving direct competition between the injected analyte and competing agent, as illustrated in Figure 7. Negative deviations from such a plot can be used to detect the presence of multi-site binding or possible negative allosteric effects, while a decrease in 1/k with the competing agent concentration would indicate that positive allosteric effects are present [14,30]. Equations similar to Eqn. (5) would then be used to describe these more complex systems. These other equations include relationships that have been developed to describe multi-site systems, allosteric systems, or systems that involve both the soluble and immobilized forms of a binding agent [14,34,35]. The information that is generated through this type of experiment is equivalent to that of a displacement study that is conducted by using equilibrium dialysis or ultrafiltration. However, the use of HPAC for this work can be conducted in much less time than these other methods and requires much smaller amounts of the binding agent [14,15].

An alternative way of analyzing zonal elution data in competition and displacement studies involves plotting the retention data according to Eqn. (6), in which k_0 is the retention factor for analyte A in the absence of any competing agent and k is the retention factor measured when a competing agent concentration of [I] is present in the mobile phase [139-141].

$$\frac{k_0}{k - k_0} = \frac{1}{\beta_{1\rightarrow A} - 1} \cdot \left(\frac{1}{K_{IL}\,[I]} + 1 \right) \tag{6}$$

This particular equation can be used in a quantitative fashion to discriminate between direct competition, non-competition and positive or negative allosteric effects. In this equation, the immobilized binding agent is viewed as having at least two sites, one for binding to the injected analyte and the second for possible binding to a competing agent if allosteric effects are present. In the case of allosteric effects, the interaction of A with the binding agent L will be altered as I also binds to this agent, causing the association equilibrium constant for A with the binding agent to change from K_{AL} to K_{AL}'. This change is represented in Eqn.

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(6) by the coupling constant $ß_{I→A}$, in which $ß_{I→A} = K_{AL}'/K_{AL}$. According to Eqn. (6), a plot of $k_0/(k-k_0)$ versus 1/[I] should give a linear relationship for a simple positive or negative allosteric interaction; this plot, in turn, can be used to provide the values for $ß_{I→A}$ and K_{IL} . An example of such a plot is shown in Figure 8 for the allosteric interactions that occur between *R*- or *S*-ibuprofen and *S*-lorazepam acetate on HSA [140]. Related work has investigated the allosteric effects that occur on HSA during the interactions of *R*- or *S*-ibuprofen with other benzodiazepine enantiomers, the interactions between warfarin and tamoxifen on HSA, and the interactions between L-tryptophan and phenytoin on HSA [139-141]. A useful feature of Eqn. (6) and these experiments is that they can be used to look independently at both directions of an allosteric effect, thus allowing separate measurements to be made for the two directions of such an interaction [139].

Frontal analysis may also be utilized as a tool to examine the competition between solutes for an immobilized binding agent such as HSA [142]. This type of experiment can be carried out by measuring the change in the breakthrough time for an analyte as different concentrations of a competing agent are placed in the mobile phase. In this situation, direct competition between the analyte and the competing agent will lead to a smaller breakthrough time for the analyte as the competing agent concentration increases. Positive or negative allosteric effects can also be observed and will lead to a positive or negative shift in the breakthrough times with an increase in competing agent concentration.

Effects of Temperature and Solution Conditions on Drug Binding

The effects of solution conditions on drug binding have also been examined by HPAC for many types of serum binding agents [14,15,30,38,59,62-64,100]. Zonal elution can be used for this purpose to see how a change in column conditions will alter the observed retention of an injected drug. Factors that can be easily investigated by this method include the pH, ionic strength, and general content of the mobile phase. This information can be valuable in determining the relative contributions of various forces to the formation and stabilization of a solute-protein complex. An example of such a study is shown in Figure 9 [93]. Adjusting the solvent polarity by adding a small amount of organic modifier to the mobile phase, as illustrated in Figure 9, can alter solute-protein binding by disrupting non-polar interactions or by causing a change in solute and protein structure. Changing the pH can affect interactions between a protein and solute by altering their conformations, net charges, or coulombic interactions. An increase in ionic strength can decrease coulombic interactions through a shielding effect but may also cause an increase in non-polar solute adsorption [14,30]. Although the same types of experiments could be conducted by techniques like equilibrium dialysis and ultrafiltration, the greater precision and reproducibility of HPAC makes it a better tool for detecting small changes in binding properties. In addition, the use of the same protein preparation for many experiments in HPAC significantly reduces the cost for such work and minimizes the effects of batch-to-batch variations.

Temperature is another factor that is often varied during zonal elution studies to study drug-protein interactions. For example, the role of temperature in chiral separations on AGP or HSA columns has been determined in many studies by this approach [14,15,37,48,58,64,143-146]. If used correctly, this type of experiment can provide not only qualitative data on the effects of temperature on binding but can also be used to determine the changes in enthalpy and entropy that are associated with the formation of a drug-protein complex. For instance, the following relationship shows how the retention factor for a drug would be expected to vary on an immobilized protein column if this system has single site binding and the analyte has no other significant source of retention on the column [30].

$$\ln \quad k = -(\Delta H/R \quad T) + \Delta S/R + \ln(m_L/V_M) \tag{7}$$

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In Eqn. (7), T is the absolute temperature at which the retention factor is measured, R is the ideal gas law constant, ΔHis the change in enthalpy for the reaction, ΔS is the change in entropy, and the other terms (m_L and V_M) are the same as defined previously. The resulting plot of ln k versus 1/T has often been employed in thermodynamic studies [99,147-154] and in reports that consider how changes in temperature affect the selectivity of albumin columns [155,156]. However, caution needs to be exercised in using Eqn. (7) for thermodynamic studies in that it requires that the value of m_L does not change with temperature. This assumption is not always valid for the binding of drugs to binding agents such as HSA [93,147]. A better approach is to use frontal analysis to obtain independent estimates of the association equilibrium constant (K_A) and the binding capacity (m_L) for the drug-protein interaction. For a system with single site binding, the resulting K_A values that are obtained from the frontal analysis data can be plotted as a function of temperature according to Eqn. (8).

$$\ln \quad K_A = -(\Delta H/R \quad T) + \Delta S/R \tag{8}$$

If the resulting plot of ln K_A versus 1/T is found to be linear, the slope and intercept can then be used to obtain the values of ΔH and ΔS [14,30]. Frontal analysis can also be used to see how the apparent activity of the immobilized protein (m_L) changes with temperature [93,147].

Examining the Location and Structure of Binding Sites

Many reports have used HPAC to determine the location and structure of binding regions for a drug or solute on protein such as HSA or AGP [14,30,40]. This is often done with zonal elution and through the use of an injected probe that has a known binding site on the protein. This type of experiment, which was illustrated earlier in Figures 3 and 7, has been used to investigate the binding of HSA and other albumins with non-steroidal anti-inflammatory drugs [157,158], *R*- and *S*-ibuprofen [159], *cis*- and *trans*-clomiphene [160], digitoxin or acetyldigitoxin [161,162], phenytoin [163], carbamazepine [147], L-thyroxine [164] and verapamil [95]. Probe compounds that have been employed in such work include *R/S*-warfarin, L-tryptophan, phenylbutazone, *R/S*-ibuprofen, 2,3,5-triiodobenzoic acid, *cis/trans*-clomiphene, acetyldigitoxin, digitoxin, and phenol red [95,147,157-164]. Other agents that have been explored for use with HSA include various coumarins as probes for Sudlow site I [165] and indoles as probes for Sudlow site II [166]. Propranolol has been used in the same manner as a site-selective probe for AGP [40]. It is possible in this approach to use multiple probes to generate maps that show the relationship between the various binding regions for a drug on a protein such as HSA [162,163]. As an example, probes for both Sudlow sites I and II have been recently used with columns containing normal HSA or glycated HSA to determine how binding by tolbutamide and acetohexamide at these regions may change during diabetes (see Figure 10) [119,120].

An additional approach for learning about the binding sites on a protein is to use a set of structurally-related compounds to see how changes in the structure of a drug or solute will affect their interactions with this protein. This method has been employed to examine the binding of HSA to L-thyroxine and related thyronines, to warfarin and various coumarins, and to a variety of indole compounds [164-166]. If a large set of test solutes are sampled, the data can be used to develop a quantitative structure-retention relationship [167-169]. This approach has been used to investigate the binding of HSA to benzodiazepines [170] and 2,3-substituted-3-hydroxy-propionic acids or related compounds [171,172]. Similar approaches have been employed for determining the structural requirements needed for the binding and stereoselective interactions of AGP with quinazolone derivatives [145], tetracyclic and

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pentacyclic vinca alkaloid analogs [173], quinolones [174], amino alcohols [175], beta-adrenolytic drugs and antihistamines [176-178].

Another technique that has been used in HPAC to characterize solute binding to serum proteins is to use altered forms of the protein that have been modified at specific sites. For instance, one report used HSA that was treated with *p*-nitrophenyl acetate, a reagent thought to mainly modify Tyr-411 (i.e., a residue located at Sudlow site II on this protein). This modification was shown to change the retention of a variety of solutes injected onto normal HSA versus modified HSA columns [121]. A similar study used *o*-nitrophenylsulfenyl chloride to modify the lone tryptophan residue on HSA, Trp-214, which is located within Sudlow site I [122]. Other studies have involved a modification of the lone free cysteine residue on HSA with ethacrynic acid [179] and the use of BSA fragments in the chiral separation of benzoin and other drugs [180,181].

Kinetic Studies of Drug Interactions

It is also possible to obtain information on the rates of drug interactions with HSA by using HPAC. Band-broadening measurements can be used as one approach for such work. This type of experiment involves injecting a small amount of a drug onto an HPAC column while carefully monitoring the retention time and width of the eluting peak. These injections are performed at several flow rates on both the HPAC column and on a column of the same size which contains an identical support but with no immobilized binding agent being present. This control column is used to correct for any band-broadening that occurs due to processes other than the binding and dissociation of the drug from the immobilized binding agent. By comparing plots of the peak widths (or plate heights) for the HPAC and control columns, the contribution to band-broadening due to the drug interaction with the binding agent can be obtained. This contribution is represented by Eqn. (9),

$$H_k = \frac{2 \cdot u \cdot k}{k_d \cdot (1+k)^2} \tag{9}$$

in which u is the linear velocity of mobile phase in the column, k is the retention factor of the injected solute, H_k is the plate height due to the drug interaction, and k_d is the dissociation rate constant between the drug and immobilized binding agent. According to this equation, a plot of H_k versus $u\ k/(1 + k)^2$ should give a slope of $2/k_d$ and an intercept of zero for a system with 1:1 binding. By using the k_d values obtained from these plots along with independent estimates of the equilibrium constants for the same system, the association rate constants for the drug and binding agent can also be obtained. This approach has been employed as a tool to examine the rate of interaction between *R*- or *S*-warfarin and D- or L-tryptophan with HSA over a variety of temperatures [97,98]. The work with D- and L-tryptophan and HSA also looked at how the association and dissociation rates for this system varied with pH, ionic strength and the organic modifier content in the mobile phase. This information has been shown to be important in optimizing chiral separations that use HSA [97,98] and has been used in the development of new assays for personalized medicine based on the measurement of free drug or hormone fractions in serum [130-132].

Several other approaches for HPAC have recently been developed that make it possible to measure the dissociation rates of drugs from serum proteins. Peak profiling is a variation on the band-broadening method that involves simultaneously determining the band-broadening for both a retained and non-retained solute on an HPAC column to examine the contributions of drug association/dissociation versus other band-broadening processes [125,126]. This approach has been used with data acquired at single flow rates [125] or at multiple flow rates [126] in studies that have determined the dissociation rate of L-

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tryptophan from HSA. Modified data analysis methods for peak profiling have also been developed for work with drugs that have significant binding to both an immobilized binding agent and with the support; this latter approach has been used to examine the kinetics of dissociation for imipramine and propranolol from HSA columns [124].

The peak decay method is another technique that has been used in HPAC to examine drug dissociation rates from serum binding agents [127-129]. This technique is performed by first equilibrating and saturating a small affinity column with a solution that contains the analyte of interest or an easily detected analog of this analyte. The column is then quickly switched to a mobile phase in which the analyte is not present. The release of the bound analyte is then monitored over time, resulting in a decay curve (see Figure 11). The slope of this decay curve is related to the dissociation rate of the analyte and the mass transfer kinetics within the column. If the mass transfer rate is known or is fast compared to analyte dissociation, then the decay curve can be used to provide the dissociation rate constant for the analyte from the immobilized ligand [127-129]. This approach has been used to examine the release of various drugs from HSA and gives good correlation with results that have been reported for the same analytes by other methods [127,128].

There are several advantages in using HPAC for kinetic studies compared to other methods, such as stopped-flow analysis or SPR. Unlike these other methods, HPAC is not limited to a particular method for signal production but can instead be used with a variety of detection formats. For instance, HPAC has already been used for drug binding studies with absorbance, fluorescence, or mass spectrometric detection. This versatility in detection format means that a wide range of drugs and drug concentrations can be examined in HPAC binding studies. It has also been noted that HPAC methods such as peak profiling, band broadening measurements and peak decay analysis can easily access reaction rates that are commonly found for drug interactions with serum proteins [97,98,124-129]. This does not appear to be the case for SPR, which has only been used to examine a limited number of these systems and even those that have been examined tend to have relatively slowest rates for such interactions [126]. In addition, there is no background signal from the binding agent in HPAC because this agent is immobilized within the column and does not pass through the detector. Many of these advantages also apply to the use of HPAC in determining association equilibrium constants or other parameters for drug interactions with serum binding agents.

High-Throughput Screening of Drug Binding

HPAC has been shown in several recent studies to be useful in the high-throughput screening and rapid analysis of drug-protein binding. Work based on frontal analysis-mass spectrometry used HPAC to screen mixtures of candidate compounds during the drug discovery process [182]. An HSA column used under gradient elution conditions (i.e., going from a pH 7.4, 0.050 M ammonium acetate buffer to a 70:30 mixture of this buffer with 2-propanol) has also been explored as a rapid means for examining the binding of drugs with HSA [183]. Another report used both frontal analysis and zonal elution to rapidly analyze the interaction of various drugs with HSA. In this work, a standard plot based on measured retention factors and association equilibrium constants was prepared for reference compounds and then used to determine the association equilibrium constants for other drugs with HSA [96].

Two recent reports have used affinity microcolumns in HPAC to quickly examine drug-protein binding [87,138]. In the first of these reports, HSA affinity microcolumns using silica monoliths were prepared and found to be useful in estimating the retention factors and plate height measurements for warfarin and carbamazepine. This report was able to take advantage of the lower backpressure of silica monoliths and the use of column lengths as

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short as 1 mm to decrease the overall analysis times to less than 10 s [87]. The second study compared binding data that was obtained with HSA affinity microcolumns to data obtained using longer HPAC columns. This comparison made use of bothzonal elution and frontal analysis methods and employed HSA affinity microcolumns of various lengths to analyze the interactions of warfarin and L-tryptophan (see Figure 12). It was again shown that column lengths as short as 1 mm in length could be used in these studies, significantly decreasing the analysis times and amount of immobilized protein that was required when compared to more traditional HPAC columns [138].

Conclusion

This review has examined various ways in which HPAC can be used as an analytical tool in the study of drug interactions with serum binding agents. The general principles of HPAC were discussed and it was shown how serum binding agents can be placed onto chromatographic supports for use in drug binding studies. It was described how HPAC has already been employed with many drugs and with binding agents such as HSA, AGP and lipoproteins. Methods that have been used in these studies have included zonal elution, frontal analysis, ultrafast affinity extraction, band-broadening measurements, peak profiling and peak decay analysis. With these approaches it has been possible to obtain a wide range of information on drug interactions with serum binding agents. Examples of the information that can be generated with HPAC are data on the overall degree of binding of a drug to a protein or serum agent, the equilibrium constants for this interaction, and the rate constants for this interaction. It is also possible to determine the number of binding sites for a drug on a particular protein and to measure the binding strength at each of these sites. The use of structural analogs of a drug or structural variants of the binding agent can make it possible to learn about structure of the binding sites. Experiments conducted at different temperatures, pH values, ion strengths or mobile phase compositions can provide additional data on the nature of these binding processes.

The many ways in which HPAC can be used in drug binding studies, and the variety of information this approach can provide, make it a powerful approach for characterizing drug interactions with serum proteins and other binding agents. Several specific examples of these applications were provided. Along with more routine drug binding studies, it was discussed how recent work with tools such as affinity microcolumns and monolithic supports has allowed for the creation of fast HPAC methods that are suitable for the high-throughput analysis and characterization of drug binding. Applications in the area of personalized medicine were also discussed. Some advantages of HPAC in these studies are its speed, ease of automation, precision, variety of detection formats, ability to use a small amount of protein for a large number of studies, and the variety of information that can be obtained with this approach. Based on these advantages and possible applications, it is expected that HPAC will continue to grow in popularity as an analytical tool for clinical or pharmaceutical research and in the study of drug interactions with proteins and other binding agents found in blood.

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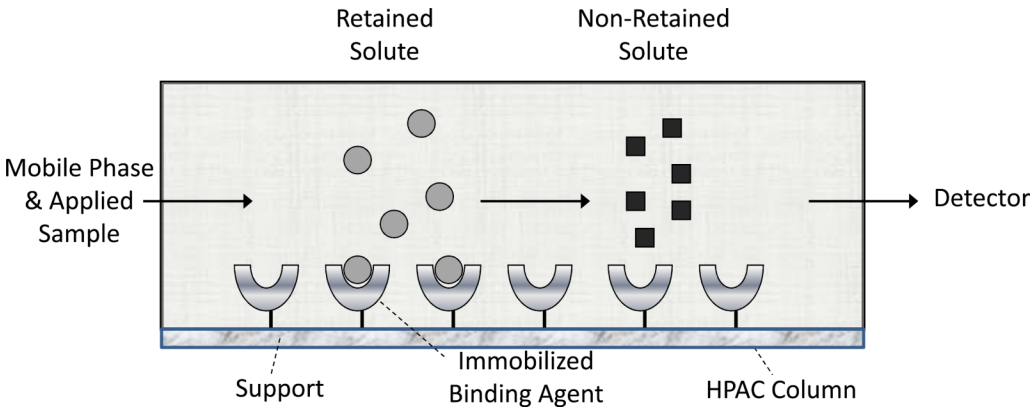

Figure 1.

General design of an HPAC system to examine the specific retention and elution of a drug from a column containing an immobilized protein or binding agent. The drug or desired analyte is applied or injected with the mobile phase at one end of the column and the elution of this drug or analyte is monitored at the other end of the column by using an on-line detector. Non-retained solutes will pass first through the column, followed later by the retained solutes and drugs.

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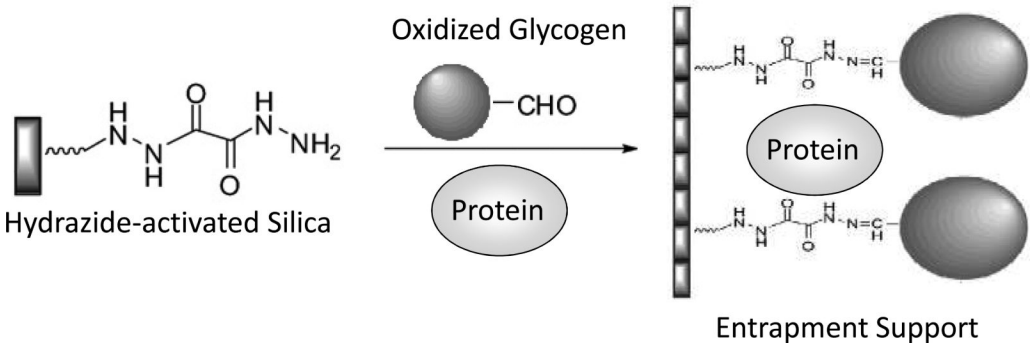

Figure 2.
General scheme for entrapment of a biomolecule such as a protein using a glycogen-capped and hydrazide-activated support. Reproduced with permission from Ref. [115].

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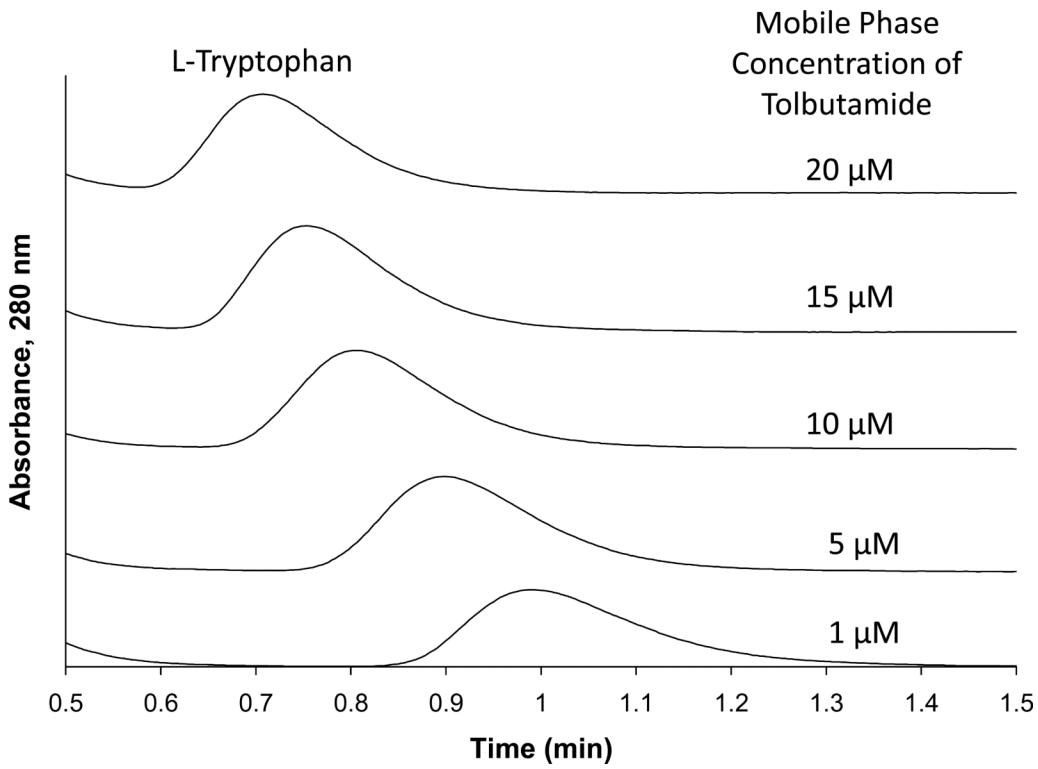

Figure 3.
Competition studies based on zonal elution for the injection of L-tryptophan as a site-selective probe onto an HSA column in the presence of various concentrations of tolbutamide in the mobile phase. Adapted with permission from Ref. [123].

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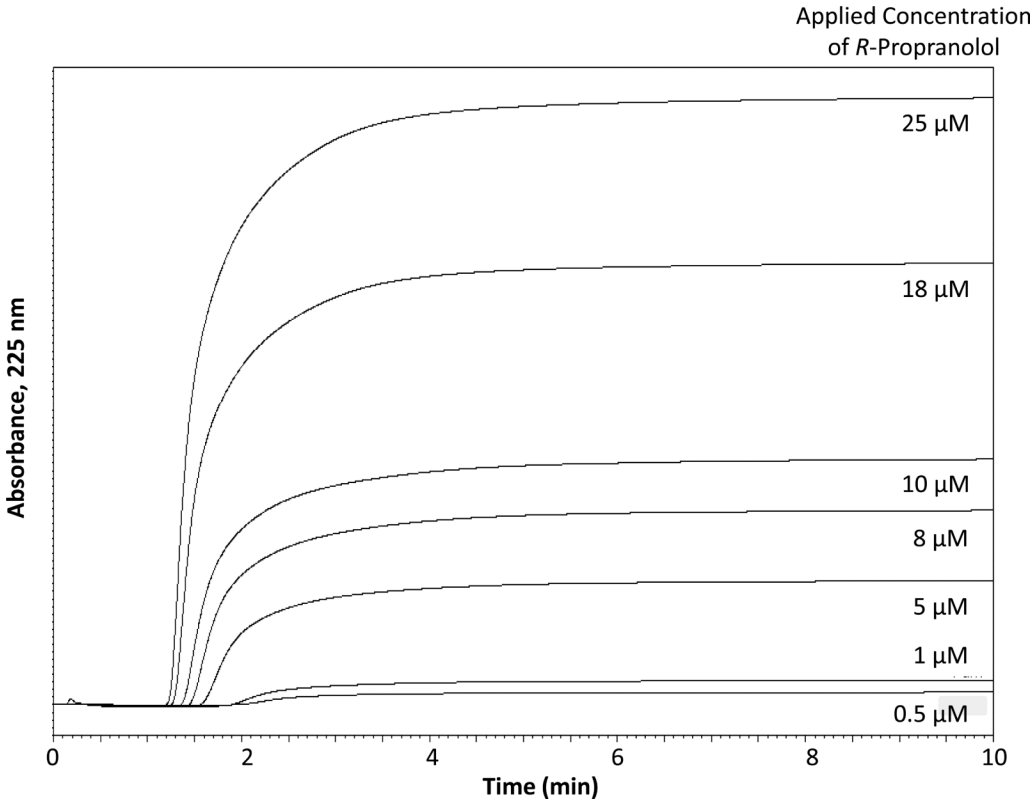

Figure 4.

Typical frontal analysis curves obtained for solutions containing various concentrations of *R*-propranolol that were applied at 1 ml/min to a 2.1 mm I.D. × 5 cm LDL column at pH 7.4 and 37°C.

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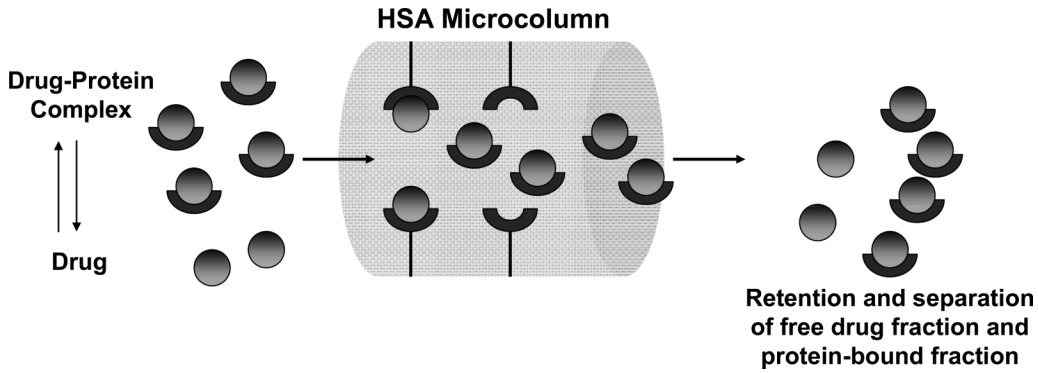

Figure 5.
General scheme for the separation of the free and protein-bound fractions of a drug in a sample through the use of an affinity microcolumn that contains immobilized HSA. Reproduced with permission from Ref. [133].

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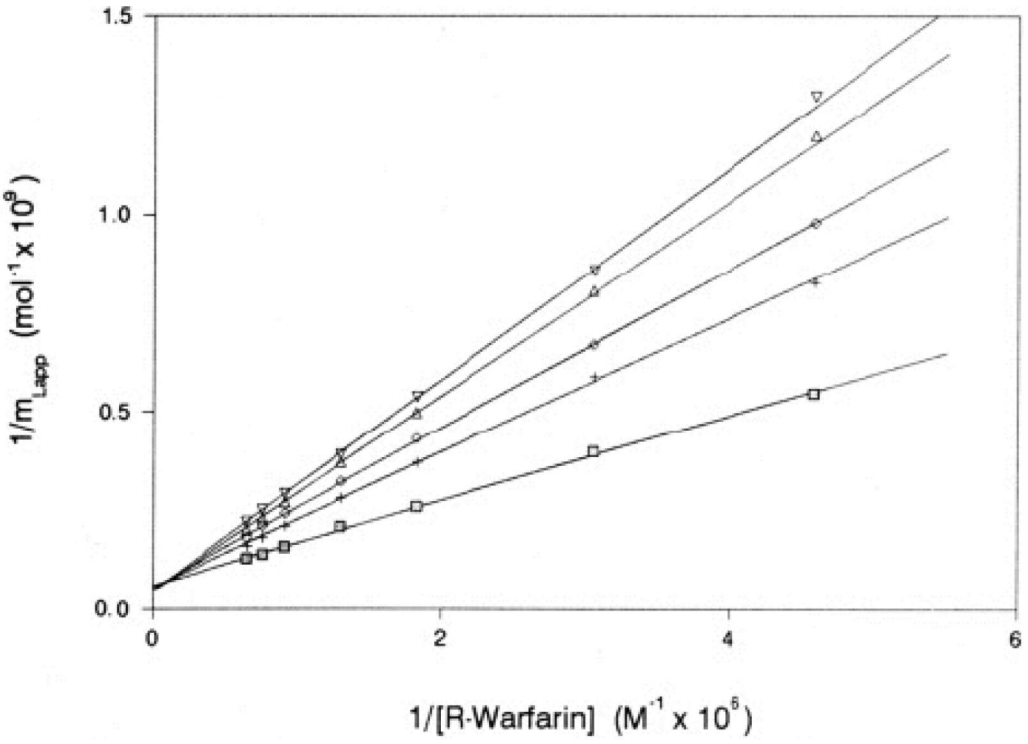

Figure 6.
Frontal analysis results plotted according to Eqn. (3) for *R*-warfarin on an HSA column at temperatures (from top-to-bottom) of 45, 37, 25, 15 and 4 °C. Reproduced with permission from Ref. [93].

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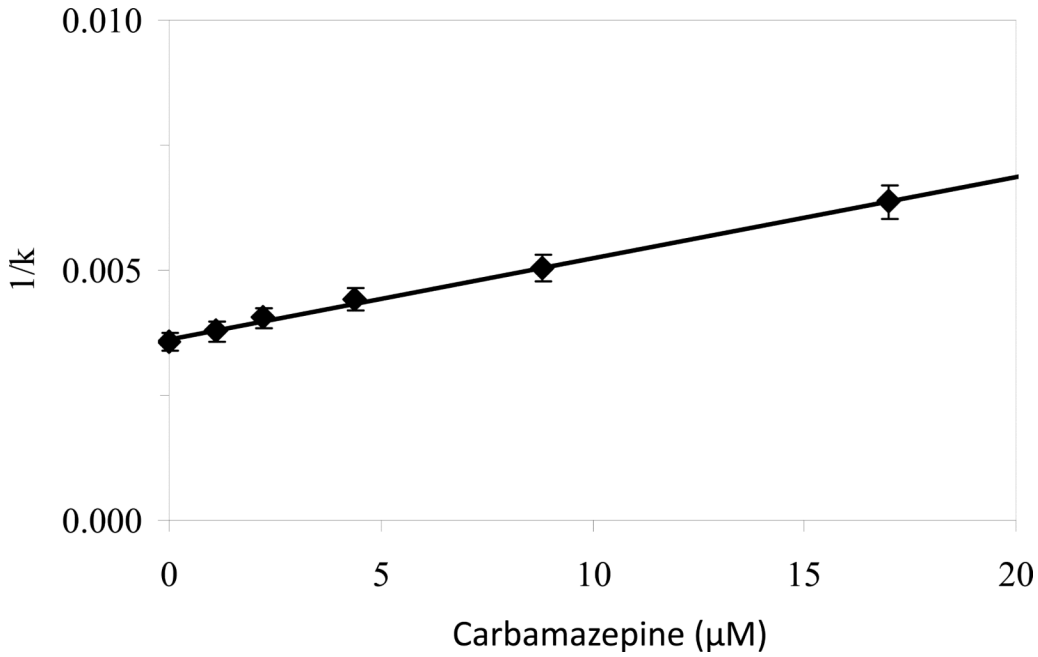

Figure 7.
Zonal elution studies examining the retention of *S*-propranolol on an AGP column as various concentrations of carbamazepine were placed into the mobile phase. These measurements were made at 37°C using pH 7.4, 0.067 M phosphate buffer and the error bars represent a range of ± 1 S.D. The solid line shows the best-fit response that was obtained when fitting Eqn. (5) to the data set. Reproduced with permission from Ref. [40].

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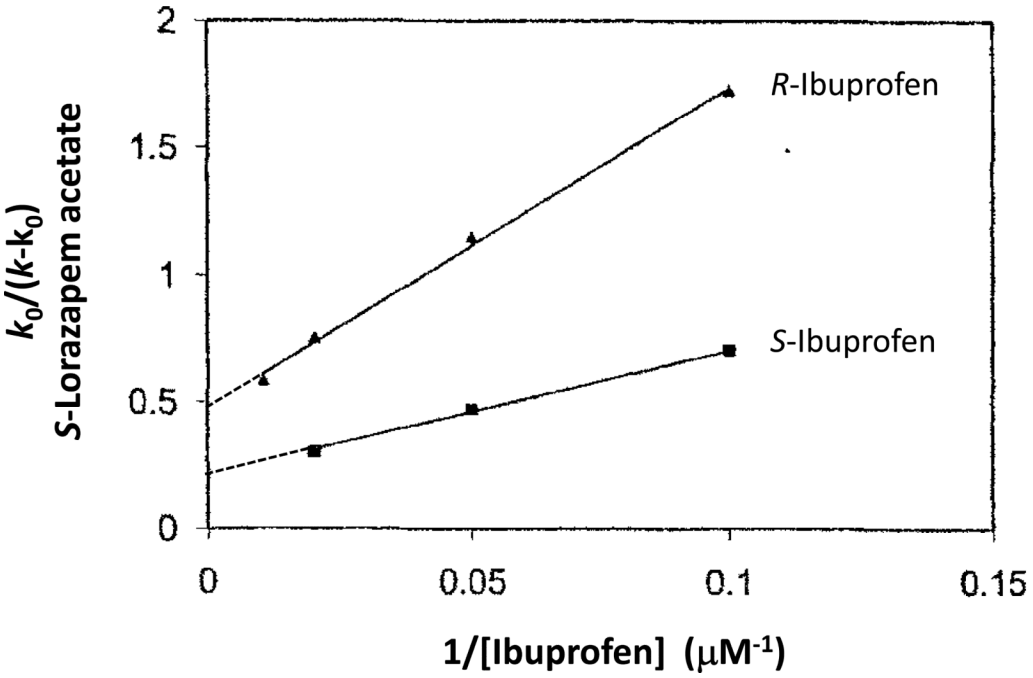

Figure 8.
Examination of the allosteric interactions between *R*- or *S*-ibuprofen and *S*-lorazepam acetate as these drugs bind to HSA, as determined by using HPAC and Eqn. (6). Adapted with permission from Ref. [140].

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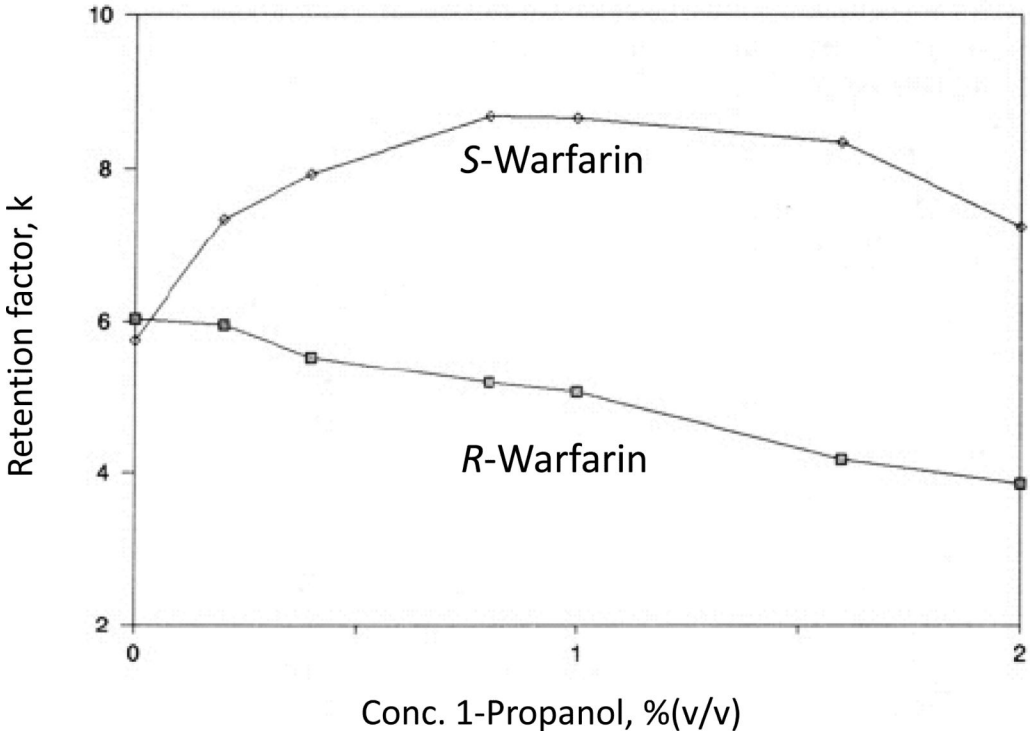

Figure 9.
Change in the retention factors for *R*- and *S*-warfarin on an immobilized HSA column as various concentrations of 1-propanol were placed into a pH 7.4, 0.067 M phosphate buffer. Adapted with permission from Ref. [93].

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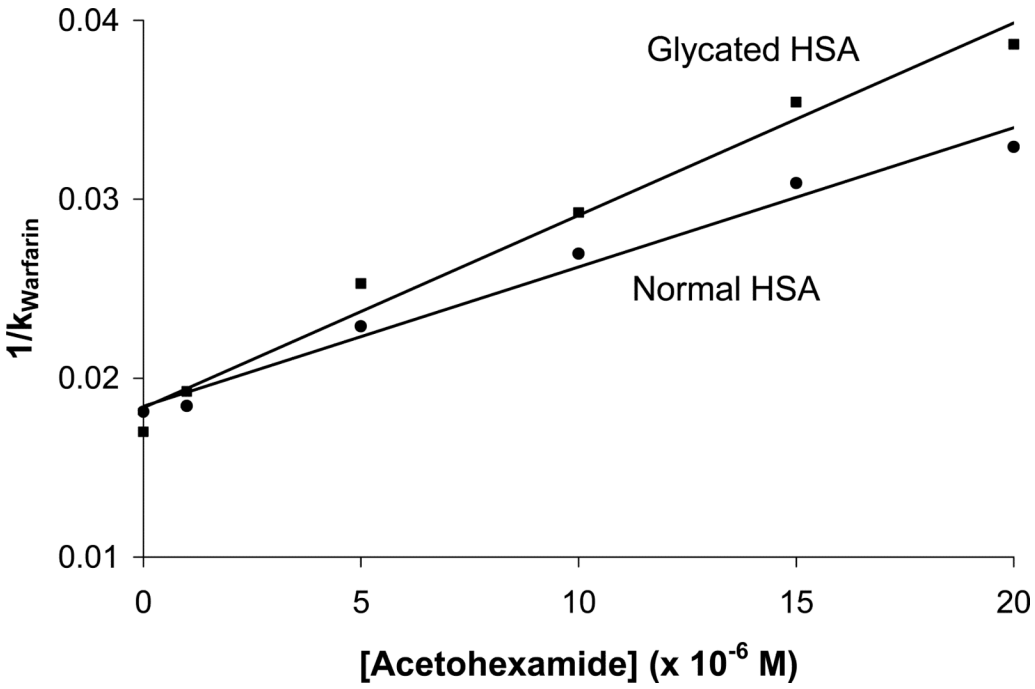

Figure 10.
Plots of 1/*k* vs. [Acetohexamide], as prepared according to Eqn. (5), for injections of *R*-warfarin as a probe for Sudlow site I on columns containing normal HSA (●) or glycated HSA (■) and in the presence of various concentrations of acetohexamide in the mobile phase. Adapted with permission from Ref. [118].

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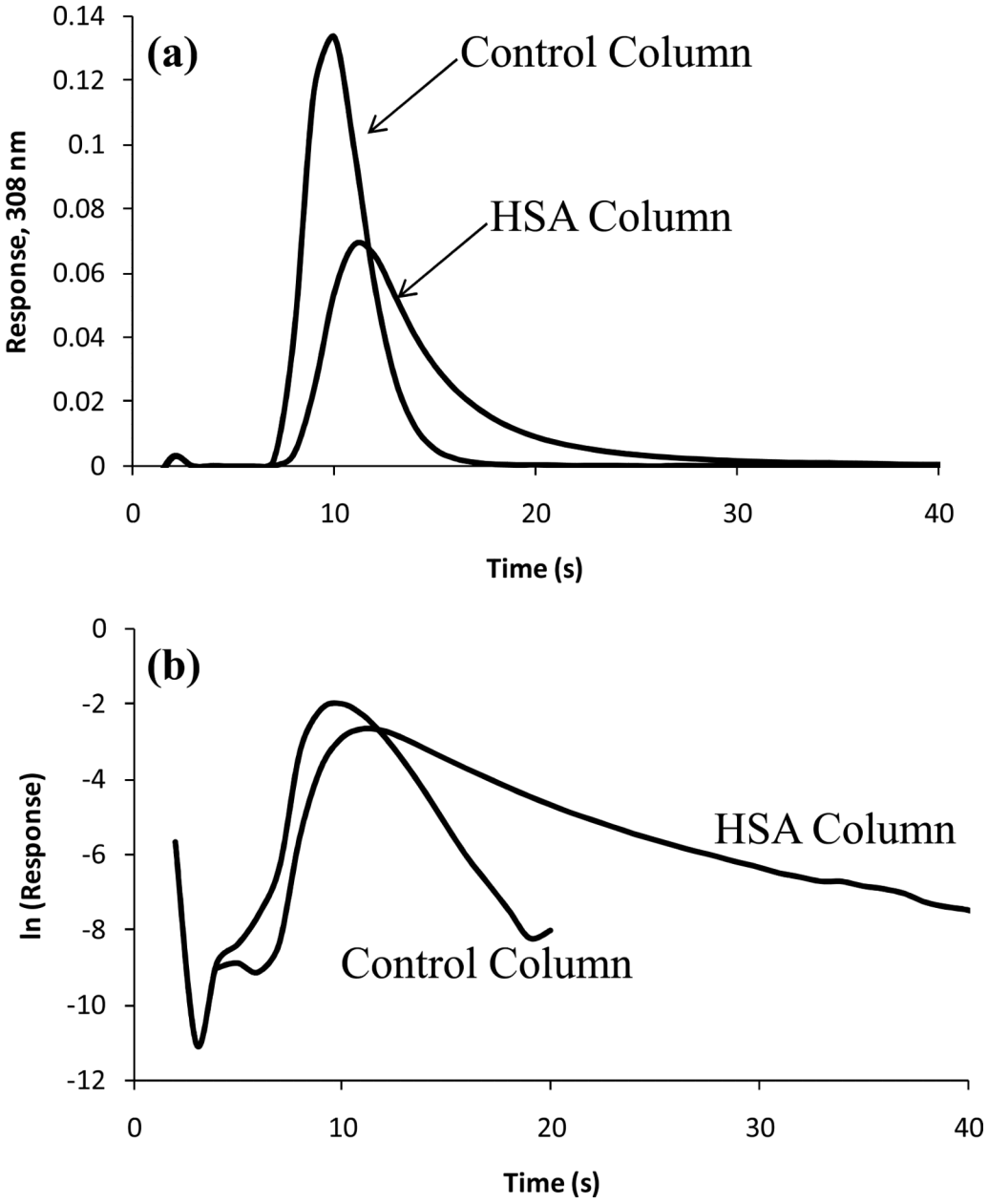

Figure 11.

(a) Elution profiles and (b) natural logarithm of the elution profiles for 100 μL injections of 10 μM racemic warfarin made at 4 mL/min onto 1 mm × 4.6 mm i.d. silica monolith columns containing immobilized HSA or a control support. These results and those shown in all later figures were obtained at 37 °C and using pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase. Reproduced with permission from Ref. [129].

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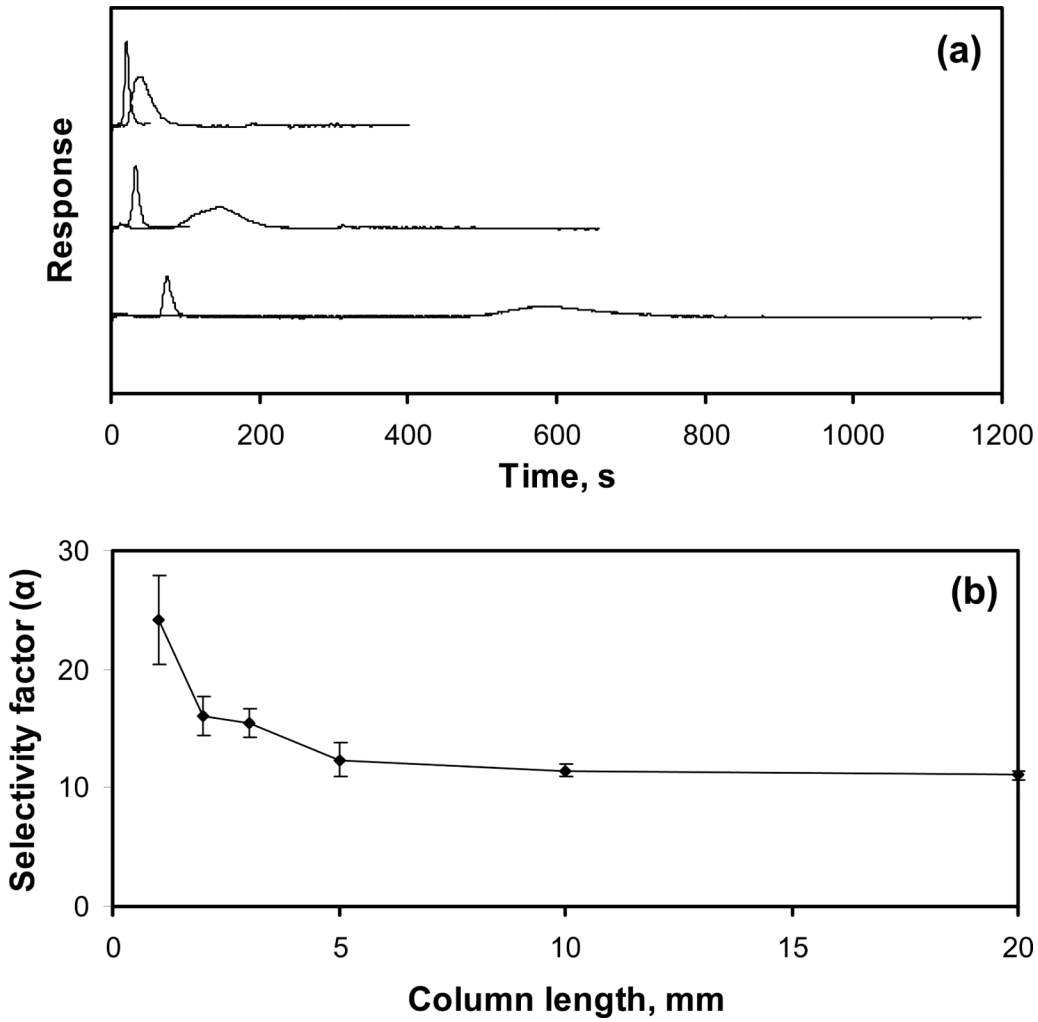

Figure 12.

(a) Typical chromatograms obtained for *R*-warfarin and L-tryptophan and (b) the resulting selectivity factors (i.e., ratio of retention factors) determined *R*-warfarin versus L-tryptophan on HSA microcolumns. The results in (a) were generated using HSA microcolumns with lengths of 20, 5 or 1 mm and an inner diameter of 2.1 mm. The error bars represent a range of ± 1 S.D. of the mean. Reproduced with permission from Ref. [138].