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Analysis of Solution-Phase Biomolecular Interactions by Liquid Chromatography: General Strategies and Recent Developments

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Abstract

The analysis of biomolecular interactions is important in characterizing and understanding many fundamental processes that occur in the body and biological systems. A variety of methods are available for studying the extent and rate of binding of these interactions. Some of these techniques are homogeneous methods, with all interacting components being present in the solution-phase, while others are heterogeneous, such as involving both solution-phase and solid-phase components. LC and HPLC have often been used to study biomolecular processes. Although these chromatographic methods make use of both a liquid phase (i.e., the mobile phase and applied samples) and a solid phase (the stationary phase and support), they can be used to study solution-phase interactions. This review examines several strategies that have been developed and employed to use LC and HPLC for this purpose. These strategies include the Hummel-Dreyer method, solution-phase frontal analysis, and the use of physical entrapment for a soluble component of a biomolecular interaction. Other strategies that are discussed are those in which the stationary phase of the column is used as a secondary component or capture agent when studying a solution-phase interaction, as occurs in normal-role affinity chromatography and ultrafast affinity extraction. The general principles for each of these strategies will be considered, along with their advantages, potential limitations, and applications.

Key words: Biomolecular interactions; Hummel-Dreyer method; frontal analysis; entrapment; normal-role affinity chromatography; ultrafast affinity extraction

1. Introduction

Biomolecular interactions take place throughout living systems [1]. Examples include the interactions of antibodies with foreign agents (i.e., antigens); the binding of nucleic acids with complementary nucleic acids or proteins; the interactions of enzymes with their substrates and co-factors; and the binding of proteins with other proteins or smaller target molecules such as drugs, hormones, and fatty acids [1-8]. These interactions are often critical in determining and regulating the fate of many endogenous substances in the body and living systems, as well as in determining how exogenous agents such as pharmaceuticals may behave and act in these systems [2,3,9-15].

A common model for describing a reversible biomolecular interaction is represented by the following reaction and equations [16-21].

$$A + L \underset{k_\mathrm{d}}{\overset{k_\mathrm{a}}{\rightleftharpoons}} AL \tag{1}$$

$$K_\mathrm{a} = \frac{[AL]}{[A][L]} \qquad \text{or} \qquad K_\mathrm{d} = \frac{[L][A]}{[AL]} \tag{2}$$

$$K_\mathrm{a} = \frac{k_\mathrm{a}}{k_\mathrm{d}} = \frac{1}{K_\mathrm{d}} \tag{3}$$

In this model, compound A interacts with binding agent L to form a 1:1 complex, as shown in eq. (1). Compound A may represent a small solute (e.g., a drug, enzyme inhibitor or co-factor) or a large biomolecule (e.g., an enzyme or antibody). Similarly, binding agent L may also be a small solute or a large biomolecule. The association equilibrium constant for this reaction, as depicted in eq. (2), is represented by the term K_a, and the dissociation equilibrium constant is given by K_d, where K_a and K_d are inversely related to each other. The rate of binding of A with L is described by the second-order association rate constant k_a, and the rate of dissociation of their complex is described by the first-order dissociation rate constant k_d. As shown in eq. (3), the ratio of these rate constants is also equal to K_a. Similar models and equilibrium or rate expressions can be used for

more complicated biomolecular interactions, such as those involving multivalent binding, other interacting components [19-21].

Many analytical methods have been employed with models like those in eqs. (1-3) to examine biomolecular interactions and to provide information on the binding strengths and rates of these processes [22-49]. For the measurement of binding constants in solution-phase systems with reversible interactions (e.g., drug-protein binding), equilibrium dialysis and ultrafiltration have long been the gold standard methods [14,23,24]. Other techniques that have been used in these cases have included various forms of spectroscopy [25-28], CE [29-32], SPR [33-35], LC [17,18,36-42], NMR [43-45], MS or LC-MS [32,46-48], CD [34,41,49], and microcalorimetry [50,51]. Methods that have been employed to study the kinetics of biomolecular interactions have included stopped flow analysis, SPR, CE, and some forms of LC (e.g., affinity methods) [52].

Various factors need to be considered when selecting between methods for binding or kinetic studies. Some of these factors are the amounts of interacting components that are required, the amount of sample that is needed and available, the range of binding or rate constants that are to be measured, and the ability to directly or indirectly detect the interacting components at the levels that are used in the method [17-19,53,54]. Other items to consider are the speed and throughput of technique, the accuracy and precision of this method, and the number of samples or solutions that need to be processed [18]. Finally, the cost of the analysis and the availability of equipment plus expertise for the measurement are practical factors to consider.

LC, especially in the form of HPLC, has many potential advantages when used for binding and kinetic studies of biomolecular interactions [17-21,36-42,52-54]. For example, HPLC is a well-established and readily-available method that is known for its efficiency, speed and precision, small sample requirements, ability to be combined with various forms of separation and detection

modes, and ease of automation [18,38-41]. However, there are also potential limitations to using HPLC or any other type of LC format as a tool for studying biomolecular interactions. For instance, LC is typically viewed as being a heterogeneous method that combines a stationary phase and solid support with a liquid mobile phase and solution-phase samples [19,20,41]. In contrast to this, many other methods that are commonly used for biomolecular interaction studies (e.g., equilibrium dialysis, CE, and many spectroscopic techniques) are homogeneous methods in which all the interacting components are in solution [41]. However, there are several strategies by which LC and HPLC can be used for studying biochemical interactions that keep all interacting species in solution. Such strategies make it possible to maintain the advantages of LC and HPLC while adding the advantages of a homogenous format for interaction studies.

The purpose of this review is to examine the various ways in which LC and HPLC have been used to examine solution-phase biomolecular interactions. This discussion will include vacancy techniques such as the Hummel-Dreyer method, as well as approaches based on solution-phase frontal analysis or the use of columns in which some of the interacting components are entrapped or encapsulated but still present in a soluble form. Other strategies that will be examined are those that use the stationary phase in an LC column as a secondary component to study interactions within solution-phase samples, such as employed in normal-role affinity chromatography and ultrafast affinity extraction (UAE). The general principles of each approach will be described. Their advantages, limitations, and reported applications will also be discussed. In addition, the extension of these methods to other further applications will be considered.

2. Hummel-Dreyer Method for Binding Studies in LC

Some of the earliest methods for examining solution-phase interactions by LC and HPLC were those based on vacancy techniques [53,55-58]. In this type of approach, the mobile phase

contains one or more of the interacting chemicals, such as the binding agent and target solute of interest. An injection is then made of a sample that lacks one or more of these chemicals. As this sample travels through the column, the local equilibrium between the binding agent and its target in the mobile phase is perturbed [18,55,56]. This results in the production of one or more negative "vacancy peaks", each of which represents the depletion of some additive to the mobile phase that is involved in the interaction being examined. The size of the vacancy peak is then used to determine the amount of binding agent or target that was depleted [18]. This information, in turn, can be used to determine the amounts of these chemicals that were originally present in the mobile phase in their bound and non-bound forms [53,55-58]. One common example of such an approach is the Hummel-Dreyer method [18,53,55-58].

This method was first described by J.P. Hummel and W.J. Dreyer in 1962 [58]. The principle of this technique is shown in Figure 1. This method can be used to examine reversible interactions of a small target (e.g., a drug) with a larger binding agent (e.g., a protein) in systems that have fast association and dissociation rates on the time scale of the analysis [53,55,56]. In this technique, the target solute of interest is put into the mobile phase at various known concentrations and passed through a column which can separate the non-bound form of the target from its complex with the binding agent [53,55]. This gives a background signal for the mobile phase that is proportional to the level of target that is present. A small amount of the desired binding agent is then injected into this mobile phase and through the column. As the sample pulse passes through the column, the target in the mobile phase interacts with the binding agent and leads to a vacancy peak due to depletion of the unbound target in the mobile phase. The area that is measured for this negative peak is then utilized to determine the concentration of the target in its complex with the binding agent [53,55,56].

The Hummel-Dreyer method has often been used to examine reversible drug-protein interactions in solution [53,55-58]. This method can be used to estimate both the binding constants of these interactions and the total number of sites for the target solute on a binding agent [53,55,56]. Once data have been acquired on the amount of unbound target that is present as the concentration of target vs binding agent is varied, this information can be analyzed by the Scatchard equation [53,59,60].

$$r/[\mathrm{D}] = K_a\,(n - r) \qquad \text{or} \qquad r/[\mathrm{D}] = nK_a - K_a\,r \tag{4}$$

In this relationship, r is the moles of drug or target bound per mole of the larger agent (e.g., a protein), [D] is the remaining unbound concentration of the target (e.g., a drug), K_a is the average association equilibrium constant for this interaction, and n is the relative moles per mole of binding agent of sites for the target [53,60]. A plot of $r/[\mathrm{D}]$ vs r based on eq. (4) should provide an intercept equal to nK_a and a slope equal to $-K_a$ for a system with reversible binding and a single class of binding sites for the target solute on the larger binding agent [53].

There are several requirements for this method to work. First, the interaction that is being examined should have rapid association and dissociation kinetics on the time scale of the chromatographic process so that a local equilibrium can be established between the target and binding agent as the binding agent passes through the column [18,53,61,62]. Second, the concentration and detection properties of the target in the mobile phase should be suitable to allow a measurable trough to be created when the binding agent is injected. Third, the column and chromatographic conditions must provide a separation between the target-binding agent complex and unbound target that allows the peaks and troughs for these components to be well-resolved. Failure to meet these requirements can result in troughs that cannot be measured or peak tailing

and poor resolution that results in inaccurate information being obtained on the target's unbound vs bound concentrations [53,62].

Both internal and external calibration have been used with the Hummel-Dreyer method [53,56,58,59,63]. The internal calibration approach that was originally employed with this method involves various concentrations of the target being placed into the mobile phase while a constant amount of binding agent is injected [56,58,59]. A curve is then obtained by plotting the areas of the troughs or peaks versus the concentration of the target to extrapolate and estimate the amount of target that is bound to the injected agent [56]. In external calibration, various concentrations of the target are placed into the mobile phase and the injected sample is simply a buffer solution with no binding agent present [18,53,63]. The areas of the troughs are again used to determine the unbound concentration of the target [56,63].

Early applications of the Hummel-Dreyer method used low-performance size-exclusion columns to separate the bound and unbound fractions of the target. As an example, a Sephadex G-25 support and column were employed in the first application of this method, in which this technique was used to measure the affinity of 2′-cytidylic acid with RNAase [58]. This method was later adapted in the late 1970s for use with HPLC-based size-exclusion supports and columns [55,59,64]. For instance, Sebille et al. used the Hummel-Dreyer method in an HPLC system with a glycerylpropylsilyl silica size-exclusion column to study interactions by warfarin and furosemide with human serum albumin (HSA) [59]. Size-exclusion HPLC columns containing diol silica have also been used for this purpose (see Figure 2) [55,59,63-69]. Other types of columns that have been used in the Hummel-Dreyer method to separate unbound and bound forms of targets have included those containing restricted access media (e.g., internal surface reversed-phase, or ISRP, supports) or anion-exchange resins [70-79].

HPLC and the Hummel-Dreyer method have been collectively used to examine the interactions for a variety of targets and binding agents. As illustrated in Figure 2, examples include the binding of drugs or small solutes with proteins such as HSA [59,60,64-68,70-72,80-83] or modified forms of HSA [74], bovine serum albumin [73], alpha$_1$-acid glycoprotein (AGP) [67-69,80-83], and lipoproteins [68,80-84]. Other examples are the interactions of ADP and ATP with chloroplast ATPase CF1 [75], the binding of soybean trypsin inhibitor with trypsin [76], the binding of drugs and substituted thiazoles with carbonic anhydrase III [77,78], and the interactions of spongistatin I with tubulin [79]. The association equilibrium constants for these systems have ranged from around 4×10^3 to 2×10^7 M^{-1} and the time per sample injection has typically been 4-20 min when using HPLC systems [59,60,64,65-84].

3. Solution-Phase Frontal Analysis

Frontal analysis is another method that can be used in LC and HPLC to study solution-phase biomolecular interactions that involve different sizes of interacting components. The general scheme for frontal analysis as a solution-phase method is shown in Figure 3. In this approach, a large-volume sample containing both small and larger interacting components (e.g., a drug and protein) is injected onto a size-exclusion column or other type column that employs a size-based separation mechanism [85,86]. The column excludes the larger binding agent but is selected to retain the smaller component in the sample mixture [85,86]. Because a relatively large-volume of sample is applied to the column, only a partial separation of the small and larger components occurs, resulting in an elution profile with several plateau regions [53,87]. There are ideally three such plateau regions in this profile, which correspond to the unbound and free form of the smaller component, the complex of this small component with the larger binding agent, and the unbound form of the larger binding agent. The heights of these plateau regions can be used to determine the

total and free concentrations of each component [88,89]. If several concentrations of the smaller component are used in such an experiment, it is also possible to determine the equilibrium constant for the interaction between the large binding agent and smaller component [90-93]. A Scatchard plot, as described earlier by eq. (4), or related plot can then be fit to the data and used to estimate the binding parameters for the system, such as the association equilibrium constant and the number of binding sites involved in the interaction.

High-performance frontal analysis (HPFA) is a form of this method in which HPLC and frontal analysis have been combined to analyze biomolecular interactions in solution [24,53,89,94]. For example, HPFA has been used to study the interactions of the serum transport protein HSA with drugs and small solutes such as warfarin, furosemide, diazepam, carbamazepine, fenoprofen, troglitazone, perillyl alcohol, *S*-ibuprofen, isoflavones, catechin hydrate, and genistein [59,63,70,89,95-100]. HPFA has further been employed to measure interactions of the protein BSA with warfarin, ephedrine, and L-tryptophan [101]. HPFA has also been coupled with ISRP supports and columns, which have been shown to provide better resolution of drug and drug-protein complex bands than more traditional size-exclusion columns and require much smaller sample volumes (i.e., μL vs mL volumes) [53,89,96].

Experiments based on frontal analysis have many of the same requirements as the Hummel-Dreyer method. For instance, the interaction being studied must again have rapid association and dissociation kinetics on the time scale of the chromatographic separation to allow a local equilibrium to be established between small and large interacting components [53]. The concentrations of these components also need to be in a range that allows detection of their bound and unbound components [24,53]. One limitation of this approach is that it can be difficult to use in systems where the interacting components have strong binding, which means the unbound

fraction for one of these components (e.g., the smaller agent) may be low and difficult to detect [24,53]. To overcome this limitation, on-line preconcentration of the unbound fraction has been used with HPFA in the study of drug-protein binding by coupling this method with a chiral HPLC column or an electrokinetic chromatographic method. The binding of HSA to chiral drugs such as warfarin, BOF-4272, fenopren, ketoprofen, nilvadipine, semotiadil, levosemotiadil, oxybutynin and N-desethyloxybutynin has been examined by using this approach (see Figure 4) [88,102–108]. This method has further been used to examine the interactions of AGP with semotiadil, levosemotiadil, oxybutynin and *N*-desethyloxybutynin [105,107,108]. The binding constants that have been measured by frontal analysis and HPFA for these and other systems have spanned over at least four orders of magnitude, with association equilibrium constants ranging from at least 7×10^3 to 5×10^7 M^{-1} [88,97-100,102-108].

4. Entrapment Techniques for Solution-Phase Binding Studies in LC

Entrapment or encapsulation is an immobilization technique in which a binding agent is placed in a soluble form within the pores or matrix of a support [109-114]. When this type of immobilization is used in binding studies, the support typically contains pores or openings smaller than the entrapped binding agent but that allow access by a smaller component that may interact with this agent (see Figure 5) [109-114]. Several studies have used the formation of a sol-gel to place potential binding agents into a silica matrix [110-114]. For instance, BSA has been encapsulated by the sol-gel method and used to study the binding of this protein to propranolol and naproxen [113]. Monolithic silica columns have been prepared by the sol-gel method to entrap dihydrofolate reductase and characterize, by LC/MS, its binding with small inhibitors for this enzyme [110].

Frontal affinity chromatography (FAC) is one approach that has been used with entrapment to study biomolecular interactions, either alone or in combination with MS [17,53,110,115-117]. FAC is carried out by continuously applying a solute of interest to a column that contains an immobilized form of a binding agent [17,53,115,118-120]. As the binding agent interacts with the applied solute, the binding sites on the immobilized agent become gradually saturated and the amount of solute that passes through the column increases [17,115]. This gives a response and chromatogram that is known as a breakthrough curve, as shown in Figure 6(a) [17,117]. The shape and position of the breakthrough curve can be used to rank and determine the affinity of various applied solutes. Solutes with strong binding to the entrapped agent will elute later, while more weakly bound solutes will elute sooner.

In FAC, the mean position of the breakthrough curve will shift to shorter times as the concentration on an applied solute is increased. This shift will also depend on the strength of binding by this solute with the immobilized agent and the amount of binding sites that are available in the column for the solute. The following equation is often used to describe this relationship in FAC for a system with reversible 1:1 binding [110,115].

$$(V_{\mathrm{R}} - V_{\mathrm{M}}) = \frac{B_t}{K_{\mathrm{d}} + [A]} \tag{5}$$

In this equation, V_{R} is the breakthrough volume of the applied solute, V_{M} is the void volume of the column, $[A]$ is the concentration of applied solute A, K_{d} is the dissociation equilibrium constant for the binding of A with the immobilized agent, and B_t is the total active sites for the solutes on the immobilized binding agent. According to eq. (5), a plot of $(V_R - V_M)$ vs. $[A]$ can be used with non-linear regression to give the values of B_t and K_{d} for a system with 1:1 interactions [115]. Alternatively, eq. (5) can be rearranged into the following form,

$$\frac{1}{(V_{\mathrm{R}}-V_{\mathrm{M}})[A]} = \frac{K_{\mathrm{d}}}{B_t[A]} + \frac{1}{B_t} \quad (6)$$

in which a plot of $\frac{1}{(V_{\mathrm{R}}-V_{\mathrm{M}})[A]}$ vs $\frac{1}{[A]}$ should provide a linear response with *x*- and *y*-intercepts that are equal to $-\frac{1}{K_{\mathrm{d}}}$ and $\frac{1}{B_t}$, respectively [115]. FAC has been used with MS (i.e., FAC-MS) and affinity microcolumns prepared by sol-gel entrapment to characterize the binding by small inhibitors for the enzyme dihydrofolate reductase [110].

Frontal analysis in the form of FAC-MS has also been used to study the competition between potential target analytes and immobilized binding agents [115,118,119], although this approach has not previously been used with entrapment. In this type of study, a competing agent is added with the target analyte to the mobile phase. The resulting chromatograms are then analyzed by measuring the shift in breakthrough time or volume of the analyte as a function of the concentration of the competing agent [119]. If the breakthrough time decreases with an increase in concentration of the competing agent, direct competition or a negative allosteric effect between the analyte and competing agent is present. If the breakthrough time increases as the concentration of the competing agent increases, a positive allosteric effect is present between the analyte and competing agent [119]. It is also possible to rank the strength of potential displacing agents by using eq. (7), which describes the interactions between a competing agent I and the target analyte during such an experiment [115,118,119].

$$(V_{\mathrm{R}} - V_{\mathrm{min}}) = \frac{P}{K_{\mathrm{d,IL}}+[\mathrm{I}]} \quad (7)$$

In this relationship, $K_{\mathrm{d,IL}}$ is the dissociation equilibrium constant for the interaction between the competing agent and the immobilized binding agent L, [I] is the concentration of the competing agent, V_{R} is the breakthrough volume of the target analyte, and V_{min} is the breakthrough volume of the target analyte when the interaction being examined is completely suppressed, as can be

determined by running the target with a high concentration of the competing agent. The term P is the product of the amount of active binding sites (B_t) and the term $\frac{K_{\mathrm{d,IL}}}{K_{\mathrm{d,AL}}}$, where $K_{\mathrm{d,AL}}$ is the dissociation equilibrium constant for the target analyte with the immobilized ligand [115,118,119].

Another means of entrapment is one in which the binding agent is placed in the pores or at the surface of an activated support. This support is then combined with a large capping agent that will prevent the binding agent from leaving the support but still allows access to smaller solutes. This scheme has been used with porous HPLC hydrazide-activated supports that have been capped with glycogen, a large biomolecule with aldehyde groups that can form a stable bond with the hydrazide-support [116,121-123]. This means of support formation has been used to entrap binding agents with molar masses ranging from at least 6 to 150 kDa, including HSA, AGP, and immunoglobulin G (IgG) [116,121,122,124,125]. In addition, slow mass transfer effects and issues with support stability, as can occur in the sol-gel method, are avoided or minimized in this entrapment method [116]. Like sol-gels, capped HPLC supports that contain entrapped proteins have been used in frontal analysis for binding studies. For instance, frontal analysis with microcolumns containing entrapped HSA has been used to measure the association equilibrium constants and the amount of binding sites of this protein for *S*-warfarin, and entrapment has been used with frontal analysis to examine binding by the protein AGP to carbamazepine [121,122].

Zonal elution format is another format that has been used to analyze biomolecular interactions by combining HPLC supports with entrapment, as illustrated in Figure 6(b) [121,122,124,125]. In this method, a narrow plug of a solute is injected onto a column containing an entrapped and soluble form of a binding agent; this plug is applied in the presence of a mobile phase with a suitable pH and composition for binding and that is applied at a fixed flow rate under linear elution condition [125]. The retention time (t_{R}) of the solute is then determined on both the

column with the entrapped agent and with a control column with no binding agent present. The void time of the column (t_M) is measured by injecting a non-retained solute and combined with t_R to get the retention factor of the solute (k) on each column, where $k = (t_R - t_M)/t_M$. Finally, the specific retention factor (k') for the solute with the entrapped binding agent is found by taking the difference in retention factors for the column with the entrapped binding agent and without this binding agent present, as a means to correct for any non-specific binding that may be present with the support [19,125,126]. If all the entrapped binding agent is fully active, the value of k' for the solute can be related to the association equilibrium constant (K_a), as shown in eq. (8) for a solute with a simple interaction with the binding agent, or to the global affinity constant (nK'_a), as described in eq. (9) for a solute with multiple independent sites on the binding agent [19,126].

$$k' = K_a \frac{m_L}{V_M} \tag{8}$$

$$k' = \frac{(n_1 K_{a1} + n_2 K_{a2} + \cdots + n_n K_{an}) m_L}{V_M} = \frac{(nK'_a) m_L}{V_M} \tag{9}$$

Other terms in these equations are the moles of active binding sites for the solute on the immobilized binding agent (m_L) and the void volume of the column (V_M). In eq. (9), the terms K_{a1} through K_{an} represent the association equilibrium constants for the solute at sites 1 through n on the entrapped agent, and n_1 through n_n are the relative amounts (in mol/mol of binding agent) for each of these sites. These equations have been used with zonal elution and entrapment in HPLC systems to evaluate the overall binding of drugs such as amitriptyline, carbamazepine, chloramphenicol, chlorpromazine, disopyramide, imipramine, lidocaine, propranolol, nortriptyline, quinidine, and sulfonylureas with HSA and AGP [117,121,122], as well as the binding of sulfonylureas with entrapped normal or modified HSA [116,124,125]. This approach

has also been used to determine the binding strengths of various pharmaceuticals with humic acid, a form of dissolved organic matter that is found in the environment [127].

Both types of entrapment that have been described in this section have the advantage of giving an immobilized binding agent that remains in soluble form and should closely mimic the behavior in its native state [109,110,116]. These approaches, combined with either frontal analysis or zonal elution, have been used to examine reversible biomolecular interactions with binding constants ranging from 2×10^4 to 2×10^7 M^{-1} [110,116,117,121,122,124,125,127]. A potential limitation of sol-gels is the need for support preparation conditions that will give an appropriate pore size to contain the binding agent but still allow access to applied and potential target solutes; in addition, the conditions used for support formation should not lead to any significant loss of binding agent activity [109,112,128]. Other issues for the sol-gel method, and especially when used with HPLC, are the high levels of shrinkage that may occur with sol-gels and the slow rates of mass transfer that may be present for solutes to binding agents that are held deep within the support [110,128]. Many of these issues, such as shrinkage and slow mass transfer, are overcome when porous HPLC supports with capping agents are instead used for entrapment [121]. However, some optimization of the pore size and entrapment conditions are also needed in this second approach to maximize the amount of binding agent that can be placed within a support for binding studies [116,123,124,127].

5. Binding Studies using Normal-Role Affinity Chromatography

Affinity chromatography and high-performance affinity chromatography (HPAC) can also be used for binding and rate studies of biomolecular interactions [21,129,130]. Affinity chromatography and HPAC are types of LC that use a biologically-related binding agent as the stationary phase [131-135]. Both affinity chromatography and HPAC have often been used as

heterogeneous methods in which one of the agents in a biomolecular interaction is placed onto a chromatographic support and examined for its retention of other components [130,131]. However, it is also possible to modify these methods to analyze solution-phase interactions by using the immobilized binding agent as a secondary component that is involved indirectly in the desired interaction. One way this can be accomplished is by using a technique known as normal-role affinity chromatography [129,136].

The basis of normal-role affinity chromatography is illustrated in Figure 7. In this example, analyte (A) is injected onto a column that contains an immobilized interacting ligand (L) for A. In addition, the mobile phase is prepared to contain a known concentration of a soluble competing agent (S) that can bind to the same single region on A as L. If the concentration of A is much smaller than L or S (i.e., [A] << [L] and [S]) and A has no non-specific binding to the column, the observed retention factor (k) for A can be described by the following equation [130].

$$\frac{1}{k} = \frac{1}{K_{\mathrm{a,AL}}[\mathrm{L}]} + \frac{K_{\mathrm{a,AS}}[\mathrm{S}]}{K_{\mathrm{a,AL}}[\mathrm{L}]} \qquad (10)$$

In this expression, $K_{\mathrm{a,AL}}$ is the association equilibrium constant for A with the immobilized ligand L, and $K_{\mathrm{a,AS}}$ is the association equilibrium constant for A with the soluble agent S. According to eq. (10), a plot of $1/k$ vs [S] for a system that involves 1:1 binding and competition between L and S for A should give a linear relationship, where the ratio of the slope to the intercept will provide $K_{\mathrm{a,AS}}$ and the intercept can be used to obtain $K_{\mathrm{a,AL}}$ [130]. Similar relationships can be derived for systems with multivalent interactions or multiple binding sites [21,130].

As its name implies, normal-role elution was the original format used in affinity chromatography when the modern version of this separation technique was developed in the late 1960's [21,136]. This format was initially used for enzyme and protein isolation based on low-performance supports that contained small, immobilized binding partners for these

biomacromolecules, such as an analog of an inhibitor or substrate for enzyme purification. However, expressions such as eq. (10) made it possible to also determine the binding constants for the biomolecule with both the immobilized agent and soluble competing agents that were added to the mobile phase. For instance, this method has been used in a zonal elution format to characterize binding and interactions in systems such as between nucleotides and Staphylococcus nuclease or ribonuclease, as well as antibody-antigen interactions between a bivalent TEPC 15 immunoglobulin A monomer and phosphorylcholine [21]. Normal-role affinity chromatography has also been combined with a frontal analysis format for such work [21]. In addition, normal-role elution has been employed with HPLC affinity columns for binding studies. For instance, this combination has been used to examine the binding of several drugs with soluble ß-cyclodextrin through the utilization of a column that contained HSA as an immobilized binding agent for the same drugs, as shown in Figure 8 [130].

A key requirement of this method, and potential limitation, is the need for some immobilized binding agent that can compete or interact with a soluble agent for the same binding partner. This binding agent may either be a general one, such as the use of HSA as a competitor for ß-cyclodextrin in binding to drugs [130]. It may also be a more specific agent, as has been used in examining binding by enzymes with their inhibitors or antibodies with their antigens [21]. It is further necessary that the interactions between all these agents be sufficiently fast to allow them to reach a local equilibrium during their passage through the column [21,130]. One additional requirement is it must be possible to detect the eluting binding partner in the presence of the soluble agent in the mobile phase [130]. If these requirements are met, it has been shown that this approach can allow a single column to be used to examine many related interactions involving the same binding partner and different soluble agents [21,130]. Binding constants that have been measured

by normal-role affinity chromatography in either a zonal elution or frontal analysis mode have been reported over at least three orders of magnitude, with association equilibrium constants spanning from at least 5×10^2 to 8×10^5 M^{-1} [21,130].

6. Ultrafast Affinity Extraction

Another form of HPAC that has been employed for the study of solution-phase interactions is ultrafast affinity extraction (UAE) [137]. UAE has been used to examine interactions between solutes and ligands in both simple solutions and biological samples [11,137-141]. In this method, samples containing the solute and ligand, along with a standard containing the solute without the ligand, are injected at various flow rates into an affinity column containing an immobilized capture agent. The free (i.e., unbound) form of the solute in the injected sample is next selectively captured by the secondary binding agent in the column, providing the apparent free fraction of the solute in the sample at the given flow rate. The ratio of retained peak areas for the solute in the sample versus the standard is then determined to get the apparent free fraction (F_t) for the solute at this flow rate. If an antibody is used as the secondary capture agent, this approach is also known as ultrafast immunoextraction [142-145].

Figure 9 shows the general scheme used in UAE. At low-to-moderate flow rates, the column residence time of the solute and sample may allow some dissociation of the solute from its bound form with the ligand in the sample, leading to an increase in the apparent free fraction as the flow rate decreases [137,146]. On the other hand, a consistent free fraction for the solute is obtained at high flow rates, because dissociation of the solute from the solute-ligand complex is limited under these conditions due to their short residence time in the column [137,146]. Flow rates that provide or approach a constant apparent free fraction value (F_0) represent the optimum flow rate range for this type of measurement. This optimum range will vary for each solute-ligand

system; however, the column residence times at which these flow rates appear commonly fall in the sub-second time domain [137]. The apparent free fraction values at low-to-moderate flow rates can also provide valuable information on the dissociation kinetics of the solute from the ligand in the sample [137,146]. The dissociation rate constant of the solute from this ligand can be estimated from the slope from a linear plot prepared according to either eq. (11) or (12).

$$ln\frac{(1-F_0)}{(1-F_t)} = k_d t \tag{11}$$

$$ln\frac{1}{(1-F_t)} = k_d t - \ln(1 - F_0) \tag{12}$$

In these equations, the terms F_t and t represent the apparent free fraction and column residence time at the given flow rate. The term F_0 is the free fraction measured at or near equilibrium (i.e., at high flow rates), and k_d is the dissociation rate constant for the solute from the binding agent in the sample [137,119]. If the free fraction at equilibrium is not directly experimentally accessible, it can also be estimated from the intercept of a plot made according to eq. (12). Examples of plots for $\ln[(1-F_0)/(1-F_t)]$ or $\ln[1/(1-F_t)]$ versus t are provided in Figure 10 [146].

The association equilibrium constant for the solute and its ligand in the sample can also be determined by using F_0 with the total known concentrations of the solute and ligand in the sample, as shown in eq. (13).

$$K_a \text{ or } nK'_a = \frac{1-F_0}{F_0([L]_0-[A]_0+[A]_0F_0)} \tag{13}$$

In this relationship, K_a and nK'_a are the association equilibrium constant and the global affinity constant for the given binding system; F_0 is the free fraction obtained or estimated at equilibrium and/or at the optimum flow rate range; and $[L]_0$ and $[A]_0$ are the total concentrations of the ligand and solute or analyte [137,119,146,147]. If multiple independent sites on the ligand are involved in binding the solute, the equilibrium constant obtained with eq. (13) represents the global affinity constant, where n is the number of binding sites for the solute on the ligand [137,146]. Related

equations have been developed for UAE when examining biomolecular systems with a mixture of different types of interactions [147] or multiple binding agents for the same solute [140].

UAE has been used to examine the binding of drugs and hormones with the transport proteins HSA, AGP, and sex-hormone binding globulin [139,140,146,148-151]. For example, UAE has been used to study binding by the drugs warfarin, verapamil, chlorpromazine, diazepam, quinidine, tolbutamide, gliclazide, acetohexamide, and glibenclamide, as well as the hormone testosterone, to normal or modified HSA [139,146,148-150]. Furthermore, interactions of AGP with the drugs carbamazepine, disopyramide, lidocaine, propafenone, warfarin, propranolol, imipramine, chlorpromazine, and verapamil have been characterized by UAE [140,147]. In addition, UAE has been used to determine the binding strength of testosterone with sex-hormone binding globulin [146].

Some advantages of UAE in this type of work are that binding and rate information can be obtained quickly (i.e., in a few minutes), and this method requires only small amounts of a binding agent (i.e., pico-to-nano molar range) [137,138]. In addition, UAE allows for the direct analysis of interactions in the solution-phase without the use of labels [137]. The dissociation rate constants that can be measured by UAE have ranged from 10^{-2} to 10^{1} s^{-1}, and binding affinities that can be determined have spanned from 10^{4} to 10^{9} M^{-1} [137,139,140,146-151].

7. Conclusion

This review has examined how LC and HPLC can be used in the analysis of reversible biomolecular systems in which all the interacting components are in solution. Various strategies were described for such work, as summarized in Table 1. For instance, vacancy techniques such as the Hummel-Dreyer method and schemes using solution-phase frontal analysis were some of the original formats used for such work. These methods have been applied with columns based on

size-exclusion or ISRP media for examining systems such as drug-protein binding and with association equilibrium constants in the general range of 10^3 to 10^7 M^{-1}. The use of a support that contains an entrapped and soluble form of one of the interacting components is a second strategy that has been used in such work. Several means of entrapment or encapsulation have been developed for this purpose. Columns prepared in this manner have been used in both frontal and zonal elution methods to screen or study binding of inhibitors to enzymes and drugs to serum transport proteins with affinities spanning from 10^4 to 10^7 M^{-1}.

Two other ways in which LC or HPLC have been used to investigate solution-phase binding are normal-role affinity chromatography and UAE. Normal-role affinity chromatography is one of the original formats for affinity chromatography and has been utilized to study interactions such as binding by an enzyme with an inhibitor, co-factor, or substrate, as well as antibody-antigen, and cyclodextrin-drug interactions. These interactions have had binding constants extending from 10^2 to almost 10^6 M^{-1}. UAE also employs a column with a secondary binding agent, but this agent is now used to quickly capture one of the components of a solution-phase interaction. This method has been used to study both the extent and rate of drug and hormone interactions with proteins such as HSA, AGP and sex hormone-binding globulin. These systems have had affinities ranging from 10^4 to 10^9 M^{-1} and dissociation rate constants spanning from 10^{-2} to 10^1 s^{-1}.

This review described the general principles for each of these strategies and the ways in which they have been used to study solution-phase biomolecular interactions. The applications for each approach were summarized, along with general requirements and potential limitations of each method. The variety of strategies that are available in LC and HPLC for examining solution-phase interactions should continue to make these methods useful in the study of other biomolecular

systems. Additional work in developing new columns and schemes for such work should further allow LC and HPLC to be extended in the future to applications involving both biological systems and other forms of interactions that involve biomolecules or that have similar reversible binding processes.

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Figure Legends

Figure 1. General scheme for the Hummel-Dreyer method.

Figure 2. Example of results from Hummel-Dreyer method including (a) a typical chromatogram and (b) a Scatchard plot used to analyze the results of this technique. The results in (a) were obtained for a 12.5 μL sample solution of 2 g/L HSA at 0.5 mL/min and 37 °C on a 15 cm × 4.2 mm I.D. column with a Glycophase G (100 Å) support and in the presence of a mobile phase containing 0.5 μM of the drug warfarin in pH 7.4 phosphate buffer. The data in (b) were also obtained for the binding of warfarin with HSA but using slightly different chromatographic conditions. These figures are adapted with permission from (a) Ref. [59] and (b) Ref. [64].

Figure 3. General scheme for solution-phase frontal analysis.

Figure 4. (a) Typical chromatogram (a) and (b) Scatchard plot for analysis of the results of solution-phase high-performance frontal analysis (HPFA). The chromatogram in (a) is for a 1.5 mL injected mixture containing the *S*-enantiomer of *N*-desethyloxybutynin (10 μM) and the protein AGP (20 μM), as acquired at pH 7.4, 37°C, and 1.0 mL/min using a 10 cm × 4.6 mm I.D. Develosil 100-Diol-5 column. The Scatchard plots in (b) are for the *R*- and *S*-enantiomers of *N*-desethyloxybutynin during their binding to AGP in these HPFA studies. Adapted with permission from Ref. [108].

Figure 5. General scheme for LC interaction studies based on entrapment of a binding agent.

Figure 6. Examples of (a) frontal analysis and (b) zonal elution studies carried out by HPLC and using protein columns prepared by entrapment. The breakthrough curves in (a) were obtained at pH 7.4, 37 ºC, and 0.50 mL/min during the application of (top-to-bottom) 20, 15, 10, 5, 2.5 and 1 μM carbamazepine onto a 1.0 cm × 2.1 mm I.D. column containing entrapped AGP. The chromatograms in (b) were also acquired at pH 7.4, 37 ºC, and 0.50 mL/min but were generated by injecting small samples of carbamazepine onto 1.0 cm × 2.1 mm I.D. columns containing entrapped AGP, entrapped HSA, or a control support. These plots are adapted with permission from Ref. [117].

Figure 7. General scheme for normal-role affinity chromatography.

Figure 8. Examples of chromatograms and analysis of binding data obtained by normal-role affinity chromatography. These results are for injections of a racemic mixture of *R*- and *S*-warfarin made at pH 7.4, 37 ºC and 0.3 mL/min onto a 5 cm × 2.1 mm I.D. column containing immobilized HSA and in the presence of various concentrations of ß-cyclodextrin (ß-CD) as a soluble binding agent. The results shown in the inset were analyzed according to eq. (10). Adapted with permission from Ref. [130].

Figure 9. General scheme for ultrafast affinity extraction (UAE).

Figure 10. (a) Results obtained by UAE for free fraction measurements of testosterone in the presence of a soluble binding agent that was either HSA or sex hormone binding globulin (SHBG). These results were obtained by injecting a sample containing 10 μM testosterone with 20 μM HSA made onto a 5 mm × 2.1 mm I.D. HSA microcolumn or by injecting 50 μL of the sample containing 42 nM testosterone

with 20 nM SHBG onto a 20 mm × 2.1 mm I.D. HSA microcolumn at 37 °C and pH 7.4. (b) Examples of plots prepared according to eq. (11) (right *y*-axis) or eq. (12) (left *y*-axis) to determine the dissociation rate constant for testosterone from SHBG. These plots are adapted with permission from Ref. [146].

Table 1. Summary of methods for the analysis of solution-phase biomolecular interactions by LC

Method & Principle	Advantages	Limitations
Hummel-Dreyer Method A binding agent is injected onto a size exclusion-based column and into a mobile phase that contains the target solute; as binding occurs between the solute and injected agent, the vacancy peak for the target is measured	• Often used to examine reversible drug-protein binding but can be applied to other types of interactions • Can provide equilibrium constants and number of interaction sites for a solute with its binding agent • Has been used for systems with affinities ranging from ~10^3-10^7 M^{-1}	• Requires rapid kinetics on the time-scale of the separation • Must use concentrations and conditions that make it possible to measure the vacancy peak • Column and chromatographic conditions must be able to separate the unbound target from its complex with the binding agent
Solution-Phase Frontal Analysis A large-volume sample containing both small and larger interacting components is injected onto a column with a size-based separation mechanism that excludes the larger binding agent but retains the smaller component; the free and bound	• Can provide equilibrium constants and number of interaction sites of a solute with its binding agent • Often used to examine reversible drug-protein binding, but can be applied to a wide variety of other interactions • Has been used for systems with affinities ranging from ~10^3-10^7 M^{-1}	• Requires rapid kinetics on the time-scale of the separation • Must be possible to measure the regions for the unbound and bound forms of interacting components • Column and chromatographic conditions must be able to separate the unbound target from its complex with the binding agent

fractions of these components are partially separated and measured		• Difficult to use for systems with strong binding and small unbound fractions
Entrapment-Based Binding Methods A binding agent is non-covalently and physically entrapped within the pores or matrix of a support and then used in binding studies based on LC	• The soluble, immobilized binding agent often closely mimics its native behavior • Used to examine several types of reversible interactions • Can be combined with frontal analysis or zonal elution formats • Can provide equilibrium constants, number of interaction sites, and information on competition of solutes for a binding agent • Has been used for systems with affinities ranging from ~10^4-10^7 M^{-1}	• Need conditions for support preparation that will give an appropriate pore size to contain the binding agent, while also providing good mass transfer properties and support stability • Loss of binding agent activity is possible if incorrect conditions are used for support preparation • High levels of shrinkage of the support may occur with some materials used for entrapment
Normal-Role Affinity Chromatography A solute is injected onto a column with an immobilized agent that competes for solute binding with a soluble agent in the mobile phase	• Has been used to examine several types of reversible interactions • Can be combined with frontal analysis or zonal elution formats • Can be used to measure equilibrium or rate constants	• Need an immobilized agent that can interact with the same target solute as the soluble binding partner • Typically requires rapid kinetics on the time-scale of the separation

	• A single column can be used to examine interactions involving the same binding partner but different soluble agents • Has been used for systems with affinities ranging from ~10^2-10^6 M^{-1}	• Must be able to detect the injected and eluting binding partner in the presence of the soluble agent in the mobile phase
Ultrafast Affinity Extraction The unbound vs bound forms of a target solute in a sample containing a binding agent are separated by rapidly capturing the unbound solute with an immobilized agent	• Can be used to measure equilibrium or rate constants • Can be used with simple solutions or complex biological samples • Requires only small amount of binding agent • Label-free method • Has been used for systems with dissociation rate constants of ~10^{-2}-10^1 s^{-1} and affinities of ~10^4-10^9 M^{-1}	• Requires short column residence times and immobilized binding agent that can capture the unbound form of the solute • Requires rapid rate of target extraction compared to rate of target dissociation from binding agent in the sample • Optimum flow-rate range can vary between systems

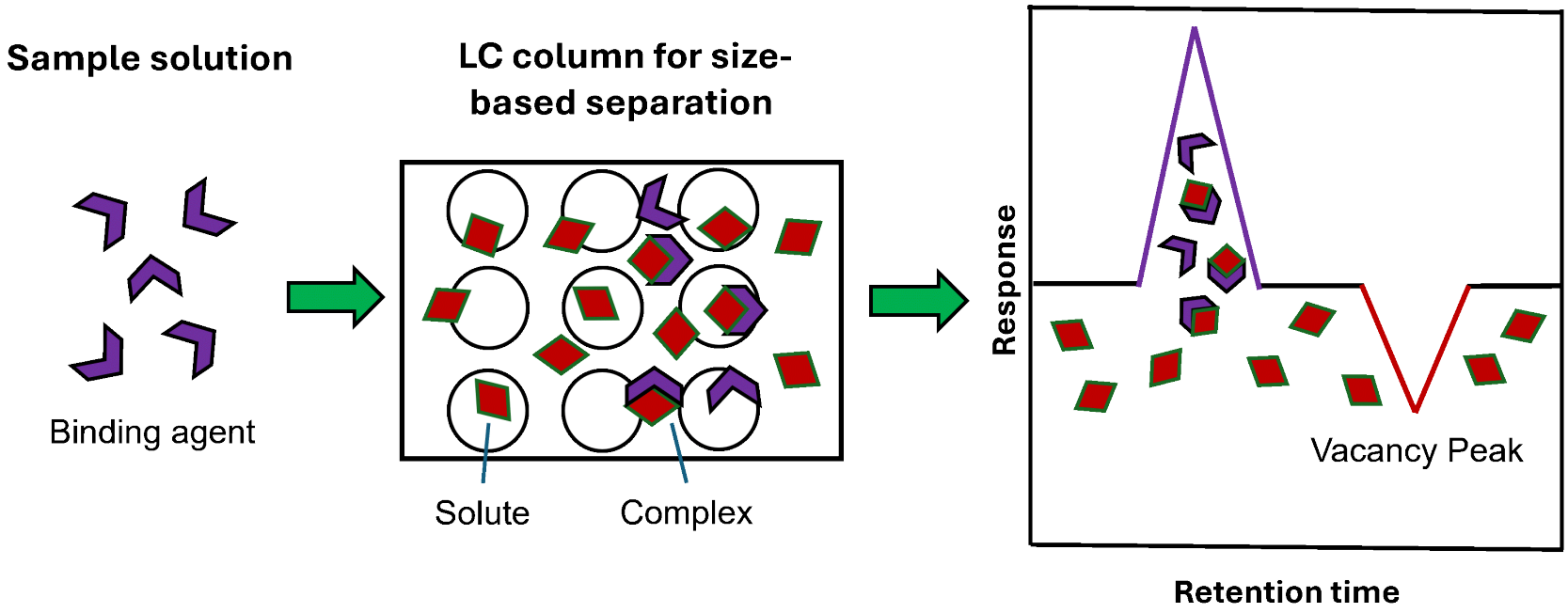
Sample solution
LC column for size-based separation
Binding agent
Solute
Complex
Response
Vacancy Peak
Retention time

Figure 1

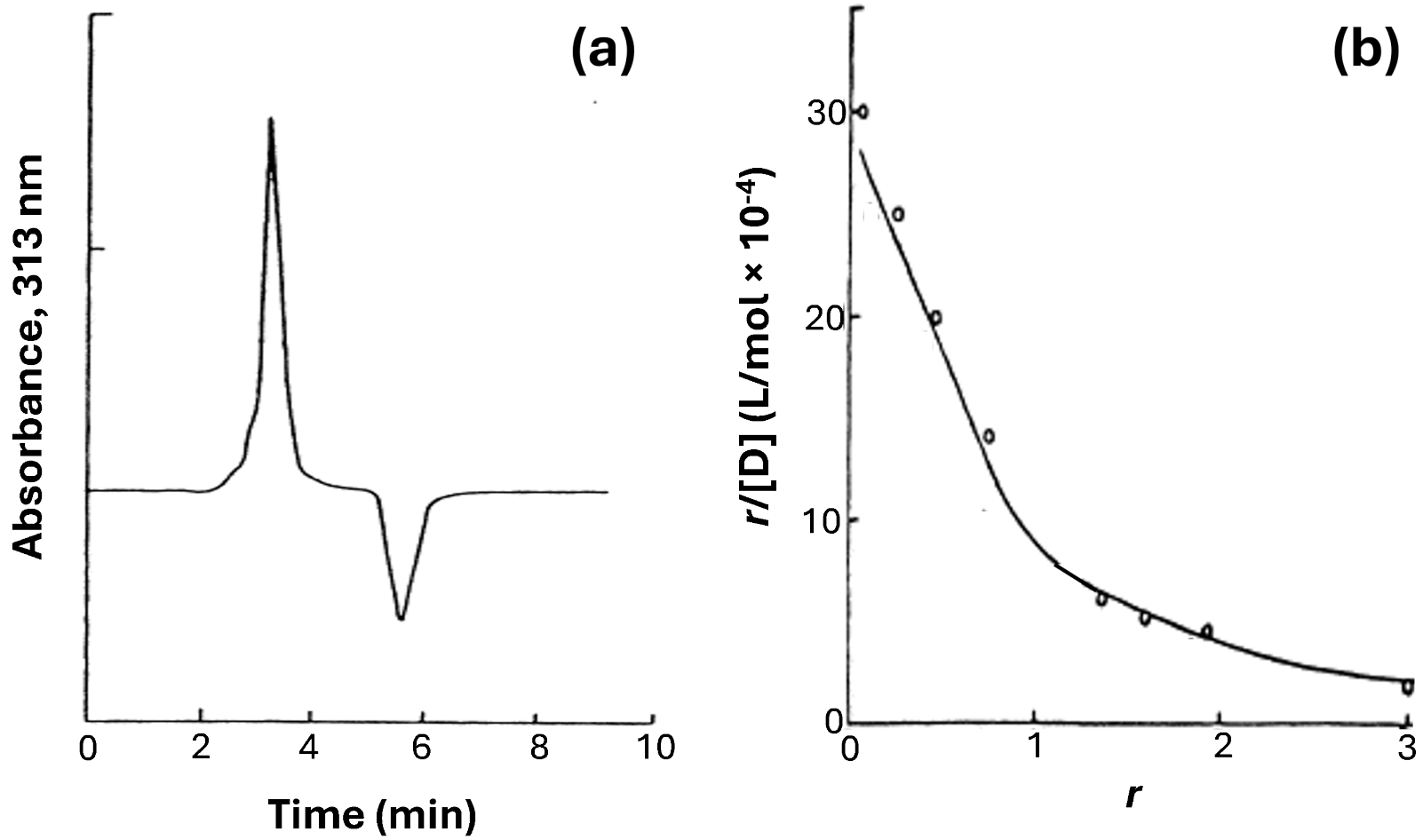
(a)
Absorbance, 313 nm
0
2
4
6
8
10
Time (min)
(b)
r/[D] (L/mol × 10-4)
30
20
10
0
0
1
2
3
r

Figure 2

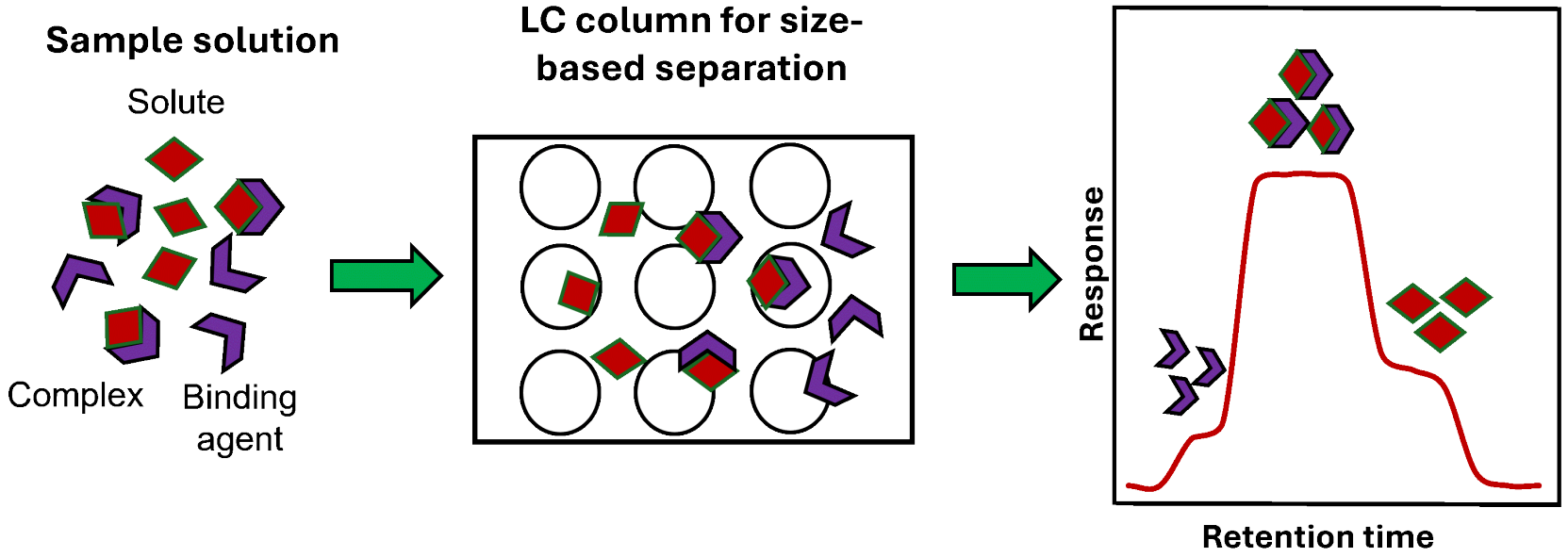
Sample solution
Solute
Complex
Binding agent
LC column for size-based separation
Response
Retention time

Figure 3

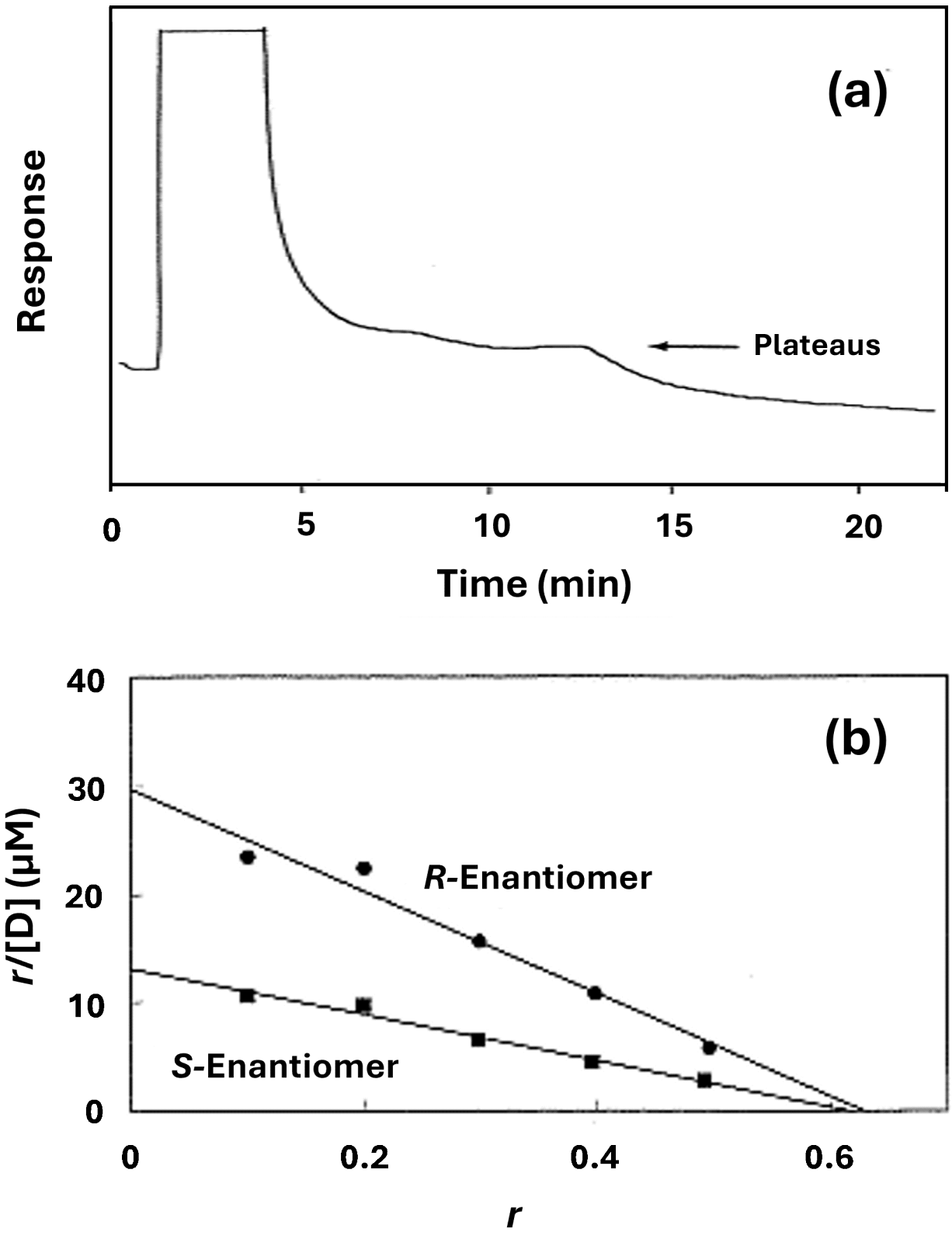
(a)
Response
Plateaus
0
5
10
15
20
Time (min)
(b)
40
30
20
10
0
r/[D] (μM)
R-Enantiomer
S-Enantiomer
0
0.2
0.4
0.6
r

Figure 4

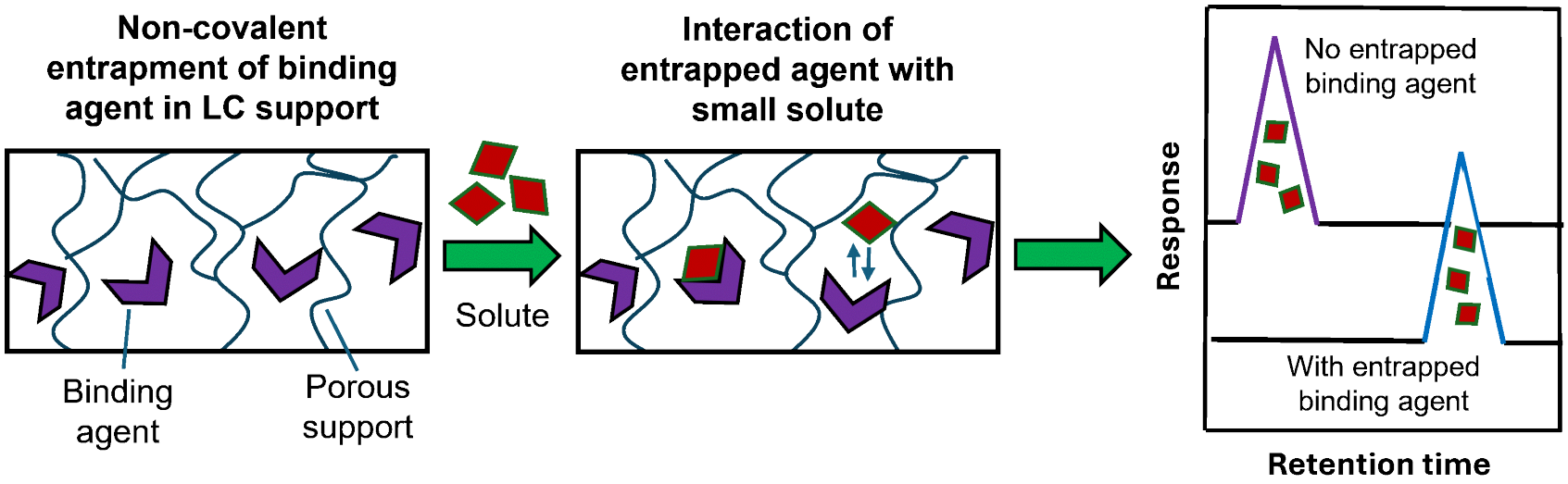
Non-covalent entrapment of binding agent in LC support
Binding agent
Porous support
Solute
Interaction of entrapped agent with small solute
Response
No entrapped binding agent
With entrapped binding agent
Retention time

Figure 5

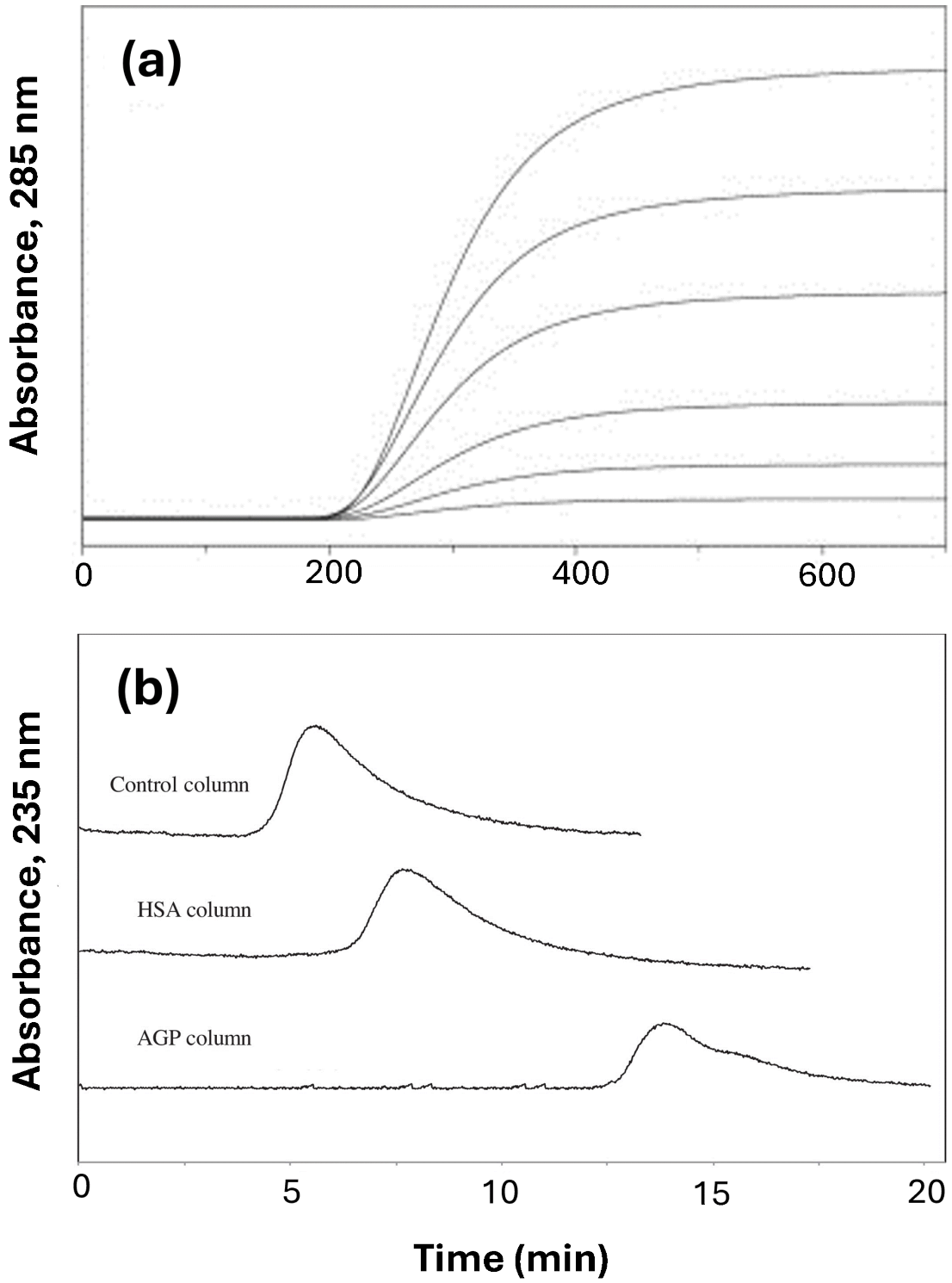
(a)
Absorbance, 285 nm
0
200
400
600
(b)
Absorbance, 235 nm
Control column
HSA column
AGP column
0
5
10
15
20
Time (min)

Figure 6

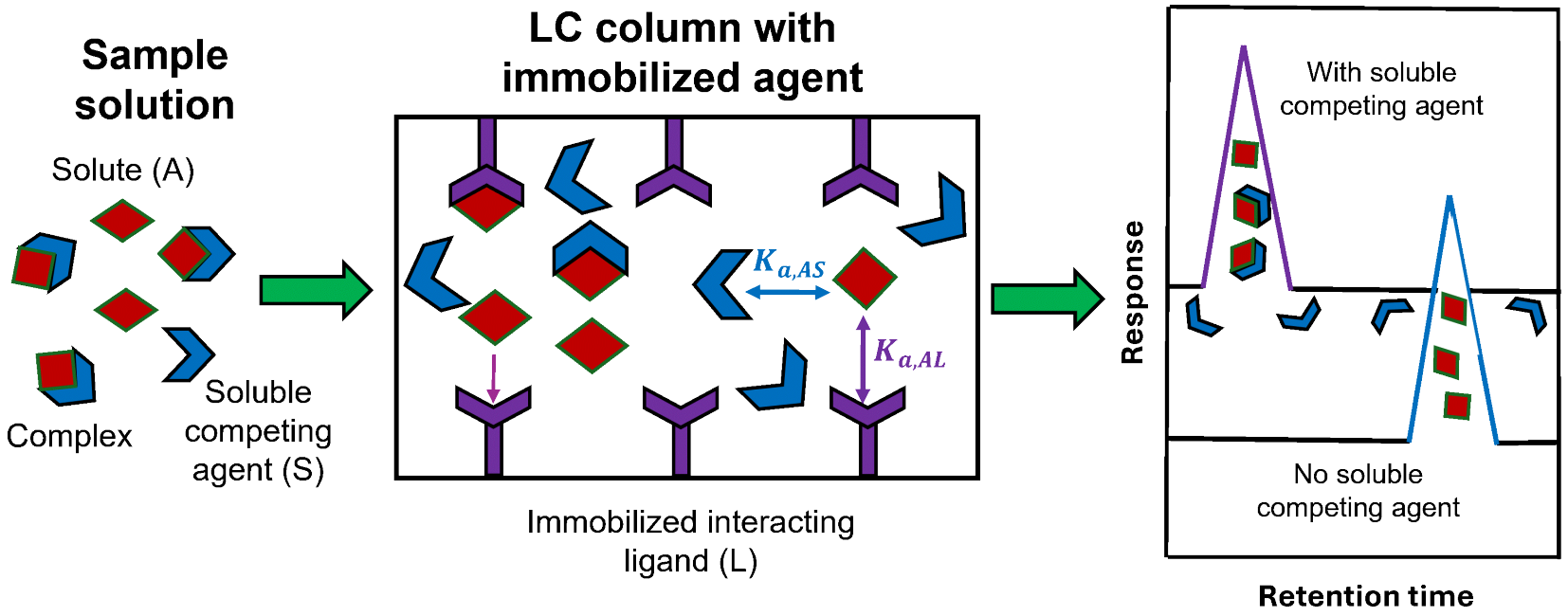
Sample solution
Solute (A)
Complex
Soluble competing agent (S)
LC column with immobilized agent
$K_{a,AS}$
$K_{a,AL}$
Immobilized interacting ligand (L)
Response
With soluble competing agent
No soluble competing agent
Retention time

Figure 7

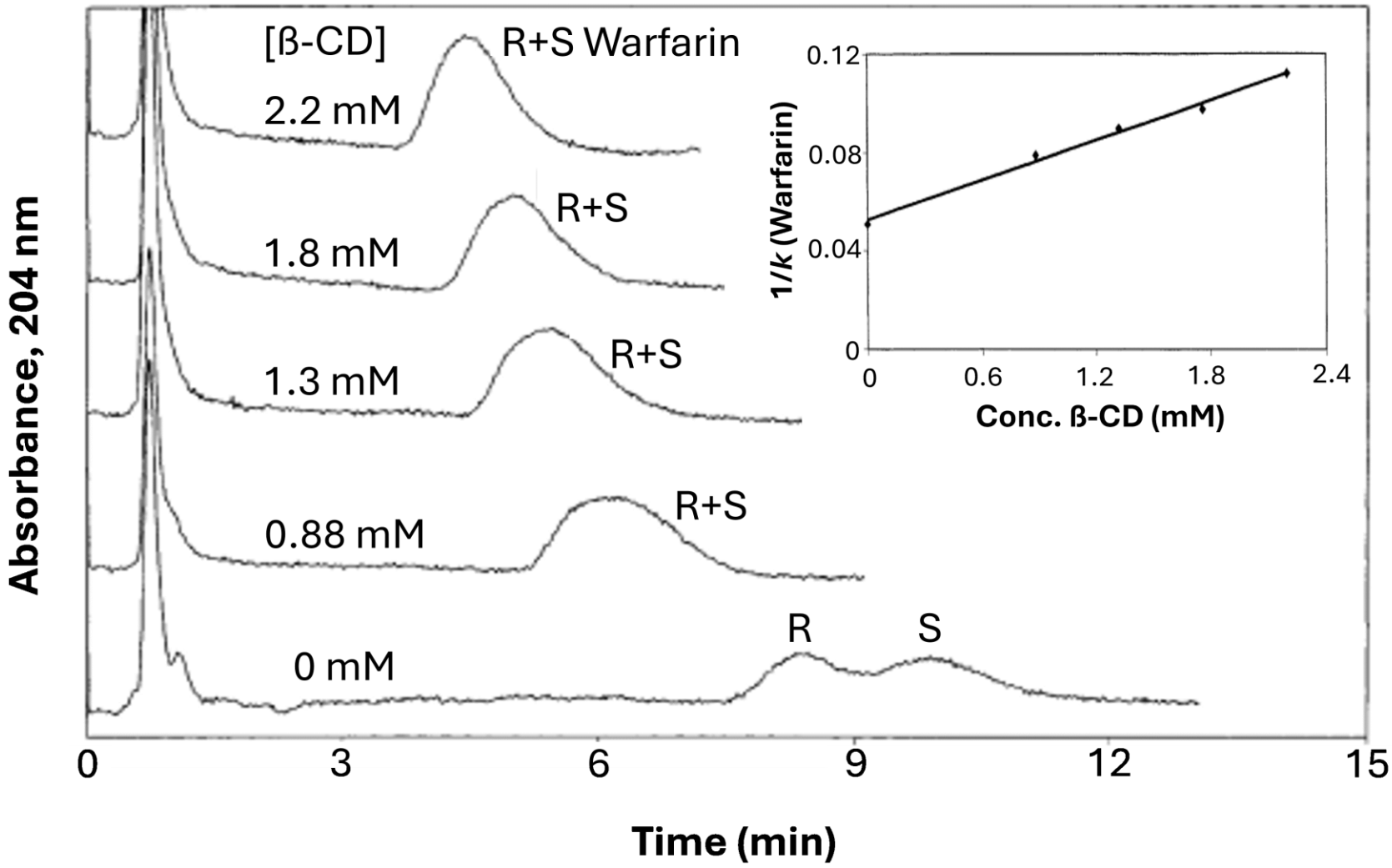
[ß-CD]
R+S Warfarin
2.2 mM
1.8 mM
R+S
1.3 mM
R+S
0.88 mM
R+S
0 mM
R
S
Absorbance, 204 nm
Time (min)
0
3
6
9
12
15
1/k (Warfarin)
0.12
0.08
0.04
0
0
0.6
1.2
1.8
2.4
Conc. ß-CD (mM)

Figure 8

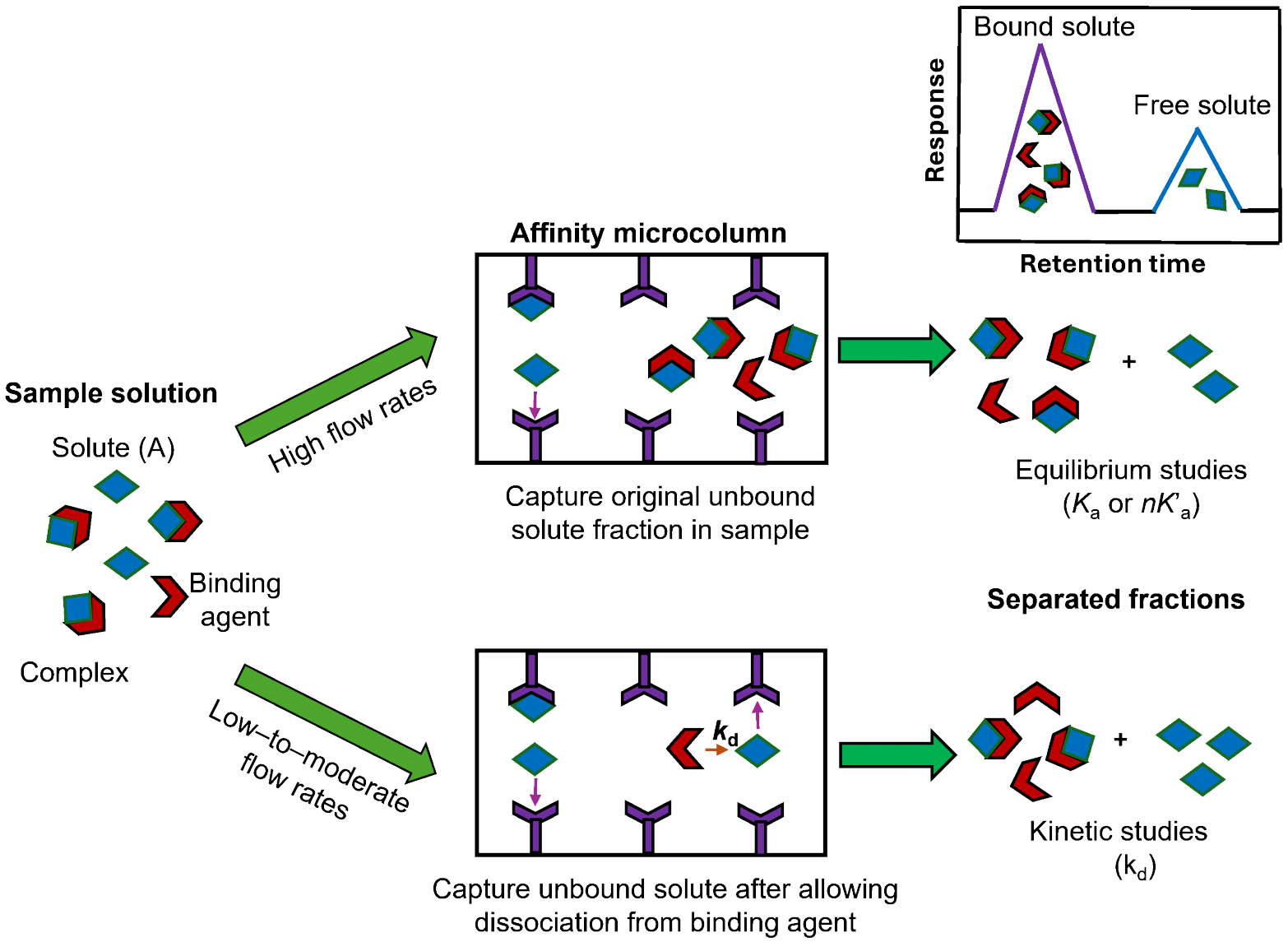
Bound solute
Free solute
Response
Retention time
Affinity microcolumn
Sample solution
Solute (A)
High flow rates
Binding agent
Complex
Capture original unbound solute fraction in sample
+
Equilibrium studies
(K_a or nK'_a)
Separated fractions
Low–to–moderate flow rates
k_d
+
Kinetic studies
(k_d)
Capture unbound solute after allowing dissociation from binding agent

Figure 9

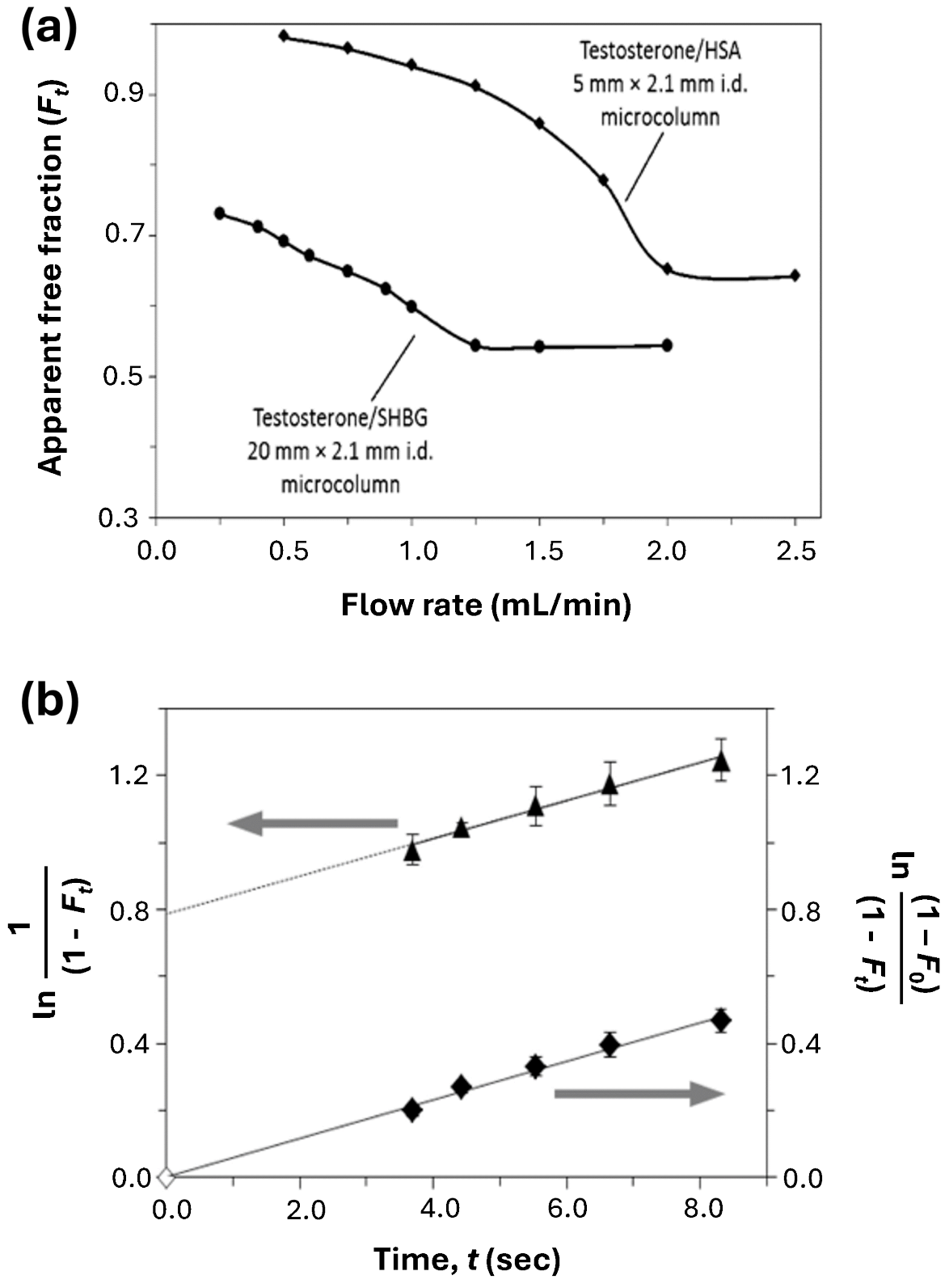
(a)
Apparent free fraction (F_t)
0.9
0.7
0.5
0.3
Testosterone/HSA
5 mm × 2.1 mm i.d.
microcolumn
Testosterone/SHBG
20 mm × 2.1 mm i.d.
microcolumn
0.0
0.5
1.0
1.5
2.0
2.5
Flow rate (mL/min)
(b)
$\ln \frac{1}{(1 - F_t)}$
$\ln \frac{(1 - F_0)}{(1 - F_t)}$
1.2
0.8
0.4
0.0
0.0
2.0
4.0
6.0
8.0
Time, t (sec)

Figure 10