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# Kinetic Analysis of Drug-Protein Interactions by Affinity Chromatography

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## Abstract

Information on the kinetics of drug-protein interactions is of crucial importance in drug discovery and development. Several methods based on affinity chromatography have been developed in recent years to examine the association and dissociation rates of these processes. These techniques include band-broadening measurements, the peak decay method, peak fitting methods, the split-peak method, and free fraction analysis. This review will examine the general principles and applications of these approaches and discuss their use in the characterization, screening and analysis of drug-protein interactions in the body.

## Graphical Abstract

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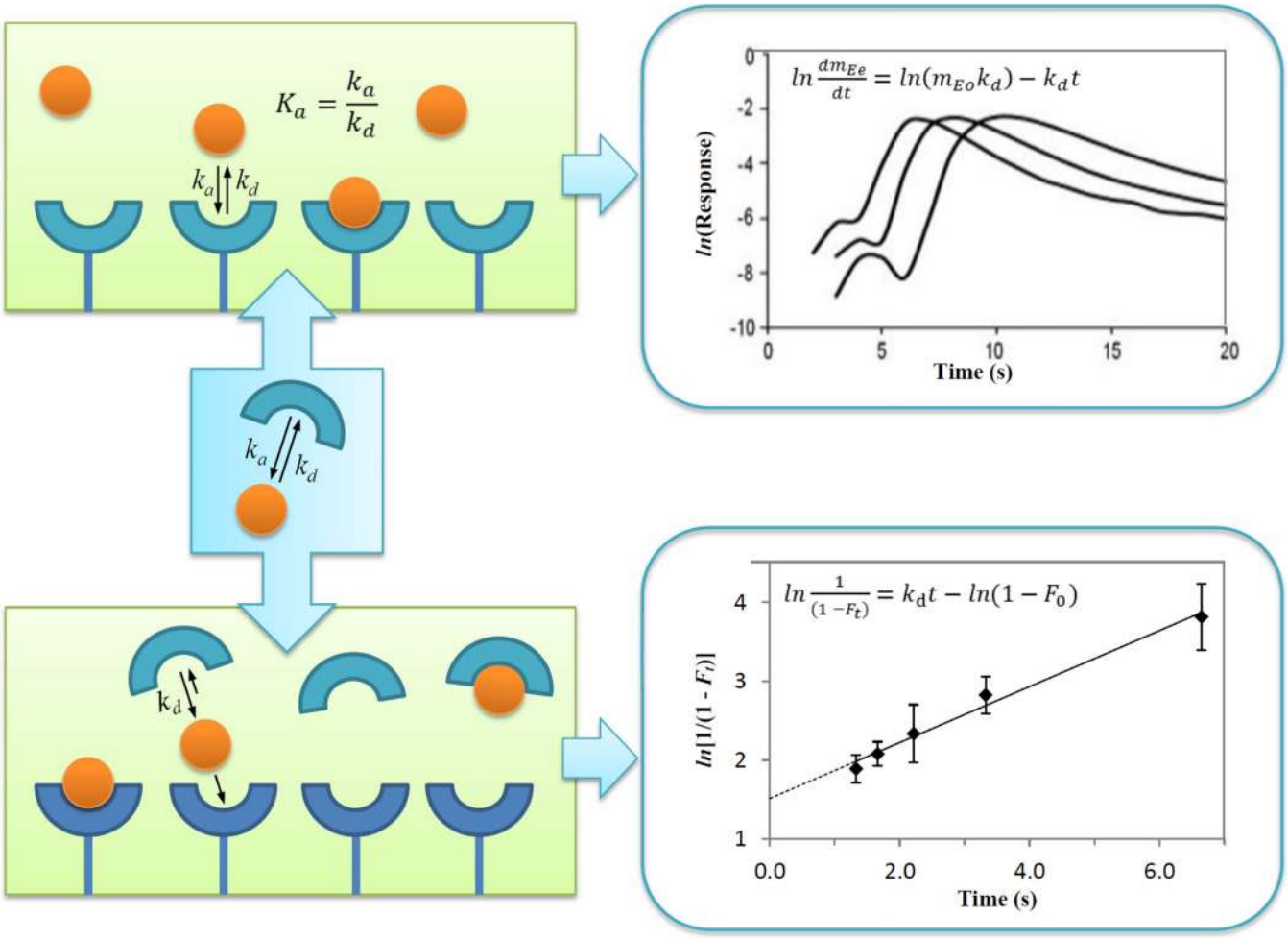
**Conflict of interest**

The authors have no conflict of interest to declare.




**Keywords**

affinity chromatography; high-performance affinity chromatography; kinetics; drug-protein interactions; peak profiling; peak decay analysis; split-peak method; ultrafast affinity extraction

## Introduction

The characterization of drug-protein interaction rates can provide important information on the pharmacokinetics and pharmacodynamics of a drug or drug candidate [1–4]. For instance, the kinetics of drug interactions with serum transport proteins, such as human serum albumin (HSA) and $\alpha_1$-acid glycoprotein (AGP), can affect the distribution, metabolism and half-life of many drugs in the body [1,5]. In addition, data on the rates of drug-receptor interactions can be used to describe the mechanism of action for some pharmaceutical agents [3,6–8].

Affinity chromatography and high-performance versions of this method have been employed in recent years to study the rates of drug-protein interactions [9–11]. This review will discuss several methods that have been developed for this work, as well as the

advantages and potential limitations of each approach. Recent applications that will be presented will include the use of these techniques in kinetic studies of drug interactions with serum proteins and receptors.

## General principles of affinity chromatography

Affinity chromatography is a type of liquid chromatography that utilizes a biologically-related binding agent as the stationary phase [12,13]. High-performance affinity chromatography (HPAC) is a form of affinity chromatography in which a rigid and efficient HPLC-type support is used to contain this immobilized binding agent within the column [12]. One way these methods can be used is to study the interactions between an applied target and the immobilized binding agent; however, the immobilized binding agent can also be used as a secondary probe to examine a solution-phase interaction (see Fig. 1) [9,14,15]. These techniques have already been used to investigate many types of biological systems, including drug-protein, hormone-protein, protein-protein, lectin-sugar, and antibody-antigen interactions [9–12,14–16].

There are a number of advantages to using affinity chromatography and HPAC for the analysis of biological interactions. These advantages include the ability to reuse the same immobilized binding agent for many experiments, which helps to provide good reproducibility, reduces the amount of binding agent that is needed, and minimizes the effects of batch-to-batch variations in the binding agent [1,12,15]. If the correct immobilization conditions are used, the behavior of the immobilized binding agent can show good agreement with the behavior of the same agent in its native form [4,9,10,17]. In addition, various detection methods (e.g., absorbance, fluorescence, or mass spectrometry) can be employed with affinity chromatography and HPAC, usually in a label-free manner, to study biological interactions [9–11]. The speed, precision and ease of automation of HPAC also make this method useful for rapid and high-throughput measurements of drug-protein interactions [9,12,16].

## Band-Broadening Measurements

Band-broadening measurements are used in one group of methods for examining the kinetics of a biological interaction by affinity chromatography [9,10,15]. This type of experiment is often carried out by applying a small sample of the target analyte onto an affinity column that contains the immobilized binding agent of interest and onto a control column with no binding agent present. The widths of the resulting peaks are then used to provide information on the rate of the interaction between the target and immobilized binding agent [9–11].

The *plate height method* is the first way in which band-broadening measurements can be used to provide kinetic information on a biological interaction. In this technique, the number of theoretical plates ($N$) for an affinity column and a control column are measured under a given set of conditions. The value of $N$ for each column is equal to the ratio ($t_R^2/\sigma_R^2$), where $t_R$ is the retention time of the target analyte, and $\sigma_R^2$ is the variance of the target's peak (i.e., a measure of the peak's width). The total plate height ($H_{total}$) for each column is then calculated by using the relationship $H_{total} = L/N$, where $L$ is the length of the column [9].

The value of the total plate height for any type of column can be viewed as the summation of plate height contributions due to the various processes that lead to the broadening of a chromatographic peak [9,18,19]. One of these terms is $H_k$, which represents the plate height contribution due to stationary phase mass transfer; this term is of particular interest in kinetic studies because it is directly related to the rate at which the target is interacting with the immobilized binding agent [9,10,18,19].

In the most common form of the plate height method, data from the affinity column and a control column are used to measure $H_{total}$ and to estimate the individual contributions of the various band-broadening processes in each column [9,10,18,19]. This combined information is used to find the value of $H_k$ for the affinity column at known or measured values for the linear velocity ($u$) of the mobile phase and the retention factor ($k$) of the target in the affinity column. This information is then used to find the dissociation rate constant ($k_d$) for the interaction of the target with the immobilized binding agent.

The plate height method has been used to examine drug-protein interactions with weak-to-moderate binding strengths (association equilibrium constant, $K_a < 10^6\ M^{-1}$) and $k_d$ values in the range of $10^{-2}$ to $10^1\ s^{-1}$. For instance, this method has been utilized to characterize the binding of *R*- and *S*-warfarin and D- and L-tryptophan with HSA [10,18,19]. This method has also been employed in determining the effects of changing the pH, temperature, and solution's ionic strength or polarity on the rates of these interactions [18–20]. The plate height method does require the use of a small amount of the injected target compared to the total amount of binding agent that is present. In addition, the association and dissociation rates of the target in its interactions with the immobilized binding agent should be reasonably fast compared to the retention time of the target to allow multiple binding and dissociation events to occur as the target passes through the affinity column [18,19].

The *peak profiling method* is a second approach that uses band-broadening measurements. This method differs from the plate height method in that it makes use of a simple difference in the total plate heights that are obtained between an affinity column ($H_R$) and a control column ($H_M$), rather than using more detailed estimates of the plate height contributions of individual band-broadening processes [21–23]. A typical plot that is used in this method and an equation that can be used to analyze such data are provided in Fig. 2 [22]. This method can be carried out by using data that are obtained at several flow rates or by using data acquired at a single flow rate. The equation shown in Fig. 2 is applicable when there is only a single type of binding site present in the affinity column. However, a modified form of this expression can be used for systems that involve multi-site interactions or binding by a drug with both the immobilized binding agent and the support [22,23].

Peak profiling has been recently used to characterize dissociation rate constants in the range of $10^{-1}$ to $10^1\ s^{-1}$ for a number of drugs and solutes (e.g., L-tryptophan, carbamazepine, imipramine, and phenytoin) with an immobilized protein such as HSA [21–23]. For instance, the example shown in Fig. 2 involved an analysis of the interaction kinetics of phenytoin metabolites with HSA [22]. An advantage of the peak profiling method is it can be used at relatively high flow rates, which makes it attractive as a possible tool for the high-throughput screening of drug-protein interactions [21]. However, care must still be taken in

using a suitably small sample size for this measurement, and the system to be examined should have reasonably fast association and dissociation rates on the time scale of the experiment [9].

## Peak decay method

In the *peak decay method*, a small amount of a target analyte is injected onto an affinity column or a control column, followed by later release of the retained target from the column [9,10,24]. A relatively high flow rate and set of mobile phase conditions are used during the elution step to prevent rebinding of the target once it has been released and to limit the movement of the released target back into the stagnant mobile phase [24,25]. The elution profile that is obtained under these conditions approaches a first-order decay curve, which can be used to obtain the dissociation rate constant for the target from the immobilized binding agent (see Fig. 3) [24–26]. One way this type of behavior can be achieved is by using a high concentration of a competing agent to displace the target analyte and prevent its re-association [9,10]. For systems with weak-to-moderate binding strengths, it is also possible to use an affinity microcolumn in a noncompetitive format, where a relatively high concentration of the target is applied to allow rapid saturation of the column and to prevent re-association of the released target [24–26].

The peak decay method has been used to measure dissociation rate constants in the range of $10^{-2}$ to $10^{1}$ $s^{-1}$ [10]. In work with drug-protein systems, this technique has been utilized to determine the dissociation rate constants for various drugs (e.g., imipramine, cisplatin, and lidocaine) that have weak-to-moderate affinities with serum proteins such as HSA or AGP [24–26]. The interactions of racemic warfarin and the separate enantiomers of warfarin with HSA have also been determined by this approach [26].

One advantage of the peak decay method is it is less sensitive than band-broadening methods to changes in the amount of applied target [9,10]. In addition, the high flow rates that are employed in this method make it attractive for the high-throughput screening of drug-protein interactions. Only small column sizes and small amounts of binding agents are needed in the non-competitive format of this method, which is another valuable feature. However, the peak decay method does require either a suitable competing agent or careful selection of the column and flow rate conditions for the elution process to provide a usable decay curve [9,10].

## Peak fitting methods

*Peak fitting* is another approach that has been applied in affinity chromatography for kinetic studies. This method involves the injection or application of a target analyte onto the affinity column at various sample concentrations or conditions, followed by fitting of the overall peaks to various chromatographic models. This fit is then used to estimate the rate constants and equilibrium constants for the interaction of the target with the immobilized binding agent [3,9,27].

Peak fitting has been used to examine the kinetics of various systems with weak-to-moderate affinities. Examples include the interactions of nicotinic acetylcholine receptors with several

non-competitive inhibitors, such as 18-methoxycoronaridine, phencyclidine, and bupropion [28,29]. The interaction of novobiocin with heat shock protein 90α has also been investigated by this method [30]. The association and dissociation rate constants that have been measured by peak fitting have been in the range of $10^4$ to $10^7$ $M^{-1}s^{-1}$ and $10^{-1}$ to 10 $s^{-1}$, respectively [3,10,27]. Although this is a fairly flexible method, it is necessary to test and verify any assumptions that are made in the models employed in this approach, such as whether mobile phase mass transfer is negligible or needs to be considered during data analysis [9,10].

## Split-peak method

The *split-peak method* can be employed to examine the kinetics of interactions that are essentially irreversible within the timescale of the application step [9]. This method is based on the fact that there is a finite probability an applied target may elute from an affinity column without interacting with the immobilized binding agent, even when this binding agent is fully active and present in a large excess versus the target. This phenomenon is known as the "splitpeak effect" and results in the formation of two peaks following the injection of a single solute: a non-retained peak and a strongly retained peak [9,31,32]. This effect is created by the presence of slow association kinetics between the target and binding agent and/or slow mass transfer kinetics for movement by the target into the pores of the support or to the support's surface. In the case of slow association kinetics, the relative size of the non-retained fraction is determined by the flow rate, the moles of binding agent that are present, and the relative amount of target that is applied to the column [9,10,31].

The split-peak method has been utilized in examining the kinetics of systems with high affinities ($K_a > 10^6$ $M^{-1}$) and association rate constants in the range of $10^4$ to $10^6$ $M^{-1}$ $s^{-1}$ [9,10,33,34]. For instance, this method has been used to measure the association rate constants for HSA and thyroxine with immobilized antibodies against these targets [33,34]. An advantage of this method is that it uses peak areas, which are easier to obtain and measure than peak variances or peak profiles. However, this method does requires that a good separation be obtained between the non-retained and retained fractions of the applied target, which makes it best suited for systems with relatively slow dissociation kinetics ($k_d <$ $10^{-1}$ $s^{-1}$) [33,34]. It is also important in this method to have experimental conditions that can lead to an observable split-peak effect [9,10].

## Free Fraction Analysis

A number of methods based on affinity chromatography have been developed that allow drug-protein interactions to be studied by measuring the free, or non-bound, fraction of a drug. *Ultrafast affinity extraction* is one technique that has been utilized for this purpose [10,35,36]. In this method, a sample that contains a mixture of the target analyte (e.g., a drug or hormone) and a soluble binding agent (e.g., a protein) is injected onto an affinity microcolumn that can bind to the target in its free form. If the sample only spends a small amount of time in the column, the other sample components and the bound form of the target will elute as a non-retained peak while the free form of the target is captured by the column.

Fig. 4 shows how this method can be employed when examining the kinetics of a solution-phase drug-protein interaction [37]. In this type of experiment, the apparent free drug fraction is measured at several flow rates, with some of these conditions providing sufficient time for the drug to dissociate from proteins in the sample as this mixture passes through an affinity column that can extract the free form of the drug. The change in the apparent free fraction with the flow rate and residence time of the sample in the column is then used to provide the dissociation rate constant for the drug-protein complex [10,37].

Ultrafast affinity extraction has been used to study the binding of various drugs with serum proteins [10,37]. Recent examples include the use of this method to examine the dissociation rates of verapamil, warfarin, tolbutamide, acetohexamide, gliclazide and chlorpromazine from their solution-phase complexes with HSA [37]. Dissociation rate constants that have been determined by ultrafast affinity extraction have been in the range of $10^{-2}$ to $10^{1}$ $s^{-1}$ [10,37]. An advantage of this technique is it can directly examine interactions that are occurring between a drug and protein or binding agent in solution. In addition, this method requires only a small amount of sample and can provide results within a few minutes of sample injection. The use of peak areas instead of peak shapes or variances again simplifies the process of data analysis. However, the column sizes and flow rates that are used in this type of experiment do need to be selected and optimized in advance to provide at least a partial separation of the free and bound forms of the target in typical samples [10,37].

## Conclusions

This review examined various approaches based on affinity chromatography that can be utilized for kinetic studies of drug-protein interactions. These methods are available in several formats and have been used to characterize biological interactions with a wide range of binding strengths and rate constants [10]. Systems that have been examined with these techniques include the interactions of drugs with serum proteins and receptors. In many of these methods, the affinity column and immobilized binding agent can be reused for many experiments and results can often be obtained in only a few minutes. These advantages make this set of techniques attractive for use in the screening or rapid analysis of interactions by drugs and drug candidates with proteins and other binding agents [8,9].

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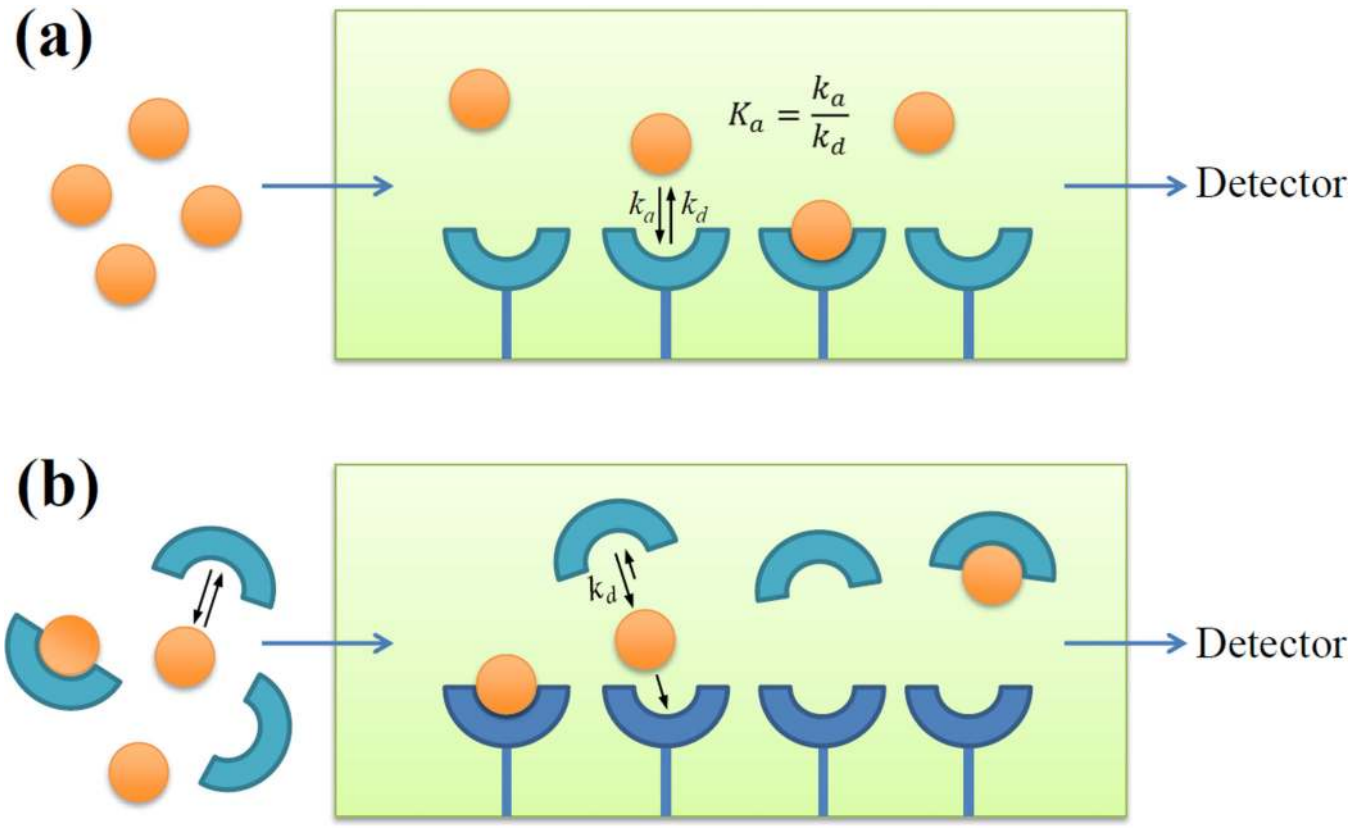


**Figure 1.**

Two general methods used in affinity chromatography and HPAC for examining the kinetics of drug-protein interactions. The method in (a) involves the application of the target drug or solute (represented by the circles) onto an affinity column that contains an immobilized protein as a binding agent; the observed retention or elution of the target is then used to provide information on the association and/or dissociation rates of the interaction between the target and immobilized binding agent. The method in (b) instead uses the immobilized binding agent as a secondary probe to examine the interaction of the target with a soluble protein or binding agent in an applied sample. Terms: $K_a$, association equilibrium constant; $k_a$, association rate constant; $k_d$, dissociation rate constant; HPAC, high-performance affinity chromatography.

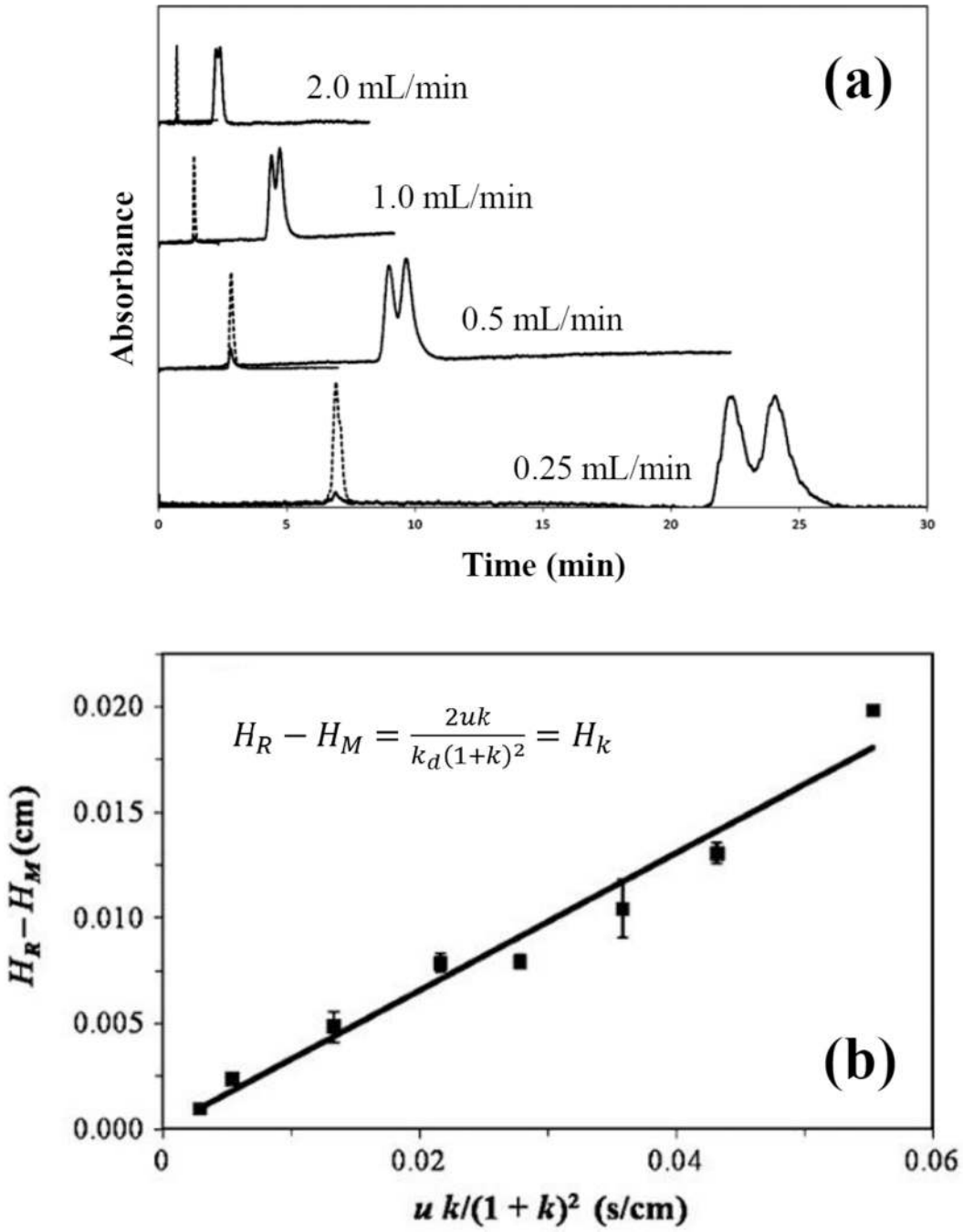


**Figure 2.**

(a) Chromatograms obtained for *m*-HPPH (solid lines; measured at 203 nm) and sodium nitrate (a non-retained solute, dashed lines; measured at 205 nm) on a 10 mm × 4.6 mm id HSA column at several injection flow rates, and (b) peak profiling plots obtained for the second eluting enantiomer of *p*-HPPH under similar conditions on an HSA column, as plotted according to the equation shown within this figure. The two peaks for *m*-HPPH in (a) represent the two enantiomers for this phenytoin metabolite. The error bars in (b) represent a range of ± 1 S.D. for triplicate injections. Terms: *m*-HPPH, 5-(3-hydroxyphenyl)-5-

phenylhydantoin; *p*-HPPH, 5-(4-hydroxyphenyl)-5-phenyl-hydantoin; $H_R$, plate height for the applied drug or target analyte on the affinity column; $H_M$, plate height for a non-retained solute on the affinity column, or for the target on a control column; $H_k$, plate height contribution due to stationary phase mass transfer; $u$, linear velocity of the mobile phase; $k$, retention factor of the target; HSA, human serum albumin. According to the equation in (b), the dissociation rate constant $k_d$ can be obtained from the slope in a plot made of $(H_R - H_M)$ versus $uk/(1)^2$. Adapted with permission from Ref. [22].

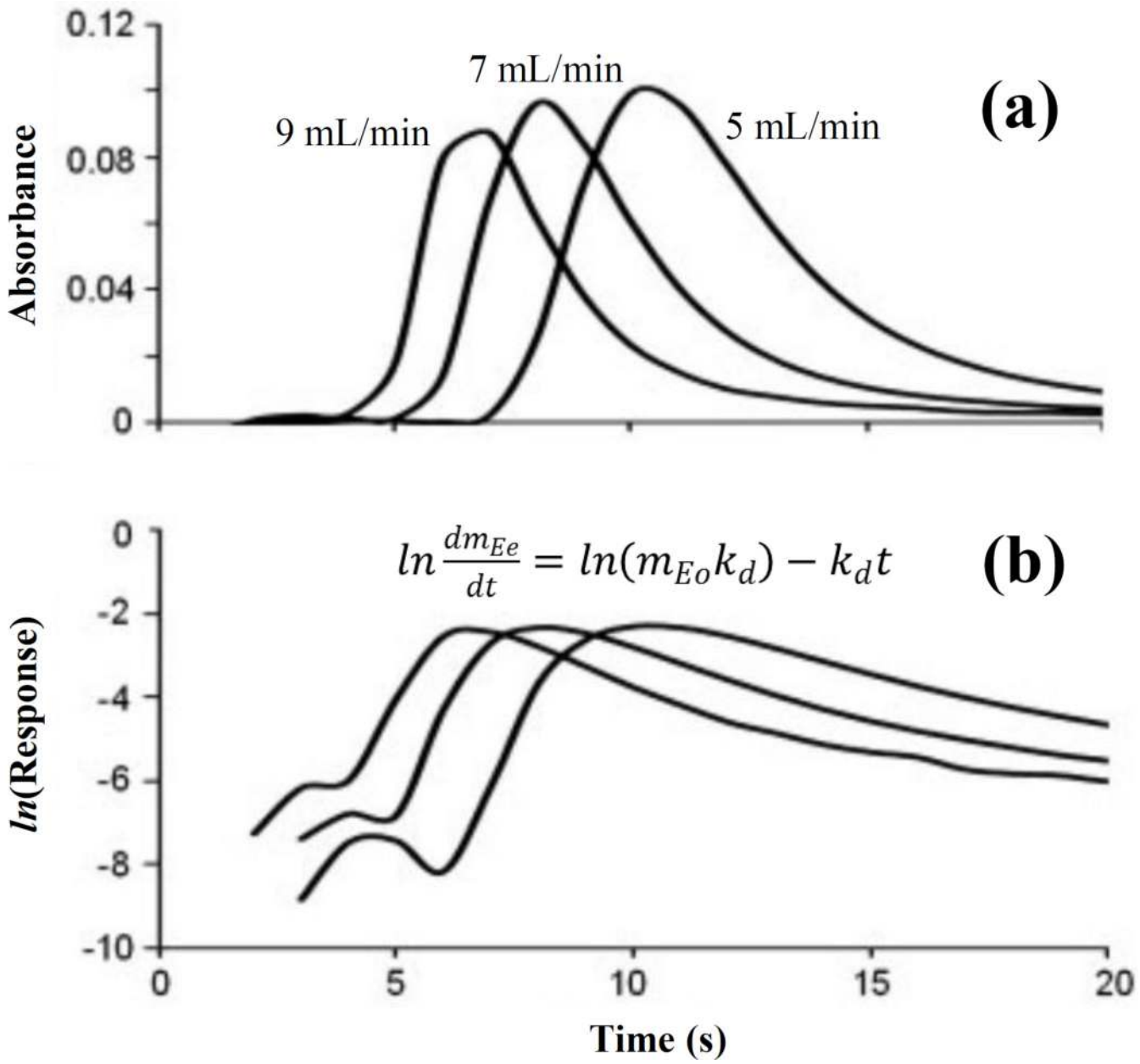


**Figure 3.**

Typical results for the peak decay method, including (a) the elution peak profiles (monitored at 209 nm) obtained for the application of nortriptyline at various flow rates onto a 1 mm × 4.6 mm id silica monolith column containing AGP and (b) the logarithmic form of these elution profiles. Terms: $m_{Ee}$, moles of target analyte eluting from the column at time $t$; $m_{Eo}$, initial moles of target bound to the column; $k_d$, dissociation rate constant for the target with the immobilized binding agent; AGP, $\alpha_1$-acid glycoprotein. According to the equation shown in (b), the slope obtained for a plot of the natural logarithm of the response versus $t$ should provide the dissociation rate constant $k_d$. Adapted with permission from Ref. [24].

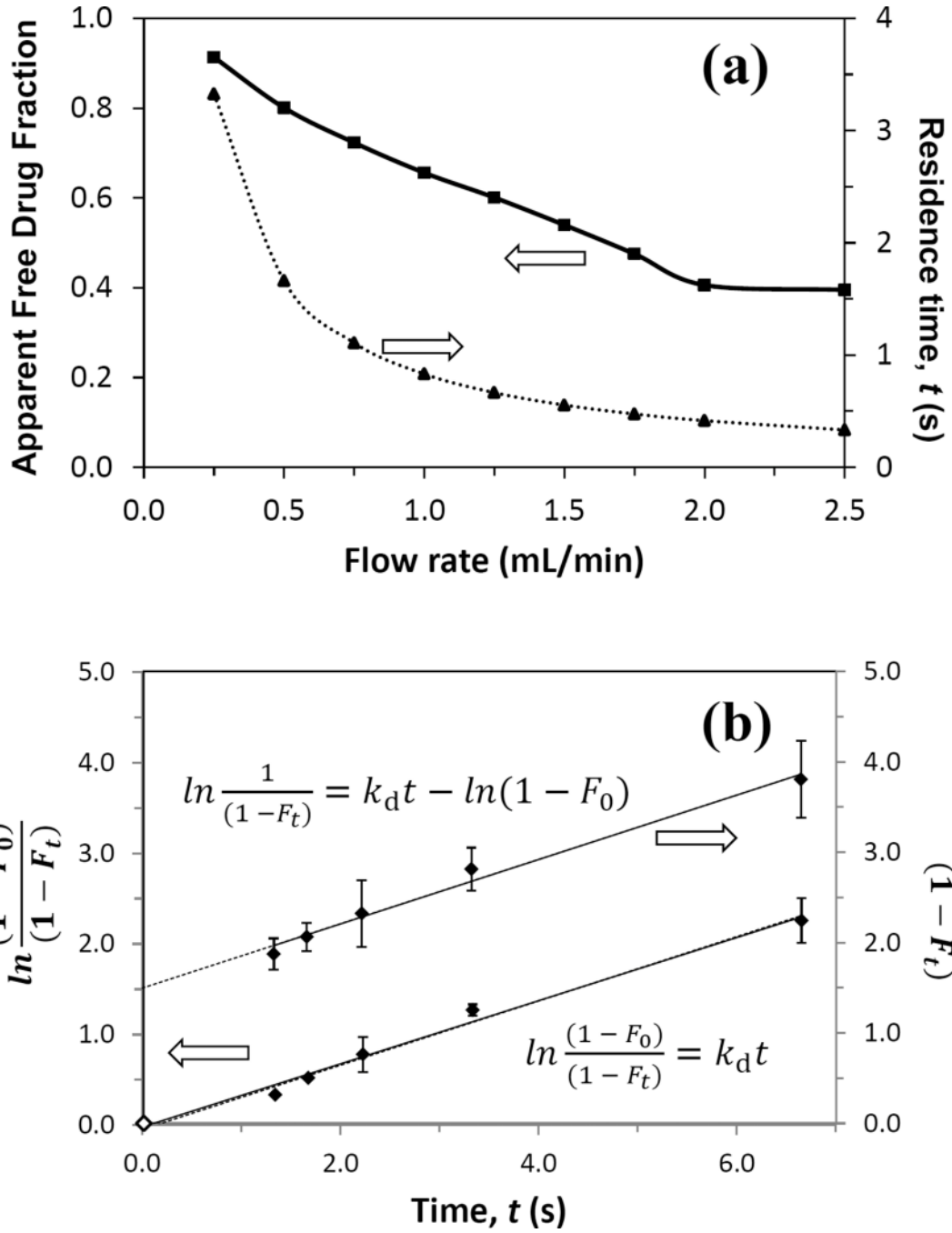


**Figure 4.**

Typical results obtained by free fraction analysis, as illustrated by (a) measurements of the apparent free fraction of verapamil in the presence of soluble HSA and as a function of flow rate or sample residence time in a 10 mm × 2.1 mm id affinity extraction column containing immobilized HSA, and (b) analysis of these results to obtain the dissociation rate constant for verapamil from soluble HSA. Terms: $F_0$, free drug fraction in the original sample at equilibrium (e.g., as measured at high flow rates, to avoid dissociation of the drug from its complex with the soluble protein); $F_t$, apparent free drug fraction measured at column

residence time $t$; HSA, human serum albumin. As indicated by the equations in (b), the slope that is obtained for a plot of either $ln[(1 - F_0)/(1 - F_t)]$ or $ln[1/(1 - F_t)]$ versus $t$ should provide the dissociation rate constant $k_d$. Adapted with permission from Ref. [37].