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Determination of Drug−Plasma Protein Binding Kinetics and Equilibria by Chromatographic Profiling: Exemplification of the Method Using L-Tryptophan and Albumin

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Drug−plasma protein binding may greatly influence the bioavailability and metabolism of a plasma-borne drug, the bound form being partially protected from the metabolic fate of the unbound drug. Traditionally, equilibrium values (e.g., percentage binding) for drug−protein binding have been measured to rationalize in vivo phenomena. However, such studies overlook the influence of kinetics. A rapid method of simultaneously determining kinetic rate constants and equilibrium constants from chromatographic profiles has been developed, based on the use of immobilized protein columns and HPLC. By measuring the chromatographic profiles (the position and width) of a retained and an unretained compound one can directly determine both the rate and equilibrium constants. Results are presented for the binding of L-tryptophan to human serum albumin to exemplify the method. The association equilibrium constant (K_a) and the association and dissociation rate constants (k_a and k_d, respectively) were thereby measured in an aqueous pH 7.4 environment at 37 °C as 0.84 10^4 M^{-1}, 5.8 10^4 M^{-1} s^{-1}, and 6.9 s^{-1}, respectively. These compare favorably with previously published results. The described method may be used in quantitative structure−property relationship-based rational drug discovery or for the rationalization of drug pharmacokinetics.

Many drugs exist in vivo largely bound to albumin or other plasma proteins. Plasma proteins provide a depot for drugs with poor aqueous solubility, maintain buffered free drug levels, and assist drug distribution. There can be further consequences of plasma protein binding, for instance, in the observed hypoglycemic action of fatty acid acylated insulins[1] (the observed duration of hypoglycemic action in animals after subcutaneous injection is correlated with their albumin binding affinities).

It is frequently assumed that the free and bound forms of the drug exist in a permanent state of equilibrium, such that the concentration of free drug is constantly and *instantaneously* maintained at a value determined by the equilibrium binding constant. This has led to the argument that plasma binding does not directly influence metabolism or renal tubular excretion.[2] However, this may be an inaccurate assumption under certain circumstances: where the kinetics of the dissociation process are significantly slower than the uptake of a strongly bound drug by the surrounding tissues, the equilibrium assumption will not be valid. Likewise, where dissociation of bound drug is slow, drug−protein binding may partially protect a drug from metabolism;[3] only if the kinetics are sufficiently rapid is the free drug level maintained constant during passage through a tissue by dissociation of protein-bound material. Thus, a strong binding equilibrium alone does not in itself determine the consequences of plasma binding; the kinetics of binding can act as a major determining factor. The terms restrictive and permissive (nonrestrictive) have been used to describe binding that reduces availability and that does not affect availability respectively.[4] Strongly bound drug molecules that have slow dissociation kinetics would be anticipated to exhibit restrictive behavior.

Serum albumin is the major extracellular protein of human plasma, accounting for 60% of total plasma protein content, having a concentration of 34−50 g L^{-1} (500−750 μM).[5] It is also present in extravascular fluid. Human serum albumin (HSA) is of particular interest in terms of its drug binding properties. The ability of albumin to bind a wide variety of ligands allows it to perform a considerable role in the transport, distribution, and metabolism of both endogenous and exogenous compounds.[4] Furthermore, it is involved in the transfer of many of these compounds across organ/circulatory interfaces such as are found in the liver, intestine, kidney, and brain. HSA circulates throughout the body about once every minute, but of this minute spends only 1−3 s in

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any particular capillary where it can exchange transported substances with the neighboring cells.[5]

Although serum albumin is a major drug binding protein, other plasma proteins may bind drug molecules to a very significant extent. One such protein is α_1-acid glycoprotein (α_1-AGP). This is an "acute-phase" protein: levels of α_1-AGP are increased in disease states. It is of particular importance in the binding of basic drugs. In contrast to HSA, which has a number of binding sites at which specific binding of drugs occur, the high-affinity binding of most drugs to α_1-AGP is mediated by only one common binding function.[6] The kinetics of drug binding to α_1-AGP will be the subject of a future publication.

Traditionally, equilibrium values (e.g., percentage binding) for drug−protein binding have been measured to rationalize in vivo phenomena. However, such studies overlook the strong influence of kinetics in dynamic systems such as living organisms; it is not just the equilibrium constant of binding that is important but the *rates* of association and dissociation of a drug to and from a protein. Some drug molecules undergo rapid dissociation from plasma proteins. Therefore, even if they have an apparently high percentage of binding they are not effectively protected from metabolism or excretion. Conversely, those compounds that undergo slow dissociation may not be able to exert their therapeutic effect before being removed from the site of action by the flow of blood. Hepatic clearance and passage of a drug across the blood−brain barrier are two examples of processes that respond to differences in the rate constants.[7,8]

The association of a drug (D) with a protein (P) to form a complex (DP) and the reverse dissociation can be represented as

$$\mathrm{D} + \mathrm{P} \underset{k_\mathrm{d}}{\overset{k_\mathrm{a}}{\rightleftharpoons}} \mathrm{DP} \qquad (1)$$

where k_a and k_d are the association and dissociation rate constants, respectively. The corresponding association equilibrium constant (K_a) is the ratio of the two rate constants:

$$K_\mathrm{a} = k_\mathrm{a}/k_\mathrm{d} \qquad (2)$$

Thus, different compounds may have the same equilibrium constant, and hence percentage binding, but substantially differ in the rates of association and dissociation, perhaps by orders of magnitude, their ratio only remaining consistent. The determination of these kinetic rates is therefore important in elucidating pharmacokinetics.

Historically, the primary methods of determining equilibrium binding constants have involved dialysis, ultracentrifugation, or direct spectroscopic methodology,[9] although more recently chromatographic methods have been successfully employed.[10] Likewise, the determination of kinetic rate constants for association and dissociation has typically involved time-consuming stopped-flow spectroscopic techniques.[11] Chromatographic methods for determining rate constants have been developed, but have required multiple measurements at various flow rates.[12] While all these approaches can give accurate results, they are inappropriate for the study of the larger number of compounds often considered in modern drug discovery within a practical time span. To this end, a method of rapidly determining the kinetic rate constants, together with the corresponding equilibrium constants, from chromatographic profiles has been developed, the application of which we exemplify here.

Chromatography has traditionally been used as a separative technique. However, it may also be used to investigate the distribution properties per se of an analyte. An example of this is in the chromatographic determination of partition coefficients,[13] which provides a rapid alternative to traditional methods, e.g., shake flask, for high-throughput work. The present work is based on the use of immobilized protein columns, although the principles apply to any stationary phase. Both the position and shape of a chromatographic peak for an eluted ligand are used to determine the equilibrium constant and the association and dissociation rate constants. In essence, the retention time is characteristic of the equilibrium constant for association, with the shape of the peak containing information about the kinetics. The analysis is based on the concept of chromatographic peaks as probability distribution functions, a concept first proposed by Giddings and Eyring.[14]

Giddings and Eyring published their statistical approach to describe molecular migration in chromatography in 1955. They treated the chromatographic process as a Poisson distribution process and the chromatographic peak as the probability density function for the elution of a solute as a function of time (in all of the following, it is assumed that the detection method is linear with concentration). Denizot and Delaage further developed this statistical approach to the analysis of peak shapes in 1975.[15] They applied it to affinity chromatography, a technique traditionally used for the separation of macromolecules. They also generalized the approach, to take into account other forms of dispersion such as diffusion.

In the Giddings and Eyring model each molecule is assumed to have a constant probability of binding per unit time, p_a, given by the product of the association rate constant and the concentration of binding sites, i.e., k_a[binding site]. Likewise, the complex is presumed to have a constant probability per unit time of dissociating, p_d, given by the dissociation rate constant, k_d. The assumption is made that there is a single mode of binding, the consequences of which will be considered further in the discussion section below. Comparison of the behavior of a chromatographically retained compound with one that is unretained allows the determination of these probabilities and thence the rate constants. Each molecule is assumed to spend the same time, t_0, in the mobile phase and to move in the mobile phase with constant velocity. Differences in retention time are accounted for by

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differences in the time spent bound to the stationary phase. Each solute molecule is eluted when it has covered a distance equal to the column length or, equivalently, spent a time t_0 in the mobile phase within the column. It was demonstrated that the probability $p\ dt$ of having a retention time between t_R and $t_R + dt$ for any n (the number of bindings) is expressed by[14,15]

$$p\ dt = \sum_{n=1}^{\infty} \frac{(p_a t_0)^n \exp[-p_a t_0](p_d t)^{n-1} \exp[-p_d t] p_d\ dt}{n!(n-1)!} \quad (3)$$

where the symbols t_R and t_0 represent the time at which a retained and an unretained molecule elute and $t = t_R - t_0$. This equation essentially represents the shape of a chromatogram for the retained compound.[14]

The shape of a chromatographic peak may be completely characterized by its moments.[16] The zero and first moments give the area and mean (center of gravity), respectively. The second, third, and fourth moments (if taken around the mean) are measures of the peak's width, asymmetry, and flattening, respectively. Denizot and Delaage subsequently derived expressions for the moments of the distribution described by eq 3 and considered other sources of dispersion, such as diffusion and differences in the flow rate of the solvent within the column. They therefore viewed t_0 as a random variable, as is t, rather than as a constant. It follows from Denizot and Delaage that

$$\text{mean time of retained peak} = E[t_R] = E[t_0]\left(1 + \frac{p_a}{p_d}\right) \quad (4)$$

and

$$\text{variance of retained peak} = {\sigma_R}^2 = \frac{2p_a}{{p_d}^2}E[t_0] + \left(1 + \frac{p_a}{p_d}\right)^2 {\sigma_0}^2 \quad (5)$$

where $E[t_R]$ and $E[t_0]$ are the mean times of the chromatographic peak of a retained and an unretained compound, respectively. Likewise σ_R^2 and σ_0^2 are the respective peak variances, these being a measure of the width of a peak (i.e., not of the error in its position), for example, as depicted in Figure 1.

Equations 4 and 5 can be solved with respect to p_a and p_d:

$$p_a = k_a[\text{receptor}] = \frac{2E[t_0](E[t_R] - E[t_0])^2}{{\sigma_R}^2(\mathrm{E}[t_0])^2 - {\sigma_0}^2(E[t_R])^2} \quad (6)$$

$$p_d = k_d = \frac{2(E[t_0])^2(E[t_R] - E[t_0])}{{\sigma_R}^2(E[t_0])^2 - \mathrm{E}\ {\sigma_0}^2(E[t_R])^2} \quad (7)$$

where [receptor] is the molar concentration of receptor sites in the volume of mobile phase within the column. Consequently, the

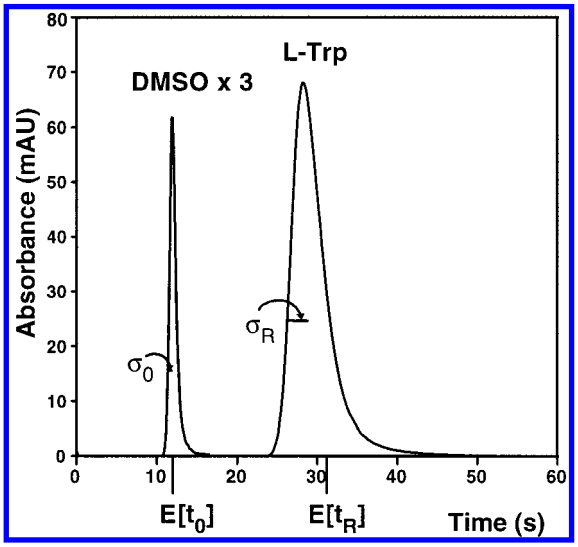

Figure 1. Illustration of the parameters employed for an unretained ($E[t_0]$, σ_0) and retained ($E[t_R]$, σ_R) compound in the chromatographic profiling analysis of DMSO and L-tryptophan, respectively. The two chromatograms were obtained from separate injections, and the DMSO chromatogram has been multiplied 3-fold for illustrative purposes.

equilibrium constant can be determined as the ratio of the rate constants:

$$K_a = \frac{k_a}{k_d} = \frac{(E[t_R] - E[t_0])}{E[t_0][\text{receptor}]} \quad (8)$$

Note that, in contrast to the individual rate constants, the equilibrium constant is independent of the peak width and is determined solely by the mean retention times.

Thus, by measuring the chromatographic profiles of a retained and an unretained compound, one can directly determine k_a, k_d, and K_a (assuming the receptor loading of the stationary phase in known). To ensure the kinetics are measurable, it is necessary to employ flow rates sufficiently fast that equilibrium on the column cannot be established, whereupon peak broadening through kinetic effects occur. Furthermore, by judicious choice of temperature and mobile-phase composition, the conditions employed may be tailored to simulate those found in vivo. Here we illustrate the technique by determining the binding characteristics of L-tryptophan, an archetypal plasma binding probe, using dimethyl sulfoxide (DMSO) as the unretained t_0 reference.

While the measured dissociation rate constant is independent of the protein concentration, the equilibrium constant and the association rate constant are dependent on this value. Therefore, measurement of the total accessible HSA concentration is required. This was performed by frontal analysis[17] (FA) using L-tryptophan and DMSO.

EXPERIMENTAL SECTION

Ultrapure grade L-tryptophan and HPLC grade DMSO were obtained from Sigma Aldrich Co. Ltd. AnalaR grade sodium dihydrogen orthophosphate monohydrate, disodium hydrogen

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Table 1. Kinetic and Equilibrium Results Obtained by Chromatographic Profiling for the Binding of L-Tryptophan to HSA and Literature Results for Comparison

	T (°C)	pH	K_a (10^4 M^{-1})	k_a (10^4 M^{-1} s^{-1})	k_d (s^{-1})	% bound[g]	$t_{d1/2}$ (s)
			Current Work				
chromatographic profiling (3.5 mL/min)	37	7.4	0.836 ± 0.002	5.8 ± 0.03	6.9 ± 0.03	85.0	0.100 ± 0.001
frontal analysis	37	7.4	0.93 ± 0.01			86.3	
			Literature				
Yang and Hage[a]	37	7.0	2.4 ± 0.3	14	6.0	94.2	0.116
Yang and Hage[a]	37	7.4	5[e]	20[e]	3[e]		
McMenamy[b]	37	7.6	1.3			89.8	
McMenamy[b]	37	7.0	0.5			77.1	
McMenamy[c]	2	7.6	2[f]			93.1	
Lagercrantz[d]	rt?	7.4	1.1			88.1	

[a] Reference 12. [b] Reference 18. [c] Reference 19. [d] Reference 20. [e] Read from graphical results, kinetic and equilibrium constants inconsistent in original paper. [f] Read from graphical results. [g] Calculated for [HSA] = 6.75×10^{-4} M.

orthophosphate dihydrate, and HPLC grade water for buffer preparation were obtained from Merck Ltd.

A Hewlett-Packard 1090 HPLC system with diode array detection was used. All chromatography was performed isocratically at 37 °C using an aqueous 50 mM phosphate buffer (pH 7.4) mobile phase. Analytical immobilized HSA columns, 50 × 4.6 mm, were obtained from Thermo Hypersil-Keystone. When not in use, the column was stored at 4 °C in an aqueous solution of 0.01% sodium azide to prevent bacterial growth. The flow rate used was 3.5 mL min^{-1}, which was confirmed by volumetric collection of eluant. Five-microliter injections of either a 100 μM stock solution of L-tryptophan or 1 mM DMSO in mobile phase were made. For chromatographic profiling and frontal analysis, the diode array detector response at 220 nm was employed.

The 5-cm column used contains silica with a median pore diameter of 300 Å and has a pore volume of 0.7 cm^3 g^{-1} and surface area of 90 m^2 g^{-1} (data supplied by the manufacturer; for reference). The mobile-phase volume of the column was determined to be 0.66 mL from measurements made for unretained compounds across a range of flow rates.

For the frontal analysis experiments, used to determine the concentration of binding sites, L-tryptophan solutions from 20 to 200 μM were run. This procedure is relatively time-consuming but provides results that are then found to remain constant over the lifetime of the column. The accessible receptor concentration (as used in eqs 6 and 8) at a flow rate of 3.5 mL min^{-1} was thereby determined as 1.81 10^{-4} M.

The HPLC instrumentation was configured to minimize extracolumn time and effects. Furthermore, as the algorithm (i.e., eqs 6−8) refers to "on-column" times, all retention times were corrected for extracolumn periods by subtracting the time taken from injection to detection when the column was replaced by a zero-volume connector.

Although the chromatographic peak position and variance could be directly determined from the raw data, it was found convenient for analysis to fit a curve to the data. The data were exported into the software program PeakFit (Jandel Scientific Software, AISN Software Inc.), an EMG + GMG (exponentially modified Gaussian + half-Gaussian modified Gaussian) function fitted from which the mean and variance were calculated. This functional form was found to fit the data to within the noise level in all circumstances of this study. Full error analysis was performed using standard propagation of errors formulas.

RESULTS AND DISCUSSION

A representative chromatogram of DMSO (the t_0 marker) and L-tryptophan is presented in Figure 1, and the kinetic rate constants and association equilibrium constant, as determined in this study, are presented in Table 1. In addition, the derived dissociation half-life ($t_{d1/2}$) and in vivo percentage bound (% bound) are tabulated. For comparison, previously reported results from the literature, employing various methods of determination, are also quoted.

The dissociation half-life is determined by the kinetic dissociation rate constant and represents the half-life of dissociation for the bound drug when placed in an infinite sink, i.e., where all unbound drug is effectively removed.

$$t_{d1/2} = \ln(2)/k_d \tag{9}$$

This may be considered to crudely simulate the case where partitioning or transport into other body compartments (tissues, etc.) rapidly occurs. As such, it provides an approximate measure of how quickly drug dissociation can occur, for comparison with, say, the few seconds blood resides in a capillary.

The in vivo percentage bound is the percentage of drug that would be bound to HSA under in vivo circumstances, ignoring other sources of binding. It is calculated from the equilibrium constant, taking the in vivo concentration of HSA in plasma as [ivHSA] = 6.75×10^{-4}M (assuming this is in great excess over the drug):

$$\%\ \text{bound} = \frac{\text{concentration of bound drug}}{\text{total concentration of drug}} \times 100\% = \frac{[\text{ivHSA}]K_a}{[\text{ivHSA}]K_a + 1} \times 100\% \tag{10}$$

The results determined by the chromatographic profiling method

reported here compare well with those from previous literature determined by equilibrium dialysis[18,19] and by chromatography,[12,20] as shown in Table 1. Furthermore, the results reported here were determined in a completely aqueous pH 7.4 environment at 37 °C and thus are directly comparable to in vivo conditions.

The results are, as shown in Table 1, in broad agreement with those found in the literature. Where differences exist, these can be explained by differences in the pH or in the temperature used for the study. L-Tryptophan is known to bind more strongly to HSA at lower temperatures[18] and at higher pHs.[11] The discrepancy with the results of Yang and Hage[12] may be due to the fact that they appear to have used results obtained using two different columns to derive their data, yet we have observed that the concentration of binding sites may vary very significantly between nominally identical columns. In support of this, the k_d is approximately the same, but k_a and K_a differ—and these are the parameters with a dependence on the protein concentration.

HSA has a variety of potential binding modes. The molecule consists of three structurally homologous domains denoted I, II, and III, with each domain composed of two smaller subdomains, A and B, giving a "heart-shaped" structure.[21,22] Two principal binding sites for drug binding have been identified from binding studies.[23,24] These are located in subdomains IIA and IIIA and are referred to as site I and site II, respectively. Site II binds a broader variety of drug molecules, including diazepam and ibuprofen, and the amino acid tryptophan. Site I is the primary binding site of warfarin and phenylbutazone.

It is assumed in the Denizot−Delaage formalism that there is a single type of receptor and mode of binding for each compound. While this is true for tryptophan interactions with HSA, it is not universal. In the case of multiple sites, the Denizot−Delaage method represents each interaction as with an equivalent single "pseudosite". Consequently, care has to be taken in interpreting results if binding is occurring at more than one site or with more than one mode (orientation, etc.). However, the kinetic and equilibrium constants for individual sites can be determined, if required, by introducing inhibitors or blockers of the unwanted sites into the mobile phase. Nonetheless, the primary use of these measurements is foreseen to be in quantitative structure−property relationship (QSPR)-based rational drug discovery and optimization or for the rationalization of pharmacokinetics. In this context, the kinetic and equilibrium constants for a pseudo single receptor are appropriate parameters to describe the overall interactions to sufficient approximation.

A typical chromatogram of L-tryptophan is presented in Figure 1. The Denizot−Delaage formalism, through the incorporation of the parameters for an unretained compound, accounts for the various sources of band broadening beyond that due to binding kinetics. In particular, it encompasses the broadening due to diffusion within the mobile phase. However, for the Denizot−Delaage equations to fully compensate for these forms of band broadening, it is necessary for the t_0 unretained reference compound (DMSO here) to have a diffusion coefficient similar to that of the retained compound under study (L-tryptophan). This is true for small compounds such as these, such that the lack of equivalence is unlikely to lead to errors, especially at higher flow rates where binding kinetics dominate. However, the diffusion coefficient of large protein molecules is significantly smaller. Therefore, if traditional affinity chromatography is used, in which the protein is passed through a column of immobilized ligand rather than vice versa, the mass-transfer term is far more likely to dominate, even at higher flow rates.

A variety of compounds purportedly thought not to bind to HSA were explored as possible unretained references, including DMSO, sodium citrate, uridine, sodium nitrate, and carbohydrate derivatives. However, all but DMSO and sodium citrate had distinct retention, albeit small, on the HSA columns used. For convenience, DMSO was chosen as the unretained reference compound.

For the kinetics to be apparent in the chromatogram, it is necessary to employ flow rates sufficiently rapid that equilibrium cannot be established before the compound is eluted. This can be quantified by the "critical ratio", deduced from the denominator of eqs 6 and 7:

$$\text{critical ratio} = \eta = \frac{\sigma_R / E[t_R]}{\sigma_0 / E[t_0]} \tag{11}$$

For the kinetics to be measurable, this ratio must be greater than 1. A value of 1 indicates that the compound has eluted under equilibrium conditions and therefore no kinetic information is available from the chromatogram, although the equilibrium constant can still be determined from the retention times via eq 8. Values greater than 1 indicate a degree of kinetic broadening in the peak shape.

$$\eta = 1 \quad \Rightarrow \text{equilibrium chromatography} \tag{12}$$

$$\eta > 1 \quad \Rightarrow \text{kinetic broadening} \tag{13}$$

By considering the effect of flow rate on eqs 6 and 7, it can be established that the kinetics are well determined when the "kinetic factor", κ, defined as

$$\kappa = (\eta^2 - 1)\sigma_R{}^2 u / \eta^2 \tag{14}$$

is constant with increasing linear flow rate u.

$$d\kappa / du \rightarrow 0 \quad \Rightarrow \text{kinetics well determined} \tag{15}$$

Comparison with the van Deemter equation for plate height[25] shows condition 15 as equivalent to requiring the van Deemter equation to be dominated by the "*C* term", the term containing kinetic dispersion, as might be expected.

At very low flow rates, the chromatography approaches equilibrium control. The kinetics are not well determined unless

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the flow rate is greater than 3.0 mL min^{-1}, under the conditions reported here, whereupon the kinetic factor, η, tends to a constant value. For intermediate flow rates, kinetics make a detectable contribution to the band broadening. However, in these circumstances, they are less well determined and poorly distinguishable from other mechanisms of band broadening or artifacts not accounted for within the Denizot−Delaage formalism, such as differential diffusion due to nonequivalent diffusion coefficients mentioned above.

Altering the conditions used, such as flow rate or length of column, increases the range of compounds for which rate constants are measurable. However, at high flow rates, one may encounter problems with HPLC pumping efficiency and increased errors due to the shortness of retention time. Therefore, there has to be a compromise between the desire to have very high flow rates in order to enhance the contribution of kinetics to the chromatogram and the ability of current instrumentation to perform at these levels. In practice, a flow rate of 3.5 mL min^{-1} was found optimal for L-tryptophan, ensuring the compound was retained for a sufficient period to be distinct from an unretained compound.

Key to the success of the method is the ability to establish effective mass transfer of the solute through diffusion from the bulk mobile phase to within the solvation shell of the stationary phase at a rate faster than the kinetics of association. The chromatographic process then becomes controlled by the rates of association and dissociation to/from the immobilized protein. This is easily achieved by small druglike molecules in solution, with their fast diffusion rates.

The application of the Denizot−Delaage model to obtain rate data in affinity chromatography has been criticized[26,27] on the basis that it is only valid when the effect of mass-transfer kinetics (i.e., diffusion) on the mean and variance of the profile is negligible. In traditional affinity chromatography, the small molecule is immobilized and the protein added to the mobile phase. Under such circumstances, the diffusion rate of the large macromolecule in the mobile phase is sufficiently slow that it causes the chromatographic process to become diffusion controlled and the kinetics of association and dissociation become obscured. Typical drug-sized molecules have diffusion coefficients of the order of 10^{-9} m^2 s^{-1} compared to some 10^{-10}−10^{-11} m^2 s^{-1} for proteins the size of HSA (the diffusion coefficient of HSA itself is 6.1×10^{-12} m^2 s^{-1} [28]). Thus, by immobilizing the protein and placing the small ligand in the mobile phase, we have overcome this diffusion limitation. Hethcote and DeLisi have described the use of immobilized protein versus the immobilized ligand as reversed-role affinity chromatography.[27]

Further benefits of using immobilized protein rather than immobilized ligand are that the smaller size of the free species leads to greater accessibility to sites within the pores of the support and the interactions are more representative of those in free solution. In order for binding to take place, the protein and ligand need to be in the correct orientation with respect to one another. This is more likely to be achieved if it is the protein rather than the ligand that is bound as the protein is a much larger molecule and the binding site may poorly accessible within the protein. An immobilized small ligand may find it difficult to access the binding site simply because it is tethered to the stationary phase. Furthermore, the linkage tethering an immobilized small ligand will, at best, impinge on the entrance to the binding site, so altering the binding characteristics from that of a free ligand or, at worst, change or eliminate the mode of binding. In contrast, with care, a protein can be tethered through positions remote from the binding site, so allowing full, unmodified, access to a ligand.

A previously reported example of the use of the Denizot−Delaage method in traditional affinity chromatography is in a study of the binding of pancreatic ribonuclease to uridine-5′-(Sepharose-4-aminophenyl phosphate)-2′(3′)-phosphate beads.[29] Chaiken found that the rate constants obtained were too small by several orders of magnitude relative to those measured in free solution. The reference relates to unpublished results of R. H. Long and I. M. Chaiken. Chaiken concluded that for valid rate data the use of ligands immobilized onto low-porosity supports would be required. However, the issue of porosity is only one of a number of factors that could have led to the results they obtained. In addition to the comments made above, Sepharose-bound ligand was used. Sepharose has a relatively large particle diameter, and mass transfer in the mobile phase is proportional to the square of the particle size.

From the study reported here, we have established that porosity is not an issue if the *accessible* receptor concentration is determined at the flow rates employed for the Denizot−Delaage analysis. Although the total protein loading may be much greater, a significant proportion, deep within porous stationary supports, will be inaccessible at higher flow rates during the time scale of the chromatographic elution. In effect, the continuous flow provides no opportunity for the molecules to diffuse deep into the porous support before being carried onward by the flow. By performing frontal analysis at a range of flow rates, it is possible to quantitate this effect. At high flow rates, where the eluted compound has little opportunity to access the inner regions of the stationary phase, the accessible concentration drops to 1×10^{-4} M for the column used for this study. At lower flow rates, the accessible concentration increases, although never to the nominal concentration calculated from the protein loading of the column. For instance, at a flow rate of 0.4 mL min^{-1}, the concentration of binding sites, as measured by frontal analysis, is some 41% greater for this study.

Previous reports[17,30] of the apparent reduction in active receptors when proteins are immobilized, determined by frontal analysis, have invoked the argument that the protein is partially denatured on immobilization. However, the results reported here indicate that flow rate is a significant factor. This phenomenon will be explored further in a future publication. On initial reflection, it may appear that the existence of inaccessible sites is contrary to the discussion above regarding the need for effective diffusion from the bulk mobile phase. However, they are actually fully consistent: the stationary phase may be imagined as divided into two zones, a core that is inaccessible and an accessible surface

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where diffusion from the mobile phase is efficient within the time scale of the experiment. The requirement of efficient diffusion thus defines the extent of the accessible receptors. All the inaccessible receptors have no influence in the chromatography and may be considered as absent.

CONCLUSIONS

The chromatographic profiling method described here has potential as a medium-throughput screen for determining the binding characteristics of drugs under aqueous conditions simulating the in vivo environment. Having first established the accessible receptor concentration of a column by frontal analysis, it is possible to subsequently determine the kinetic and equilibrium binding parameters of a wide range of compounds by simple chromatographic analysis. Typical retention times are in the 0.1−20-min range, depending on the strength of binding. To minimize potential interference, the tryptophan and DMSO were run as separate injections for this study. However, to maximize throughput, they may be co-injected or a single DMSO t_0 reference run for multiple samples.

Furthermore, by the use of multiple peak fitting to determine the mean and variance of each component chromatographic peak, it is also possible to study multiple compounds simultaneously co-injected, providing they have sufficiently different retention times such that their individual chromatographic peaks are discernible. However, care should be taken to ensure that the components of a mixture are not interacting either with each other or allosterically with the receptor. Although inferior to separate injections, it holds the possibility of determining the individual binding characteristics of both enantiomers of a chiral molecule from the racemic mixture without the need for prior resolution of each stereoisomer. As a corollary, by incorporation of additives into the mobile phase, it is possible to investigate their role and effects, including the modulation of allosteric changes in receptors and action of binding inhibitors. Moreover, by the use of immobilized serum albumin from different species (e.g., rat), it is possible to identify potential species differences in animal studies and validate animal models for biometabolism and pharmacokinetic investigations. We are testing this method with a wide variety of compounds with different binding strengths. The results will be presented in forthcoming publications.

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