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REVIEW

Drug–protein binding: a critical review of analytical tools

**Karine Vuignier · Julie Schappler · Jean-Luc Veuthey ·
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Abstract The extent of drug binding to plasma proteins, determined by measuring the free active fraction, has a significant effect on the pharmacokinetics and pharmacodynamics of a drug. It is therefore highly important to estimate drug-binding ability to these macromolecules in the early stages of drug discovery and in clinical practice. Traditionally, equilibrium dialysis is used, and is presented as the reference method, but it suffers from many drawbacks. In an attempt to circumvent these, a vast array of different methods has been developed. This review focuses on the most important approaches used to characterize drug–protein binding. A description of the principle of each method with its inherent strengths and weaknesses is outlined. The binding affinity ranges, information accessibility, material consumption, and throughput are compared for each method. Finally, a discussion is included to help users choose the most suitable approach from among the wealth of methods presented.

Keywords Plasma proteins · Binding affinity constants · Protein binding methods · Method selection

Abbreviations

ACE	Affinity capillary electrophoresis (mobility shift assay)
AGP	α_1-Acid glycoprotein
BGE	Background electrolyte
BSA	Bovine serum albumin
CD	Circular dichroism
CE	Capillary electrophoresis
CE/FA	Capillary electrophoresis/frontal analysis
CZE	Capillary zone electrophoresis
D	Drug
DP	Drug–protein complex
DSC	Differential scanning calorimetry
ED	Equilibrium dialysis
FA	Frontal analysis
FACCE	Continuous capillary electrophoresis frontal analysis
HD	Hummel–Dreyer method
HDL	High-density lipoproteins
HPAC	High-performance affinity chromatography
HSA	Human serum albumin
IR	Infrared
ISRP	Internal-surface reversed phase
ITC	Isothermal titration calorimetry
K_a	Association constant
K_d	Dissociation constant
k_{off}	Dissociation rate constant
k_{on}	Association rate constant
LC	Liquid chromatography
LDL	Low-density lipoproteins
LIF	Laser-induced fluorescence
m	Total number of different classes of binding sites
MS	Mass spectrometry
n	Number of binding sites with the same affinity per protein molecule
NMR	Nuclear magnetic resonance
NSB	Nonspecific binding
ORD	Optical rotatory dispersion
P	Protein
PAMPA	Parallel artificial membrane assay
r	Number of total drugs bound per protein
SEC	Size-exclusion chromatography

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SPR	Surface plasmon resonance
UC	Ultracentrifugation
UF	Ultrafiltration
VACE	Vacancy affinity capillary electrophoresis
VP	Vacancy peak method
ZE	Zonal elution
ΔCp	Heat capacity change
ΔG	Gibbs free energy
ΔH	Enthalpy of the binding reaction
ΔS	Entropy change
μ	Electrophoretic mobility

Introduction

After being distributed in circulating blood, drugs bind to plasma proteins in varying degrees. In general, such binding is reversible, and an equilibrium exists between bound and free molecular species. It is commonly stated that unless there is a specific transport system, only the free drug molecules are able to cross membrane barriers and be distributed to tissues to undergo metabolism and glomerular filtration. Only the free drug fraction is able to exert pharmacological and/or toxicological effects [1–3] (Fig. 1). Thus, drug–plasma-protein binding is critically involved in drug pharmacokinetics (i.e., absorption, distribution, metabolism, and elimination) and pharmacodynamics (pharmacological effects). Plasma-protein binding has, hence, been considered, along with solubility, lipophilicity, ionization, and metabolic properties, as a key piece of the data characterizing any compound, and must be evaluated in the early stages of drug discovery [4]. Although there may be many components in plasma that are capable of binding drugs, two major proteins, human serum albumin (HSA) and α_1-acid glycoprotein (AGP), are present in relatively high quantities and able to bind a broad variety of drugs with sufficient affinity to have a significant effect on drug disposition and action [5]. Globulins and lipoproteins may also play a role to a lesser degree [6]. Many methods have been proposed to assess protein-binding abilities based on diverse analytical tools that can be divided into separative and non-separative approaches. This review deals with the main methods developed to characterize drug–plasma protein interactions. The strengths and weaknesses of each method and a comparison of these different assays in terms of binding affinity ranges, information accessibility, material consumption, and throughput are treated. Finally, a discussion is included to help users to choose the most suitable approach for their interacting system.

Drug–protein binding: theoretical description

The binding of a drug to a protein can be viewed as a reversible and rapid equilibrium process governed by the law of mass action. Irreversible binding of some drugs to plasma proteins has nevertheless also been demonstrated [7, 8].

In the simplest case, assuming there is only one reversible-binding site on the protein for a drug molecule, binding between the drug and the protein can be described by the following equilibrium [9]:

$$[\mathrm{D}] + [\mathrm{P}] \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} [\mathrm{DP}] \qquad (1)$$

where [D], [P], and [DP] are the free drug, free protein, and drug–protein complex concentrations, respectively, and k_{on} and k_{off} are the association and dissociation rate constants. At

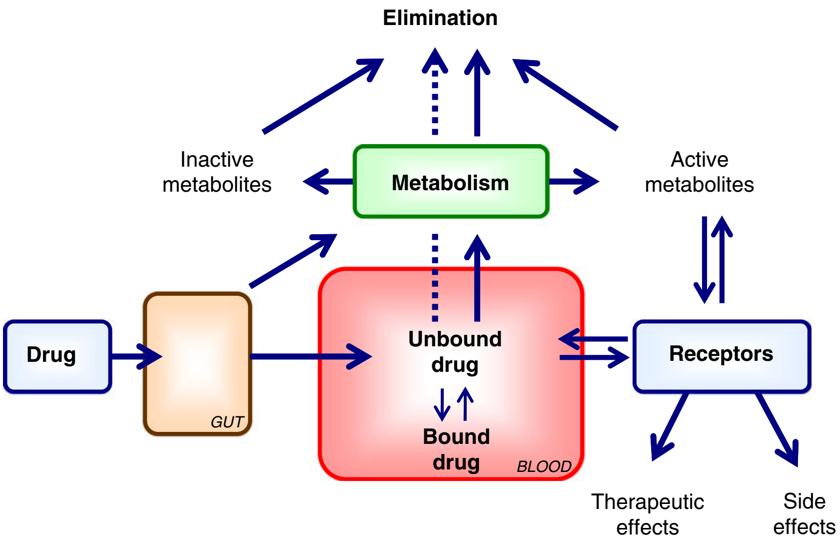

Fig. 1 Protein binding and drug distribution

equilibrium, the rate of association is equal to the rate of dissociation. Thus the association constant (K_a) can be defined as follows:

$$K_a = \frac{k_{on}}{k_{off}} = \frac{[DP]}{[D] \cdot [P]} \tag{2}$$

The dissociation constant, K_d, which is the reciprocal of K_a is also often reported. It represents the concentration of free drug that occupies half of the overall acceptor sites at equilibrium [8].

Another important property to introduce is the number of total drugs bound per protein or the fraction of total binding sites occupied. This is represented by the letter *r* (Eq. 3):

$$r = \frac{[\mathrm{DP}]}{[\mathrm{P}] + [\mathrm{DP}]} = \frac{\mathrm{n} \cdot \mathrm{K_a} \cdot [D]}{1 + \mathrm{K_a} \cdot [D]} \tag{3}$$

where *n* is the maximum number of binding sites on the protein. In Eq. 3, it is assumed that the *n* binding sites on the protein have the same affinity constant for the drug but a protein can have several classes of *n* binding sites, each with its own K_a value. Equation 3 can thus be rewritten as Eq. 4, where *m* is the total number of different classes of binding sites:

$$r = \sum_{i=1}^{m} \frac{\mathrm{n_i} \cdot \mathrm{k_{ai}} \cdot [D]}{1 + \mathrm{K_{ai}} \cdot [D]} \tag{4}$$

Equation 4 assumes that the individual binding regions on the protein have independent affinities for the solute. Such an assumption is true when allosteric interactions are not present. If this is not the case, then more complex reaction models have to be used. Another assumption in these equations is that the binding of the drug to each region on the protein can be described by a single-step, reversible process [10].

The different approaches used to assess drug–protein binding

The approaches used to investigate drug–protein interactions are divided into separative and non-separative methods (Fig. 2). The first group involves the separation of the free ligand from the bound species and is used to determine directly either the unbound drug or the bound drug concentration [11]. The second group relies on detection of a change in a physicochemical property of either the ligand or the protein because of the binding [12].

Separative methods

Equilibrium dialysis and related techniques

Equilibrium dialysis (ED) and related techniques are based on differences in molecular size and/or weight. In a standard ED experiment, two compartments are separated by a semipermeable membrane that acts as a molecular sieve to allow only molecules smaller than a certain molecular weight to permeate through it. It is thus (ideally) perfectly permeable to the drug and impermeable to the protein and the drug–protein complex. One compartment contains the protein sample and the second, the tested drug. After a defined incubation time, equilibrium is reached, and the free drug fraction can be measured in the second compartment.

ED has been the method most widely used to study drug–protein interactions. Moreover, it is performed in solution, and true equilibrium is maintained during the whole experiment. Thus, ED is regarded as the reference method. ED suffers, however, from many drawbacks. Equilibration times are long (typically 12–48 h), and an initial set of studies has to be performed to determine the time necessary for the system to reach equilibrium [13]. Devices based on a 96-well format have been proposed to enhance the throughput [14, 15] and are now commercially available (Equilibrium Dialyzer-96 from Harvard Biosciences (Holliston, MA, USA), Rapid Equilibrium Device from Thermo Scientific/Pierce (Rockford, IL, USA), Micro Equilibrium Dialysis Device from HTdialysis LLC (Gales Ferry, CT, USA)). Another potential problem associated with ED is the volume shift associated with the oncotic pressure that takes place because of the semi-permeable membrane and the presence of proteins [16]. This volume change can be as large as +10 to +30% [17]. Nonspecific adsorption of drugs or proteins on the cell walls and on the dialysis membrane can also occur [18]. This "parasitic" binding can be higher than 50% of the total concentration [8]. Moreover, the Donnan effect may arise for charged proteins and distort the interaction measurements [19]. Finally, poor aqueous solubility of the compounds may also be problematic and limit the use of ED.

Ultrafiltration (UF) has been proposed as a rapid alternative to ED. This method is very similar except that the analysis speed is increased by application of pressure to force the solution through the membrane. Nevertheless, issues also encountered in ED, such as the potential nonspecific binding of the compounds to the filter membrane, the Donnan effect, and protein leakage may arise [6]. Although some authors [18, 20] considered the application of a pressure during the separation process to be detrimental for the stability of the binding equilibrium, other work showed that UF is indeed performed under equilibrium conditions [21]. Because kits are commercially available even in a 96-well format (Millipore, Bedford, MA, USA; Harvard Bioscience, Holliston, MA, USA) and because this technique is simple to perform, it is still often used at the drug discovery stage to rank compounds on the basis of plasma protein binding, and for drug therapeutic

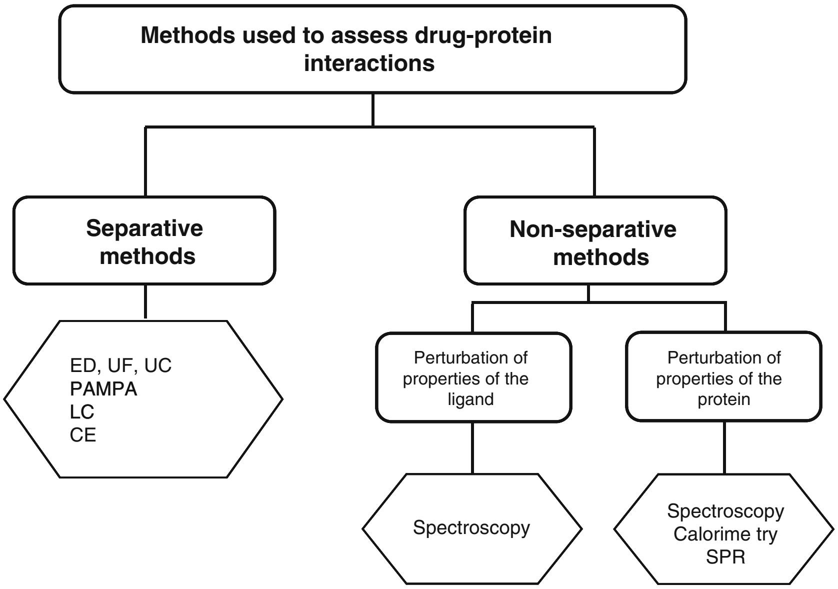

Fig. 2 Separative and non-separative methods used for investigation of drug–protein binding. *ED*, equilibrium dialysis; *UF*, ultrafiltration; *UC*, ultracentrifugation; *PAMPA*, parallel artificial membrane permeability assay; *LC*, liquid chromatography; *CE*, capillary electrophoresis; *SPR*, surface plasmon resonance-based assays

monitoring, and pharmacokinetic and pharmacodynamic studies in clinical laboratories.

In ultracentrifugation (UC), another related technique, a solution of drug and protein mixed together is placed in a centrifugal field. Centrifugation is continued until all the protein and the drug–protein complex sediment to the bottom of the tube. Because the sedimentation coefficient of the drug is generally very small compared with that of the protein, the free drug remains in the supernatant where it can be quantified. UC has the advantage of eliminating problems associated with membrane effects (e.g., the Donnan effect and membrane adsorption). However, the equipment used for UC is expensive in contrast with that used for ED and UF. Moreover, comparative studies with different types of drug revealed that quantitative discrepancies occurred between results obtained by ED and UC because of estimation of the free drug concentration, which can be affected by physical phenomena such as sedimentation, back diffusion and viscosity [8, 22]. For example, the error due to sedimentation of the drug can be as large as 10% for drugs of 300 Da and up to 40% for high-mass drugs (e.g., suramin, 1297 Da) [23]. These issues, combined with its low throughput (even for modern apparatus), have rendered this approach unattractive.

Parallel artificial membrane assay

The parallel artificial membrane permeability assay (PAMPA) was originally developed to enable prediction of drug passive permeability through biological membranes. The assay is based on a 96-well filter plate coated with an artificial membrane (made of phospholipids [24, 25] or solvent(s) [26, 27]) used to separate two compartments, one containing a buffer solution of the compounds to be tested (defined as the donor compartment) and the other containing only fresh buffer (defined as the acceptor compartment).

Làzaro et al. [28] used the PAMPA technique to measure the kinetics of permeation of a compound through a hexadecane or 1-octanol membrane in the presence and absence of protein in the donor compartment. The free drug concentration in the acceptor compartment is measured at different times and the difference between the two experiments is used to estimate the binding constant. The key assumption in this approach is that only the free drug is able to cross the membrane whereas both the protein and the drug–protein complex are unable to do so. This assay has been used to study the interaction between HSA and 11 drugs with different lipophilicity, and acidic and basic character, and K_d values in the mmol L^{-1} to μmol L^{-1} range.

Use of PAMPA in assessment of binding constants has several advantages:

1. the absence of an equilibration time requirement and the 96-well format render the assay faster than traditional ED;
2. there is no volume change due to oncotic pressure because the chemical membranes used are not water-permeable; and
3. nonspecific adsorption is self-corrected.

However, the liquid membrane (hexadecane or 1-octanol) must be chosen to allow the free drug to permeate through it while the protein is retained in the donor compartment. The membrane has to be chosen depending on the compound studied, which can be problematic during batch analysis. Finally, in comparison with ED, the analytical burden is more or less identical as both methods use liquid chromatography–mass spectrometry (LC–MS) or LC–MS–MS as readout.

The use of PAMPA for drug–protein binding studies is able to furnish the equilibrium binding constants. According to the mathematical treatment of the data proposed, however, only a 1:1 model has been applied and no stoichiometric information is available.

Liquid chromatographic techniques

Liquid chromatographic methods used to assess drug–protein interactions can be divided into two main approaches depending on whether both interacting species are free in solution (size-exclusion chromatography) or whether one component, generally the protein, is immobilized on the chromatographic support (affinity chromatography). Zonal elution (small-plug injection) or frontal analysis (large-plug injection) can be applied to both approaches. In zonal elution (ZE), the retention time or the peak area is used to obtain the association constants whereas frontal analysis (FA) quantification is based on plateau height [29].

In size-exclusion chromatography (SEC), molecules in solution are separated on the basis of their size or, more precisely, on their hydrodynamic volume. Typically, a mixture of drug and protein elutes through a column packed with porous particles. The protein and drug–protein complex molecules, which are too large to penetrate the pores of the packing, elute first. Drug molecules, however, which can penetrate or diffuse into the pores, elute later [30]. Internal-surface reversed-phase (ISRP) supports, based on the same principle of drug–protein separation, have also been used to this end [31].

In SEC, proteins and ligands are both used free in solution. As previously mentioned, different modes have been reported, depending to the experimental setup. Drug and protein can either be mixed and then injected in a large plug into the column (FA) or small-plug injections (ZE) can be applied to either the mixture of drug and protein (direct separation method), to one of the interaction partners while the other species is dissolved in the eluent (Hummel–Dreyer, or HD, method), or to neat buffer while both components are dissolved in the mobile phase (vacancy peak, or VP, method) [29].

In drug–protein binding assays, SEC has attracted only limited attention. Its drawbacks, for example low column efficiency and poor protein recovery, have made this approach rather inconvenient and rarely used nowadays [18]. Furthermore, short column life-times have also been criticized, even if modern columns are more robust.

High-performance affinity chromatography (HPAC) is based on immobilization of a protein on a support and injection of an interacting solute into the column. Drugs with high affinity will interact with the immobilized protein and will eluate later than drugs with no or less affinity.

One of the main advantages that made this approach valuable is its ability to use only small amounts of protein for a large number of studies, because the same protein preparation can be reused for multiple experiments. This minimizes run-to-run variations. For example, columns containing HSA immobilized on silica particles have been used for 500–1000 injections [32]. Other interesting features of HPAC include its ease of automation and ability to study the behavior of both enantiomers of chiral drugs by using a racemate once the enantiomeric resolution is obtained on the column.

The main debated and critical aspect to take into consideration when performing binding studies with HPAC is the immobilization of the protein on the chromatographic support. It is therefore important to consider the extent to which this support will model the behavior of the same protein in its soluble form. This is of crucial concern, because the immobilization process can affect protein activity by denaturation, improper orientation, or steric hindrance at the binding sites to be studied. It can either impede or artificially enhance the recognition process [33]. Moreover, the biochromatographic support detects any ligand interactions with the stationary phase that can affect the retention time. The matrix anchoring the protein has its own potential retention capacity, which creates nonspecific binding inducing lower precision for low-affinity compounds [34, 35]. Very strong affinities may also be challenging, because organic modifiers (up to 30–40% [36, 37]) have to be used to elute compounds with very strong affinity (>99% binding). These modifiers can alter the conformation of the protein and the drug–protein binding by disrupting non-polar interactions. This usually reduces the measured binding of solutes to protein.

Albumin is the most studied plasma protein in HPAC. Among the reported anchoring procedures, those employing the covalent binding of HSA to a silica diol or to a silica epoxy matrix seemed to mimic well the process in solution [38–40]. This lends support to the validity of using immobilized HSA as a model for albumin in solution. In contrast, in most studies there is only fair correlation between results obtained by use of immobilized AGP and those obtained in solution. Furthermore, the immobilization procedure is more complex than for HSA [41, 42]. However, Xuan et al. [43] recently optimized the procedure, and better results were obtained, which suggests it is possible to use AGP columns to directly model the binding of drugs to soluble AGP. Hage's group [44] has also recently immobilized high-density lipoproteins (HDL) on silica columns with promising results.

As previously mentioned, HPAC can be performed in two different setups (ZE and FA). ZE is most commonly used to study drug interactions with serum proteins because of its simplicity and, probably, because it is performed in the same mode as used for most analytical applications of chromatography (i.e., a narrow plug of solute is injected into a column while the solute's elution time is monitored). ZE is mainly used to provide information on the bound fraction of a drug because the relative quantity of drug at equilibrium bound to the immobilized protein vs. free in the mobile phase is directly measured by the retention factor ($k' = t_r - t_m/t_m$, where t_r is the retention time of the drug and t_m is the retention time of an unretained solute) [29]. It has been shown that a relative strong correlation exists between the term $k'/(k'+1)$ and the amount (%) of protein binding observed in solution-phase studies. Therefore, a quantitative reference curve linking $k'/(k'+1)$ and the protein binding percentage can be generated with a series of well-known compounds. The binding of an unknown compound can thus be determined using this reference curve.

ZE is a relatively rapid approach to access drug–protein binding. It is an interesting method, yet some experimental conditions (e.g., drug concentration, flow-rate, column length, and back pressure) have to be optimized for each compound before starting the binding study [32]. ZE can also be used to study the kinetic rate of binding interactions [45, 46].

FA is based on the continuous infusion of ligand over a protein target immobilized on the column while the amount of eluted ligand is monitored. The saturation of the column with the ligand results in a breakthrough curve in which the mean position is related to the binding capacity of the column as long as rapid association and dissociation kinetics occur [47]. If experiments are repeated at different drug concentrations, the association equilibrium constants can be obtained, because the position of the breakthrough curve is related to the concentration of applied solute, the amount of protein in the column, and K_a [29]. Nevertheless, this approach inconveniently requires a fairly large amount of solute (~50 mL of μmol L^{-1} drug solution) [48, 49].

Several papers [50–52] used MS as a detection method to improve detection sensitivity, which, in turn, increased the range of accessible affinity constants (K_a up to 10^{12} $\mathrm{mol}^{-1}\mathrm{L}$ with MS vs. $10^{8}\mathrm{mol}^{-1}\mathrm{L}$ with UV). As a result, several screening applications demonstrated the capability of HPAC–FA–MS to assess strong interactions between biological systems, together with a relatively high throughput [53]. The limiting factor with MS in affinity studies, however, is the use of MS-friendly buffers capable of retaining protein stability at the same time.

Capillary electrophoretic techniques

General advantages of capillary electrophoresis (CE) include:

1. high efficiency and separation selectivity;
2. low sample and reagent consumption;
3. high speed of analysis;
4. ease of automation; and
5. ability to work under near-physiological conditions (buffer pH and ionic strength).

Furthermore, in the particular field of drug–protein binding studies, it provides the favorable possibility of evaluating interactions in free solution [33, 54, 55]. However, CE also has drawbacks, for example the risk of protein adsorption on the capillary walls and the low detection limits of commonly used UV detectors. In order to enhance sensitivity, a laser-induced fluorescence (LIF) detection system has been used in the early stages of drug discovery, as exemplified by the CE-based platform developed at Cetek (Marlborough, MA, USA) to screen crude natural extract libraries [56, 57]. Unfortunately, because only few molecules possess native fluorescence, labeling is often required. This can interfere with the binding interactions, as illustrated in a recent study by Pedersen et al. [58]. Another valuable alternative to enlarge the range of binding constants assessable by CE is an MS detection system. One issue of MS is the use of MS-compatible buffers that will not alter the binding process, just as in HPAC. MS hyphenation to CE in the drug–protein binding field is not well developed [59–61], and progress is needed to make this technique more reliable, even though some groups have published promising results.

As for LC, both ZE (small-plug injection) and FA (large-plug injection) can be applied in CE. In FA, both species are mixed prior to injection. When large plugs are injected, the resulting electropherograms consist of plateaus instead of thin peaks, and quantification is made by measuring the height of the resulting plateau. In ZE, a mixture of both interacting species can be injected into the capillary containing neat buffer. Another possibility is the injection of only one of the binding partners while the other is dissolved in the electrophoresis buffer. It is also possible to inject neat buffer (without any drug and protein) into a capillary filled with both interacting species. K_a can be obtained either by quantification of peak areas or by migration shift. All the different CE approaches with their advantages and drawbacks are summarized in Table 1. Because they have been extensively reviewed [33, 54, 62–68], only capillary electrophoresis/frontal analysis (CE/FA) and affinity capillary electrophoresis (ACE) will be discussed below. These approaches are the most widely used, because of their simplicity and reliability.

In CE/FA, the interacting species are first mixed in the sample vial. Upon attainment of the binding equilibrium, a volume of sample sufficiently large to maintain equilibrium during the electrophoretic run, generally 10–20% of the effective capillary length, is introduced into the capillary [69,

Table 1 Comparison of the different capillary electrophoretic approaches used to assess drug–protein binding

	Frontal Analysis (large-plug injection)		**Zonal Elution (small-plug injection)**					
	CE/FA	**FACCE**	**CZE**	**ACE**	**HD**	**VP**	**VACE**	
Setup	 Injection of a large plug	Continuous injection						
How to obtain K_a	$[D_{free}]$: plateau height	$[D_{free}]$: plateau height	$[D_{free}]$: peak area	Change in μ of the injected compound	$[D_{bound}]$: peak area	$[D_{free}]$: peak area	Change in μ of one of the species	
Main Advantages and Drawbacks	- slow and fast kinetics systems: analysis possible - multiple equilibria easily studied - stoichiometric information - binding percentage easily obtained		- slow kinetics systems ($K_a > 10^7\ M^{-1}$) required - multiple equilibria easily studied - stoichiometric information - binding percentage easily obtained - highly purified samples not required	- knowledge of the exact [D] not needed - highly purified samples not required - enantiomeric separation -[P] >> [D] - stoichiometric information not obtainable - multiple equilibria : difficult to deal with	- multiple equilibria easily studied - stoichiometric information - binding percentage easily obtained - highly purified samples not required	- require higher amount of P and D than other CE set-ups - BGE absorption too low : poor sensitivity - BGE absorption too strong : detector saturation - binding percentage easily obtained by VP but not by VACE - highly purified samples not required		
Information obtained	K_1, n_1, K_2, n_2		K_1, n_1, K_2, n_2	K_1, (K_2 : difficult)	K_1, n_1, K_2, n_2	K_1, n_1, K_2, n_2	P-vacancy K_1	D-vacancy K_1, n_1, K_2, n_2
Condition	$\mu_{DP} = \mu_p$ but $\mu_D \neq \mu_{P;}\ \mu_{DP}$	$\mu_{DP} = \mu_p$ and $\mu_D > \mu_{DP}$	$\mu_D \neq \mu_{P;}\ \mu_{DP}$	$\mu_{DP} \neq \mu_D$	$\mu_{DP} = \mu_p$	$\mu_{DP} = \mu_p$	$\mu_{DP} \neq \mu_P$	$\mu_{DP} \neq \mu_D$
Case 1 $\mu_{DP}=\mu_p>\mu_D$	K_a ↔	Impossible : μ_D has to be > μ_{DP}	K_a ↔[1]	Impossible if P injected, D in the BGE K_a ↑ if D injected, P in the BGE	K_a ↔	K_a ↑	Impossible	K_a ↑
Case 2 $\mu_{DP}>\mu_p>\mu_D$	K_a ↓		K_a ↔[1]	K_a ↑	K_a ↑	K_a ↑	K_a ↓	K_a ↑
Case 3 $\mu_{DP}<\mu_p>\mu_D$	K_a ↑		K_a ↔[1]	K_a ↓	K_a ↓	K_a ↓	K_a ↓	K_a ↑
Binding kinetics required	Fast and slow	Fast and slow	Slow	Fast	Fast	Fast	Fast	Fast

[a] As long as there is no complex dissociation during the analysis time, i.e. high affinity needed.

BGE, background electrolyte; D, drug; P, protein; [ ], concentration; *K*, equilibrium binding constant; *n*, number of binding sites with the same affinity per protein molecule; μ_{DP}, complex mobility; μ_D, drug mobility; μ_P, protein mobility; ↔, adequate *K* values; ↓, underestimated *K* values; ↑, overestimated *K* values; CE/FA, capillary electrophoresis/frontal analysis; FACCE, continuous capillary electrophoresis frontal analysis; CZE, capillary zone electrophoresis; ACE, affinity capillary electrophoresis (mobility shift assay); HD, Hummel–Dreyer method; VP, vacancy peak method; VACE, vacancy affinity capillary electrophoresis.

70]. The introduction of large volumes gives rise to the appearance of plateaus. If the electrophoretic mobility of the protein equals that of the drug–protein complex ($\mu_P = \mu_{DP}$), the height of the free drug plateau is proportional to the free drug concentration in the original sample [71]. By repeating the analysis at different drug/protein ratios, both K_a and the stoichiometry of the reaction can be assessed via non-linear regression.

CE/FA remains popular for several reasons:

1. its ability to deal with multiple equilibria and to characterize the reaction stoichiometry;
2. its robustness, because of to the plateaus formed;
3. its low material consumption (nL); and
4. the possibility offered to work with systems with fast and slow kinetic reactions [72].

In ACE, the capillary is filled with buffer containing the protein (P) in varying concentrations and a small amount of drug (D) is injected into the capillary [66, 73]. K_a can be calculated from the change in the electrophoretic mobility of D upon complexation. Its migration time (t_m) is confined between two extreme values—the t_m of D without any P in the running buffer and the t_m of D at high protein concentration (saturation). If performed at different drug/protein ratios, non-linear regression treatment of the data provides K_a [33, 65, 73–76].

One protein sample can be used for screening many different putative ligands in different buffers [65]. This makes the approach valuable when material is scarce. Moreover, protein preparations do not need to be highly purified, and protein binding of racemic drugs may be performed by injecting the racemic mixture directly. A precise value of the drug concentration is not required. Notably, calculation of K_a requires that the protein concentration at equilibrium be approximated to the added P concentration in the capillary. If this were held to be true, the protein concentration must be larger than the drug concentration. It thus becomes difficult to correctly assess the value of K_a for moderate-to-high-affinity systems ($K_a > 10^5 \mathrm{mol}^{-1} \mathrm{L}$). Other disadvantages of ACE are its inability to yield the reaction stoichiometry and the difficulty of dealing with multiple equilibria. The different K_a values can only be obtained when the change in mobility with increasing complexation ($P \rightleftarrows PD \rightleftarrows PD_2$) is proportional to the number of ligands. If this is not the case, an appropriate model relating the mobility of all the interacting species involved should be incorporated in the data-processing procedure, which inevitably complicates the interpretation of these data [64].

Non-separative methods

Spectroscopic techniques

Spectroscopic methods (UV–visible, fluorescence, infrared (IR), nuclear magnetic resonance (NMR), optical rotatory dispersion (ORD), and circular dichroism (CD)) are based on the perturbation of the electronic and spectroscopic energy levels of the ligand or the protein by the binding.

These methods can be performed in solution, which enables true equilibrium measurements. Spectroscopic methods have the great advantage of providing a better understanding of the binding mechanism, in addition to binding affinity constant measurement. They also facilitate insight into three-dimensional protein structure, which in turn enables elucidation of some complementary structural and conformational variations of a protein molecule resulting from ligand attachment [18]. Changes in the UV or visible absorption spectrum of a drug may be interpreted in terms of the polarity of a drug binding site. Fluorescence spectroscopy helps in identification of the binding site of a drug and can also be used to calculate the binding distance between the fluorophore on the protein and the drug [77]. IR is an excellent tool to study secondary structure of the protein. NMR spectroscopy indicates which groups or parts of a protein molecule are involved in the binding process, and CD yields information about the three-dimensional structure of the drug-binding site [78–81]. A new trend in drug–protein interaction research is the use of different spectroscopic analyses combined with computational methods (molecular docking) to obtain a clear picture of the mode of interaction of the binding partners [77, 82].

Spectroscopic approaches are successful mainly for high-affinity binding sites and are not very powerful for studying multiple equilibria. This is because the analytical response is not a direct measure of the extent of binding but instead is rather proportional to it [13, 83]. Another critical point is the lack of sensitivity of these methods, which seriously limits their wider use. For example, in IR, samples of mmol L^{-1} concentration have to be used. Such concentrations may lead to solubility problems and nonspecific aggregation [18, 84].

Calorimetric techniques

Two calorimetric approaches are used to study drug–protein interactions: isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC).

ITC is the calorimetric approach most used to investigate biomolecular interactions. It measures one of the most fundamental characteristics of molecular complex formation, i.e. heat uptake or release. Typically, an ITC experiment consists of successive additions of a drug to a solution of protein contained in a reaction cell. Each addition of ligand to the protein sample leads to the formation of a specific amount of ligand–protein complex, according to the binding affinity which can be evaluated by monitoring the heat release [85–89].

ITC experiments can be designed with molecules of arbitrary size and “spectroscopically silent” compounds. Moreover there is no need for derivatization or protein immobilization on a support. However, very high and very low-affinity processes cannot be studied by ITC, and sometimes the large amount of material required for accurate measurements makes ITC experiments impracticable [86]. Its low throughput may also be an issue, because the time required to run a full titration experiment is at least 2.5 h and usually even longer. In this setup, the thermal equilibration of the measurement cell after ligand addition can easily take an additional 30–60 min [90]. Finally, as for all non-separation techniques, the samples have to be highly purified.

DSC was primarily developed to characterize protein stability and folding. The instrumentation used for DSC experiments is very similar to that used for ITC. The setup is nevertheless different, because the reaction cell containing a mixture of drug and protein is heated at a controlled rate. When a small molecule binds preferentially to the native form of a protein, the drug stabilizes the protein and the transition midpoint of the protein–ligand complex (the temperature at which 50% of the protein is in its native conformation and the other 50% is denatured) thus occurs at a higher temperature than the midpoint in the absence of ligand. DSC is, therefore, an indirect method for measuring binding constants, because K_a is estimated from measurements of equilibrium between folded and unfolded protein rather than bound and unbound forms [91].

The main interest in DSC is in its ability to estimate very large binding constants (up to $10^{15} \mathrm{mol}^{-1} \mathrm{L}$) [92] that cannot be conveniently measured by other techniques. Its main disadvantages are the very low throughput and the large sample consumption.

The real strength of calorimetric methods is their ability to provide a complete thermodynamic picture of the binding reaction. They can therefore be depicted as powerful tools with a high information content. The direct thermodynamic observable is the heat associated with the binding event, i.e. the enthalpy of the binding reaction (ΔH). These methods can yield the equilibrium binding constants, the entropy change (ΔS), the Gibbs free energy (ΔG), and the stoichiometry of the association [93, 94]. Use of these thermodynamic data makes it possible to deduce the interaction mechanism [95]. Moreover, because the heat capacity change (ΔC_p), obtained from experiments performed at different temperatures reflects the burial of polar and non-polar surfaces as a consequence of the

Table 2 Main features, advantages and disadvantages of the different methods used to assess drug–protein binding

	ED	UF	UC	ITC	DSC	Spectro.	SPR	HPAC	CE	PAMPA
Throughput	Low	Low	Low	Low	Low	Moderate	Moderate	Moderate to high	Moderate to high	High
Immobilization	No	No	No	No	No	No	Yes	Yes	No	No
Multiple binding sites	Yes	Yes	Yes	Yes (difficult)	No	No	No	Yes	Yes	No
Usual volume of sample	500-1000 µL	500-1000 µL	500-1000 µL	200 µL – 1.4 mL	0.5 mL	low mL	D: 30-500 µL	–ZE: a few µL -FA: ~10 mL	nL	300 µL
Usual order of concentration	µmol L^{-1}	µmol L^{-1} - mmol L^{-1}	mmol L^{-1}	mmol L^{-1}	mmol L^{-1}	µmol L^{-1}- mmol L^{-1}	D: µmol L^{-1} P : 50-100 µg (reusable)	–ZE: D: µmol L^{-1} -FA: D: µmol L^{-1} ~30 mg P / g of silica^3	µmol L^{-1}	µmol L^{-1}
Plasma protein studied	BSA HSA AGP HDL/LDL Plasma	BSA HSA AGP HDL/LDL Plasma	BSA HSA AGP Plasma	BSA HSA	BSA^1 HSA^1 Plasma^2	BSA HSA AGP Lipoproteins Plasma	BSA HSA AGP	BSA HSA AGP HDL	BSA HSA AGP HDL/LDL Plasma	HSA
Kinetic data	No	No	No	No	No	Yes	Yes	Yes (rare)	Yes (rare)	No
Information	K_a, n	K_a, n	K_a, n	K_a, n ΔH, ΔS, ΔG, ΔCp	K_a, n ΔH, ΔS, ΔG, ΔCp	K_a, n Structural info.	K_a, n k_{on} / k_{off}	K_a, n	K_a, n	K_a
(+)	- Simple - Inexpensive - 96-well format available	- Simple - 96-well format available	- Simple	- Thermodynamic information	- Thermodynamic information - High affinity systems assessable	- Additional information on the nature of the interaction, structural information	- Binding kinetic information - Very low amount of sample - Real .time data of binding	- Immobilized P sample reusable - Enantiomeric affinity on racemate - Pure sample not required	- Very low material - Rapid and simple method development - Pure sample not required	- 96-well format
(-)	- Long equilibration time - Volume shifts - $MW_{protein}$ >> MW_{drug} - NSB - Donnan effect - Highly pure reagents	- Filter obstruction - May disturb the equilibrium - Sieve effect - $MW_{protein}$ >> MW_{drug} - NSB - Highly pure reagents	- Lipoproteins float - Risk of D sedimentation, back diffusion, dissociation of the complex - Highly pure reagents	- Large amount of sample - Experimental setup largely dependant on the system of interest - Highly pure reagents	- Lack of validity performance - Highly pure reagents	- Poor reproducibility - False negatives - Highly pure reagents	- Immobilization step → long method development - Stoichiometric information not directly available - Highly pure reagents -Cost	- Immobilization step → long method development	- Sensitivity -possible adsorption on capillary wall	- Highly pure reagents

ED: equilibrium dialysis, UF: ultrafiltration, UC: ultracentrifugation, ITC: isothermal titration calorimetry, DSC: differential scanning calorimetry, Spectro.: spectroscopic assays, SPR: surface plasmon resonance-based assays, HPAC: high-performance affinity chromatography, ZE: zonal elution, FA: frontal analysis, CE: capillary electrophoresis, PAMPA: parallel artificial membrane permeability assay, K_a: equilibrium binding constant, n: stoichiometry, ΔH: enthalpy of the binding reaction, ΔS: entropy change, ΔG: Gibbs free energy, ΔCp: heat capacity change, k_{on}: association rate constant, k_{off}: dissociation rate constant, NSB: nonspecific binding, BSA: bovin serum albumin, HSA: human serum albumin, AGP: α1-acid glycoprotein. HDL: high density lipoproteins, LDL: low density lipoproteins, (+): main advantages, (-): main drawbacks, P: protein, D: drug, MW : molecular weight.

[1] not in the binding field but to characterize stability and folding [95].

[2] not to calculate binding affinity but to get a signature thermogram [100].

[3] for HSA [48].

binding reaction, calorimetric methods provide a link between thermodynamic data and structural information about macromolecules [86, 96].

Nowadays, ITC is regaining popularity in medicinal chemistry laboratories, because knowledge of thermodynamic data can aid rational drug design. A typical example of a drug optimization strategy combining structural and thermodynamic approaches is the development of high-potency inhibitors of the HIV-1 protease [97]. In contrast, only a few papers have studied the interaction between small molecules and proteins by DSC [91, 92, 98, 99]. Questions may arise concerning the real strength of DSC in this domain. A unique recent paper in the protein-binding field [100] used DSC to show that plasma from healthy individuals yields a reproducible signature thermogram whereas DSC analysis of plasma from diseased individuals reveals significant changes because of the interaction of small molecules with plasma proteins. DSC might thus provide a tool for disease screening and monitoring.

Surface plasmon resonance-based assays

Surface plasmon resonance (SPR)-based techniques involve immobilizing one reactant (the protein) on a surface and monitoring its interaction with a second component (the drug) in solution that flows over the surface [101, 102]. Basically, an SPR detector monitors changes in the refractive index that occur as molecular complexes form or break during the binding reaction at the sensor surface anchoring one of the interaction partners [103]. This response, also proportional to the mass of bound material, is recorded in a sensorgram. Mathematical treatment of the signal obtained gives the binding data.

Qualitatively, visual inspection of the response curves indicates if complex formation takes place (increase of response), reaches equilibrium and/or saturation (plateau), and is reversible (decrease of response). It can also give kinetic stability information about the complex formed. Analysis of time and concentration-dependant response curves can also provide quantitative information about the stoichiometry of binding, equilibrium binding constants, and kinetic rate constants [104].

Methods based on SPR are interesting for several reasons:

1. the lack of labeling requirement;
2. their ability to characterize binding reactions in real time
3. the assessable quantitative information about the binding events (affinity and kinetic rate constants); and

4. the small amount of material used (for typical analyses a few micrograms of a protein are required to make a single surface in typical analyses).

Furthermore, a wide range of affinity constants ($K_d \approx$ mmol L^{-1}–pmol L^{-1}) and kinetic rate constants (k_{on} from 10^3 to 10^8mol^{-1}L s^{-1}, k_{off} from 10^{-6} to 1 s^{-1}) can be assessed [105]. However, as for HPAC, an often criticized aspect is the immobilization of the protein on the sensor surface [104, 106–109]. SPR has been used successfully to rank drug molecules into weak, medium, and strong binders to HSA and AGP but encountered limitations in K_a determination for some compounds, because of complex data and behavior [110, 42]. It is worth remembering that the refractive index change per molecule is related linearly to the molecular weight of the compound that binds the immobilized protein. Therefore, when working with small-molecular-weight drugs that bind to high molecular weight proteins, it might be difficult to obtain reliable data without specific equipment. Finally, SPR-based technology has a moderate throughput rate and is currently not suited to the study of a large number of compounds. The commercialization of higher-throughput devices such as the Biacore A100 with its parallel flow cells on a single chip may open new application fields in the drug-discovery process. Nevertheless, the equipment and maintenance costs of this technique might be discouraging.

Method selection

There are probably no general rules or flowcharts for selecting the best experimental method to study drug–protein binding, yet it may be helpful to present some of the pertinent factors to take into account when choosing a method to study an interaction system. Many of these considerations are deduced from comparison of the advantages and limitations of each technique summarized in Table 2. Additionally, because of the complementary nature of the techniques, often only a combination of different approaches enables the scientist to catch a glimpse of the complex world of protein-binding studies.

The first considerations are the type of information required and the number of compounds to test. In early drug discovery, rather simple information is needed. The binding percentage may be sufficient so long as the throughput is high enough. At that stage, PAMPA and HPAC could be good approaches, because of the 96-well technology and the information about the amount of binding (%) available from a single injection, respectively. In HPAC and in CE, the binding affinity constants can be obtained relatively rapidly. Moreover, CE experiments can be performed in parallel because of the multiplexed CE instruments available (e.g., the 96-capillary format instrument from CombiSep (Ames, IA, USA)). Recently, microchip CE has also been used to characterize interactions, in order to reduce reagent consump-

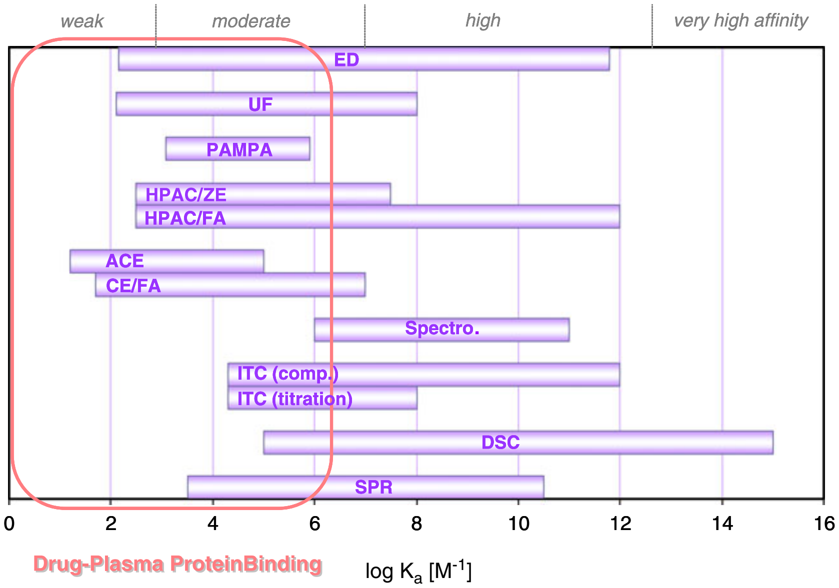

Fig. 3 Range of binding constants (log K_a) assessable by the different methods used to study drug–protein interactions. *ED*, equilibrium dialysis; *UF*, ultrafiltration; *PAMPA*, parallel artificial membrane permeability assay; *HPAC/ZE*, high-performance affinity chromatography/zonal elution approach; *HPAC/FA*, high-performance affinity chromatography/frontal analysis approach; *ACE*, affinity capillary electrophoresis (mobility shift assay); *CE/FA*, capillary electrophoresis/frontal analysis; *Spectro.*, spectroscopic assays; *ITC*, isothermal titration calorimetry; *comp.*, competition studies; *titration*, titration studies; *DSC*, differential scanning calorimetry; *SPR*, surface plasmon resonance-based assays

tion and analysis time [111–113]. HPAC, however, requires the immobilization of one of the interacting partners on the chromatographic support, which can require significant time to optimize the anchoring procedure. Moreover, preliminary studies have to be performed to check the extent to which the support models the protein behavior in solution. For some proteins, the problem of protein immobilization can also lead to the complete loss of its binding properties. The same is true for SPR techniques. In contrast, when rapid and simple method development is a priority, CE is a really good choice [114].

In advanced drug discovery or development stages, spectroscopic and/or calorimetric approaches are the methods of choice to obtain a complete view of the binding mechanisms, as illustrated elsewhere [115, 116]. Kinetics information, obtained mainly by SPR, might be of great importance in the understanding of some biological phenomena. In the particular case of drug–protein binding, it is still a matter of debate whether or not the kinetics should be considered when modeling a drug pharmacokinetic profile. In most cases, however, it seems not to be necessary because the time scale of protein-binding equilibration is much shorter than that of other pharmacokinetic processes, for example drug distribution and elimination. Thus, the assumption of an instantaneous binding equilibrium is valid in most practical situations [117]. The stoichiometry of the system should also be taken into consideration, because some methods (spectroscopic approaches, SPR) are quite successful when applied to binding systems of simple 1:1 stoichiometry but are relatively ineffective or imply complicated data analysis for systems having multiple equilibria [13].

Another important consideration is the range of K_a values assessable with each method (Fig. 3). Spectroscopic approaches are better suited to high-affinity systems whereas PAMPA, ED, CE, and HPAC are adequate for measurement of weak to moderate affinity. More specific interactions, i.e. higher affinities, can easily be characterized by ITC whereas very-high-affinity systems require DSC. Most studies dealing with calorimetry are indeed found in the drug design field, i.e. interaction with a defined target (strong interaction), and only few have reported interactions of small-molecular-weight drugs with plasma proteins (polyphenol–BSA [118], Ni^{2+}–HSA [119], penicillins–HSA [94], surfactants–HSA [120]).

The physicochemical properties of the studied compounds and the amount of material available may also condition the choice of the method. If solubility is low, spectroscopic and calorimetric approaches may fail whereas PAMPA, HPAC, or SPR may be adequate, because of their greater sensitivity. A limitation of PAMPA could, nevertheless, be the analysis of very lipophilic drugs that might stay trapped in the membrane. When material is scarce, CE should be considered as it consumes only minute amounts of sample, in contrast with spectroscopic and calorimetric approaches.

The classical methods (ED, UF, and UC) are not suited to study interactions between compounds of approximately the same size, because they are based on differences in molecular size. Typically, protein–protein interactions cannot be analyzed by such systems. SPR that senses changes proportional to the mass of bound material on the sensor chip may be a fine alternative.

Samples of low purity require separation techniques, such as HPAC or CE, unless a purification step is performed beforehand. Finally, the instruments available in the operator’s laboratory, his/her own experience with a type of method, and the costs involved may also be considered.

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