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Review

Estimation of drug–protein binding parameters on assuming the validity of thermodynamic equilibrium

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Abstract

This contribution focuses the reader's attention on the pitfalls usually emerging during the phase of evaluation of experimental data of drug–protein binding studies. To overcome the occurrence of problem(s) apparently defying solution, the concept of ''affinity spectra'' is recommended to be implemented for data evaluation. A (general) ''binding study protocol'' is also suggested, which can prevent the formation of inadequate conclusions and the generation of unrealistic drug–protein binding parameters.

Keywords: Drug–protein binding; Thermodynamic equilibrium; Bjerrum plot; *S*-Propafenone; α_1-Acid glycoprotein; Reviews

Contents

1. Introductory remarks

In the blood circulation, a drug (D) is usually in two forms: free (F) and reversibly bound (B) to a protein (P). Pharmacologically effective is only the free form, which may permeate the vessel wall and

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reach its site of action — the target tissue, receptor. In a limit situation, when the given drug molecules would be (hypothetically) fixed-in-full (i.e. 100%), there would not be any unbound drug left to trigger the pharmacological action at the extravascular/receptor site.

Serum albumin binds mostly acidic and neutral drugs, while α_1-acid glycoprotein (α_1-AGP) is responsible for binding predominantly basic drugs. Lipoproteins, immunoglobulins, and other blood constituents (erythrocytes, blood platelets) may also act as drug-complexing macrobiomolecules. Studies investigating the drug binding interaction with whole blood, plasma or serum, as well as with selected plasma proteins, are thus an indispensable phase of early (preclinical) pharmacokinetics of any new/prospective drug.

The two most common solution–phase methods for the evaluation of the extent of drug binding are ultrafiltration (UF) and equilibrium dialysis (ED). When the value of the extent of binding reaches ≥90% a more detailed evaluation of the drug–protein interaction is to be performed: The parameters investigated are, e.g. the (equilibrium) association (K) and/or dissociation ($K' = 1/K$) constants, the number of specific/saturable binding sites (n) within the protein macromolecule, the first-order dissociation and/or the second-order association rate constants.

To establish these parameters, the instantaneous chemical activities (molar concentrations) of each component of the ternary mixture should be determined without deranging the equilibrium described by the following equation:

$$\mathrm{P} + \mathrm{D} \leftrightarrow \mathrm{P{:}D} \tag{1}$$

In relation to the above specified conditions, both UF and ED, often classified as standard/reference methods, have however some drawbacks [1,2]:

- On employing the UF method, one should take into account that during the process of filtration the equilibrium is being permanently deranged. (A 10% disintegration of the P:D complex characterized by the association constant $K = 10^4$–10^7 l/mol lasts only for fractions of seconds [3]). Although the attempt to carry out UF within the shortest possible time and to separate only the minimally required volume of the free drug fraction is correct, nevertheless the true, thermodynamic equilibrium can not be fully preserved!
- When working with the ED method, during equilibration (lasting hours) a partial denaturation of the protein and/or degradation of the sample components induced by the growth of the ubiquitous microorganisms occurs rather frequently!

Several gel filtration methods, later on high-performance liquid chromatographic (HPLC) as well as capillary electrophoretic (CE) methods have been implemented for the investigation of the interacting drug–protein systems. Yet only a few of them allow to perform (solution–phase) measurements at the conditions prescribed in Eq. (1). Of the latter, at present the most frequently used are the gel filtration design method of Hummel and Dreyer [4] or that of the equilibrium saturation chromatographic method introduced by Sébille et al. [5], as well as the HPLC/CE arrangement of frontal analysis. The strengths, weaknesses, and the application potential of these and of a number of further chromatographic and electrophoretic methods were overviewed by Hage and Tweed [6].

This communication attempts to address non-specialists and relevant interested readers by bringing into their conscious awareness some of the pitfalls usually emerging in the phase of evaluating drug–protein binding data, i.e. the determined set of the [B], [F] values.

2. Evaluation of drug–protein binding interaction

The reversible binding interaction has been usually described by the following relationship:

$$[\mathrm{B}]/[\mathrm{P}] \equiv r = \sum_{i=1}^{N} n_i K_i [\mathrm{F}]/(1 + K_i[\mathrm{F}]) \tag{2}$$

in which N represents the number of individual regions of (specific/saturable) binding sites within the protein macromolecule [7].

The task to establish the values of n_i and K_i parameters from a set of known j-pairs of the r and [F] values is primarily limited by the unknown value of the (integer) N.

$N=1$: By this choice, Eq. (2) is transformed into the following form:

$$r = \frac{nK[\mathrm{F}]}{1 + K[\mathrm{F}]} \tag{3}$$

The correctness/incorrectness of the choice $N=1$ as well as the validity of the calculated values of the n and K parameters is simply verifiable by applying a criterion of identity (ξ) such as absolute or relative (percentual) difference between the set of experimental and of recalculated r values (The use of the obsolete graphical approach to determine the n and K values by means of Eq. (3) modified as:

$$r/[\mathrm{F}] = nK - Kr \tag{4}$$

is invalid! Actually, the relationship (4) is by itself not the function of the type $y = f(x)$, rather the (dependent) variable r depends on the (independent) variable, which is again r?).[1]

$N=2$: Having established that on selecting $N=1$ the calculated ξ significantly exceeds the set limit value of this criterion, the apparently most logical subsequent step would be the choice $N=2$ and a repetition of the calculation procedure by using the relationship:

$$r = \frac{n_1K_1[\mathrm{F}]}{1 + K_1[\mathrm{F}]} + \frac{n_2K_2[\mathrm{F}]}{1 + K_2[\mathrm{F}]} \tag{5}$$

However, since in both terms of Eq. (5) the n_1K_1 and n_2K_2 products are in the numerators while the K_1 and K_2 parameters are in the denominators, by the regression method more than one single set of suitable n_1, K_1, n_2, K_2 values can be found. Thus although by applying the above calculation procedure the existence of two individual regions of specific/saturable binding sites within the protein macromolecule has not been excluded, the true, unambiguously objective n_1, K_1, n_2, and K_2 values remain unknown. This fact presents one of the most crucial pitfalls, appearing on evaluating the drug–protein binding interaction.[2]

Such an apparently insoluble situation can partially be overcome by presenting the j-pair(s) of the experimental r and [F] values in a tabulated form and/or (better) as a binding isotherm: r vs. [F]. On inspecting the binding data observed by different methods within one or various laboratories, it is recommended to compare the shapes and positions of such binding isotherms [10].

3. Binding isotherm(s)

3.1. Bjerrum plot

Presentation of the binding data in the form r_j vs. logarithm of $[\mathrm{F}]_j$ — the so-called ''Bjerrum plot'' [11] — is characterized by the following features:

(1) The plot has a symmetrical S-shape with clearly recognizable inflex.

(2) The location of the inflection point in relation to the ordinate ''r'' represents exactly one half of the total number of specific/saturable binding sites on the protein, i.e.:[3]

$$\frac{1}{2}\sum_{i=1}^{N} n_i$$

3.2. Affinity spectra

In 1983, Tobler and Engel [12] published an original iterative procedure for the analysis of the equilibrium binding experiments, the result of which is the so-called ''affinity spectrum''. The input data for the computation are represented exclusively by

[1] Although the shortcomings of this so-called ''Scatchard graph'' analysis have been critically discussed [8,9], numerous papers still appear reporting extraction of the n, K parameters from such a graph. The authors interpret the frequently observed curvilinear trend of the Scatchard graph as a proof for the presence of two (or more) individual regions of specific/saturable binding sites on the protein examined.

[2] Processing Eq. (2) with $N=2$, or any one greater (integer) N value, is enormously sensitive not only to the values of the computation initiating n_i, K_i guesses, to the length of one iteration step, and to the selected statistical weight, but often also to the sequence of input pairs of starting n_i, K_i estimates, as well as to the values of their lower and upper limits.

[3] The Bjerrum plot does however not allow to establish the actual N value.

the j-pairs of the r and [F] values. The treatment of the data is derived from the equation:

$$r = \sum_{i=1}^{N} n_i[\mathrm{F}]/(K_i' + [\mathrm{F}]) \quad (6)$$

expanded to the following form:

$$r = \sum_{i=1}^{100} n_i[\mathrm{F}]/(K_i' + [\mathrm{F}]) + \mathrm{Const}_1[\mathrm{F}] + \mathrm{Const}_0 \quad (7)$$

where $\mathrm{Const}_1[\mathrm{F}]$ represents the non-specific/unsaturable binding term and Const_0 relates to ''irreversible''/chemical binding [12].

The result of computation, based on the linear programing principle, is that n_i is significantly greater than zero for only some K_i' values. Statistical evaluation produces one (or more) bell-shaped curve(s), the features of which provide information concerning the presence of one (or more) individual region(s) of specific/saturable binding sites within the protein macromolecule. The location and the width of the ith bell-shaped curve correspond to the estimate of the dissociation constant K_i' and to the error of this estimate, respectively; the curve height relates to the value of n_i.[4]

4. Proposal of a (general) ''binding study protocol''

On summarizing the above discussed facts, the following (general) ''binding study protocol'' should be observed:

4.1. Materials

The protein examined should be as pure as possible; the drug should be represented by one single type of molecule. (In the case of a chiral drug the investigation should be performed with one single enantiomer).

4.2. Method

The [B] and [F] values should be determined by an analytical method without deranging the thermodynamic equilibrium of the (interacting) system. The measurements should be performed within an appropriately wide range of drug concentrations; minimally at one, preferably at more levels of the protein.

4.3. Data analysis

The set(s) of the r, [F] values should be processed primarily by the ''affinity spectra'' approach. (The finding that $\mathrm{Const}_0 = 0$ indicates reversibility of the process studied).

5. Results

(A) If $\mathrm{Const}_0 = 0$, the term $\mathrm{Const}_1[\mathrm{F}] = 0$ along with one single bell-shaped curve means that $N = 1$ and thus the binding data can be analyzed in greater detail (also) by applying Eq. (3).

(B) If $\mathrm{Const}_0 = 0$, the term $\mathrm{Const}_1[\mathrm{F}] = 0$ along with two (or more) bell-shaped curves means that $N = 2$ (or 3, 4, etc.) and thus the value of

$$\frac{1}{2}\sum_{i=1}^{N} n_i$$

can be derived also from the Bjerrum plot.

(C) If $\mathrm{Const}_0 = 0$, the term $\mathrm{Const}_1[\mathrm{F}] \neq 0$ along with one single bell-shaped curve means that by using the equation:

$$r = \frac{nK[\mathrm{F}]}{1 + K[\mathrm{F}]} + \mathrm{const}_1[\mathrm{F}] \quad (8)$$

one objective set of the binding parameters (n, K, const_1) can also be computed.[5]

[4] The main advantage of the ''affinity spectra'' approach is that no starting (guess) values (n_i, K_i' estimates) have to be supplied.

[5] Such a situation is exemplified by the ''Illustrative Example''.

(D) If $Const_0 = 0$, the term $Const_1[F] \neq 0$ along with two (or more) bell-shaped curves means that the ''affinity spectrum'', within which also the Bjerrum plot has been implemented, represents the only way to interpret the binding process studied.

6. Illustrative example

The extent of binding of the basic antiarrhythmic drug (*RS*)-propafenone to human plasma proteins is known to be high, with α_1-acid glycoprotein playing a major role in this process [13]. The reversible binding interaction of individual propafenone enantiomers (*R*-, *S*-) with pure human α_1-AGP was thus investigated by an HPLC method applying the Hummel and Dreyer arrangement (for details, see Ref. [14]):

Columns 2, 3 in Table 1 represent a set of the [F], *r* values determined on assaying the system containing the drug *S*-enantiomer. The recalculated $r_{rec.}$ values are given in column 4. Along with these values, the output data (see Table 1) of the ''affinity spectrum'' display the parameters computed on applying Eq. (7).

As evident from both Table 1 and Fig. 1, the most plausible binding isotherm valid for the system studied is the one described by Eq. (8). Such a binding model is applicable when along with one single region of the specific/saturable binding sites another non-specific/unsaturable region is manifest, and when within the investigated drug concentration range no saturation of the protein binding sites can be achieved. On exploiting Eq. (8), the non-linear regression analysis of the experimental data showed a very good stability of iterations yielding invariably optimal *n*, *K*, and $const_1$ values listed in Table 2.

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Table 1
Input and output data of the ''affinity spectrum'' of the reversible bimolecular interaction between *S*-propafenone enantiomer[a] and human α_1-acid glycoprotein

No.	[F; mol/l]	*r*	$r_{rec.}$	Difference (%)
1	5.00×10^{-4}	6.46	6.46	−0.0
2	2.50×10^{-4}	4.03	3.73	8.1
3	2.00×10^{-4}	2.88	3.18	−9.4
4	1.25×10^{-4}	2.06	2.35	−12.5
5	1.00×10^{-4}	2.18	2.08	5.0
6	5.00×10^{-5}	1.51	1.51	−0.0
7	2.50×10^{-5}	1.12	1.20	−6.6
8	1.00×10^{-5}	1.08	0.94	14.6
9	5.00×10^{-6}	0.99	0.78	27.3
10	2.50×10^{-6}	0.58	0.62	−6.3
11	1.00×10^{-6}	0.39	0.44	−11.6
12	5.00×10^{-7}	0.35	0.35	−0.0

Parameter	Raw results	[Unit]	Significance (%)
n	0.777		96.0
K′	2.86×10^{-6}	[mol/l]	96.0
Non-specific			84.5
$Const_0$	0.229		$\ll$; $\rightarrow$0.0

[a] The drug enantiomeric purity was 99.0%.

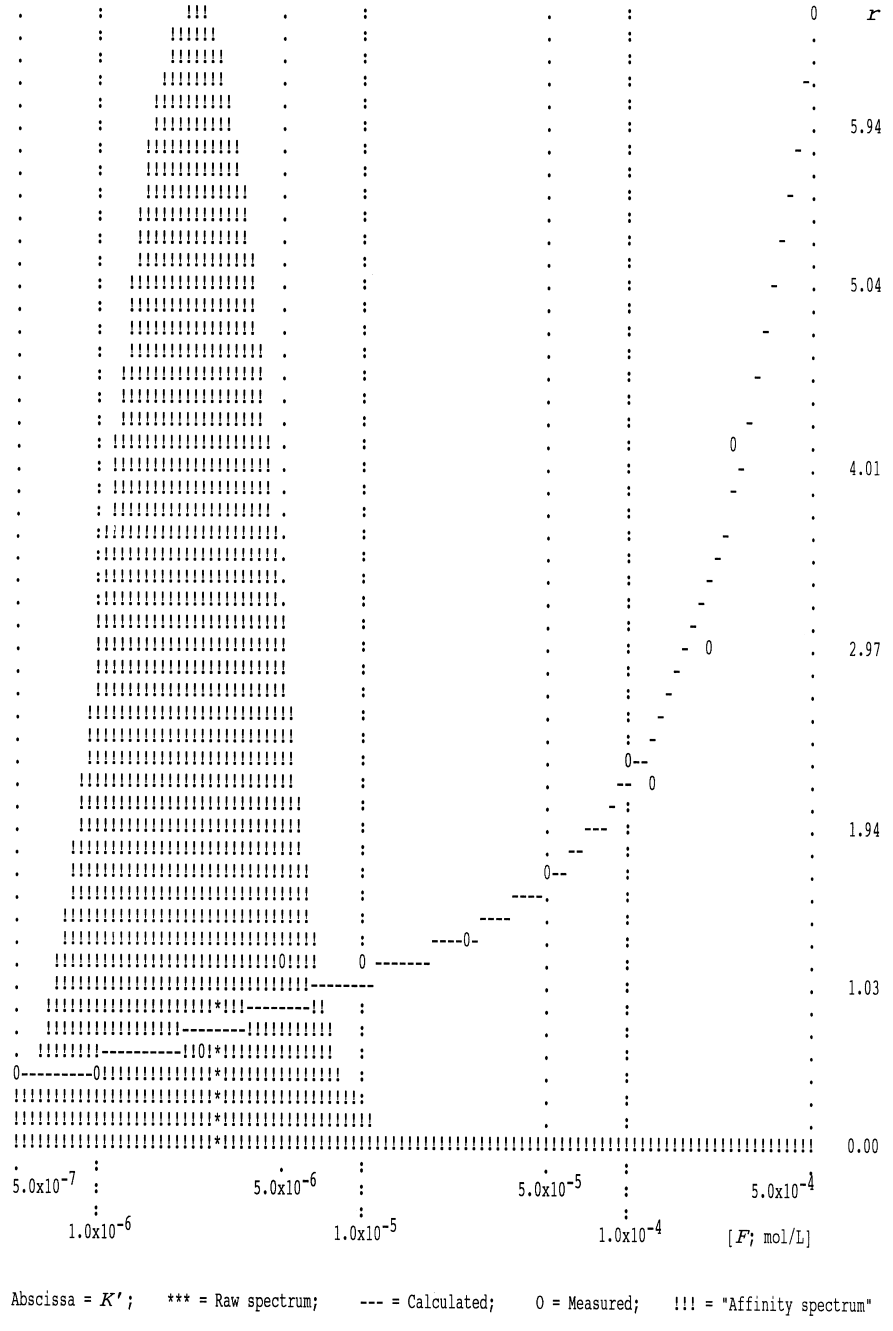

Fig. 1. ‘‘Affinity spectrum’’ of the reversible bimolecular binding interaction between *S*-propafenone and human α_1-acid glycoprotein. Experimental set of the r_j vs. $[F]_j$, i.e. the binding isotherm in the form of the Bjerrum plot (-- O --) is included. As evident from the Bjerrum plot, even at the highest [F] concentration (5.0×10^{-4} mol/l) no saturation of the α_1-AGP binding sites can be achieved.

Table 2
Binding parameters of the reversible bimolecular interaction between *S*-propafenone enantiomer and human α_1-acid glycoprotein

Parameter		[Unit]
Iteration starting value		
n	1.0	
K	5.0×10^5	[l/mol]
$const_1$	5.0×10^4	[l/mol]
Optimalized value[a]		
n	0.98 ± 0.08	
K	$(9\pm1.88)\times10^5$	[l/mol]
$const_1$	$(1.07\pm0.09)\times10^4$	[l/mol]

[a] Parameters given as mean±SEM.

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