J. Pharm. Pharmacol. 1999, 51: 41–48
Received May 27, 1998
Accepted September 2, 1998

Determination of the Affinity of Drugs toward Serum Albumin by Measurement of the Quenching of the Intrinsic Tryptophan Fluorescence of the Protein

DENNIS E. EPPS, THOMAS J. RAUB*, VALERIA CAIOLFA, AUGUSTO CHIARI, AND MORENO ZAMAI

*Discovery Technologies, *Drug Absorption and Transport , Pharmacia and Upjohn, 301 Henrietta Street, Kalamazoo, MI 49007, USA and †Biotechnology, Pharmacia and Upjohn, Nerviano, Italy*

Abstract

Binding of new chemical entities to serum proteins is an issue confronting pharmaceutical companies during development of potential therapeutic agents. Most drugs bind to the most abundant plasma protein, human serum albumin (HSA), at two major binding sites. Excepting fluorescence spectroscopy, existing methods for assaying drug binding to serum albumin are insensitive to higher-affinity compounds and can be labour-intensive, time-consuming, and usually require compound-specific assays. This led us to examine alternative ways to measure drug-albumin interaction.

One method described here uses fluorescence quenching of the single tryptophan (Trp) residue in HSA excited at 295 nm to measure drug-binding affinity. Unfortunately, many compounds absorb, fluoresce, or both, in this UV wavelength region of the spectrum. Several types of binding phenomenon and spectral interference were identified by use of six structurally unrelated compounds and the equations necessary to make corrections mathematically were derived and applied to calculate binding constants accurately. The general cases were: direct quenching of Trp fluorescence by optically transparent ligands with low or high affinities; binding of optically transparent, non-fluorescent ligands to two specific sites where both sites or only one site result in Trp fluorescence quenching; and chromophores whose absorption either overlaps the Trp emission and quenches by energy transfer or absorbs light at the Trp fluorescence excitation wavelength producing absorptive screening as well as fluorescence quenching.

Unless identification of the site specificity of drug binding to serum albumin is desired, quenching of the Trp fluorescence of albumin by titration with ligand is a rapid and facile method for determining the binding affinities of drugs for serum albumin.

Many drugs and other small molecules bind reversibly to albumin and other serum components which then function as de-facto carriers. Serum albumin, in particular, often increases the apparent solubility of hydrophobic drugs in plasma and modulates their delivery to cells in-vivo and in-vitro, but reduces the concentration of free pharmacophore, which determines the physiological activity. Because albumin is the most abundant protein (35–50 mg mL^{-1}) in plasma, it can play a dominant role in drug disposition and efficacy (Olson & Christ 1996). Consequently, it is important to know the affinity of a drug for serum albumin even if it is not the only factor predictive of serum concentrations of the free drug. Other serum components, such as α_1-acid glycoprotein and lipoproteins, might also act as carriers (Hervé et al 1994).

Fluorescence spectroscopy has been the most widely used method of choice for monitoring drug-binding to plasma albumin (Oravcová et al 1996) because of its sensitivity, accuracy, rapidity and ease of use. In comparison, other conventional approaches reviewed by Oravcová et al (1996) using affinity and size-exclusion HPLC, equili-

Correspondence: D. E. Epps, Discovery Technologies, Pharmacia and Upjohn, Inc., Mailstop 7295-209-1, 301 Henrietta Street, Kalamazoo, MI 49007, USA.

brium dialysis, ultrafiltration and ultracentrifugation all suffer from lack of sensitivity or long analysis times, or both, making them impractical for determination of affinity constants for a large number of compounds. Also, low sensitivity imposes the use of protein concentrations far in excess of the dissociation constant for the drug–protein complex, possibly precluding the calculation of real affinity constants.

We previously described experimental methods for calculating binding affinities for non-fluorescent ligands toward serum albumin by displacement of site-specific fluorescent probes—warfarin, dansylamide, or danyslsarcosine (Epps et al 1995). These fluorescent probes enable one to work at longer wavelengths and, hence, with fewer chances of interference by chromophores on the test ligands. Although the displacement of a site-specific probe determines the identity of the binding site, this method also is limited to the two primary binding sites of albumin.

It has been reported that the binding of certain ligands to albumin is accompanied by changes in the intrinsic tryptophan (Trp) fluorescence of albumin (Jun et al 1971, 1972; Sudlow et al 1975, 1976; Soilene & Means 1979; He & Carter 1992). In our current work we explored whether this fluorescence could provide the basis of a convenient method for measuring the affinity of non-fluorescent ligands toward albumin, irrespective of the site-specificity of binding. The method, being a direct titration, lends itself to the testing of different binding models and it is ideally suited for detecting binding to more than one specific site. However, because of the short wavelength of the useful range of the Trp excitation spectrum where the numerous tyrosines are not excited (Peters 1996), e.g. 295–305 nm, optical interference, especially from inner filter effects, by the test compound is more likely to occur. We show here that the Trp fluorescence quench method provides a signal strong enough for study of the binding of a variety of non-fluorescent ligands to one or more sites on albumin, thus obviating the need for assays specific to the test compounds. In addition, we have also developed means of correcting for optical interferences when the method is used to measure affinity constant more accurately.

Materials and Methods

Materials

Essentially fatty acid- and globulin-free human serum albumin and Fraction V powdered human serum albumin (96–99%) were both obtained from Sigma (St Louis, MO; #A-3782 and A-1653, respectively), as was Trizma base. All new chemical entities reported in this manuscript were synthesized and purified by chemists at Pharmacia and Upjohn. They are identified below by their compound numbers and spectral properties; their chemical structures are proprietary and incidental to the theme of this report, namely novel experimental methods and data analysis.

Fluorescence measurements

A Cary 2200 spectrophotometer was used to measure absorption spectra; fluorescence was measured by means of an ISS K2 spectrofluorimeter in the ratio mode with temperature maintained by a Lauda RM6 circulating bath. The intrinsic Trp fluorescence of HSA was observed through an Ealing 340 ± 5 nm centre-wavelength filter with excitation at 285 nm. Albumin stock solutions were prepared the day of the experiment and were never stored. Concentrated drug solutions were prepared in dimethylsulphoxide (DMSO) or buffer and diluted when appropriate. Initial fluorescence readings of HSA were obtained in 2 mL 25 mM Tris + 25 mM NaCl, pH 7·4, followed by subsequent readings after the sequential addition of small amounts of drugs. The final DMSO concentrations never exceeded 1% (v/v), and all fluorescence readings were corrected for the dilution effect. The presence of this volume of DMSO in the assay mixtures had no effect on the fluorescence measurements.

Theory and data analysis

Binding of an optically transparent, non-fluorescent compound to a single, specific site on albumin. The binding of a compound or ligand, at concentration L, to a given binding site on albumin, at concentration H, to form a complex, at concentration C, occurs, in general, through a single-step, rapid thermodynamic equilibrium according to the scheme:

$$H + L \overset{K_1}{\Longleftrightarrow} C \qquad (1)$$

where K_1 is the dissociation constant of the ligand–albumin complex. The simplest experimental method for determining K_1 consists in keeping one of the analytical concentrations, either H_0 or L_0, constant at a value lower than K_1 and an excess of the other reagent at a series of concentrations covering the range from below K_1 to above K_1. Under these conditions, the concentration-dependence of the complex is given by a simple Langmuir isotherm:

$$C = H_0L_0/(K_1 + H_0) \quad (2)$$

for L_0 constant and H_0 variable, or:

$$C = H_0L_0/(K_1 + L_0) \quad (3)$$

for H_0 constant and L_0 variable.

If the binding of a non-fluorescent ligand changes the molar emissivity of albumin from α to β, the fluorescence of the reaction mixture, F, is given by:

$$F = \alpha H + \beta C = \alpha H_0 + (\beta - \alpha)C \quad (4)$$

and thus:

$$C = (F - \alpha H_0)/(\beta - \alpha) \quad (5)$$

For H_0 constant and L_0 variable, equation 3 then becomes:

$$F = F_0 - [(F_0 - F_\infty)L_0]/(K_1 + L_0) \quad (6)$$

where F_0 is the fluorescence emission intensity of the albumin solution in the absence of the ligand, $F_0 = \alpha H_0$, and F_∞ is that of the solution of albumin saturated with ligand, $F_\infty = \beta H_0$. By use of equation 6 the experimental dependence of F on L_0 can be analysed by non-linear least-squares regression to yield best-fit values for F_0, F_∞, and K_1.

However, the method using L_0 constant and H_0 variable is not well-suited for measuring ligand binding by the changes induced in albumin fluorescence, because upon binding of the ligand the fluorescence of the solution:

$$F = \alpha H_0 - [(\alpha L_0 - \beta L_0)H_0]/(K_1 + L_0) \quad (7)$$

undergoes a small change overlaid by a large, linearly increasing background contributed by the excess albumin.

For optimum data analysis the experimental conditions should be chosen such that the albumin concentration does not exceed the value of K_1 and such that the ligand concentration is much higher than its limit of detectability. Thus, for ligands with high affinity toward albumin, one is forced to use conditions when H_0, L_0 and K_1 are all of comparable magnitude. The concentration of the complex is then given by the quadratic equation:

$$C = \tfrac{1}{2}[H_0 + L_0 + K_1 - \sqrt{\{(H_0 + L_0 + K_1)^2 - 4H_0L_0\}}] \quad (8)$$

and the fluorescence of the solution is:

$$F = F_0 - [(\alpha - \beta)/2](H_0 + L_0 + K_1 - \sqrt{\{(H_0 + L_0 + K_1)^2 - 4H_0L_0\}}) \quad (9)$$

Equation 9 can then be used to analyse, by non-linear least-squares regression, the experimental plots of F against L_0 obtained at constant H_0 to calculate the best-fit values not only for α, β, and K_1, but also H_0. When the concentrations of albumin and ligand are comparable, one can also use conditions of constant L_0 and variable H_0, because the background albumin emission will not overwhelm the fluorescence changes induced by ligand binding. The same equation 9 is also applicable to the analysis of such data.

Binding of an optically transparent, non-fluorescent compound to two independent specific sites on albumin. If the affinity toward both sites is low enough to enable the use of the experimental conditions $L_0 \gg H_0 \approx K_1$, the experimentally observed ligand concentration-dependent changes in albumin fluorescence are simply additive:

$$F = F_0 - \Delta F_1 L_0/(K_1 + L_0) - \Delta F_2 L_0/(K_2 + L_0) \quad (10)$$

where K_1 and K_2 are the dissociation constants associated with sites 1 and 2 and ΔF_1 and ΔF_2 are the changes in albumin fluorescence associated with ligand binding at the two sites. The experimental dependence of F on L_0 is readily analysed by non-linear least-squares regression using equation 10 to yield K_1 and K_2. If ligand binding to one of the two sites does not produce any fluorescence changes, then the data obtained under the conditions $L_0 \gg H_0 \approx K_1$ conform to equation 6 and provide no indication whatsoever about the presence of a second binding site.

If the bimodal binding occurs to one high-affinity site and one low-affinity site, then the experiments may be performed under the conditions $H_0 > K_1$ and $H_0 \ll K_2$. Then, if binding to both sites produces a fluorescence change, titration with ligand should reflect both equation 6 and equation 9, i.e., it should obey the relationship:

$$F = F_0 - \tfrac{1}{2}\Delta F_1(H_0 + L_0 + K_1 - \sqrt{\{(H_0 + L_0 + K_1)^2 - 4H_0L_0\}}) - \Delta F_2 L_0/(K_2 + L_0) \quad (11)$$

High-affinity binding to both sites imposes the use of conditions when H_0, L_0 and K_1 are all of comparable magnitude. The rapid, reversible binding to the two independent binding sites is represented by the equilibria:

$$H_1 + L \overset{K_1}{\Longleftrightarrow} C_1 \quad (12)$$

$$H_2 + L \overset{K_1}{\Longleftrightarrow} C_2 \quad (13)$$

where H_1 and H_2 represent the concentrations of the two unoccupied sites and C_1 and C_2 are the con-

centrations of the corresponding ligand–albumin complexes. The analytical concentrations of the two sites are equal to that of the albumin molarity:

$$H_0 = H_1 + C_1 = H_2 + C_2 \quad (14)$$

and the dissociation constants are defined as:

$$K_1 = H_1L/C_1 \text{ and } K_2 = H_2L/C_2 \quad (15)$$

By substitution we obtain:

$$K_2 = [(H_0 - C_2)L]/C_2 \quad (16)$$

Thus, C_2 can be expressed as a function of L:

$$C_2 = H_0L/(K_2 + L) \quad (17)$$

From the definition of K_1:

$$K_1 = H_1L/(H_0 - H_1) \quad (18)$$

one can express L as a function of H_1:

$$L = K_1(H_0/H_1 - 1) \quad (19)$$

With $C_1 = H_0 - H_1$ and L_0, the analytical ligand concentration, being $L_0 = L + C_1 + C_2$, we obtain an expression for L_0 as a function of H_1:

$$L_0 = K_1H_0/H_1 - K_1 + H_0 - H_1 + H_0(1 + K_2[K_1H_0/H_1 - K_1]) \quad (20)$$

or

$$L_0 = (H_0 - H_1)[1 + K_1/H_1 + H_0(H_0 + H_1 \{K_2/K_1 - 1\})] \quad (21)$$

If the albumin fluorescence does not change upon binding to the H_2 site, then the fluorescence of the reaction mixture, F, depends only on the ligand binding to the H_1 site: $F = \alpha H_1 + \beta C_1$ where $\alpha \gg \beta$. In terms of F_0 and F_∞ we then obtain:

$$F_0 = F_\infty + [(F_0 - F_\infty)/H_0]H_1 \quad (22)$$

and thus:

$$H_1 = [(F - F_\infty)/(F_0 - F_\infty)]/H_0 \quad (23)$$

Substitution into equation 20 yields:

$$L_0 = (F_0 - F)[H_0/(F_0 - F_\infty) + K_1/(F - F_\infty) + H_0/\{F_0 - F + K_2/K_1(F - F_\infty)\}] \quad (24)$$

Thus, F as a function of L_0 is a third-order equation and it is not practical to use the solution of equation 23 for F as a function of L_0 for data analysis. Rather, the experimental dependence of F on L_0 can be analysed by non-linear least-squares regression using equation 23 directly, thereby reversing the independent and dependent variables. If the experimental error is truly random, reasonably small, and non-proportional, the best-fit values of K_1 and K_2 do not change significantly upon such reversal of the variables.

Compounds containing chromophores that absorb at the wavelength of excitation or emission, or both. Some ligands contain chromophores that, in the concentration range used, absorb a significant amount of the light used to excite the albumin Trp or the light emitted by it, or both. The usual geometry of the spectrofluorimeter consists in the exciting beam being collimated to the centre of a square cuvette with the detector also focused to that small region. Using this configuration and assuming that the absorptivities are the same for the free and the albumin-bound ligands, the experimentally measured F can be expressed as the uncorrected fluorescence, F_u, given by equation 6 or equation 9, multiplied by a term representing the absorption by the ligand. According to Beer's law, we then have:

$$F = F_u e^{-2.303\varepsilon l L_0} \quad (25)$$

where ε is the molar absorptivity of the ligand, and l is the path-length over which the exciting or emitted light is absorbed— $\sim 0{\cdot}5$ cm with the usual geometry. It cannot be overemphasized that a given set of experimental data should be analysed by all the possible models. With non-linear least-squares regression analyses the goodness of the fits to the various binding models should be judged using information theoretical criteria or AIC values (Akaike 1972), or an appropriate *F*-Test. Also, plotting of the residuals is recommended for revealing systematic errors and, hence, the inadequacy of the model. The first indication that a given model has too many parameters is often that the standard deviations of the calculated parameters are exceedingly large.

Results

Binding of an optically transparent compound to serum albumin

Ligands can quench the intrinsic Trp fluorescence of HSA upon binding as demonstrated by compound **1**. As shown in Figure 1, titration of HSA with compound **1** produces a saturable, concentration-dependent decrease in the Trp fluorescence of the protein. These data, analysed using equation 9, were fully consistent with this equation as shown by the goodness of fit yielding a reasonable K_1 value (Table 1) and a predicted stoichiometry of $0{\cdot}7 \pm 0{\cdot}3$ mol binding sites mol^{-1} HSA.

The reproducibility of the method can be demonstrated by comparing K_1 values calculated using equation 6 for another compound on three

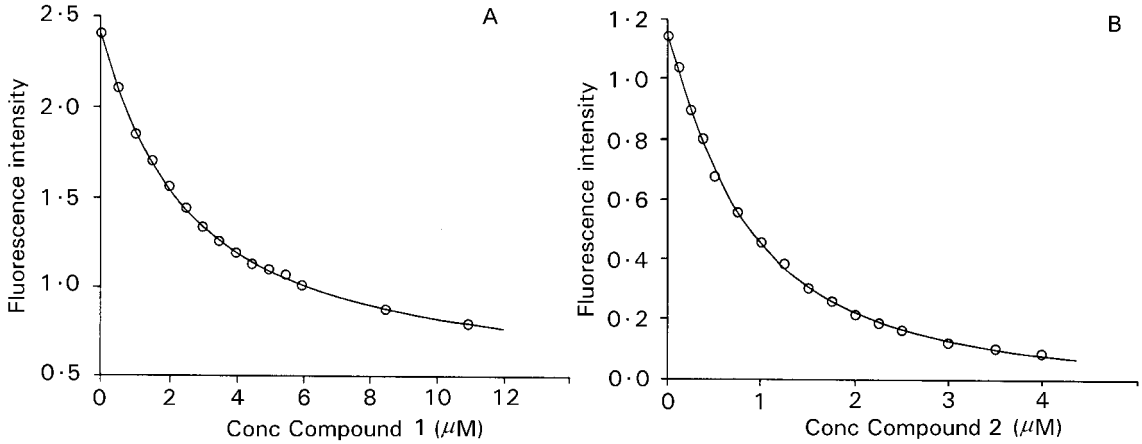

Figure 1. Binding of optically transparent compounds to HSA and inner filter effects. HSA, 1 μM, was titrated with optically transparent compound **1** (A) and with compound **2** which contains a chromophore that absorbs at the Trp emission wavelength (B) and the dose-dependent decrease in Trp fluorescence was followed. Data for both compounds were analysed by use of equation 9.

Table 1. Parameters for binding of structurally unrelated compounds to human serum albumin.

Compound	K_{11}* (μM)	K_{12} (μM)	$\varepsilon_{calc} \times 10^{-3}$ ($M^{-1}cm^{-1}$)	$\varepsilon_{expt} \times 10^{-3}$† ($M^{-1}cm^{-1}$)	Model equation
1	2·2 ± 0·2	–	–	–	9
2	0·48 ± 0·07	–	–	–	9
3	<0·10	3·6 ± 0·8	–	–	11
4	0·54 ± 0·05	–	40·8	42·5 ± 1·5	9 and 25
5	18·5 ± 2·1	–	2·90	3·00 ± 0·40	6 and 25
6	0·10 ± 0·02	2·1 ± 0·08	–	–	23

*Mean value calculated from the non-linear least-squares fit of the data (± s.d.). †Mean value of results from three measurements using different concentrations of HSA (± s.d.).

different occasions using different HSA and drug-stock solutions (data not shown). Values of 1·2, 1·3 and 1·5 μM were obtained. To examine whether the presence of fatty acids affects the Trp fluorescence quench response, measurements were made with this same compound using Fraction V HSA. A K_1 of 1·0 ± 0·3 μM was in good agreement with the mean K_1 of 1·37 ± 0·15 μM obtained using fatty acid-free HSA. Similar K_1 can be obtained using bovine serum albumin also (result not shown). The influence of other endogenous ligands, e.g. bilirubin, on Trp fluorescence was not examined.

Binding of a compound for which the UV absorption overlaps the Trp emission spectrum

A compound with UV absorption that overlaps the Trp emission spectrum should quench that emission not only by the effects of subtle changes in the environment, but also by energy transfer, provided that the binding occurs in close proximity to the Trp residue. With energy transfer operating, the data should still conform to equation 6 or equation 9, but the changes in fluorescence should be much more pronounced and could, in fact, reach $F_\infty = 0$. We found an example of such a behaviour in compound **2**, which has an absorption maximum at 325 nm, but a negligible fluorescence emissivity at the high-wavelength end of the UV spectrum. Addition of compound **2** to a fixed amount (1 μM) of HSA results in a concentration-dependent decrease in the Trp fluorescence of HSA which approaches zero at high ligand concentrations (Figure 1). The data analysed using equation 9 were in good agreement with this model, as shown by the fit of experimental data points with the theoretical curve. The analysis indicated that compound **2** bound HSA with high affinity (Table 1) and that a predicted concentration of HSA of 0·7 ± 0·1 μM is in good agreement with the analytical concentration of 1 μM.

Binding of a compound to multiple sites on serum albumin

Figure 2 shows that when a fixed amount (1 μM) of HSA is titrated with compound **3**, the concentration-dependent decrease in Trp fluorescence is biphasic with a discontinuity at $\sim$1 μM ligand. Because the ligand does not absorb in the spectral region of the experiment, the biphasic behaviour is

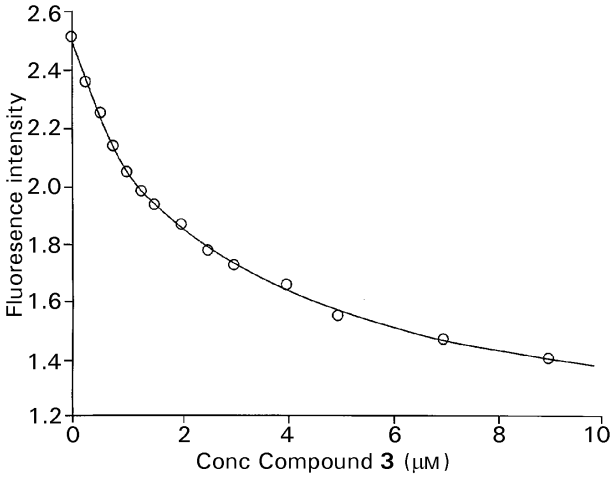

Figure 2. Stoichiometric binding of an optically transparent compound to two specific sites on HSA. HSA, 1 μM, was titrated with compound **3**. Data were analysed by use of equation 11.

probably because of binding of the ligand to two independent sites with each interaction producing some change in Trp fluorescence. Indeed, when the data were analysed by the various binding models, we obtained the best fit with equation 11. In addition to one low- and one high-affinity binding site (Table 1), values of 0·075 and 0·53 were calculated for $\Delta F_1/F_0$ and $\Delta F_2/F_0$, respectively. The predicted stoichiometry of $0{\cdot}95 \pm 0{\cdot}11$ mol binding sites per albumin molecule was again in good agreement with the analytical HSA concentration of 1 μM.

High-affinity binding of a compound that absorbs light at the excitation wavelength

At the concentrations used in the binding experiments the absorbance of compound **4** was significant at 295 nm, the wavelength used for excitation of the Trp residue. Thus this compound is expected to cause significant absorptive screening. As shown in Figure 3, addition of increasing amounts of compound **4** to 0·5 μM HSA results in a biphasic fluorescence decrease. An initially steep decrease, indicative of high-affinity binding, is followed by a more shallow decrease approaching zero at high ligand concentrations. The data were analysed by a variety of models with a combination of equation 9 and equation 25 giving the best fit to the experimental data. The analysis calculated a low affinity K_1, $F_0 - F_\infty$ of 4·2, and an H_0 of $0{\cdot}39 \pm 0{\cdot}08$ μM (Table 1). Further validation of this model is obtained by comparing the calculated and measured values of ε (Table 1).

Another example illustrating low-affinity binding with inner filter effect

Addition of compound **5** to 1 μM BSA produces a decrease in Trp fluorescence, again, with F approaching zero at high ligand concentrations (Figure 3). The data were best fit by a combination of equation 6 and equation 25 yielding a low-affinity K_1, $F_0 - F_\infty$ of 0·9, and an ε value identical with the independently-measured value (Table 1).

Thus equation 25 accounts quantitatively for the drift that follows the fluorescence changes produced upon binding. The hallmark of an inner filter effect is the absence of a clear plateau at high ligand concentrations. In any case, when the inner filter effect is suspected it should be confirmed independently by measuring the UV absorption spectrum of the ligand or by measuring the fluorescence emission of a Trp solution in the presence of increasing concentrations of the ligand.

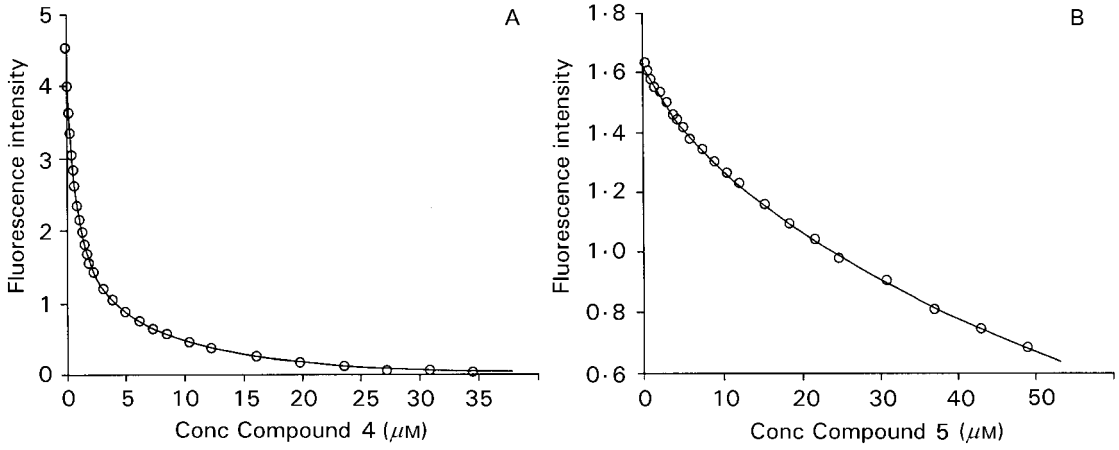

Figure 3. Stoichiometric binding of compound to HSA with external quenching (narrow-beam model). Both compounds contain a chromophore that absorbs at the excitation wavelength. A. HSA, 0·5 μM, was titrated with compound **4** and the data were analysed using equation 9 corrected for Beer's Law (equation 25). B. The binding of compound **5** to HSA, 1 μM, was fit to a simple Langmuir isotherm (equation 6) corrected for Beer's Law (equation 25).

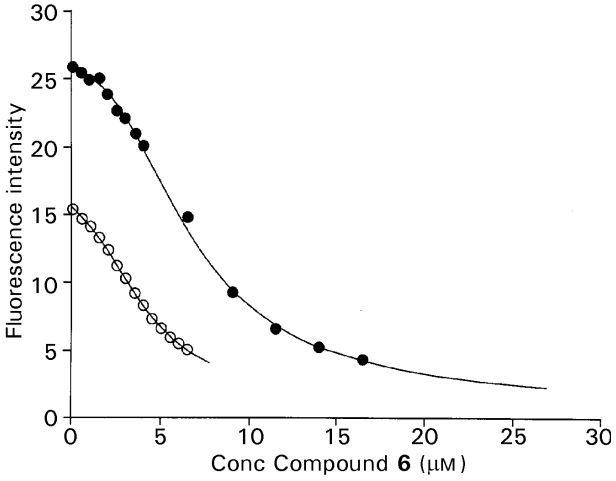

Figure 4. Two-site binding with only one site quenching Trp fluorescence. HSA (●, 4 μM; ○, 2 μM) was titrated with optically transparent compound **6** and the change in Trp fluorescence was monitored as a function of added ligand. The data were analysed by use of equation 23.

Binding of a compound to two HSA sites with only one site causing a change in fluorescence

Another type of unexpected behaviour was observed when HSA was titrated with compound **6**. The decrease in the Trp emission of HSA as a function of the added ligand produced a sigmoidal response (Figure 4). Similar data were obtained when the experiment was repeated using one-half the amount of HSA. These data were analysed by the various single and multiple site models, but were found to be best fit by use of equation 23 corresponding to a two-site binding model with quenching produced by only one of the binding sites. Simultaneous analysis of the two data sets shown in Figure 4 identified a low- and higher-affinity binding site (Table 1) and H_0 concentrations of $1{\cdot}6 \pm 0{\cdot}1$ and $3{\cdot}5 \pm 0{\cdot}2$ μM. These values are in good agreement with the analytical concentrations of 2 and 4 μM HSA that were used.

Discussion

Warfarin and dansylsarcosine (Wanwimolruk & Birkett 1986) are fluorescent probes specific for the Sudlow site I or domain IIA and Sudlow site II or domain IIIA binding sites, respectively, of albumin (He & Carter 1992). These sites bind the majority of compounds with a large amount of structural heterogeneity. We have previously reported that the affinity constants and site-specificity of ligand binding to HSA can be determined by displacing these fluorescent probes with non-fluorescent ligands (Epps et al 1995). Often, especially during the early phase of template selection of drug candidates, the site-specificity of the HSA-bound ligand is not essential information. The project team often only desires to know whether the ligand binds to serum albumin and, if so, the affinity of this interaction. To provide these data in a timely manner by avoiding compound-dependent assays it is easier to titrate albumin with the test compounds and use quenching of the intrinsic Trp fluorescence as the signal. However, these measurements are made at an excitation wavelength of 295 nm; a spectral region where there is a far greater likelihood of encountering compounds the absorption of which lies close to or within this range. Compounds that absorb in this region will cause absorptive screening and, if their absorption wavelengths are sufficiently toward the red (> 300 nm), they can quench the Trp fluorescence by a combination of changing protein conformation and energy transfer. Furthermore, a compound might bind to more than one site on HSA, but produce a spectral change at only one of the sites. Thus, although the optical properties of the compound can produce obstacles to the calculation of accurate affinity constants from Trp fluorescence quenching data, these problems are not insurmountable.

Three amino acid residues—Trp214, Lys199 and Tyr411—are probably involved in the binding of most ligands to HSA (Sudlow et al 1975; Soilene & Means 1979; Ozeki et al 1980; Kurono et al 1987). The single Trp residue of HSA is conserved in all mammalian albumins and probably limits solvent accessibility to the IIA binding site while participating in hydrophobic interactions at the interface between sites IIA and IIIA (He & Carter 1992; Peters 1996). Consequently, this Trp residue is positioned strategically where quenching of its fluorescence can be used to monitor ligand binding at either major binding site, albeit more sensitively for site-IIa binding (Chignell 1970; Sudlow et al 1973). The exact mechanism(s) by which the fluorescence is quenched is unclear, but might result from increased solvent accessibility, reduced hydrophobic interactions, etc., as a result of ligand-induced changes in protein conformation. The concept in itself is not new because steroids have been shown to dose-dependently quench bovine serum albumin fluorescence excited at 285 nm (Attallah & Lata 1968). Thus, with the appropriate experimental conditions and data analysis, saturable quenching of HSA Trp fluorescence can be an ideal signal for easily demonstrating the binding of ligands to HSA.

We present examples of structurally unrelated ligands, encountered during the application of this method to various projects, that have optical or other properties which interfere with analysis of

these simple Langmuir binding data. We used the tools of quantitative analysis to derive the equations necessary to account for optical interferences and other deviations from these simple binding models and apply these models using non-linear least-squares numerical analysis. Although we have not shown the chemical structures of the ligands discussed, nor identified their specific sites of binding to HSA, the cases outlined will enable the user to demonstrate compound-albumin interaction for a variety of chemical entities and to calculate affinity constants. The researcher must know the spectral properties of the test compound to identify the possible optical effects. This assay and the analytical approaches described are being used routinely within the drug-discovery process early during lead selection. The information is often used to help interpret in-vitro potencies where albumin is present in the assay, such as cell-based assays containing dilute foetal bovine serum, and in-vivo efficacy whereby albumin can have an impact on the pharmacokinetics and pharmacodistribution of a chemical class. On average it takes 1 h (approx.) per compound to titrate albumin and to analyse the data in the absence of custom software. The Trp fluorescence quench assay is not a high-throughput procedure, but future identification or synthesis of albumin-interacting ligands with fluorescence in the visible wavelengths would make competitive binding assays suitable in a 96-well plate-reader format.

Acknowledgement

We are grateful to Dr Ferenc Kézdy for some of the mathematical derivations used in this work and to Dr Maria Grandi for her support of the research.

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