

Fluorometric Determination of Drug-Protein Association Constants: Binding of Pamaquine by Bovine Serum Albumin

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Abstract □ The binding of pamaquine to bovine serum albumin is accompanied by the enhancement of the fluorescence efficiency of the former but without shifting its fluorescence energy. This phenomenon was used to evaluate the stoichiometry and strength of the binding. The results indicate that three singly protonated pamaquine molecules are bound by each bovine serum albumin molecule. The individual binding constants were calculated by using the Bjerrum technique. The average values of the three constants were $K_1 = 6.4 \times 10^7$, $K_2 = 3.1 \times 10^6$, and $K_3 = 1.9 \times 10^5$, indicating that, compared to anionic drugs and fluorescent probes, pamaquine is very strongly bound by the protein.

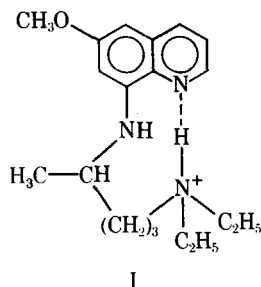
Keyphrases □ Pamaquine—binding by bovine serum albumin, fluorometric determination of drug-protein association constants □ Bovine serum albumin—pamaquine binding, fluorometric determination of drug-protein association constants □ Binding—pamaquine to bovine serum albumin, fluorometric determination of association constants □ Fluorometry—determination, drug-protein association constants, binding of pamaquine by bovine serum albumin

Recently, it was observed in this laboratory that the fluorescence intensity of the antimalarial pamaquine was enhanced in the presence of bovine serum albumin at pH 7.4. Consequently, it was felt that by systematically studying the variation of the quantum yield of fluorescence of pamaquine as a function of relative protein concentration, the parameters of the binding of the drug to the protein could be quickly and reasonably accurately established.

EXPERIMENTAL

Purified pamaquine phosphate¹ and crystalline bovine serum albumin of A grade² were used. The concentrations of bovine serum albumin solutions were calculated from the weight of the sample and then checked by determining absorbance at 280 nm. The average molecular weight of 69,000 and the molar absorptivity of 4.55×10^4 at 280 nm were used in the calculations. The bovine serum albumin concentrations determined directly by weighing were in excellent agreement with the concentrations determined photochemically.

All bovine serum albumin and pamaquine solutions were prepared 2 hr prior to fluorometric titration from distilled, deionized water buffered to pH 7.4 with a total phosphate ($[\text{H}_2\text{PO}_4^-] +$



I

$[\text{HPO}_4^{2-}]$ concentration of 0.1 M. The fluorometric titrations were carried out as follows: 2.0 ml of the protein solution of appropriate concentration in a 1-cm silica cell was titrated by successive additions of 1- μl volumes of a 1×10^{-3} M solution of pamaquine, delivered from an air- and liquidtight microsyringe³. The temperature during the titrations was maintained at 25°. The fluorescent intensity measurements were made on an uncorrected fluorescence spectrophotometer⁴ with excitation at 367 nm and fluorescence monitored at 518 nm.

DISCUSSION

Pamaquine (I) exists in its singly protonated form at pH 7.4, and an intramolecular hydrogen bond is present between the protonated diethylamino group and the ring nitrogen (1).

It has been suggested (1) that in the thermally equilibrated, excited state, the proton would be covalently bonded to the ring nitrogen and electrostatically hydrogen bonded to the diethylamino group. When pamaquine is bound to bovine serum albumin at pH 7.4, the fluorescence band does not shift but the fluorescence intensity is enhanced. The ratio of the quantum yields of the bound pamaquine to that of the pamaquine is 2.5. The fact that the fluorescence band of pamaquine does not shift on binding indicates that the intramolecular hydrogen bond of pamaquine is not broken in the process of binding to the protein, because any change in the electron density around the ring nitrogen would result in shifting of the fluorescence band.

For substrates like pamaquine, which have some fluorescence in the free form and whose fluorescence is enhanced without a change in the emission wavelength when bound to a protein, the fraction of substrate bound, α , can be determined by using Eq. 1 (see Appendix for derivation):

$$\alpha = \frac{F_p - F_f}{F_b - F_f} \quad (\text{Eq. 1})$$

where F_p and F_f are the fluorescence intensities of a given concentration of substrate in solutions of low protein concentration and in solutions without any protein, respectively; and F_b is the fluorescence intensity of the same concentration of the fully bound substrate. The latter is taken to be the fluorescence intensity of the substrate in the presence of excess protein. Such a treatment will yield values of α , provided the fluorescence intensities of the bound pamaquine and of the free pamaquine are linear functions of the respective concentrations.

The value of F is calculated using:

$$F = \phi I_0 \left[2.3A - \frac{(2.3A)^2}{2!} + \frac{(2.3A)^3}{3!} - \frac{(2.3A)^4}{4!} + \dots \right] \quad (\text{Eq. 2})$$

where ϕ is the quantum yield of the emitting species, I_0 is the intensity of the exciting radiation, and A is the absorbance. The linearity of F with the concentration of the emitting species can only be taken for granted at very low absorbance (<0.02) at the exciting wavelength, in which case the higher power terms in Eq. 2 become negligible by comparison with the first term. For example, when $A > 0.02$, the deviation from linearity is greater than 2%. At absorbances of $0.02 < A < 0.15$, the second term in Eq. 2 results in 115% deviation from linearity at any point in the fluorometric titration. At higher absorbances ($A > 0.15$), the third term in Eq. 2 generally also becomes large enough to have to be considered.

Several fluorometric titrations were carried out by increasing

* Control No. N432UE, Sterling-Winthrop Research Institute, Rensselaer, N.Y.

² Lot No. 300597 (Pentex brand product of Miles Laboratory), Calbiochem, San Diego, Calif.

³ Unimetrics.

⁴ Model MPF-2A, Perkin-Elmer, Norwalk, Conn.

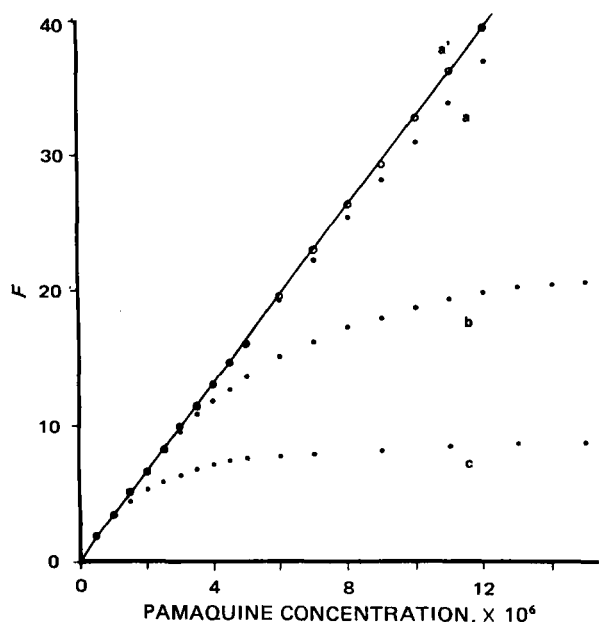


Figure 1—Plots of relative fluorescence intensities, F , as a function of total pamaquine concentration for the bovine serum albumin-pamaquine titrations with a constant amount of the protein (P_T). Key: curve a, $P_T = 2.5 \times 10^{-5}$ and 5×10^{-5} M; curve b, $P_T = 2.5 \times 10^{-6}$ M; and curve c, $P_T = 1 \times 10^{-6}$ M. The straight line a' is obtained after correcting the fluorescence intensities in curve a for the absorbance effect (see text).

the initial protein concentration. The titrations with the protein concentrations of 2.5×10^{-5} and 5.0×10^{-5} M both gave exactly the same net enhancement in fluorescence intensity for a given amount of pamaquine, indicating that at these concentrations of bovine serum albumin the added pamaquine was essentially in the bound form. At the excitation wavelength, 367 nm, the molar absorptivity for the pamaquine-bovine serum albumin system was determined to be 5.2×10^3 liters mole $^{-1}$ (pamaquine) cm $^{-1}$. Hence, at pamaquine concentrations greater than 3.85×10^{-6} M ($A = 0.02$), the deviation from linearity will be greater than 2%, and appropriate corrections to the observed intensities should be applied to compensate for the second term in Eq. 2.

For the two titrations with the high protein concentrations (2.5×10^{-5} and 5.0×10^{-5} M) and for the titration with no protein, the observed fluorescence intensities (F_b and F_f , respectively) at every point during the titration were corrected by applying the correction factor 1.15A. Curve a in Fig. 1 was plotted using the observed values of $F_b - F_f$. After applying the correction to the observed fluorescence intensities, line a' was obtained, verifying that the deviation from linearity of curve a was indeed due to the absorbance effect.

When the titrations are carried out at low protein concentration so that the substrate is only partially bound, the fluorescence intensity, F_p , is the sum of the fluorescence intensities of both the bound and unbound forms of pamaquine; therefore, F_p cannot simply be corrected for the second term in Eq. 2 unless the concentration of bound (or free) pamaquine is known in advance. To circumvent this problem, the concentration of bound pamaquine was estimated from Eq. 1 using the uncorrected F_p values and these approximate values were then used to correct the F_p values for the absorbance effect. Curves b and c in Fig. 1 are the plots of corrected $F_p - F_f$ values versus total concentration of pamaquine for bovine serum albumin concentrations of 2.5×10^{-6} and 1.0×10^{-6} M, respectively. The binding parameters were calculated by using the corrected values of F_f , F_p , and F_b .

The maximum number of binding sites, N , was determined from Job's plot (2, 3) as follows. First, the fluorescence intensity of pamaquine in pH 7.4 buffer was measured for solutions having concentrations from 0 to 1.0×10^{-5} M (line a in Fig. 2). The total fluorescence intensities of several pamaquine-bovine serum albumin solutions, made by varying the mole fraction (χ) of pamaquine and

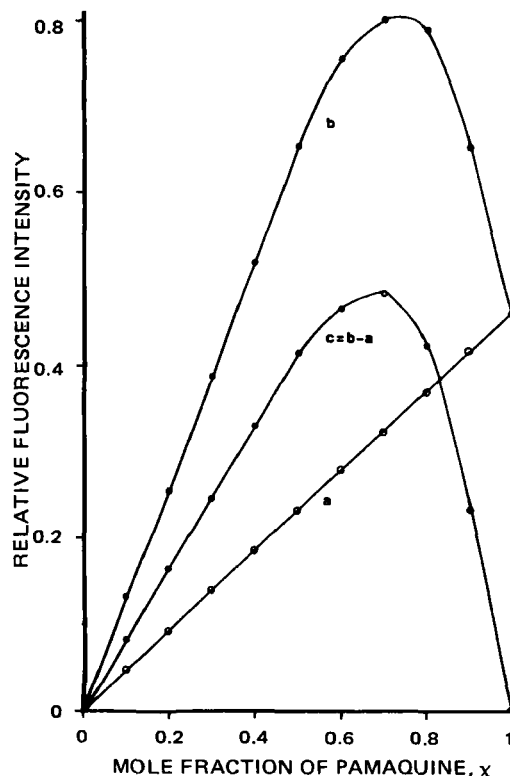


Figure 2—Job's plot for the bovine serum albumin-pamaquine binding. Line a is the variation of the fluorescence intensity with the concentration of pamaquine in the $0-1 \times 10^{-5}$ M range. Curve b is the total observed fluorescence intensity as a function of the mole fraction (χ) of pamaquine. Curve c is the apparent enhancement in the fluorescence intensity as a function of χ . The total concentration of the protein plus pamaquine was kept constant at 1×10^{-5} M.

keeping the total concentration of pamaquine plus bovine serum albumin at 1.0×10^{-5} M (curve b in Fig. 2), were then measured. The apparent enhancement of fluorescence intensity due to the binding at different mole fractions of pamaquine is the difference between curve b and line a in Fig. 2. Curve c is plotted with the apparent enhancement of fluorescence intensity as a function of mole fraction of pamaquine. The point of inflection is at $\chi = 0.74$, which corresponds to a ratio of pamaquine to bovine serum albumin of 2.85. This value indicates that the maximum number of binding sites on the protein molecule for pamaquine is three.

In the evaluation of protein binding equilibria, the commonly used equation is the Scatchard equation (4):

$$\frac{\bar{n}}{[D]} = Nk - \bar{n}k \quad (\text{Eq. 3})$$

where \bar{n} represents the ratio of moles of bound substrate per mole of protein, $[D]$ is the concentration of unbound substrate, N is the maximum number of binding sites on a protein molecule, and k is the binding constant. The value of \bar{n} can be calculated from the fraction of pamaquine bound by using the relationship:

$$\bar{n} = \alpha \frac{D_T}{P_T} \quad (\text{Eq. 4})$$

where D_T and P_T are the formal concentrations of pamaquine and bovine serum albumin, respectively. The concentration of unbound pamaquine was obtained by multiplying D_T by $(1 - \alpha)$. When the N binding sites are noninteracting and have the same binding affinities, a plot of $\bar{n}/[D]$ versus \bar{n} (known as the Scatchard plot) gives a straight line with the x -axis intercept at $\bar{n} = N$, the y -axis intercept at $\bar{n}/[D] = Nk$, and the slope of $-k$. However, if the individual binding sites have different binding affinities, due either to the distinct nature of the sites or to the interaction be-

Table I—Equilibrium Constants for the Binding of Pamaquine to Bovine Serum Albumin at 25°^a

	$P_T = 2.5 \times 10^{-6} M$		$P_T = 1.0 \times 10^{-6} M$		Average
	Scatchard Treatment	Bjerrum Treatment	Scatchard Treatment	Bjerrum Treatment	
$\log k_1$	7.81	7.82	7.80	7.78	7.80
$\log k_2$	6.55	6.37	6.60	6.43	6.49
$\log k_3$	5.36	5.15	5.45	5.21	5.29

^a P_T is the total concentration of the protein.

tween the bound substrates at different sites, the Scatchard plot gives a downward curve instead of a straight line.

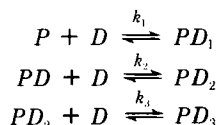
If the binding strength of the individual binding sites are significantly different from each other, the Scatchard plot may still be used to estimate binding parameters by appropriately analyzing the curve in relation to the following modified form of Eq. 3, where k_i is the binding constant for the i th site. Figure 3 shows the Scatchard plot for the bovine serum albumin-pamaquine system at the protein concentration of $2.5 \times 10^{-6} M$.

The curve obtained indicates that the three binding sites on bovine serum albumin for pamaquine have different binding strengths. Graphical analysis of this plot according to:

$$\bar{n} = \sum_{i=1}^{i=N} \frac{k_i [D]}{1 + k_i [D]} \quad (\text{Eq. 5})$$

yielded binding constants for the three sites. The values are listed in Table I along with the values obtained for another titration at $P_T = 1.0 \times 10^{-6} M$.

Multiple equilibria involving a small number of ligands can be best treated by the Bjerrum technique (5). This approach is applicable to any system in thermodynamic equilibrium and makes no presupposition concerning the equivalence of binding sites or the relative magnitude of successive equilibrium constants. In the case of the bovine serum albumin-pamaquine system, the three successive equilibria (Scheme I, P = protein and D = pamaquine) are characterized by equilibrium constants k_1 , k_2 , and k_3 such that:



Scheme I

$$k_1 = \frac{[PD]}{[P][D]} \quad (\text{Eq. 6})$$

$$k_2 = \frac{[PD_2]}{[PD][D]} \quad (\text{Eq. 7})$$

$$k_3 = \frac{[PD_3]}{[PD_2][D]} \quad (\text{Eq. 8})$$

The average number of bound pamaquine molecules per mole of protein can now be expressed as:

$$\bar{n} = \frac{[PD] + 2[PD_2] + 3[PD_3]}{[P] + [PD] + [PD_2] + [PD_3]} \quad (\text{Eq. 9})$$

which, when combined with Eqs. 6-8, gives:

$$\bar{n} = \frac{k_1 [D] + 2k_1 k_2 [D]^2 + 3k_1 k_2 k_3 [D]^3}{1 + k_1 [D] + k_1 k_2 [D]^2 + k_1 k_2 k_3 [D]^3} \quad (\text{Eq. 10})$$

Equation 10 predicts that if k_1 , k_2 , and k_3 are widely different from each other so that essentially only one equilibrium is experimentally detectable at any point in the titration, a plot of $-\log [D]$ versus \bar{n} (known as the Bjerrum plot) would show (for the present system) three distinct inflection regions corresponding to $-\log [D] = \log K_1$ when $\bar{n} = 0.5$, $-\log [D] = \log K_2$ when $\bar{n} =$

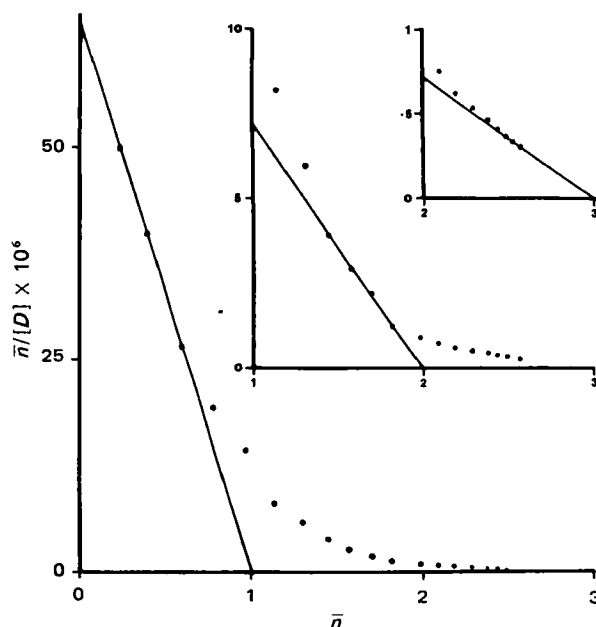


Figure 3—Scatchard plot of $\bar{n}/[D]$ versus \bar{n} for the bovine serum albumin-pamaquine titration with the protein concentration of $2.5 \times 10^{-6} M$. Insets are the plots with an expanded y-axis.

1.5, and $-\log [D] = \log K_3$ when $\bar{n} = 2.5$.

Figure 4 shows the Bjerrum plot of $-\log [D]$ versus \bar{n} for the bovine serum albumin-pamaquine titration ($P_T = 2.5 \times 10^{-6} M$). The absence of sharp inflection regions is indicative of overlapping successive equilibria. However, the plot does show shallow inflection regions at the expected values of \bar{n} ; therefore, $-\log [D]$ values at $\bar{n} = 0.5$, 1.5, and 2.5 can be taken as a fair estimate of $\log k_1$, $\log k_2$, and $\log k_3$, respectively. The results obtained from this Bjerrum plot are given in Table I along with the results obtained for another titration at $P_T = 1.0 \times 10^{-6} M$. The binding constants thus obtained are in good agreement with those estimated from the Scatchard treatment.

The overall magnitude of the binding constants obtained for the bovine serum albumin-pamaquine system is quite large compared, for example, to the binding of negatively charged 8-anilino-1-naphthalenesulfonate (6), indicating that pamaquine binds strongly to bovine serum albumin. Because of the positively charged nature of pamaquine, it is probable that the binding site on the protein for pamaquine is negatively charged. The most common anionic sites on bovine serum albumin consist of ionized carboxyl groups of various amino acids. There are about 37 free glutamic

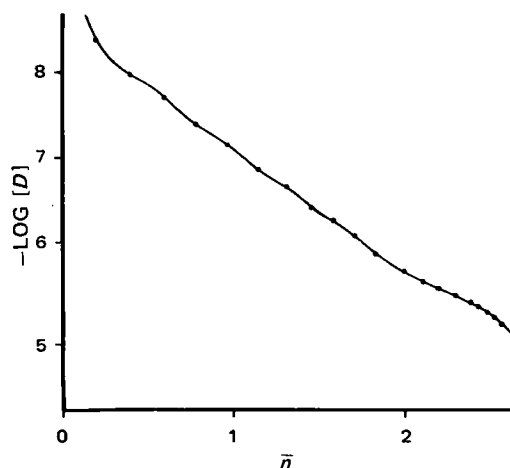


Figure 4—Bjerrum plot of $-\log [D]$ as a function of \bar{n} for the bovine serum albumin-pamaquine titration with the protein concentration of $2.5 \times 10^{-6} M$.

acid residues and 56 free aspartyl acid groups (7).

However, in spite of the presence of such a large number of negative centers on bovine serum albumin, only three pamaquine molecules bind to it. Apparently, not all negative centers are available for the binding of pamaquine. Clearly, many are buried in the interior of the protein. Moreover, it is possible that the binding sites of pamaquine are ionized carboxyl groups on the outside of the protein with a suitable juxtaposition of other substituents or side chains so as to make the pamaquine-bovine serum albumin interaction energetically favorable. Even though pamaquine is positively charged, the quinoline ring, the alkyl side chain, and the ethyl groups on the terminal amino nitrogen could make its interaction with water less favorable than with a site on the protein which is negatively charged and has nonpolar side groups.

APPENDIX

The following illustrates the derivation of the equation to calculate the fraction of substrate bound when bound and free substrate both fluoresce, but with different intensities, at the analytical emission wavelength.

If F_p is the observed fluorescence intensity of a solution containing a total protein concentration of P and a total substrate concentration of S , then:

$$F_p = F_U + F_B \quad (\text{Eq. A1})$$

where F_U and F_B are the fluorescence intensities due to the unbound and bound fractions of substrate, respectively.

Assuming that the absorbance, A , by the emitting species at the wavelength of excitation is less than 0.02, the fluorescence intensity is related to the concentration of emitting species as:

$$F = 2.303 I_0 \phi \epsilon c l \quad (\text{Eq. A2})$$

where I_0 = intensity of incident radiation, ϕ = quantum yield of the emitter, ϵ = molar absorptivity, c = concentration of the emitter, and l = path length.

Since $I_0 l$ is an instrumental constant, Eq. A2 can be rewritten as:

$$F = k \phi \epsilon c \quad (\text{Eq. A3})$$

Substituting Eq. A3 into Eq. A1 yields:

$$F_p = k(\phi_U \epsilon_U [U] + \phi_B \epsilon_B [B]) \quad (\text{Eq. A4})$$

where subscripts U and B stand for unbound and bound fractions of substrate, respectively. But since $S = [U] + [B]$, Eq. A4 can be rewritten as:

$$F_p = k[\phi_U \epsilon_U (S - [B]) + \phi_B \epsilon_B [B]] \quad (\text{Eq. A5})$$

Similarly, when all of the substrate is free, i.e., in the absence of protein, the fluorescence intensity F_f will be given by:

$$F_f = k \phi_U \epsilon_U S \quad (\text{Eq. A6})$$

and when all of the substrate is bound to protein, i.e., in presence of excess protein, the fluorescence intensity F_b will be given by:

$$F_b = k \phi_B \epsilon_B S \quad (\text{Eq. A7})$$

Taking the ratio of Eqs. A6 and A7 gives:

$$\phi_U \epsilon_U = \frac{F_f}{F_b} \times \phi_B \epsilon_B \quad (\text{Eq. A8})$$

Substituting Eq. A8 into Eq. A5 yields:

$$F_p = k \left[\frac{F_f}{F_b} \phi_B \epsilon_B (S - [B]) + \phi_B \epsilon_B [B] \right] \quad (\text{Eq. A9a})$$

$$F_p = k \phi_B \epsilon_B \left\{ \frac{F_f}{F_b} S - \frac{F_f}{F_b} [B] + [B] \right\} \quad (\text{Eq. A9b})$$

Dividing Eq. A9 by Eq. A7 gives:

$$\frac{F_p}{F_b} = \frac{\frac{F_f}{F_b} S - \frac{F_f}{F_b} [B] + [B]}{S} \quad (\text{Eq. A10})$$

Dividing throughout by S gives:

$$\frac{F_p}{F_b} = \frac{F_f}{F_b} - \frac{F_f [B]}{F_b S} + \frac{[B]}{S} \quad (\text{Eq. A11})$$

But $[B]/S = \alpha$, the fraction of substrate bound, and Eq. A11 becomes:

$$\frac{F_p}{F_b} = \frac{F_f}{F_b} - \frac{F_f}{F_b} \alpha + \alpha \quad (\text{Eq. A12})$$

Solving for α yields:

$$\alpha = \frac{F_p - F_f}{F_b - F_f} \quad (\text{Eq. A13})$$

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