

Study of the Interaction between Xylazine and Bovine Serum Albumin by Fluorescence Quenching Measurements

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The binding of xylazine to bovine serum albumin (BSA) was studied by fluorescence quenching, as a function of temperature. The experimental data could be fitted to both the Stern–Volmer equation and the Stern–Volmer equation modified by Lehrer. The temperature dependence of the Stern–Volmer constant, K_{sv} suggests that the mechanism of the quenching process is mainly dynamic in origin. The thermodynamic parameters were estimated based on such temperature dependence.

The positive values found for the enthalpy and entropy changes seem to indicate that the hydrophobic contribution is the predominant intermolecular force stabilizing the xylazine–BSA complex.

Fluorescence quenching was also used to calculate the binding constants by the Scatchard procedure. The values of these constants are of the same order of magnitude as the Stern–Volmer constants.

These results, together with the spectral changes in the fluorescence emission spectra of BSA induced by xylazine, suggest that the interaction may take place in subdomains IIA and IIIA since these subdomains have been proposed to bind drugs and other hydrophobic materials.

Key words binding; BSA; xylazine; quenching; fluorescence

Xylazine, 2-(2,6-dimethylphenylamino)-4*H*-5,6-dihydro-1,3-thiazine, is a non-narcotic sedative, analgesic, and muscle relaxant that acts on the central and autonomic nervous system.¹⁾ The drug's effects depend on the dose and route of administration, and the sedative effect can vary from animal to animal.²⁾ Another property of theoretical interest is its potent anesthetic effect when injected locally.³⁾ Xylazine strongly potentiates the effects of all tranquilizers, sedatives, cataleptics, dissociative anesthetics and anesthetic agents, and should be used in combination with drugs in these groups.⁴⁾ However, xylazine is an effective and relatively safe drug and has been used in combination with various neuroleptics, narcotics and barbiturates for short-term anesthesia in most domestic animals and many wild animal species.^{5,6)} The drug is rapidly absorbed from intramuscular injection sites, attaining highest levels in the brain and kidney. About 70% of the drug is eliminated *via* the kidneys, and the residues are below 0.1 ppm after 10 h in most of the tissues.⁷⁾

Bovine serum albumin (BSA) is the most abundant plasma protein available and it is able to bind drugs and other exogenous and endogenous substances.⁸⁾ Such interactions can produce changes in the UV-Vis absorption and fluorescence spectra^{9,10)}; therefore, spectroscopic methods may be useful for studying the interaction between xylazine and BSA.

In this paper, we have used the fluorescence-quenching method to study the binding of xylazine to BSA following the works of Lehrer¹¹⁾ and the review by Eftink and Ghiron.¹²⁾

Materials and Methods

Materials BSA, essentially fatty acid free, was obtained from Fluka Biochemika and xylazine from Chemical Pharmaceutics Bayer, S. A. (Spain). These compounds were of the highest available commercial purity and were used as received.

The concentration of xylazine was calculated gravimetrically, assuming

a molecular weight of 256.5 g/mol. A stock solution of xylazine was prepared in a 6.1 mM phosphate buffer containing 0.25 mg/ml, pH = 7.4 ± 0.1, measured by a Crison 2001 pH meter at 25 °C.

Stock solution of BSA was prepared in the same phosphate buffer and its concentration was determined spectrophotometrically with an extinction coefficient at 280 nm of 36000 l/(mol·cm).¹³⁾

Apparatus Absorbance measurements were taken in a UVIKON-940 spectrophotometer, and fluorescence measurements were carried out using a Perkin-Elmer MPF-44A spectrofluorimeter, equipped with a jacketed cell-holder connected to a constant temperature bath thermostated at 15, 25, 30, 37, 40 ± 0.5 °C. Two excitation wavelengths were used: 280 nm, which is the maximum absorption wavelength of the protein, and 295 nm, with is the maximum absorption wavelength of tryptophan. The emission wavelength in both cases was at 335 nm. The slit widths were placed at 7 nm for excitation and 6 nm for emission.

Procedures For fluorescence measurements, typically 2.0 ml of 0.01 mM BSA solution were placed in a 1 cm square quartz sample cell and 2.0 ml of buffer were placed in the reference cell. Aliquots of 50 or 100 μl of a solution containing 0.5 mM xylazine and 0.01 mM BSA were added to the sample cell. After mixing, relative fluorescence was measured following the same procedure described previously.¹⁴⁾

The correction factor for the inner filter effect can be calculated quite simply¹⁵⁾ as:

$$F_{\text{corrected}} = F_{\text{measured}} H \quad (1)$$

$$H = \text{antilogarithm} \{ (\epsilon_{\text{excitation}} + \epsilon_{\text{emission}}) [Q] (\text{pathlength}/2) \} \quad (2)$$

where $\epsilon_{\text{excitation}}$ is the molar absorptivity of xylazine at the excitation wavelength, $\epsilon_{\text{emission}}$ is that at the fluorescence wavelength, and $[Q]$ is the molar concentration of added xylazine.

In the present work, Eq. 3 was used in the correction of inner filter effect:

$$H = \text{antilogarithm} \{ \epsilon_{280 \text{ nm}} [\text{xylazine}] \cdot 0.5 \} \quad (3)$$

since $\epsilon_{\text{emission } 335 \text{ nm}} \approx 0$.

Analysis of the Results Quenching data were initially treated by the usual Stern–Volmer Equation,¹⁶⁾ expressed as:

$$F_0/F = 1 + K[Q] \quad (4)$$

where F_0 and F are, respectively, the fluorescence intensities in the absence and presence of the quencher, Q is the concentration of quencher and K_{sv} is the Stern–Volmer quenching constant.

Quenching data were also interpreted according to the Stern–Volmer equation modified by Lehrer¹¹⁾ to analyze the quenching of heterogeneous systems, and were expressed as:

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$$F_0/(F_0 - F) = 1/f_a + 1/(f_a k [Q]) \quad (5)$$

where f_a is the fraction of the initial fluorescence which is accessible to the quencher.

In such analyses, $F_0/(F_0 - F)$ is plotted vs. $1/[Q]$, and the fractional accessibility is obtained from the ordinate intercept by extrapolating the straight-line of the plot to infinite $[Q]$.

Scatchard Plot This method is based on the equation¹⁷:

$$v/L = nK - Kv \quad (6)$$

where v is the moles of drug bound per mole of protein, L is the molar concentration of free drug, n is the binding sites, and K is the equilibrium binding constant. The saturation function v has been obtained from the decrease in relative fluorescence intensity as the relation between $\Delta F/\Delta F_{\max}$.¹⁸

Estimation of Thermodynamic Parameters for the Binding Process The temperature dependence of the binding constant allows us to estimate the binding enthalpy change according to the van't Hoff relationship:

$$d(\ln K_{sv})/d(1/T) = -(\Delta H^\circ/R) \quad (7)$$

The Gibbs' free energy changes, ΔG , were determined from the binding constant by the equation:

$$\Delta G = RT \ln K_{sv} \quad (8)$$

The entropy change, ΔS , was estimated from the Gibbs' free energy and the enthalpy as:

$$\Delta S = (\Delta H - \Delta G)/T \quad (9)$$

Results and Discussion

Fluorescence intensity data were analyzed according to the Stern–Volmer law by plotting F_0/F versus the xylazine concentration in the range of 0–10 mM. Figure 1 shows the Stern–Volmer plots obtained under different temperatures. In these titrations, carried out at a constant protein concentration of 0.01 mM, there is a linear dependence between F_0/F and Q that is indicative of the homogeneity of quenching. The slope yields the Stern–Volmer constant, K_{sv} .

Although an unambiguous assignment of dynamic and static quenching requires the measurements of the change in fluorescence lifetime in the presence the quencher,¹⁶ the temperature dependence on K_{sv} is often used to differentiate between collisional and static quenching as a possible mechanism for fluorescence quenching in proteins.^{19,20} As can be seen from Fig. 1, the slopes increase with increasing temperature, and such temperature dependence is consistent with a collisional or dynamic fluorescence quenching mechanism. Moreover, this fact is consistent with the good linearity obtained in the Stern–Volmer plots, because a positive deviation from the straight-line would be due to static quenching.

BSA contains two tryptophan residues, at positions 134 and 212.²¹ However, the absence of downward curvature in the Stern–Volmer plot suggests the homogeneity of quenching and therefore can be considered to have a number of fluorophores that have a similar degree of exposure. If one makes the assumption that there is a population of identically accessible fluorophores, as is the case for aldolase, β -trypsin and pepsin,²² and the remainder are completely shielded, Eq. 5 gives f_a , which is the fractional fluorescence due to the residues accessible to the solvent.

Figure 2 shows the result of the fluorescence titrations at 15, 25, 30, 37 and 40 °C; linear plots with values for f_a between 0.45 and 1 are obtained. It is important to notice

that f_a increases with increasing temperature, suggesting that the chromophore groups are more exposed to the solvent as the temperature increases, and thus, the accessibility of the xylazine to the binding sites on BSA is increased.

When the fluorescence spectra were recorded in the presence of 8.5 mM xylazine (Fig. 3), a considerable reduction in the fluorescence emission of BSA was observed. If the excitation wavelength is at 280 nm, about 70% of the native fluorescence is quenched, but if the excitation wavelength is at 295 nm only about 30% is

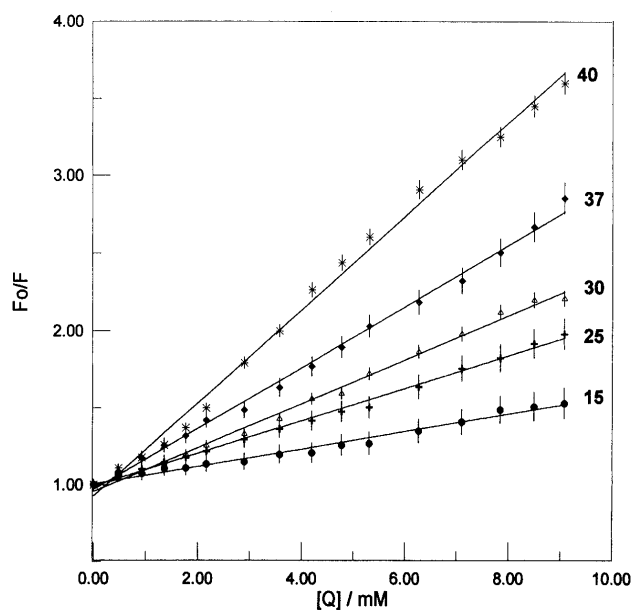


Fig. 1. The Stern–Volmer Plot as a Function of Temperature for Xylazine–BSA Interaction

The excitation and emission wavelengths were, respectively, 280 and 335 nm. The BSA concentration was kept constant at 0.01 mM. Five temperatures were tested, 15, 25, 30, 37 and 40 ± 0.5 °C.

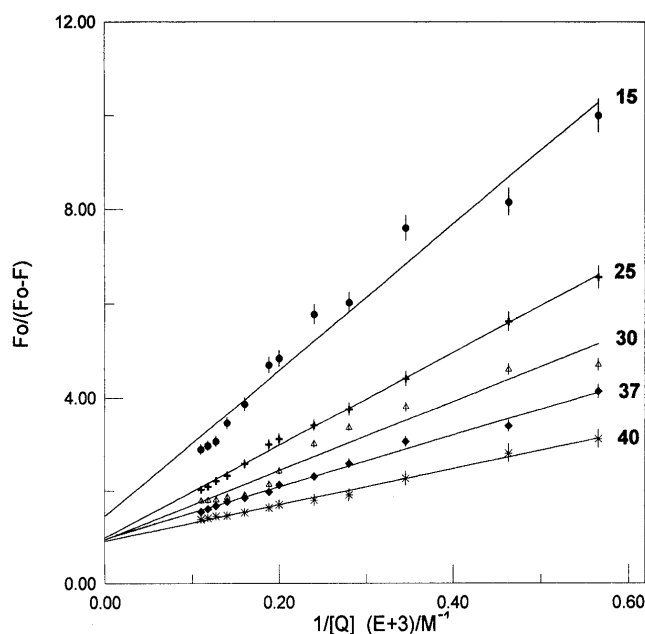


Fig. 2. Quenching Data Plotted According to the Equation of Lehrer

The experimental conditions are the same as those given in the insert of Fig. 1. From the slopes of this representation it is possible to obtain the fraction of fluorescence quenchable at each temperature.

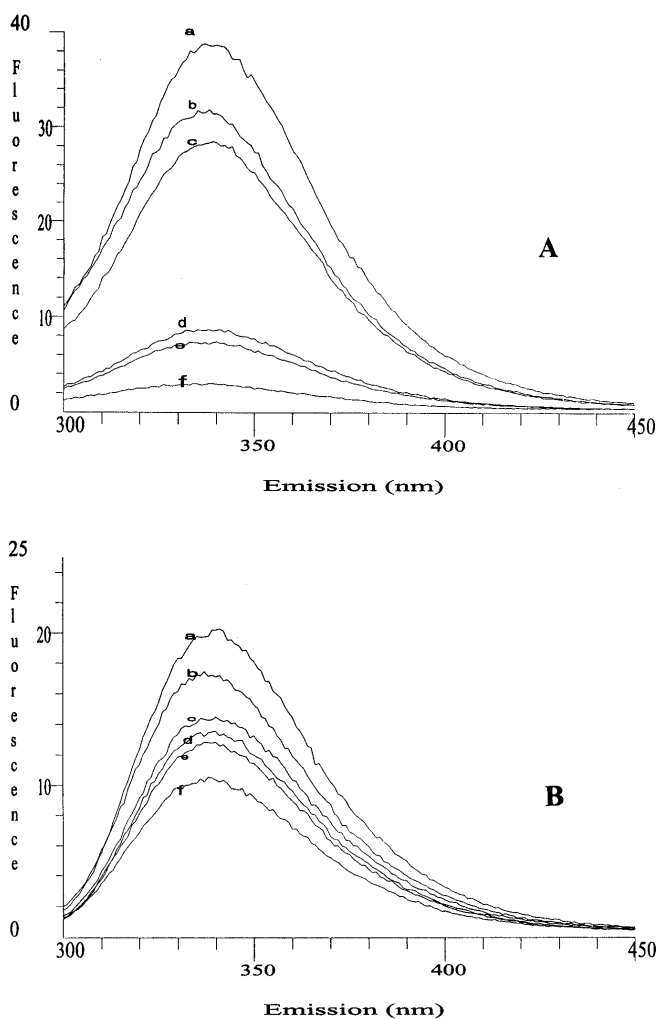


Fig. 3. Effect of Xylazine and Temperature on the Fluorescence Emission Spectrum of BSA 0.01 mM

The excitation wavelengths were A) 280 nm and B) 295 nm, (a, b, c) in the absence of xylazine and (d, e, f) in the presence of 8.5 mM of xylazine. The spectra were registered (a, d) at 15°C, (b, e) at 25°C and (C, f) at 37°C.

quenched. In addition, when the excitation takes place at 280 nm, the emission spectrum shows a perturbation in the band position that is not observed at an excitation at 295 nm. These marked differences in both the shape and wavelength of maximum emission as a function of the excitation wavelength are not particularly surprising considering that BSA contains 19 tyrosines and only 2 tryptophans, and that the tryptophan region of the spectrum did not reveal any significant differences.

The binding of xylazine to BSA was also determined by fluorescence quenching measurements, assuming $v = \Delta F / \Delta F_{\max}$.¹⁸⁾ As shown in Fig. 4, the experimental points are well fitted to a straight line, suggesting a single class of binding sites. From the slope of these plots, the association constant can be calculated for each experimental temperature. Such values, given in Table 1, are in good agreement with the K_{sv} values calculated from the Stern–Volmer plots, and in addition they show a similar temperature dependence. Thus, Stern–Volmer constants can be taken as the binding constant, independently of their static or dynamic character.¹⁴⁾ The values of the binding constant found in this work indicate that xylazine shows a small affinity to BSA under our experimental conditions, since such values range from 100 to 200 l/mol depending on temperature. Such relatively low values could be explained by xylazine's pharmacokinetic parameters *in vivo*,⁷⁾ because in cattle, after an i.m. injection of 0.1 mg/kg, xylazine reaches the highest levels

Table 1. Stern–Volmer Constants, f_a Values and Binding Constants for the Interaction of Xylazine with BSA

T (°C)	K_{sv} (l/M)	f_a (%)	K (l/M)
15	56.94 ± 0.022	44.25 ± 0.741	50 ± 0.02
25	105.64 ± 0.022	83.54 ± 0.174	105.59 ± 0.017
30	145.35 ± 0.020	97.18 ± 0.115	137.23 ± 0.020
37	197.44 ± 0.038	97.94 ± 0.083	179.23 ± 0.022
40	299.61 ± 0.030	100 ± 0.080	285.15 ± 0.020

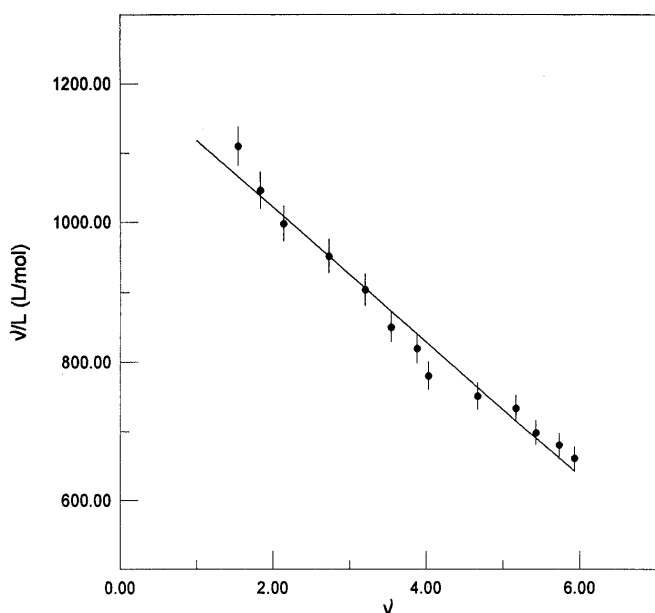


Fig. 4. Scatchard Plots for Binding Xylazine to BSA at 25°C

From the slope of this representation the binding constants can be obtained.

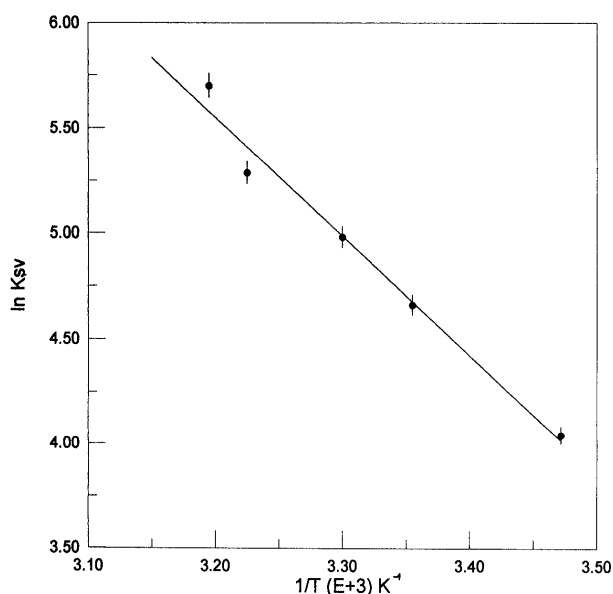


Fig. 5. Arrhenius Plot for BSA Quenching by Xylazine

Five temperatures were tested: 15, 25, 30, 37 and 40 ± 0.5 °C. From the slope of this representation and enthalpy value of 46.85 ± 0.02 kJ/mol was obtained.

Table 2. Thermodynamic Parameters for the Interaction of Xylazine with BSA

T (°C)	ln K_{sv}	ΔG (kJ/mol)	ΔS (J/mol K)
15	4.402	-9.673	196.32
25	4.660	-11.539	196.00
30	4.979	-12.537	196.06
37	5.285	-13.614	195.11
40	5.702	-14.831	197.13

The value of enthalpy is 46.85 ± 0.02 kJ/mol, and it was obtained from the slope of van't Hoff plot in Figure 5. The regression coefficient is 0.987.

in the brain and kidney, and after 10 h, the xylazine levels in all tissues, except the liver and kidneys, are less than 0.1 ppm. We have also used the temperature dependence of the K_{sv} to estimate the thermodynamic parameters for the quenching of BSA by xylazine. Figure 5 shows the van't Hoff plot used to estimate the enthalpy change. The data fits to a straight line, indicating a small value of heat capacity change in analogy with several protein-ligand interactions.²³⁾ There is a negative linear dependence between $\ln K_{sv}$ and $1/T$ which is consistent with a positive change in the enthalpy of 46.85 kJ/mol.

The entropy change estimated from the Gibbs free energy and the enthalpy is positive and practically independent of the temperature due to the small dependence between the Gibbs free energy and temperature. Positive entropic and enthalpic contributions are expected from hydrophobic forces associated with the transfer of the drug molecule from the solvent to the binding site.²³⁾ Hydrophobic interactions might be the dominant physical force between BSA and xylazine, although not the only one, because there are essentially four types of noncovalent interactions that could participate in the binding of ligands to proteins. Of these, the first type is accompanied by positive values of both entropy and enthalpy changes.²⁴⁾ The same type of interactions have been found for the porphyrin-HSA association.²⁵⁾ On the other hand, it has been demonstrated by spectrofluorimetric measurements that the average association constant of the dye 1-anilino-naphthalene-8-sulphonate (ANS) for different proteins is related to their hydrophobicity.¹³⁾ Therefore, it seems likely that xylazine binds to HSA mainly through hydrophobic interactions.

Recently, a three-dimensional structure of HSA has been determined crystallographically²⁶⁾ and it is able to explain numerous physical phenomena. These authors have pointed out that the principal regions of ligand binding are located in hydrophobic cavities in subdomains IIA and

IIIA, which exhibit similar chemistry. Moreover, the same authors had previously published that the tryptophan residues in both albumins play an important structural role in the formation of the IIA binding site and participate in additional hydrophobic packing interaction between the IIA and IIIA interface. Taking into account that both albumins, HSA and BSA, differ only in four amino acids, many of which are homologous,²⁷⁾ the reversible changes in the spectral properties of BSA could arise from the binding of the xylazine to the hydrophobic environments, mainly in the subdomains IIA and IIIA, because they are the binding loci for free fatty acids, drugs and other hydrophobic materials.

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