Tetracycline (TC) derivatives comprise a group of bacteriostatic antibiotics extensively used in human and veterinary medicine and in aquaculture, and as animal growth promoters due to their safe toxicological profile.\(^1\) More recently, they have been proposed as anti-amyloidogenic drugs since they can interfere with protein conformational changes and self-assemblies associated with protein misfolding and deposition diseases.\(^2\)–\(^4\)

Despite their popular use, TC derivatives are highly unstable and some degradation products formed during the storage or normal use of the drugs are pharmacologically toxic or inactive compounds.\(^5\) Anhydrotetracycline (AHTC) is one of the major degradation products of TC that has been linked to several side effects of the drug.\(^6\) We evaluated the interaction of AHTC with bovine serum albumin (BSA), one of the main carriers of amphiphilic molecules in blood, using three complementary analytical methods: fluorescence spectroscopy, isothermal titration calorimetry and differential scanning calorimetry.\(^7\) Drug binding was enthalpically and entropically driven and seemed to involve hydrophobic interactions.\(^8\) Fluorescence enhancement and hypsochromic shifts observed upon binding suggested a low-polarity location excluded from water for the bound drug. Our data are useful for evaluating the biodisponibility of the pharmacophore and the dynamic distribution of the toxic derivative.

**Key words** protein stability; ligand binding; bovine serum albumin; tetracycline; differential scanning calorimetry; isothermal titration calorimetry

Tetracycline (TC) derivatives are extensively used as antibiotics in human and animal medicine and, very recently, they have been screened as anti-amyloidogenic drugs. Anhydrotetracycline (AHTC) is one of the major degradation products of TC that has been linked to several side effects of the drug. We evaluated the interaction of AHTC with bovine serum albumin (BSA), one of the main carriers of amphiphilic molecules in blood, using three complementary analytical methods: fluorescence spectroscopy, isothermal titration calorimetry and differential scanning calorimetry. AHTC bound to BSA with an association constant in the order of $10^5 \text{M}^{-1}$. Drug binding was enthalpically and entropically driven and seemed to involve hydrophobic interactions. AHTC fluorescence enhancement and hypsochromic shifts observed upon binding suggested a low-polarity location excluded from water for the bound drug. Our data are useful for evaluating the biodisponibility of the pharmacophore and the dynamic distribution of the toxic derivative.

Key words protein stability; ligand binding; bovine serum albumin; tetracycline; differential scanning calorimetry; isothermal titration calorimetry

![Chemical Structures of Tetracycline (TC) and Anhydrotetracycline (AHTC) Indicating the Degradation Pathway of TC in Acid Solution](image-url)

**Fig. 1.** Chemical Structures of Tetracycline (TC) and Anhydrotetracycline (AHTC) Indicating the Degradation Pathway of TC in Acid Solution
Additional isothermal titration calorimetry (ITC) measurements were performed in order to get relevant thermodynamic binding parameters, confirmed by complementary differential scanning calorimetry (DSC) experiments. The results are discussed in terms of the available data for other TC derivatives interacting with proteins.

MATERIALS AND METHODS

Chemicals  Fatty acid and globulin-free BSA and tetracycline were purchased from Sigma Chemical Co. and used without further purification. AHTC was synthesised using the following procedure\textsuperscript{12}; a solution of 2-propanol (20.2 ml), methanol (1.5 ml) and concentrated hydrochloric acid (7.2 ml) was added to tetracycline hydrochloride (2.3975 g). After refluxing for 20 min, the resulting yellow precipitate was filtered, washed with 2-propanol (3×10 ml) and dried under vacuum for 2.5 h. The crude product was then refluxed in benzene (250 ml). The remaining precipitate was filtered and dried under vacuum to yield 2.0457 g of product. The product was identified by infrared spectroscopy using a KBr disk, giving the following characteristic wavelenth signals (cm\textsuperscript{-1}): 3409, 3318, 3181, 3096, 3065, 1646, 1614, 1568, 1461, 1440, 1400, 1369, 1340, 1243, 1137, 1076, 818.

BSA and AHTC were dissolved in 100 mM phosphate buffer pH 7.4 prepared in ultra pure water. The concentrations for dilution, were determined by UV absorption from diluted solutions using the extinction coefficients for the free ligand used. AHTC was selectively excited at 430 nm.

ITC Measurements  Thermograms were obtained using a MicroCal VP-DSC calorimeter from MicroCal Llc. BSA concentration was 9 μM. The reference cell was filled with buffer and a 26 p.s.i. pressure was applied to both cells. A scan rate of 60 °C/h was used for all the experiments.

The calorimetric data were analyzed using the Origin 7.5 software supplied by the manufacturer. A reversible non-two-state denaturation model was used to obtain the midpoint of the thermal unfolding (T\textsubscript{m}), the actual heat absorption during protein unfolding (ΔH\textsubscript{m}) and the van’t Hoff (ΔH\textsubscript{vH}) enthalpy or effective enthalpy, which represents the theoretical heat of the transition assuming a two-state model:

\[ \Delta H_{\text{vH}} = RT^2 \frac{d \ln f_L}{dT} \]

The cooperative unit was calculated as the ratio \[ \Delta H_{\text{m}}/\Delta H_{\text{vH}} \]\textsuperscript{22}. If the assumption that denaturation is a transition between only two states is a valid one, \textit{i.e.} without intermediates significantly populated, the effective enthalpy should be equal to the real one and the ratio between them should be close to unity.

The applicability of equilibrium thermodynamic analysis to irreversible DSC transitions has been discussed previously.\textsuperscript{23} Reversible unfolding models were employed to fit the data after checking that the calorimetric signal was reduced by about 40% after heating a BSA solution up to the T\textsubscript{m} then cooled it to room temperature and then reheated it to T\textgreater T\textsubscript{m} and that different thermograms and results were obtained either (i) at different protein concentrations (ref. 24 and this work) and (ii) by fitting the entire DSC curve using only the first half of the experimental data from the endotherm.

DSC has proven to be very useful for characterizing the energetics of protein unfolding and biomolecular interactions.\textsuperscript{25–27} We have employed the formalism described in ref. 28 to estimate the apparent binding constant (K\textsubscript{app}) of AHTC to BSA from DSC curves acquired at different ligand/protein molar ratios. Briefly, the model assumes a two-state protein unfolding transition coupled to ligand binding to n independent sites in the native state. For every temperature, the enthalpies and constants for the unfolding and binding reactions were evaluated. Mass balance equations were solved by iterative techniques. Once the relative population of the unfolded state and the overall ligand-linked enthalpy change were computed, the average excess heat capacity change was evaluated by numerical differentiation. This sequence was coupled to a multidimensional optimization routine in order to get K\textsubscript{app} (see ref. 28 and references therein for details). The intrinsic unfolding parameters were obtained from an endotherm of free BSA. The binding enthalpy
(ΔH), determined by ITC, was kept constant. Individual thermograms measured at $[\text{AHTC}]_{\text{tot}}/[\text{BSA}]_{\text{tot}} = 10$ were fit and output $K^\text{app}$ values were averaged. The robustness of the fits was assessed from the agreement of the $K^\text{app}$ values obtained by fitting the DSC curves considering the entire range of the heat absorption peak or only the 12.5, 25 and 50% of the endotherms where potential irreversible states were expected to be less populated.

RESULTS AND DISCUSSION

Fluorescence Changes Upon AHTC–BSA Interaction

Fluorometric studies of several TC derivatives indicate that these compounds share the polarity sensitivity of classical solvatochromic probes, such as arylaminonaphthalene sulfonates (ANS). The fluorescence intensity is enhanced in non-polar environments as a consequence of an increase in the quantum yield, which is accompanied by a spectral shift towards lower wavelengths.

We found similar polarity sensitivity for AHTC fluorescence. In comparison with the emission in buffer, the spectrum of AHTC in butanol was blue-shifted by 30 nm and the intensity increased about twofold (Fig. 2). This enhancement suggested an increase in the quantum yield since the absorbance of AHTC was the same in these two solvents (data not shown). Similarly, a marked increase in the fluorescence intensity of the drug along with a hypsochromic shift of 26 nm was observed in the presence of BSA (Fig. 2). The correspondence between the fluorescence signatures of AHTC found in media of different polarity and upon binding to BSA (Fig. 2) suggests that the bound drug is located in a low-polar environment.

Numerous binding studies indicate that the major regions of ligand binding sites in albumin are located in hydrophobic and positively charged cavities in subdomains IIA and IIIA. Site I is located at subdomain IIA and has been characterized as a big and flexible pocket with positive charges in the entrance and hydrophobic chain residues inside. Site I is capable of the binding of negatively charged heterocyclic ligands as bulky as bilirubin. Site II, located in subdomain IIIA, has similar physicochemical characteristics as Site I but the pocket is smaller and less flexible. Therefore, Site II usually binds smaller ligands, such as diazepam. Displacement experiments employing warfarin and bilirubin (Site I) and diazepam (Site II) suggested that TC derivatives bind to the Site I at subdomain IIA of BSA. On the basis of our fluorescence results, we postulate that AHTC would bind to a buried binding site excluded from water in subdomain IIA. This tentative binding location correlates with the big, planar and aromatic structure of AHTC and the predominant anionic form of the drug.

Binding Properties Assessed by Fluorescence Spectroscopy

We took advantage of the distinctive fluorescence signatures of AHTC upon interacting with BSA in order to estimate the binding parameters. Figure 3A shows the fluorometric titration of BSA with AHTC. The data correspond to the area under the curves of the spectra shown in (A), in the absence (black) or the presence of BSA (red). The lines represent the fits obtained using Eq. 1.

Fig. 2. Spectroscopic Behaviour of AHTC in Different Media

Fluorescence emission spectra of 25 μM AHTC in buffer (——) and in butanol (——) and of 1.2 μM AHTC in buffer (——–) and in the presence of BSA at $[\text{AHTC}]_{\text{tot}}/[\text{BSA}]_{\text{tot}} = 0.5$ (——–). AHTC was excited at 430 nm.

Fig. 3. Analysis of AHTC–BSA Interaction by Fluorescence Spectroscopy

(A) Fluorescence emission spectra of solutions of BSA alone (1) and at increasing concentrations of AHTC, $[\text{AHTC}]_{\text{tot}}/[\text{BSA}]_{\text{tot}}$: 0.5 (2), 1 (3), 2 (4), 3.5 (5), 5 (6), 7.5 (7), 10 (8), 15 (9), 20 (10), 30 (11) and 40 (12). Inset: Fluorescence emission spectra of solutions at the same concentrations of AHTC in buffer alone. The initial protein concentration was 2.5 mM. Spectra were corrected for inner filter effects. (B) Titration curve of BSA with AHTC. The data correspond to the area under the curves of the spectra shown in (A), in the absence (black) or the presence of BSA (red). The lines represent the fits obtained using Eq. 1.
The binding reaction had a favorable enthalpy, $\Delta H_b$, of about $-6 \text{ kcal mol}^{-1}$ (Table 1). This global parameter involves numerous contributions such as bulk hydration effects, direct non-covalent interactions at the protein–ligand interface and conformational changes of the interacting partners. 

By way of comparison, $\Delta H_b$ ranging from 2 to 3 kcal mol$^{-1}$ have been reported for various TC derivatives interacting with BSA. 

We obtained a somewhat different value for AHTC, but the methods of determination and the experimental conditions may not be directly comparable. The $\Delta H_b$<0 was accompanied by a favorable entropy contribution ($\Delta S_b$>0) as determined by the titration experiments (Table 1). On binding, an entropic penalty accounting for the reduction in the number of particles and their translational and conformational degrees of freedom in the complex are expected. 

In this sense, we have reported that aniline naphthalene sulfonate (ANS) derivatives induce an overall tightening of the BSA structure which may contribute to an unfavorable conformational entropy change. The positive entropy contribution observed for AHTC–BSA interaction suggests the release of structural water molecules involved in a network of interactions.

**Thermodynamic Binding Parameters Obtained by ITC**

ITC experiments were carried out in order to get thermodynamic binding parameters. Figure 4 shows a representative calorimetric titration experiment. The exothermic heat peaks exhibited a monotonic decrease with the addition of drug until saturation was reached (Fig. 4A). The dependence of heat evolved upon BSA–AHTC interaction was characteristic for a single set of binding sites (Fig. 4B). The affinity and stoichiometry for AHTC binding determined by ITC agreed quite well with those values estimated by fluorescence (Table 1).

**Table 1. Thermodynamic Binding Parameters for AHTC–BSA Interaction at 25 °C**

<table>
<thead>
<tr>
<th></th>
<th>$K_b^{\text{eq}} \times 10^7 M^{-1}$</th>
<th>$\Delta H_b$/kcal mol$^{-1}$</th>
<th>$\Delta S_b$/kcal mol$^{-1}$</th>
<th>$T\Delta S_b$/kcal mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence</td>
<td>0.97±0.02</td>
<td>6.6±0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITC</td>
<td>1.27±0.04</td>
<td>2.2±0.3</td>
<td>−6.1±0.3</td>
<td>1.2</td>
</tr>
<tr>
<td>DSC</td>
<td>1.6±0.1</td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* The standard error only considers the fitting dispersion. 

In this sense, we have reported that aniline naphthalene sulfonate (ANS) derivatives induce an overall tightening of the BSA structure which may contribute to an unfavorable conformational entropy change. The positive entropy contribution observed for AHTC–BSA interaction suggests the release of structural water molecules involved in a network of interactions.

**DSC Analysis**

Protein stability is modulated by ligands that bind with a moderate or high affinity as a thermodynamic consequence of the coupling of the unfolding and binding equilibria. This effect is accounted for by a shift of $T_m$ along with a change in $\Delta H_m$, being more marked for high affinity protein–ligand interactions.

We employed DSC as an analytical tool to estimate the binding parameters. Figure 5 shows representative experimental endotherms obtained for BSA alone and at increasing [AHTC]$_{tot}$/[BSA]$_{tot}$ before (inset) and after baseline subtraction. The thermal stability of an AHTC solution was confirmed by the invariability of the absorbance spectra throughout the temperature range of the DSC scans (data not shown). The thermally induced unfolding of free BSA was characterized by a denaturation temperature ($T_m^\text{DSC}$) of 60.9 °C, an unfolding enthalpy ($\Delta H_m^\text{DSC}$) of 129 kcal mol$^{-1}$ and a heat capacity change ($\Delta C_p^\text{DSC}$) of 5.9 kcal mol$^{-1}$ K$^{-1}$, respectively (Fig. 5, Table 2). The protein unfolding parameters were in excellent agreement with those reported previously. The cooperative unit was near 1 (Table 2) suggesting a two-state unfolding process (see DSC measurements section in Materials and Methods).

**Table 2. Thermal Unfolding Parameters of BSA at Different Ligand/Protein Molar Ratios**

<table>
<thead>
<tr>
<th>[AHTC]$<em>{tot}$/[BSA]$</em>{tot}$</th>
<th>$T_m$/°C</th>
<th>$\Delta H_m$/kcal mol$^{-1}$</th>
<th>$\Delta H_m^\text{DSC}$/kcal mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>60.9</td>
<td>129</td>
<td>129</td>
</tr>
<tr>
<td>1</td>
<td>61.8</td>
<td>90</td>
<td>162</td>
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<tr>
<td>5</td>
<td>63.7</td>
<td>102</td>
<td>159</td>
</tr>
<tr>
<td>10</td>
<td>64.8</td>
<td>144</td>
<td>167</td>
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<td>15</td>
<td>65.6</td>
<td>155</td>
<td>159</td>
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</tr>
<tr>
<td>65</td>
<td>68.9</td>
<td>150</td>
<td>196</td>
</tr>
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</table>
equation that entails mial are supplied. 36) At constant \( n \) ciation from the native protein, \( m \) keeps increasing with a moderate affinity. \( \) trend indicates that native BSA preferentially binds AHTC 21 kcal mol \(-1\) in \( T_m \) and \( \Delta H_m \) respectively, was seen at \([\text{AHTC}]_{\text{tot}}/[[\text{BSA}]_{\text{tot}}]=65\) (Fig. 5, Table 2). The calorimetric trend indicates that native BSA preferentially binds AHTC with a moderate affinity.

For protein unfolding processes that involve ligand disso-

![Image](https://via.placeholder.com/150)

Fig. 6. Analysis of Binding Stoichiometry for the AHTC–BSA Complex from DSC Data

(A) Preferential binding of AHTC to native BSA. Fluorescence emission spectra of AHTC in buffer alone at 25 °C (---), and 75 °C (----), and in the presence of BSA at 25 °C (——) and 75 °C (-----) (Fig. 5, Table 2), the emission spectra of free and bound AHTC at different temperatures were compared. At \([AHTC]_M/ [\text{BSA}]_M=65\), the fluorescence features of AHTC at \( T>m\), i.e., in the presence of the denatured protein, were essentially the same as those found in buffer (Fig. 6A) indicating that binding to the unfolded state, if any, can be neglected.

In view of this, \( n \) was estimated from the plot of \( \ln([\text{AHTC}]_{\text{tot}})/M) \) versus \( 1/T_m \) (Fig. 6B) according to Eq. 2. The values of \( \Delta C_p^o, \Delta H_m, \) and \( T_m \) were obtained from the experimental thermogram of free BSA, whereas the \( \Delta H_m \) was obtained from ITC measurements. These parameters were held constant during the nonlinear curve fit of the data in Fig. 6B. The best fit yielded a value of \( n=1.6 \pm 0.1 \) (Table 1). To minimize the number of parameters, the data were also fit with a similar equation but assuming a temperature-independent \( K_{\text{app}} \), i.e., neglecting the \( \Delta H_m \) term in Eq. 2. In this case, we obtained a value of \( n=1.5 \pm 0.1 \). Despite the approximations inherent in this method, the DSC analysis suggests that between one and two molecules of AHTC bind to native BSA, in good agreement with previous determinations (Table 1).

In addition, \( K_{\text{app}} \) was assessed from the DSC experiments as described in Experimental. Individual endotherms acquired at \([AHTC]_{\text{tot}}/[[\text{BSA}]_{\text{tot}}]=10 \) were fit assuming one binding site on the native protein. Figure 5 shows a comparison between the experimental and fit curves. The discrepancies at \( T>T_m \) probably arise from some aggregation process which was not taken into account in the theoretical model. Additional fits using up to the 50% of the data of the experimental unfolding traces yielded similar \( K_{\text{app}} \) values. With this procedure, we estimated an average affinity constant of about \( 2 \times 10^9 M^{-1} \), in satisfactory agreement with that directly determined by ITC (Table 1).

Species Distribution Profile Analysis The apparent binding constant of the AHTC–BSA complex was approxi-
mately one order of magnitude higher than those reported for TC and other TC derivatives.17–30 Although there may be a correlation between the higher hydrophobicity of AHTC relative to other TC derivatives16,17 and the higher affinity, further contributions such as electrostatic forces should be considered.40–42 Indeed, the relevance of electrostatic interaction in TC binding to BSA has been discussed previously.51 Thus, the species distribution profile as a function of pH (Fig. 7) revealed a notable difference between AHTC and its parent compound. At physiological pH, AHTC is mainly found as a monovalent anion and only a 3% (δAHTC = 0.03) is present in the neutral form (Fig. 7B). Having in mind that $K_{app} = \alpha K_b$, $K_b$ being the intrinsic thermodynamic binding constant, the distribution profile indicates that under our experimental condition BSA would bind a negatively charged AHTC molecule with a $K_b$ of $10^5 \text{M}^{-1}$ or a neutral form with a much higher affinity such that the $K_{app}$ of $10^6 \text{M}^{-1}$. In addition, the ratios between the total anionic and neutral species for the two drugs are quite different at physiological pH (Fig. 7): ca. 30 and 0.5 for AHTC and TC, respectively. Therefore, ionic interactions may be involved in the distinct binding of these drugs to serum albumins.

CONCLUSION

In this work we have characterized the interaction of the highly toxic TC derivative, AHTC, with BSA. By using three complementary analytical techniques, we showed that one molecule of AHTC interacts with BSA with a moderate affinity, in a binding event favored by enthalpic and entropic contributions. Drug binding probably involves both hydrophobic and electrostatic interactions. The fact that the affinity of AHTC for albumin is higher than that exhibited by other TC derivatives may have an impact on the biodisponibility of pharmacological drugs and the dynamic distribution of the toxic derivative due to mechanisms of competition for the binding site. This is particularly interesting due to the extensive use of TC derivatives in medicine and the increasing number of applications differing from their classical anti-microbial actions.

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