Binding of Naproxen and Amitriptyline to Bovine Serum Albumin: Biophysical Aspects

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Binding of the drugs naproxen (which is an anti-inflammatory) and amitriptyline (which is an anti-depressant) to bovine serum albumin (BSA) has been studied using isothermal titration calorimetry (ITC), in combination with fluorescence and circular dichroism spectroscopies. Naproxen is observed to bind more strongly to BSA than amitriptyline. The temperature-dependent ITC results indicate the interaction of one molecule of naproxen with more than one protein molecule. On the other hand, amitriptyline binds to BSA with a reaction stoichiometry that varies from 1:1.2 to 1:2.9. The van't Hoff enthalpy, which is calculated from the temperature dependence of the binding constant, agrees well with the calorimetric enthalpy in the case of naproxen binding to BSA, indicating adherence to a two-state binding process. However, their disagreement in the case of amitriptyline indicates conformational changes in the protein upon ligand binding, as well as with the rise in temperature. The spectroscopic results did not suggest appreciable conformational changes as a result of binding; hence, the discrepancy could be attributed to the temperature-induced conformational changes. With increases in the ionic strength, a reduction in the binding affinity of naproxen to BSA is observed. This suggests the prevailing electrostatic interactions in the complexation process. The preponderance of the hydrophobic interactions in the binding of amitriptyline to BSA is indicated by the absence of any dependence of the ionic strength. A predominance of electrostatic interactions in the case of naproxen binding to BSA and that of hydrophobic interactions in the case of amitriptyline binding to BSA is further strengthened by the results of the binding experiments performed in the presence of ionic and nonionic surfactants. The binding parameters indicate that Triton X-100 blocks the hydrophobic binding sites on BSA, thereby altering the binding affinity of amitriptyline toward BSA. A partial overlap of the binding sites for these drugs is indicated by the binding parameters obtained in the titration of naproxen to the amitriptyline−BSA complex and vice versa. Thus, the results provide a quantitative understanding of the binding of naproxen and amitriptyline to BSA, which is important in understanding their effect as therapeutic agents individually and in combination therapy.

1. Introduction

It is known that the distribution, free concentration, and metabolism of various drugs are strongly affected by the drug−protein interactions in the blood stream.[1] Most of the administered drugs are extensively and reversibly bound to plasma proteins, such as serum albumin and α_1-acid glycoprotein, and are transported mainly as a drug−protein complex. To understand the physicochemical basis of drug−protein interactions, serum albumins are the best studied models, because of their abundance and versatile binding properties. Albumins are the most abundant proteins in the circulatory system of a wide variety of organisms, being the major macromolecule that contributes to the osmotic blood pressure, and they are chiefly responsible for maintaining the blood pH.[2,3] Many drugs and other bioactive small molecules bind reversibly to albumin and other serum components. It has been shown to shuttle a broad range of endogenous and exogenous ligands, including more than 70% of drugs.[4,5] Hence, they can have a dominant role in the drug disposition and their efficiency.[6] The capability of serum albumins to bind aromatic and heterocyclic compounds is largely dependent on the existence of two major binding regions, namely Sudlow's site I and site II,[7,8] which are located within specialized cavities in subdomains IIA and IIIA, respectively.[2,9] Warfarin and benzodiazepine are selective probes for site I and site II, respectively.[10,11] These hydrophobic binding pockets enable the serum albumins to increase the apparent solubility of the hydrophobic drugs in plasma and modulate their delivery to the cells in vivo and in vitro.[12] The effectiveness of drugs as pharmaceutical agents is dependent on their binding ability and can also influence the drug stability and toxicity during the chemotherapeutic process.[1] The information on the interaction of serum albumins and drugs can make us better understand the absorption and distribution of drugs in vivo. In addition, the drug−albumin complex may be considered as a model for gaining general fundamental insights into the drug−protein binding. Consequently, it is important in regard to improving our understanding of the biomolecular recognition and it forms an essential part of the rational drug-design process.

Naproxen belongs to the nonsteroidal anti-inflammatory class of drugs. These drugs are used for the management of mild to moderate pain, fever, and inflammation. They work by reducing the levels of prostaglandins, which are chemicals that are responsible for the aforementioned conditions. Naproxen blocks the enzyme cyclooxygenase-2, which makes prostaglandins, resulting in its lower concentration, thereby reducing inflammation, pain, and fever.

Amitriptyline is a popular drug of choice from the tricyclic antidepressant clan. Depression is an all-pervasive sense of

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sadness and gloom. In some patients with depression, abnormal levels of serotonin and noradrenaline may relate to their depression. Amitriptyline elevates mood by reducing the reuptake of these neurotransmitters, thus raising their levels in the brain tissue. A combination of these two drugs has been well-used for the trearment of diseases such as fibromyalgia.[13]

To the best of our knowledge, the binding thermodynamics of amitriptyline with bovine serum albumin (BSA) is not reported in the literature and reports on naproxen−BSA interactions are only qualitative in nature. The present study examines the thermodynamics of the binding of these two drugs to BSA, using isothermal titration calorimetry (ITC), and the consequent conformational changes have been monitored using fluorescence and circular dichroism spectroscopies. Experiments have been performed as a function of temperature to extract the binding thermodynamic parameters. The binding of these drugs to BSA has been studied in the presence of salt and surfactants (cationic, anionic, and nonionic), to evaluate the different forces and contributions responsible for the binding process. The experiments have also been performed to deduce whether the drugs share common binding sites on the protein. The calorimetric results are then coupled with spectroscopic observations to understand the mechanism underlying these interactions.

2. Experimental Section

2.1. Materials. Fatty-acid-free BSA, naproxen, sodium salt, and amitriptyline hydrochloride were purchased from Sigma−Aldrich Chemical Co. The water used to prepare the solution was double-distilled and further deionized using a Cole−Parmer mixed-bed ion-exchange column. All the experiments were performed at pH 7 in 10 mM phosphate buffer. The protein stock solution was prepared by extensive overnight dialysis at 4 °C against the buffer. The reported pH is that of the final dialyzate, determined on a standard Control Dynamics pH meter at ambient temperature. The concentration of the protein was determined on a Shimadzu model UV-265 double-beam spectrophotometer, using a value of $E^{1\%}_{1cm} = 6.8$ at 280 nm.[14]

2.2. Isothermal Titration Calorimetry (ITC). The energetics of the binding of naproxen and amitriptyline to BSA were assessed using an isothermal titration calorimeter (VP-ITC, MicroCal, Northampton, USA). All the solutions were thoroughly degassed before loading, and the consequent water loss was compensated using degassed deionized water. The sample cell was loaded with the buffer or 0.045 mM protein solution. A 250-μL autopipet was filled with the respective drug solution (0.31 mM naproxen or 1.13 mM amitriptyline) and its stirring speed was fixed at 300 rpm. Each experiment consisted of 10-μL consecutive injections at durations of 20 s each with a 4-min interval. To correct the heat effects of dilution and mixing, control experiments were performed at the same concentrations of the protein and drugs and subtracted from the respective drug−protein titrations. The heat released or absorbed upon each injection was measured, and the data were plotted as integrated quantities. The data were analyzed with a single set of identical binding sites model using the Origin 7 software provided by MicroCal. The ITC profiles were best-fitted to a single set of identical binding sites model. The total heat content Q of the solution contained in the active cell volume V_0 (determined relative to zero for the unligated species) at fractional saturation Θ is given by

$$Q = n\Theta M_t \Delta H V_o \tag{1}$$

where ΔH is the molar heat of ligand binding, M_t the total concentration of the macromolecule, and n the number of binding sites in the macromolecule. The heat released from the ith injection for an injection volume dV_i ($\Delta Q(i)$) is then given by the following equation:

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_o}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1) \tag{2}$$

The plot of change in enthalpy against temperature was used to calculate the change in heat capacity upon binding, according to

$$\Delta C_p = \left(\frac{\partial \Delta H}{\partial T}\right)_p \tag{3}$$

The value of the van't Hoff enthalpy at each temperature was calculated, including ΔC_p values, using the following equation:

$$\Delta_{vH}H(T_1) = \frac{\{\ln(K(T_2)/K(T_1)) - (\Delta C_p/R)\ln(T_2/T_1) + (\Delta C_p T_1/R)[(1/T_1) - (1/T_2)]\} \times R}{[(1/T_1) - (1/T_2)]} \tag{4}$$

2.3. Fluorescence Spectroscopy. Intrinsic fluorescence measurements of BSA, in the presence of naproxen and amitriptyline, were performed on a Perkin−Elmer model LS-55 spectrofluorimeter with a 3-mL quartz cell that had a path length of 1 cm. The protein concentration in all the experiments was kept at 7.25×10^{-7} mol dm^{-3}. The excitation and emission slit widths were fixed at 5 nm. The excitation wavelength was set at 295 nm to selectively excite the tryptophan residues, and the emission spectra were recorded in the wavelength range of 300−400 nm at a scan rate of 100 nm/min. The experiments were performed using [drug]/[BSA] molar ratios of 0.2, 0.4, 0.8, 1.0, 1.6, and 2.0.

2.4. Circular Dichroism (CD) Spectroscopy. The alterations in the secondary and tertiary structure of the protein in the presence of the drugs were studied on a Jasco-810 CD spectropolarimeter. The protein concentration and path lengths used were 5 μM and 0.2 cm, respectively, for the far-UV CD experiments and 20 μM and 1 cm, respectively, for the near-UV CD experiments. The spectropolarimeter was sufficiently purged with 99.9% nitrogen before starting the instrument. The spectra were collected at a scan speed of 500 nm/min and a response time of 1 s. Each spectrum was baseline-corrected, and the final plot was taken as an average of three accumulated plots. The molar ellipticity was calculated from the observed ellipticity θ as

$$[\theta] = 100 \times \left(\frac{\theta}{c \times l}\right) \tag{5}$$

where c is the concentration of the protein in mol dm^{-3} and l is the path length of the cell (in centimeters). These experiments were performed for the [drug]/[BSA] molar ratios of 0.2, 0.4, 0.8, and 2.0.

3. Results and Discussion

3.1. Isothermal Titration Calorimetry of the Binding of Naproxen to BSA at pH 7 and Various Temperatures. A representative calorimetric titration profile of 0.31 mM naproxen with 0.045 mM BSA at pH 7 and 298.15 K is shown in Figure 1. Each peak in the binding isotherm (see Figure 1, panel A) represents a single injection of the drug into the protein solution.

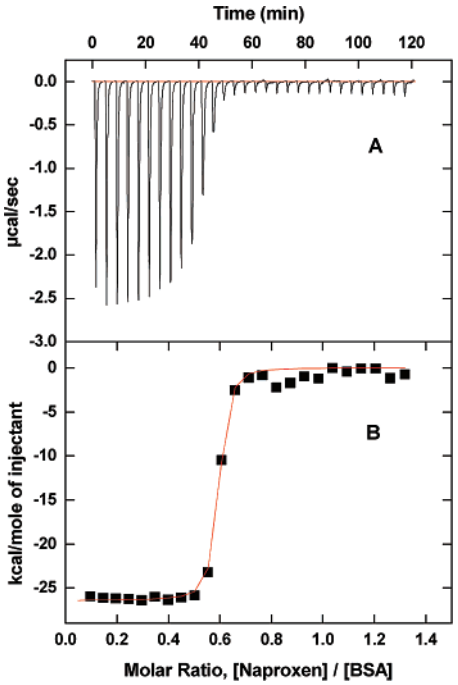


Figure 1. (A) Raw data for the titration of 0.31 mM naproxen with 0.045 mM BSA at pH 7 and 25 °C, showing the calorimetric response as successive injections of the ligand are added to the sample cell. (B) Integrated heat profile of the calorimetric titration shown in panel A. The solid line represents the best nonlinear least-squares fit to a single-binding-site model.

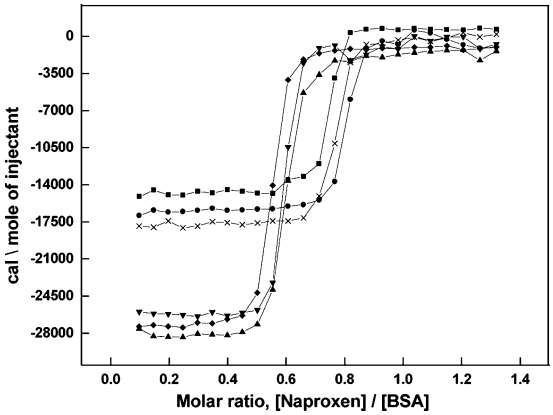


Figure 2. Binding isotherms for the titration of 0.31 mM naproxen with 0.045 mM BSA at pH 7 and various temperatures: (■) 10, (●) 15, (×) 20, (▼) 25, (◆) 30, and (▲) 35 °C.

Panel B of the figure shows the plot of the amount of heat liberated per injection as a function of the molar ratio of the drug to the protein. A standard nonlinear least-squares regression binding model, involving a single class of noninteracting sites fitted well to the data. The smooth solid line shown in Figure 1 (panel B) is the best fit to the experimental data. The binding profiles from 10 °C to 35 °C are shown in Figure 2. The temperature dependence of the thermodynamic parameters accompanying the binding of naproxen to BSA is summarized in Table 1. Each value in this table is an average of two to three independent experiments. The observed enthalpy does not have significant contribution from the buffer ionization, because phosphate has a small value for the enthalpy of ionization ($\Delta H_{\text{ioniz}} = 3.6$ kJ/mol).[15] Therefore, the observed enthalpy is practically the binding enthalpy of the drug to the protein.

As seen in Figures 1 and 2, the ITC titrations of 0.31 mM naproxen with 0.045 mM BSA yielded negative heat deflection, which indicated that the binding is an exothermic process with

TABLE 1: Binding Parameters Accompanying the Titration of 0.31 mM Naproxen with 0.045 mM Bovine Serum Albumin (BSA) at pH 7 and Various Temperatures[a]

temperature, T (°C)	N	K ($\times 10^7$ M^{-1})	change in enthalpy, ΔH ($\times 10^4$ cal/mol)	change in entropy, ΔS (cal K^{-1} mol^{-1})
10	0.76	8.33 ± 2.67	−1.41 ± 0.02	−13.8
15	0.82	4.75 ± 1.52	−1.58 ± 0.19	−19.8
20	0.77	3.06 ± 0.06	−1.76 ± 0.01	−25.8
25	0.61	3.68 ± 1.18	−2.31 ± 0.03	−42.9
30	0.58	1.80 ± 0.52	−2.48 ± 0.04	−48.4
35	0.57	1.14 ± 0.05	−2.89 ± 0.07	−61.3

[a] Note that 1 cal = 4.18 J.

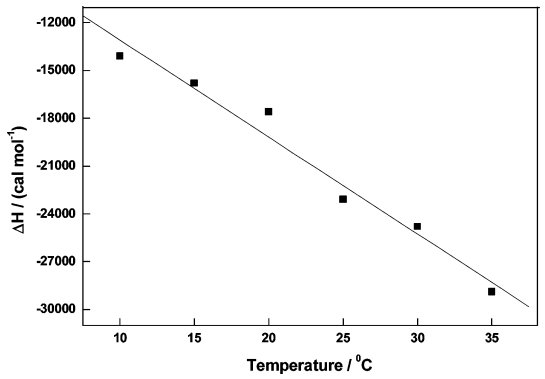


Figure 3. Plot of enthalpy against temperature for the binding of naproxen to BSA at pH 7 and 25 °C.

a high affinity constant at all of the studied temperatures. The value of K varies from $(8.33 \pm 4.97) \times 10^7$ M^{-1} at 10 °C to $(1.14 \pm 0.05) \times 10^7$ M^{-1} at 35 °C. The binding is entropically opposed but enthalpically favored in the studied temperature range. The enthalpy of binding is in the range of -14.1 ± 0.02 kcal/mol to -28.9 ± 0.07 kcal/mol. Naproxen is a negatively charged molecule at pH 7 ($pK_a = 4.9$)[16] and it is expected to bind at the sites that are comprised of the positively charged amino acid residues and hydrophobic side chains on the protein. The strong exothermicity observed in the binding suggests the involvement of electrostatic interactions in the binding process. The binding of naproxen to BSA is primarily driven by the enthalpic contribution, whereas $T\Delta S$ contributes unfavorably (see Table 1). Generally, the value of the stoichiometry of the binding is <1. With increases in temperature, there can be an expansion of the binding site that affects the favorable interaction of the protein with naproxen. This leads to a reduction in the binding affinity and a decrease in the stoichiometry of binding. This could possibly be assigned to the interaction of the negatively charged ester group of naproxen with one protein molecule and via hydrophobic interactions of the aromatic naphthalene group with the other protein molecule and thus decreasing the stoichiometry from 0.76 at 10 °C to 0.57 at 35 °C. This explanation is consistent with a decrease in the entropy of binding with an increase in temperature, indicating more restriction of the protein molecule, as a result of binding. The value of the van't Hoff enthalpy was calculated from the temperature dependence of the binding constant K and ΔC_p, using eq 4. It agrees well with the calorimetric enthalpy at each temperature, indicating adherence to two-state binding process and that the binding of naproxen to the protein does not alter its conformation appreciably. A plot of ΔH against temperature (Figure 3) yields a straight line with a correlation coefficient $R = 0.985$ and yielding a value of $\Delta C_p = -609 \pm 53$ cal K^{-1} mol^{-1} from the slope, which implies that there is a burial of hydrophobic groups due to the binding process. Excellent

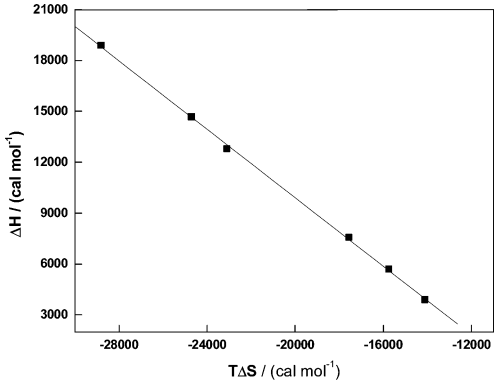


Figure 4. Plot of enthalpy−entropy compensation for the binding of naproxen to BSA at pH 7 and 25 °C.

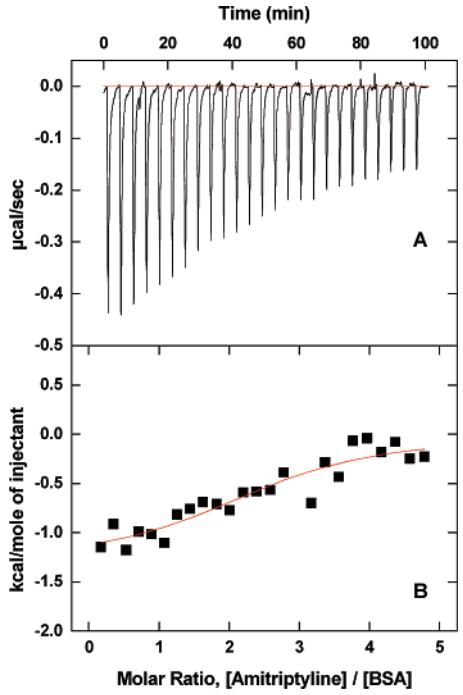


Figure 5. (A) Raw data for the titration of 1.13 mM amitriptyline with 0.045 mM BSA at pH 7 and 25 °C, showing the calorimetric response as successive injections of the ligand are added to the sample cell. (B) Integrated heat profile of the calorimetric titration shown in panel A. The solid line represents the best nonlinear least-squares fit to a single-binding-site model.

enthalpy−entropy compensation is observed in the plot of ΔH against $T\Delta S$ with the slope = 1.01 and the correlation coefficient $R = 0.9997$ (Figure 4). This means that, in the case of naproxen binding to BSA, only single molecular interactions are occurring, which are consistent with our conclusions on the equivalence of the van't Hoff and calorimetric enthalpy.

3.2. Isothermal Titration Calorimetry of the Binding of Amitriptyline to BSA at pH 7 and Various Temperatures. Figure 5 shows representative calorimetric titrations of 1.13 mM amitriptyline with 0.045 mM BSA at pH 7 and 25 °C. These titrations also exhibited exothermic characteristics. The data obtained at different temperatures, fitted to a single set of binding site model and the binding parameters thus obtained, are given in Table 2. With an increase in temperature from 10 °C to 30 °C, the stoichiometry and the association constant also increase. This could be due to a slight expansion of the binding site, to allow more amitriptyline molecules to be accommodated on a single set of identical binding sites with increased affinity. It is possible that the expansion of the binding site also offers

TABLE 2: Binding Parameters Accompanying the Titration of 1.13 mM Amitriptyline with 0.045 mM BSA at pH 7 and Various Temperatures[a]

temperature, T (°C)	N	K ($\times 10^4$ M^{-1})	change in enthalpy, ΔH ($\times 10^3$ cal/mol)	change in entropy, ΔS (cal K^{-1} mol^{-1})
10	1.2	2.16 ± 1.17	−3.54 ± 1.68	7.30
15	1.8	3.92 ± 1.71	−2.33 ± 0.46	12.9
20	2.7	4.25 ± 2.00	−0.82 ± 0.22	18.4
25	2.9	4.65 ± 2.19	−1.00 ± 0.26	18.0
30	2.1	21.3 ± 9.1	−1.11 ± 0.08	20.7

[a] Note that 1 cal = 4.18 J.

a larger hydrophobic area for the binding, which accounts for the increase in the value of the equilibrium constant. The change in enthalpy is favorable in all the cases but decreases as the temperature increases. Amitriptyline, being an amphiphilic molecule, has a critical micelle concentration of 36 mM.[17] In all our experiments, the concentration of the drug in solution was well below its critical micelle concentration. Therefore, the observed binding is essentially that of the monomeric drug molecules to the protein. The unfavorable decrease in the binding enthalpy is compensated by a steady increase in the positive entropy values, as observed in Table 2. A plot of ΔH against $T\Delta S$ yielded a straight line with a slope = 0.80 and $R = 0.998$, which also supports this observation. Here, the van't Hoff enthalpy, which is calculated from the temperature dependence of the binding constant, did not match with the calorimetric enthalpy. Possible reasons for this mismatch could be assigned to alteration in the conformation of the protein upon binding, as well as with the increase in temperature.[18] The value of the heat capacity of binding, calculated from a plot of ΔH against T, is 124 ± 30 cal K^{-1} mol^{-1} with a correlation coefficient R = 0.947. The positive value of ΔC_p indicates that the binding leads to an exposure of the hydrophobic surfaces of the protein. The increase in positive entropy with increases in temperature indicates that the hydrophobic interactions are more dominant during the binding process and that there is an increase in the degree of freedom upon binding.

3.3. Ionic Strength Dependence of the Binding of Naproxen and Amitriptyline to BSA. The salt dependence of a bimolecular association is often used to assess the contribution of charge−charge interactions to the free energy of binding.[19] To understand the role of electrostatic interactions in the binding process, the ionic strength dependence of the binding of naproxen and amitriptyline with BSA was studied. The experiments were performed in the presence of 0.2, 0.5, and 1.0 mol dm^{-3} NaCl at pH 7 and 25 °C. The binding parameters thus obtained are listed in Table 3.

For naproxen, an increase in the ionic strength from 0.2 mol dm^{-3} to 1.0 mol dm^{-3} leads to a decrease in the binding affinity of the drug to the protein, as reflected by the decrease in the value of K by a factor of 10. The stoichiometry of the binding increases from 0.65 to 0.98 with an increase in the ionic strength from 0.2 mol dm^{-3} to 1.0 mol dm^{-3}. However, in the case of amitriptyline, the value of the binding constant remains the same within the standard deviation with an increase in ionic strength. The binding becomes more exothermic, as shown by the increase in the value of ΔH compared to that in the absence of NaCl. The values of ΔS shows a decrease, as a result of these ionic interactions, and, in the presence of 1 mol dm^{-3} NaCl, it becomes negative, which indicates unfavorable entropic contributions; however, the interaction still proceeds favorably, because of the larger exothermic enthalpic contribution. For naproxen, the slope of a double logarithmic plot of log K against

TABLE 3: Binding Parameters Accompanying the Titration of 0.31 mM Naproxen and 1.13 mM Amitriptyline with 0.045 mM BSA at pH 7 in the Presence of NaCl at 25 °C[a]

NaCl (mol dm^{-3})	N	K (M^{-1})	change in enthalpy, ΔH (cal/mol)	change in entropy, ΔS (cal K^{-1} mol^{-1})
		Naproxen		
0.2	0.65	$(1.73 \pm 0.35) \times 10^7$	$(-1.97 \pm 0.02) \times 10^4$	−32.9
0.5	0.94	$(7.57 \pm 0.45) \times 10^6$	$(-1.34 \pm 0.01) \times 10^4$	−13.6
1.0	0.98	$(1.61 \pm 0.27) \times 10^6$	$(-1.84 \pm 0.03) \times 10^4$	−33.4
		Amitriptyline		
0.2	1.4	$(1.53 \pm 0.49) \times 10^4$	$(-3.83 \pm 1.11) \times 10^3$	6.27
0.5	1.5	$(1.90 \pm 0.38) \times 10^4$	$(-3.28 \pm 0.01) \times 10^3$	8.56
1.0	1.1	$(1.28 \pm 0.40) \times 10^4$	$(-6.59 \pm 2.85) \times 10^3$	−3.30

[a] Note that 1 cal = 4.18 J.

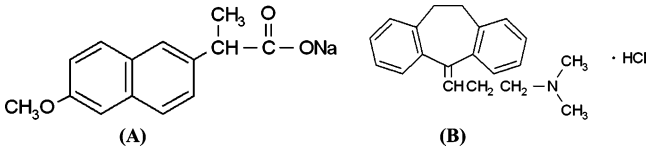


Figure 6. Structure of naproxen sodium salt (A) and amitriptyline hydrochloride (B).

log [Na^+] was −1.45, which is generally equivalent to the number of counterions released upon the drug binding.[20] However, in the case of amitriptyline, the value of the slope is almost zero, which indicates the absence of significant electrostatic contributions in the binding process. The possibilities of the ionic strength dependence include the binding to the charged amino acid residues and also the formation of an ion pair with the drug. Naproxen has a carboxyl group with a free negative charge (see Figure 6A) and is able to interact strongly with the positively charged sites available on the protein. Although amitriptyline is also ionic, it has a larger hydrophobic content (see Figure 6B). This results in a stronger electrostatic interaction of naproxen with the protein, compared to that of amitriptyline at the same pH value.

3.4. Effect of Surfactants on the Binding of Naproxen and Amitriptyline to BSA. To understand the effect of hydrophobic moieties on the binding of drugs to BSA and the extent of overlap of binding sites on BSA for the drugs and surfactants, experiments were performed on the binding of naproxen and amitriptyline to BSA in the presence of anionic, cationic, and nonionic surfactants at pH 7 and 25 °C. The ITC data for the binding of drugs to BSA in the presence of the surfactant micelles are given in Table 4.

Figure 7 shows the ITC binding profiles obtained for binding of naproxen to BSA in the presence of the surfactants. In the presence of the anionic surfactant sodium dodecyl sulfate (SDS), no typical binding pattern is observed (see Figure 7A). SDS, being an anionic surfactant, can interfere in the binding of naproxen to the positively charged residues on the protein. Gelamo and Tabak[21] used CD spectroscopy to demonstrate that the normalized ellipticity of the native BSA at 198 nm decreases to 60% of the initial ellipticity above 2 mM, up to 10 mM of the surfactant. The ellipticity at 222 nm decreases to 80% of the initial ellipticity in the same concentration range. This partial denaturing capability of SDS at our experimental concentration manifests in the reduction of the integrity of the binding sites. The combined result of these two effects leads to the no-binding profile of naproxen for BSA in the presence of SDS, as observed in Figure 7A. Naproxen is observed to bind to BSA in the presence of a cationic surfactant, such as hexadecyltrimethylammonium bromide (HTAB), in a cooperative manner (Figure 7B). The data presented in this figure have been corrected for the dilution effects of ligand, protein, and for the mixing effects of the buffer. As seen in this figure, the heat signals toward saturation cross from exothermic to endothermic effect and do not become zero. This is due to the reason that the pK_a value of naproxen is 4.9 and therefore at pH 7 it exists as negatively charged molecule and forms a complex with the positively charged HTAB. In the dilution experiment of naproxen, the syringe contained a solution of naproxen and HTAB, which was added to the cell that contained only HTAB solution. Figure 8 represents the enthalpy profile obtained from the dilution of ligand. It is possible that the alteration in the complexation behavior of the naproxen−HTAB complex is different in aqueous HTAB than in aqueous solution that contains HTAB and BSA, thereby leading to a nonzero saturating heat signal in the binding profile. Contrary to SDS, HTAB is a positively charged surfactant and is not expected to block the binding sites for naproxen via electrostatic interactions. It also appears that, at the experimental concentration, the denaturing effect of HTAB does not lead to a loss of the integrity of binding sites and, hence, the typical binding profile is observed (see Figure 7B). Figure 7C shows the binding of naproxen to BSA in the presence of the nonionic surfactant Triton X-100 (TX-100). The number of moles of naproxen molecules binding per mole of BSA increases from 0.61 to 0.94 with a reduction in the binding affinity from $(3.68 \pm 1.18) \times 10^7$ to $(5.53 \pm 1.31) \times 10^6$ M^{-1}. There is a significant change in the entropy of binding. It changes from an unfavorable value of −42.9 cal K^{-1} mol^{-1} in the absence of TX-100 to 17.7 cal K^{-1} mol^{-1} in its presence. Changes in the values of both ΔH and ΔS indicate a reduction in the extent of electrostatic interaction between the ligand and the protein.

These results also indicate an overlap between the binding site of TX-100 and naproxen on BSA. The increase in the value of N from 0.61 in the absence of TX-100 to 0.94 in the presence of this surfactant indicates that TX-100 leads to 1:1 binding of naproxen with BSA. This reduction in the extent of electrostatic interaction could be due to the interaction of polar groups of TX-100 with those which constitute the binding sites on BSA.

Figure 9 represents the binding of amitriptyline to BSA in the presence of the surfactants at 25 °C. The presence of SDS (Figure 9A) is also observed to reduce the binding of amitriptyline with BSA, as reflected by a decrease in the value of the affinity constant K from $(4.65 \pm 2.19) \times 10^4$ M^{-1} to $(1.14 \pm 0.42) \times 10^4$ M^{-1}.

SDS at higher concentrations mainly interacts via hydrophobic interactions; therefore, at the micellar concentration, it can interfere in the hydrophobic associations of amitriptyline with BSA. Also, as discussed in the case of naproxen, SDS has the ability to partially denature the protein in the studied concentration, which disturbs the binding site of the protein for the drug molecules. It is also observed, in Tables 2 and 4, that the binding changes from exothermic to endothermic in the absence and

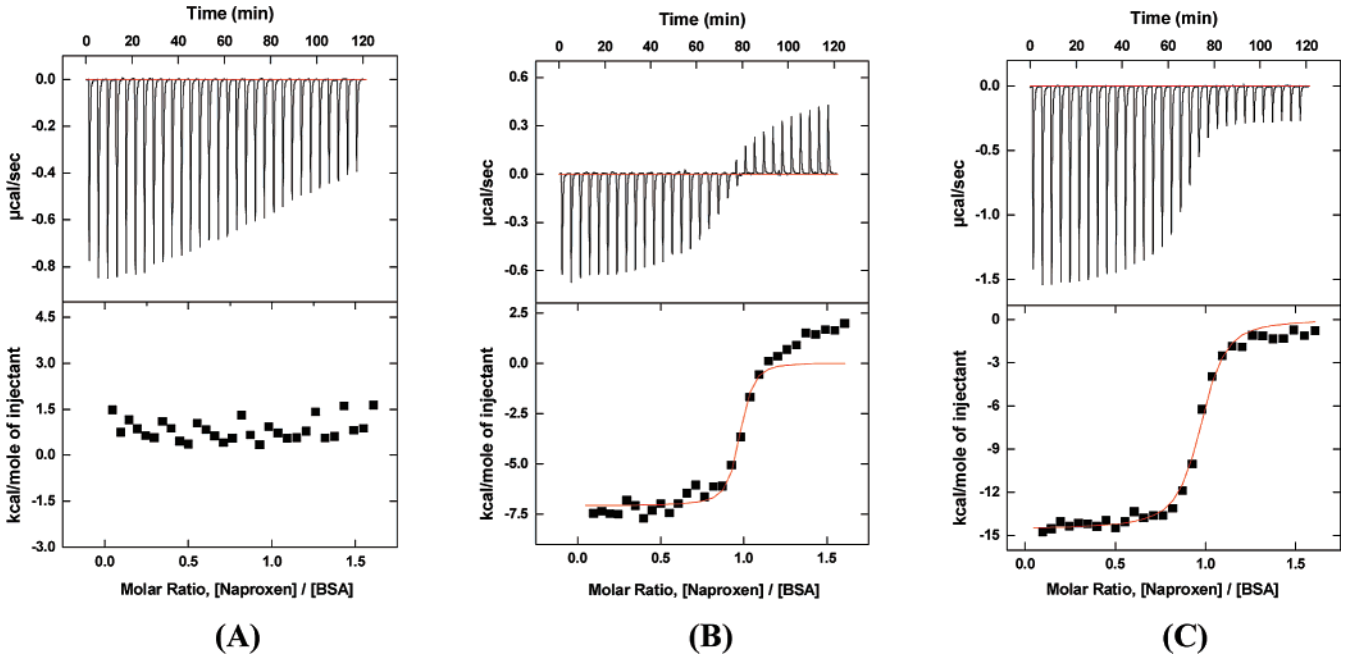


Figure 7. Isothermal titration calorimetry (ITC) profile for the titration of 0.31 mM naproxen to 0.045 mM BSA in the presence of (A) 8.5 mM SDS, (B) 1.10 mM HTAB, and (C) 0.35 mM TX-100 at pH 7 and 25 °C.

TABLE 4: Binding Parameters Accompanying the Titration of 0.31 mM Naproxen and 1.13 mM Amitriptyline with 0.045 mM BSA at pH 7 in the Presence of Surfactants at 25 °C[#]

surfactant	concentration of surfactant (mM)	N	K (M^{-1})	change in enthalpy, ΔH (cal/mol)	change in entropy, ΔS (cal K^{-1} mol^{-1})
			Naproxen		
SDS	8.50			no binding	
HTAB	1.10	0.91 ± 0.03	$(2.27 \pm 4.81) \times 10^7$	$(-7.11 \pm 0.46) \times 10^3$	9.74
TX-100	0.35	0.94 ± 0.01	$(5.53 \pm 1.31) \times 10^6$	$(-1.45 \pm 0.02) \times 10^4$	17.7
			Amitriptyline		
SDS	8.50	3.99 ± 0.24	$(1.14 \pm 0.42) \times 10^4$	$(2.32 \pm 0.39) \times 10^3$	26.3
TX-100	0.35			no binding	

[a] Note that 1 cal = 4.18 J.

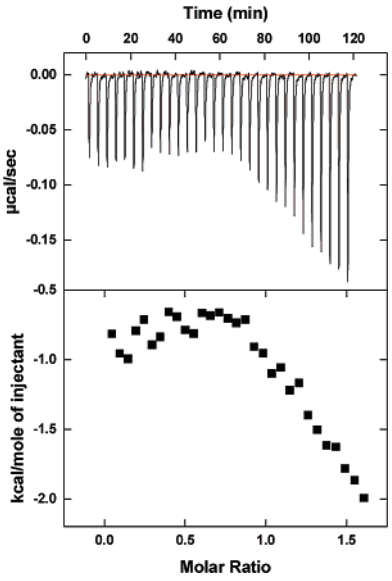


Figure 8. ITC profile for the dilution of 0.31 mM naproxen in the presence of 1.10 mM HTAB at 25 °C.

presence of SDS, respectively. This indicates a reduction in the extent of electrostatic interaction in the binding process. It has been reported, based on structural three-dimensional data, that the binding sites for many diverse ligands are localized within subdomain IIA or IIIA of the albumin molecule.[2,22] These binding regions, using the nomenclature of Sudlow et al.,[7,8] corresponds to sites I and II, respectively. Both of these domains are characterized by the presence of a central cavity formed from six amphipathic helices arranged in a myoglobin-like fold.[23] Based on the equilibrium dialysis and intrinsic fluorescence spectroscopic studies, Kragh-Hansen et al.[23] proposed that there are nine binding sites for amphipathic dodecyl compounds on human serum albumin. With this, they predicted that all the domains of serum albumin have the capacity to bind amphipathic dodecyl compounds. Gelamo et al.[24] suggested that the fluorescence quenching observed in the case of SDS binding to BSA was due to direct contact of the surfactant molecule with the indole of Trp131 residue. Thus, taking into account the type of association SDS can have with the serum albumins, it is obvious that it will influence any other hydrophobic associations, as in the case of amitriptyline binding to BSA, which results in the accommodation of more drug molecules with lower affinity.

Similar to the results obtained in the presence of naproxen, the interaction of amitriptyline with HTAB also does not show zero saturating heat signals (see Figure 9B).

The ionization constant (pK_a) of amitriptyline in the free molecular state is 9.4.[25,26] At pH 7, amitriptyline exhibits a cationic character. The tricyclic portion of the amitriptyline molecule is hydrophobic, and the tertiary amine portion is hydrophilic. The tertiary amine becomes protonated (cationic) at low pH and deprotonated (neutral) at high pH values.[27] Amitriptyline also seems to form a complex with HTAB via

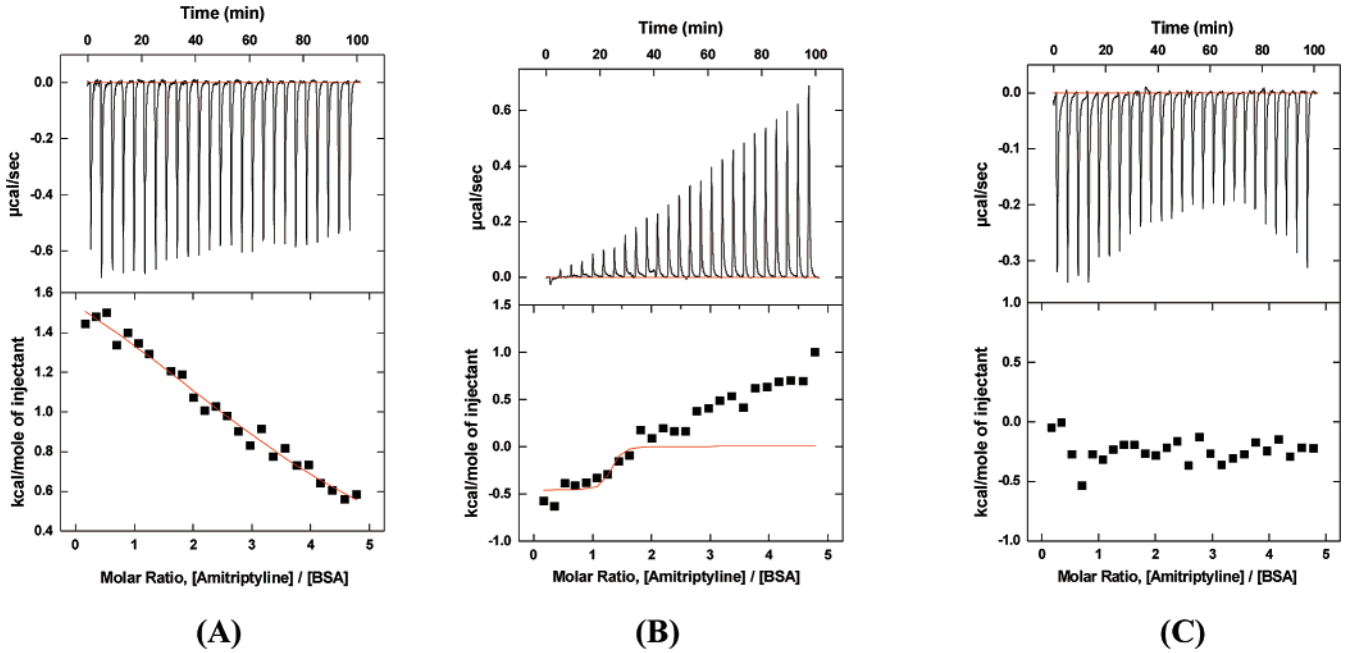
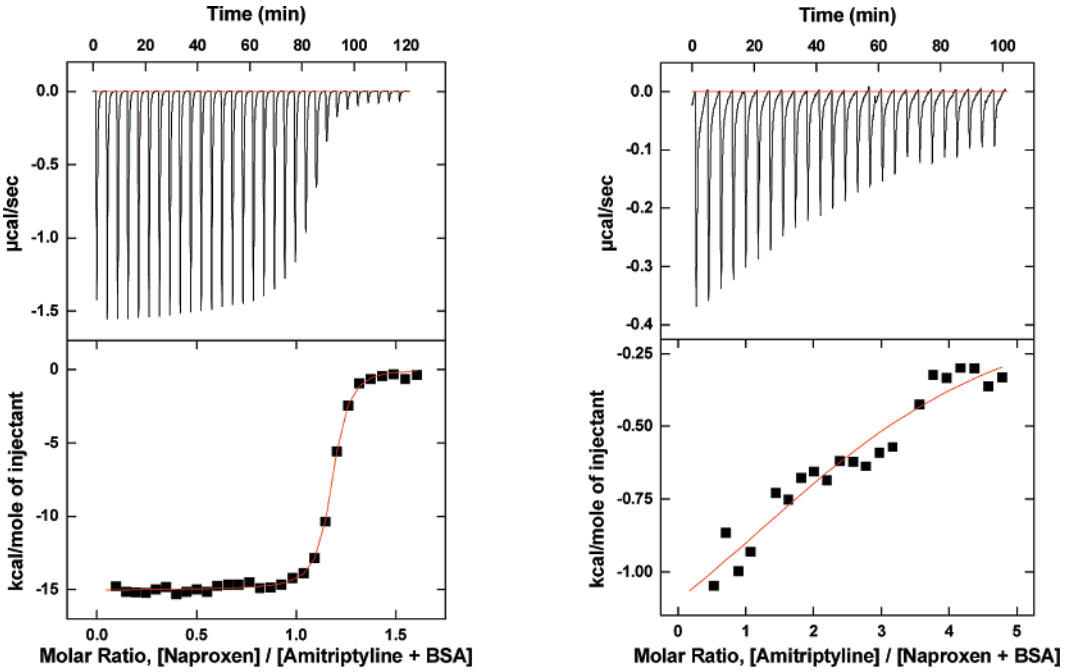


Figure 9. ITC profile for the titration of 1.13 mM amitriptyline to 0.045 mM BSA in the presence of (A) 8.5 mM SDS, (B) 1.10 mM HTAB, and (C) 0.35 mM TX-100 at pH 7 and 25 °C.

hydrophobic interactions. The dissociation of the complex, upon its addition to a buffer or protein solution, may be different, thereby leading to nonzero saturating heat signals. In the presence of the nonionic surfactant TX-100, amitriptyline does not bind to the protein (see Figure 9C). Comparison of the data on the binding of naproxen and amitriptyline with BSA (see Tables 1, 2, and 4) shows that the extent of electrostatic interaction, in the case of naproxen, is greater. Because TX-100 is a neutral molecule and it can bind via polar and nonpolar interactions with equal preference, it seems, from the data, that the hydrophobic groups of TX-100 are blocking the binding sites for amitriptyline on BSA where the drug can bind to the protein via hydrophobic interactions.

3.5. Comparison of the Binding of Naproxen and Amitriptyline to BSA in the Presence of Anionic, Cationic, and Nonionic Surfactants. SDS interacts with BSA via electrostatic and hydrophobic interactions.[28] Titrations of naproxen to BSA in the presence of SDS show that the drug does not bind to BSA in the presence of SDS micelles, whereas appreciable binding of amitriptyline is observed, which suggests stronger involvement of electrostatic interactions between naproxen and BSA, which are interrupted in the presence of SDS. The combined effect of the partial denaturing action of SDS and its ability to interfere with the naproxen binding results in no binding of the drug to the protein. Because amitriptyline still shows appreciable binding and its enthalpy of interaction is endothermic, it indicates nonoverlapping binding sites for SDS and amitriptyline on BSA and also that the involvement of hydrophobic interactions are more predominant in the case of amitriptyline−BSA binding. The data on the binding of drugs to BSA in the presence of HTAB suggests that the protein−ligand binding is affected due to the formation of a drug−surfactant complex. Appreciable binding of naproxen to BSA in the presence of TX-100 and no binding of amitriptyline in the presence of the surfactant confirms that involvement of hydrophobic interactions are more prominent in the case of amitriptyline−BSA binding.

3.6. Binding of Naproxen and Amitriptyline to BSA in the Presence of Each Other. The binding of naproxen with BSA in the presence of amitriptyline and vice versa was also studied to understand if these drugs share common binding sites on the protein. This is expected to provide information on the nature of the binding sites and the relative affinity of the drug for the protein in the presence of each other. Figure 10 shows the ITC profiles obtained for the binding of naproxen to BSA in the presence of amitriptyline and vice versa. The drug−protein complex in the cell was prepared in a 2:1 molar ratio. The integrated heat profiles best-fit a single-binding-site model, and the results thus obtained are summarized in Table 5. The binding profile for the titration of 0.31 mM of naproxen to the amitriptyline−BSA complex also fitted to a single-binding-site model. The results show that naproxen binds to BSA in the presence of amitriptyline with reduced affinity ($N = 1.2$). A decrease in the favorable negative enthalpy is observed, whereas the entropy of binding is lessr negative than that obtained at 25 °C in the absence of amitriptyline. Similar titrations of 1.13 mM amitriptyline with the naproxen−BSA complex also fitted to a single-binding-site model. Here also, the values show a decrease in the binding affinity and an insignificant change in the value of the stoichiometry of the reaction. The binding enthalpy and entropy remained same within the limits of standard deviation. The slight fall in the binding affinity of naproxen to BSA in the presence of amitriptyline is accompanied by a reduction in the exothermicity of the reaction and an increase in the number of naproxen molecules binding to BSA by a factor of 2. The structures of naproxen and amitriptyline (see Figure 6) suggest that naproxen can interact via electrostatic as well as hydrophobic interactions with the protein binding sites, whereas amitriptyline, being more hydrophobic in nature, can make stronger hydrophobic contact at the binding sites. The value of $N = 0.61$ in the case of naproxen binding to BSA in the absence of amitriptyline at 25 °C suggests that one molecule of naproxen can bind to two molecules of the protein. This includes a possibility that the negatively charged end group of naproxen binds via electrostatic interaction with one molecule and by hydrophobic interaction with the other molecule of BSA, thus sandwiching itself between the two protein molecules. However, when naproxen is interacting with the amitriptyline-bound BSA, the hydrophobic binding sites probably are already blocked by amitriptyline and, hence, forces naproxen to bind



(A) (B)

Figure 10. ITC profiles for the titration (A) of 0.31 mM naproxen into the cell containing 0.09 mM amitriptyline and 0.045 mM BSA, and (B) of 1.13 mM amitriptyline into the cell containing 0.09 mM naproxen and 0.045 mM BSA, each at pH 7 and 25 °C.

TABLE 5: Binding of Naproxen and Amitriptyline with 0.045 mM BSA at pH 7 and 25 °C[a]

N	K (M^{-1})	ΔH (cal/mol)	ΔS (cal K^{-1} mol^{-1})
Titration of 0.31 mM Naproxen to Amitriptyline−BSA Complex			
1.2	$(1.88 \pm 0.23) \times 10^7$	$(-1.52 \pm 0.01) \times 10^4$	−17.6
Titration of 1.13 mM Amitriptyline to Naproxen−BSA complex			
3.1	$(1.34 \pm 1.10) \times 10^4$	$(-1.70 \pm 0.80) \times 10^3$	13.2

[a] Note that 1 cal = 4.18 J.

only via electrostatic interactions at a single site with a relatively reduced affinity, resulting in the $N = 1.2$ value. These results also indicate that amitriptyline binds to BSA mainly via hydrophobic interactions. The hydrophobic cavity could be large enough to accommodate the hydrophobic rings of both naproxen and amitriptyline, because the binding affinity of amitriptyline with BSA is not affected in the presence of naproxen. This indicates the absence of the significant electrostatic interactions of amitriptyline with BSA and that the major force of interaction is hydrophobic in nature. Our observations are consistent with those of Leis et al.,[29] who studied the pH-dependent adsorption of amitriptyline on human serum albumin, using conductometric measurements, and concluded that the adsorption was a consequence of hydrophobic effects.

3.7. Fluorescence Spectroscopy. To understand the effect of drugs on the tryptophan environment of the protein, the intrinsic fluorescence of 7.25×10^{-7} mol dm^{-3} of BSA in the presence of increasing concentrations of drugs in the molar ratio of [drug]/[BSA] was studied. The fluorescence spectra of the protein in the presence of naproxen and amitriptyline are shown in Figures 11A and B, respectively. The maximum intensity for BSA in the absence of the drug is observed at 346.5 nm, which is consistent with those reported in the literature.[30]

The addition of naproxen at a molar ratio of 0.2 leads to a decrease in the intensity of the emission maxima with no change in the value of λ_{max}. Further increases in the drug concentration show a steady increase in the fluorescence intensity. The value of λ_{max} shows a blue shift of 2 nm at a molar ratio of 1.0 and remains the same up to a molar ratio of 2.0. The presence of amitriptyline in the molar ratios of 0.2 and 0.4 shows a slight decrease in the fluorescence intensity without any change in the value of λ_{max} (Figure 11B). Beyond this point, up to a molar ratio of 2.0, the spectra exhibit a slight increase in the fluorescence intensity again without any change in the value of λ_{max}. These fluorescence results indicate that the binding of naproxen and amitriptyline does not cause significant alteration in the local environment of the tryptophan moieties, which are located at positions 134 and 212 in subdomains IA and IIA, respectively.[2]

3.8. Circular Dichroism Spectroscopy. The changes in the secondary and tertiary structures of the protein in the presence of the drugs were studied in the far ultraviolet (UV) and near UV CD region at increasing molar ratios of [drug]/[BSA]. The spectrum of intact BSA showed two minima at 209 and 222 nm, which is in accord with the literature.[31] In the presence of naproxen, no appreciable perturbation of the secondary and tertiary structures in the protein is observed (see Figure 12). Similarly, no significant change in the conformation of the protein is observed in the presence of amitriptyline also (Figure 13). Thus, for both amitriptyline and naproxen, the aromatic and peptide regions do not show any appreciable change in the CD spectra, which rules out significant conformational changes in the protein on drug binding, the results of which are consistent with the fluorescence observations.

4. Mode of Interaction of Drugs with BSA. A reduction in the binding affinity of naproxen to BSA with increasing ionic strength indicates a predominance of electrostatic interactions in the complexation. However, the binding affinity of amitriptyline for BSA is not altered, which suggests the involvement of non-Coulombic interactions in the binding process. The predominance of ionic interactions in the binding of naproxen to BSA is further supported by an absence of a typical binding pattern in the presence of SDS, which blocks the binding sites

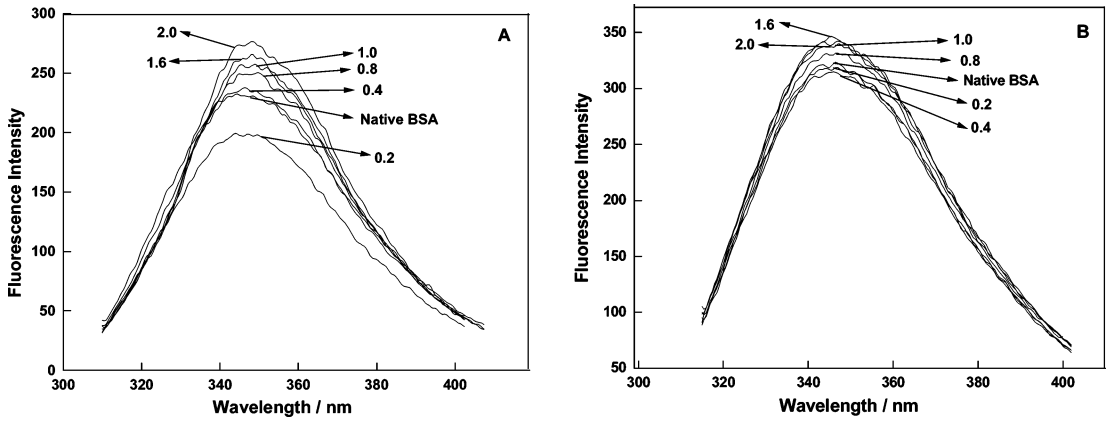


Figure 11. Intrinsic fluorescence emission spectra of BSA in the presence of (A) naproxen and (B) amitriptyline. The numbers indicate the [drug]/[BSA] molar ratio.

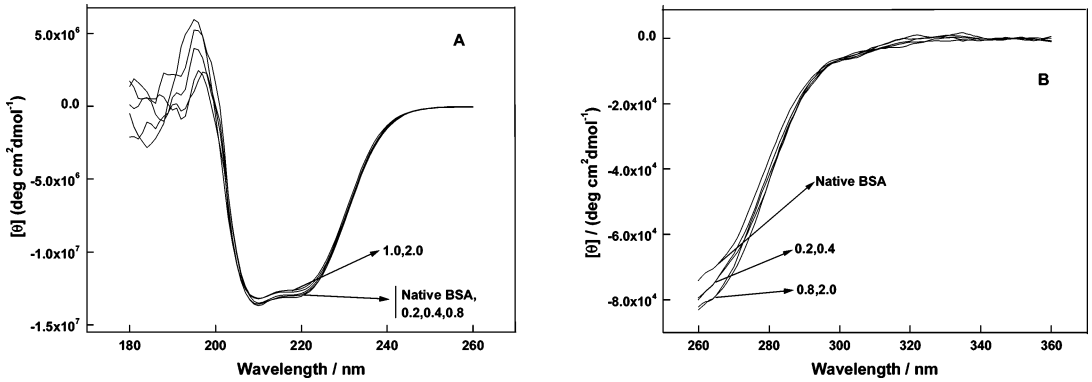


Figure 12. Circular dichroism (CD) spectra of BSA in the presence of naproxen: (A) far-UV CD spectroscopy and (B) near-UV CD spectroscopy. The concentrations used are represented as [naproxen]/[BSA] molar ratios (0.2, 0.4, 0.8, and 2.0).

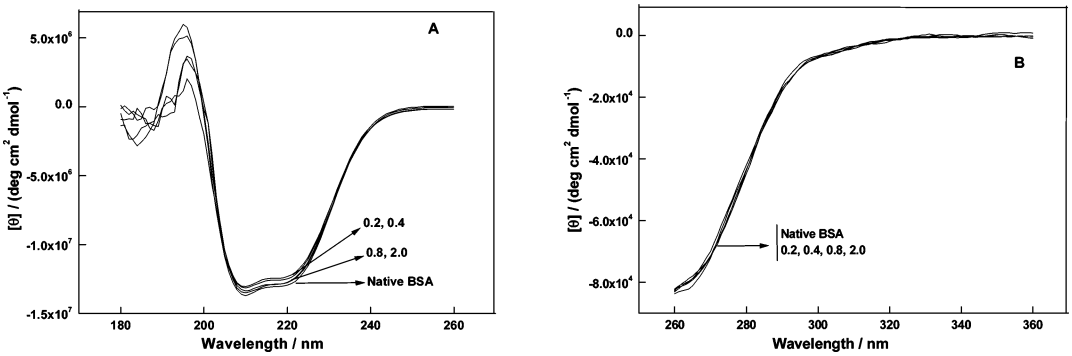


Figure 13. CD spectra of BSA in the presence of amitriptyline: (A) far-UV CD spectroscopy and (B) near-UV CD spectroscopy. The concentrations used are represented as [amitriptyline]/[BSA] molar ratios (0.2, 0.4, 0.8, and 2.0).

for the drug via electrostatic interactions, in addition to its ability to partially denature the protein. The presence of TX-100 seems to interfere with the binding of naproxen to the protein and shifts the value of *N* from 0.61 to 0.94. On the other hand, amitriptyline binds to BSA in the presence of SDS and does not bind to the protein in the presence of TX-100. Being a neutral molecule, TX-100 can block the hydrophobic binding sites, which are observed in the titration profile of amitriptyline with BSA in its presence. These results show the predominance of hydrophobic interactions in the binding of amitriptyline with BSA. The predominance of electrostatic interactions in the binding of naproxen to BSA and that of hydrophobic interactions in the binding of amitriptyline with BSA are further supported by the results of binding experiments performed in the presence of each other. Also, the binding parameters obtained in the titration of naproxen to the amitriptyline−BSA complex, and vice versa, indicate a partial overlap of the binding sites for these drugs on BSA.

A study by Goldenberg et al.[13] on 62 patients with fibromyalgia indicated that patients taking the combined amitriptyline−naproxen regimen experienced minor, but not significant, improvement in pain, when compared with patients who took amitriptyline alone. They concluded that amitriptyline, or a combination of amitriptyline and naproxen, is an effective therapeutic regimen for patients with fibromyalgia. Our results support this observation that the binding affinity of amitriptyline for BSA is not altered in the presence of naproxen. Therefore, it is able to execute its action, even in the presence of naproxen.

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References and Notes

(1) Hu, Y. J.; Liu, Y.; Shen, X. S.; Fang, X. Y.; Qu, S. S. *J. Mol. Struct.* **2005**, *738*, 143.

(2) He, X. M.; Carter, D. C. *Nature (London)* **1992**, *358*, 209.

(3) Peters, T., Jr. *All about Albumin: Biochemistry, Genetics and Medical Applications*; Academic Press: San Diego, CA, 1996.

(4) Kratochwil, N. A.; Huber, W.; Muller, F.; Kansy, M.; Gerber, P. R. *Biochem. Pharmacol.* **2002**, *64*, 1355.

(5) Kragh-Hansen, U. *Pharmacol. Rev.* **1981**, *33*, 17.

(6) Olson, R. E.; Christ, D. D. *Annu. Rep. Med. Chem.* **1996**, *31*, 327.

(7) Sudlow, G.; Birkett, D. J.; Wade, D. N. *Mol. Pharmacol.* **1975**, *11*, 824.

(8) Sudlow, G.; Birkett, D. J.; Wade, D. N. *Mol. Pharmacol.* **1976**, *12*, 1052.

(9) Sugio, S.; Kashima, A.; Mochizuki, S.; Noda, M.; Kobayashi, K. *Protein Eng.* **1999**, *12*, 439.

(10) Kragh-Hansen, U.; Chuang, V. T. G.; Otagiri, M. *Biol. Pharm. Bull.* **2002**, *25*, 695.

(11) Petitas, I.; Bhattacharya, A. A.; Twine, S.; East, E.; Curry, S. *J. Biol. Chem.* **2001**, *276*, 22804.

(12) Shaikh, S. M. T.; Seetharamappa, J.; Kandagal, P. B.; Ashoka, S. *J. Mol. Struct.* **2006**, *786*, 46.

(13) Goldenberg, D. L.; Felson, D. T.; Dinerman, H. *Arthritis Rheum.* **1986**, *29*, 1371.

(14) Sober, H. A.; Harte, R. A. In *Handbook of Biochemistry, Selected Data for Molecular Biology*, 2nd Edition; Sober, H. A., Harte, R. A., Eds.; CRC Press: Cleveland, OH, 1973; p C-71.

(15) Goldberg, R. N.; Kishore, N.; Lennen, R. M. *CRC Handbook of Chemistry and Physics*, 83rd Edition; Lide, D. R., Ed.; CRC Press: Boca Raton, FL, 2002.

(16) Okimoto, K.; Rajewski, R. A.; Uekama, K.; Jona, J. A.; Stella, V. J. *Pharm. Res.* **1996**, *13*, 256.

(17) Attwood, D.; Florence, A. T. *Surfactant Systems: Their Chemistry, Pharmacy and Biology*; Chapman and Hall: New York, 1983.

(18) Chaires, J. B. *Biophys. Chem.* **1997**, *64*, 15.

(19) Tanaka, M.; Asahi, Y.; Masuda, S. *J. Macromol. Sci.−Pure Appl. Chem.* **1995**, *32*, 339.

(20) Barcelo, F.; Capo, D. Portugal, J. *Nucleic Acids Res.* **2002**, *30*, 4567.

(21) Gelamo, E. L.; Tabak, M. *Spectrochim. Acta A* **2000**, *56*, 2255.

(22) Carter, D. C.; Ho, J. X. *Adv. Protein Chem.* **1994**, *45*, 153.

(23) Kragh-Hansen, U.; Hellec, F.; de Foresta, B.; le Maire, M.; Moller, J. V. *Biophys. J.* **2001**, *80*, 2898.

(24) Gelamo, E. L.; Silva, C. H. T. P.; Imasato, H.; Tabak, M. *Biochim. Biophys. Acta* **2002**, *1594*, 84.

(25) Aquila, C. D. *J. Pharmacol. Biomed. Anal.* **2002**, *30*, 341.

(26) Hansch, C. *Comprehensive Medicinal Chemistry: The Rational Design, Mechanistic Study and Therapeutic Application of Chemical Compounds*; Pergamon Press: Oxford, U.K., 1990.

(27) Kim, E. J.; Shah, D. O. *Colloids Surf. A* **2003**, *227*, 105.

(28) Nielsen, A. D.; Borch, K.; Westh, P. *Biochim. Biophys. Acta* **2000**, *1479*, 321.

(29) Leis, D.; Barbosa, S.; Attwood, D.; Taboada, P.; Mosquera, V. *J. Phys. Chem. B* **2002**, *106*, 9143.

(30) Kamat, B. P.; Seetharamappa, J. *J. Chem. Sci.* **2005**, *117*, 649.

(31) Shin, W. S.; Yamashita, H.; Hirose, M. *Biochem. J.* **1994**, *304*, 81.