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Interaction of weakly bound antibiotics neomycin and lincomycin with bovine and human serum albumin: biophysical approach

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The thermodynamics of interaction of neomycin and lincomycin with bovine serum albumin (BSA) and human serum albumin (HSA) has been studied using isothermal titration calorimetry (ITC), in combination with UV–visible, steady state and time resolved fluorescence spectroscopic measurements. Neomycin is observed to bind weakly to BSA and HSA whereas lincomycin did not show any evidence for binding with the native state of these proteins, rather it interacts in the presence of surfactants. The ITC results suggest 1 : 1 binding stoichiometry for neomycin in the studied temperature range. The values of the van't Hoff enthalpy do not agree with the calorimetric enthalpy in the case of neomycin, suggesting conformational changes in the protein upon ligand binding, as well as with the rise in the temperature. Experiments at different ionic strengths, and in the presence of tetrabutyl ammonium bromide and surfactants suggest the predominant involvement of electrostatic interactions in the complexation process of neomycin with BSA and HSA, and non-specific interaction behaviour of lincomycin with these proteins.

Keywords: binding of antibiotics to serum albumin/bovine serum albumin/isothermal titration calorimetry/lincomycin/Neomycin.

Abbreviations: BSA, bovine serum albumin; HSA, human serum albumin; HTAB, hexadecyltrimethyl ammonium bromide; SDS, sodium dodecyl sulphate; TBAB, tetrabutylammonium bromide; TX-100, triton X-100.

Knowledge of the mechanisms of interaction between drugs and plasma proteins is of crucial importance in understanding the pharmacodynamics and pharmacokinetics of a drug (*1*). Therefore studies on drug–protein interactions have increasingly attracted the research attention of chemists, pharmacists and biologists (*2*). Microcalorimetry has been widely used in understanding the thermodynamic aspects of drug–protein interactions (*3–9*). Serum albumin is the major extracellular protein of plasma, accounting for 60% of the total plasma protein content, having a concentration of 34–50 g dm^{-3} [(500–750) × 10^{-6} mol dm^{-3}]. Serum albumin possesses a unique capability to bind, covalently or reversibly, a great number of various endogenous and exogenous compounds (*10*). An understanding of the features of drug interactions with albumin can provide insights into drug therapy and design. Bovine serum albumin (BSA) has been one of the most extensively studied proteins, particularly because of its 76% structural homology with human serum albumin (HSA) (*11*). It has been shown that the distribution, free concentration and the metabolism of various drugs may be strongly affected by drug–protein interactions in the blood stream (*12*).

Neomycin (Fig. 1A) has been a widely used antibiotic which belongs to the water soluble aminoglycoside family, produced by *Streptomyces fradiae*. It is known to prohibit the growth of Gram-positive bacteria and Gram-negative bacteria (*13*, *14*), though it may cause ototoxicity and nephrotoxicity. Neomycin can disturb protein synthesis in bacteria by binding 30S subunit of ribosomal RNA. Therefore, it can cause misreading of the genetic code and inhibit translation (*15*). It is also used as a preventive measure for hepatic encephalopathy and hypercholesterolemia. Neomycin is used to treat gastrointestinal infections of cattle, sheep, pigs, goats and poultry by the oral route and to treat mastitis by intramammary administration (*16*). Therefore the interaction behaviour of neomycin with the plasma proteins is crucial to the health of humans and animals.

Lincomycin (Fig. 1B) is a lincosamide antibiotic isolated from the bacteria of the genus *Streptomyces*. Although similar in structure, antibacterial spectrum, and in mechanism of action to macrolides they are also effective against other species as well i.e. actinomycetes, mycoplasma (*17*), and some species of *Plasmodium* (*18*). A lincosamide antibiotic produced by *S. lincolnensis* is used as the hydrochloride salt (*18*). Lincomycin hydrochloride is a well-established antibiotic drug used in human and veterinary medicine. It is effective primarily against Gram-positive pathogens and has been used in a considerable variety of illnesses, including infections of the mouth and upper respiratory tract, as well as skin infections (*19*).

To the best of our knowledge the binding thermodynamics of neomycin and lincomycin with serum albumin has not been reported in literature, though some qualitative studies are available (*20*, *21*).

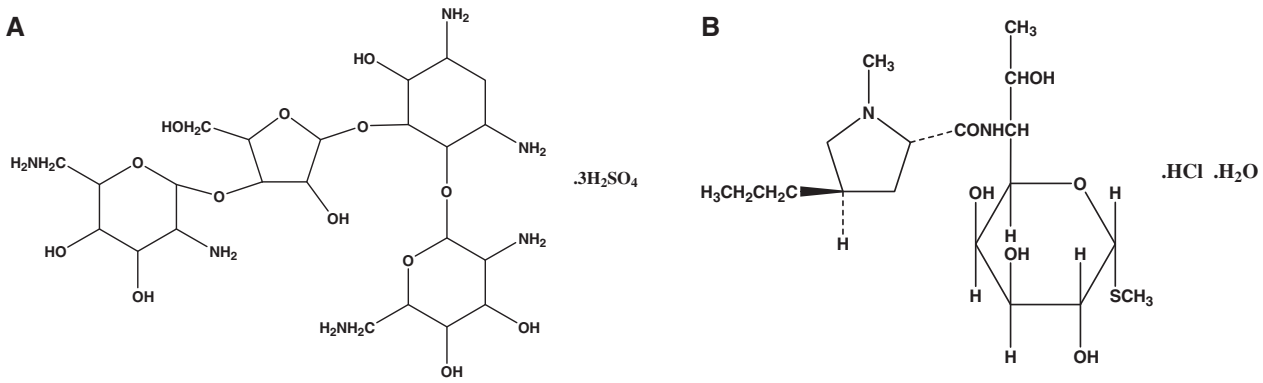

Fig. 1 Structure of (A) neomycin trisulphate and (B) lincomycin hydrochloride.

The binding of drugs to albumin has physiological significance in the transport, modulation and inactivation of metabolites and drug activities, such as a protective device in the binding and inactivation of potential toxic compounds to which the body is exposed. The nature and strength of interaction not only give an idea of drug delivery of the studied antibiotics in the body but, enable us to understand the pharmacokinetics and pharmacodynamics of the drugs.

The present study examines the thermodynamics of interaction of these two drugs with BSA and HSA using a combination of isothermal titration calorimetry (ITC), fluorescence and UV–visible spectroscopic techniques. Experiments have been performed as a function of temperature to extract the binding thermodynamic parameters. Effects of salt, tetrabutyl ammonium bromide (TBAB) and surfactants (cationic, anionic and non-ionic) have been studied on the interaction of the drug with the proteins in order to evaluate the contributions of different forces responsible for the interactions, and also to understand how the conformationally altered protein modifies the interaction pattern. The calorimetric results have been coupled with spectroscopic observations to understand the mechanism underlying the interaction of these drugs with the proteins.

Materials and Methods

Materials

Fatty acid free BSA, HSA, neomycin trisulphate, lincomycin hydrochloride, sodium chloride, sodium dodecyl sulphate (SDS), hexadecyltrimethylammonium bromide (HTAB) and triton X-100 (TX-100) were purchased from Sigma-Aldrich Chemical Co., USA. Tetrabutylammonium bromide of extra pure research quality grade was purchased from Spectrochem, India. The water used to prepare the solutions was double-distilled and further deionized using a Cole-Parmer mixed-bed ion-exchange column. All the experiments were performed at pH 7.4 in 20×10^{-3} mol dm^{-3} phosphate buffer. The protein stock solution was prepared by extensive overnight dialysis at 277 K against the buffer. The reported pH is that of the final dialyzate, determined on a standard Control Dynamics pH meter at the ambient temperature. The concentration of the protein was determined on a Jasco V-550 double-beam spectrophotometer, using a value of $E^{1\%}_{1cm} = 6.8$ at 280 nm (*22*).

ITC

The energetics of the binding of neomycin and lincomycin to BSA and HSA were studied by 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 protein solution at the desired concentration. A 250×10^{-6} dm^3 autopippet was filled with the drug solution and its stirring speed was fixed at 300 r.p.m. Each experiment consisted of 10×10^{-6} dm^3 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 the drug and subtracted from the respective drug–protein titrations. 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 is the total concentration of the protein, and n *is* the number of binding sites in the protein. The heat released from the ith injection $\Delta Q(i)$ for an injection volume dV_i is then given by the following equation:

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1). \tag{2}$$

The heat released or absorbed upon each injection was measured, and the data were plotted as integrated quantities. The data fitted well to a single set of identical binding sites model analysed using the Origin 7 software provided by MicroCal.

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

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

Fluorescence spectroscopy

Intrinsic fluorescence measurements of BSA and HSA in the presence of neomycin and lincomycin were performed on a Perkin-Elmer model LS-55 spectrofluorimeter with a 3×10^{-3} dm^{-3} quartz cell that had a path length of 1 cm. The protein concentration in all the experiments was kept at 0.725×10^{-6} mol dm^{-3} for BSA and 1.45×10^{-6} mol dm^{-3} for HSA. 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^{-1}. The experiments were performed at several [drug]/[protein] molar ratios.

Time resolved fluorescence spectroscopy

Time resolved fluorescence studies were performed at the magic angle using a pulsed Nano-LED based time correlated single photon counting fluorescence spectrophotometer from IBH, UK with $\lambda_{ex} = 295$ nm and $\lambda_{em} = 344$ nm. Full width at half maximum of the instrument response function is 250 ps and the resolution is 56 ps per channel. Experiments were performed in the absence and presence of both these drugs at ambient temperature. The data obtained were fitted with a biexponential decay function, after deconvolution of instrument response function by an iterative reconvolution technique by IBH DAS 6.0 data analysis software using reduced χ^2 and weighted residuals as parameter for goodness

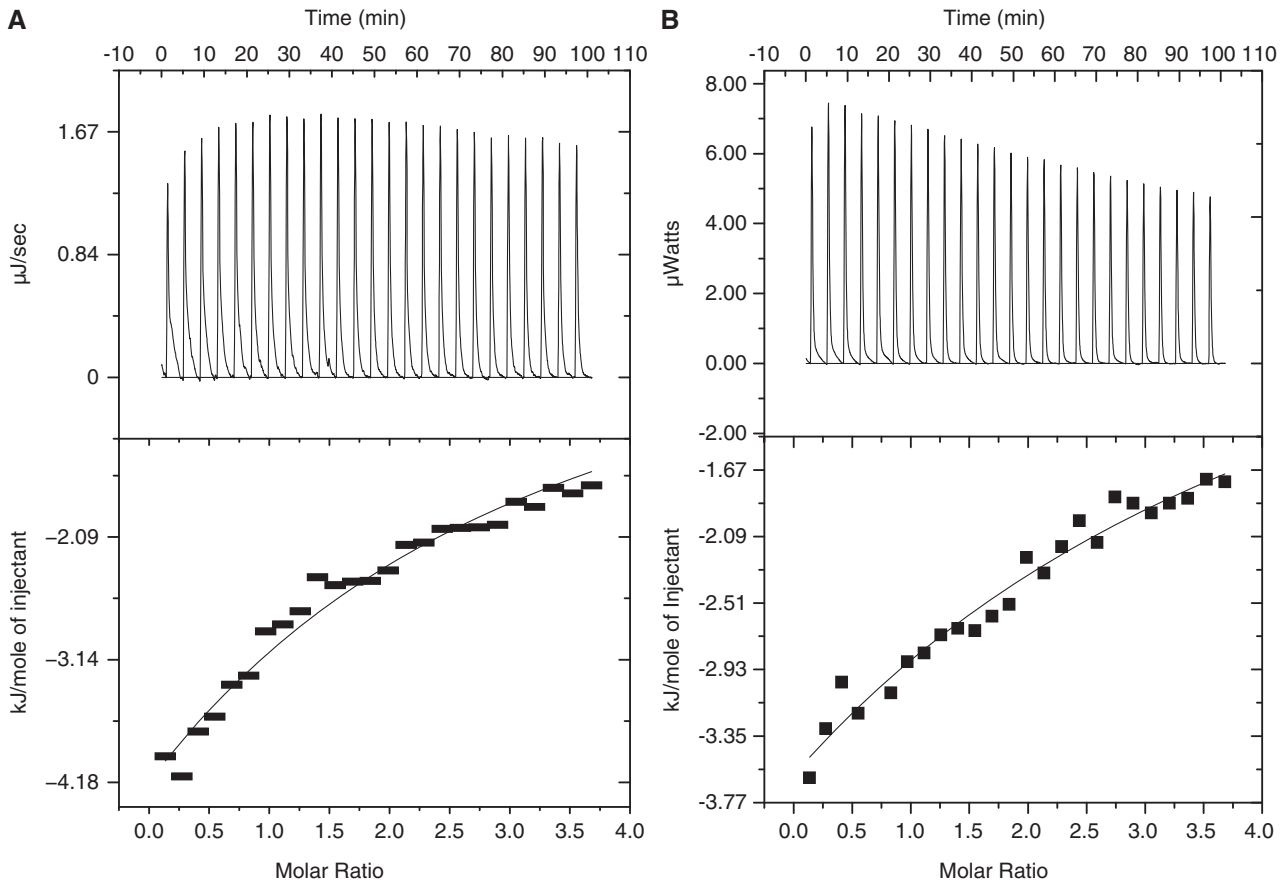

Fig. 2 Raw data for the titration of 2.5×10^{-3} mol dm^{-3} neomycin with 0.13×10^{-3} mol dm^{-3} BSA (A) and 0.13×10^{-3} mol dm^{-3} HSA (B) at pH 7.4 and 298.15 K, showing the calorimetric response as successive injections of the ligand are added to the sample cell. Integrated heat profiles of the calorimetric titration are shown in lower panel.

of the fit. The decay curves were fitted using a non-linear iterative least square fit method using the following equation:

$$G(t) = \sum_i B_i \exp\left(-\frac{t}{\tau_i}\right) \quad (4)$$

$G(t)$ is the fitted decay curve usually assumed to be a sum of exponentials, where B_i is the pre-exponential factor for the ith component, t is the time and τ_i is the corresponding fluorescence lifetime. The data fitted well to biexponential decay where the intensity is assumed to decay as the sum of individual single-exponential decays

$$I(t) = I(0)[\alpha_1 e^{-t/\tau}{}_1 + \alpha_2 e^{-t/\tau}{}_2]. \quad (5)$$

Here τ_1 and τ_2 are the decay times and α_1 and α_2 represent the amplitudes of the components at $t = 0$. The average lifetime $\langle\tau\rangle$ was calculated by using following relation:

$$\langle\tau\rangle = \alpha_1\tau_1 + \alpha_2\tau_2 \quad (6)$$

Results and discussion

ITC of the binding of neomycin to BSA and HSA

The representative heat profiles accompanying the titration of 2.5×10^{-3} mol dm^{-3} neomycin with 0.13×10^{-3} mol dm^{-3} BSA and HSA at pH 7.4 and 298.15 K are shown in Fig. 2. Each peak in the binding isotherm (Fig. 2) represents a single injection of the drug into the protein solution. Figure 2 also 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 non-linear least-squares regression

Table I. Thermodynamic parameters accompanying the binding of 2.5 mM neomycin with 0.13 mM BSA at different temperatures.

Temperature/ K	K/(mol^{-1} dm^3)	ΔH°/(kJ mol^{-1})	ΔS°/ (J K^{-1} mol^{-1})
BSA			
288.15	$(1.22 \pm 0.04) \times 10^3$	-30.54 ± 2.51	-47.70
293.15	$(1.27 \pm 0.08) \times 10^3$	-30.12 ± 1.26	-43.10
298.15	$(1.18 \pm 0.09) \times 10^3$	-30.54 ± 1.67	-43.93
303.15	$(0.78 \pm 0.09) \times 10^3$	-74.06 ± 2.09	-189.12
308.15	$(0.79 \pm 0.11) \times 10^3$	-95.81 ± 9.20	-234.30
HSA			
298.15	$(1.20 \pm 0.05) \times 10^3$	-30.30 ± 1.56	-47.00

binding model, involving a single class of non-interacting sites fitted well to the data. The smooth solid line shown in the Fig. 2 is the best fit to the experimental data. The temperature dependence of the thermodynamic parameters accompanying the binding of neomycin to BSA are summarized in Table I. Each value in this table is an average of two to three independent measurements. The reported standard deviation with each value in the tables is equal to 1SD obtained from the standard deviations associated with the data of independent measurements fitted to the chosen binding model using Origin 7.0. Buffer ionization can contribute to the experimentally

determined enthalpies of binding. Since phosphate has a small value of the enthalpy of ionization ($3.6\,kJ\,mol^{-1}$) (*23*), the observed values of enthalpy are practically the binding enthalpies of the drug to the protein. Experiments were also done at concentration of neomycin 100 times that of the BSA at 298.15 K (Supplementary Fig. S2), which provided the values of K, ΔH°, and ΔS° as $(1.10 \pm 0.08) \times 10^3$, $-30.27 \pm 0.95\,kJ\,mol^{-1}$ and $-43.50\,J\,K^{-1}\,mol^{-1}$, respectively. These values are in excellent agreement with those reported at 298.15 K in Table I.

Since albumin has a capacity to offer multiple binding sites to a ligand, we have tried fitting the experimental data to a two site sequential binding model (see Supplementary Fig. S1 in Supplementary Data, which is a fit to the experimental data points of Fig. 2A). Though the fit appears to be good, there is a large error associated with the thermodynamic parameters accompanying the binding of second ligand. For example, these values are, $K_2 = 173 \pm 59$ and $\Delta H_2^{\circ} = -90.78 \pm 34.06\,kJ\,mol^{-1}$ at $T = 298.15$ K. These parameters have unacceptably large uncertainty values. Further, fitting the data to sequential model with three or more binding sites did not yield good results. Therefore, Fig. 2 represents the best fitting to the experimental data points according to a single binding site model.

As seen in Fig. 2, the heats of titration of $2.5 \times 10^{-3}\,mol\,dm^{-3}$ neomycin with $0.13 \times 10^{-3}\,mol\,dm^{-3}$ BSA or HSA showed positive heat deflection. However, the dilution of $2.5 \times 10^{-3}\,mol\,dm^{-3}$ neomycin into the buffer was observed to be endothermic at each injection. The integrated heat profiles shown in Fig. 2 have been corrected for dilution effects of neomycin and the proteins. The dilution corrected titration profiles show that the binding is an exothermic process. Fitting of these data to the single site binding model described earlier provides a value of binding constant of the order of 10^3 for both BSA and HSA. Neomycin binds with HSA at 298.15 K with $K = (1.20 \pm 0.05) \times 10^3$, $\Delta H^{\circ} = -30.30 \pm 1.56\,kJ\,mol^{-1}$ and $\Delta S^{\circ} = -47.00\,J\,K^{-1}\,mol^{-1}$ which are almost the same values as those observed with BSA. The value of binding constant remains almost the same in the temperature range of 288.15–303.15 K. The binding becomes more exothermic with rise in temperature, though it is entropically opposed. The value of heat capacity of binding calculated from the plot of enthalpy of binding versus temperature is $-3.5 \pm 1.0\,kJ\,K^{-1}\,mol^{-1}$. The negative value of heat capacity of binding is generally associated with burial of hydrophobic residues upon ligand binding, which in the present case appears to be a small contribution.

Neomycin contains six amino groups (Fig. 1A) with pK_a values between 5.7 and 8.8 (*24*, *25*). Therefore, at pH 7.4 neomycin will be a mix of positively and negatively charged amino groups and is expected to bind at sites that are comprised of both positively and negatively charged amino acid residues. Since neomycin has seven hydroxyl groups in addition to amino groups, it can also interact via hydrogen bonding. The observed exothermicity suggests a predominant involvement of electrostatic interactions in the binding process. The binding of neomycin to BSA or HSA is primarily driven by enthalpic contributions and $T\Delta S$ contributes unfavourably. The unfavourable entropic effect most likely arises due to the ligand binding and reorientation of the solvent structure to a different extent at deferent temperatures. The value of binding affinity only reduces slightly with the change in temperature in the range of 288.15–308.15 K. However, at temperature >298.15 K, the binding is more exothermic with a larger unfavourable compensation from entropy.

The two-state behaviour of the binding reaction can be assessed by comparing the values of van't Hoff enthalpy with the calorimetric enthalpy. Since the binding of neomycin is accompanied by a slight change in heat capacity $-3.5 \pm 1.0\,kJ\,K^{-1}\,mol^{-1}$, the value of the van't Hoff enthalpy was calculated by using the following equation:

$$\Delta H_{vH}(T_1) = \left[\frac{\left\{ \begin{array}{l} \ln K(T_2)/K(T_1) - \Delta C_p/R \ln T_2/T_1 \\ +\Delta C_p T_1/R(1/T_1 - 1/T_2) \end{array} \right\} xR}{(1/T_1 - 1/T_2)} \right]. \quad (7)$$

Equation (*7*) is integrated form of the van't Hoff equation:

$$\left(\frac{\partial \ln K}{\partial T}\right)_p = \frac{\Delta_{vH} H^o}{RT^2}.$$

Here

$$\Delta_{vH} H^o(T) = \Delta_{vH} H^o(T_1) + \Delta Cp(T - T_1).$$

The value of ΔC_p was calculated from a plot of ΔH° against T.

Generally for an exothermic reaction an increase in temperature leads to reduction in the value of binding constant in accordance with the Le-Chatlier principle. Using the data presented in Table I and Equation (*7*), it is observed that the values of van't Hoff enthalpy do not agree with the observed calorimetric enthalpy. The calculated value of van't Hoff enthalpy using Equation (*7*) at 298.15 K is $-13.44\,kJ\,mol^{-1}$. A plot of calorimetric enthalpy against temperature yields an average van't Hoff enthalpy of $-20.25\,kJ\,mol^{-1}$ which is also far away from the calorimetric enthalpy. This further suggests that the protein may undergo a conformational change induced either by ligand binding or by an increase in temperature.

Ionic strength dependence of the binding of neomycin to BSA and HSA

The salt dependence of a bimolecular association is often used to assess the contribution of charge–charge interactions to the free energy of binding (*26*). To understand the role of electrostatic interactions in the binding process, the ionic strength dependence of the binding of neomycin with BSA and HSA was studied. The experiments were performed in the presence of 0.1, 0.5 and $1.0\,mol\,dm^{-3}$ NaCl at pH 7.4 and 298.15 K with BSA and $1.0\,mol\,dm^{-3}$ NaCl with HSA (Fig. 3).

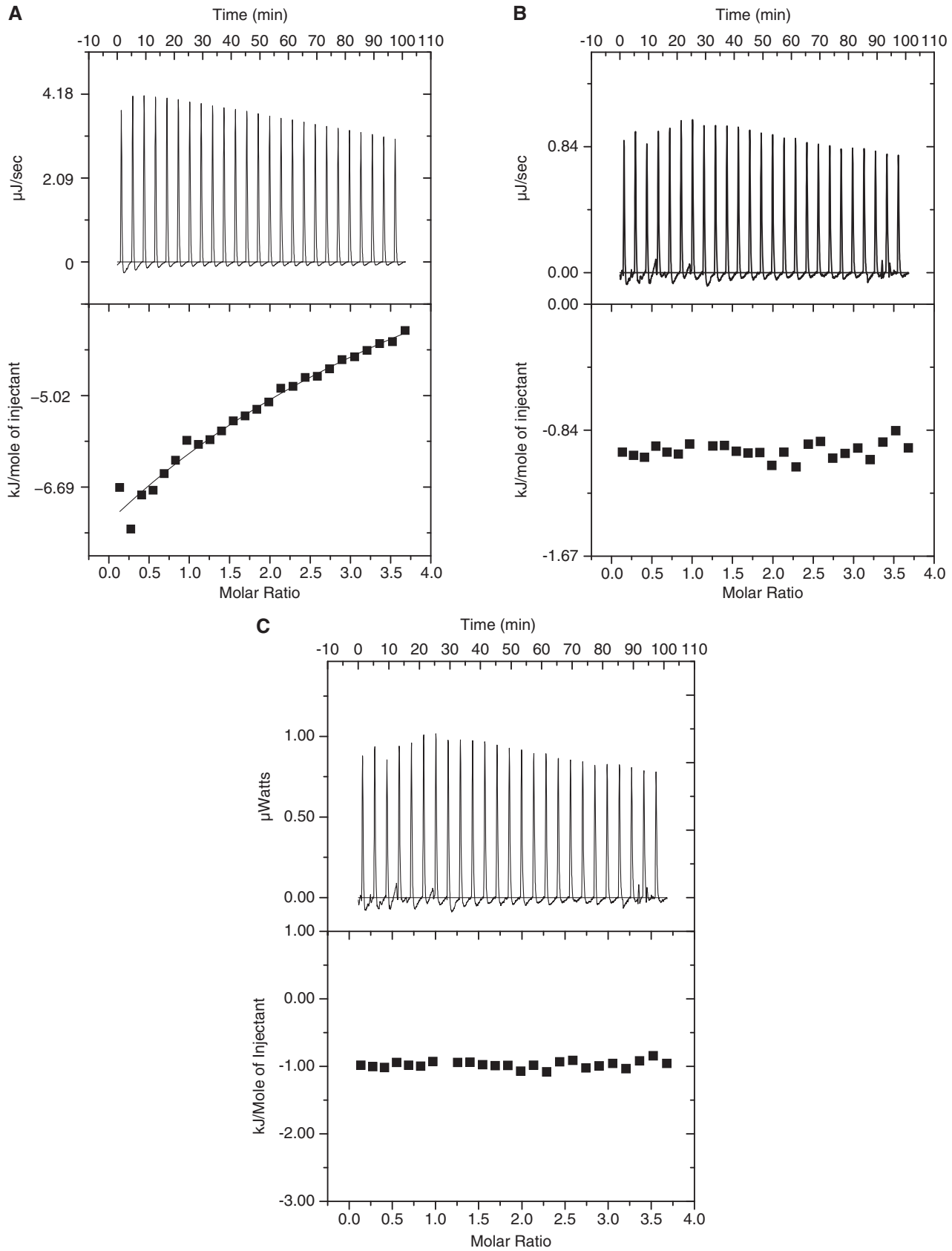

Fig. 3 Raw data for the titration of 2.5×10^{-3} mol dm^{-3} neomycin with 0.13×10^{-3} mol dm^{-3} BSA with in presence of (A) 0.1, (B) 1 mol dm^{-3} NaCl and (C) with 0.13×10^{-3} mol dm^{-3} HSA in presence of 1 mol dm^{-3} NaCl.

For the binding of neomycin to these proteins, an increase in ionic strength up to 0.5 mol dm^{-3} leads to a reduction in the binding affinity of the drug to the protein as reflected by a decrease in the value of K from 1.18×10^3 to an average of $\sim 0.4 \times 10^3$. Further increase in the ionic strength up to a value of 1.0 mol dm^{-3} leads to no binding both for BSA and HSA. The binding becomes more exothermic as shown by the increase in the negative value of enthalpy compared to that in the absence of NaCl. The value of ΔS becomes more negative as a result of these ionic interactions, which indicates unfavourable entropic contribution. The negative value of the entropy of binding most likely results from solvent reorganization as a result of ionic interference. However, the binding still proceeds favourably due to the larger exothermic contributions. Additional reasons for the ionic strength dependence include the binding of the ions to the charged amino acids and also the formation of an ion pair with the drug.

Effect and tetrabutylammonium bromide on binding of neomycin to BSA and HSA

To understand the effect of molecules, which are capable of interfering in hydrophobic interactions, on the extent of binding, experiments were done in the presence of tetrabutylammonium bromide TBAB at pH 7.4 and 298.15 K. The ITC binding profiles in presence of TBAB are shown in Fig. 4.

The values of the binding constant decreased with an increase in the concentration of TBAB from 0.02 to 0.05 mol dm^{-3} (Fig. 4A and B). However, with further increase of the concentration of TBAB to 0.1 mol dm^{-3} no typical binding pattern (Fig. 4C and D) is observed. TBAB is a molecule with mixed hydrophobic and ionic character, and can interfere in the binding of neomycin at both the charged and hydrophobic amino acid residues. The enhanced effects of TBAB at higher concentration are most likely due to larger interference in ionic interactions. The possible contributions of hydrophobic interactions are judged to be less likely due to the lack of hydrophobic groups in the drug molecule.

Effect of surfactants on binding of neomycin to BSA and HSA

In order to understand the contribution of non-columbic interactions in the binding, experiments were conducted in presence of the non-ionic surfactant TX-100. Figure 5 shows the dilution corrected integrated heat profile accompanying the titrations of 2.5 mM neomycin with 0.13×10^{-3} mol dm^{-3} BSA and HSA containing 0.35×10^{-3} mol dm^{-3} TX-100, where the surfactant is in the pre-miceller form. The values of K, ΔH° and ΔS° are $(1.10 \pm 04) \times 10^3$, -44.87 ± 0.84 and -91.21 J K^{-1} mol^{-1}, respectively. Thus it is observed that the presence of TX-100 in solution does not alter the binding affinity of the drug for the protein, though the binding is slightly more exothermic and is compensated by a larger entropic loss. Since TX-100 is a neutral surfactant capable of forming H-bonds and interfering in the hydrophobic interactions through hydrophobic groups, no change in binding affinity suggests lesser involvement of non-columbic interactions in the binding.

We have recently reported (*27*) that the antibiotic drug carbenicillin binds to BSA with the values of K, ΔH° and ΔS° as $(4.98 \pm 0.11) \times 10^3$, -39.58 ± 0.46 kJ mol^{-1} and -61.9 J K^{-1} mol^{-1}, respectively, at 298.15 K. These values are close to those observed for the binding of neomycin with BSA and HSA (Table I). It is further observed that in both the cases, the value of association constant remains of the order of 10^3 in the studied temperature range. Effects of ionic strength, TBAB and the surfactant TX-100 on the thermodynamic quantities accompanying the binding of neomycin compared with that of carbenicillin (*27*) suggest predominant involvement of electrostatic interactions in both the cases, though involvement of hydrophobic interactions is also indicated in the binding of latter.

ITC of the interaction of lincomycin with BSA and HSA

Figure 6 shows integrated injection heats for the titration of 4.52×10^{-3} mol dm^{-3} lincomycin with 0.09×10^{-3} mol dm^{-3} BSA and HSA. Almost negligible heat effects were observed after correction for dilution effects in the whole titration range at 298.15 K (Fig. 6). Experiments conducted at 308.15 K with both the proteins, also did not show any typical binding profile with liberation or absorption of heat (Fig. 6B and C). Experiments were also conducted by raising the concentration of the protein in the cell to 0.45×10^{-3} mol dm^{-3} and that of the drug to 22.6×10^{-3} mol dm^{-3} in order to confirm that the heat signals are not missed due to lower concentration of the protein or of the drug. Here also no change in the heat profile was observed.

Effect of surfactants on lincomycn-BSA and HSA interaction

Surfactants are known to interact with proteins and cause partial denaturation, leading to substantial changes in protein conformation and hence modification of binding sites. Thus experiments in presence of surfactants are expected to reveal if the partially unfolded BSA or HSA has more or fewer binding sites created for lincomycin. Titrations of lincomycin with BSA and HSA were performed in the presence of anionic (SDS), cationic (HTAB) and non-ionic (TX-100) surfactants. The representative heat profiles are shown in Figs 7 and 8, respectively. A typical binding profile is observed with both proteins in the presence of SDS and HTAB with the proteins. However, in the case of TX-100, no binding pattern was observed. We attempted to use different binding models to fit the ITC data on the interaction of lincomycin with BSA and HSA in presence of SDS. However, no reasonable binding model was able to represent the data. This rules out well-defined binding sites on BSA or HSA in the structurally modified form of the proteins and suggests a non-specific interaction behaviour.

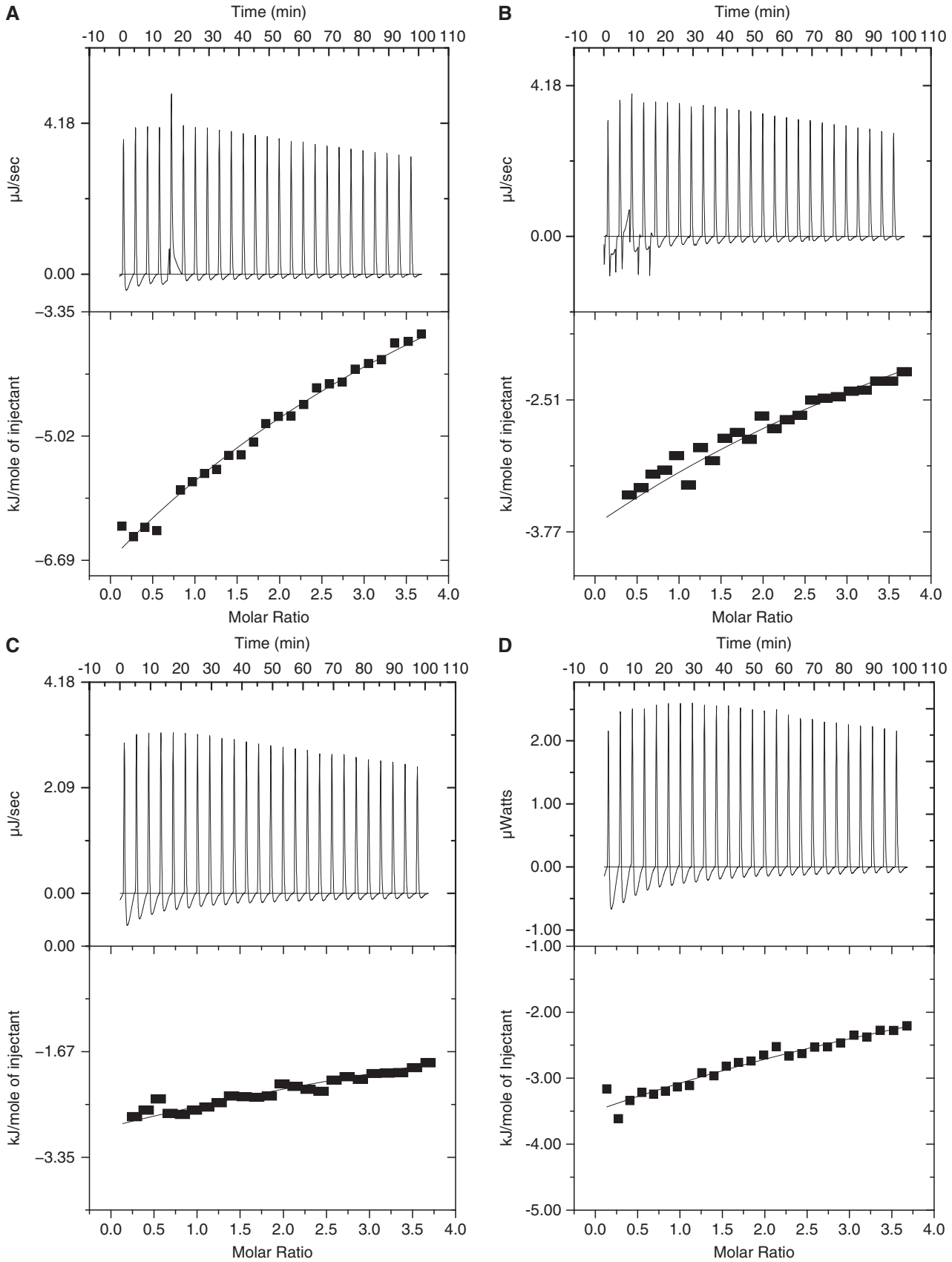

Fig. 4 Raw data for the titration of 2.5×10^{-3} mol dm^{-3} neomycin with 0.13×10^{-3} mol dm^{-3} BSA in presence of (A) 0.02, (B) 0.05, (C) 0.1 mol dm^{-3} tetrabutylammonium bromide and (D) with 0.13×10^{-3} mol dm^{-3} HSA in presence of 0.1 mol dm^{-3} tetrabutylammonium bromide.

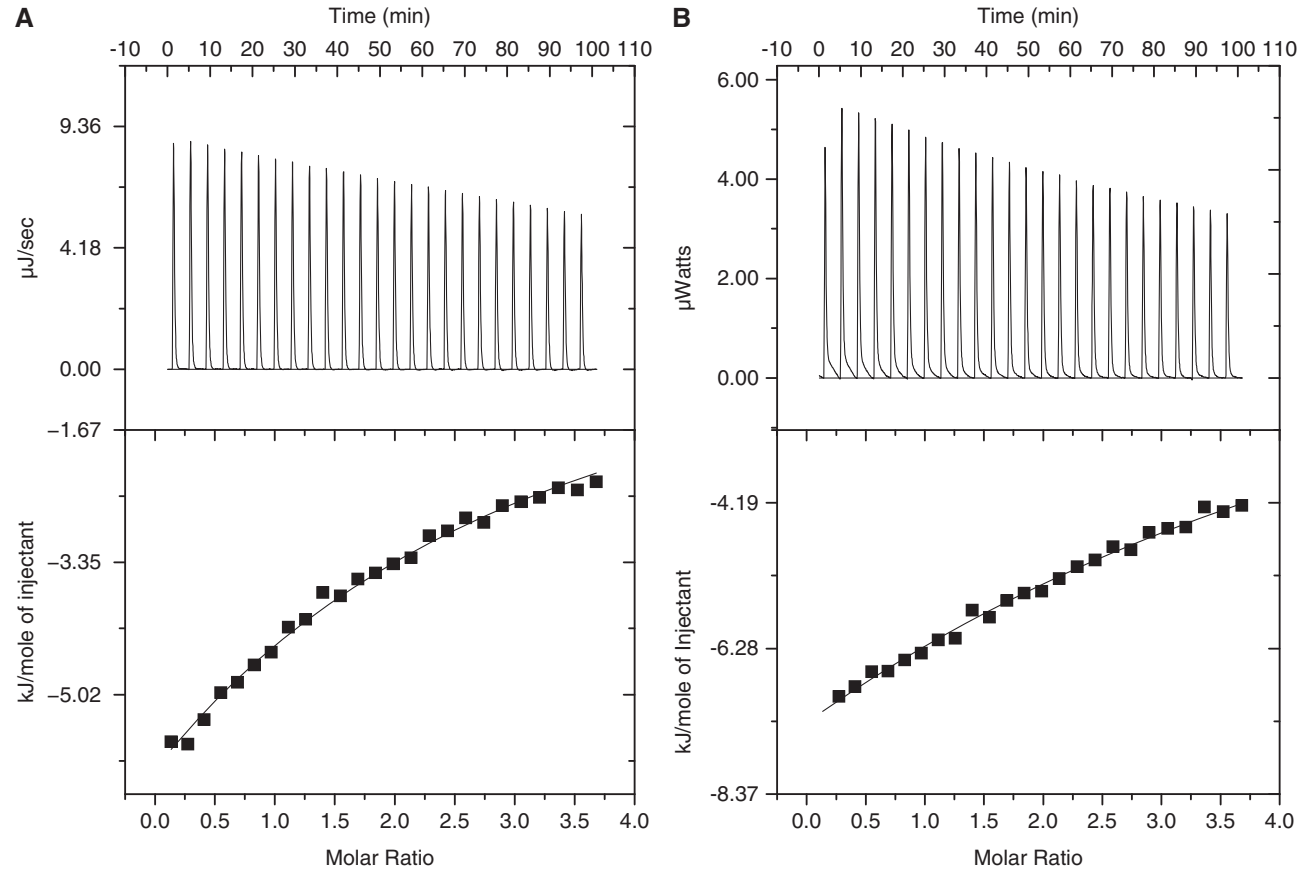

Fig. 5 Raw data for the titration of 2.5×10^{-3} mol dm^{-3} neomycin in presence of 0.35×10^{-3} mol dm^{-3} TX-100 with 0.13×10^{-3} mol dm^{-3} (A) BSA and (B) HSA.

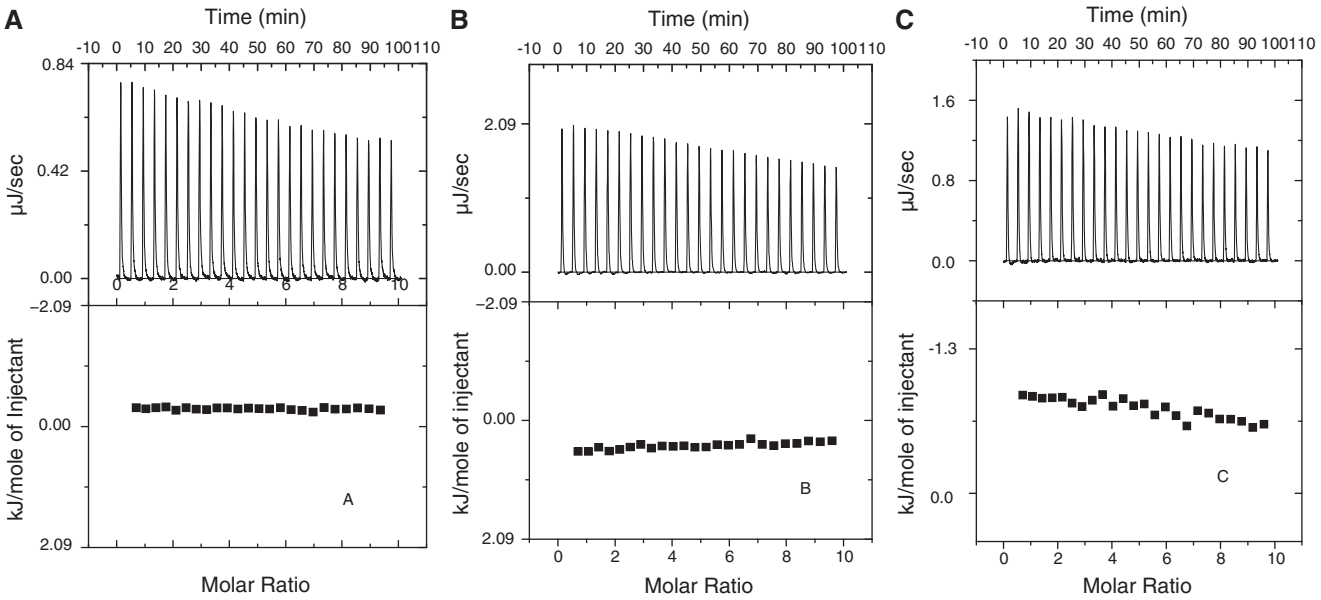

Fig. 6 Raw data for the titration of 4.51×10^{-3} mol dm^{-3} lincomycin to 0.09×10^{-3} mol dm^{-3} BSA (A) 298.15 K and (B) 308.15 K and to 0.09×10^{-3} mol dm^{-3} HSA at (C) 308.15 K at pH 7.4.

Thermal denaturation of BSA and HSA in the presence of lincomycin

Generally when a ligand binds to the native state of a protein, it imparts thermal stability to it thereby enhancing its thermal stability index. In the present case, since the ITC showed typical binding profile only in presence of anionic and cationic surfactants, the thermal unfoldings of BSA and HSA were studied in the absence and presence of the drug to check the

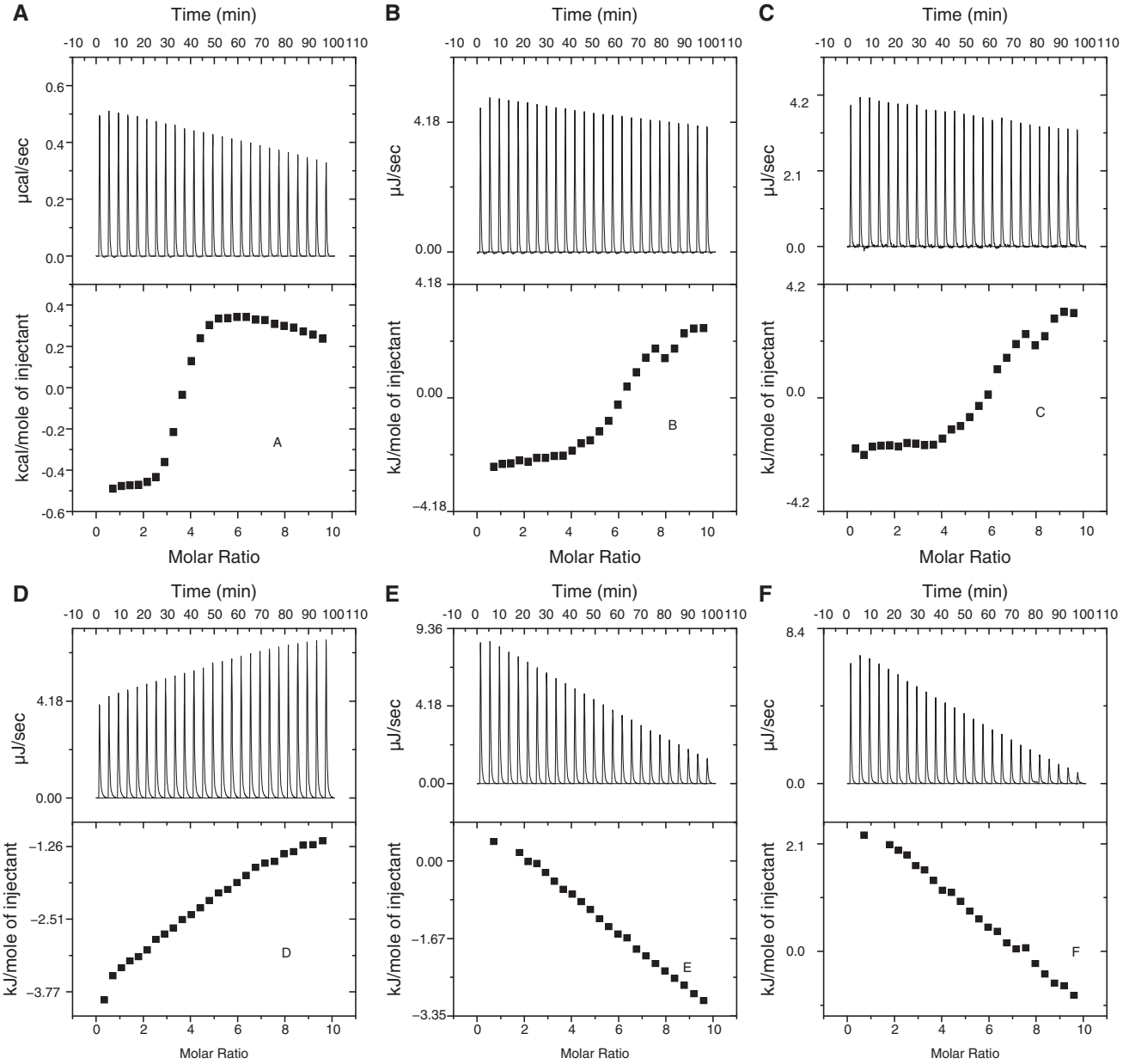

Fig. 7 Raw data for the titration of 4.51×10^{-3} mol dm^{-3} lincomycin with 0.09×10^{-3} mol dm^{-3} BSA (A) 298.15 K, (B) 308.15 K and with 0.09×10^{-3} mol dm^{-3} HSA (C) 308.15 K in presence of 2×10^{-3} mol dm^{-3} SDS, and titration of 4.51×10^{-3} mol dm^{-3} lincomycin with 0.09×10^{-3} mol dm^{-3} BSA (D) 298.15 K, (E) 308.15 K and with 0.09×10^{-3} mol dm^{-3} HSA (F) 308.15 K in presence of 5×10^{-3} mol dm^{-3} HTAB at pH 7.4.

extent of thermal stabilization provided if binding does take place. The thermal unfolding curves were obtained by measuring the absorbance changes of the protein at 293 nm as a function of temperature. The thermal denaturation experiments were carried out at different molar ratios of BSA or HSA to lincomycin from 1 : 0 to 1 : 10. Figure 9 shows thermal melting profiles of BSA and HSA at different molar ratios of protein to drug. The corresponding thermodynamic parameters accompanying the thermal unfolding are reported in Table II. In the absence of SDS, the value of the temperature where the transition is half complete ($T_{1/2}$), and the enthalpy of unfolding do not change upon addition of lincomycin from the molar ratio of 0.20–10 in solution. In the presence of 2×10^{-3} mol dm^{-3} SDS, the transition temperature of BSA decreased by ~12 K with a reduction in the binding enthalpy of ~70 kJ mol^{-1}. This suggests that SDS is leading to partial denaturation of the protein and hence partial exposure of various groups to the solvent environment. Addition of lincomycin to this SDS induced partially denatured BSA enhances the thermal stability of the protein. There is an increase of 9.5 K in transition temperature of protein when lincomycin to BSA molar ratio in protein is increased from 0.25 to 10. This is also accompanied with an endothermicity of unfolding by ~70 kJ mol^{-1}. These observations are consistent with the ITC results that the addition of

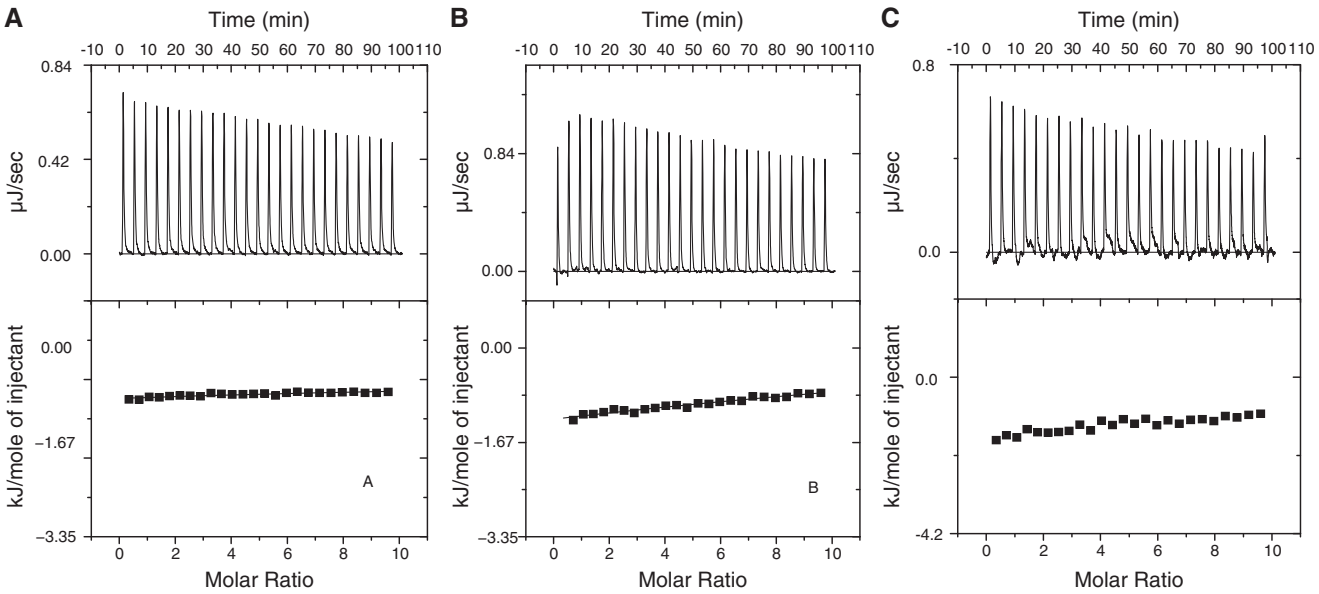

Fig. 8 Raw data for the titration of 4.51×10^{-3} mol dm^{-3} lincomycin to 0.09×10^{-3} mol dm^{-3} BSA (A) 298.15 K, (B) 308.15 K, and to 0.09×10^{-3} mol dm^{-3} HSA (C) 308.15 K in presence of 1×10^{-3} mol dm^{-3} TX-100 at pH 7.4.

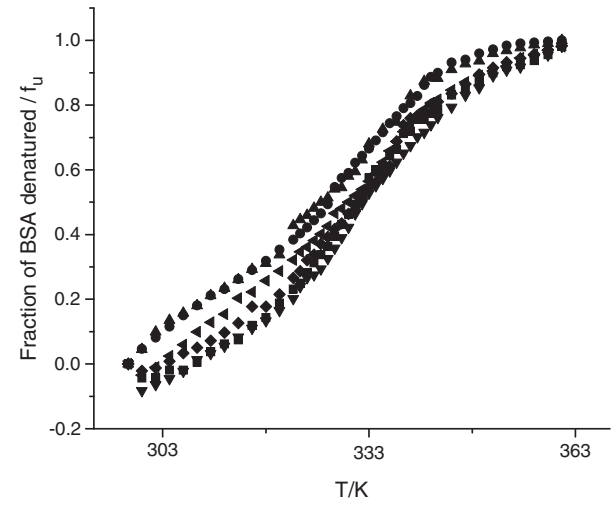

Fig. 9 Thermodynamic parameters accompanying the thermal unfolding of 15×10^{-6} mol dm^{-3} BSA in the absence and presence of different [lincomycin]/[BSA] molar ratios (exclamatory) 0, (filled circle) 0.25, (filled triangle pointed upward) 0.5, (inverted filled trianlge) 1, (filled rhombus) 5 and (sideward) 10.

SDS induces conformational modification in the protein thereby creating binding sites for lincomycin. Thermal unfolding experiments could not be carried out in presence of HTAB and TX-100 since the aggregation of the protein started during the transition leading to precipitation.

Fluorescence studies

To understand the effect of neomycin and lincomycin on the tryptophan environment of these proteins and to determine the binding constant of interaction by another method, the intrinsic fluorescence of 0.725×10^{-6} mol dm^{-3} BSA and 1.55×10^{-6} mol dm^{-3} HSA in the presence of increasing concentration of drugs was studied. The fluorescence spectra of protein in the presence of neomycin and lincomycin are shown in Figs 10 and 11, respectively. The maximum intensity of BSA and HSA in the absence of the drugs is observed at 345 nm and that of HSA at 350 nm which are in good agreement with that reported in literature (*28*, *29*).

The addition of neomycin at increasing molar ratio of the [drug]/[protein] from 0 to 100 does not lead to appreciable changes either in the maximum intensity of emission or in the value of λ_{max}. This indicates that either neomycin does not bind to BSA and HSA or that binding of the drug is not happening in the close vicinity of tryptophan residues, which are located at positions 134 and 212 in sub domains IA and IIA of BSA and 214 of HSA, respectively (*30*, *31*). Since the ITC results clearly show that binding of neomycin to BSA and HSA is of the order of 10^3, comparison with fluorescence observations are in support of latter possibility. The addition of lincomycin to BSA or HSA at molar ratios from 0 to 50 also did not lead to significant changes in the fluorescence emission spectra. These observations are consistent with the ITC results that the lincomycin does not bind to BSA or HSA at pH 7.4. Even in the presence of SDS (Fig. 12) and HTAB (figure not shown), the fluorescence intensity and the value of λ_{max} did not change upon addition of lincomycin to protein. Similar results were obtained with HSA (Fig. 13). Since the ITC results in the presence of SDS and HTAB show binding pattern with the change of heat without fitting any reasonable binding model (Fig. 7), it may be inferred that the interaction of lincomycin with BSA or HSA are non-specific.

Time resolved fluorescence lifetime measurements

Fluorescence life time measurements were carried out for BSA and HSA in the absence and presence of neomycin and lincomycin (Figs 14 and 15). A sample containing 15×10^{-6} mol dm^{-3} BSA was excited at 295 nm

Table II. Thermal unfolding of BSA in the presence of varying [lincomycin]/[BSA] molar ratios represented as [D]/[P] at pH 7.4 in absence and presence of 2 mM SDS.

[D]/[P] [SDS] = 0 mM	$T_{1/2}$/K	ΔH°/(kJ mol^{-1})	[D]/[P] [SDS] = 2 mM	$T_{1/2}$/K	ΔH° (kJ mol^{-1})
0.00	333.9 ± 1.1	201	0.00	321.7 ± 1.8	129
0.25	333.8 ± 2.1	193	5	331.5 ± 2.7	128
0.50	333.7 ± 1.7	188	0.5	331.7 ± 2.2	176
1.00	333.5 ± 1.6	200	1.00	331.1 ± 1.8	166
5.00	333.6 ± 2.0	183	5.00	331.1 ± 1.7	176
10.00	333.6 ± 1.5	187	10.00	331.2 ± 2.9	196

$T_{1/2}$ represents the temperature at half the denaturation.

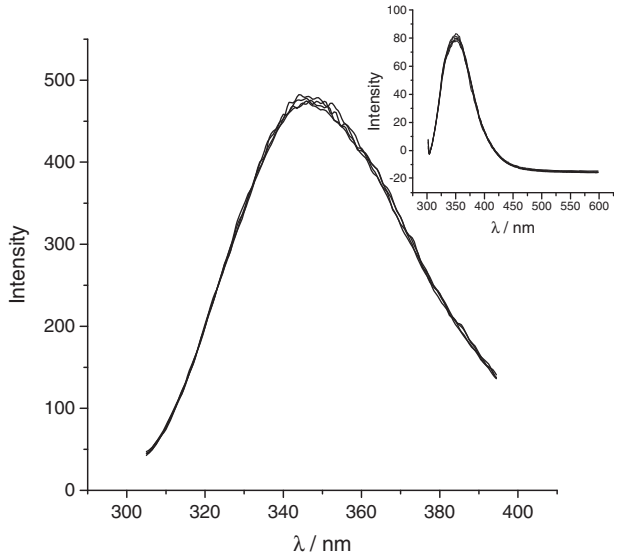

Fig. 10 Intrinsic fluorescence spectra at different molar ratios of [neomycin]/[protein] ranging from 0 to 100 for 0.72×10^{-6} mol dm^{-3} BSA and for 1.5×10^{-6} mol dm^{-3} HSA (inset).

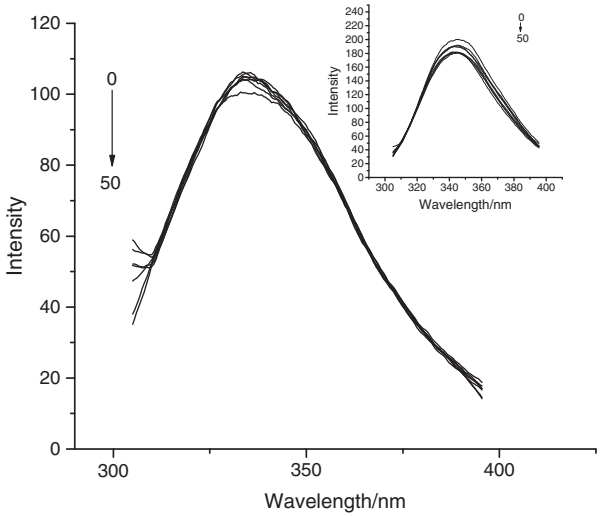

Fig. 12 Intrinsic fluorescence of 0.72×10^{-6} mol dm^{-3} BSA in the presence of varying [lincomycin]/[BSA] ratios at pH 7.4 with 2×10^{-3} mol dm^{-3} SDS and without SDS (in inset) at 308.15 K.

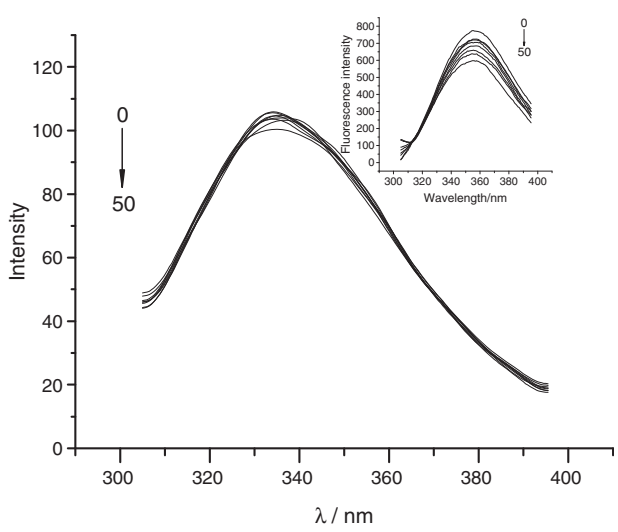

Fig. 11 Intrinsic fluorescence of 0.72×10^{-6} mol dm^{-3} BSA in the presence of varying [lincomycin]/[BSA] ratios at pH 7.4 with 2×10^{-3} mol dm^{-3} SDS and without SDS (inset) at 298.15 K.

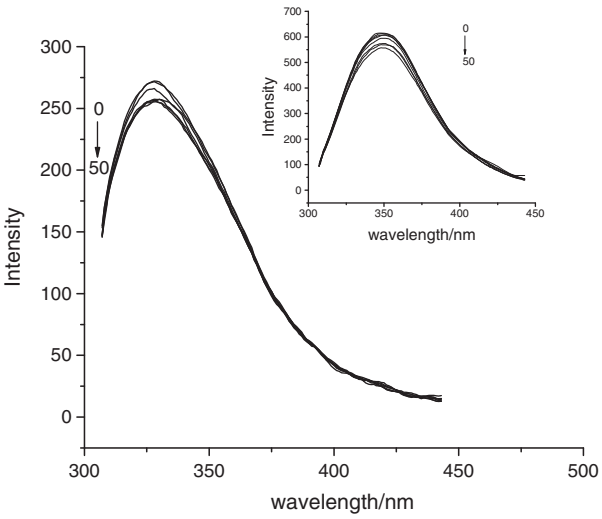

Fig. 13 Intrinsic fluorescence of 1.50×10^{-6} mol dm^{-3} HSA in the presence of varying [lincomycin]/[HSA] ratios at pH 7.4 with 2×10^{-3} mol dm^{-3} SDS and without SDS (inset) at 308.15 K.

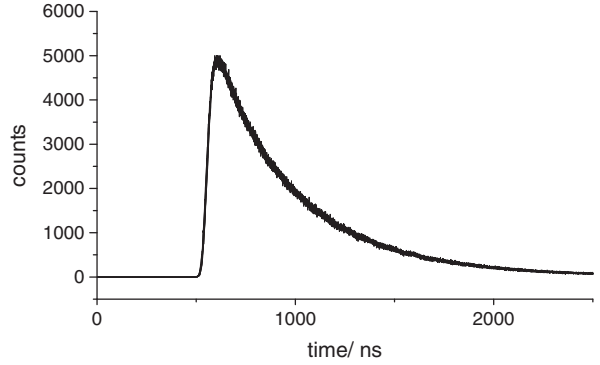

Fig. 14 Fluorescence decay profiles of 1.5×10^{-6} mol dm^{-3} BSA in the presence of different [neomycin]/[protein] molar ratios ranging from 0 to 25.

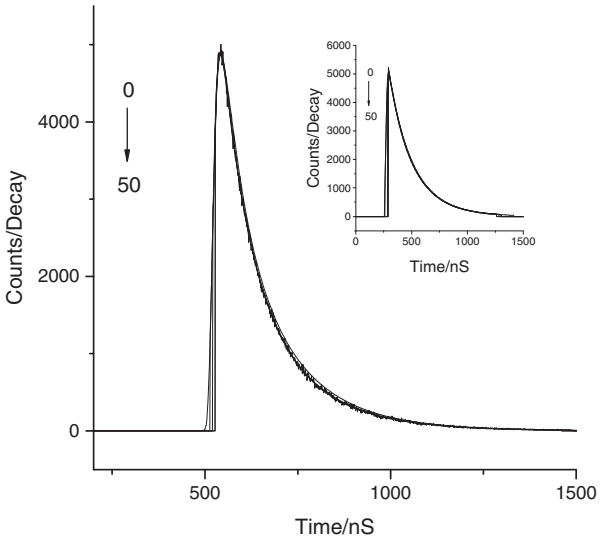

Fig. 15 Lifetimes of fluorescence decay of 15×10^{-6} mol dm^{-3} BSA in the presence of varying [lincomycin]/[BSA] ratios ranging from 0 to 50 at pH 7.4 with 2×10^{-3} mol dm^{-3} SDS and without SDS (inset).

and emission was monitored at 344 nm. The decay curves were well fit by a bi-exponential function in the entire concentration range of drugs studied. It is seen in Table III that the fluorescence life time of BSA did not change significantly with the rise in the concentration of neomycin. It is consistent with the steady-state fluorescence results that binding does not involve tryptophan residues. On the other hand though the average life time of tryptophan changes from 6.08 to 3.20 ns upon addition of lincomycin in presence of 2×10^{-3} mol dm^{-3} SDS at 1: 1 molar ratio (Fig. 15), it further remains constant up to [drug]/[BSA] molar ratio of 50 (Table IV). It should be noted in Table IV that in the absence of SDS, the average life time of tryptophan does not change upon addition of lincomycin in the entire concentration range studied. Even in the presence of HTAB and TX-100 (Fig. 16) no change in life time was observed (Table V). These observations provide further support to the ITC, thermal denaturation, and steady-state fluorescence results that lincomycin does not bind to BSA in the native conformation but interacts non-specifically in the presence of surfactants (SDS and HTAB).

Table III. Fluorescence lifetime and amplitude measurements for 0.13 mM BSA in presence of neomycin.

[Drug]/[protein]	Lifetime/(ns) τ_1	τ_2	Amplitude α_1	α_2	Avg. lifetime/(ns) $\langle\tau\rangle$	χ^2
0	3.03	6.58	0.203	0.79	5.80	1.00
1	2.17	6.33	0.18	0.81	5.13	1.09
2	1.59	6.21	0.19	0.80	5.28	1.06
5	1.92	6.19	0.16	0.83	5.48	1.03
7	1.44	6.14	0.18	0.81	5.25	1.08
10	1.03	6.13	0.20	0.79	5.13	1.07
25	1.34	6.21	0.19	0.80	5.25	1.12

Table IV. Lifetimes of fluorescence decay of 15 μM BSA in the presence of varying [lincomycin]/[BSA] ratios represented as [D]/[P] at pH 7.4 (a) in buffer, and (b) in presence of 2 mM SDS.

[D]/[P]	Lifetime (ns) τ_1	τ_2	Amplitude a_1	a_2	Avg. lifetime (ns) d $\langle\tau\rangle$	χ^2
[SDS] = 0 mM						
0	1.73	6.24	0.849	0.151	5.56	1.19
1	5.09	8.46	0.702	0.297	6.08	1.09
5	5.12	10.8	0.821	0.178	6.17	1.16
10	5.41	9.95	0.839	0.161	6.13	1.14
50	5.46	1.26	0.893	0.107	6.23	1.14
[SDS] = 2 mM						
0	2.24	4.93	0.628	0.372	3.24	1.06
1	2.18	4.86	0.618	0.382	3.20	1.07
5	2.28	4.94	0.631	0.369	3.28	1.09
10	2.09	4.74	0.580	0.420	3.20	1.16
50	2.27	4.92	0.588	0.412	3.36	1.14

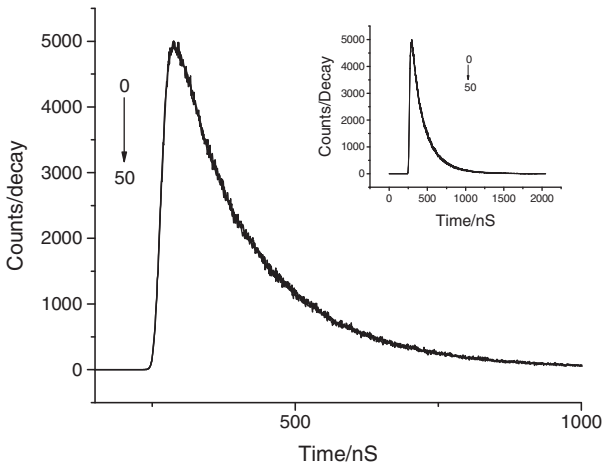

Fig. 16 Lifetimes of fluorescencedecay of 15×10^{-6} mol dm^{-3} BSA in the presence of varying [lincomycin]/[BSA] ratios ranging from 0 to 50 at pH 7.4 in the presence of 5×10^{-3} mol dm^{-3} HTAB and 1×10^{-3} mol dm^{-3} TX-100 (inset).

Possible binding sites for the drug

The crystal structure of serum albumin is reported in the literature at a resolution 2.5×10^{-10} m (*32*). It is well known that there is 76% homology between BSA and HSA. It has two major binding sites in the

Table V. Lifetimes of fluorescence decay of 15×10^{-6} mol dm^{-3} BSA in the presence of varying [lincomycin]/[BSA] molar ratios represented as [D]/[P] at pH 7.4 (a) in presence of 5 mM HTAB and (b) with 1 mM TX-100.

[D]/[P]	Lifetime (ns)		Amplitude		Avg. lifetime (ns)	χ^2
	τ_1	τ_2	a_1	a_2	$\langle\tau\rangle$	
[HTAB] = 5×10^{-3} mol dm^{-3}						
0	1.52	4.59	0.347	0.653	3.53	1.09
5	1.55	4.58	0.333	0.667	3.57	1.02
50	1.52	4.62	0.340	0.625	3.55	1.08
[TX-100] = 1×10^{-3} mol dm^{-3}						
0	1.96	5.57	0.347	0.653	4.32	1.09
5	1.43	4.94	0.233	0.767	4.12	1.00
50	1.31	4.94	0.222	0.778	4.13	1.09

subdomains IIA and IIIA known as warafarin and diazepam binding sites, respectively. Site 1 is believed to be a binding site for salicylates, sulphonamides and several other similar types of drugs. The inside wall of this binding pocket is formed by hydrophobic side chains and the entrance of pocket is surrounded by Arg257, Arg222, Lys199, His242, Arg218 and Lys195 (*33*). Site 2, which is known as a binding site for tryptophan, thyroxin, octanoate and other similar drugs, corresponds to pocket of subdomain IIIA. This pocket is lined by hydrophobic side chains and double disulphide bridge of helix IIIA-h_3 (*34*). The side chain of Arg410 is located at the mouth of the pocket whereas the hydroxyl of Tyr411 faces towards the inside of the pocket (*32*). The structure of neomycin (Fig. 1A) and corresponding pK_a values of various amine groups suggest that the drug can bind to the sites which are composed of polar/ionic residues on serum albumin at pH 7.4.

Since in the presence of NaCl and TBAB there is a reduction in the binding ability of neomycin to BSA and HSA, the contribution of ionic residues in binding are indicated. Furthermore, no changes in the binding affinity of neomycin for BSA and HSA in the presence of sucrose rules out the involvement of residues which are capable of forming H-bonds. These observations suggest that neomycin binds to serum albumin at site 1 where the binding pocket is surrounded by positively charged residues. This further supports fluorescence results that binding of neomycin does not alter the local environment of tryptophan residues which are located in the interior hydrophobic core.

As discussed earlier, based on the thermal unfolding experiments, BSA is in the partially unfolded state in the presence of SDS and HTAB, and hence partial exposure of various groups can lead to interaction of lincomycin with the polar and hydrophobic groups of the protein in a non-specific manner.

Conclusions

A combination of isothermal titration calorimetric, UV–visible, steady state and time resolved spectroscopic measurements have been made to understand the interaction of antibiotics neomycin and lincomycin with BSA and HSA qualitatively and quantitatively. Neomycin has been observed to bind to BSA and HSA with an affinity constant of the order of 10^3. The binding of neomycin is enthalpically favoured but entropically opposed with a stoichiometry of binding of 1 : 1. The results on the binding of neomycin to BSA and HSA in the presence of additives suggest predominance of electrostatic interactions in the complexation. Lincomycin, on the other hand does not show any evidence for binding to the native state of these proteins. However, lincomycin demonstrates a non-specific interaction behaviour with surfactant induced conformationally modified BSA and HSA. These observations suggest that serum albumin is not the primary binding component for lincomycin in the plasma protein.

Supplementary Data

Supplementary Data are available at *JB* Online.

Acknowledgements

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Conflict of interest

None declared.

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