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Mechanism of interaction of vincristine sulphate and rifampicin with bovine serum albumin: A spectroscopic study

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Abstract. The mechanism of interaction of vincristine sulphate (VS) and rifampicin (RF) with bovine serum albumin (BSA) has been studied by quenching of BSA fluorescence by RF/VS. The Stern–Volmer plot indicates the presence of a static component in the quenching mechanism. Results also show that both the tryptophan residues of BSA are accessible to VS and RF. The high magnitude of rate constant of quenching indicates that the process of energy transfer occurs by intermolecular interaction and VS/RF-binding site is in close proximity to the tryptophan residues of BSA. Binding studies in the presence of a hydrophobic probe, 8-anilino-1-naphthalene-sulphonic acid sodium salt (ANS) indicate that the VS and RF compete with ANS for hydrophobic sites on the surface of BSA. Small decreases in critical micellar concentrations (CMC) of anionic surfactants in presence of VS/ RF show that the ionic character of VS/RF also contributes to binding. The temperature dependence of the association constant is used to estimate the values of the thermodynamic parameters involved in the interaction of VS/RF with BSA and the results indicate that hydrophobic forces play a significant role in the binding. Circular dichroism studies reveal that the change in helicity of BSA are due to binding of VS/RF to BSA.

Keywords. Vincristine sulphate; rifampicin; fluorescence quenching mechanism.

1. Introduction

Drug–protein interactions play a key role in understanding the distribution, elimination and transport of small molecules in biological systems. These factors in turn partly determine the time-course and intensity of drug effects. Understanding the molecular basis of drug-protein interaction is important in designing new therapeutic agents for improved drug activity.[1] Vincristine sulphate (VS), which belongs to the class of vinca alkaloids, is an anticancer drug given intravenously for the treatment of acute leukemia, rhabdomyosarcoma, neuroblastoma, Hodgkin's disease and non-Hodgkin's lymphoma. Rifampicin (RF) is used as a antimycobacterial; it acts by interfering with the DNA-dependent RNA polymerase of bacterial cells. It is now widely used together with isoniazid and streptomycin for the chemotherapy of tuberculosis. Several adverse effects were observed when the patients were administered more the normal dose of VS and RF. So, it is important to study the interactions between VS/RF and BSA. In view of this, studies have been undertaken to elucidate the nature of interactions between the selected drugs and serum transporter protein (BSA). Two common methods that have been used in evaluating the binding of drugs to albumin are equilibrium dialysis and ultrafiltration.[2–5] These methods are laborious and time-consuming and the results, at times, are not reproducible. Also, these conventional methods are often inapplicable to the analyses of strongly bound drugs because of technical problems such as drug adsorption on the membrane and leakage of bound drug through membrane. To overcome these problems, we have employed fluorometric and circular dichroism methods to investigate the mode of interaction of VS and RF with BSA. Fluorescence techniques are very useful in the study of macromolecules such as proteins because of their high sensitivity, ease of application and ability to complement data obtained from other spectroscopic techniques.

2. Experimental

2.1 *Chemicals and reagents*

Serum albumin bovine (BSA, Fraction V, approximately 99%; protease-free and essentially *g*-globulin

*For correspondence

free) and 8-anilino-1-naphthalein-sulphonic acid, sodium salt were obtained from Sigma Chemical Company, USA. Vincristine sulphate and rifampicin were obtained as gift samples from Cipla, India. All other materials were of analytical reagent grade. The solutions of drug and BSA were prepared in 0·1 M phosphate buffer of pH 7·4 containing 0·15 M NaCl. BSA solution was prepared based on its molecular weight of 65,000.

2.2 *Apparatus*

Fluorescence measurements were performed on a Hitachi spectrofluorometer Model F-2000 equipped with a 150W xenon lamp and 10 nm slit width. CD measurements were made on a Jasco-810 using a 1·00 cm quartz cell at 0·2 nm wavelength intervals, with 3 scans averaged for each CD spectrum in the range of 190–250 nm.

2.3 *General procedure*

2.3a *VS/RF-BSA interaction:* Some preliminary studies were carried out to select optimum protein and drug concentrations for drug–protein interaction. On the basis of preliminary experiments, BSA concentration was kept fixed at 10 *m*M and drug concentration was varied from 10 to 70 *m*M for VS and 10 to 40 *m*M for RF. Fluorescence spectra were recorded at room temperature (27°C) in the range 300–500 nm upon excitation at 296 nm in each case. The absorbances of drug–protein mixtures in the concentration range employed for the experiment did not exceed 0·05 at the excitation wavelength in order to avoid inner filter effect.

2.3b *Procedure for thermodynamics of drug–protein interaction:* Thermodynamic parameters for the binding of VS/RF to BSA were determined by carrying out binding studies at three different temperatures, 13°, 29° and 35°C by the spectrofluorometric method.

2.3c *Procedure for binding studies in the presence of a hydrophobic probe:* Experiments were carried out in the presence of ANS. In the first set of experiments, the interaction of drug/ANS with BSA was studied under identical conditions. BSA concentration was kept fixed at 10 *m*M and drug/ANS concentration was varied (4–25 *m*M). Fluorescence spectra were recorded in the range of 300–500 nm upon excitation at 296 nm. In the second set of experiments, BSA–ANS interaction was studied in the absence and presence of 5, 10, 15, and 20 *m*M of drug. Concentrations of BSA and ANS were fixed at 10 and 20 *m*, respectively. Fluorescence spectra were recorded in the range 390–550 nm upon excitation at 370 nm.

2.3d *Critical micellar concentration (CMC):* CMC of an anionic surfactant, sodium dodecyl sulphate (SDS) was determined in the presence and absence of 40 *m*M drug and in the presence of ANS.[6] ANS concentration was kept fixed at 40 *m*M and the SDS concentration was varied from 1·5 to 9·0 mM. Fluorescence spectra were recorded in the range 390–500 nm upon excitation at 370 nm.

2.3e *Effect of paracetamol on drug–protein interaction:* Protein–drug binding was studied in presence and absence of paracetamol using fluorescence spectroscopy. Emission spectra were recorded in the range of 300–500 nm.

2.3f *Surface tension measurement:* Surface tension of drug solutions (0·8%) prepared in phosphate buffer of pH 7·4 containing 0·15 M NaCl solution at 27°C was determined by drop weight and drop number method using a stalagmometer.

2.3g *Circular dichroism (CD) studies:* CD measurements of BSA were made in the presence and absence of the drug. A stock solution of 0·1 *m*M BSA was prepared in 0·01 M phosphate buffer containing 0·15 M NaCl solution. BSA to drug concentration was varied (1 : 1, 1 : 3 and 1 : 5) and CD spectra were recorded.

3. Results and discussion

The structures of VS and RF employed in the present study are shown in figure 1.

3.1 *Fluorescence studies*

We have measured the competitive absorbance of protein and drug molecules at the excitation wavelength (296 nm) and observed that both of them do not contribute to the inner filter effect as evident from their very low absorbance values. Fluorescence spectra of BSA were recorded in the presence of increasing amounts of VS/RF (figure 2) upon excitation at 296 nm. In the case of VS, interaction may be

through an energy transfer mechanism, as seen by the appearance of a new peak at 462–464 nm with increasing drug concentration with clear isoemmissive point. However, RF quenched the fluorescence intensity of tryptophan of BSA with a red-shift in the emission wavelength (from 344 to 361 nm). The fraction of drug that was bound, q, was determined according to Weber and Young,[7] and Maruyama *et al*,[8] using the equation,

$$q = (F_0 - F)/F_0, \tag{1}$$

where F and F_0 denote the fluorescence intensities of protein in a solution with a given concentration of drug and without the drug respectively. The fluorescence data were analysed using the method described by Ward.[9] It has been shown that for n equivalent and independent binding sites,

$$1/[(1 - q)K] = [D_t]/q - n[P_T], \tag{2}$$

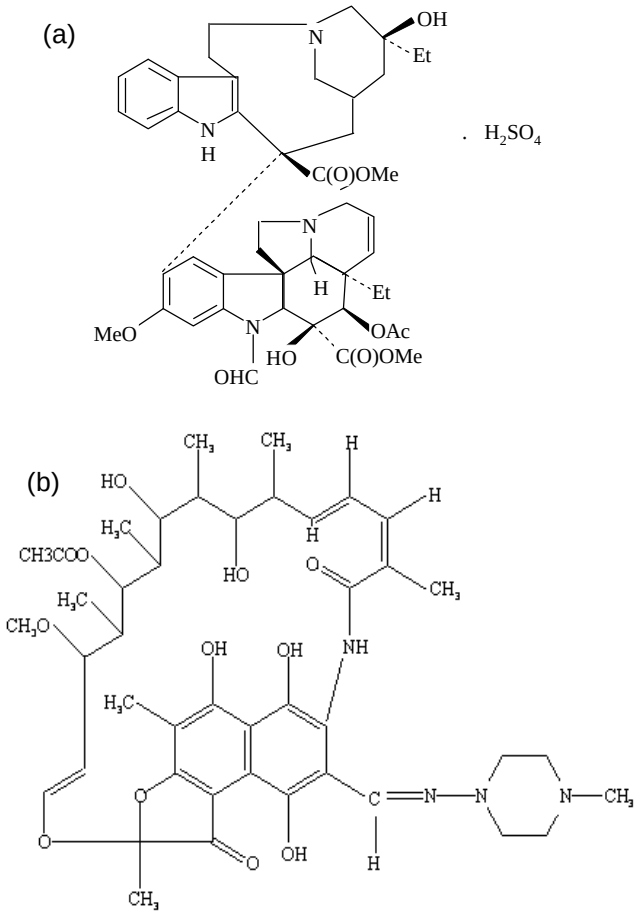

Figure 1. Structures of (**a**) vincristine sulphate and (**b**) rifampicin.

where K is the association constant for drug–protein interaction, n is the number of binding sites, $[Dt]$ is the total drug concentration and $[P_T]$ is the total protein concentration. A plot of $1/(1 - q)$ versus $[Dt]/q$ is shown in figure 3 for VS and RF. The values of K and n obtained from the slope and intercept of such plot are $3{\cdot}17 \times 10^4\ M^{-1}$ and 4·7 for VS and $1{\cdot}83 \times 10^5\ M^{-1}$ and 1·0 for RF respectively. Standard free energy change (ΔG_0) values were obtained from the relationship, $\Delta G_0 = -2{\cdot}303\ RT\log K$ and were be –26·0 and –30·21 kJ mol^{-1} at 29°C for VS and RF respectively. This shows that the VS/RF moieties interact strongly with the protein.

3.2 *Stern–Volmer analysis*

Fluorescence intensity data are also analysed according to Stern–Volmer law,[10,11]

$$F_0/F = 1 + K_{SV}[Q], \tag{3}$$

by plotting F_0/F versus $[Q]$, where F_0 and F are the steady state fluorescence intensities at 344 nm in the absence and presence of the quencher (drug) respectively, K_{SV} is the Stern–Volmer constant and $[Q]$ is the total drug concentration. The Stern–Volmer plot (figure 4a) shows a positive deviation from a straight line, suggesting the presence of a static component in the quenching mechanism.[12] A modified form of the Stern–Volmer equation[12] that describes quenching data when both dynamic and static quenching are operative is

$$F_0/F = 1 + K_{SV}[Q] \exp V[Q], \tag{4}$$

where K_{SV} is the collisional quenching constant or Stern–Volmer quenching constant and V is the static quenching constant. The value of V is obtained from 4 by plotting $[F_0/F \exp\{(V[Q])\}]$ versus $[Q]$ for varying V until a linear plot is obtained. The K_{SV} value is then obtained from the slope of $[F_0/F \exp\{(V[Q])\}]$ versus $[Q]$ plot passing through the intercept equal to 1 (figure 4b). The values of V and K_{SV} so obtained are observed to be $4{\cdot}01 \times 10^3$ and $4{\cdot}65 \times 10^3\ M^{-1}$ and, $2{\cdot}96 \times 10^3$ and $3{\cdot}21 \times 10^3\ M^{-1}$ for VS and RF respectively.

According to Eftink and Ghiron,[12] upward curvature in the Stern–Volmer plot indicates that both tryptophan residues of BSA are exposed to the quencher and the quenching constants of both tryptophan residues are nearly identical, while a down-

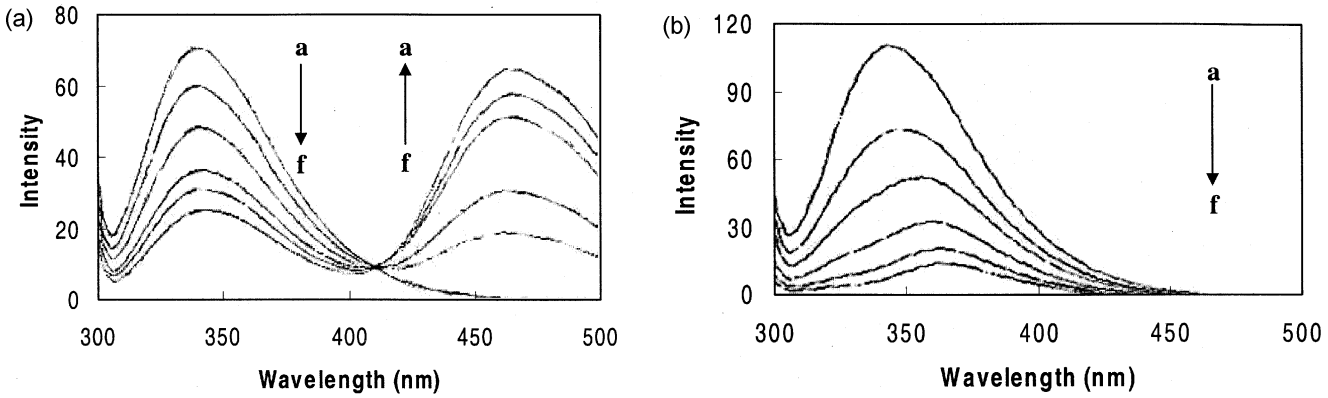

Figure 2. Fluorescence spectra of BSA (10 *m*M) in the presence of (**a**) VS (a – 0, b – 10, c – 20, d – 40, e – 50, and f – 70 *m*M) and (**b**) RF (a – 0, b – 5, c – 10, d – 20, e – 30 and f – 40 *m*M).

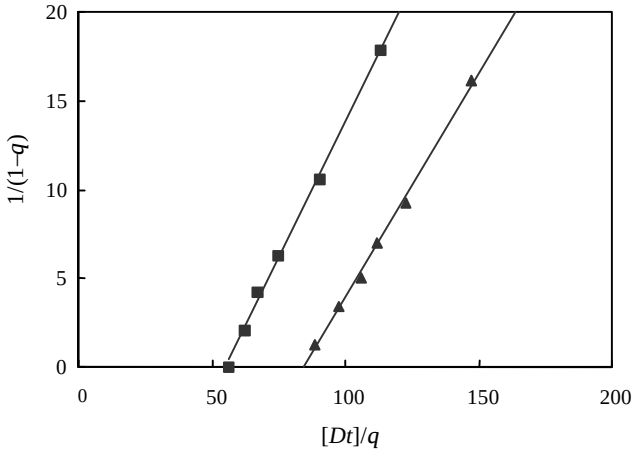

Figure 3. $1/(1 - q)$ versus $[Dt]/q$ plot for the binding of VS (–■–) and RF (–▲–) to BSA.

ward curvature indicates buried tryptophan residues. Fluorescence quenching data are also analysed by the modified Stern–Volmer plot, $F_0/(F_0 - F) = 1/f_a + 1/[Q]\ f_a\ K_{SV}$, where F_0 and F are fluorescence intensities at 344 nm in the presence and absence of the quencher, respectively, at $[Q] = D_t$, the total concentration of drug, K_{SV} is the Stern–Volmer quenching constant and f_a is the fraction of fluorophore (protein) accessible to the quencher (drug). From the plot of $F_0/(F_0 - F)$ versus $1/D_t$ (figure 5) the values of f_a and K_{SV} are determined. The value of f_a is close to unity (1·02) indicating thereby that both tryptophan residues of BSA are involved in the interaction. For a bimolecular quenching process, $K_{SV} = k_q t_0$, where t_0 is the lifetime in the absence of quencher and k_q is the rate constant for quenching. As t_0 value for tryptophan fluorescence in proteins is known to be equal to 10^{-9} s,[9] the rate constant, k_q would be of the order of $10^{12}\ M^{-1}\ s^{-1}$. The k_q depends on the probability of a collision between the fluorophore and the quencher. This probability depends on their rate of diffusion (D), their size and concentration. It can be shown that

$$k_q = 4p\ aDN_a \times 10^{-3}, \qquad (5)$$

where D is the sum of the diffusion coefficients of quencher and fluorophore, a is the sum of molecular radii and N_a is the Avogadro number. The large magnitude of k_q in the present study ($10^{12}\ M^{-1}\ s^{-1}$) can probably be attributed to increase in the encounter radii of tryptophan and drug. This can happen only if the process of energy transfer takes place by intermolecular interaction forces and is possible only when the drug binding site is in close proximity to the tryptophan residues of BSA.

3.3 *Binding studies in the presence of ANS*

Fluorescence spectra of 10 *m*M BSA in the presence of increasing amounts of the drug/ANS (4–25 *m*M) were determined after excitation at 296 nm. Both the drug and ANS quench the fluorescence of BSA, but the magnitude of decrease in the fluorescence intensity is larger for ANS when compared to that for the drugs. ANS bound to BSA calculated from the fraction of occupied sites (q) is 81% whereas the drug bound to BSA is only 48% in case of VS and 33.2% in case of RF under identical conditions. It is known that excitation at 296 nm involves the fluorescence due only to tryptophan residues of BSA. Further, under conditions of the experiment, tryptophan resi-

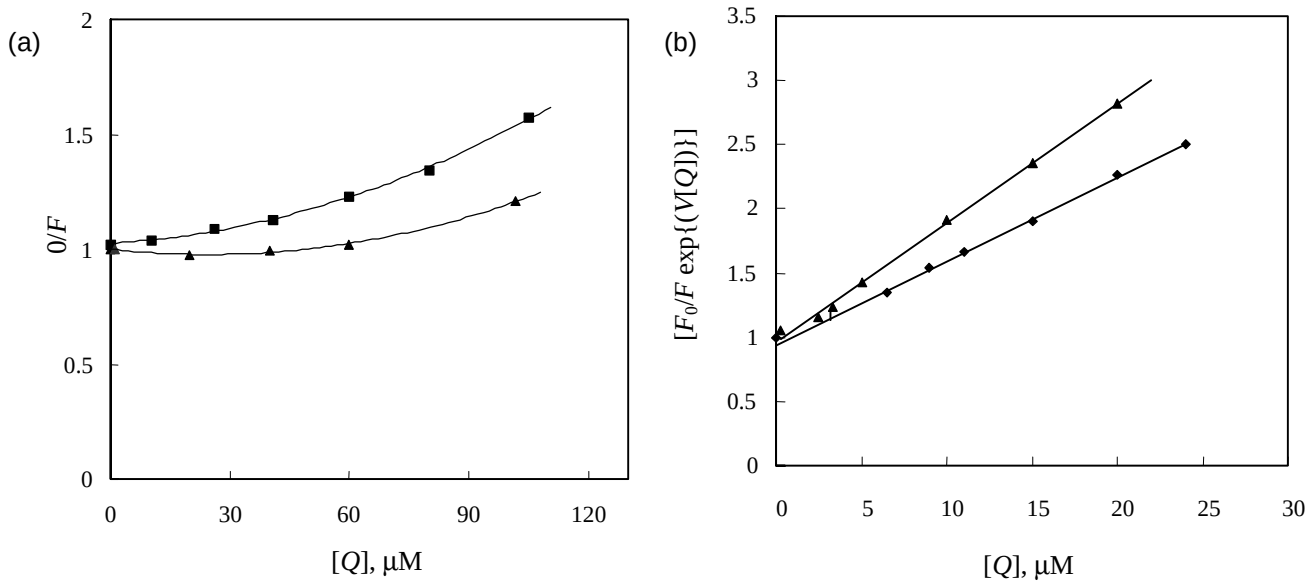

Figure 4. Plot of F_0/F versus [Q] for the binding of (**a**) VS (–■–) and RF (–▲–) and (**b**) plot of [F_0/F exp{(V[Q])}] versus [Q] for VS (–▲–) and RF (–■–).

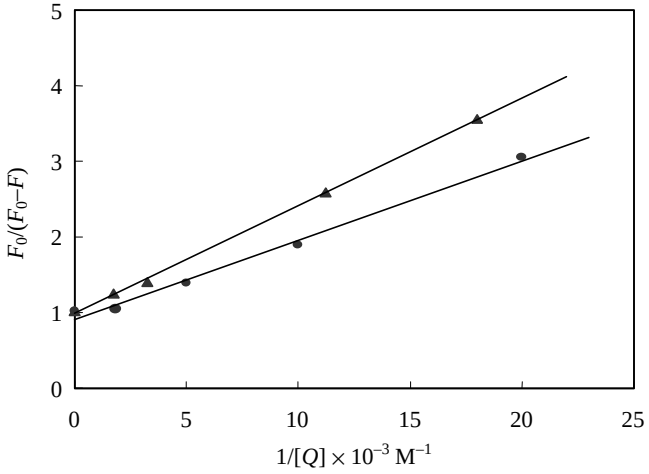

Figure 5. Plot of $F_0/(F_0 - F)$ versus 1/[Q] for VS (–▲–) and RF (–●–).

dues of BSA are partially exposed and their accessibility depends upon the nature of the interacting species.[13]

In the second set of experiments, BSA–ANS interaction was studied in the absence and presence of 5, 10, 15, and 20 *m*M of drug by monitoring ANS fluorescence upon excitation at 370 nm. We observe that fluorescence intensity decreases very slightly when VS/RF is added to the BSA (10 *m*M)-ANS (20 *m*M) system thereby indicating that the VS/RF may compete with ANS for hydrophobic sites on the protein surface.

To understand further the nature of interaction involved, the CMC of an anionic surfactant, SDS was determined in the absence and presence of 40 *m*M drug. ANS is virtually non-fluorescent in aqueous solution and becomes highly fluorescent in non-polar solvents. Large increase in the intensity of ANS fluorescence on association of surfactant monomers to form micelles is employed as the basis of CMC determination. Fluorescence intensity of ANS (40 *m*M) was determined in the presence of increasing concentration of SDS (1·5–9·0 mM) upon excitation at 370 nm. Relative fluorescence intensity at l_{max} (482–486 nm) was plotted against the concentration of SDS. We find that the presence of VS/RF decreases the CMC of SDS slightly. The small decrease in CMC value in the presence of the VS/RF shows that both of them have some ionic character in addition to the hydrophobic character.

3.4 *Thermodynamics of drug–protein interaction*

Thermodynamic parameters for the binding of drug to BSA were determined. using the relation,

$$\log K = -\Delta H_0/2{\cdot}303RT + \Delta S_0/2{\cdot}303R. \quad (6)$$

Log K versus 1/T plot enables the determination of standard enthalpy change, ΔH_0 and standard entropy change, ΔS_0 for the binding process. ΔH_0, and ΔS_0 values are –12·44 kJ mol^{-1} and –11·48 kJ mol^{-1},

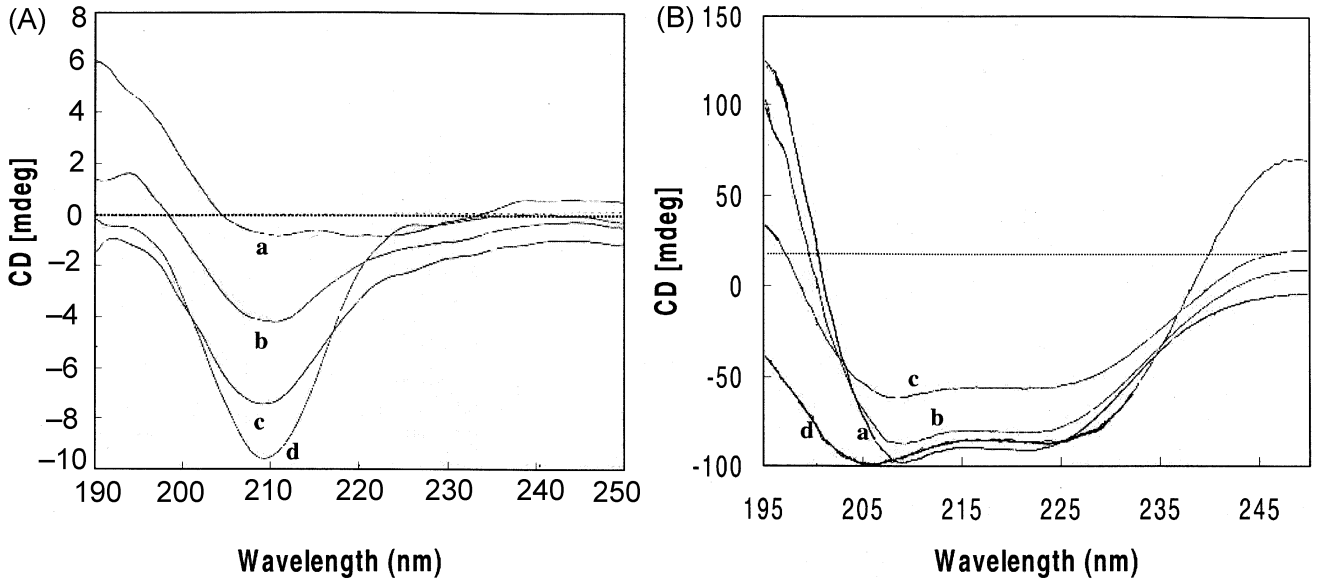

Figure 6. Circular dichroism spectra of (**A**) BSA, 0·1 *m*M (a); BSA: VS = 1 : 1(b); 1 : 3 (c) and 1 : 5 (d); (**B**) BSA, 0·1 *m*M (a); BSA: RF = 1 : 1(b); 1 : 3 (c) and 1 : 5 (d).

+44·61 $JK^{-1}\,mol^{-1}$ and +61·84 $JK^{-1}\,mol^{-1}$ for VS and RF, respectively.

3.5 *Surface activity*

Surface tension data are expressed as surface activity, which in turn is expressed as surface pressure, p, which is the difference between surface tension of the solvent and that of the solution. Thus, surface activity values, expressed as surface pressure, $p = l_{solvent} - l_{solution}$, are $2{\cdot}6 \times 10^{-4}$ and $3{\cdot}36 \times 10^{-2}\ Nm^{-1}$ for VS and RF respectively. Reduction in the surface tension of solvent or increase in the surface pressure is attributed to the hydrophobic nature of the drug molecule.

3.6 *Circular dichroism method*

Interaction of VS/RF with BSA is also confirmed by CD spectra (figure 6). The CD spectra of 0·1 *m*M BSA in buffer (a) and varied ratios of BSA-drug, 1 : 1 (b); 1 : 3 (c) and 1 : 5 (d) show marked changes. As expected, the a-helices of protein show a strong double minimum at 220 nm and 209 nm.[14] The intensities of this double minimum reflect the helicity of BSA and further indicate that BSA contains more than 50% of á-helical structure. On increased addition of VS to BSA (1 : 1, 1 : 3 and 1 : 5), the second minimum at 220 nm vanishes and the intensity of the first minimum starts increasing with a blue shift of 2 nm (209 to 207 nm). Upon the addition of RF to BSA (1 : 1 and 1 : 3), the extent of a-helicity of the protein decreases and, hence, the intensity of double minimum is reduced. On further addition of RF to BSA (1 : 5), the second minimum at 220 nm is reduced and intensity of the first minimum start increasing with a blue shift of 2 nm (209 to 207 nm).[14]

4. Conclusions

The binding of an anticancer drug, VS and an antimycobacterial drug, RF to BSA have been investigated by spectrofluorometry and CD methods. The results obtained suggest that VS and RF are bound to BSA possibly by hydrophobic forces. Fluorescence quenching studies reveal the presence of a static component in the quenching mechanism. The Stern–Volmer plot indicates that both tryptophan residues of BSA are exposed to the drug, VS/RF. Binding studies in the presence of ANS show that VS and RF compete with ANS for hydrophobic sites on the surface of BSA. The large magnitude of k_q observed in the present work can probably be attributed to increase in the encounter radii of the tryptophan and the drug. The shape and intensity of negative CD bands at 209 and 220 nm show major differences in the presence of VS and RF due to change in the chemical environment of the helical content of the protein close to the surface.

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