Spectroscopic Studies and Life Time Measurements of Binding of a Bioactive Compound to Bovine Serum Albumin and the Effects of Common Ions and Other Drugs on Binding

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The mechanism of binding of anti-inflammatory drug, nimesulide (NIM) with bovine serum albumin (BSA) was investigated by fluorescence, absorption, circular dichroism (CD) and lifetime measurements under simulative physiological conditions. The analysis of fluorescence data indicated the presence of both dynamic and static quenching mechanism in the binding. Various binding parameters have been evaluated. The CD spectral data revealed the decrease in α-helical content of BSA from 70.9% (in free BSA) to 42.03% (in bound form) thereby indicating the conformational change in BSA upon binding. The binding of NIM to BSA was also confirmed by absorption spectra. Based on the Förster's theory of non-radiation energy transfer, the binding average distance, r between the donor (BSA) and acceptor (NIM) was found to be 2.17 nm. The association constants of NIM-BSA decreased in presence of the common ions and other drugs thereby indicating the availability of higher concentration of free drug (NIM) in plasma.

Key words bovine serum albumin; anti-inflammatory drug; spectroscopic study

The interaction of a drug with blood components influences its bioavailability and can affect the functions of several biomolecules.[1] Clinical effect of the plasmatic level of a drug is an important pharmacological parameter determined by drug absorption, distribution and elimination. Serum albumins, the most abundant proteins in circulatory system of a wide variety of organisms, have been well studied for many years. They account for about 60% of the total protein corresponding to a concentration of 42 g l^{-1} [2,3] and provide about 80% of osmotic pressure of blood.[2] Albumins have been used as a model protein for diverse biophysical, biochemical and physicochemical studies[3] to gain general fundamental insights into drug–protein binding. The remarkable binding properties of albumin accounts for the central role it can play in both the efficacy and rate of delivery of drugs. Several classes of drugs including tranquillizers, anti-coagulants and general anesthetics are transported in the blood while bound to albumin. This kind of work has stimulated many researchers to carry out the research on the nature of the drug binding sites and investigations of whether natural metabolites, drugs and fatty acids compete with one another for binding to the protein.[4] These results provide salient information of the structural features that determine the therapeutic effectiveness of drugs, and hence become an important research field in chemistry, life sciences and clinical medicine.[1,4—8]

Two common methods that have been used in evaluating the binding of drugs to albumin include equilibrium dialysis and ultrafiltration.[9,10] These methods are laborious and time consuming and the results at times are not reproducible. However, 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 the leakage of bound drug through membrane. To overcome these problems, we have employed fluorimetric, lifetime measurements, absorption and circular dichroism methods to investigate the mode of binding of NIM with BSA in the present investigation.

Nimesulide (NIM), chemically *N*-(4-nitro-2-phenoxyphenyl) methanesulphonamide, is a relatively new non-steroidal anti-inflammatory (NSAID), antipyretic and analgesic drug.[11] It is widely used for the treatment of inflammatory conditions associated with rheumatoid arthritis, respiratory tract infections, soft tissue and oral cavity inflammations. The therapeutic effect of NSAIDs is the result of their ability to inhibit prostaglandin synthesis via inhibition of cyclooxigenase. The analgesic potency of NIM is similar to that of ibuprofen and indomethacin. Nonetheless, NIM has shown a higher antipyretic potency relatively than indomethacin, ibuprofen, aspirin and paracetamol. Because of its biological importance, we thought of investigating the mechanism of binding of NIM with BSA by fluorescence, UV–visible absorption, circular dichroism and fluorescence lifetime measurements. The energy transfer between the drug and protein is reported for the first time. This is the first study wherein three spectroscopic methods are employed to investigate the interaction of NIM with BSA.

Experimental

Materials Bovine Serum Albumin (BSA, Fraction V, approximately 99%; protease free and essentially γ-globulin free) was obtained from Sigma Chemical Company, St. Louis, U.S.A. Nimesulide was obtained as gift sample from the Aurobindo Pharma, India. Pure samples of drugs *viz.*, ciprofloxacin (CIP), paracetamol (PCT), atenolol (ATE), ceterizine dihydrochloride (CTH) and piroxicam (PIX) were obtained as gift samples from different firms. The solutions of NIM 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 65000. All other materials were of analytical reagent grade and double distilled water was used throughout.

Spectral Measurements Fluorescence measurements were performed on a Hitachi spectrofluorimeter Model F-2000 equipped with a 150 W Xenon lamp and slit width of 10 nm. A 1.00 cm quartz cell was used for measurements. The CD measurements were made on a JASCO-J-715 spectropolarimeter using a 0.1 cm cell at 0.2 nm intervals, with 3 scans averaged for each CD spectrum in the range of 200—250 nm. The absorption spectra were recorded on a double beam CARY 50-BIO UV–visible spectrophotometer equipped with a 150 W Xenon lamp and slit width of 10 nm. A

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quartz cell of 1.00 cm was used for measurements.

Fluorescence decays were recorded using TCSPC method using the following setup. A diode pumped millena CW laser (Spectra Physics) 532 nm was used to pump the Ti:sapphire rod in Tsunami picoseconds mode locked laser system (Spectra Physics). The 750 nm (80 MHz) was taken from the Ti:sapphire laser and passed through pulse picker (Spectra Physics, 3980 2S) to generate 4 MHz pulses. The third harmonic output (296 nm) was generated by flexible harmonic generator (Spectra Physics, GWU 23 PS). The vertically polarized 296 nm laser was used to excite the sample. The fluorescence emission at magic angle (54.7°) was dispersed in a monochromator (f/3 aperture), counted by a MCP PMT (Hamamatsu R 3809) and processed through CFD, TAC and MCA. The instrument response function for this system is *ca.* 52 ps. The fluorescence decay was analyzed by using the software provided by IBH (DAS-6) and PTI Global Analysis Software. A 1.0 cm quartz cell was used throughout the study.

NIM-BSA Interactions From the preliminary experiments on fluorescence studies, the BSA concentration was kept fixed at 12 μM and drug concentration was varied from 20 to 120 μM. Fluorescence spectra were recorded at 295, 301 and 308 K 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 to avoid inner filter effect.

Circular Dichroism (CD) Measurements The CD measurements of BSA in the presence and absence of NIM were made in the range of 200—250 nm using a 0.1 cm cell at 0.2 nm intervals, with 3 scans averaged for each CD spectra. A stock solution of 150 μM BSA was prepared in 0.1 M phosphate buffer containing 0.15 M NaCl. The BSA to drug concentration was varied (1 : 9, 1 : 21, 1 : 36, 1 : 42) and the CD spectrum was recorded.

Lifetime Measurements The Fluorescence lifetime measurements of BSA in the presence and absence of NIM were recorded by fixing 296 nm as the excitation wavelength and 344 nm as the emission wavelength. A stock solution of 150 μM BSA was prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. The BSA concentration was kept fixed at 30 μM and NIM concentration was varied from 50 to 125 μM.

Effects of Some Common Ions and Other Drugs The fluorescence spectra of NIM-BSA were recorded in presence and absence of various common ions *viz.*, SO_4^{2-}, F^-, I^-, CH_3COO^-, PO_4^{3-}, Fe^{2+}, Co^{2+}, K^+, Ni^{2+}, V^{5+} and Cu^{2+} and other drugs *viz.*, ciprofloxacin (CIP), paracetamol (PCT), atenolol (ATE), ceterizine dihydrochloride (CTH) and piroxicam (PIX) at 344 nm upon excitation at 296 nm. The concentration of BSA and that of common ion/drug was fixed at 12 μM while the concentration of NIM was varied from 20 to 120 μM.

Results and Discussion

Fluorescence Measurements The fluorescence intensity of a compound can be decreased by a variety of molecular interactions *viz.*, excited-state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisional quenching. Such decrease in intensity is called quenching. In order to investigate the binding of NIM to BSA, the fluorescence spectra were recorded at 344 nm upon excitation at 296 nm. NIM causes a concentration dependent quenching of the intrinsic fluorescence of BSA (Fig. 1) without changing the emission maximum indicating that there is no change in the local dielectric environment. The interaction of NIM to BSA was further confirmed by absorption and circular dichroism techniques.

The fluorescence quenching data are analyzed by the Stern–Volmer equation,

$$F_o/F = 1 + K_{SV}[Q] \quad (1)$$

where F_o and F are the steady state fluorescence intensities in the absence and presence of quencher, respectively, K_{SV} is the Stern–Volmer quenching constant and [Q] is the concentration of quencher (NIM). The plot of F_o/F *versus* [Q] showed positive deviation (concave towards the *Y* axis) indicating the presence of both static and dynamic quenching[12] by the same fluorophore (Fig. 3). The dynamic portion of the

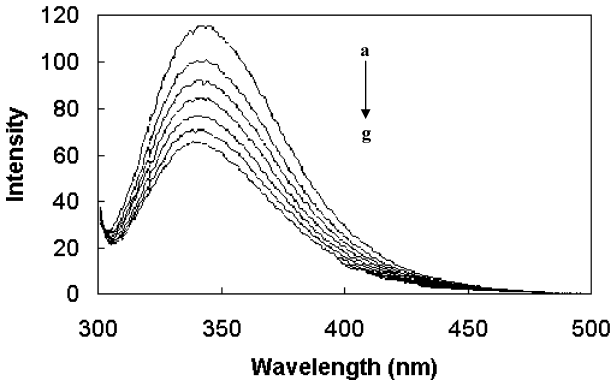

Fig. 1. Fluorescence Spectra of BSA in the Presence of NIM

BSA concentration was fixed at 12 μM (a). NIM concentration was 20 (b), 40 (c), 60 (d), 80 (e), 100 (f), 120 μM (g).

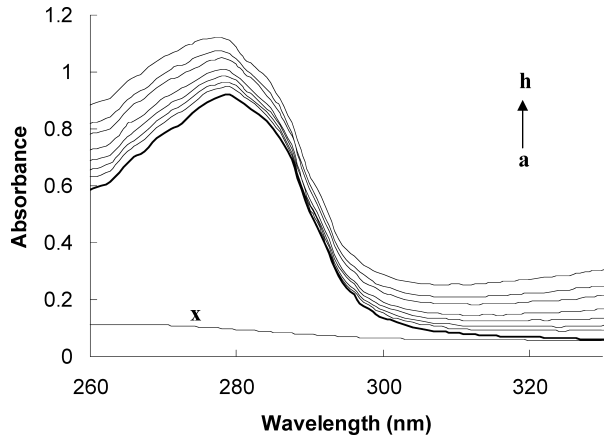

Fig. 2. Absorbance Spectrum of BSA, NIM and BSA–NIM System

BSA concentration was kept fixed at 12 μM (a). NIM concentration for NIM–BSA system was 13.2 (b), 19.8 (c), 33.3 (d) 46.6 (e), 60 (f), 73.3 (g) and 90 μM (h). Concentration of 12 μM (x) was used for NIM only.

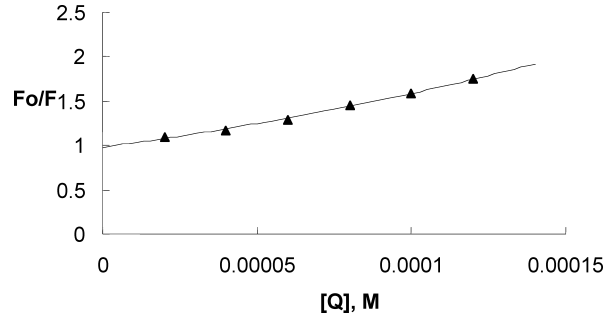

Fig. 3. Stern–Volmer Plot for the Binding of NIM with BSA

observed quenching was determined by lifetime measurements using the equation,

$$\tau_o/\tau = 1 + K_D[Q] \quad (2)$$

where τ_o and τ are the fluorescence lifetimes of BSA in absence and presence of NIM, respectively and K_D is the dynamic quenching constant. The fluorescence decay of BSA in presence of different concentrations of the quencher was found to be biexponential (Fig. 4). The lifetime (τ), relative amplitudes (A) and χ^2 of the various decay analysis of the BSA–NIM system are listed in Table 1. The value of K_D was found to be 398.14 M^{-1} from the plot of τ_o/τ *versus* [Q]. The

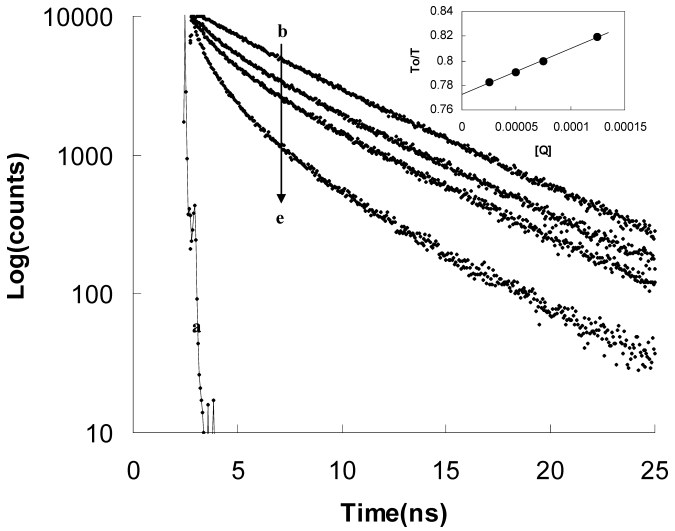

Fig. 4. Fluorescence Decay Profiles of BSA in the Absence and Presence of NIM in 0.1 M Phosphate Buffer of pH 7.4, λ_{ex}=296 nm and λ_{em}=344 nm

(a) Laser profile, BSA concentration was fixed at 30 μM and (b) in NIM–BSA system, BSA concentration was 30 μM and that of NIM was 50 (c), 75 (d) and 125 μM (e). Inset shows the plot of τ_o/τ *versus* [Q].

Table 1. Lifetimes of Fluorescence Decay of BSA (30 μM) in Phosphate Buffer of pH 7.4 at Different Concentrations of NIM

System	NIM (μM)	Analysis	Lifetime (ns)		Amplitude		χ^2
			τ_1	τ_2	A_1	A_2	
BSA	—	biexp.	4.65	7.26	52.32	47.68	1.11
BSA–NIM	25	biexp.	5.88	1.64	87.23	12.77	1.25
BSA–NIM	50	biexp.	5.89	1.54	84.83	15.17	1.21
BSA–NIM	75	biexp.	5.77	1.49	82.54	17.46	1.20
BSA–NIM	125	biexp.	5.62	1.39	78.90	21.10	1.40

value of static quenching constant, K_S was obtained using the equation,

$$\frac{(F_o-F)/F}{[Q]} = (K_S+K_D)+K_S K_D[Q] \quad (3)$$

by plotting the graph of $\{(F_o-F)/F\}/[Q]$ *versus* [Q] and using the value of K_D obtained from lifetime measurements. It was found to be $4.53\times10^3\,M^{-1}$.

The fluorescence data obtained at room temperature was further examined using modified Stern–Volmer equation,

$$F_o/(F_o-F)=1/f_a+1/\{([Q]f_aK_{SV})\} \quad (4)$$

where f_a is the fraction of fluorophore (protein) accessible to the quencher. From the plot of $F_o/(F_o-F)$ *versus* 1/[Q], the values of f_a and K_{SV} were obtained from the values of intercept and slope, respectively (Fig. 5). The value of f_a was found to be 0.77 at 301 K indicating that 77.0% of the total fluorescence of BSA is accessible to quencher. The Stern–Volmer quenching constant was found to be $9.05\times10^3\,M^{-1}$. The quenching rate constant of the biomolecule, K_q was evaluated using the equation,

$$K_q=K_{SV}/\tau_o \quad (5)$$

where τ_o is the average lifetime of the protein without the quencher. The τ_o value for BSA in the present study was found to be 4.65 ns (which was selected on the basis of higher amplitude value) and hence the value of K_q was observed to be $1.95\times10^{12}\,l\,M^{-1}\,s^{-1}$. The upper limit of k_q expected for a diffusion-controlled bimolecular process[13] is $10^{10}\,l\,M^{-1}\,s^{-1}$. The magnitude of k_q in the present study ($10^{12}\,l\,M^{-1}\,s^{-1}$) indicates that the quenching is not initiated by dynamic collision but from compound formation.[13]

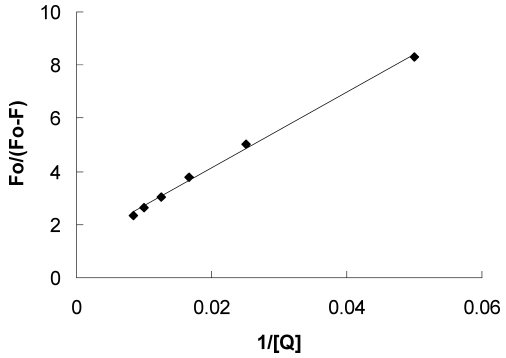

Fig. 5. Modified Stern–Volmer Plot for the Binding of NIM with BSA

Parachor, which is a measure of molar volume of drug, was calculated for NIM from the atomic parachors and other structural features.[14] The value was found to be $810.49\,(N\,m^{-1})^{1/4}\,m^3$. The value of parachor indicates that the larger size of drug molecule probably has larger hydrophobic area, which can interact with hydrophobic surface on the protein molecule.

Analysis of Binding Equilibria When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the equation[15],

$$\lg(F_o-F)/F=\lg K+n\lg[Q] \quad (6)$$

where K and n are the binding constant and the number of binding sites, respectively. Thus, a plot of $\lg(F_o-F)/F$ *versus* lg[Q] yielded the K and n values to be $3.34\times10^5\,l\,M^{-1}$ and 0.94, respectively. The value of n is approximately equal to 1 indicating that there is one class of binding site to the NIM in BSA. From this n value it is proposed that NIM most likely binds to the hydrophobic pocket located in subdomain II A; that is to say tryptophan-214 is near or within the binding site.[16]

Type of Interaction Force between NIM and BSA Considering the dependence of binding constant on temperature, a thermodynamic process was considered to be responsible for the formation of the complex. Hence, the thermodynamic parameters depend on the temperatures and were analyzed to characterize the acting forces between NIM and BSA. The acting forces between a small molecule and macromolecule include hydrogen bond, Van der Waals force, electrostatic force, hydrophobic interaction force, and so on. The thermodynamic parameters were determined using the following equations

$$\lg K=-\Delta H^\circ/2.303RT+\Delta S^\circ/2.303R \quad (7)$$

$$\Delta G^\circ=\Delta H^\circ-T\Delta S^\circ \quad (8)$$

where ΔH°, ΔG° and ΔS° are respectively enthalpy change, free energy change and entropy change. The binding studies were carried out at 295, 301 and 308 K. At these tempera-

Table 2. Effects of Common Ions and Other Drugs on Binding Constants of BSA–NIM System

System	Binding constant (M^{-1})
BSA+NIM	3.34×10^5
BSA+NIM+SO_4^{2-}	5.35×10^4
BSA+NIM+F^-	1.98×10^5
BSA+NIM+I^-	2.19×10^5
BSA+NIM+CH_3COO^-	1.91×10^5
BSA+NIM+Fe^{2+}	7.66×10^4
BSA+NIM+Co^{2+}	9.19×10^4
BSA+NIM+K^+	2.31×10^5
BSA+NIM+V^{5+}	8.27×10^4
BSA+NIM+Ni^{2+}	2.97×10^3
BSA+NIM+Cu^{2+}	7.70×10^3
BSA+NIM+CIP	4.49×10^3
BSA+NIM+PCT	3.16×10^3
BSA+NIM+ATE	6.06×10^3
BSA+NIM+CTH	4.61×10^3
BSA+NIM+PIX	3.89×10^3

Table 3. Thermodynamic Parameters of BSA–NIM System

Drug	Temperature (K)	Binding constant ($K\times10^{-5}$, M^{-1})	ΔG° ($kJ\,mol^{-1}$)	ΔH° ($kJ\,mol^{-1}$)	ΔS° ($J\,mol^{-1}\,K^{-1}$)
NIM	295	4.13	−31.72	−24.05	+25.84
	301	3.34	−31.83		
	308	2.72	−32.05		

tures the BSA doesn't undergo any structural degradation. The values of $\lg K$, ΔH°, ΔS° and ΔG° are summarized in Table 3. The negative value of ΔG° reveals that the interaction process is spontaneous. The positive entropy change occurs because the water molecules that are arranged in an orderly fashion around the drug and protein acquire the more random configuration as a result of hydrophobic interactions. Negative ΔH° value can not be attributed to electrostatic interactions since electrostatic interactions the ΔH° value is very small, almost zero.[17] Negative ΔH° and positive ΔS° values showed that both hydrogen bond and hydrophobic interactions play a role in the binding of NIM to BSA.[18,19]

Energy Transfer between NIM and BSA The overlap of the UV absorption spectra of NIM with the fluorescence emission spectra of BSA is shown in the Fig. 6. The importance of the energy transfer in biochemistry is that, the efficiency of transfer can be used to evaluate the distance between the ligand and the tryptophan residues in the protein. The efficiency of energy transfer, E, was studied according to Förster's energy transfer theory.[20] The efficiency of energy transfer, E, is calculated using the equation

$$E = 1 - \frac{F}{F_o} = \frac{R_0^6}{R_0^6 + r^6} \tag{9}$$

where F and F_o are the fluorescence intensities of BSA in the presence and absence of NIM, r is the distance between acceptor and donor and R_0 is the critical distance when the transfer efficiency is 50%.

$$R_0 = 8.8\times10^{-25} k^2 N^{-4} \Phi J \tag{10}$$

where k^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor and J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. J is given by

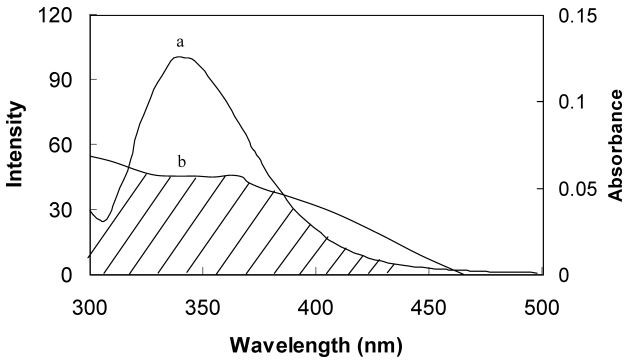

Fig. 6. The Overlap of the Fluorescence Spectrum of BSA and the Absorbance Spectrum of NIM (λ_{ex}=296 nm, λ_{em}=344 nm, [BSA]/[NIM]=1 : 1)

(a) The fluorescence spectrum of BSA and (b) the absorption spectrum of NIM.

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{11}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength, λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength, λ. In the present case, $K^2=2/3$, $n=1.336$ and $\Phi=0.15$[19—21] From Eqs. 9 to 11, we could able to calculate that $J=1.52\times10^{-15}\,cm^3\,1_M{}^{-1}$, $R_o=1.86$ nm, $E=0.28$ and $r=2.17$ nm. The donor-to-acceptor distance, $r<8$ nm[22] indicated that the energy transfer from BSA to NIM occurs with high possibility. BSA has two tryptophan residues namely Trp-214, located in a hydrophobic fold and Trp-135 located on the surface of the molecule.[23,24] In the present study, NIM is probably bound to Trp-214 residue mainly through the hydrophobic interaction as evident from thermodynamic results. However, the interaction between Trp-135 and NIM cannot be precluded, so the distance, r calculated is actually the average value between the bound NIM and the two tryptophan residues in BSA. Similar observation is also reported in the literature.[25]

The Effect of Ions on the Binding Constant of NIM–BSA The fluorescence intensity was changed before and after the addition of some common ions such as SO_4^{2-}, F^-, I^-, CH_3COO^-, PO_4^{3-}, Fe^{2+}, Co^{2+}, K^+, Ni^{2+}, V^{5+} and Cu^{2+}. The effect of common ions on the binding constants was investigated at 301 K and the results are summarized in Table 2. Some times a subject may be administered two or more drugs simultaneously. Such simultaneous administration of two or more strongly bound drugs can compete with one another for the binding sites on albumin and so result in displacement interactions.[26] Hence, we have examined the effect of other drugs *viz.*, ciprofloxacin (CIP), paracetamol (PCT), atenolol (ATE), ceterizine dihydrochloride (CTH) and piroxicam (PIX) on the binding constant of NIM–BSA system at 301 K by recording the fluorescence intensity in the range 300—500 nm upon excitation at 296 nm. It was noticed that the addition of drug to BSA decreased the fluorescence intensity of BSA. The association constants of NIM–BSA were also determined in presence of the above drugs and the

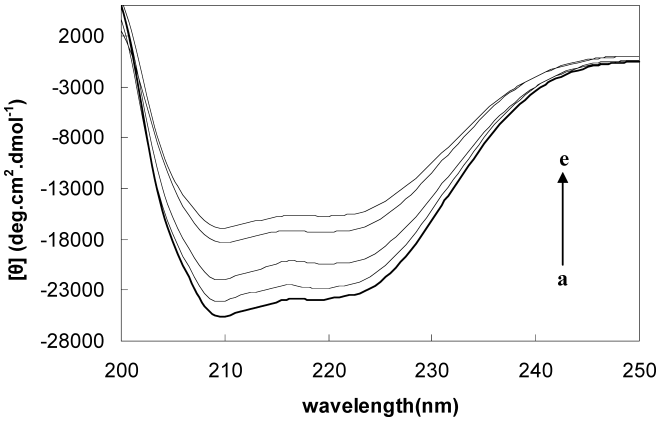

Fig. 7. CD Spectra of the BSA–NIM System Obtained in 0.1 M Phosphate Buffer of pH 7.4 at Room Temperature

BSA concentration was kept fixed at 10 μM (a). In NIM–BSA system, NIM concentration was 90 (b), 210 (c), 360 (d) and 420 μM (e).

results are shown in Table 2. The values of K decreased in presence of all the common ions and other drugs. This indicated the availability of higher concentration of free drug in plasma in the presence of common ions and other drugs.[26] Increase in the concentration of free drug enhances the pharmacological effects and may produce toxic reactions. So, there is a need for an adjustment in dosage of NIM to be administered to the subjects in the presence of the above common ions/drugs.

Conformation Investigations To explore the structural changes of BSA upon the addition of NIM, we have recorded the absorbances of BSA (12 μM) in presence of different amounts of NIM (Fig. 2). It is evident from the figure that the BSA undergoes conformational changes in presence of NIM. The UV absorption intensity of BSA was increased with the variation of NIM concentration. Further, a slight blue shift of maximum peak position was also noticed possibly due to complex formation between NIM and BSA.[27]

When drugs bind to a globular protein, the intramolecular forces responsible for maintaining the secondary and tertiary structures can be altered, resulting in a conformational change of the protein.[28]

Circular dichroism is a sensitive technique to monitor the conformational changes in the protein upon interaction with the ligand. The CD spectra of BSA in the absence and presence of NIM are shown in the Fig. 7. The CD spectra of BSA exhibited two negative bands in the UV region at 208 and 220 nm, characteristic of an α-helical structure of protein.[6] The CD results were expressed in terms of mean residue ellipticity (MRE) in deg cm^2 dmol^{-1} according to the following equation,

$$\text{MRE} = \frac{\text{observed CD (m deg)}}{C_p n l \times 10} \tag{12}$$

where C_P is the molar concentration of the protein, n is the number of aminoacid residues and l is the path length. The α-helical contents of free and combined BSA[7] were calculated from MRE value at 208 nm using the equation

$$\alpha\text{-helix (\%)} = \frac{[-\text{MRE}_{208} - 4000]}{[33000 - 4000]} \times 100 \tag{13}$$

where MRE_{208} is the observed MRE value at 208 nm, 4000 is the MRE of the β-form and random coil conformation cross at 208 nm, and 33000 is the MRE value of a pure α-helix at 208 nm. From the above equation, the quantitative analysis results of the α-helix in the secondary structure of BSA were obtained. They differed from that of 70.9% in free BSA to 42.03% in the BSA–NIM complex, which was indicative of the loss of α-helical. The percentage of protein α-helix structure decreased indicated that the drug, NIM bound with the amino acid residue of the main polypeptide chain of protein and destroyed their hydrogen bonding networks.[28] In other words, the binding of NIM to protein caused significant reduction of protein α helix structure, which signified the rearrangement of carbonyl hydrogen bonding network of the main polypeptide chain.

Conclusions

This paper provided an approach for studying the binding of protein with NIM using absorption, fluorescence, CD and lifetime measurements. The results showed that BSA fluorescence quenched by NIM through both dynamic and static quenching for the same quencher. The spectral data revealed the conformational changes of BSA upon interaction with NIM. The common ions and other drugs increased the concentration of free NIM in plasma. This work also reports the distance between tryptophan and bound NIM for the first time based on Förster's energy transfer theory. This report has a great significance in pharmacology and clinical medicine as well as methodology.

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