ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Interaction Between a Antiretroviral Drug – Navirapine with Bovine Serum Albumin : A Fluorescence Quenching and Fourier Transformation Infrared Spectroscopy Study

Gunagi SD, Nandibewoor ST, Chimatadar SA*

P.G. Department of Studies in Chemistry, Karnatak University, Pavate Nagar, Dharwad 580003, India.

ABSTRACT

The interaction between navirapine drug to bovine serum albumin(bsa) has been studied by different spectroscopic methods under physiological conditions. The experimental results revealed a static quenching mechanism in the interaction of navirapine with bsa. The number of binding sites close to unity for navirapine–bsa indicated the presence of single class of binding site for navirapine in protein. The binding constant values of navirapine–bsa were obtained as $1.98x10^4$, $1.74\ x10^4$, and $1.38\ x10^4$ at 288K, 298K and 308K respectively. Thermodynamic parameters indicated that the hydrophobic forces played the major role in the binding of navirapine to bsa. The distance of separation between the serum albumin and navirapine was obtained from the Förster's theory of non-radioactive energy transfer. The effect of some metal ions on binding of navirapine to bsa were also investigated. The fluorescence spectra, UV absorption spectra and FT-IR spectral results revealed the changes in the secondary structure of bsa upon interaction with navirapine.

Key words: bovine serum albumin, navirapine, fluorescence spectroscopy, binding constant, metal ions.

Corresponding author:
E-mail: schimatadar@gmail.com

ISSN: 0975-8585

INTRODUCTION

Serum albumin, the most abundant protein in the circulatory system, has been one of the most extensively studied of all proteins [1]. Serum albumins are prone to bind effectively many small organic molecules. Bovine serum albumin (bsa), a large globular protein (65,000 Daltons), consists of a single chain of 583 amino acids residues [1]. It is known that the distribution, free concentration and metabolism of several drugs might be strongly affected by drug–protein interactions in the blood stream. This type of interaction can also influence the drug stability and toxicity during the chemotherapeutic process. Therefore, the studies on the binding of a drug with protein will facilitate interpretation of the metabolism and transporting process of drug. In this regard, bsa has been studied extensively, partly because of its structural homology with human serum albumin (hsa) [2-5].

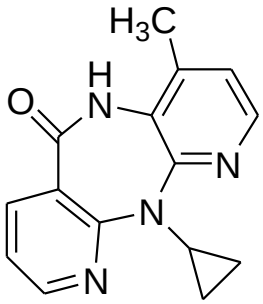

Fig. 1: The structure of navirapine.

Navirapine (navp) (Fig. 1) falls in the non-nucleoside reverse transcriptase inhibitor class of antiretroviral drug [6]. Both nucleoside and non-nucleoside reverse transcriptase inhibitors inhibit the same target, the reverse transcriptase enzyme, an essential viral enzyme which transcribes viral rna into dna. Navirapine is used in combination with other medications to treat human immuno deficiency virus infection in patients with or without acquired immuno deficiency syndrome. Navirapine is in a class of medications called non-nucleoside reverse transcriptase reverse inhibitors. In view of its biological importance, we planned to study the interaction of navirapine with bsa by using different spectroscopic techniques viz., fluorescence, UV–Vis absorption and FT- IR. The present study reveals the changes in the fluorescence and structural properties of bsa upon binding with navirapine, which have been utilized to characterize the interaction parameters. As it is not reported in the literature so far.

Warfarin is an anticoagulant. It is most likely to be the drug popularly refered to as a blood thinner, since it does not affect the thickness or viscosity of blood. Instead it acts on the liver to decrease the quantity of a few key proteins in blood that allow blood to clot. Digitoxin is a cardiac glycoside. It has similar structure and effects to digoxin. It could be used in patients with poor or erratic kidney function. Both warfarin and digitoxin are used for the site probe study in this topic.

ISSN: 0975-8585

MATERIALS AND METHODS

Materials:

Bovine serum albumin (bsa,) was obtained from Sigma Chemical Company, St. Louis, USA. Navirapine was obtained as gift sample from Micro Labs Ltd. The solutions of navirapine and bsa were prepared in 0.1M phosphate buffer of 7.4 pH which contains 0.15M sodium chloride. Millipore water was used throughout the study.

Apparatus:

Fluorescence spectra were recorded using a RF-5301 PC Hitachi spectro fluorometer Model F-2000 (Tokyo, Japan) with a 150 W Xenon lamp, a 1 cm quartz cell and thermostatic cuvette holder. The excitation and emission bandwidths were both 5 nm. The temperature of the sample was maintained by recycling water throughout the experiment. The absorption spectra were recorded on a double beam CARY 50-BIO UV–vis. Spectrophotometer (Victoria, Australia), FT-IR Nicolet-5700; USA was used to record infrared spectra.

Fluorescence studies:

Based on preliminary investigations, the concentration of bsa was kept constant at 5 µM , while that of the navirapine was varied from 5 to 45µM. The fluorescent intensity of bsa was recorded at 340nm upon excitation at 296nm.The interactions were carried out at three different temperatures (288, 298, and 308K).

Ultraviolet absorption studies:

The UV–vis spectra were obtained by scanning the solution on the spectrophotometer in the wavelength region of 200–400 nm. Bovine serum albumin concentration was fixed at 5 µM while that of navirapine was varied from 5 to 45µM in presence of phosphate buffer.

FT-IR Measurements:

The FT-IR spectra of bsa in presence and absence of navirapine at 298 K were recorded in the range of 1400–1800cm^{-1}. Bovine serum albumin concentration was fixed at 5 µM while that of navirapine was varied from 5 to 45µM in presence of phosphate buffer.

Displacement studies:

The displacement experiments were performed using different site probes viz., warfarin, ibuprofen and digitoxin for site I, II and III, respectively [7] by keeping the concentration of bsa and the probe constant(5 µM each).The fluorescence quenching titration was used as before to determine the binding constant of navirapine – bsa in presence of above site probes.

ISSN: 0975-8585

Effect of common ions:

The effects of some common ions viz., Co^{+2}, Cu^{+2}, Ni^{+2}, Ca^{+2} and Zn^{+2} were investigated on navirapine– bsa interactions. The fluorescence spectra of navirapine– bsa system were recorded in presence of above ions at 340nm upon excitation at 296nm. The overall concentration of bsa and that of the common ions was fixed at 5 µM.

RESULTS AND DISCUSSION

Fluorescence quenching of bsa by navirapine:

The fluorescence spectroscopy was used to determine the nature of interaction between navirapine and bsa. In bsa, tryptophan and tyrosine residues contribute to fluorescence spectra. The decrease in fluorescence intensity of a compound by a variety of molecular reactions viz., energy transfer, ground state complex formation, excited state reactions, collisional quenching and molecular rearrangements is called quenching. In order to know the binding of navirapine to bsa, the fluorescence spectra were recorded upon excitation at 296nm (Fig. 2).As the concentration of navirapine gradually increases, the fluorescence intensity of bsa decreases. A well defined isobactic point was observed at 405 nm which is direct evidence of navirapine- bsa complex formation. All these results indicated that there were strong interactions between navirapine and bsa. In order to predict the possible quenching mechanism, the fluorescence quenching data were subjected to Stern–Volmer analysis [6] using the following equation.

$$\frac{Fo}{F} = 1 + K_{SV}[Q] = 1 + Kq\,\Gamma_o\,[Q] \qquad (1)$$

Where, F and F_0 are the fluorescence intensity of bsa with and without the quencher, respectively. The values of K_{sv} for navirapine- bsa system at different temperatures were found to be 3.515×10^4, $2.591 \times 10^{4,}$ 2.199×10^4 L mol^{-1} respectively. This indicates the static quenching interaction between nevirapine and bsa. K_q is the quenching rate constant of the bio-molecule, K_{SV} is the dynamic quenching constant, Γ_o is the average lifetime of bio-molecule without the quencher and [Q] is the concentration of the quencher . Obviously,

$$K_{SV} = Kq/\Gamma_o \qquad (2)$$

Sinece the fluorescence lifetime of the biopolymer is 10^{-8} s [8], the value K_q for navirapine- bsa system was observed to be 3.515×10^{12} , 2.591×10^{12} , 2.199×10^{12} L mol^{-1} s^{-1} respectively at 288,298 and 308K.However, the maximum scatter collision quenching constant , K_q of various quenchers with the biopolymer is 2×10^{10} L mol^{-1} s^{-1}[9]. Thus the rate constant calculated by protein quenching procedure is greater than Kq of scatter procedure. This indicates that a static quenching mechanism is operative[10].

ISSN: 0975-8585

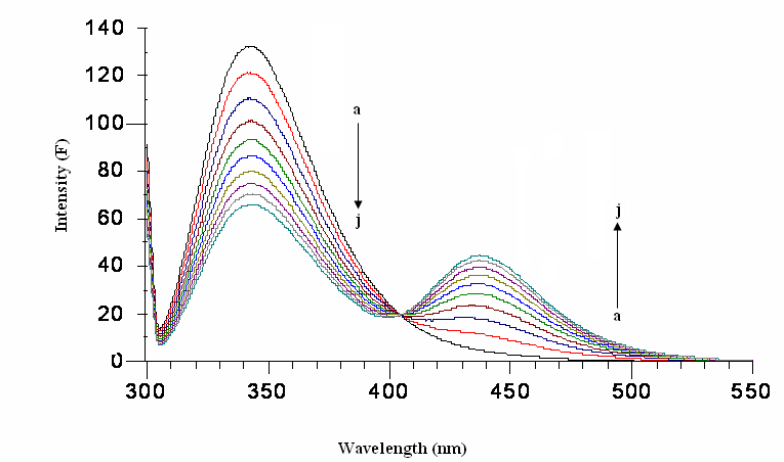

Fig. 2: Fluorescence spectra of bsa (5 µM) in presence of navirapine:(a)0 µM, (b)5 µM, (c)10 µM, (d) 15 µM, (e)20 µM, (f)25 µM, (g)30 µM, (h)35 µM, (i)40 µM, (j)45 µM

The Stern–Volmer plots for the results of interactions carried out at different temperatures (288, 298 and 308K) were observed to be linear with slopes (K_{SV} values) decreasing with increasing temperature (Fig. 3). This indicated the presence of static quenching mechanism in the interaction between navirapine and bsa. Further, the values of Kq were evaluated using the Eq.(2).The values of K_{SV} and Kq are given in Table1. The maximum scatter collision-quenching constant of various kinds of quenchers to biopolymer is reported to be 1.0×10^{10} L mol^{-1} s^{-1} [11]. The higher values of Kq noticed in the present investigation revealed that the quenching was not initiated by dynamic collision but from the formation of a complex. This phenomenon might be the result of the radiation less energy transfer between navirapine and bsa.

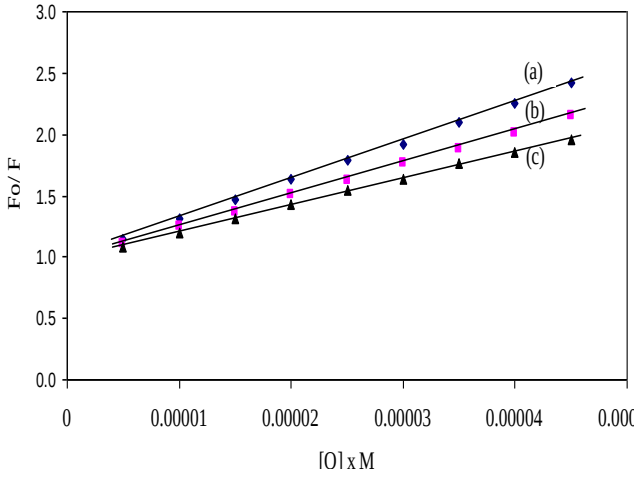

Fig. 3: The Stern–Volmer curves for quenching of navirapine with BSA at 288 K (a), 298 K (b) and 308 K (c).

Binding constant and number of binding sites:

Fluorescence intensity data can also be used to obtain the binding constant, K and the number of binding sites, n. When small molecules bind independently to a set of equivalent

sites on a macromolecule, the equilibrium between free and bound molecules is given [7] by the following equation.

$$\frac{\log (Fo - F)}{F} = \log K + n\log[Q] \qquad (3)$$

The values of K and n were obtained from the intercept and slope of the plot of log [(F_0 -F)/F] vs. log [Q] (Fig. 4). The values of K were found to be (1.98 x10^4, 1.74 x10^4 and 1.38 x10^4 LM^{-1}) and those of n were noticed to be 0.96, 0.95 and 0.98 respectively, at 288, 298 and 308 K(Table 1). The decreased binding constant with increase in temperature, resulted possibly due to the reduction of the stability of navirapine– bsa complex. The values of binding sites close to unity indicated that there was only one independent class of binding site on bsa for navirapine.

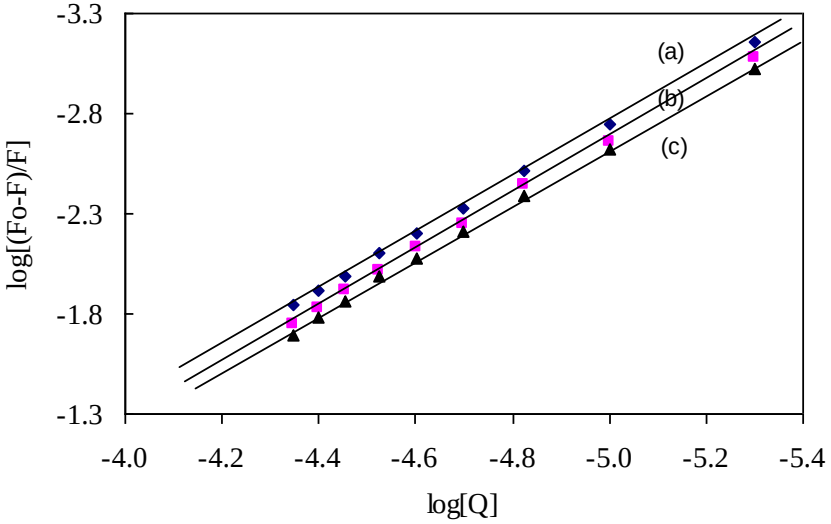

Fig. 4: The plot of log (F_0-F)/F Vs log[Q] for quenching of BSA by navirapine at 288 K, 298 K and 308 K (λ_{ex} = 296 nm; λ_{em} = 340 nm.[navirapine] = 5-45 µM; [bsa] = 5 µM.

Table 1: Thermodynamic parameters of navirapine – bsa system

System	Temp(T) K	Binding constant (K x10^{-4}) L mol^{-1}	No. of binding sites (n)	ΔH^0 (k J mol^{-1})	ΔS^0 (J K^{-1} mol^{-1})	ΔG^0 (k J mol^{-1})
bsa - navirapine	288	1.98 ± 0.01	0.96	-13.2	36.5	-24.5
	298	1.74 ± 0.02	0.95			
	308	1.38 ± 0.01	0.98			

Thermodynamic parameters and the nature of binding forces:

Considering the dependence of binding constant on temperature, a thermodynamic process was considered to be responsible for the formation of the complex. Therefore, the thermodynamic parameters (enthalpy change ΔH^0, entropy change ΔS^0 and free energy change ΔG^0 (Table 1) dependent on temperatures were analyzed in order to further characterize the acting forces between navirapine and bsa, as these are the main evidences to propose the binding mode. Primarily, four types of forces take part in drug–protein interaction, viz.,

ISSN: 0975-8585

electrostatic forces, hydrophobic forces, van der Waals force of interactions and hydrogen bonding [12]. Signs and magnitudes of thermodynamic parameters determine the nature of forces actually taking part in protein–drug interaction. The binding studies were carried out at three different temperatures viz., 288, 298 and 308K and thermodynamic parameters were evaluated using the following Van't Hoff equation and Gibbs–Helmholtz equation:

$$\log K = - \Delta H^0 / 2.303\ RT + \Delta S^0 / 2.303\ R \quad (4)$$

and

$$\Delta G^0 = \Delta H^0 - T\ \Delta S^0 \quad (5)$$

The values of ΔH^0 and ΔS^0 were obtained from the slope and intercept of the plot of log K vs. 1/T (Fig. 5). The values of ΔH^0, ΔG^0 and ΔS^0 are listed in Table 1. The negative sign of ΔG^0 values supported the assertion that all binding processes are spontaneous. The values of ΔH^0 are negative and large, while the values of ΔS^0 are positive. According to Ross and Subramanian [13], the positive ΔS^0 value was frequently taken as an evidence for hydrophobic interaction, while small negative ΔH^0 values reveal the presence of electrostatic interactions [10]. Since $\Delta H^0<0$ and $\Delta S^0>0$, the acting force between navirapine and bsa is believed to be predominantly hydrophobic force [14].

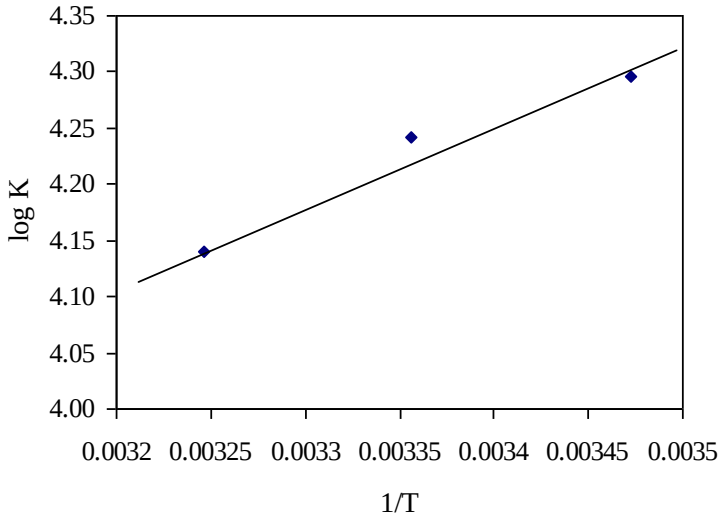

Fig. 5: Van't Hoff plot for the binding of navirapine with bsa

Energy transfer between navirapine and bsa:

There is a considerable overlap between absorption spectrum of navirapine (acceptor) and fluorescence spectrum of bsa (donor) which forms the basis of fluorescence resonance energy transfer(FRET) (Fig. 6). Energy transfer phenomena have wide applications in energy conversion process [15]. Photodynamic action, which is often used in the treatment of cancer, is also a consequence of energy transfer [16]. According to Förster's non radiative energy transfer theory [17], the energy transfer will happen under the following conditions: (i)the donor can produce fluorescence light,(ii) fluorescence emission spectrum of the donor and UV absorption spectrum of the acceptor have more overlap and (iii) the distance between the donor(bsa) and the acceptor(navirapine) is lower than 8 nm. The fluorescence quenching of bsa

upon binding with navirapine indicated the energy transfer between navirapine and bsa. The efficiency of energy transfer, E, was calculated using the equation

$$E = 1 - \frac{F}{Fo} = \frac{R_o^6}{R_o^6 + r^6} \quad (6)$$

where F and F_0 are the fluorescence intensities of bsa in presence and absence of navirapine, r the distance between acceptor and donor and R_0 the critical distance when the transfer efficiency is 50%. The value of R_0 is calculated using the equation

$$R^6_o = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \quad (7)$$

where k^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ 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 the following equation

$$J = \frac{\sum F(\lambda) E(\lambda) \lambda^4 \Delta\lambda}{\sum F(\lambda) \Delta\lambda} \quad (8)$$

where F(λ) is the fluorescence intensity of the fluorescent donor of wavelength, λ, ε(λ) is the molar absorption coefficient of the acceptor at wavelength, λ. For ligand– bsa interaction, k^2 = 2/3, N – 1.336 and Φ – 0.15 [15]. The values of J, R_0, E and r were calculated to be 4.85×10^{-14} cm^3 L mol^{-1} , 3.32 nm, 0.0952, 1.527 nm respectively at 298 K. Larger bsa – navirapine distance, r compared to that of R_0 observed in the present study also revealed the presence of static quenching mechanism between drug and protein. [18, 19]. The donor to acceptor distance, r < 7 nm indicated that the energy transfer from bsa to navirapine occurs with high possibility [20].

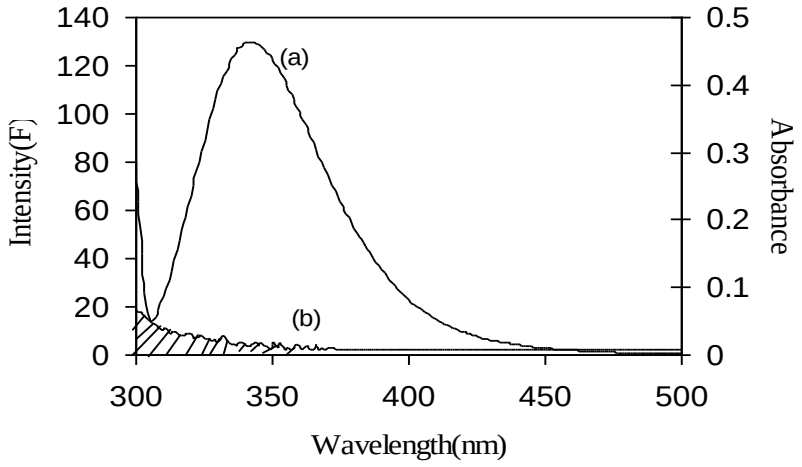

Fig. 6: The overlap of fluorescence spectrum of bsa (a) and the absorbance spectrum of navirapine (b), {[bsa]:[navirapine] = 1:1}.

ISSN: 0975-8585

Absorption spectroscopic studies:

UV–visible absorption spectroscopy is employed to explore the structural change [12] and to know the formation of complex between the drug and protein [21]. The λ_{max} of bsa observed at around 280nm was mainly due to the presence of tryptophan and tyrosine residues in bsa. It was evident from the spectrum of bsa (Fig. 7) that the absorption intensity of bsa increased regularly with increasing concentration of navirapine. Further, the red shift in absorption maximum indicated the change in polarity around tryptophan residue and changes in the peptide strand of bsa molecule and hence the change in hydrophobicity [12,22].

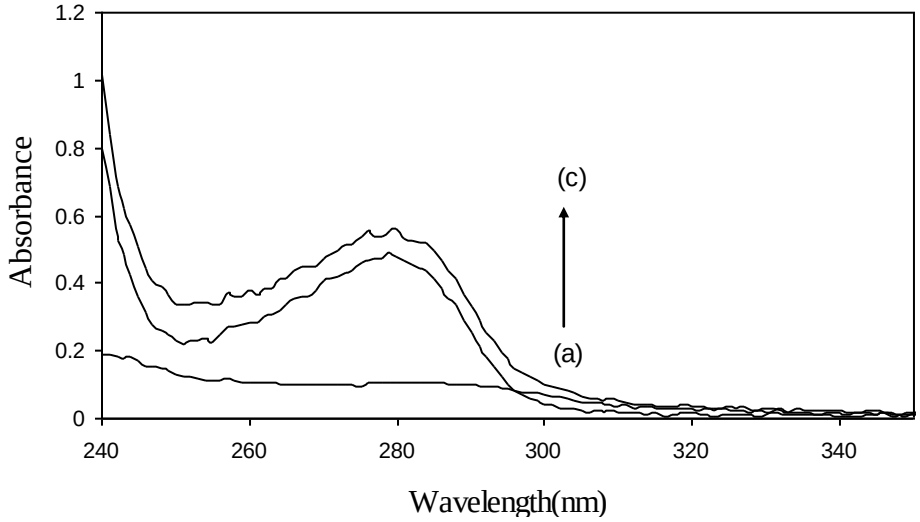

Fig. 7: Absorption spectra of navirapine, bsa and bsa –navirapine system. Navirapine concentration was at 5 µM(a), bsa concentration was at 5 µM (b), bsa –navirapine system was at 5 µM (c)

FT-IR spectroscopic studies:

Additional evidence for navirapine – bsa interaction was obtained from FT-IR spectra. Infrared spectrum of protein exhibited a number of amide bands due to different vibrations of the peptide moiety. Of all the amide modes of the peptide group, the single most widely used one in studies of protein secondary structure is the amide I. The amides I and II peaks occurred in the region of 1600–1700 cm^{-1} and 1500–1600 cm^{-1}, respectively. Amide I band is more sensitive to changes in protein secondary structure compared to amide II. Hence, the amide Ib and is more useful for studies of secondary structure [23-27]. The FT-IR spectrum of free bsa in phosphate buffer solution and the difference spectra after binding with navirapine are given in Fig. 8. It was noticed that the peak position of amide I was shifted from 1668 to1673 cm^{-1}, while that of amide II was shifted from 1525 cm^{-1} to 1536 cm^{-1} in the IR spectrum of bsa upon interaction with navirapine. This indicated that the navirapine interacted with bsa and the secondary structure of bsa was changed.

ISSN: 0975-8585

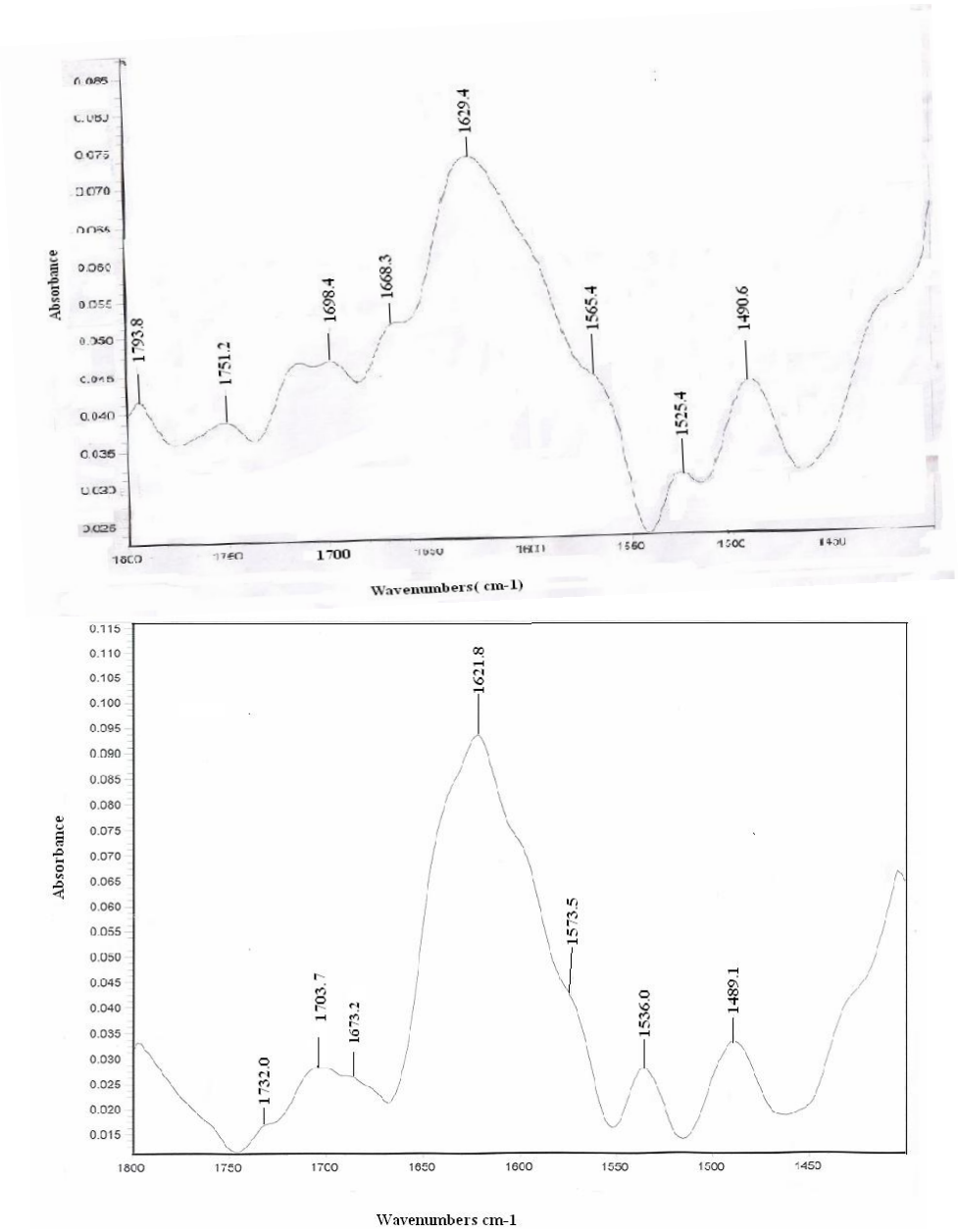

Fig. 8: FT-IR spectra and difference spectra of bsa;(a) the FT-IR spectra of free bsa (subtracting the absorption of the buffer solution from the spectrum of the protein solution) and (b) the FT-IR difference spectra of bsa (subtracting the absorption of the navirapine-freeform from that of navirapine– bsa bound form) in phosphate buffer ;[bsa] = 5 µM and [navirapine] = 5 µM.

Site probe studies:

Sudlow et al. [28] have suggested two main distinct binding sites (sites I and II) in bsa. Site I of bsa has affinity for warfarin, phenylbutazone, etc. and site II for ibuprofen, flufenamic acid, etc. It is reported that digitoxin binding is independent of sites I and II [29] and binds to site III. In order to establish the binding site in bsa for navirapine, competitive binding studies were performed using site probes, warfarin, ibuprofen, and digitoxin. For this, emission spectra

of ternary mixtures of navirapine, bsa and site probes were recorded, separately. The corresponding binding constant values were evaluated and these are recorded in Table 3. The binding constant of navirapine – bsa decreased remarkably in presence of warfarin while this value remained almost same in presence of ibuprofen and digitoxin. These results revealed that the warfarin displaced navirapine from the binding site while ibuprofen and digitoxin had a little effect on the binding of navirapine to bsa. Hence, we have concluded that the navirapine was bound to site I of BSA.

Table 2: Effect of common ions on binding constant of bsa –navirapine

Systems (cations)	Binding constant ($L\ M^{-1}$)
bsa + navirapine	$1.743 \pm 0.003 \times 10^4$
bsa + navirapine +Co^{2+}	$1.374 \pm 0.006 \times 10^4$
bsa + navirapine +Ni^{2+}	$5.046 \pm 0.006 \times 10^3$
bsa + navirapine +Ca^{2+}	$1.188 \pm 0.003 \times 10^4$
bsa + navirapine +Zn^{2+}	$8.260 \pm 0.002 \times 10^3$
bsa + navirapine +Cu^{2+}	$4.347 \pm 0.002 \times 10^4$

Table 3: Effect of warfarin, ibuprofen, digitoxin on binding constant of bsa-navirapine

Systems	Binding constant ($L\ M^{-1}$)
bsa + navirapine	$1.743 \pm 0.01 \times 10^4$
bsa + navirapine + warfarin	$1.321 \pm 0.02 \times 10^3$
bsa + navirapine + ibuprofin	$1.765 \pm 0.04 \times 10^4$
bsa + navirapine + digitoxin	$1.731 \pm 0.01 \times 10^4$

Effect of metal ions on the interactions of navirapine with bsa:

In plasma, there are some metal ions, which can affect the interactions of the drugs and serum albumins. Trace metal ions, especially the bivalent type are essential in the human body and play an important structural role in many proteins. It is reported [30] that Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} and Ca^{2+} and other metal ions can form complexes with serum albumins. Hence, the effects of some metal salt solutions viz., $CuCl_2$, $ZnCl_2$, $NiCl_2$, $CoCl_2$ and $CaCl_2$ on the binding of navirapine with bsa were investigated in the present study. Under the experimental conditions, none of the cation gave the precipitate in phosphate buffer. The binding constant of navirapine – bsa in presence of above ions was evaluated and the corresponding results are shown in Table 2. The binding constant of navirapine – bsa system decreased in presence of Co^{2+}, Ca^{+2}, Zn^{+2} and Ni^{2+} . This was likely to be caused by a conformational change in the vicinity of the binding site. The decrease in the binding constant in presence of above metal ions would shorten the storage time of the drug in blood plasma and hence more amount of free drug would be available in plasma [19]. This led to the need for more doses of drug to achieve the desired therapeutic effect in presence of above ions. The binding constant increased in presence of Cu^{2+}, thereby indicating the strong binding between the navirapine and bsa and

availability of more drug for action. This led to the need for less dose of drug for desired therapeutic effect.

CONCLUSION

The interaction of navirapine with bsa by employing various spectroscopic techniques. The results showed that the bsa fluorescence was quenched by navirapine through the static quenching mechanism. The thermodynamic parameters revealed that the hydrophobic forces are the main forces in the interaction process. The UV absorption and FT-IR studies showed that there is a change in the secondary structure of the protein after binding. The biological significance of this work is evident since albumin serves as a carrier molecule for multiple drugs and the interaction of navirapine with albumin was not characterized so far.

ACKNOWLEDGEMENT

One of the authors (S. D. Gunagi) thanks to UGC, New Delhi for the award of Research Fellowship in Science for Meritorious Students.

REFERENCES

[1] Peter TJ. All about Albumin. Biochemistry, Genetics and Medical Applications, Academic Press; California: 1996; pp. 133–87.
[2] Shang L, Jiang X, Dong S. J Photochem Photobiol A 2006; 184: 93-97.
[3] Zhou N, Liang Y, Wang P. J Photochem Photobiol A 2007; 185:271-276.
[4] Naik PN, Chimatadar SA, Nandibewoor ST . J Photochemistry and Photobiology B 2010; 100: 147-159.
[5] Naik PN, Chimatadar SA, Nandibewoor ST. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 2009; 73: 841–845.
[6] Patel SS, Benfield P. Clinical Immunotherapeutics 1996; 6 (4): 307–317.
[7] Sudlow G, Birkett DJ, Wade DN. Mol Pharmacol 1975; 11: 824-832.
[8] Heberer T. Toxicol Lett 2002; 131: 5-17.
[9] Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB,Buxton HT. Environ Sci Technol 2002; 36: 1202-1211.
[10] S Deepa and AK Mishra. J Pharm Biomed Anal 2005; 38: 556-563.
[11] Lakowicz JR. Principles of Fluorescence Spectroscopy, 3rd Ed.,Springer Publications; New York, USA: 2006; pp. 2598-2606
[12] Hu Y, Yi L, Wang J, Xiao HX, Qu SS. J Pharma Biomed Anal 2004; 36: 915-919.
[13] PD Ross, S Subhramanian. Biochem 1981; 20: 3096-3102.
[14] Liu JQ, Tian JN, Zhang JY, Hu ZD, Chen XG. Anal Bioanal Chem 2003; 37: 6864-867.
[15] Naik DB, Moorty PN, Priyadarsini KI. Chem Phys Lett 1990; 168: 533-538.
[16] Baptista MS, Indig GL. J. Phys. Chem. B 1998; 102: 4678-4688.
[17] Förster T, Sinanoglu O , Modern Quantum Chemistry, vol. 3, Academic Press;New York: 1996; pp136-146.
[18] Cui F, Fan J, Li J, Hu Z . Bioorg Med Chem 2004; 12:151-157.
[19] He W, Li Y, Xue C, Hu Z, Chen X, Sheng F. Bioorg Med Chem 2005; 13: 1837-1845.

[20] Valeur B. in: Molecular Fluorescence: Principle and Applications, Wiley Press;New York: 2001; pp 69-80.
[21] Bi S, Song D, Tian Y, Zhou X, Liu Z, Zhang H. Spectrochim Acta A 2005; 61: 629-636.
[22] Ulrich KH. Pharmacol Rev 1981; 33: 17-53.
[23] Wi S, Pancoska P, Keiderling TA. Biospectroscopy 1998; 4: 93-106.
[24] Rahmelow K, ubner WH. Anal Biochem 1996; 241: 5-13.
[25] Lin S, Wei Y, Li M, Wang S. Eur J Pharm Biopharm 2004; 57: 457-464.
[26] Brauner JW, Flach CR, Mendelsohn R. J Am Chem Soc 2005; 127: 100-109.
[27] Tian J, Liu J, Hu Z, Chen X. Bioorg. Med. Chem 2005; 13: 4124-29.
[28] Sudlow G, Birkett DJ, Wade DN. Mol Pharmacol 1976; 12: 1052-61.
[29] Sjoholm I, Ekman B, Kober A, Pahlman IL, Seiving B, Sjodin T. Mol Pharmacol 1979; 16 : 767-777.
[30] Zhang G, Wang Y, Zhang H, Tang S, Tao W. Pest Biochem Phys 2007; 87: 23-29.