

## SPECTROSCOPIC AND ELECTROCHEMICAL INVESTIGATION OF BINDING OF NEVIRAPINE WITH BOVINE SERUM ALBUMIN

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### ABSTRACT

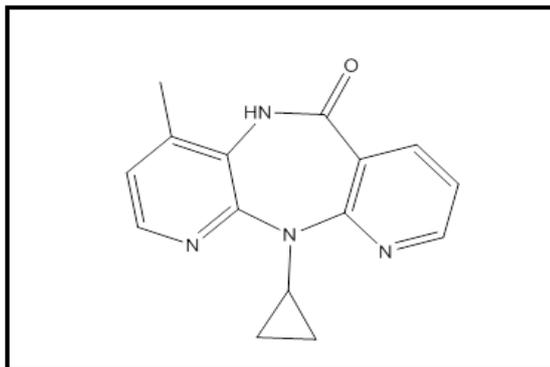
Nevirapine is a non-nucleoside reverse transcriptase inhibitor used for the treatment of Human Immunodeficiency Virus Type-1 infection and AIDS. A systematic interaction between nevirapine and bovine serum albumin (BSA) has been investigated by UV-Vis absorption, infra-red (IR) spectra, fluorescence, circular dichroism (CD), cyclic voltammetry (CV), and nuclear magnetic resonance (NMR) spectroscopy. UV-Vis absorption and fluorescence quenching is attributed to the formation of nevirapine-BSA complex. The fluorescence analysis indicates the quenching of BSA by nevirapine occurs through static procedure. Synchronous fluorescence, CD, and IR spectra revealed the conformation and microenvironment of BSA were altered by interaction with nevirapine. NMR spectroscopic results suggested hydrogen bonding and hydrophobic interactions played an important role in nevirapine-BSA complex formation. Cyclic voltammetry (CV) proved the interaction of nevirapine with BSA forming an electrochemically inactive complex.

**KEYWORDS:** BSA, nevirapine, fluorescence quenching, circular dichroism, cyclic voltammetry, NMR.

### 1. INTRODUCTION

Nevirapine is a non-nucleoside reverse transcriptase inhibitor (NNRTI) which belongs to dipyridodiazepinones chemical class. <sup>[1, 2]</sup> Nevirapine is used in combination with nucleoside analogs for treatment of Human Immunodeficiency Virus Type-1(HIV-1) infection and AIDS. Both the 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. Nevirapine binds directly to reverse transcriptase (RT) and blocks the RNA-dependent and DNA-dependent DNA polymerase activities by causing a disruption of enzyme activity site. The structure of nevirapine is shown in **Fig.1**



**Fig.1 Structure of Nevirapine**

Bovine serum albumin has been studied extensively because of its structural homology to human serum albumin (HSA), high aqueous solubility, easy availability, and low cost. <sup>[3, 4]</sup> BSA is most abundant protein in blood circulatory system which plays an important role in the transport and disposition of variety of substances in blood. BSA has a wide range of physiochemical functions such as binding, delivery, and transport of variety of chemicals in blood. BSA is also used as blocking agent, carrier protein, and stabilizer. <sup>[5]</sup> BSA consists of 582 amino acid residues cross linked with 17 cysteine residues. BSA has two tryptophan residues, located at position 134 and 213. Trp-213 is located within hydrophobic binding pocket of the protein and Trp-134 is located on the surface of the protein. <sup>[6]</sup> The intrinsic fluorescence of BSA is obtained mainly from tyrosine (Tyr), phenylalanine (Phe), and tryptophan (Trp) residues. The fluorescence of tyrosine is quenched by tryptophan residue and phenylalanine shows very weak fluorescence emission. Therefore, tryptophan residue is used to investigate the interaction of ligand with BSA. The interaction of BSA with ligands has been extensively studied. <sup>[7-10]</sup> there is a single report on the study of interaction of BSA with nevirapine by Chimatdar *et.al.* <sup>[11]</sup> Using FT-IR and fluorescence quenching. However, there are no reports on the binding of nevirapine with BSA using synchronous fluorescence, circular dichroism (CD), cyclic voltammetry (CV), and NMR. The present work describes the studies of binding of nevirapine with BSA using spectrofluorimetry, IR spectroscopy, UV-Vis absorption, circular dichroism, cyclic voltammetry, and NMR. Thus, extensive study has been conducted to understand the binding mechanism of nevirapine with BSA.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Bovine serum albumin (BSA, lyophilized powder,  $\geq 98\%$ , M.W.  $\approx 66000$  Da) was purchased from Sigma-Aldrich, India and used without further purification. Nevirapine (anhydrous) was obtained from Sigma-Aldrich, India. The stock solution of BSA was prepared by dissolving appropriate amount of BSA in phosphate buffer with pH= 7.0. The extinction coefficient ( $43,824 \text{ L mol}^{-1}\text{cm}^{-1}$ ) at 280 nm was used to determine the concentration of BSA solution. <sup>[12]</sup> All other reagents and solvents were of analytical reagent grade. Double distilled water was used in all the experiments.

### 2.2. Fluorescence Measurements

The fluorescence measurements were recorded on SHIMADZU RF-5301 PC spectrofluorophotometer. The excitation wavelength was set at 280 nm and emission was recorded in the range of 281-450 nm. The excitation and emission slit widths were kept at 5 nm each. The fluorescence emission of BSA was recorded at 293, 298, and 310 K with different concentration of nevirapine solution in a 1cm quartz cell. All the measurements were recorded after 25 min of incubation.

### 2.3. Synchronous Fluorescence Measurement

For this measurement, the initial excitation wavelength was fixed at 200 nm and scanned up to 550 nm. The difference between excitation and emission wavelength ( $\Delta\lambda$ ) was set at 15 nm for tyrosine residue and 60 nm for tryptophan residues. The excitation and emission slit width was set at 5 nm.

### 2.4. UV-Vis Measurements

UV-Vis absorption spectra of BSA were recorded on SHIMADZU UV-2401 PC spectrophotometer in the absence and presence of nevirapine at 298 K in the range of 250-330 nm. The quartz cuvette with 1cm path length was used.

### 2.5. FT-IR Measurements

FT-IR spectra were recorded on PerkinElmer FT-IR spectrometer. The FT-IR measurements of BSA in the absence and presence of nevirapine were recorded in the range of 1400-1800  $\text{cm}^{-1}$ . The transmittance of phosphate buffer and free nevirapine solution were recorded and subtracted.

## 2.6. Circular Dichroism Measurement

The CD measurements were recorded using JASCO, J-815, CD spectrometer. The CD measurement of BSA in absence and presence of nevirapine were recorded with three accumulations for each spectrum. The measurements were taken in the far UV region in the range of 190-260 nm with scan speed of 10 nm/min. The band width was fixed at 5 nm.

## 2.7. Cyclic Voltammetric Measurements

All the electrochemical measurements were recorded on AUTOLAB PGSTAT 30 electrochemical workstation. A glassy carbon electrode, platinum wire, and Ag/AgCl were used as working, auxiliary, and reference electrode respectively. The scan rate was fixed at  $100 \text{ mV S}^{-1}$ . Nitrogen is purged in all solutions for 15 min before the voltammetric measurements.

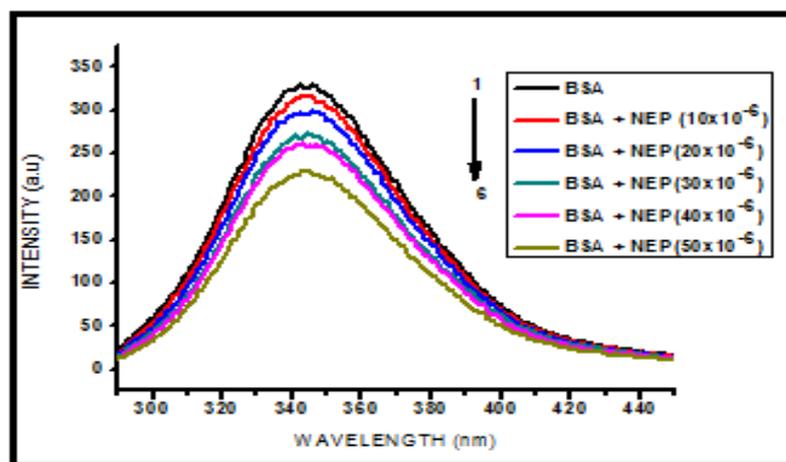
## 2.8. NMR Measurements

NMR experiments were recorded on Avance 300 MHz spectrometer (Bruker).  $^1\text{H}$  and  $^{13}\text{C}$  spectra of nevirapine in absence and presence of BSA was measured in  $\text{DMSO-d}_6$  at 298K.

## 3. RESULTS AND DISCUSSION

### 3.1. Fluorescence Quenching of BSA In Presence of Nevirapine

The fluorescence technique is the most sensitive and an efficient method to study the interaction between BSA and ligand. The ligand binding sites of BSA is located in subdomain IIA. <sup>[13]</sup> The fluorescence study was carried to investigate the structural changes of BSA caused upon addition of nevirapine. The effect of nevirapine on the fluorescence intensity of BSA is shown in **Fig.2**

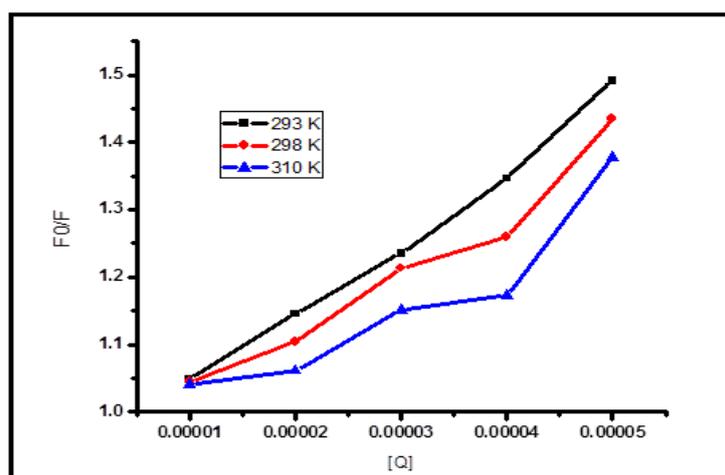


**Fig-2.** Fluorescence quenching spectra of BSA in absence and presence of different concentration of nevirapine at room temperature.

It indicates that fluorescence intensity of BSA gradually decreased with increasing concentration of nevirapine. The maximum of fluorescence peak is also shifted from 342 to 346 nm revealing that the interaction between nevirapine and BSA occurred by forming nevirapine-BSA complex. Fluorescence quenching occurs by two different mechanism, dynamic quenching and static quenching. <sup>[14, 15]</sup> Dynamic quenching involves increase in diffusion coefficient with increase in temperature, however, static quenching involves decreasing quenching constant ( $K_q$ ) with increase in temperature accompanying decreased stability of complex. <sup>[16, 17]</sup> The fluorescence quenching experiments were performed at 293, 298, and 310 K, to investigate the fluorescence quenching mechanism of the interaction between nevirapine and BSA which could be explained by Stern-Volmer equation. <sup>[14,18,19]</sup>

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \quad (1)$$

Where  $F_0$  and  $F$  denotes the fluorescence intensities of BSA in absence and presence of nevirapine,  $K_{sv}$  is the Stern-Volmer quenching constant,  $[Q]$  is the concentration of the nevirapine,  $K_q$  is the biomolecular quenching rate constant,  $\tau_0$  is the average lifetime of the molecule without the quencher which is equal to  $10^{-8}$  s.<sup>20</sup> The stern-volmer plot of  $F_0/F$  vs.  $[Q]$  at three different temperatures is shown in **Fig.3**



**Fig.3** Stern-Volmer plots for the quenching of BSA by nevirapine at three different temperatures.

The values of  $K_{sv}$  and  $K_q$  were calculated and presented in **Table.1**

**Table-1. Binding Parameters of Nevirapine with BSA at Different Temperatures.**

T (K)	K <sub>sv</sub> (M <sup>-1</sup> )	K <sub>q</sub> (M <sup>-1</sup> s <sup>-1</sup> )	K (LM <sup>-1</sup> )	n
293	10.08×10 <sup>4</sup>	10.08×10 <sup>12</sup>	5.95×10 <sup>5</sup>	1.4
298	9.36×10 <sup>4</sup>	9.36×10 <sup>12</sup>	4.73×10 <sup>5</sup>	1.5
310	7.87×10 <sup>4</sup>	7.87×10 <sup>12</sup>	3.75×10 <sup>5</sup>	1.3

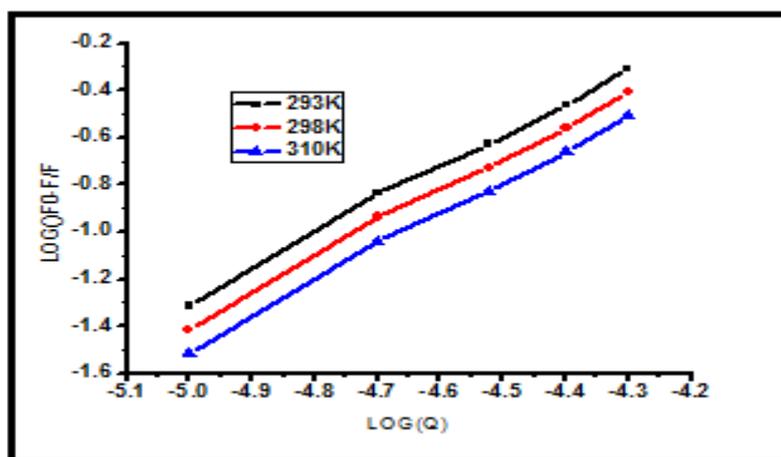
The values of K<sub>q</sub> were much higher than that of the maximum scattering collision quenching constant (2.0×10<sup>10</sup> L mol<sup>-1</sup>), indicating that the interaction between BSA and nevirapine occurs through static quenching and not due to dynamic quenching.

### 3.2. Number of Binding Sites and Binding Constant of BSA- Nevirapine Complex.

The number of binding sites (n) and binding constant (K) between the nevirapine and BSA was calculated using the equation. <sup>[21]</sup>

$$\log \frac{F_0 - F}{F} = \log K + n \log [Q] \quad (2)$$

The binding constant (K) and number of binding sites (n) were obtained from the plot of log [(F<sub>0</sub>-F)/F] vs. log [Q] as shown in **Fig .4**



**Fig.4 Plot of log [(F<sub>0</sub>-F)/F] vs. log [Q] of quenching of BSA by nevirapine at three different temperatures.**

The values of K and n are summarized in **Table.1**. The binding constant (K) decreases with increase in temperature and value of binding number (n) is almost unity. These results show that the formation of unstable complex between nevirapine and BSA. <sup>[22]</sup>

### 3.3. Mode of binding

Usually the small molecules bind to the biomacromolecule through hydrogen bonding, van der Waals force, hydrophobic interactions, and electrostatic interactions.<sup>[23]</sup> The change in entropy ( $\Delta S^\circ$ ) and enthalpy ( $\Delta H^\circ$ ) were obtained from Vant Hoff's equation.

$$\ln K = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (3)$$

Where K is the binding constant, R is the gas constant and T is the temperature. The Gibbs free energy is calculated from the equation.

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = -RT \ln K \quad (4)$$

The values of ( $\Delta S^\circ$ ) and ( $\Delta H^\circ$ ) were obtained from slope and intercept of the plot of  $\ln(K)$  vs.  $1/T$  respectively. The values of  $\Delta G^\circ$ , ( $\Delta S^\circ$ ), and ( $\Delta H^\circ$ ) were reported in **Table.2**.

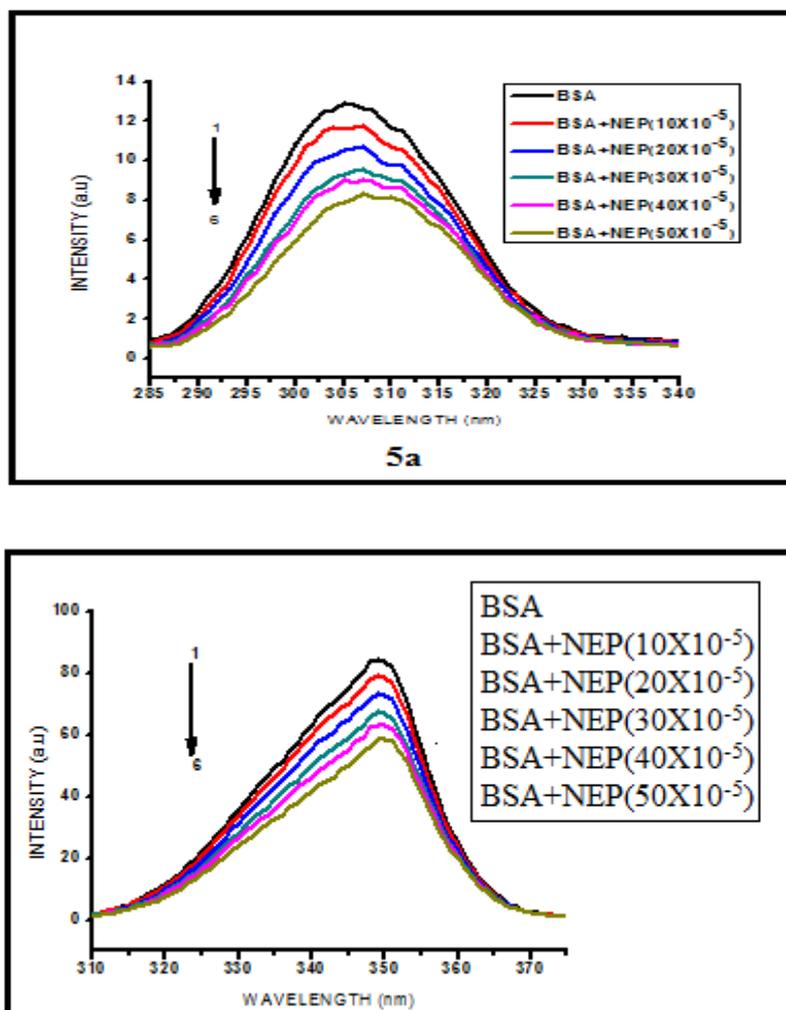
**Table-2. Thermodynamic Parameters of Nevirapine-Bsa Interaction.**

Temperature (k)	$\Delta H^\circ$ (KJmol <sup>-1</sup> )	$\Delta S^\circ$ (KJmol <sup>-1</sup> )	$\Delta G^\circ$ (Jmol <sup>-1</sup> K <sup>-1</sup> )
293			-32.38
298	-9.40	14.00	-32.37
310			-33.07

The positive value of  $\Delta S^\circ$  (14.00 Jmol<sup>-1</sup>K<sup>-1</sup>) and negative value of  $\Delta H^\circ$  (-9.40 KJmol<sup>-1</sup>) indicate that hydrogen bonding and hydrophobic interactions are involved in the binding.<sup>23, 24</sup> The negative value of  $\Delta G^\circ$  suggests that the binding between nevirapine and BSA occurred spontaneously.

### 3.4. Conformational Investigation by Synchronous Fluorescence Spectra

The synchronous fluorescence spectra give the information about conformational changes of BSA by measuring the fluorescence emission wavelength shift of tryptophan and tyrosine.<sup>25</sup> When the  $\Delta\lambda$  between excitation and emission wavelength are fixed at 15 and 60 nm, it gives the characteristic information about tyrosine and tryptophan residue respectively.<sup>26</sup> The change in conformation of BSA upon addition of nevirapine is shown in **Fig.5**.

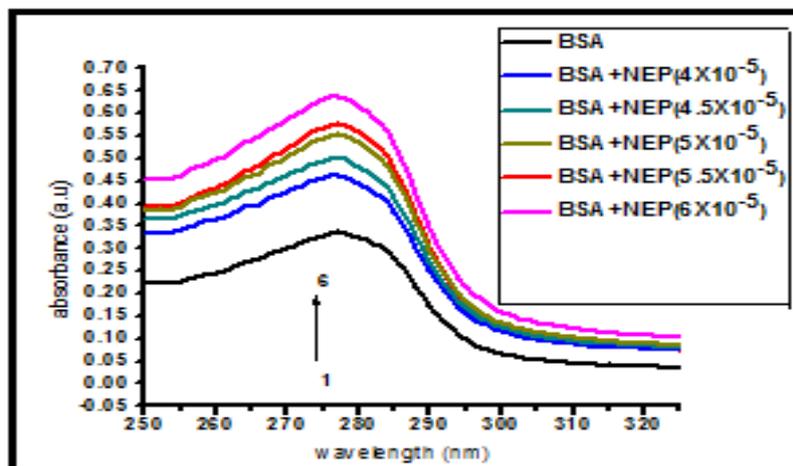


**Fig.5 Synchronous fluorescence spectrum of BSA with different concentration of nevirapine at (5a)  $\Delta\lambda=15$  and (5b)  $\Delta\lambda=60$ .**

The maximum emission wavelength shift of tyrosine residues did not alter significantly, indicating that no considerable changes observed in the microenvironment of tyrosine residue. However, maximum emission wavelength of tryptophan residue blue shifted by 2-3 nm revealing the polarity around tryptophan residue is decreased and hydrophobicity is increased. <sup>[27]</sup>

### **3.5. UV-Vis spectra of BSA-nevirapine complex**

UV-Vis spectra of BSA in absence and presence of nevirapine was recorded at 298K as shown in **Fig.6**.

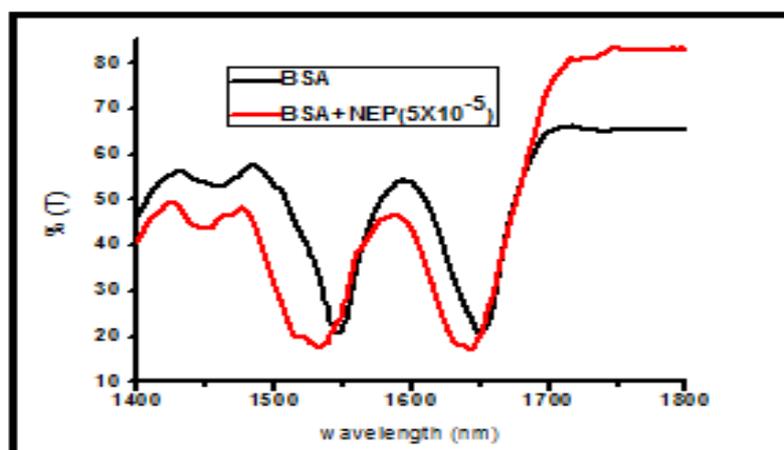


**Fig.6** UV-Vis absorption spectra of BSA in absence and presence of different concentration of nevirapine at 298 K.

The BSA shows absorption peak at 280 nm which increases gradually upon increasing concentration of nevirapine and shifted slightly toward lower wavelength indicating the formation of ground state complex between BSA and nevirapine.

### 3.6. Effect of Nevirapine on Secondary Structure of Protein Using IR Spectra.

IR spectra were used to study the change in conformation of protein secondary structure. The IR spectra before and after binding of nevirapine to BSA are shown in **Fig.7**.



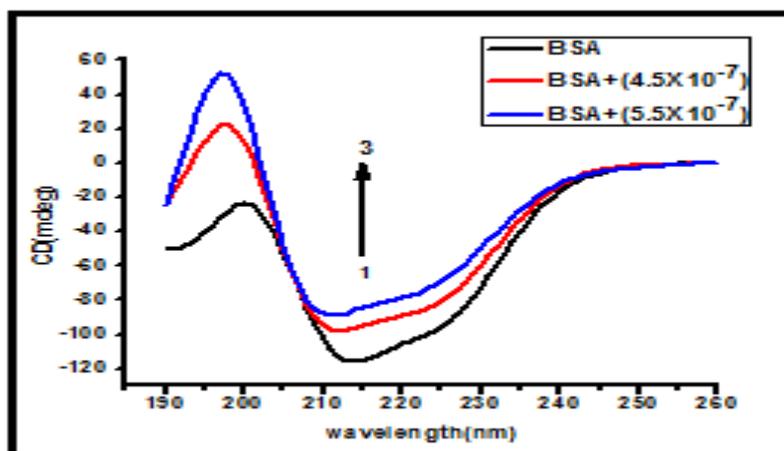
**Fig.7** Infrared spectrum of BSA in absence and presence of nevirapine.

Proteins show number of amide bands in IR spectra. Among those amide I and amide II bands ranging from 1600-1700 and 1500-1600  $\text{cm}^{-1}$  respectively mainly give the information about secondary structure of proteins.<sup>28, 29</sup> The amide I band arises from C=O stretching vibration and amide II band arises due to C-N stretching coupled with N-H vibration. However, amide I band is more sensitive than amide II band. The hydrogen bonding and

geometry are mainly responsible for disturbing the amide I band of protein. The amide I band shifted from  $1650\text{ cm}^{-1}$  to  $1641\text{ cm}^{-1}$  and amide II band shifted from  $1557\text{ cm}^{-1}$  to  $1551\text{ cm}^{-1}$  indicating change in secondary structure of BSA due to the formation of hydrogen bonding between nevirapine and BSA.

### 3.7. Effect of Nevirapine on the Conformation of BSA Using Circular Dichroism

The primary structure of BSA contains a single chain of about 582 amino acid residues.<sup>[30]</sup> The secondary structure of BSA is formed by 67% of  $\alpha$ -helix of six turns and 17 disulphide bridges.<sup>[31]</sup> When ligand binds to BSA, the intermolecular forces responsible for maintaining the secondary structure gets altered, leading to conformational changes in the BSA.<sup>[32]</sup> Circular dichroism (CD) is a useful technique to explore the interaction between protein and drugs. The far UV region (190-250 nm) is used to investigate the secondary structure of protein. Peptide bond is mainly responsible for the absorption in far UV region. Because of the asymmetric protein environment and inherent chirality, the induced asymmetry of achiral ligands was observed during their binding with proteins. The CD spectra of BSA shows two negative bands at 208 and 222 nm which are characteristic of  $\alpha$ -helical structure of protein.<sup>[33]</sup> The band intensity of BSA decreases with the addition of nevirapine as shown in **Fig.8**.



**Fig.8** Circular dichroism spectrum of BSA in absence and presence of different concentration of nevirapine at 298 K.

The CD results are expressed in terms of molar residual ellipticity using the following equation:

$$[\theta] = (\theta \times 100 \times M) / (C \times l \times n) \quad (5)$$

Where,  $\theta$  is ellipticity in degrees,  $l$  is optical path in cm,  $C$  is the concentration in mg/ml,  $M$  is molar mass and  $n$  is the number of residues in the protein.

The values of  $\alpha$ -helical content of free and bound BSA at 208 and 222 nm were calculated using the equations:

$$F(H)_{208\text{ nm}} = ([\theta]_{208} - 4,000) / (-33,000 - 4,000) \quad \text{and} \quad (6)$$

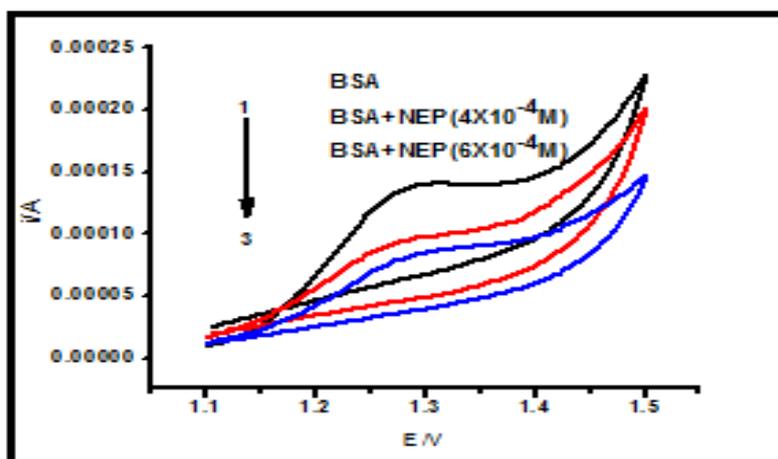
$$F(H)_{222\text{ nm}} = ([\theta]_{222} - 3,000) / (-36,000 - 3,000) \quad (7)$$

The % of  $\alpha$ -helix values are reported in **Table.3**

**Table-3. The Change in % of A- Helix of BSA upon Interaction with Nevirapine. The symbol "alpha"**

Substances	Molar ratio	% of $\alpha$ -helix	
		at 208 nm	at 222 nm
Free BSA		42	32
BSA: NEVIRAPINE	1:1	37	29
	1:2	21	17

It was observed that the  $\alpha$ -helicity decreased from 42 to 21% at 208 nm and 32 to 17% at 222 nm indicating the change in secondary structure of BSA upon interaction with nevirapine. Moreover, shape of peak and peak maximum remained almost same. This reveals that BSA retains its  $\alpha$ -helix structure after binding to the nevirapine. <sup>[34]</sup>



**Fig.9 Cyclic voltammograms of nevirapine in absence and presence of different concentration of BSA.**

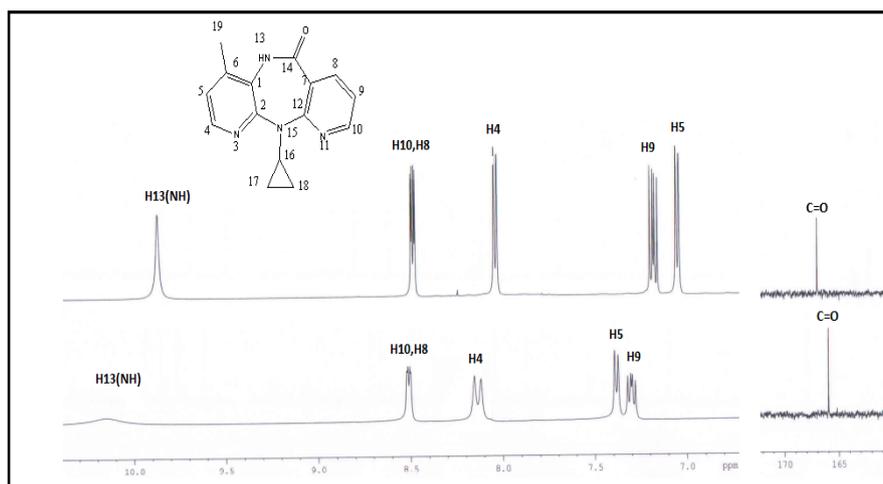
### 3.8. Electrochemical Behavior of Nevirapine

As shown in **Fig.9**, the electrochemical behavior of nevirapine in absence and presence of BSA was recorded on glassy carbon electrode in 0.4 M BR buffer with pH = 4 using scan rate of 100 mV s<sup>-1</sup>. Cyclic voltammogram of nevirapine gives rise to an oxidation peak at 1.29 V and no reduction peak is observed, which indicated that the reaction is irreversible in nature.

Upon the addition of BSA, peak current of nevirapine decreases, but peak potential remains almost same and no new peaks were observed in potential range. Thus, it is concluded that nevirapine forms an electrochemically inactive complex with BSA. The concentration of nevirapine decreased on the surface of electrode; hence the oxidation peak current of nevirapine gets reduced.

### 3.9. Interaction of nevirapine with BSA using NMR

The  $^1\text{H}$  and  $^{13}\text{C}$  spectrum were recorded to assign chemical shift of nevirapine in the absence and presence of BSA as shown in the **Fig.10**.



**Fig.10** NMR spectra of nevirapine [200  $\mu\text{M}$ ] in absence and presence of BSA [25  $\mu\text{M}$ ] at 298 K

The change in chemical shifts of nevirapine is summarized in **Table.4**. It was observed that the chemical shifts and line widths of nevirapine gets altered upon addition of BSA.

**Table. 4**  $^1\text{H}$  Chemical Shifts (In ppm) For Nevirapine in Absence and Presence of BSA.

Proton position Of NEVIRAPINE NEVIRAPINE	$^1\text{H}$ chemical shift (in ppm)		
	NEVIRAPINE	NEVIRAPINE + BSA	{(NEVIRAPINE + BSA) - NEVIRAPINE
4	8.06	8.16	0.1
5	7.03	7.39	0.36
8	8.47	8.50	0.03
9	7.17	7.32	0.15
10	8.49	8.32	0.03
13	9.86	10.15	0.29
16	0.88	0.96	0.08
17	0.37	0.46	0.09
18	0.37	0.46	0.09
19	2.06	2.42	0.36

When ligand binds to a protein, the chemical shifts gets changed either by direct interaction between ligand and protein or by inducing conformational change in the protein. Upon the addition of BSA, H-13 of nevirapine gets deshielded and carbonyl carbon is shielded. This indicates both H and O- atom of amide bond in the nevirapine are involved in the hydrogen bonding with amino acid side chain of BSA. This is due to the electron intensity around H-atom decreases and that of O-atom increases because the H and O-atom act as a hydrogen bond donor and acceptor respectively. All the aromatic protons also show significant chemical shifts on addition of BSA, revealing that  $\pi$ - $\pi$  stacking between nevirapine and aromatic ring residue of BSA occurs. [35] After addition of BSA, H-4, H-8, and H-10 get deshielded whereas H-5 and H-9 are shielded due to the change in local environment as a consequence of nevirapine- BSA interactions. As shown in **Fig.10**, H-5 and H-9 shift to upfield prominently, revealing that they are closer to aromatic ring residue of nevirapine but H-4, H-8, and H-10 show little downfield shifts. Thus, it can be concluded that aromatic ring residue of BSA stacks with the region of nevirapine away from the N-atoms present in the pyridine rings, which might be due to repulsive forces between the electronegative N-atoms of nevirapine and  $\pi$  electron cloud of aromatic ring residues of BSA. The broadening of proton signals arise from spin-spin coupling overlapped into one another, leading to decrease in spin-spin relaxation time and increased correlation time. [36]

#### 4. CONCLUSION

The interaction between nevirapine and BSA was studied to derive important parameters like quenching constant, binding constant, number of binding sites, % of  $\alpha$ -helix, hydrogen bonding, and electrostatic interactions. UV-Vis spectra confirmed the formation of complex between nevirapine and BSA. Based on the fluorescence quenching data, we calculated the values of  $K_{SV}$ ,  $K_q$ , and  $K$  revealing that nevirapine binds to BSA by static quenching mechanism. CD spectra showed that conformation of BSA gets altered upon interacting with nevirapine. Further, from cyclic voltammetry, we concluded that the complex formed between nevirapine and BSA was electrochemically inactive. Finally, IR and NMR data indicates the hydrogen bonding and electrostatic interactions played a major role in complex formation.

#### ACKNOWLEDGEMENTS

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