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Interaction of different prototropic species of an anticancer drug Ellipticine with HSA and IgG proteins: multispectroscopic and molecular modeling studies

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Abstract: Studies on interactions between an anticancer alkaloid, ellipticine and various carrier proteins in blood serum show tangible results to gain insight into the solubility and transport of the drug in physiological conditions. In this report, we extensively studied the interactions of different prototropic species of ellipticine with two prominent serum proteins namely human serum albumin (HSA) and immunoglobulin G (IgG) in their native and partially unfolded states using steady state and time resolved fluorescence spectroscopy, molecular docking and circular dichroism (CD). Both the fluorescence techniques and molecular modeling study elucidate that only neutral species of ellipticine binds with HSA in the sudlow site II. Unlike HSA, IgG in native state mostly binds with cationic species of ellipticine. However, in partially unfolded configuration, IgG binds with the binding of neutral ellipticine molecules. Molecular docking studies indicate the prevalence of electrostatic interactions involving charged residues in the binding process of cationic species of ellipticine with native IgG at its F_{ab} region. In native conformation, the hydrophobic residues of F_{ab} region are found to be buried completely by the ligand. This implies that the hydrophobic interaction will be favored by unfolding of IgG through which the hydrophobic pocket will be more accessible to neutral species of ellipticine. The circular dichroism measurements reveal that upon interaction with ellipticine, heat and acid treated HSA resumes its α-helical content. This conclusive comparative study on interactions of IgG and HSA with ellipticine yields in the result that native HSA is responsible for transport of neutral species of ellipticine whereas IgG carries cationic ellipticine in its native form.

1. Introduction:

Binding of drugs with the proteins present in blood stream is a significant process to determine their eventual activities and fate of the drugs once they have entered into the circulatory system. In general, interactions of drug molecules with various serum proteins can improve their solubility and control the rate of distribution, excretion as well as their toxicity.[1] These interactions become relevant as drugs have maximum affinity towards serum proteins.[2] The major serum proteins are albumin and globulins. Albumin mainly helps in transportation of various agents whereas globulins play role in inflammatory response.[3] Human serum albumin (HSA) is the major component of blood plasma and constitutes approximately half of the proteins found in blood. It serves as transport protein for quite a lot of endogenous and exogenous ligands as well as for various drug molecules.[4] It also maintains the colloid osmotic

pressure of the circulation and buffers the pH of plasma.[5] HSA consists of 585 amino acids and composed of a single polypeptide chain. It contains three α-helical domains I to III, each containing two subdomains.[6] The principal regions for ligand binding in albumin are located in hydrophobic cavities of sub domain IIA and IIIA. Nobleness of HSA lies in its interaction with various drugs and intracellular trafficking due to the presence of various binding sites in this protein.[7]

Along with albumins, another significant component of serum proteins includes globulins. In normal serum, globulins constitute about 36% of the total plasma proteins. The serum globulins are classified as α, β and γ globulins. Gamma globulins are the major one among globulins and play crucial role in defense mechanism and are commonly known as immunoglobulin.[8] The major γ-globulin in human blood is immunoglobulin G (IgG), which defend against variety of infectious pathogens. IgG contains four polypeptide chains of which two are heavy and two are light. These chains are cross linked by disulphide bonds in a Y-shaped structure, which is characterized by three fragments (two F_{ab} and one F_c). They have domains that are structurally independent. Specific amino acid sequence in F_{ab} of IgG determines its ability for selective binding with a particular antigen.[9] A prime attribute of these proteins is that non-polar residues are buried in a core, to avoid contact with water. The disclosure of these hydrophobic residues crops up upon heat and acid treatment.[10] According to several reports; hydrophobic domains are located both in F_{ab} and F_c region of IgG. DSC measurements proposed that F_{ab} domains are mostly affected by heating at 63ºC due to its lower melting temperature as compared with that of F_c[11] while upon decreasing the pH, F_c fragment is primarily affected and thus become partially unfolded.

Recently several groups reported interaction of drug molecules with various proteins.[12] These studies reveal the mere conformational changes of proteins upon interaction with different chemicals which include surfactant and drug molecules. However, knowledge on the interaction of drug molecules with proteins at molecular level is lagging behind. Moreover, the specific affinity of a protein for a particular species or conformation of a drug molecule is very important from the drug transport point of view. The present study is designed to understand the interactions of HSA and IgG in their native and partially unfolded conformations with different

prototropic species of an anticancer alkaloid namely ellipticine using steady state and time resolved fluorescence spectroscopy, molecular modeling and circular dichroism (CD) techniques.

Ellipticine, a pyridocarbazole type of plant alkaloid exhibits cytotoxic activity against various tumor cells. It is cytotoxic towards tumors of breast, kidney and lungs. Significance of ellipticine lies in its biological activity i.e. it interacts with DNA of tumor cells and treats solid tumors.[13] Recently, ellipticine and structurally related compounds were reported to have application in the treatment of obesity and tested for human pre-AIDS treatment in association with other drugs.[13d-13e] Various groups reported the photophysical characteristics of eliipticine.[14] It can exist as protonated or deprotonated species depending on the pH of the medium.[15] However, the major drawback of use of neutral ellipticine as pharmaceutical agent is its low solubility in aqueous system, whereas the cationic species i.e., ellipticinium is relatively more soluble in water than its neutral form. At pH lower than 7.4, due to protonation of pyridine ring nitrogen of ellipticine, cationic species are formed. To surmount the drawback of low solubility of ellipticine in aqueous media, the drug is attached to polymers, peptides, lipids or micelles which boost the bioavailability of drug.[16]

Our present study will elaborate the mechanism of possible transportation of the drug through various serum components. The results obtained from this study will likely to shed critical light in to the fundamental understanding of binding of drug to native and partially unfolded conformations of protein to get a detailed picture of interaction.

2. Experimental methods

2.1. Materials:

Ellipticine, HSA and IgG were purchased from Sigma-Aldrich. Na_2HPO_4 and NaH_2PO_4 were purchased from Merck. All the chemicals were used without any further purification. All the experiments were performed in MilliQ water. The structural formulae of ellipticine and three dimensional structures of HSA and IgG are shown in Scheme 1.

Stock solution of ellipticine was prepared in methanol. Required amount of solution was taken in a volumetric flask and dried under vacuum to create a thin film of ellipticine. An appropriate amount of phosphate buffer (10 mM) was added to it and was sonicated for two hours.

2.2. Preparation of protein solution:

All the protein solutions were freshly prepared on the day of experiment. For heat denaturation experiment, HSA and IgG were dissolved in appropriate buffers and was heated on a water bath at 70ºC for 10-15 minutes and then were cooled at room temperature. For acid denaturation both the proteins were dissolved at pH 3.5. All the protein solutions were incubated for an hour before the experiment.

2.3. Circular Dichroism (CD): CD spectra of proteins and their complexes were recorded with a Jasco J-815 spectrometer (Jasco, Tokyo, Japan). For measurements in the far-UV region (200-270 nm), a quartz cell with a path length of 0.1 cm (Hellma, Muellheim/Baden, Germany) was used in nitrogen atmosphere. The protein concentration was kept constant (5 μm) while the concentration of drug molecule was varied up to 2 μm. An accumulation of five scans with a scan speed of 20 nm/min was performed for data collection for each sample. The sample temperature was maintained at 20ºC using Escy temperature controller circulating water bath connected to the water-jacketed quartz cuvettes. Spectra were corrected for buffer signal.

2.4. Molecular docking studies:

The crystal structure of HSA, F_{ab} and F_c fragments of IgG were downloaded from Protein Data Bank (PDB entry 1AO6, 1N0X and 4BYH respectively). The energy minimized conformation of ellipticine was generated by using a web-server known as *FROG*[17a] (Frog2 version), which generates energy-minimized conformations using *AMMOS*.[17b] This conformation of ligand was used further for docking with the proteins. Preparation of the target proteins for docking using Autodock tools involved removal of all water molecules from PDB files, addition of polar hydrogen atoms and assigning Gasteiger charges to the proteins. Docking was carried out with Autodock 4.2 Lamarckian Genetic Algorithm. All other docking parameters were kept as default. *PyMol* was used for visualization and measurement of distances between the atoms of ligand and the residues of protein.

The solvent accessible surface area (ASA) of all the amino acid residues in uncomplexed proteins and in protein-ellipticine docked complexes of HSA, F_{ab} and F_c regions of IgG were calculated using *NACCESS*.[17c] The best docked conformation of the ligand in each case of docking was selected and composite coordinates were generated to form the docked complex. The change in ASA (ΔASA) for a particular residue of a protein due to interaction with ligand was calculated as a difference between the ASA of the residue before and after interaction. If ASA of a residue lost significantly on interaction with the ligand, it was considered as being involved in protein-ligand interaction.

2.5. Steady state and time resolved measurements:

Absorption spectra were taken in a Varian UV-Vis spectrophotometer (Model: Cary 100). Emission spectra were collected using Fluoromax-4p spectrofluorimeter from Horiba Jobin Yvon (Model: FM-100). The samples were excited at 375 nm. The fluorescence spectra were corrected for the spectral sensitivity of the instrument. The excitation and emission slits were kept as 2 nm for each. All the measurements were performed at 25° C.

For the time resolved studies, we have used a picosecond time correlated single photon counting (TCSPC) system from IBH (Model: Fluorocube-01-NL). The experimental setup for TCSPC has been described elsewhere.[18] The samples were excited at 375 nm using a picosecond diode laser (Model: Pico Brite-375L). The repetition rate was 5 MHz. The signals were collected at magic angle (54.70°) polarization using a photomultiplier tube (TBX-07C) as detector which has a dark counts less than 20 cps. The full width half maximum (fwhm) of instrument response function of our setup is ~140 ps. The data analysis was done using IBH DAS (version 6) decay analysis software.

The fluorescence decay was described as a sum of exponential functions:

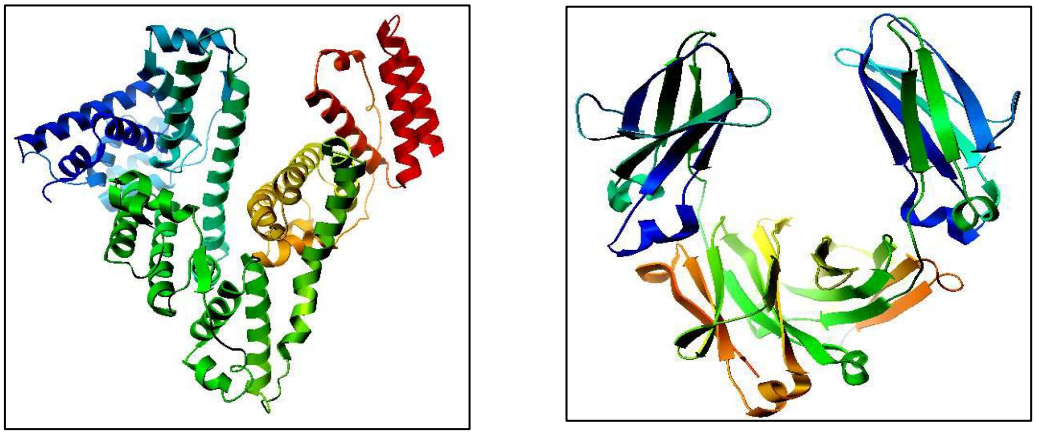
$$D(t) = \sum_{i=1}^{n} a_i \exp\left(\frac{-t}{\tau_i}\right) \quad (1)$$

Where $D(t)$ is the normalized fluorescence decay and τ_i are the fluorescence lifetimes of various fluorescent components and a_i are the normalized pre-exponential factors.

The amplitude weighted lifetime can be given by:

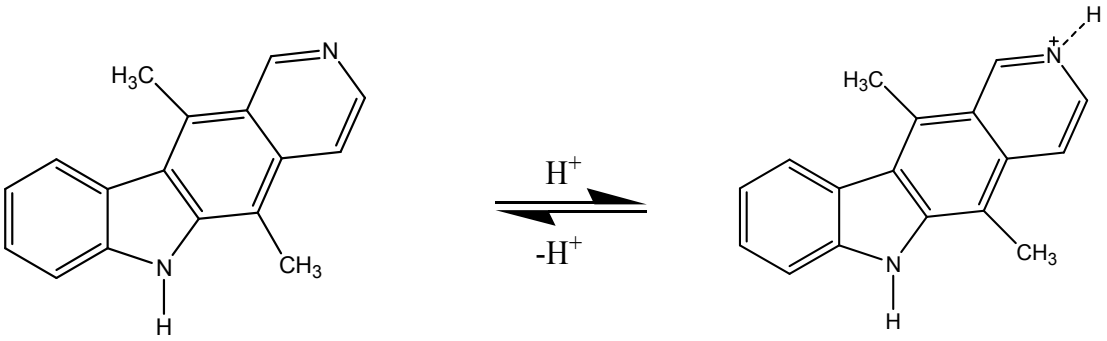
$$\langle \tau \rangle = \sum_{i=1}^{n} a_i \tau_i \quad (2)$$

The quality of the fit was judged by reduced χ-square (χ^2) values and corresponding residual distribution. The acceptable fits have a χ^2 near to unity.



A B

Crystal structure of (A) HSA and (B) IgG.



Prototropic forms of ellipticine

Scheme 1.

Results and Discussion:

3.1. Circular Dichroism Study:

To gain a structural insight on interaction of ellipticine with both the proteins, CD spectra of HSA and IgG in their native and partially unfolded states at different concentration of ellipticine were recorded. The CD spectra of native HSA exhibit two negative minima at 208 and 217 nm (Figure 1). The second one is typical characterization of α-helical structure of proteins.[19] Heat and acid treatment drops the peak intensity at all wavelengths. This reduction in the band

intensity indicates partial unfolding through conversion of α-helix into unordered structures. Table 1 shows quantitative decreases in α-helical structure due to heat and acid treatment. Addition of ellipticine (2 μM) increases the band intensity at all wavelengths in the far-UV CD region and stabilizes the helical structure of heat shocked and acid treated HSA (Table 1).

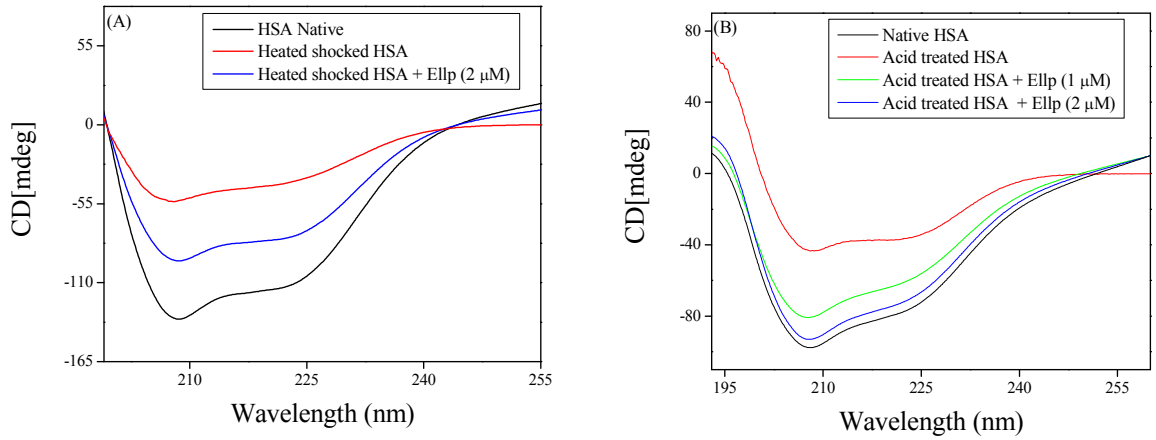


Figure 1. *Circular Dichroism spectra of native and partially unfolded HSA in presence of ellipticine (A) CD spectra of heat shocked HSA (B) CD spectra of HSA in acidic unfolding condition.*

CD spectra of IgG exhibit a negative maxima at 217 nm and a positive maxima at 202 nm, which is characteristic of β-sheet.[20] We found that the intensity at 202 nm decreases without any significant shift in wavelength in case of heat shocked IgG and this signifies increase in random coil (Figure 2). Quantitative analysis of secondary structure also reveals that in heat shocked IgG, there is a reduction of β-sheet and β-turns conformations with increase in random and helical structures (Table 1), which is supported by earlier report. Addition of ellipticine to partially unfolded IgG (heated) helps to resume β-sheet conformation with simultaneous reduction in random coil content. The increment in random coil perhaps results in binding of neutral species of ellipticine to IgG in its partially unfolded state. This assertion is also supported by steady state emission spectra which reveal a massive enhancement of peak at 440 nm upon interaction ellipticine with IgG in its partially unfolded state.

The CD spectra of acid treated IgG (pH~ 3.5) reveals that band intensity at positive maxima i.e. at 202 nm decreases whereas the negative peak at 217 was relatively less affected (Figure 2B). Quantitative analysis signifies that acid denaturation of IgG increase the percentage of random coils from 11% to 18.7% (Table 1). Since 202 nm wavelength in CD measurement corresponds to the F_c region of IgG[20], the decrement in ellipticity at 202 nm signifies that acid denaturation of IgG mainly affect F_c region. Addition of ellipticine increases in band intensity at 202 nm along with a reduction in the percentage of random coils from 18.7% to 10.6% and followed by an increment in α-Helix from 2% to 8.2%. This observation signifies that ellipticine binds to F_c region of acid treated IgG and help in resuming the ellipticity of IgG by increasing the intensity at 202 nm.

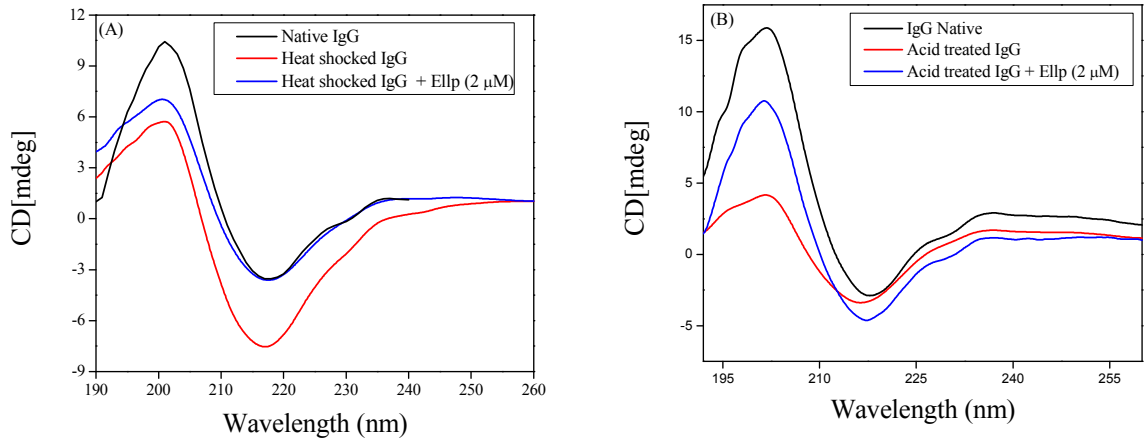


Figure 2. *Circular Dichroism spectra of native and partially unfolded IgG in presence of ellipticine (A) CD spectra of heat unfolded IgG (B) CD spectra of acid unfolded IgG.*

Table 1. *Secondary structure analysis of HSA and IgG at native and partially unfolded states. (calculated by CDNN software).*

System	α-Helix (%)	β-Sheet (%)	β-Turns	Random coils
		HSA		
Native	57.7 %	13.8 %	15.2 %	12.6 %
Heated	31.5 %	19.8 %	18.5 %	30.1 %
Heated + 1μM Ellp	47.0 %	19.1 %	15.8 %	17.9 %
Heated + 2μM Ellp	47.0 %	19.1 %	15.8 %	17.9 %
pH 3.5	30.6 %	20.8 %	16.8 %	31.7 %
pH 3.5 + 1μM Ellp	33.2 %	24.1 %	18.6 %	24.0 %

pH 3.5 + 2 μM Ellp	40.1 %	18.8 %	17.9 %	23.0 %
IgG				
Native	3 %	74 %	12 %	11 %
Heated	7 %	70 %	9 %	14 %
Heated + 1μM Ellp	5 %	73.7 %	10 %	11.5 %
pH 3.5	2 %	72 %	7.3 %	18.7 %
pH 3 + 1μM Ellp	8.2 %	73.5 %	7.7 %	10.6 %

Interaction studies between ellipticine and partially unfolded proteins are pertinent to confirm the identified ligand binding regions in those proteins in their respective native conformations. As evident from CD data, due to either heat shock or acid treatment, HSA and IgG do not unfold completely and both the proteins arrives some other secondary structural conformation (called as partially unfolded). These conformations are different from their native one. Stabilizing contributions from different atomic interactions, entropic factor and minimal structural frustration stabilize a native conformation as compared to its various partially unfolded conformations.[21] From minor changes in CD of IgG due to heat or acid treatment, the partially unfolded IgG can be visualized as slightly affected global "native" conformation.[21] This will lead the hydrophobic interacting residues to get better exposure for interaction with neutral ellipticine. For HSA, a significant change over from α-helical structure to random structure occurs. Predicted ligand binging region (site 2) is also on helical structure. Therefore, due to heating, the site may get highly exposed and in this partially unfolded condition polar/charged interactions with ellipticine will be compromised**.**

3.2. Steady state measurements:

It is already reported that ellipticine has pK_a around 7.40.[15] Therefore; in aqueous solution at physiological condition, ellipticine exists in neutral and protonated species. At low pH, the cationic species dominate due to protonation of nitrogen atom on pyridine moiety, whereas the neutral species are prevalent at higher pH. We reported the detailed emission properties of ellipticine molecules at different pH in our earlier publication.[18] Ellipticine exhibits emission bands at 540 and 440 nm. The band at shorter wavelength (440 nm) was attributed to the neutral species and longer wavelength band (540 nm) was attributed to the cationic species.

In our earlier publication,[18] we reported that addition of HSA to aqueous solution of ellipticine enhances the intensity at 440 nm. This implies that neutral species of ellipticine preferentially

binds with HSA. We repeated this experiment (Figure 3). The result is consistent with our earlier report. The monotonous increase in intensity at 440 nm band strengthen the fact that neutral species of ellipticine binds with the hydrophobic pocket of HSA. We had already reported that ANS and warfarin displacement experiments confirmed that the binding site of ellipticine in HSA is sudlow site 2.[18c] This experimental finding is further substantiated by the results of molecular modeling study which is discussed in the next section. Theoretical distance between Trp 214 and ellipticine as obtained from the docking study matches nicely with the experimental results. Therefore, it is confirmed that HSA entraps the neutral species of ellipticine.

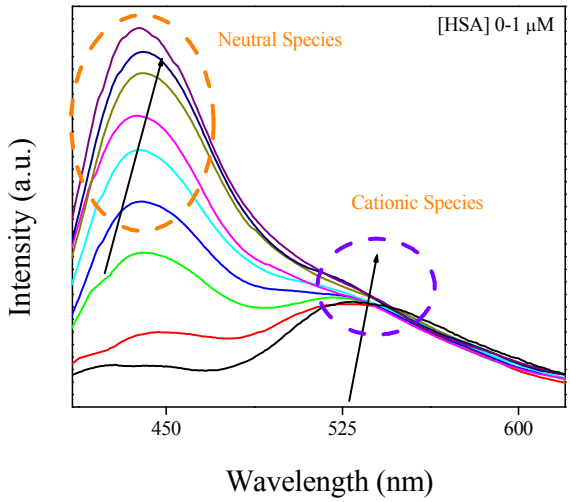


Figure 3. *The emission spectra of ellipticine at different concentration of HSA (0-1 μM). The upward arrow indicates the increase in emission intensity with increasing concentration of HSA.*

Figure 3 also shows that upon addition of HSA to aqueous solution of ellipticine, no significant change in intensity takes place at 540 nm. This observation provides evidence supporting the fact that cationic species does not bind significantly with HSA. On the other hand, Figure 4A shows that addition of IgG to aqueous solution of ellipticine yields a three times rise in intensity at 540 nm. The significant increment in quantum yield at 540 nm implies that cationic species of ellipticine binds with native IgG at physiological condition. IgG has an isoelectric point around 6.50[8-10], therefore, it remains as negatively charged in physiological condition. Hence, the cationic ellipticine binds native IgG through electrostatic interactions. The little increment in intensity at 440 nm indicates that the hydrophobic residues of IgG are not very much accessible in native condition to capture neutral species of ellipticine. Surprisingly different results were

further obtained when ligand binding experiments were conducted with thermally and acid treated IgG. Figure 4B reveals that the addition of heat shocked IgG to the aqueous solution of ellipticine results a significant enhancement in intensity at 440 nm as compared to that at 540 nm.

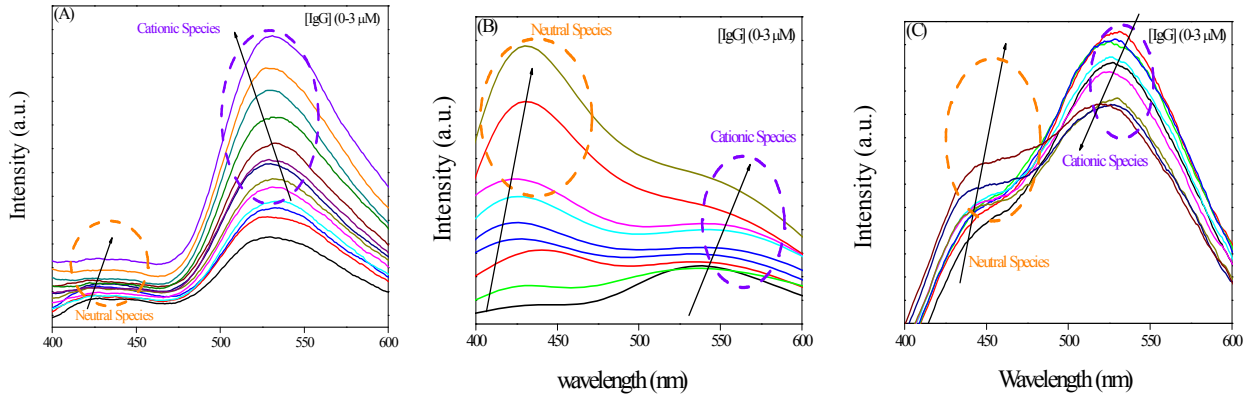


Figure 4. *(A) Steady state emission spectra of ellipticine at different concentration of native IgG (0-3 μM) at pH~7.40 (B) Heat shocked IgG (0-3 μM) at pH~7.40 (C) Acid treated IgG (pH~3.50).*

The boost in intensity at 440 nm with addition of heat shocked IgG may be due to the entrapment of ellipticine neutral species in the partially unfolded IgG. This fact indicates that upon heating, the hydrophobic pockets of IgG become exposed and thus capture neutral species of ellipticine. Surprisingly in Figure 4C, we observed that the cationic peak at 540 nm exhibits a drop in intensity upon addition of acid treated IgG till 3 μM (shown by downward arrow in Figure 4C). This result is contradictory to what observed in case of native and heat shocked IgG. However, further addition of acid treated IgG to aqueous solution of ellipticine raises intensity at 540 nm (data not shown). In this case, we speculate that the acid treated IgG initially deprotonates ellipticine and increases the population of neutral species which binds with the IgG and thus the emission intensity at 440 nm increases. We reported earlier that[18e] the addition of lysozyme to aqueous solution of ellipticine causes a similar type of decrement in intensity at 540 nm.

We have also plotted I_{440}/I_{540} for native, heat and acid treated IgG (Figure 5). It is observed that I_{440}/I_{540} decreases initially for native IgG indicating that cationic species primarily bind with

native IgG. However, at higher concentration of IgG (above 2 μM) when all cationic species are grabbed by negatively charged IgG, the neutral species start to bind with hydrophobic pocket in native IgG resulting in an enhancement in I_{440}/I_{540}. On the other hand I_{440}/I_{540} increases from very beginning upon addition of partially unfolded IgG. This indicates that mostly neutral species bind with heat and acid treated IgG.

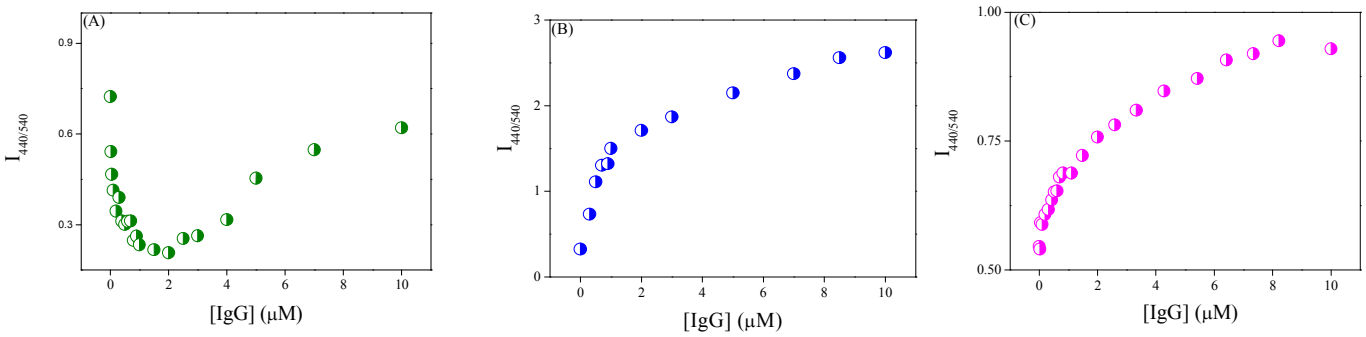


Figure 5. *I_{440}/I_{540} plot of ellipticine in (A) Native IgG (0-10 μM) at pH~7.40 (B) Heat shocked IgG at pH~7.40 (0-10 μM) and (C) acid treated IgG at pH ~3.0 ((0-10 μM).*

It has already been reported that treatment of IgG with heat and acid causes exposure of buried apolar groups and due to heat and acid treatment the F_{ab} and F_c fragments of IgG are exposed respectively.[11] This exposure helps in binding of neutral ellipticine molecules with IgG. Our results also unambiguously reveals that neutral species of ellipticine binds with the hydrophobic pocket of IgG in its partially unfolded state. Unlike IgG, when binding studies were conducted with heat shocked HSA, the quantum yield at Sudlow site 2 was reduced. To gain a more insight regarding the binding of ellipticine to IgG and HSA, we have plotted the quantum yield of ellipticine at 440 nm at different concentration of native and partially unfolded IgG and HSA (Figure 6). It is revealed from Figure 6 that quantum yield of neutral species of ellipticine in presence of partially unfolded IgG (by heating) is much higher compared to that in presence of native IgG. This clearly supports our finding that neutral species of ellipticine is entrapped in the hydrophobic site of partially unfolded IgG. On the other hand, the quantum yield of neutral ellipticine was found to be higher in presence of native HSA than that in presence of partially unfolded HSA. The lower quantum yield in presence of unfolded HSA may be due to the fact that upon heat treatment the protein transforms into a less ordered structure and therefore, the

captured ellipticine molecules experience a less confinement. However, we cannot compare the quantum yield of neutral species of ellipticine in presence of acid treated proteins with that one in presence of native protein. This is because of the fact that at pH~ 3, ellipticine molecules turn into cationic species and thus negligible amount of neutral species are left in the solution.

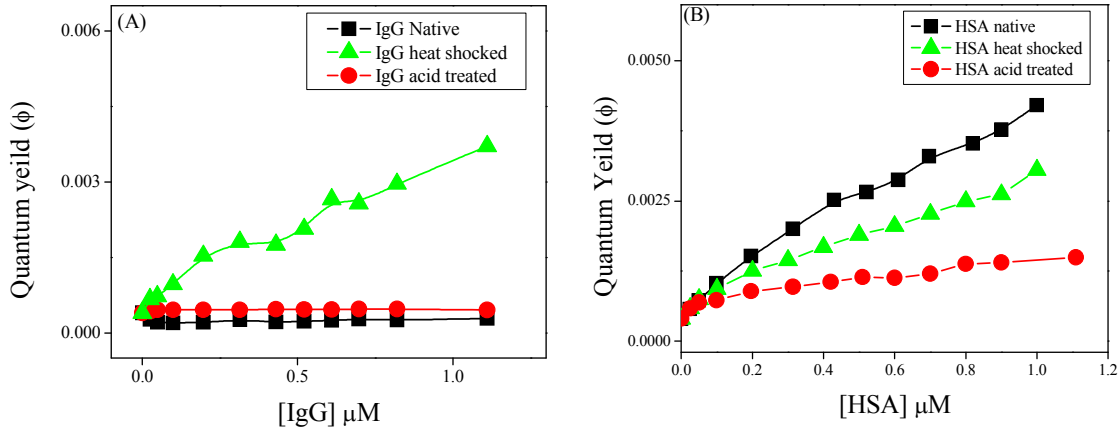


Figure 6. *Quantum yield plots of neutral species of ellipticine at various concentration of native, heat shocked and acid treated (A) IgG (B) HSA.*

3.3. Molecular docking studies:

In absence of crystal structure of a protien-ligand complex, docking studies are generally carried out to obtain detailed information about the binding sites and the binding interactions involved during complexation of ligand with the protein. Docking results can substantiate the experimental results to a large extent by finding preferred binding region of ellipticine in the respective protein.

The emission spectra of ellipticine in (Figure 3) suggest that the ligand is going to a hydrophobic pocket of HSA. Subsequent experimentations suggest that the ligand is not interacting with site 1 (subdomain IIA), rather it is binding with other hydrophobic cavity (site 2) present in subdomain IIIA.[18c] This binding site (site 2) is composed of six helices from subdomain IIIA and has a preformed hydrophobic cavity with some distinct polar features. The prominent polar region in site 2 is situated close to one side of the entrance to the binding pocket. These polar residues include Arg 410, Lys 414 and Ser 489. The distances between the interacting residues of HSA with the ligand are given in supporting information (supporting information Table S1). It is

evident from Table S1 that the polar nitrogen atoms of the ligand are in proximity to the polar residues like Arg 410, Lys 414 and Ser 489. The earlier report states that site 2 preferentially binds the drugs having peripherally located electronegative groups.[22] The compounds like difluisal, diazepam, ibuprofen, indoxyl sulphate etc., which bind specifically to site 2 in such a way that at least one oxygen atom is in vicinity of the polar patch of site 2. A similar type of binding of the ligand with HSA is also observed in case of ellipticine (Figure 7), which strongly supports and substantiates the experimental observations of binding of the ligand in site 2.

To identify the residues taking part in interaction, we have also calculated the ASA of all the residues in free protein and in protein-ellipticine complex. The changes in ASA of the interacting residues of HSA are given in supporting information (supporting information Table S2). The major change in ASA was observed for the polar and charged residues like Arg 410, Lys 413, Lys 538, Lys 540. Most notably, the residues like Arg 410, Lys 414, which are accessible before interaction, become buried by the ligand. The distance from the ligand to Trp 214 (residue responsible for the intrinsic fluorescence of HSA in a major way) of site 1 was also measured and found that the ligand is 30.47 Å apart from Trp 214. This distance is very close to the valued determined experimentally, which suggests a only weak energy transfer between Trp 214 and the ligand.

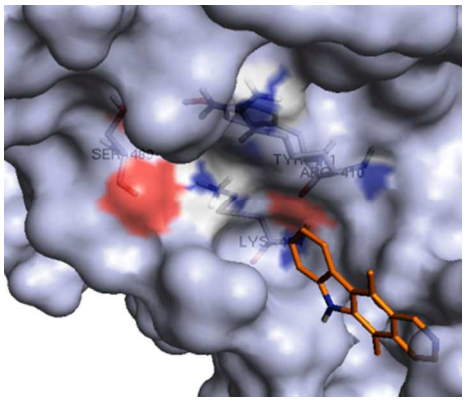


Figure 7. *Docked conformation of ellipticine with HSA.*

To find preferred binding site of ellipticine within IgG, the ligand was docked individually with F_{ab} and F_c fragments of the protein. In the docked conformation of ellipticine in F_{ab} region (Figure 8A) proximity of the ligand is found with residues like Phe 83, Ala 84, Leu 85, Arg 106,

Gln 166, Asp 167 and Ser 168. CASTp (Computed Atlas of Surface Topography of proteins) program was used to identify the surface accessible pockets as well as interior inaccessible cavities for proteins. Using CASTp, total 65 pockets has been found to be located in F_{ab} region of IgG, out of which the largest pocket have area 1316.1 $Å^2$. Interestingly, we have found that the above mentioned residues are included in this pocket where hydrophobic residues are buried toward inside and polar residues are close to the entrance to the pocket. Whereas, ellipticine interacts with Ile 377, Ala 378, Val 379, Tyr 391, Leu 406, Thr 393, Pro 395 residues of F_c region of IgG (Figure 8B).

For precise identification of binding site of ellipticine in native IgG (whether in F_{ab} or F_c region), we have compared the interactions of the ligand in F_{ab} and F_c. From steady state fluorescence data, it is found that the addition of native IgG to ellipticine causes much significant change in 540 emission peak compared to 440 nm emission peak. This indeed suggests the preferential interaction of cationic species of ellipticine with native IgG. Now in F_{ab} binding pocket, Asp 167 is present along with other hydrophobic residues. In Asp 167 residue, a deprotonated acid group will exist in our working pH (pKa of the side chain of Asp is ~3.9) and this negative charge from deprotonated acid group can interact significantly with the cationic species of ellipticine. Whereas, in F_c region no such residue is found that can favor electrostatic interactions with cationic ellipticine. So, the binding of ellipticine in F_{ab} region as suggested by docking studies agrees well with the experimental findings. Involvement of both hydrophobic and electrostatic interactions in binding of ligand at F_{ab} pocket is also substantiated by the fact that Phe 83, Ala 84 of F_{ab} region which are accessible before interaction become completely unaccessible and Asp 167 becomes ~90% buried due to ligand binding (supporting information Table S3). On contrary, only hydrophobic residues are found to be almost buried by the ligand for F_c binding (Table S4). Now during partial unfolding of the protein due to heating, it can be speculated that the less accessible hydrophobic residues in F_{ab} binding pocket will come out and in that case both hydrophobic as well as electrostatic interactions will occur. This is supported by the steady state fluorescence of ellipticine with heat shocked IgG, where intensity of both the emission peaks increased with predominance in neutral species binding.

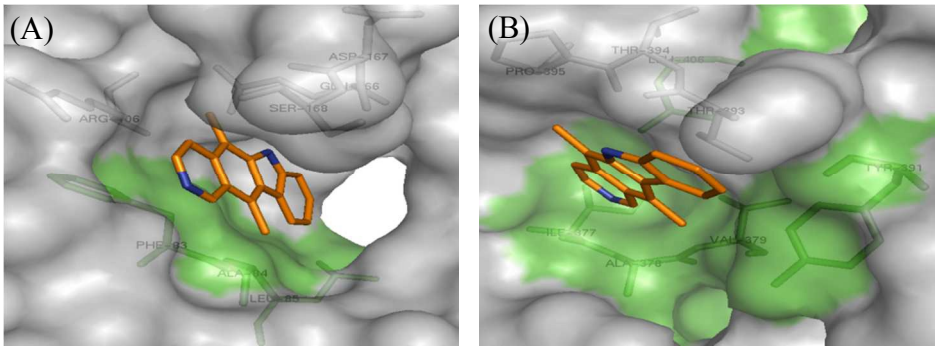


Figure 8. *Docked conformation of ellipticine with (A) F_{ab} and (B) F_c regions of IgG protein.*

3.4. Time Resolved Study:

In the present study, the time resolved data reveals the explicit binding of ellipticine with IgG and HSA. We took time resolved decays of ellipticine in aqueous buffer solution and in presence of different concentration of native and heat shocked proteins. In the steady state experiment, we did not find notable changes at 540 nm upon addition of HSA (Figure 3). So; we collected decays of ellipticine at 440 nm in presence of HSA. The results are summarized in Table S5 (supporting information). Ellipticine exhibits bi-exponential decay at 440 nm in aqueous solution at physiological pH. The lifetime components consist of shorter component (τ_1) around 0.42 ns (84%) and longer component (τ_2) around 4.50 ns (16%). The average lifetime is 1.10 ns. Addition of 10 μM HSA to aqueous solution of ellipticine significantly increases the average lifetime (from 1.10 ns to 7.18 ns) and a long component is generated around 14.50 ns at maximum concentration HSA (10 μM). This fact indicates that ellipticine strongly binds with hydrophobic pocket of protein. It is already reported that pka of ellipticine is reduced in nonpolar solvent.[14a,14b] Thus it can be assumed ellipticine molecules are strongly buried in the hydrophobic pocket of HSA. This conjecture is further supported by the fact that ellipticine in nonpolar solvent like hexane ellipticine exhibits a very long lifetime around 11 ns. .[14a-b, 18b]

In case of IgG, decays were collected at 440 nm as well as at 540 nm concurrently to supervise binding of ellipticine with native and heats shocked proteins. The representative decays of ellipticine at different concentration of IgG are shown in Figure 9.

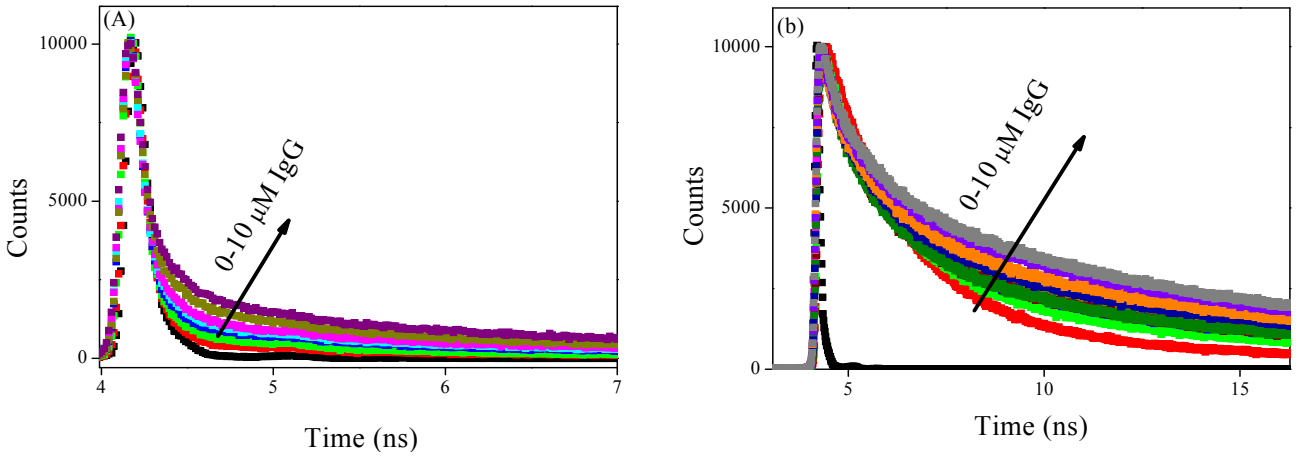


Figure 9. Time resolved decays of ellipticine in presence of IgG at (A) 440 nm (B) 540 nm.

The decay of ellipticine in aqueous buffer solution at 540 nm consists of the components around 1.80 ns (84%) and 5.80 ns (16%). The component around 1.80 ns is ascribed to the cationic species which is prevalent in the aqueous solution at physiological condition.[18] Upon addition of native and heat shocked heat IgG, the average lifetime increases from 2.44 ns to 7.58 and 5.10 ns respectively (Table S6A (supporting information)). Further a long component is generated (around 12.50 ns at 10 μM IgG) and population of this component increases continuously. The fact indicates that ellipticine bind with IgG. The plot of average lifetime at 540 nm as a function of concentration of native IgG is shown in Figure 10A. Interestingly, the population of the longer component is significantly higher (54%) in 10 μM native IgG than that in heat unfolded IgG (32%) (Supporting information, Table S6A). The ratio of population of slower and fast component (a_2/a_1) (540 nm decays) shows much higher values for native IgG (Figure 10B) than compared to heat treated one. Here, it is difficult to assign the individual time component to any particularly species. The decay parameters correspond to 540 nm wavelength represents the cationic species. Therefore, we assume that the higher population of longer component primarily be attributed to the fact that the native IgG in its native state capture more number of cationic ellipticine than in unfolded state.

Similar kind decay parameters were obtained when decays were collected at 440 nm in presence of native and heat shocked IgG. We found that addition of native and heat shocked IgG increases the average lifetime of ellipticine at 440 nm (Figure 10C) and a long component is generated. The observation supports the fact that ellipticine binds with the hydrophobic pocket of IgG. Unlike 540 nm decays, ratio of population of slower and fast component (a_2/a_1) reveals much higher values for partially unfolded IgG than that for native IgG (Figure 10D). As 440 nm decay parameters represent the neutral species, therefore, we assume that unfolding of IgG enables the hydrophobic pockets to capture more number of ellipticine molecules. It is interesting to compare the decay parameters for IgG at 440 nm and 540 nm. Figure 10A and 10B reveal that at 540 nm decay, average lifetime as well as a_2/a_1 are higher for native IgG as compared to that for unfolded one. On the contrary, Figure 10C and 10D reveal that at 440 nm decay, average lifetime as well as a_2/a_1 are lower for native IgG as compared to that for unfolded one. Therefore, it can be easily documented that unfolded IgG is more capable of encapsulating neutral species. Moreover, denaturation reduces the binding efficiency of IgG with the cationic species. Due to unfolding, the hydrophobic pockets become exposed, which screens the negative part of IgG from interaction with cationic species of ellipticine.

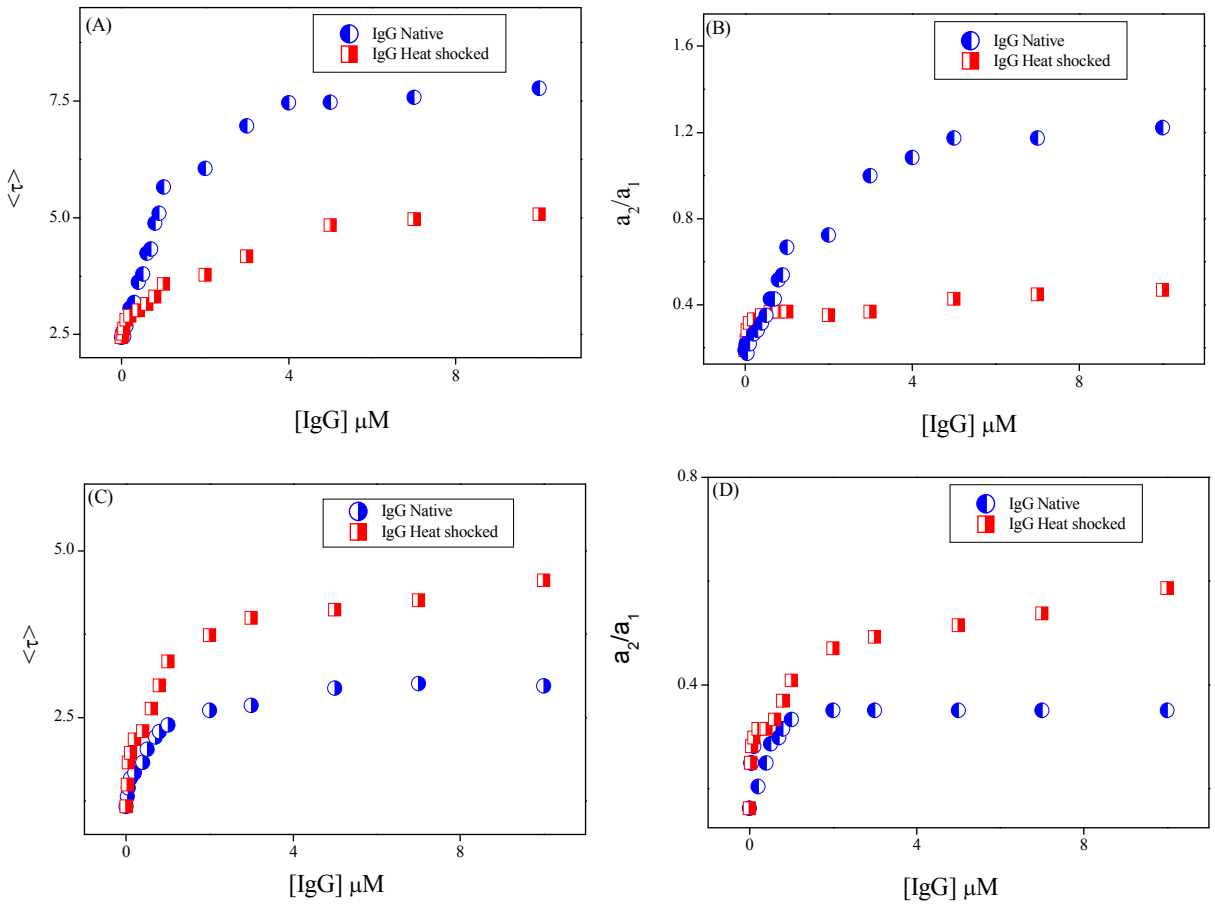


Figure 10. *Plot of average lifetime [(A) and (C])* and *population ratio of fast and slower components* (a_2/a_1) *[(B) and (D)] against concentration of native and heat unfolded IgG. For (A) and (B) decays were collected at 540 nm, whereas for (C) and (D) decays were collected at 440 nm.*

4. Conclusion:

The present study elucidates the binding of ellipticine with HSA and IgG proteins. It is observed that neutral species of ellipticine has more affinity towards native HSA due to hydrophobic interaction in comparison with heat and acid unfolded HSA. The binding of the ligand is reduced in acidic condition due to electrostatic repulsion between cationic ellipticine and positively charged protein. Unlike HSA, neutral ellipticine has more affinity towards partially unfolded IgG in comparison to native IgG. In native state of IgG, electrostatic attraction between negatively charged protein and cationic ellipticine exists. From steady state, time resolved fluorescence and

CD results it is evident that cationic species of ellipticine binds with native IgG (preferably in F_{ab} region), whereas neutral species of ellipticine binds with native HSA. CD measurements confirm renaturation of partially unfolded protein due to addition of ellipticine. So, present study reveals that serum albumin is responsible for transport of neutral species of ellipticine, whereas serum globulin is responsible for transport of cationic ellipticine.

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