Spectroscopic and Docking Studies on Binding of Bovine Serum Albumin with Antipsychotic Fluphenazine Drug

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Abstract

Proteins show conformational changes on binding with ligands. Bovine serum albumin (BSA) is a plasma protein binds with a remarkable variety of ligands like drugs. To clarify the structural change of BSA–drug complexes UV and other spectroscopic studies are helpful. Fluphenazine is a antipsychotic drug, used in chronic psychoses treatment like schizophrenia.

The present work explores the binding of fluphenazine with the bovine serum albumin (BSA) by UV-visible and fluorescence quenching spectra, synchronous fluorescence spectra measurement studies. Fluorescence intensity of BSA was quenched on increasing concentration addition of fluphenazine. BSA structure alteration by fluphenazine was shown by UV-vis and synchronous fluorescence studies. Change in stability of native BSA in presence of fluphenazine was studied by spectroscopic analysis. CD Spectra Measurements indicates change in BSA after binding with fluphenazine at secondary structure level. Molecular Docking results revealed that fluphenazine showed significant binding affinity towards the Sudlow's sites present within subdomain IIA of domain II and subdomain IIIB of domain III.

This study explains interaction and modification at molecular level between fluphenazine and BSA and helps to understand the mechanism of drug binding effect in increasing concentrations.

Keywords: Fluphenazine, BSA, Fluorescence, Quenching, Spectroscopy.

I. Introduction

Serum proteins binds with drug molecules is an important study because of binding interactions affects the drug distribution, elimination, clinical response, duration and pharmacological effect. Not only drugs but serum protein plays major role in transport of small hydrophobic or sparingly soluble molecules such as steroids, bilirubin, fatty acids, hormones [1] and also transfers metal ions [2]. BSA [Fig. 1] [3] is a single chain serum protein obtained from blood serum of bovine. It is a globular heart shaped protein with approximate dimensions of $80 \times 80 \times 30$ Å and a molecular weight of ~66.4 kDa. Albumin contains 3 domains (I, II, III), which are separated into 9 loops (L1–L9) by 17 disulfide bridges. Each domain carries two subdomains A and B. It contains 583 amino acids with two tryptophan residues Trp-134 and Trp-213 in subdomain IB and IIA respectively and 20 tyrosine residues dispersed over 3 domains [4]–[8].

Binding of ligands with carrier proteins has great interest in the medicinal chemistry field. BSA is generally used as model protein to study binding of several small molecules including drugs

with plasma protein since its similarity with human serum albumin, less cost and no ethical issue. Small modification in BSA leads to a significant change in its biochemical response as on interaction with ligand like drug molecules. Thus, formation of a Drug–BSA complex may be useful as a model to gain fundamental insights into drug-protein interaction.

Fig. 1 BSA Structure

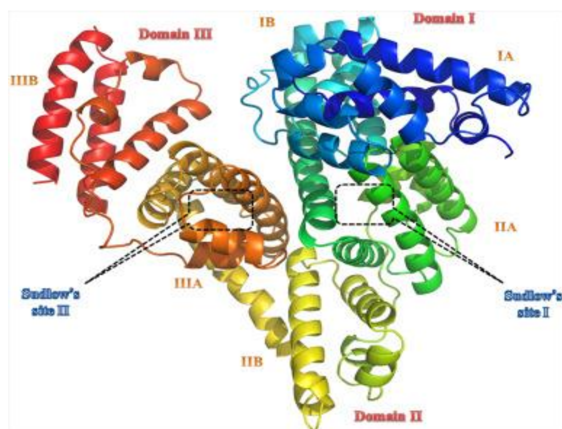
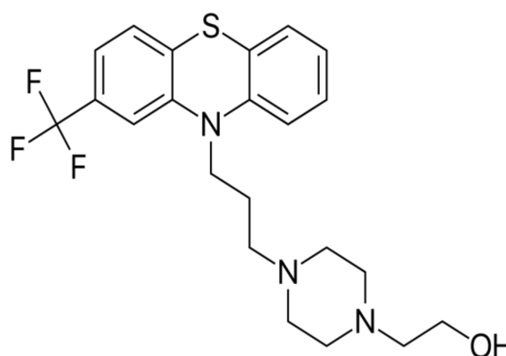


Fig. 2 Fluphenazine structure



Fluphenazine [Fig. 2][9] belongs to phenothiazine sold with brand name modecate or prolixin a high potency antipsychotic drug[10] used in treatment of chronic psychoses like schizophreni [11]. Routes of administrations are mouth and intramuscular, its injectable form is included in the WHO's essential medicines list [12]. Fluphenazine is available in its dihydrochloride, decanoate and enanthate forms [13].

Fluphenazine blocks post-synaptic D2 receptors of basal ganglia, cortical and limbic system. It also blocks alpha-1 adrenergic, muscarinic-1 and histamine-1 receptors to exhibit its antipsychotic action [14] [15]. Drug binding to plasma proteins affects its distribution and elimination as well as the duration and intensity of its physiological effects [16]. It is well known that the interaction of drug with blood components influences its bioavailability.

Few reports are available on interactions of BSA with tranquilizer drugs, such as alprazolam [17], clonazepam, [18], Lorazepam, oxazepam, bromazepam [19] chlorpromazine [20]. In present work we studied the high concentration Fluphenazine interaction with albumin. Interactions were studied, using UV-visible, fluorescence spectroscopy.

II. Materials and Methods

A. Reagents and Solutions

Bovine Serum Albumin (M.W. ~66 KDa, ≥99% essential fatty acid, endotoxin, protease, DNase, RNase free) and Phosphate Buffered Saline (PBS) 10X (pH = 7.40) was purchased from SRL India. Fluphenazine (≥98%) was purchased from Sigma Aldrich. Stock solutions of BSA (200 µM) and fluphenazine (200 µM) were prepared by using 1X PBS buffer solution (pH = 7.40).

B. UV-visible Spectra Measurements

UV-visible absorption spectra of albumin, fluphenazine, and albumin in presence of varying concentrations of fluphenazine were recorded at room temperature on UV-1600 spectrophotometer (Shimadzu, Kyoto, Japan) with 10 mm quartz rectangular cells from 200 to 400 nm. The 1X PBS solution (pH=7.40) was used as the reference solution.

C. Fluorescence Quenching Spectra Measurements

The steady-state fluorescence spectra of BSA solutions in absence and presence of fluphenazine with varying concentrations were measured at pH 7.40 on an FP-8300 Spectrofluorometer (Jasco, Japan) with 10 mm quartz rectangular cell from scan range 270 to 500 nm while λ_{ex} was set at 278 nm. Scan speed was maintained as 500 nm/min and both slit widths were kept at 5 nm.

D. Synchronous Fluorescence Spectra Measurements

The synchronous fluorescence spectra of BSA solution and BSA in presence of varying concentrations of fluphenazine were measured on FP-8300 Spectrofluorometer with 10 mm quartz rectangular cell (Jasco, Japan) instrument at different scanning intervals of $\Delta\lambda$ ($\Delta\lambda = \lambda_{em} - \lambda ex$, λ_{em} and λ_{ex} are the emission and excitation wavelengths, respectively) at room temperature. The $\Delta\lambda$ values were set at 15 nm and 60 nm, respectively, which describe the properties of Tyr and Trp residues, respectively. The scan range was set at 270 to 500 nm with λ_{ex} as 278 nm. Scan speed was maintained as 500nm/min and both slit widths were kept at 5 nm.

E. CD Spectra Measurements

The independent BSA and fluphenazine CD measurements were made on a J-1500 CD spectrometer (Jasco, Japan) with a 2 mm quartz cell in 190 – 350 nm. The BSA vs fluphenazine concentration was in proportion of 1:0, 1:1, and 1:2, and CD spectra recorded at scan speed 1000nm /min.

F. Molecular Docking

Molecular docking studies were employed in order to investigate binding modes of ligand to bovine serum albumin (BSA) and their molecular interactions. The AutoDock Tool (ADT) was used for docking studies. The 3D crystal structure of BSA was retrieve from protein data bank (PDB ID: 4OR0). The 3D structure (Fig. 8) of Fluphenazine (FPZ; PubChem ID: 3372), was retrieved in SDF format from PubChem Database [15] (https://pubchem.ncbi.nlm.nih.gov/).

The 3D crystal structure of BSA was retrieve from protein data bank (PDB ID: 4OR0) and prepared in UCSF chimera to remove crystal water and Hetroatoms. Then 3D structures of ligand were prepared by optimization using MMFF94 force field in Chem3D 15.0. The ligand structure were saved in pdb file format and used for docking studies with BSA. Lowest binding energy conformations were selected for analysis of molecular interactions by using UCSF Chimera and Discovery Studio [21][22].

III. Results and Discussion

A. UV spectra measurements

UV absorption measurement is frequently used to measure the change in the conformation of protein binding interaction between protein and drug and to know the complex formation. The UV absorption spectra of BSA and fluphenazine were shown in Fig. 3. In the results weak band near 280 nm belongs to the $\pi \rightarrow \pi^*$ transition of the aromatic amino acids [23][24].

The absorption intensity of these bands near 278 nm increased with increasing the concentration of fluphenazine. In addition, the blue shift of the band near 278 nm was observed by 1nm. This indicates that fluphenazine bind to BSA, resulting in forming Fluphenazine–BSA complex and a change in the conformation of BSA[25]. Strong absorption peaks around 260 nm are observed; these are characteristic peaks of fluphenazine that do not affect the protein's absorption peak at 278 nm.

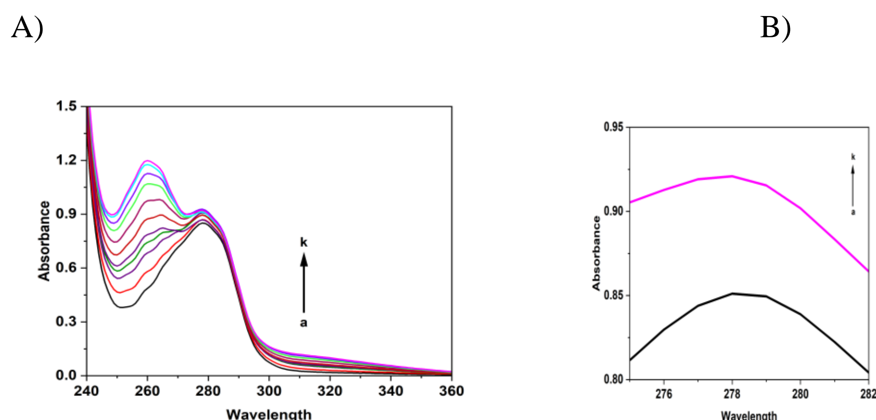


Fig. 3. A) UV absorption spectra of BSA (20 μM) with various concentrations of fluphenazine from 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μM respectively at pH 7.4. **B)** Zoom view of BSA and Fluphenazine-BSA at 278nm.

B. Fluorescence Quenching of BSA

Trp, Tyr and Phe residues can make the protein generate endogenous fluorescence. The most sensitive to the changes in the surrounding micro-environment is Trp which has strongest fluorescence intensity [26] and is used as an endogenous fluorescent probe to understand the binding interaction between protein and drugs. Steady-state fluorescence spectra of BSA solutions in absence and presence of fluphenazine were shown in Fig. 4.

Strong fluorescence emission peak is obtained at 339nm at λ ex =280 nm, which mainly comes from Trp residues. Fluorescence intensity of BSA decreased after addition of fluphenazine from 0 to 150 μM along with the blue shift of the maximum emission wavelength (λ em) (~ 9 nm). This shows that there is an alteration in the microenvironment surrounding Trp residues due to binding of fluphenazine to BSA.

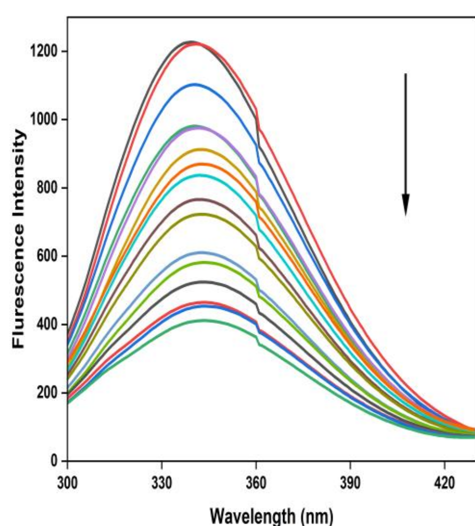


Fig. 4. Fluorescence spectra of BSA (20 μM) with various concentrations of fluphenazine from 0, 10, 20, up to 150 μM increasing order concentrations (pH 7.4).

C. Binding Site and Constant Study

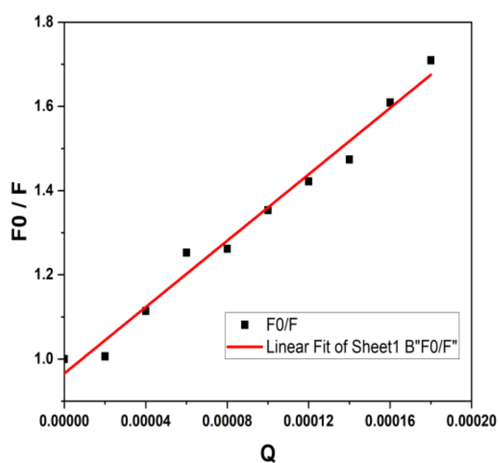
To clarify the quenching mechanism of BSA induced by fluphenazine, fluorescence quenching experiments were performed and the quenching parameters of BSA induced by fluphenazine were calculated according to the Stern–Volmer equation. Binding constants (K_b) and binding site (n) for the Fluphenazine-BSA complex can be determined with double logarithmic plots [27].

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

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

F_0 and F are the BSA's fluorescence intensities in the fluphenazine absence and presence, and [Q] denotes the fluphenazine concentration. K_{SV} is a quenching constant. The k_q is quenching rate constant of protein, K_b is binding constant, n is binding sites number, it could be mathematically determined from the plot of log[$(F0 - F)/F$] vs. log [Q] and τ_0 is the protein's fluorescence lifetime with a quencher, about two ns for BSA [28][29]. The '*n*' value for complex fluphenazine BSA at the studied temperature is near to two, representing the existence of double binding site on BSA for fluphenazine. The K_b value in order of 10^5 at studied temperature indicates a robust fluphenazine binding interaction with BSA.

A)



B)

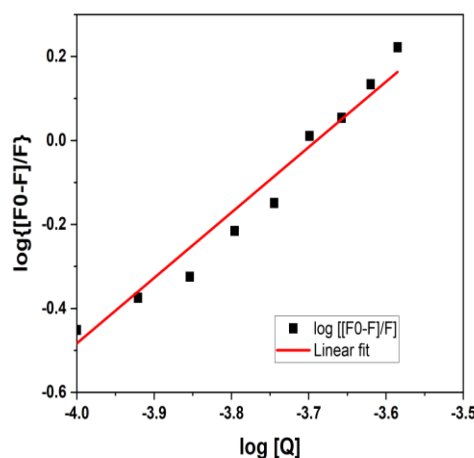


Fig. 5. A) Stern-Volmer and **B)** Modified Stern-Volmer graph for BSA with varying concentration of fluphenazine.

D. Thermodynamic Study

The dominant binding forces between drugs and proteins are hydrogen bonds, Van der Waals forces, electrostatic forces, hydrophobic interactive forces usually thermodynamic parameter signs and magnitudes of the ΔH^0; enthalpy change and ΔS^0; entropy change, the main forces can be accounted in the binding reaction is encountered. Experiments were done out at 298K. Here ΔH^0 and ΔS^0 can be assumed constants. The Van't Hoff equation is applied for calculation.

$$lnK_b = \frac{-\Delta H^0}{RT} - \frac{\Delta S^0}{R} \quad (3)$$

Where R is gas constant, T is experimental temperature, and K_b is binding constant of corresponding T. Then ΔG^0 can be obtained from the given equation:

$$\Delta G^0 = -RTlnK_b = \Delta H^0 - T\Delta S^0 \quad (4)$$

Table.1 specifies fluorescence data results. Negative ΔS^0 and ΔH^0 value suggest that H - bonds and Van der Waal's forces are significantly involved in fluphenazine binding to BSA. However, $\Delta G^0<0$, $\Delta H^0<0$, and $|\Delta H^0| > |T\Delta S^0|$ indicate sudden fluphenazine binding with BSA; reaction is an enthalpy driven and exothermic process.

Table 1. Different constants, binding sites, and energy obtained from fluorescence data.

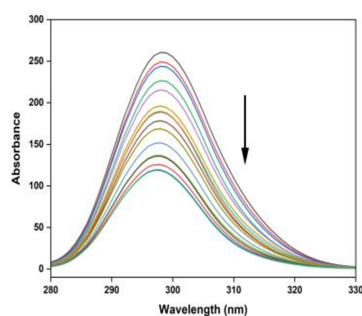
K_{sv}/M^{-1}	$K_q/M^{-1}s^{-1}$	K_b/M^{-1}	*n*	$\Delta G^0/J\ M^{-1}$
3.940×10^3	1.970×10^{13}	5.59654×10^5	2	-3.2793×10^4

E. Synchronous fluorescence measurements

The synchronous fluorescence spectrum is used to obtain molecular environment information in a chromosphere vicinity. When the wavelength interval between λ em and λ ex is stabilized at 15 nm or 60 nm, the synchronous fluorescence spectra is used to distinguish the characteristics of Tyr residues and Trp residues of BSA, respectively. Usually, shift of the λ em represents the change in Tyr or Trp residues surrounding microenvironment polarity [30].

Blue shift of λ em suggests the Trp or Tyr residues surrounding polarity decreases and increases the hydrophobicity with the increase of the macromolecules folding state [31]. The synchronous fluorescence spectra of BSA along with fluphenazine were shown in Fig. 6 A) & B). After addition of fluphenazine it is observed that gradually decrease in fluorescence intensities of Tyr and Trp residues with 1nm blue shifts for 15nm and 60nm. It is determined by decreasing the surrounding polarity of Tyr or Trp residue and increasing hydrophobicity with an increase of folding state of macromolecule due to binding of fluphenazine to BSA.

A) **B)**



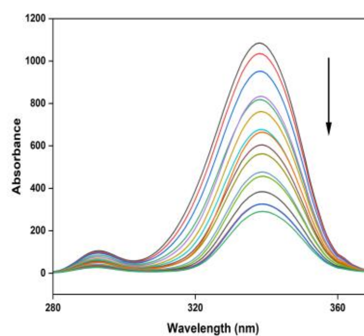


Fig. 6 Synchronous fluorescence spectra of Tyr residue with Δλ = 15 nm **(A)** and Trp residue with Δλ = 60 nm **(B)** in BSA of 20 μM with various concentrations of fluphenazine from 0, 10, 20 up to 120 μM increasing order concentrations (pH 7.4).

F. CD Spectra Measurements

Conformational study of proteins is carried out with CD spectroscopy. BSA's and BSA-fluphenazine CD spectra with 7.4 pH were shown in Fig. 7. As per result, 222 and 208 nm negative bands were obtained, which is accountable for $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ electron transfer for α helix peptide bond [32][33]. These bands' intensity decreased with fluphenazine addition. Protein's secondary structural level analysis was done by a quantitative multivariate analysis program supplied with a Jasco J-1500 CD spectrometer [34][35]. Results can be found in Table 2 shows percentage change in the BSA structure with different conformations.

Table 2. Conformational composition of BSA in absence and presence of fluphenazine.

Conformation	BSA	BSA- fluphenazine (1:1)	BSA- fluphenazine (1:2)
Alpha Helix	41.3 %	41 %	41.8 %
Beta sheet	23.9%	23.9 %	23.1 %
Beta turn	8.4 %	7.7 %	7.4 %
Random coil	26.5 %	27.4 %	27.7 %
Total	100	100	100

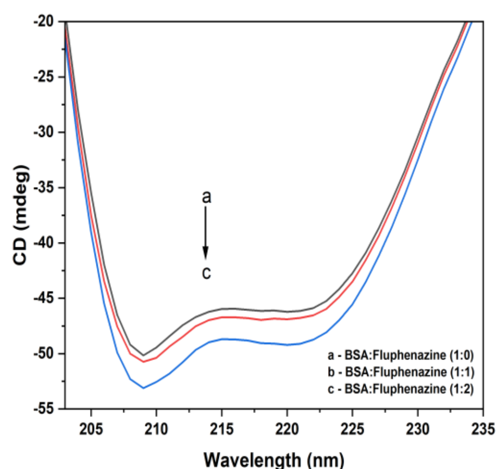


Fig. 7 CD spectra of BSA solutions (20 μM) in the presence of fluphenazine at 298K. The concentrations of fluphenazine 0, 20, and 40 μM respectively.

G. Molecular Docking

Molecular docking studies were applied to evaluate the binding poses and affinity of fluphenazine to BSA with concomitant interactions using AutoDock and 2D interaction diagrams were drawn by Discovery studio Visualizer (BIOVIA, 2021) [29]. Fluphenazine showed significant binding affinity towards the sudlow's sites present within subdomain IIA of domain II and subdomain IIIB of domain III (Fig.9). Binding Energy and Ki values are -6.81 kcal/mol and 10.11µM are obtained during the study of fluphenazine-BSA interactions.

To understand the molecular interactions involved in binding of fluphenazine to the BSA, further lowest energy conformation was analysed. Four hydrogen bonds were involved in binding of fluphenazine with BSA, two in between Arg208 and Leu480 with fluorine and amino group of fluphenazine respectively and two between Asp323 with a distance of 2.12 Å and 2.29 Å with hydroxyl and amino group of fluphenazine (Fig.10). Residues of BSA involved in hydrophobic interactions with fluphenazine are Ala212, Asp323, Glu353, Leu346, and Val481 (Fig.10).

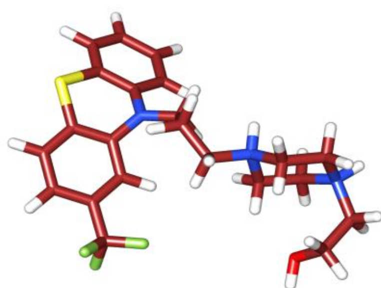


Fig. 8 3D structure of fluphenazine (FPZ).

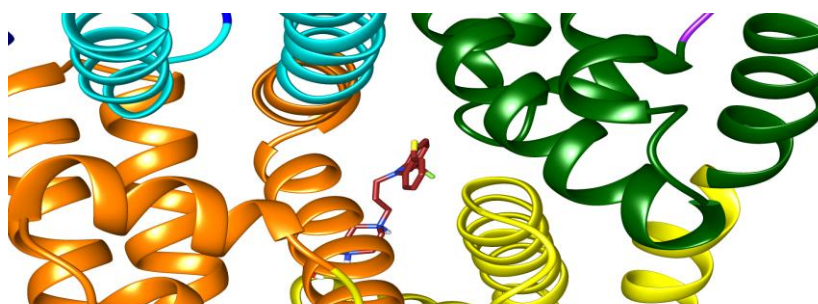


Fig. 9 Docked complex BSA and fluphenazine (FPZ) in ribbon and stick representations with lowest energy pose.

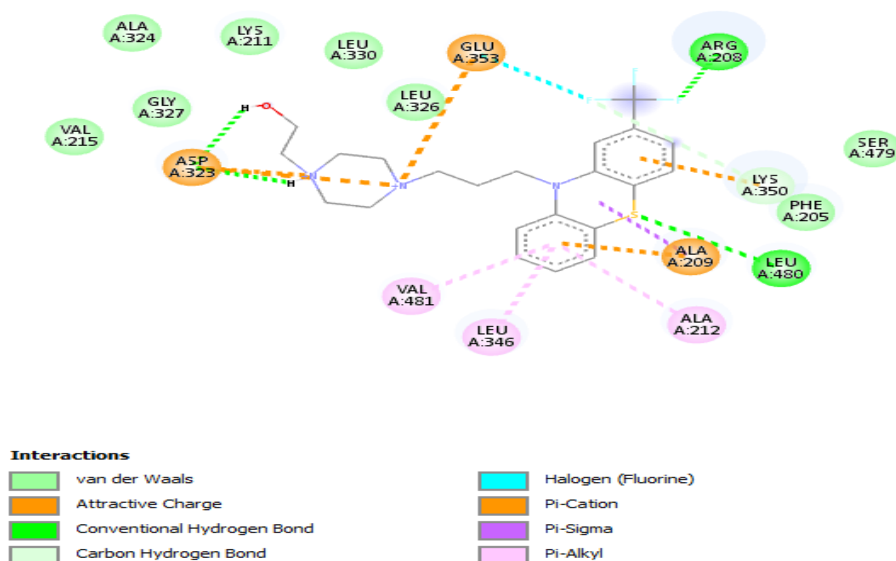


Fig. 10 2D Docking interactions between active site residues of BSA with fluphenazine (FPZ) showing types of interactions.

IV. Conclusion

This work describes the interaction between fluphenazine and BSA by using multispectroscopic techniques. The Stern-Volmer quenching constant K_{sv} and quenching constant K_q indicate that fluphenazine BSA binding reaction occurs by dynamic quenching mechanism. The value of n is two revealed that two binding sites are on BSA for fluphenazine binding. The results of synchronous fluorescence spectra and three dimensional fluorescence spectra are indicative of conformational changes of BSA upon binding with fluphenazine.

The hydrophobic interaction plays an important role during the fluorescence quenching of BSA by fluphenazine. CD Spectra Measurements indicates change in the secondary structures of BSA. Molecular Docking analysis revealed that hydrophobic interaction along with four hydrogen bonds were involved in binding of fluphenazine with BSA

Binding of drugs to serum protein is an important factor in determining their pharmacokinetics and pharmacological effect. Hence such study of interaction between BSA and fluphenazine would be useful in clinical medicine, medicinal chemistry, pharmaceutical industry, and life sciences studies.

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