Concise Review on Fluorescence Spectroscopy – A Commonly Utilized Technique in Study of Drugs-Protein Interaction

Bhutkar Shraddha P., *Dhande Swati R., Kadam Vilasrao J.

Bharati Vidyapeeth's College of Pharmacy, Sector-8, CBD Belapur, Navi Mumbai, Maharashtra, India.
Email Address: dswatir@gmail.com

Abstract:

Human Serum Albumin (HSA) is the most abundant plasma protein with molecular weight 67kDa. X-ray crystallography studies infer that it is constituted of three homologous domains namely I, II, III. These domains are further subdivided into subdomains A and B respectively. Subdomain IIA represents Sudlow's site I that is Warfarin binding site and Subdomain IIIA represents Sudlow's site II that is Ibuprofen binding site. Serum albumin exhibits excellent binding properties and hence it has main physiological function of transport of drug molecules to the tissues. Upon absorption of drugs into the systemic circulation, they bind to serum albumin and exist in HSA-drug complex form. This review focuses upon the application of Fluorescence Quenching Techniques by enlightening the binding studies of Diflunisal, Captopril and Tenofovir to the Human Serum Albumin. Fluorescence spectroscopic techniques are preferred because of the ease of use, greater sensitivity and rapidness.

Key words: Human Serum Albumin, Sudlow's sites, Fluorescence Spectroscopy, Diflunisal, Captopril, Tenofovir.

Human Serum Albumin (HSA) is a most abundant multifunctional non-glycosylated, negatively charged plasma protein at physiological pH due to its low isoelectric point [1], with inherent ligand-binding and transport properties, antioxidant and enzymatic functions. It is synthesized in the liver and has molecular weight of 67kDa [2]. Normal concentration of albumin in human plasma is 35–50 g/l which represents 50% of normal person's plasma protein [3, 4]. Physiologically, albumin is responsible for functions such as maintaining colloid osmotic pressure and may affect the microvascular integrity and related aspects of the inflammatory pathway [2]. Albumin structure allows it to bind and transport diverse metabolites such as metal ions, fatty acids, bilirubin and drugs [5, 6]. Indeed, binding of this protein with drugs having long half-life, enhances their pharmacokinetic properties [7].

STRUCTURAL FEATURES OF HSA

1) HSA is a small globular protein composed of 585 amino acids, having few tryptophan or methionine residues but has abundant of charged amino acid residues such as lysine, and aspartic acid and does not show presence of prosthetic groups or carbohydrate moiety [2].

2) X-ray crystallography studies has shown HSA to possess a heart-shaped tertiary structure, with approx. dimensions of 80 × 80 × 30 Å [2, 8-10].

3) The structure of HSA is organized into three homologous domains, I, II and III, which is further subdivided into two sub domains A and B which comprises of 4 and 6 alpha helices respectively [2,9].

4) HSA consists of 35 cysteine amino acid residues, which form disulfide bridges, contributing to stabilization of overall tertiary structure of protein [10], except one free cysteine-derived, redox active, thiol (-SH) moiety (Cys-34) in domain I, which is responsible for 80% of thiols (500 μmol/L) in plasma [2,10].

5) The main binding sites of the protein site I and site II are located on subdomains IIA and IIIA, respectively (Sudlow et al. 1976) [11].

6) According to Sudlow's, bulky heterocyclic anions bind to site I (located in subdomain IIA), whereas aromatic carboxylates bind to site II (located in subdomain IIIA) with an extended configuration [12].

7) Warfarin, an anti-coagulant agent, and ibuprofen, a NSAID, are stereotypical ligands for Sudlow's site I (subdomain IIA) and Sudlow's site II (subdomain IIIA), respectively [12].

8) HSA binds to seven equivalents of long-chain fatty acids (FAs) at various binding sites with different binding affinities [9, 12].

9) FA1 forms a salt bridge with Arg117 residue of polypeptide linker in the subdomain IB-IIA, whereas FA2 forms hydrogen bonds with Tyr150 residue from subdomain IB and with Arg257 and Ser287 residue from subdomain IIA [12].

10) FA2 is unique for its placement amidst between three subdomains, viz IA, IB, and IIA [12].

11) FA3 and FA4 are placed in a large void in subdomain IIIA that comprises Sudlow's site II [12].

12) FA3 forms salt linkages with Arg348 and Arg485, and a hydrogen bond with Ser342; FA4 forms a salt linkage with Arg410 and hydrogen bonds with Tyr411 and Ser489 [12].

13) FA5 is located in subdomain IIIB with the polar head directed towards subdomain IIIA and forms both salt linkages and hydrogen bond with Tyr401 and Lys525 [12].

14) Polar interactions are not demonstrated by FA6 and FA7, suggesting that FA6 and FA7 are low affinity FA binding sites [12].

15) The void holding FA7 represents the Sudlow's site I (the warfarin site), whereas Sudlow's site II overlays with FA3 and FA4 (the diazepam site) [12].

16) Doctility of Human Serum Albumin (HSA) permits its interaction with various compounds [4].

17) Eg.: Paracetamol, most common analgesic drug, binds to amino acid residues in the subdomain IIIA [4, 13].

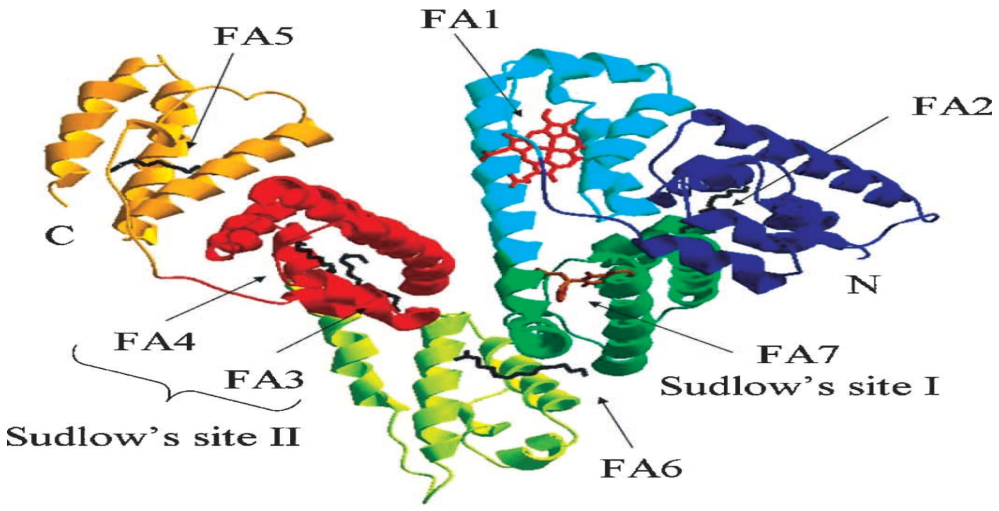

Fig 1. : Different binding sites of Human Serum Albumin showing presence of 7 fatty acids (FAs)

IMPORTANCE OF STUDYING HSA – DRUG INTERACTIONS

Generally, after the fate of absorption of drugs, they usually exists in two forms in circulation, namely, bound form or free form to serum proteins (HSA). The free drugs exhibits passive diffusion through the capillary walls constituted of endothelial cells into the organs where they are metabolized, and excreted via biliary excretion or glomerular filtration in kidney. Only free drug molecules can interact and associate with therapeutic targets to produce therapeutic effects. Mostly free drug concentration in the tissue depends on the unbound drug concentration in the plasma [14]. Binding of drug molecules to HSA is a reversible process, therefore the HSA-drug complex serves as freely circulating reservoir in systemic circulation thereby facilitating release of free drug molecules [15]. Thus, it is necessary to study HSA–drug interactions in order to understand the pharmacokinetics and pharmacological effects of drugs molecules [8].

For studying the interaction of drugs and plasma proteins, Fluorescence Spectroscopic Techniques are commonly used as they have high level of sensitivity, rapidity and ease of use. The above methods are advantageous and superior over conventional approaches such as affinity and size exclusion chromatography (SEC), equilibrium dialysis, ultra-filtration and ultra-centrifugation, which lacks sensitivity, requires long

analysis time, or both, and requires excess of protein concentrations of the dissociation constant for the drug–HSA complex [16, 17].

PRINCIPLES AND INSTRUMENTATION OF SPECTROFLOUROMETER

Principle:

Fluorescence refers to the phenomenon of re-emission of longer wavelength photons by a molecule that has absorbed photons of shorter wavelengths. Due to absorption light by molecules, electrons from ground state gets excited to a higher electronic state. Electrons remain in the excited state for about 10-8 seconds and then returns to the ground state and emits energy in the form of fluorescence during this process. Emitted photons have a longer wavelength than the absorbed photons [18].

Fluorescent compounds exhibits two characteristic spectra: an excitation spectrum and an emission spectrum. These spectra are often referred to as a compound's fluorescence fingerprint. No two compounds have the same fluorescence fingerprint. This principle makes fluorescence spectroscopy a highly specific analytical technique [18].

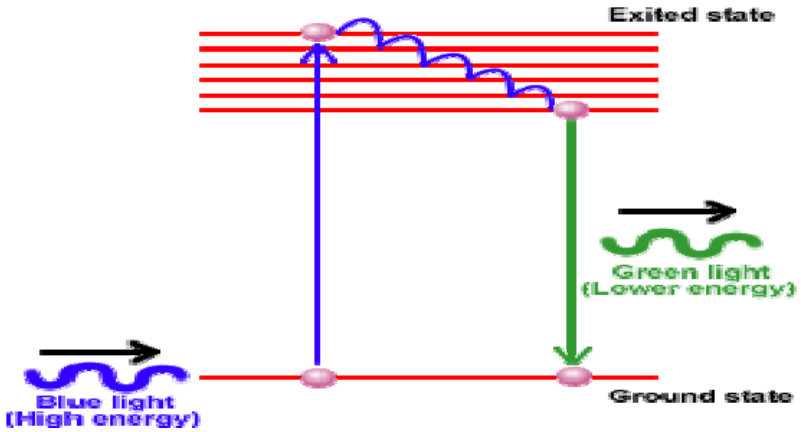

Figure 2. : Mechanism of Fluorescence.

Quenching of Fluorescence:

Any process, which decreases the intensity of fluorescence shown by a sample, is called Fluorescence quenching. The basic processes such as excited state reactions, molecular rearrangement reactions, energy transfer, ground state complex formation, and collisional quenching involved in molecular interaction, can result in quenching [18].

Fluorescence can be lost by:

1. Collisional quenching:

It involves collisions with other molecules that result in the loss of excitation energy as heat instead of as emitted light. (E.g. iodide ions, oxygen, nitroxide radical) [18].

2. Static quenching:

It is interaction of the fluorophore with the quencher which forms a stable non-fluorescent complex. This complex has a different absorption spectrum from the fluorophore, change in absorption spectrum is an important diagnostic of this type of quenching. A special case of static quenching is self-quenching, in this fluorophore and quencher are the same species. Self-quenching is particularly seen in concentrated solutions of tracer dyes [18].

Instrumentation:

A Spectrofluorometers consists of an Excitation Monochromator and an Emission Monochromator [18].

Components of Spectrofluorometer [18]:

1) Light Source: The light source provides the energy that excites the compound of interest. Examples include xenon lamps, high pressure mercury vapor lamps, xenon-mercury arc lamps, lasers, and LED's. Lamps emit a wide range of light with higher wavelengths than those required to excite the compound whereas Lasers and LED's emit more specific wavelengths.

a) Xenon lamps are very powerful since they emit light from 190-1200 nm.

b) Mercury vapor lamps are highly intense than xenon lamps.

Various fluorescent phosphors coatings are used on the lamps to provide the desired wavelength of exciting light [18].

2) Excitation Filter: The excitation filter is used to eliminate out the wavelengths of light not absorbed by the compound of interest. It allows only selected wavelength of light energy to pass through and excite the sample [18].

3) Cuvette: The cuvette is utilized to hold the sample of interest. The cuvette material must such that it should allow the compound's absorption and emission light to pass through it. The dimensions of the sample cell drastically affects the measurement. The greater the path length, the lower the concentration is recorded [18].

Fluorometers commonly hold 10 mm square cuvettes, and/or 13 mm or 25 mm test tubes [18].

4) Emission Filter: Stray lights such as Rayleigh and Raman scatter can also be emitted from the sample. In addition, background stray light may be present that does not pass through the sample and directly falls over emission filter. The role of emission filter is to screen out these extra unwanted components, thereby only allowing primarily wavelengths of light to fall on detector [18].

5) Detector: The detector employed in fluorescence spectroscopy is mostly a photomultiplier tube. The light intensity, directly proportional (linear) to the compound's concentration, is shown as a digital readout [18].

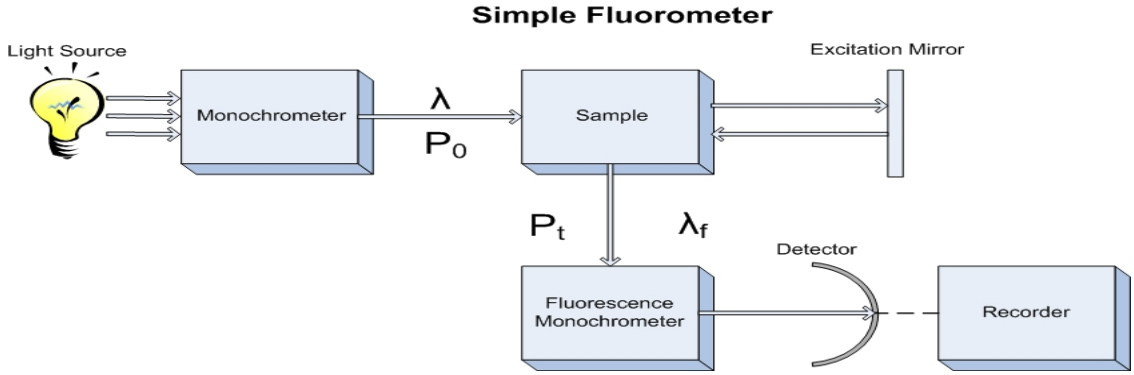

Figure 3. : Schematic diagram of Spectrofluorometer

Advantages on Fluorescence Spectroscopy [18]:

1) It is highly sensitive than spectrophotometer.

2) Spectrofluorometers are highly specific and less susceptible to interferences because fewer materials exhibit fluorescence.

3) Fluorescence output is linear to sample concentration over a very broad range.

4) Reagent and instrumentation costs are low when compared to many other analytical techniques, such as GC and HPLC. Reagent used for this type of analysis less costly, due to the high sensitivity of Spectrofluorometers, hence less reagent can be used.

Disadvantages [18]:

1) Very few molecules exhibit fluorescence hence cannot be used for analysis for wide range of compounds.

2) Has complex instrumentation as compared to UV-VIS Spectrophotometers.

INTERACTION STUDIES OF HUMAN SERUM ALBUMIN AND VARIOUS DRUG MOLECULES:

1) Diflunisal and its prodrug Acetyl Diflunisal:

Diflunisal is an aspirin-like NSAID that acts by inhibiting COX-2 (cyclooxygenase-2) enzyme involved in prostaglandin synthesis [19]. It has high caliber than aspirin as an anti-inflammatory agent because of its higher potency, less adverse effects, and longer duration of action [20]. However, it is 99% bound to serum albumin, necessitating a regimen of at least 250 mg twice daily or more for efficacy [21].

The binding ability of Diflunisal and Acetyl Diflunisal to HSA was tested by Fluorescence Quenching Technique. Increase in drug concentrations led to increase in quenching of the fluorescence signal of HSA–Fatty Acids, suggesting that both Diflunisal and acetyl Diflunisal interact with HSA. The binding constants of HSA for above drug and its prodrug were predicted from the modified Stern–Volmer plot (Fig. 4). The binding constant of HSA for Acetyl Diflunisal was found to be K = 0.747 ± 0.035 x 10^5 L/mol, which is equivalent to -27.801 kJ/mol of free energy and the binding constant of HSA for Diflunisal was found to be K = 1.443 ± 0.021x10^5 L/mol, which is equivalent to -29.433 kJ/mol of free energy [22]. Mao et al. displayed that Diflunisal binds to subdomain IIIA with a strong binding affinity [23].

The above fluorescence quenching results shows that Diflunisal also strongly binds to subdomain IIA of HSA (K = 1.443 ± 0.021 x 10^5 L/mol). The prodrug Acetyl Diflunisal acts by acetyling the lysine199 in subdomain IIA of HSA. Also above results infer that acetylated albumin has a low binding affinity for Diflunisal; thereby, along with acetylation of HSA it releases the active drug Diflunisal simultaneously [22].

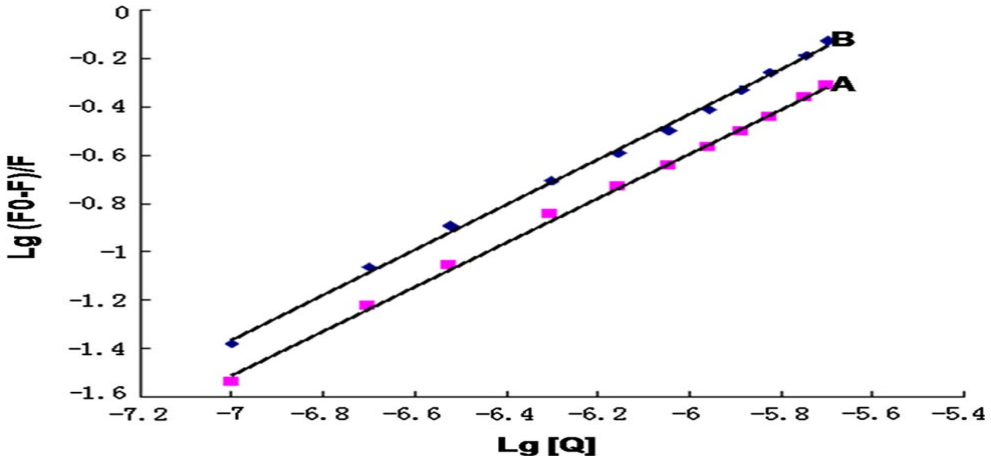

Fig 4. Plots of log [(F_o- F/F)] vs log [Q] for drug bound to HSA–Fatty Acid. (Line A-Acetyl Diflunisal and Line B-Diflunisal)

2) Captopril

Captopril, 1-[(2S)-3-mercapto-2- methyl-1-oxopropyl]-L-proline, is an orally active ACE I inhibitor having short duration of action [24]. It has numerous clinical therapeutic applications, and is widely used for treatment of (CHF) chronic heart failure and hypertension [24, 25, 26]. It contains two centers of asymmetry, one linked with the (S)-proline part of the molecule and the other with the 3-mercapto-2-methyl- propionic acid side chain [25].

To understand the pharmacological activities of captopril at molecular level, the inter-action between captopril and HSA is studied by different spectroscopic methods such as UV spectroscopy and Fluorescence Spectroscopy. Interaction between HSA is studied by investigating the quenching mechanism, binding constants, binding sites, binding mode, and binding distance [25].

Absorption spectra was recorded on TU1901 UV/Vis Spectrophotometer (PGeneral, Beijing, China) and fluorescence spectra was recorded on RF- 5301PC fluorescence spectrometer (Jasco, Japan) [25]. The interaction of captopril with HSA was studied by observing the changes in intrinsic fluorescence intensity of HSA upon addition of captopril.

Upon addition of captopril solution (5.0×10^{-3}mol . L^{-1}) to HSA solution (1.0×10^{-4}mol . L^{-1}), led to a concentration-dependent quenching of intrinsic fluorescence intensity of HSA, suggesting that captopril binds to HSA and the microenvironment around the chromophore of HSA changes.

Increase in concentration of captopril, the fluorescence intensity of HSA decreases gradually. Such strong quenching clearly indicates the binding of captopril with HSA (Fig 5) [25].

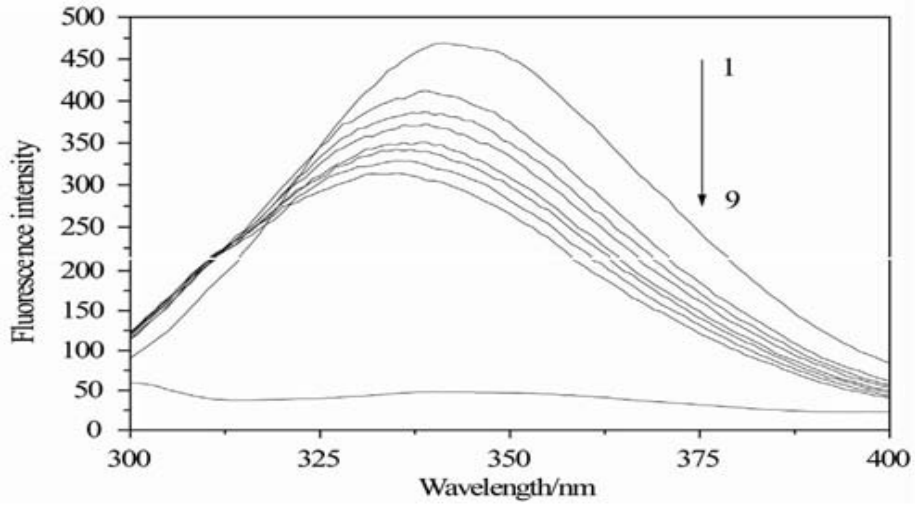

Fig 5. : Quenching of intrinsic fluorescence intensity of HSA upon addition of Captopril.

C_{HSA} = 1.0 x 10^{-6} mol . L^{-1};

$C_{Captopril}$ = (0.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5 and 2.75) x 10^{-4} mol . L^{-1}.

3) Tenofovir

Disoproxilfumarate (prodrug of Tenofovir) is a fumaric acid salt derivative of bisisopropoxycarbonyloxymethyl ester derivative of Tenofovir (Ten). In body, tenofovir disoproxilfumarate is converted to tenofovir, an acyclic nucleoside phosphonate (nucleotide) analog of adenosine 50-monophosphate. Tenofovir is used for the treatment of HIV infection. It is also an experimental treatment for hepatitis B [27].

The binding of Ten to Human Serum Albumin is studied using quenching mechanism, binding constants, and the number of binding sites were evaluated by the Fluorescence Quenching method.

The fluorescence quenching of HSA is caused by the tryptophan, tyrosine and phenylalanine residues. Interaction of Ten can change the microenvironment of tryptophan residues, which can generate changes of intrinsic fluorescence intensity of HSA. Ten most likely binds to the hydrophobic pocket located in subdomain IIA (site I). The fluorescence spectra of HSA with varying concentrations of Ten are shown in Fig. 6. The fluorescence of HSA regularly decreased with the increasing concentration of Ten, but no significant shift of the emission maximum wavelength was observed, indicating that Ten interacted with HSA and quenched its intrinsic fluorescence [28].

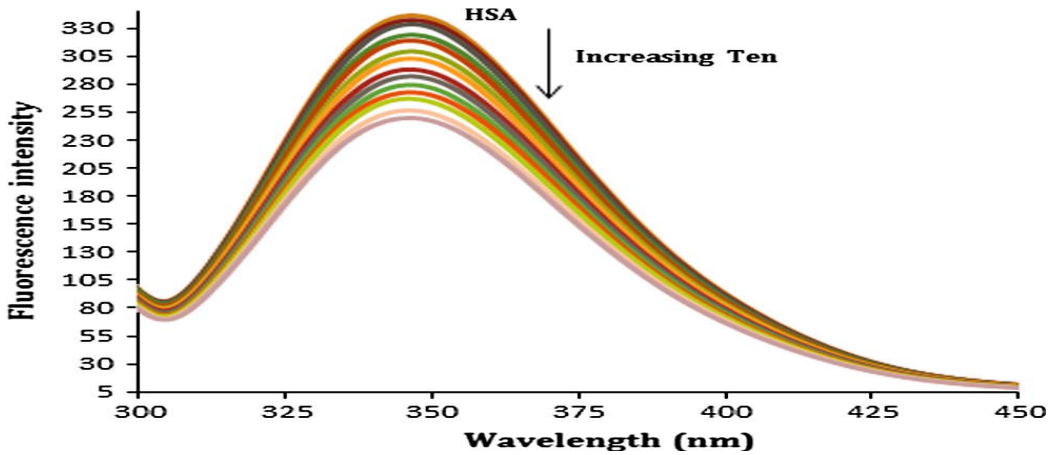

Fig 6: Fluorescence spectra of HSA in the absence and the presence of Tenofovir,

[HSA] = 5 x 10^{-6} M and [Ten] = 0.0 to 9.5 x 10^{-5} M at 298 K

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