

Biophysical Interaction of Propylthiouracil with Human and Bovine Serum Albumins

Husain Alsamamra*, Musa Abuteir and Saqer Darwish

Department of Physics, Al-Quds University, Palestine

*Corresponding author:

Husain Alsamamra

✉ hsamamra@staff.alquds.edu

Associate Professor, Department of Physics, Al-Quds University, Palestine.

Tel: +970658881802

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Abstract

The physical interaction of antithyroid drug, propylthiouracil was studied with bovine and human serum albumins through UV absorption and fluorescence spectroscopic techniques. The obtained values of the quenching constants were calculated by the Stern-Volmer equation are in the order of $1012 \text{ Lmol}^{-1} \text{ s}^{-1}$ indicating that both serum albumins were quenched by the drug in a static manner. The binding constants of the drug interaction with both HSA and BSA proteins are found to be relatively weak and are in the order of 103 M^{-1} . The tryptophan residues of HSA and BSA are most perturbed by the binding process which was authenticated by the fluorescence spectra of both proteins in the presence of propylthiouracil. The importance behind this study is to clarify the mechanism of the interaction between propylthiouracil with HSA and BSA, as well as providing additional values in order to study drug-protein interaction which may facilitate the study of drug metabolism and transportation.

Keywords: Propylthiouracil; HAS; BSA; Binding constant; UV absorption; Fluorescence emission; Static quenching

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Introduction

One of the common diseases of this age is Graves' disease (GD), GD is an autoimmune thyroid disorder and it is one of the main causes of hyperthyroidism in areas with iodine abundance, typically affects women than men and usually characterized by thyroid poisoning [1,2]. GD reported 20-30 cases per 100,000 people each year, with 3% of women and 0.5% of men acquiring GD during their lifetime [3]. The antithyroid drug (ATD), Propylthiouracil (6-propyl-2-thiouracil) (PTU), its chemical structure provided in **Figure 1** [4], It is exclusively prescribed to control hyperthyroidism in GD that inhibits the function of an enzyme to stimulate the synthesis of thyroid hormones [5-8]. ATDs obstructs the iodination of tyrosyl residues in thyroglobulin catalyzed by thyroid peroxidase, thereby arresting the production of thyroid hormones [9-11]. PTU is usually used only when methimazole, surgery, and radioactive iodine is not possible, as recommended for pregnant women [12-16].

Serum Albumin

A drug binding to plasma proteins plays the major factor on

the distribution, metabolism, pharmacological. Under different physiological and pathological conditions plasma proteins can be reduced, and severely restricted drugs can have a risk of enhanced activity and unwanted side effects [17]. Serum albumin is responsible for maintaining oncotic pressure in human plasma. Human Serum Albumin (HSA), its secondary structure presented in the top part of **Figure 2** [18], is a single monomeric polypeptide of 585 amino acid residues, it is a major protein component of human plasma with a typical concentration of 0.6 mM in the bloodstream [19] contributing significantly to the colloidal oncotic pressure and antioxidative capacity of human serum. X-ray crystallographic studies showed that HSA is composed of three homologous domains (I-III), each domain is further divided into subdomain A and B [20]. HSA has the possibility and the ability to bind in a non-covalent mode with a wide range of chemically different endogenous and exogenous ligands. The therapeutic efficiency of the ligands depends on their ability to bind to serum albumin and the mechanism involved in the binding. HSA- ligand complexes as provided by crystallographic studies have shown that drug-like compounds mainly interact with one of two high-affinity binding sites on the protein subdomains IIA and IIIA,

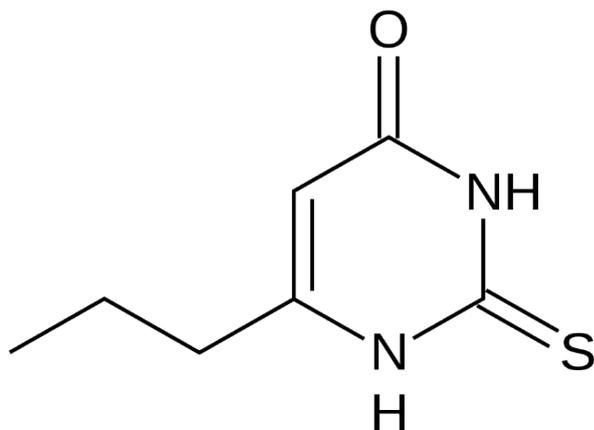


Figure 1 Chemical structure of Propylthiouracil.

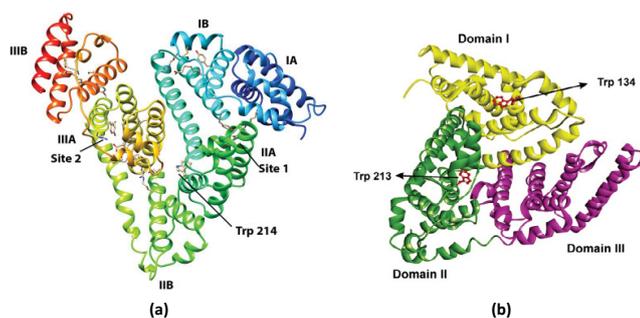


Figure 2 Secondary structure of (a) HSA; (b) BSA.

which make it potential for different drugs delivery [21]. These binding sites empower the HSA with an exceptional ability in binding various ligands which adds to its biological importance in delivering a number of drugs in the blood system to their targeting organs/tissues within the human body [22,23]. The intrinsic property of HSA that it contains a single tryptophan residue (Trp 214) in domain IIA and its fluorescence is highly sensitive to the ligands bounded nearby, therefore, this property is used as a probe to elucidate the binding properties of drugs with HSA. The fact that HSA possesses multiple binding sites for a number of ligands with a moderate binding affinity (104-106) suggests a high degree of flexibility of HSA [24-26], according to the recent biophysical studies, this flexibility is due to that HSA have the fastest hydrogen exchange rate in aqueous solution [27]. One of the most sequences related to HSA is Bovine Serum Albumin (BSA), the secondary structure provided in the bottom part of **Figure 2** [28]. The similarity between HSA and BSA is 76% in terms of identity and 88% in terms of amino acid sequences [29]. It is likely that depending on the content of fatty acids, pH, and ionic forces these three domains denature regardless of each other or can unite. The primary structure of BSA was elucidated by Brown in 1975, BSA contains the 607 amino acid residue, twenty-one of which is tyrosine (Tyr) residues and two of which are tryptophan (Trp) residues located at positions 134 and 213, respectively which cause BSA to have intrinsic fluorescence [30,31].

The reversible drug-protein complex formation has attained a great concern in pharmacokinetics because this formation made through weaker chemical bonds, for example, hydrogen bonds or Van der Waals forces [32,33].

Different studies and experiments reported in the literature were used to clarify the interaction of serum albumin with small molecules, drugs and toxic chemicals using the fluorescence method [34,35]. But there has been no work study the interaction of propylthiouracil with protein albumin. Thus, the present work aimed to obtain a detail and insightful information of HSA and BSA binding with propylthiouracil using UV-visible and fluorescence spectroscopy. Fluorescence spectroscopy is a powerful technique in providing structural changes information due to quenching upon intermolecular forces. Thus, the fluorescence technique provides data to calculate binding constants and binding sites. The study of the interaction of PTU with HSA and BSA may facilitate the mechanism of the transportation of hyperthyroid drug and to understand the binding type of drug-protein interaction in addition to the precise knowledge of drug-biomolecular interactions which is crucial for a rational design of pharmaceuticals.

In this work, we study the interactions of propylthiouracil with human and bovine serum albumins applying different supportive data including a binding parameter, quenching, and nature of binding. However, this data obtained from UV absorption and fluorescence spectroscopic techniques. The importance of this investigation lies in the well-recognized role of the two serum albumins as a drug transporter to target specified organs.

Materials and Samples Preparation

Propylthiouracil (170.2 g/mol), human and bovine serum albumins (66.5 kDa) in powder form were purchased from Sigma Aldrich chemical company and were used without any further purifications. HSA and BSA stock solutions were prepared and dissolved in phosphate buffer saline with pH of 7.4 to obtain a concentration of 80 mg/ml which corresponds to 1.2 mM. The final concentration of HSA and BSA in the HSA/BSA-PTU complex was 40 mg/ml equivalent to the molarity of 0.6 mM which is comparable to its concentration in the blood. The stock solution of PTU with a molarity of 2.6 mM was prepared by dissolving the drug in ethanol. PTU standard solutions were prepared by successive dilutions. The HSA/BSA-PTU complexes were prepared by mixing equal volumes of 1.2 mM HSA/BSA molarity and each of the standard PTU solutions. The final concentration of HSA and BSA were kept at (40 mg/ml) in all samples while the concentrations of propylthiouracil in the final HSA/BSA-PTU solutions were reduced to be as (0.5 mM, 0.7 mM, 0.9 mM, 1.1 mM, and 1.3 mM).

Instruments

UV-VIS absorption spectra

A spectrophotometer is a powerful technique which is used to measure the absorption spectrum of the samples in the range between 220-750 nm, with high accuracy and reproducibility. The data was collected using 5 μ L samples using NanoDrop ND-

1000 Spectrophotometer for the free HSA/BSA (0.6 mM) and for the HSA/BSA-PTU complexes 'solutions with the following concentrations (0.5, 0.7, 0.9, 1.1 and 1.3) mM. UV measurements were repeated for all the samples and no significant differences were observed. The UV-absorption spectra for both complexes HSA-PTU and BSA-PTU are obtained at the wavelength of 280 nm.

Fluorescence

Fluorescence data were obtained by a NanoDrop ND-3300 Fluoroscpectrophotometer for the following complexes concentration (0.5, 0.7, 0.9, 1.1 and 1.3) mM at 25°C. The excitation source comes from one of three solid-state Light Emitting Diodes (LEDs) which have options that include: UV LED with maximum excitation 365 nm, Blue LED with excitation 470 nm, and white LED from 500 to 650 nm excitation. A 2048-element CCD array detector covering 400-750 nm, is connected by an optical fiber to the optical measurement surface. The excitation is done for both complexes HSA-PTU and BSA-PTU at the wavelength of 390 nm and the maximum emission wavelength is at 440 nm.

Results and Discussions

UV-VIS absorption spectra

Ultraviolet and visible spectrometers have been used for many years and have become one of the important analytical instruments in modern laboratories because of its simplicity, versatility, speed, accuracy, and cost-effectiveness [36]. This experimental technique was used to calculate the binding constants for several drug-protein complexes [37]. The UV absorbance data of HSA-PTU and BSA-PTU complexes were obtained and presented in **Figure 3**. The figure shows a maximum absorption peak at a wavelength of 280 nm, the spectrums provide a direct relationship between the increase in peak intensity of the HSA-PTU (top) and BSA-PTU (bottom) complexes and the concentration of the PTU. The increase in the intensity is due to the interaction between HSA/BSA and PTU as monitored in buffered solution by scanning the wavelengths. As indicated in the figure, there is a clear absorption signal for free PTU.

From Eq. 5, the reciprocal plot of $1/(A-A_0)$ versus $(1/L)$ is linear as presented in **Figure 4**. When a fixed concentration of HSA or BSA was allowed to complex with different amounts of PTU, there is a linear increase in the UV absorbance of HSA or BSA. The binding constants for the HSA-PTU and BSA-PTU complexes were calculated using UV spectra according to published methods [22,38] with the assumption that there is one-to-one interaction between HSA/BSA and PTU in aqueous solution to establish chemical equilibrium as in the following equations.



The corresponding formation/binding constants for both proteins are given by:

$$K = [\text{HSA: PTU}]/[\text{PTU}] [\text{HSA}] \quad (3)$$

$$K = [\text{BSA: PTU}]/[\text{PTU}] [\text{BSA}] \quad (4)$$

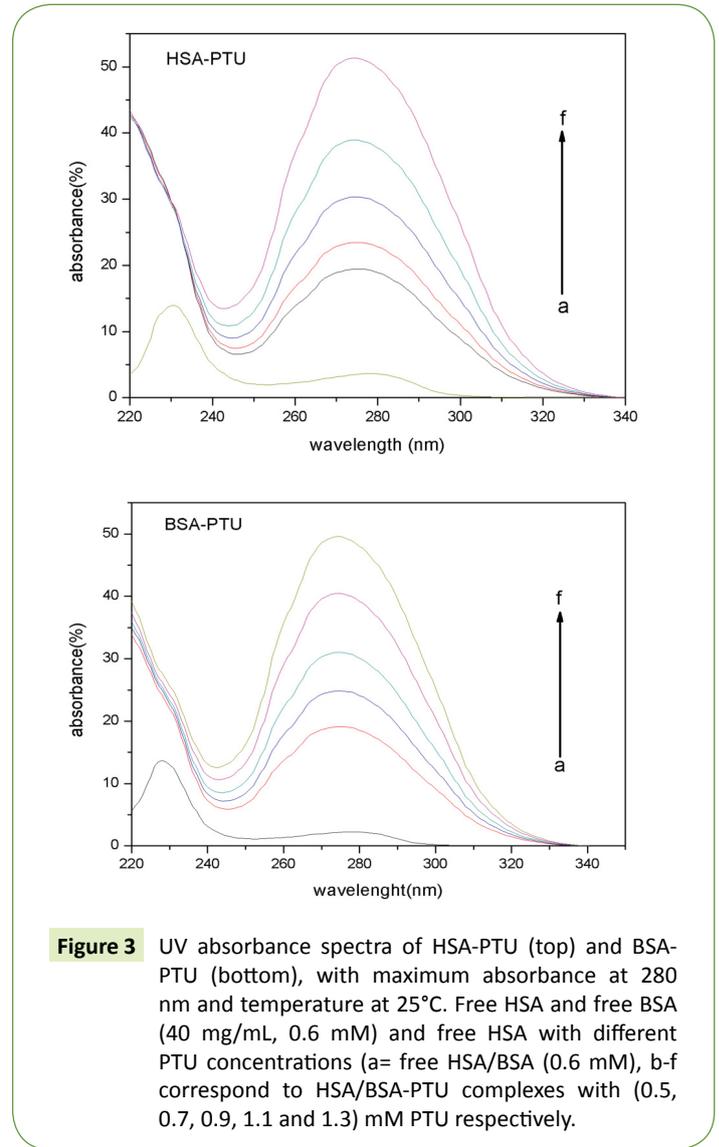


Figure 3 UV absorbance spectra of HSA-PTU (top) and BSA-PTU (bottom), with maximum absorbance at 280 nm and temperature at 25°C. Free HSA and free BSA (40 mg/mL, 0.6 mM) and free HSA with different PTU concentrations (a= free HSA/BSA (0.6 mM), b-f correspond to HSA/BSA-PTU complexes with (0.5, 0.7, 0.9, 1.1 and 1.3) mM PTU respectively.

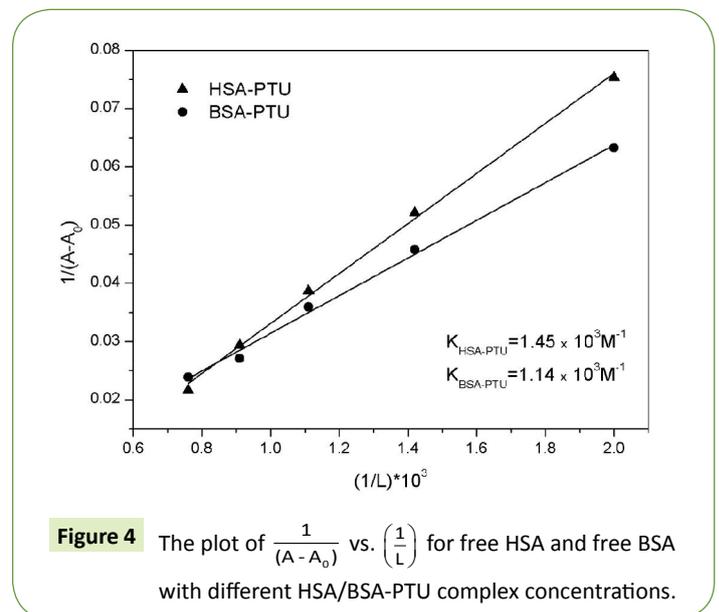


Figure 4 The plot of $\frac{1}{(A-A_0)}$ vs. $\left(\frac{1}{L}\right)$ for free HSA and free BSA with different HSA/BSA-PTU complex concentrations.

Accordingly, the UV absorbance spectra were treated using linear double reciprocal plots based on the following equation.

$$\frac{1}{A - A_0} = \frac{1}{A_\infty - A_0} + \frac{1}{k(A - A_0)} \times \frac{1}{L} \quad (5)$$

Where A_0 corresponds to the initial absorption of HSA or BSA at 280 nm in the absence of ligand, A_∞ is the final absorption of the HSA or BSA, and A is the recorded absorption at different PTU concentration (L).

The binding constants (K) for both protein interactions with propylthiouracil were estimated from the ratio of the intercept to the slope found from **Figure 4**. It was found that both binding constant values are close to each other with KHSA-PTU equals to $1.45 \times 10^3 \text{ M}^{-1}$ and $K_{\text{BSA-PTU}}$ found to be $1.14 \times 10^3 \text{ M}^{-1}$. The value for both binding constants is an indication of weak intermolecular force interaction between the HSA/BSA and PTU when compared to reported protein-drug interaction with a binding constant in the range of 10^4 and 10^6 M^{-1} [39]. The importance of the binding constant value stems from its valuable application in drug delivery and pharmacokinetics [23] therefore, absorbance measurements were constantly repeated and similar results were obtained consistently.

Fluorescence emission spectroscopy

In order to study the reactivity of chemicals and biological systems; fluorescence technique is an effective and powerful tool because it allows noninvasive measurements of substances in low concentration under physiological conditions. Since the amino acids tryptophan, tyrosine and phenylalanine are fluorescent in nature, thus contributing to the fluorescence of the protein in which they are present [40]. Among the two amino acid residues, Trp is highly fluorescent and sensitive to the micro-environmental changes that might occur in its vicinity whereas phenylalanine does not get excited (for the most part) easily and has a low quantum yield. Owing to these reasons, Trp is usually used in quenching studies.

The effect of propylthiouracil on the fluorescence emission spectrum of HSA and BSA, excited at 390 nm are presented in **Figure 5**. The emission spectrum for the two complexes showed a maximum emission wavelength at 440 nm. The definition of fluorescence quenching is the reduction of the quantum yield of fluorescence from a fluorophore. When a fluorophore is substantially induced with quencher molecule by a variety of molecular interaction (i.e. excited state reactions, energy transfer, molecular rearrangements, and ground-state complex formation) quenching of fluorescence is evident [41]. The quenching took place in a concentration-dependent manner; as the concentration of PTU increases result in a decrease in HSA and BSA fluorescence intensity.

The HSA and BSA fluorescences are decreased upon increasing concentrations of PTU, which indicates that the PTU is interacting with HSA and BSA in the surrounding of the fluorescent residues Trp214 (HSA), Trp213 and Trp134 (BSA). This interaction of PTU affects the two proteins fluorescence upon binding to PTU due to the quenching ability of tryptophan residues upon colliding with PTU.

Fluorescence quenching can be processed through different mechanisms, typically classified as dynamic quenching and static

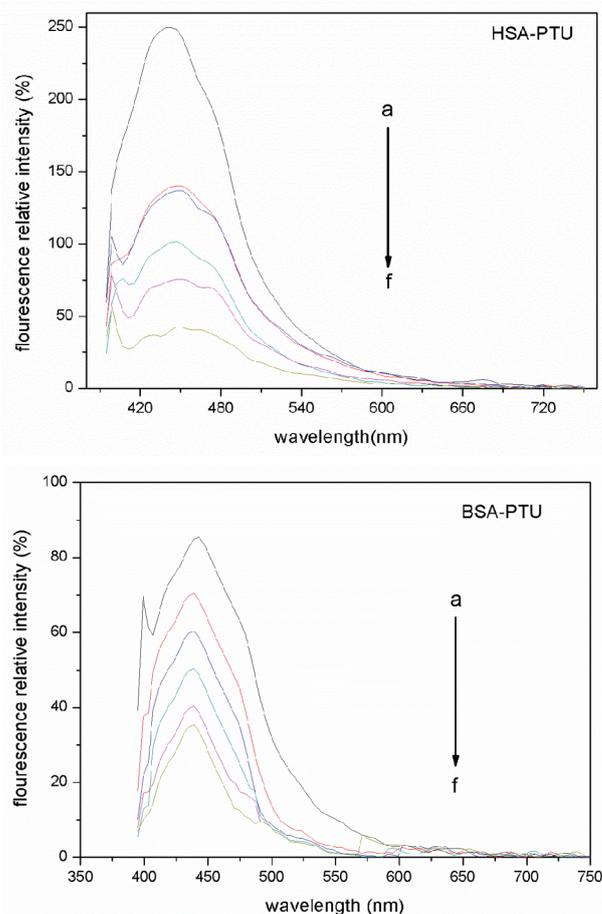


Figure 5 Fluorescence emission spectra of HSA-PTU (top) and BSA-PTU (bottom), with maximum absorbance at 340 nm and temperature at 25°C. Free HSA and free BSA (40 mg/mL, 0.6 mM) and free HSA with different PTU concentrations (a= free HSA/BSA (0.6 mM), b-f correspond to HSA/BSA-PTU complexes with (0.5, 0.7, 0.9, 1.1 and 1.3) mM PTU respectively.

quenching. In order to ensure the quenching mechanism, the fluorescence data between quenching efficiency (F_0/F) and the concentration of quencher (L) for the complete static or dynamic quenching mode should satisfy the following Stern-Volmer equation [42].

$$\frac{F_0}{F} = 1 + K_{sv} [L] = 1 + k_q \tau_0 [L] \quad (5)$$

where F_0 and F are the fluorescence intensity of fluorophore in the absence and in the presence of quencher, respectively, (L) is the concentration of quencher (PTU), K_{sv} in M^{-1} is the Stern-Volmer constant corresponding to the slope of the plot for F_0/F versus (L) as shown in **Figure 5**, k_q is the quenching rate constant for biomolecular quenching in $\text{M}^{-1}\text{s}^{-1}$, and τ_0 is the average fluorescence lifetime of fluorophore without quencher evaluated at about 10^{-8} [40].

As seen in **Figure 6**, the curves are linear which suggests the existence of a single type of quenching (dynamic or static) and/or a single binding site for both complexes., the fluorescence quenching constant values for HSA-PTU and BSA-PTU are

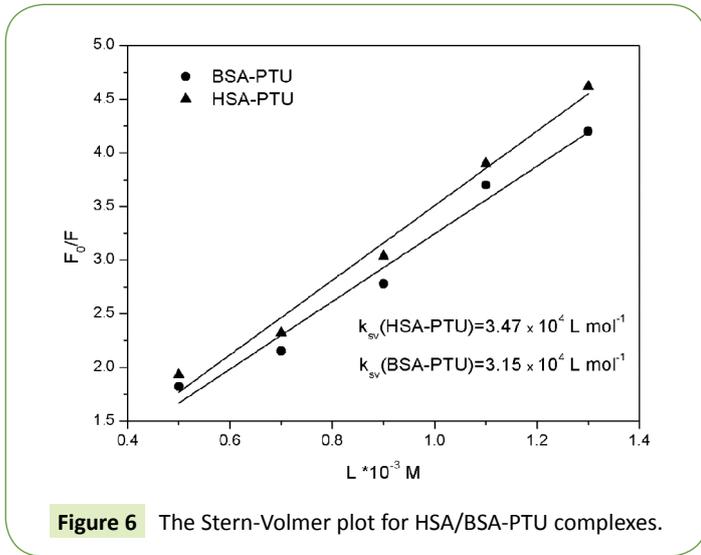


Figure 6 The Stern-Volmer plot for HSA/BSA-PTU complexes.

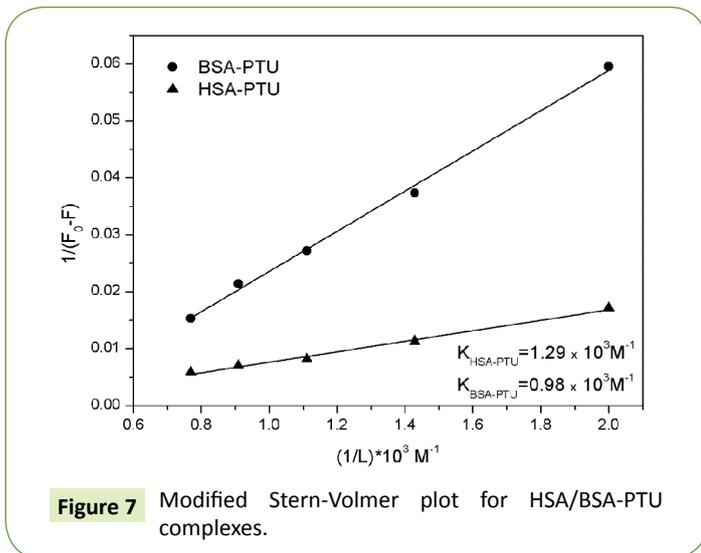


Figure 7 Modified Stern-Volmer plot for HSA/BSA-PTU complexes.

calculated by applying Eq. 5 and are equal to $(3.47 \times 10^4, 3.15 \times 10^4) \text{ L mol}^{-1}$ respectively. The obtained values of k_q ($3.47 \times 10^{12}, 3.15 \times 10^{12}) \text{ L mol}^{-1} \text{ s}^{-1}$ for HSA-PTU and BSA-PTU respectively, the attained values of k_q were more than the limiting diffusion constant of the biomolecule ($2 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$) [43]. Thus, it is proposed that the complex formation takes a major role rather than a dynamic collision for the interaction of HSA/BSA-PTU. In addition, this evidence indicated that a static quenching was dominant in the system [44].

One of the most frequently used equations used to calculate the binding constant of the drug-protein interaction is the modified Stern-Volmer equation [45].

$$\frac{1}{F_0 - F} = \frac{1}{F_0 K (L)} + \frac{1}{F_0} \quad (6)$$

In order to calculate the binding constant K of HSA-PTU and BSA-PTU complexes, a plot of the reciprocal of the difference between the unquenched fluorescence and the quenched fluorescence $\left(\frac{1}{F_0 - F}\right)$ vs the reciprocal concentration of PTU $\left(\frac{1}{L}\right)$ as shown in

Figure 7. The presented curves are linear and have a slope of $\frac{1}{F_0 K}$ and intercept $\frac{1}{F_0}$ according to Eq. 5.

The obtained values of K were found to be $(1.29 \times 10^3 \text{ M}^{-1}, 0.98 \times 10^3 \text{ M}^{-1})$, which are very close to the values obtained by UV spectroscopic technique and supports the effective role of static quenching.

The obtained binding constant values illustrated that there was a weak binding force between PTU and the two serum albumins and this is due to the limited number of binding sites for endogenous and exogenous ligands that has serum albumin. Because these ligands have binding constants ranging from 10^4 to 10^8 (significant interaction) and reversibly bound [46].

Conclusion

To conclude, the complex interaction between PTU with HSA and BSA has been investigated by using UV absorption and fluorescence spectroscopic techniques in a steady-state manner. The values of the binding constants ($\sim 10^3 \text{ M}^{-1}$) for both complexations obtained from both techniques were found to be relatively weak. The quenching constant values for both interactions were found to be in the order of $10^{12} \text{ L mol}^{-1} \text{ s}^{-1}$ which justifies that the intrinsic fluorescence of both HSA and BSA were quenched by PTU in a static mode, and the linear plot of Stern-Volmer equation supports the idea of static quenching.

The results obtained in this work facilitate the understanding of the HSA and BSA interaction with PTU. The biological significance of this investigation lies in the well-recognized role of the two serum albumins as a drug transporter to target specified organs in such a way that the target organ could be cured therefore it is very important to understand the various types of molecular interactions of HSA and BSA with drugs which will be essential in the drug delivery and in promotion alternative foundations for new pathways of drug delivery.

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