European Journal of Biophysics
2023; 11(1): 1-16
http://www.sciencepublishinggroup.com/j/ejb
doi: 10.11648/j.ejb.20231101.11
ISSN: 2329-1745 (Print); ISSN: 2329-1737 (Online)

Studies of Thyroid Hormones (Propylthiouracil and L-Thyroxine) Interaction with Human Serum Albumin-Spectroscopic Approach

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To cite this article:

Israa Ismail Abu Katteh, Husain Rashad Alsamamra, Musa Mahmoud Abu Teir. Studies of Thyroid Hormones (Propylthiouracil and L-Thyroxine) Interaction with Human Serum Albumin-Spectroscopic Approach. *European Journal of Biophysics*. Vol. 11, No. 1, 2023, pp. 1-16. doi: 10.11648/j.ejb.20231101.11

Received: February 25, 2023; **Accepted**: March 21, 2023; **Published**: March 31, 2023

Abstract: In this work, UV-absorption, fluorescence spectroscopy and Fourier transform infrared (FTIR) spectroscopy were used to investigate the relationship between Propylthiouracil and L-Thyroxine with human serum albumin. The binding constants of propylthiouracil and L-Thyroxine have been determined of both UV-absorption and fluorescence spectroscopy. The binding Constants values measured at 293k are 1.659×10^{3} M^{-1} for propylthiouracil and 1.013×10^{4} M^{-1} for L-Thyroxine. The constant values of the Stern–Volmer quenching were determined to be 2.144×10^{3} L mol^{-1} for propylthiouracil and 1.937×10^{3} L mol^{-1} L-for Thyroxine. The UV-absorption intensity of HSA- hormones complexes has increased with increasing of propylthiouracil and L-Thyroxine concentration. With the rise in propylthiouracil and L-thyroxine concentrations, the fluorescence results indicate a decrease in HSA- hormone emission intensity. To determine the effects of protein secondary structure and hormone binding mechanisms, we have used FTIR spectroscopy with Fourier self-deconvolution technique and second derivative resolution enhancement, as well as curve-fitting methods for the investigation of the amide I, II, and III regions. The peak positions within the three amide regions (amide I, amide II and amide III) were allocated and any results were explored due to changes in concentration. Due to variations in hormone concentrations, the measurements of the FTIR spectra indicate a difference in the intensity of absorption bands.. FTIR Spectra measurements reflect a difference in the strength of the absorption bands due to changes in hormone concentrations.

Keywords: Thyroid Hormones (Propylthiouracil and L-Thyroxine), Human Serum Albumin, Binding Constant, Fourier Transform IR, UV/Visible Fluorescence Spectroscopy

1. Introduction

Propylthiouracil (6-propyl-2-thiouracil) belongs to the class of organic compounds known as pyrimidines. In July 1947, it was used for medicinal use and played an important part in the treatment of hyperthyroidism or Graves' disease [1]. The molecular formula of PTU is $C_7H_{10}N_2OS$ and chemical structure shown in Figure 1. Propylthiouracil is an inhibitor of thyroid hormone synthesis; it functions by inhibiting the thyroperoxidase enzyme, which adds Iodide to the thyroxine hormone precursor thyroglobulin residues of tyrosine [2]. The enzyme tertaiodothyronine 5 'deiodinase, which changes thyroxine to triiodothyronine, is also inhibited by propylthiouracil [3].

L-Thyroxine belongs to the Phenylalanine family of organic compounds. It was first made in 1927, and has played a significant role in hypothyroidism care and suppressive therapy to shrink or prevent growth of abnormal thyroid tissue [4]. The molecular formula of L-thyroxine is $C_{15}H_{11}I_4NO_4$ and chemical structure shown in Figure 2. L-Thyroxine is synthetic version of the natural hormone thyroxine, naturally formed by the thyroid gland. When the thyroid gland is not producing enough natural thyroxine, L-Thyroxine relieves the symptoms associated with thyroxine deficiency since it increases serum thyroxine concentrations and mimics the action of thyroxine in the body [5].

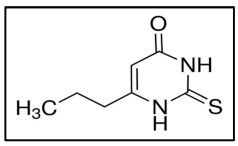


Figure 1. *Propylthiouracil Chemical Structure.*

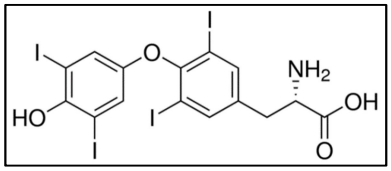


Figure 2. *Chemical structure of L –Thyroxine.*

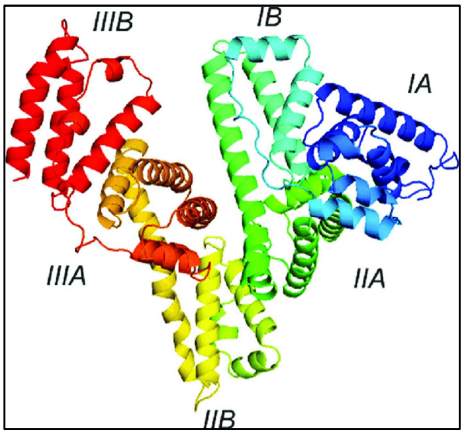


Figure 3. *Heart shaped Human Serum Albumin and its subdomains (Brij M. et al, 2009).*

The main protein part of blood plasma is the human serum albumin as shown in Figure 3. It is present in all body fluids, For example its blood concentration is about 40 mg ml^{-1}($\sim$0.6m mol^{-1}). It is synthesized and has a half-life of 19 days in the liver [2, 6]. A single polypeptide chain of 585 amino acids in length consists of human serum albumin, which in a heart-shaped conformation integrates three domains of homology (labeled I, II and III). Every domain is subdivided into smaller subdomains, A and B, each having six and four alpha α-helices [7].

Human serum albumin has a high plasma concentration (35-50 g / L human serum) and is used in various essential bio-functions, such as osmotic pressure maintenance and nutritional transfer to cells from the circulation system [8]. The osmotic pressure required for the proper distribution of body fluids between the body tissues and intravascular compartments is important for controlling and maintaining [9].

Human serum albumin has capable of binding numerous endogenous and exogenous ligands in the physiological system, counting fatty acids, hormones, pharmaceuticals, metabolites, metal ions, water, various drugs and many other compounds. Such intrinsic properties make human serum albumin a desirable protein material for medicinal use mainly in the delivery of a wide variety of medications or diagnostics. In addition, human serum albumin has become the most widely used biopolymer-based nanocarriers because of its attractive stability, biodegradability, promising distribution of size and easily regulated surface chemistry due to drug delivery and medical diagnosis [6, 10-12].

Several high and low affinity ligand binding sites have been differentiated on human serum albumin, the primary of which are responsible for the binding of most protein-associated pharmaceuticals to be known as sites I and II. Site I appears to bind relatively large heterocyclic or dicarboxylic acid compounds [13]. Furthermore, this site is large and capable of binding bulky endogenous substances. By differentiating, because binding is more stereo-specific, location II is smaller and less flexible in nature. Within human serum albumin, extra high affinity binding regions are vital for certain drugs and compounds that do not adhere to either position I or II [14].

One of the special feature of human serum albumin 's primary structure is that it has a single tryptophan at amino acid position 214, which is situated within center of subdomain IIA. Since of the location of tryptophan amino acids in Human serum albumin and characteristic fluorescence properties. It provides protein chemists and biochemists with a profitable investigative method. Since the single tryptophan residue, fluorescence quenching tests, molar concentration determination, fluorescence lifetime studies, etc., several spectroscopic and fluorescence studies have been possible [6, 11, 15].

A spectroscopic technique, including (FT‐ IR) Spectroscopy, fluorescence spectroscopy, ultraviolet visible absorption spectroscopy, also investigates the molecular interactions between human serum albumin and certain compounds. Due to their high sensitivity, precision, rapidity and fast implementation, these methods have been used to investigate the interaction of small molecule substances and proteins.

The aim of the present work is to study the interaction between Propylthiouracil and L-Thyroxine and human serum albumin (HSA). The importance of the study comes from the fact that Propylthiouracil and L-Thyroxine applying different supportive data including a binding parameter, quenching, and nature of binding. Therefore, Propylthiouracil and L-Thyroxine and transportation by proteins in the blood plasma becomes an important issue which requires investigations of the interaction mechanisms and determining the binding constant between Propylthiouracil and L-Thyroxine and HSA. In order to attain these objectives, UV-Vis absorption spectroscopy, fluorescence spectroscopy and FTIR spectroscopy were employed to carry out detailed investigation of Propylthiouracil and L-Thyroxine -HSA association.

2. Materials, Sample Preparations and Instrumentations

2.1. Materials and Sample Preparations

Human serum albumin, Propylthiouracil (6-propyl-2-thiouracil), and L-Thyroxine were acquired from

Sigma Aldrich Company and utilized without advance purification.

Human serum albumin (HAS), its molecular weight is 66.478 kilo Dalton (KDa). Propylthiouracil (6-propyl-2-thiouracile) with a Formula of chemicals ($C_7H_{10}N_2OS$) and a weight of molecule is 170.23 $g.mol^{-1}$, L-Thyroxine with a Formula of chemicals ($C_{15}H_{11}I_4NO_4$) and a weight of molecule is 776.87 $g.mol^{-1}$, and Dulbecco's phosphate buffered saline is used to prepare the sample. As spectroscopic cell windows, optical grade silicon windows (NICODOM Ltd) are used. Sigma Aldrich Company bought these windows.

The information were collected utilizing samples within the shape of thin films of HSA, Propylthiouracil mixed with HSA, and L-Thyroxine mixed with HSA.

2.1.1. Preparation of HSA Stock Solution

In phosphate buffer Saline (pH 7.4) HSA was dissolved, with concentration of (80mg/2ml).

2.1.2. Preparation of Propylthiouracil Stock Solution

The molar concentration of stock propylthiouracil solution is 0.01M. The desired concentrations achieved by using the molarity dilution equation ($M_iV_i = M_fV_f$). the following concentrations of propylthiouracil (0.35, 0.3, 0.25, 0.2 and 0.1 mM) were prepared in phosphate buffer solution (pH 7.4) by using the molarity dilution equation.

2.1.3. Preparation of L-Thyroxine Stock Solution

The molar concentration of stock L-Thyroxine solution is 0.01M. The desired concentrations achieved by using the molarity dilution equation ($M_iV_i = M_fV_f$). the following concentrations of L-Thyroxine ($0.35, 0.3, 0.25, 0.2$ and 0.1 mM) were prepared in phosphate buffer solution (pH 7.4) by using the molarity dilution equation.

2.1.4. HSA- Propylthiouracil Solutions

The final concentration of HSA-propylthiouracil solutions was arranged to make the HSA-Hormone complex by mixing equal volumes of HSA and propylthiouracil. The HSA concentration was maintained at 40mg/ml in all samples. The propylthiouracil concentration in the last solution is (0.35, 0.3, 0.25, 0.2 and 0.1 mM) in any case.

2.1.5. HSA- L-Thyroxine Solutions

The final concentration of HSA-L-Thyroxine solutions was arranged to make the HSA- Hormone complex by combining the same amount of HSA and L-Thyroxine. The HSA concentration was maintained at 40mg/ml in all samples. In either case, the L-thyroxine concentration is (0.35, 0.3, 0.25, 0.2 and 0.1 mM) in the final solution.

2.1.6. Thin Film Preparations

Silicon windows (NICODOM Ltd) are used as windows for spectroscopic cells. The 100% line of a NICODOM silicon window indicates that the silicon bands do not show add-up absorption in the mid-IR region and can be efficiently subtracted. A silicon widow was dispersed with 20µl of each HSA-propylthiouracil or L-Thyroxine sample and the dissolvable was evaporated using an incubator, to get a clear thin film on the silicon window. At a room temperature of 25°C, both solutions were arranged at the same time.

2.2. Instrumentations

2.2.1. Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR measurements were acquired with a liquid KBr beam splitter and a nitrogen-cooled MCT detector on a Bruker IFS 66 / S spectrophotometer. During the measurements, the spectrometer was persistently washed with dry air. Information was analyzed using the software Bruker Uncertainties 66 / S and the origin program.

Within the wave number range of 400-4000 cm^{-1}, the absorption spectrum was obtained. To broaden the signal to noise ratio, a spectrum of 126 scans was taken as an average. Baseline correction, normalization, Fourier self-deconvolution and measurements of peak areas were performed by Optic Consumer (OPUS) software for all the spectra. The second derivative of the continuum was used to determine the peak positions [16, 17].

The HSA, HSA-propylthiouracil or L-thyroxine complex infrared spectra were obtained within the 1200-1800 cm^{-1} region. By subtracting the buffer solution absorption spectrum from the protein solution spectrum, the FT-IR spectrum of free HSA was procured. The differential spectra (protein and propylthiouracil or L-thyroxine-protein solution) were produced using the featureless locale of the 1800-2200 cm^{-1} protein solution as an inside standard solution for the net interaction effect [6, 12, 18].

2.2.2. UV-VIS Spectrophotometer (NanoDrop ND-1000)

Use the NanoDrop ND-1000 spectrophotometer, the absorption spectrum of HSA with propylthiouracil or L-Thyroxine was obtained. It is used to calculate, with high precision, the sample absorption spectrum is in the range of 220-750 nm.

2.2.3. Fluorospectrometer (NanoDrop 3300)

NanoDrop ND-3300 Fluoro-spectrophotometer at 25°C was used for the fluorescence measurements. One of the three solid-state LEDs produces the excitation source. The excitation source options include: 365 nm UV LED with greatest excitation, 470 nm Blue LED with excitation, and excitation from 500 to 650 nm white LED.

3. Results and Discussion

UV absorption spectrums are discussed and analyzed in the first section. The second section deals with the effects of Fluorescence spectroscopy. Spectroscopy charts and information analysis of Fourier transform infrared (FT-IR) are given in the last section.

3.1. UV-Absorption Spectroscopy and Binding Constant (K)

One of the successful techniques used to investigate the interaction between hormones and HSA was UV-absorption spectroscopy. The absorption spectra of different concentration

of propylthiouracil and L-Thyroxine with fixed amount of HSA are shown in Figure 4 and Figure 5. On the other hand, the excitation was done at 210 nm, while the absorption was recorded at 280 and 277 nm. The figures show that the UV intensity of HSA increases as the concentration of propylthiouracil and L-Thyroxine increases, and the absorption peaks of this solutions showed moderate shifts.

The spectrum of pure hormones propylthiouracil and L-thyroxine showed little or no absorption of UV. These results support the assumption that the peak shifts between the free HSA solution and hormones are due to the interaction between hormones and HSA complexes.

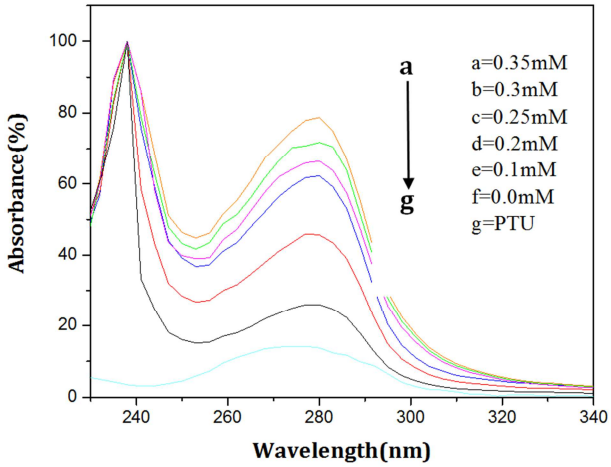


Figure 4. *UV-absorbance HSA spectra at varying propylthiouracil concentrations* $(a = 0.35\ mM, b = 0.3mM, c = 0.25mM, d = 0.2mM, e = 0.1\ mM, f = 0.0mM\ and\ g = propylthiouracil)$

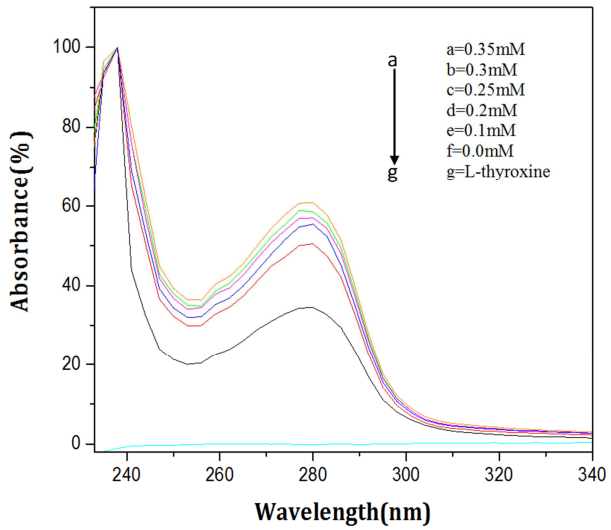


Figure 5. *UV-absorbance HSA spectra at varying concentrations of L-thyroxine* $(a = 0.35\ mM, b = 0.3mM, c = 0.25mM, d = 0.2mM, e = 0.1\ mM, f = 0.0mM\ and\ g = L - Thyroxine)$.

(Propylthiouracil or L-Thyroxine)-HSA complexes binding constants were decided utilizing UV- absorption comes about concurring to published method [20-23], by accepting there is only one sort of interaction between Propylthiouracil or L-Thyroxine and HSA in aqueous solution, which leads to set up equations (1) and (2) as follows:

$$\text{HSA+Propylthiouracil} \leftrightarrow \text{Propylthiouracil: HAS} \quad (1)$$

$$\text{HSA+ L-Thyroxine} \leftrightarrow \text{L-Thyroxine: HSA}$$

$$K = [\text{Propylthiouracil: HSA}]/[\text{Propylthiouracil }][\text{HSA}] \quad (2)$$

$$K=[\text{L-Thyroxine: HSA}]/[\text{L-Thyroxine }][\text{HSA}]$$

The absorption data on the following equation were treated utilizing linear double reciprocal plots [19].

$$\frac{1}{A-A_0} = \frac{1}{A_\infty - A_0} + \frac{1}{K(A_\infty - A_0)} \times \frac{1}{L} \quad (3)$$

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

The double reciprocal plot of $1/(A - A_0)$ vs. 1/L is Linear for HSA with different concentrations of propylthiouracil or L-Thyroxine as shown in the Figure 6 and Figure 7. It is possible to calculate the binding constant (K) from the ratio of the intercept to the slope to be $(1.659 \times 10^3 M^{-1})$ for propylthiouracil-HSA complexes, while that $(1.013 \times 10^4 M^{-1})$ for L-Thyroxine-HSA complexes, The binding constant values obtained are indicative of a weak propylthiouracil-HSA and L-Thyroxine complexes interaction with respect to other strong drug-HSA complexes with the binding constant in the range of 10^5 and $10^6 M^{-1}$ [6, 19, 23, 24].

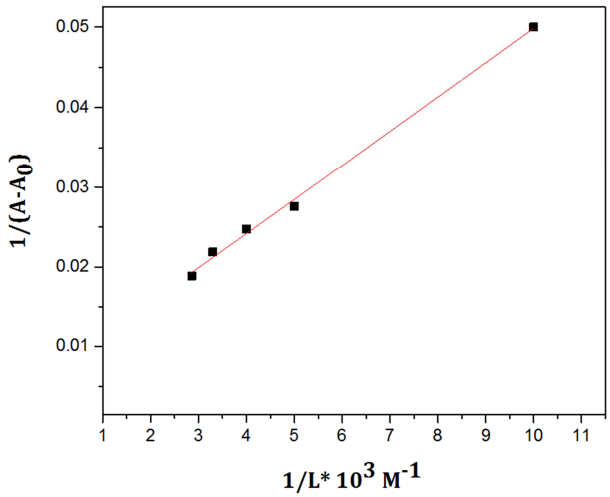


Figure 6. *The plot of* $1/(A - A_0)$ *vs.* $1/L$ *for HSA with different concentrations of propylthiouracil.*

3.2. Fluorescence Spectroscopy

Fluorescence spectroscopy has long been one of the most useful biophysical procedures accessible to researchers considering the structure and work of biological molecules, especially proteins.

Protein comprises three fluorescent aromatic amino acids: tryptophan, tyrosine and phenylalanine. Tryptophan, which is the dominant intrinsic fluorophore, is normally present in

proteins at around% mol. If it is ionized or near to an amino group, a carboxyl group, or a tryptophan residue, tyrosine fluorescence is almost completely quenched [25]. In most studies, phenylalanine is not excited since phenylalanine's quantum yield in proteins is low, so emissions from phenylalanine residues in proteins are rarely observed [26].

The fluorescence emission spectra of HSA at different concentrations of Propylthiouracil (0.35, 0.3, 0.25, 0.2, 0.1)×10^{-3} mol L^{-1} are shown in Figure 8, and the fluorescence emission spectra of HSA at different concentrations of L-Thyroxine (0.35, 0.3, 0.25, 0.2, 0.1)×10^{-3} mol L^{-1} are shown in Figure 9.

So far we see in Figures 8 and 9 the fluorescence emission spectra of HSA at different concentration of propylthiouracil or L-Thyroxine showed that HSA had a strong fluorescence emission band at wavelength 394 nm. Moreover it can be seen that, increasing the concentration of propylthiouracil or L-Thyroxine from (0.1mM to 0.35mM) in the system with HSA causes a decrease in fluorescence intensity without changing the shape of the peaks. These results indicated that there was an interaction between propylthiouracil or L-Thyroxine and HSA.

3.2.1. Determination of Stern-Volmer Quenching Constants (K_{sv}) and the Quenching Rate Constant of Biomolecule (K_q)

Fluorescence quenching includes any type that reduces the fluorescence intensity of a sample. Different mechanisms, generally divided into dynamic quenching and static quenching, may cause fluorescence quenching.

Dynamic quenching results from collisional between the fluorophore and the quencher and static quenching resulting from the formation of a dynamic ground state between the fluorophore and the quencher [19, 27].

The Stern-Volmer equation explains collisional quenching of fluorescence:

$$\frac{F_0}{F} = 1 + K_q\tau_0[L] = 1 + K_{sv}[L] \quad (4)$$

Where K_{sv} is the Stern- Volmer constant, and τ_0 is the mean fluorescence life time of the fluorophore in the absence of quencher, and L is the quencher concentration. In addition to F_0 and F, respectively, the intensity of fluorescence in the absence and presence of a quencher.

The K_{sv} value indicates the importance of the accessibility of Fluorophores to the quencher. Although the meaning of K_q gives an indication of the nature of the quencher's diffusion within the medium [28].

As shown in Figures 10 and 11, linear curves have been plotted according to the Stern-Volmer equation for propylthiouracil-HSA and L-Thyroxine- HSA complexes, respectively. From the curve slope obtained in Figures 10 and 11, the quenching constant K_{sv} was obtained. The K_{sv} values for propylthiouracil-HSA or L-Thyroxine- HSA complexes equal to $(2.144 \times 10^3\ \text{L mol}^{-1},\ 1.937 \times 10^3 \text{L mol}^{-1})$, respectively.

The Stern- Volmer quenching constant can be represented as $K_{sv} = K_q t_0$ that we can obtain the value of K_q using the fluorescence life time of 10^{-8}s for HSA. we calculated the K_q values for propylthiouracil-HSA and L-Thyroxine-HSA complexes equal to $(2.144 \times 10^{11}\ \text{L mol}^{-1}\ \text{S}^{-1},\ 1.937 \times 10^{11}\ \text{L mol}^{-1}\text{S}^{-1})$, respectively. There are larger than the maximum dynamic quenching constant for different quenchers with biopolymer ($2 \times 10^{10}\text{L mol}^{-1}\ \text{S}^{-1}$). It could indicate that the quenching is not initiated by a dynamic collision, but by the formation of a complex that results in static quenching dominance [19, 23].

3.2.2. Determination of Binding Constant (k) by Fluorescence Spectroscopy

The modified Stern-Volmer could be used when static quenching is dominant [29].

$$\frac{1}{F_0 - F} = \frac{1}{F_0 K(L)} + \frac{1}{F_0} \quad (5)$$

Where K is the binding constant of Propylthiouracil or L-Thyroxine with HSA, and can be obtained by plotting $1/(F_0\text{-}F)$ vs. 1/L of HSA hormones system as shown in Figure 12 and Figure 13. The obtained values of K for Propylthiouracil or L-Thyroxine –HSA complexes are $(1.756 \times 10^3\text{M}^{-1}, 1.017 \times 10^4\text{M}^{-1})$, respectively. That agrees well for the value results provided via UV spectroscopy and confirms the successful function of static quenching.

We can see L-Thyroxine has higher binding constant of $(1.013\text{-}1.017 \times 10^4\text{M}^{-1})$ than propylthiouracil $(1.659 - 1.756 \times 10^4\text{M}^{-1})$. For the two molecules, the contrast is based on molecular structure variation. The binding constant of L-Thyroxine arises from the electrostatic interaction of the anionic phenolate group of thyroxine with cationic lysyl ϵ-amino groups in HAS [17, 30].

Propylthiouracil is sulfhydryl compound, Sulfhydryl (-SH) is a functional group that may perform a role in the control of binding processes for several ligands. In secondary, tertiary or quaternary protein structures, disulfide (-S-S-) bonds bind the required amino acids together for functional purposes [6, 31]. Propyl group is also needed for the binding of propylthiouracil to HSA. This binding is strengthened by hydrophobic interaction between the propyl group in propylthiouracil and a polar substituents on HSA.

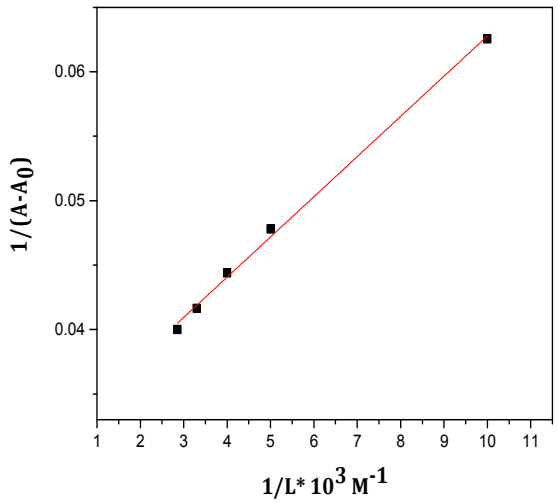


Figure 7. *The plot of $1/(A - A_0)$ vs. $1/L$ for HSA with different concentrations of L-Thyroxine.*

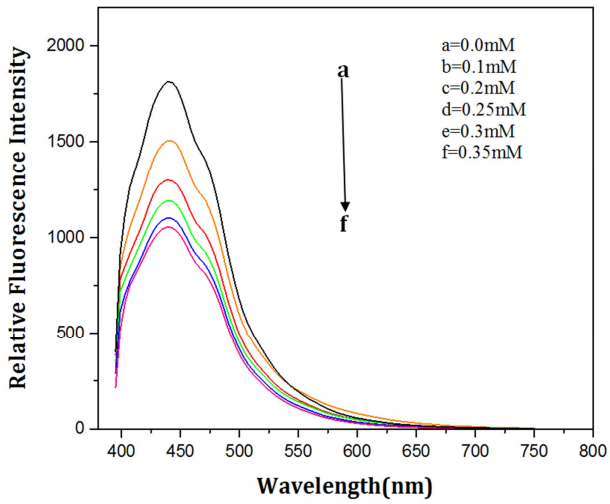


Figure 8. *In the absence and presence of Propylthiouracil in these concentrations, the fluorescence emission spectra of HSA*($a = 0.0\ mM, b = 0.1mM, c = 0.2mM, d = 0.25mM, e = 0.3\ mM, f = 0.35mM$).

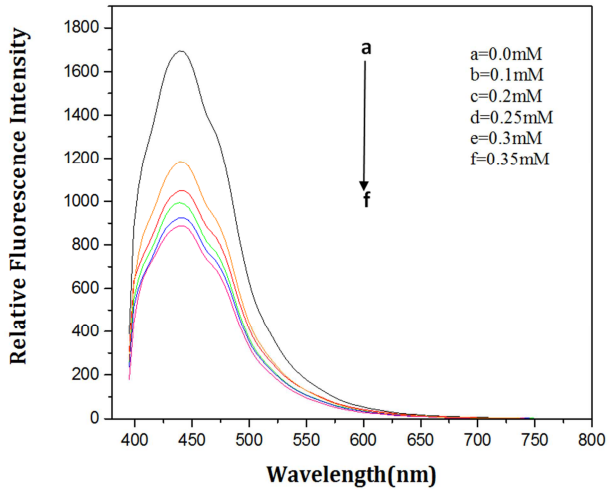


Figure 9. *In the absence and presence of L-Thyroxine in these concentrations, the fluorescence emission spectra of HSA* ($a = 0.0\ mM, b = 0.1mM, c = 0.2mM, d = 0.25mM, e = 0.3\ mM, f = 0.35mM$)

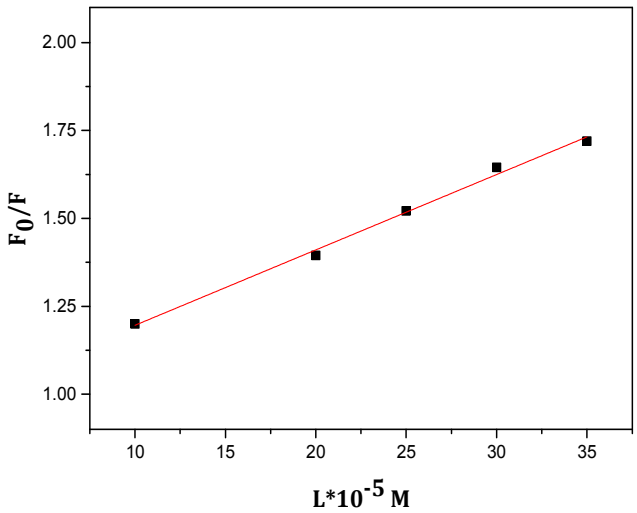


Figure 10. *The Stern –Volmer plot propylthiouracil-HSA system.*

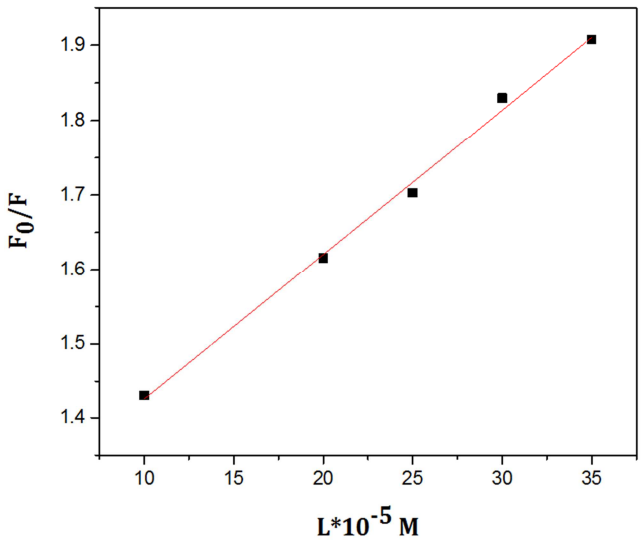


Figure 11. *The Stern –Volmer plot L-Thyroxine-HSA system.*

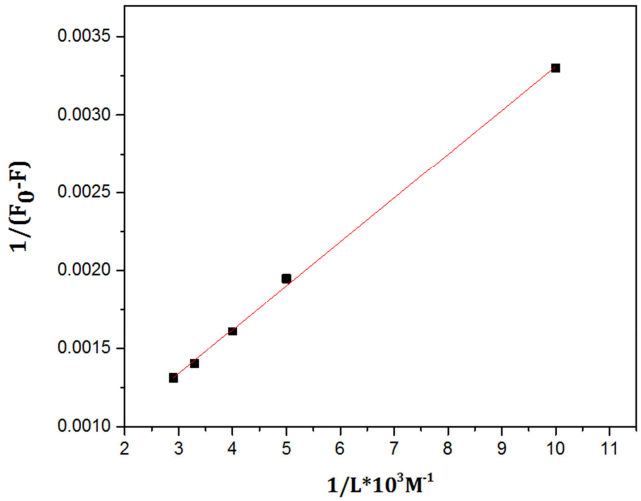


Figure 12. *The plot of plot* $1 / (F_0 - F)$ *vs.* $1 / [L] * 10^3 M^{-1}$ *of HSA-Propylthiouracil system.*

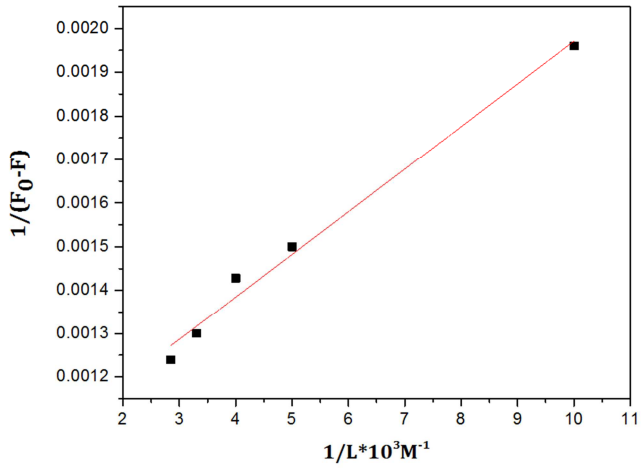


Figure 13. *The plot of plot* $1 / (F_0 - F)$ *vs.* $1 / [L] * 10^3 M^{-1}$ *of HSA- L-Thyroxine system.*

3.3. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy is a sensitive and useful technique

which may be relevant to protein structure investigations and studies. It is useful non-destructive, requires less planning for samples, and can be used in a wide range of conditions. It provides data on the protein content in the secondary structure. This could arise from the amide band that comes from the vibrations of the groups around the protein peptide. The attachment between peptides involves changes in hydrogen bonding. When drugs and globular proteins such as HSA are associated, this may alter the amide modes' vibrational frequency [23, 32].

The FTIR was used in this research study to investigate changes in the HSA secondary structure after the binding of propylthiouracil and L-Thyroxine to HSA. Infrared protein spectral data is commonly interpreted in terms of structural unit vibrations. These repeated units give rise to nine characteristic bands of infrared absorption, called A, B, and I-VII amide. This is a product of the groups vibrating around the protein peptide.

Protein structure analysis by FTIR spectroscopy is mainly performed using the amide I, II and III band. With a significant contribution from C=O stretching vibration and a small contribution from C-N stretching vibration, Amide I absorption is directly connected to the backbone conformation. The absorption of amide I occurs in $1600-1700\ cm^{-1}$. The amide II absorption results both from $N-H$ bending vibration and $C-N$ stretching vibration. The absorption of this band occurs in $1480-1600\ cm^{-1}$. Amide III absorption occurs in $1220-1330\ cm^{-1}$. This band originates from the $C-N$ stretching vibration as well as $N-H$ in-plane bending vibration, with weak contribution from $C-C$ stretching and $C=O$ in-plane bending vibration [19].

The spectra of HSA-propylthiouracil with concentrations (0.0, 0.1, 0.2, 0.25, 0.3, 0.35 mM), and the spectra of HSA-L-Thyroxine with concentrations (0.0, 0.1, 0.2, 0.25, 0.3, 0.35 mM) are shown in Figure 14 and Figure 15, respectively.

The peak positions of HSA with different propylthiouracil and L-Thyroxine concentrations for amide I, II and III regions are listed in Table 1, and Table 2, respectively. For HSA-propylthiouracil system, the amide I bands of HSA infrared spectrum shifted as listed in table 1: 1613 to 1609 cm^{-1}, 1627 to 1625 cm^{-1}, 1641 to 1639 cm^{-1}, 1658 to 1656 cm^{-1}, 1675 to 1674 cm^{-1}, 1691 to 1689 cm^{-1} after interaction with propylthiouracil. In amide II region of the peak positions have shifted in the following order: 1514 to 1513 cm^{-1}, 1530 to 1528 cm^{-1}, 1548 to 1546 cm^{-1}, 1564 to 1565 cm^{-1}, 1582 to 1580 cm^{-1}, 1598 to 1593 cm^{-1}. In amide III region of the peak position have shifted in the following order: 1224 to 1225 cm^{-1}, 1247 to 1242 cm^{-1}, 1264 to 1266 cm^{-1}, 1293 to 1299 cm^{-1}, 1308 to 1307 cm^{-1}.

For HSA- L-Thyroxine interaction, the amide I bands of HSA infrared spectrum shifted as listed in table 2: 1613 to 1609 cm^{-1}, 1627 to 1625 cm^{-1}, 1641 to 1639 cm^{-1}, 1658 to 1656 cm^{-1}, 1675 to 1674 cm^{-1}, 1691 to 1689 cm^{-1} after interaction with propylthiouracil. In amide II region of the peak positions have shifted in the following order: 1514 to 1513 cm^{-1}, 1530 to 1528 cm^{-1}, 1548 to 1546 cm^{-1}, 1564 to 1565 cm^{-1}, 1582 to 1580 cm^{-1}, 1598 to 1593 cm^{-1}. In amide III region of the peak position have shifted in the following order: 1224 to 1225 cm^{-1}, 1247 to 1242 cm^{-1}, 1264 to 1266 cm^{-1}, 1293 to 1299 cm^{-1}, 1308 to 1307 cm^{-1}.

Changes in peak positions and peak shapes have explained that the secondary structures of HSA have been influenced by the interaction of propylthiouracil or L-thyroxine with HSA.

The difference spectra for [(HSA+ Propylthiouracil or L-Thyroxine)-(protein)] were collected in able to monitor the differences in intensity of these vibrations. The results are display in Figures 16, 17, 18 and 19). For HSA-Propylthiouracil interaction, Figure 16 shows FTIR spectra (top two curves) and difference spectra of HSA and its complexes with different propylthiouracil concentrations in the amide I and amide II regions.

In the amide I region, a strong negative feature appears at 1654 cm^{-1}, In amide II a strong negative feature appears at 1545 cm^{-1}. for amide III two negative features appear at 1308 cm^{-1} and 1243 cm^{-1} as shown in Figure 17. These features were detected at a low concentration of Propylthiouracil (0.2mM).

For HSA-L-Thyroxine interaction, Figure 18 shows FTIR spectra (top two curves) and difference spectra of HSA and its complexes with different L-Thyroxine concentrations in the amide I and amide II regions.

In the amide I region, a strong negative feature appears at 1656 cm^{-1}, In amide II a strong negative feature appears at 1545 cm^{-1}. for amide III two negative features appear at 1318 cm^{-1} and 1242 cm^{-1} as shown in Figure 19. These features were observed at low L-Thyroxine concentration (0.3mM).

The C-O stretching vibration in propylthiouracil has main contribution at $1627\ cm^{-1}$ and at $1626\ cm^{-1}$. The FTIR bands in amide III of propylthiouracil assigned to C-N stretching mode of vibrations [33].

In dehydrated films, in order to obtain quantitative data of the protein secondary structure for free HSA, HSA-Propylthiouracil, HSA-L-Thyroxine complexes are calculated from the amide I, II and III band shape. In the range of (1700-1600 cm^{-1}), (1600-1480 cm^{-1}), and (1330-1220 cm^{-1}), baseline correction was performed to obtain amide bands I, II and III. For the purpose of increasing spectral resolution and thus estimating the numbers, location and each component band's area, to such three ranges, Fourier self-deconvolution and second derivative were then applied respectively. To bring the best Lorentzian-shaped curves to match the original HSA spectrum, the curve-fitting method has been done by origin.

In keeping with the frequency of its maximum, Amide I, II and III regions were allocated to secondary structures after Fourier self-deconvolution was applied.

Amide I was divided as follows: 1610- 1640 cm^{-1} representing β -sheets, 1640–1650 cm^{-1} to random coil, 1650–1658 $cm^{-1}$to α-helix, and 1660–1700 cm-1 to β-turn structure.

In the case of amide II, The absorption band consists of four parts and is distributed in the order below: 1488-1500 $cm^{-1}$to β-sheets, 1504-1525 cm^{-1} to random coil, 1527-1560

$cm^{-1}$to α-helix and 1564-1585 to turn structure.

The component bands of Amide III have been allocated as follows: α-helix 1330– 1290 cm^{-1}, β-turn 1290–1270 cm^{-1}, random coil 1270–1250 $cm^{-1}$and β-sheet 1250–1220 cm^{-1}.

Identifying the secondary structure of the free HSA and its propylthiouracil or L-thyroxine complexes with different hormone concentrations is given in (Table 3 and Table 4). secondary structure determinations of the free human serum albumin and its propylthiouracil or L-Thyroxine complexes and the second derivative resolution enhancement and curve-fitted amide I, amide II, and amide III regions are shown in Figures 20, 21, 22, 23, 24, 25, 26, 27, 28, and 29.

However the following results were observed for propylthiouracil or L-thyroxine -HSA interaction:

For amide I: HSA free consisted of α-helical (55%), β sheet (16%), random coil (15%), and β-turn structure (14%). After propylthiouracil-HSA interaction, α-helical decreased from (55%) to (46%), βsheet increased from (16%) to (23%), random coil increased from (15%) to (16%), and β-turn structure increased from (14%) to (19%). For L-thyroxine-HSA interaction α-helical decreased from (55%) to (46%), βsheet increased from (16%) to (23%), random coil increased from (15%) to (19%), and β-turn structure increased from (14%) to (19%).

For amide II: HSA free consisted of α-helical (50%), β-sheet (18%), random coil (13%), and β-turn structure (19%). After propylthiouracil-HSA interaction, α-helical decreased from (50%) to (45%), βsheet increased from (18%) to (20%), random coil decreased from (13%) to (12%), and β-turn structure increased from (19%) to (23%). For L-thyroxine-HSA interaction α-helical decreased from (50%) to (48%), β-sheet decreased from (18%) to (15%), random coil decreased from (13%) to (11%), and β-turn structure increased from (19%) to (25%).

For amide III: HSA free consisted of α-helical (50%), β-sheet (19%), random coil (14%), and β-turn structure (17%). After propylthiouracil-HSA interaction, α-helical decreased from (50%) to (41%), βsheet increased from (19%) to (23%), random coil increased from (14%) to (19%), and β-turn structure increased from (17%) to (20%). For L-thyroxine-HSA interaction α-helical decreased from (50%) to (41%), βsheet increased from (19%) to (25%), random coil increased from (14%) to (21%), and β-turn structure increased from (17%) to (18%).

The decrease of the α-helical percentage with the increase of propylthiouracil or L-thyroxine concentration is evident and this trend is consistent for three Amide regions. For the β-sheet the relative percentage increased with the increase of propylthiouracil or L-thyroxine concentration.

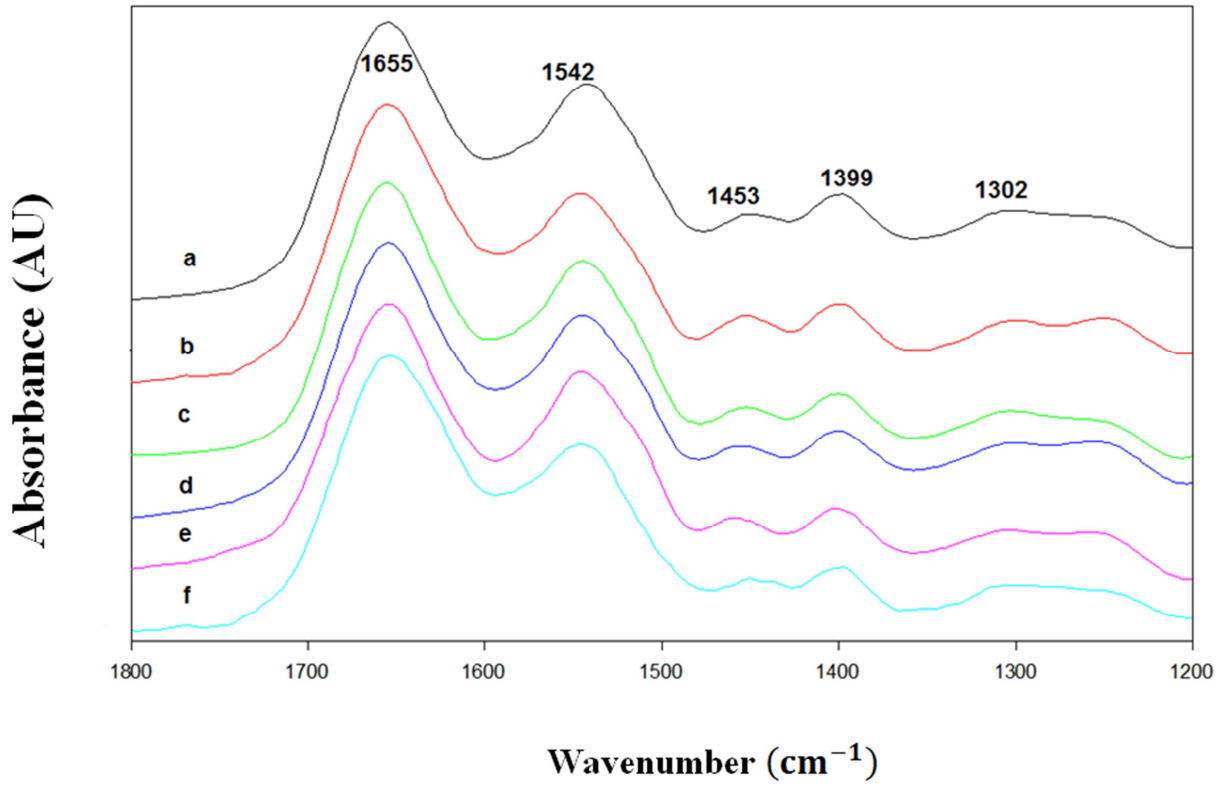


Figure 14. *The spectra of HSA-propylthiouracil with concentrations* ($a = 0.0mM, b = 0.1mM, c = 0.2mM, d = 0.25mM, e = 0.3, f = 0.35\ mM$).

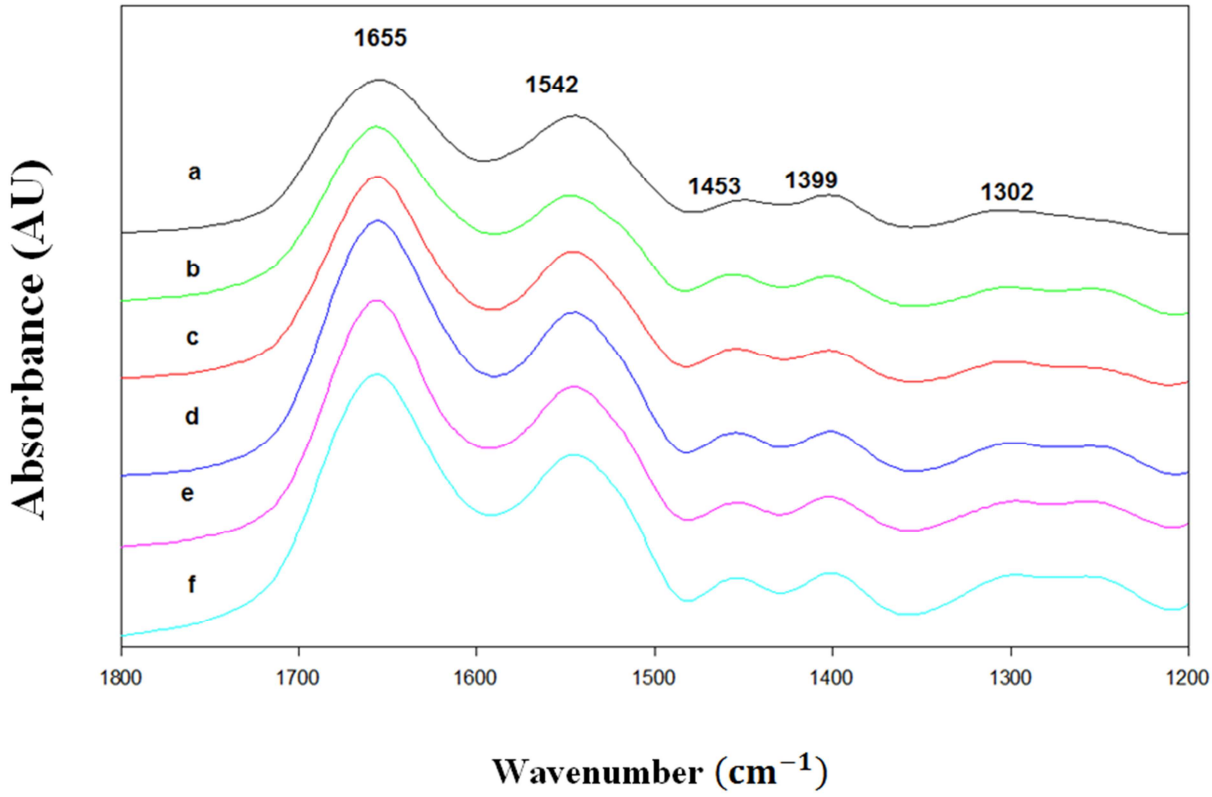


Figure 15. *The spectra of HSA-L-Thyroxine with concentrations* ($a = 0.0mM, b = 0.1mM, c = 0.2mM, d = 0.25mM, e = 0.3, f = 0.35\ mM$).

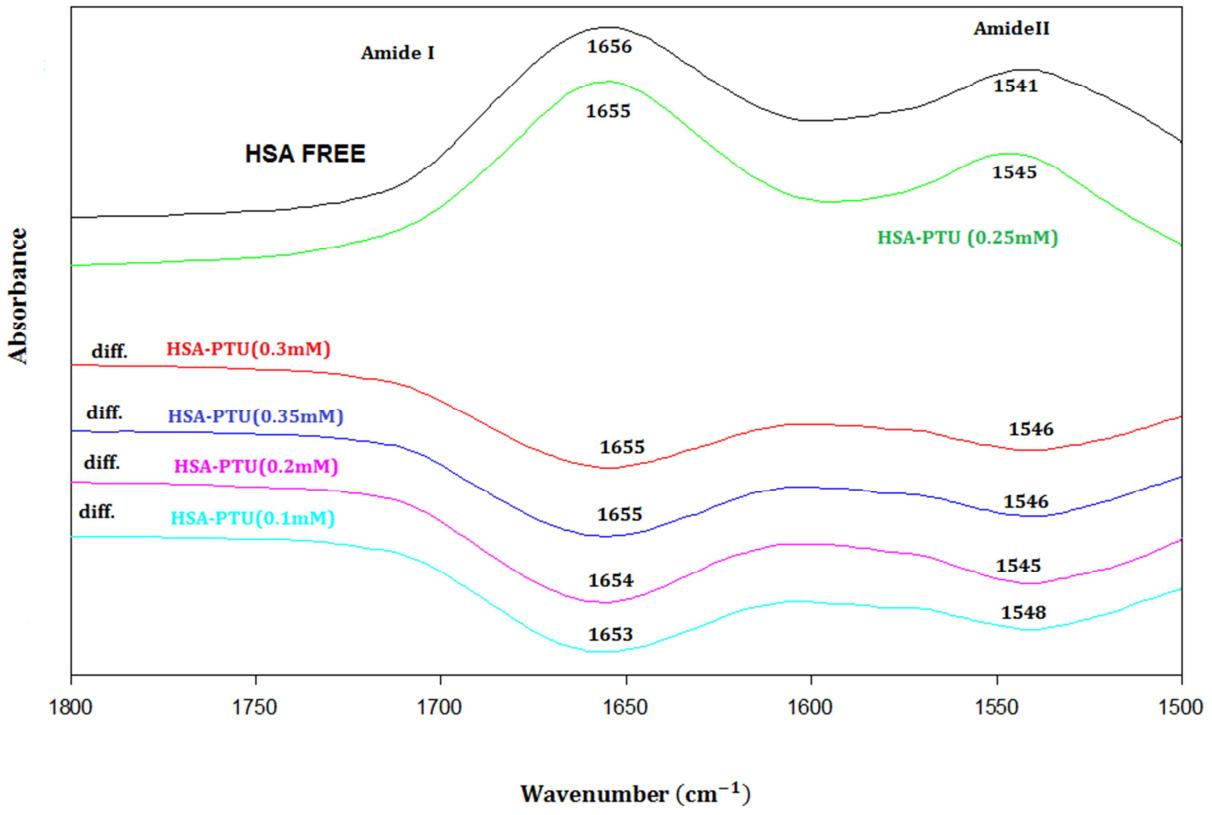


Figure 16. *FTIR spectra* (*top two curves*) *and difference spectra of HSA and its complexes with different propylthiouracil concentrations in the region of 1800-1500* cm^{-1}.

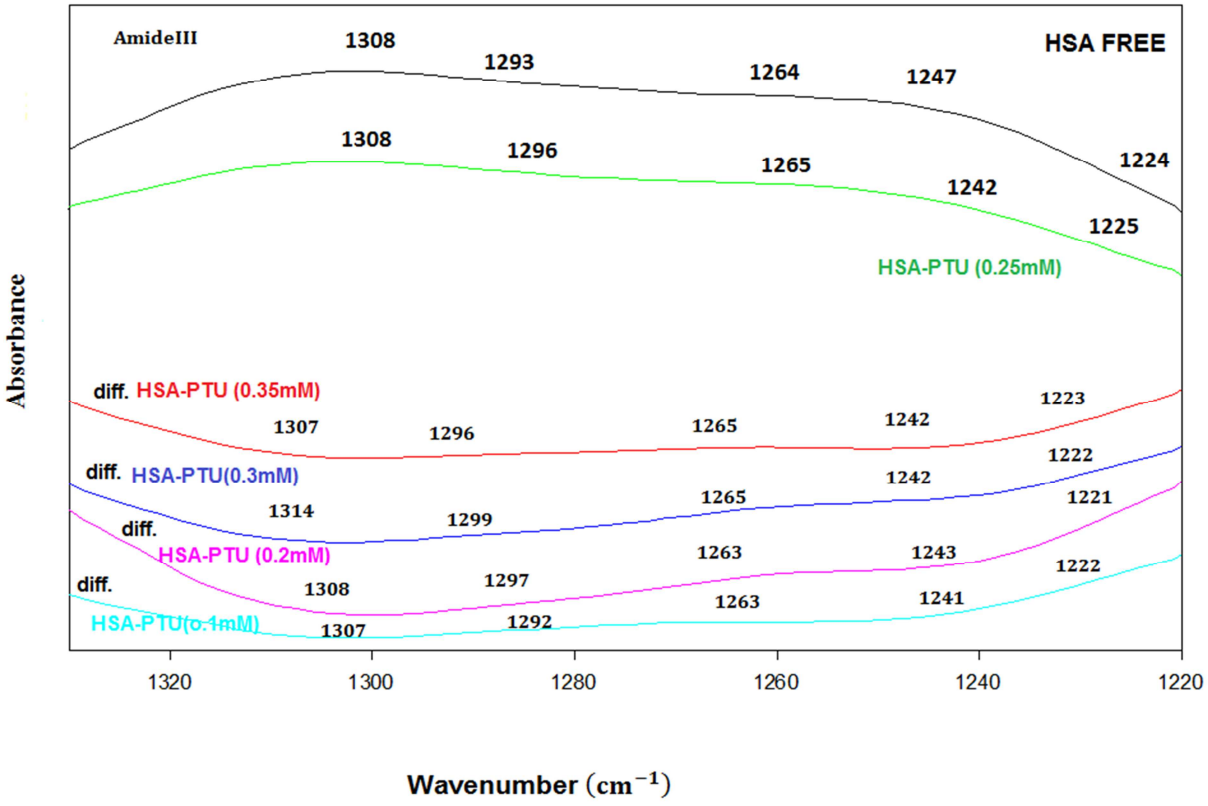


Figure 17. *FTIR spectra (top two curves) and difference spectra of HSA and its complexes with different propylthiouracil concentrations in the region of 1330-1220 cm* $^{-1}$.

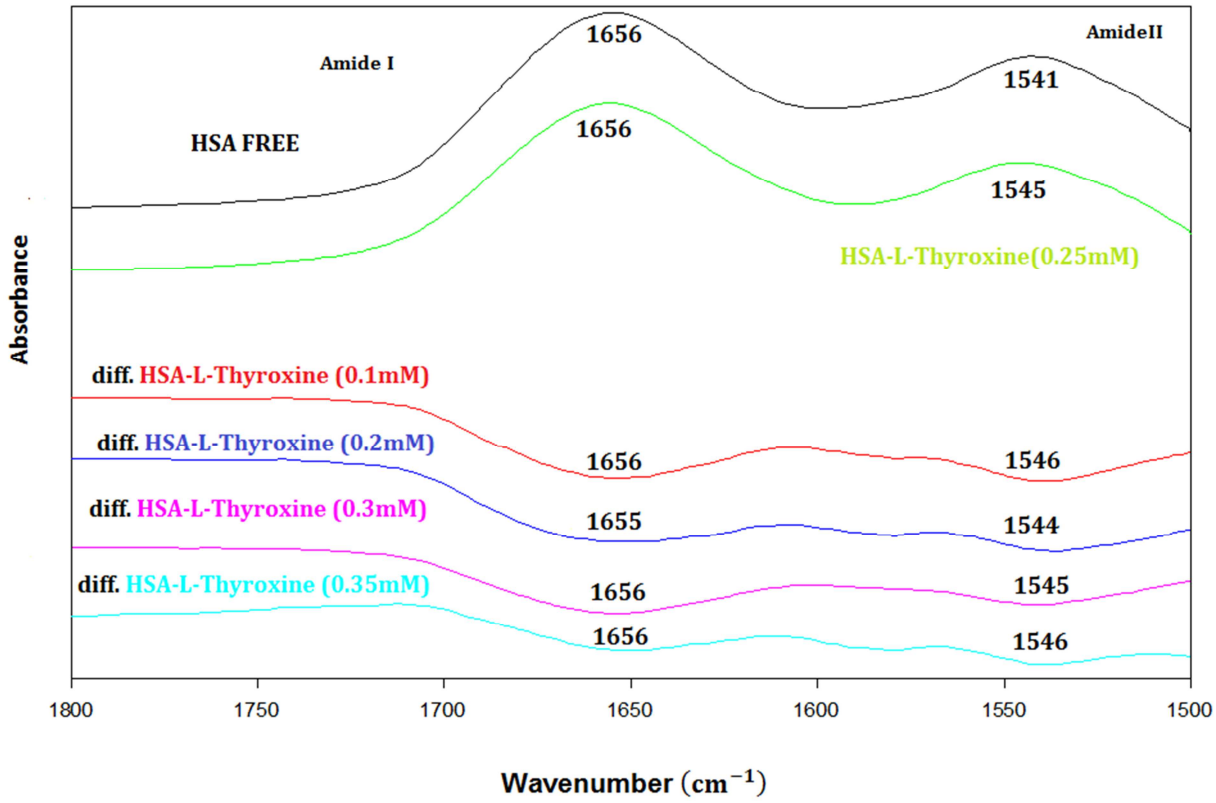


Figure 18. *FTIR spectra (top two curves) and difference spectra of HSA and its complexes with different L-Thyroxine concentrations in the region of 1800-1500 cm* $^{-1}$.

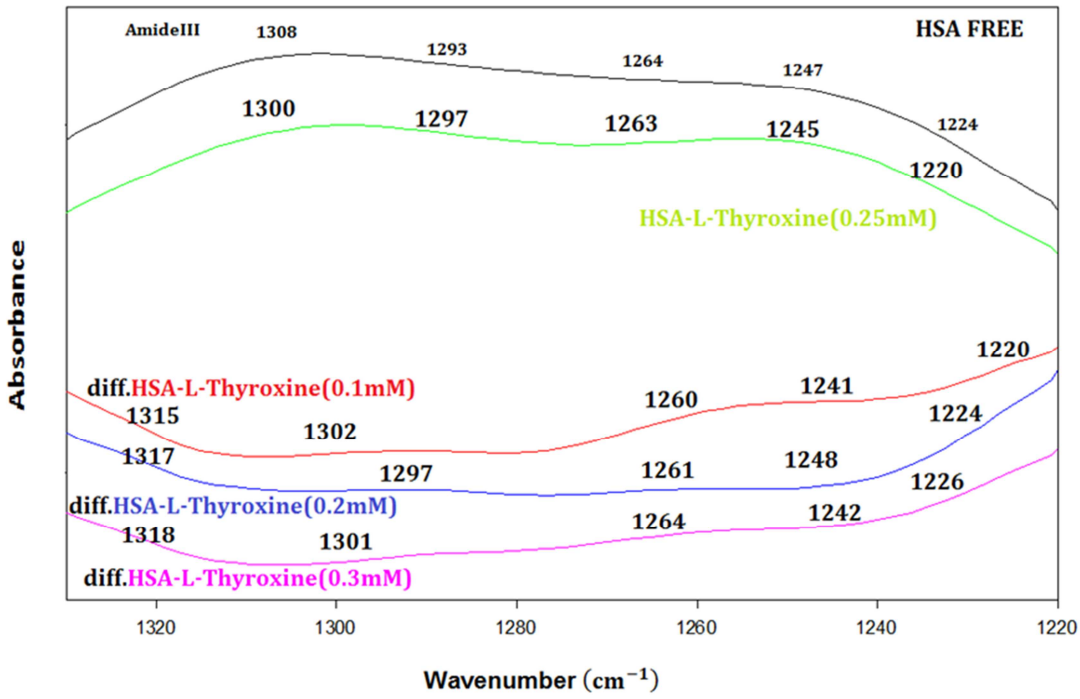


Figure 19. *FTIR spectra (top two curves) and difference spectra of HSA and its complexes with different L-Thyroxine concentrations in the region of 1330-1220 cm $^{-1}$.*

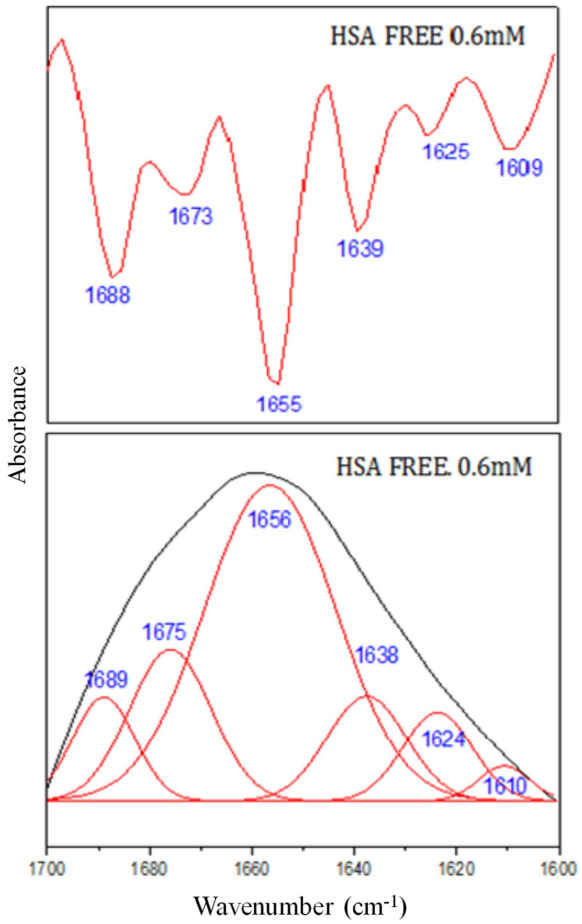


Figure 20. *Second-derivative resolution and curve-fitted area of amide I (1700-1612cm^{-1}) and secondary structure assessment of the free human serum albumin.*

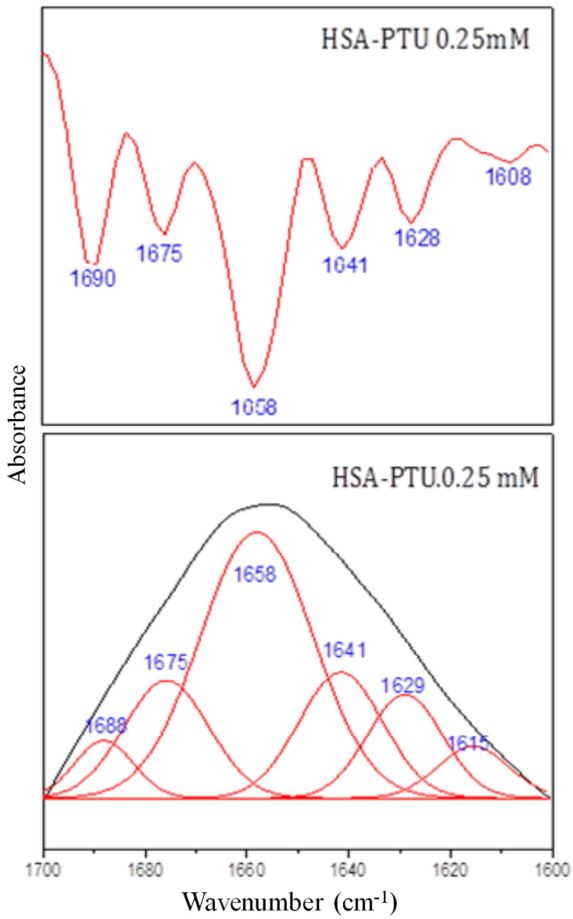


Figure 21. *Second-derivative resolution and curve-fitted area of amide I region (1700-1612cm^{-1}) and secondary structure assessment of the 0.25mM concentration of HSA-Propylthiouracil complexes.*

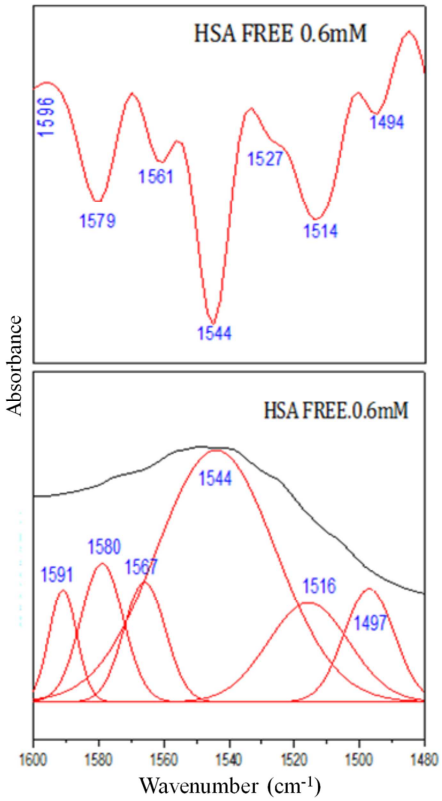


Figure 22. *Second-derivative resolution and curve-fitted area of amide I (1600-1480cm^{-1}) and secondary structure assessment of the free human serum albumin.*

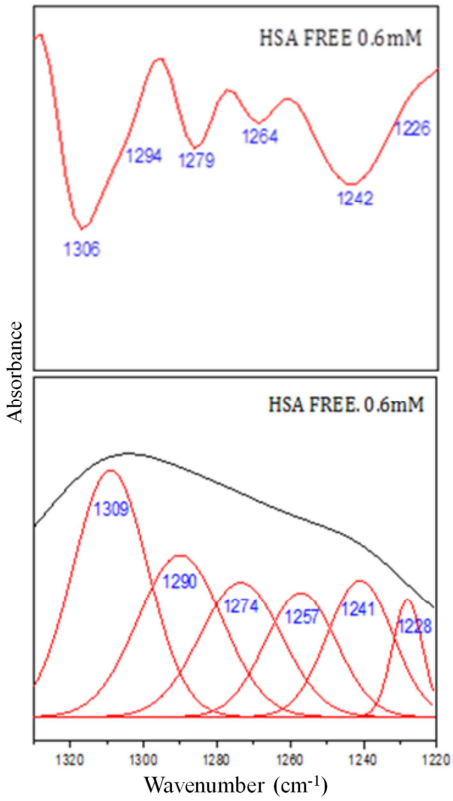


Figure 24. *Second-derivative resolution and curve-fitted area of amide I (1330-1220cm^{-1}) and secondary structure assessment of the free human serum albumin.*

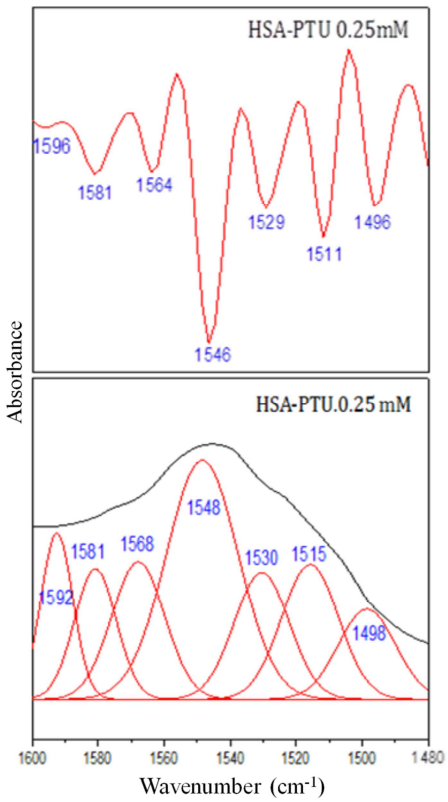


Figure 23. *Second-derivative resolution and curve-fitted area of amide I region (1600-1480 cm^{-1}) and secondary structure assessment of the 0.25mM concentration of HSA-Propylthiouracil complexes.*

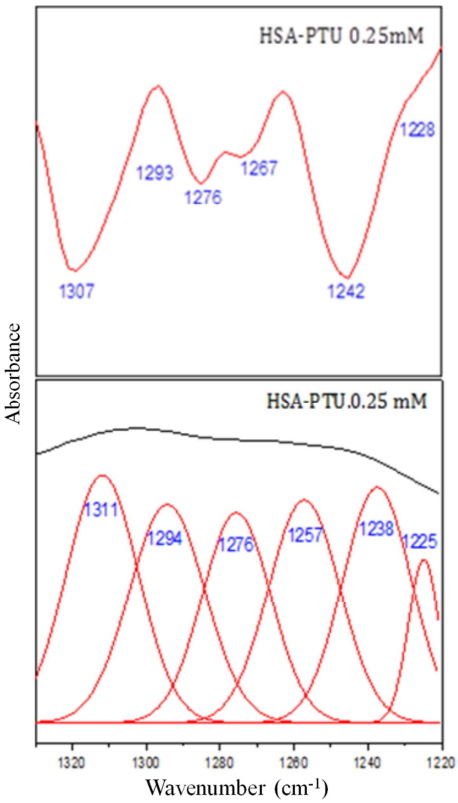


Figure 25. *Second-derivative resolution and curve-fitted area of amide I region (1330-1220 cm^{-1}) and secondary structure assessment of the 0.25mM concentration of HSA-Propylthiouracil complexes.*

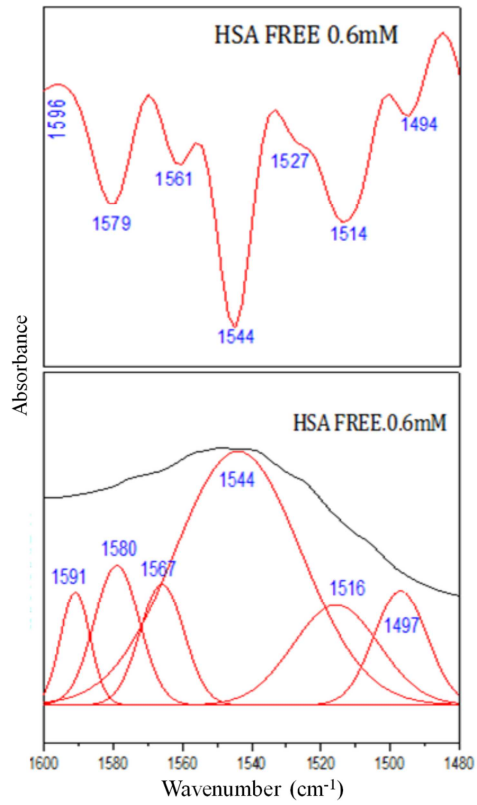


Figure 26. *Second-derivative resolution and curve-fitted area of amide II (1600-1480cm^{-1}) and secondary structure assessment of the free human serum albumin.*

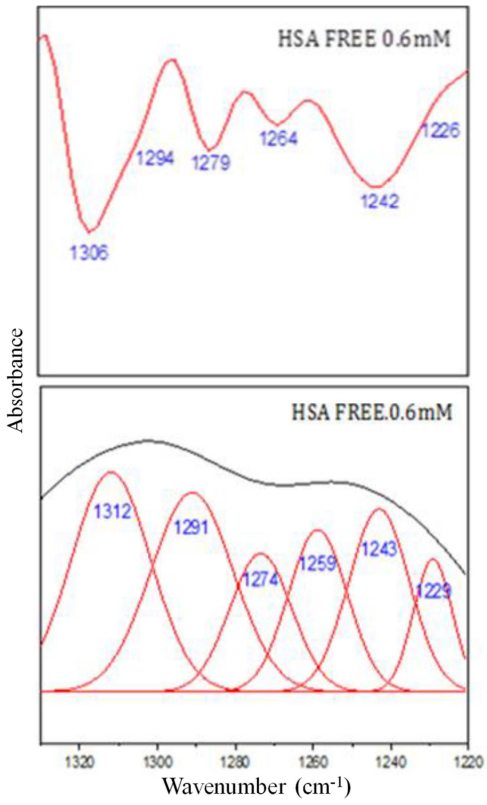


Figure 28. *Second-derivative resolution and curve-fitted area of amide III (1330-1220cm^{-1}) and secondary structure assessment of the free human serum albumin.*

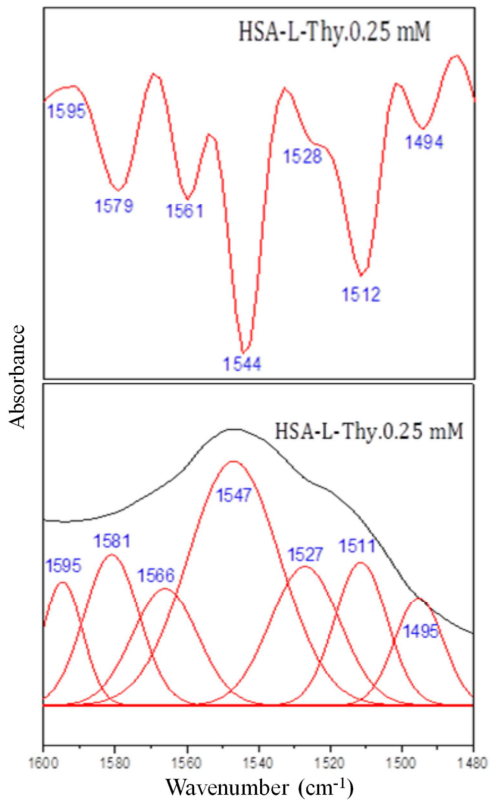


Figure 27. *Second-derivative resolution and curve-fitted area of amide II region (1600-1480 cm^{-1}) and secondary structure assessment of the 0.25mM concentration of HSA-Propylthiouracil complexes.*

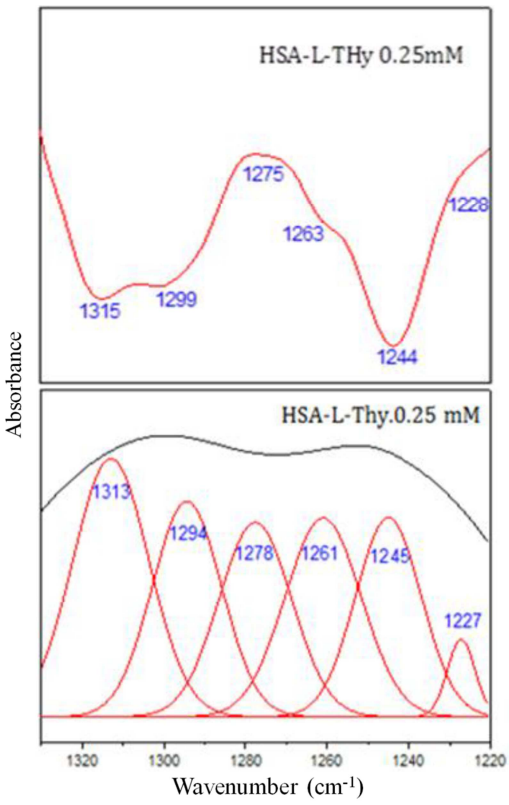


Figure 29. *Second-derivative resolution and curve-fitted area of amide III region (1330-1220 cm^{-1}) and secondary structure assessment of the 0.25mM concentration of HSA-Propylthiouracil complexes.*

Table 1. *Band assignments in the absorbance spectra of HSA with different Propylthiouracil concentrations for amide I, II and III regions.*

Bands	**HSA FREE**	**HSA-PTU. 0.1mM**	**HSA-PTU. 0.2mM**	**HSA-PTU. 0.25mM**	**HSA-PTU. 0.3mM**	**HSA-PTU. 0.35mM**
	1615	1612	1612	1609	1611	1613
	1625	1626	1625	1625	1627	1627
Amide I	1639	1639	1639	1639	1641	1641
$(1600-1700)cm^{-1}$	1657	1657	1656	1656	1658	1658
	1674	1677	1675	1675	1675	1675
	1689	1689	1690	1690	1691	1691
	1513	1511	1514	1513	1513	1514
	1529	1528	1529	1530	1530	1530
AmideII	1546	1549	1547	1546	1548	1541
$(1480-1600)cm^{-1}$	1564	1565	1564	1565	1565	1564
	1581	1580	1581	1581	1582	1582
	1598	1593	1598	1596	1598	1598
	1222	1223	1225	1223	1223	1224
AmideIII	1243	1242	1244	1242	1245	1247
$(1220-1330)cm^{-1}$	1264	1266	1265	1265	1263	1264
	1294	1291	1295	1299	1295	1293
	1307	1308	1308	1308	1308	1308

Table 2. *Band assignments in the absorbance spectra of HSA with different L-Thyroxine concentrations for amide I, II and III regions.*

Bands	**HSA FREE**	**HSA-PTU. 0.1mM**	**HSA-PTU. 0.2mM**	**HSA-PTU. 0.25mM**	**HSA-PTU. 0.3mM**	**HSA-PTU. 0.35mM**
	1609	1608	1608	1607	1608	1613
	1624	1624	1623	1622	1623	1627
Amide I	1638	1638	1638	1637	1637	1641
$(1600-1700)cm^{-1}$	1654	1655	1655	1654	1654	1658
	1672	1672	1672	1671	1672	1675
	1687	1687	1687	1689	1688	1691
	1511	1511	1511	1509	1511	1514
	1527	1526	1527	1524	1527	1530
AmideII	1544	1544	1544	1543	1543	1541
$(1480-1600)cm^{-1}$	1564	1566	1564	1565	1564	1564
	1579	1579	1579	1578	1579	1582
	1595	1595	1595	1594	1595	1598
	1221	1220	1220	1224	1220	1224
AmideIII	1244	1248	1245	1242	1247	1247
$(1220-1330)cm^{-1}$	1261	1263	1263	1264	1263	1264
	1301	1297	1301	1302	1302	1293
	1316	1300	1317	1318	1315	1308

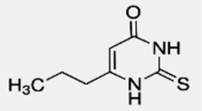
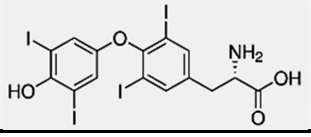
Table 3. *Secondary structure determination for amide I, II and III regions in HSA and its propylthiouracil complexes.*

Bands	**HSA FREE**	**HSA-PTU. 0.1mM**	**HSA-PTU. 0.2mM**	**HSA-PTU. 0.25mM**	**HSA-PTU. 0.3mM**	**HSA-PTU. 0.35mM**
$Amide\ I(1600-1700)cm^{-1}$						
β-sheets$(cm^{-1})(1610-1640)(1680-1700)$	16	20	19	23	20	21
Random$(cm^{-1})(1640-1650)$	15	15	15	16	16	16
$\alpha\text{-}helix(cm^{-1})(1650-1660)$	55	52	49	46	48	47
Turn $(cm^{-1})(1660-1680)$	14	15	19	15	16	16
$Amide\ II(1480-1600)cm^{-1}$						
β-sheets$(cm^{-1})(1488-1500)(1587-1598)$	18	19	20	19	17	19
Random$(cm^{-1})(1504-1525)$	13	14	12	14	13	14
$\alpha\text{-}helix(cm^{-1})(1527-1560)$	50	45	46	44	47	45
Turn $(cm^{-1})(1564-1585)$	19	22	22	23	23	22
$Amide\ III(1220-1330)cm^{-1}$						
β-sheets$(cm^{-1})(1220-1250)$	19	23	22	23	22	21
Random$(cm^{-1})(1250-1270)$	14	16	18	19	16	18
Turn $(cm^{-1})(1270-1290)$	17	20	18	17	19	19
$\alpha\text{-}helix(cm^{-1})(1290-1330)$	50	41	42	41	43	42

Table 4 *Secondary structure determination for amide I, II and III regions in HSA and its L-Thyroxine complexes.*

Bands	HSA FREE	HSA-PTU. 0.1mM	HSA-PTU. 0.2mM	HSA-PTU. 0.25mM	HSA-PTU. 0.3mM	HSA-PTU. 0.35mM
$Amide\ I(1600-1700)cm^{-1}$						
β-sheets$(cm^{-1})(1610-1640)(1680-1700)$	16	20	19	23	17	20
Random$(cm^{-1})(1640-1650)$	15	14	14	16	19	18
α-$helix(cm^{-1})(1650-1660)$	55	51	48	46	49	47
Turn $(cm^{-1})(1660-1680)$	14	15	19	15	15	15
$Amide\ II(1480-1600)cm^{-1}$						
β-sheets$(cm^{-1})(1488-1500)(1587-1598)$	18	17	17	15	18	17
Random$(cm^{-1})(1504-1525)$	13	12	11	12	13	13
α-$helix(cm^{-1})(1527-1560)$	50	49	50	48	48	48
Turn $(cm^{-1})(1564-1585)$	19	22	22	25	21	22
$Amide\ III(1220-1330)cm^{-1}$						
β-sheets$(cm^{-1})(1220-1250)$	19	22	25	20	24	22
Random$(cm^{-1})(1250-1270)$	14	21	17	19	17	21
Turn $(cm^{-1})(1270-1290)$	17	16	14	18	18	14
α-$helix(cm^{-1})(1290-1330)$	50	41	44	43	41	43

Table 5. *Propylthiouracil and L-Thyroxine with HSA comparison.*

Hormone- HSA binding	Molecular structure	Binding Constant (UV-VIS)	Stern-Volmer constant (K_{sv})	Binding Constant (fluorescence)
Propylthiouracil		$1.756\times10^{3}\mathrm{M}^{-1}$	$2.144\times10^{3}\mathrm{L\ mol}^{-1}$	$1.659\times10^{3}\mathrm{M}^{-1}$
L-Thyroxine		$1.017\times10^{4}\mathrm{M}^{-1}$	$1.937\times10^{3}\mathrm{L\ mol}^{-1}$	$1.013\times10^{4}\mathrm{M}^{-1}$

4. Conclusions

Spectroscopic techniques (UV-VIS spectroscopy, Fluorescence spectroscopy and FT-IR spectroscopy) have been used to investigate the interaction between thyroid hormones (propylthiouracil and L-thyroxine) and HSA.

The determined binding constant for propylthiouracil-HSA complexes was K=$1.659\times10^{3}\mathrm{M}^{-1}$ using the UV spectrum, while that for L-Thyroxine-HSA complexes $1.013\times10^{4}\mathrm{M}^{-1}$. The fluorescence spectrum analysis yielded the binding constant to be K= $1.756\times10^{3}\mathrm{M}^{-1}$, $1.017\times10^{4}\mathrm{M}^{-1}$ for propylthiouracil-HSA and L-Thyroxine-HSA complexes (Table 5). That is well in line with the value previously acquired by UV spectroscopy and promotes static quenching's effective function. In addition, the Stern- Volmer values and the constants of the quenching rate for propylthiouracil and L-thyroxine are calculated to be (2.144×10^{3} L mol^{-1}, 2.144×10^{11} L $\mathrm{mol}^{-1}\mathrm{S}^{-1}$) and ($1.937\times10^{3}$ L mol^{-1}, 1.937×10^{11} L $\mathrm{mol}^{-1}\mathrm{S}^{-1}$), respectively. This research indicates that static quenching is responsible of fluorescence quenching (decrease in intensity), which is an indicator of complex protein and hormone formations.

Our FT-IR spectrum study shows that increasing propylthiouracil and L-thyroxine concentration contributes to protein unfolding and a decrease in the percentage of alpha-helical structure in favor of β-sheet structure.

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