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RESEARCH ARTICLE

SPECTROSCOPIC APPROACH OF PROTEIN- ANDROGEN COMPLEXES: STUDY OF TESTOSTERONE INTERACTIONS WITH HUMAN SERUM ALBUMIN "HSA"

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ABSTRACT

The molecular interactions between HSA and Testosterone have been successfully investigated. The absorption, distribution and metabolism of many molecules can be altered based on their affinity to HSA. HSA is often increases the apparent solubility of hydrophobic ligands in plasma and modulate their delivery to cells. In this study, the interaction between Testosterone and HSA has been investigated using UV-VIS absorption spectrophotometry and FT-IR spectroscopy; binding constant and the effects on the protein secondary structure have been confirmed. From UV-VIS absorption spectrophotometry which showed an increase in the absorption intensity with increasing the molecular ratios of testosterone to HSA, it is found that the value of the binding constant of testosterone to HSA, K equals 34.9×102 M^{-1}. FT-IR spectroscopy in the mid infrared region with Fourier self deconvolution, second derivative, difference spectra, peak picking and curve fitting were used to determine the effect of Testosterone on the protein secondary structure in the amides I, II and III regions. From the FTIR absorbance spectra, it is found that the intensity of the absorption bands increased with increasing the molecular ratios of Testosterone, where from the deconvoluted and curve fitted spectra, it is found that the absorbance intensity for α- helices decreases relative to β-sheets; this decrease in intensity is related to the formation of H- bonding in the complex molecules.

INTRODUCTION

Hormones are the most familiar to the general public, due probably to the widespread pharmacological use and abuse of steroid hormones for diverse purposes, such as contraception and body building (Hardie 1991). Steroids hormones mainly can act as a chemical messenger in a wide range of species and target tissues to produce both slow genomic responses and rapid non-genomic responses (Norman *et al.,* 2004). They have many physiological effects on human body; their incorrect concentration may cause abnormalities in human body (Abu Teir *et al.,* 2010). Steroid hormones help control metabolism, inflammation, immune functions, salt and water balance, development of sexual characteristics and the ability to withstand illness and injury (Frye 2009). In human all steroid hormones are derived from cholesterol and differ only in the ring structure and side chains attached to it (Brandt 1999) (Brandt 1999), so as cholesterol is a non-polar and hydrophobic molecule steroid hormones are insoluble in water but lipid soluble thus they have to be carried in the blood bound to specific carrier proteins such as sex hormone-binding globulin or corticosteroid-binding globulin. Sex steroid binding globulin carries testosterone and estradiol (Frye 2009). Testosterone is a steroid hormone from the androgen group which is found in males and in smaller amount in female. It is 7-8 times concentrated in human males' plasma than in human females. The metabolic consumption of testosterone in males is greater. Testosterone is classified as a strong androgen and secreted primarily from the testicles of males and the ovaries of females, while small amounts of testosterone and weak androgens such as anabolic steroids are secreted by the adrenal gland (Reed *et al.,* 2006).

The chemical structure of testosterone is as shown in Figure (1) ($C_{19}H_{28}O_2$) (Brandt 1999). It is classified as Δ4 steroid as the double bond (un-saturation site) is located at 4-5 position. Testosterone chemical structure lacks the 2-carbon side-chain attached to the 17 position existed in the cholesterol structure, making it a 19-carbon steroid, also the side-chain has been replaced by a 17β-hydroxyl (Brandt 1999). Human serum albumin HSA is an abundant plasma protein that binds a wide variety of hydrophobic ligands including fatty acids, bilirubin, thyroxine and hemin and also drugs (Carter *et al.,* 1989; Abu Teir *et al.,* 2010). The most important physiological role for the protein is to bring such solutes into blood stream and then deliver them to the target organs, as well as to maintain the PH and osmotic pressure of plasma (Norman *et al.,* 2004). HSA concentration in human plasma is 40 mg/ml (Tushar *et al.,* 2008). The molecular interactions between HSA and some compounds have been investigated successfully (Gudrun *et al.,* 2002; Ouameur *et al.,* 2004; Ji-Sook *et al.,* 2006; Abu *et al.,* 2014; Darwish *et al.,* 2010). It has recently been proved that

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serum albumin plays a decisive role in the transport and disposition of variety of endogenous and exogenous compound such as fatty acids, hormones, bilirubin, drugs (Tang *et al.,* 2006).

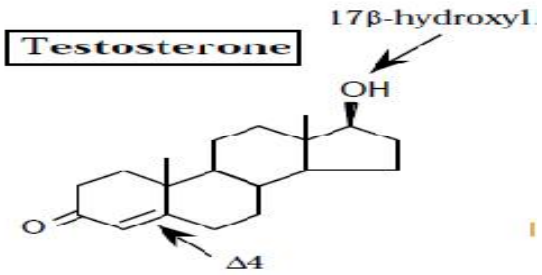

Figure 1. Chemical structure of Testosterone

The distribution and metabolism of many biologically active compounds in our body whether drugs or natural products are correlated with their affinities toward serum albumin which is the most abundant protein carrier in our plasma. So the study of the interaction of such molecules for example testosterone with albumin is of a fundamental importance. Some investigations have been applied on Testosterone-HSA interaction but none of them determined in details neither Testosterone-HSA binding constant (k) nor the effect of testosterone complexes on the protein structure. Some investigations only indicated that the interaction occurred and others used the equilibrium dialysis method to calculate the binding constant (k) (Pearlman and Crepy 1967; Litwack and Axelord 1970). Infrared spectroscopy provides measurements of molecular vibrations due to the specific absorption of infrared radiation by chemical bonds. It is known that the form and frequency of the Amide I band, which is assigned to the C=O stretching vibration within the peptide bonds is very characteristic for the structure of the studied protein. From the band secondary structure, components peaks (α-helix, β-strand) can be derived and the analysis of this single band allows elucidation of conformational changes with high sensitivity (Darwish *et al.,* 2010).

This work will be limited to the mid-range infrared, which covers the frequency range from 4000 to 400 cm^{-1}. This wavelength region includes bands that arise from three conformational sensitive vibrations within the peptide backbone (Amides I, II and III) of these vibrations, Amide I is the most widely used and can provide information on secondary structure composition and structural stability (Cui *et al.,* 2008; Kang *et al.,* 2004; Rondeau *et al.,* 2007; Abu Teir *et al.,* 2010). Other spectroscopy techniques are usually used in studying the interaction of drugs and proteins, fluorescence and UV spectroscopy are commonly used because of their high sensitivity, rapidity and ease of implementation (Wybranowski *et al.,* 2008; Li *et al.,* 2008; Li *et al.,* 2006). The binding of testosterone to HSA was investigated by means of UV-absorption spectroscopy, fluorescence spectroscopy, and FT-IR spectroscopy. Spectroscopic evidence regarding the drug binding mode, drug binding constant and the effects of testosterone on the protein secondary structure are provided in this work.

MATERIALS AND METHODS

Materials

HSA (fatty acid free), testosterone in powder form were purchased from Sigma Aldrich chemical company and used without further purifications. The data were collected using samples in the form of thin films for FT-IR measurements and liquid form for UV-VIS and fluorescence measurements.

Preparation of stock solutions

HSA was dissolved in phosphate buffer saline, at physiological pH 7.4, to a concentration of (80 mg/ml), to get a final concentration of (40 mg/ml) in the final hormone-HSA solution. Testosterone with molecular weight (288.42 g/mol) was dissolved in phosphate buffer saline (0.7622 mg/ml), the phosphate buffer saline was at room temperature, the solution was placed on a shaker for one hour in order to dissolve the testosterone powder with buffer, then it was placed in ultrasonic water path (SIBATA AU-3T) for 8 hours to ensure that the entire amount of testosterone was completely dissolved. The solution was placed in a water path with a temperature range 37-40c° for one hour to let the solution completely dissolved and became homogenous. The final concentrations of HSA-Testosterone solutions were prepared by mixing equal volume of HSA and hormone. HSA concentration in all samples kept at 40 mg/ml. However, the concentration of hormone in the final protein hormone solutions was reduced such that the molecular ratios (HSA: Testosterone) are 10:18, 10:14, 10:10, 10:6 and 10:2. Silicon windows (NICODOM Ltd) were used as spectroscopic cell windows. The optical transmission is high with little or no distortion of the transmitted signal. The 100% line of a NICODOM silicon window shows that the silicon bands in the mid-IR region do not exhibit total absorption and can be easily subtracted. 60 µl of each sample of HSA-Testosterone was spread on a silicon window and an incubator was used to evaporate the solvent, to obtain a transparent thin film on the silicon window. All solutions were prepared at the same time at room temperature.

UV-absorption spectroscopy

The absorption spectra were obtained by the use of a NanoDrop ND-1000 spectrophotometer. It is used to measure the absorption spectrum of the samples in the range between 220-750 nm, with high accuracy and reproducibility. The absorption spectra of different ratios of testosterone with fixed amount of HSA are displayed in Fig. (2). The excitation has been done on 210 nm and the absorption is recorded at 278 nm. The figure shows that the UV-VIS intensity of HSA increases with the increasing of testosterone percentage, and that the absorption peaks of these solutions showed moderate shifts indicating that with the addition of testosterone, the peptide strands of HSA molecules extended more and the hydrophobicity of testosterone was decreased (Peng *et al.,* 2008). The results indicated that an interaction occurred between testosterone and HSA. Obviously, it was seen from the spectrum that pure hormone have little or no UV absorption. This result support that the peak shifts between free

HSA solution and testosterone-HSA complexes are due to the interaction between testosterone and HSA. Repeated measurements were done for all the samples showing consistent results and no significant differences were observed.

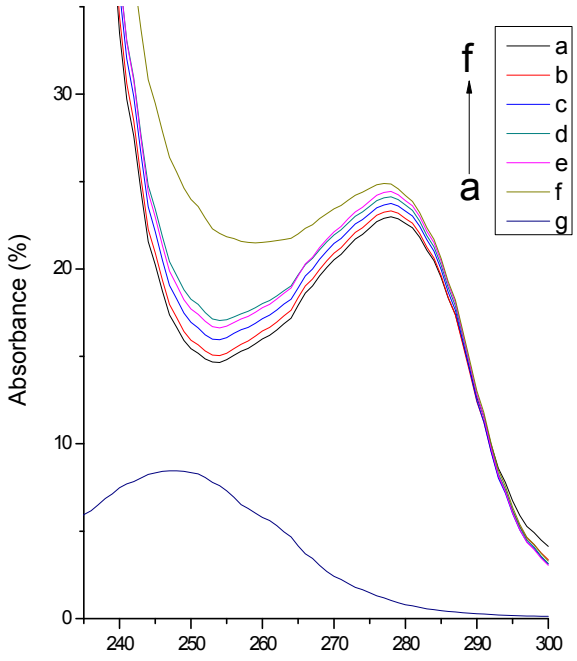

Figure 2. UV-absorbance spectra of HSA with different molar ratios of testosterone (a=0:10, b=2:10, c=6:10, d=10:10, e=14:10, f=18:10, g= free testosterone)

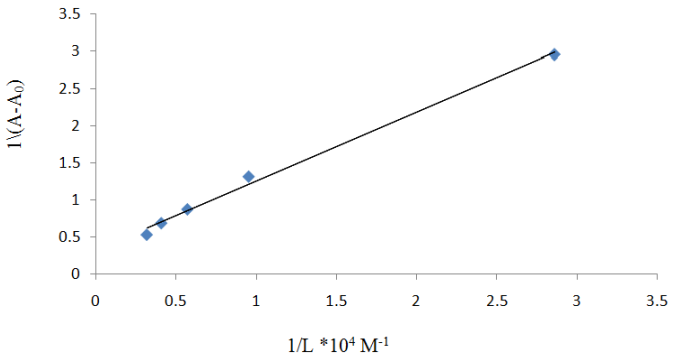

Figure 3. The plot of $1/(A-A_0)$ vs 1/L for HSA with different concentrations of testosterone

Fluorescence spectrophotometer

The fluorescence measurements were performed by a NanoDrop ND-3300 Fluorospectrophotometer at 25°C. The excitation source comes from one of three solid-state light emitting diodes (LED's). The excitation source options include: UV LED with maximum excitation 365 nm, Blue LED with excitation 470 nm, and white LED from 500 to 650nm 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 at the wavelength of 360 nm and the maximum emission wavelength is at 439 nm. In this work for HSA-Testosterone complexes excitation wavelength at 360nm was used, and the observed wavelength emission was at 439 nm. The fluorescence sensor is based on intramolecular charge transfer (ICT), which is highly sensitive to the polarity of microenvironment. Therefore, it is expected to act as fluorescent probe for some biochemical systems like proteins (Tian *et al.,* 2003). The fluorescence quenching spectra of HSA at various percentage of testosterone is shown in Fig. (4). Obviously from the results, the fluorescence intensity of HSA gradually decreased while the peak position shows little change upon increasing the percentage of testosterone, indicating that testosterone binds to HSA. Under the same condition, no fluorescence of testosterone was observed. Which indicates that testosterone could quench the auto fluorescence of HSA, and that the interaction between testosterone and HSA exists, leading to a change in the microenvironment around the tryptophan residue and further exposure of tryptophan residue to the polar solvent (Petitpas *et al.,* 2001; Wang *et al.,* 2008).

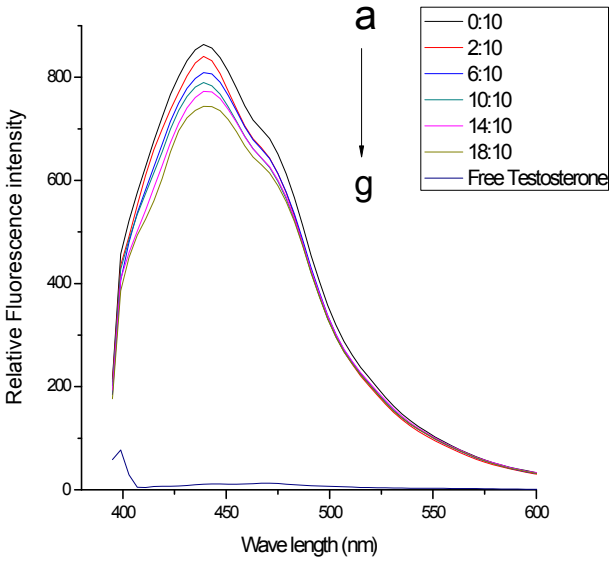

Figure 4. Fluorescence emission spectra of HSA in the absence and presence of testosterone in these ratios (T: HSA a=0:10, b=2:10, c=6:10, d=10:10, e=14:10, f=18:10, g=free testosterone)

FT-IR Spectroscopy Experimental Procedures

The FT-IR measurements were obtained on a Bruker IFS 66/S spectrophotometer equipped with a liquid nitrogen-cooled MCT detector and a KBr beam splitter. The spectrometer was continuously purged with dry air during the measurements. The absorption spectra were obtained in the wave number range of 400–4000 cm^{-1}. A spectrum was taken as an average of 60 scans to increase the signal to noise ratio, and the spectral resolution was at 4 cm^{-1}. The aperture used in this study was 8 mm, since we found that this aperture gives best signal to noise ratio. Baseline correction, normalization and peak areas calculations were performed for all the spectra by OPUS software. The peak positions were determined using the second derivative of the spectra The infrared spectra of HSA, Testosterone-HSA complexes were obtained in the region of 1000-1800 cm^{-1}. The FT-IR spectrum of free HSA was acquired by subtracting the absorption spectrum of the

buffer solution from the spectrum of the protein solution. For the net interaction effect, the difference spectra {(protein and testosterone solution)-(protein solution)} were generated using the featureless region of the protein solution 1800-2200 cm^{-1} as an internal standard (Surewicz *et al.,* 1993). The accuracy of this subtraction method is tested using several control samples with the same protein or drug concentrations, which resulted into a flat base line formation. The obtained spectral differences were used here, to investigate the nature of the drug–HSA interaction.

RESULTS AND DISCUSSION

Analysis of UV-absorption spectroscopy of HSA by Testosterone

Testosterone –HSA complexes binding constant were determined using UV-VIS spectrophotometer results according to published method (Stephanos and Inorg 1996; Klotz and Hunston 1971; Ouameur *et al.,* 2004), by assuming that there is only one type of interaction between testosterone and HSA in aqueous solution, which leads to establish equation (1) and (2) as follows:

HSA + Testosterone ⟷ Testosterone: HSA .....(1)

K = (Testosterone: HSA)/(Testosterone) (HSA) ……..(2)

The absorption data were treated using linear double reciprocal plots based on the following equation (Lakowicz 2006):

$$\frac{1}{A-Ao} = \frac{1}{A\infty-Ao} + \frac{1}{K(A\infty-Ao)} \times \frac{1}{L} \qquad \text{……..(3)}$$

Where A_0 corresponds to the initial absorption of protein at 280 nm in the absence of ligand , A_∞ is the final absorption of the legated protein, and A is the recorded absorption different testosterone concentrations (L). The double reciprocal plot of $1/(A-A_0)$ vs. 1/L is linear as shown in Fig(3) and the binding constant (K) can be estimated from the ratio of the intercept to the slope to be $(0.349 \times 10^4\ M^{-1})$ for testosterone-HSA complexes. The value obtained is indicative of a weak testosterone protein interaction with respect to the other drug-HSA complexes with binding constant in the range of 10^5 and $10^6\ M^{-1}$ (Kargh-Hanse 1981).

Analysis of Fluorescence spectroscopy of HSA by Testosterone

Fluorescence quenching can be defined as a bimolecular process that reduces the fluorescence intensity without changing the fluorescence emission spectrum; it can result from transient excited-state interactions (collisional quenching) or from formation of non-fluorescent ground-state species. As discussed mentioned before assuming dynamic quenching is dominating, then the decreased in intensity is described by the well-known Stern-Volmer equation:

$$F_0/F = 1 + K_q \tau_0 (L) = 1 + K_{sv}(L) \qquad \text{……..(4)}$$

In this expression F and F_0 are the fluorescence intensities with and without quencher, K_{sv} is the Stern-Volmer quenching constant, K_q is the bimolecular quenching constant, τ_0 is average lifetime of the biomolecule without quencher, and (L) is the quencher concentration. The Stern-Volmer quenching constant K_{sv} indicates the sensitivity of the fluorophore to a quencher.

Linear curves were plotted according to the Stern-Volmer equation as shown in Fig. (5) for testosterone-HSA complexes. The Stern-Volmer quenching constant K_{sv} was obtained by the slope of the curves obtained in Fig.(5), and its value equals $(0.0456\times10^4\ L\ mol^{-1})$ for testosterone-HSA complexes. From equation 4 the value of $K_{sv} = K_q \tau_0$, from which we can calculate the value of K_q using the fluorescence life time of 10^{-8} s for HSA (Cheng *et al.,* 2006), to obtain K_q values of $(4.5\times10^{10}\ L\ mol^{-1}s^{-1})$ for testosterone-HSA complexes. Which is larger than the maximum dynamic quenching constant for various quenchers with biopolymer $(2\times10^{10}\ L\ mol^{-1}\ s^{-1})$ (Lakowicz 2006). This implies that the quenching is not initiated by dynamic collision but from formation of a complex, so static quenching is dominant (Cui *et al.,* 2008; Wang *et al.,* 2008).

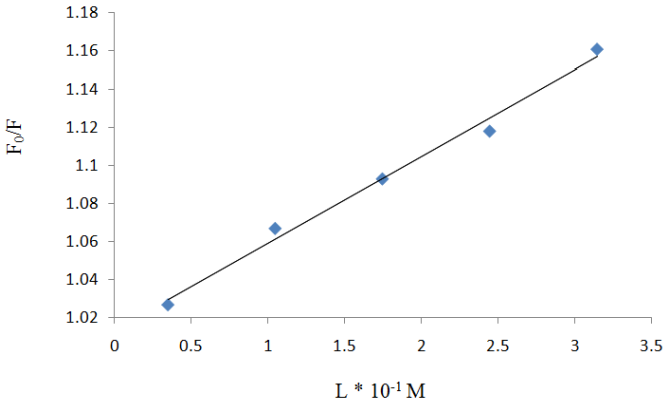

Figure 5. The Stern Volmer plot for testosterone –HSA complexes

When static quenching is dominant the modified Stern-Volmer equation could be used (Yang *et al.,* 1994)

$$\frac{1}{Fo-F} = \frac{1}{FoKL} + \frac{1}{Fo} \qquad \text{……..(5)}$$

Where K is the binding constant of testosterone with HSA, and can be calculated by plotting $1/(F_0-F)$ vs 1/L, see Fig.(6). The value of K equals the ratio of the intercept to the slope. The obtained value of K equals (0.382×10^4) from Fig.(6), which agrees well with the value obtained earlier UV spectroscopy and supports the effective role of static quenching. The highly effective quenching constant in this case has lead to a lower value of binding constant between the hormone and HSA, due to an effective hydrogen bonding between testosterone and HSA (Darwish *et al.,* 2010).

The acting forces between a small molecule substance and macromolecule mainly include hydrogen bond, van der- waals force, electrostatic force and hydrophobic interaction force. It was more likely that hydrophobic and electrostatic interactions were involved in the binding process. However, testosterone

might be largely unionized under the experimental conditions, as expected from its structure. Hence, electrostatic interaction could be precluded from the binding process. Thus, the binding of testosterone to HSA includes the hydrophobic interaction (Cui *et al.,* 2008). Hydrophobic interaction is mostly an entropic effect originating from the disruption of highly dynamic hydrogen bonds between molecules of liquid water by the nonpolar solutes. Minimizing the number of hydrophobic side chains exposed to water is the principal driving force behind the folding process (Pace *et al.,* 1996) formation of hydrogen bonds within the protein stabilizes protein structure (Rose *et al.,* 2006).

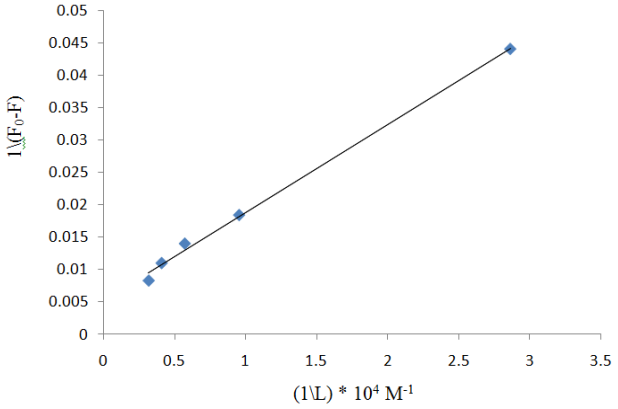

Figure 6. The plot of $1/(F_0-F)$ vs $(1/L) \times 10^4$ for testosterone –HSA complexes

Previous experiment have been applied to investigate the interaction of progesterone and cholesterol with HSA (Abu *et al.,* 2010; Abu *et al.,* 2012) by UV –VIS spectroscopy in addition to investigation of the fluorescence quenching for both hormones. In this experiment the same experimental procedure has been applied. The constant obtained were the binding constant k using UV-VIS spectrophotometer, the Stern-Volmer quenching constant K_{sv} and the binding constant using fluorescence spectrophotometer. Those constants for the two hormones and their parent (cholesterol) are listed in Table (1).

FT-IR Spectroscopy

FT-IR transform spectrophotometers have greatly extended the capabilities of infrared spectroscopy and have been applied to many areas that are very difficult or nearly impossible to analyze by dispersive instruments (Shernan 2014). FT-IR spectroscopy provides information about secondary content of proteins, this information arises from the amide band which is a result from the vibrations of the groups around the peptide bonds of proteins (Haris and Severcan 1999). Changes in hydrogen bonding involves in the peptide linkages would occur, this results in changes of vibrational frequency of the different amide modes when the binding between drugs and globular protein like HSA occurs. The infrared of the protein is characterized by a set of absorption regions known as the amide region and the C-H region. The modes most widely used in protein structural studies are amide I, amide II and amide III. Amide I band ranging from 1700 to 1600 cm-1 and arises principally from the C=O stretching. Amide II band is primarily N-H bending with a contribution from C-N stretching vibrations, amide II ranging from 1600-1480 cm^{-1}. Amide III band ranging from 1330-1220 cm^{-1} which is due to the C-N stretching mode coupled to the in-plane N-H bending mode (Abu Teir *et al.,* 2010). This absorption is normally weak.

It is obviously seen in Fig.(7) as testosterone ratio was increased the intensities of the amide I, amide II and amide III bands decreased further in the spectra of all testosterone HAS complexes. The reduction in the intensity of the three amide bands is related to the testosterone HSA interaction. Also as shown in Fig. (8) the spectra are dominated by absorbance bands of amide I and amide II at peak positions 1656 cm-1 and 1545cm-1 respectively. In Table (1) the peak positions of HSA with different ratios of testosterone are listed. For testosterone-HSA interaction the amide bands of HSA infrared spectrum shifted as listed in the table, for amide I band the peak positions have shifted as

Table 1. Band assignment in the absorbance spectra of HSA with different testosterone molecular ratios for amide I, amide II, and amide III regions

Bands	HSA Free	T:HSA	T:HSA	T:HSA	T:HSA	T:HSA
		2:10	6:10	10:10	14:10	18:10
		1607		1610	1610	
	1622	1622	1622	1624	1625	1626
	1636	1636	1636	1637	1637	1638
Amide I	1654	1655	1655	1656	1657	1657
(1700-1600)	1680	1680	1680	1680	1679	1679
	1695	1695	1695	1695	1694	1694
	1497	1497	1496	1497	1497	1497
	1515	1515	1516	1515	1514	1515
					1533	1533
Amide II	1546	1546	1546	1548	1548	1549
(1600-1480)	1577	1577	1576	1577	1578	1578
					1570	1569
	1594	1594	1594	1594	1594	1594
	1232	1232	1231	1231	1232	1232
	1244	1244	1244	1244	1245	1244
	1267	1267	1267	1267	1266	1267
Amide III	1278	1279				1276
(1330-1220)	1294	1294	1294	1294	1294	1294
	1309	1309	1309	1309	1309	1309
	1325	1325	1325	1325	1325	1325

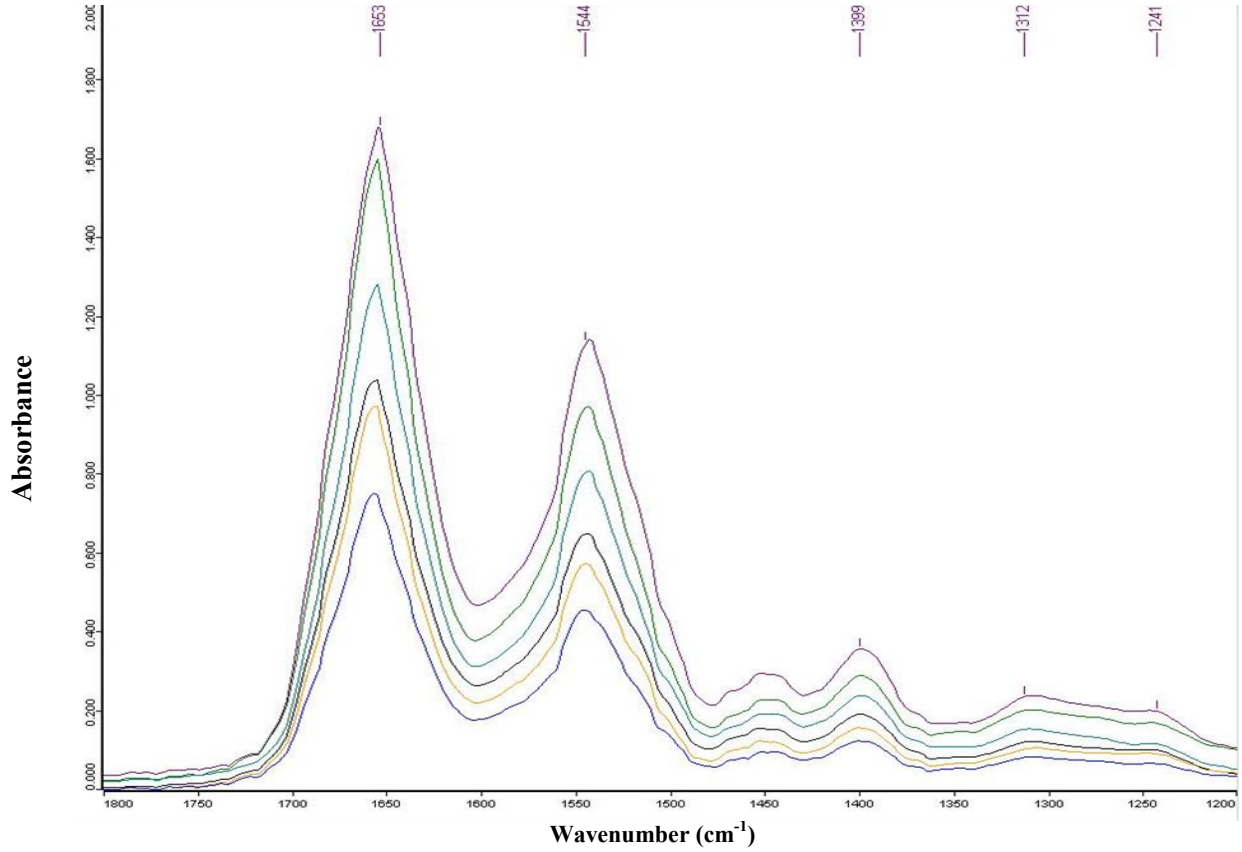

Figure 7. The spectrum of Testosterone-HSA complexes with different percentage of testosterone

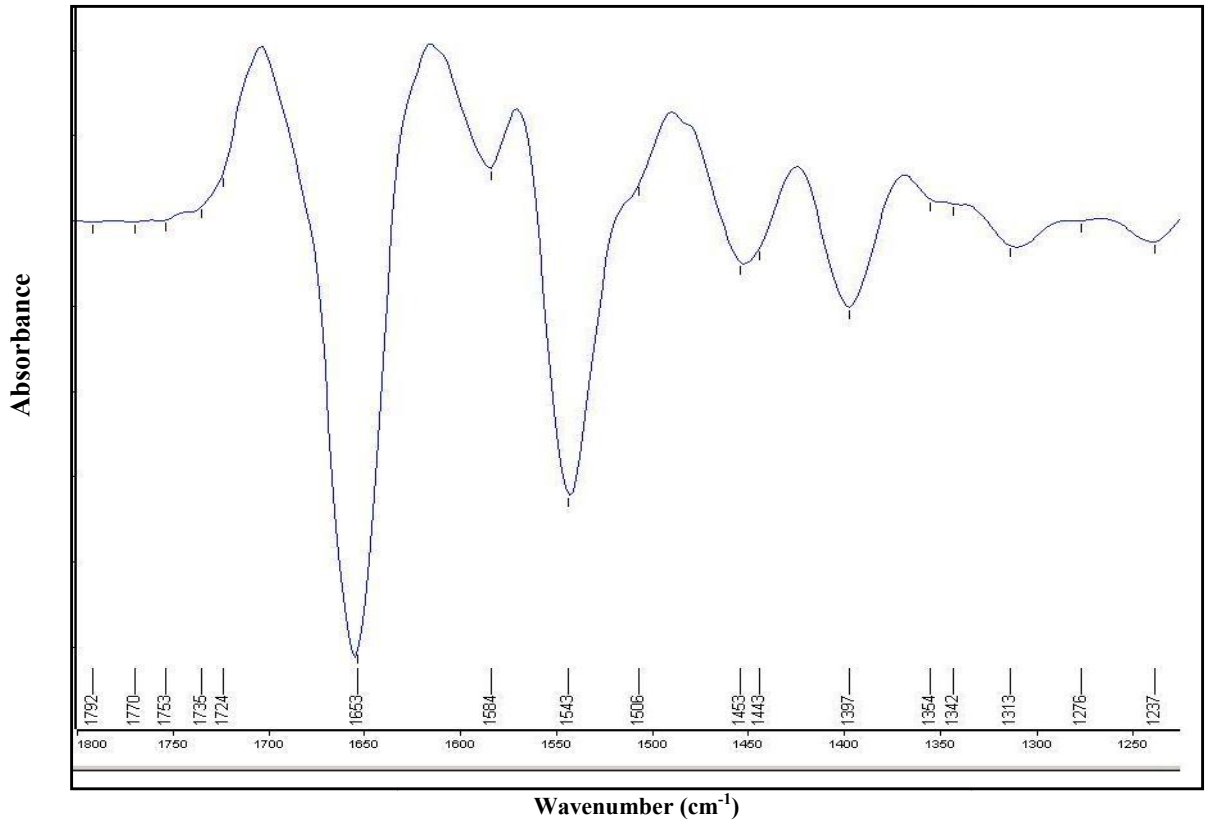

Figure 8. The second derivative of free HSA. The spectra are dominated by absorbance bands of amide I and amide II at peak positions 1656 cm^{-1} and 1545cm^{-1} respectively

follows: 1622 cm^{-1} to 1626 cm^{-1}, 1636 cm^{-1} to 1638 cm^{-1} , 1654 cm^{-1} to 1657 cm^{-1}, 1680 cm^{-1} to 1679 cm^{-1} and 1695 cm^{-1} to 1694 cm^{-1} . In amide II the peak positions have shifted as follows: 1546 cm^{-1} to 1549 cm^{-1} and 1577 cm^{-1} to 1578 cm^{-1}, in addition new peaks have been appeared at high molecular ratios of testosterone at 1533 cm^{-1} and 1569. In amide III region the peak positions are also have been shifted as the following order: 1278 cm^{-1} to 1276 cm^{-1}. Shifts in peak shape of certain elements can occur due to difference in chemical bonding, between different samples/standards. The shifts in peaks shape of HSA after the interaction with testosterone has been occurred are due to the changes in protein secondary structure. Those shifts are attributed to the newly imposed hydrogen bonding between testosterone (on both =O and –OH sites) and the protein (Sarver and Krueger 1991). It has been observed for the testosterone-HSA complexes in amide I band, that the shift to higher frequency for the second peak (1622-1626 cm^{-1}) and then for the major peak (1654-1657 cm^{-1}). In amide II the higher shift occurs at the major peak (1546-1549 cm^{-1}). The only peak shift in amide III has been observed at (1278-1276 cm^{-1}), and it is not observed in all complexes.

Hydrogen bonding may affect the bond strength, may have impact on the IR, causing the peak shift, larger or smaller. In made I the observed characteristic band shifts often allow the assignment of these bands to peptide groups or to specific amino-acid side-chains. An additional advantage is the shift of the strong water absorbance away from the amide I region (1610–1700 cm^{-1}) which is sensitive to protein structure. The minor but reproducible shift indicates that a partial unfolding of the protein occurs in HSA, with the retention of a residual native-like structure. It has been observed that the shifts in peaks are going toward a higher wave number, this implies that the strength of the bond has been increased but with a small percentage (Uversky and Permykov 2007).

The difference spectra for ((HSA +Testosterone) – (Free HSA)) have been obtained so as to monitor the intensity variations of these vibrations, the results are shown in Fig. (9) and Fig. (10). Fig. (9) shows FT-IR spectra (two top curves) and difference spectra of HSA and its complexes with different testosterone percentage in amide I and amide II regions. Fig. (10) illustrates the difference spectra of HSA and its complexes with different testosterone percentage in amide III region. In amide I region a strong negative feature is obvious at 1654 cm^{-1} as well another negative feature appeared clearly in amid e II region at 1542 cm^{-1}. In amide III region two negative features were observed at 1308 $cm^{-1}$and 1245 cm^{-1}, with a little shift as testosterone ratios were increased. It is clearly shown that the strong negative features in the difference spectra became stronger as percentage of testosterone was increased with a little shift in their positions, which are attributed to the intensity decrease in the amide I, II and III bands in the spectra of the testosterone-HSA, that is due to the interaction (H-bonding) of the testosterone with protein C=O and C-N groups, and the reduction of the proteins α-helix structure upon testosterone –HSA interaction (Purcell *et al.,* 2000; Krimm and Bandekar 1986).

The shape of the amide I band of globular proteins is characteristic of their secondary structure, with a publication by Byler and Susi (1986). The determination of secondary structures in proteins from FT-IR spectra actually started. This had become possible by the availability of high signal to noise ratio digitalized spectra obtained by FT-IR spectrometer. In those experiments, the basic analytical tools used are the infrared Fourier Self Deconvolution in addition to curve fit and second derivative resolution. So as to obtain quantitative analysis of the protein secondary structure for each different secondary structure forming HSA. The aim of Fourier Self-Deconvolution is to enhance the apparent resolution of a

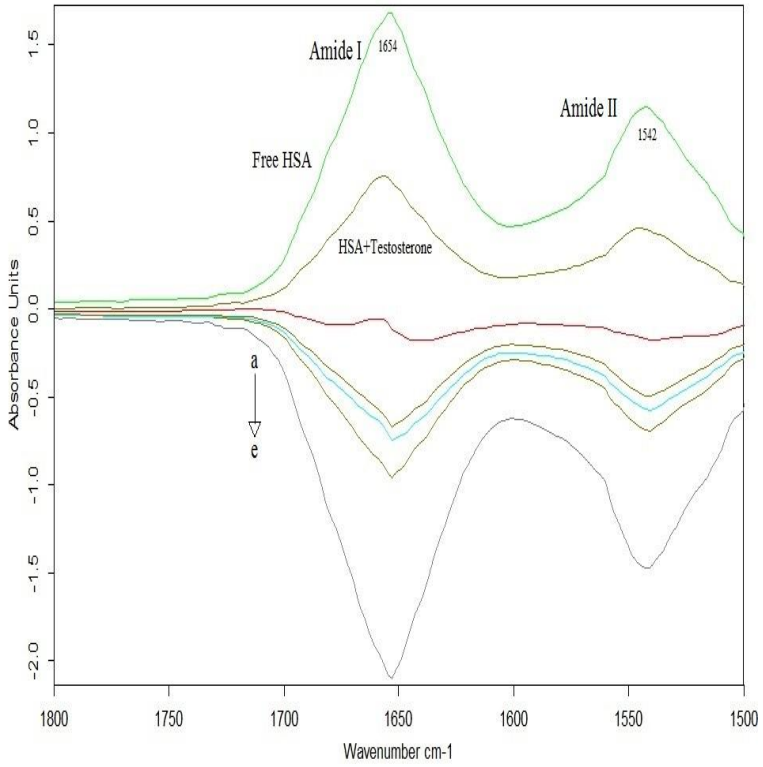

Figure 9. FT-IR spectra (top two curves) and difference spectra (from a to e) of HSA and its complexes with different testosterone concentrations in the region 1800-1500 cm^{-1}. (a= Diff (T+HSA) [2:10], b=Diff (T+HSA) [6:10], c= Diff (T+HSA) [10:10], d= Diff T+HSA [14:10], e= Diff T+HSA [18:10])

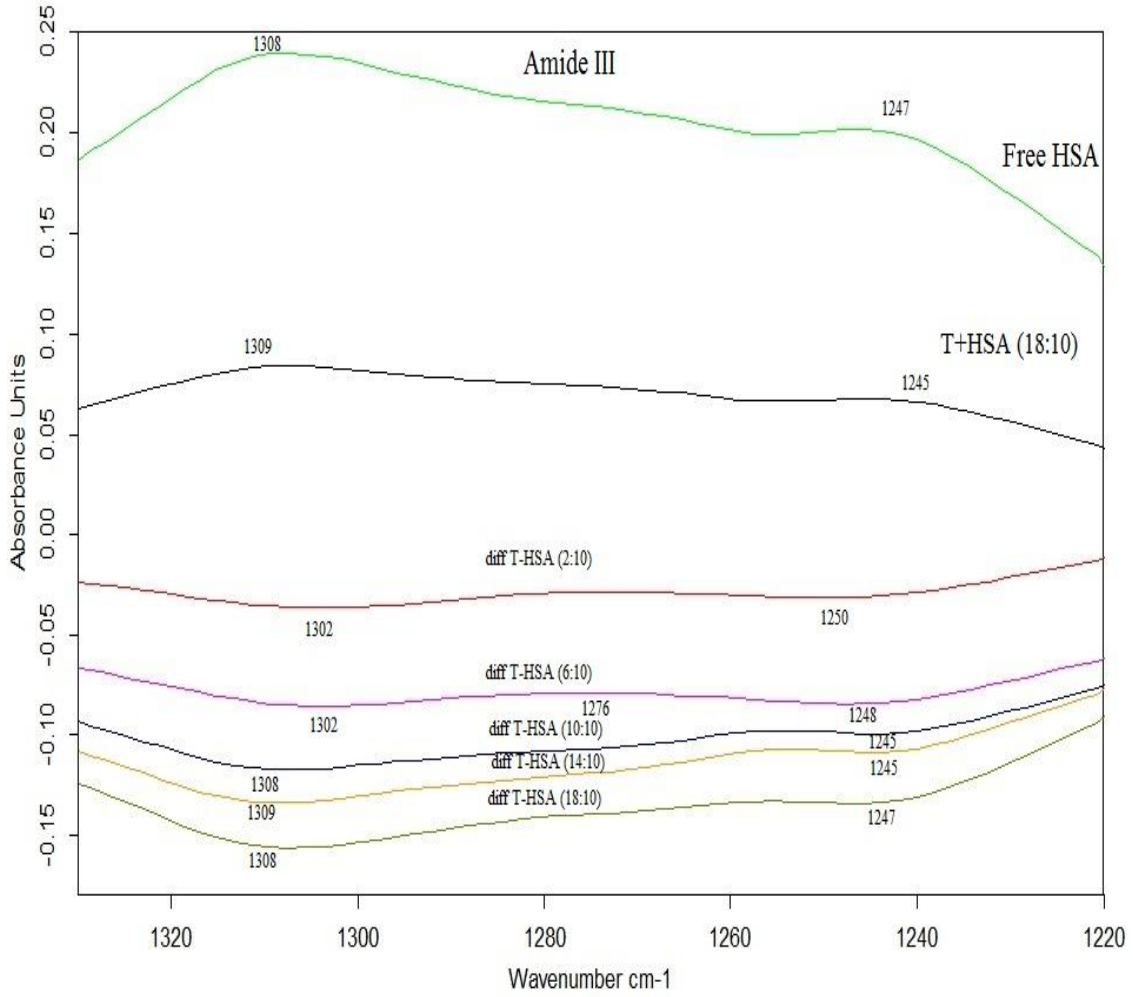

Figure 10. FT-IR spectra (top two curves) and difference spectra of HSA and its complexes with different testosterone concentrations in the region of 1330-1220 cm^{-1}

Table 2. Secondary structure determination for Amide I, amide II, and amide III regions in HSA and its testosterone complexes

2nd Structure	HSA free (%)	HSA-T 2:10 (%)	HSA-T 6:10 (%)	HSA-T 10:10 (%)	HSA-T 14:10 (%)	HSA-T 18:10 (%)
			Amide I			
β-sheets (cm^{-2}) (1610-1624) (1689-1700)	17	19	20	21	22	22
Random (cm^{-2}) (1625-1640)	10	10	10	10	9	8
α-hilex (cm^{-2}) (1646-1669)	63	62	59	58	59	57
Turn (cm^{-2}) (1672-1687)	10	9	11	11	11	13
			Amide II			
β-sheets (cm^{-2}) (1488-1504) (1585-1598)	31	34	35	34	35	37
Random (cm^{-2}) (1508-1523)	9	7	7	8	7	6
α-hilex (cm^{-2}) (1528-1560)	49	47	46	46	44	44
Turn (cm^{-2}) (1562-1585)	11	12	12	12	13	13
			Amide III			
β-sheets (cm^{-2}) (1220-1256)	34	38	43	46	47	52
Random (cm^{-2}) (1257-1285)	12	11	11	7	7	8
α-hilex (cm^{-2}) (1302-1329)	50	47	42	41	41	35
Turn (cm^{-2}) (1287-1301)	4	4	4	6	5	5

spectrum, or to decrease the line width. Spectral ranges comprising broad and overlapping lines can thus be separated into sharp single lines. The Fourier Self-Deconvolution is only useful in case of lines which are substantially broader than the spectral resolution (OPUS bruker manual version 2004). The component bands of amide I, amide II and amide III regions were assigned to secondary structure according to the frequency of its maximum a raised after Fourier self deconvolution have been applied for amide I band ranging from 1610 to 1700 cm^{-1} assigned as follows: 1610-1624 cm^{-1} are basically represent β-sheets, 1625-1640 cm^{-1} random coil, 1646-1671 cm^{-1} α-helix, 1672-1787 cm^{-1} turn structure, and 1689-1700 cm^{-1} to β- sheets (antiparallel). For amide II ranging from 1480-1600 cm^{-1}, the absorption band assigned as follows: 1488-1504 cm^{-1} to β-sheet, 1508-1523 cm^{-1} to random coil, 1528-1560 cm^{-1} to α-helix, 1562-1585 cm^{-1} to turn structure, and 1585-1598 cm^{-1} to β- sheet (antiparallel). For amide III ranging from 1220 to 1330 cm^{-1} have been assigned in the following order: 1220-1256 cm^{-1} to β-sheet, 1257-1285 cm^{-1} to random coil, 1287-1301 cm^{-1} to turn structure, and 1302-1329 cm^{-1} to α-helix.

It has been assigned that investigation in amide I absorption is primarily determined by the backbone conformation and independent of amino acid sequence, so most investigations have been concentrated on amide I band (Krimm and Bandekar 1986). For amide II and III band more information is to be gained as various type of chemical bond are involved so investigation in those bands are useful in predicting the protein secondary structure. Based on the above assignments, the percentages of each secondary structure of HSA were calculated by integrated areas of the component bands in amide I, amide II and amide III then summed and divided by the total area. The obtained number is taken as the proportion of the polypeptide chain in that conformation. The secondary structure determination for the free HSA and its testosterone complexes are given in Table (2). This table shows the content of each secondary structure of HSA before and after the interaction with testosterone at different ratios. It is obviously seen that α-helix percentage decreases with the increase of testosterone ratios in the calculations. This trend is consistent in the three Amide regions.

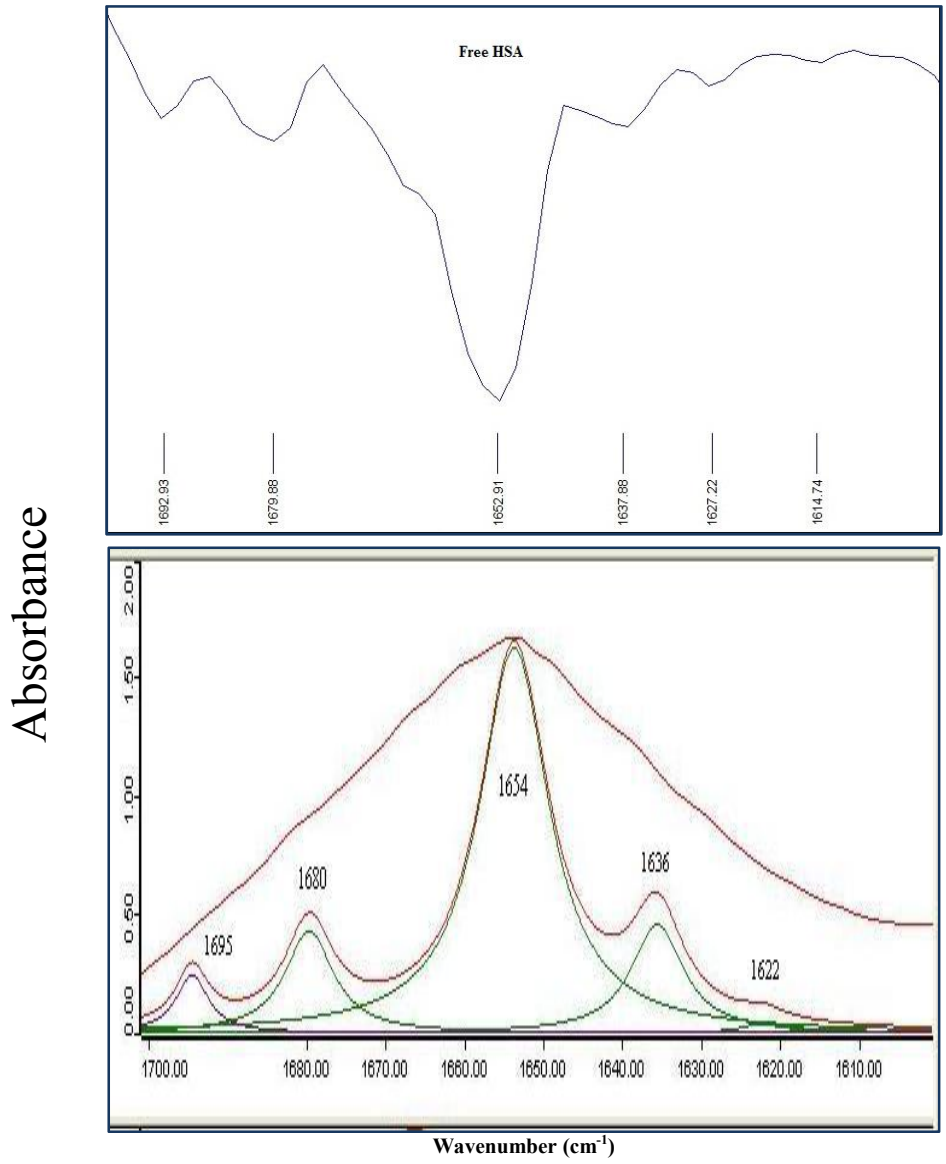

Figure 11. Second derivative enhancement and curve fitted Amide I region (1610-1700 cm^{-1}) and secondary structure determination of the free human serum albumin

The second derivative resolution enhancement and curve-fitted Amide I, Amide II and Amide III regions and secondary structure determinations of the free HSA and its testosterone complexes (T-HSA 18:10) with the highest of testosterone in dehydrated films are shown in Figures (11 to 16). For testosterone- HSA interaction: in amide I region, HSA free consists of (17%) β-sheet, (10%) random coil, (63%) α-helical structure, and (10%) turn structure while after testosterone HSA complexation , α-helical structure reduced from 63% to 57%, β-sheets increased from 17% to 22%, turn structure increased from 10% to 13% and random coil decreased from 10% to 8%, as shown in Table (2), Fig. (11) and Fig.(12).

In amide II region, HSA free consists of (31%) β-sheets, (9%) random coil, (49%) α-helical structure, and (11%) turn structure as shown in Table (2) and Fig. (13) while after testosterone –HSA interaction, α-helical structure reduced from 49% to 44% , β-sheets increased from 31% to 37% , random coils decreased from 9% to 6% while turn structure increased from 11% to 13% as shown in Fig.(14). In amide III region, HSA free consists of (34%) β-sheet, (12%) random coil, (50%) α-helical structure and (4%) turn structure while after testosterone-HSA interaction, α-helical structure reduced from 50% to 35%, β-sheet increased from 34% to 52%, random coil decreased from 12% to 8% and turn structure increased from 4% to 5% as shown in Fig.(15), Fig.(16) and Table (2).

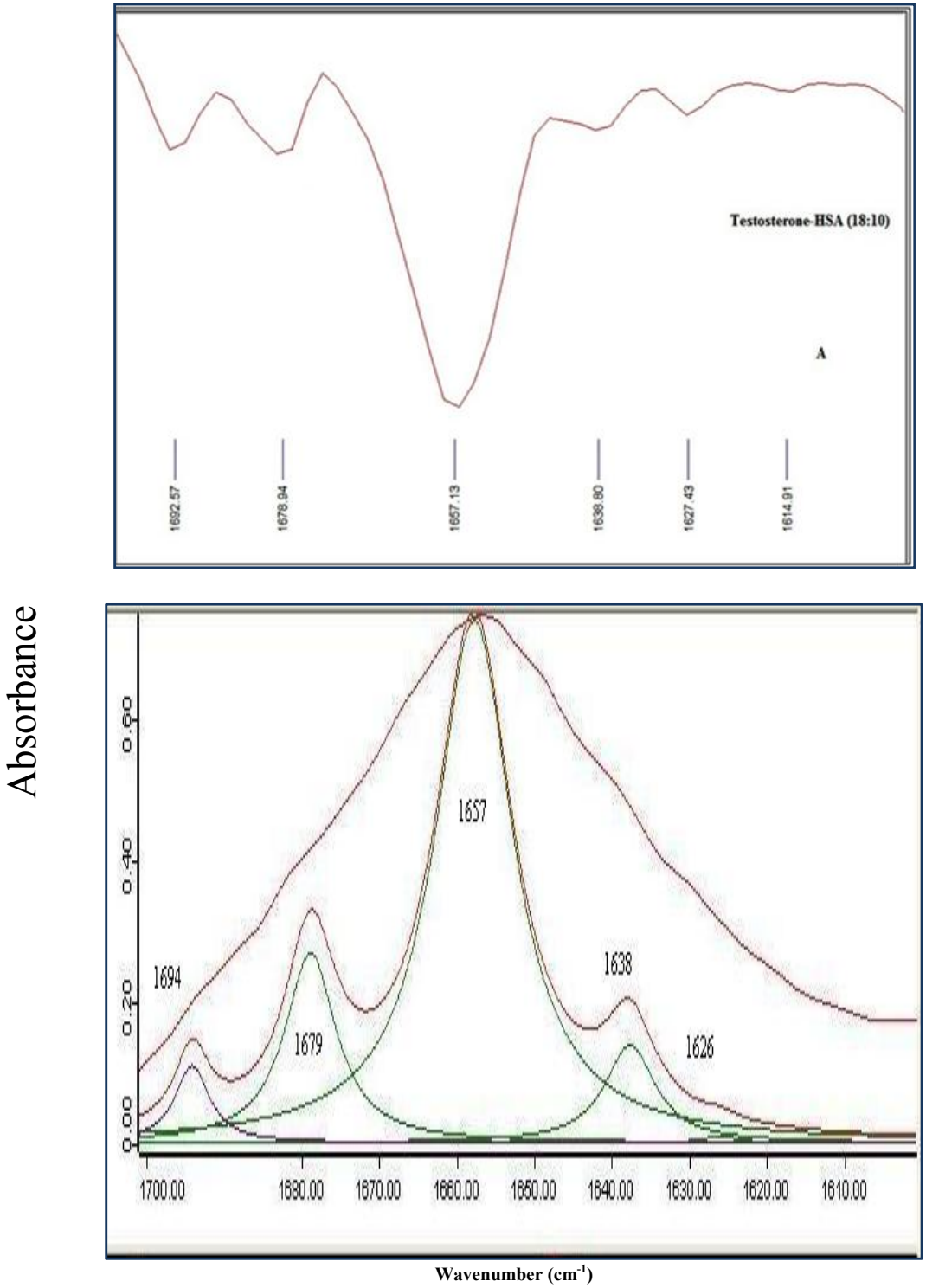

Figure 12. Second derivative enhancement and curve fitted Amide I region (1610-1700 cm^{-1}) and secondary structure determination of human serum albumin and its testosterone complexes with 18:10 testosterone:HSA ratio

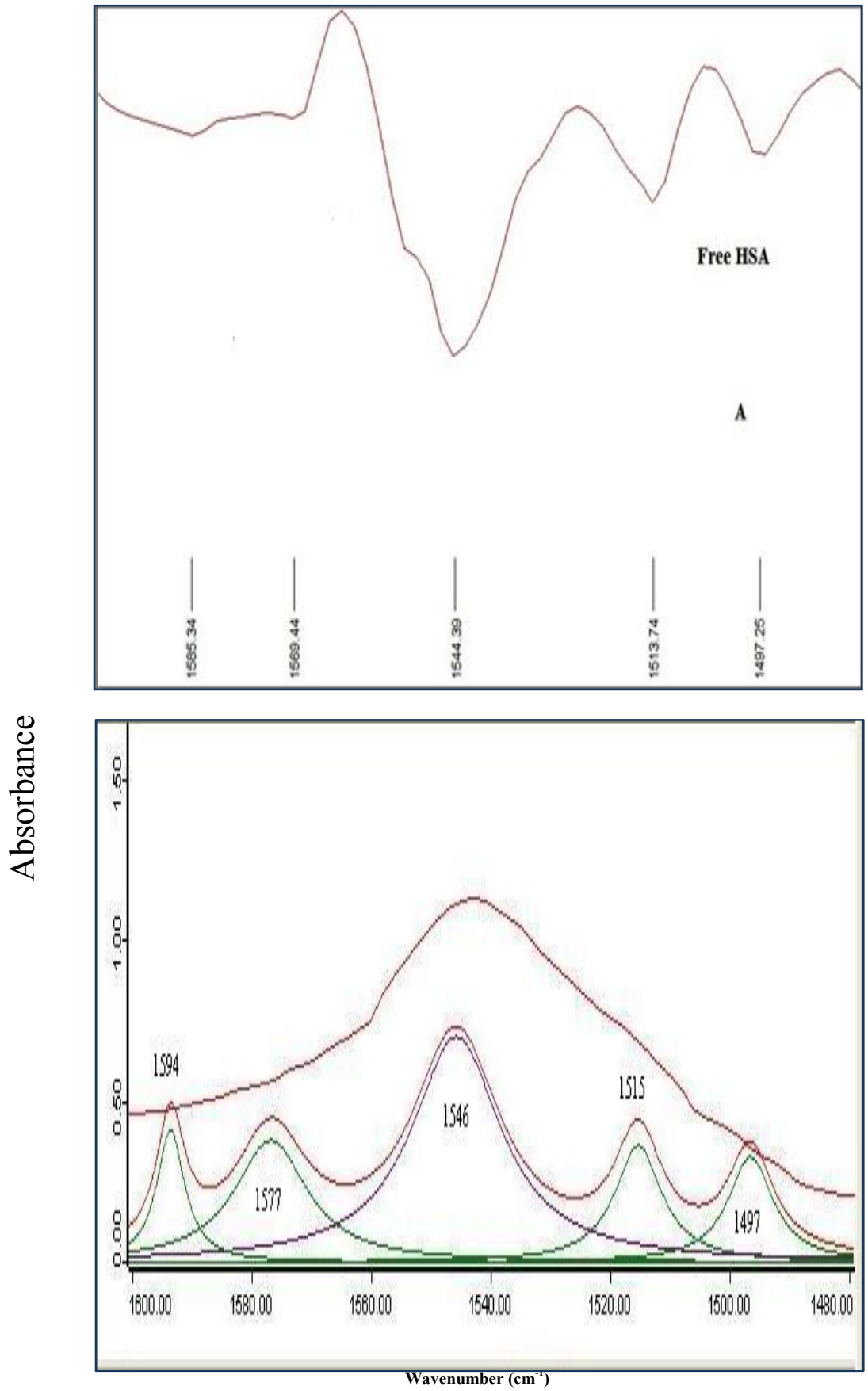

Figure 13. Second derivative enhancement and curve fitted amide II region (1600-1480 cm^{-1}) and secondary structure determination of the free human serum albumin

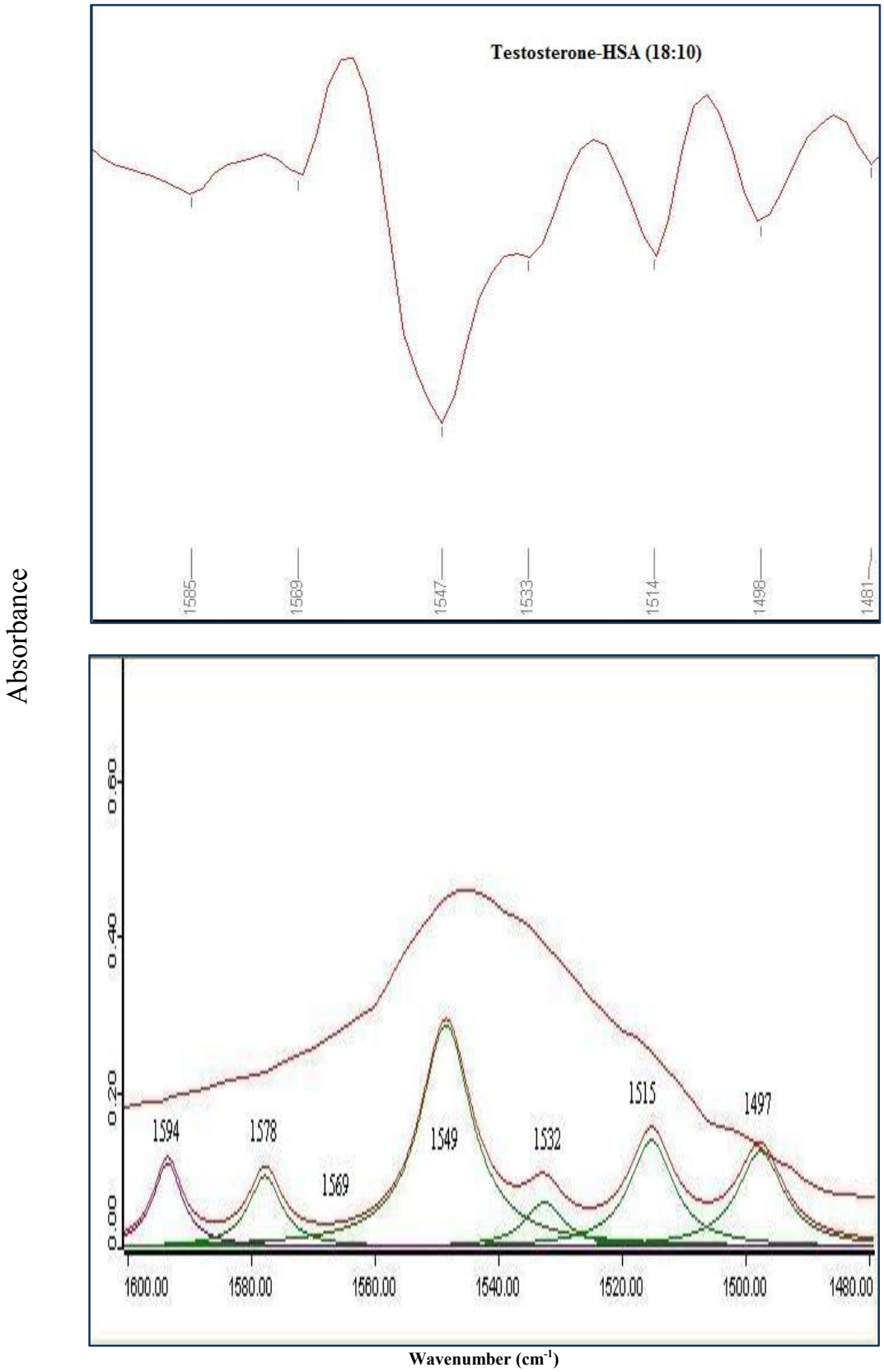

Figure 14. Second derivative enhancement and curve fitted Amide II region (1600-1480 cm^{-1}) and secondary structure determination of t human serum albumin and its testosterone complexes with 18:10 testosterone:HSA ratio

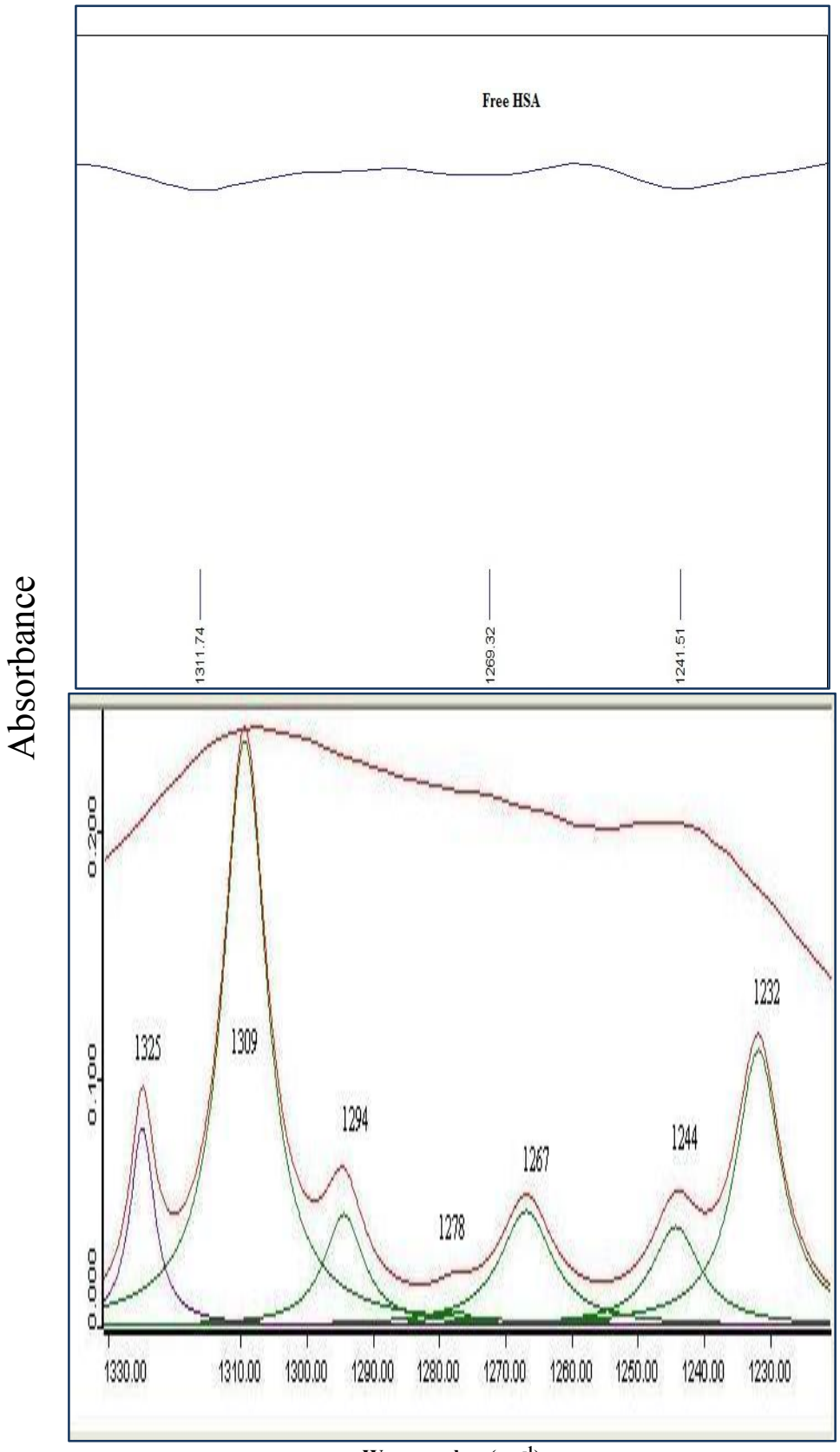

Figure 15. Second derivative enhancement and curve fitted amide III region (1330-1220 cm^{-1}) and secondary structure determination of the free human serum albumin. Complexes with 18:10 testosterone: HSA ratio

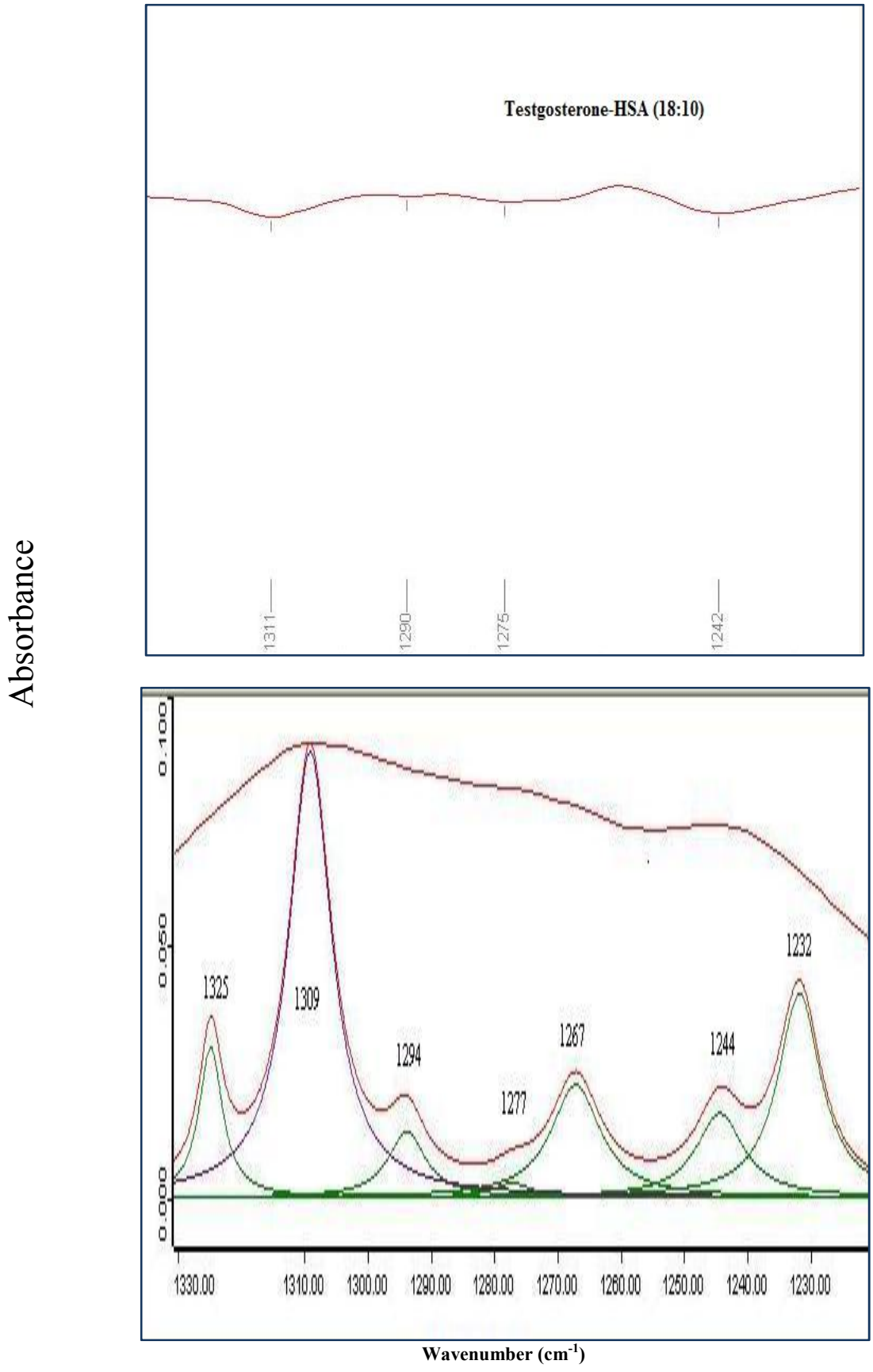

Figure 16. Second derivative enhancement and curve fitted Amide III region (1330-1220 cm^{-1}) and secondary structure determination of human serum albumin and its testosterone complexes with 18:10 testosterone:HSA ratio

The decrease of α-helix percentage with the increase of testosterone concentration is evident in the calculations from table above and this trend is consistent in the three Amide regions. However, for β-sheet, relative percentage increased has been observed with the increase of testosterone concentration. The reduction of α-helix intensity percentage in parallel to the increase of β-sheets have been remarkably noticed and believed due to unfolding of the protein in the presence of testosterone as a result of the formation of H bonding between HSA and the hormone. The newly formed H-bonding results in the C-N bond assuming partial double bond character due to a flow of electrons from the C=O to the C-N bond which decreases the intensity of the original vibrations (Krimm and Bandekar 1986; Jackson and Mantsch 1991). As discussed before, the hydrogen bonds in α-helix are formed outside the helix (the amino-acid side-chains are on the outside of the helix) and parallel to the helix axis, while for β-sheet the hydrogen bonds take position in the planes of β-sheets as the preferred orientations especially in the antiparallel sheets, so the restrictions on the formation of hydrogen bonds in β-sheet relative to the case in α-helix explains the larger effect on reducing the intensity percentage of α-helix to that of β-sheet. Similar conformational transitions from an α-helix to β-sheet structure were observed for the protein unfolding upon protonation and heat denaturation (Surewicz *et al.,* 1987; Holzbaur *et al.,* 2005).

Conclusion

In this work, the interaction between testosterone (male hormone) and HSA Albumin (the universal hydrophobic molecule carrier) has been investigated using spectroscopic techniques, including (FT-IR, Fluorescence and UV-VIS spectrophotometers). Our experimental work showed relatively high binding affinity between testosterone and HSA. Referring to UV spectrum the calculated binding constant for testosterone-HSA is ($K= 34.9\times10^2$ M^{-1}). The analysis of fluorescence spectrum yield binding constant for testosterone-HSA interaction, it has been measured to be ($K=38.23\times10^2$ M^{-1}). The binding constant obtained by different methods has very close values. Comparing the binding constant for testosterone with the previously measured binding constants of cholesterol and progesterone, regarding to that one can conclude, that testosterone-HSA interaction is the strongest as it is considered to be both hydrogen donor and acceptor. Cholesterol interaction with albumin was substantially weaker than testosterone-HSA interaction but stronger than progesterone-HSA interaction. The value of Stern-Volmer quenching constant and quenching rate constant for testosterone have been measured to be ($K_{sv}= 4.5\times10^2$ L Mol^{-1}, $K_q= 4.5\times10^{10}$ L $Mol^{-1}s^{-1}$). These experimental results static quenching is responsible for the fluorescence quenching (decrease of intensity). This is an indication for complex formation between the protein and hormone. Analysis of FT-IR spectrum indicates that increasing the concentration of testosterone leads to protein unfolding, reduction of α-helical structure percentage in favor of β-sheet structure. It has been observed that HSA-Testosterone complexes caused shifts in the peaks of HSA this is due to the change in bond strength and partial unfolding of the protein. The newly imposed hydrogen bonds results in the C-N bond assuming partial double bond character due to flow of electrons from the C=O to the C-N bond which decreases the intensity of the original vibrations.

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