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Investigation of the interaction between vitamin C and vitamin B_{12} with human serum albumin using spectroscopic techniques

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Abstract: Vitamin C is an important regulatory for iron uptake and vitamin B_{12} is essential for proper functioning of folic acid. Human serum albumin is an abundant plasma protein, the major soluble protein constituent of the circulatory system and has many physiological functions including transport of a variety of compounds. In this work, the molecular interaction between vitamin C and $B_{12}$with human serum albumin was investigated using constant protein concentration and various drug concentrations at pH 7.4. Three different spectroscopic methods were used; fluorescence spectroscopy, UV absorption and FT-IR spectroscopy. From spectral analysis, both vitamins showed a strong ability to quench the intrinsic fluorescence of human serum albumin through a static quenching procedure.The binding constant (k) is estimated from UV-absorption as $k=1.28\times10^4$ M^{-1} for HSA-Vitamin C and $k=2.21\times10^4$ M^{-1} for HSA-vitamin B_{12}. Both results showed a good agreement with the binding constants obtained from the modified Stern-Volmer equation using florescence technique. The appearance or disappearance of the bands is a good sign to understand the mechanisms at the molecular level. The FT-IR spectral changes indicates an increase of intensity for HSA-vitamin C interaction and a reduction of intensity for HSA-vitamin B_{12} interaction. For HSA-vitamin C complexes, positive features are related to increase in intensity of the amide I and II bands upon drug-complexation due to drug binding to protein C=O, C-N and N-H groups. While in the

difference spectra of vitamin B_{12}-HSA complexes, intensity decreases as the concentration of vitamin B_{12} increases for amide I band at 1656cm^{-1}, and amid II band at 1544 cm^{-1}, this results in stabilization by hydrogen bonding by having the C-N bond assuming partial double character due to a flow of electrons from the C=O to the C-N.

Keywords: Vitamin C, Vitamin B_{12}, Human serum albumin, UV-spectroscopy, Fluorescence spectroscopy, FT-IR.

1. INTRODUCTION

Vitamins are organic molecules required in very small quantities in the diet for health, growth, and survival[1-4]. Most vitamins are used for the synthesis of coenzymes, complex organic molecules that assist enzymes in catalyzing biochemical reactions, so the absence of a vitamin from the diet or an inadequate intake results in characteristic deficiency signs and symptoms reflect an inability of cells to carry out certain reactions[2, 3].

Ascorbic acid (vitamin C) is a water-soluble micronutrient required for multiple biological functions **(Fig. 1),**cannot be synthesized by humans and incorporated into our diet[5]. Vitamin C is an important regulator of iron uptake[1, 5]; reduces ferric to ferrous ions, thus promoting dietary non-iron absorption from the gastrointestinal tract, and stabilizes iron-binding proteins[6].The two main components of vitamin C are ascorbate and dehydroascorbic acid[7] .The transport of ascorbate through the human body involves two sodium-dependent vitamin C transporters[8-10] which work as powerful antioxidant capacity[11]. In a general sense, vitamin C acts as a cofactor and reduces certain enzymes by providing them with electrons, due to its chemical structure[12]. Those enzymes can react with biomolecules known as lipids, proteins and DNA, and cause harm[10, 13, 14]. In order to help prevent that, vitamin C reduces oxygen species when lipid peroxidation is formed, reduces radical inhibitors in protein oxidation, and prevents nitrosamine formation to reduce DNA damage[15].

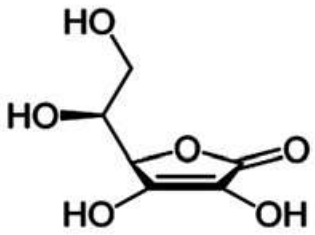


Fig. 1: Chemical structure of Vitamin C (ascorbic acid)

Vitamin B_{12}, also called cyanocobalamin **(Fig. 2),** is one of 8 B vitamins[16]. All B vitamins help the body convert food (carbohydrates) into fuel (glucose), which is used to produce energy[17]. These B vitamins help the body use fats and protein[18]. B complex vitamins are needed for healthy skin, hair, eyes, and liver. They also help the nervous system function properly[19] .Vitamin B_{12} is the only vitamin containing metal ion (trivalent cobalt) [20]. It is needed in many body processes; in the manufacturing and the maintenance of red blood cells, the synthesis of DNA, the simulate of nerve cells, the growth promotion and energy releases, and the proper functioning of folic acid[21-24]. Characteristic signs of B_{12} deficiency include fatigue, weakness, constipation, loss of appetite, and weight loss[25].

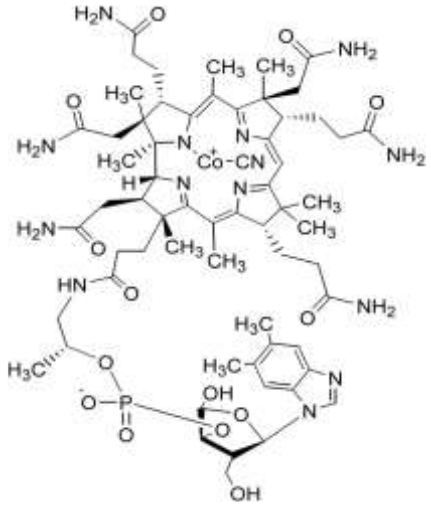


Fig. 2: Chemical structure of vitamin B_{12}

Serum albumins are the most crucial protein constituents of the human blood circulation system, which fulfill a variety of physiological functions fundamental to human metabolism[26-28]. One of the substantial features of this class of proteins is their ability to perform effective delivery role in case of drugs and other biologically active substances[29]]. Human serum albumin (HSA) is the most abundant protein in blood plasma and is able to bind and thereby transport various compounds such as fatty acids, hormones, steroids, metal ions, therapeutic ions and a large number of drugs. Strong binding of albumin can decrease the concentrations of free drugs in plasma, whereas weak binding can lead to a short lifetime or poor distribution[30]. The interaction between HSA and drugs has been investigated using techniques such as electrochemistry[31], chromatography[32], nuclear magnetic resonance[33] and spectral analysis. Spectral analysis is widely applied because of its easy operation, low cost, and abundant theoretical formulation[34].

The principal objective of this work was to investigate the interaction of HSA complexes with vitamin C and vitamin B_{12} by using Fourier transform infrared spectroscopy (FTIR), thus, the work will be limited to the mid-range infrared which covers the frequency range from 4000 to 400 cm^{-1}. Other spectroscopic techniques have been used in studying the interaction of drugs with proteins, fluorescence and UV spectroscopy are commonly used because of their sensitivity, rapidity and ease of implementation.

2. MATERIALS AND METHODS

Human serum albumin (fatty acid free) was purchased from Sigma chemical company. Vitamin C and vitamin B_{12} in powder form were also purchased from Sigma chemical company and used without further purification.

2.1. Preparation of stock solutions*:* HSA was dissolved in 25% ethanol in phosphate buffer Saline and at physiological pH of 7.4), to a concentration of (80mg/ml), and used at final concentration of (40 mg/ml) in the final vitamin- HSA solution. Vitamin C (molecular weight of 176.13 g.mol-1) and vitamin B_{12} (molecular weight of 1355.4 $g.mol^{-1}$), were dissolved in 25% ethanol in phosphate buffer Saline and, then the solution was placed in ultrasonic water path (SIBATA AU-3T) for one hour to ensure that all the amount of vitamins were completely dissolved. The final concentrations of HSA-

Vitamins complexes were prepared by mixing equal volume of HSA to equal volume from different concentration of vitamins C and B_{12}. HSA concentration in all samples kept at 40 $mg.ml^{-1}$. However, the final concentrations of the vitamins in solutions are (40 $mg.ml^{-1}$, 20 $mg.ml^{-1}$, 10 $mg.ml^{-1}$, 5 $mg.ml^{-1}$, 2 $mg.ml^{-1}$and 1 $mg.ml^{-1}$).The solution of vitamins and HSA were incubated for 1 h (at 25^0C) before spectroscopic measurements were taken.

2.2. ***UV-VIS spectrophotometer****:* The absorption spectra were obtained by the use of a Nano Drop 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 were recorded for free HSA 40 $mg.ml^{-1}$ and for its complexes with vitamin C and B_{12}solutions with the concentrations of (40 $mg.ml^{-1}$, 20 $mg.ml^{-1}$, 10 $mg.ml^{-1}$, 5 $mg.ml^{-1}$, 2 $mg.ml^{-1}$and 1 $mg.ml^{-1}$). Repeated measurements were done for all samples.

2**.3.** ***Fluorescence spectrometer****:* The fluorescence measurements were performed by a Nano Drop ND-3300 Fluoro-spectrophotometer at 25°C. The excitation source comes from one of three solid-state light emitting diodes (LEDs). 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. The emission spectra were recorded for free HSA 40 $mg.ml^{-1}$ and its complexes with vitamins C and B_{12} solutions with the concentrations of (40 $mg.ml^{-1}$, 20 $mg.ml^{-1}$, 10 $mg.ml^{-1}$, 5 $mg.ml^{-1}$, 2 $mg.ml^{-1}$and 1 $mg.ml^{-1}$).

2.4. ***FT-IR spectroscopy:*** The FT-IR measurements were obtained on a Bruker IFS 66/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, and vitamins-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 vitamins solutions) - (protein solution)] were generated using the featureless region of the protein solution 1800-2200 cm^{-1}as an internal standard[35]. The accuracy of this subtraction method is tested using several control samples with the same protein or vitamins concentrations, which resulted into a flat base line formation. The obtained spectral differences were used here, to investigate the nature of the vitamins-HSA interaction.

3. RESULTS AND DISCUSSION

3.1. ***UV-absorption spectroscopy****:* UV-absorption spectroscopy was used to determine the binding constants between HSA and a drug (Vitamin C and Vitamin B_{12}). The strength of interaction between HSA and drugs is dependent on the binding constant which can be calculated using graphical analysis of the absorbance spectrum. The excitation has been done on 210 nm and the absorption is recorded at 268 nm for vitamin C and at 278 nm for vitamin B_{12}.The absorption spectra of different concentrations of vitamin C **(Fig.3.a)** and vitamin B_{12}**(Fig.3.b)** with HSA showed an increase of the

intensity as the vitamin C concentration increases; this is due to major ligand protein interaction at protein surface which does not limit the mobility of ligand around HSA molecule while the intensity deceases with increasing vitamin B_{12} concentration which originates from the existence of aromatic amino acids being the components of a protein molecule as well as its complicated molecular structure.

The absorption data were treated using linear reciprocal plots based on the following equation[35].

$$\frac{1}{A - A_0} = \frac{1}{A_\infty - A_0} + \frac{1}{K[A_\infty - A_0]} \cdot \frac{1}{L} \qquad (1)$$

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 ligated protein, and A is the recorded absorption at different Retinol concentrations (L). **Fig. 4.a and Fig. 4.b** represents the double reciprocal plots of 1/(A- A_0) vs. 1/L for HSA-vitamin C and HSA-vitamin B_{12} complexes respectively. The binding constant (K) can be estimated from the ratio of the intercept to the slope. The obtained values of the binding constants indicates a relatively weak interaction of vitamin C (K= 1.28×10^4 M^{-1}) and vitamin B_{12} (K= 2.21×10^4 M^{-1}) when compared to other drug-HSA complexes with binding constants in the range[13] of 10^5 and 10^6 M^{-1}. The reason for the low stability can be attributed to the presence of mainly hydrogen-bonding interaction or an indirect vitamin-HSA interaction through water molecules[36].

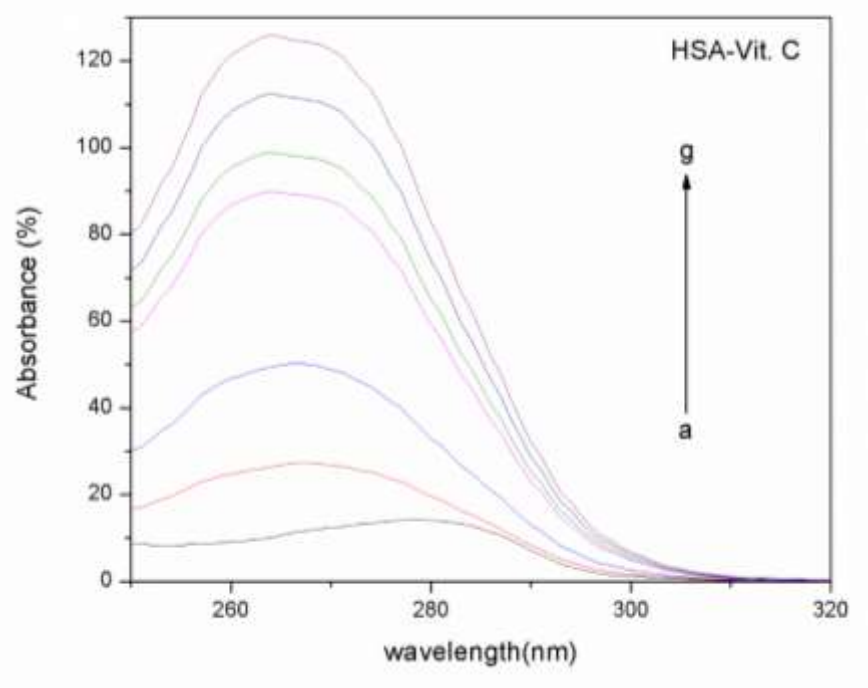


Fig.3.a:UV-absorbance spectra of HSA with different concentrations of Vitamin C (a=free HSA, b=1mg.ml^{-1},c=2mg.ml^{-1}, d=5mg.ml^{-1}, e=10mg.ml^{-1}, f=20mg.ml^{-1}, g=40 mg.ml^{-1})

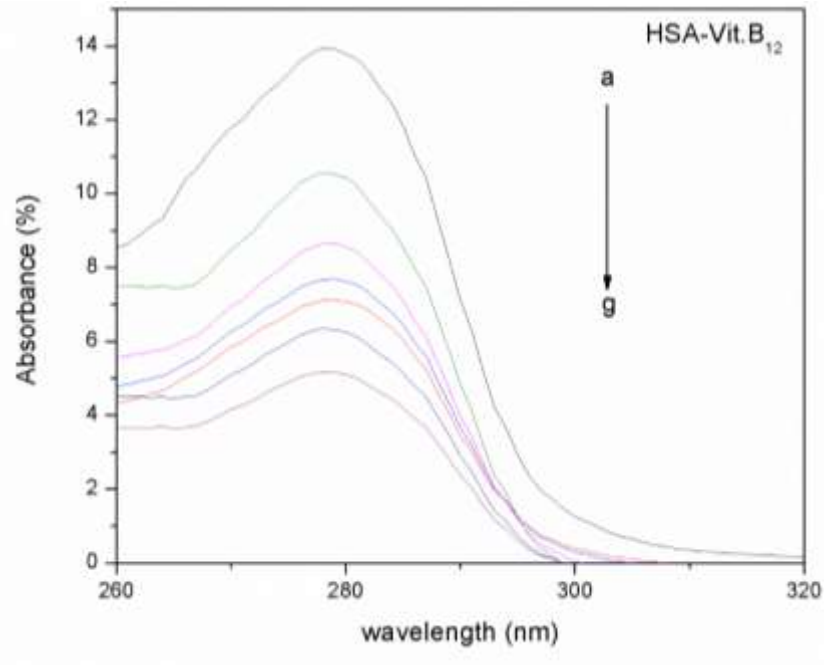


Fig.3.b. UV-absorbance spectra of HSA with different concentrations of Vitamin B_{12} (a=free HSA, b=1mg.ml^{-1},c=2mg.ml^{-1}, d=5mg.ml^{-1}, e=10mg.ml^{-1}, f=20mg.ml^{-1}, g=40 mg.ml^{-1})

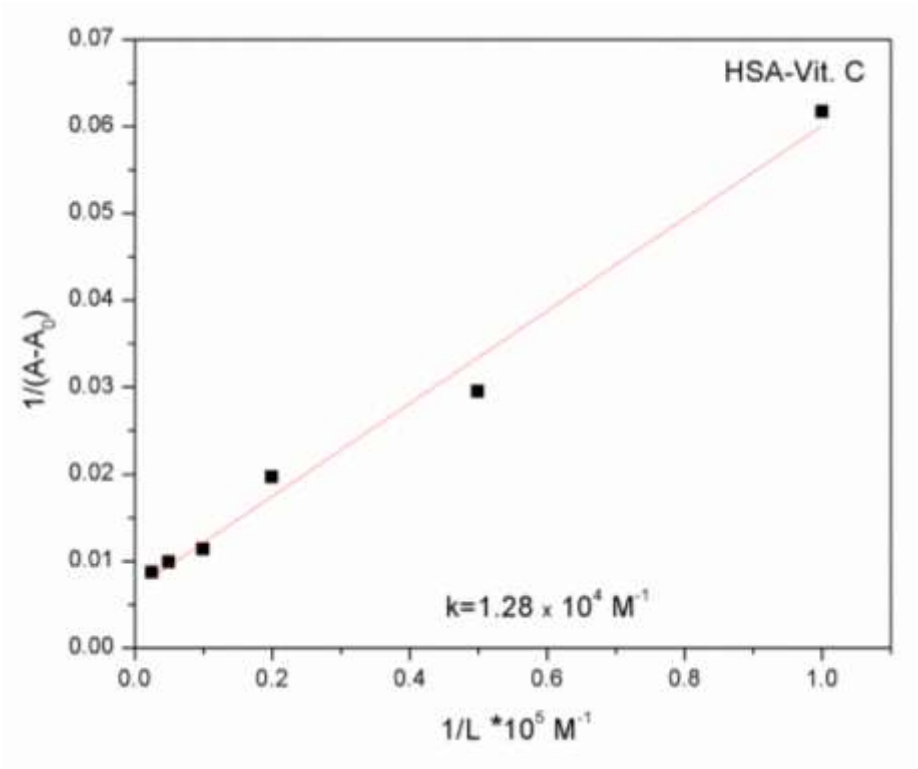


Fig.4.a: The plot of $1/(A-A_o)$ vs. 1/L for HSA with different concentrations of vitamin C.

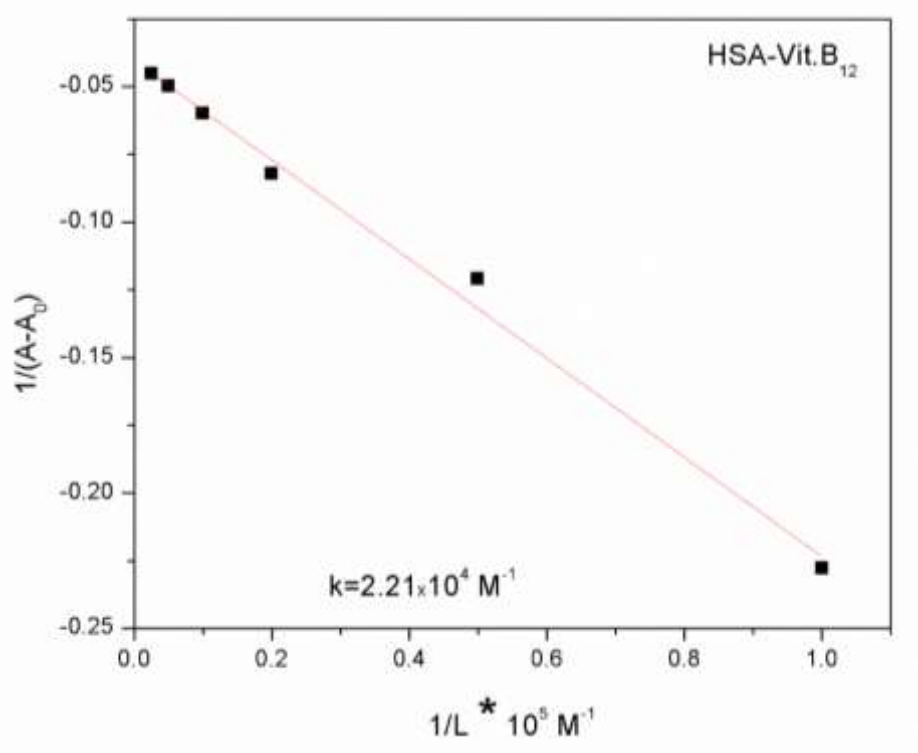


Fig.4.b: The plot of $1/(A-A_o)$ vs. 1/L for HSA with different concentrations of vitamin B_{12}.

3.2. Fluorescence spectroscopy: It was found that the protein fluorescence quenching can be the result of both the interactions, collisional or binding, with other molecules, as well as the inner filter effect pertaining to the absorption of light at the excitation or emission wavelength by the compounds presented in the solution. Fluorescence measurements can give some information on the binding mechanism of small molecule substances to protein, including binding mode, binding constants, binding sites and intermolecular distances. Various molecular interactions can decrease the fluorescence intensity of a compound such as molecular rearrangements, exited state reactions, energy transfer, ground state complex formation, and collisional quenching[37].

The fluorescence of HSA results from the tryptophan, tyrosine, and phenylalanine residues. The intrinsic fluorescence of many proteins is mainly contributed by tryptophan alone, because phenylalanine has very low quantum yield and the fluorescence of tyrosine is almost totally quenched if it is ionized or near an amino group, a carboxyl group, or a tryptophan residue[19, 20, 39, 40].In this work for HSA-vitamins complexes excitation wavelength at 360nm was used. 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[41].

As was observed, the HSA fluorescence spectrum exhibiting the peak maximum at 460 nm (**Fig.5.a)** and the intensity increases as the vitamin C concentration increased while the peak maximum appears to be at 440 nm **(Fig.5.b)** and the fluorescence intensity decreased regularly with increasing of vitamin B_{12} concentration. The peak positions shows little or no change at all.

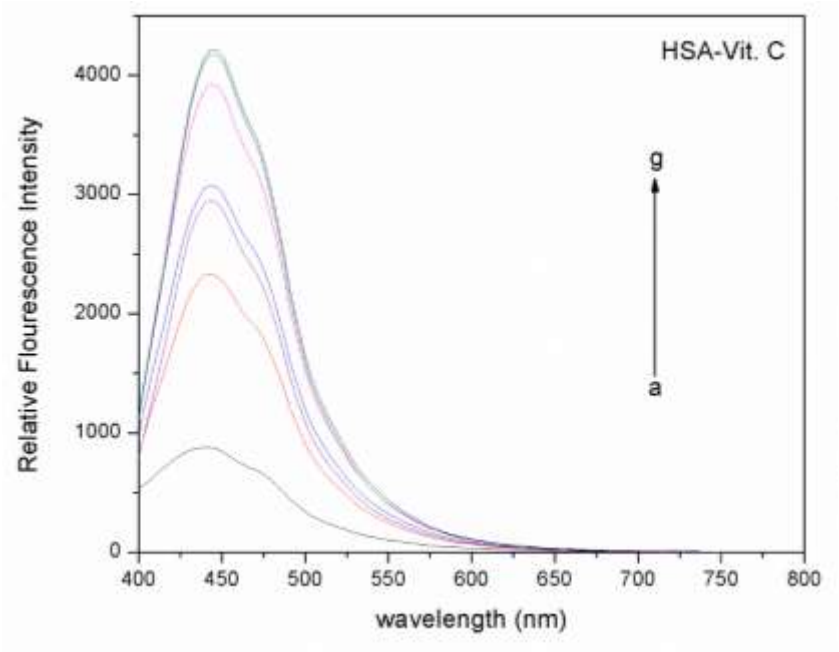


Fig. 5.a: Fluorescence emission spectra of HSA in the absence and presence of vitamin C in these concentrations (a=free HSA, b=1mg.ml^{-1},c=2mg.ml^{-1}, d=5mg.ml^{-1}, e=10mg.ml^{-1}, f=20mg.ml^{-1}, g=40 mg.ml^{-1})

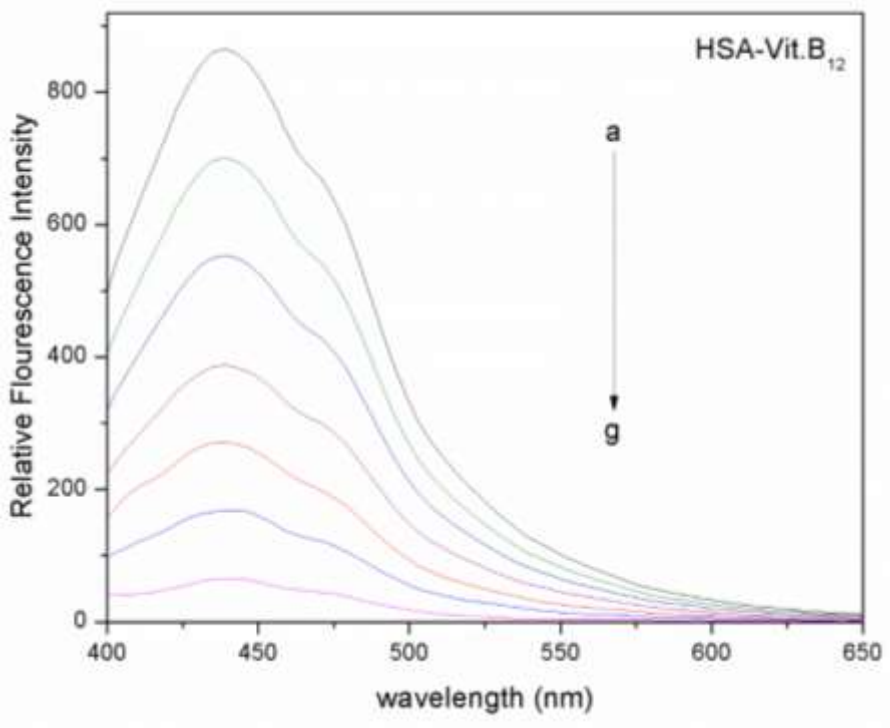


Fig. 5.b: Fluorescence emission spectra of HSA in the absence and presence of vitamin B_{12} in these concentrations (a=free HSA, b=1mg.ml^{-1},c=2mg.ml^{-1}, d=5mg.ml^{-1}, e=10mg.ml^{-1}, f=20mg.ml^{-1}, g=40 mg.ml^{-1})

To elucidate the mechanism of fluorescence quenching, the steady state fluorescence quenching data were examined based on the classic Stern-Volmer equation[36]:

$$\frac{F_0}{F} = 1 + K_{sv}[L] = 1 + k_q\tau_0[L] \qquad (2)$$

where F_0 and F are the HSA fluorescence intensities in the absence and presence of quencher (Vitamins C and B_{12}); k_q is the HSA bimolecular quenching rate constant; τ_0 is the average fluorescence lifetime of the HSA molecule without quencher[38] (of the order of 10^{-8}); [L] is the concentration of quencher, k_{sv} is Stern-Volmer fluorescence quenching constant.

As it was already found, the process of fluorescence quenching can proceed via two mechanisms: dynamic (collisional, diffusion-limited) and static (diffusion-independent). The Stern-Volmer plots, which are linear within certain concentration, may either expose the presence of a single type of quenching, or showthe occurrence of just a single binding site for quencher in the fluorophoreneighbourhood[41-43]. The Stern-Volmer plots demonstrate sometimes an upward curvature, mainly at higher values of quencher concentration, what could be ascribed to the combined type of quenching (both static and dynamic) [44]. It appeared that in proteins case the positive deviations from the Stern-Volmer equation are also recorded when the extent of quenching is large or the fluorescence process is dominated by one single residue (distance-dependent quenching due to a single molecular interaction)[45]. **Fig. 6.a and Fig.6.b**represents the Stern-Volmer plot of HSA fluorescence intensities of Vitamin C and vitamin B_{12} respectively.

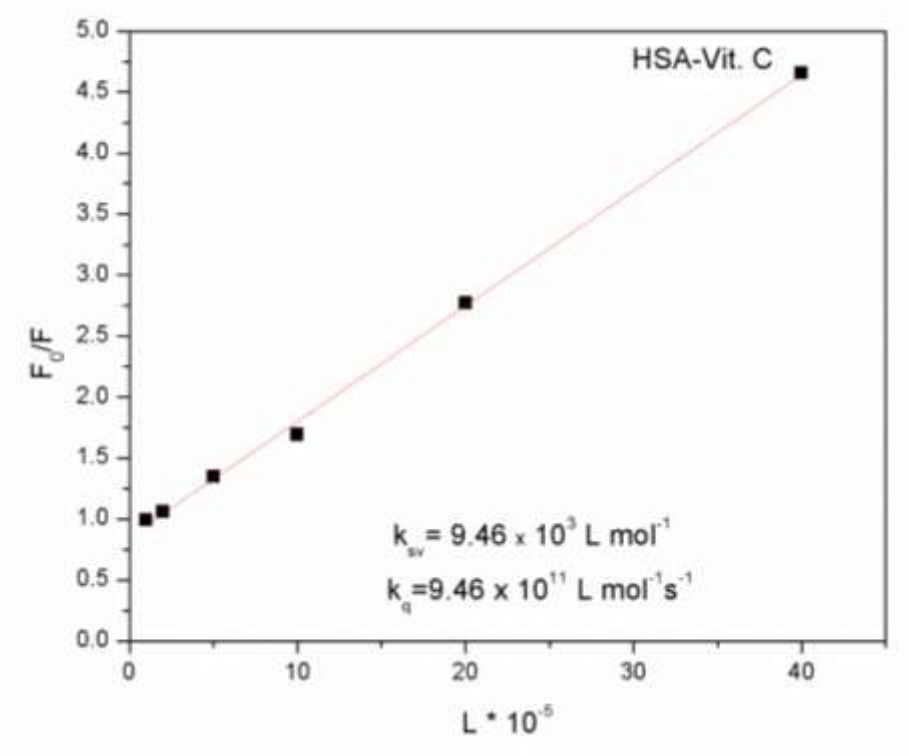


Fig.6.a: The Stern-Volmer plot for vitamin C- HSA complex

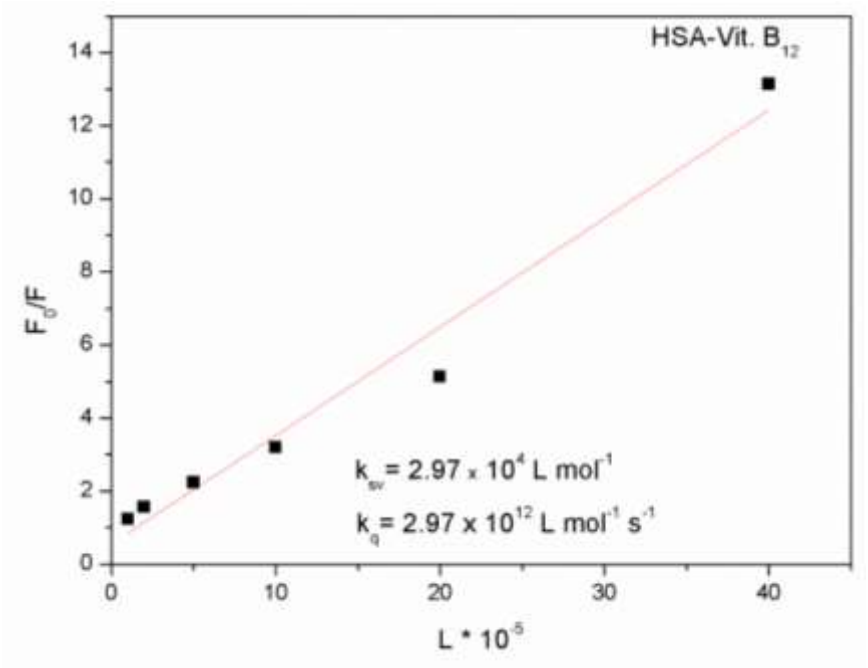


Fig.6.b: The Stern-Volmer plot for vitamin B_{12}- HSA complex

The curves are linear, suggesting the existence of a single type of quenching (dynamic or static) and/or a single binding site for both vitamins in the HSA neighborhood., the values ofthe fluorescence quenching constant determined applying Eq. (2) areequal to (9.46×10^3, 2.97×10^4) L mol^{-1} for vitamins C andB_{12} respectively, both values are much lower than other k_{sv} values for the similar systems signalized earlier in literature[1,44]. The calculated k_{sv} valuesare much too large to be caused by collisional quenching in water (two-three orders of magnitude larger), as well as the values of kq, which are equal to(9.46×10^{11}, 2.97×10^{12}) L mol^{-1} s^{-1} for vitamins C and B_{12} . These values confirms clearly the existence of static (diffusion- independent) mechanism of fluorescence quenching[10, 44].

Obviously, the values of kq were greater than that of the maximum dynamic quenching constant. This suggested that the fluorescence quenching was not the result of dynamic quenching, but the consequence of static quenching[43, 45]. When static quenching is dominant, the modified Stern- Volmer equation could be used[35].

$$\frac{1}{F_0 - F} = \frac{1}{F_0 K(L)} + \frac{1}{F_0} \qquad (3)$$

Where K is the binding constant of vitamin-HSA. To determine the binding constants of HSA-vitamins system, a plot of $\frac{1}{F0-F}$vs$\frac{1}{L}$ for different vitamins ratios are made and shown in **Fig. 7.a and Fig. 7.b** for vitamins C and B_{12} respectively. The plots are linear and have a slope of $\frac{1}{F0\,K}$ and intercept $\frac{1}{F0}$according to eq. (3). The values of K were found to be ($1.3\times 10^4 M^{-1}$, 2.29×10^4 M^{-1}), which agrees well with the values obtained earlier by UV spectroscopy and supports the effective role of static quenching.

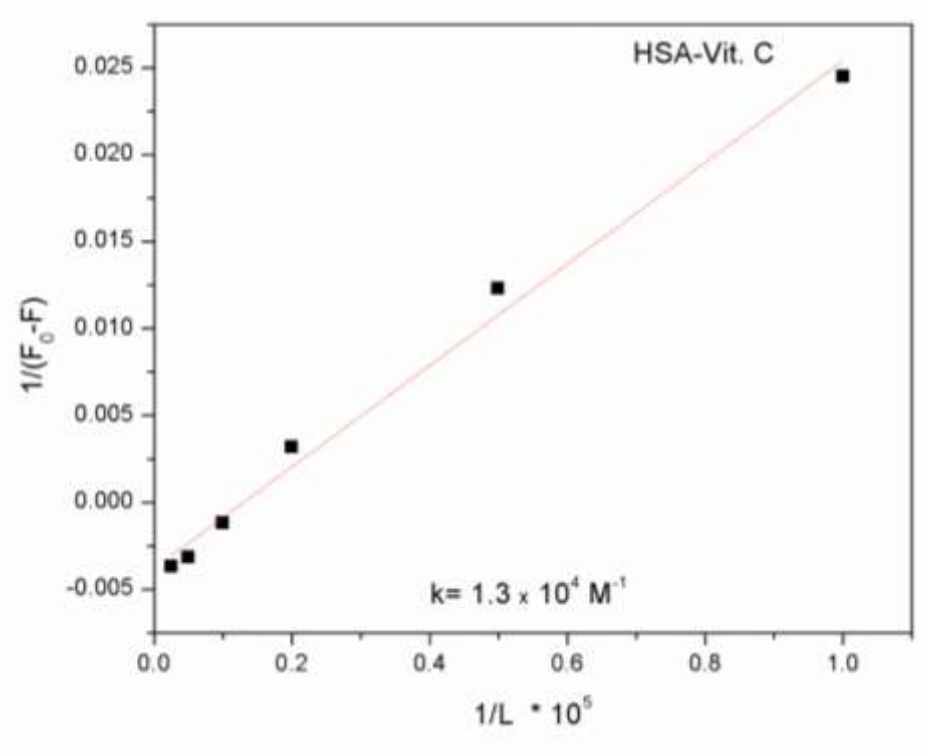


Fig. 7.a: The plot of $1/(F_0\text{-}F)$ vs. $1/[L\times 10^5]$ for HSA-Vitamin C

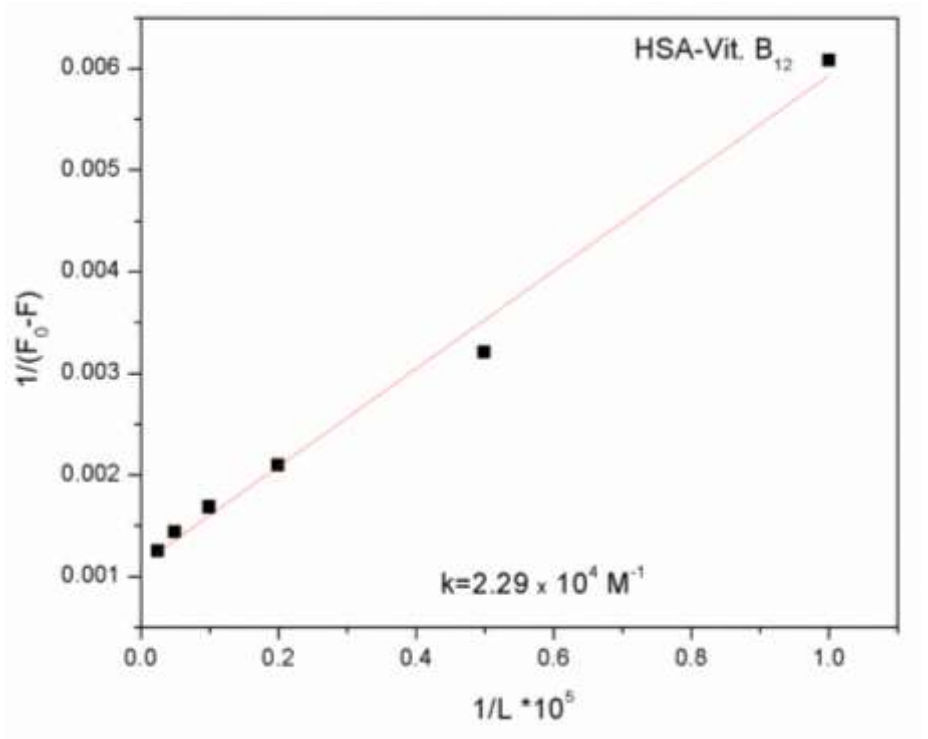


Fig. 7.b: The plot of $1/(F_0\text{-}F)$ vs. $1/[L\times 10^5]$ for HSA-Vitamin B_{12}

3.2. FT-IR spectroscopy: FT-IR spectroscopy is a powerful technique for the study of hydrogen bonding[46], and has been identified as one of the few techniques that is established in the determination of protein secondary structure at different physiological systems[8, 37]. The information on the secondary structure of proteins could be deduced from the infrared spectra. Proteins exhibit a number of amide bands, which represent different vibrations of the peptide moiety. The amide group of proteins and polypeptides presents characteristic vibrational modes (amide modes) that are sensitive to the protein conformation and largely been constrained to group frequency interpretations[14, 23].

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[44],has been widely accepted to be used [46]. The amide II band is primarily N-H bending with a contribution from C-N stretching vibrations; amide I ranging from 1600 to 1480 cm^{-1} while amide III band ranging from 1330 to 1220 cm^{-1} which is due to the C-N stretching mode coupled to the in-plane N - H bendingmode[47].

The second derivative of free HSA is shown in **Fig. 8.a**, where the spectra is dominated by absorbance bands of amide I and amide II at peak positions 1656 and 1544cm^{-1}. **Figures (8.b&8.c)** respectively shows the spectrum of HSA-vitamin C and HSA-vitamin B_{12} complexes with different concentrations.

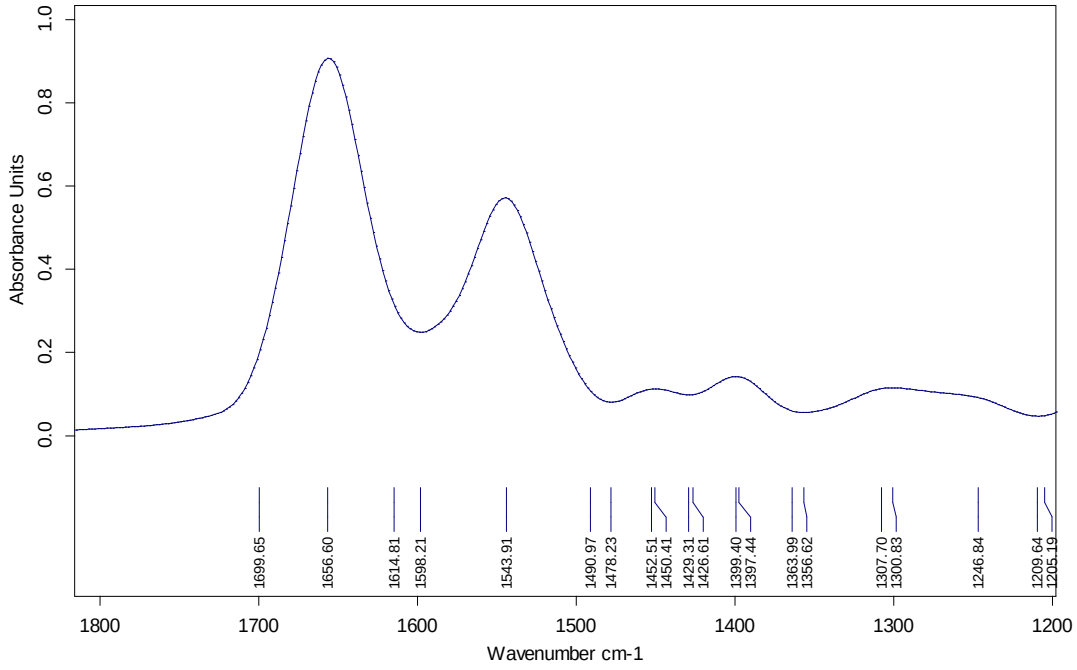


Fig. 8.a: Second derivative of free HSA.

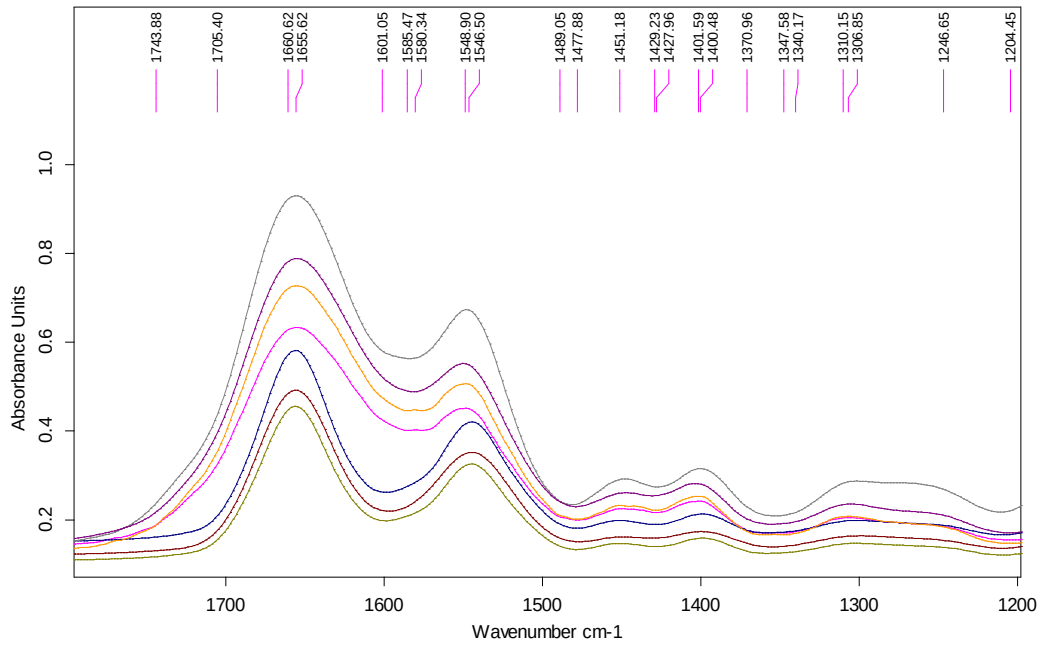


Fig. 8.b: Different spectra of HSA and its complexes with different vitamin C concentrations in the region 1800-1200 cm^{-1}.

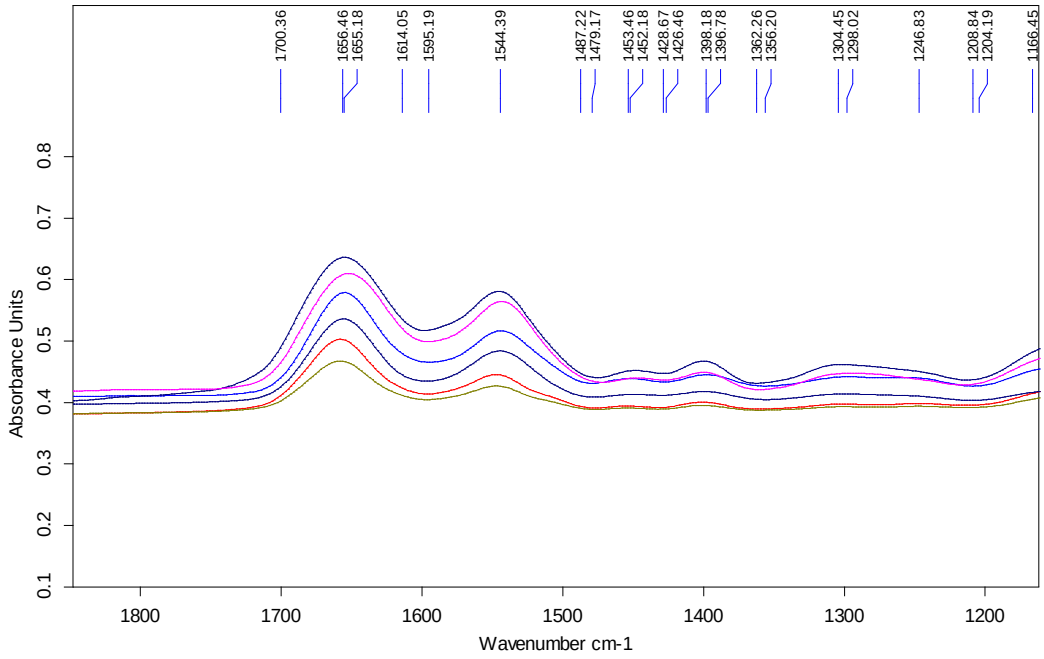


Fig. 8.c: Different spectra of HSA and its complexes with different vitamin B_{12} concentrations in the region 1800-1200 cm^{-1}.

The peak positions of HSA with different concentrations of vitamins C and B_{12} are listed in **tables 1& 2 r**espectively. It is clearly that the amide bands of HSA infrared spectrum are shifted in two different manner.

Table 1:. Band assignment in the absorbance spectra of HSA with different vitamin C concentrations for Amid I-III regions

Band regions (cm^{-1})	HSA Free	HSA-Vitamin C 1 $mg.ml^{-1}$	HSA-Vitamin C 2 $mg.ml^{-1}$	HSA-Vitamin C 5 $mg.ml^{-1}$	HSA-Vitamin C 10 $mg.ml^{-1}$	HSA-Vitamin C 20 $mg.ml^{-1}$	HSA-Vitamin C 40 $mg.ml^{-1}$
Amid I (1600-1700)	1658	1658	1658	1655	1654	1651	1650
	1620	1615	1598	1614	1597	1620	1607
	1598	1598	1597	1596	1595	1593	1591
Amid II (1480-1600)	1547	1546	1546	1546	1545	1544	1544
	1489	1489	1487	1485	1484	1484	1482
	1479	1478	1477	1477	1476	1473	1473
	1455	1452	1452	1451	1451	1451	1449
	1429	1427	1427	1426	1426	1426	1426
	1400	1399	1399	1398	1398	1398	1397
	1368	1368	1367	1366	1366	1366	1362
	1363	1361	1361	1358	1358	1356	1355
Amid III (1220-1330)	1310	1310	1309	1308	1308	1306	1304
	1248	1247	1247	1246	1246	1245	1244
	1216	1214	1214	1210	1209	1206	1203

Table 2: Band assignment in the absorbance spectra of HSA with different vitamin B_{12} concentrations for Amid I-III regions

Band regions (cm^{-1})	HSA Free	HSA-Vitamin B_{12} 1 $mg.ml^{-1}$	HSA-Vitamin B_{12} 2 $mg.ml^{-1}$	HSA-Vitamin B_{12} 5 $mg.ml^{-1}$	HSA-Vitamin B_{12} 10 $mg.ml^{-1}$	HSA-Vitamin B_{12} 20 $mg.ml^{-1}$	HSA-Vitamin B_{12} 40 $mg.ml^{-1}$
	1704	1705	1705	1706	1710	1710	1712
Amid I (1600-1700)	1656	1656	1656	1658	1658	1659	1661
	1601	1606	1608	1615	1616	1624	1617
Amid II (1480-1600)	1569	1569	1571	1573	1576	1578	1580
	1543	1544	1544	1544	1545	1545	1546
	1478	1478	1481	1481	1484	1485	1490
	1451	1451	1451	1451	1451	1453	1457
	1398	1400	1399	1400	1401	1400	1415
Amid III (1220-1330)	1302	1307	1310	1309	1310	1311	1312
	1246	1247	1248	1247	1247	1250	1256
	1204	1209	1210	1212	1214	1216	1220

An intensity increase in the difference spectra of the vitamin C-HSA complexes was observed for the protein amide I band at 1656 $cm^{-1}$and amide II at 1544 cm^{-1}. Positive features are located in the difference spectra for amide I and II bands at 1660 cm^{-1} and 1548 cm^{-1}(vitamin C). These positive features are related to increase in intensity of the amide I and II bands upon drug-complexation due to drug binding to protein C=O, C-N and N-H groups. Additional evidence comes from the decrement of the peak positions as the concentration of vitamin C increases as shown in **table 1.**

In the difference spectra of vitamin B_{12}-HSA complexes, the intensity decreases as the concentration of vitamin B_{12}increases for amide I band at 1656cm^{-1}, and amid II band at 1544 cm^{-1}. Table 2. Provides an increment of the peak positions as the concentration of vitamin B_{12} increases; this results in stabilization by hydrogen bonding by having the C-N bond assuming partial double character due to a flow of electrons from[44] the C=O to the C-N.

SUMMARY AND CONCLUSIONS

In summary, the binding of vitamin C and vitamin B_{12} to HSA has been investigated by UV-absorption spectroscopy, fluorescence spectroscopy and by FT-IR spectroscopy. The binding constant values indicate a relatively weak binding of both vitamins with HSA and the quenching constant indicate that the intrinsic fluorescence of HSA was quenched by both vitamins through static quenching mechanism. Analysis of FT-IR spectra reveals that HSA-vitamin C interaction induces intensity increment while HSA-vitamin B_{12} interaction induces intensity reduction.

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