ISSN: 0976-3376

Available Online at http://www.journalajst.com

ASIAN JOURNAL OF SCIENCE AND TECHNOLOGY

Asian Journal of Science and Technology
Vol. 09, Issue, 07, pp.8340-8347, July, 2018

RESEARCH ARTICLE

COMPARATIVE STUDIES ON THE INTERACTIONS BETWEEN HUMAN SERUM ALBUMIN, BOVINE SERUM ALBUMIN AND CHOLESTEROL: FTIR AND FLUORESCENCE SPECTROSCOPY

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ARTICLE INFO

Article History:
Received 06th April, 2018
Received in revised form
09th May, 2018
Accepted 20th June, 2018
Published online 30th July, 2018

Key words:
BSA , HSA, binding constant, IR and Fluorescence Spectroscopy.

ABSTRACT

The interaction of the human serum albumin (HSA), bovine serum albumin (BSA) with cholesterol has been investigated. The basic binding interaction was studied by FTIR and fluorescence spectroscopy. From spectral analysis cholesterol showed a strong ability to quench the intrinsic fluorescence of HSA and BSA through a static quenching mechanism. The binding constant (k) between HSA and cholesterol is estimated to be $K=2.14 \times 10^3\ M^{-1}$ at 293 K while between BSA and cholesterol is estimated to be $K=.1.12 \times 10^3\ M^{-1}$ at the same temperature. FTIR spectroscopy with Fourier self-deconvolution technique was used to determine the protein secondary structure and cholesterol binding mechanisms. The observed spectral changes indicate a higher percentage of H-bonding between cholesterol and α-helix compared to the percentage of H-bonding to cholesterol and β-sheets.

INTRODUCTION

Cholesterol is an extremely important biological molecule being an essential component of cell membrane as well as a precursor for the synthesis of a number of essential vitamins, steroid hormones and bile acids, its chemical structure is shown in Figure 1 (Hardie, 1991). Human serum albumin (HSA) (Figure 2A) is the most abundant carrier protein of the body with a high affinity for a wide range of metabolites and drugs. The most important physiological role of HSA is to carry such solutes in the blood stream and then deliver them to the target organs, as well as to maintain the pH and osmotic pressure of plasma (Pani, 2003).The molecular interactions between HSA and many compounds have been investigated (Korkmaz, 2005; Maiti *et al*., 2008; Jürgens *et al*., 2002). It has been recently proved that serum albumin plays a decisive role in the transport and disposition of a variety of endogenous and exogenous compound such as fatty acids, hormones, bilirubin, drugs (Ouameur *et al*., 2004). The distribution and metabolism of many biologically active compounds in the body whether drugs or natural products are correlated with their affinities toward serum albumin. Thus, the study of the interaction of such molecules with albumin is of imperative and fundamental importance (Ha *et al*., 2006). Extensive studies on different aspects of drug–HSA interactions are still in progress because of the clinical significance of the process (Tang 2006).

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Numerous analytical techniques are used for ligand protein binding studies and they are continuously extending our knowledge about the complex mechanisms involved in the drug–HSA binding process (Protein, 2008). Serum albumin is a major soluble protein constituent of the circulatory system and has many physiological functions such as acting as a plasma carrier by nonspecifically binding to several hydrophobic steroid hormones and as a transport protein for hemin and fatty acids (Ha, 2006). Albumins are characterized by a low content of tryptophan and methionine and a high content of cystine and charged amino acids (Tang *et al*., 2006; Protein, 2008). Bovine serum albumin (BSA),its molecular structure shown in Figure (2B) which is an example of a mammalian albumin, has been studied extensively because of its stability, neutrality in many biochemical reactions, and low cost (Darwish *et al*., 2010). Brown elucidated the 607 amino acid residue, primary structure of BSA (Figure 2B) in 1975, twenty one of which are tyrosine (Tyr) residues and two of which are tryptophan (Trp) residues located at positions 134 and 212, respectively (Tang, 2006; Darwish, 2010). These two Trp residues cause BSA to have intrinsic fluorescence. Bovine serum albumin (BSA) is constituted by 582 amino acid residues and, on the basis of the distribution of the disulfide bridges and of the amino acid sequence, it can be regarded as composed of three homologous domains (I, II and III) linked together. Each domain can be subdivided into two subdomains, A and B. BSA has two tryptophans, as mentioned above which they are embedded in subdomains IB and IIA, respectively (Moriyama *et al*., 1996). In this work, we have compared the behavior interaction of cholesterol with both BSA and HSA by means of FTIR and fluorescence spectrometers.

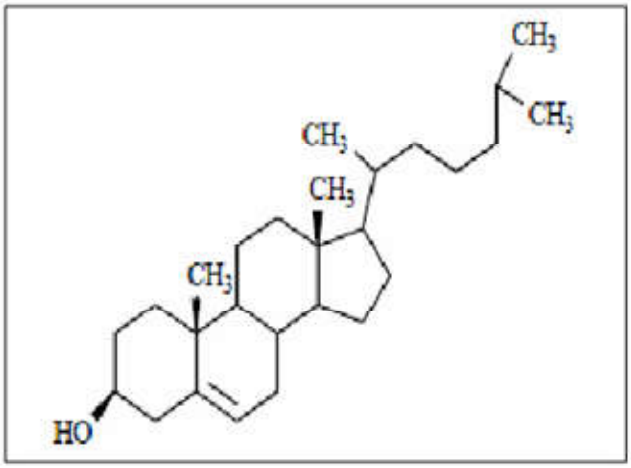


Fig.1. Chemical structure of cholesterol

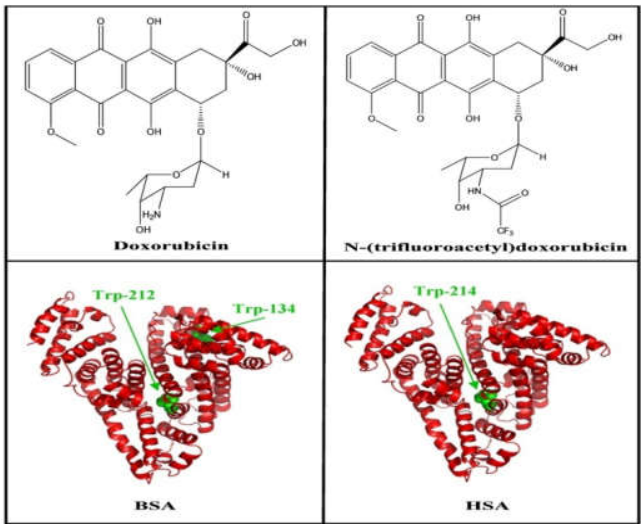


Fig. 2. Chemical structure of HSA and BSA

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). 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 of the secondary structure, components peaks (α-helix, β-sheets) can be derived and the analysis of this single band allows elucidation of conformational changes with high sensitivity (Darwish, 2010). Other spectroscopy techniques are usually used to study the interaction of many small molecules to proteins, fluorescence spectroscopy is commonly used because of its high sensitivity, rapidity and ease of implementation (Wybranowski *et al*., 2008; Sereikaite, 2006; Jianghong *et al*., 2008).

MATERIALS AND METHODS

HSA (fatty acid free), BSA, and Cholesterol in solid form were purchased from Sigma Aldrich chemical company and used without further purifications. The data was taken using thin films samples for FTIR measurements and solution samples for fluorescence measurements.

Preparation of HSA, BSA stock solution: HSA and BSA were dissolved in phosphate buffer saline pH 7.4 (80mg/ml) .

Preparation of Cholesterol stock solution: Cholesterol was dissolve in phosphate buffer saline (0.0009373g/ml), the solution was put in ultrasonic water path (SIBATA AU-3T) for six hours to ensure that all the amount of cholesterol was dissolved completely.

HSA-Cholesterol solutions: The final concentration of HSA-Cholesterol solutions were prepared by mixing equal volume of HSA solution and Cholesterol solution with various concentration. HSA concentration in all samples is 40mg/ml. However, the concentration of Cholesterol in the final protein cholesterol mixtures were decreased such that the molecular ratio (HSA:Cholesterol) started at 10:18, 10:14, 10:10, 10:6, and ended at 10:2.

BSA-Cholesterol solutions: The same procedure was done for the BSA-Cholesterol solutions by mixing equal volume of BSA solution and Cholesterol solution with various concentration. BSA concentration in all samples is 40mg/ml. However, the concentration of Cholesterol in the final protein cholesterol mixtures were decreased such that the molecular ratio (BSA:Cholesterol) started at 0.125, 0.25, 0.5, and 1 mM.

Thin film preparations: 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 Infra Red (MIR) region do not exhibit total absorption and can be easily subtracted. 50µl of each sample of HSA-Cholesterol mixture were placed on a silicon window and incubated at temperature 25 ^{0}C to evaporate the solvent. To obtain a transparent thin film on the silicon windows, all the samples were prepared at room tempreture. The same procedure was done for the BSA-Cholesterol mixture under the same conditions.

Fluorospectrometer (NanoDrop 3300): The fluorescence measurements were performed by a NanoDrop ND-3300 Fluorospectrometer at 25 ^{o}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.

FTIR Spectroscopy Experimental Procedures: The FTIR 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.

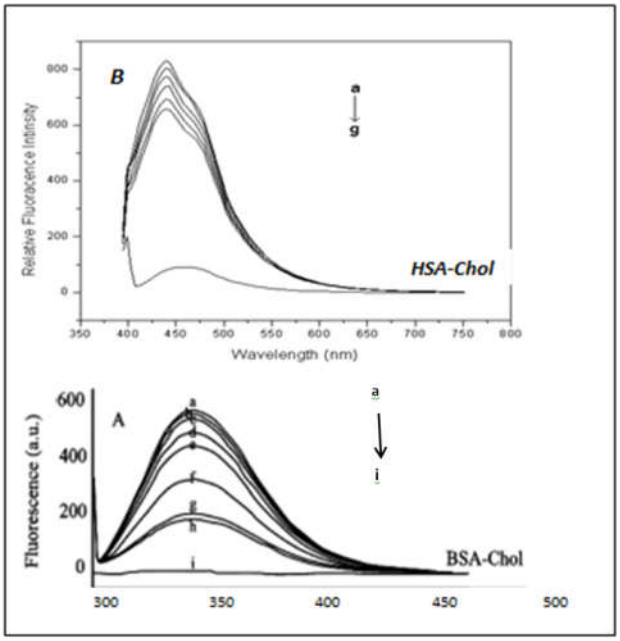


Fig.3. (A) Fluorescence emission spectra of BSA in the absence and presence of cholesterol in these ratios (chol: BSA a=0:125, b=0.25, c=0.5, d=1, e=1.4, f=1.8, g=2 ,h=2.2 and i=free cholesterol), (B) Fluorescence emission spectra of HSA in the absence and presence of cholesterol in these ratios (chol: HSA a=0:10, b=2:10, c=6:10, d=10:10, e=14:10, f=18:10, g=free cholesterol).

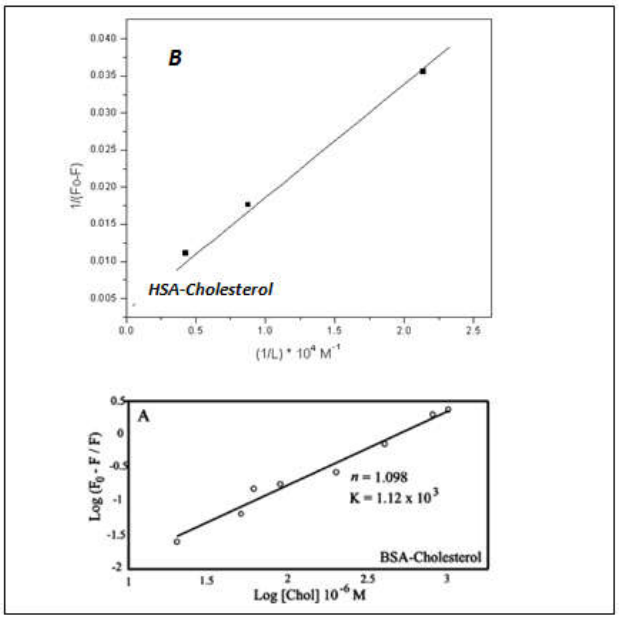


Fig. 4. (A) The plot of log((Fo-F)/F) vs log(L) x10-6 for cholesterol- BSA complexes. (B) The plot of 1/(Fo-F) vs (1/L)×104 for cholesterol- HSA complexes

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, BSA, BSA-Cholesterol and Cholesterol–HSA complex were obtained in the region of 1000–1800 cm^{-1}. The FTIR spectrum of free HSA and free BSA 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 Cholesterol 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).

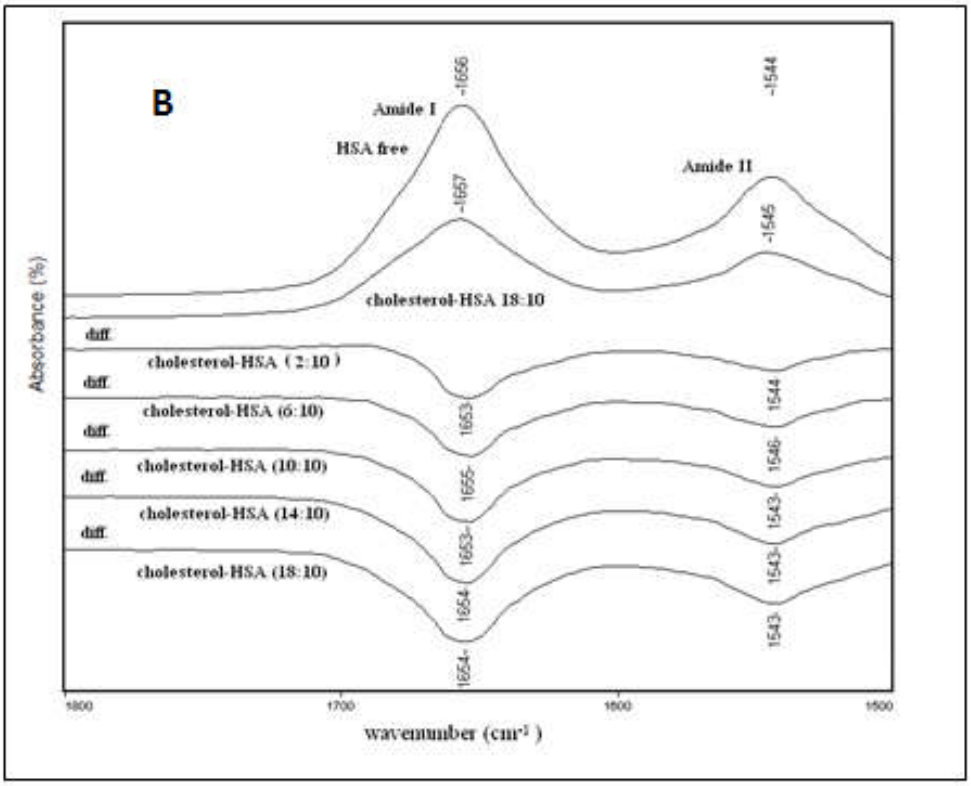
RESULTS AND DISCUSSION

Fluorescence spectroscopy: Fluorescence spectroscopy is one of the most widely used spectroscopic techniques in the fields of biochemistry and molecular biophysics today (Royer, 1995). The auto fluorescence of HSA comes from the tryptophan, tyrosine and phenylalanine residues. Actually, the intrinsic fluorescence of HSA is almost contributed by tryptophan alone, because phenylalanine has a 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 (Sulkowska, 2002). In our investigation of HSA- cholesterol mixtures we have done the excitation at 360nm wavelength and the observed emission was at 439nm wavelength. 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 system like proteins (Tian, 2003). The fluorescence quenching spectra of HSA at various contents of Cholesterol is shown in Figure (3B).

Obviously the fluorescence intensity of HSA gradually decreased while the peak position shows little or no change at all upon increasing the contents of cholesterol, indicating the binding of cholesterol to HSA. Under the same condition, no fluorescence of cholesterol was observed. Which indicates that cholesterol could quench the inner fluorescence of HSA and that the interaction between cholesterol and HSA indeed existed 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*., 2007; Gerbanowski *et al*., 1999). Fluorescence quenching refers to any process, which decreases the fluorescence intensity of a sample (Cui *et al*., 2007). Variety of molecular interactions can result in quenching, these include excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching (Cheng *et al*., 2006). Fluorescence quenching can be induced by different mechanisms, usually classified into dynamic and static quenching (Lakowica, 1973). So, the quenching is not initiated by dynamic collision but from formation of a complex, so static quenching is dominant (Lakowica, 1973; Eftink, 1991) When static equilibrium is dominant one can use the modified Stern-Volmer equation (Lakowicz, 1973)

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

Where K is the binding constant of cholesterol with HSA, and can be calculated by plotting $1/(F_0\text{-}F)$ vs 1/L, Figure (4A). The value of K equals the ratio of the intercept to the slope. The obtained values of K equals $K=2.14 \times 10^3$ M^{-1} for Cholesterol - HSA mixtures from Figure (4A) supports the effective role of static quenching.



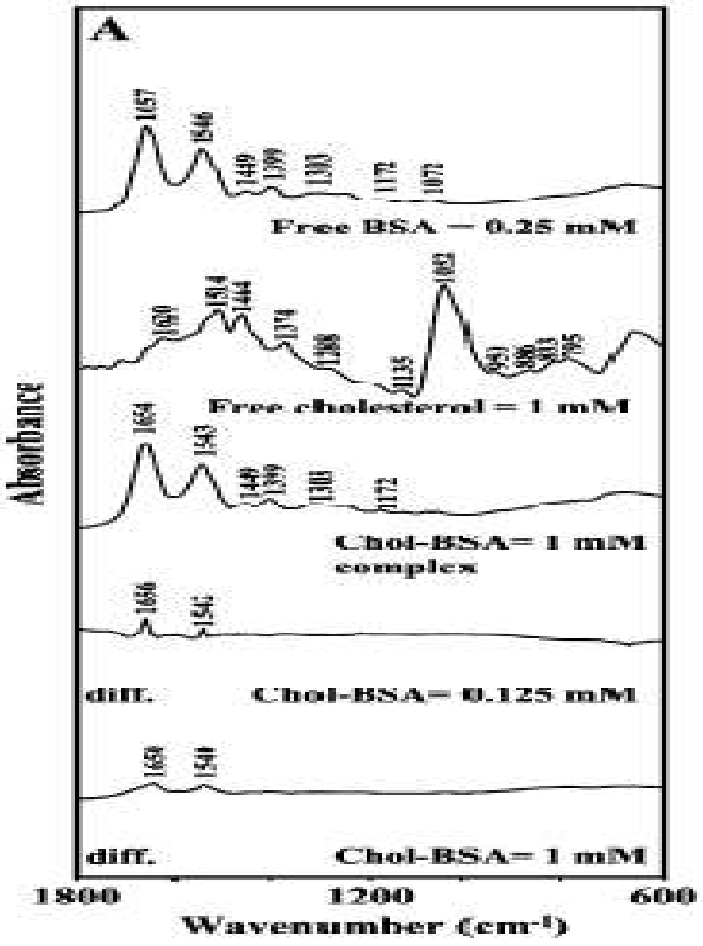


Fig.5. (A) FT-IR spectra (top two curves) and difference spectra of HSA and its complexes with different cholesterol concentrations in the region 1800-1500 cm^{-1}. (B) FT-IR spectra (top two curves) and difference spectra of BSA and its complexes with different cholesterol concentrations in the region 1800-600 cm^{-1}

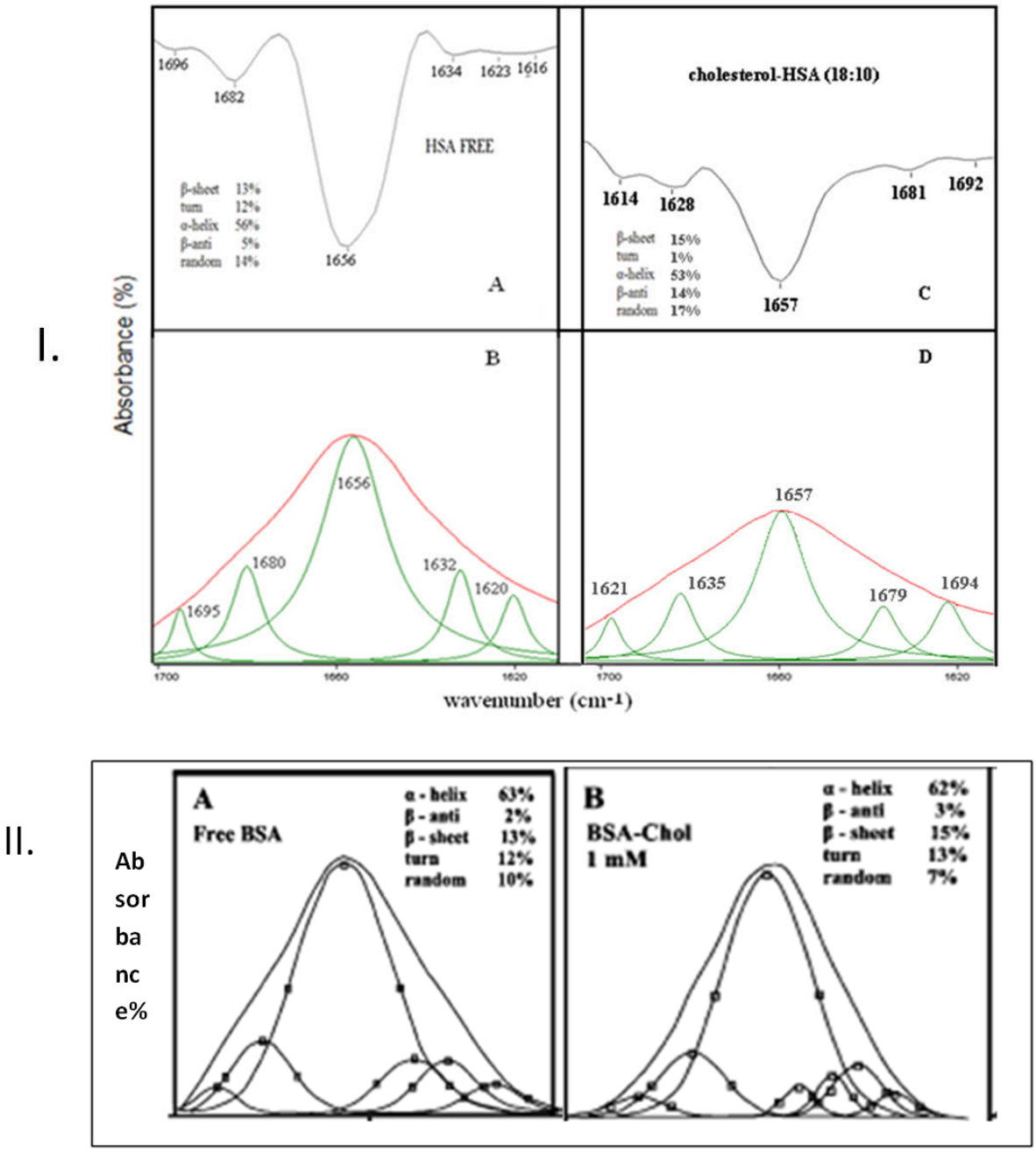


Fig. 6. (I) Second-derivative enhancement and curve-fitted Amide I region (1610-1700 cm^{-1}) and secondary structure determination of the free human serum albumin (A and B) and it cholesterol complexes (C and D) with 18:10 cholesterol: HSA ratios. (II) Second-derivative enhancement and curve-fitted Amide I region (1600-1700 cm^{-1}) and secondary structure determination of the free bovine serum albumin (A and B) and it cholesterol complexes (C and D) with 18:10 cholesterol: BSA ratios.

The highly effective quenching constant in this case has lead to a lower value of binding constant between the cholesterol and HSA due to an effective hydrogen bonding (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 beside the hydrogen bonds were involved in the binding process. However, cholesterol 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 cholesterol to HSA includes the hydrophobic interaction and hydrogen bonds (Wang *et al*., 2008).

The fluorescence of BSA 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 (Petitpas *et al*., 2001). The fluorescence spectra of BSA at various ratios of Cholesterol (0.125, 0.25, 0.5, and 1 mM) are shown in Figure (3A). The fluorescence intensity of BSA decreased regularly with the increasing of Cholesterol concentration. The peak position shows little or no change at all. When the static quenching equation is used (Wang *et al*., 2007)

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

Where K is the binding constant of cholesterol with BSA, and can be calculated by plotting log((F_0-F)/F) vs log(L), figure (4B). The value of K can be determined from the slope and the intercept shows that the value of K is *K*-Chol $=1.12 \times 10^3$ M^{-1}, which agrees well with the value obtained earlier by 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 drug and BSA due to an effective hydrogen bonding between cholesterol and BSA. From figures (4 A and B) it was obvious that the binding constant between the cholesterol with HAS and with BSA almost the same which means a weak interaction of the cholesterol with both of them.

FT-IR Spectroscopy: FT-IR spectroscopy is a powerful technique for the study of hydrogen bonding (Yang *et al*., 1994), and has been identified as one of the few techniques that is established in the determination of protein secondary structure at different physiological systems (Li *et al*., 2006; Sirotkin *et al*., 2001). Infrared spectra were recorded on a Fourier transform infrared (FTIR) spectrometer. (Impact 420 model), equipped with deuterated triglycine sulfate (DTGS) detector and KBr beam splitter, using AgBr windows. A solution of lipid was added dropwise to the BSA solution with constant stirring to ensure the formation of homogeneous solution and to reach the target lipid concentrations of 0.125, 0.25, 0.5, and 1 mM with a final BSA concentration of 0.25 mM (20 mg/mL). Spectra were collected after 2 h incubation of BSA with lipid solution at room temperature, using hydrated film. Interferograms were accumulated over the spectral range of 600-4000 cm^{-1} with a nominal resolution of 4 cm^{-1} and 100 scans. The difference spectra ((BSA solution + lipid solution) - (BSA solution)) were generated using the polypeptide antisymmetric and symmetric C-H stretching bands, located at 2800-2900 cm^{-1}, as internal standard.

The bands due to protein C-H stretching vibrations do not undergo spectral changes (shifting or intensity variation) upon lipid complexation and, therefore, are commonly used as internal standard. For producing difference spectra these bands were adjusted to the baseline level, in order to normalize difference spectra. Details regarding infrared spectral treatment are given in our recent publication (Arrondo, 1993). The information on the secondary structure of proteins arises from the amide bands which is a result from the vibrations of the peptide groups of proteins. When small molecules bind to a globular protein like HSA, changes in hydrogen bonding which is involved in the peptide linkages would occurs, resulting in changes in the vibrational frequency of the different amide modes (Beauchemin *et al*., 2007; Ganim, 2006). The modes most widely used in protein structural studies are amide I, amide II and amide III. Amide I band ranging from 1600 to 1700 cm^{-1} and arises principally from the C=O stretching (Haris, 1999) and has been widely accepted to be used for two purposes (Vandenbussche *et al*., 1992). Amide II band is primarily N-H bending with a contribution from C-N stretching vibrations, amide II ranging from 1480 to 1600 cm^{-1}. Amide III band ranging from 1220 to 1330 cm^{-1} which is due to the C-N stretching mode coupled to the in-plane N - H bending mode.

The difference spectra for ((HSA + cholesterol) - (HSA)) were obtained in order to monitor the intensity variations of these vibrations; the results are shown in Figures (5A and 5B). Figure (5B) shows FT-IR spectra (top two curves) and difference spectra of HSA and its mixtures with different cholesterol contents in amide I and amide II regions and figure(5B) shows FT-IR spectra (top two curves) and difference spectra of HSA and its mixtures with different cholesterol contents in amide III region. In amide I region clearly there is a strong negative feature at 1654cm^{-1} with a little shift in its position, and in amide II region one negative feature was also observed at 1543cm^{-1} also with a little shift in its position (Figure 5B). For amide III region two negative feature was observed at 1245cm^{-1} and at 1311cm^{-1} with a little shift in their positions as cholesterol contents was increased, another weak negative feature was observed at 1287cm^{-1} at low content of cholesterol and disappeared as the contents of cholesterol was increased (Figure 5B). It is clearly shown that the strong negative features in the difference spectra became stronger as contents of cholesterol 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 cholesterol– HSA mixtures, that is due to the interaction (H bonding) of the cholesterol with protein C=O and C-N groups, and the reduction of the proteins α-helix structure upon cholesterol–HSA interaction (Krimm, 1986; Purcell *et al*., 2001). The band at 1517 cm-1 of the free HSA, related to the tyrosine amino acid side-chain vibration (Fabian *et al*., 1994; Yamamoto, 1991; Matsuura, 1986), exhibited no spectral changes upon cholesterol mixing, which is indicative of no perturbation of the tyrosine residue in the cholesterol– HSA mixtures. The second derivative resolution enhancement and curve – fitted Amide I, Amide II, and Amide III regions and secondary structure determinations of the free human serum albumin (A, B) and its cholesterol mixtures (C, D) with the highest contents of cholesterol in dehydrated films are shown in (Figure 6 (I A and B)). Determination of the secondary structure of HSA and its cholesterol mixtures was carried out on the basis of the procedure described by Byler and Susi (44). In this work, a quantitative analysis of the protein secondary structure for the free HSA and cholesterol– HSA mixtures in dehydrated films is determined from the shape of Amide I, amide II and amide III bands. Infrared Fourier self-deconvolution with second derivative resolution and curve fitting procedures, were applied to increase spectral resolution and therefore to estimate the number, position and area of each component bands. The procedure was in general carried out considering only components detected by second derivatives and the half widths at half height (HWHH) for the component peaks are kept around 5cm^{-1} , the above procedure was reported in our lab recent publication (Darwish *et al*., 2010). 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 reduction of α-helix intensity percentage in favor of the increase of β-sheets are believed to be due to the unfolding of the protein in the presence of cholesterol as a result of the formation of H bonding between HSA, BSA and the cholesterol. The newly formed H-bonding result 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(45) and

the hydrogen bonds in α-helix are formed inside 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 anti-parallel 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*., 1996). The Cholesterol -BSA complexation was characterized by infrared spectroscopy. Since there was no major spectral shifting for the protein amide I band at 1656 cm-1 and amide II band at 1544 cm-1 (C-N stretching coupled with N-H bending modes)[11-13] upon lipid interaction, the difference spectra ((BSA solution + cholesterol solution) - (BSA solution)) were obtained, in order to monitor the intensity variations of these vibrations, and the results are shown in Figures 5A and 5B.

Similarly, the infrared self-deconvolution with second derivative resolution enhancement and curve-fitting procedures[13] were used to determine the protein secondary structure in the presence of cholesterol (Figure 5B). At low cholesterol concentration (0.125 mM), intensity changes were observed for the protein amide I at 1657 cm-1 and amide II at 1546 cm-1, in the difference spectra cholesterol-BSA complexes (Figures 5 A and B; difference, 0.125 mM). Positive features are observed in the difference spectra for amide I and II bands at 1655-1656 for Chol-BSA, (Figure 5A). The positive features are related to an increase in the intensity of the amide I and amide II bands upon lipid complexation, while negative features are due to the reduction of intensity of the amide I band due to the loss of protein R helix structure (Figures 6(II) A and B; difference, 0.125 mM). The spectral changes observed for the amide I and amide II bands (CdO and C-N vibrations) come from lipid-BSA interaction, which aletrs protein conformation. Additional evidence for lipids interaction comes from the shifting of the protein amide A band at 3295 cm-1 (N-H stretching mode) in the free BSA to 3299 (Chol-BSA) upon lipid complexation. As lipid concentration increased to 1 mM, positive features in amide I and II bands were observed at 1651, 1650 (amide I) cm-1 and 1539, 1540 cm-1 (amide II) for both Chol- BSA complexes. (Figures 6Aand 6B; difference, 1 and 0.25 mM). The observed increase in intensity of the amide I band at 1657 cm-1 in the spectra of Chol- BSA suggests a minor alteration, caused by structural changes in intramolecular bonding. Similar infrared spectral changes were observed for the protein amide I band in several drug-protein complexes, where major protein conformational changes occurred (Ahmed Ouameur *et al*., 2006).

Conclusion

The binding of cholesterol to HSA and BSA have been investigated by fluorescence spectroscopy and by FTIR spectroscopy. From the Fluorescence Investigations we determined values for the binding constant and the quenching constant. The results indicate that the intrinsic fluorescence of HAS and so for BSA was quenched by cholesterol through static quenching mechanism. Analysis of the FTIR spectra reveals that HSA – cholesterol and BSA-Cholesterol interaction results in major protein secondary structural changes in the compositions of α-helix to that of the β-sheets.

Acknowledgement: This work is supported by the German Research Foundation DFG grant No. DR228/24-2

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