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Multispectroscopic and Molecular Docking Study on the Interaction of Human Serum Albumin with the Copper Complex [Cu(HEAC)Br]Br

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Abstract

In this article the interaction of human serum albumin (HAS) with a copper complex [Cu(HEAC)Br]Br containing of bromide and 2-(2-(2-Hydroxyethylamino)ethylamino)cyclohexanol (HEAC) ligands was investigated. To have a better understanding over the interaction mode multispectroscopic methods such as UV–vis, fluorescence, CD spectroscopy along with molecular docking studies were used. The results of both UV–vis spectroscopy and fluorescence test indicated the existence of interaction between human serum albumin and the copper complex. The binding and quenching constants were calculated and indicated that the quenching type was static and mechanism was through the formation of the none fluorescence complex. Also, the results of the competitive replacement test with ibuprofen and warfarin showed that the complex binds to human serum albumin in site II (subdomain IIIA) and from only one site. The results of the CD indicated that the addition of the copper complex to the HSA solution stabilizes the α-helix structure of HSA.

Introduction

The application of copper compounds in biology and medicine science is an important and common topic. Nature is eventually known as the first user of the copper compounds to sustain and stabilize life for a long period of time. For instance, the copper chemistry of cobalamin (better known as vitamin B12) and derivatives has been investigated for decades, along with that of a variety of enzymes and cofactors containing metal–carbon bonds. Copper-based complexes are among the most promising entities for target-specific next-generation anticancer and NSAIDS therapeutic agents. Copper has a key role in numerous physiological cellular processes. However, because of its high redox activity the free copper ions are highly cytotoxic, and for this reason the intracellular level of copper must be strongly regulated [1]. It is well known that in neoplastic diseases, copper metabolism is severely altered. The connection between copper elevated level and disease like serum progression, tumor burden, and recurrence in a variety of cancers (such as liver, lung, prostrate, breast, sarcoma, and Hodgkin's lymphoma) has been the center of many studies and is well known. A large number of copper complexes with various sets of ligands have been prepared for this cause and displayed noteworthy *in-vitro* cytotoxicity [2-4]. Copper complexes are considered among the best non-Pt compounds as anticancer agents [10]. It is reported that the properties of copper-coordinated compounds are highly dependent to the nature of ligands and donor atoms bounded to the metal ion. Copper is found in all living organisms and is a crucial trace element in redox chemistry, growth and development. It is essential for the best function of several proteins that involved in energy metabolism, respiration

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and DNA synthesis, and enzymes such as cytochrome oxidase, superoxide dismutase (SOD), ascorbate oxidase and tyrosinase. The major functions of copper–biological compounds involve oxidation–reduction reactions where copper containing biological molecules react directly with molecular oxygen to produce free radicals [5-7]. The copper and its complexes known as a very important bioactive compounds in-vitro and in-vivo. They always have been interesting as potential drugs for therapeutic and preventing agents in various diseases. The schematic observed coordination of copper in complexes is presented in Fig. 1. In its non-complex form, copper is bound to ceruloplasmin, albumin, and other proteins, while in the complex form with different ligands it interacts with biomolecules, mainly proteins and nucleic acids mostly DNA and HSA [8].

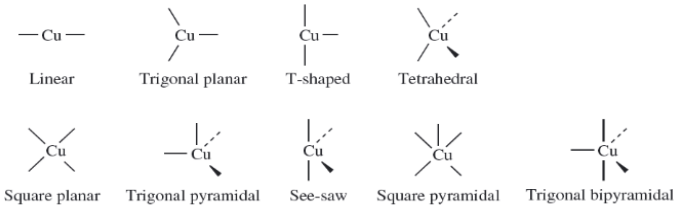

Figure 1: The observed coordination geometries for the copper.

The main reason for the interest in Cu complexes is because of their potential use as antimicrobial, antiviral, anti-inflammatory, antitumor agents, enzyme inhibitors, or chemical nucleases. Numerous Cu(II) complexes of NSAIDs showed enhanced anti-inflammatory and antiulcerogenic activity, as well as, reduced gastrointestinal toxicity compared to the non-complexed copper [9,10].

β-Amino alcohols are of interest in medicinal chemistry. They have a variety of biological functions, including tuberculostatic and antibacterial roles [11]. They can also serve as active pharmaceutical ingredients (APIs), some of which are α- adrenergic agonist, β-adrenergic agonist [12], inhibitors of HIV protease [13] and anti-hypertensive agent by inhibiting α- adrenergic receptor and/or β-adrenergic receptor [14]. Some of γ-amino alcohols showed γ-secretase inhibitory effect and notch-sparing performance [15].

Human serum albumin (HSA) is the most abundant protein in plasma and originally is a monomeric multi-domain macromolecule, representing the main determinant of plasma oncotic pressure and the main modulator of fluid distribution between body compartments. HSA presents an amazing ligand binding capacity, which provides a depot and carrier for many endogenous and exogenous compounds. It is also known as the main carrier for fatty acids that affects pharmacokinetics of many drugs. It provides the metabolic modification of some ligands, renders potential toxins harmless, accounts for most of the anti-oxidant capacity of human plasma, and displays enzymatic properties [16]. Human Albumin serum is the major serum protein; and it plays a vital role in physiological functions (such as maintenance of colloidal osmatic pressure, binding of a wide set of compounds, provision of the bulk of plasma antioxidant activity, etc.). HSA is the most abundant protein in human blood plasma, making half of the serum proteins volume. Albumin serum plays major roles in human blood. Some of the bold roles can be such as: transportation of hormones and fatty acids, it provides a strong ligand-binding property, buffers pH of blood, and maintains oncotic pressure of blood, Serum albumin level in human blood can directly affect the half-life of drugs and the N-terminal portion of the human serum albumin binds Cu, Ni, and Co ions with a high affinity.

The transporting and binding properties of human serum albumin in blood is a crucial role in the distribution of drugs in the body. For this reason, the interaction and the binding mode of the drugs to human serum albumin has been the subject of many studies in the past decades.it was observed that in the cases which the drug was not enable to bind to the HSA a much higher amount of the drug was necessary to use and major Complications were observed on the patient. In this study the interaction between the copper complex [Cu(HEAC)Br]Br and HSA was observed using multispectroscopic methods such as UV–Vis, fluorescence and CD spectroscopy. The molecular docking study is conducted to determine the exact site of binding between the copper complex and HAS.

Materials & methods

Materials

Human serum albumin (HSA) (content > 98 %), NaH_2PO_4, Na2HPO4 (content > 98 %), Tris- (hydroxymethyl)-amino-methane–hydrogen chloride (content > 98 %), warfarin and Ibuprofen were purchased Sigma Aldrich. The Tris_HCl buffer solution was made from Tris- (hydroxymethyl)-amino-methane–hydrogen chloride (pH = 7.40), and was stored at 4 _C in the dark. The phosphate buffer solution was prepared from NaH2PO4, Na2HPO4, and NaOH (pH = 7.40), and was stored in the dark. The stock solution of HSA protein was prepared in phosphate buffer.

Preparation of the copper complex [Cu(HEAC)Br]Br

In 2018, Mardani et al. [17] synthesized a copper complex of HEAC is 2-(2-(2-Hydroxyethylamino)ethylamino)cyclohexanol and bromide ligands. This complex was prepared as follows and after purification has been used (Fig. 2): HEAC (0.69 g, 3.4 mmol) and CuBr2 (0.22 g, 1 mmol) were separately dissolved in ethanol (20 mL), and were irradiated inside an ultrasonic bath for 20 minutes at room temperature. The ligand solution was added to the metallic solution before being sonicated for 30 minutes and removing the solvent by rotary evaporation. Suitable blue prisms for X-ray diffraction studies were obtained by slow evaporation of the solution for a week and collected by Iteration. Yield: 0.27 g, 63%; mp 180–184 ºC. Anal. calcd for C10H22Br2CuN2O2 (%): C, 28.22; H, 5.21; N, 6.58. Found: C, 28.32; H, 5.20; N, 6.64. IR (KBr, cm-1): 3242 (ν O–H), 3200 (ν N–H), 2929 (ν C–H and/or vas CH2), 2881 (ns CH2), 1448 (δas CH2), 1328 (δs CH2), 1243 and 1204 (ν C–O), 1100 and 1050 (ν C–N). UV-Vis (H2O, λmax (nm)/ε): 680/76 (d -d). The complex solution was prepared at the concentration of 1×10^{-3}.

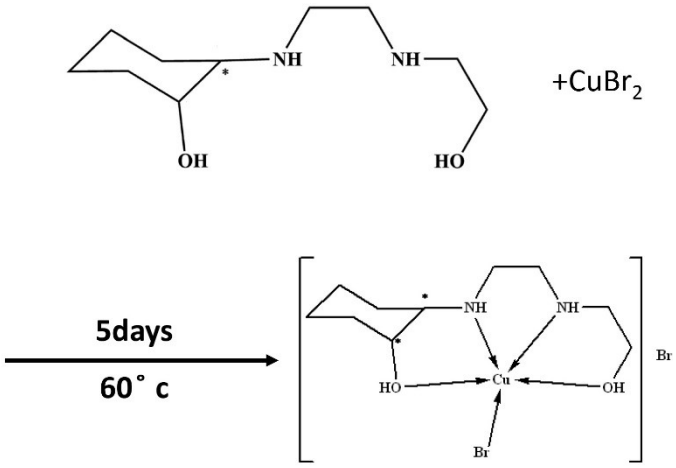

Figure 2: Schematic reaction of the synthesis of the copper complex.

Methods of study

Electronic Absorption Spectral Studies

The ultraviolet-visible absorption spectra were caried out on a Nordantec T80 UV-Visible spectrophotometer along with a 1- cm path length quartz cuvette. The solution of the human serum albumin and phosphate buffer was titrated by the adding amounts of the copper complex [Cu(HEAC)Br]Br at the room temperature and range of 200-400 nm.

Fluorescence Emission Spectra Analysis

Investigate and study of the intrinsic fluorescence of HSA is one of the most common ways to detect any changes in the structure of the human serum albumin [18]. The reason for the fluorescence of human serum albumin is mainly the presence of residues like tryptophan and then tyrosine and phenylalanine in its structure [19, 20]. The emission spectrum studies were performed in the range of 300-500 nm with the excitation at 295 nm at medium sensitivity levels. Fluorescence studies of the interaction between copper complex ([Cu(HEAC)Br]Br) and Human serum albumin was carried out using a JASCO Fluorescence spectrophotometer (FP6200) using a 1 cm path length cell. The concentration of the HAS were kept constant at (1×10^{-4} M) and various amounts of the copper complex with concentration of (1×10^{-3} M) was added to it. these studies were repeated at 288, 303, and 310 K. the excitation wavelength was at 295 nm, and emission spectra were noted in the range of 300-550 nm with medium sensitivity. The quenching constant and the stern-volmer constant were measured applying the Stern-Volmer equation (Equation 1):

$$F_0/_F = 1 + K_{SV}[Q] \qquad \textbf{Eq .1}$$

$$F_0/_F = 1 + K_q\tau_0[Q] \qquad \textbf{Eq .2}$$

Whereas F_0 and F display intensities of fluorescence in the absence and existence of the applied quencher respectively. K_q is the constant of the fluorophore quenching rate, K_{SV} is the quenching constant, τ_0 is defined as the fluorophore lifetime in the vicinity of quencher ($\tau_0 = 10^{-8}$) , and [Q] is the concentration of quencher.in order to estimate the binding constant of the complex and the number of active binding sites the following equation was used [21]:

$$\boldsymbol{Log(F - F_0)/_F = Log\, K_b + n\, Log\,[Complex]}$$

Here F0 and F are the fluorescence intensities of the fluorophore in the absence and presence of different concentrations of the copper complex. Thermodynamic parameters such as enthalpy and entropy (ΔH, and ΔS) were calculated employing the Van't Hoff equation (Equation 3 and Equation 4) [22].

$$\Delta G = -RT\,Ln\,K_b \qquad \textbf{Eq. 3}$$

$$\Delta G = \Delta H - T\Delta S \qquad \textbf{Eq. 4}$$

Determination of the binding site of the copper complex on HSA

To determine the binding site for copper complex on HSA a competitive displacement experiment was used. Two important binding sites on human serum albumin are Sudlow sites I and II. Sudlow site I is located in subdomain IIA and Sudlow site II is located in subdomain IIIA. Sudlow site I has a preferential binding affinity for bulky heterocyclic compounds such as Azapropazone, Phentylbutazone and Warfarin. Sudlow site II seems to preferentially bind to aromatic compounds such as Ibuprofen, Flufenamic acid, Chlorpheniramine maleate and other drugs [23–25]. A series Fluorescence emission test was performed with both drugs of Ibuprofen and Warfarin in the absence and persence of the coppor complex to compare the binding constant and affinity of the drugs to HSA and to learn the binding site of the oppor complex.

Circular dichroism (CD) measurements

To have a better understanding on the structural changes of human serum albumin following the addition of the copper complex the CD spectroscopy was used. The test was carried out using a Jasco J-715 spectropolarimeter by a 0.1-cm quartz cuvette in the wavelength range of 220–300 nm. with a solution of the complex and HSA (1×10-4) at room temperature.

Molecular Docking Simulation

The open-source AutoDock Vina (version 1.1.2) and MGL tools 1.5.6 were used to perform docking simulations. The partial charges of Gasteiger and polar hydrogens were added to the copper(II) complex and all rotatable bonds were defined. The HSA 3D structure (PDB id: 1AO6, chain A) obtained by X-ray crystallography was used as a template. The HSA was enclosed in a box with the number of points in x, y, and z dimensions of 48, 40, and 30 and center grid box of 32.861, -11.806, and 37.722 with a grid spacing of 1.00 Å. The docking calculation was performed using the Lamarckian genetic algorithm (LGA).

Results and Discussions

UV-Vis Absorption Studies

The UV-Vis spectroscopy is one of the most common methods that is been used in the past decades and can simply define any changes in the structure of biomacromolecules such as HSA. This method can also be used to estimate binding mode and binding constant between biomacromolecules and various ligands [26-28]. Human serum albumin has a certain characterized spectrum which include two absorption peaks: one is near 210 nm and is due to the helical structure of HSA and second is the peak near 280 nm due to the π-π* transition of the aromatic amino acids [29]. To have a better understanding on the binding mode of the copper complex [Cu(HEAC)Br]Br] and human serum albumin, the Uv-Vis absorption spectroscopy observations was caried out at the range of 200-400 nm. Uv-Vis spectrometric studies of the interaction between copper complex ([Cu(HEAC)Br]Br) and Human serum albumin was carried out using an Agilent Uv-Vis spectrophotometer (8453). The absorption spectrum of HSA in the absence and presence of different concentrations of the Cu(II) complex has been displayed in Fig. 3. After the addition of various amounts of the complex a significant decrease in the intensity of HSA peaks was observed even though the shape and max point of the peaks almost remained the same. The results indicate that there was an interaction between the copper complex and HSA. The decrease in the intensity of the peaks can be attributed to the binding of the copper complex to the amino acid residues chromophores located in subdomains IIA or IIIA of HSA [30].

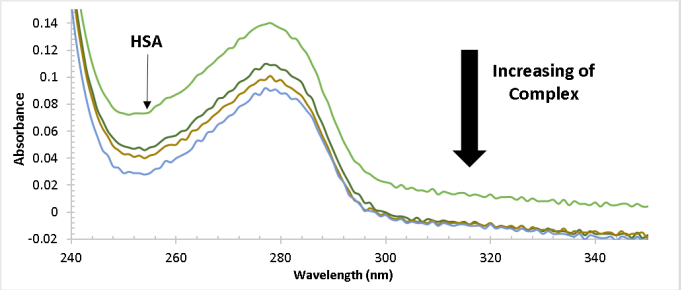

Figure 3: Absorption spectra of HSA at the presence of different concentrations of copper complex ([Cu(HEAC)Br]Br).

Fluorescence Emission Analysis

Fluorescence quenching Analysis is One of the most common ways to detect any change in the structure of the human serum albumin is to investigate and study the intrinsic fluorescence of HSA. The reason for the fluorescence of human serum albumin is mainly the presence of residues like tryptophan and then tyrosine and phenylalanine in its structure [19, 20]. The emission spectrum studies were performed in the range of 300-500 nm with the excitation at 295 nm at medium sensitivity levels. These observations were done in various temperatures with different amounts of the complex. Fluorescence emission spectra of the HSA in the presence of different amounts of the copper complex ([Cu(HEAC)Br]Br) was shown in the Fig. 4. Upon the addition of the complex, a significant decrease in the fluorescence intensity was observed indicating that there was an interaction between the complex and HSA resulting the quench of the emission. Quenching is often categorized into two categories:

- Dynamic quenching
- Static quenching

The difference between these two types can be detected by the observation of emission intensity in various temperatures. In the static quenching with the increase of the temperature the K_{SV} will decrease and in dynamic quenching with the rise in temperature the K_{SV} increase. The emission observations and the (KSV) calculated with the Stern-Volmer equation (Eq. 1) along with increase in the Kq indicates that the copper complex has a dynamic quenching mechanism. But the maximum Kq for the dynamic mechanism is 2×1010 [31–33]. Since the calculated numbers **(Table 1)** are much higher than this amount it indicates the formation of non-fluorescence complex and the static quenching mechanism **(Figure 5 and Figure 6)**.

Also we can find out the type of mechanism quenching by carefully examining the UV spectrum, because dynamic and static quenching give us two different UV spectra of protein. A novel complex formed between protein and ligand in the static quenching can be the main causes of change in the UV protein spectrum [33].

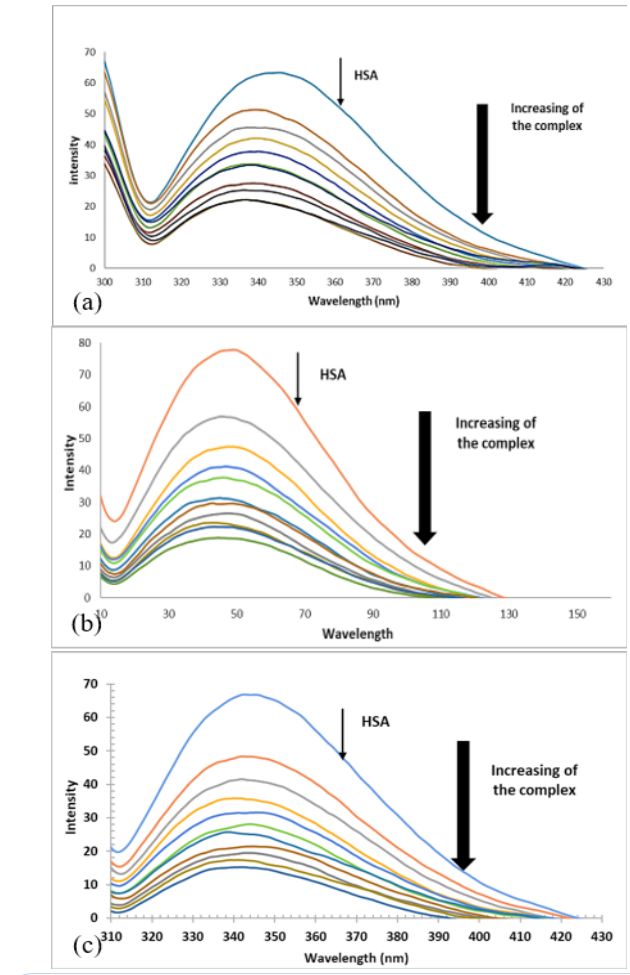

Figure 4: Fluorescence emission spectra of the HSA in the presence of different amounts of the copper complex at various (a) 288 (b) 303 and (c) 310 K.

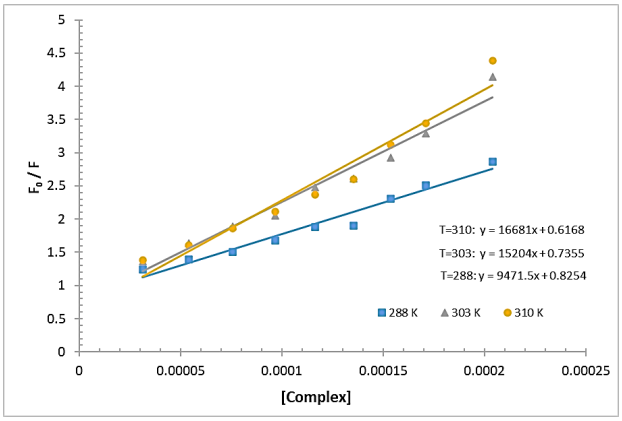

Figure 5: Stern-Volmer plot for the binding of the complex to HSA at 288, 303, and 310 K.

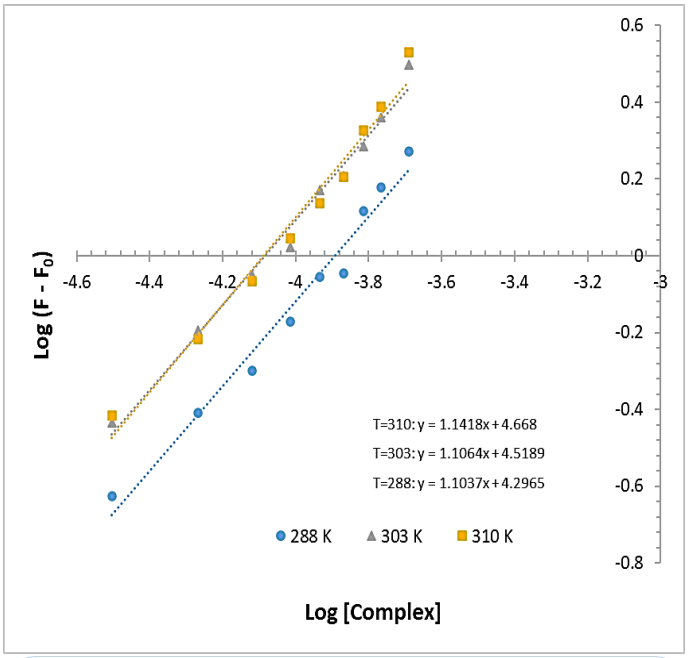

Figure 6: Scat-Chard plots for the interaction between the copper complex and HSA at 288, 303 and 310 K.

The results indicate that there is an interaction between the protein and the complex and that there is one active binding site. The increase in the binding constant with the rising temperature indicates the increase in the binding affinity of complex-protein. The fact that the n is roundly one show that the copper complex is a single binding drug.

Determination of the thermodynamic parameters

There are various possible interactions between the copper complex and human serum albumin to have a better understanding of the interaction type the calculation of the thermodynamic parameters such as free energy changes (ΔG), enthalpy changes (ΔH) and entropy changes (ΔS) of the interaction [34] is necessary. In addition, the Vant-Hoff plots, that ploted ΔG against T where the slope is ΔS and the intercept is ΔH, are used, as follows (Eq. 3 Fig. 7).

According to the variation of the enthalpy (ΔH) and entropy (ΔS), the type of the interaction between drug and the macromolecule could be investigated. The main conditions are listed in the following [22].

$\Delta H > 0 \; and \; the \; \Delta S > 0$ indicates the existence of hydrophobic forces.

$\Delta H < 0 \; and \; the \; \Delta S < 0$ indicates the existence of Van der Waals interactions and the hydrogen bonds.

$\Delta H < 0 \; or \; \Delta H \approx 0 \; and \; the \; \Delta S > 0$ indicates the existence of the electrostatic interactions.

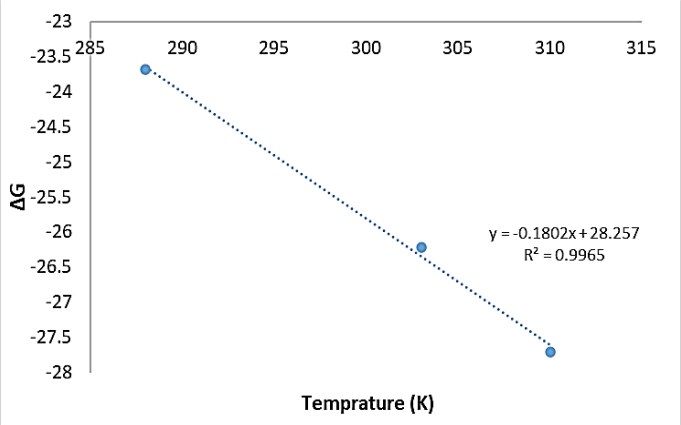

Figure 7: Vant-Hoff plot for the interaction between the copper complex and HSA.

The negativity of the ΔG indicates that the reaction is spontaneous and the interaction between copper complex ([Cu(HEAC)Br]Br) and human serum albumin is mainly of the hydrophobic interactions [35].

Determination of Binding sites

To determine the binding site for copper complex on HSA a competitive displacement experiment can be use. The human serum albumin is mostly composed of α-helix with an overall structure that resembles a heart shape. Human serum albumin has nine double loops spanning three homologous domains [23, 36]. The domains are named as domain I, II and III, respectively. Each domain has two long loops with one shorter loop. Two important binding sites on human serum albumin are Sudlow sites I and II. Sudlow site I is located in subdomain IIA and Sudlow site II is located in subdomain IIIA. Sudlow site I has a preferential binding affinity for bulky heterocyclic compounds such as Azapropazone, Phentylbutazone and Warfarin. Sudlow site II seems to preferentially bind to aromatic compounds such as Ibuprofen, Flufenamic acid, Chlorpheniramine maleate and other drugs [23–25]. It was observed in many studies that the complexes and ligands usually prefer to bind to site I or site II over the other binding sites of HSA. It was also observed that most of the ligands binds only to one of these sites; while some others are able to bind to the both of them. To find the complex-HSA binding site, in this study, a competitive experiment with Warfarin and Ibuprofen was employed. the binding constant with the presence of copper complex and with site markers was calculated. The calculated binding constants are presented in Table 3.4. It was observed that the binding constant in the presence of ibuprofen had a significant drop while in the presence of warfarin the change was minor and small. This results indicate that the copper complex ([Cu(HEAC)Br]Br) has the affinity to bind to the site II(subdomain IIIA) of HSA. The results are presented in **Figure 8 to Figure 10** and **Table 2.**

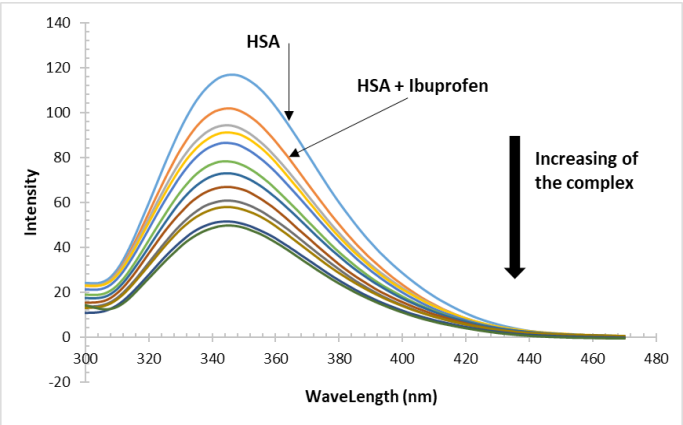

Figure 8: Effect of the ([Cu(HEAC)Br]Br) complex on the fluorescence of Ibuprofen + HAS.

Table 1: Vant-Hoff plot for the interaction between the copper complex and HSA.

T (K)	K_{SV} (M^{-1})× 10^{-4}	R^2	K_q ($M^{-1}S^{-1}$)×10^{11}	K_b (M^{-1})	n	ΔG^0 (kJmol^{-1})	R	ΔH^0 (kJmol^{-1})	ΔS^0 (JM^{-1}K^{-1})
288.15	0.9471	0.96	9.47	19792.47	1.10	-23.70	8.314	28.257	1802
303.15	1.5204	0.97	15.2	33029.35	1.11	-26.37			
310.15	1.6681	0.95	16.7	46558.61	1.14	-27.71			

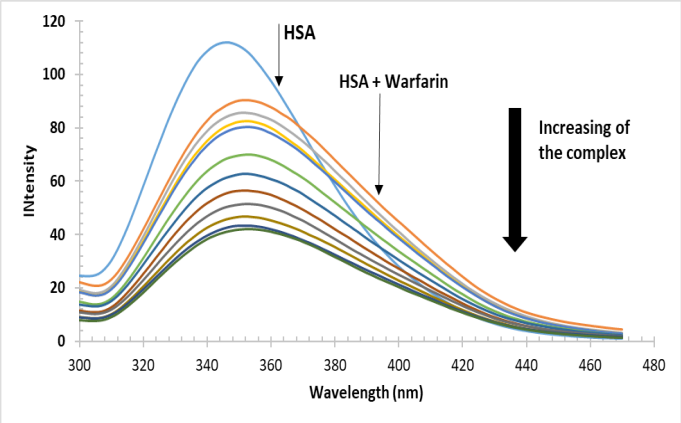

Figure 9: Effect of the ([Cu(HEAC)Br]Br) complex on the fluorescence of Warfarin + HAS.

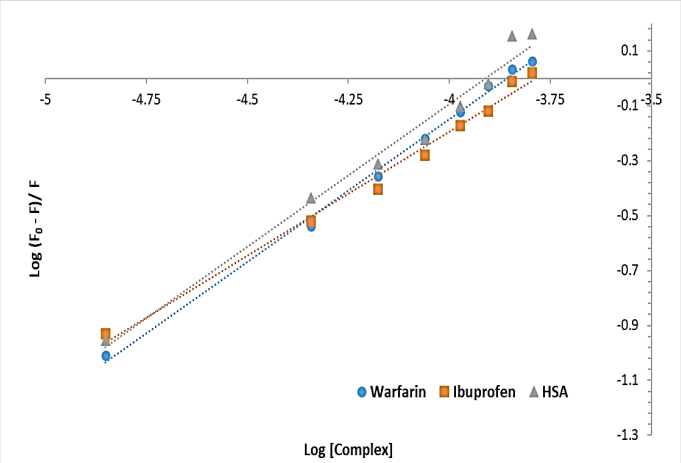

Figure 10: Scat-Chard plots for interaction between the copper complex and HSA, after addition of Warfarin, and Ibuprofen.

Table 2: Binding Constants of competetive experiments for the copper ([Cu(HEAC)Br]Br) complex -HAS.

Site Marker	K_b (M^{-1})	R^2
System	1.19×10^4	0.98
Warfarin	1.02×10^4	0.99
Ibuprofen	2.61×10^3	0.98

Circular dichroism study of the interaction of copper complex and HSA

CD is defined as the differential absorption of the left- and right-circularly polarized light when such light beams are passed through a chiral sample, such as a protein solution. In the CD measurements of the chiral molecules like proteins, AL (which is the absorbance of the left circularly light) does not equal AR(which is the absorbance of the right circularly light) at some wavelengths, resulting in either positive or negative ΔA [37]. Since this method is able to reveal any change in the structure of the HSA protein we can use it to observe the interaction effect between HSA and the copper complex. TheCD spectrum of HSA exhibits two negative bands at 208 nm and 220 nm, which is characteristic of α-helix structure of the protein [38]. The observations of the CD test indicates that the addition of the copper complex to the HSA solution results in the increase in the intensity which means that that the concentration of the α–helix is increased but since the shape of the spectrum was same and had no changes, so the alpha structure was remained and kept same **(Figure 11)**.

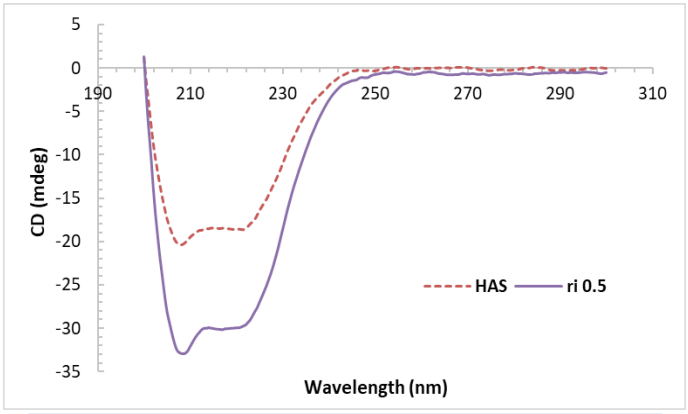

Figure 11: CD spectra of HSA and HSA+ copper complex.

Molecular docking simulation of the interaction of the ([Cu(HEAC)Br]Br) complex and HSA

In order To confirm the experimental results, molecular docking simulation was performed with HSA. From the docking calculation, the best docking energy result is picked up from the 20 minimum energy conformers from the 500 runs and is shown in **Figure 12**. As can be seen, the copper complex binding site is situated in in site II. This result is supported by our site-specific probe studies, which found that the binding site for the complex and ibuprofen was the same in HSA.

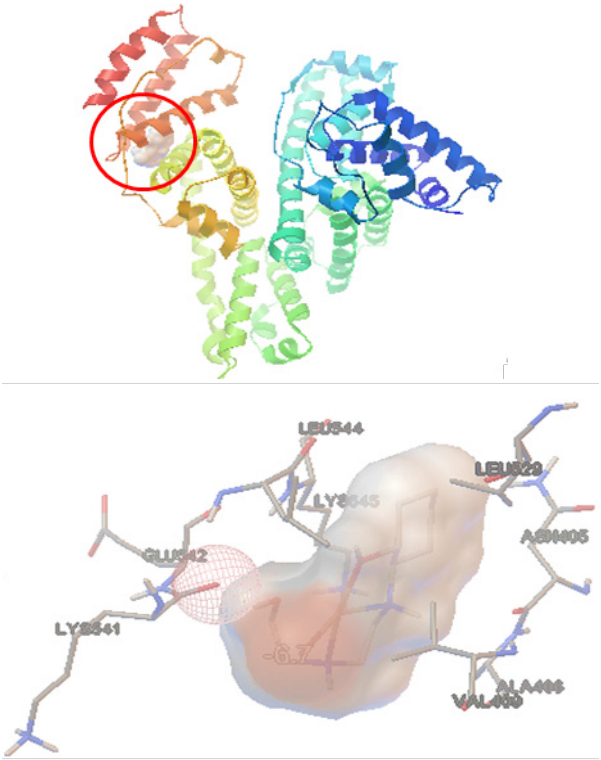

Figure 12: Molecular modeling of the interaction between the complex([Cu(HEAC)Br]Br) and HSA (PDB ID: 1AO6) (right) Amino acids residues surrounding bound the Cu(II) complex (left).

Conclusions

In this study for the first time the interaction between the copper complex [Cu(HEAC)Br]Br with human serum albumin was investigated.The investigations exhibited that the copper complex interacts with HSA via the site II(subdomain IIIA) of HSA. The results are as follows: The results of both ultraviolet spectroscopy and fluorescence test indicated the existence of interaction between human serum albumin and the copper

complex. Binding constant calculations and the number of binding sites as well as the competitive replacement test with ibuprofen and warfarin showed that the complex binds to human serum albumin in site II (subdomain IIIA) and from only one site. Also, the quenching mechanism type has been static. The results of the Circular dichroism (CD) test show that the α-helix of human serum albumin increased and it is proposed that the copper complex binding stabilizes the α-helix structure of HSA. The results of molecular docking simulations indicated that the interaction with human serum albumin is through site II (subdomain IIIA) and from only one site proving the results of the fluorescence probe test.

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