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Evaluation the Interaction of Human Serum Albumin with Anticancer Compound of Palladium(II) Complex Using Competitive Site Markers

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ABSTRACT

In pharmaceutical field, the effect of drugs on biological substances is of particular importance. Therefore, in this research, the interaction between human plasma carrier protein of Albumin (HSA) and a newly synthesized complex of palladium was tested by using site marker competitive tests and various spectroscopic methods such as ultraviolet, fluorescence, and circular dichroism (CD) at different temperatures of 25 and 37 °C. The study of fluorescence spectroscopy showed that this complex reduced the inherent fluorescence emission of HSA, which had eventually quenched by dynamic method mechanism. As well, binding constants were calculated on the palladium compound. According to the obtained thermodynamic parameters of ΔH° and ΔS° values, it was shown that the interaction of palladium complex to the protein occurs via hydrophobic force. The analysis of the circular dichroism (CD) spectrum also showed that the palladium complex caused no significant changes in the main structure of the human serum albumin. These results thermal stability the protein in the presence of palladium complex showed a decrease in thermal stability of human serum albumin. Additionally, to recognize the complex connection points site in protein used spectroscopic methods in the competitive experiments in other words warfarin for Sudlow's site one, ibuprofen for Sudlow's site two, and digitoxin for Sudlow's site three were determined competitive experiments have shown that palladium complex along with digitoxin has a common position on HSA at site three. Finally, according to the above datum, it might be understood which the synthesized palladium complex can bind on Sudlow's site three on HSA by hydrophobic force devoid of activating any alteration in the regular secondary construction of carrier protein.

Keywords: Competitive binding, HSA, Fluorescence quenching, Oxalato palladium, Site marker

INTRODUCTION

Previous studies have shown that albumin is one of the most outstanding proteins in the blood. Its concentration is about 3/6-5/2 mg ml^{-1} in adult blood. This protein is a spherical with weight of 66.3 kDa and makes up about 65% of the total plasma. [1-3]. The protein structure consists of a single polypeptide chain and multiple domains, including 585 amino acid residues that facilitate exogenous, endogenous distribution, and transportation [4-6]. Human albumin serum is non-glycosylated albumin formed of three same domains (one to three); each amplitude has two sub amplitude. containing similar building components [7]. The modular structure of this protein provides its diverse ligand binding sites and it is stabilized by 17 disulfide bridges [8].

The three-domain structure of this molecule makes it flexible, which consequently changes under different conditions such as changes in the pH of the environment [9-13]. Flexibility of HSA can be considered as the reason of its loop-link-loop structure [1,3]. Previous studies have shown that human serum albumin is accountable for the forwarding of liver ligands and is widely responsible for some significant biological functions. These studies have also shown the effect resulted from the drug's interaction with blood-carrier

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protein [3,14]. Normally, HSA can be bind up to two moles of fatty acids under physiological conditions, and in some diseases, it may contain up to six moles. So, for fatty acids, a total of seven sites have been identified in the structures of this protein [15].

Albumin sites are located in three homologous domains [16]. Accordingly, these sites are distributed in three homologous HSA domains. One site is under the IB domainthe second situation is at the jointing amid IA and IIA, the third and fourth sites are at IIIB, the fifth site is at IIIB, the sixth junction is amid IIA and IIB, at last as the seventh site is in a hydrophobic pocket in IIA. However, there are some obvious changes in how fatty acids bind to different places [17-21].

One of the important factors in pharmacokinetics is the increased albumin content, which consequently leads to an increase in drug delivery rate. Therefore, it can be said that balance competition between drugs in the availability of drugs as well as albumin determines both the stimulus in the binding of drugs.

Metal compounds are used for the treatment of various health problems, including gastrointestinal diseases and different types of cancer, and chronic inflammation. Among metal-based anti-cancer compounds, platinum compounds were found to play an important role. Of note, the first generation of these drugs are cisplatin, carboplatin, and oxaliplatin, respectively [22-25]. In many research, the biological effect of the metal compounds has been evaluated [26].

Previous reports have shown that palladium(II) metal is effectively used in analyses and also for catalytic applications such as hydrogenation, oxidation, and the formation of carbon-carbon bonds, as well as electrochemical reactions in fuel cells [1,2,4,5,13,27]

Therefore, in the current study, the interaction between the newly synthesized compound of 2,2′-bipyridine-3,3′-dicarboxylic acid oxalato palladium(II) (Fig. 1), as a newly synthesized antitumor compound with less toxicity compared to other platinum complexes or palladium complexes drugs, and human serum albumin protein was investigated. Since HSA is a pliability molecule, the connection of each medicine to this protein consequently affects the location of other drugs. So, changes and interactions between HSA and other drug molecules should

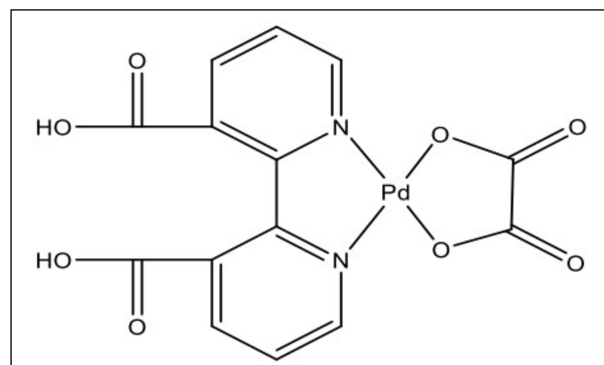

Fig. 1. The molecular structure of 2,2′-bipyridine-3,3′-dicarboxylic acid, oxalato palladium(II).

be investigated as well [28]. Then, in the present study, we applied different site *markers e.g.* warfarin, digitoxin, and ibuprofen in competitive reactions, to determine the position of palladium complex on protein [29] by helping diverse spectroscopic techniques *e.g.* UV-Vis, fluorescence, and CD.

MATERIAL AND METHODS

Reagents

(HSA) with high purity was bought from Sigma. Pd compounds were made according to previous reports. Moreover, site markers of Ibuprofen, Digitoxin and Warfarin were purchased from Nano Zist Company. Afterward, NaCl solution (5 mM) was used as a solvent. To calculate the concentration of HSA, the spectrophotometric method and molecular attraction factor $\varepsilon^{10\%}$ at 278 nm = 5.3 M^{-1} cm^{-1} were used.

Fluorescence Measurements

Changes in Fluorescence amount of protein was obtained using a Cary spectrophotometer. The excited wavelength was calculated as 295 nm and the emission range was set between 301 to 501 nm.

Fluorescence intensity was measured using a cuvette with a path length of 1 cm at different temperatures of 25 and 37 °C. As well, 5 μM HSA was used in the intrinsic fluorescence studies. Additionally, competitive studies were performed in the presence of 5 μM of warfarin, digitoxin, and ibuprofen as the site markers.

Circular Dichroism Measurements

The two-color circular spectrum was measured with an Avio 215 spectrometer. To evaluate the effects of the drug on the secondary structure of the protein, its changes in the secondary structure of the protein (5 µM) in the presence and absence of palladium(II) complex were analyzed. (0, 49.3 and 147.9 µM) in the UV range (between 190 and 260 nm) using a 0.1 cm path cuvette.

Next, to determine the content of the HSA secondary structure elements, using the CDNN software interaction of Pd(II) complex and HSA at different concentrations of Pd(II) complex was used to predict the content and amount of secondary structure of HSA [30].

Thermal Denaturation Study

Thermal denaturation of 16.4 µM HSA in the presence and absence of 106.6 µM Pd(II) complex was analyzed using a UV-Vis CARY-100-Bio spectrophotometer.

Then, we increased the temperature from 20 to 90 °C. At the desired temperature range, absorption changes were recorded at the reference wavelength of 280 nm in the sample cuvette. and the values of melting temperatures and $DG°_{25}$ were calculated [29].

RESULTS AND DISCUSSION

Fluorescence Studies

Previous reports and X-ray crystallographic data have shown that Tryptophan-214 is responsible for the inherent fluorescence in HSA in the hydrophobic cavity below the IIA domain [31-34]. Figure 2a-b shows the changes in diffusion spectra of human serum albumin at 25 and 37 °C in the absence and presence of different concentrations of palladium complex.

By increasing the concentration of Pd(II) complex at both temperatures, reducing the emission of intrinsic fluorescence Trp-214 regularly, and then quenched the intrinsic fluorescence of the protein [25,35,36].

Alterations in maximum fluorescence emission of the HSA at wavelength of 340 nm at different concentrations of palladium complex at two different temperatures of 25 and 37 °C, was shown in Fig. 3a. As the concentration of the Pd(II) complex increases, the fluorescence emission of protein decreases, so it is known the quenching rate increases

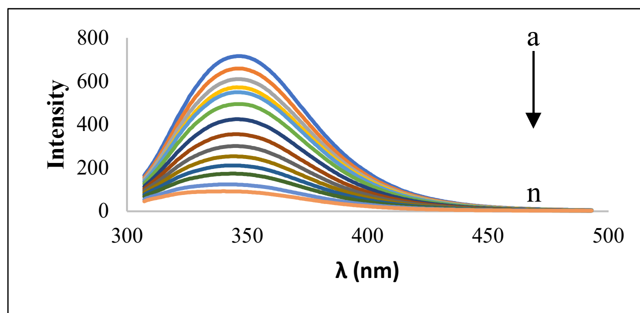

Fig. 2a. Examination diagram of five micromolar HSA fluorescence spectrum in the absence and presence of different concentrations of Pd(II) complex from concentration (a) 0 to (n) 57.5 µM in 5 mM of NaCl solution, pH 7.4, at 25 °C.

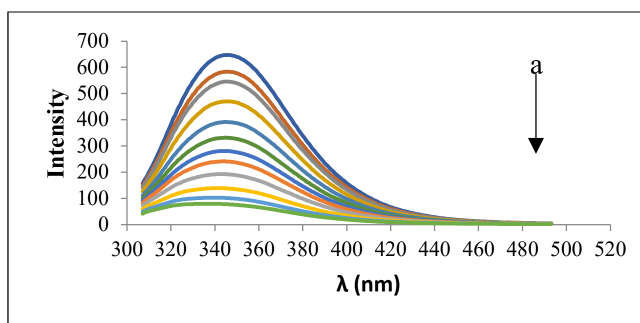

Fig. 2b. Examination diagram of five micromolar HSA fluorescence spectrum in the absence and presence of different concentrations of Pd(II) complex from concentration (a) 0 to (l) 50 µM in 5 mM of NaCl solution, pH 7.4, at 37 °C

[25].

The quenching effect phenomenon generally occurs via static or dynamic mechanism, which can be detected considering their specific dependence on temperature. To determine the exact type of the quenching mechanism, the dependence of each reaction must be measured at two different temperatures. In the dynamic quenching mechanism, increasing the temperature will increase the quenching constant rate, while in the static quenching mechanism, increasing the temperature will reduce the quenching constant rate.

The quenching mechanism on fluorescence data was analyzed by using the Stern-Volmer equation (Eq. (1))

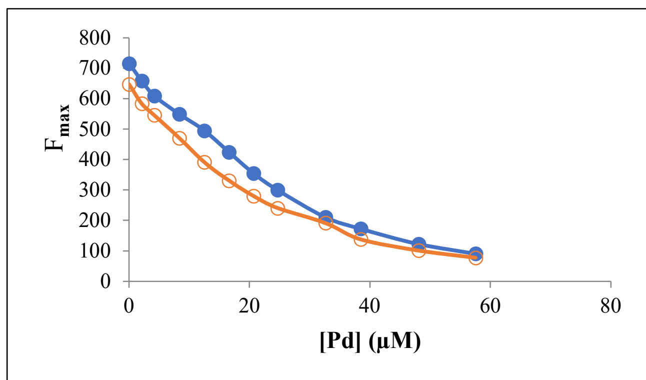

Fig. 3a. Diagram of maximum fluorescence emission at different concentrations of Pd(II) complex at two temperatures of 25 °C (●) and 37 °C (○).

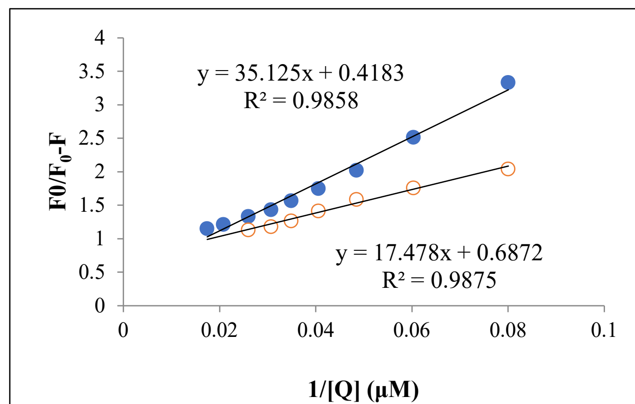

Fig. 4. The modified Stern-Volmer plot of HSA quenching by Pd(II) complex in 5 mM NaCl solution at 25 (●) and 37 °C (○).

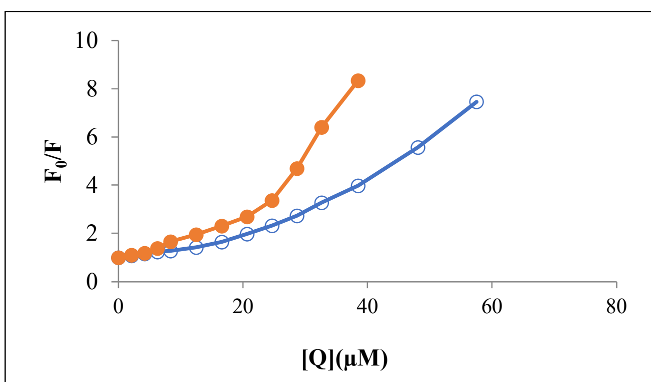

Fig. 3b. Plot of $\frac{F_0}{F}$ *versus* Pd(II) complex or quencher concentration ([Q]) at two temperatures of 25 (●) and 3 °C.

[19,20,37]:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \tag{1}$$

In this equation, F_0 and F indicate the intensity of HSA fluorescence in the absence and presence of quencher (palladium complex), respectively. In addition, K_{sv} and [Q] refer to the Stern-Volmer quenching constant and the quencher concentration, respectively. According to the Stern-Volmer diagram (Fig. 3b), F_0/F *versus* Pd(II) concentration [Q] was shown at different temperatures of 25 and 37 ° C.

According to Fig. 3b, which is a nonlinear diagram showing a positive slope, it can be concluded that the quenching mechanism of protein is a combination of both static and dynamic mechanisms or due to the high concentration of ligand around fluorophore [28].

Since the Stern-Volmer diagram is nonlinear, we used the modified Stern-Volmer equation to determine the type of quenching mechanism as well as determining the constant values of Stern-Volmer quenching at the two temperatures (Eq. (2)) [38,39]:

$$\frac{F_0}{\Delta F} = \frac{F_0}{F_0 - F} = \frac{1}{f_a K_{SV}} \frac{1}{[Q]} + \frac{1}{f_a} \tag{2}$$

According to Fig. 4, it was shown that ($F_0/F_0 - F$) and the reciprocal quencher concentration (1/[Q]) have a linear relationship. The values of intercept and slope of the plot were used to calculate the values of f_a and K_{SV}, respectively [40].

The values of f_a in the presence of palladium complex were calculated 2.39 and 1.45 at different temperatures of 25 and 37 °C, respectively (Table 1). Also, the Stern-Volmer constant values at both of the temperatures were represented in Table 1 and show that the values of Stern-Volmer constants were temperature-dependent [28]. So, by increasing the temperature from 25 to 37 °C, the values of K_{SV} increased, indicating the type of the dynamic quenching mechanism (Najaran *et al.,* 2016).

Various binding parameters such as the binding constant values (K) as well as the number of binding site (n) in the interaction of palladium complex with human albumin serum can be obtained using the Eq. (3) [41-43]

Table 1. The Calculated Binding Parameters of Pd(II) Complex to HSA with and without Site Markers of Digitoxin (dig), Ibuprofen (ibu), and Warfarin (war) at the Two Temperatures of 25 and 37 °C

[dig] (μM)	[ibu] (μM)	[war] (μM)	T (°C)	n	K (M^{-1})	K_{SV} (μM^{-1})	f_a	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (kJ mol^{-1} K^{-1})
0	0	0	25	1.2	0.93×10^{-6}	0.012	2.4	-34.12		
0	0	0	37	1.4	20×10^{-6}	0.039	1.45	-45.45	+197.9	+0.7
5	0	0	25	1.4	8×10^{-16}	0.03	0.71	+51.9		
0	5	0	25	1	2×10^{-14}	0.04	0.66	+43.9		
0	0	5	25	0.9	6×10^{-2}	0.07	2.3	-21.6		

$$\log[F_0 - F/F] = \log K + n\log[Q] \quad (3)$$

The plot of $\log[F_0 - F/F]$ *versus* $\log[Q]$ is a linear diagram, then, the values of n and binding constants can be calculated from slope and Y-intercept, respectively (Fig. 5 and Table 1) [6,44,45].

As it is shown in Table 1, there is one binding site for Pd(II) complex on HSA at both of the temperatures of 25 and 37 °C.

As well, with increasing temperature, the binding affinity of complex on protein increased which indicative of endothermic interaction of complex and protein (Kazemi *et al.* 2021).

Previous studies have shown that HSA has three homologous α- helical domains, including I, II, and III. Hydrophobic cavities under subdomains IIA and IIIA are known as the major binding sites for albumin drug, namely Sudlow's sites I and II, respectively [28].

A hydrophilic cavity is under the IB domain, which can be known as site III, and for small molecules, it is known as a protein pocket. Warfarin specifically binds to serum albumin at the site I, while ibuprofen and digitoxin binds to site II and III, respectively. Warfarin, ibuprofen, and digitotoxin as albumin site markers markers were used to determine the specific site of the human serum for palladium complex. Warfarin can bind to Sudlow's sites I, whereas ibuprofen and digitotoxin bind to Sudlow's sites II and III, respectively [46].

So, in the present study, firstly, the fluorescence emission spectrum of the incubated protein with each of the site markers of Warfarin, ibuprofen, and digitotoxin was recorded. Next, the solution was titrated using a different

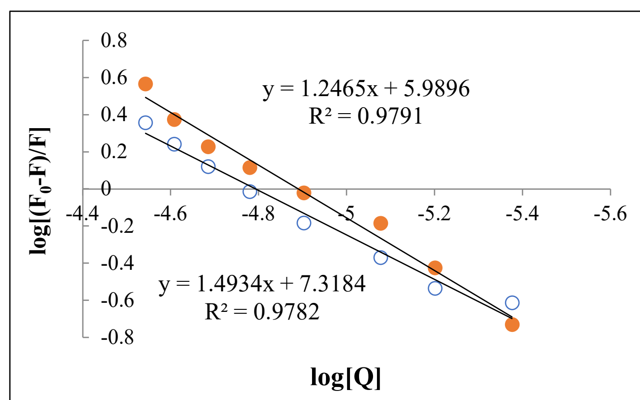

Fig. 5. The best linear plot of log $[F_0 - F/F]$ *versus* log[Pd] based on the Eq. (3) at 25 (●) and 37 °C (○).

concentration of the palladium complex and the results were shown in Fig. 5. The competitive fluorescence emission results indicate that the emission of the incubated protein with warfarin site markers was quenched by increasing the concentrations of palladium complex. Correspondingly, similar results were observed for the incubated protein in the presence of both ibuprofen and digitotoxin (Data not shown).

By using of Eq. (3), the number of binding sites and binding affinity of Pd complex to incubated HSA with various site markers calculated (As shown as in Fig. 6 and Table 1). By comparing the binding constant values between the binary combination of the palladium-protein complex with ternary complexes, including warfarin-protein-palladium, ibuprofen-protein-palladium, and digitoxin-protein-palladium, it was determined that the lowest constant binding value is related to the digitoxin complex. On the other hand, the data indicated that the positions of digitoxin

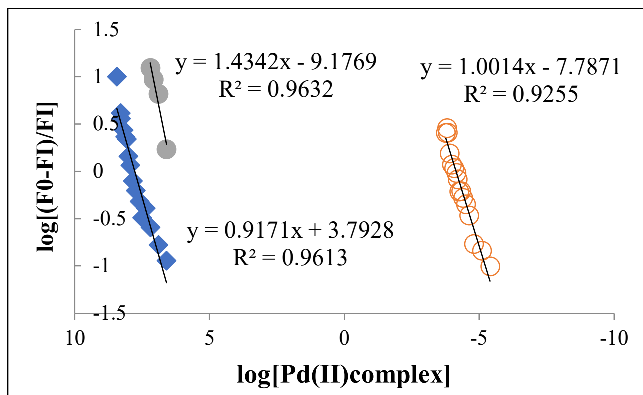

Fig. 6. The best linear plots of $logF_0$ - F/F *versus* log[Pd(II) complex] based on Eq. (3) in the presence of site markers of warfarin (♦), ibuprofen (○), and digitoxin (●) at 2 5 °C.

and palladium complexes in human serum albumin are common, *i.e.* it is in the Sudlow's sites III position. Therefore, it can be concluded that the Pd(II) complex and digitoxin compete for binding to the protein at a common site.

According to the results shown in Table 1, it was found that ibuprofen and warfarin did not occupy all protein binding sites. Thus, as demonstrated, the Pd(II) complex can still bind to the protein, indicating that the Pd(II) complex, warfarin, and ibuprofen could bind to different HSA positions (as shown in Fig. 6). These findings can be used during chemotherapy, when administrating multiple medications. According to Table 1, by examining the binding site of the three binary compounds as well as the binding site of palladium complex with incubated albumin protein with site markers, the value of binding site was determined about one binding site.

By calculating the thermodynamic parameters at different temperatures and then by analyzing them, the binding forces, which were involved in the interaction of the protein-ligand, were identified.

Considering enthalpy changes (ΔH°), free energy changes (ΔG°), and entropy changes (ΔS°) based on Vant Hoff equation ((4) and (5)), and by knowing the K values at 25 and 37 °C, the entropy and enthalpy values were determined (Table 1).

$$lnK = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R} \quad (5)$$

$$\Delta G° = \Delta H° - T\Delta S° = -RTlnK \quad (6)$$

Where R is the gas constant [44].

So, the values of various thermodynamic parameters were calculated and listed in Table 1.

According to Table 1 and Ross and Subramanian suggestions, the symptoms and thermodynamic values related to the process of ligand-protein binding reactions can be identified as different types of reactions in this ligand-protein binding process as follows:

When both parameters (ΔS°) and (ΔH°) are both positive, the binding is hydrophobic.

If the two parameters (ΔS°) and (ΔH°) are both negative, the binding formed is one of the types of hydrogen bond and van der Waals.

If the above-mentioned two parameters as (ΔS°) is positive and (ΔH°) is negative, which is a specific condition between ionic species in aqueous solution, the binding is of the electrostatic type (Ross and Sabramarnin 1981).

The negative parameter (ΔG°)) also indicates the spontaneity of the reaction. As well, positive ΔS° along with positive (ΔH°) are indicators of an entropy-driven and endothermic process. Altogether, these data indicate that a hydrophobic interaction exists between HSA and Pd(II) complexes [47].

Circular Dichroism Studies

The circular dichroism spectra (CD) of the human serum albumin in the far-UV region and in the presence of different concentrations of Pd(II) complex (including 0, 98.6 and 295.8 μM) at 25 °C, are shown in Fig. 7.

CD analysis can be used to assess the protein structural changes caused by ligand binding accurately. Based on the α-helical structure of HSA, two negative bands in the UV region at 208 nm (π→π*) and 222 nm (n→π*) were obtained (Najaran *et al*., 2016).

According to Fig. 8 results, native HSA contains 53.4% α-helix, 24.2% β-sheet, and 22.4% random coil structures. By increasing the concentration of Pd(II) complex in the protein solution, the amount of ellipticity at both 208 and 222 nm showed no significant decrease, indicating that the secondary structure of the protein had no significant changes (Fig. 7). The spectra obtained from the circular bipolar data were analyzed using CDNN software, and it was found that the

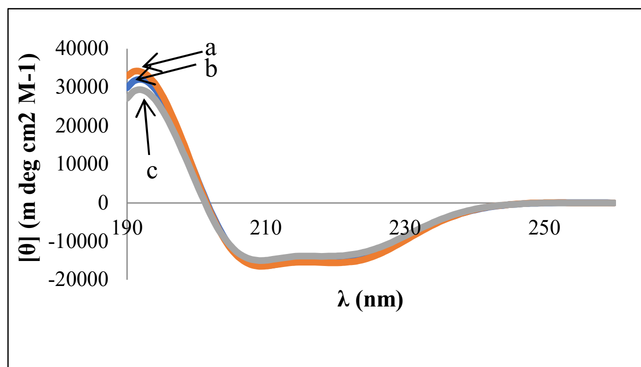

Fig. 7. Far-UV-CD analysis of (a) 5 µM pure HSA; in the presence of (b) 98.6 and (C) 295.8 µM Pd(II) complex at 25 °C.

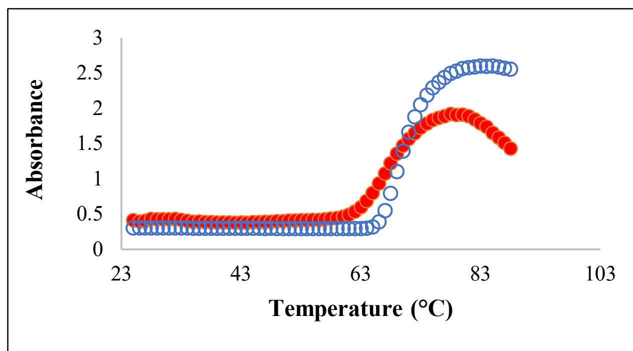

Fig. 8. Thermal behavior of human albumin serum in the absence (○) and presence of palladium complex (●).

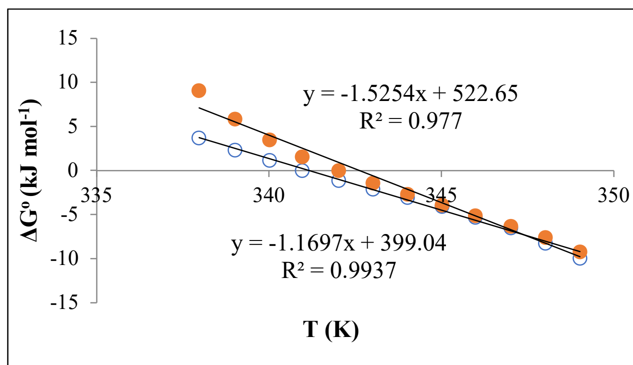

Fig. 9. Alterations of Gibbs free energy against temperature of human albumin serum in the absence (○) and presence of palladium complex (●).

Pd(II) complex caused very minor changes in the helix structure and beta sheet structure, but the original structure of the protein was preserved.

Thermal Denaturation Analysis

In order to obtain more information on the effect of palladium complex on the stability of HAS, the thermal denaturation studies of human albumin serum protein were performed using temperature scan spectroscopy analysis [48]. The results of these studies showed (Fig. 8) that the presence of palladium leads to changes in the transition temperature (T_m) of human albumin serum (Table 2). Thereafter, using Pace analysis, the Gibbs standard free energy of denaturation value of the protein ($\Delta G°_{25}$) at 25 °C was calculated from the thermal abnormality curves. Moreover, the Gibbs standard free energy based on the two-state theory was used to determine the conformational stability of a human albumin protein. According to the two-state theory (Eq. (7)),

Native (N) ⇔Denatured (D)

$$F_d = \frac{Y_N - Y_{obs}}{Y_N - Y_D} \quad (7)$$

Y_{obc} is the unstable parameter; for example, absorption, Y_N, and Y_D are the characteristic values of normal and denatures conformations, respectively.

In a two-state mechanism used protein abnormalities by temperature, the fraction of denatured protein (F_d) as well as the equilibrium constant (K_D) are calculated by considering the absorption changes at different temperatures (Eq. (8)) [48]

$$K_D = \frac{F_d}{1 - F_d} \quad (8)$$

As well, the free Gibbs free energy change ($\Delta G°_D$) for denaturation process was obtained using the following equation. (Eq. (9))

$$\Delta G°_D = -RT\ln K \quad (9)$$

Where, R is the general constant, gases, and T is the absolute temperature. If the temperature is increased, $\Delta G°_D$ will also change linearly in a certain range, as shown Figs. 8-9. Therefore, some calculations were done, as a result, the value of $\Delta G°_{25}$ was calculated, as indicated in Table 2.

Table 2. T_m and ΔG^o_{25} Values for Human Albumin Serum in the Absence and Presence of Palladium Complex (106.6 μM)

	ΔG^o_{25} (kJ mol^{-1})	T_m (°C)
HSA	72.1	69
HSA+Pd	53.2	67

If the temperature is increased, ΔG°_D will also change linearly in a certain range, as shown in Figs. 8-9. Thereafter, some related calculations performed, and the value of ΔG°_{25} was calculated, as recorded in Table 2. In addition, the transition temperature (T_m) of human albumin serum changed in the presence of palladium analog, indicating a change in the thermal stability of the protein in the presence of this drug [49].

CONCLUSION

Platinum and palladium metal complexes were demonstrated to have the potential of attacking cancer cells as anti-tumors. Therefore, in a recent study, the effects of palladium analogs (as the newly designed drugs) on the most important carrier protein in the blood (which is the human serum albumin protein) were investigated from a molecular perspective.

In this study, changes in the structure, and stability of this carrier protein resulted from the binding of the palladium complex at an ambient temperature and a physiological temperature were investigated, in order to study the induced changes and side effects of this complex on body proteins. As well, the binding parameters of complex to serum albumin protein determined. Moreover, the determination and prediction of the binding site of the palladium complex on HSA were performed using the competitive interaction studies in the presence of site markers of warfarin, ibuprofen, and digitoxin.

To reduce the therapeutic dose of these drugs, it is necessary to understand the drug-protein binding capacity as well as the protein-drug interaction, which is considered as one of the determining factors in drugs in pharmacodynamics and pharmacokinetics. Additionally, competitive binding of drugs given to the patient at the same time increased the level of free drugs, thereby increasing the potential side effects of the drug in the patient. Drugs distributed during the multi-drug therapy process may consequently alter the binding of the carrier protein. Therefore, the determination of the position of the binding sites of both drugs as well as the possible interactions for each drug is necessary. Therefore, the substitution effects of drugs that are simultaneously distributed in the blood, should be investigated in order to avoid any unwanted side effect for the patient. For this purpose, to identify the binding of palladium complex, which is known as a candidate for chemotherapy, the human albumin serum was examined to indicate the effects of its interaction with other drugs.

In regard to Digitoxin-protein-palladium, it was found that the binding site of the palladium complex is similar to the digitoxin and located in the III position. Furthermore, the warfarin binding site was different from that of the palladium complex, and for ibuprofen with the palladium complex, it was determined that palladium complex did not occupy the entire site. Also this complex can decrease the thermal stability of the protein.

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