



Detection of interaction between ornidazole and albumin by using thermodynamic parameters

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Abstract

The binding of 1-chloro-3-(2-methyl-5-nitro-1H-imidazole-1-yl) propan-2-ol (Ornidazole) to human serum albumin (HSA) was studied by fluorescence and UV-visible spectroscopy. Interaction of ornidazole (OR) with HSA was identified by Stern-Volmer and Van't Hoff equations. The binding constant, K_b and the thermodynamic parameters, ΔH , ΔS , and ΔG at different temperatures were calculated by several equations. Data shows that the fluorescence quenching mechanism of HSA with ornidazole may occur via static quenching. The thermodynamic parameters showed that van der Waals interactions and hydrogen bonds are the major forces for the interaction of ornidazole with HSA. The spectral changes of synchronous fluorescence suggested that both the microenvironment of OR and the conformation of HSA concerning their concentrations have changed during binding.

Keywords: Ornidazole; Human Serum Albumin (HSA); Fluorescence Quenching; Stern Volmer Equation; Thermodynamic Parameter.

1. Introduction

Albumin is the main protein for the transportation of a wide variety of substances like metals, fatty acids, amino acids, hormones, and a large list of drugs [1]. Amino acids number and sequences of the albumins from different sources are well known. Therefore, albumin is widely used in the studies of interactions between drugs and proteins *in vitro*. Database related to the albumin leads to ease of explaining the binding mechanism of albumin to any substances. Several types of albumin which are from human, rat, and bovine are the most commonly studied proteins to identify the interaction between proteins and drugs for many decades. However, the tridimensional structure is only available for human serum albumin (HSA). HSA contains about 580 amino acid residues in a single polypeptide chain with a molecular weight of 67,000 Da [2]. HSA has 67% of alpha-helix with six turns and 17 disulfide bridges in the secondary structure [3-4]. The rest of the chain is composed of an extended peptide chain with 10% of β turns. The X-ray crystal structure of HSA shows three homologous domains (I, II, and III). Each domain includes two subdomains (IA and B, IIA and B, IIIA and B) which possess structural patterns. Ligands bind to two subdomains, IIA and IIIA in Sudlow site I and Sudlow site II. These sites have hydrophobic cavities [5-7]. A lot of Tyr residues, from 18 to 21 depending on the species are involved in the sequence of HSA. A single Trp residue appears as Trp-214 in the sequence of HSA [2].

A large number of drugs and bioactive molecules bind reversibly to all types of albumin [8-13]. Albumin usually increases the solubility of hydrophobic drugs in plasma and serum, which is a very important protein for the drug delivery to target cells *in vivo* and *in vitro* [14]. The knowledge of the reaction between a drug molecule and albumin is useful for interpreting the pharmacokinetics and transport of the drugs. Particularly, the structural and energetic aspects of the interaction provide knowledge to understand interaction mechanisms for the further development of new therapeutic agents [15-16].

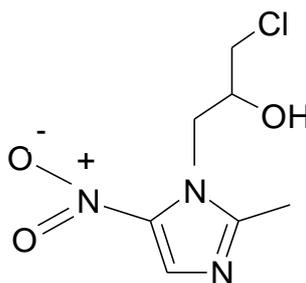
Ornidazole which is one of the 5-nitroimidazole derivatives belongs to a group of medicines called antiprotozoal. It is used to treat infections caused by protozoa such as amoebiasis, trichomoniasis. It is also used in the poultry industry [17-18]. The efficacy and safety of ornidazole, as a nitroimidazole antibiotic, has been investigated for use in Crohn's disease which almost inevitably recurs after ileocolonic resection, and effective prophylactic therapy has not been identified. However, an important side effect of ornidazole is well known. Ornidazole is a type of concomitant administration of oral anticoagulants which may increase the risk of hemorrhage due to diminished hepatic metabolism [19-21]. Fluorescence spectroscopy is a powerful tool for the investigation of the reaction of drugs with proteins and makes it possible to analyze the spectrum of a protein at specific excitation and emission wavelengths [15-16]. In the present work, we demonstrated the binding of ornidazole to human serum albumin by using fluorescence and UV-Visible spectroscopy. The nature of the binding of the drugs with the proteins were described by using the Stern Volmer equation. Synchronous fluorescence was also used to estimate environmental changes of HSA corresponding to interaction. This work aimed to determine the affinity of ornidazole to HSA and to investigate the thermodynamic parameters of their interaction.

2. Materials and methods

Materials: 1-chloro-3-(2-methyl-5-nitro-1H-imidazole-1-yl) propan-2-ol (Ornidazole), HCl, NaOH, albumin from human serum (97-99%), ethanol, and Tris (hydroxymethyl) aminomethane were purchased from Sigma–Aldrich (USA). The ultra-pure water was purified by the Milli-Q water purification system (Millipore, Bedford, MA, USA). All reagents and chemicals were of molecular and analytical grade.

Apparatus: All fluorescence measurements were done by a Perkin Elmer LS-55 spectrofluorometer (Perkin Elmer, USA) equipped with a xenon lamp source and a 1.0 cm quartz cell. Absorption spectra were recorded on a Shimadzu-1700 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) using a 1.0 cm quartz cell.

Fluorescence spectroscopy: Ornidazole (OR) (Scheme 1) was prepared as 1 mM of stock solution in a water-ethanol solvent system (V:V, 50:50%) for all experiments. 0.1 mM of HSA solution was prepared by directly dissolving the protein in a Tris buffer including 20 mM of Tris at pH of 7.4. All aqueous solutions using in testing contained 5% (v/v) ethanol for all measurements. An appropriate volume of OR solution was added to HSA ranging from 1 μg to 20 μg , then mixed for 10 minutes at RT. The fluorescence spectra from 200 to 500 nm were scanned by fluorescence spectroscopy using the 10 nm of slit in a quartz cell. The appropriate volume of HSA (10 μg) was mixed with different amounts of OR ranging from 1 to 80 μM and then, diluted to 2.0 mL with the Tris buffer. The fluorescence spectra were collected with the excitation wavelength at 280 nm.



Scheme 1: Structure of ornidazole.

Synchronous fluorescence spectroscopy: Appropriate volume of HSA in the Tris buffer was mixed with OR at a concentration range between 1 and 80 μM . The fluorescence spectra were recorded at the $\Delta\lambda=15\text{nm}$ and $\Delta\lambda=60\text{nm}$ for all OR-HSA.

Absorption of OR against HAS: The appropriate volume of HSA in 20 mM of Tris buffer (pH:7.4) was added to different amounts of OR ranging from 5 to 60 μM . Absorption spectra of the solution were collected by Shimadzu 1700 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan).

3. Results and discussion

Fluorescence quenching: A good linear response of fluorescence intensity concerning to HSA concentrations was also obtained at 280 nm of excitation and 342 nm of emission wavelengths. The linear equation is $IF = 10.969 \text{ HSA} - 27.146 \times 10^{-6}$ ($R = 0.9965$) while the concentration of HSA ranging from 1 to 20 μg . The emission spectra of HSA at a fixed concentration of OR (20 μM) were done in 20 mM of Tris buffer (pH 7.4) and is presented in Fig. 1. Interaction of OR with HSA results in enhancement of the fluorescence intensity with a slight blue shift at 280 of excitation wavelength when amounts of HSA enhanced. The blue shift means a decrease in the polarity of the micro-environment of OR. This result suggests that an interaction between OR and HSA occurs in the hydrophobic microenvironment of HSA.

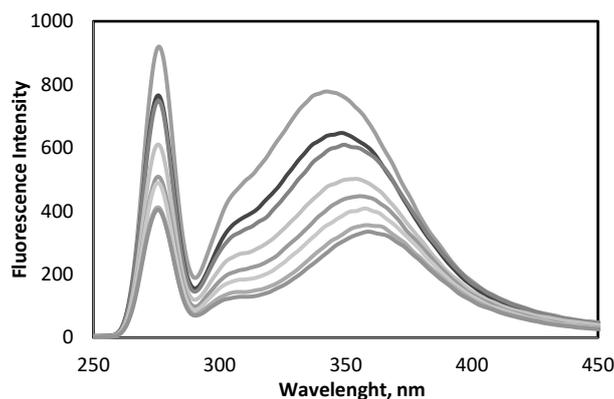


Fig. 1: Fluorescence Spectrum of HSA at A Fixed Concentration of Ornidazole ($T = 296 \text{ K}$, $\lambda_{\text{Ex}} = 280 \text{ Nm}$). [OR]: 20 μM , [HSA]: 20, 15, 12.5, 10, 7.5, 5, 2.5 and 1 Mg.

The fluorescence intensity of HSA decreased and the emission band exhibited a slight red shift when the amounts of OR were increased. The fluorescence intensity of HSA regularly decreased with an increase in OR concentration ranging from 1 to 80 μM , as shown in (Fig. 2). The red shift of the maximum emission can prove the changes in the residues' surrounding environments in polarity which is increased, suggesting the decrease in hydrophobicity and extension of HSA molecule [22-23].

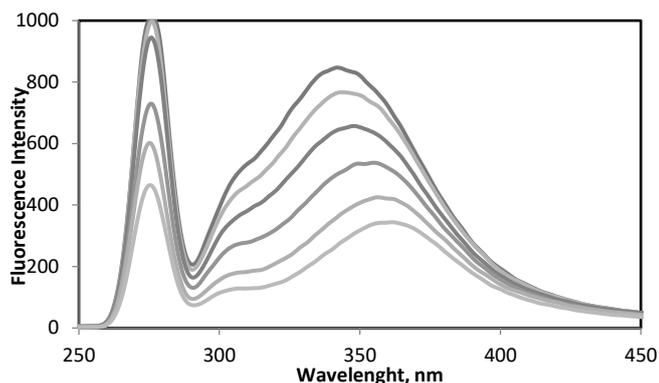


Fig. 2: Fluorescence Spectrum of HSA (10 Mg) at Different Concentrations of Ornidazole (T = 296 K, λ_{Ex} = 280 Nm). [OR] = 5, 10, 20, 30, 40, 60 and 80 μ m.

Synchronous fluorescence of HSA: It is well known that the fluorescence characteristic of protein results from the protein's tyrosine and tryptophan residues. The synchronous fluorescence spectrum gives the intrinsic fluorescence of the proteins related to tyrosine and tryptophan residues of which fluorescence is recorded at a specific wavelength, $\Delta\lambda$, between excitation and emission wavelengths. When $\Delta\lambda$ is set as 60 nm, the synchronous fluorescence spectrum belongs to the characteristic information of the tryptophan residues. When set at 15 nm, the characteristic spectrum of the tyrosine residues appears [22]. The fluorescence intensity of HSA with the addition of OR at a concentration range between 1 and 80 μ M was measured. Fig. 3a and Fig. 3b show that the fluorescence intensity of tyrosine residues ($\Delta\lambda=15$ nm) decreased with a slight blue shift while that of tryptophan residues ($\Delta\lambda= 60$ nm) exert a slight red shift at the emission spectra with decrease in OR concentration. However, it is seen that the pitch of quenching at $\Delta\lambda$ 60 nm is higher than that of $\Delta\lambda$ 15 nm with the addition of OR (Fig. 4). OR molecule was presented to be closer to tryptophan residues than tyrosine residues. The result indicates that the binding sites are mainly on tryptophan moiety [23].

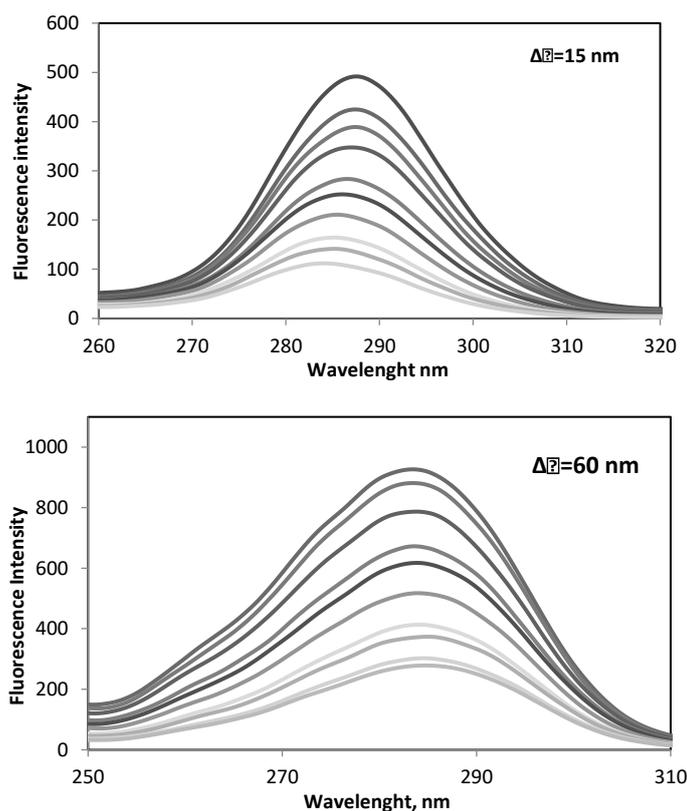


Fig. 3: (A) Synchronous Fluorescence Spectrum of HSA (10 Mg) in the Presence of Different Amount of Ornidazole with $\Delta\lambda= 15$ Nm. (B) Synchronous Fluorescence Spectrum of HSA in the Presence of Different Amount of Ornidazole with $\Delta\lambda= 60$ Nm, [OR] = 1, 4, 8, 10, 20, 30, 40, 50, 60, 70 and 80 μ m.

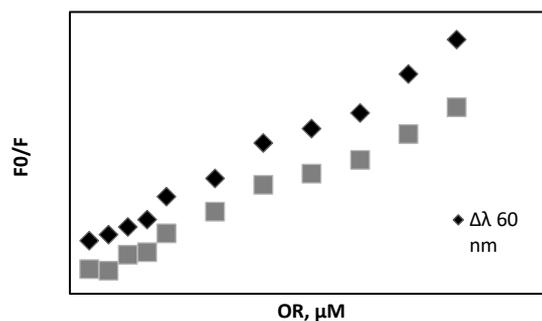


Fig. 4: The Quenching Degree of HSA (10 Mg) Synchronous Fluorescence by Ornidazole. [OR] = 2, 4, 6, 8, 10, 15, 20, 25, 30, 35 and 40 μM .

Binding Mechanism of OR and HSA: Reduction of the fluorescence intensity by any molecule is referred to as fluorescence quenching. A large number of molecular interactions which can lead to quenching are molecular rearrangements, excited-state reactions, energy transfer, complex formation, and collisional quenching. Quenching can occur via two mechanisms as dynamic and static quenching. Both quenching mechanisms depend on temperature and excited-state lifetime. Higher temperatures result in faster diffusion, thus larger amounts of collisional quenching. The fluorescence quenching is assumed to be a dynamic quenching process first to explain the binding mechanism of HSA by OR. The dynamic quenching mechanism is usually analyzed via the Stern-Volmer equation (Eq 1) as follows [23]:

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{SV}[Q] \quad (1)$$

Where F_0 and F are the steady-state fluorescence intensities in the absence and the presence of quencher, K_{SV} is the Stern–Volmer quenching constant, and $[Q]$ is the concentration of quencher. τ_0 is the average biomolecules fluorescence lifetime which is known to be 10^{-8} s without the addition of quencher [24].

The Stern-Volmer plots at four temperatures are shown in Fig. 5. The values of K_{SV} and K_q at 296, 298, 303, and 308 K were calculated from the regression equations of which slopes are presented in Table 1. K_q values were found to be 2.87, 3.14, 3.66, and 4.11 $\times 10^{12}$ $\text{L mol}^{-1} \text{s}^{-1}$. Stern-Volmer plots for the quenching of HSA-OR at different temperatures. K_{SV} values were determined as 0.287, 0.321, 0.369 and 0.410 $\times 10^{-5}$ L mol^{-1} . Data indicate that the K_q values are much larger than the maximum scattering collision quenching constant which is considered as 2.0×10^{10} $\text{L mol}^{-1} \text{s}^{-1}$ [25]. Higher K_q values suggest that the interaction between OR and HSA may come from static quenching via forming complexes [26].

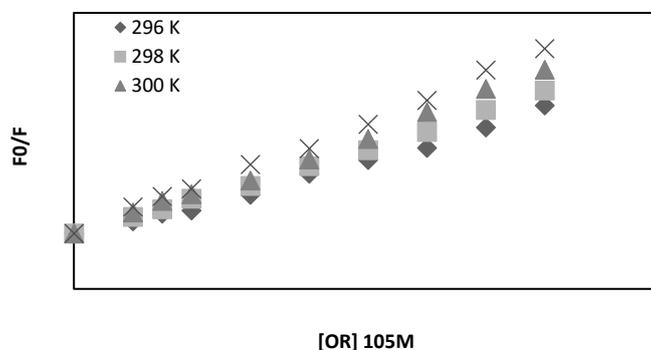


Fig. 5: Stern-Volmer Plots for the Quenching of HSA by OR at Different Temperatures.

UV-visible spectroscopy is a useful tool to explain structural changes in proteins and to study interactions between proteins and small molecules. The UV-visible spectral measurements also distinguish the quenching mechanisms as static and dynamic quenching [22]. According to the principles of absorption spectroscopy, dynamic quenching is only induced by the excited states of the fluorophores. There is no change in absorption spectra, which suggests that absorption spectra of fluorescent molecules are not affected by a quencher. In contrast, static quenching is affected by the formation of ground-state complexes, lead to changes in absorption spectra [25-28]. In the range of 250–400 nm in Fig. 6, the absorption peak at 325 nm shows a slightly shift. Therefore, the fluorescence quenching between OR and HSA was mainly attributed to being static quenching. However, the absorbance corresponding to OR increased upon the addition of the HSA molecule. Because of this increase, an interaction between OR and HSA is considered to occur. So, peptide strands of HSA may be extended, resulting in a conformational change in the protein structure.

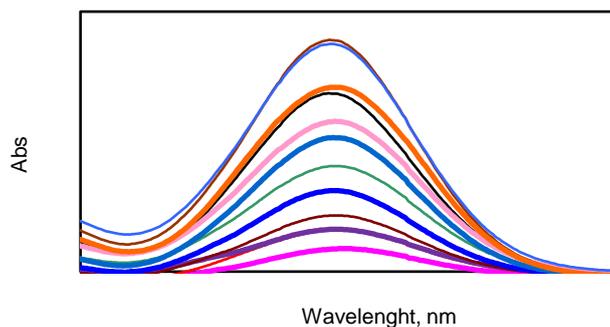


Fig. 6: Absorption Spectra of OR at Different Concentration in the Presence and Absence of HSA (10 Mg) at 5, 10, 20, 30 50, 60 Mm Free OR and OR-HSA are Shown by Thick and Thin Lines, Respectively.

Analysis of binding equilibria: HSA is considered a biomacromolecules. OR binds independently on the equivalent sites of HSA. Therefore, the equilibrium between free and bound molecules is given by the equation (Eq 2) as follows [28]:

$$\text{Lg} (F_0 - F)/F = \text{lg} K_b + n \text{lg}[Q] \quad (2)$$

Where K_b is the binding constant and n is the number of binding sites per protein. These values are estimated from Eq. 2 at different temperatures. The linear quantities are given in Table 1 and are larger than 0.99, indicating that the assumptions underlying the derivation of Eq. 1 are satisfied. (Table 1) presents values of K_b ($2.18, 1.77, 1.41, \text{ and } 1.20 \times 10^{-3} \text{ mol L}^{-1}$) and n (1.46, 1.41, 1.36 and 1.34) which decreased with an increase in temperatures (296, 298, 300, and 302 K) as viewed in Fig.7. This may be indicative for forming an unstable compound that would be partly decomposed when the temperature increased. Therefore, the values of K_b and n were low at high temperatures.

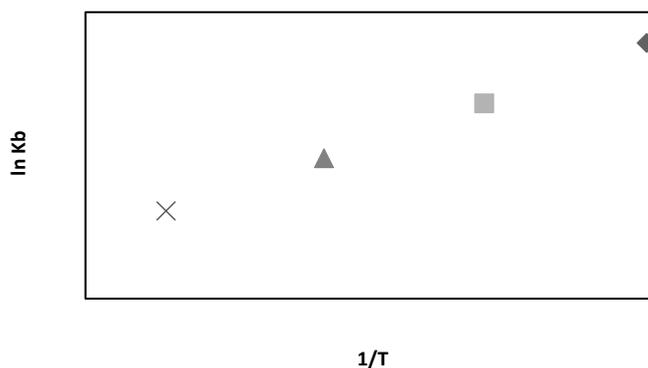


Fig. 7: Van't Hoff Plot for the Quenching of HSA by OR at Different Temperatures.

Table 1: Quenching and Binding Constant K_b and Binding Sites n at Different Temperatures

pH	T (K)	$K_b, \text{ M}$	n	R^a	$K_q \text{ L mol}^{-1} \text{ s}^{-1}$	$K_{sv} \text{ L mol}^{-1}$
7,4	296	2.18e-3	1.46	0.9910	2.87e12	296e5
	298	1.77e-3	1.41	0.9944	3.14e12	298e5
	300	1.47e-3	1.37	0.9913	3.66e12	300e5
	302	1.20e-3	1.34	0.9954	4.11e12	302e5

^(a)R is the linear quotiety.

Thermodynamic parameters and nature of the binding forces: Thermodynamic parameters provide an idea about the molecular mechanism of the interaction between OR and HSA. The thermodynamic parameters have been estimated from binding constants (K_b) at four temperatures [29]. The Van't Hoff plot of $\ln K_b$ vs. $1/T$ and Eq. 3 gives the thermodynamic parameters such as ΔG , ΔH , and ΔS corresponding to this interaction. Enthalpy (ΔH) and entropy change (ΔS) can be determined from the following Van't Hoff equation (Eq s) [22]:

$$\ln K_b = -\Delta H/RT + \Delta S/R \quad (3)$$

Where K_b is binding constant at the different temperatures, R is the gas constant; T is the experimental temperature [7], [8]. The free energy change (ΔG) is estimated from the following relationship (Eq. 4) [25]:

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

Data at different temperatures (296, 298, 302, and 304 K) are represented in Table 2. The slope and intercept of Van't Hoff plot give values of the enthalpy and entropy which are independent of temperature according to the basic assumption of Van't Hoff equation. The large negative changes in entropy and enthalpy ($\Delta S = -100.56 \text{ J mol}^{-1} \text{ K}^{-1}$ and $\Delta H = -0.44 \text{ J mol}^{-1}$) suggest that van der Waals interactions and hydrogen bonds are major forces for binding [26, 30]. The free energy changes, ΔG in different temperatures were calculated as 35.01 (298 K), 6.37 (303 K), 6.17 (308 K), and 5.97 kJ mol^{-1} (304 K). The values of ΔG were positive at all experimental temperatures, indicating that the reaction is energy-dependence. The values of ΔH and ΔS are negative at the experimental temperatures, suggests that the van der Waals interactions and hydrogen bonds play major roles in the binding mechanism.

Table 2: Relative Thermodynamic Parameters

pH	T (K)	R	ΔH (kJ mol ⁻¹)	ΔG (kJ mol ⁻¹)	ΔS (j mol ⁻¹ T ⁻¹)
7.4	296	0.9929	-36.43	6.57	-100.56
	298	0.9974		6.37	
	300	0.9945		6.17	
	302	0.9966		5.97	

^(a) R is the linear quotiety.

4. Conclusions

The interaction study of drugs with proteins have great importance in molecular biology, biochemistry, pharmacy, and pharmacology. Our study shows that fluorescence spectroscopy can be very useful in investigating the molecular interactions between drugs and HSA. From the fluorescence measurements, the binding constant and binding ratio allowed to be measured. Thermodynamic parameters related to the interaction were also estimated by using fluorescence spectroscopy. In this study, the binding of ornidazole to human serum albumin was evaluated by fluorescence measurements. The result indicated that ornidazole interacted with HSA by a static quenching procedure. The reaction between HSA and OR which occurs via van der Waals interactions and hydrogen bonds is energy dependent.

This study indicated that interaction between drugs and proteins can be studied by the fluorescence quenching technique. This technique may give us a better understanding of pharmacokinetics such as distribution of a drug, drug metabolism, and the dosage in therapeutics *in vitro*. Particularly, the study on the binding sites of proteins will help to synthesize new drugs and to find their targets in the human body as a specific task. Further studies on protein binding of drugs similar molecules will be in progress in our laboratory.

Acknowledgments

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