Regular Article

PHYSICAL CHEMISTRY RESEARCH
Published by the
Iranian Chemical Society
www.physchemres.org
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Phys. Chem. Res., Vol. 9, No. 3, 525-538, September 2021
DOI: 10.22036/pcr.2021.247363.1829

The Influence of 6-Shogaol, a Therapeutic Agent of *Zingiber Officinale*, on the Interaction of Ciprofloxacin with Bovine Serum Albumin

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(Received 27 December 2020, Accepted 7 April 2021)

The binding of ciprofloxacin (CIP) to bovine serum albumin (BSA) in the presence and absence of 6-shogaol was investigated by multiple techniques. Fluorescence spectroscopy results indicated that CIP was able to quench BSA in the presence and absence of 6-shogaol through a static mechanism. The CIP and 6-shogaol binding to BSA leads to the increase of the binding constant (K_a) value of CIP to BSA. In accordance to the negative values of $\Delta H°$ and $\Delta S°$, the functions of hydrogen bonding and van der Waals forces are very important in this special binding. In addition, the negative values of $\Delta H°$ and $\Delta S°$ for BSA-CIP in the presence of 6-shogaol have been larger than those of the absence of 6-shogaol, highlighting the role of hydrogen bonding and van der Waals forces. As indicated by the synchronized fluorescence spectroscopy at $\Delta\lambda = 60$ nm, the location of CIP with mixed BSA in binary and trinary systems has been nearer to Tyr residues.

Keywords: Binding constant, Fluorescence quenching, Drug binding, Three-dimensional fluorescence

INTRODUCTION

The biological, therapeutic activity and toxicity of a drug in pharmacology can remarkably affected by the protein-drug interaction [1]. A decreased lifetime of a drug is due to its weakly binding to albumin and may in addition induce its weak distribution. Nevertheless, a powerful binding with the drug results in a decreased free drug concentrations. The enhancement of the concentration of the free drug can lead to further drug-drug or drug-food interactions. The investigation of interaction among drug and albumin is essential in assessment of the biological activity of the drug [2].

Due to low purchase price of BSA, and the structural homology of BSA with human serum albumin (HSA), the human serum albumin is commonly replaced by bovine serum albumin to illustrate the serum albumins in protein-ligand binding investigations [3]. BSA is composed of three construction ally similar domains (I, II, III) and every domain contains two sub-domains (A, B). BSA possesses two tryptophan residues (Trp-134 and Trp-212) that are placed in the subdomains IB and IIA severally [4] (Scheme 1a). The tryptophan residues are very susceptible to their surrounding areas which assist to combine fluorescence characteristics with BSA. Therefore, the drug binding to BSA can induce conformally changes in protein which causes changes in emission spectra [5].

Ciprofloxacin [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(piperazinyl)-quinolone-3-carboxylic acid] (CIP) (Scheme 1b) is a greatly popular fluoroquinolone with a broad-spectrum of bactericidal antibiotic activity, and being effective against the multitude gram-positive and gram-negative bacteria and microorganisms. Many medical and epidemiological studies show a powerful relation between prohibition of human cancers and dietary agents [6]. Thus, chemoprophylaxis of cancers by phytochemicals and nutraceuticals has become a prosperous field of research over the past years.

Zingiber officinale, often known as ginger, is used as a

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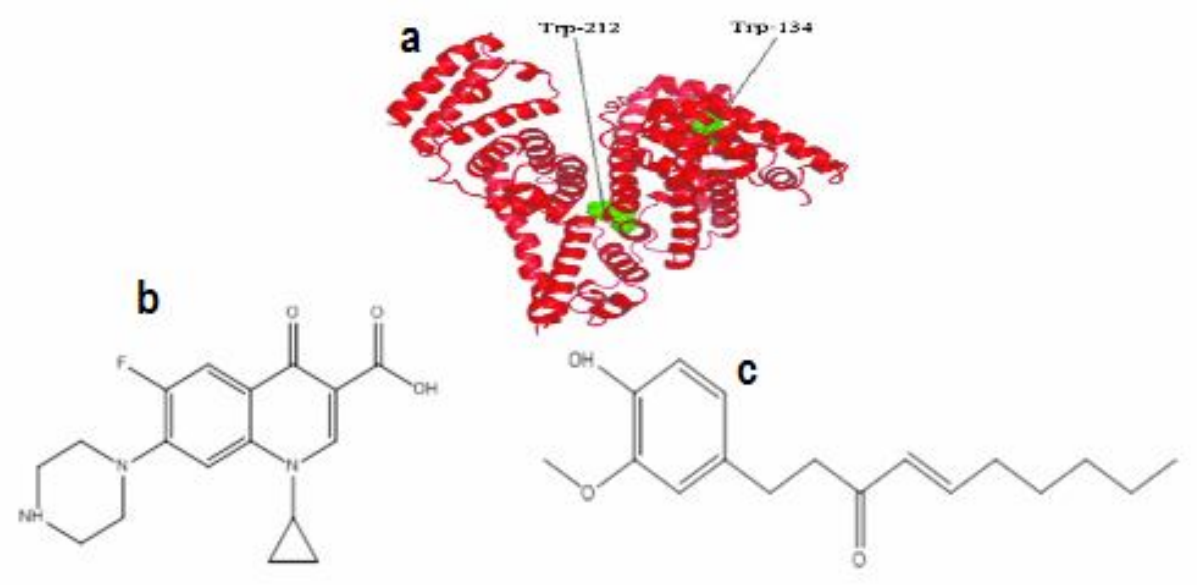

Scheme 1. (a) Chemical 3D structure of BSA showing Trp-212 and Trp-134 in green color(taken from the PDB), (b) structural formula of Ciprofloxacin (drown by ChemDraw Ultra12.0), and (c) 6-shogaol (drown by ChemDraw Ultra12.0)

spice and a flavor agent. Ginger has powerful phenolic materials known as gingerols. This compound displays numerous therapeutically properties such dyspepsia, gripes, qualm, vomit, gastritis, diarrhea, arthritis, migraine, blood pressure and treatment of colds. 6-Shogaol (Scheme 1c) is the essential component of dried ginger [7]. Many investigations have displayed anticancer actuality shown by extracts of phenolic compounds of ginger against cancers of skin, gastrointestinal, colon and breast [8]. The use of complimentary or alternative remedies is on the increase globally because most people believe that the natural agents are safer than the conventional therapeutic agents. On the other hand, the indiscriminate use of CIP for gram-positive and gram-negative bacteria problems is high. Given that ginger is also widely used by a vast majority of the populace for various ailments including gastrointestinal [9], there is a high probability of concomitant consumption of ginger and CIP. Our present study therefore attempts at elucidating the possible herb-drug interaction between CIP and 6-shogaol with a focus on the estimation of the binding modes and evaluation of thermodynamic parameters through spectroscopic methods.

EXPERIMENTAL

Chemicals

BSA (bovine serum albumin, fatty acid free, >98%) was bought from SRL (India) and used without further purification. Ciprofloxacin was obtained from Samen Pharmaceutical Co. Ciprofloxacin and BSA were solved in a solution of potassium phosphate buffer (pH = 7.4, 50 mM). 6-Shogaol was separated from the rhizomes of *Zingiber officinale* similarly as elucidated by S.R. Feroz *et al.* [10]. The identifying of 6-shogaol occurred by spectroscopic methods further by confirmation of results with those reproduced previously [11]. The concentrations of Protein, drug and Shogaol used in all experiments were $C_{BSA} = 4.52 \times 10^{-3}$ mM, $C_{CIP} = 9.05 \times 10^{-5}$ M and $C_{shogaol} = 4.81 \times 10^{-5}$ M, respectively.

Methods

Fluorescence measurements have been carried out on a spectrophotometer (HITACHI F-2500) at 280 and 295 nm excitation wavelengths, with the emission and excitation wavelengths that had been recorded from 300 to 500 nm.

The emission and excitation bandwidths have been set at 5 nm. All pH determinations were carried out with a Metrohm 744 pH-meter.

The data of fluorescence quenching are generally evaluated through the Stern-Volmer equation [12,13]:

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

$$K_{SV} = K_q \tau_0 \quad (2)$$

where F and F_0 are the intensities of fluorescence in the presence and absence of quencher, respectively, k_q is the quenching rate constant for the protein, K_{SV} is the Stern-Volmer dynamic quenching constant, τ_0 is the mean lifetime of the fluorophore in the absence of quencher ($\tau_0 = 10^{-8}$ s), and [Q] is the quencher concentration.

Values of the binding constant (K_a) and the attachment sites number (n) can be obtained by this equation:

$$\log \frac{(F_0 - F)}{F} = \log K_a + n \log [Q] \quad (3)$$

A chart of log[(F_0 - F)/F] against log[Q] resulted a direct line through least squares examination and its slope was adequate to n with an intercept of logK_a [14].

The thermodynamic variables for CIP-BSA interaction without and with 6-shogaol were specified from using the subsequent equation (van't Hoff plot):

$$\ln K_a = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (4)$$

Somewhere R is the universal gas constant, T is the experiential temperature (K), K_a is the binding constant. The entropy ($\Delta S°$) and the enthalpy ($\Delta H°$) changes can be achieved from the intercept and slope of the fitted curve of lnK_a against 1/T, respectively. Later, the $\Delta G°$ (Gibbs free energy), was obtained employing the subsequent equation [14]:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (5)$$

Fluorescence quenching experiments were performed at different temperatures of 298, 303 and 308 K.

Synchronous fluorescence spectroscopy has been performed through concurrently scanning the emission and excitation monochromators. The acquired spectra have revealed the Tyr and Trp residues of BSA when the $\Delta\lambda$ had been 60 and 15 nm, respectively.

The UV-Vis spectra were registered at ambient temperature on a SPEKOL 1500 spectrophotometer at 200-500 nm. Synchronous fluorescence experiments were performed at 298 K.

To assess the binding constant, K_a, between protein and ligand at 298 K, the absorption information were analyzed using Equation [15]:

$$\frac{A_0}{A - A_0} = \frac{\varepsilon_P}{\varepsilon_B} + \frac{\varepsilon_P}{\varepsilon_B \times K_a} \times \frac{1}{[Q]} \quad (6)$$

where A and A_0 are the absorbance of protein with and without of drug, and ε_P and ε_B are the extinction coefficients of protein and its complex with drug. K_a is the binding constant at the related temperature and [Q] is the concentration of drug. So, the double reciprocating chart of $A_0/(A - A_0)$ against 1/[Q] is a straight line and K_a could be evaluated from the proportion of the intercept to the slope.

The fluorescence spectra overlapping of BSA and UV spectra of CIP without and with 6-shogaol were employed to estimate the amount of energy transfer.

The energy transfer phenomenon is classified into radiative and non-radiative energy transfer modes in fluorescence. In accordance with Forster's theory [16], the energy transfer is influenced by the distance amongst the accepter and donor, (r) and the critical energy transfer distance (R_0).

The relationship between FRET efficacy and distance is as follows:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (7)$$

where E is energy transfer efficiency, and F_0 and F are intensities of fluorescence of bovine serum albumin (BSA) in the absence and existence of CIP. R_0 is calculated using the subsequent equation:

$$R_0^6 = 8.79 \times 10^{-25} K^2 N^{-4} \Phi J \quad (8)$$

where K^2 implies the special orientation factor, and medium refractive index is "*N*" that N = 1.336. Φ is the donor's fluorescence quantum yield without acceptor which is equal to Φ = 0.118, R_0 = 1.32 nm and J is the result of spectra overlapping between absorption spectrum of the acceptor and the fluorescence emission spectrum of the donor and is presented by:

$$J = \Sigma F(\lambda)\, \varepsilon(\lambda)\, \lambda 4\, \Delta\lambda / \Sigma F(\lambda)\, \Delta\lambda \quad (9)$$

where F(λ) is the emission spectrum fluorescent of donor, and ε(λ) implies extinction coefficient of the acceptor.

3-D fluorescence spectra were carried out on a HITACHI F-2500 spectrophotometer under the excitation and emission wavelengths that had been fixed amongst 220 and 500 nm with the enhancements of 5 nm.

RESULTS AND DISCUSSION

Fluorescence Quenching Mechanism

The Fluorescence procedure is a very sensitive and powerful implement for investigating the interaction amongst small ligands and proteins. The BSA fluorescence is bringing about three inherent fluorophores existing in its structure such as Tyr (tyrosine), Trp (tryptophan) and Phe (phenylalanine) [17]. The fluorescence of BSA is excited at about 280 nm or greater wavelengths. The BSA absorption at 280 nm is due to the each of two tyrosine and tryptophan residues, whiles at wavelengths higher than 295 nm, the absorption is because of tryptophan. Therefore, the intrinsic fluorescence of proteins can provide remarkable details on the dynamics and structure, and it is mostly used in the investigation of association reactions and protein folding [18].

The fluorescence spectra of BSA-CIP in the existence and absence of 6-shogaol at λ_{ex} = 280 and 295 nm are exhibited in Fig. 1. CIP showed intrinsic fluorescence at 380-470 nm. The spectrum displays that with enhancing CIP concentrations, the fluorescence severity of BSA was reduced and the CIP peak enhanced in an orderly way (fluorescence spectra of BSA and CIP are in two various areas and do not overlap).

The red shift in the fluorescence spectra is correlated with rising the microenvironmental polarity after binding, indicating that the protein chromophore was brought around less hydrophobic environment. In addition, an isosbestic point can be considered, whereat BSA-CIP has the similar fluorescence intensity over 390 nm. The isosbestic point indicates an equilibrium between free and bound shapes of the CIP. All these results display an BSA-CIP complex formation in the absence and presence of 6-shogaol [18]. Stern-Volmer plots were applied to clarify the quenching mechanism (Fig. 2).

Table 1 indexed the K_{SV} and k_q values achieved from the Stern-Volmer procedure, and as the results show, the k_q values were bigger than the maximal scatter collision quenching constant of different quenchers with biopolymers (2.0×10^{10} M^{-1} s^{-1}). This demonstrated that the fluorescence quenching mechanism is static.

In accordance with the modified Stern-Volmer procedure, the f_a amounts at λ_{ex} = 280 and 295 nm of BSA-CIP in the absence and presence of 6-shogaol are listed in Table 1. As could be considered, the existence of 6-shogaol influences the f_a values. A f_a value equivalent to 1 displayed that whole of Trp residues were accessible for the quencher. Therefore, an alter in the amount of f_a caused changing in the portion of fluorescent elements that were accessible for the quencher [19]. The values of K_a and n are included in Table 2.

As the outcomes show, the interaction amongst BSA and CIP in the absence and presence of 6-shoagol is different. K_a is a criterion of the attachment of BSA to CIP. Data propose that the amount of K_a for BSA-CIP in the presence of 6-shogaol at λ_{ex} = 280 and 295 nm increased. As a result, the presence of 6-shogaol led to a powerful complex to be formed. The interaction treatment of BSA-CIP altered with the existence of 6-shogaol. The binding affinity for the ternary and binary systems at λ_{ex} = 295 nm was greater than that at λ_{ex} = 280 nm [13].

Thermodynamic Analysis

Thermodynamic variables of a binding interaction are vital for validating the binding forces. Four types of non-covalent interactions comprising van der Waals forces, hydrogen bonds, hydrophobic and electrostatic interactions are identified [20]. With the purpose of elucidate the CIP interacting with BSA in the absence and presence of 6-shogaol, the thermodynamical variables were investigated

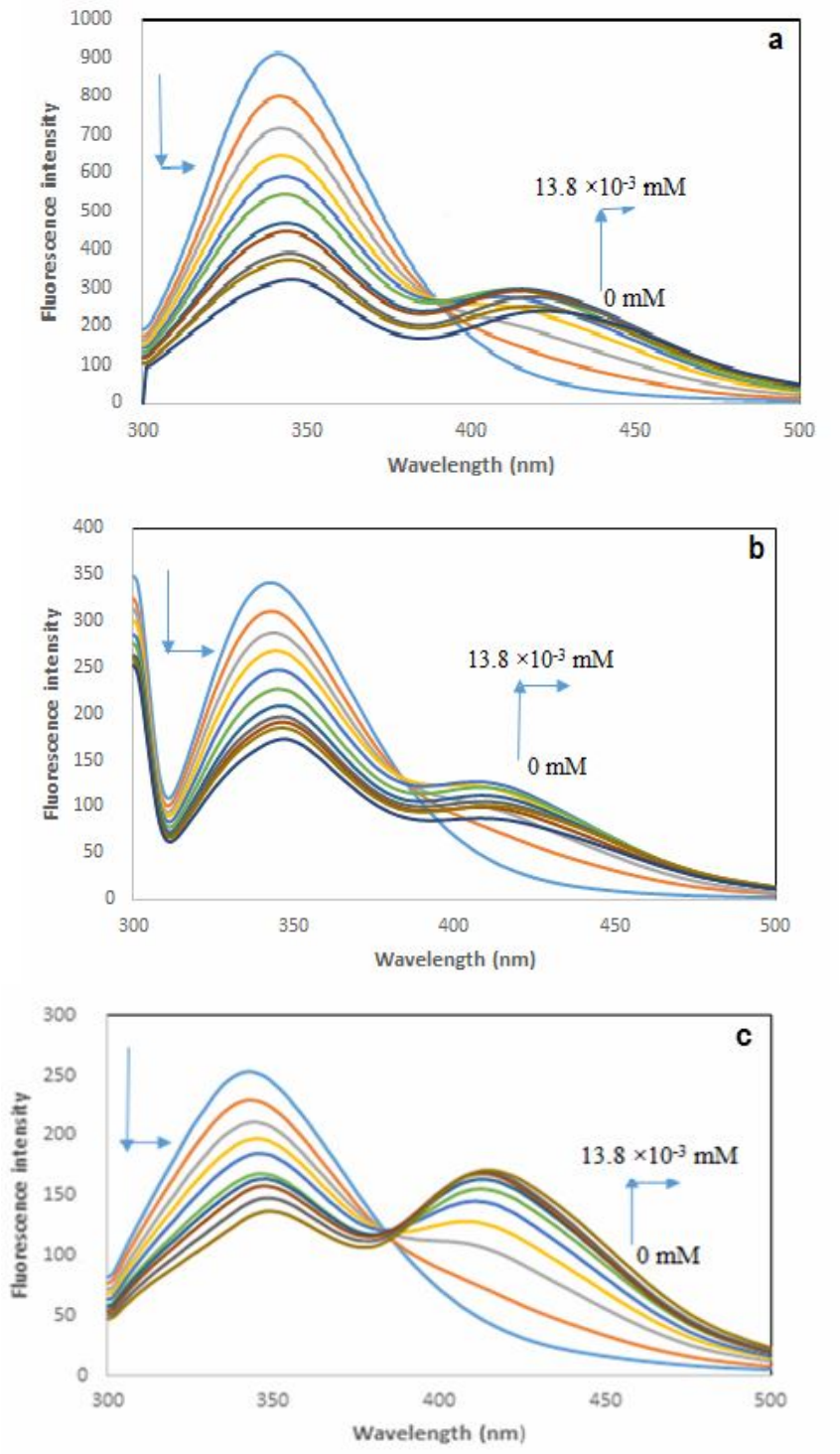

Fig. 1. Emission fluorescence spectra of BSA at different concentrations of CIP at (a) BSA-CIP, λ_{ex} = 280 nm, (b) BSA-CIP, λ_{ex} = 295 nm, (c) BSA-Shogaol-CIP, λ_{ex} = 280 nm and (d) BSA-Shogaol-CIP, λ_{ex} = 295 nm. T = 298 K, pH 7.4, $C_{BSA} = 4.52 \times 10^{-3}$ mM.

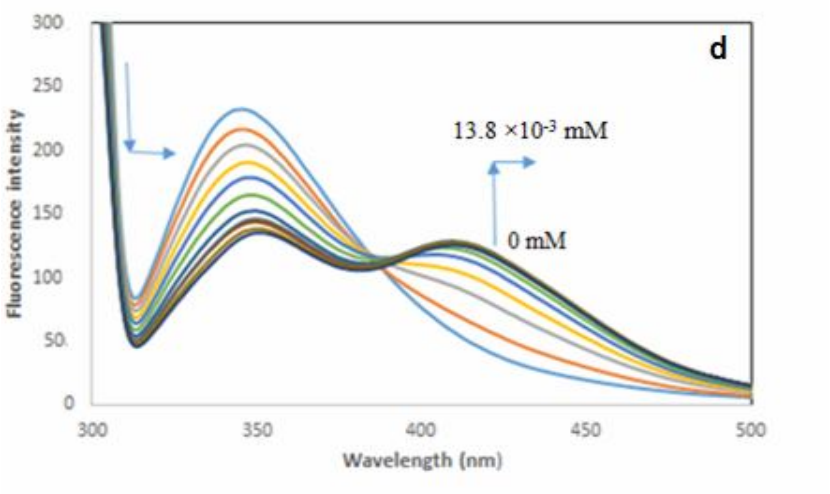

Fig. 1. Continued.

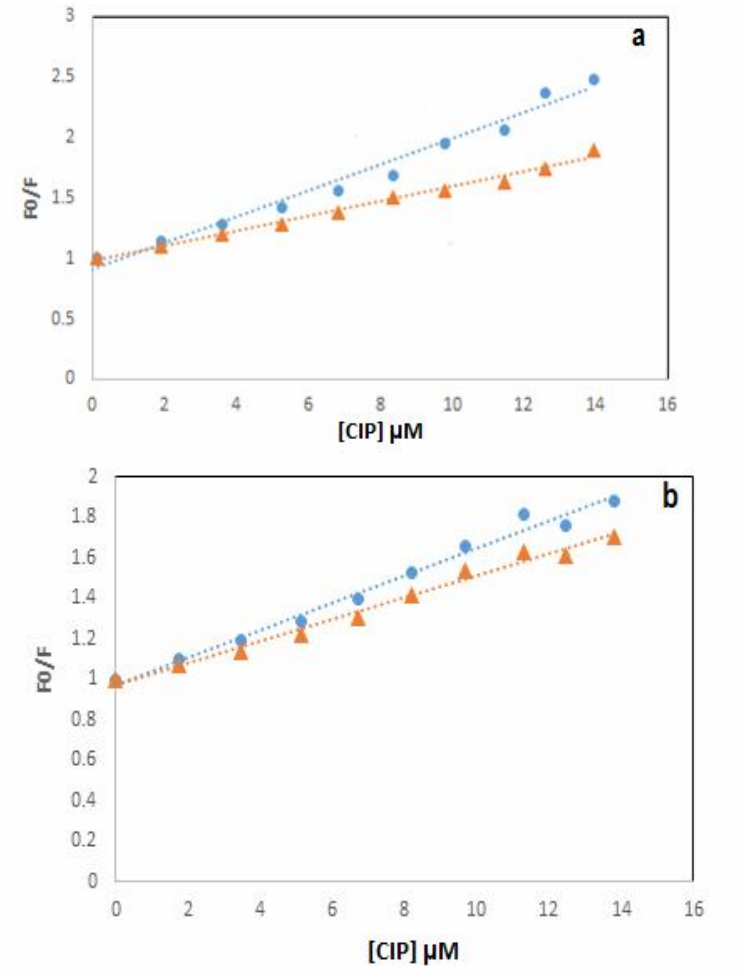

Fig. 2. Stern-Volmer plots for fluorescence quenching of BSA at (a) λ_{ex} = 280 nm, and at (b) λ_{ex} = 295 nm. (●)BSA-CIP; (▲) BSA-Shogaol-CIP.

Table 1. Stern-Volmer Quenching Constants at λ_{ex} = 280 and 295 nm for Interaction of CIP with BSA in Binary and Ternary Systems (pH = 7.4)

System	λ_{ex} (nm)	k_q ($M^{-1} S^{-1}$)	K_{sv} (M^{-1})	R^2	f_a
CIP-BSA	280	1.08×10^{13}	1.08×10^{5}	0.9769	1.228
Shogaol-CIP-BSA	280	0.616×10^{13}	0.616×10^{5}	0.9884	0.988
CIP-BSA	295	0.67×10^{13}	0.67×10^{5}	0.9835	1.315
Shogaol-CIP-BSA	295	0.538×10^{13}	0.538×10^{5}	0.9853	1.484

Table 2. Binding Parameters of BSA-CIP Complex at λ_{ex} = 280 and 295 nm in Binary and Ternary Systems (pH = 7.4)

System	λ_{ex} (nm)	K_a (M^{-1})	n	R^2
CIP-BSA	280	4.565×10^{3}	1.16	0.9912
Shogaol-CIP-BSA	280	5.194×10^{3}	1.028	0.9946
CIP-BSA	295	2.273×10^{3}	1.172	0.9932
Shogaol-CIP-BSA	295	4.090×10^{3}	1.096	0.9913

at three various temperatures of 298, 303 and 308 K and the amounts were computed from van't Hoff charts (Fig. 3), as indicated in Table 3.

The negative sign of ΔG° demonstrates that the binding operation has occurred spontaneously. As indicated in Table 3, the negative values of ΔH°, concerning the CIP interaction and BSA complex, without and with 6-shogaol, implicates the binding has been mostly enthalpy motivated, whereas the ΔS° values have not been good in these conditions. The values of ΔS° and ΔH° of the binding between BSA-CIP and BSA-Shogaol-CIP were found to be negative, displaying that the functioning of hydrogen bond interactions and van der Waals forces were quite significant [21]. Additionally, the negative ΔH° and ΔS° for the BSA-Shogaol-CIP complex were greater than those for the BSA-CIP, indicating the greater importance of hydrogen bonding and van der Waals forces.

Synchronous Fluorescence Spectroscopy

Figures 4a and b display the main peaks for the BSA-CIP and BSA-Shogaol-CIP systems at Δλ = 15 and 60 nm, respectively. As indicated, an enhancement in CIP concentration from 0 to 13.8×10^{-3} mM resulted in the fluorescence intensity to reduce in overall systems. Based on Fig. 4, the synchronous fluorescence of BSA-CIP without and with 6-shogaol does not show any wavelength shift at Δλ = 15 nm. A little red shift was considered in the systems at Δλ = 60 nm which indicated that when the CIP was added, the polarization about Trp residues was enhanced and the hydrophobic character was reduced [22].

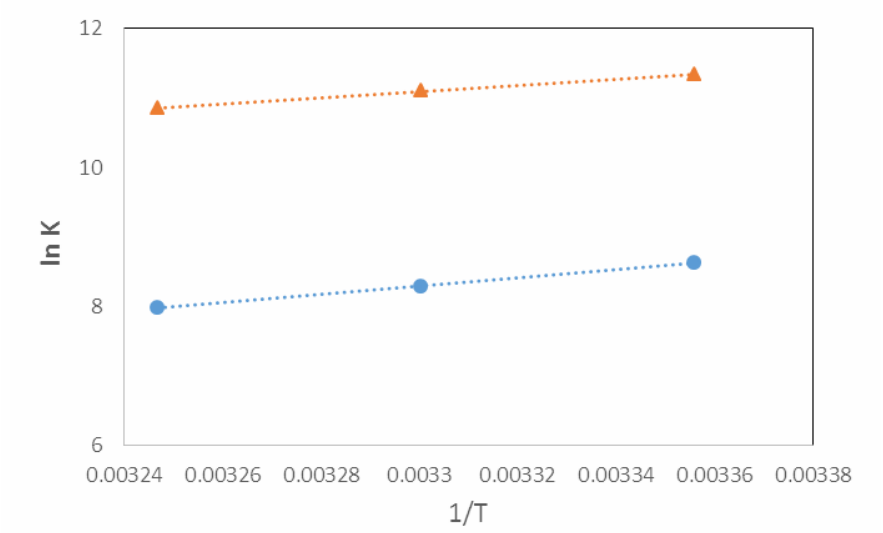

Fig. 3. The van't Hoff plot for the interaction of BSA-CIP (●) and BSA-Shogaol-CIP (▲) complex.

Table 3. Thermodynamic Parameters for CIP on BSA in Binary and Ternary Systems at Various Temperatures (pH = 7.4)

System	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1})	ΔG° (kJ mol^{-1})	R^2
CIP -BSA	-49.165	-93.333	-21.351	0.998
			-20.885	
			-20.418	
Shogaol-CIP -BSA	-37.273	-30.741	-28.112	0.999
			-27.958	
			-27.804	

Therefore, the interaction of CIP with BSA affected the conformation of BSA slightly.

Figures 4a and b indicates the slope of the BSA-CIP complex plot, without and with 6-shogaol, respectively. As indicated, the slope was greater when $\Delta\lambda$ = 60 nm, which resulted in the inference that Trp perform a significant function throughout fluorescence quenching of BSA. This indicated that CIP was nearer to the tryptophan than the tyrosine residues.

UV Spectroscopy

The UV-Vis absorption measuring is a remarkably beneficial and easy procedure, that is frequently utilized to affirm static quenching procedure. This technique is a procedure to understand the complex details and generally used to investigate the structural changes. Dynamic

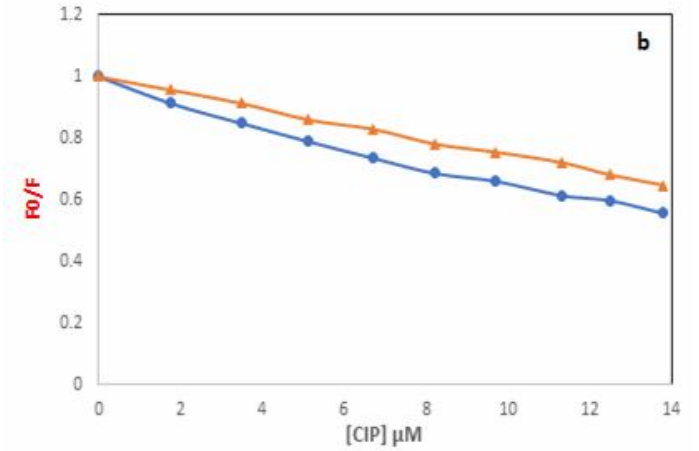

Fig. 4. A comparison of curves of F/F_0 *vs*. [CIP] for BSA-CIP (a) and BSA-Shogaol-CIP (b), at $\Delta\lambda$ = 15 nm (▲) and $\Delta\lambda$ = 60 nm (●).

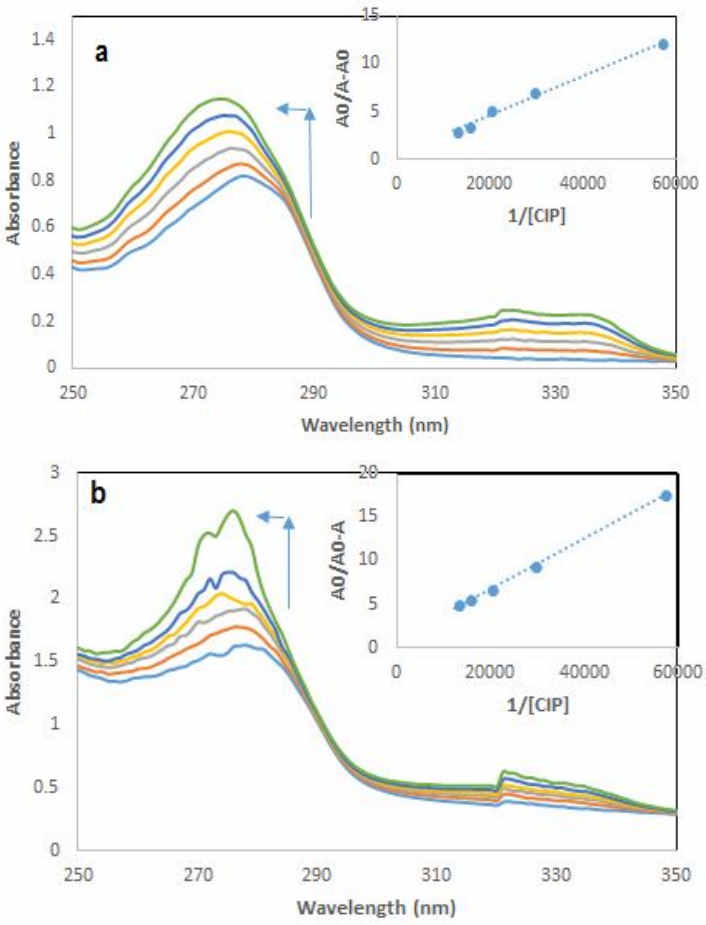

Fig. 5. (a) UV-Vis spectra of BSA-CIP and (b) BSA-Shogaol-CIP at 298 K and pH 7.40 (inset) The plots of $A_0/(A - A_0)$ *vs*. [1/CIP].

quenching does not change the absorption spectra and just influences the fluorophores excited states. In contrast, in the static quenching, a protein-drug complex forms and alters the UV spectral pattern of the protein. The UV-Vis spectra of BSA-CIP without and with of 6-shogaol are shown in Fig. 5 [23].

According to Fig. 5, a powerful peak about 278 nm is seen, which could be related to the absorption of the tryptophan, tyrosine and phenylalanine residues of BSA. With the increase of CIP, the absorption peak of BSA which is located about 278 nm moderately increased.

The double reciprocal plot of Eq. (7); *i.e.*, $A_0/(A - A_0)$ *vs.* 1/[CIP] (inset of Figs. 5a and b), plotted was linear; by adopting the ratio of intercept with slope. The estimated K_a values are 2.94×10^3 and 3.69×10^3 M^{-1} for BSA-CIP complex without and with 6-shogaol, respectively. The K_a values are of the similar order of magnitude achieved by fluorescence spectroscopy. Because $K_a > 1$, the concentrations of BSA-CIP and BSA-Shogaol-CIP complexes are higher than free molecules [24].

Energy Transferring Measurements (FRET)

Fluorescence resonance energy transferring (FRET) is a forceful device that has been widely utilized for investigating the intervals among chromophores bounded and protein. The spectral overlapping between the absorption of CIP and fluorescence emission of BSA with and without 6-shogaol is shown in Fig. 6.

The r values of BSA and CIP were obtained from Eqs. ((8)-(10)) and experimental data. The binding distance, r, amongst acceptor and donor, were 3.25 nm and 2.83 nm in the BSA-CIP and BSA-Shogaol-CIP, respectively. Obviously, the aforesaid intervals were less than 7 nm after interaction, confirming the presence of a static quenching mechanism. As can be understood, the distance from CIP to BSA in the presence of 6-shogaol is smaller than that of its binary match (BSA-CIP), indicating that 6-shogaol can cause conformational alterations in (BSA-CIP) and cause a greater affinity in the BSA binding to CIP [25].

3D Fluorescence Determinations

3D fluorescence spectroscopy is a modern analyzing and forceful manner for investigating the conformational alterations of macromolecules [26]. Furthermore, fluorescence features can be entirely obtained by concurrently variation the emission and excitation wave-lengths [26]. Fluorescence severity of residues and the highest emission wave length have a nearby relationship to the polarization of their microenvironment [27]. Figure 7 displays the 3D fluorescence spectra of BSA complexes without and with 6-shogaol.

In accordance with Figs. 7a, b and c, peak a ($\lambda ex = \lambda em$) displays the Rayleigh scattering peak, where obviously its fluorescence intensity is enhanced after the addition of CIP without and with 6-shogaol. This could be due to the formation of BSA-CIP complex after CIP addition, leading to the increase in scattering effect. Peak b ($\lambda em = 2\lambda ex$) is considered as the second-order scattering peak. Peak 1 has displayed the spectral properties of tyrosine and tryptophan residues for the reason that when a protein is stimulated at 280 nm, it principally indicates the inherent fluorescence of these two residues whereas the fluorescence of phenylalanine residue may be unimportant. Peak 2 mainly demonstrates the fluorescence spectra situation of polypeptide framework structure regarding BSA, which are principally due to the π-π* shift of BSA feature polypeptide framework structure C=O [28]. Comparison of the fluorescence intensity alterations of peak 1 and peak 2 shows that they have had a clear reduction in the presence of CIP in the dual and trinary systems. In accordance to the results, the interaction of CIP with BSA has caused the little polypeptide unrolling of protein, leading to the conformational alterations, also enhancing the disposal of several hydrophobic regions that were hidden before. All these results indicate that the CIP binding to BSA without and with 6-shogaol can induce certain microenvironmental and conformational alterations in protein.

CONCLUSIONS

The study of CIP binding to BSA in the presence of 6-shogaol is of significant importance in pharmacology and biochemistry for elucidating the transport and storage of drug during bacterial infections treatment. It is noteworthy that in pharmacology, when the effect of a mixture of two compounds equals or exceeds the sum of the effects of the individual component, this is defined as synergism.

In this study, we demonstrated the CIP binding to BSA

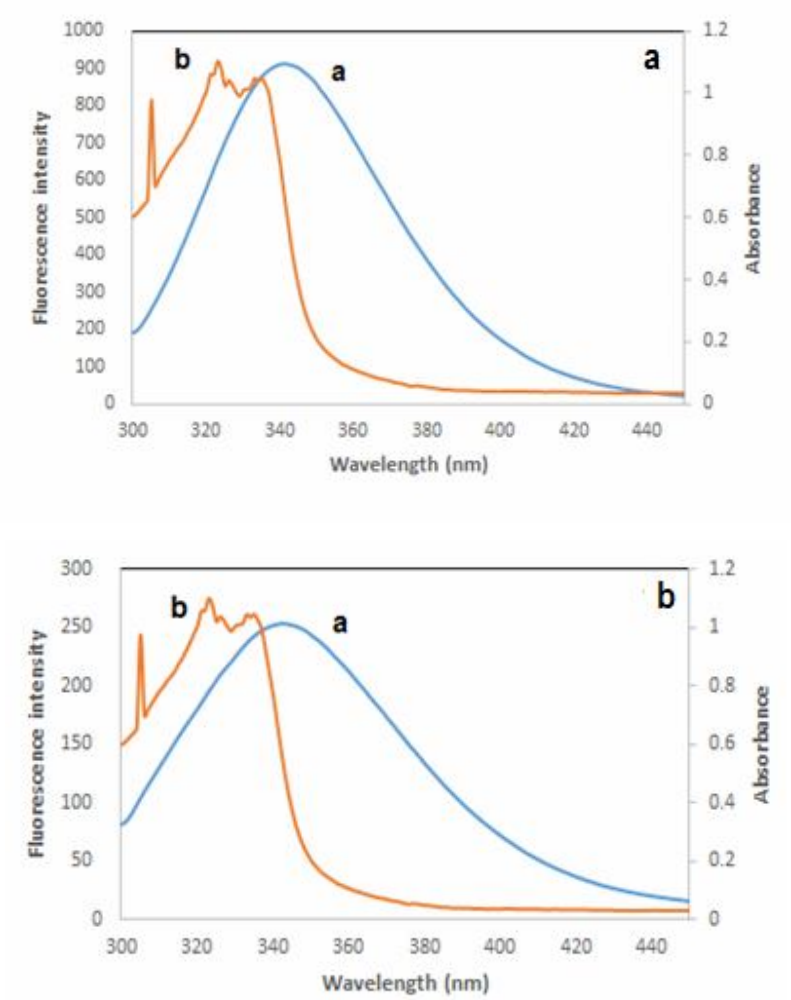

Fig. 6. (a) Overlap of the fluorescence emission spectrum (curve a) of BSA with the absorption spectrum (curve b) for CIP, and (b) overlap of the fluorescence emission spectrum (curve a) of BSA-Shogaol with the absorption spectrum (curve b) for CIP, T = 298 K, pH 7.4, [BSA] = [CIP] = 4.5×10^{-3} mM**.**

in the presence and absence of 6-shogoal through different spectroscopic techniques. 6-Shogaol could increase the binding affinity between the CIP and BSA in the Shogaol-CIP-BSA system, indicating that the synergism between 6-shogaol and CIP should increase the effect of the drug when they coexist. The increase of binding constant should extend the retention time of the drug in blood plasma. On the other hand, based on the evidence of an increase in affinity between drug and BSA, when 6-shogaol is present, relatively low doses of CIP is required in patients. Consequently, dosage should be controlled to inhibit unpleasant side effects.

ACKNOWLEDGEMENTS

This work is financially assisted by the Islamic Azad University- Bandar Abbas Branch. The authors are grateful for that.

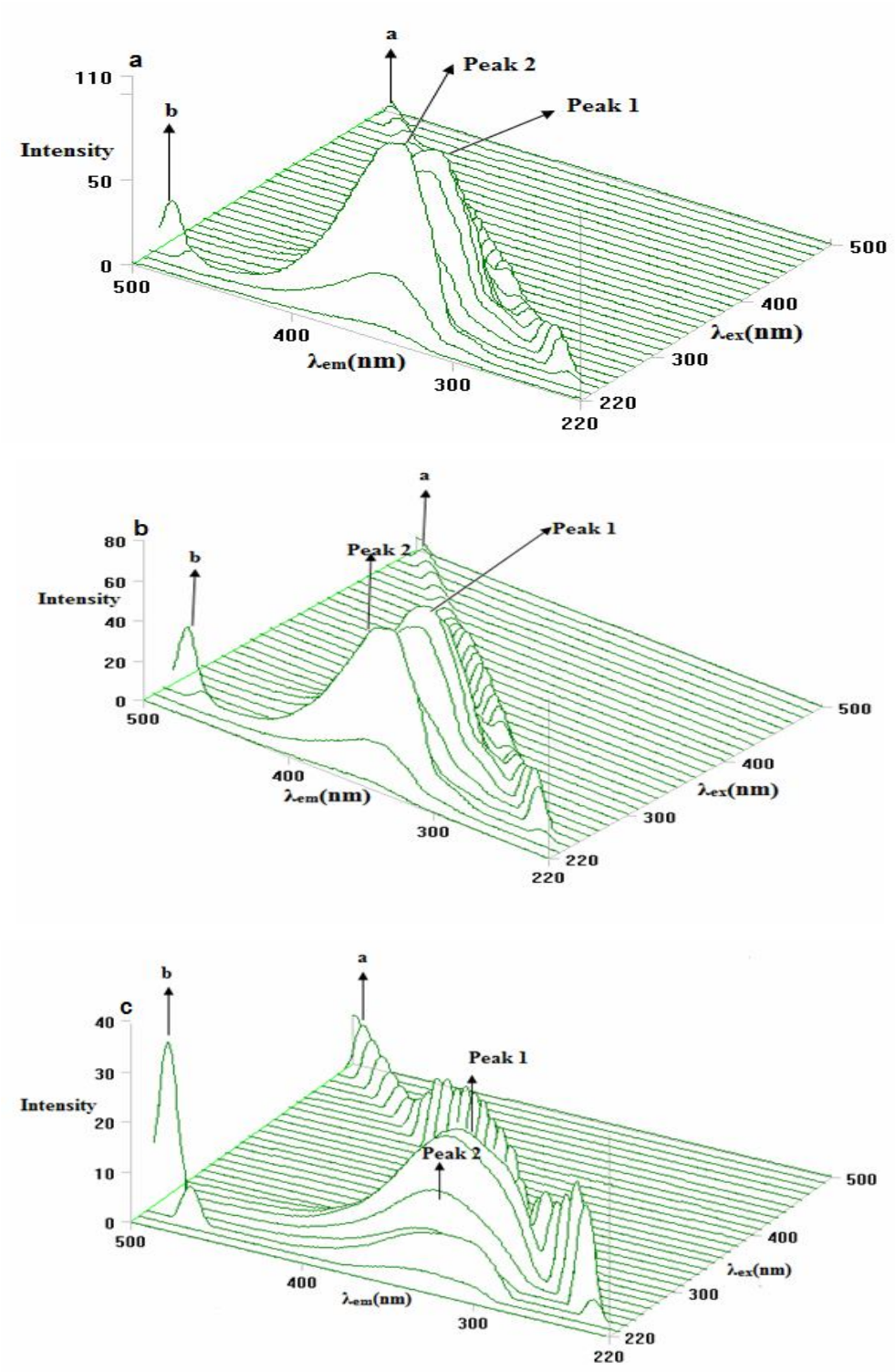

Fig. 7. Three-dimensional fluorescence and the corresponding contour spectra of (a) free BSA, (b) BSA-CIP and (c) BSA-Shogaol-CIP, at T = 298 K, pH 7.4 and [BSA] = 4.5×10^{-3} mM.

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