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Spectroscopic study on the interaction of losartan potassium and bovine serum albumin

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ABSTRACT

The interaction of losartan potassium (LP) with bovine serum albumin (BSA) was studied by fluorescence quenching in combination with UV–Vis spectroscopic method under near physiological conditions. The fluorescence quenching rate constants and binding constants for BSA–LP system were determined at different temperatures. The fluorescence quenching of BSA by LP is due to static quenching and energy transfer. The results of thermodynamic parameters, ΔH (–134.3 kJ mol^{-1}), ΔS (–368 J mol^{-1} K^{-1}) and ΔG (–24.52 to –20.83 kJ mol^{-1}), indicated that van der Waals interaction and hydrogen bonding played a major role for LP–BSA association. The competitive experiments demonstrated that the primary binding site of LP on BSA was located at site II in sub-domain IIIA of BSA. The distance between LP and a tryptophane unit was estimated to be 3.183 nm based on the Förster resonance energy transfer theory. The binding constant (K_a) of BSA–LP at 298K was 1.932×10^4 L mol^{-1}. Synchronous fluorescence and three-dimensional fluorescence studies showed that the presence of LP could change the conformation of BSA during the binding process.

Key words: Bovine serum albumin, Losartan potassium, Fluorescence quenching, Binding constant, Three-dimensional fluorescence

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INTRODUCTION

Serum albumins (SA) make a significant contribution to colloid osmotic blood pressure and aid in the transport, distribution and metabolism of many endogenous and exogenous ligands. Protein–drug binding greatly influences the absorption, distribution, metabolism and excretion properties of typical drugs [1,2]. Thus, it is necessary to study the interaction of drug with serum albumins at molecular level.

Losartan potassium (LP) is a nonpeptide angiotensin II receptor antagonist used for the treatment of hypertension [3]. It binds competitively and selectively to the angiotensin II subtype 1 (AT1) receptor, thereby blocking angiotensin II-induced physiological effects [4]. Following oral administration, losartan is well absorbed with a systemic bioavailability of approximately 33% and the mean peak plasma concentrations of losartan and its metabolite are reached in 1–2 h and in 3–4h, respectively[5]. The determination of losartan potassium in human urine [6] and rabbit plasma [7], and human plasma [8,9] were carried out. It was reported that high serum potassium levels after using losartan can reflect more severe renal disease [10]. Although the losartan determination and pharmacokinetic study were carried out [7-9], the interaction of losartan with serum albumin was barely studied. To our knowledge, there is only a report for studying the interaction of LP at the binding sites of bovine serum albumin by equilibrium dialysis method in presence or absence of palmitic acid using ranitidine and diazepam as site-1 and site-2 specific probe,

respectively[11]. However, no the information regarding energy transfer, binding forces and BSA conformation change for the interaction between LP and serum albumin was reported.

Human and bovine serum albumins exhibit similar binding chemistry due to the high percentage of sequence identities between the two proteins [12]. In this work, bovine serum albumin (BSA) was used because of its low cost and easy availability, and LP was used as a model drug. The interaction characteristic between LP and BSA under physiological conditions was examined by the fluorescence quenching in combination with the UV–vis spectroscopic method.

EXPERIMENTAL SECTION

Drugs and reagents

Commercially available bovine serum albumin (BSA, catalog no.A-7030, purity: 98%, M: 68,000) was purchased from Sigma Chemical Company. BSA stock solution (1.0×10^{-4} M) was prepared by dissolving an appropriate amount of BSA with 0.1 M Tris–HCl (pH 7.4) buffer solution, and kept in the dark at 4 °C. BSA working solutions were prepared by diluting the stock solution with water. Losartan potassium (LP, purity: 98.7%) was purchased from the National Institutes for Food and Drug Control (Beijing, China). A stock solution (1.0×10^{-3} M) of LP was prepared in water, and stored in refrigerator at 4 °C. Tris–HCl buffer (pH 7.40) consists of Tris (0.1 M), HCl (0.1 M), and NaCl (0.5 M). NaCl solution was used to maintain the ion strength. All chemicals were of analytical reagent grade or better. Purified water was prepared by an XGJ- 30 highly pure water machine (Yongcheng purification Science & Technology Co. Ltd., Beijing, China). Equipment All fluorescence measurements were performed on an F-7000 Fluorescence spectrophotometer (Hitachi, Japan) which was equipped with a 1 cm quartz cell and thermostat bath. The spectrum data points were collected from 280 to 500 nm. The widths of the excitation and the emission slit were both set at 5 nm. Fluorescence measurements were carried out at room temperatures.

The absorption spectra were performed on a TU-1900 double light Spectrophotometer (Beijing TAYASAF Science & Technology Co., Ltd., China) using a 1 cm quartz cell in the wavelength range of 200–500 nm. All pH measurements were performed with a pHS-3C pH meter (Shanghai, China).

Determination of fluorescence intensity

Five 10-mL clean and dried test tubes were taken, and 2 mL of 0.5 M NaCl, 2.0 mL Tris–HCl buffer (pH 7.40), 0.25 mL of 4.0×10^{-5} M BSA, and different volumes (0.5–2.5 mL) of LP standard solution of 1.0×10^{-4} M were added in each test tube, and diluted to 10 mL with water. The concentration of BSA was 1.0×10^{-6} M, and that of LP was 0.5, 1.0, 1.5, 2.0, and 2.5×10^{-5} M. Sixth test-tube containing only BSA solution at pH 7.4 was marked as ‘‘control’’, and seventh test-tube containing only 1×10^{-5} M LP was used for the comparison. After mixing the solutions, these were allowed to stand for 15 min for maximum binding of LP to BSA. The fluorescence intensity after the correction of inner-filter effect was calculated by the equation[13]: $F_{cor} = F_{obs}\ \exp(½A_{ex} + ½\ A_{em})$, where F_{obs} is fluorescence intensity measured before the correction of inner-filter effect, A_{ex} and A_{em} are absorbance of the test solution at excitation and emission wavelengths, respectively. The corrected fluorescence intensity was used for studying on the interaction of LP and BSA. After corrected inner-filter effect, fluorescence intensity (F_0) in the absence of quencher LP and the fluorescence intensity (F) in the presence of quencher LP were measured at a wavelength of λ_{ex}=280 nm and λ_{em}=340 nm under temperature of 298, 303 and 308 K for estimating the interaction between LP and BSA.

RESULTS AND DISCUSSION

Fluorescence quenching mechanism

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in fluorescence quenching of excited state fluorophores. These include molecular rearrangements, energy transfer, ground state complex formation and collisional quenching. The quenching of fluorescence may be static or dynamic, and can be recognized by temperature dependence studies. The quenching rate constants are expected to decrease with increase in temperature for static quenching, while for dynamic quenching a reverse effect was observed [13].

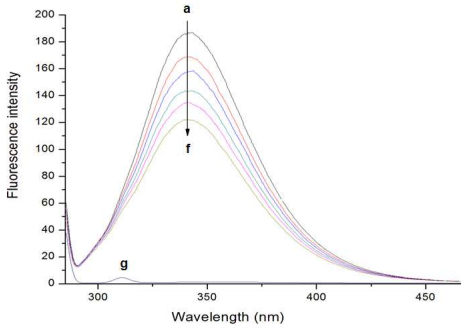

Figure 1: Fluorescence spectra of BSA in the absence or presence of LP at λ_{ex}

280 nm after corrected inter inner-filter effect
BSA, 1.0×10^{-6} M; LP ($\times10^{-5}$ M), a→f: 0, 0.5, 1.0, 1.5, 2.0 and 2.5; g:
LP, 1×10^{-5} M.

Figure 1 shows the fluorescence spectra of BSA in the absence and presence of LP after corrected inner-filter effect. No fluorescence of LP was observed at λ_{ex}=280 nm. The fluorescence spectra of BSA show a broad band with maximum at ~340 nm. It is observed that both fluorescence intensity of BSA decrease with increasing concentration of LP. A maximum fluorescence emission of BSA underwent spectral shift from 343.4.0 to 341.4 nm. It is suggested that BSA and LP formed a complex. The fluorescence quenching data are analyzed by the Stern–Volmer equation[14]:

$$F_0/F = 1 + k_q\tau_0[Q] = k_{sv}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensity in the absence and presence of quencher, respectively. k_q is the quenching rate constant, τ_0 is the fluorescence life time of biopolymer BSA ($\tau_0 = 10^{-8}$ s) [15], k_{sv} and $[Q]$ are the Stern–Volmer quenching constant and concentration of quencher, respectively. In this work, the Stern–Volmer plots of F_0/F vs concentration of LP were obtained (Fig. 2). The values estimated are given in Table 1.

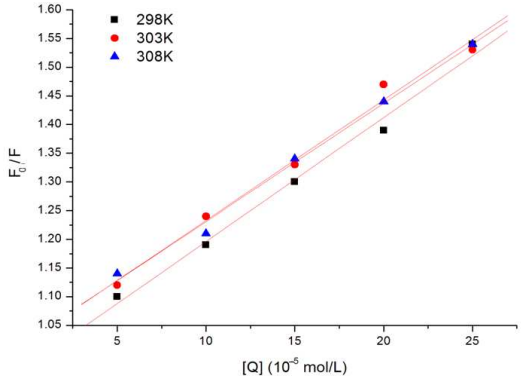

Figure 2: Stern–Volmer plots of F_0/F vs concentration of LP

Table 1: Quenching parameters of BSA–losartan potassium at different temperatures.

T (K)	Equation	R	*Ksv* (L mol^{-1})	*Kq* (L mol$^{-1}\cdot$s^{-1})
298	$F_0/F = 0.98 + 2.16\times10^4[Q]$	0.9954	2.16×10^4	2.16×10^{12}
303	$F_0/F = 1.023 + 2.10\times10^4[Q]$	0.9945	2.10×10^4	2.10×10^{12}
308	$F_0/F = 1.025 + 2.06\times10^4[Q]$	0.9970	2.06×10^4	2.06×10^{12}

The variation of F_0/F against LP concentration Q fits in the equation of y = c + mx with correlation coefficient (R) of ≥0.9945. Because the quenching rate constant (k_q) decreased with increase in temperature and k_q values were

greater than the maximum scatter collision quenching constants of various quenchers with the biomolecules (2.0×10^{10} L mol^{-1} s^{-1}), indicating that the fluorescence quenching was mainly arisen from static quenching by complex formation instead of dynamic quenching[13,16].

Binding constant and binding site number

The binding of LP with BSA to form complex in the ground state is further understood on the basis of available binding site number and binding constant of the complex formation process.

Assuming the quenching belongs to a dynamic quenching. the following equation was used to calculate the binding constant and binding sites[17,18]:

$$\log [(F_0 - F)/F] = \log K_a + n \log [Q] \quad (2)$$

where K_a and n are the binding constant and binding site number, respectively. Figure 3 shows the plots of log $[(F_0–F)/F]$ vs log $[Q]$. Binding constant (K_a) and the binding site number (n) could be calculated from the intercept and slope, as shown in Table 2.

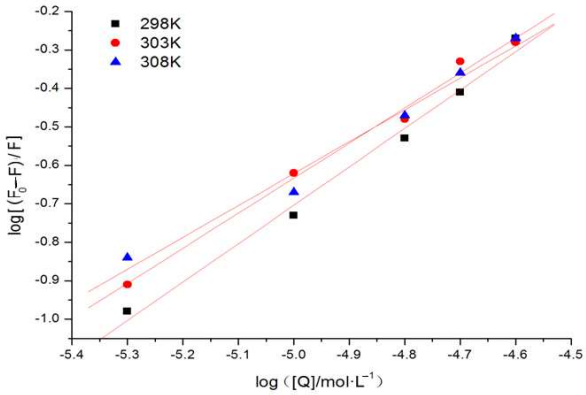

Figure 3: The plots of log $[(F_0–F)/F]$ vs log $[Q]$ under different temperatures

Table 2: Regression equation, correlation coefficient (R), binding constant K_a and the number of binding site n between losartan potassium and BSA at different temperature

T (K)	Equation	R	K_a(L mol^{-1})	n
298	$\log[(F_0–F)/F]=4.286 + 0.998 \log[Q]$	0.9950	1.932×10^4	0.998
303	$\log[(F_0–F)/F]=3.919+ 0.910 \log[Q]$	0.9960	8.299×10^3	0.910
308	$\log[(F_0–F)/F]=3.521+ 0.829 \log[Q]$	0.9906	3.319×10^3	0.829

It is shown that the correlation coefficient of the regression equations for the curves of $\log[(F_0–F)/F]$ vs $\log[Q]$ is approximately equal to 1. The binding constant decreased with the increase of temperature, showing that the quenching does not belong to a dynamic quenching, but belongs to a static quenching. The K_a values calculated for LP–BSA indicate a significant interaction between ligand and protein[19,20].

Interaction forces between LP with BSA

The interaction forces between drug and biomolecules include hydrogen bonds, van der Waals forces, electrostatic and hydrophobic interactions[21]. The temperature dependence of the interaction of LP with BSA was investigated at 298, 303 and 308 K. The thermodynamic parameters can be evaluated from the Van't Hoff equation:

$$\mathrm{Ln}K_a = -\Delta H/RT + \Delta S/\mathrm{R} \quad (3)$$

where K_A is the binding constant at corresponding temperature T, and R is the gas constant. The enthalpy change (ΔH) and entropy change (ΔS) can be obtained from the slope and the ordinates at the origin of the Van't Hoff plot, respectively (Fig. 4).

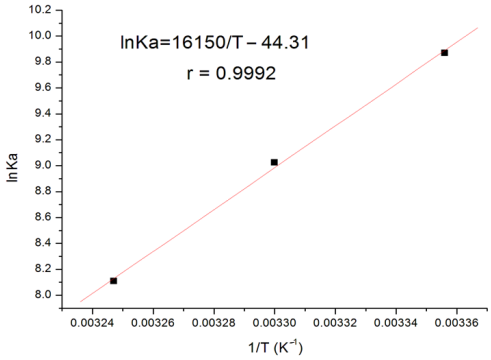

Figure 4: Van't Hoff plot for the interaction of LP with BSA at pH 7.40

The free energy change, ΔG is determined from the following relationship

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

The values of ΔG, ΔS and ΔH are calculated and summarized in Table 3.

Table 3: Thermodynamic parameters of the interaction between losartan potassium and BSA at different temperatures

T (K)	K_a(L mol^{-1})	ΔH（kJ mol^{-1}）	ΔS (J mol$^{-1}\cdot$K^{-1})	ΔG (kJ mol^{-1})
298	1.932×10^4	-134.3	-368.4	-24.52
303	8.299×10^3			-22.67
308	3.319×10^3			-20.83

As can be seen in Table 3, a negative value of free energy (ΔG) supports the assertion that the binding process is spontaneous. The negative enthalpy (ΔH) and entropy (ΔS) values of the interaction of LP and BSA indicate that the binding is mainly enthalpy-driven and the entropy is unfavorable for it. van der Waals interactions and hydrogen bonding played major role in the reaction [22].

Binding sites of LP on the BSA

On the basis of the probe-displacement method, there are at least three relatively-high specific drug-binding sites on the BSA molecules. These sites, commonly called warfarin, ibuprofen and digoxin-binding sites, are also denoted as Site I, Site II, and Site III, respectively[23,24]. To further determine the binding site of LP, the competitive experiments were carried out at 298 K using warfarin, ibuprofen and digoxin as a Site I-, Site II- and Site III specific probe, respectively. Binding constant K_a and the binding site numbers n for the system in the presence of site specific probe are listed in Table 4.

Table 4: Binding constant K_a and binding site number n between losartan potassium and BSA in the presence of site-specific probe at 298 K

System	K_a(L mol^{-1})	n	R
LP–BSA	1.932×10^4	0.998	0.9950
LP–BSA–warfarin	4.977×10^3	0.887	0.9948
LP–BSA–ibuprofen	2.924×10^3	0.809	0.9984
LP–BSA–digoxin	8.831×10^3	0.914	0.9922

In the presence of site specific probe, the binding constants for LP–BSA system decreased. In the case of ibuprofen probe, it is 2.924×10^3 L mol^{-1}, obviously lower than that for the LP–BSA system without any specific probe, showing the competition of ibuprofen with LP at a same site. These results demonstrated that the primary binding site of LP at site II within subdomain IIIA of BSA. It is same with the result obtained with diazepam as site II specific probe[11].

Energy transfer from BSA to LP

Fluorescence resonance energy transfer is an important technique for investigating a variety of biological phenomena including energy transfer processes[25]. Here the donor and acceptor are BSA and LP, respectively. The

fluorescence emission of BSA–LP solution at an excitation wavelength of 280 nm is only from BSA since LP is a non-fluorescence drug molecule. However, at this wavelength LP has weak absorption. It was observed that there was spectral overlap between fluorescence emission of BSA and absorption spectra of LP (Fig. 5). It is suggested the possibility of fluorescence resonance energy transfer from BSA to LP molecules in solution.

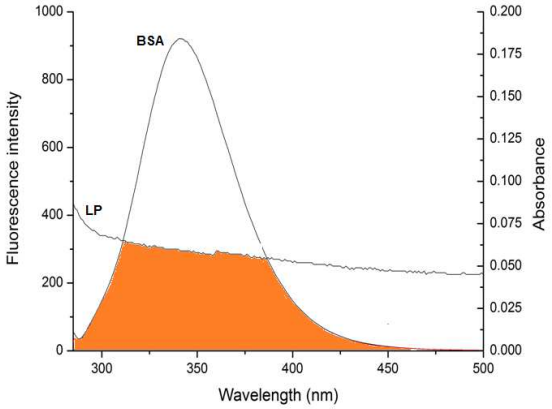

Figure 5: Spectral overlap of absorption of LP with fluorescence of BSA

The region of integral overlap can be used to calculate the critical energy transfer distance (R_0) between BSA (donor) and LP (acceptor) according to Föster's non-radiative energy transfer theory using Förster's equation[19, 26]. Based on this theory, the efficiency (E) of energy transfer between donor (BSA) and acceptor (LP) can be calculated by Eq. 5:

$$E = R_0^6(R_0^6 + r^6) \quad (5)$$

where, r is the binding distance between donor and acceptor, and R_0 is the critical binding distance when the efficiency (E) of energy transfer is 50%, which can be calculated by Eq. (6):

$$R_0^6 = 8.8\times10^{-25}k^2n^{-4}\,\Phi_D J \quad (6)$$

where, the k^2 is the spatial orientation factor of the dipole; n is the refractive index of medium; Φ_D is the quantum yield of the donor in the absence of acceptor; and J is the overlap integral of the emission spectrum of the donor and the absorption spectrum of the acceptor. The J value can be calculated by Eq. (7):

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda / \sum F(\lambda)\Delta\lambda \quad (7)$$

where, $F(\lambda)$ is the fluorescence intensity of the fluorescent donor, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor. In the present case, k^2, n and Φ_D are 2/3, 1.336 and 0.118, respectively[12]. The efficiency (E) of energy transfer can be determined by Eq. (8):

$$E = 1 - F/F_0 \quad (8)$$

where, F_0 and F are the fluorescence intensities of BSA solutions in the absence and presence of LP, respectively. From the overlapping we found R_0=2.352 nm from Eq. (7) using K^2=2/3, n=1.336 and Φ_D=0.118 (tryptophan residue) for the aqueous solution of BSA. J value could be calculated from Eq. 7 and the corresponding result was 7.80×10^{-15} cm^3 L mol^{-1}. E value calculated from Eq. 8 was 14.01%. At the same time, the binding distance (r=3.23 nm) between BSA and LP was obtained by Eq.5. Apparently, it is less than 7.0 nm. This result indicates the nonradiative energy transfer from BSA to LP occurs with high possibility[27,28]. It also suggested that the bindings of LP to BSA molecules were formed through energy transfer, which quenched the fluorescence of BSA molecules, indicating the presence of static quenching interaction between BSA and LP [29].

Change of BSA conformation

Synchronous fluorescence is a kind of simple and sensitive method to measure the fluorescence quenching. It can provide the information of polarity change around the chromophore microenvironment. Δλ, representing the difference between excitation and emission wavelengths, is an important operating parameter. When Δλ is 15 nm, synchronous fluorescence is characteristic of tyrosine residue, while when Δλ is 60 nm, it provided the characteristic information of tryptophan residues [28]. The synchronous fluorescence spectra of tyrosine residue and tryptophan residues in BSA with addition of LP were observed.

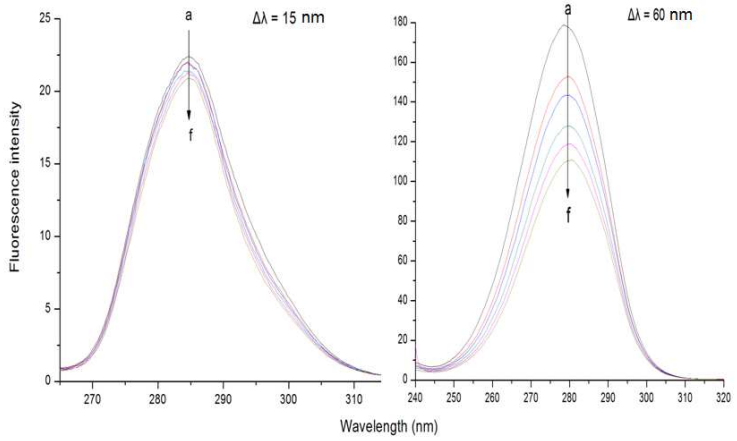

Figure 6: Synchronous fluorescence spectra of BSA with different amount of LP

T = 298 K; BSA, 1.0×10^{-6} M; LP (10^{-5} M), a→f: 0, 0.5, 1.0, 1.5, 2.0, 2.5

Figure 6 shows that the fluorescence intensity of tyrosine increased lightly with increasing LP content, but no obviously change of maximum emission wavelength was observed, while the fluorescence intensity of tryptophan decreased regularly, with the change of maximum emission wavelength from 338.8 to 341.2 nm. The red shift of fluorescence spectrum suggests the polarity of the surrounding environment increasing [30]. The fluorescence intensity and the pitch of quenching for Δλ= 60 nm was much higher than those for Δλ=15 nm. Such result means LP is closer to tryptophan residues than tyrosine residues, namely binding sites mainly are focused on tryptophan moiety. Above result showed that LP could change the conformation of BSA because the microenvironment around tryptophan residue changed during the binding process.

The three-dimensional fluorescence spectrum is another powerful method for studying conformation change of BSA. In this work, the three-dimensional fluorescence spectra and the contour spectra of BSA and BSA–LP systems were observed, as shown in Fig. 7, and the characteristic parameters are summarized in Table 5.

Table 5: Three-dimensional fluorescence spectra characteristic parameters of the BSA and BSA–losartan potassium systems

System	Parameters	Peak 1	Peak 2
BSA	Peak position (λex/λem, nm)	275.0/340.0	225.0/340.0
	Relative intensity	163.5	154.5
	Stokes shift Δλ (nm)	65	115
BSA-Losartan	Peak position (λex/λem, nm)	280.0/ 340.0	225.0/340.0
	Relative intensity	130.2	88.57
	Stokes shift Δλ (nm)	60	115

From Fig. 7, peak 1 ($\lambda_{ex}/\lambda_{em}$: 275.0/340.0 nm) reveals the spectral characteristic of tryptophan and tyrosine residues. After the addition of LP, the fluorescence intensity of BSA decreased from 163.5 to 130.2. This suggests a less polar environment of both residues and almost all the hydrophobic amino acid residues of BSA were buried in the hydrophobic pocket. Less polar environment means that the binding position between LP and BSA located within this hydrophobic pocket, the addition of LP changed the polarity of this hydrophobic microenvironment and the conformation of BSA[31].

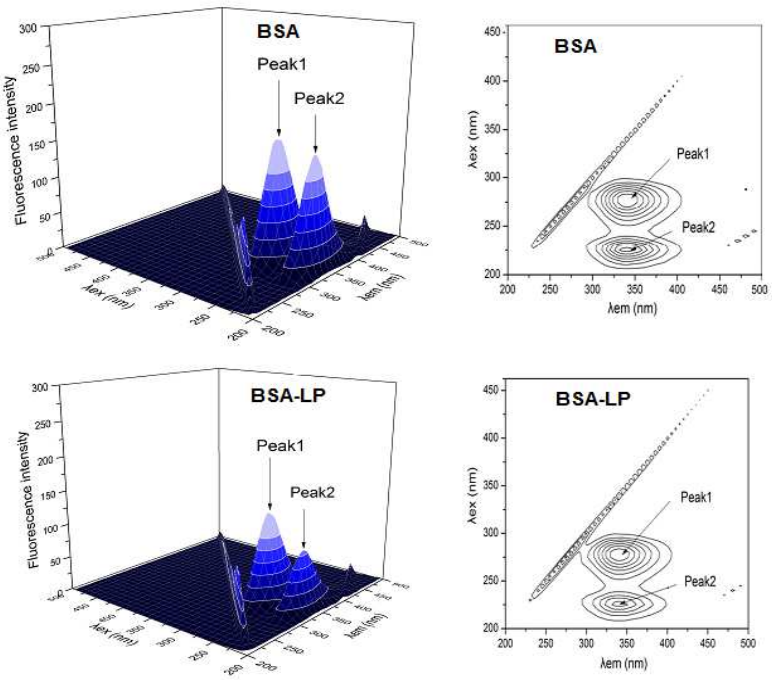

Figure 7: Three-dimensional fluorescence spectra (left) and contour spectra (right) of BSA and BSA–LP systems BSA, 1×10^{-6} M; LP, 1×10^{-5} M

In Fig. 7, peak 2 ($\lambda_{ex}/\lambda_{em}$: 225.0/340.0 nm) reveals the fluorescence spectra behavior of polypeptide backbone structures, which is caused by the transition of π–π* of BSA's characteristic polypeptide backbone structure C=O [32]. After the addition of LP, peak 2 decreased dramatically with increasing concentration of LP. The fluorescence intensity of BSA decreased from 154.5 to 88.47. After addition of LP in BSA, fingerprint lines in below part of contour spectra changed to be sparse. These revealed that the microenvironment and conformation of BSA were changed in the binding reaction. The interaction between LP and BSA induced the unfolding of the polypeptides chains of BSA and conformational change of BSA.

CONCLUSION

The results showed that losartan potassium can bind to bovine serum albumin by hydrogen bonding and Van der Waals forces. The primary binding for losartan potassium was located at site II in subdomain IIIA of BSA. The result observed by synchronous fluorescence and three-dimensional fluorescence spectra demonstrated that the presence of LP induced some microenvironmental and conformational changes of BSA molecules. The results are of great importance in pharmacy, pharmacology and biochemistry, and are expected to provide important insight into the interactions of the physiologically important protein BSA with drugs.

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