Interaction of rofecoxib with human serum albumin: Determination of binding constants and the binding site by spectroscopic methods

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Abstract

The interaction of rofecoxib with human serum albumin (HSA) under physiological condition was investigated by fluorescence, UV–vis absorbance and Fourier transfer infrared (FT-IR) spectroscopy. Fluorescence data revealed that the fluorescence quenching of HSA by rofecoxib was the result of the formed complex of HSA–rofecoxib, and the site binding constants (K_a) were 4.840×10^4, 3.450×10^4, and $2.325 \times 10^4 \, M^{-1}$ at 298, 304, and 310 K, respectively. The thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS) for the reaction were calculated to be $-46.90 \, kJ \, mol^{-1}$ and $-67.59 \, J \, mol^{-1} \, K^{-1}$ according to van't Hoff equation. The spectroscopic measurements and the thermodynamic parameters suggested that van der Waals interaction and hydrogen bonds were the predominant intermolecular forces to stabilize the complex. The distance $r = 5.1$ nm between donor (Trp^{214}) and accepter (rofecoxib) was obtained according to the Förster theory of non-radiative energy transfer. FT-IR spectra and UV–vis absorbance showed that the change of protein secondary structures resulted from the rofecoxib binding to several amino acids on the hydrophobic pocket of HSA. Furthermore, it is observed from the probe of competitive experiments that the binding location of rofecoxib with HSA could be the same as the warfarin site I of HSA, which was also revealed by fluorescence anisotropy.
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Keywords: Rofecoxib; Human serum albumin; Fluorescence quenching; Site competitive binding

1. Introduction

Serum albumin, as the most abundant protein constituent of blood plasma [1], facilitates the disposition and transportation of varieties of exogenous and endogenous ligands. The protein is capable of binding an extraordinarily broad range of pharmaceuticals, including fatty acids, amino acids, steroids, metal ions, etc. It is also responsible for the maintenance of blood pH [2], the drug disposition and efficacy [3], and the contribution of colloid osmotic blood pressure. Much of the clinical and pharmaceutical interest in the serum albumin derives from its effects on the drug pharmacokinetics [4]. HSA is considered to have at least three specific binding sites for high-affinity binding of drugs, sites I, II and III [5,6] and a single tryptophan (Trp^{214})

in the subdomain IIA. X-ray measurements have revealed that ligands binding to HSA were located in hydrophobic cavities in subdomains IIA and IIIA. It is important to study the interaction of the drugs with HSA owing to the interaction of drugs with HSA influence the drugs' pharmacology and pharmacodynamics.

Rofecoxib(4-[4-(methylsulfonyl)phenyl]-3-phenyl-2(5H)-furanone), a prescription COX-2 selective, non-steroidal anti-inflammatory drug (NSAID) was proved by FDA in May 1999 for the relief of signs and symptoms of osteoarthritis, for the management of acute pain in adults, and for the treatment of menstrual symptoms, and was later approved for the relief of signs and symptoms of rheumatoid arthritis in adults and children. As its structure shown in Fig. 1, it has been developed for treating acute pain and chronic inflammatory disorders without gastric side effects associated with the use of COX-1 inhibitors [7–9].

It is currently approved for the treatment of acute and chronic symptoms of osteoarthritis, rheumatoid arthritis, acute pain and menstrual pain [10]. Furthermore, rofecoxib can also slow the

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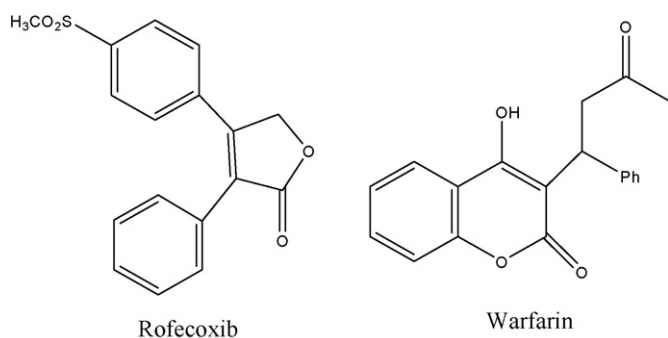*E-mail addresses:* prof.liuyi@263.net, liuyi@chem.whu.edu.cn (Y. Liu).

Fig. 1. Molecular structure of rofecoxib and warfarin.

growth of human pancreatic cancer by changing the gene expression that favors cell cyclic arrest [11]. On 30 September 2004, however, Merck & Co., Inc. announced a voluntary withdrawal of rofecoxib (Vioxx) from the U.S. and worldwide market due to the safety concerns of an increasing risk of cardiovascular events (including heart attack and stroke) in patients on rofecoxib [12].

It is widely accepted that the distribution, metabolism, and efficacy of many drugs can be altered based on their affinity to serum albumin. Significantly, the determination and understanding of rofecoxib interacting with serum albumin are important for the therapy and design of the drug [2]. The knowledge of the interaction and binding to HSA is poorly understood but may open new avenues for the design of the most suitable rofecoxib derivatives without side-effects. Investigating the influence of the drug on protein not only provide the pharmacological action of rofecoxib, but also can illuminate its binding mechanisms.

In this paper, we have studied the interaction of rofecoxib with human serum albumin (HSA) at three temperatures (298, 304, and 310 K) under physiological condition. Spectroscopic data were used to quantify the binding constants of rofecoxib to HSA and the action distance which was based on the Förster energy transference (FET). UV–vis and FT-IR spectroscopy revealed that the change of protein secondary structure resulted from the rofecoxib binding to several amino acids on the hydrophobic pocket of HSA. What is more, the interaction of the mainly acting forces and the binding site of the location were characterized by optical spectroscopy.

2. Materials and methods

2.1. Materials and solutions

Human serum albumin (HSA, fatty acid free), purchased from Sigma Chemical Company, was used without further purification. Tris–Base had a purity of no less than 99.5%, and NaCl, HCl, etc., were all of analytical purity. HSA was dissolved in Tris–HCl buffer solution (50 mM Tris–Base, 100 mM NaCl, pH 7.4 ± 0.1). Rofecoxib was obtained from USA of Merck & Co., Inc., prepared by absolute dimethyl sulfoxide (DMSO) to form 5 mM solution, and the DMSO in the solution of HSA was at a concentration of 0.1–1.5% (v/v). Warfarin was obtained from Medicine Co. Ltd., Jiangsu (China), prepared by the Tris–HCl

buffer (pH 7.4 ± 0.1) to form 5 mM solution. All other solutions were also prepared in a Tris–HCl buffer (pH 7.4 ± 0.1).

To evaluate the effect of DMSO on the conformation of HSA and fluorescence quenching, a 2.0 mL of 10 μM HSA was titrated by DMSO which was in the range of concentration 0.1–1.5% (v/v), and the spectra of UV–vis absorbance and fluorescence quenching was studied. There was no change observed in the spectral profiles (data not shown), which suggests no change in HSA conformation and can be considered negligible in the amount used [13].

2.2. Fluorescence measurements

Fluorescence spectra were measured with a F-2500 Spectrofluorimeter (Hitachi, Japan) equipped with a 1.0 cm quartz cell and a thermostat bath. The excitation wavelength was 295 nm, and the emission spectra was read at 300–450 nm, using 2.5 nm/2.5 nm slit widths. To quantify the binding constants of rofecoxib to HSA, a 2.0 mL solution containing 10 μM HSA was titrated by successive additions of rofecoxib solution using trace syringes (to give a concentration ranging from 0 to 50 μM), and the fluorescence intensity was measured ($\lambda_{ex} = 295$ nm). All experiments were measured at each temperature (298, 304, and 310 K) with recycle water keeping the temperature constant. The appropriate blanks corresponding to the buffer were subtracted to correct background of fluorescence. The results obtained were analyzed by using the Stern–Volmer equation or modified Stern–Volmer equation to calculate binding constants.

2.3. UV absorbance measurement

UV–vis absorption spectra of 10 μM of free rofecoxib in buffer solution (pH 7.4 ± 0.1, 0.2% of DMSO), and as well as the UV–vis absorption spectra of rofecoxib/HSA varieties of molar ratio complexes were recorded on a TU-1901 UV–vis spectrometer (Puxi Analytic Instrument Ltd. of Beijing, China) from 220 to 400 nm. To accomplish this, a 2.0 mL solution of 10 μM HSA was titrated by successive additions of rofecoxib solution.

2.4. FT-IR measurements

FT-IR was measured on a Perkin-Elmer spectrometer equipped with a MCT-B detector. Solution spectra were taken, using CaF_2 windows with resolution of 2 cm^{-1} and 400 scans. HSA was prepared using a deuterated water to prevent the interference of hydrogen bonds in the amide region of the protein spectra. The amount of DMSO (0.5–1.0%) used as solvent in the HSA solution in the spectra of IR has not absorbance in the range 1600–1700 cm^{-1}. The subtraction of D_2O from the protein solution was carried out as Ref. [14].

2.5. Fluorescence anisotropy measurement

The steady state anisotropy, however, gives information about the rotational rate of the solute molecule. Upon excitation with vertically polarized light the emitted fluorescent light is

completely depolarized. Coupling of the chromophore to a larger molecule reduces its mobility resulting in an increased anisotropy.

The anisotropy (r) is defined as the difference between the fluorescence intensity emitted parallel and perpendicular ($I_{||}$ and $I_{\perp}$) divided by the total intensity. Fluorescence anisotropy was calculated from fluorescence intensity measurement employing a vertical excitation polarizer and vertical and horizontal emission polarizers according to Eq. (1) [15]:

$$r = \frac{I_{||} - GI_{\perp}}{I_{||} + 2GI_{\perp}} \tag{1}$$

where $I_{||}$ is the intensity of emitted light measured in the direction parallel to excitation, $I_{\perp}$ the intensity of emitted light measured in the direction perpendicular to excitation and $G = I_{\perp}/I_{||}$ is the instrument grating correction factor. Fluorescence anisotropy measurement was measured by using the automatic polarization device of a LS50B spectrofluorometer (Perkin-Elmer) equipped with a polarization. Excitation and emission bandwidths were all adjusted to 10 nm. Each titration point of the sample of equilibration at least four times with an integration time of 1 min was collected.

3. Results and discussions

3.1. Fluorescence quenching mechanism and binding constant

The fluorescence of protein was a sensitive method to investigate the conformation of protein when its environment and structure change. Fluorescence quenching can be dynamic, resulting from collisional encounters between the fluorophore and quencher, or static, resulting from the formation of a ground state complex between the fluorophore and quencher [16], which gives information about the changes of the molecular microenvironment in a vicinity of the chromophore molecules and the binding to serum albumin. Static and dynamic quenching can be distinguished by their different binding constants dependence on temperature and viscosity, or preferably by lifetime measurements. In this paper, we have used the binding constants dependence on the temperature to elucidate the quenching mechanism.

The effect of rofecoxib on the fluorescence spectra of HSA was explored and the spectral changes were shown in Fig. 2. Upon addition of rofecoxib into the protein solution, the fluorescence intensity of HSA at around 340 nm decreased along with the emission peak of a slight red shift and a concomitant increase in emission at 395 nm. This phenomenon can be the result of the
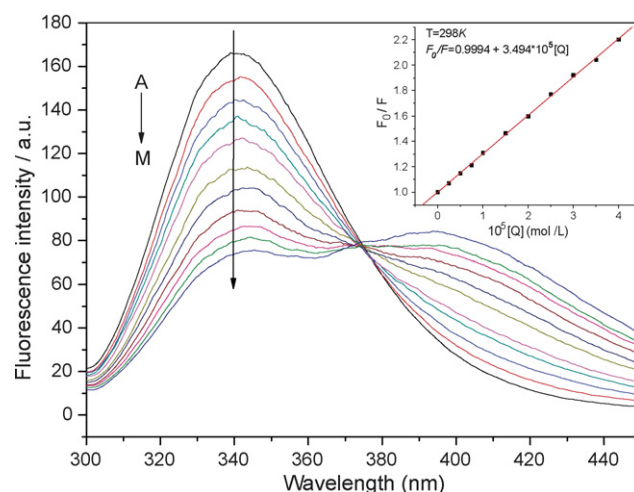

Fig. 2. Effect of rofecoxib on fluorescence spectrum of HSA ($T = 298$ K, $\lambda_{ex} = 295$ nm). c(HSA) = 10 μM; c(rofecoxib)/(10 μM), A–M: 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0.

radiationless energy transfer between the Trp[214] and rofecoxib bound to the HSA [17]. The occurrence of an isoacitinic point at 374 nm indicated that the quenching of protein fluorescence depended on the formation of compounds between rofecoxib and HSA [18].

For collisional quenching can be described by the well-known Stern–Volmer equation (2) to confirm the mechanism.

$$\frac{F_0}{F} = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] \tag{2}$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, k_q the biomolecular quenching constant, τ_0 the life time of the fluorescence in absence of quencher, $[Q]$ the concentration of quencher, and K_{SV} is the Stern–Volmer quenching constant. Herein, Eq. (2) was applied to determine K_{SV} by a linear regression of the plot of F_0/F against $[Q]$ at different temperatures. The results are shown in Table 1. It indicates that the calculated K_{SV} at each temperature studied shows the binding constants (K_{SV}) decreased with the rising temperature. The binding constants is inversely with the correlating temperature and the values of k_q ((3.512 ± 0.034) $\times 10^{12}$ M^{-1} s^{-1} at 298 K) being larger than the limiting diffusion constant K_{dif} of the biomolecule ($K_{dif} = 2.0 \times 10^{10}$ M^{-1} s^{-1}), which suggested that the fluorescence quenching was caused by a specific interaction between HSA and rofecoxib, and the quenching mechanism was mainly arisen from the predominant of complexes formation, while dynamic collision could be negligible in the concentration studied [19]. We further studied the difference of UV–vis absorbance

Table 1
Stern–Volmer quenching constant (K_{SV}) of the interaction of rofecoxib with HSA at three different temperatures

pH	T (K)	K_{sv} ($\times 10^{-4}$ M^{-1})	R	S.D.
7.4	298	3.512 ± 0.034	0.9985	0.050
	304	3.390 ± 0.061	0.9992	0.036
	310	3.255 ± 0.053	0.9975	0.057

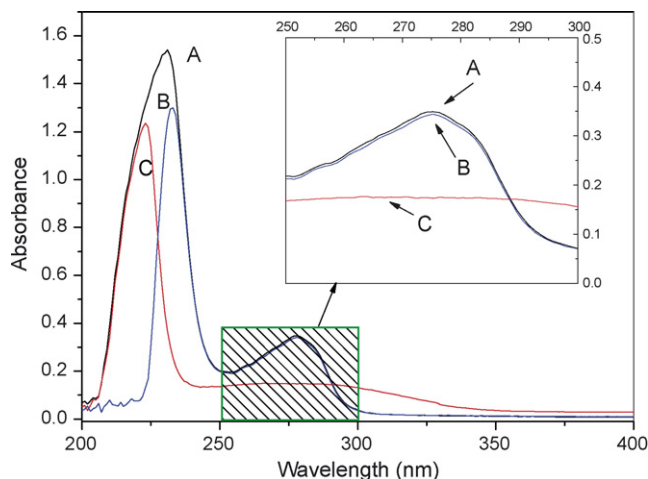R: linear correlated coefficient; S.D.: standard deviation.

Fig. 3. UV–vis spectra of HSA in the absence and presence of rofecoxib. (A) The absorption spectrum of HSA only; (B) the difference absorption spectra between rofecoxib–HSA and rofecoxib at the same concentration; (C) the absorption spectrum of rofecoxib only; $c(\mathrm{HSA}) = c(\mathrm{rofecoxib}) = 10\,\mu\mathrm{M}$.

Fig. 4. Van't Hoff plot, pH 7.40, $c(\mathrm{HSA}) = 10\,\mu\mathrm{M}$.

spectra of the complex of HSA–rofecoxib in aqueous solution (Fig. 3). The main changes that we can observe in the spectra of the complex were not due to the experimental error, especially in the range of 200–250 nm in the UV–vis absorbance spectra. Therefore, the mechanism of fluorescence quenching was a static quenching procedure. So, the quenching data must be analyzed according to the modified Stern–Volmer equation (3) [20].

$$\frac{F_0}{\Delta F} = \frac{F_0}{F_0 - F} = \frac{1}{f_a K_a} \frac{1}{[Q]} + \frac{1}{f_a} \qquad (3)$$

In this case, ΔF is the difference in fluorescence in the absence and presence of the quencher at concentration $[Q]$, f_a the fraction of accessible fluorescence and K_a is the effective quenching constant for the accessible fluorophores.

The dependence of $F_0/\Delta F$ on the reciprocal value of the quencher concentration $1/[Q]$ is the linear with slope equal to the value of $1/f_a K_a$. The value of $1/f_a$ is fixed on the ordinate. The constant K_a is the quotient of an ordinate $1/f_a$ and slope $1/f_a K_a$. The binding constants at different temperatures were summarized in Table 2.

3.2. Binding mode

Small molecules binding to biomolecules are mainly four binding modes: H-bonds, van der Waals, electrostatic and hydrophobic interactions. The thermodynamic parameters, enthalpy (ΔH) and entropy (ΔS) of reaction, are important for
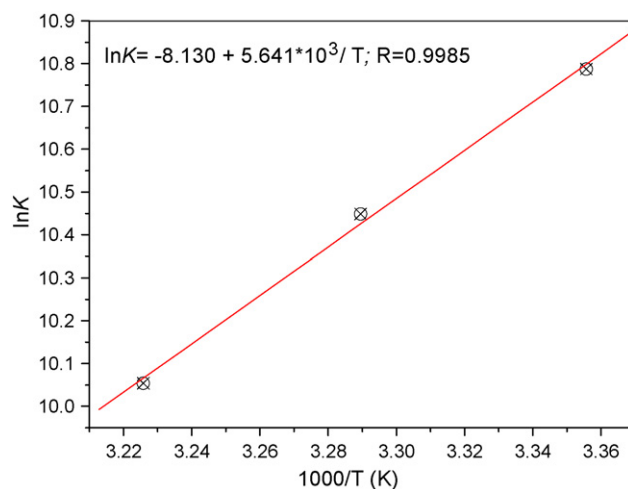
confirming binding modes. For this purpose, the slope of a plot of the bimolecular quenching constant versus $1/T$ (T, absolute temperature) gave a straight line according van't Hoff equation (4).

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \qquad (4)$$

where constants K are K_a at the corresponding temperature (the temperatures used were 298, 304 and 310 K); ΔH, ΔG and ΔS are enthalpy change, free energy change and entropy change, respectively. The temperature dependence of the binding constants was studied at three different temperatures (298, 304, and 310 K) and the slope of a plot of the bimolecular quenching constant versus $1/T$ (T, absolute temperature) are linear within experimental error (Fig. 4), which allows one to calculate the energy change in the quenching process.

If the temperature changes a little, the enthalpy change is regarded as a constant. The free energy change (ΔG) is estimated from the following relationship:

$$\Delta G = \Delta H - T\Delta S \qquad (5)$$

According to Eqs. (4) and (5), we could obtain the value of ΔH, ΔG and ΔS shown in Table 2.

As can be seen from Table 2, the negative sign for free energy (ΔG) means that the interaction process is spontaneous. The formation of rofecoxib–HSA complex compound is an exothermic reaction accompanied by the negative enthalpy (ΔH) and entropy (ΔS) values. Ross and Subramanian have characterized the sign and magnitude of the thermodynamic parameter of varieties of biomolecules/ligands interactions that may take place in

Table 2
Modified Stern–Volmer association constant K_a and relative thermodynamic parameters at pH 7.4

T (K)	K_a ($\times 10^{-4}\,\mathrm{M}^{-1}$)	R	S.D.	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (kJ mol^{-1})
298	4.840	0.9925	0.065	−46.90	−26.75	−67.59
304	3.450	0.9918	0.058		−26.34	
310	2.325	0.9916	0.078		−25.94	

R: the linear correlated coefficient; S.D.: standard deviation.

protein association processes based only upon calorimetrically determined enthalpy change [21]. From the point of view, the negative ΔH and ΔS value are frequently taken as evidence for van der Waals and H-bond formation in low dielectric medium. From the structure of rofecoxib, H-bond is easily formed in the atom oxygen. That is, rofecoxib bound to HSA was mainly based on van der Waals and hydrogen bonds playing major role in the acting forces, and the binding is mainly entropy-driven.

3.3. Energy transfer between HSA and rofecoxib

HSA has one tryptophan (Trp[214]) in subdomain IIA, we can calculate the drug binding site distance between the site and the fluorophore (Trp[214]) according to Förster theory of molecular resonance energy transfer [22]. According to this theory, the efficiency of energy transfer between the donor and the acceptor, E, could be calculated by Eq. (6) [23,24]:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{6}$$

where r is the distance between the donor and the acceptor [25], and R_0 is the critical distance, at which the efficiency of transfer is 50%.

$$R_0^6 = 8.79 \times 10^{-25} \, K^2 \, N^{-4} \, \phi \, J \tag{7}$$

In Eq. (7), K^2 is the orientation factor related to the geometry of the donor and acceptor of dipoles and $K^2 = 2/3$ is for random orientation as in fluid solution [26]; N is the refracted index of medium; ϕ is the quantum yield of the donor in the absence of acceptor; J expresses the degree of spectral overlap between the donor emission spectrum (a) and the acceptor absorption spectrum (b), which could be calculated by the equation:

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F(\lambda) \, d\lambda} \tag{8}$$

where $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range from λ to $\lambda + \Delta\lambda$ and $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ.

The overlap of the absorption spectra of the rofecoxib and the fluorescence emission spectra of HSA–rofecoxib has been shown in Fig. 5. In the present case, $N = 1.36$, $\phi = 0.074$ [27], according to Eqs. (6)–(8), we could estimate $R_0 = 4.0$ nm, and $r = 5.1$ nm. The donor-to-acceptor distance is $2 < r < 8$ nm [28], and $0.5R_0 < r < 1.5R_0$, indicating that the energy transferring from HSA to rofecoxib occurs with high possibility [29].

3.4. Changes of the protein's secondary structure induced by drug binding

For exploring the rofecoxib of influences on the structural changes of HSA, we measured UV–vis spectra (Fig. 6) of HSA by adding various amounts of rofecoxib. The figure showed that the absorption peaks of these solutions had a slight shift (from 278 nm to 281 nm) toward long wavelengths with the increasing addition of rofecoxib. The results can be explained by that the environment of tryptophan residues was changed, and the
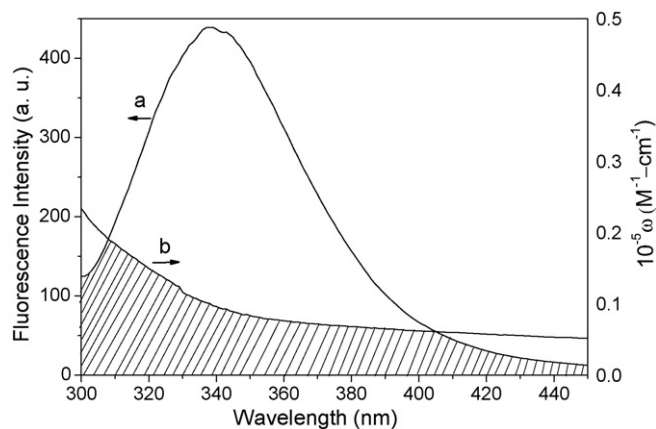
Fig. 5. Spectral overlap of rofecoxib absorption (a) with HSA fluorescence (b). [HSA] = [rofecoxib] = 10 μM.

hydrophobicity of the microenviroment of tryptophan residues was increased. The absence of water in the molecular interior increases the conformational stability, the rigidity, mechanical strength, and the contributions of electrostatic interactions [30].

Infrared spectroscopy is useful, either as an option or as a complement to the high-resolution techniques in the protein studies in a wide variety of environment [31]. When drugs bind to a globular protein, the intramolecular forces responsible for maintaining the secondary and tertiary structures can be altered, resulting in a conformational change of the protein [32]. In the IR region, the frequencies of bands due to the amide I and II vibrations are sensitive to the secondary structure of proteins. The amide I and amide II peaks position occur in the region $1600–1700 \, cm^{-1}$ (mainly C=O stretch) and $1550–1500 \, cm^{-1}$ (C–N stretch coupled with N–H bending mode). The protein amide bands have a relationship with the secondary structure of protein, and amide I band is more sensitive to the change of protein secondary structure than amide II [33].
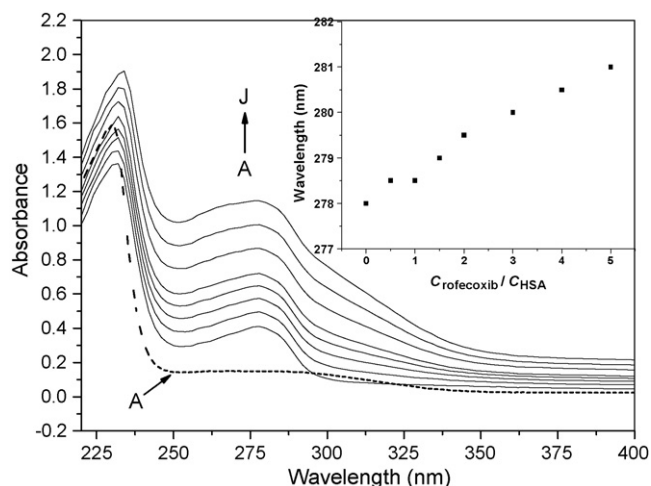
Fig. 6. UV–vis absorbance spectra of HSA in the presence of rofecoxib. Curve A: $c(HSA) = 0$; $c(rofecoxib) = 10$ μM, B–K: $c(HSA) = 10$ μM, $c(rofecoxib)/(10$ μM): 0, 0.5, 1, 1.5, 2.0, 3.0, 4.0, 5.0. The inset shows the relationship of absorbance spectra maximum wavelength at about 279 nm and the mole ratio between rofecoxib and HSA.
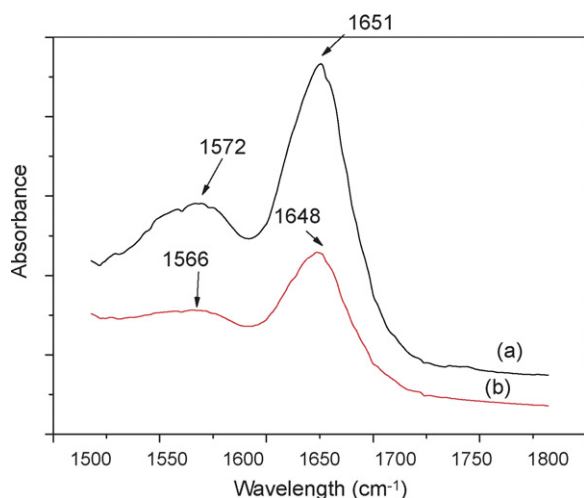
Fig. 7. FT-IR spectra and difference spectra [(HSA solution + rofecoxib solution)–rofecoxib solution] of free HSA (a) and HSA–rofecoxib complexes (b) in D_2O solution in the region of 1800–1500 cm^{-1} (1 mM HSA; 1 mM rofecoxib).

It is apparent that there was no major spectral shifting of the protein amide I at 1651 cm^{-1} and amide II band at 1572 cm^{-1} (Fig. 7). The spectrum in Fig. 7a was obtained by subtracting the absorption of D_2O from the spectrum of protein solution. Different spectrum (Fig. 7b) was obtained by subtracting the rofecoxib-free form from that of the rofecoxib-bound form. As a result, the absorbance of solvent (DMSO) on the spectra can be negligible. The evident peak shift of amide I band from 1651 cm^{-1} to 1648 cm^{-1} indicates that the secondary structure of HSA is changed when rofecoxib is added. From the result, a major reduction in the intensity of the amide I band was observed as a result of the reduction of the protein α-helical structure in favor of β-sheet (Fig. 7) [34]. Because the loop region of HSA of each subdomian contain three helical segments, parts of the surface and pocket being hydrophobic, such as several amino acids, like His, Arg, and Lys, involved in the binding process [35], make the protein in favor of β-sheet and β-turn structures. These also explain why the UV–vis spectra of HSA shift from 278 nm to 281 nm

due to the increasing of the hydrophobicity of subdomians in HSA.

3.5. Competitive binding of warfarin and rofecoxib on HSA

Serum albumin is composed of several mostly helical domains which are structured by disulfide bridges [3]. In the subdomains IIA and IIIA regions are characterized by a hydrophobic surface on one side, and a positively charged surface on the other, which allow them to specifically bind negatively charged heterocyclic ligands of average sizes and small aromatic carboxylic acids, respectively. The binding constants for the HSA high-affinity site are in the range 8.9×10^4–3.4×10^5 M^{-1} for warfarin, 3.0×10^5–3.6×10^6 M^{-1} for ibuprofen [36].

Most studies of two site markers, warfarin and ibuprofen, by means of equilibrium dialysis, found that both warfarin and ibuprofen possesses one high-affinity sites (HAS) and several low-affinity binding sites (LAS) on the HSA. Moreover, HAS and LAS are both located in site I for warfarin [37], but HAS and LAS for ibuprofen lie in site II and site I [38], respectively. Generally, at a ligand/protein molar ratio not larger than 1, the ligand is mainly bound in its HAS but otherwise, it begins to bind to in its LAS.

To identify the binding site location of rofecoxib on the region of HSA, the competitive ligand binding to HSA is employed. One of the most thoroughly characterized ligands of HSA is warfarin (see Fig. 1), a widely prescribed anticoagulant [39], which is located in the region of subdomain IIA corresponding to Sudlow site I [40]. Petitpas et al. [41] reported that warfarin occupies two chambers of the "sock-shaped" binding pocket formed by all six helices of subdomain IIA. As a result, the site marker of the displacement experiment was carried out, using drugs which specifically bind to known sites or regions on HSA. The interaction of warfarin with the rofecoxib–HSA complex (rofecoxib/HSA molar ratio = 1) was investigated at excitation 320 nm (the maximum absorption wavelength of warfarin). The ternary complex compared to the binary one was also investigated in the same condition. The result may be explained by energy transfer from warfarin to rofecoxib in the protein matrix
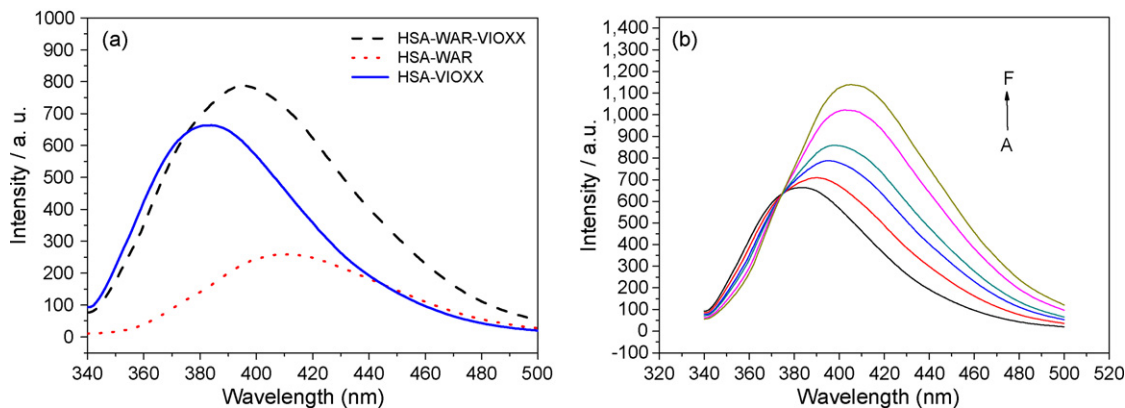

Fig. 8. (a) Fluorescence emission spectra of a HSA–rofecoxib solution (solid), a HSA–rofecoxib–warfarin solution (dashed), a HSA–warfarin solution (dots) following excitation at 320 nm. Concentration for all species was 10 μM and all spectra were recorded in the 50 mM Tris–HCl buffer at pH 7.4. (b) Effect of rofecoxib on the fluorescence emission spectra of complexes (HSA–warfarin) solution (A), c(rofecoxib)/(10 μM), A–L: 0, 0.5, 1.0, 1.5, 2.5, 3.5. Excitation at 320 nm. Concentration for HSA and warfarin were 10 μM and all spectra were recorded in the 50 mM Tris–HCl buffer at pH 7.4.
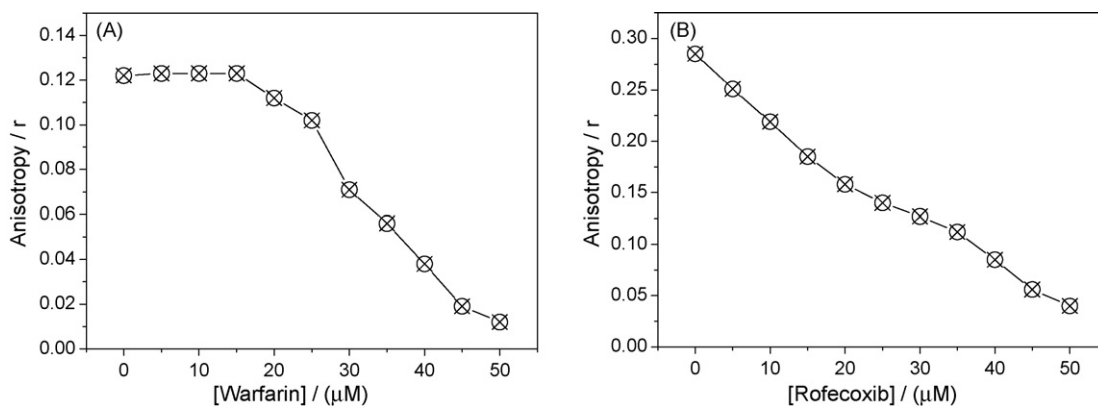
Fig. 9. (A) Correct fluorescence anistropy values of rofecoxib–HSA complex when different aliquots of warfarin were added; (B) correct fluorescence anistropy values of warfarin–HSA complex when different aliquots of rofecoxib were added. Excitation wavelength 350 nm. Emission wavelength 420 nm. Each experiment was repeated four times, $T = 298$ K, c(HSA) = 10 μM.

[42]. Fig. 8a shows the emission spectra ($\lambda_{exc} = 320$ nm) of the equimolar binary mixture of warfarin or rofecoxib with HSA and that of the ternary mixture in which warfarin, rofecoxib and HSA were present at the same concentration (10 μM). The intensity of warfarin emission in the ternary complex was less than that in its binary complex with HSA by a factor of 0.14, which suggests that rofecoxib is replaced by warfarin in the site I. We further investigated the addition of the rofecoxib into the complexes of warfarin–HSA–rofecoxib (molar ratio 1:1:1) with excitation at 320 nm (Fig. 8b). The results show that the effective of fluorescence was enhanced with the addition rofecoxib, indicating the energy of warfarin transferring to rofecoxib and rofecoxib sharing a same common binding site with warfarin.

Furthermore, the intensity of the emission from rofecoxib in the ternary complex was a factor 1.80 higher than that in the binary complex. We can be able to use the observation of energy transfer from warfarin to rofecoxib to estimate the distance between the two bound molecules [23]. The relationship between R and the energy transfer efficiency, E is given by Eq. (6). If we assume the reduced intensity in the ternary complex result solely from energy transfer, then E is approximately 0.8 and $R/R_0 \sim 0.79$. R_0 is the characteristic distance related to the properties of donor and acceptor which can be estimated within Förster formalism [43]. We can apply this formalism to the energy transfer between warfarin and rofecoxib and to estimate the apparent distance $R = 2.0$ nm, which is shorter than the distance between rofecoxib and Trp[214].

Now we come to the anisotropy of rofecoxib competitive with warfarin on the binding region of HSA. The interaction of warfarin and rofecoxib with HSA of complexes is following at the excitation and emission wavelengths: 350 nm and 420 nm (the wavelengths of maxima absortion and emission for rofecoxib), respectively.

As we can see from Fig. 9A, the anisotropy kept a constant with warfarin titrated into the complex of rofecoxib–HSA in the range of 0 and 20 μM, but the anisotropy decreased gradually, which can be explained by the affinity of ligands binding to HSA [44,45]. The turning point of the anisotropy are approximate the equimolar of the ternary mixture, indicating that the rofecoxib was displaced by warfarin with the increasing concentration of

warfarin. Rofecoxib was now used as displacer and warfarin as ligand, however, the result showed that the anisotropy of rofecoxib decreased gradually (Fig. 9B). Warfarin competing with rofecoxib in the binding site I of HSA is possible to occur, because of the estimated distance between the two compounds around 2.0 nm, and the binding constant of warfarin to HSA was higher than that of rofecoxib [46]. The value of anisotropy from Fig. 9B reveals that warfarin cannot be displaced by rofecoxib when it has bound to HSA [47]. To evaluate the effect of DMSO on the competitive binding site, fluorescence anisotropy measurement was employed to study the change of anisotropy by additions of DMSO. It is observed that the anisotropy of the complex (rofecoxib/HSA) keep a constant in the range of 0.1–1.5% (v/v), which suggests that DMSO is impossible to participate in the competitive binding to the hydrophobic pocket of HSA as a ligand.

4. Conclusions

The interaction of rofecoxib with HSA under physiological condition was studied by spectral methods. The results indicated that the probable mechanism of rofecoxib interaction with HSA is a static quenching process. The binding process was exothermic, enthalpy driven and spontaneous, as indicated by the thermodynamic parameters analyzed, and the major part of the action force is van der Waals and H-bonds. On the efficiency of energy transfer between the donor and acceptor, the distance between the fluorophore (Trp[214]) of HSA and the drug of rofecoxib was estimated to be $r = 5.1$ nm. Emission and anisotropy data show that warfarin and rofecoxib share a common binding site I correspoding to the subdomain IIA of HSA. Under certain condition, warfarin and rofecoxib cobound to HSA and energy transfer occurs from the excited warfarin to rofecoxib. On the basis of the efficiency of energy transfer, the distance between two drugs were estimated to be at most 2.0 nm.

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