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Interaction of isofraxidin with human serum albumin

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Abstract—This study was designed to examine the interaction of isofraxidin with human serum albumin (HSA) under physiological conditions with drug concentrations in the range of 3.3×10^{-6} mol L^{-1}–3.0×10^{-5} mol L^{-1} and HSA concentration at 1.5×10^{-6} mol L^{-1}. Fluorescence quenching methods in combination with Fourier transform infrared (FT-IR) spectroscopy and circular dichroism (CD) spectroscopy were used to determine the drug-binding mode, the binding constant and the protein structure changes in the presence of isofraxidin in aqueous solution. Spectroscopic evidence showed that the interaction results in one type of isofraxidin–HSA complex with binding constants of 4.1266×10^5 L mol^{-1}, 3.8612×10^5 L mol^{-1}, 3.5063×10^5 L mol^{-1}, 3.1241×10^5 L mol^{-1} at 296 K, 303 K, 310 K, 318 K, respectively. The thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS) were calculated to be -10.08 kJ mol^{-1} and 73.57 J mol^{-1} K^{-1} according to van't Hoff equation, which indicated that hydrophobic interaction played a main role in the binding of isofraxidin to HSA. The experiment results are nearly in accordance with the calculation results obtained by Silicon Graphics Ocatane2 workstation.

1. Introduction

Human serum albumin (HSA) is the most abundant protein constituent of blood plasma and serves as a protein storage component. The three-dimensional structure of human serum albumin has been determined through X-ray crystallographic measurements.[1] This globular protein consists of a single polypeptide chain of 585 amino acid residues, which has many important physiological functions.[2] HSA considerably contributes to colloid osmotic blood pressure and realize transport and distribution of many molecules and metabolites, such as fatty acids, amino acids, hormones, cations and anions, and many diverse drugs. HSA can bind and carry through the bloodstream many drugs, which are poorly soluble in water. It has been shown that the distribution, free concentration and the metabolism of various drugs can be significantly altered as a result of their binding to HSA.[3] Drug interactions at protein binding level will in most cases significantly affect the apparent distribution volume of the drugs and also affect the elimination rate of drugs; therefore the studies on this aspect can provide information of the structural features that determine the therapeutic effectivity of drugs, and have been an interesting research field in life sciences, chemistry and clinical medicine. In a series of study methods concerning the interaction of drugs and protein, fluorescence techniques are great aids in the study of interactions between drugs and plasma proteins in general and serum albumin in particular because of their high sensitivity, rapidity and ease of implementation. FT-IR, a powerful technique for the study of hydrogen bonding, has recently become very popular for structural characterization of proteins. The most important advantage of FT-IR spectroscopy for biological studies is that spectra of almost any biological system can be obtained in a wide variety of environments. For secondary-structure analysis of protein, Circular dichroism (CD) spectroscopy is a technique used most frequently. At present, there have been some reports that investigate the interaction of proteins with drugs by fluorescence technique,[4,5] FT-IR[6–8] and CD spectroscopy,[9] but studies on the binding of Chinese herbal active component to HSA are little.

Isofraxidin (7-hydroxy-6, 8-dimethoxycoumarin, structure shown in Fig. 1) is one of the major bioactive components isolated from *Sarcandra glabra* (Thunb.) Nakai used for antibacterial, anti-inflammatory, antitumor, etc.[10] Many of these agents can bind to serum proteins, especially serum albumin. The binding affects

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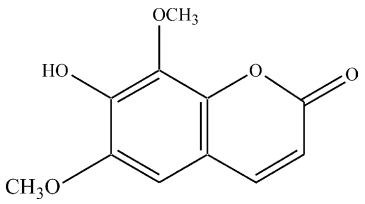

Figure 1. The chemical structure of isofraxidin.

their pharmacological and pharmacokinetic properties, and therefore a study on the binding of isofraxidin to HSA is very significant. Nowadays, some researches on the binding of coumarin derivatives to HSA have been carried out,[5,11] yet the study on the binding interaction of isofraxidin and HSA has not been reported.

In this paper, we studied the interaction of isofraxidin with human serum albumin (HSA) at four temperatures under physiological conditions utilizing fluorescence method in combination with FT-IR and CD technique. In the meantime, the binding mechanism of isofraxidin to HSA is discussed, and partial binding parameters of the reaction were calculated through Silicon Graphics Ocatane2 workstation.

2. Materials and methods

2.1. Materials

Human serum albumin (HSA, fatty acid free $<0.05\%$), purchased from Sino-American Biotechnology Company, was used without further purification and its molecular weight was assumed to be 66,500. All HSA solution were prepared in the pH 7.40 buffer solution, and HSA stock solution was kept in the dark at 4 °C. Isofraxidin was of analytical grade, and purchased from the National Institute for Control of Pharmaceutical and Bioproducts, China, and the stock solution was prepared in absolute ethanol. NaCl (analytical grade, 1.0 mol L^{-1}) solution was used to maintain the ion strength at 0.1. Buffer (pH 7.40) consists of Tris (0.2 mol L^{-1}) and HCl (0.1 mol L^{-1}), and the pH was adjusted to 7.40 by adding 0.5 mol L^{-1} NaOH when the experiment temperature was higher than 296 K. The pH was checked with a suitably standardized pH meter.

2.2. Apparatus and methods

Fluorescence spectra were measured with a RF-5301PC spectrofluorophotometer (Shimadzu), using 5 nm/5 nm slit widths. The excitation wavelength was 280 nm, and the emission was read at 300–500 nm.

Fluorometric titration experiments: 3.0 mL solution containing appropriate concentration of HSA was titrated by successive additions of a 3.0×10^{-3} mol L^{-1} ethanol stock solution of isofraxidin (to give a final concentration of 3.3×10^{-6} mol L^{-1}–3.0×10^{-5} mol L^{-1}). Titrations were done manually by using trace syringes, and the fluorescence intensity was measured (excitation at 280 nm and emission at 340 nm). All experiments were measured at four temperatures (296, 303, 310, 318 K). The temperature of sample was kept by recycle water throughout experiment. The data thus obtained were analyzed by using the Scatchard equation to calculate the binding constants.

Circular dichroism (CD) measurements were made on a Jasco-20c automatic recording spectropolarimeter (Japan) in cell of pathlength 2 mm at room temperature. The induced ellipticity was defined as the ellipticity of the drug–HSA mixture minus the ellipticity of drug alone at the same wavelength and is expressed in degrees.

FT-IR measurements were carried out at room temperature on a Nicolet Nexus 670 FT-IR spectrometer (America) equipped with a Germanium attenuated total reflection (ATR) accessory, a DTGS KBr detector and a KBr beam splitter. All spectra were taken via the Attenuated Total Reflection (ATR) method with resolution of 4 cm^{-1} and 60 scans. Spectra processing procedures: spectra of buffer solution were collected at the same condition. Then, subtract the absorbance of buffer solution from the spectra of sample solution to get the FT-IR spectra of proteins. The subtraction criterion was that the original spectrum of protein solution between 2200 and 1800 cm^{-1} was featureless.[12]

3. Results and discussion

3.1. Interaction of isofraxidin with HSA

To investigate whether isofraxidin interacts with HSA, fluorescence measurements are carried out, which can give information about the molecular environment in a vicinity of the chromophore molecules. Fig. 2 is the fluorescence emission spectra of HSA in the absence and presence of isofraxidin. HSA has a strong fluorescence emission with a peak at 340 nm on excitation at 280 nm. Its fluorescence intensity decreases in the presence of isofraxidin and the maximum emission wavelength were shifted from 340 to 328 nm, suggesting that the microenvironment around HSA is changed after the addition of isofraxidin. For isofraxidin, its fluorescence intensity increases in the presence of HSA and the maximum emission wavelength was slightly shifted from 463 to 467 nm. These results indicated that there were strong interactions and energy transfer between isofraxidin and HSA. Also, the equivalent emission point at 417 nm implies that the quenching of protein fluorescence depends on the formation of enveloped- compound of HSA and isofraxidin.

Further experiments were carried out with CD and FT-IR techniques to verify the interaction between isofraxidin and HSA. As Figure 3 showed, HSA exhibited negative bands at 208 and 217 nm, a characteristic of the typical ($\alpha\beta$) helix structure.[13] When isofraxidin was added to the solution of HSA, the intensity of negative Cotton effect of HSA at 217 nm decreased which indicated the helix structure content of HSA has been changed and it may be the result of the formation of complex between the HSA and isofraxidin.

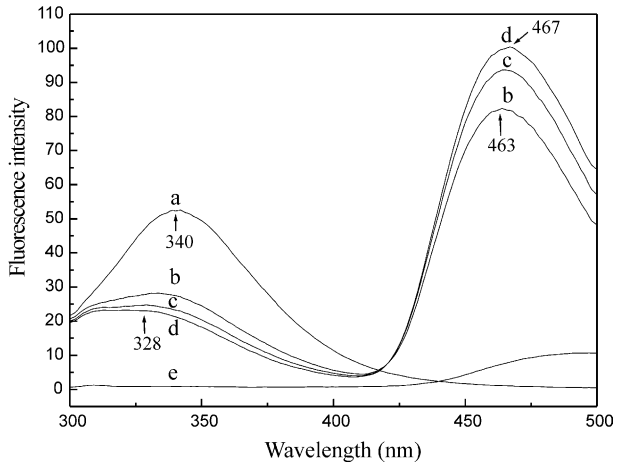

Figure 2. Fluorescence Emission Spectra excited at 280 nm (pH = 7.40). (a) 1.5×10^{-6} mol L^{-1} HSA; (b) to (d) 1.5×10^{-6} mol $\cdot L^{-1}$ HSA in the presence of 0.6×10^{-5}, 1.2×10^{-5}, 1.8×10^{-5} mol L^{-1} isofraxidin; (e) 1.2×10^{-5} mol $\cdot L^{-1}$ isofraxidin.

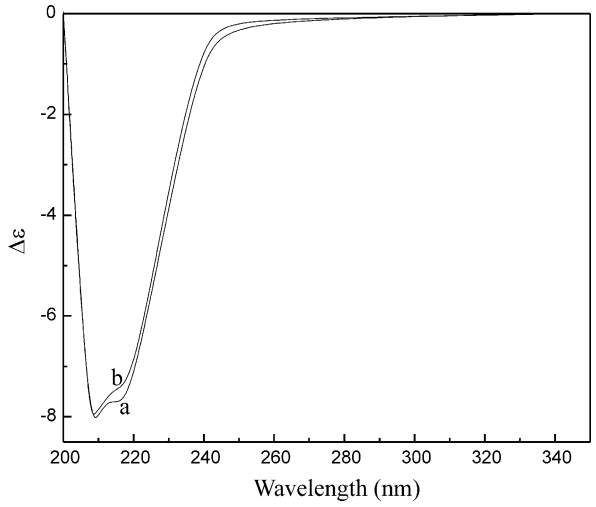

Figure 3. CD Spectra of the HSA-isofraxidin System. (a) 1.5×10^{-6} mol L^{-1} HSA; (b) 1.5×10^{-6} mol L^{-1} HSA in the presence of 3.3×10^{-6} mol L^{-1} isofraxidin.

Hydrogen bonding and the coupling between transition dipoles are amongst the most important factors governing conformational sensitivity of amide bands. 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.[14,15] Figure 4 showed the FT-IR spectra of the isofraxidin-free and isofraxidin-bound form of HSA with its difference absorption spectrum. The spectrum in Figure 4a was obtained by subtracting the absorption of Tris buffer from the spectrum of protein solution. Difference spectrum in present paper (Fig. 4b) was obtained by subtracting the spectrum of the isofraxidin-free form from that of the isofraxidin-bound form. The evident peak shift of amide I band from 1641.46 to 1654.35 cm^{-1} and the appearance of new peaks (1512.46 and 1458.98 cm^{-1}) in Fig. 4b indicate that the secondary structure of HSA is changed when isofraxidin was added. That is, there is an interaction between isofraxidin and HSA.

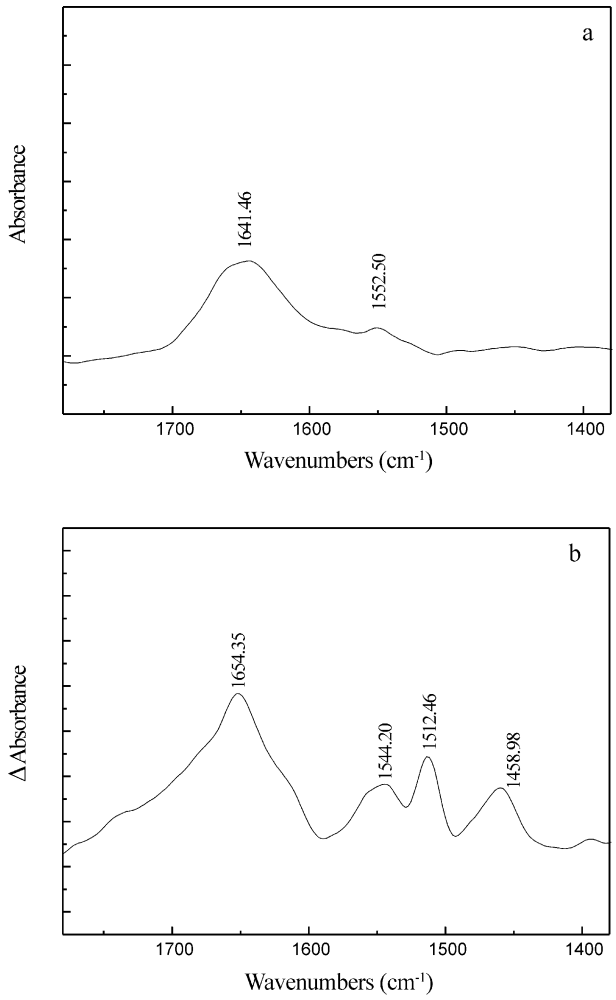

Figure 4. FT-IR spectra and differennce spectra of HSA in aqueous solution (a) FT-IR spectrum of HSA; (b) FT-IR difference spectrum of HSA obtained by subtracting the spectrum of the isofraxidin-free form from that of the isofraxidin-bound form in the region of 1780–1380 cm^{-1} at physiological pH (HSA: 3.0×10^{-5} mol L^{-1}; isofraxidin: 4.0×10^{-5} mol L^{-1}).

3.2. Binding constant

In drug-protein binding studies, several equations have been used for binding constant calculation. One frequently used is Scatchard equation:[16]

$$r/D_f = nK - r\,K$$

where r is the number of mol of bound drug per mol of protein, D_f is the concentration of unbound drug, K is the binding constant, and n is the number of binding sites.

To estimate the binding parameters of isofraxidin–HSA interaction, the fluorometric titration curves were constructed by measuring the greatly differing fluorescence intensities at 340 nm, followed by the making of Scatchard plots from the results. For this treatment to be valid, the fluorescence intensity of the bound probe must be a linear function of concentration. This is the

case only when the absorbance of the complex at the exciting wavelength is low. A correction for this absorption can be made by the method of Naik et al. [17] and was made for all the data when the absorbance at 280 nm was greater than 0.02. Figure 5 shows the titration curves of the isofraxidin–HSA system at low HSA concentration. When the titrations were carried out, isofraxidin was only partially bound. The plateau in the titration curve obtained indicates saturation of the binding sites in HSA. The linearity of Scatchard plots for the isofraxidin–HSA system obtained from the titration curve in Figure 5 indicates that isofraxidin binds to a single class of binding sites on HSA (Fig. 6). The binding constants, K, at four temperatures estimated from Scatchard plots were summarized in Table 1. As shown in Table 1, the binding constants decreased with increasing of the reaction temperature, which is consistent with the molecular modelling study of the interaction between HSA and isofraxidin.

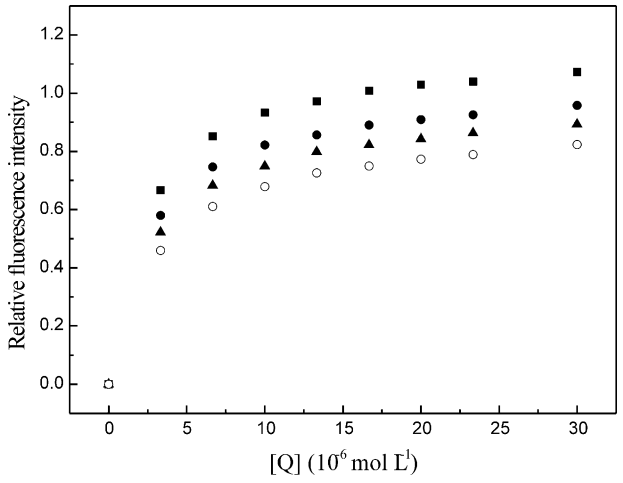

Figure 5. Relative fluorescence intensity in the isofraxidin–HSA interaction obtained by titration with isofraxidin. $C_{HSA} = 1.5\times10^{-6}$ mol L^{-1}; pH 7.40; λex = 280 nm, λem = 340 nm at 296 K (squares); 303 K (solid circles); 310 K (triangles); 318 K (hollow circles).

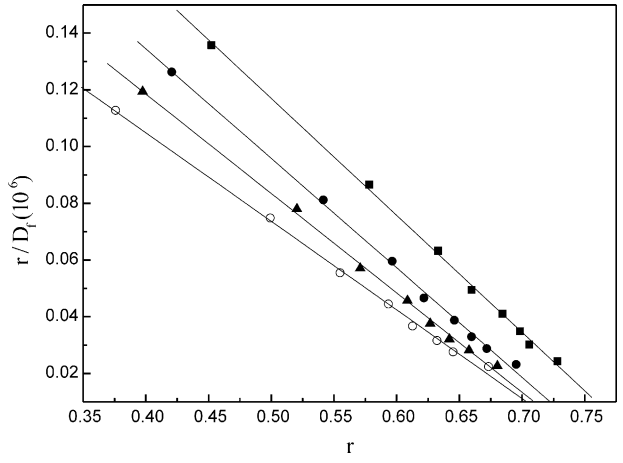

Figure 6. The Scratchard plots of the HSA–isofraxidin. (λex = 280 nm, λem = 340 nm, pH = 7.40, $C_{HSA} = 1.5\times10^{-6}$ mol L^{-1}, $C_{isofraxidin} = 3.3\times10^{-6}$–$3.0\times10^{-5}$ mol L^{-1}) at 296 K (squares); 303 K (solid circles); 310 K (triangles); 318 K (hollow circles).

3.3. Binding mode

Generally, small molecules are bound to macromolecule by 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 mode. For this purpose, the temperature-dependence of the binding constant was studied. The temperatures chosen were 296, 303, 310, and 318 K so that HSA does not undergo any structural degradation. From the temperature dependence of binding constants it is possible to calculate values for the thermodynamic functions involved in the binding process. First, the binding constants of isofraxidin to HSA at the four temperatures were estimated. Then, by plotting the binding constants according to van't Hoff equation, the thermodynamic parameters were determined from linear van't Hoff plot (Fig. 7) and are presented in Table 1. As shown in Table 1, the formation of isofraxidin–HSA complex is an exothermic reaction accompanied by positive ΔS value. Consequently, the binding process is entropically controlled, and larger positive entropy makes a much greater contribution to the free energy term for HSA. Nemethy and Scheraga [18], Timasheff [19] and Ross and Subramanian [20] have characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes, as described below. From the point of view of water structure, a positive ΔS value is frequently taken as evidence for hydrophobic interaction. Furthermore, specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive value of ΔS and

Table 1. Binding constant (K), the numbers of the binding sites (n) and thermodynamic parameters for the binding of isofraxidin to HSA

Temperature (K)	K ($\times10^5$ L mol^{-1})	n	ΔG (kJ mol^{-1})	ΔS (J·mol^{-1}·K^{-1})	ΔH (kJ·mol^{-1})
296	4.1266	0.78	−31.86		
303	3.8612	0.75	−32.37	73.57	−10.08
310	3.5063	0.74	−32.89		
318	3.1241	0.74	−33.48		

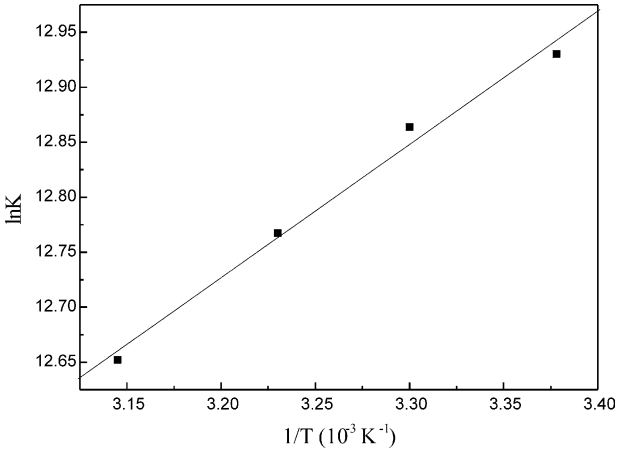

Figure 7. Variation of lnK as a function of 1/T.

a negative ΔH value. Accordingly, it is not possible to account for the thermodynamic parameters of isofraxidin–HSA complex on the basis of a single intermolecular force model. It is more likely that hydrophobic, electrostatic interactions are involved in its binding process. However, ionic interactions cannot be expected for isofraxidin-binding because isofraxidin might be considered to be largely unionized under the conditions here (pH 7.40), as can be expected from its structure. Thus electrostatic interaction cannot play a major role in the binding, and isofraxidin bound to HSA was mainly based on the hydrophobic interaction.

3.4. Molecular modeling study of the interaction between HSA and isofraxidin

Some studies have shown that HSA is able to bind many ligands in several binding sites.[1,21–23] Descriptions of the 3-D structure of crystalline albumin have revealed that HSA comprises of three homologous domains (denoted I, II, and III), each with two subdomains, A and B possessing common structural motifs. It is suggested that the principal regions of ligand binding to HSA are located in hydrophobic cavities in subdomains IIA and IIIA, which are consistent with site I and site II, respectively, and one tryptophan residue (Trp-214) of HSA is in subdomain IIA.[1,24] The crystal structure of HSA in complex with R-warfarin was taken from the Brookhaven Protein Data Bank (entry codes 1 h9z).[23] The potential of the 3-D structures of HSA was assigned according to the Amber 4.0 force field with Kollman-all-atom charges. The initial structures of all the molecules were generated by molecular modelling software Sybyl 6.9.[25] The geometries of these compounds were subsequently optimized using the Tripos force field with Gasteiger–Marsili charges. AutoDock3.05 program[26,27] was used to calculate the interaction modes between the ligands and HSA. Lamarckian genetic algorithm (LGA) implemented in Autodock was applied to calculate the possible conformation of the ligands that binds to the protein. During docking process, a maximum of 10 conformers was considered for this compound. The conformer with the lowest binding free energy was used for further analysis.

Here, partial binding parameters of the HSA–isofraxidin system were calculated through Silicon Graphics Ocatane2 workstation. As shown in Figure 8, isofraxidin binds within the subdomain IIA of the protein (The Warfarin Binding Pocket). The calculated binding Gibbs free energy (ΔG) is −29.09 kJ mol^{-1} and the corresponding binding constant is 1.2739×10^5 L mol^{-1}, which are close to the experimental data. The interaction between isofraxidin and HSA is dominated by hydrophobic force, but there is also a hydrogen bond between the drug and the residues Arg257 of HSA.

4. Conclusion

In this paper, the binding of isofraxidin to human serum albumin (HSA) under physiological condition has been presented by fluorescence methods in combination with FT-IR and CD techniques. According to Scatchard equation the binding constants K for the reaction at 296 K, 303 K, 310 K and 318 K were obtained. The thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS) indicate that hydrophobic force plays a main role in the binding of isofraxidin to HSA. The results calculated through Silicon Graphics Ocatane2 workstation are nearly in accordance with the experiment results.

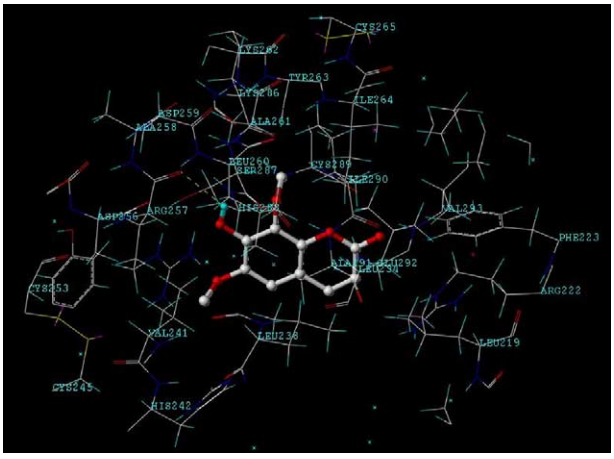

Figure 8. The interaction mode between isofraxidin and HSA, only residues around 8 Å of isofraxidin is displayed. The residues of HSA are represented using line and the isofraxidin structure is represented using ball and stick model. The hydrogen bond between isofraxidin and HSA is represented using yellow dashed line.

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