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Binding of daunorubicin to human serum albumin using molecular modeling and its analytical application

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Abstract

This study was designed to examine the interaction of daunorubicin with human serum albumin (HSA) for the first time by fluorescence spectroscopy in combination with UV absorption and molecular modeling under simulative physiological conditions. The quenching mechanism was suggested to be static quenching according to the fluorescence measurement and the linearity of Scatchard plot indicated that daunorubicin bound to a single class of binding sites on HSA. The thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS) were calculated to be −16.13 kJ/mol and 27.86 J/(mol K), according to the Vant'Hoff equation. These data suggested that hydrophobic interaction was the predominant intermolecular forces stabilizing the complex, which was in good agreement with the results of molecular modeling study. In addition, the effects of common ions on the binding constant of daunorubicin–HSA complex were also discussed at room temperature. Moreover, the synchronous fluorescence technique was successfully employed to determine the total proteins in serum, urine and saliva samples at room temperature under the optimum conditions with a wide linear range and satisfactory results.

Keywords: Daunorubicin; Human serum albumin (HSA); Molecular modeling

1. Introduction

Daunorubicin (Fig. 1) is one kind of new approved anticancer drug, which is indicated for remission induction in acute nonlymphocytic leukemia (myelogenous, monocytic, erythroid) of adults and for remission induction in acute lymphocytic leukemia of children and adults. It is recommended that daunorubicin must be administered only by physicians who are experienced in leukemia chemotherapy and in facilities with laboratory and supportive resources adequate to monitor drug tolerance and protect and maintain a patient compromised by drug toxicity. The physician and institution must be capable of responding rapidly and completely to severe hemorrhagic conditions and/or overwhelming infection. It is non-specific and reversible that many of these agents can bind to serum proteins. The binding affects their pharmacological and pharmacokinetic properties, and therefore a study on the binding of daunorubicin to HSA is very significant. Nowadays, there have been some reports that investigate the interaction of proteins with drugs by fluorescence techniques [1,2], yet the investigation on the binding interaction of daunorubicin and HSA has not been reported at the molecular level.

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 [3]. This globular protein consists of a single polypeptide chain of 585 amino acid residues, which has many important physiological functions [4]. 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, including anticoagulants, tranquilizers, and general anesthetics [5,6] are transported in the blood while bound to albumin, which are poorly soluble in water. It has been shown

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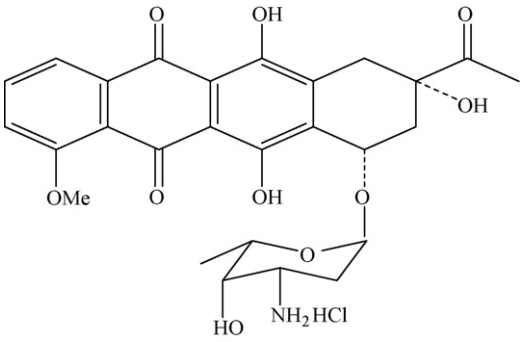

Fig. 1. The structural of daunorubicin.

that the distribution, free concentration and the metabolism of various drugs can be significantly altered as a result of their binding to HSA [7]. 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. Up to now, extensive investigations into interactions between protein and internal compounds or pharmaceutical molecules have been stimulated [8–10]. Because 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 interaction between drugs and plasma proteins in general and serum albumin in particular because of their high sensitivity, rapidity and ease of implementation.

The determination of proteins is very important in clinical medicine, biochemistry, and laboratory tests. The most frequently used methods are the Biuret [11], Bromocresol Green (BCG) [12], Coomassie Brilliant Blue (CBB) [13], and Bromophenol Blue [14]. In recent years, novel methods such as spectrophotometry [15], spectrofluorimetry [16], RLS [17], and chemiluminescence [18] were developed. The most quantitative analysis for proteins is generally based on their fluorescence enhancement effect on organic dyes. However, the organic fluorophores often suffer from photobleaching, low signal intensities, and random on/off light emission (blinking) [19]. Photobleaching is usually caused by sudden decomposition of the emitter; it is the main factor limiting the maximum number of photons obtained from a fluorophore. Low signal intensities reduce the accuracy of determination. Intermittent light emission causes problems in real-time studies of biomolecular dynamics such as protein folding, signal transduction, and enzymatic catalysis. Synchronous fluorescence scan (SFS) analysis has become a new attractive method for the determination of biomolecules [20] since its introduction by Lioyd and Evett [21], who initially applied it in the field of forensic science. The main characteristics of SFS are narrowing of the spectral band, simplification of the emission spectra, and contraction of the spectral range. Because of its sharp, narrow spectrum, SFS serves as a very simple, effective method of obtaining data for quantitative determination in a single measurement [22]. Recently, there have been some reports devoted to studies of the interaction between drugs and serum albumin [20,23]. However, the interaction between daunorubicin and HSA, and the synchronous fluorescence determination of HSA have not been reported. Compared with other molecular probe reported, an obvious characteristic was that the binding mode was investigated by using molecular modeling, the sensitivity and selectivity was higher, or the photostability was higher because the fluorescence intensity was basically kept at a constant value for at least 7 h, and or there was a wider linear range.

In this paper, the interaction of daunorubicin with HSA have been studied under physiological conditions utilizing the fluorescence method in combination with UV absorption spectra and molecular modeling. The binding mechanism of daunorubicin to HSA was discussed. According to the mechanism of Förster energy transference, the transfer efficiency of energy and distance between daunorubicin and the acceptor HSA were found. The binding sites and main sorts of binding force have been suggested. A novel method was established using synchronous fluorescence technology for the determination of the proteins in biology samples, based on the binding interaction of daunorubicin with proteins.

2. Materials and methods

2.1. Reagents

Appropriate amounts of human serum albumin (Hualan Biological Engineering Limited Company) was directly dissolved in water to prepare stock solution at final concentration of 2.0×10^{-5} M and stored in the dark at 0–4 °C. 8.1×10^{-4} M daunorubicin (synthesized), 0.5 M NaCl working solution, 0.1 M Tris–HCl buffer solution of pH 7.4 and other ionic solutions were prepared. Human serum sample was obtained from the Hospital of Henan Normal University. The serum sample was diluted 100-fold with double water before determination. All chemicals were of analytical reagent grade and were used without further purification. Double distilled water was used throughout.

2.2. Apparatus

All fluorescence spectra were recorded on an FP-6200 spectrofluorimeter (JASCO, Japan) and a RF-540 spectrofluorimeter (Shimadzu, Japan) equipped with a thermostat bath, using 5 nm/5 nm slit widths. The UV absorption spectra were performed on a Tu-1810 ultraviolet-visible spectrophotometer (Beijing General Instrument, China). The pH values were measured on a pH-3 digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass electrode. All calculations were performed on SGI workstation while studying the molecular model.

2.3. Optimization of experimental conditions

In order to get the best results, the optimal conditions were investigated. Various experimental parameters including medium, pH, addition order, reaction temperature and $\Delta\lambda$ were

studied with daunorubicin concentration being 8.1×10^{-4} M in all conditions. The experimental results shown us that 0.1 M Tris–HCl buffer solution of pH 7.4 was chosen as the supporting media; the Tris–HCl + NaCl + HSA + daunorubicin was selected in this work; 22 °C was suggested as the preferable reaction temperature; $\Delta\lambda = 20$ nm was selected while scanning synchronous fluorescence spectroscopy.

2.4. Measurements of spectrum

Under the optimum physiological conditions described above, 2.0 mL Tris–HCl buffer solution, 2.0 mL NaCl solution, appropriate amounts of HSA and daunorubicin were added to 10.0 mL standard flask and diluted to 10.0 mL with double distilled water. Fluorescence quenching spectra of HSA were obtained at excitation wavelength (280 nm) and emission wavelength (300–450 nm). Fluorescence spectra in the presence of other ions were also measured at the same conditions. In addition, the UV absorption and synchronous spectra of system were recorded.

2.5. Molecular modeling study

The potential of the 3D 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 modeling software Sybyl 6.9.1 [24]. The geometries of this drug were subsequently optimized using the Tripos force field with Gasteiger–Marsili charges. The AutoDock3.05 program was used to calculate the interaction modes between the drug and HSA. Lamarckian genetic algorithm (LGA) implemented in Autodock was applied to calculate the possible conformation of the drug that binds to the protein. During docking process, a maximum of 10 conformers was considered for the drug. The conformer with the lowest binding free energy was used for further analysis. All calculations were performed on SGI FUEL workstation.

3. Results and discussion

3.1. The binding mechanism analysis of daunorubicin and HSA

The aim of the present work was to investigate whether daunorubicin interacts with HSA and changes the conformation of HSA. The conformation changes in HSA were evaluated by measuring the intrinsic fluorescence intensity of protein tryptophan residues [25] in the absence and presence of daunorubicin. The addition of daunorubicin caused a dramatic decrease in the fluorescence emission intensity of HSA with a conspicuous change in the emission spectra (Fig. 2). HSA has a strong fluorescence emission with a peak at 337 nm on excitation at 280 nm, with gradual increase in drug concentrations, the maximum emission wavelength were shifted from 337 to 348 nm. It can be seen that a higher excess of daunorubicin led to more effective quenching of the chromophore molecules fluorescence.

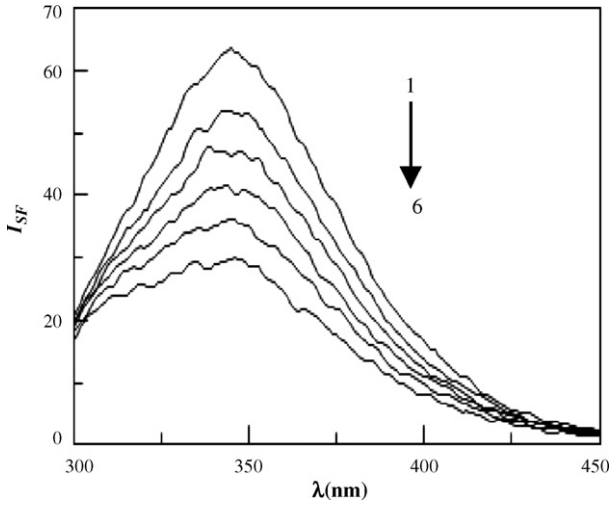

Fig. 2. The fluorescence spectra of daunorubicin–HSA system. From 1 to 6: $C_{HSA} = 2.0 \times 10^{-5}$ M; $C_{daunorubicin} = 0$, 0.8, 1.6, 2.4, 3.2, 4.0×10^{-5} M.

The strong quenching of the fluorescence clearly indicated that the binding of the drug to HSA changed the microenvironment of tryptophan residue and the tertiary structure of HSA.

Further experiment was carried out with UV technique to verify the binding of daunorubicin to HSA. Fig. 3 shows the UV absorption spectra of HSA in the absence and presence of daunorubicin. The absorption of HSA (about 210 nm) represents the helix structure of HSA [26]. As can be seen in Fig. 3, HSA has strong absorbance with a peak at 209 nm and the absorbance of HSA increased with the addition of daunorubicin; the chromophore of daunorubicin–HSA gives a very specific pattern of the UV–vis spectrum with slight dual absorbance spectra at higher concentration of daunorubicin in the system from 238 to 290 nm, and the addition of daunorubicin results in the slightly shift of daunorubicin–HSA spectrum toward a longer wavelength. The obvious enhancement of UV absorbency intensity (A) and the change of absorption spectra verified the formation of a new complex between daunorubicin and HSA.

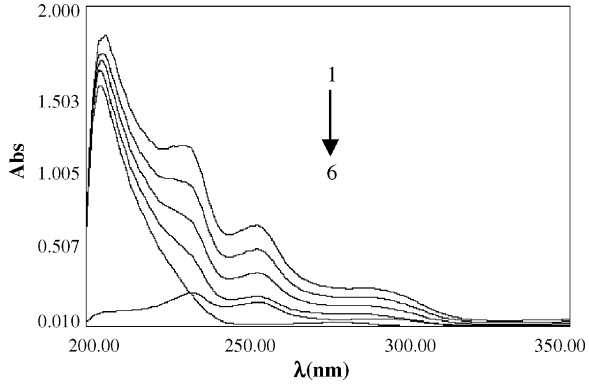

Fig. 3. UV absorption spectra of HSA in the absence and presence of daunorubicin (1–4) The UV absorption of daunorubicin–HSA, $C_{HSA} = 8 \times 10^{-7}$ M; $C_{daunorubicin} = 3.2 \times 10^{-5}$ M, $C_{daunorubicin} = 2.4 \times 10^{-5}$ M, $C_{daunorubicin} = 1.6 \times 10^{-5}$ M, $C_{daunorubicin} = 0.8 \times 10^{-5}$ M. (5) The UV absorption of HSA, $C_{HSA} = 8 \times 10^{-7}$ M. (6) The UV absorption of daunorubicin, $C_{daunorubicin} = 0.8 \times 10^{-5}$ M.

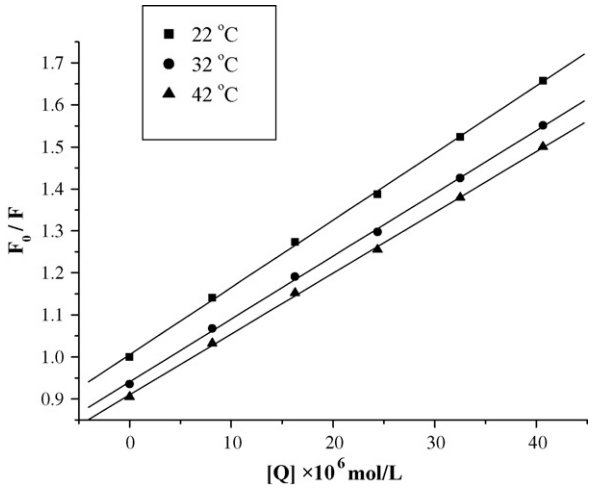

Fig. 4. The Stern–Volmer curves for quenching of daunorubicin with HSA.

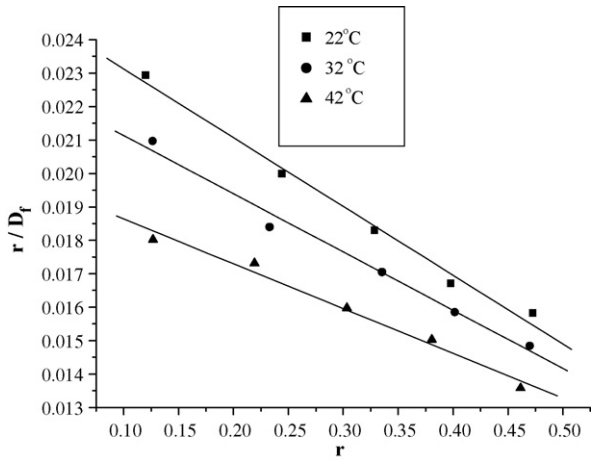

Fig. 5. Scatchard plot for the daunorubicin–HSA.

3.2. Quenching mechanism and binding constants

The dynamic and static quenching can be distinguished by their different dependence on temperature [27]. The quenching rate constants decreased with increasing temperature for the static quenching, while the reverse effect was observed for the dynamic quenching [22]. The possible quenching mechanism can be interpreted by fluorescence quenching spectra of HSA and the F_0/F–C (Stern–Volmer) curves of HSA with daunorubicin at different temperatures (22, 32, 42 °C) as shown in Fig. 4. It could be found that the Stern–Volmer plots were linear and the slopes decreased with the temperature increasing, indicating the static quenching interaction between daunorubicin and HSA occurred.

The fluorescence quenching data at 22, 32 and 42 °C were analysed using the Stern–Volmer equation:

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

where F_0 and F are the steady state fluorescence intensities in the absence and presence of quencher, respectively, K_q is the quenching rate constant of the biomolecule, K_{sv} is the Stern–Volmer quenching constant and $[Q]$ is the concentration of quencher (daunorubicin); τ_0 is the average fluorescence lifetime of the biomolecule without daunorubicin. The K_{sv} values and correlation coefficient, R (the linear relationship between F_0/F and $[Q]$) obtained at different temperatures are listed in Table 1. The fluorescence lifetime of the HSA is 10^{-8} s [22], K_{sv} is the slope of linear regression equation of Fig. 4. According to Eq. (1), the quenching constant K_q was calculated to be about 10^{12} L/(mol s) as listed in Table 1. However, the maximum scatter collision quenching constant K_q of various quenchers with the biopolymer is 2.0×10^{10} L/(mol s) [28]. Obviously, the rate constant of protein quenching procedure initiated by daunorubicin was greater than the K_q value of the scatter procedure, which demonstrated that the quenching was initiated not by dynamic collision but by compound formation, which was in accordance with the results from the obvious enhancement of UV absorbency intensity (A) and the change of absorption spectra as shown in Fig. 3 [29,30]. Therefore, the quenching of HSA fluorescence by daunorubicin depended on the formation of the new daunorubicin–HSA complex.

In drug–protein binding studies, several equations have been used for binding constant calculation. One frequently used is Scatchard equation [31]:

$$\frac{r}{D_f} = nK - rK \quad (2)$$

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. Fig. 5 shows the Scatchard plots for the daunorubicin–HSA system at different temperatures. The linearity of the Scatchard plot indicated that daunorubicin bound to a single class of binding sites on HSA, which was full agreement with the number of binding site n; and the binding constants (K, Table 2) agree very closely with those obtained by the modified Stern–Volmer equation. In addition, it was found that there was a strong interaction between daunorubicin and HSA, and the binding constant decreased with the increasing temperature, resulting in a reduction of the stability of the daunorubicin–HSA complex, but the effect of temperature is very small. Thus, the quenching efficiency of daunorubicin to HSA is not reduced obviously when difference in temperature is

Table 1
The dynamic quenching constants (L/(mol s)) between daunorubicin and HSA

T (°C)	Stern–Volmer equation	K_q (L/(mol s))	R
22	$Y = 1.006 + 1.599 \times 10^4\ [Q]$	1.599×10^{12}	0.9997
32	$Y = 0.941 + 1.496 \times 10^4\ [Q]$	1.496×10^{12}	0.9997
42	$Y = 0.910 + 1.447 \times 10^4\ [Q]$	1.447×10^{12}	0.9997

Table 2
The binding constant (K, M) between daunorubicin and HSA

T (°C)	Scatchard equation	K (L/mol)	n	R
22	$Y = 0.0175 - 0.0202r$	2.02×10^4	0.8674	0.9924
32	$Y = 0.0166 - 0.0195r$	1.95×10^4	0.8490	0.9922
42	$Y = 0.0160 - 0.0189r$	1.89×10^4	0.9902	0.9898

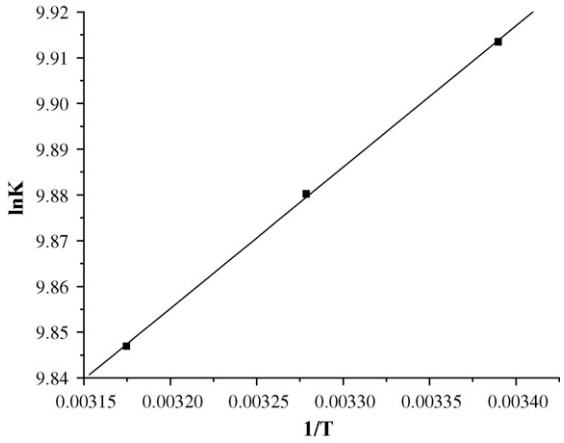

Fig. 6. Van't Hoff plot for the interaction of daunorubicin and HSA.

not wide. In this work, the binding constants obtained with the modified Stern–Volmer equation are applied in the discussion of binding modes.

3.3. Binding modes

The molecular forces contributing to protein interactions with small molecules are bound to macromolecule by four binding modes: hydrogen bonds, van der Waals, electrostatic and hydrophobic interactions. The signs and magnitudes of thermodynamic parameters for protein reactions can be accounted for the main forces contributing to protein stability. Because the temperature effect is very small, the reaction enthalpy change can be regard as a constant if the temperature range is not too wide. The plot of $\ln K$ versus $1/T$ gave a straight line according to the Van't Hoff equation [32]:

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

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

where R is the gas constant. By plotting the binding constants (K values in Table 2) according to the Van't Hoff equation, the thermodynamic parameters were determined from a linear Van't Hoff plot (Fig. 6) and listed in Table 3. As shown in Table 3, the formation of a daunorubicin–HSA coordination compound involved an exothermic and spontaneous reaction accompanied by a positive ΔS value. Nemethy and Scheraga [33], Timasheff [34] and Ross and Subramanian [35] 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 and a negative ΔH value is frequently taken as evidence for hydrophobic interaction [36]. But it has been pointed out that specific electrostatic interactions between ionic species in aqueous solution were characterized by a positive value of ΔS and a negative ΔH value. Accordingly, it was not possible to account for the thermodynamic parameters of the daunorubicin–HSA compound based on a single intermolecular force model. Furthermore, the main source of ΔG value is derived from a large contribution of ΔS term with little contribution from the ΔH factor, so the main interaction is by hydrophobic contact, but the electrostatic interaction cannot be excluded.

Table 3
The thermodynamic parameters for the binding daunorubicin to HSA

T (°C)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/(mol K))
22	−24.31		
32	−25.05	−16.13	27.86
42	−25.80		

3.4. Molecular modeling

The complementary applications of molecule modeling have been employed by computer methods to improve the understanding of the interaction of daunorubicin and HSA. Descriptions of the 3D structure of crystalline albumin have revealed that HSA comprises three homologous domains, (I–III): I (residues 1–195), II (196–383), III (384–585), each domain is a product of subdomains that posses common structural motifs. The crystallographic analysis reveals that the principal regions of ligand binding to HSA are located in hydrophobic cavities in subdomains IIA and IIIA, respectively, which exhibit similar chemistry [37]. Despite very high stability, HSA is a flexible protein with the 3D structure susceptible to environmental factors such as pH, ionic strength, etc. [38]. It was proposed that the binding took place near Trp-214 and led to a conformational change with a local perturbation of the IIA binding site in HSA [1,39] There is a large hydrophobic cavity present in subdomain IIA to which many drugs can bind [1].

The crystal structure of HSA in complex with warfarin was taken from the Brookhaven Protein Data Bank (entry codes 1h9z). The potential of the 3D structure 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 modeling software Sybyl 6.9.1 [24]. The geometry of the molecule was subsequently optimized to minimal energy using the tripos force field with Gasteiger–Marsili charges. Then it was used to replace warfarin in the HSA-warfarin crystal structure. The Flexx program was applied to calculate the possible conformation of the ligands that bind to the protein. The conformer with (rootmeans-square) (RMS) was used for further analysis. Based on this type of approach, a computational model of the target receptor has been built, partial binding parameters of the daunorubicin–HSA system were calculated through SGI FUEL workstations. The best energy ranked results are shown in Fig. 7. As shown in Fig. 7, the drug molecule was located within the binding pocket and the four rings were practically coplanar. The E-ring of daunorubicin was inserted in the hydrophobic cavity of site I, and it was important to note that the trptophan residue of HSA (Trp-214) is in close proximity to daunorubicin suggesting the existence of hydrophobic interaction between them. Further, this finding provides a good structural basis to explain the efficient fluorescence quenching of HSA emission in the

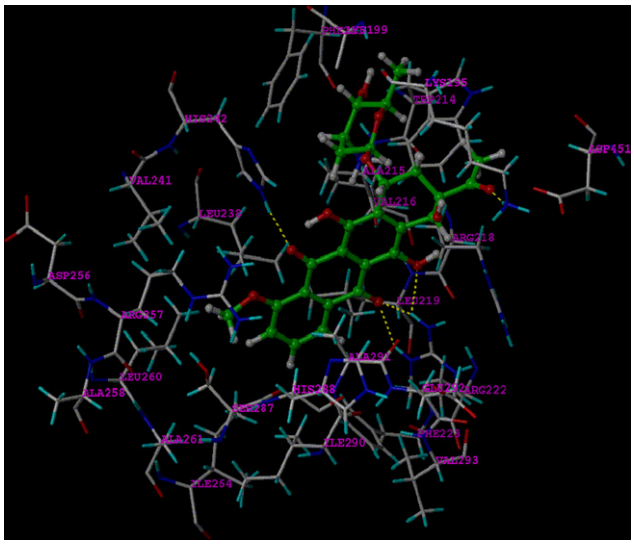

Fig. 7. The interaction model between daunorubicin and HSA. The residues of daunorubicin and HSA are represented using different tinctorial stick model. The hydrogen bond between the ligand and the protein is indicated by dashed line.

presence of the daunorubicin. There were also hydrogen bonds between daunorubicin and the residues LEU-219 and ALA-291 of HSA, the result indicated that the formation of hydrogen bond decreased the hydrophilicity and increased the hydrophobicity to stability in the daunorubicin–HSA system. On the other hand, the amino acid residues with a benzene ring can match that of the daunorubicin in space in order to firm the conformation of the complex. The ligand binding regions of HSA located in hydrophobic cavities in subdomains IIA were large enough to accommodate the daunorubicin. The results obtained from modeling indicated that the interaction between daunorubicin and HSA was dominated by hydrophobic force.

3.5. Distance measurement between tryptophan and daunorubicin binding site

According to the Förster's theory [40], the efficiency of energy transfer is related to not only the distance between tryptophan residue (donor) and cytidine (acceptor), but also to the critical energy transfer distance. That is:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{5}$$

where r represents the distance between donor and acceptor. R_0 is the critical distance when transfer efficiency is 50%, which can be calculated by

$$R_0^6 = 8.8 \times 10^{-25} k^2 n^{-4} \Phi J \tag{6}$$

where k^2 is the orientation factor related to the geometry of the donor–acceptor of dipole, n is the refractive index of medium, Φ is the fluorescence quantum yield of the donor, J is the spectra overlap of the donor emission and the acceptor absorption. J is given by

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{7}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescence reagent when wavelength is λ, $\varepsilon(\lambda)$ is the molar absorbance coefficient of the acceptor at the wavelength of λ. From these equations, J, E and R_0 can be calculated, so the value of r also can be evaluated. The overlap of the fluorescence spectrum of HSA and the absorption spectrum of daunorubicin is shown in Fig. 8.

From Fig. 8, the overlap integral calculated according to the above relationship were 2.811×10^{-14} cm^3 L/mol. It had been reported that: $k^2 = 2/3$, $n = 1.336$, $\Phi = 0.118$ for HSA [41]. Based on these data, the distance between daunorubicin and tryptophan residue in HSA was 3.78 nm. Obviously, it was lower than 7 nm after interaction between daunorubicin and HSA. This accorded with conditions of Förster's non-radiative energy transfer theory indicating that the energy transfer happened when binding and again a static quenching interaction between them, and energy transfer depend on the distance between the tryptophan residue and daunorubicin bound to HSA.

3.6. Effect of co-ions on binding of daunorubicin to HSA

The binding of ions to proteins is of great interest in biological science (catalytic function, structural stability) and a good understanding of this relationship is needed for the control of the structure and functionality of proteins. The previous studies indicated that HSA has a high-affinity metal-binding site at N-terminus. The multiple binding sites underlie the exceptional ability of HSA to interact with many organic and inorganic molecules and make this protein an important regulator of intercellular fluxes and the pharmacokinetic behavior of many drugs [38]. Therefore, we are interested in examining the effect of inorganic cations and anions on the solution system of daunorubicin–HSA that can be used as a model for investigating the interaction of daunorubicin to HSA. Table 4 is the result of the effect of common ions on the binding constants at 295 K. It is shown that the binding constants between daunoru-

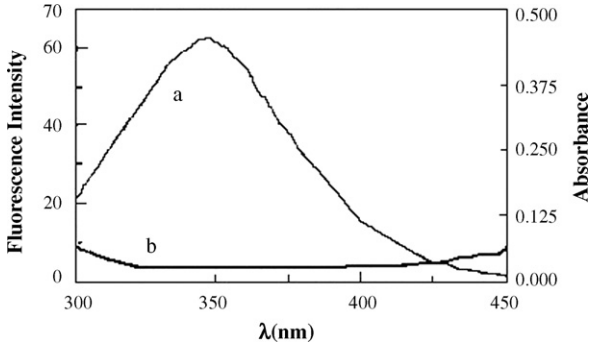

Fig. 8. The overlap of UV absorption spectrum of daunorubicin with the fluorescence emission spectrum of HSA. (a) The fluorescence emission spectrum of HSA (8×10^{-7} M); (b) the UV absorption spectrum of daunorubicin (8.1×10^{-6} M).

Table 4
The binding constants between daunorubicin and HSA in the presence of other ions

Ions	K	R	Ions	K	R
Zn^{+}	1.38×10^4	0.9997	K^{+}	1.36×10^4	0.9987
SO_4^{2-}	0.75×10^4	0.9970	NH_4^{+}	0.70×10^4	0.9991
CO_3^{2-}	1.93×10^4	0.9997	Na^{+}	1.97×10^4	0.9989
PO_4^{3-}	1.34×10^4	0.9999	Cl^{-}	0.98×10^3	0.9990
Ca^{2+}	1.46×10^4	0.9993	Mn^{2+}	1.41×10^4	0.9982
NO_3^{-}	0.91×10^4	0.9999	Pb^{2+}	0.80×10^4	0.9996

bicin and protein have changed in the presence of common ions, implying there was a binding between metal ions and HSA and the presence of metal ions directly affected the binding between daunorubicin and HSA. As a result, the binding force between protein and pharmaceutical also decreased, shortened the stored time of pharmaceutical in blood plasma and improved maximum reaction intensity of pharmaceutical.

4. Determination of HSA in biology samples

4.1. Precision, limits of detection and working curve

Based on the binding of HSA to daunorubicin, we employed synchronous fluorescence spectra to quantitative determine the human serum albumin. Fig. 9 shows the synchronous spectra of HSA in the presence of appropriate daunorubicin. It could be seen that: the synchronous fluorescence intensity (I_{SF}) of daunorubicin was very weak, so that the effect of daunorubicin on the determination of HSA could be eliminated, and the intensity of the synchronous fluorescence increased noticeably with increasing of the concentration of HSA. The enhancement intensity of synchronous fluorescence was proportion to the concentration of HSA. The linear range determined was 1.1–469.2 μg/mL, the linear regression equations was $I_{HSA} = 6.2059 + 1.3854 \times 10^7\, C_{HSA}$ (M) with a correlation coefficient (R) of 0.9997. The detection limit for HSA as defined by IUPAC was determined to be 0.4997 μg/mL [42]. The relative standard deviation (R.S.D.) was 1.35% for HSA, as obtained

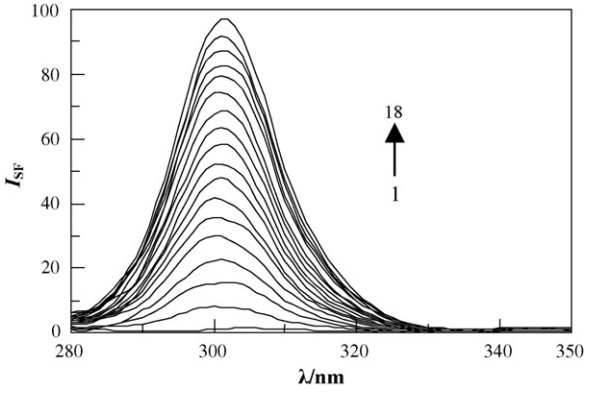

Fig. 9. Synchronous fluorescence spectra of HSA. $C_{daunorubicin} = 8.13 \times 10^{-6}$ M; from 1 to 18, $C_{HSA} = 0$, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, 4.4, 4.8, 5.2, 5.6, 6.0, 6.4, 6.8×10^{-6} M.

Table 5
Determination result of serum, urine and saliva samples ($n = 6$)

Samples	Added (g/mL)	Found (μg/mL)	Recovery (%)	R.S.D. (%)
Serum	0	60.23		0.48
	55.2	117.87	102.1	1.05
	110.4	178.4	104.5	0.77
	165.6	228.2	101.1	0.96
Urine	0	2.55		0.38
	55.2	55.22	95.6	0.45
	110.4	109.21	96.7	0.79
	165.6	163.43	97.2	1.02
Saliva	0	25.67		1.23
	55.2	83.64	103.4	0.88
	110.4	133.36	98.0	0.71
	165.6	188.33	98.5	0.69

from six replicate determinations of 8.13×10^{-4} M for daunorubicin.

4.2. Analysis of biology samples

Because the present method showed protein specificity, we thought it suitable for determining the total content of protein in complex samples containing different types of proteins (e.g.: serum albumin). Thus, this method was applied to the determination of total protein in serum, urine, saliva samples. Standard human serum, which was used to construct a calibration curve, was obtained by mixing 40 normal serum samples. Construction of the calibration curve and analysis of the serum, urine, saliva samples were then performed according to the procedures described above. Serum sample was diluted 100-fold with double distilled water just before determination without any other pretreatment, urine and saliva samples were also diluted appropriate folds. Table 5 displayed the results of determination by a standard addition method for biology samples, which were very satisfying. Therefore, the proposed method has potential for the sensitive and rapid determination of total protein in biology samples.

5. Conclusions

The interaction between daunorubicin and HSA has been investigated by different spectroscopy techniques under simulative physiological conditions. This study showed that the intrinsic fluorescence of HSA was quenched through static quenching mechanism and the binding of daunorubicin to HSA was predominantly owing to hydrophobic interaction estimated from the signs of ΔH and ΔS, which was consistent with the result from molecular modeling study. Experimental results showed that the binding of daunorubicin to HSA induced a conformational change of HSA, which was proved by the qualitative analysis data of UV absorbance. This study also showed that daunorubicin was a strong quencher and bound to HSA with high affinity. Based on this phenomenon, a new method by synchronous fluorescence for the rapid and simple determi-

nation of the proteins in biology samples was provided. The results showed that the present method was comparable with other methods in terms of sensitivity, rapid, simplicity and linear range. This method might be expanded to the application in biochemistry and clinic practice.

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