Study of the Interaction of Aglycon of Daunorubicin with Human Serum Albumin by Spectroscopy and Modeling

Fengling Cui,* Lixia Qin, Guisheng Zhang,* Xiaojun Yao, Beilei Lei

The interaction between aglycon of daunorubicin (DNR-A) and human serum albumin (HSA) was investigated using fluorescence quenching and modeling. Results shown that fluorescence quenching of HSA by DNR-A resulted from the formation of DNR-A-HSA complex. The quenching constants were determined via measurement of the binding affinity between DNR-A and HSA using the Stern-Volmer equation. The thermodynamic parameters \( \Delta G \), \( \Delta H \), \( \Delta S \) and the binding distance \( r \) were calculated. Furthermore, SFS and UV spectra suggested that the complex changed the conformation of HSA and that hydrophobic interactions played a major role in DNR-A-HSA association, which was in good agreement with the results of the modeling study. Moreover, the SFS technique was successfully applied to determine the total proteins in biology samples with satisfactory results.

Introduction

Human serum albumin (HSA) is a principal extracellular protein with a high concentration in blood plasma (40 mg·mL\(^{-1}\) or 0.6 \( \times \) 10\(^{-3}\) M). It is a globular protein and crystallographic analyses of HSA have revealed that the protein is a 585 amino acid residue monomer, composed of three homologous \( \alpha \)-helical domains (I–III), each containing two subdomains (A and B) and stabilized by 17 disulfide bridges.\(^{[1–3]} \) Aromatic and heterocyclic ligands were found to bind within two hydrophobic pockets in subdomains IIA and IIIA, namely site I and site II.\(^{[1–3]} \) Seven binding sites are localized for fatty acids in subdomains IIB, IIIA and IIIB and on the subdomain interfaces.\(^{[4]} \) HSA contains a single tryptophan (Trp214).

HSA contributes to colloid osmotic blood pressure and is chiefly responsible for the maintenance of blood pH. Moreover, it is known to play an important role in the transport and disposition of endogenous and exogenous ligands present in blood.\(^{[1]} \) Its remarkable capacity to bind a variety of drugs results in its prevailing role in drug pharmacokinetics and pharmacodynamics. Its primary...
pharmacokinetic function is to participate in absorption, distribution, metabolism and excretion of drug, of which the drug distribution is the one that HSA controls, because most drugs travel in plasma and reach the target tissues by binding to HSA.\(^5\) Hence, binding of drugs to plasma proteins is an important pharmacological parameter, since it frequently affects the distribution and elimination of a drug, as well as the duration and intensity of its physiological action.\(^6\)–\(^8\) Generally, weak binding can lead to a short lifetime or poor distribution, while strong binding can decrease the concentration of free drug in plasma. Because of this, many pharmaceutical firms have developed and standardized screens for HSA binding as the first step of new drug design. Therefore, studies of the interactions of a bioactive compound with HSA possess significance in chemistry, life sciences and clinical medicine.

Daunorubicin and other anthracycline analogs have been used clinically since the 1970s,\(^9\) and, to date, they are still some of the most effective drugs against a variety of solid tumors. Over the past 30 years, many reports have been published on the SAR of aglycon (DNR-A, Figure 1) and monosaccharides of daunorubicin. The aglycon is critical for the bioactivity of anthracycline drugs. The fused aromatic rings B, C and D of the aglycon serve as a DNA intercalating unit and the external (non-intercalating)
moieties, involving the cyclohexane ring A and the side chain, serve as an enzyme interacting domain. These are recognized as crucial moieties for the therapeutic efficacy of anthracyclines. It is very important to study the interaction of DNR-A with HSA because the interaction can help us to better understand the absorption and distribution of the drug. DNR-A was readily prepared from the hydrolysis of daunorubicin hydrochloride with dilute HCl at 90 °C for 1 h.[10] DNR-A was obtained after filtration as a red powder which was pure enough to be used directly in the study of the interaction with HSA.

The interactions of drugs with proteins are often investigated by spectroscopic techniques as these methods are sensitive and relatively easy to use. They have several advantages over conventional approaches, such as affinity and size exclusion chromatography, equilibrium dialysis, ultra filtration and ultracentrifugation, which suffer from lack of sensitivity or long analysis time or both, and use of protein concentrations far in excess of the dissociation constant for the drug-protein complex[11,12] and for drug-protein interaction studies.

The quantitative analysis of protein continues to be a very active area because it can be used as a reference for the measurement of other components in biochemical analysis and clinical diagnoses. There are many techniques, such as ultraviolet and visible absorption (UV-vis) spectroscopy,[13] fluorescence spectroscopy,[14,15] calorimetry,[16] light scattering[17] and capillary electrophoresis,[18] to detect the protein. Among them, the fluorescence method stands out from the rest since it is more widely used in peptide and protein chemistry than any other spectral detection method.[19] Since the fluorescence emitted from the native protein is very weak, the emphasis of the fluorescence method for the detection of protein focuses on the probe of proteins. However, few researchers have been involved in using a compound with intramolecular charge transfer (ICT) behavior as a probe for the determination of protein in biology samples.

In the present paper, the mechanism of interaction between DNR-A and HSA has been studied using a fluorescence spectroscopic technique and a molecular modeling method under physiological conditions. Spectroscopic data were used to quantify the binding constants of DNR-A to HSA and the action distance, which was based on the Förster’s energy transfer (FET). UV-vis and synchronous spectroscopy revealed that changes in the protein structure resulted from the DNR-A 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. Based on these results, a means of sensitive determination for protein was established. Detecting the protein in biology samples validates its reliability and these results were satisfactory.

### Experimental Part

#### Materials

Appropriate amounts of human serum albumin (Hualan Biological Engineering Limited Company) were directly dissolved in water to prepare a stock solution at a final concentration of 2.0 × 10⁻⁸ M. This was stored in the dark at 0–4 °C. 6.33 × 10⁻⁶ M DNR-A (synthesized), 0.5 m NaCl working solution, 0.1 m Tris-HCl buffer solution at pH 7.4 and other ionic solutions were prepared. A 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.

#### 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 obtained on a UV-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 an SGI workstation while studying the molecular model.

#### Measurement of Spectra

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

#### Characteristics of Synchronous Fluorescence Method

The synchronous fluorescence spectra were obtained by simultaneously scanning the excitation and emission monochromators. Thus, the synchronous fluorescence applied to the equation of synchronous luminescence:[20]

\[
F = kcdE_{ex}(\lambda_{em} - \Delta \lambda)E_{em}(\lambda_{em})
\]

where \( F \) is the relative intensity of synchronous fluorescence, \( \Delta \lambda = \lambda_{em} - \lambda_{ex} \) is a constant, \( E_{ex} \) is the excitation function at the given excitation wavelength, \( E_{em} \) is the normal emission function at the corresponding emission wavelength, \( c \) is the analytical concentration, \( d \) is the thickness of the sample cell and \( k \) is the characteristic constant comprising the “instrumental geometry
factor” and related parameters. Since the relationship between the synchronous fluorescence intensity \( (F) \) and the concentration of DNR-A should follow the Equation (1), \( F \) should be in direct proportion to the concentration of DNR-A.

The optimal values of the wavelength intervals \((\Delta \lambda)\) are important for the correct analysis and interpretation of the binding mechanism. When the wavelength interval \((\Delta \lambda)\) was fixed at 60 nm of protein, the synchronous fluorescence had the same intensity as the emission fluorescence following excitation at 280 nm, and just the emission maximum wavelength and shape of the peaks changed\(^{[21–23]}\). Thus, the synchronous fluorescence measurements can be applied to calculate association constants similar to the emission fluorescence measurements. Therefore, the synchronous fluorescence measurements can deduce the binding mechanism as the emission fluorescence measurements did. In this study, the synchronous fluorescence spectra of tyrosine residues and tryptophan residues were measured at \( \lambda_{em} = 280 \text{ nm} \) (\( \Delta \lambda = 15 \) and 60 nm) in the absence and presence of various amounts of DNR-A.

**Protein-Ligand Docking 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 the molecular modeling software Sybyl 6.9.1. 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. The Lamarckian genetic algorithm (LGA) implemented in Autodock was applied to calculate the possible conformations of the drug that binds to the protein. During the docking process, a maximum of 10 conformers were considered for the drug. The conformer with the lowest binding free energy was used for further analysis. All calculations were performed on an SGI FUEL workstation.

**Results and Discussion**

**Fluorescence Quenching of HSA Induced by DNR-A**

Water soluble globular proteins have hydrophilic functional groups on the exterior and hydrophobic groups in the interior. Therefore, probes featuring hydrophobic and hydrophilic functionalities are likely to bind selectively at the hydrophobic/hydrophilic interfaces of proteins. Burstein\(^{[24]}\) proposed that the maximum fluorescence emission of trp residues is extremely sensitive to its environment. When they are exposed to the aqueous phase, the maximum wavelength is within 350–353 nm. When they are completely or partially in a hydrophobic environment, the maximum wavelength is between 330–333 nm and within 340–342 nm, respectively. As can be seen from Figure 2, the maximum wavelength was shifted from 353 to 333 nm. So, it was deemed that trp residues passed from the aqueous phase into a hydrophobic environment gradually as the concentration of DNR-A increased. That is to say, the DNR-A complex was likely to bind selectively at the hydrophobic interfaces of HSA and the blue shift of the emission maximum can be rationalized by the binding of DNR-A to a less polar site in HSA. The enhancement effect concluded that the DNR-A molecule was included in the hydrophobic pocket of the host HSA. The size of the pocket is consistent with a DNR-A molecule where little rotation of the probe occurs during the excited state. The noticeable change in the fluorescence was in proportion to the concentration of HSA. This work has resulted in a new method for the determination of protein in biology samples.

**Quenching Mechanism and Binding Constants**

Fluorescence quenching refers to any process which decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching, including excited state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisional quenching. The different mechanisms of quenching are usually classified as either dynamic quenching or static quenching. Dynamic and static quenching can be distinguished by their differing dependence on temperature and viscosity. Dynamic quenching depends upon diffusion. Since higher temperatures result in larger diffusion coefficients, the bimolecular quenching constants are expected to increase with increasing temperature. In contrast, increased temperature is likely to result in decreased stability of complexes, and thus lower values of the static quenching constants.

In order to speculate on the fluorescence quenching mechanism, the fluorescence quenching data at different concentrations of DNR-A were fitted to the Stern-Volmer equation: \( F/F_0 = 1 + K_D [Q] \). The relationship between the static quenching constants \((K_D)\) and related parameters, including the static quenching constants \((K_D)\), the concentration of quencher \((C)\), the concentration of HSA \((C_{HSA})\), and the concentration of DNR-A \((C_{DNR-A})\), is shown in Table 1. The optimal values of the wavelength intervals \((\Delta \lambda)\) are important for the correct analysis and interpretation of the binding mechanism. When the wavelength interval \((\Delta \lambda)\) was fixed at 60 nm of protein, the synchronous fluorescence had the same intensity as the emission fluorescence following excitation at 280 nm, and just the emission maximum wavelength and shape of the peaks changed\(^{[21–23]}\). Thus, the synchronous fluorescence measurements can be applied to calculate association constants similar to the emission fluorescence measurements. Therefore, the synchronous fluorescence measurements can deduce the binding mechanism as the emission fluorescence measurements did. In this study, the synchronous fluorescence spectra of tyrosine residues and tryptophan residues were measured at \( \lambda_{em} = 280 \text{ nm} \) (\( \Delta \lambda = 15 \) and 60 nm) in the absence and presence of various amounts of DNR-A.
temperatures (293, 303 and 313 K, Figure 3) were firstly analyzed using the classical Stern-Volmer equation:\[25\]

\[ F_0 / F = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q] \]  

(2)

where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence of quencher, respectively, \( k_q \) is the biomolecular quenching constant, \( \tau_0 \) is the lifetime of the fluorescence in the absence of quencher, \([Q]\) is the concentration of quencher and \( K_{SV} \) is the Stern-Volmer quenching constant. The results in Table 1 show that the Stern-Volmer quenching constant, \( K_{SV} \), is inversely correlated with temperature, and the values of \( k_q \) were larger than the limiting diffusion constant, \( K_{dif} \), of the biomolecule (\( K_{dif} = 2.0 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1} \)).\[26\] This suggested that the fluorescence quenching was caused by a specific interaction between HSA and DNR-A, and that the quenching mechanism mainly arose from the predominance of complex formation, while dynamic collision could be negligible at the concentration studied.\[27\] Therefore, the quenching data were analyzed according to the Scatchard equation:\[28\]

\[ r / D_f = nK - rK \]  

(3)

where \( r \) is the number of moles of bound drug per mole of protein, \( D_f \) is the concentration of unbound drug, \( K \) is the binding constant and \( n \) is the number of binding sites. Figure 4 shows the Scatchard plots for the DNR-A-HSA system at different temperatures. The linearity of the Scatchard plot indicated that DNR-A bound to a single class of binding sites on HSA, which was full agreement with the number of binding sites, \( n \), and the binding constants \( K \) (Table 2) agree very closely with those obtained using the modified Stern-Volmer equation. In addition, there was a strong interaction between DNR-A and HSA. The binding constant decreased with increasing temperature, resulting in a reduction in the stability of the DNR-A-HSA complex, but the effect of temperature was very small. Thus, the quenching efficiency of DNR-A to HSA is not reduced obviously when the difference in temperature is not large. In this work, the binding constants obtained using the modified Stern-Volmer equation are applied in the discussion of binding modes.

### Thermodynamic Parameters and Nature of the Binding Forces

There are essentially four types of non-covalent interactions which could play a role in ligand binding to proteins. They are hydrogen bonds, van der Waals forces, electrostatic and hydrophobic bond interactions\[29\]. To obtain...
such information, the implications of the present results have been discussed in conjunction with thermodynamic characteristics obtained for DNR-A binding, and the thermodynamic parameters were calculated. If the enthalpy changes ($\Delta H$) do not vary significantly over the temperature range studied, then its value and that of $\Delta S$ can be determined from the Van’t Hoff equation:

$$\ln K = -\Delta H / RT + \Delta S / R$$  \hspace{1cm} (4)

In Equation (4), $K$ is the effective quenching constant at the corresponding temperature and $R$ is the gas constant. The temperatures used were 293, 303 and 313 K. The enthalpy change ($\Delta H$) and entropy change ($\Delta S$) are calculated from the slope and ordinate of the Van’t Hoff relationship (Figure 5). The free energy change ($\Delta G$) is estimated from the following relationship:

$$\Delta G = \Delta H - T \Delta S$$  \hspace{1cm} (5)

From Table 3, it can be seen that $\Delta H$ is a negative value ($-20.30$ kJ mol$^{-1}$), whereas $\Delta S$ is a positive value ($13.52$ J mol$^{-1}$ K$^{-1}$). In these experiments, DNR-A-HSA complexes were accompanied by negative enthalpy changes ($\Delta H$) and a positive entropy change ($\Delta S$, Table 3), which indicates that the binding processes are entropically driven. The negative sign for $\Delta G$ indicates the spontaneity of the binding of DNR-A with HSA. Based on the characteristic signs of the thermodynamic parameters for the various interactions$^{[30,31]}$ for the DNR-A-HSA system, the main source of the $\Delta G$ value is derived from a large contribution of the $\Delta S$ term with little contribution from the $\Delta H$ factor, so the main interaction is hydrophobic contact, but the electrostatic interaction cannot be excluded. Therefore, the thermodynamic parameters for the interaction of DNR-A and HSA can be explained on the basis of plural intermolecular forces, such as hydrophobic and electrostatic interactions, rather than single inter-molecular forces.$^{[31]}

### Energy Transfer from HSA to DNR-A

HSA has a single tryptophan residue (Trp 214) and the fluorescence of HSA mainly comes from Trp 214. So, the distance between Trp 214 and the bound DNR-A can be determined using fluorescence resonance energy transfer theory (FRET). The overlap of the UV absorption spectrum of a representative drug, DNR-A, with the fluorescence emission spectrum of HSA is shown in Figure 6. The distance between the donor and acceptor and extent of spectral overlaps determines the extent of energy transfer. The distance $r$ between a protein residue (donor) and a bound drug molecule (acceptor) can be calculated from Förster’s theory.$^{[32,33]}$ The efficiency of energy transfer ($E$) is related to the distance ($R_0$) between donor and acceptor by:$^{[34,35]}

$$E = 1 - F/F_0 = R_0^6/(R_0^6 + r^6)$$  \hspace{1cm} (6)

### Table 2. The binding constant (K/M) between DNR-A and HSA.

<table>
<thead>
<tr>
<th>$T$ (K)</th>
<th>Scatchard equation</th>
<th>$K$ (L · mol$^{-1}$)</th>
<th>$n$</th>
<th>$R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>293</td>
<td>$Y = 0.0252 - 0.0206r$</td>
<td>$2.0600 \times 10^4$</td>
<td>1.2223</td>
<td>0.9951</td>
</tr>
<tr>
<td>303</td>
<td>$Y = 0.0229 - 0.0174r$</td>
<td>$1.7410 \times 10^4$</td>
<td>1.3149</td>
<td>0.9934</td>
</tr>
<tr>
<td>313</td>
<td>$Y = 0.0198 - 0.0134r$</td>
<td>$1.3390 \times 10^4$</td>
<td>1.4922</td>
<td>0.9913</td>
</tr>
</tbody>
</table>

### Table 3. The thermodynamic parameters for the binding of DNR-A to HSA.

<table>
<thead>
<tr>
<th>$T$ (K)</th>
<th>$\Delta G$ (kJ · mol$^{-1}$)</th>
<th>$\Delta H$ (kJ · mol$^{-1}$)</th>
<th>$\Delta S$ (J · mol$^{-1}$ · K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>293</td>
<td>$-24.22$</td>
<td>$-20.30$</td>
<td>13.52</td>
</tr>
<tr>
<td>303</td>
<td>$-24.36$</td>
<td>$-20.30$</td>
<td>13.52</td>
</tr>
<tr>
<td>313</td>
<td>$-24.49$</td>
<td>$-20.30$</td>
<td>13.52</td>
</tr>
</tbody>
</table>
where \( r \) represents the distance between the donor and acceptor. \( R_0 \) is the critical distance when transfer efficiency is 50%, which can be calculated from:

\[
R_0^6 = \frac{8.8 \times 10^{-25} k^2 n^{-6} \Phi J}{C_0^2} \tag{7}
\]

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

\[
J = \frac{\sum F(\lambda) e(\lambda)\lambda^4 \Delta \lambda}{\sum F(\lambda)\Delta \lambda} \tag{8}
\]

where \( F(\lambda) \) is the fluorescence intensity of the fluorescence reagent when the wavelength is \( \lambda \) and \( e(\lambda) \) is the molar absorbance coefficient of the acceptor at the wavelength of \( \lambda \). From these equations, \( J, E \) and \( R_0 \) can be calculated, so the value of \( r \) can also be evaluated.

From Figure 6, the overlap integral calculated according to the above relationship was 1.599 \( \times \) \( 10^{-14} \) cm\(^3\) M\(^{-1}\). It had been reported that \( k^2 = 2/3, n = 1.336 \) and \( \Phi = 0.118 \) for HSA \cite{36}. Based on these data, the distance between DNR-A and the tryptophan residue in HSA was 4.71 nm. Obviously, it was lower than 7 nm after interaction between DNR-A and HSA. This accorded with the conditions of Förster’s non-radiative energy transfer theory and indicated 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 DNR-A bound to HSA.

### Conformation Investigation

To explore the structural changes of HSA on addition of DNR-A, we measured synchronous fluorescence spectra (Figure 7 and 8) of HSA with various amounts of DNR-A. Trp214, conserved in mammalian albumins, plays an important structural role in the formation of HSA \cite{2}. Spectroscopy is an ideal tool to observe conformational changes in proteins since it allows non-intrusive measurements of substances in low concentrations under physiological conditions. It is advantageous to use intrinsic fluorophores for these investigations to avoid complicated labeling with an extrinsic dye \cite{37}. Synchronous fluorescence spectroscopy, introduced by Lloyd \cite{38}, involves the simultaneous scanning of excitation and the fluorescence monochromators of a fluorimeter, while maintaining a fixed wavelength difference \((\Delta \lambda)\) between them. The synchronous fluorescence spectra give information about the molecular environment in the vicinity of the chromophore molecules. Yuan et al. \cite{39} suggested a useful method to study the environment of amino acid residues by measuring the possible shift in the wavelength emission maximum, \( K_{max} \), the shift in position of the emission maximum corresponding to changes in the polarity around the chromophore molecule. When the \( \lambda \) value \((\Delta \lambda)\) between the excitation wavelength and emission wavelength was stabilized at 15 or 60 nm, the synchronous fluorescence gave the characteristic information of tyrosine residues or tryptophan residues \cite{40}. The effects of DNR-A on HSA synchronous fluorescence spectroscopy are shown in Figure 7 and Figure 8.

It is apparent that the little stronger red shift of tryptophan residues exhibits fluorescence upon addition of drug, whereas the emission maximum of tyrosine kept...

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**Figure 6.** The overlap of the UV absorption spectrum of DNR-A with the fluorescence emission spectrum of HSA: (a) the fluorescence emission spectrum of HSA \((8 \times 10^{-7} \text{ M})\); (b) the UV absorption spectrum of DNR-A \((6.3 \times 10^{-6} \text{ M})\).

**Figure 7.** Synchronous fluorescence spectrum of HSA \((T = 293 \text{ K}, \ \text{pH} = 7.40)\), \( C_{\text{HSA}} = 2 \times 10^{-5} \text{ M}, C(\text{DNR-A})/(10^{-5} \text{ M})\); a–f: 0, 1.266, 2.53, 3.798, 5.064, 6.330, \( \Delta \lambda = 15 \text{ nm} \).
its position. The red shift of the emission maximum indicates that the conformation of HSA was changed and the polarity around the tryptophan residues was increased and the hydrophobicity decreased.\[41\]

UV-vis absorption measurements are a very simple method which are applicable for the exploration of structural changes\[28\] and to determine the complex formation.\[42\] In order to reconfirm the structural changes in HSA on addition of DNR-A, we measured the UV-vis absorbance spectra of HSA with various amounts of DNR-A. Figure 9 displays the UV-vis absorbance spectra of HSA at different contents of DNR-A. The baselines of the UV-vis absorbance spectra at 550–250 nm are raised and the absorption spectra maximum blue shift (from 302 to 281 nm). These results indicate that an interaction occurs between DNR-A and HSA, and the HSA molecules associate with DNR-A to form a DNR-A-HSA complex, while the hydrophobicity is decreased. This conclusion agrees with the results of conformational changes shown by synchronous fluorescence spectra, which indicates that the approach using synchronous fluorescence spectroscopy is scientific.\[28\]

Molecular Modeling

The complementary applications of molecular modeling have been employed using 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 sub-domains that possess common structural motifs. The crystallographic analysis reveals that the principal regions of ligand binding to HSA are located in hydrophobic cavities in sub-domains IIA and IIIA, respectively, which exhibit similar chemistry.\[1\] Despite very high stability, HSA is a flexible protein with a 3D structure susceptible to environmental factors such as pH, ionic strength, etc.\[43\] It has been 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.\[44,45\] There is a large hydrophobic cavity present in sub-domain IIA to which many drugs can bind.\[45\]

The crystal structure of HSA in complex with warfarin was taken from the Brookhaven Protein Data Bank (entry codes 1h92). 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 the molecular modeling software Sybyl 6.9.1.\[46\] 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 was built, and partial binding parameters of the DNR-A-HSA system were calculated through SGIFUEL workstations. The best energy ranked results are shown in Figure 10. As shown in Figure 10, the drug molecule was located within the binding pocket and the four rings were practically coplanar. The A-ring of DNR-A was inserted in the hydrophobic cavity of site I, and it is important to note that the tryptophan residue of HSA (Trp-214) was in close proximity to the A- and B-rings, suggesting the existence of
hydrophobic interactions between them. Furthermore, this finding provides a good structural basis to explain the efficient fluorescence quenching of HSA emission in the presence of the DNR-A. There were also hydrogen bonds between DNR-A and the residues LEU-219 and ARG-218 of HSA. This result indicated that the formation of hydrogen bonds decreased the hydrophilicity and increased the hydrophobicity to give stability in the DNR-A-HSA system. On the other hand, the amino acid residues with a benzene ring can match that of the DNR-A in space in order to confirm the conformation of the complex. The ligand binding regions of HSA located in hydrophobic cavities in sub-domain IIA were large enough to accommodate the DNR-A. The results obtained from modeling indicate that the interaction between DNR-A and HSA was dominated by hydrophobic forces.

The Effect of Common Ions on the Binding Constant

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 control of the structure and functionality of proteins. Previous studies have indicated that HSA has a high affinity metal binding site at the 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. Therefore, we are interested in examining the effect of inorganic cations and anions on the solution system of DNR-A-HSA, which can be used as a model for investigating the interaction of DNR-A with HSA. The results of the effect of common ions on the binding constants at 293 K are summarized in Table 4. Table 4 shows that the binding constants between DNR-A and protein changed in the presence of common ions, and implies stronger binding between DNR-A and HSA in the presence of common ions. As a result, the storage time of the pharmaceutical in blood plasma is prolonged and the maximum effectiveness of the drug is enhanced. Therefore, in the presence of common ions, DNR-A can be stored and removed by the proteins.

**Determination of HSA in Biology Samples**

**Precision, Limits of Detection and Working Curve**

Based on the binding of HSA to DNR-A, we employed synchronous fluorescence spectroscopy to quantitatively determine the human serum albumin and the synchronous spectra of HSA in the presence of appropriate DNR-A. This is exhibited in Figure 11. It can be seen that the synchronous fluorescence intensity \( I_{SF} \) of DNR-A was very

![Figure 10. The interaction model between DNR-A and HSA. The residues of DNR-A and HSA are represented using different tinctorial stick model. The hydrogen bond between the ligand and the protein is indicated by a dashed line.](image)

![Figure 11. Synchronous fluorescence spectra of HSA. \( C_{DNR-A} = 6.33 \times 10^{-4} \text{ M} \); from 1 - 20, \( C_{HSA} = 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, 4.4, 4.8, 5.2, 5.6, 6.0, 6.4, 6.8, 7.2, 7.6 \times 10^{-4} \text{ M} \).](image)

<table>
<thead>
<tr>
<th>Ions</th>
<th>( K \times 10^4 )</th>
<th>( R )</th>
<th>Ions</th>
<th>( K )</th>
<th>( R )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+)</td>
<td>4.24</td>
<td>0.9993</td>
<td>Fe(^{3+})</td>
<td>10.38</td>
<td>0.9985</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>4.37</td>
<td>0.9997</td>
<td>Cd(^{2+})</td>
<td>5.36</td>
<td>0.9976</td>
</tr>
<tr>
<td>F(^-)</td>
<td>5.16</td>
<td>0.9992</td>
<td>SO(_4^{2-})</td>
<td>4.88</td>
<td>0.9988</td>
</tr>
<tr>
<td>PO(_4^{3-})</td>
<td>4.27</td>
<td>0.9995</td>
<td>NH(_2^+)</td>
<td>6.53</td>
<td>0.9978</td>
</tr>
<tr>
<td>Pb(^{2+})</td>
<td>5.48</td>
<td>0.9960</td>
<td>K(^+)</td>
<td>5.60</td>
<td>0.9997</td>
</tr>
<tr>
<td>CO(_3^{2-})</td>
<td>5.74</td>
<td>0.9977</td>
<td>Zn(^{2+})</td>
<td>6.55</td>
<td>0.9992</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>5.43</td>
<td>0.9979</td>
<td>Hg(^+)</td>
<td>5.83</td>
<td>0.9970</td>
</tr>
<tr>
<td>SO(_4^{2-})</td>
<td>5.82</td>
<td>0.9968</td>
<td>C(_2O_4^{2-})</td>
<td>7.98</td>
<td>0.9978</td>
</tr>
</tbody>
</table>
weak, so that the effect of DNR-A on the determination of HSA could be eliminated, and the intensity of the synchronous fluorescence increased noticeably with increasing concentration of HSA. The enhancement intensity of synchronous fluorescence was proportional to the concentration of HSA. The linear range determined was 2.2–524.4 μg·mL⁻¹, and the linear regression equation was

\[ I_{HSA} = 2.919 + 1.433 \times 10^7 C_{HSA} \]  

with a correlation coefficient (R) of 0.9997. The detection limit for HSA, as defined by IUPAC, was determined to be 2.049 μg·mL⁻¹ [47] The relative standard deviation (RSD) was 1.05% for HSA, as obtained from 6 replicate determinations of 6.33 × 10⁻⁴ M for DNR-A.

Analysis of Biology Samples

Because the present method shown 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 and 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 and 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 pre-treatment. Urine and 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 pre-treatment. Urine and saliva samples were also diluted appropriately. Table 5 displays 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.

## Conclusion

The interaction of DNR-A with HSA has been investigated in vitro under simulated physiological conditions (pH 7.4, ionic strength 0.1) using molecular modeling and different optical techniques. The data from fluorescence and synchronous fluorescence spectra indicated that the changes in the microenvironment of HSA are induced by the binding of DNR-A. The molecular docking study and thermodynamic analysis also suggested that DNR-A could bind to HSA through hydrophobic forces, electrostatic interactions and hydrogen bonding between DNR-A and the HSA residue. According to competitive binding experiments, the binding site is located in the hydrophobic pocket of sub-domain IIA. Based on this, a means of sensitive determination of protein was established. Detecting the protein in real samples validated its reliability and the results were satisfactory. Therefore, this method has potential for application in biochemistry and clinical practice. Further work is necessary to obtain a more basic understanding of the mechanism of the binding action.

### Acknowledgements

This work was sponsored by the Nature Science Foundation of China (No. 20673034), the Young Backbone Teacher Sustentation Plan of Henan Universities (No. 200470) and the Department of Education of Henan Province (No.2006150012) (to F.C.), and the National Nature Science Foundation of China (20672031) and a fund from the Department of Education of Henan Province (2006-HACET-06) (to G. Z).

Received: April 29, 2008; Revised: July 13, 2008; Accepted: July 15, 2008; DOI: 10.1002/mabi.200800105

## Keywords

fluorescence; molecular modeling; proteins; spectroscopy; thermodynamics

```
<table>
<thead>
<tr>
<th>Samples</th>
<th>Added (g·mL⁻¹)</th>
<th>Found (μg·mL⁻¹)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
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<tr>
<td>Serum</td>
<td>0</td>
<td>97.91</td>
<td>0.53</td>
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<tr>
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<td>55.2</td>
<td>157.88</td>
<td>103.1</td>
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<tr>
<td></td>
<td>110.4</td>
<td>210.33</td>
<td>100.97</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>165.6</td>
<td>270.32</td>
<td>102.58</td>
<td>0.78</td>
</tr>
<tr>
<td>Urine</td>
<td>0</td>
<td>16.36</td>
<td>0.42</td>
<td></td>
</tr>
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<td></td>
<td>55.2</td>
<td>70.10</td>
<td>97.96</td>
<td>0.56</td>
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<tr>
<td></td>
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<td>123.35</td>
<td>97.31</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>165.6</td>
<td>177.56</td>
<td>97.58</td>
<td>0.98</td>
</tr>
<tr>
<td>Saliva</td>
<td>0</td>
<td>71.69</td>
<td>0.23</td>
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</tr>
<tr>
<td></td>
<td>55.2</td>
<td>130.15</td>
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</tr>
<tr>
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<td>110.4</td>
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<td>0.85</td>
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<td>165.6</td>
<td>239.76</td>
<td>101.04</td>
<td>1.13</td>
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</table>
```

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Macromol. Biosci. 2008, 8, 1079–1089
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