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Spectroscopic and thermodynamic characterization of the chemotherapy drug Epirubicin interaction with human serum albumin

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In the present work we studied the interactions of the chemotherapy drug Epirubicin (Epi) with human serum albumin (HSA) by fluorescence spectroscopy and differential scanning calorimetry (DSC). The Epi-HSA binding parameters were evaluated by the fluorescence quenching method. As shown by intensity measurements, Epi strongly quenched the intrinsic HSA fluorescence. The calculated Stern-Volmer quenching constant Ksv, the association binding constant K_a of the Epi-HSA complex, as well as the thermodynamic parameters, including ΔH°, ΔS° and ΔG° indicated that hydrophobic forces play a major role in the interaction of Epi with HSA. These measurements point to a quenching mechanism based on Epi-HSA static complex formation rather than to dynamic collisions. This conclusion well agrees with the DSC heat capacity profiles which showed a strong increase of the post-denaturation HSA aggregation with increase of the Epi concentration. This work provides new insights into the mechanism of Epi-HSA interactions and contributes to a better understanding of the effects of chemotherapy drugs on the stability and structural modifications of the carrier blood plasma proteins.

Keywords: Epirubicin; human serum albumin; fluorescence spectroscopy; differential scanning calorimetry; quenching; drug binding

INTRODUCTION

The pharmacokinetics and pharmacodynamics of any drug will strongly depend on its interaction with human serum albumin (HSA), the most abundant plasma protein. HSA is an important carrier of exogenous and endogenous molecules in human blood plasma. It has high binding affinity to many drugs and thus facilitates their transport throughout the blood circulation [1-3]. The nature of the binding has direct consequences on drug delivery, pharmacokinetics, pharmacodynamics, and therapeutic efficacy. Epirubicin hydrochloride (Epi**,** Fig. 1**)** is an anthracycline drug used in the treatment of a range of neoplastic conditions including breast carcinoma and gastric cancer. It can be administered in combination with other medications to treat breast cancer in patients who have had surgery to remove the tumor. Epirubicin acts by intercalating DNA strands and generating free radicals that cause cell and DNA damage. Binding to cell membranes and plasma proteins may be involved in the compound's cytotoxic effects.

Several reports have shown that the mechanism of Epi action appears to be related to its ability to bind to nucleic acids. Epi was found to bind differently to the various plasma proteins *in vitro*, and *in vivo* [5-7].

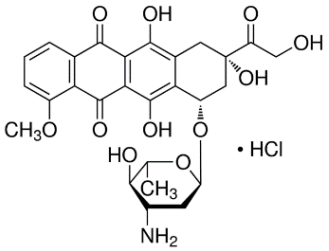

Figure 1. Chemical structure of Epirubicin hydrochloride (synonyms 4′-Epidoxorubicin hydrochloride, Epidoxorubicin hydrochloride [4]).

The *in vitro* binding rate of Epi to plasma proteins, namely alpha-HSA, gamma-HSG, alpha+beta-HSG and with isolated human red blood cells was shown to depend on the concentration of the matrix components, but no evidence is available on how Epi interacts with blood plasma proteins upon intravenous infusion. We thus decided to investigate here the fluorescence and DSC profiles of HSA upon binding of medication Epi and to use these profiles for the characterization of the Epi-HSA interaction parameters. With regard to its biological relevance, the present study demonstrated that there are significant changes in the fluorescence

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and DSC properties/parameters that may serve to evaluate the therapeutic effectiveness of anti-cancer drugs such as Epirubicin.

MATERIALS AND METHODS

Stock solutions of fatty acid-free human serum albumin and Epirubicin hydrochloride solution for injection (2 mg/ml) were prepared in PBS, pH 7.4, and in saline for the fluorescence spectroscopy study and for DSC measurements, respectively.

Fluorescence spectroscopy

A Scinco 2 South Korea spectrofluorimeter was used to measure fluorescence spectra with a 1.0 cm quartz cuvette. Bandwidths for both excitation and emission were 5 nm. Epi–HSA fluorescence measurements were carried out by keeping the concentration of HSA fixed at 4 μM and those of Epi were 7, 14, 27, 54, 68 μM. Fluorescence spectra were recorded at two different temperatures of 25 and 37 °C in the spectral emission range 300–500 nm upon excitation at 283 nm (n = 5 replicates).

Differential scanning calorimetry (DSC)

Thermal denaturation of HSA in the absence and presence of different Epi concentrations was studied by Nano DSC from Thermal Analysis Instruments with 300 μl measuring cell volume. For each sample, two consequent heating scans were performed at a scan rate of 1 K/min in the range of 0-110°C. To investigate the thermal transitions ongoing in the native samples, the second heating scans corresponding to denatured samples were used as baseline and were subtracted from the first heating scans of the native samples.

RESULTS AND DISCUSSION

Fluorescence spectroscopy

Fluorescence quenching. It is well known that HSA intrinsic fluorescence is due to the presence of specific amino acids, mainly tryptophan (Trp) and tyrosine (Tyr) residues [8]. The binding of a ligand to a protein may directly affect the fluorescence of a tryptophan residue by acting as a quencher or by interacting with the fluorophore, dynamically changing the polarity of its surrounding conditions. The fluorescence intensity of a compound can be decreased by several types of molecular interactions including excited-state reactions, energy transfer, collisional quenching, molecular rearrangements, and ground state complex formation. The different mechanisms of quenching are usually classified as either dynamic quenching or static quenching. Dynamic and static quenching can be distinguished by their temperature and viscosity dependencies [9, 10].

In our study, the interaction between Epi and HSA was investigated upon exciting HSA at 283 nm where both Trp and Tyr residues get excited [11, 12] The variations of the emission spectra of solutions at fixed HSA concentration and varying Epi concentrations at 25 and 37 °C are presented in Figure 2.

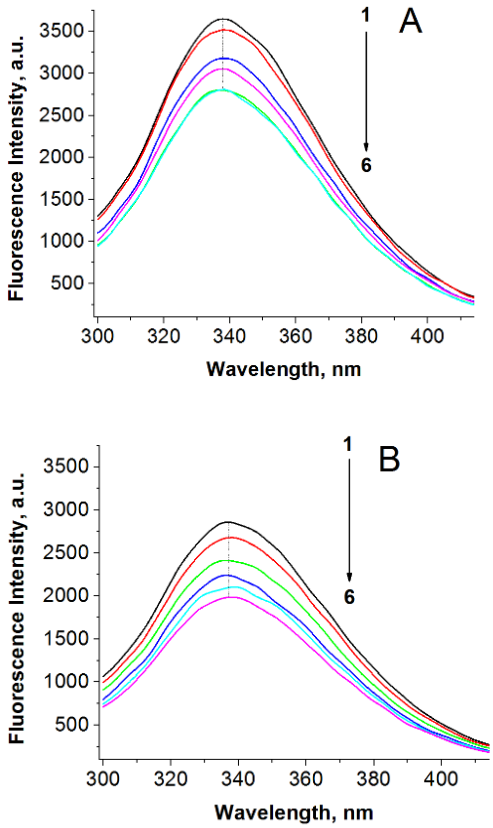

Figure 2. Fluorescence titration curve of HSA (4 μM) with the Epi complex in PBS, pH 7.4, at A. 25 °C and B. 37 °C. Curves 1-6 correspond to Epi concentrations of 0, 7, 14, 27, 54, 68 μM, respectively.

As is clear from Figures 2A and 2B, the intrinsic fluorescence spectra of HSA were strongly quenched by Epi addition at both temperatures. There is no significant shift of the maximum emission wavelength at 338 nm implying that the interaction of Epi with HSA does not affect the conformation of the region surrounding the Tyr and Trp residues [9].

To analyze the fluorescence quenching mechanism, the quenching data about HSA were evaluated using the classical Stern-Volmer equation (eq. 1):

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

where F_0 and F are the fluorescence intensities in the absence and presence of a quencher, respectively, Q is the total concentration of the quencher (Epi), and K_{SV} is the Stern-Volmer quenching constant. Kq and τ_0 are the quenching rate constant, and the average lifetime of the biomolecule without quencher, respectively. Since the fluorescence lifetime of the biopolymer is ~10^{-8} s [13], the quenching rate constant, Kq can be calculated using equation (2):

$$K_q = \frac{K_{SV}}{\tau_0} \quad (2)$$

The intensity changes are given in Stern–Volmer representation (Figure 3). From the plots of F_0/F *vs.* Q the type of quenching can be determined, i.e., these plots can be either linear for static or dynamic quenching, or display upward bent curve for mixed quenching types [9].

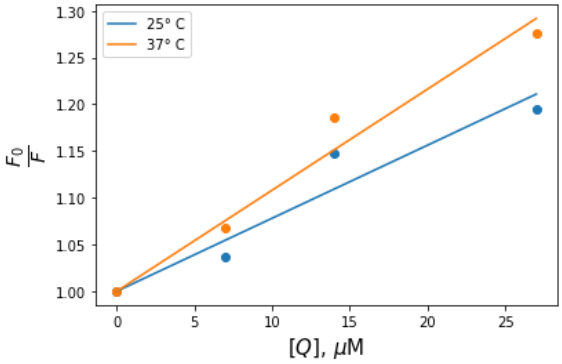

Figure 3. Stern-Volmer plot for quenching of different Epi concentrations (7-27 μM) to HSA (4 μM) in PBS solution at 25 and 37 °C.

The Stern–Volmer plot of the fluorescence quenching shows complex or mixed interactions. If the interaction is either poorly dynamic or static, the Stern–Volmer plot of the intensity gives a linear correlation between the quencher concentration and the intensity.

For the linear plots obtained in the present study, the Stern-Volmer quenching constant K_{SV} can be calculated using equation (1). In order to obtain values for the fluorescence quenching constant we used the Nedler-Mead simplex algorithm to fit a linear regression model to F_0/F and Q for the first four values of Epi (0-27 μM). The values for K_{SV} at two temperatures are given in Table 1.

Table 1. Stern-Volmer quenching constants K_{sv}, and quenching rate constant K_q for HSA complexes with Epi.

Temperature, [°C]	K_{SV}, [10^4 M^{-1}]	Kq, [10^{12} $M^{-1}s^{-1}$]
25	0.78	0.78
37	1.08	1.08

It can be seen that, upon increasing the temperature from 25 to 37 °C, the values of K_{SV} were not significantly increased suggesting that the Epi does not act as a dynamic quencher (1). The calculated values of K_q were greater than the limiting diffusion rate constant of the biomolecule (2×10^{10} $L \cdot mol^{-1} \cdot s^{-1}$). This implied that the quenching was not induced by dynamic collisions but rather originated from the formation of a ground-state complex, and indicated that the probable quenching mechanism of fluorescence of HSA by the Epi is a static quenching procedure [14].

The quenching results were also analyzed using the modified Stern-Volmer equation [13]:

$$\frac{\log(F_0 - F)}{F} = \log K_a + n \log[Q] \quad (3)$$

where K_a and n are the binding constant and number of binding sites, respectively.

The value of K_a is important to understand the affinity of the drug to plasma proteins. The binding of Epi to HSA is of importance as it changes the pharmacological activity of the drug. It is known that protein-binding may alter drug activity in two different ways: by changing the plasma drug effective concentration at its site of action or by changing the rate at which the drug is eliminated, thus changing the length of time for which effective concentrations are maintained. Thus, weak binding can lead to a short-lived protein-ligand complex, while strong binding can decrease the concentrations of the free drug in plasma.

The values of K_a and *n* for the Epi-HSA complex were calculated from the intercept and slope of the plot of log (F0- F)/F *vs.* log[Q] (Table 2).

Table 2. Binding constants Ka of Epi–HSA at two different temperatures.

Temperature, [°C]	Ka, [10^{-4} M^{-1}]
25	5.29
37	6.88

The plots for a representative system, Epi–HSA at the two different temperatures are given in Figure 4.

It should be noted that the binding constant values slightly increased with increasing temperature which suggests that at higher temperatures the increased mobility inside the serum albumin structure allows the ligand to find improved binding within the protein pocket. The binding constants calculated for Epi–HSA suggest low affinity of the drug-albumin binding.

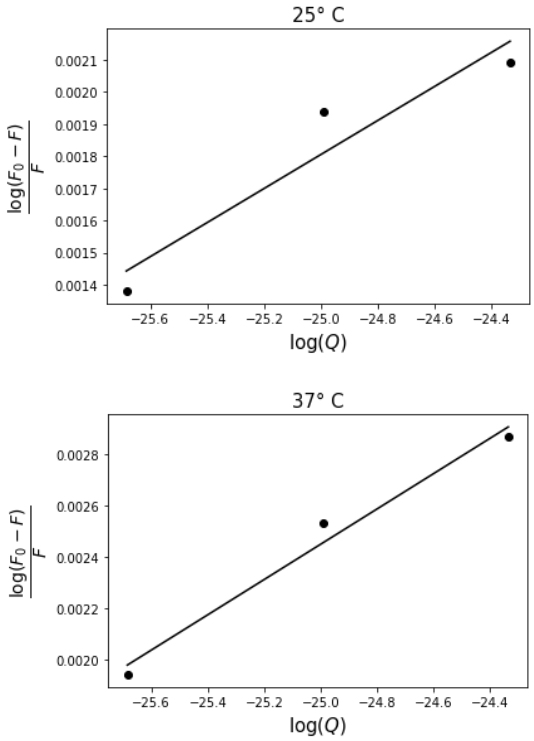

Figure 4. Modified Stern-Volmer plots for the EPI-HSA complex at A. 25 and B. 37 °C.

Thermodynamic parameters. There are several kinds of binding forces that might occur between the drug and the biomolecule. Thermodynamic enthalpy change (ΔH^0), entropy change (ΔS^0), and free energy change (Gibbs energy ΔG^0), provide estimable information in studying the interaction between the biomolecule and the drug. These values are an indicator of the prevalent driving forces associated with the binding [14].

To depict the intermolecular forces existing between Epi and HSA, the temperature-dependent thermodynamic system was utilized and evaluated at 25 and 37 °C. After estimating the binding parameters, the van't Hoff equation (equation (4)) provides the different thermodynamic parameters of the complexes [14]:

$$\ln K_a = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{T} \quad (4)$$

The values of ΔG^o can be obtained from the binding constants K_a at the two different temperatures [16]:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = -RT \ln K_a \quad (5)$$

where K_a represents the binding constant at its corresponding temperature and R is the gas constant. ΔG can be determined using van't Hoff plot, where ΔH^0 is the slope and ΔS the intercept.

In our experiments the binding process was connected with positive changes in enthalpy and Gibbs energy ($\Delta H^0>0$ and $\Delta G^0>0$), that indicate a non-spontaneous hydrophobically associated process [16]. In our study, a partial immobilization of the protein and the ligand is related to the obtained positive ΔH^0=11.77 kJ/M due to hydrophobic association processes, and partially offset by the negative ΔS^0= -6.45 $J.M^{-1}$ due to the loss of translation and rotation (if any) in the hydrophobically associated species [16]. The non-spontaneous binding of drug to the biomolecule is represented by the positive sign of ΔG^0 = 18.69 kJ M^{-1} and ΔG^0= 18.76 kJ M^{-1} at the two temperatures (25 and 37 °C), respectively [16].

Differential scanning calorimetry (DSC)

The DSC results of the influence of increasing Epi concentrations on the HSA denaturation heat capacity profile are summarized in Figure 5.

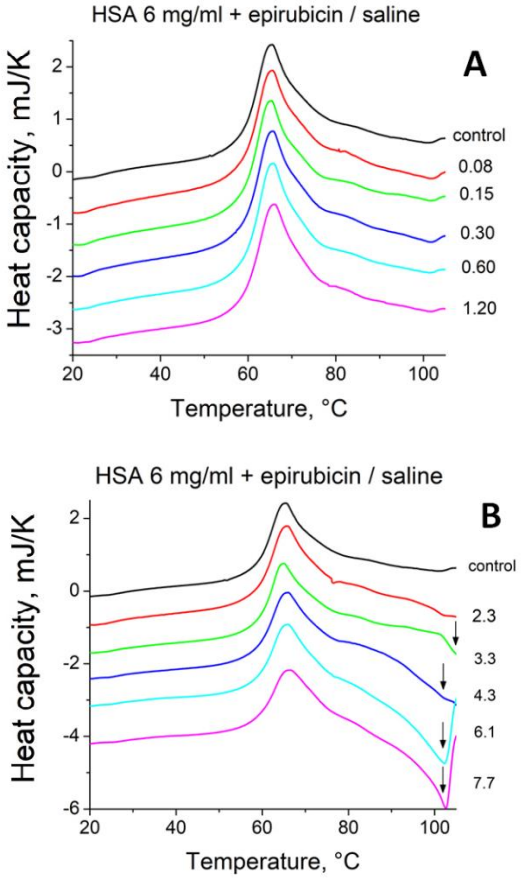

Figure 5. Heat capacity profiles obtained by DSC of Epi-HSA mixtures. The numbers on the right of the thermograms show the Epi-HSA molar ratio. The arrows in panel B point to the regions of post-denaturation HSA aggregation at high Epi-HSA ratios. The thermograms in panels A and B are vertically displaced for clarity.

They show that in the range of low Epi-HSA molar ratios (up to about 1.2 Epi molecules per one HSA molecule, Fig. 5A) Epi does not noticeably

affect the HSA denaturation profiles relative to the control, in accord with the conclusion derived from the fluorescence data about low affinity binding of the drug to HSA. However, a stronger Epi-HSA interaction takes place at higher Epi-HSA molar ratios in the range of Epi-HSA above 2 (Fig. 5B). In the latter range Epi brings about a well expressed post-denaturational HSA aggregation displayed in the downward exothermic slopes of the heat capacity curves at high temperatures (indicated by the arrows in Fig. 5B). The enhanced HSA aggregation might result from exposure of additional non-polar HSA regions upon denaturation taking place at high Epi concentrations that substantially exceed the 1:1 Epi-HSA molar ratio. This conclusion is also in accord with the fluorescence results indicating a predominant role of the hydrophobic interactions in the formation of the Epi-HSA complexes.

CONCLUSIONS

The interaction between Epirubicin, a frequently used antitumor drug, and human serum albumin, a blood plasma carrier protein, was studied at different temperatures by fluorescence spectroscopy and differential scanning calorimetry (DSC) techniques.

Fluorescence quenching results revealed the formation of static complexes between Epi and HSA. The binding was determined to be due to hydrophobic interactions. The Epi binding to HSA resulted in significant alterations of the HSA structure and conformation displayed in decreasing protein stability and an increase of the non-polar or accessible hydrophobic surface of HSA to solvent. The significant post-denaturation HSA aggregation at high Epi concentrations revealed by DSC well agrees with the conclusions based on the fluorescence quenching data suggesting low binding affinity of Epi to HSA. The observed effects are of certain interest as they may influence the balance *between bound and freely circulating drugs in cases of simultaneous Epi application with other drugs, thus affecting the drug pharmacokinetics, efficacy and adverse side impact. This study helps to gain useful insights into the significance of the binding of anti-cancer drugs with the most abundant plasma carrier protein, serum albumin, on the drug overall distribution and pharmacological activity.

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