Study of the Interaction of Carbamazepine with Bovine Serum Albumin by Fluorescence Quenching Method

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The interaction between carbamazepine (CBZ) and bovine serum albumin (BSA) was studied using fluorescence spectroscopy (FS) and ultraviolet spectroscopy (UV). The experimental results showed that the CBZ could insert into the BSA and quench the inner fluorescence of BSA by forming the CBZ-BSA complex. It was found that both static quenching and non-radiation energy transfer were the main reasons leading to the fluorescence quenching. The apparent binding constants (K) between CBZ and BSA were found to be 1.8×10^4 (27°C) and 2.8×10^4 (37°C) and the binding site values (n) were 0.97 (27°C) and 1.01 (37°C), respectively. According to the Förster theory of non-radiation energy transfer, the binding distances (r) between CBZ and BSA were 3.6 nm and 3.4 nm at 27°C and 37°C, respectively. The process of the binding was a spontaneous molecular interaction in which entropy increased and Gibbs free energy decreased, indicating that the interaction between CBZ and BSA was mainly driven by the hydrophobic force.

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Introduction

In recent years, the interaction between protein and drug molecules has increasingly attracted the research attention of chemists, pharmacists and biologists. Among proteins, serum albumins are the major soluble protein constituents of the circulatory system and have many physiological functions.^{1,2} The most important property of this group of proteins is that they serve both as a depot protein and as a transport protein for a variety of compounds, including exogenous compounds such as drugs. Bovine serum albumin (BSA) has been one of the most extensively studied of this group of proteins, particularly because of its structural homology with human serum albumin (HSA).^{3,4} It has been shown that the distribution, free concentration and the metabolism of various drugs may be strongly affected by drug-protein interaction in the blood stream.⁵ Therefore, study of the interaction between protein and drug molecules will help provide basic information on the pharmacological actions, bio-transformation, bio-distribution, *etc.* of drugs. The binding phenomena will also be useful to explain the relationship between the structures and functions of proteins.

Carbamazepine (CBZ, its molecular structure is shown in Fig. 1) is a tricyclic neutral lipophilic compound that is widely used in the treatment of partial epilepsy, trigeminal neuralgia and as an adjunct to neuroleptic therapy in psychosis. Due to its favorable therapeutic profile, it has become one of the most widely used anticonvulsant drugs.⁶

Fluorescence spectroscopy (FS) and ultraviolet spectroscopy (UV) have been widely used to study the interactions between the small molecule ligands and bio-macromolecules. In this study, the interaction between CBZ and BSA has been investigated by using FS and UV. Our experimental results

showed that the CBZ could insert into the BSA and quench the inner fluorescence by forming the CBZ-BSA complex and that static quenching and non-radiation energy transfer were the main reasons leading to the fluorescence quenching. In addition, the binding constants and binding mechanism of the process were also proposed in this work. To our knowledge, this is the first report about the study on interactions between CBZ and BSA.

Experimental

Apparatus

A Hitachi F-4500 fluorescence spectrophotometer equipped with a 1.0 cm quartz cell and a thermostat bath was used to measure the fluorescence spectra and the fluorescence intensity. The excitation wavelength and emission wavelength for BSA were set at 285 and 343 nm, respectively, with the excitation and emission slit widths set as 5.0 nm. The absorption spectrum was recorded with a UV-250 recording spectrophotometer (Shimadzu).

Reagents

Carbamazepine was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), BSA (electrophoresis grade reagents) from Sigma, and Tris (no less than 99.5% pure) from Serva (New York, USA). NaCl solution (0.1 mol l⁻¹) was used to keep the ionic strength

Fig. 1 Molecular structure of CBZ.

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Fig. 2 The quenching effect of CBZ on BSA fluorescence. $\lambda_{\text{ex}}/\lambda_{\text{em}} = 285 \text{ nm}/343 \text{ nm}$, $C_{\text{BSA}} = 1.0 \times 10^{-5} \text{ mol l}^{-1}$, C_{CBZ} from 1 to 9: 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 and $6.0 (\times 10^{-5} \text{ mol l}^{-1})$.

Fig. 3 The Stern-Volmer curves of fluorescence quenching of BSA by CBZ.

and 0.05 mol l^{-1} Tris-HCl buffer solution was selected to keep the pH of the solution at 7.4. BSA solution ($1.0 \times 10^{-5} \text{ mol l}^{-1}$) was prepared in pH 7.4 Tris-HCl buffer solution. CBZ ($2.0 \times 10^{-3} \text{ mol l}^{-1}$) was obtained by dissolving appropriate amounts of it in ethanol. All other chemicals were of analytical reagent grade and all solutions were prepared with doubly distilled water.

Procedure

Appropriate quantities of $2.0 \times 10^{-3} \text{ mol l}^{-1}$ CBZ ethanol solution were transferred to a 10 ml flask and evaporated to dryness under a gentle stream of nitrogen gas, then 10 ml of $1.0 \times 10^{-5} \text{ mol l}^{-1}$ BSA solution was added. The resultant mixture was subsequently ultrasonicated for 5 min and incubated at 27°C or 37°C for 3 h. A blank system containing only 10 ml $1.0 \times 10^{-5} \text{ mol l}^{-1}$ BSA solution was prepared similarly. Fluorescence quenching spectra and fluorescence intensity were obtained with a fluorescence spectrophotometer.

For absorption spectra (UV) experiments, $1.0 \times 10^{-5} \text{ mol l}^{-1}$ CBZ solution was prepared in pH 7.4 Tris-HCl buffer solution. The spectral scanning curve was taken at both 27°C and 37°C .

Results and Discussion

Fluorescence quenching spectra

Figure 2 shows the emission spectra of BSA in the presence of various concentrations of CBZ. It was observed that the fluorescence intensity of BSA decreased regularly with the increasing concentration of CBZ, but that there was no

Fig. 4 Double-log plot of CBZ quenching effect on BSA fluorescence at different temperatures.

significant λ_{em} shift with the addition of CBZ, which indicated that CBZ can quench the inner fluorescence of BSA and that the interaction between CBZ and BSA has occurred.

Quenching can occur by different mechanisms, usually classified as dynamic quenching and static quenching. Dynamic and static quenching can be distinguished by their different dependence on temperature and excited-state lifetime. Higher temperatures will result in faster diffusion and hence larger amounts of collisional quenching and higher temperatures will typically result in the dissociation of weakly bound complexes and hence smaller amounts of static quenching. For the dynamic quenching, the mechanism can be described by the Stern-Volmer equation:^{7,8}

$$F_0/F = 1 + K_q \tau_0 [Q] = 1 + K_{\text{SV}} [Q] \quad (1)$$

where F_0 and F represent the fluorescence intensities in the absence and in the presence of quencher, K_q is the quenching rate constant of the bimolecule, K_{SV} is the dynamic quenching constant, τ_0 is the average lifetime of the molecule without quencher and $[Q]$ is the concentration of the quencher. In order to confirm the quenching mechanism, the procedure of the fluorescence quenching was first assumed to be dynamic quenching.

Figure 3 displays the Stern-Volmer plots of the quenching of BSA by CBZ at different temperatures. From the experimental data, the corresponding dynamic quenching constants for the interaction between CBZ and BSA were $K_{\text{SV}} = 2.62 \times 10^4$ (27°C , $R = 0.9963$) and $K_{\text{SV}} = 2.58 \times 10^4$ (37°C , $R = 0.9959$), respectively. Because the fluorescence life time of the biopolymer is 10^{-8} s ,⁹⁻¹¹ the quenching constants K_q at 27°C and 37°C were calculated to be 2.62×10^{12} and $2.58 \times 10^{12} \text{ l mol}^{-1} \text{ s}^{-1}$, respectively. According to the literature,^{12,13} for dynamic quenching, the maximum scatter collision quenching constant of various quenchers with the biopolymer is $2.0 \times 10^{10} \text{ l mol}^{-1} \text{ s}^{-1}$, and the K_{SV} increases with increasing temperature. Considering that in our experiment the rate constant of the protein quenching procedure initiated by CBZ is much greater than $2.0 \times 10^{10} \text{ l mol}^{-1} \text{ s}^{-1}$ and that the K_{SV} decreased with increased temperature, it can be concluded that the quenching is not initiated by dynamic quenching, but probably by static quenching resulting from the formation of CBZ-BSA complex.

Binding constant and binding sites

For static quenching, the relationship between fluorescence quenching intensity and the concentration of quenchers can be

Fig. 5 Overlap spectra of CBZ's UV absorption spectra (1) and BSA's fluorescence emission spectra (2): $C_{\text{BSA}} = 1.0 \times 10^{-5} \text{ mol l}^{-1}$, $C_{\text{CBZ}} = 1.0 \times 10^{-5} \text{ mol l}^{-1}$.

described by the binding constant formula:^{14,15}

$$\log(F_0 - F)/F = \log K + n \log [Q] \quad (2)$$

where K is the binding constant, and n is the number of binding sites per BSA. After the fluorescence quenching intensities on BSA at 343 nm were measured, the double-logarithm algorithm was assessed by Eq. (2). Figure 4 shows the double-logarithm curve and Table 1 gives the corresponding calculated results. The correlation coefficients are larger than 0.995, indicating that the interaction between CBZ and BSA agrees well with the site-binding model underlying the Eq. (2). The result illustrates that there is a strong binding force between CBZ and BSA, and a binding site would be formed.

Binding distance between the drug and the amino acid residues of BSA

According to the Förster non-radiation energy transfer theory,^{16,17} the energy-transfer effect is related not only to the distance between the acceptor and donor, but also to the critical energy-transfer distance (R_0).

$$E = R_0^6 / (R_0^6 + r^6) \quad (3)$$

$$E = 1 - F/F_0 \quad (4)$$

where E is the energy transfer efficiency, R_0 is the critical distance when the transfer efficiency is 50%, and r is the binding distance between donor and acceptor.

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^4 \Phi J \quad (5)$$

where K^2 is the spatial orientation factor of the dipole, N the refractive index of the medium, Φ the fluorescence quantum yield of the donor, and J the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. Therefore,

$$J = (\sum I_F(\lambda) \epsilon_D(\lambda) \lambda^4 \Delta\lambda) / (\sum I_F(\lambda) \Delta\lambda) \quad (6)$$

Here $I_F(\lambda)$ is the fluorescence intensity of the fluorescence donor at wavelength λ and $\epsilon_D(\lambda)$ the molar absorptivity of the acceptor at wavelength λ .

The overlap of the absorption spectrum of CBZ and the fluorescence emission spectrum of BSA is shown in Fig. 5. The overlap integral was calculated to be $3.24 \times 10^{-15} \text{ cm}^3 \text{ L mol}^{-1}$ at 27°C by integrating the spectra for 300–500 nm in Fig. 5. The critical distance, R_0 , corresponding to 50% energy transfer from

Table 1 The binding parameters for the system of CBZ-BSA

Temp./°C	Binding constant/ l mol^{-1}	Binding site	Correlation coefficient
27	1.8×10^4	0.97	0.9966
37	2.8×10^4	1.01	0.9956

Table 2 The thermodynamic parameters of CBZ-BSA binding procedure

Temp./°C	$\Delta H/\text{kJ mol}^{-1}$	$\Delta G/\text{kJ mol}^{-1}$	$\Delta S/\text{J mol}^{-1} \text{ K}^{-1}$
27	33.9	−24.36	194.85
37	33.9	−26.31	194.85

BSA to CBZ was calculated to be 2.11 nm from the Eq. (5) when $K^2 = 2/3$, $N = 1.336$ and $\Phi = 0.15$.^{9,18} The binding distance, r , between CBZ and the amino acid residue in BSA was found to be 3.6 nm and 3.4 nm at 27°C and 37°C, respectively, which are much smaller than 7 nm, a criterion value for energy transfer phenomenon to occur,^{16,17} suggesting that the energy transfer from BSA to CBZ may occur with high possibility.

Thermodynamic parameters and nature of the binding forces

The interaction forces between drug and biomolecule may involve hydrophobic forces, electrostatic interactions, van der Waals interactions, hydrogen bonds, etc. In order to elucidate the interaction of CBZ with BSA, we calculated the thermodynamic parameters from Eqs. (7)–(9). If the temperature does not vary significantly, the enthalpy change (ΔH) can be regarded as a constant. The free energy change (ΔG) can be estimated from the following equation, based on the binding constants at different temperatures:

$$\Delta G = -RT \ln K \quad (7)$$

where R is the gas constant, T is the experimental temperature, and K is the binding constant at the corresponding T . Then the enthalpy change (ΔH) and entropy change (ΔS) can be calculated from the Eqs. (8) and (9):

$$\ln K_2/K_1 = [1/T_1 - 1/T_2] \Delta H/R \quad (8)$$

where K_1 and K_2 are the binding constant at the experiment temperatures T_1 and T_2 , respectively.

$$\Delta G = \Delta H - T \Delta S \quad (9)$$

The thermodynamic parameters for the interaction of CBZ with BSA are shown in Table 2. The negative sign for ΔG means that the interaction process is spontaneous. The positive ΔH and ΔS values indicate that the binding between CBZ and BSA is mainly ΔS -driven, with little contribution from the enthalpy factor, and the hydrophobic force may play a major role in the reaction.¹⁹

Conclusion

In this paper, the interaction of CBZ with BSA has been studied by FS and UV. The experiment results indicated that CBZ can

interact strongly with BSA through the hydrophobic force, and that the probable quenching mechanism of BSA fluorescence by CBZ is static quenching and non-radiation energy transfer. The apparent binding constants (K) between CBZ and BSA were 1.8×10^4 (27°C) and 2.8×10^4 (37°C), and the binding site values (n) were 0.97 (27°C) and 1.01 (37°C), respectively. According to the Förster theory of non-radiation energy transfer, the binding distances (r) between CBZ and BSA were 3.6 nm and 3.4 nm at 27°C and 37°C.

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