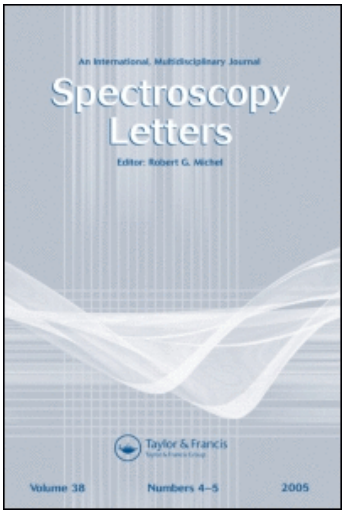

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Spectroscopic Study of the Interaction Between Folic Acid and Bovine Serum Albumin

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Spectroscopic Study of the Interaction Between Folic Acid and Bovine Serum Albumin

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Abstract: The interaction between folic acid and bovine serum albumin (BSA) was investigated by spectroscopic methods including fluorescence, circular dichroism (CD), and UV-Vis absorption adsorption spectroscopies. The binding constant K_A and the number of binding sites, n, were determined based on the fluorescence quenching of BSA in the presence of folic acid. The thermodynamic parameters ΔH, ΔG, ΔS at different temperatures were calculated, and the results indicated that the acting force between folic acid and BSA was mainly hydrogen bonding. The distance between the donor (BSA) and acceptor (folic acid) was obtained according to Förster's theory of nonradiational energy transfer. The quenching mechanism of the BSA–folic acid system is a combination of static quenching and nonradiative energy transfer. The CD and synchronous fluorescence spectra were used to investigate the structural change of BSA upon addition of folic acid, and the result indicated that the secondary structure of BSA changed in the presence of folic acid.

Keywords: Bovine serum albumin, fluorescence quenching, folic acid, interaction

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INTRODUCTION

Serum albumin can bind to many endogenous and exogenous materials. The interaction between drugs and serum albumin greatly effects the transportation and metabolic process of drugs in organisms. When drugs get into the human body, they reach a receptor site and produce pharmacological always by the deposition and transportation of plasma. Most organic drugs will bind to albumin more or less in plasma. The bound drugs don't easily penetrate the capillary wall and this not only confines further transportation but also effects the metabolism and distribution of drugs in the human body. Moreover, the binding of drugs to albumin decides the residual quantity of drugs in plasma and affects the maximum action intensity of drugs but contributes to prevent the action of drugs from fluctuating severely and prolonging of the action time.[1] So the study of interaction between drugs and proteins has been an important content of drug dynamics and clinical pharmacology. The molecular interactions are often monitored by using optical techniques. These methods are sensitive and relatively easy to use, whereas fluorescence spectroscopy is a more valuable technique for studying the interaction between biomolecules and micromolecules.

The primary structure of bovine serum albumin (BSA) is well-known for a long time, and its tertiary structure was determined a few years ago by X-ray crystallography. BSA consists of 585 amino acids and has two tryptophans (Trp212 and Trp134). One (Trp212) of the two tryptophans in BSA is located in a chemical microenvironment, and the other tryptophan in BSA (Trp134) is located on the surface of the molecule like other serum albumin. BSA has three basis repeat units named structural domains. Each domain can be divided into two subdomain's that include a "trpC" loop and a fused subdomain like "trpA and trpB." The six subdomains assemble to form a column-shaped structure, and all the hydrophobic amino acid lie at the bottom of the column-shaped structure. So BSA has an affinity for negatively-charged hydrophobic molecules.[2]

Folic acid (FA), also named vitamin B_{11} or vitamin Bc, belongs to group B vitamins.[3] Its chemical name is pteroylalutamic acid (PGA). It is composed of 2-amino-4-hydroxy-6-methyl pterin, *para*-aminobenzoic acid, and L-glutamic acid (Fig. 1). Folic acid will lose its activity of vitamin without glutamic acid. Watson[4] proved in 1946 that folic acid has high therapeutic effect on the treatment of malignant anemia. Folic acid is necessary to maintain the normal vital movement of an organism. Biochemical and medical study has confirmed that folic acid as a coenzyme is very important to the biosynthesis of purine, pyrimidine, nucleic acid, and protein, and to the division and formation of cells.[5] Shortage of folic acid easily results in the declining of physiological function and some diseases such as megaloblastic anemia, gastrointestinal dysfunction, mental retardation, and neurovascular abnormal newborn.[3]

At present, investigation about the interaction between folic acid and bovine serum albumin has not been reported. In this study, we studied the

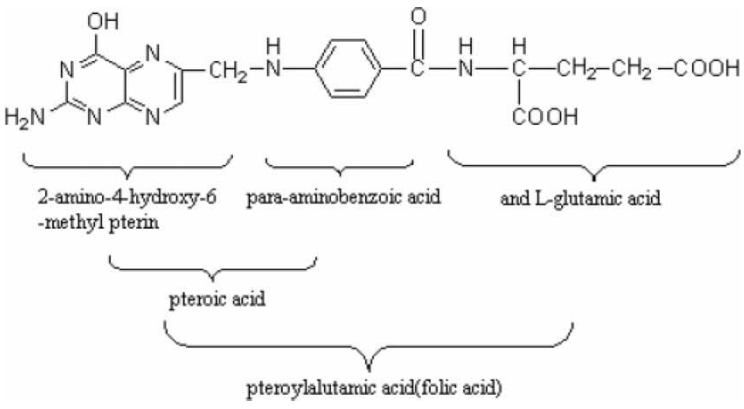

Figure 1. The structure of folic acid.

binding reaction between folic acid and bovine serum albumin using the theory of energy transfer and quenching of fluorescence. Moreover, we also discuss the conformational change of BSA with synchronous fluorescence spectrum and CD spectrum. The current work supplies useful information for studying the deposition, transportation, and pharmacologic action of folic acid in the body.

MATERIALS AND METHODS

Apparatus

All fluorescence measurements were carried out on an LS-55 recording spectrofluorimeter (PE, USA) and appropriate blanks corresponding to the buffer were subtracted to correct background fluorescence. The UV-Vis spectra were recorded on a HP8453 UV-Vis spectrophotometer (HP, USA) equipped with 1.0-cm quartz cells. CD spectra were recorded in a Jasco J-810720 spectropolarimeter. All pH measurements were made with a pHS-3 digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glass–calomel electrode. CS501AB temperature thermostat was used to control the temperature of solution.

Reagents

BSA (Chemical Reagents Company, Shanghai, China) and folic acid (Dongfeng Biotechnological Incorporated Company, Shanghai, China) were directly dissolved in water to prepare 1.0×10^{-5} mol/L and 8.0×10^{-5} mol/L working solutions, respectively. Tris-HCl buffer solution (0.2 mol/L, pH = 7.4) and sodium chloride (0.5 mol/L) solution, which was used to

keep the ion strength, were prepared. ANS was dissolved into alcohol to prepare 6.0×10^{-4} mol/L solution. SDS was dissolved in water to prepare 1.0×10^{-2} mol/L solution. All reagents were of analytical grade, and double-distilled water was used throughout the experiment.

Experimental Method

BSA solution (0.40 mL) and appropriate amounts of folic acid solution were added to a 10-mL volume calibrated tube. The mixture was diluted to 10 mL with Tris-HCl buffer solution, shaken thoroughly, and placed for 20 min.

Fluorescence spectrum was obtained at excitation and emission wavelengths of 279 nm and $300 \sim 500$ nm, respectively. The excitation and emission bandwidths were 10 nm and 5 nm.

The synchronous fluorescence spectrums of various molar ratio between BSA and folic acid were determined at $\Delta\lambda = 15$ nm and $\Delta\lambda = 60$ nm, respectively.

The absorption spectrum of folic acid with molar ratio n(FA):n(BSA) = 1:1 was obtained on the spectrophotometer.

RESULTS AND DISCUSSION

Mechanism of Fluorescence Quenching

Protein can produce endogenous fluorescence (BSA: $\lambda_{ex}/\lambda_{em} = 279$ nm/349 nm) in the presence of tryptophane and tyrosine. Figure 2 shows the emission spectra of BSA in the presence of various concentrations of folic acid. It is apparent that the fluorescence intensity of BSA decreased regularly with the increase of concentration of folic acid at 349 nm. Moreover, an equal emission point appeared at 412 nm and the emission peak (450 nm) after equal emission point is the fluorescence peak of folic acid.

Figure 3 indicates that BSA itself has almost no fluorescence and ANS and folic acid produce weak fluorescence at 515 nm and 450 nm with excitation at 390 nm, respectively. The fluorescence intensity of BSA was enhanced greatly using ANS as probe. Addition of folic acid made the fluorescence intensity of BSA–ANS system stronger and addition of SDS made it weaker. But the fluorescence intensity of BSA–ANS–SDS was enhanced in the presence of folic acid and the emission peak blue shift. From above, we inferred that there was transfer of energy and interaction between folic acid and BSA.

Quenching process can be divided into two broad categories: dynamic quenching and static quenching. If the relationship between the fluorescence intensity and concentration of the quencher completely fitted in with Stern–Volmer equation, the quenching type could be sorted to dynamic quenching or static quenching.

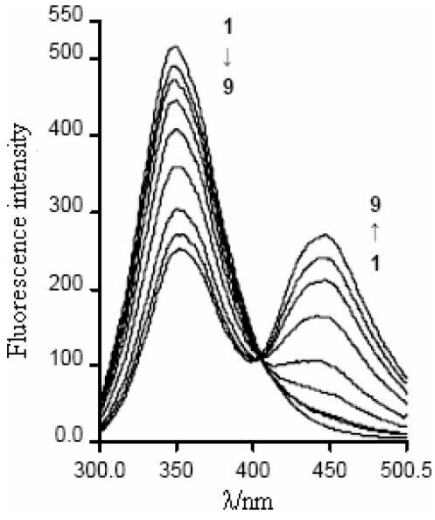

Figure 2. Effect of FA on the fluorescence spectrum of BSA. *C*BSA $= 4.0 \times 10^{-7}$ mol/L, C_{FA} of $1 \sim 9$: 0, 0.40, 0.80, 1.60, 3.20, 6.40, 9.60, 12.8, 16 ($\times 10^{-6}$ mol/L).

In order to study the quenching process of BSA, we first assume that it is dynamic. The quenching equation is as follows:[6]

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q] \tag{1}$$

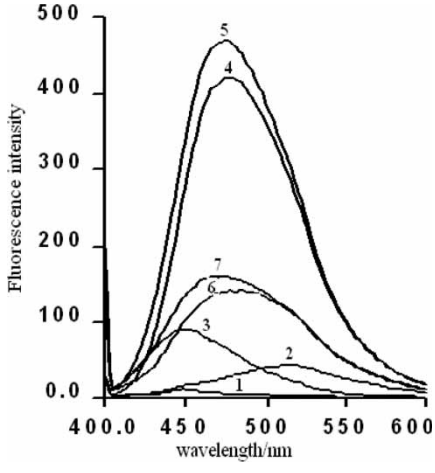

Figure 3. Fluorescence spectrum: 1, BSA; 2, ANS; 3, folic acid; 4, BSA + ANS; 5, BSA + ANS + folic acid; 6, BSA + ANS + SDS; 7, BSA + ANS + SDS + folic acid. $C_{BSA} = 4.0 \times 10^{-7}$ mol/L, $C_{FA} = 1.6 \times 10^{-5}$ mol/L, $C_{ANS} = 4.8 \times 10^{-5}$ mol/L, $C_{SDS} = 7.5 \times 10^{-4}$ mol/L.

where K_q is the quenching rate constant of bimolecule, K_{sv} is the dynamic quenching constant and $K_{sv} = K_q\tau_0$, τ_0 is the average lifetime of fluorescence molecule without quencher, $[Q]$ is the concentration of quencher, and F and F_0 are the fluorescence intensity with and without quencher, respectively.

The Stern–Volmer graph at different temperature is shown in Fig. 4. The slope of the Stern–Volmer curve is the dynamic quenching constant K_{sv}. The fluorescence lifetime of biomolecule is about 1.0×10^{-8}s[7] and thus the quenching rate constant K_q can be calculated. The results are listed in Table 1. In general, the maximum scatter collision quenching constant of various quenchers to the biomolecule is $2.0 \times 10^{10}\,\mathrm{L \cdot mol^{-1} \cdot s^{-1}}$.[7] Obviously, the quenching rate constant of BSA initiated by folic acid is much larger than the K_q of the scatter collision process. This means that the quenching is not initiated by dynamic collision but by the formation of a complex, namely static quenching.

Static and dynamic quenching can often be distinguished by the effect of temperature. Dynamic quenching depends on diffusion. Higher temperature results in faster diffusion and then the quenching rate constant increases with increasing temperature. In contrast, increasing temperature is likely to result in decreasing of complex stability and hence lower value of the static quenching constant.[8] Figure 4 shows the Stern–Volmer plot for the folic acid–BSA system at different temperatures. The slopes decrease with increasing temperature. This indicates further that the quenching process is static quenching.

The Number of Binding Sites n and Binding Constant K_A

The relationship between the fluorescence intensity and the concentration of quencher can be deduced from the expression of binding constant for the static quenching.[9] The binding process can be described by the following reaction equation:

$$nQ + P = Q_nP$$

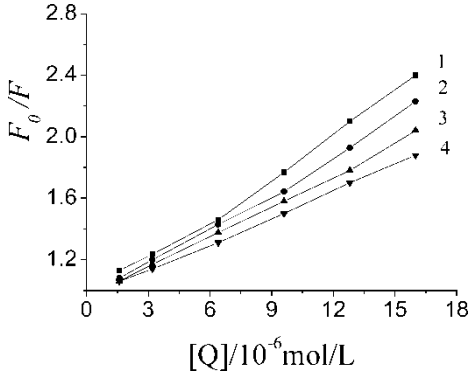

Figure 4. Stern–Volmer curves: 1, 12°C; 2, 20°C; 3, 33°C; 4, 45°C.

Table 1. The Stern–Volmer quenching constant K_{sv} and quenching rate constant K_q

$T/^{\circ}C$	r	K_{sv} ($L \cdot mol^{-1}$)	K_q ($L \cdot mol^{-1} \cdot s^{-1}$)
12	0.9943	8.94×10^4	8.94×10^{12}
20	0.9962	7.58×10^4	7.58×10^{12}
33	0.9969	6.23×10^4	6.23×10^{12}
45	0.9975	4.95×10^4	4.95×10^{12}

Assuming that each biomolecule (P) has n equal and independent binding sites available for accepting quencher and the complex Q_nP has no fluorescence, we can have the following equations:

$$[P_0] = [Q_nP] + [P] \tag{2}$$

$$K_A = [Q_nP]/[Q]^n[P] = ([P_0] - [P])/[Q]^n[P] \tag{3}$$

where $[P]$, $[Q]$, $[Q_nP]$ are the concentration of free biomolecule fluorophor, quencher, and complex, respectively, $[P_0]$ is the total concentration of fluorophor, and K_A is the binding constant.

The fluorescence intensity is direct proportion to the concentration of free biomolecule fluorophor for the static quenching, so

$$[P]/[P_0] = F/F_0 \tag{4}$$

According to the Equations (2), (3), and (4), we can obtain following equation:

$$\log(F_0 - F)/F = \log K_A + n \log[Q] \tag{5}$$

Plotting $\log(F_0 - F)/F$ vs. $\log[Q]$ and fitting the curve with equation, we would simultaneously obtain two parameters: K_A and n. The results are listed in Table 2. The results suggest that BSA and folic acid binds with molar ratio 1:1. Greater binding constants suggest that stronger binding force existed between BSA and folic acid, and folic acid can be stored and transported by protein.

The Acting Force Between Folic Acid and BSA

The acting force for the binding between drugs and biomolecules includes hydrogen bond, van der Waals forces, electrostatic force, hydrophobic interaction force, and so on. In order to study the interaction of folic acid with BSA, the thermodynamic parameters were calculated from the van't Hoff plots.[10] If the enthalpy change ΔH does not vary significantly

Table 2. The binding constant K_A at different temperature and in different medium

	Tris-HCl	$H_2PO_4^-$-HPO_4^{2-}
12°C		
K_A	2.57×10^5	3.09×10^5
n	0.992	1.03
20°C		
K_A	2.04×10^5	2.14×10^5
n	1.04	0.984
33°C		
K_A	1.66×10^5	1.95×10^5
n	1.03	0.988
45°C		
K_A	1.07×10^5	1.21×10^5
n	1.02	1.06

over the temperature range studied, then its value and that of ΔS can be determined from the van't Hoff equation:

$$\ln K_A = -\Delta H/RT + \Delta S/R \tag{6}$$

In Eq. (6), K_A is the binding constant at the corresponding temperature and R is the gas constant. The enthalpy change ΔH is calculated from the slope of the van't Hoff relationship. The free energy change is estimated from the following relationship:

$$\Delta G = \Delta H - T\Delta S \tag{7}$$

Figure 5 is the van't Hoff plot of the folic acid-BSA system. Table 3 shows the values of ΔH and ΔS obtained from the slopes and the ordinates

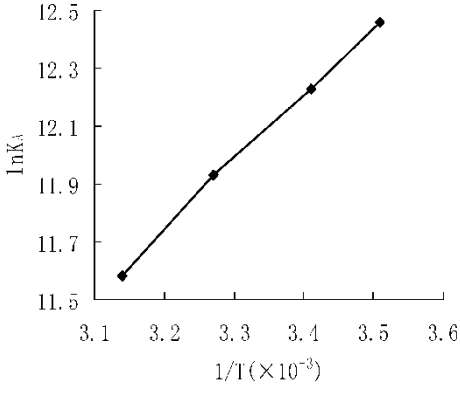

Figure 5. van't Hoff plot.

Table 3. Thermodynamic parameters of folic acid–BSA interaction

$T/^{\circ}C$	K_A	ΔG (KJ/mol)	ΔH (KJ/mol)	ΔS (J/mol · K)
12	2.57×10^5	−29.54		
20	2.04×10^5	−29.82		
33	1.66×10^5	−30.27	−19.57	34.97
45	1.07×10^5	−30.69		

at the origin of the fitted lines. From Table 3 it can be seen that ΔH and ΔS have a negative value (-19.57 kJ/mol) and a positive value (34.92J/mol · K), respectively. The negative value for ΔG means that the binding process is spontaneous. For drug–protein interaction, $\Delta H > 0$, $\Delta S > 0$ is taken as evidence for hydrophobic interaction; $\Delta H < 0$, $\Delta S < 0$ for hydrogen bond; $\Delta H \approx 0$, $\Delta S > 0$ for electrostatic force.[11] In general, positive entropy is frequently taken as evidence for hydrophobic interaction, but negative enthalpy is a manifestation of hydrogen bond. Furthermore, the main source of ΔG value is derived from a large contribution of ΔH term with a little contribution from the ΔS factor, so the main interaction is hydrogen bond, but the hydrophobic interaction cannot be excluded.

The Energy Transfer Between Folic Acid and BSA

According to Förster's nonradiative energy transfer,[12] the rate of energy transfer depends on the relative orientation of the donor and acceptor dipoles, the extent of overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, and the distance between the donor and the acceptor. The energy transfer efficiency is related not only to the distance (r) between the acceptor and the donor but also to the critical energy transfer distance (R_0), that is,

$$E = R_0^6/(R_0^6 + r^6) \tag{8}$$

When the energy transfer efficiency is 50%, the critical energy transfer distance can be calculated by

$$R_0^6 = 8.8 \times 10^{-25}(K^2 N^{-4} \Phi J) \tag{9}$$

where K^2 is a factor describing the relative orientation in space of the transition dipoles of the donor and acceptor, N is the refractive index of the medium, and Φ is the fluorescence quantum yield of the donor in the absence of acceptor. The overlap integral J expresses the degree of spectral overlap between the donor emission and the acceptor absorption.

$$J = \Sigma F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda / \Sigma F(\lambda)\Delta\lambda \tag{10}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor in wavelength λ, and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor in wavelength λ and its unit is $L \cdot mol^{-1} \cdot cm^{-1}$. The energy transfer efficiency is frequency calculated by the following equation:

$$E = (F_0 - F)/F_0 \tag{11}$$

J can be evaluated by integrating the spectrum in Fig. 6 for $\lambda = 300 \sim 500$ nm, and the values of R_0, E, and r can be obtained from Eqs. (8), (9), (10), and (11) using $K^2 = 2/3$, $N = 1.336$, $\varphi = 0.118$.[13] The results are $J = 1.07 \times 10^{-14} cm^3 \cdot L \cdot mol^{-1}$, $R_0 = 2.48$ nm, $E = 0.0562$, $r = 3.98$ nm. The data of R_0 and r are reasonable. This means that the nonradiative energy transfer comes into being between folic acid and BSA. Thus, the quenching mechanism of FA–BSA systems is a combination of static quenching and nonradiative energy transfer.

Conformation Investigation

Fixing the space ($\Delta\lambda$) between excitation and emission wavelength and scanning the monochromators of excitation and emission simultaneously, we can obtained the synchronous fluorescence spectrum, which is often used to analyze the conformational change of protein. When $\Delta\lambda = 15$ nm, the obtained synchronous fluorescence spectrum only indicates the spectral property of tyrosine residues, but when $\Delta\lambda = 60$ nm, only of tryptophane residues. The change of emission wavelength can be used to judge the conformational change of protein because the maximum emission wavelength of residues is related to the polarity of circumstance in which residues exist.[14]

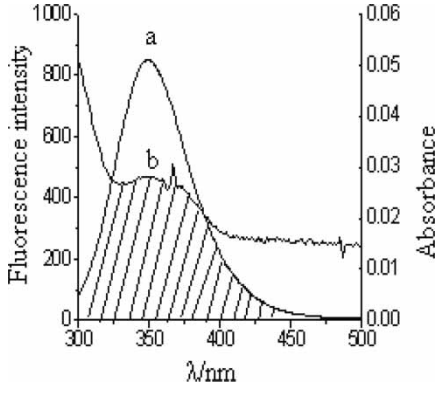

Figure 6. Overlap between fluorescence spectrum of BSA line (a) and the absorption spectrum of FA line (b).

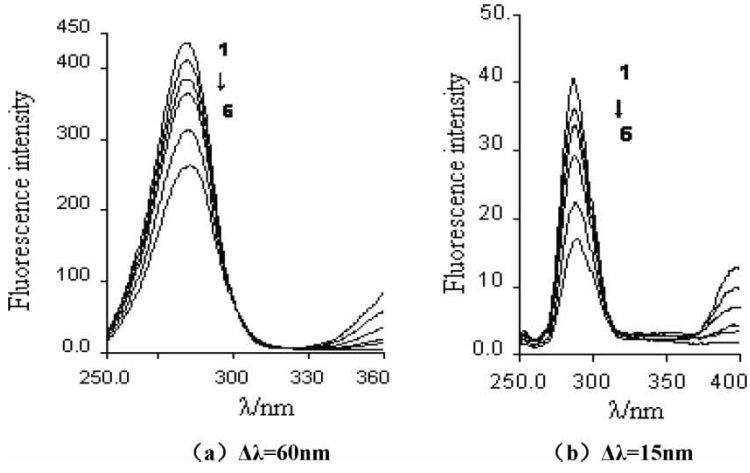

Figure 7. Effect of folic acid on the synchronous fluorescence spectra of BSA. $C_{BSA} = 4.0 \times 10^{-7}$ mol/L, C_{FA} (10^{-6} mol/L) 1 ~ 6: 0, 0.80, 1.6, 3.2, 6.4, 9.6.

Figures 7(a) and 7(b) are the synchronous fluorescence spectrums of tryptophane and tyrosine residues. Comparing Figs. 7(a) and 7(b), we can conclude that the fluorescence of BSA mainly originated from that of tryptophane residues. Moreover, it had a slight red shift of maximum emission wavelength of tryptophane when folic acid was added. Figure 8 is the absorption spectrum of BSA, and we found that it had a slight red shift of maximum absorption peak of BSA in the presence of folic acid by comparing the lines 2 and 3. This suggested that the conformation of BSA changed with the binding of BSA to folic acid.

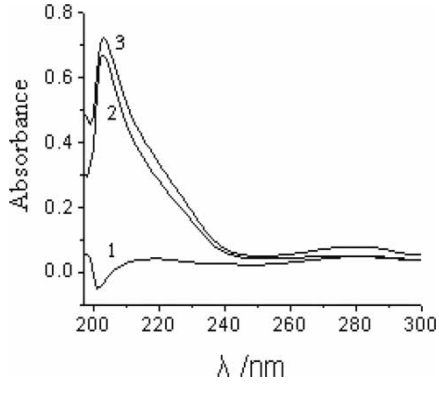

Figure 8. Absorption spectrum: 1, folic acid (1.6×10^{-5} mol/L); 2, BSA (4.0×10^{-7} mol/L); 3, folic acid (1.6×10^{-5} mol/L) + BSA (4.0×10^{-7} mol/L).

To obtain an insight into the structure of BSA, CD spectra were studied for the folic acid–BSA system. In the far ultraviolet region, such spectra relate to the polypeptide backbone structures. The comparison of the spectra of folic acid–BSA with BSA is shown in Fig. 9 at pH 7.40. The CD spectra of BSA exhibited two negative peaks at 208 and 222 nm, which is typical of the a-helix structure of class proteins. The interaction between folic acid and BSA caused only a decrease in band intensity without any significant shift of the peaks, indicating that this drug induces a slight decrease in the helix structure content of the protein. From CD and fluorescence spectra results, we can conclude that the interaction of folic acid with BSA induced the slight unfolding of the constitutive polypeptides of protein, which resulted in a conformational change of the protein that increased the exposure of some hydrophobic regions that were previously buried.

The Effect of Other Materials on the Binding Constant Between Drug and Protein

The UV absorption spectrum of folic acid in the presence of metal ions shows there is no interaction between metal ions and folic acid. But there is a binding reaction between metal ions and protein, and the presence of metal ions directly affects the binding between folic acid and protein. Further, in order to study the effect of metal ions on the binding between folic acid and BSA, some binding constants were determined at 20°C in presence of various metal ions. The results are shown in Table 4. The competition between metal ions and folic acid decreased the binding constant between BSA and folic acid, implying that the acting force between folic acid and BSA also decreased. But the binding constants between the BSA and folic acid increased in the presence of some organism. The higher binding constant obtained in the presence of organism might have

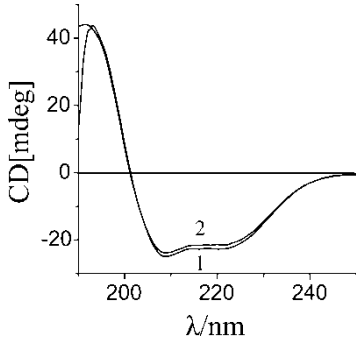

Figure 9. Circular dichorism spectra of BSA: 1, BSA; 2, BSA + folic acid.

Table 4. Effect of other materials on the binding constant between folic acid and BSA

Metal ion	K'_A/K_A	Organism	K'_A/K_A
Ca^{2+}	0.537	Starch	1.291
Mg^{2+}	0.541	Glucose	1.413
Zn^{2+}	0.851	Lauric acid	0.339
Al^{3+}	0.214	Dextrose	2.09
Fe^{3+}	0.794	Urea	6.167

resulted from the organism facilitating the hydrophobic interaction between folic acid and BSA.

CONCLUSIONS

The binding study of drugs with proteins is of great importance in pharmacy, pharmacology, and biochemistry. In this paper, the interaction of folic acid with BSA was studied by spectroscopic methods including fluorescence spectrum, CD spectra, and UV-Vis absorption spectrum. The results of CD spectrum and synchronous spectra indicate that the secondary structure of BSA changed in the presence of folic acid. The experimental results also indicate that the quenching mechanism of fluorescence of BSA by folic acid is a static quenching procedure. The thermodynamic parameters ΔH, ΔG, ΔS at different temperatures were calculated, and the results indicated that the acting force between folic acid and BSA is mainly hydrogen bond, but the hydrophobic interaction cannot be excluded.

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