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Molecular Spectroscopic Study on Site-selective Binding of Benorilate to Bovine Serum Albumin

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Abstract: The interaction between benorilate (BEN) and bovine serum albumin (BSA) was investigated under physiological condition by molecular spectroscopic techniques, including fluorescence spectroscopy, UV-visible spectroscopy, synchronous fluorescence spectroscopy and three-dimensional fluorescence spectroscopy. The intrinsic fluorescence of tryptophan in BSA was significantly quenched by BEN via dynamic quenching. The hydrophobic interaction did favor the interaction of BSA with BEN. The apparent binding constants and binding sites number at the tryptophan site were 1 050 L · mol^{-1} and 0.88, respectively. Thermodynamic parameters such as enthalpy change (ΔH), entropy change (ΔS) and free energy change (ΔG) were also obtained. The conformation changes of BSA in the presence of BEN were proved by the evidences of synchronous fluorescence spectroscopy and three-dimensional fluorescence spectroscopy. Two site-specific fluorescence probes, dansylamide (DA) and dansyl-L-proline (DP), were employed in competitive binding experiments to monitor the BEN binding sites of BSA. The apparent binding constants at site Ⅰ and Ⅱ were 4 300 and 21 200 L · mol^{-1}, respectively.

Key words: benorilate; bovine serum albumin; fluorescence quenching; thermodynamic parameters; three-dimensional fluorescence spectroscopy

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贝诺酯与牛血清白蛋白位点选择性结合的分子光谱研究

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摘要: 采用荧光光谱、紫外可见光谱、同步荧光光谱及三维荧光光谱等分子光谱方法,研究了生理条件下贝诺酯(BEN)与牛血清白蛋白(BSA)的相互作用。结果表明,BEN对BSA的内源荧光有显著的猝灭作用,猝灭机理为动态猝灭,二者之间的作用力类型以疏水作用为主,BEN与BSA发生反应后,使BSA的疏水环境极性增强,疏水性减弱,荧光强度降低。测得的表观结合常数和结合位点数分别是1 050 L · mol^{-1}和0.88,同时测

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得了焓变(ΔH)、熵变(ΔS)和自由能变(ΔG)等热力学参数。同步荧光和三维荧光光谱的结果表明,BEN 使 BSA 的构象发生改变。利用荧光特异性位点探针 DA 和 DP,通过竞争结合实验,监测 BEN 与 BSA 的结合位点,测得了位点Ⅰ和位点Ⅱ的表观结合常数分别为 4 300 L·mol^{-1}和 21 200 L·mol^{-1},表明 BEN 与 BSA 优先在位点Ⅱ结合。

关　键　词: 贝诺酯; 牛血清白蛋白; 荧光猝灭; 热力学参数; 三维荧光光谱

1　Introduction

Benorilate (benorylate, (4-acetamidophenyl) 2-acetyloxybenzoate, BEN, Fig. 1), a non-steroidal anti-inflammatory drug, is a combination of paracetamol and aspirin[1]. It possesses a synergistic effect of the two drugs, and has analgesic and antipyretic activities[2]. Therefore BEN has been widely used in clinical medicine for the treatment of inflammation in rheumatoid arthritis, osteoarthrosis and other musculoskeletal conditions[3]. The effectiveness of drugs depends on their binding ability to protein, especially serum albumin. Since studying drug/protein interactions is not only important for explaining the relationship between the structures and functions of proteins but also crucial for drug development within the pharmaceutical industry[4], it is necessary to investigate the interaction between drug and plasma protein. To our knowledge, however, there was no report about the interaction between BEN and protein. BEN's effect on plasma protein still remains unclear.

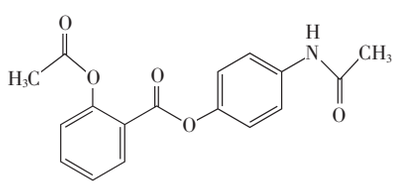

Fig. 1　Structure of benorilate

Bovine serum albumin (BSA) is a type of model protein that widely used in the field of drug/protein interactions, as it is the most abundant protein in bovine plasma and its structure is very analogous to human serum albumin and has been well characterized[5]. BSA is a 607 amino acid monomer containing three homologous domains (Ⅰ, Ⅱ and Ⅲ), each subdivided into A and B subdomains. It is well-known that BSA has two high-affinity drug binding sites, namely site Ⅰand site Ⅱ[6]. Site Ⅰ is located in subdomain ⅡA and site Ⅱ is located in subdomain ⅢA. In order to study the binding pro-perties of drug to serum albumin, a number of site-specific fluorescence probes are widely used (*e. g.* site Ⅰ for warfarin, phenylbutazone, dansylamide (DA), iodipamide, iophenoxic acid and cumarins, and site Ⅱ for ibuprofen, dansyl-L-proline (DP), fluofenamic acid, diazepam, and benzodiazepines)[7-8].

Many techniques, including UV-visible absorption spectroscopy, fluorescence spectroscopy[9], circular dichroism spectroscopy[9], nuclear magnetic resonance[10], equilibrium dialysis[11], chromatography[12] and electrochemistry[13], have been developed to study the interaction between non-steroidal anti-inflammatory drugs and serum albumin. Among these techniques, fluorescence spectroscopy has emerged as a feasible and powerful technique for probing the affinity of biomolecules due to its distinct advantages such as extreme sensitivity and high selectivity.

In our previous work, the binding affinities between diclofenac sodium, another non-steroidal anti-inflammatory drug, and BSA were measured by fluorescence spectroscopy and capillary electrophoresis[14]. The fluorescence results were found to be comparable to those obtained with other techniques. In this paper, the interaction of BSA with BEN under physiological condition was studied by fluorometric titration combined with UV-visible, synchronous fluorescence, and three-dimensional fluorescence spectroscopy. Much more attention was given to site-selective binding of BEN to BSA. DA and DP were employed as site-specific probes to monitor the BEN binding sites of BSA. The detailed binding information,

including quenching mechanism, binding parameter, binding mode, thermodynamic parameter, conformational variation and binding sites, was obtained.

2 Experiments

2.1 Materials

BSA (fraction V, lyophilized power, ≥98.0% purity), DA, and DP were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). BEN was acquired from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 7H_2O$, KCl, and ethanol were analytical grade and were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). All of the reagents were used as received.

BSA was prepared in pH 7.40 PBS buffer (0.01 mol · L^{-1} of $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 7H_2O$ each in 0.15 mol · L^{-1} KCl), at a concentration of 0.1 mmol · L^{-1}, and stored at 4 ℃. BSA was filtered through a 0.22 μm membrance filter (Shanghai Youqi Co., Ltd., China) prior to use. Stock solutions of BEN, DA, and DP ware prepared at a concentration of 5.0 mmol · L^{-1} (in ethanol). All the samples were diluted to the desired concentration with PBS. Double deionized water with resistivity of more than 18.2 MΩ · cm^{-1} was used throughout the experiments.

2.2 Apparatus

Fluorescence measurements were carried out on a RF-5301PC spectrophotometer equipped with 1 cm path cells (Shimadzu Co., Japan). The excitation and emission slide width were both set to 3 nm. Data were collected with the RF-5301PC Personal Fluorescence Software (version 2.02). UV-visible absorption measurements were preformed on a 760 CRT dual-beam UV-visible spectrophotometer (Shanghai Precision & Scientific Instrument Co., LTD, China) between 200 and 600 nm using a 1 cm path length quartz cuvette.

2.3 Binding of BEN and BSA

Fluorometric titration measurements were preformed to study the binding of BSA with BEN. The concentration of BSA solution was kept at 5.0 μmol · L^{-1}, while the final concentration of BEN varied from 0 to 0.12 mmol · L^{-1} by successive addition of appropriate concentration of BEN. The protein solutions in BEN were incubated for 5 min. Then the emission spectra were recorded from 300 to 400 nm at excitation wavelength of 295 nm. Blank titration in the PBS buffer was made to correct the fluorescence of free drug in the absence of protein. All the experiments were repeated at least three times and carried out at room temperature. The fluorescence intensity was corrected for dilution and inner-filter effects using the following equation[15]:

$$F_{corr} = F_{obs}(V/V_0) \times 10^{\frac{A_{ex}+A_{em}}{2}}, \quad (1)$$

where F_{corr} is the corrected fluorescence intensity, F_{obs} the observed fluorescence intensity, V_0 the initial volume of the sample, V the volume after adding drug, A_{ex} the absorption value at excitation wavelength and A_{em} the absorption value at emission wavelength.

2.4 Competitive Binding of BEN and Site Specific Probe to BSA

Two site specific probes DA (site Ⅰ probe) and DP (site Ⅱ probe) were used to monitor the BEN binding sites of BSA. BEN was gradually added to the mixture solution of BSA and site probes with equimolar concentrations at 5.0 μmol · L^{-1}. The excitation wavelength was set at 350 nm for DA and 375 nm for DP[16]. The emission spectra were recorded from 400 to 650 nm.

3 Results and Discussion

3.1 The Binding Haracteristics of BEN to BSA

BSA has two tryptophan residues: Trp-212, located in the subdomain IIA, and Trp-134, located in the subdomain IB[5]. The intrinsic fluorescence of tryptophan in serum album is mainly derived from Trp-212 and very sensitive to the environment around the amino acid residue. Binding of drug molecules with BSA always causes its fluorescence changes. Consequently, tryptophan fluorescence has been widely used to investigate drug-protein interactions[17].

The interaction between BEN and BSA was studied by fluorometric titration. Tryptophan fluorescence of BSA was monitored by exciting the protein

solution at 295 nm because only tryptophan emission will be observed and the contribution from tyrosine residues can be ignored at the excitation wavelength[15]. The fluorometric titration results of BEN in the presence of BSA are shown in Fig. 2. As can be seen in Fig. 2, the fluorescence intensity of BSA was progressively quenched by addition of BEN, which indicated that BEN may interact with BSA. No significant shifts of the emission maximum were observed.

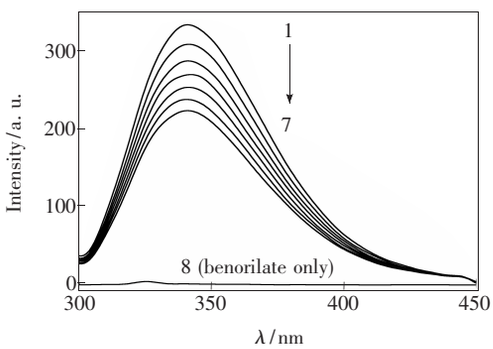

Fig. 2　Fluorescence emission spectra of BSA in the presence of difference concentration of BEN (25℃, λ_{ex} = 295 nm, c(BSA) = 5.0 μmol · L^{-1}, curve 1 ~ 7: c(BEN) = 0 ~ 0.12 mmol · L^{-1} at intervals of 0.2 μmol · L^{-1}, curve 8: 5.0 μmol · L^{-1} BEN only).

The mechanisms of fluorescence quenching usually include dynamic and static quenching. To explore the quenching mechanism, the fluorescence quenching results were analyzed by the well-known Stern-Volmer equation (Eq. 2)[15].

$$F_0/F_{corr} = 1 + k_q\tau_0[Q] = 1 + K_{sv}[Q], \quad (2)$$

where F_0 is the steady-state fluorescence intensity in the absence of quencher (BEN), F_{corr} the corrected fluorescence intensity in the presence of quencher, K_q the quenching rate constant, τ_0 the average lifetime of the protein without quencher ($\tau_0 = 10^{-8}$ s[18]), $[Q]$ the concentration of quencher, and K_{sv} the Stern-Volmer dynamic quenching constant.

Fig. 3 shows the Stern-Volmer plots for the BEN-BSA system at different temperatures (298 and 310 K). The corresponding results are listed in Table 1. A good linear relationship between $\frac{F_0}{F_{corr}}$ and $[Q]$ was found. As can be seen in Table 1, the K_{sv} values were direct dependence on temperature, which suggested that the probable quenching mechanism of BEN to BSA was initiated by dynamic collision rather than by complex formation. It was worth noting that the K_q values from Eq. 2 at 298 and 310 K were both large enough. The reason may be concerned with the increment of BSA fluorescent quantum yield[19] and the effect of ion strength[20].

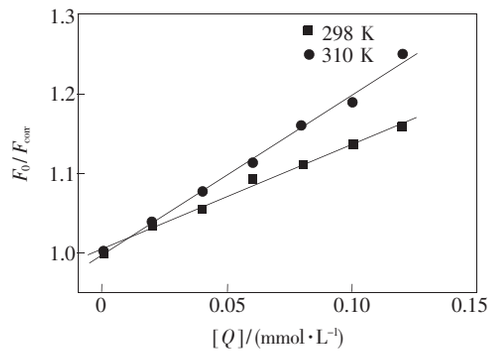

Fig. 3　Stern-Volmer plots of the BEN-BSA system at different temperatures

Another approach to distinguish dynamic and static quenching is to study the absorption spectra of the fluorophore[7,15]. Dynamic quenching only affects the excited states of the fluorophores, and thus no change in the UV-visible absorption spectra is expected[19]. To further confirm the quenching mechanism, the absorption spectra of BSA in the absence and presence of BEN were measured and plotted in Fig. 4. The absorbance of a series of BEN-BSA mixtures at 275 nm in the inset of Fig. 4 shows a good linear relation, indicating a small experimental error.

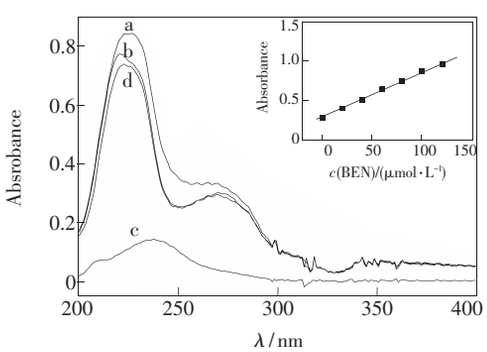

Fig. 4　UV absorbance spectra of BSA in the presence of BEN. (a) V(BEN)/V(BSA) = 1∶1 complex; (b) BSA; (c) BEN; (d) difference between V(BEN)/V(BSA) = 1∶1 complex and BEN. c(BEN) = c(BSA) = 5.0 μmol · L^{-1}. Inset: absorbance of BSA as a function of BEN concentration at 275 nm.

The UV absorption spectrum of BSA (Fig. 4, curve b) and the difference absorption spectrum between equimolarity of the BEN-BSA mixture (Fig. 4, curve a) and BEN (Fig. 4, curve c) could be overlapped within experimental error. This result reconfirms that the probable quenching mechanism of BEN to BSA is a dynamic quenching procedure.

3.2 Binding Parameters

The apparent binding constant K_A and binding sites number n can be calculated from the following equation[21]:

$$\lg\frac{F_0 - F_{corr}}{F_{corr}} = n\lg K_A - n\lg\left(\frac{1}{[Q_t] - (F_0 - F_{corr})[P_t]/F_0}\right), \quad (3)$$

Here, $[Q_t]$ is the total quencher concentration, and $[P_t]$ is the total protein concentration. The free drug concentration is not known in the experiment, and hence, $[Q_t]$ is used for data processing instead. By the plot of $\lg(F_0 - F_{corr})/F_{corr}$ versus $\lg(1/([Q_t] - (F_0 - F_{corr})[P_t]/F_0))$, the apparent binding constant K_A and binding sites number n can be estimated (see Table 1). The binding sites number n approximately equal to 1 which indicates the existence of only one binding site in BSA for BEN. The values of K_A as calculated are of the order of 10^3. Such a weak binding suggests that drug and protein do not actually form a ground-state complex[15], reconfirming the dynamic quenching mechanism of BEN to BSA.

Table 1 Stern-Volmer quenching constant K_{SV}, apparent binding constant K_A and thermodynamic parameters of the BEN-BSA system at different temperatures

	Eq. 2		Eq. 3		Eq. (4~6)		
T/K	K_q/ (L·mol^{-1}·s^{-1})	K_{SV}/ (L·mol^{-1})	K_A/ (L·mol^{-1})	n	ΔH/ (kJ·mol^{-1})	ΔG/ (kJ·mol^{-1})	ΔS/ (J·mol^{-1}·K^{-1})
298	1.32×10^{11}	1.32×10^3	1.05×10^3	0.88	41.56	-17.24	197
310	2.01×10^{11}	2.01×10^3	2.01×10^3	1.01	41.56	-19.60	197

3.3 Binding Mode

The interaction forces between drugs and protein molecules may include hydrogen bonds, Van der Waals forces, electrostatic, and hydrophobic interactions[22]. Thermodynamic parameters such as enthalpy change (ΔH), entropy change (ΔS) and free energy change (ΔG) were used to further characterize the interaction between BEN and BSA. If ΔH does not vary signiflcantly over the temperature range studied, thermodynamic parameters can be calculated using the following equations[23-25]:

$$\ln\frac{K_{A2}}{K_{A1}} = \frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right), \quad (4)$$

$$\Delta G = -RT\ln K_A, \quad (5)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T}. \quad (6)$$

According to the apparent binding constant K_A from Eq. 3, thermodynamic parameters for BEN binding to BSA at different temperatures were obtained and listed in Table 1. Since both ΔH and ΔS were larger than zero, it can be concluded that the combination between BEN and BSA is mainly via the hydrophobic interaction[26].

3.4 Conformation Investigation

Synchronous fluorescence spectroscopy has been demonstrated to be a powerful and simple technique for conformation studies of biomolecules[27]. The technique is based on measuring the shift in the emission maximum, which corresponds to changes of polarity around the fluorophore molecules[28]. When the difference value ($\Delta\lambda$) between emission wavelength and excitation wavelength was set at 15 nm or 60 nm, the synchronous fluorescence may give the characteristic information of tyrosine or tryptophan residues[29].

Synchronous fluorescence spectra of BSA with various concentration of BEN were shown in Fig. 5. A significant red shift on the emission maximum of tyrosine residues is observed, which indicates that the conformation of BSA was changed by BEN. The polarity around the tyrosine residues was increased,

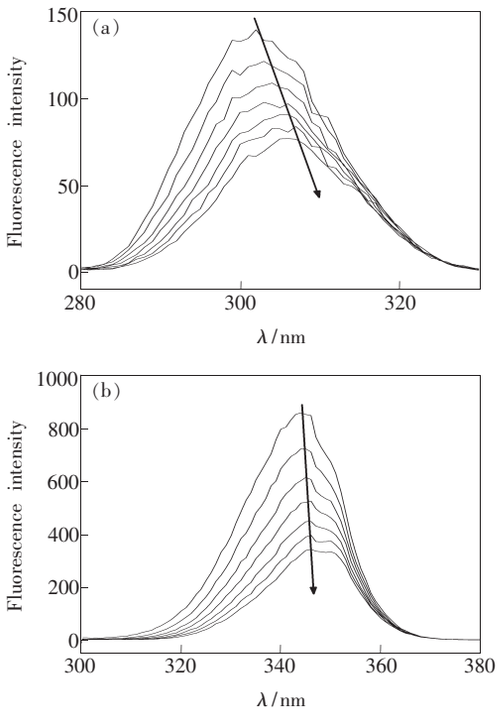

Fig. 5　Synchronous fluorescence spectra of BSA in the presence of difference concentration of BEN (298 K, c(BSA) = 5.0 μmol · L^{-1}, curve (from up to down): c(BEN) = 0 ~ 0.12 mmol · L^{-1} at intervals of 20 μmol · L^{-1}). (a) $\Delta\lambda$ = 15 nm and (b) $\Delta\lambda$ = 60 nm.

and thus the hydrophobicity was decreased. However, only a slightly red shift on the emission maximum of tryptophan residues is found, which suggests that the tryptophan residues are located in a hydrophilic microregion and exposed to the solvent molecules during the interaction process[30].

Furthermore, the quenching of the fluorescence intensity of tryptophan residues is stronger than that of tyrosine residue, suggesting that tryptophan residues contribute greatly to the quenching of intrinsic fluorescence of BSA. BEN was closer to tryptophan residues compared to tyrosine residues.

Additional evidence on the conformational changes of BSA binding to BEN was presented in the three-dimensional fluorescence results. The fluorescence contour plots of BSA in the presence of different concentrations of BEN were shown in Fig. 6. As can be seen in Fig. 6(a), the maximum excitation (λ_{ex}) and emission (λ_{em}) wavelengths of BSA solution are 280 and 341 nm. The peak (280/341 nm) corresponds to the spectral behavior of tryptophan and tyrosine residues[31]. After the introduction of BEN, the fluorescence intensities decrease gradually with

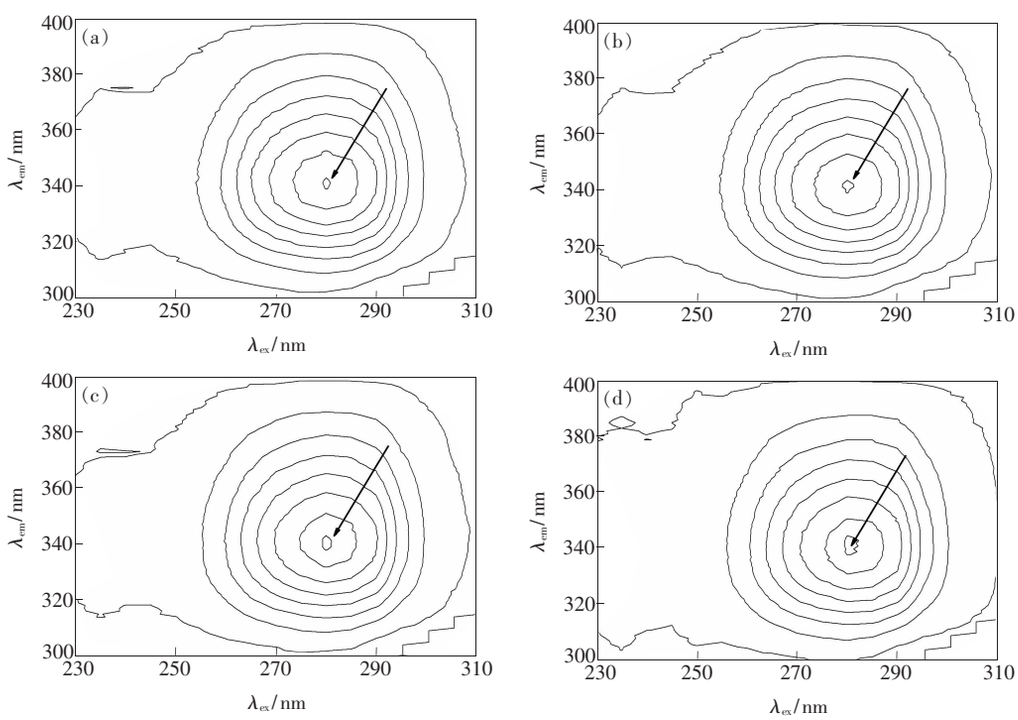

Fig. 6　Contour plots of BSA in the presence of difference concentration of BEN (298 K, c(BSA) = 5.0 μmol · L^{-1}, a ~ d: c(BEN) = 0, 5.0, 10.0, 27.5 μmol · L^{-1}). The arrow indicates the fluorescence peak.

the increment of BEN concentrations, which were consistent with the results of fluorometric titration measurements. The maximum emission wavelengths (341 nm) have no obvious shift (see Fig. 6(b) ~ (d)). This indicates that the tryptophan residues exposed in solvent completely and thus no other change in the immediate environment of the tryptophan residues occurs except that BEN molecules get close enough to the tryptophan residue for the generation of fluorescence quenching effect. This finding was in accordance with the results of synchronous fluorescence experiments.

3.5 Site-specific Binding of BEN on BSA

BSA has two high-affinity drug-binding sites, called as site Ⅰ and site Ⅱ. The binding affinity from site I is mainly through hydrophobic interactions while site II involves synergistic effects of hydrophobic, hydrogen bonding, and electrostatic interactions. DA and DP were used as site marker fluorescence probes for the two binding sites on BSA, respectively. A competitive experiment was carried out to study the site-specific binding of BEN on BSA. As can be seen from Fig. 7(a), increasing BEN to the DA-BSA mixture resulted in substantial decrease in fluorescence intensity and then reached saturation at 20 $\mu mol \cdot L^{-1}$. The intensity reduced to about 79% of its initial value. The peak wavelength shifted from 514 to 526 nm. Further addition of BEN did not cause to any obvious change in either the intensity or the wavelength. Free DA did not emit fluorescence, and thus displacement of DA by BEN from BSA reduced its fluorescence, which indicate that BEN did bind competitively to the same site as the probe DA. Given the fact that the fluorescence intensity reached saturation and did not reduce to zero even at high drug concentrations, it can be deduced that BEN did not thoroughly displace the probe DA from BSA. Using Eq. 3, the apparent binding constant K_A and binding sites number n can be calculated to be 4 300 $L \cdot mol^{-1}$ and 0.55, respectively.

Titration of BEN against BSA with the binding site Ⅱ probe DP showed gradual reduction of the probe's fluorescence (see Fig. 7b). In the limited

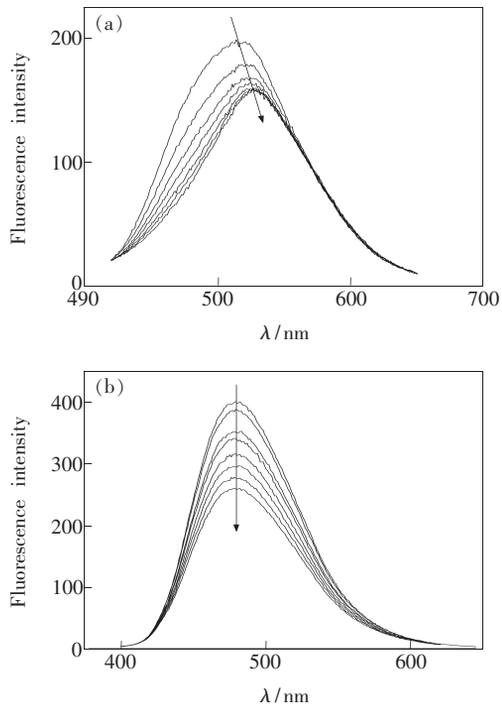

Fig. 7 (a) Fluorescence emission spectra of 5.0 $\mu mol \cdot L^{-1}$ DA mixed with 5.0 $\mu mol \cdot L^{-1}$ BSA with addition of BEN (0, 5.0, 10.0, 15.0, 20.0, 25.0, 30 $\mu mol \cdot L^{-1}$). λ_{ex} = 350 nm; (b) 5.0 $\mu mol \cdot L^{-1}$ DA mixed with 5.0 $\mu mol \cdot L^{-1}$ BSA with addition of BEN (0, 2.0, 7.5, 10.0, 15.0, 20.0, 25.0, 30.0 $\mu mol \cdot L^{-1}$). λ_{ex} = 375 nm. The arrow indicates the increasing concentration of BEN.

concentration range of BEN, the fluorescence intensity reduced to about 65% of its initial value whereas the peak wavelength did not significantly shift. The results can be interpreted by a competitive binding mode in which BEN binds to the same site on BSA as DP. The values of K_A and n were obtained to be 21 200 $L \cdot mol^{-1}$ and 1.00, respectively. Among the three binding sites investigated in this work, *i.e.* the tryptophan site, drug-binding site Ⅰ and site Ⅱ, the apparent binding constant at the tryptophan site is the smallest (1 050 $L \cdot mol^{-1}$, 298 K) and at site Ⅱ is the biggest (21 200 $L \cdot mol^{-1}$, 298 K). These results indicate that BEN has high site selectivity and binds to BSA at site Ⅱ preferentially.

4 Conclusion

In this work, the interaction between BEN and BSA was investigated by fluorescence emission

spectroscopy, UV-visible absorption spectroscopy, synchronous fluorescence spectroscopy, and three-dimensional fluorescence spectroscopy. The experimental results indicate that the intrinsic fluorescence quenching of tryptophan in BSA by BEN is a dynamic procedure and the hydrophobic interaction plays a major role in the binding of BEN to BSA. The results of synchronous fluorescence spectroscopy and three-dimensional fluorescence contour plots confirm the conformation change of BSA in the interaction between BEN and BSA. Site marker competitive binding experiments indicate that BEN has high site selectivity and binds to BSA at site Ⅱ, preferentially.

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