Brazilian Journal of Medical and Biological Research (2008) 41: 589-595
ISSN 0100-879X

Spectroscopic studies on the interaction of efonidipine with bovine serum albumin

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Efonidipine hydrochloride is an antihypertensive and antianginal agent with fewer side effects and is better tolerated in the treatment of hypertension with renal impairment. Its interaction with bovine serum albumin (BSA) is of great use for the understanding of the pharmacokinetic and pharmacodynamic mechanisms of the drug. The binding of efonidipine to BSA was investigated by fluorescence spectroscopy and circular dichroism. BSA fluorescence was quenched by efonidipine, due to the fact that efonidipine quenched the fluorescence of tryptophan residues mainly by the collision mode. The thermodynamic parameters ΔH^0 and ΔS^0 were 68.04 kJ/mol and 319.42 $J \cdot mol^{-1} \cdot K^{-1}$, respectively, indicating that the hydrophobic interactions played a major role. The results of circular dichroism and synchronous fluorescence measurements showed that the binding of efonidipine to BSA led to a conformational change of BSA. The fraction of occupied sites (θ) for the 8-anilino-1-naphthalein-sulfonic acid (ANS)-BSA system is 85%, whereas for the NZ-105-BSA system, it is 53%, which suggests that the interaction of ANS with BSA is stronger than that of NZ-105 with BSA. Binding studies in the presence of ANS indicated that efonidipine competed with ANS for hydrophobic sites of BSA. The effects of metal ions on the binding constant of the efonidipine-BSA complex were also investigated. The presence of metal ions Zn^{2+}, Mg^{2+}, Al^{3+}, K^+, and Ca^{2+} increased the binding constant of efonidipine-BSA complex, which may prolong the storage period of NZ-105 in blood plasma and enhance its maximum effects.

Key words: Efonidipine; Bovine serum albumin; Fluorescence quenching; Synchronous fluorescence; Circular dichroism

Research supported by Beijing Natural Science Foundation of China (#5073043), Scholastic Natural Science Foundation of Capital Medical University (#2005ZR06), and Beijing Municipal Project for Developing Advanced Human Resources for Higher Education.

Received November 8, 2007. Accepted June 18, 2008

Introduction

It is known that drug-protein interactions greatly influence the absorption, distribution, metabolism, and excretion properties of drugs (1). Serum albumin is the most abundant protein in the circulatory system of a wide variety of organisms and plays an important role in the transport and deposition of many drugs (2-4). Thus, an understanding of the features of drug interactions with albumin can provide insights into drug therapy and design. Consequently, attention has been focused on the binding of drugs to albumin. Bovine serum albumin (BSA) is usually employed as a model protein because of its low cost and availability and because it is structurally homologous with human serum albumin (5).

Efonidipine hydrochloride (efonidipine, NZ-105; see structure in Figure 1) is a recently synthesized 1,4-dihydropyridine derivative that is used as an antihypertensive and antianginal agent. It has a potent vasodilating effect on vascular smooth muscle and a long-lasting hypotensive effect with a very slow onset (6). NZ-105 also has a chronotropic effect which may suppress tachycardia (7,8). It has few side effects and is well tolerated for the treatment of hypertension in the presence of renal impairment (9). Thus, NZ-105 is a member of a new generation of drugs for the treatment of hypertensive patients with chronic renal disease. However, detailed information on the binding of NZ-105 to BSA is not available.

Spectral methods can reveal the binding of drugs with albumin at low concentrations. The fluorescence quenching technique is often used to monitor the molecular interactions because of its high sensitivity, reproducibility and relatively easy use (10,11). In the present study, we investigated the interactions of NZ-105 with BSA as well as the effect of common metal ions on these interactions by fluorescence spectroscopy. Additionally, binding of NZ-105 to BSA in the presence of the fluorescence probe 8-anilino-1-naphthalein-sulfonic acid (ANS) was also determined. The effects of NZ-105 on the conformational changes of BSA were investigated by synchronous fluorescence spectroscopy and circular dichroism (CD).

Material and Methods

Chemicals

BSA (>96%, essentially fatty acid free, lyophilized powder) and ANS were purchased from Sigma (USA). Efonidipine was obtained from Garlin Pharmaceutical Co. (China). All other chemicals (KCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2$, $ZnCl_2$, $AlCl_3 \cdot 6H_2O$) were of analytical grade and used without further purification.

Instruments

Fluorescence and synchronous measurements were performed using a 1-cm quartz cell on an RF-5301PC spectrofluorophotometer (Shimadzu, Japan) equipped with an SB-11 water bath (Eyela). The absorption spectrum was obtained with a UV-2250 UV-Vis spectrophotometer (Shimadzu). The CD measurements were recorded on a JASCO-J-810 spectropolarimeter (JASCO, Japan) using a 0.1-cm quartz cell.

Efonidipine-BSA interactions

A 2.5-mL amount of 10 µM BSA (based on a molecular mass of 68 kDa) was added to the quartz cell, NZ-105 was then gradually added to the cell and the NZ-105 concentrations ranged from 5 to 50 µM. Fluorescence emission spectra were recorded at three temperatures, i.e., 298, 304, and 310 K upon excitation at 295 nm. The excitation and emission slit widths were 3 nm each.

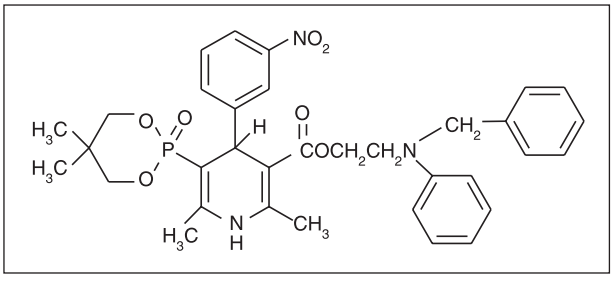

Figure 1. Structure of efonidipine ((±)-5-(5,5-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3-carboxylic acid 2-(N-benzyl-N-phenylamino) ethyl ester P-oxidehydrochloride ethanol).

Synchronous fluorescence measurements

Synchronous fluorescence spectra of BSA with various concentrations of NZ-105 were obtained from 300 to 400 nm when $\Delta\lambda$ = 60 nm and from 280 to 350 nm when $\Delta\lambda$= 15 nm. The excitation and emission slit widths were 3 and 1.5 nm, respectively.

Circular dichroism measurements

CD spectra of BSA in the absence and presence of NZ-105 were recorded from 200 to 280 nm at 0.2-nm intervals with five scans averaged for each spectrum. The overall concentration of BSA was kept at 1 µM, while the molar ratios of NZ-105 to BSA ranged from 0:1 to 3:1 and 10:1.

Binding studies in the presence of ANS

In the first set of experiments, the binding of NZ-105 or ANS to BSA was studied under the same experimental conditions. The final BSA concentration was 10 µM and the concentration of NZ-105 or ANS varied from 2.5 to 25 µM. Fluorescence spectra of BSA were recorded from 300 to 500 nm with excitation at 295 nm. In the second set of experiments, the binding sites of NZ-105 and ANS to BSA were determined simultaneously. BSA and ANS concentrations were fixed at 10 and 20 µM, respectively, and the concentration of NZ-105 varied from 0 to 20 µM. The fluorescence spectra of ANS were recorded from 390 to 550 nm with excitation at 370 nm.

Effects of some common ions

The fluorescence spectra of NZ-105-BSA were recorded in the presence of metal ions (Zn^{2+}, Mg^{2+}, Al^{3+}, K^+, and Ca^{2+}) from 300 to 500 nm upon excitation at 295 nm. Both BSA and metal ion concentrations were 10 µM.

Results and Discussion

Fluorescence studies

The intrinsic fluorescence of BSA when excited at 295 nm is mainly due to the presence of the two tryptophan residues: Trp-134 and Trp-212. Trp-212 is located within a hydrophobic binding pocket in the IIA sub-domain of the protein, whereas Trp-134 is located on the surface of the albumin molecule and more exposed to the environment (12). Though the tyrosine residue can also contribute to fluorescence, it presents very weak emission when ex-

cited at 295 nm.

The fluorescence spectra of BSA were recorded in the presence of increasing amount of NZ-105. As shown in Figure 2, the fluorescence intensity of BSA decreased regularly with increasing concentration of NZ-105, while the emission maximum and shape of the peaks remained almost unchanged. This indicated that NZ-105 could bind to BSA without altering the environment in the vicinity of the chromophore tryptophan residues.

The binding parameters were then obtained according to the following equation:

$$\log(\frac{F_0 - F}{F}) = \log K_A + n\log[Q] \qquad \text{(Equation 1)}$$

where n is the number of binding sites, K_A is the binding constant or the apparent association constant for drug-protein interaction. Values of n and K_A can thereby be determined from the intercept and slope by plotting $log(F_0 - F) / F$ versus log[Q]. The data in Table 1 show that the value of n is approximately equal to 1, indicating that there is one class of binding sites for NZ-105 in BSA (13). The value of the association constant increased from 5.58 to 16.14 x 10^4 M^{-1} with increasing temperature from 298 to 310 K. The increase in K_A may be caused by a slight expansion of the binding site which might accommodate more NZ-105 molecules. The expansion of the binding site may also provide a larger hydrophobic area for the binding, thus leading to the increase in the value of K_A.

Stern-Volmer analysis

Fluorescence quenching is usually classified as dynamic quenching and static quenching (14). Dynamic quenching, or collisional quenching, results from collision between fluorophore and quencher. Static quenching is due to ground-state complex formation between fluorophore and quencher (15). To determine the mechanism of binding between BSA and NZ-105, fluorescence intensity data were analyzed by the Stern-Volmer equation (16):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \qquad \text{(Equation 2)}$$

where F and F_0 are the fluorescence intensity with and without the quencher (drug), K_{sv} the Stern-Volmer quenching constant, and [Q] the concentration of the drug. If the resulting plots exhibit a good linear relationship, this is generally indicative of the purely collisional quenching process or static quenching process (17).

However, the Stern-Volmer plot showed a minor positive deviation (Figure 3) indicating the presence of both static and dynamic quenching. Therefore, the data were processed based on a modified Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + K_{SV}[Q]\exp(V[Q] \qquad \text{(Equation 3)}$$

where V is the static quenching constant, whose value can be obtained from Equation 3 by plotting $\frac{F_0 - F}{F\exp(V[Q]}$ versus [Q] by varying V until a linear plot is acquired (Figure 3). The K_{sv} can then be obtained from the slope. The values of V and K_{sv} at different temperatures (298, 304, and 310 K) are presented in Table 2. As can be seen from Table 2, the values of K_{sv} are much higher than the value of V, suggesting that the overall quenching is dominated by dynamic quenching, though a small static quenching component contributes to the positive deviation of the Stern-Volmer plot (18). This small part of static quenching can be explained by the fact that a minor fraction of the quencher molecule is very close to, or in contact with the fluorescent molecule at the exact moment it happens to be excited. The mechanism of efonidipine binding to BSA is similar to that of azelnidipine binding to BSA (19) due to the very similar structures of the two drugs.

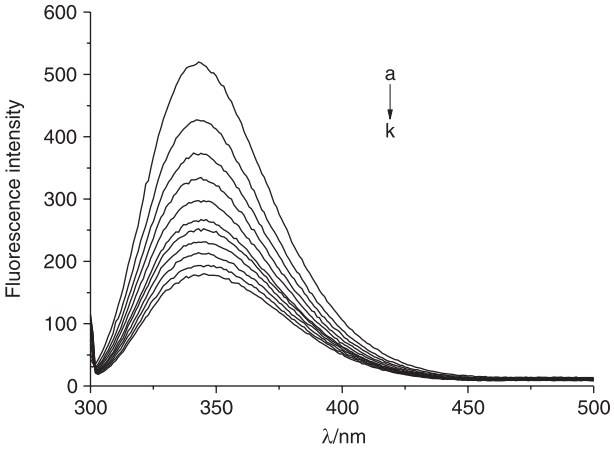

Figure 2. Fluorescence quenching spectra of BSA at different concentrations of efonidipine at 298 K; C_{BSA} = 10 µM; $C_{efonidipine}$ from a to k: 0 (a), 5 (b), 10 (c), 15 (d), 20 (e), 25 (f), 30 (g), 35 (h), 40 (i), 45 (j), and 50 (k) µM.

Table 1. Binding parameters obtained from the interaction of efonidipine with bovine serum albumin.

T (K)	K_A (x 10^4 M^{-1})	n	R^2	SD
298	5.58	1.02	0.9997	0.0057
304	10.38	1.08	0.9992	0.0104
310	16.14	1.14	0.9995	0.0090

The parameters were calculated from the data in Figure 2 using Equation 1. T = temperature.

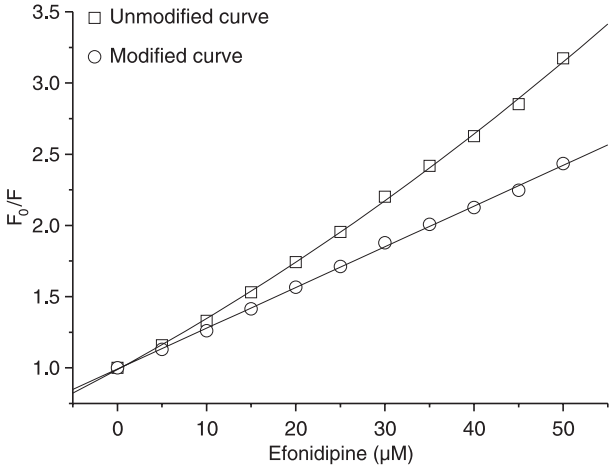

Figure 3. Modified and unmodified Stern-Volmer plots of the interaction of efonidipine with BSA. Original data are given in Figure 2.

Table 2. Quenching constants at three different temperatures for the efonidipine-bovine serum albumin system.

T (K)	K_{SV} (x 10^4 M^{-1})	V (x 10^3 M^{-1})	R^2	SD
298	3.45	4.00	0.9989	0.0264
304	3.24	5.20	0.9997	0.0093
310	2.84	5.30	0.9987	0.0177

Equation 3 was used to calculate the data given in this table from those in Figure 3. T = temperature.

Table 3. Thermodynamic parameters of the efonidipine-bovine serum albumin interaction.

T (K)	K_A (x 10^4 M^{-1})	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS^0 ($J \cdot mol^{-1} \cdot K^{-1}$)
298	5.58	-27.08		
304	10.38	-29.19	68.04	319.42
310	16.14	-30.91		

Data were calculated with Equation 5. T = temperature.

Type of interaction force between efonidipine and BSA

The interaction forces between drugs and biomolecules include hydrophobic force, electrostatic interactions, van der Waals interactions, hydrogen bonds, etc. (20). In order to identify the interaction force of NZ-105 with BSA, the thermodynamic parameters, i.e., free energy changes (ΔG^0), enthalpy changes (ΔH^0), and entropy changes (ΔS^0) of the interactions were calculated from the following equations:

$$\ln K = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \quad \text{(Equation 4)}$$

$$\Delta G^0 = -RT \ln K = \Delta H^0 - T\Delta S^0 \quad \text{(Equation 5)}$$

where, K corresponds to the binding constant K_A and R is the gas constant. The thermodynamic parameters are summarized in Table 3. The negative value of ΔG^0 identifies NZ-105 binding to BSA as a spontaneous process. The positive ΔH^0 and ΔS^0 values indicate that the force acting between NZ-105 and BSA is mainly a hydrophobic interaction (21). Thus, the non-polar hydrophobic groups of serum albumins may be responsible for the main effect determining the binding of NZ-105 and serum albumin. This, together with the number of binding sites, suggest that the binding site for NZ-105 on BSA is primarily on the Trp-212 residue, which is located within a hydrophobic binding pocket of the protein.

Conformational investigations

Synchronous fluorescence spectra (Figure 4) of BSA with different amounts of NZ-105 were measured to explore the conformational change of BSA upon the addition of NZ-105. The synchronous fluorescence spectra provide information about the molecular environment in the vicinity of the chromosphere molecules and the changes of maximum emission wavelength reflect the conformational changes of BSA (22). When the $\Delta\lambda$ between excitation wavelength and emission wavelength was fixed at 15 and 60 nm, the synchronous fluorescence spectra gave the characteristic information of tyrosine and tryptophan residues, respectively (23).

The quenching of the fluorescence intensity of tryptophan residues is stronger than that of tyrosine residues, indicating that tryptophan residues contribute greatly to the intrinsic fluorescence of BSA. It is also noted that there was a red shift of tryptophan residue fluorescence emission maximum upon the addition of NZ-105, whereas the emission maximum of tyrosine remained unchanged. The red shift indicates that the conformation of BSA is changed and the hydrophobicity decreases. The decreased hydrophobicity may be attributed to the binding of NZ-105 to BSA, which causes the extension of peptide strand of the BSA.

To confirm that there is a conformational change of BSA upon the addition of NZ-105, the CD spectra of BSA with and without NZ-105 were measured. As shown in Figure 5, the CD spectra of BSA exhibit two negative bands in the UV region at 209 and 222 nm, characteristic of the α-helical structure of the protein (24). The binding of

NZ-105 to BSA reduced the intensity of both bands, indicating a decrease of the α-helical content in the protein. That is, NZ-105 binds to the amino acid residue of the polypeptide chain of BSA and destroys their hydrogen bonding networks (25). Nevertheless, the CD spectra of BSA in the presence and absence of NZ-105 are similar in shape, implying that the structure of BSA after the binding of NZ-105 continues to be predominantly α-helical.

Energy transfer from BSA to efonidipine

According to Föster's theory, the energy transfer effect is related not only to the distance between the acceptor and donor (r_0), but also to the critical energy transfer distance (R_0):

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad \text{(Equation 6)}$$

where E is the energy transfer efficiency and R_0 is the critical distance when the transfer efficiency is 50% and can be calculated from the following equation:

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \quad \text{(Equation 7)}$$

In Equation 7, k^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, and ϕ is the fluorescence quantum yield of the donor. J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor (Figure 6). Therefore,

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad \text{(Equation 8)}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at wavelength λ, and $\varepsilon(\lambda)$ is the molar absorptivity of the acceptor at wavelength λ.

Under the present experimental conditions, $k = 2/3$, $n = 1.336$, $\phi = 0.15$ (26). According to Equations 6 to 8, we can calculate that $J = 2.32 \times 10^{-15}$ cm^3 L mol^{-1}, $R_0 = 2.00$ nm, $E = 0.301$, and $r = 2.30$ nm. Since the distance between donor and acceptor for the NZ-105-BSA complex is in the 2- to 8-nm scale, non-radiative energy transfer occurs between NZ-105 and BSA with high probability.

Binding studies in the presence of ANS

When two different kinds of drugs are administered simultaneously, it is possible that one will compete with the

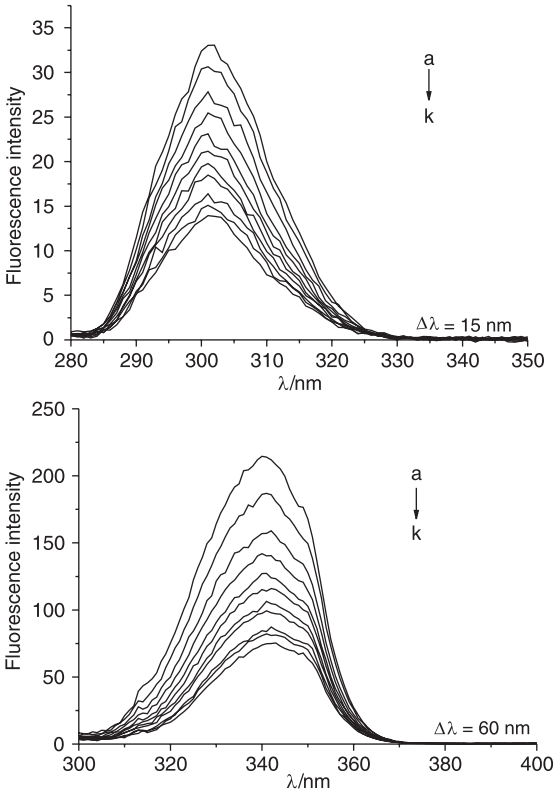

Figure 4. Synchronous fluorescence spectra of BSA upon the addition of efonidipine at 298 K. C_{BSA} = 10 μM; $C_{efonidipine}$ from a to k: 0 (a), 5 (b), 10 (c), 15 (d), 20 (e), 25 (f), 30 (g), 35 (h), 40 (i), 45 (j), and 50 (k) μM.

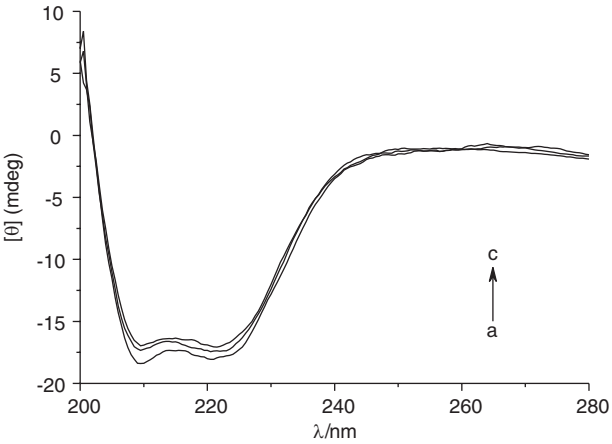

Figure 5. CD spectra of BSA in the presence of efonidipine. C_{BSA} = 1 μM; $C_{efonidipine}$:C_{BSA} from a to c are 0:1, 3:1, and 10:1.

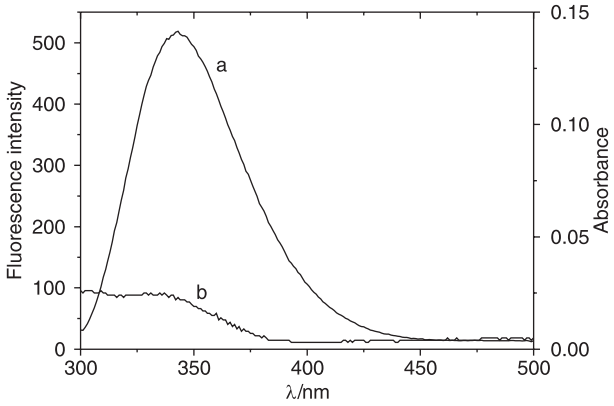

Figure 6. Spectral overlap between BSA fluorescence spectrum (a) and efonidipine UV-Vis absorbance spectrum (b). T = 298 K. $C_{efonidipine}$:C_{BSA} = 1:1.

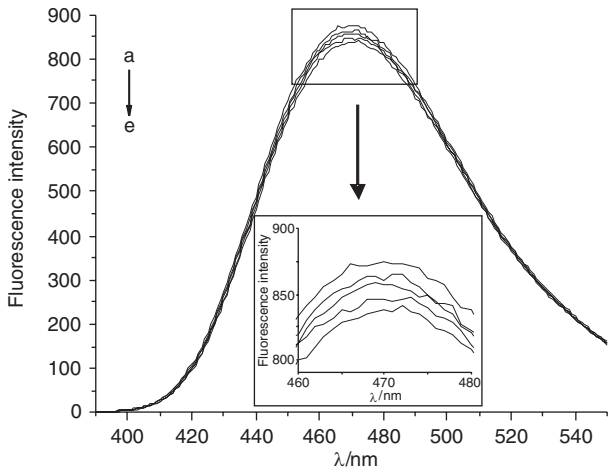

Figure 7. Fluorescence quenching spectra of ANS in the ANS-BSA system at different concentrations of NZ-105 at 298 K; C_{BSA} = 10 μM; C_{ANS} = 20 μM; $C_{efonidipine}$ from a to e: 0 (a), 5 (b), 10 (c), 15 (d), and 20 (e) μM.

Table 4. K_A binding constants of the efonidipine-bovine serum albumin complex in the presence of various metal ions at 298 K.

System	K_A (x 10^4 M^{-1})	R^2	SD
BSA-efonidipine	5.58	0.9997	0.0057
BSA-efonidipine-Al^{3+}	11.6	0.9987	0.0131
BSA-efonidipine-Ca^{2+}	10.1	0.9964	0.0219
BSA-efonidipine-K^{+}	7.41	0.9998	0.0046
BSA-efonidipine-Mg^{2+}	13.9	0.9956	0.0248
BSA-efonidipine-Zn^{2+}	6.03	0.9950	0.0250

The concentrations of bovine serum albumin (BSA), efonidipine, and metal ion were 10 μM, 0-50 μM, and 10 μM.

other for the binding sites on albumin in blood. The fluorescence studies were supplemented with the experiment of competitive binding of the fluorescent probe ANS. In the presence of NZ-05, ANS is one of the most universally used drugs in protein research. The fluorescence spectra of BSA show that both NZ-105 and ANS quench the fluorescence of BSA. The binding capacity of BSA to ANS/NZ-105 can be evaluated by the fraction of occupied sites (θ) described as follows:

$$\theta = 1 - F / F_0 \qquad \text{(Equation 9)}$$

where F_0 and F are fluorescence intensities measured at the maximum of the emission band, in the absence and presence of ANS/NZ-105, respectively. The value of θ for the ANS-BSA system is 85%, whereas for NZ-105-BSA system, it is 53%. This indicates that the magnitude of decrease in fluorescence intensity is much larger for ANS than for NZ-105, which suggest that the interaction of ANS with BSA is stronger than that of NZ-105 with BSA. This may be explained by the fact that the tryptophan residues are fully accessible to the hydrophobic probe ANS, but are only partially accessible to NZ-105.

Next, the BSA-ANS interaction was studied upon gradual addition of NZ-105 by monitoring the changes in ANS fluorescence intensity upon excitation at 370 nm. The fluorescence intensity of ANS exhibited a slight decrease upon the addition of NZ-105 (see Figure 7). Note that methanol did not affect the fluorescence intensity of ANS. The decrease observed in ANS fluorescence intensity upon the addition of NZ-105 suggests that NZ-105 displaced ANS from its binding site. Thus, NZ-105 can compete with ANS for hydrophobic sites on the surface of BSA, which means that ANS and NZ-105 do share same binding site in BSA.

Influences of common ions on the binding constant

Common ions are widely distributed in human blood and they have a definite ability to bind proteins (26), hence affecting the binding of drugs to albumin. To investigate the effect of ions on drug-albumin binding, the binding constants were determined in the presence of various ions. The values of the apparent binding constant K_A as acquired are listed in Table 4.

As shown in Table 4, the presence of Zn^{2+}, Mg^{2+}, Al^{3+}, K^{+}, and Ca^{2+} increases the binding constant of the NZ-105-BSA complex, indicating stronger binding of NZ-105 to BSA. The higher binding constants may result from the formation of metal ion-NZ-105 complexes via a metal ion bridge, which further interacts with the protein. This may

prolong the time during which NZ-105 remains in blood and thus enhance its maximum effects (25).

The values of the thermodynamic parameters ΔH^0, ΔS^0 and ΔG^0 at different temperatures demonstrate that the hydrophobic interaction force plays a major role in the interaction of NZ-105 with BSA. The results of synchronous fluorescence spectroscopy and CD show that the conformation of BSA changes in the presence of NZ-105. The interaction of NZ-105 with BSA is weaker than that of ANS with BSA. However, NZ-105 can compete with ANS for hydrophobic sites on the surface of BSA. Furthermore, the presence of metal ions such as Zn^{2+}, Mg^{2+}, Al^{3+}, K^+, and Ca^{2+} increases the binding constant of the efonidipine-BSA complex.

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