ISSN : 0974 - 7435 Volume 11 Issue 6

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 11(6), 2015 [233-239]

Investigation of the interaction between aspirin and bovine serum albumin using fluorescence spectroscopy

Baosheng Liu*, Lihui Zhang, Zhiyun Li, Ying Guo

Key Laboratory of Medical Chemistry and Molecular Diagnosis, Ministry of Education, College of Chemistry & Environmental Science, Hebei University, Baoding 071002, Hebei Province, (P.R.CHINA)

E-mail: lbs@hbu.edu.cn

ABSTRACT

Under simulated physiological conditions (pH = 7.4), the reaction mechanism between aspirin and bovine serum albumin (BSA) at different temperatures (293 K, 303 K, 310 K) was investigated by the fluorescence spectroscopy (method 1) with focus on the fluorescence change of protein, as well as the fluorescence spectroscopy (method 2) with focus on the fluorescence change of drug. The results indicated that the electrostatic force played an important role on the conjugation reaction between aspirin and BSA. The binding constant obtained from method 2 was larger than the one obtained from method 1 by two orders of magnitude for the aspirin-BSA system. This indicated that the method 2 with focus on the fluorescence change of drug was a more accurate and more reasonable method. At last the correctness of method 2 was verified by UV-vis absorption spectroscopy and synchronous fluorescence spectroscopy with focus on the fluorescence change of drug.

KEYWORDS

Fluorescence spectroscopy;
Aspirin;
Bovine serum albumin;
Interaction.

INTRODUCTION

The fluorescence spectroscopy (method 1) with focus on the fluorescence change of protein studies the reaction mechanism of small molecule drugs and proteins, by studying the change of fluorescence intensity of protein at the maximum emission wavelength before and after adding the drugs as well as the derived binding constants, binding sites and the donor-to-acceptor distance etc between proteins and drugs[1-3]. Protein consists 20 kinds of amino-acids and only aromatic tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) emit fluorescence and exhibits different fluorescence due to their individual chromophore (side groups)[4]. In method 1, the main source of BSA fluorescence is Trp-212. Method 1 does not reflect interaction of the other non-fluorescence-emitting residues with drugs[5], and the fluorescence spectrogram only reflects partial information of the interaction of BSA with drug, the obtained information is thus of insufficient accuracy. Fluorescence changes of small molecules drugs will reflect the whole information of interaction between drug and protein. As a result, the fluorescence of small molecule drug reflects the overall fluorescence in the interaction. Because of this, a new method by taking the drug as the object of detection is applied, to study the interaction between

FULL PAPER

drugs and proteins in order to cover the shortcomings of method 1.

Acetylsalicylic acid (aspirin) is a common analgesic and an antipyretic[6]. It is metabolised and transformed to salicylic acid, which plays an important role in human and animal metabolism[7]. The binding mechanism of drugs and proteins was studied by utilizing fluorescence methods with studied objects as proteins and drugs, respectively, followed by UV-vis absorption spectroscopy and synchronous fluorescence spectroscopy with focus on the fluorescence change of drug to verify the latter. The new method is a useful supplement to the application of fluorescence spectroscopy on the research of combination mechanism of proteins and drugs, and helps to further properly understand about the interactions mechanism of drug-protein system.

EXPERIMENTAL

Apparatus

All fluorescence spectra were recorded with a Shimadzu RF-5301PC spectro- fluorophotometer. Absorption was measured with an UV-Vis recording spectrophotometer (UV-265 Shimadzu, Japan). All pH measurements were made with a pHS-3C precision acidity meter (Leici, Shanghai). All temperatures were controlled by a CS501 super-heated water bath (Nantong Science Instrument Factory).

Materials

Aspirin is of purity grade inferior 98.5%. Bovine serum albumin (BSA) was purchased from Sigma Company and of purity grade inferior 99%. Stock solutions of BSA (1.0×10^{-5} mol·L^{-1}), and aspirin (1.0×10^{-3} mol·L^{-1}) were prepared. All the stock solutions were further diluted as working solutions prior to use. Tris-HCl (0.05 mol·L^{-1}) buffer solution containing NaCl (0.15 mol·L^{-1}) was used to keep the pH of the solution at 7.40, and NaCl solution was used to maintain the ionic strength of the solution. All other reagents were analytical grade and all aqueous solutions were prepared with newly double-distilled water and stored at 277 K.

General procedures

Method 1 measurements

In a typical fluorescence measurement, 1.0 mL of Tris-HCl buffer, pH 7.40, 0.4 mL of 1.0×10^{-5} mol·L^{-1} BSA solution and different concentrations of aspirin were added into 10 mL colorimetric tube successively. The samples were diluted to scaled volume with water, mixed thoroughly by shaking, and kept static for 30 minutes at different temperatures (293, 303 and 310 K). Excitation wavelength for BSA was 280 nm, with excitation and emission slit at 5nm. The solution was subsequently scanned on the ûuorophotometer and the fluorescent intensity F at the maximum fluorescent peaks was recorded.

Method 2 measurements

1.0 mL of Tris-HCl buffer, pH 7.40, 1.0 mL of 1.0×10^{-3} mol·L^{-1} aspirin solution and different concentrations of BSA were added into 10 mL colorimetric tube successively. The samples were diluted to scaled volume with water, mixed thoroughly by shaking, and kept static for 30 minutes at different temperatures (293, 303 and 310 K). Excitation wavelength for aspirin was 296 nm, with excitation and emission slit at 5 nm. The solution was subsequently scanned on the ûuorophotometer and the fluorescent intensity I_F at the maximum fluorescent peaks was recorded.

Synchronous fluorescence measurements

The Volume of aspirin is 2.0 mL, the others were the same as in Section 2.3.2. The fluorescence spectra were scanned when the value of $\Delta\lambda$ was between the excitation and emission wavelengths stabilized at 30 nm in the range from 250 nm to 550 nm, and recorded the fluorescent intensity I_S at the maximum fluorescent peaks.

UV-vis absorption measurements

The solution preparation was the same as in Section 2.3.3, with corresponding concentration of BSA solution as the reference. The UV-vis absorption spectra of aspirin in the presence and absence of BSA were scanned with 1cm quartz cells in the range from 190 nm to 370 nm, and the absorption intensity A at the maximum absorption peak was recorded.

RESULTS AND DISCUSSION

Fluorescence quenching spectra of BSA-aspirin system

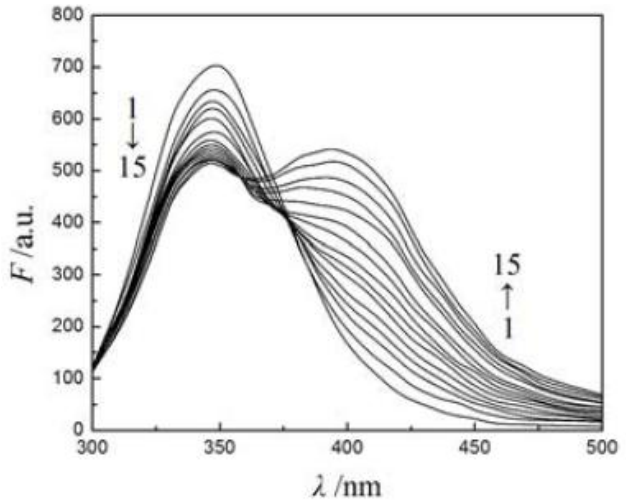

Figure 1 : Fluorescence spectra of BSA-aspirin system (T = 303 K); C_{BSA} = 4.0×10^{-7} mol L^{-1}; 1~15 $C_{aspirin}$ = (0, 0.75, 3.0, 5.0, 7.0, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60) × 10^{-5} mol L^{-1}

Proteins were considered to have intrinsic fluorescence due to the presence of amino acids, mainly Trp, Tyr and Phe. When the excitation wavelength was at 280 nm, BSA has a strong fluorescence emission peak at 340 nm. The fluorescence spectra of BSA-aspirin system was shown in Figure 1. As shown in Figure 1, the fluorescence intensity of BSA decreased gradually with the addition of aspirin, however, the fluorescence intensity of aspirin increased gradually, and a launch point was observed between the fluorescence peak of aspirin and the fluorescence peak of BSA. This result showed that aspirin could quench the intrinsic fluorescence of BSA significantly and there was an interaction between aspirin and BSA, it could also reveal that a new complex is being formed[8].

In order to confirm the quenching mechanism, the fluorescence quenching data are analyzed by the Stern-Volmer equation[9]:

$$F_0 / F = 1 + K_q \tau_0 [L] = 1 + K_{sv}[L] \quad (1)$$

Where, F_0 and F are the fluorescence intensities of BSA before and after the addition of the aspirin, respectively. τ_0 is the average lifetime of fluorescence without ligand, which is about 10^{-8} s. K_{sv} is the Stern-Volmer quenching constant. K_q is the bimolecular quenching constant, and [L] is the concentration of the ligand. Based on the linear regression plot of F_0/F versus [L], the K_q values can be obtained. The calculated results were shown in TABLE 1. Different mechanisms of quenching are usually classified as dynamic quenching and static quenching. Dynamic and static quenching can be distinguished by their different dependence on temperature. The quenching rate constant decreases with rising temperature for static quenching, but the reverse trend is observed for the dynamic quenching. In TABLE 1, the values of K_{sv} decreased with the rising temperature in all systems, which indicated that the probable quenching mechanism of the interaction between BSA and aspirin was initiated by complex formation rather than by dynamic collision[10]. In addition, all the values of K_q were much greater than the maximum scatter collision quenching constant of various quenchers (2×10^{10} L·mol^{-1} s^{-1}), this also suggested that the quenching was a static process[11].

For static quenching process, the relationship between the fluorescence intensity and the concentration of quencher can be usually described by Eq. (2) [12] to obtain the binding constant (K_a) and the number of binging sites (n) in most paper:

$$\log\{(F_0 - F)/F\} = n \lg K_a + n \log\{[D_t] - n(F_0 - F)/F_0[B_t]\} \quad (2)$$

Where, [D_t] and [B_t] are the total concentrations of aspirin and BSA, respectively. On the assumption that n in the bracket is equal to 1, the curve of log[(F_0-F)/F] versus log{[D_t]-[B_t](F_0-F)/F_0} is drawn and linear regression equation is plotted, then the value of n can be obtained from the slope of the plot. If the n value obtained is not equal to 1, then it is substituted into the bracket and the curve of log[(F_0-F)/F] versus log{[D_t]-

TABLE 1 : Quenching related parameters of BSA and aspirin at different temperatures

T/(K)	K_q /(L·mol^{-1} s^{-1})	K_{sv} / (L·mol^{-1})	r_1	K_a /(L·mol^{-1})	n	r_2
293	2.79×10^{11}	2.79×10^3	0.996 6	4.53×10^3	0.67	0.996 5
303	2.69×10^{11}	2.69×10^3	0.998 8	3.55×10^3	0.74	0.996 2
310	2.33×10^{11}	2.33×10^3	0.996 2	2.87×10^3	0.63	0.998 0

K_q is the quenching rate constant; K_a is the binding constant; n is the number of binding site. r_1 is the linear relative coefficient of F_0/F~[L]; r_2 is the linear relative coefficient of log(F_0-F)/F~log{[D_t]-n[B_t](F_0-F)/F_0}.

FULL PAPER

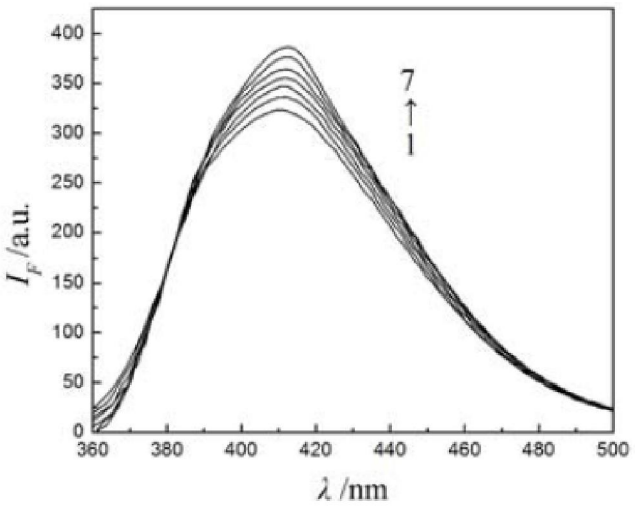

Figure 2 : Fluorescence spectrum of aspirin-BSA system (T = 303 K); $C_{aspirin}$ =1.0×10^{-5} mol L^{-1}; 1~7 C_{BSA}= (0, 0.4, 0.8, 1.0, 1.5, 2.0, 3.0) ×10^{-7} mol L^{-1}

$n[B_t](F_0\text{-}F)/F_0\}$ is drawn again. The above process was repeated again and again till n obtained was only a single value. Based on the n obtained the binding constant K_a can be also obtained. In this work, a calculation program was developed. The calculation process can be finished with calculator based on the simple program and the calculated result can be obtained by inputting F, $[D_t]$ and $[B_t]$. The calculated result was shown in TABLE 1. As shown in TABLE 1, the fact that the values of n were all approximately to 1 implied that just one binding site for aspirin existed in BSA. Meanwhile, binding constants K_a decreased with the rising temperature, further suggested that the quenching was a static process[13].

Fluorescence spectra studies

According to the experiment as discussed Section 2.3.2, the interaction between BSA and aspirin with aspirin as the detection object is investigated. With gradual addition of BSA to aspirin solution, the intensity of the peak at 410 nm increases with red shift of 4 nm, the results indicate that the interaction between BSA and aspirin leads to the formation of a complex between drug and protein. After deducting the corresponding concentration of BSA, the fluorescence spectra of the system is shown in Figure 2. Figure 2 shows that with the increasing concentration of BSA, the fluorescence intensity of aspirin at 410 nm increases. The results indicate that the aspirin-BSA system is a fluorescence enhancement reaction when drug aspirin is treated as the studied object. The fluorescence enhancement equation[14]:

$$\left(\mathbf{I_F} - \mathbf{I_F}^0\right)^{-1} = \Delta\mathbf{I}_{\mathbf{F\,max}}{}^{-1} + \mathbf{K}_{\mathbf{a1}}{}^{-1} \cdot \Delta\mathbf{I}_{\mathbf{F\,max}}{}^{-1} \cdot [\mathbf{L}]^{-1} \quad (3)$$

Where, $I_F{}^0$ and I_F are the steady-state fluorescence intensity in the absence and presence of BSA, respectively. $[L]$ is the concentration of the ligand. $I_{F''}$ is the fluorescence intensity when BSA and aspirin reaches saturation. Based on the linear regression plot of $(I_F\text{-}I_F)^{-1}$ versus $[L]^{-1}$, the K_{a1} values can be obtained. The calculated results were shown in TABLE 2. As shown in both TABLE 1 and TABLE 2, the K_{a1} value of method 2 is much greater than the K_a value of method 1 at the same temperature, which shows that in addition to Trp-212 of BSA peptides, the others also interacts with aspirin. And in addition to the "point to point" interaction between aspirin and Trp-212, the "point to side" interaction between aspirin and the other peptides in BSA hydrophobic sub-domain also exists[15]. This shows that compared to method 1 with protein as detection object, treating drugs as detection object can give more complete and more accurate expression of the interaction information of proteins and drugs.

TABLE 2 : The binding constants of aspirin-BSA system by method 2 at different temperatures

T/(K)	K_{a1}/(L·mol^{-1})	Linear regression equation	r_3
293	5.46×10^5	$(I_F\text{-}I_F{}^0)^{-1}$ = 0.00667+ 1.223×10^{-8}$[L]^{-1}$	0.999 3
303	3.82×10^5	$(I_F\text{-}I_F{}^0)^{-1}$ = 0.01035 + 2.706×10^{-8}$[L]^{-1}$	0.998 9
310	3.00×10^5	$(I_F\text{-}I_F{}^0)^{-1}$ = 0.01114 + 3.708×10^{-8}$[L]^{-1}$	0.996 9

K_{a1} is the binding constant; r_3 is the linear relative coefficient of $(I_F\text{-}I_F{}^0)^{-1}$~$[L]^{-1}$.

Synchronous fluorescence spectra studies

Synchronous fluorescence method has the advantages of good selectivity, high sensitivity, less interference etc, and it can be used for the simultaneous determination of multicomponent mixture[16]. One of the most important parameter for using synchronous fluorescence technology is choosing the correct wavelength interval Δλ, which directly influences the sensitivity and selectivity[17]. The effects of Δλ on synchronous fluorescence intensity at 20, 30, 40, 50,

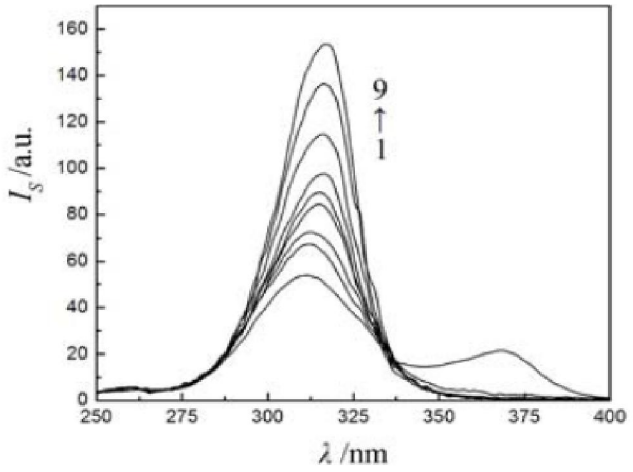

Figure 3 : Synchronization fluorescence spectrum of aspirin-BSA system (T = 303 K); $C_{aspirin}$ =2.0×10^{-5} mol L^{-1}; 1~9 C_{BSA}= (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0) ×10^{-7} mol L^{-1}

TABLE 3 : The binding constants of aspirin-BSA system by synchronous fluorescence method at different temperatures

T/(K)	K_{a2}/(L·mol^{-1})	Linear regression equation	r_4
293	6.50×10^5	$(I_s\text{-}I_s^0)^{-1}$ = 0.00261+ 4.014×10^{-9}[L]$^{-1}$	0.997 0
303	4.34×10^5	$(I_s\text{-}I_s^0)^{-1}$ = 0.00228 + 5.258×10^{-9}[L]$^{-1}$	0.998 2
310	3.50×10^5	$(I_s\text{-}I_s^0)^{-1}$ = 0.00093 + 2.645×10^{-9}[L]$^{-1}$	0.999 3

K_{a2} is the binding constant; r_4 is the linear relative coefficient of $(I_s\text{-}I_s^0)^{-1}$~[L]$^{-1}$.

60, 70, 80, 90, 100, 110, 100 and 120 nm were tested and it turned out that when Δλ is 30 nm, the synchronous fluorescence signal of the influence is the strongest. With increasing concentration of BSA, the synchronous fluorescence intensity of aspirin-BSA also increases. After deducting the corresponding concentration of BSA, the fluorescence spectra of the system is shown in Figure 3. According to the Eq. (3), the binding constants K_{a2} are calculated and listed in TABLE 3. Comparing TABLE 1, TABLE 2 and TABLE 3, we can see that the K_{a2} value is observed to be much larger than K_a of the method 1 and close to K_{a1} obtained by the method 2. This phenomenon also shows that treating the drug as detection objects can give more complete and more accurate expression the interaction information of proteins and drugs. In this way, the correctness for method 2 is proved.

UV-vis absorption spectra studies

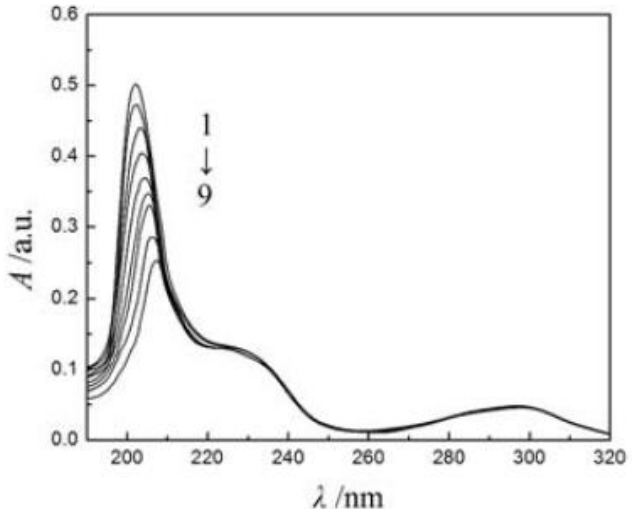

Figure 4 : Absorption spectra of aspirin-BSA system (T = 303 K); $C_{aspirin}$ =2.0×10^{-5} mol L^{-1}; 1~9 C_{BSA}= (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0) ×10^{-7} mol L^{-1}

The binding constant K_b of protein and drug can be calculated on the following equation[18]:

$$(A_0 - A)^{-1} = A_0^{-1} + K_b^{-1}A^{-1}_0[L]^{-1} \quad (4)$$

Where, A_0 and A are the absorption values in the absence and presence of ligand, respectively. And [L] is the concentration of the ligand. The UV-vis absorption spectra of aspirin in the absence and presence of BSA are shown in Figure 4. As shown in Figure 4, with gradual addition of BSA to aspirin solution, the intensity of the peak at 202 nm decreases with red shift of 7 nm, indicating that the interaction between BSA and aspirin leads to the formation of a complex between drug and protein[19]. Based on the linear regression plot of $(A_0\text{-}A)^{-1}$ versus $[L]^{-1}$, the K_b values can be obtained. The calculated results are shown in TABLE 4. As seen in TABLE 4, the binding constant K_b decreases with rising temperature, which is consistent with the results of fluorescence methods. The K_b values are close to $K_{a1}0K_{a2}$ of the fluorescence spectroscopy methods with focus on the fluorescence change of drug, which indicates that the method 2 is correct. The differences of binding constants may be caused by the differences between fluorescence method and UV-vis absorption method.

Type of interaction force of BSA-aspirin systems

Generally, the interaction force between the small drug molecule and biological macromolecule include hydrogen bond, Van der Waals force, electrostatic in-

FULL PAPER

TABLE 4 : The binding constants of aspirin-BSA system by UV-vis absorption method at different temperatures

T/(K)	K_b/(L·mol^{-1})	Linear regression equation	r_5
293	6.06×10^5	$(A_0\text{-}A)^{-1} = 0.797 + 1.316\times10^{-6}[L]^{-1}$	0.997 9
303	4.23×10^5	$(A_0\text{-}A)^{-1} = 1.140 + 2.697\times10^{-6}[L]^{-1}$	0.998 5
310	3.10×10^5	$(A_0\text{-}A)^{-1} = 0.414 + 1.338\times10^{-6}[L]^{-1}$	0.997 2

K_b is the binding constant; r_5 is the linear relative coefficient of $(A_0\text{-}A)^{-1}$~$[L]^{-1}$

teractions and hydrophobic force, etc. Ross and Subramanian[20] have characterized the sign and magnitude of the thermodynamic parameter, enthalpy change (ΔH), free energy (ΔG) and entropy change (ΔS) of reaction, associated with various individual kinds of interaction. When temperature varies in a small range, the ΔH could be considered as a constant[21]. Negative ΔH and positive ΔS indicate electrostatic interaction plays a major role in the binding reaction. Positive ΔH and ΔS are generally considered as the evidence for typical hydrophobic interactions. In addition, Van der Waals force and hydrogen bonding formation in low dielectric media are characterized by negative ΔH and ΔS[22]. The thermodynamic parameters can be calculated on the basis of the following equation[23]:

TABLE 5 : The thermodynamic parameters of aspirin-BSA at different temperatures

Method	T /(K)	K /(L·mol^{-1})	ΔH /(KJ·mol^{-1})	ΔS /(J·mol^{-1}·K^{-1})	ΔG /(KJ·mol^{-1})
Method 1	293	4.53×10^3	-20.10	1.42	-20.51
	303	3.55×10^3		1.66	-20.59
	310	2.87×10^3		1.39	-20.52
Method 2	293	5.46×10^5	-26.58	19.10	-32.18
	303	3.82×10^5		19.13	-32.38
	310	3.00×10^5		19.10	-32.50
UV-vis absorption method	293	6.06×10^5	-29.51	9.97	-32.43
	303	4.23×10^5		10.30	-32.64
	310	3.10×10^5		9.92	-32.59
Synchronous fluorescence method	293	6.50×10^5	-27.69	16.79	-32.61
	303	4.34×10^5		16.55	-32.70
	310	3.50×10^5		16.83	-32.90

$$\mathbf{R \ln K = \Delta S - \Delta H / T} \quad (5)$$

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

According to the relevant thermodynamic parameters of small molecule drugs and biological macromolecules, the type of interaction force can be simply judged[24]. Based on the linear regression plot of $R\ln K$ versus $1/T$, the ΔH value can be obtained. Therefore, the values of ΔH, ΔS and ΔG were listed in TABLE 5. The reaction process of aspirin and BSA was a spontaneous molecular interaction procedure in which entropy increased and Gibbs free energy decreased[25]. The entropy is a measure of the degree of disorder of the system. Each spontaneous and irreversible process is a change from order to disorder, and towards the direction of higher degree of confusion. Under the isothermal, isobaric and no non-expansion work conditions, spontaneous variation of the system is always carried out towards decreasing the Gibbs free energy[26]. From TABLE 5 it can be seen that the ΔS values obtained were smallest and the ΔG values obtained were largest by method 1, which indicated that the reaction using aspirin as the focus was easier. It is more accurate and reasonable that we take the fluorescence changes of drugs as the studied object. The positive value of ΔS and ΔH showed that the electrostatic interaction play a major role in the binding process[27]. The conclusions of four methods were consistent, which indicated that when studying the type of interaction force between drug and protein, the conclusions of the method 1 were consistent with methods with focus on the

fluorescence change of drug.

CONCLUSIONS

In this paper, the interaction between aspirin and BSA was studied at different temperatures with the methods of method 1 and method 2, and verified by UV-vis absorption and synchronous fluorescence method. Comparing binding constants of the four methods shows that the K_a values of method 2, synchronous fluorescence method and UV-vis absorption method were greater than method 1 and the three values are close to each other. That means that taking drugs as detection object for method 2 can be more comprehensive and more accurate when expressing the interaction information between protein and drug in terms of fluorescence. Linear phase relations of linear regression equation of three methods are above 0.99. Although the values for the methods with focus on the fluorescence change of drug and UV-vis absorption method of binding constant are slightly different, the difference is minimal. It also suggests that the method 2 is reasonable. The method 2 is a challenge for method 1, and it provides a new way to study the interaction more accurately between drugs and proteins, which will further improve the study of the reaction mechanism between drugs and proteins.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of National Science Foundation of China (Grant no. 20675024) and Hebei Provincial Key Basic Research Program (Grant no. 10967126D).

REFERENCES

[1] B.S.Liu, X.N.Yan, C.Yang, Y.Q.Zhang, Y.K.Lv; J.Hebei University.Nat.Sci., **31**, 262 **(2011)**.
[2] A.Varlan, M.Hillebrand; Rev.Roum.Chim., **55**, 69 **(2010)**.
[3] J.Yang, Z.H.Jing, J.J.Jie, P.Guo; J.Mol.Struct., **920**, 227 **(2009)**.
[4] J.R.Lakowicz; Principle of Fluorescence Spectroscopy.New York: Plenum Press, **(1983)**.
[5] T.Tan, R.Huang, Z.N.Xia; Chin.J.Anal.Chem., **35**, 1415 **(2007)**.
[6] Z.aciong; Thromb.Res., **110**, 361 **(2003)**.
[7] J.R.Paterson, G.Baxter, J.S.Dreyer, J.M.Halket, R.T.Flynn, J.R.Lawrence; J.Agric.Food Chem., **56**, 11648 **(2008)**.
[8] R.Huang, Z.N.Xia, P.Gong; Spectrosc.Spect.Anal., **28**, 161 **(2008)**.
[9] Q.L.Guo, R.Li, F.L.Jiang, J.C.Tu, L.W.Li, Y.Liu; Acta Phys.Chim.Sin., **25**, 2147 **(2009)**.
[10] Y.J.Hu, Y.Liu, J.B.Wang, X.H.Xiao, S.S.Qu; J.Pharm.Biomed.Anal., **36**, 915 **(2004)**.
[11] T.W.Cao, J.D.Yang; Acta Phys.Chim.Sin., **24**, 715 **(2008)**.
[12] X.N.Yan, B.S.Liu, B.H.Chong, S.N.Cao; J.Lumin., **142**, 155 **(2013)**.
[13] S.A.Markarian, M.G.Aznauryan; Mol.Biol.Rep., **39**, 7559 **(2012)**.
[14] M.H.Luo, C.W.Li, C.P.Yu, Z.H.Li, H.Y.Guan, L.C.Lu; J.Pediatric Pharm., **18**, 1 **(2012)**.
[15] S.Y.Bi, Y.T.Sun, C.Y.Qiao, H.Q.Zhang, C.M.Liu; J.Lumin., **129**, 541 **(2009)**.
[16] G.Z.Chen, X.Z.Huang, J.G.Xu; Analysis of Fluorescence Spectroscopy.Second Ed.Bei Jing: Science Press, 115 **(1990)**.
[17] A.N.Liang, L.Wang, H.Q.Chen, B.B.Qian, B.Ling, J.Fu; Talanta, **81**, 438 **(2010)**.
[18] X.J.Chang, Y.Huang, Q.He; china Acta.Chim.Sin., **63**, 223 **(2005)**.
[19] F.Ding, J.L.Huang, J.Lin, Z.Y.Li, F.Liu, Z.Q.Jiang, Y.Sun; Dyes Pigm., **82**, 65 **(2009)**.
[20] P.D.Ross, S.Subramanian; Biochemistry, **20**, 3096 **(1981)**.
[21] Y.J.Hu, Y.Liu, R.M.Zhao, J.X.Dong, S.S.Qu; J.Photochem.Photobiol., A: Chem., **179**, 324 **(2006)**.
[22] H.X.Zhang, X.Huang, P.Mei, K.H.Li, C.N.Yan; J.Fluoresc., **16**, 287 **(2006)**.
[23] I.Matei, M.Hillebrand; J.Pharm.Biomed.Anal., **51**, 768 **(2010)**.
[24] X.Y.Yu, R.H.Liu, R.Q.Yi, F.X.Yang, H.W.Huang, J.Chen, D.H.Ji, Y.Yang, X.F.Li, P.G.Yi; Spectrochim.Acte Part A: Mol.Biomol.Spectrosc,, **78**, 1329 **(2011)**.
[25] J.Mariam, P.M.Dongre, D.C.Kothari; J.Fluoresc., **21**, 2193 **(2011)**.
[26] W.X.Shen; Physical Chemistry: A Key Course.Second Ed.Bei Jing: Science Press, 84 **(2009)**.
[27] Q.Yao, X.Y.Yu, T.Y.Zheng, H.T.Liu, Y.Yang, P.G.Yi; Spectrochim.Acte Part A: Mol.Biomol.Spectrosc., **113**, 447 **(2013)**.