ISSN(PRINT) : 2320 -1967
ISSN(ONLINE) : 2320 -1975

FULL PAPER

CHEMXPRESS 9(4), 303-309, (2016)

Comparative studies on the interaction of melizide with bovine serum albumin by fluorescence quenching spectroscopy and synchronous fluorescence spectroscopy

Shaotong Duan, Baosheng Liu*, Mengmeng Cui, Tongtong Li
College of Chemistry & Environmental Science, Hebei University, Baoding 071002, Hebei Province, (P.R.CHINA)
E-mail: lbs@hbu.edu.cn

Abstract : The reaction mechanism of Melizide to bovine serum albumin was investigated by both fluorescence quenching and synchronous fluorescence spectroscopy in different temperature (293, 303 and 310 K). The results demonstrated that Melizide caused strong fluorescence quenching of bovine serum albumin by a dynamic quenching mechanism, during which the hydrophobic interaction played a dominant role in this system. And the order of magnitudes of binding constant is 10^4, the number of binding site in the system was closed to 1. It also showed that the primary binding site for CFS was sub-hydrophobic domain IIA. The UV-Vis absorption spectra also showed that the quenching process is dynamic quenching.

Keywords : Bovine serum albumin; Melizide; Fluorescence quenching spectrometry; Synchronous fluorescence spectroscopy; Interaction.

INTRODUCTION

As the improvement of human living conditions and the change of diet, diabetes has become "the third biggest killer" after the tumor and cardio-cerebrovascular disease which endangers human's health. Melizide is an oral hypoglycemic agent which belongs to the second-generation sulfonylureas[1]. Its structure was shown in Figure 1. Melizide is used for the treatment of type II diabetes, can directly stimulate the islet cells and make them release endogenous insulin, thereby reducing blood glucose levels and glycosylated hemoglobin. The study of the interaction between proteins and endogenous compounds as well as many drug molecules has been attracting the attention of people. Studing the binding mechanism of protein-ligands has very important significance for the life sciences, chemistry, pharmacology and clinical medicine. The interaction between bovine

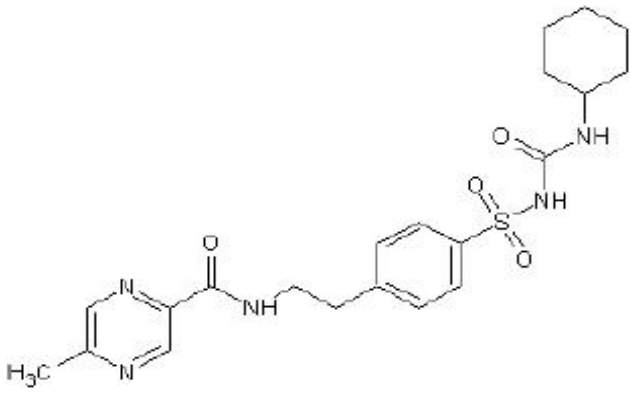


Figure 1 : Chemical structure of melizide

serum albumin (BSA) and various drugs has been reported numerously[2,3]. However the interaction between Melizide and BSA has not yet been reported.

EXPERIMENTAL

Apparatus and materials

FULL PAPER

a. Apparatus

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

b. Materials

BSA was purchased from Sigma-Aldrich (purity grade inferior 99%, Shanghai, China). Stock solutions of Melizide standard substance (CAS#, 29094-61-9) (5.6×10^{-5} M) and BSA (1.0×10^{-5} M) were prepared. All the stock solutions were further diluted as working solutions prior to use. Tris-HCl buffer solution containing NaCl (0.15 M) 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 of analytical grade, and all aqueous solutions were prepared with fresh double-distilled water and stored at 277 K.

The fluorescence intensities were corrected for the absorption of excitation light and re-absorption of emitted light to decrease the inner filter using the following relationship[4]:

$$F_{cor} = F_{obs} \times e^{(A_{ex} + A_{em})/2} \quad (1)$$

Where F_{cor} and F_{obs} are the corrected and observed fluorescence intensities, respectively, and A_{ex} and A_{em} are the absorbance values of Melizide at excitation and emission wavelengths, respectively. The fluorescence intensity used in this article was corrected.

Procedures

1 Fluorescence measurements

In a typical fluorescence measurement, 1.0 mL Tris-HCl (pH = 7.40), 0.3 mL BSA solution (1.0×10^{-5} M) and different concentrations of Melizide were added into 10 mL colorimetric tube successively. The samples were diluted to scaled volume with double-distilled water, mixed thoroughly by shaking, and kept static for 40 min at different temperatures (298, 303 and 310 K). The excitation wavelength for BSA was 280 nm and 295 nm, respectively, with a 1.0 cm path length cell. The excitation and emission slits were set at 5 nm. The solution was subsequently scanned on the fluorophotometer and determined the fluorescent intensity at 340 nm.

2 Synchronous fluorescence measurements

Solution preparation was as detailed above. We recorded the synchronous fluorescence spectra of the BSA-Melizide system when the $\Delta\lambda$ value between the excitation and emission wavelengths was stabilized at 15 and 60 nm, respectively.

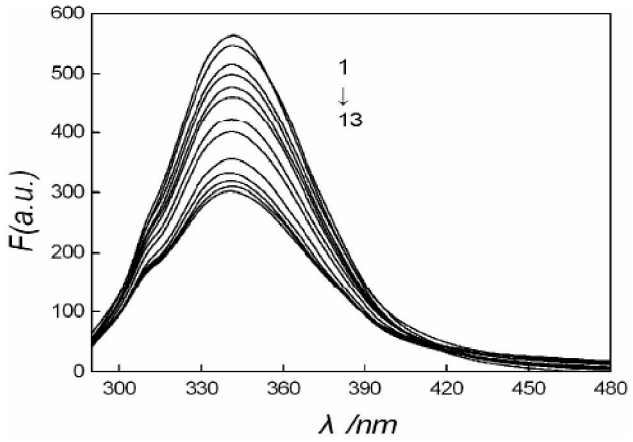
3 UV-Vis absorption measurements

1.0 mL Tris-HCl (pH = 7.40), 2.0 mL BSA solution (1.0×10^{-5} M) and different concentrations of Melizide were added into 10 mL colorimetric tube successively. The reference was different concentrations of Melizide solution. The samples were diluted to scaled volume with double-distilled water, mixed thoroughly by shaking, and kept static for 40 min at 298 K. The UV-Vis absorption spectra of BSA in the presence and absence of Melizide were recorded with 1.0 cm quartz cells in the range from 190 nm to 350 nm.

RESULTS AND DISCUSSION

Fluorescence quenching mechanism studies of BSA-Melizide system

Fluorescence quenching measurement is an effective method for studying the binding interactions of protein with ligand at a molecular level[5]. A variety of molecular interactions can result in fluorescence quenching of excited state fluorophores. These include molecular rearrangement, energy transfer, ground state complex formation and collision quenching[6]. BSA is considered to have intrinsic fluorescence due to the presence of amino acids, mainly tryptophan and tyrosine. When the excitation wavelength was 280 nm (or 295 nm), BSA had a strong fluorescence emission peak at 340 nm. The fluorescence spectra of BSA-Melizide system (λ_{ex} = 280 nm) was shown in Figure 2 (it was similar to 295 nm), which showed that the fluorescence intensity of BSA decreased regularly with the addition of Melizide when the excitation wavelength was 280 nm (similar to 295 nm). This result showed that Melizide could quench the intrinsic fluorescence of BSA and that there was an interaction between Melizide and BSA[7]. The fluorescence quenching is usually classified as dynamic quenching and static quenching,



C_{BSA} = 3.0 × 10^{-7} M, $C_{Melizide}$ = (0, 0.028, 0.056, 0.28, 0.56, 1.12, 1.68, 2.24, 2.8, 3.36, 3.92, 4.48, 4.76) × 10^{-5} M

Figure 2 : Fluorescence spectra of BSA-Melizide system (*T* = 293 K, λ_{ex} = 280 nm)

dynamic quenching constant increases with the increasing temperature while the static quenching constant decreases. To confirm the quenching mechanism, the fluorescence quenching data were analyzed using the Stern-Volmer eqn (2)[8]:

$$F_0 / F = 1 + K_q \tau_0 [L] = 1 + K_{SV} [L] \quad (2)$$

Where, F_0 and F represent the fluorescence signals in the absence and presence of quencher, respectively. τ_0 is the average lifetime of fluorescence without quencher, 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 quencher. According to eqn (2), based on the linear fit plot of F_0/F versus [L], the K_{sv} values can be obtained. The results were shown in TABLE 1. TABLE 1 showed that, the values of K_q increased with increasing temperature in all systems. As we all know, higher temperature will increase the diffusion coefficient, and then increase the bimolecular quenching constant K_q [9]. This can be inferred that the process is dynamic quenching. In addition, all the values of K_q were much greater than the maximum scatter collision quenching constant values of various quenchers (2×10^{10} L mol^{-1} s^{-1}) in different temperature. The measured values of K_q is larger may be affected by ionic strength[10].

The relationship between the fluorescence intensity and the concentration of quencher can usually be described using eqn (3) to obtain the binding constant (K_a) and the number of binging sites (n)[11]:

$$\log(F_0 / F - 1) = n \log K_a + n \log\{[L] - n(1 - F / F_0)[B_t]\} \quad (3)$$

Where [L] and [B_t] are the total concentrations of Melizide and protein, respectively. On the assumption that n in the bracket is equal to 1, the curve of log(F_0/F-1) versus log{[L]-n(1-F/F_0)[B_t]} is drawn and linearly fitted, then the value of n can be obtained from the slope of the plot. If the value of n is not equal to 1, it is substituted into the bracket and the curve of log(F_0/F-1) versus log{[L]-n(1-F/F_0)[B_t]} is drawn again. This process is repeated again and again till a single value is obtained for n. On the basis of the value of n obtained, the binding constant K_a can also be determined. The calculation can be completed using a calculator based on the simple program developed, and the results can be obtained by substituting values of F, [L], and [B_t]. The results were shown in TABLE 1. The results showed that all the values of n were approximately equal to 1 at different temperatures, implying that there was just one binding site for Melizide existed in BSA. The results were shown in TABLE 1. The results showed that all the values of n were approximately equal to 1 at different temperatures, implying that there was just one binding site for Melizide existed in BSA. Meanwhile, the binding constant between Melizide and BSA increased with increasing temperature, which indicated that high

TABLE 1 : Quenching reactive parameters of BSA and Melizideat different temperatures

λ_{ex}/(nm)	T/K	K_q/(L·mol^{-1}·s^{-1})	K_{sv}/(L·mol^{-1})	r_1	K_a/(L·mol^{-1})	n	r_2
280	293	1.64×10^{12}	1.64×10^{4}	0.9960	1.45×10^{4}	0.86	0.9960
	303	3.04×10^{12}	3.04×10^{4}	0.9961	3.09×10^{4}	0.89	0.9940
	310	3.91×10^{12}	3.91×10^{4}	0.9932	4.51×10^{4}	0.80	0.9989
295	293	1.24×10^{12}	1.24×10^{4}	0.9925	1.17×10^{4}	0.85	0.9936
	303	2.20×10^{12}	2.20×10^{4}	0.9963	2.20×10^{4}	0.83	0.9958
	310	2.72×10^{12}	2.72×10^{4}	0.9932	2.96×10^{4}	0.80	0.9947

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 oflog(F_0/F-1) ~log{[L]-n(1-F/F_0)[B_t]}

FULL PAPER

temperature enhanced the collision of BSA and Melizide, further suggesting that the quenching was a dynamic process[12].

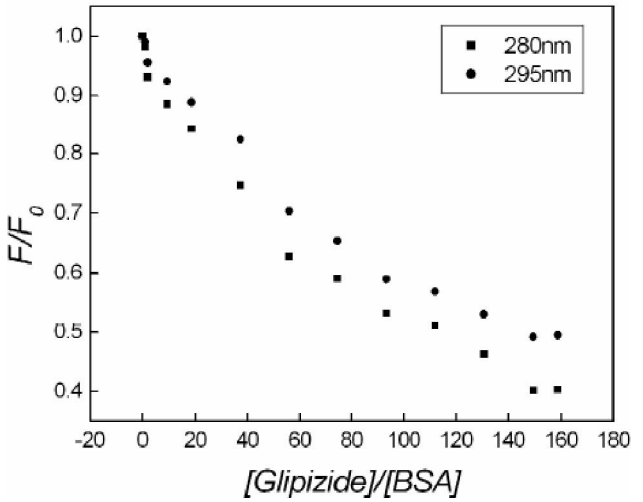
To further confirm the quenching mechanism is dynamic quenching, the UV-Vis absorption spectra of BSA in the presence and absence of Melizide were measured in the Tris-HCl buffer solution with pH = 7.4. The result showed that the UV-Vis absorption spectra of BSA in the presence of Melizide were not changed, which showed that the fluorescence quenching between BSA and Melizide is not caused by the interaction between the ground state molecules. So, the quenching process is not static but dynamic quenching[13].

The influence of Melizide to tryptophan and tyrosine of BSA

For the same protein, the fluorescence effect is generated by the chromophores of tryptophan, tyrosine and phenylalanine amino acid residues[14]. Because amino acid residues have different chromophores, their fluorescence excitation and emission wavelengths are also different. At 280 nm wavelength the tryptophan and tyrosine residues in protein are excited, whereas at 295 nm wavelength, only tryptophan residues are excited[15]. In bovine serum albumin sub-hydrophobic domain, IIA (containing both tryptophan and tyrosine) and IIIA (containing only tyrosine) are the major binding sites of small-molecule ligands[16]. Based on the Stern-Volmer equation, participation of tyrosine and tryptophan groups in BSA-Melizide system was assessed by comparing the fluorescence quenching of BSA excited at 280 and 295 nm, and then to determine the specific binding site[17]. Figure 3 showed that in the presence of Melizide, the quenching curves of BSA excited at 280 and 295 nm did not overlap, and that the quenching curve of BSA at 280 nm was much greater than that at 295 nm. This phenomenon showed that tryptophan and tyrosine residues were essential in the interaction between Melizide and BSA. In addition, it implied that the primary binding site for Melizide was sub-hydrophobic domain IIA.

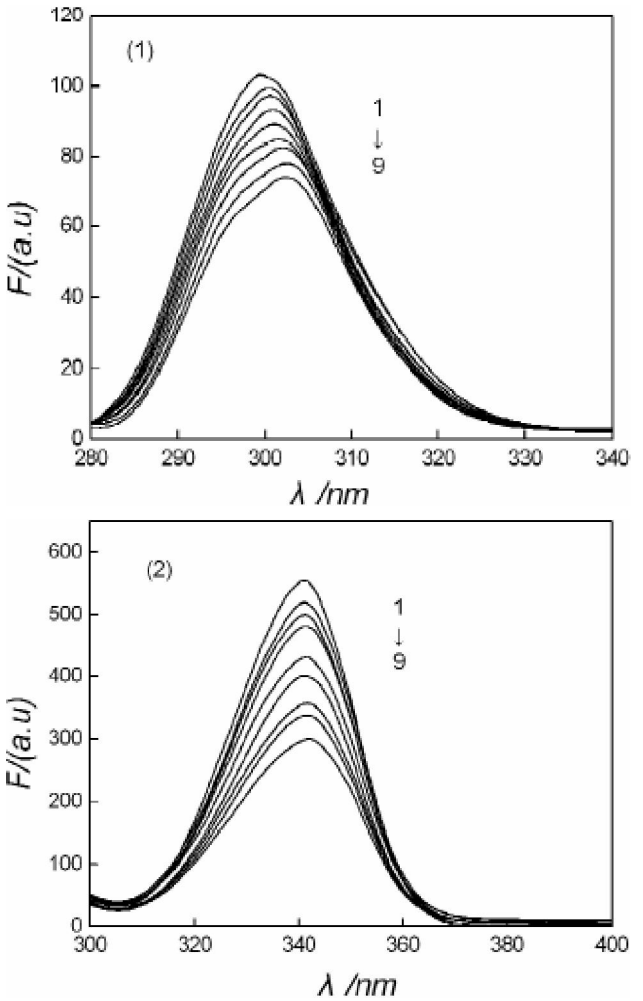
Synchronous fluorescence spectra studies of BSA-Melizid system

For synchronous fluorescence spectra of proteins, when the $\Delta\lambda$ value between the excitation and emission wavelengths is stabilized at 15 or 60 nm, the



C_{BSA} = 3.0 × 10^{-7} M; $C_{Melizide}$ = (0, 0.028, 0.056, 0.28, 0.56, 1.12, 1.68, 2.24, 2.8, 3.36, 3.92, 4.48, 4.76) × 10^{-5} M

Figure 3 : Quenching curves of BSA-Melizide system at λ_{ex} = 280 nm and 295 nm



C_{BSA} = 3.0 × 10^{-7} M, $C_{Melizide}$ = (0, 0.056, 0.28, 0.56, 1.12, 2.24, 2.8, 3.36, 4.48) × 10^{-5} M; (1) $\Delta\lambda$ = 15 nm; (2) $\Delta\lambda$ = 60 nm

Figure 4 : Synchronous fluorescence spectra of BSA-Melizide system (T = 293 K)

synchronous fluorescence gives characteristic information for tyrosine residues or tryptophan residues, respectively[18]. The study of crystal structure shows that the secondary structure of BSA includes three α-helical domains which are called I, II and III. And each domain is divided into two sub-domains which are called A and B. There are three binding sites of the drugs on the BSA, one is located in the domain I A (containing the tyrosine and tryptophan residues), at the same time, the other two are located in II A (only contains tyrosine residues)[19]. Fixed the concentration of BSA, increasing the concentration of Melizide gradually, the synchronous fluorescence spectroscopy of BSA-Melizide system was shown in Figure 4. It can be seen from Figure 4 that when $\Delta\lambda$ was 15 nm or 60 nm, a gradual decrease of the fluorescence intensity of BSA and a slight red shift at the maximum emission wavelength were observed upon addition of Melizide. This incated that the microenvironment of tyrosine residues and tryptophan residue were both changed, the polarity around the tryptophan residues and the tyrosine residues increased, the hydrophobicity decreased[20], the extension degree of the peptide chain increased and the structure of proteins became loose[21]. The result showed that tryptophan and tyrosine residues were both involved in the reaction, the primary binding site for Melizide was sub-hydrophobic domain IIA. This is in accordance with the results obtained from 3.2.

In addition, synchronous fluorescence spectroscopy can be used to calculate the binding parameters. The corresponding results for $\Delta\lambda$ = 60 nm and $\Delta\lambda$ = 15 nm, according to eqn (2) and (3), were shown in TABLE 2. The obtained data can be used to estimate the quenching mechanism of BSA-Melizide system. From TABLE 2 it can be seen that the values of K_{sv} increased with the increase in temperature in all systems, indicating that the probable quenching mechanism of the interaction between BSA and Melizide being a dynamic process. The value of *n* approached unity, indicating that one molecule of Melizide combines with one molecule of BSA. The values of K_a increased regularly with increasing temperature, which also indicated that the process was dynamic quenching. The quenching mechanism obtained using the synchronous fluorescence method was coincident with that obtained using the traditional fluorescence quenching method, which confirmed the accuracy of synchronous fluorescence spectrometry.

Type of interaction force in BSA-Melizide systems

In general, the interaction forces between a small drug molecule and a biological macromolecule include hydrogen bonds, Vander Waal's force, electrostatic interactions and hydrophobic force[22]. Generally, the nature of the interaction forces between the quencher and biomacromolecule can be obtained from the thermodynamic parameters. The thermodynamic parameters can be calculated on the basis of Van't Hoff equation[23]:

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (4)$$

$$\Delta G = \Delta H - T\Delta S = -RT\ln K_a \quad (5)$$

Where, ΔH and ΔS represent the standard variation of the enthalpy and, respectively, entropy of the binding process. R is the gas constant (R = 8.314 J mol^{-1} K^{-1}). T is the absolute temperature. From the thermodynamic standpoint, $\Delta H > 0$ and $\Delta S > 0$ imply a hydrophobic interaction. $\Delta H < 0$ and $\Delta S < 0$ reflect the vander Waals force or hydrogen bond formation. And $\Delta H < 0$ and $\Delta S > 0$ suggest an electrostatic force[24]. When the temperature varies over a small range, ΔH can be considered to be constant[25]. According to eqn (4), (5), the values of ΔH, ΔS and ΔG can be obtained at each temperature, and were listed in TABLE 3. As can be seen from TABLE 3, the value of ΔG was negative, and the value of ΔH and ΔS were positive. The negative

TABLE 2 : Quenching reactive parameters of BSA and Melizideat different temperatures

Δλ/(nm)	T/K	K_q/(L·mol^{-1}·s^{-1})	K_{sv}/(L·mol^{-1})	r_1	K_a/(L·mol^{-1})	n	r_2
15	293	1.15×10^{12}	1.15×10^{4}	0.9934	1.35×10^{4}	0.88	0.9963
	303	1.65×10^{12}	1.65×10^{4}	0.9951	2.38×10^{4}	0.90	0.9962
	310	2.11×10^{12}	2.11×10^{4}	0.9936	3.02×10^{4}	0.82	0.9958
60	293	1.84×10^{12}	1.84×10^{4}	0.9987	1.75×10^{4}	0.87	0.9991
	303	3.26×10^{12}	3.26×10^{4}	0.9975	3.35×10^{4}	0.91	0.9954
	310	3.98×10^{12}	3.98×10^{4}	0.9922	4.85×10^{4}	0.92	0.9995

FULL PAPER

TABLE 3 : The thermodynamic parameters

	T / (K)	K_a / (L·mol^{-1})	ΔH /(KJ·mol^{-1})	ΔS /(J·mol^{-1}·K^{-1})	ΔG /(KJ·mol^{-1})
	293	1.45×10^4		253.17	-23.34
λ_{ex} =280 nm	303	3.09×10^4	50.84	253.73	-26.04
	310	4.51×10^4		253.10	-27.62
	293	1.17×10^4		220.07	-22.82
λ_{ex} =295 nm	303	2.20×10^4	41.66	220.63	-25.19
	310	2.96×10^4		220.00	-26.54
	293	1.35×10^4		202.83	-23.17
$\Delta\lambda$=15nm	303	2.38×10^4	36.26	203.47	-25.39
	310	3.02×10^4		202.74	-26.59
	293	1.75×10^4		236.48	-23.80
$\Delta\lambda$=60nm	303	3.35×10^4	45.49	236.77	-26.25
	310	4.85×10^4		236.45	-27.81

value of ΔG confirmed a spontaneous reaction between BSA and Melizide. The positive value of ΔH and ΔS showed that Melizide mainly bound to BSA by a hydrophobic interaction. The conclusions drawn from the synchronous fluorescence method were consistent with the traditional fluorescence quenching method.

In addition, $\Delta H > 0$ indicated that the process of the interaction between BSA and Melizide was endothermic. Therefore, in this reaction, high temperature will promote the interaction of BSA and Melizide. From the perspective of fluorescence quenching, as the temperature increases, the extent of BSA fluorescence quenching enhanced, the values of K_q increased. This was coincident with the result obtained from 3.1.

CONCLUSIONS

The interaction between Melizide and BSA under simulated physiological conditions had been investigated using fluorescence quenching and synchronous fluorescence spectroscopy in this study, which used the same equation for processing the data. We could see that all data obtained using both the methods were in the same order of magnitude and very similar, showing the quenching mechanism and the type of interaction force of two methods were consistent. This indicated synchronous fluorescence spectrometry was a new method of studying the binding mechanism between drug and protein, and it was a useful supplement to the conventional method. It is of great significance to study the transportation and metabolism progress of drugs and reveal the essence of the drug efficacy, especially the physiological toxicity of drugs on the human's body.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support of National Science Foundation of China (No. 206275024) and Hebei Provincial Key Basic Research Program (No. 10967126D).

REFERENCES

[1] W.A.Kradjan, K.A.Kobayashi, L.A.Bauer et al; J.Clin.Pharmaco, **29(12)**, 1121-1127 **(1989)**.
[2] N.Wang, L.Ye, F.Yan, et al; Int.J.Pharm, **351(1-2)**, 55-60 **(2008)**.
[3] Y.M.Yu, J.C.Feng, Y.Liu; Acta.Chimica.Sinica, **69(2)**, 190-198 (in Chinese) **(2011)**.
[4] Q.Wang, J.W.He, J.Yan, D.Wu, H.Li; Luminescence, **30(2)**, 240-6 **(2015)**.
[5] J.H.Shi, J.Wang, Y.Y.Zhu, J.Chen; J.Lumin, **145**, 643-650 **(2014)**.
[6] Q.Wang, X.Y.Liu, M.Su, Z.H.Shi, H.W.Sun; Spectrochimica.Acta.A., **136**, 321-326 **(2015)**.
[7] C.N.Yan, J.Liu, Y.Liu; Journal of Yangtze university, **7(3)**, 41-46 **(2010)**.
[8] Y.M.Yang, D.J.Li, C.Xu; J.Mol.Struct, **1084**, 229-235 **(2015)**.
[9] J.R.Lakowicz; Principles of Fluorescence Spectroscopy, Second EdўNew York : Plenum Press, 237 **(1999)**.

[10] J.G.Xu, Z.M.Wang; Fluorescence Analytical Methods, Science Press (Beijing), 65 **(2006)**.
[11] N.Sudha, Y.Sameena, S.Chandrasekaran et al; Spectrosc.Lett., **48**, 112-119 **(2014)**.
[12] S.Ghosh, S.Jana, N.Guchhait; J.Phys.Chem., **116(3)**, 1155-1163 **(2012)**.
[13] Q.Wang, S.R.Zhang, X.H.Ji; Spectroc.Acta.A., **124**, 84-90 **(2014)**.
[14] X.F.Zhang, G.Yang, Y.Dong, Y.Q.Zhao, X.R.Sun, L.Chen, H.B.Chen; Spectrochimic.Acta.A., **137**, 1280-1285 **(2015)**.
[15] L.H.Zhang, B.S.Liu, Z.Y.Li, Y.Guo; Spectrosc.Lett., **48**, 441-446 **(2014)**.
[16] A.Sułkowska, M.Maciążek-Jurczyk, B.Bojko et al.; J. Mol. Struct., **881(1-3)**, 97-106 **(2008)**.
[17] M.Maciazek-Jurczyk, A.Sulkowska, B.Bojko et al; J.Mol.Struct., **924-926**, 378-384 **(2009)**.
[18] Y.Y.Hu, S.Q.Xu, X.S.Zhu; Spectrochimic.Acta.A., **74(2)**, 526-531 **(2009)**.
[19] F.L.Cui, Y.R.Cui, H.X.Luo et al; Chin. Sci. Bull., **51(18)**, 2201-2207 **(2006)**.
[20] A.D.Bani-Yaseen; J.Lumin., **131**, 1042-1047 **(2011)**.
[21] G.W.Aodeng, Y.C.Jin; Chin.J.Lumin., **32(4)**, 404-410 (in Chinese) **(2011)**.
[22] G.F.Shen, T.T.Liu, Q.Wang, M.Jiang, J.H.Shi; J. Photoch. Photobio., **153**, 380-390 **(2015)**.
[23] A.Q.Gong, X.S.Zhu,Y.Y.Hu, S.H.Yu; Talanta., **73(4)**, 668-673 **(2007)**.
[24] A.Iovescu, A.Băran, G.Stîngă, J.Photoch.Photobiol.B., **153**, 198-205 **(2015)**.
[25] X.Y.Yu, B.F.Jiang, Z.X.Liao, X.F.Li; Spectroch. Acta. A., **142**, 260-265 **(2015)**.