Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(5):968-974

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Study of the combination reaction between drugs and bovine serum albumin with methyl green as a fluorescence probe

Ying Guo, Baosheng Liu*, Zhiyun Li, Lihui Zhang and Yunkai Lv

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

---

ABSTRACT

We studied the combination between seven kinds of drugs and the bovine serum albumin (BSA) using Methyl Green (MG) as fluorescent probe. These drugs were Streptomycin sulphate, Kanamycin sulfate, Gentamicin, Amikacin, Neomycin, Thiamphenicol and Florfenicol. And after reaction of these drugs, the fluorescence intensity of BSA had no obvious change. Research has shown that in Tris-HCl (pH=7.40) buffer solution, MG made BSA fluorescence quenching, quenching mechanism belongs to static quenching, and the binding constant, binding points and interaction force type between MG and BSA were obtained under different temperature. Fluorescence intensity of BSA was recovered when seven kinds of drugs were added to the BSA-MG system, respectively. The degree of recovery were SM> AM> FN> TN> KM> GM> NM. This suggested that the reaction mechanism between MG, drugs and BSA was competition binding reaction, and the binding sites between seven kinds of drugs and BSA were determined on sub-domain IIA (site I) using labeled drugs with specific binding sites.

Keywords: Methyl Green, bovine serum albumin, drugs, Spectroscopy, Bonding mechanism

---

INTRODUCTION

Methyl green (MG) is a basic triphenylmethane and dicationic dye usually used for staining of solutions in medicine and biology [1]. Kanamycin sulfate (KM), Streptomycin (SM), Amikacin (AM), Gentamicin (GM), Neomycin (NM) belongs to aminoglycoside antibiotics, they were the long-sought remedy for tuberculosis and other serious bacterial infections [2]. Thiamphenicol (TN), Florfenicol (FN) belongs to the chloramphenicol of broad-spectrum antibiotic. Serum albumin, the most abundant protein constituent in blood plasma, can be combined with a lot of endogenous and exogenous compounds and plays a fundamental role in the disposition and transportation of various molecules. Therefore, investigating the binding mechanism of endogenous or exogenous compounds and serum albumins has very significant implications for the life sciences, chemistry, pharmacy and clinical medicine. In recent decades investigations of the interaction between drugs and bovine serum albumin (BSA) by use of the fluorescence method have been extensively reported [3-6]. The fluorescence spectrum change is obvious in the literature reported [7-10]. However, in many cases the required experimental data cannot be measured directly or calculated indirectly because of a lack of experimental results or the inconclusive nature of experimental results for the pharmaceutical molecules and the bio-macromolecules. The problem can be effectively solved by using the fluorescent probe method [11-13]. In this paper, we using MG as a fluorescence probe to research seven drugs and BSA binding reaction and reaction mechanism under the physiological conditions. Proof of seven drugs and BSA binding in vivo, and the binding capacity were SM> AM> FN> TN> KM> GM> NM. Determine the combination regional of these seven drugs and BSA. This study provides a method for the study of reaction mechanism that some drugs (similar to TN, KM, SM, AM, GM, NM and FN) reacting with protein.

EXPERIMENTAL SECTION

Apparatus and Materials

All fluorescence spectra were recorded on a Shimadzu RF-5301PC spectro-fluorophotometer. All spectrophotometric measurements were made with a Shimadzu UV-265 spectrophotometer. 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).

Bovine serum albumin was purchased from Sigma(the purity grade inferior 99%) and stock solutions (1.0×10^{-5} mol L^{-1}) were prepared by doubly distilled water. Methyl Green (2.0×10^{-3} mol L^{-1}) were prepared by doubly distilled water, respectively. All the stock solutions were further diluted as working solutions prior to use. Warfarin, ibuprofen, and digoxin were all obtained from the Chinese Institute of Drug and Biological Products and further diluted as working solution (1.0×10^{-5} mol L^{-1}). The Tris-HCl buffer (0.05 mol L^{-1}, pH=7.4) containing 0.15 mol L^{-1} NaCl was selected to keep the pH value constant and to maintain the ionic strength of the solution. All other reagents were of analytical reagent grade and double-distilled water was used during the experiment. And all the stock solutions were 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 [14]:

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

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

Procedures

Fluorescence spectra and Synchronous fluorescence spectra

In the experiment we use the 1.0 mL of pH 7.40 Tris-HCl, a certain of 1.0×10^{-5} mol L^{-1} BSA solution, and different amount of MG (1.0×10^{-3} mol L^{-1}) was added into 10 mL colorimetric tube sequentially. The samples were diluted to scaled volume with double-distilled water, mixed thoroughly by shaking, and kept static for 30 minutes. The fluorescence emission spectra were measured at 293, 303, and 310 K with the width of the excitation and emission slit adjusted at 5.0 and 5.0 nm, respectively. An excitation wavelength of 280 nm was chosen and the emission wavelength was recorded from 285 to 500 nm.The synchronous fluorescence spectra were obtained by simultaneously scanning the excitation and emission monochromators. It were recorded at $\Delta\lambda$=15 nm and 60 nm in the absence and presence of various amounts of MG over a wavelength range of 280-400 nm.

Effects of drugs on BSA-MG system

At 293K, first we adding 0.3 mL1.0×10^{-3} mol L^{-1} MG solution, then adding the different amount of drugs(2.0×10^{-3} mol L^{-1}), respectively. After the method according to the above operation.

Determination of the binding sites

At 293K, different concentrations (1.0×10^{-5} mol L^{-1}) of site marker I (WF), II (IB), or III (DG) were added to the mixture of BSA-MG system. After the method according to the above operation.

RESULTS AND DISCUSSION

Fluorescence quenching spectra of BSA-MG system

The intrinsic fluorescence of protein is a sensitive tool to study the conformation of protein when its environment or structure gets change. The quenching mechanism of fluorescence can be classified into static quenching and dynamic quenching [15]. Dynamic quenching is mainly caused by collisional encounters between the fluorophore and the quencher, static quenching is mainly resulted from the formation of stable compound between fluorophore and quencher [16]. Fig. 1 shows the fluorescence emission spectra of BSA in the presence of various concentrations of MG at 293 K. The fluorescence emission intensity of BSA decreased regularly with the gradual addition of MG. This result indicates that MG can interact with BSA and quench its intrinsic fluorescence, changing the microenvironment of the fluorophores.

If it is assumed that the fluorescence quenching mechanism of BSA by MG is dynamic quenching process, fluorescence quenching can be described by Stern-Volmer equation [17].

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

where F and F_0 are the relative fluorescence intensities in the presence and absence of quencher, respectively; [Q] is the concentration of quencher, K_{sv} is the Stern-Volmer quenching constant, which measures the efficiency of quenching. K_q is the quenching rate constant of the biomolecule, τ_0 is the average lifetime of the biomolecule in absence of quencher evaluated at about 10^{-8} s [18]. According to the Stern-Volmer plots of F_0/F versus quencher concentration at different temperatures (293, 303, and 310 K). The quenching rate constant K_q was obtained and listed in Table 1. It is obvious K_q decreases with rising temperatures, revealing that the quenching is initiated by static quenching process. Moreover, the values of K_q between BSA and MG are all greater than 2×10^{10} L·mol^{-1}·s^{-1}. Therefore, MG binding BSA was a static quenching process proved to be true [19].

For the static quenching interaction, under the assumption that there are similar and independent binding sites in the biomolecule, the binding constant and the number of binding sites can be derived from the double logarithm regression curve (Eq. (3)) [20]

$$\lg[(F_0 - F) / F] = n \lg[Q] + \lg K_a \quad (3)$$

where K_a is the binding constant , n is the number of binding sites. [Q] is the total concentrations of MG. The curve of log [(F_0-F)/F] versus log [Q] is drawn and fitted linearly, then the value of n and K_a can be obtained from the plot. And Table 1 gives the corresponding calculated results.The value of n almost equals to 1, indicating that there is one class of binding site for MG to BSA molecule. In other words, MG and BSA form a complex with molar ratio 1:1. According to the results shown in Table 1, the binding constants of the interaction between MG and BSA decreases with the rising temperature, further suggested that the quenching was a static process [21].

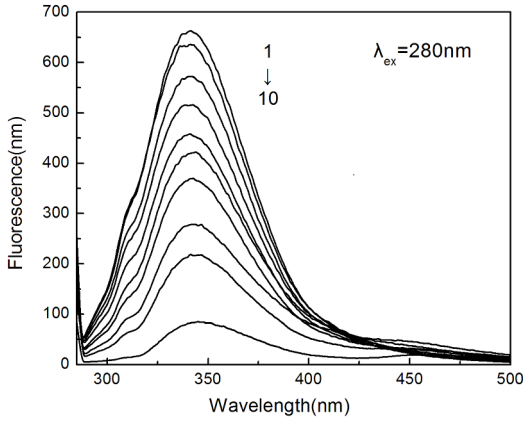

Figure 1. Fluorescence emission spectra of BSA-MG(T=293K)
1~10 C_{BSA} (3×10^{-7} mol L^{-1})+C_{MG} (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 4.0)×10^{-4} mol L^{-1}

Table 1 Quenching reactive parameter of BSA and MG at different temperatures

T/(K)	K_q/(L mol^{-1} s^{-1})	r_1	SD_1	K_a/(L mol^{-1})	r_2	SD_2	n
293	9.80×10^{11}	0.9967	0.031	1.91×10^{4}	0.9938	0.023	1.01
303	8.67×10^{11}	0.9989	0.015	1.68×10^{4}	0.9978	0.031	1.04
308	8.09×10^{11}	0.9953	0.019	0.98×10^{4}	0.9922	0.044	1.06

r_1、r_2 are the linear relative coefficient of F_0/F~[Q]、lg(F_0-F)/F~lg[Q]，respectively. SD_1 、SD_2 are the standard deviation of F_0/F~[Q]、lg(F_0-F)/F~lg[Q]，respectively.

Synchronous Fluorescence Spectra

The synchronous fluorescence spectra can provide information on the molecular microenvironment, particularly in the vicinity of the fluorophore functional groups [22]. When Δλ was 15 nm, synchronous fluorescence detects characteristics of tyrosine (Tyr) residues, but when Δλ was 60 nm, characteristic information from tryptophan (Trp) residues is highlighted [23].

The synchronous fluorescence spectra of BSA-MG systems shown in Fig. 2. As seen in Fig. 2, when Δλ was fixed at 15nm, no shift of λ_{max} was apparent. But the λ_{max} had red shifted when Δλ=60nm. This suggested that the interaction of BSA with MG have a distinct effect on the conformation of the microenvironment around Trp residues, and did

not changed the microenvironment of Tyr residues [24]. High concentrations of dyes make protein molecules extend, reducing the energy transfer between the amino acid residues, and reducing their fluorescence intensity.

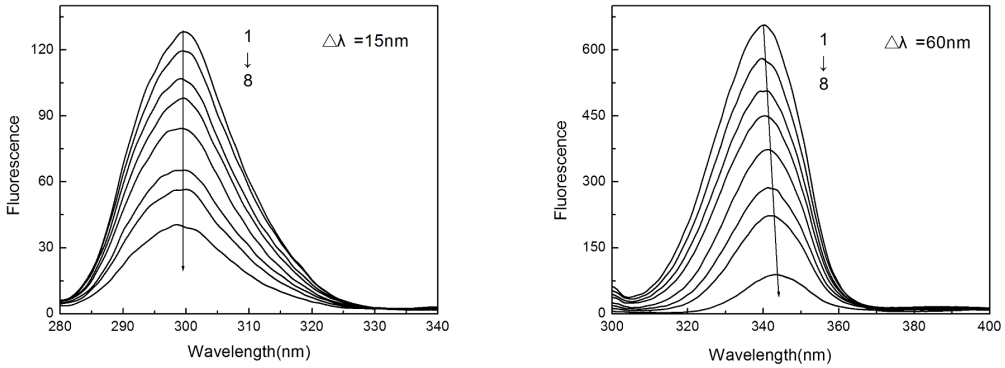

Figure 2. Synchronous fluorescence spectra of BSA-MG system (T=293K)
1-8 C_{BSA} (3.0×10^{-7} mol L^{-1})+C_{MG} (0, 0.1, 0.2, 0.4, 0.6, 1.0, 1.5, 2.0)$\times10^{-4}$ mol L^{-1}

Type of interaction force of BSA-MG systems

Basically, four main types of interactions, hydrogen bonds, electrostatic forces, van der Waals forces, and hydrophobic forces play critical roles in the interactions between small molecules and macromolecules [25]. In order to characterize the force between MG and BSA, thermodynamic parameters on the temperatures were analyzed. The thermodynamic parameters, free energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) are important for confirming the binding mode. The thermodynamic parameters can be calculated using Eqs. (4) and (5) [26-27].

$$R\ln K = \Delta S - \Delta H / T \quad (4)$$

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

In the present case, K is analogous to the effective quenching constants K_a for the quencher-acceptor system at the corresponding temperature and R is gas constant. If it is assumed that the enthalpy change (ΔH) nearly had no change within the investigated temperature, there should be a good linear relationship between ln K and 1/T. If ΔH<0 and ΔS<0, van der Waals interactions and hydrogen bonds play major roles in the binding reaction. If ΔH>0 and ΔS>0, hydrophobic interactions are dominant. If ΔH<0 and ΔS>0, electrostatic forces are more important in the binding reaction [28]. The result of ΔH, ΔS and ΔG were -28.02 KJ mol^{-1}, -13.07 KJ mol^{-1}, -24.19 KJ mol^{-1} (T=293 K), respectively. This showed that the van der Waals interactions and hydrogen bonds play major roles in the binding process between MG and BSA. The negative value of ΔG indicated a spontaneous reaction occurred between MG and BSA.

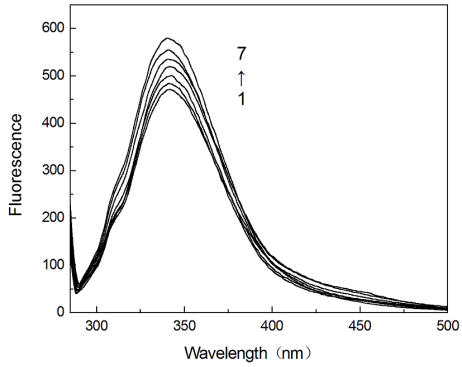

Figure 3. Fluorescence emission spectra of BSA-MG-TN(T=293K)
1-7 C_{BSA} (3.0×10^{-7} mol L^{-1})+C_{MG} (4.0×10^{-5} mol L^{-1})+C_{TN} (0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0)$\times10^{-4}$ mol L^{-1}

Effect of drugs on the fluorescence emission spectra of the BSA-MG system

The emission spectra of BSA-MG in the absence and presence of TN are shown in Fig. 3 (KM, SM, AM, GM, NM and FN are similar to TN). As shown in Fig. 3, when more and more drugs added to the system, the fluorescence intensity at 340 nm increased gradually, which indicative of a competitive binding reaction between drugs, MG and

BSA, and made the fluorescence intensity of BSA recovered. Comparing the recovery of BSA fluorescence intensity of different drugs at the same concentration: SM> AM> FN> TN> KM> GM> NM, it indicates that the capability of competitive binding reaction between drugs and MG-BSA system: SM> AM> FN> TN> KM> GM> NM.

Fluorescence recovery mechanism

When TN (KM, SM, AM, GM, NM and FN are similar to TN) was added into the BSA-MG complex system the fluorescence intensity of BSA recovered (Fig. 3). This phenomenon indicated there was a competitive interaction between TN and MG for BSA. Eqs. (6) and (7) show the competitive reaction:

BSA-MG+TN→BSA-TN+MG (6)

Or BSA-MG+TN→MG-TN+BSA-TN (7)

The absorption spectra of BSA-MG in the absence and presence of TN were recorded to confirm the competitive reaction according to Eqs. (6) or (7) (Fig. 3). As it can be seen from Fig. 4, TN and BSA had no absorption in the range 500-700 nm and λ_{max} of TN was at 630 nm. The absorption of MG decreased with increasing concentration of TN. If the competitive reaction was according to Eq. (6), the absorption of MG should be increased with increasing concentration of TN, which is not observed (Fig. 4). If the competitive reaction was according to Eq. (7) TN had bound MG and caused the absorption of MG to gradually decrease with increasing concentration of TN, which is consistent with Fig. 4. There was no obvious fluorescence intensity change on combining BSA and TN, so the relative fluorescence intensity of BSA gradually recovered with increasing concentration of TN, which is consistent with Fig. 3. Therefore, the competitive reaction was according to Eq. (7).

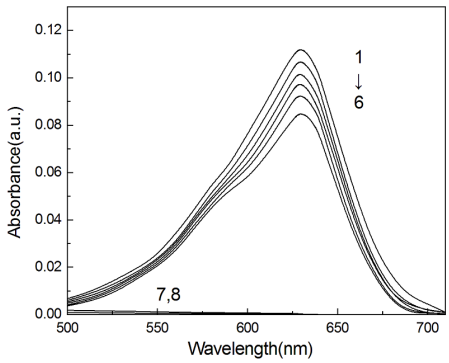

Figure 4. Absorption spectra of BSA-MG-TN(T=293K)

1, C_{MG} (1.0×10^{-5} mol L^{-1}); 2~6, C_{MG} (1.0×10^{-5} mol L^{-1})+C_{BSA} (3.0×10^{-7} mol L^{-1})+C_{TN} (0,1.0, 2.0, 3.0, 4.0)$\times10^{-3}$ mol L^{-1}; 7, C_{BSA} (3.0×10^{-7} mol L^{-1}); 8, C_{TN} (4.0×10^{-3} mol L^{-1})

Identification of the binding site

At 280 nm wavelength the Trp and Tyr residues in BSA are excited, whereas the 295 nm wavelength excites only Trp residues. In BSA sub-hydrophobic domain, □A (containing both Trp 212 and Tyr 263) and □A (containing only Tyr: Tyr 401, Tyr411, Tyr 497) is the major binding site of small molecule ligands [29]. Based on the Stern-Volmer equation, comparing the fluorescence quenching of BSA excited at 280 nm and 295 nm allows to estimate the participation of Trp and Tyr groups in the system [30]. As seen in Fig. 3, in the presence of MG, the quenching curves of BSA excited at 280 nm and 295 nm overlap approximately. This phenomenon showed that Trp residues played an important role in the interaction between MG and BSA. BSA has two tryptophan moieties (Trp 134 and Trp 212). Trp 134 is embedded in the first sub-domain IB and is more exposed to a hydrophilic environment, whereas Trp 212 is embedded in sub-domain IIA and deeply buries in the hydrophobic loop. So, it is considered that MG most likely binds to the hydrophobic pocket located in sub-domain IIA [31].

The crystal structure of BSA is a heart-shaped helical monomer composed of three homologous domains named I, II, and III, with each domain including two sub-domains called A and B to form a cylinder [32]. The principal ligand-binding regions of albumin are hydrophobic cavities in sub-domains IIA and IIIA, which have similar chemical properties. These two binding cavities are also referred to as sites I, II, and III (site I in sub-domain IIA, sites II and III in sub-domain IIIA). To identify the binding site on BSA, site marker competitive experiments were carried out, using the drug which specially binds to a known site or region on BSA. X-ray crystallography studies have shown that warfarin (WF) binds to sub-domain IIA whereas ibuprofen (IB) and digoxin (DG) are believed to bind to IIIA binder sites II and III, respectively [33]. Information about BSA-MG binding site can therefore be obtained by monitoring changes in the fluorescence of MG-bound BSA caused by binding by site I (WF), site II (IB),

and site III (DG) markers. Binding constants determined on the basis of Eq. (3) show the effect of WF, IB, and DG on BSA-MG at 293 K. It is observed that binding constants for the ternary system ($K_{BSA\text{-}WF\text{-}MG}$=5.26×10^3 L mol^{-1}; $K_{BSA\text{-}IB\text{-}MG}$=9.91×10^3 L mol^{-1}; $K_{BSA\text{-}DG\text{-}MG}$=9.64×10^3 L mol^{-1}) were lower than that for the binary system BSA-MG ($K_{BSA\text{-}MG}$=1.91×10^4 L mol^{-1}). It can be seen that the binding constant for the ternary system BSA-WF-MG) was the most different, indicating that WF hinders the formation of BSA-MG and can compete for the same binding site in sub-domain IIA (site I).

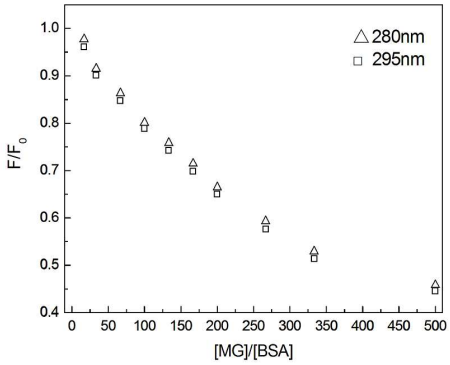

Figure 5. Fluorescence emission spectra of BSA-MG (T =293 K)
C_{BSA} (3.0×10^{-7} mol L^{-1}), C_{MG}($5.0\times10^{-6}\sim4.0\times10^{-4}$) mol L^{-1}

CONCLUSION

The binding sites(sub-domain IIA site I) of MG on BSA is determined by competitive reagent, proves the specific binding between MG and BSA, and explains that MG can study the combination of drugs and proteins as a probe instead of competition reagent. Due to After reaction of BSA and KM, SM, AM, GM, NM, TN, FN, respectively, the fluorescence intensity of BSA has no obvious change, so there is no way to directly research drugs and BSA binding reaction with fluorescence spectrometry. In this paper using MG as fluorescent probe to study the drugs to respond to the combination of the BSA, it proves that there is a reaction between drugs and BSA, and it determines their binding site on sub-domain IIA (site I). The study of using fluorescent probes for the interaction between no or weak fluorescence intensity change of drug molecular and BSA opens new avenues of research, broadens the scope of drugs research.

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] BG Berg; DM Green, *J. Acoust. Soc. Am.*, **1990**, 88(2), 758-766.
[2] A Forge; J Schacht, *Audiology and Neurotology*, **2000**, 5, 3-22.
[3] A Rogozea; I Matei; IM Turcu, *J. Phys. Chem. B.*, **2012**, 116(49), 14245-14253.
[4] U Katrahalli; S Jaldappagari; SS Kalanur, *J. Lumin.*, **2010**, 130(2), 211-216.
[5] B Kaboudin; K Moradi; MR Faghihi, *J. Lumin.*, **2013**, 139(7), 104-112.
[6] YN Ni; S Du; S Kokot, *Anal. Chim. Acta.*, **2007**, 584(1), 19-27.
[7] PN Naik; SA Chimatadar, *Spectrochim. Acta Part A.*, **2009**, 73(5), 841-845.
[8] H Xu; Q Liu; Y Wen, *Spectrochim. Acta Part A.*, **2008**, 71(3), 984-988.
[9] N Zhou; YZ Liang; P Wang, *J. Mol. Struct.*, **2008**, 872(2-3), 190-196.
[10] D Ran; X Wu; J Zheng; J Yang; H Zhou; M Zhang; Y Tang, *J. Fluoresc.*, **2007**, 17(6), 721-726.
[11] Hu YJ; Y Liu; LX Zhang; RM Zhao; Qu SS, *J. Mol. Struct.*, **2005**, 750(1-3), 174-178.
[12] A Sulkowska, *J. Mol. Struct.*, **2002**, 614(1), 227-232.
[13] Q Yao; XY Yu; TY Zheng, *Spectrochim. Acta Part A.*, **2013**, 113(9), 447-451.
[14] RF Steiner, I Weinryb. Excited States of Proteins and Nucleic Acids, Plenum Press, New York, **1971,** 40.
[15] B Ahmad; S Parveen; RH Khan, *Biomacromolecules*, **2006**, 7 (4), 1350-1356.
[16] S Deepa; AK Mishra, *J. Pharm. Biomed. Anal.*, **2005**, 38(1), 556-563.
[17] EL Gelamo; CH Silva; H Imasato; M Tabak, *Biochim. Biophys. Acta.*, **2002**, 1594(1), 84-99.
[18] JR Lakowicz; G Weber, *Biochemistry*, **1973**, 12 (21), 4161-4170.
[19] H Xu; QW Liu; YQ Wen, *Spectrochim. Acta Part A.*, **2008**, 71(3), 984-988.

[20] DH Ran; X Wu; JH Zheng; JH Yang; HP Zhou; MF Zhang; YJ Tang, *J. Fluoresc.*, **2007**, 17(6), 721-726.
[21] JC Li; N Li; QH Wu; Z Wang; JJ Ma; C Wang; LJ Zhang, *J. Mol. Struct.*, **2007**, 833(1-3), 184-188.
[22] YJ Hu; Y Liu; RM Zhao; JX Dong; SS Qu, *J. Photochem. Photobiol. A*, **2006**, 179 (3), 324-329.
[23] YZ Zhang; XX Chen; J Dai; XP Zhang; YX Liu; Y Liu, *Luminescence*, 2008, 23(3), 150-156.
[24] J Chamani; N Tafrishi; M Momen-Heravi, *J. Lumin.*, **2010**, 130(7), 1160-1168.
[25] PD Ross; S Subramanian, *Biochemistry*, **1981**, 20(11), 3096-3102.
[26] M Gharagozlou; DM Boghaei, *Spectrochim. Acta Part A.*, **2008**, 71(4), 1617-1622.
[27] Y Teng; RT Liu; C Li; Q Xia; PJ Zhang, *J. Hazard. Mater.*, **2011**, 190(1-3), 574-581.
[28] YZ Zhang; J Dai; XP Zhang; X Yang; Y Liu, *J. Mol. Struct.*, **2008**, 888(1–3), 152-159.
[29] K Yamasaki; T Maruyama; U Kragh-Hansen; M Otagiri, *Biochim. Biophys. Acta.*, **1996**, 1295(2), 147-157.
[30] Y Yang; XY Yu; WH Tong; SY Lu; HT Liu; Q Yao; H Zhou, *J. Solution Chem.*, **2013**, 42(3), 666-675.
[31] A Sułkowska; B Bojko; J Równicka; D Pentak; W Sułkowski, *J. Mol. Struct.*, **2003**, 651-653(1), 237-243.
[32] YZ Zhang; B Zhou; B Zhang, *J. Hazard. Mater.*, **2009**, 163(2-3), 1345-1352.
[33] QL Zhang; YN Ni; S Kokot, *J. Pharm. Biomed. Anal.*, **2010**, 52(2), 280-288.