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Improved spectrophotometric study of the reaction mechanism between pioglitazone hydrochloride and bovine serum albumin

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ABSTRACT

Under simulated physiological conditions, the reaction mechanism between pioglitazone hydrochloride (PGH) and bovine serum albumin (BSA) at different temperatures (298 K, 310 K and 318 K) was investigated by the classical fluorescence spectroscopy with focus on the fluorescence change of protein, as well as the elastic scattering fluorescence spectroscopy with focus on the fluorescence change of drug. The results indicated that PGH could quench the intrinsic fluorescence of BSA strongly by a static quenching process. The electrostatic force played an important role on the conjugation reaction of PGH with BSA, and the number of binding site (*n*) in the binary system was approximately equal to 1. The value of Hill's coefficients (n_H) was approximately equal to 1, which suggested no cooperativity in BSA-PGH system. In addition, the binding constant obtained from elastic scattering fluorescence spectroscopy was larger than the one obtained from classical fluorescence spectroscopy with two orders of magnitude for the BSA-PGH system. The results show that the research is more accurate and reasonable with focus on the fluorescence change of drug and is also speculated that "point to side" interaction between drugs and proteins is existed.

KEYWORDS

Classical fluorescence spectroscopy;
Elastic scattering fluorescence spectroscopy;
Pioglitazone hydrochloride;
Bovine serum albumin;
Reaction mechanism.

INTRODUCTION

The classical fluorescence spectroscopy 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[1-2]. Elastic light scattering is a kind of light scattering, which the radiation light wavelength is the same with incident light wavelength[3] and is called Rayleigh scattering when the scale of the scattering particles is much smaller than the wavelength of incident light. When Rayleigh scattering is located in the vicinity of the absorption band, it is possible to cause a sharp increase in scattering intensity, and this resonance phenomenon is called Rayleigh

scattering, also known as resonance light scattering (resonance light scattering, RLS). In classical fluorescence spectroscopy, the main source of bovine serum albumin fluorescence is Trp-212. Classical fluorescence spectroscopy does not reflect interaction of the other non-fluorescence-emitting residues with drugs and the fluorescence spectrogram only reflects partial information of the interaction of bovine serum albumin with drug[4]. As a result, the obtained information is insufficient accuracy. However, fluorescence changes of small molecule drugs can reflect the whole information of interaction between drugs and proteins, so that the fluorescence of small molecule drug reflects the overall fluorescence in the interaction. In order to cover the shortcomings of classical fluorescence spectroscopy, a new method by taking the drug as the object of detection is applied to study the interaction between drugs and proteins.

Pioglitazone hydrochloride (PGH) is chemical hypoglycemic composition for treating diabetes, and is often associated with the traditional Chinese medicine for treating diabetes. The structure of PGH is shown in Figure 1. Combination of Chinese and western medicines is more popular in the current market. However, chemical hypoglycemic drugs are strictly limited in dose. When dose is too much will lead to coma and even death. But some manufacturers illegally added chemical hypoglycemic composition to Chinese patent medicines and health products for reaping significant effect and huge profits, and their behaviors are seriously harmful to people's health[5]. The study of interaction of PGH with BSA is helpful to understand the reaction mechanism of PGH in the human body, and take the drug and health products safely.

EXPERIMENTAL

Apparatus

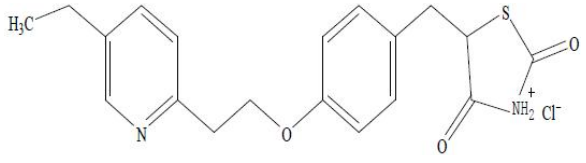

Figure 1 : Chemical structure of pioglitazone hydrochloride

All fluorescence spectra were recorded with a Shimadzu RF-5301PC spectrofluorophotometer. 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 SYC-15_B superheated water bath (SangLi, Nanjing).

Materials

Bovine serum albumin (BSA) was purchased from Sigma Co. and was of the purity grade inferior 99%. Pioglitazone hydrochloride (PGH) was purchased from Beijing Taiyang Pharmaceutical Co., Ltd. Stock solutions of BSA (1.0×10^{-4} mol·L^{-1}) and PGH (5.0×10^{-4} mol·L^{-1}) were prepared. All the stock solutions were further diluted for use as working solutions. Tris-HCl buffer solution containing 0.15 mol·L^{-1} NaCl was used to maintain the pH of solutions 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 newly double-distilled water and stored at 277K.

Procedures

Classical fluorescence spectrometry

In a typical fluorescence measurement, 1.0 mL of Tris-HCl solution, 1.0 mL of 2.0×10^{-6} mol·L^{-1} BSA solution and different concentrations of PGH were successively added to a 10-mL colorimetric tube. The samples were diluted to scaled volume with water, mixed thoroughly by shaking, and kept static for 40 min at different temperatures (298K, 310K and 318K). The excitation and emission slits were set at 5 nm. The excitation wavelength for BSA was 280 nm and 295 nm, respectively, with a 10 nm path length cell. We recorded the intensity of fluorescence at 340 nm.

Elastic scattering fluorescence spectroscopy measurements

1.0 mL of Tris-HCl solution, 0.5 mL of 5.0×10^{-4} mol·L^{-1} PGH solution and different concentrations of BSA were successively added to a 10-mL colorimetric tube. The samples were diluted to scaled vol-

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ume with water, mixed thoroughly by shaking, and kept static for 30min at different temperatures (298K, 310K and 318K). The excitation and emission slits were set at 5 nm. The fluorescence spectra were measured ($\Delta\lambda$ at 0 nm and emission wavelengths of 220-450 nm) with a 10 nm path length cell. The fluorescent intensity I was recorded.

RESULTS AND DISCUSSION

Classical fluorescence spectra of BSA-PGH system

The classical fluorescence spectra of BSA-PGH system were shown in Figure 2 with focus on the fluorescence change of protein. As shown in Figure2, the fluorescence intensity of BSA decreased gradually with the addition of PGH. The result showed that PGH could quench the intrinsic fluorescence of BSA significantly and there was an interaction between PGH and BSA[6-7].

In order to confirm the quenching mechanism, the fluorescence quenching data were analyzed by the Stern–Volmer equation (1)[8-9]:

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

Where, F_0 and F are the fluorescence intensities of BSA in the absence and presence of the PGH, respectively. τ_0 is the average lifetime of fluorescence without quencher, which is about 10^{-8} s[10]. K_{sv} is the Stern-Volmer quenching constant. K_q is the bimolecular quenching constant and [L] is the concentration of the quencher. Based on the linear fit plot of F_0/F *versus* [L], values of K_{sv} and K_q could be obtained at different temperatures. The calculate results were shown in TABLE 1. From TABLE 1, we could see that the K_{sv} values were inversely correlated with temperatures, that is to say, the extent of fluorescence quenching of PGH to BSA was reduced with rising temperature, which suggested that the fluorescence quenching of BSA were initiated by the formation of ground-state complex rather than by dynamic collision[11]. Furthermore, K_q values were much greater than the maximum scatter collision quenching constant (2.0×10^{10} L·mol^{-1}·s^{-1}[12]) for various quenchers with biomolecules. Considering that in our experiment the rate constants of the protein quenching procedure initiated by PGH were higher than the maximum value possible for diffusion limited quenching in solution (~10^{10} L·mol^{-1}·s^{-1}). It suggested that the static quenching was dominant in the interaction between PGH and BSA[13].

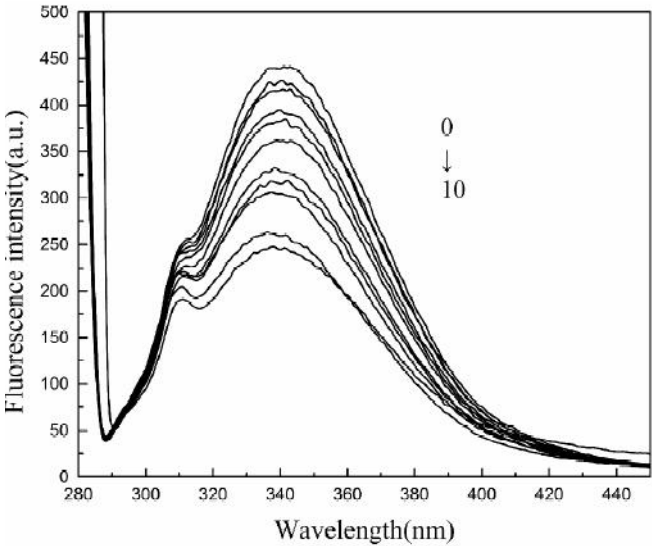

Figure 2 : Fluorescence spectra of BSA-PGH system (T = 298 K, λ_{ex} = 280 nm); C_{BSA}= 2.0×10^{-7} mol·L^{-1}, 0~10 C_{PGH}= (0, 0.5, 2.0, 3.0, 4.0, 5.0, 7.0, 8.0, 10.0, 15.0, 20.0) ×10^{-5} mol·L^{-1}

For static quenching process, the relationship between the fluorescence intensity and the concentration of quencher can be usually described by derived Eq. (2)[14-15] to obtain the binding constant (K_a) and the number of binging sites (n) in most papers:

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

Where, K_a and n are the binding constant and the number of binding sites, respectively. From Eq. (2), the binding parameters can be obtained by the plot of lg [($F_0 - F$)/F] versus lg [L]. The values of K_a and n at 298 K, 310 K, and 318 K are listed 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 PGH existed in BSA. Meanwhile, the K_a decreased with the rising temperature, further suggested that the quenching was a static process[16].

RLS of BSA-PGH system

According to the experiment as discussed section elastic scattering fluorescence spectroscopy measurements, the interaction between BSA and PGH with PGH as the detection object was investigated. The elastic scattering fluorescence spectros-

TABLE 1 : Quenching reactive parameters of BSA and PGH at 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	298	4.8×10^{11}	4.8×10^{3}	0.9981	4.8×10^{3}	1.09	0.9981
	310	3.7×10^{11}	3.7×10^{3}	0.9934	4.3×10^{3}	1.07	0.9945
	318	3.0×10^{11}	3.0×10^{3}	0.9943	3.2×10^{3}	0.93	0.9928
295	298	3.0×10^{11}	3.0×10^{3}	0.9986	3.2×10^{3}	1.05	0.9981
	310	2.8×10^{11}	2.8×10^{3}	0.9950	2.8×10^{3}	0.95	0.9974
	318	2.5×10^{11}	2.5×10^{3}	0.9942	2.2×10^{3}	0.86	0.9988

K_q is the quenching rate constant; K_{sv} is the Stern-Volmer quenching 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 lg [$(F_0$-$F)/F$]~lg [L]

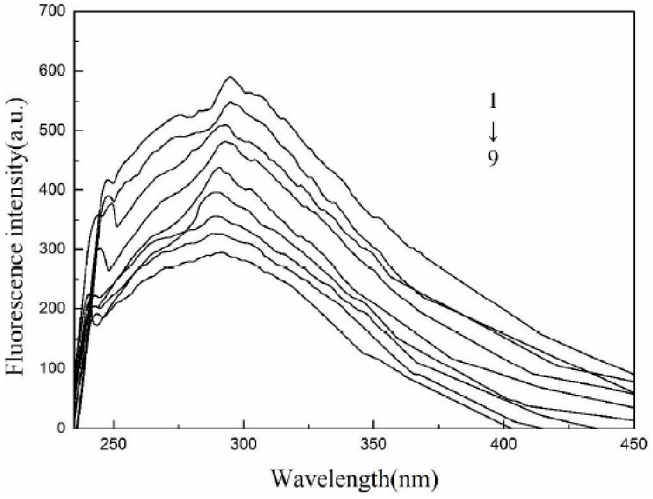

Figure 3 : Elastic scattering fluorescence spectra of BSA-PGH system (T = 298 K); C_{PGH} = 2.5×10^{-5} mol·L^{-1}, 1~9C_{BSA} = (0, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5) ×10^{-7} mol·L^{-1}

copy of BSA-PGH system was shown in Figure 3. As seen in Figure 3, the scattering intensity of PGH decreased gradually with the addition of BSA to PGH solution, which suggested that there was an interaction between PGH and BSA. According to the Eq. (1) and (2), the calculated results were shown in TABLE 2. As seen in TABLE 2, the number of binging site (n) was all approximately to 1. Meanwhile, K_{a1} and K_{sv1} all were reduced with the rising temperatures, and 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})under different temperatures, further suggested that the quenching was a static process, which were consistent with the results of classical fluorescence spectroscopy. This indicated that taking protein or drug as the object of detection, although detection methods were different, it could get the same interaction mechanism between drugs and protein. Moreover, the K_{a1} values of elastic scattering fluorescence spectroscopy with drug as detection object were much greater than the K_a values of classical fluorescence spectroscopy with protein as detection object at the same temperature, which showed that the others also interacts with PGH besides Trp-212 of BSA. In addition to the "point to point" interaction between PGH and Trp-212, the "point to side" interaction between PGH and the other peptides in BSA hydrophobic sub-domain also exists[17]. This shows that treating drugs as detection object can give more complete and more accurate expression of the interaction information of protein and drugs than classical fluorescence spectroscopy with protein as detection object.

Type of interaction force of BSA–PGH system

The interaction forces acting between a small molecule and biological macromolecule mainly in-

TABLE 2 : Quenching reactive parameters of PGH and BSA at different temperatures

T/(K)	K_{q1} /(L· mol^{-1}·s)	K_{sv1} / (L· mol^{-1})	r_3	K_{a1} /(L· mol^{-1})	n	r_4
298	8.04×10^{13}	8.04×10^{5}	0.9976	7.48×10^{5}	0.99	0.9985
310	3.72×10^{13}	3.72×10^{5}	0.9991	6.54×10^{5}	1.04	0.9927
318	2.93×10^{13}	2.93×10^{5}	0.9946	4.16×10^{5}	1.03	0.9975

K_{q1} is the quenching rate constant; K_{sv1} is the Stern-Volmer quenching constant; K_{a1} is the binding constant; n is the number of binding site; r_3 is the linear relative coefficient of F_0/F~[L]; r_4 is the linear relative coefficient of lg [$(F_0$-$F)/F$]~lg [L]

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TABLE 4: The thermodynamic parameters of BSA-PGH at different temperatures

Method	T /(K)	K_a/(L·mol^{-1})	ΔH /(KJ·mol^{-1})	ΔS/(J·mol^{-1}·K^{-1})	ΔG /(KJ·mol^{-1})
Classical fluorescence spectroscopy	298	4.8×10^3	-14.74	21.03	-21.00
	310	4.3×10^3		22.03	-21.56
	318	3.2×10^3		20.77	-21.34
Elastic scattering fluorescence spectroscopy	298	7.48×10^5	-21.76	39.42	-33.51
	310	6.54×10^5		41.13	-34.51
	318	4.16×10^5		39.14	-34.21

TABLE 5 : Hill's coefficient of BSA-PGH systems at different temperatures

T / (K)	Classical fluorescence spectroscopy		Elastic scattering fluorescence spectroscopy	
	n_H	r_6	n_H	r_6
298	1.03	0.9985	1.01	0.9983
310	1.01	0.9906	1.00	0.9993
318	0.98	0.9995	0.98	0.9975

r_6 is the linear relative coefficient of lg [Y/(1-Y)]~lg [L]; n_H is the hill's coefficient.

clude hydrogen bond, van der Waals force, electrostatic and hydrophobic interactions, etc. According to the relevant thermodynamic parameters of small molecule drugs with biological macromolecules, the type of interaction force can be simply judged[18]. If the temperature does not vary significantly, the ΔH can be regarded as a constant[19], then its value and that of the entropy change (ΔS) can be determined from Eq. (3)[20]:

$$\mathbf{R\ln K = \Delta S - \Delta H / T} \quad \mathbf{(3)}$$

Where, K_a is the binding constant at corresponding temperature and R is the gas constant. The enthalpy change (ΔH) and the entropy change (ΔS) were obtained from the slope and intercept of Eq. (3) plot based on $RlnK$ versus T^{-1}.

The Gibbs energy change (ΔG) was estimated from the following relationship:

$$\mathbf{\Delta G = -RT\ln K = \Delta H - T\Delta S} \quad \mathbf{(4)}$$

The values of thermodynamic parameters K_a, ΔH, ΔS and ΔG are listed in TABLE 4. As shown in the TABLE 4, the negative value of ΔG clarified a spontaneous reaction between BSA and PGH. The negative value of ΔH and positive value of ΔS showed that PGH mainly bound to BSA by the electrostatic attraction[21]. Comparing the thermodynamic parameters obtained by elastic scattering fluorescence spectroscopy and classical fluorescence spectroscopy, the results of two methods were consistent, so that we can make a conclusion that the study of reaction mechanism of PGH with BSA was feasible with treating drugs as detection object by elastic scattering fluorescence spectroscopy.

Hill's coefficient of BSA–PGH system

According to the Hill's coefficient of BSA–PGH system, we can make a quantitative analysis for cooperativity between protein and ligands on the basis of the following equation[22]:

$$\mathbf{\lg\frac{Y}{1-Y} = \lg K + n_H \lg[L]}, \quad \mathbf{(5)}$$

Where, Y is the fractional binding saturation; fraction of sites occupied with the ligand; n_H is the Hill's coefficient; K is the binding constant. Hill's coefficient is greater than 1, which exhibits positive cooperativity and its role is enhanced with increasing n_H. Conversely, Hill's coefficient is less than 1, which exhibits negative cooperativity and its role is enhanced by decreasing n_H. A coefficient of 1 indicates non-cooperative reaction[23].

For fluorescence measurement

$$\mathbf{\frac{Y}{1-Y} = \frac{Q}{Q_m - Q}}, \quad \mathbf{(6)}$$

$$\mathbf{Q = \frac{F_0 - F}{F_0}}, \quad \mathbf{(7)}$$

Where 1/Qm is intercept of the plot 1/Q versus 1/[L]. According to the formula (5), Hill's coefficient of BSA–PGH system can be gained from the slope of the plot of lg [Y/(1-Y)]versus lg [L]. The results were presented in TABLE 5. From TABLE 5, it could be seen that the values of n_H were both equal to 1 approximately at different temperatures by classical fluorescence spectroscopy and elastic scattering fluorescence spectroscopy, which indicated that there was non-cooperative reaction between BSA and PGH. Meanwhile, it illustrated elastic scattering fluorescence spectroscopy is correct to determine synergy between drug and protein.

CONCLUSIONS

The reaction mechanism between PGH and BSA was investigated by classical fluorescence spectroscopy and elastic scattering fluorescence spectroscopy. Comparing binding constants obtained by classical fluorescence spectroscopy and elastic scattering fluorescence spectroscopy, shows that the K_a values of classical fluorescence spectroscopy was smaller than elastic scattering fluorescence spectroscopy. That means taking drugs as detection object by elastic scattering fluorescence spectroscopy can be more comprehensive and more accurate expressing the interaction information between protein and drug in terms of fluorescence. In addition to Trp residues of bovine serum albumin, the others also interact with PGH. Elastic scattering fluorescence spectroscopy 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.

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