SPECTROSCOPIC STUDY OF SITE-SELECTIVE BINDING OF PIOGLITAZONE HYDROCHLORIDE TO TRYPSIN

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Site-selective binding between pioglitazone hydrochloride (PGH) and trypsin (TRP) was investigated by fluorescence spectroscopy, ultraviolet spectroscopy, synchronous fluorescence spectroscopy, and circular dichroism spectroscopy. The results demonstrated that PGH could quench the intrinsic fluorescence of TRP strongly by a static quenching process. The microenvironment of tryptophan (Trp) and tyrosine (Tyr) residue was both changed, and the polarity of the hydrophobic environment in the TRP cavity was enhanced and the hydrophobicity was weakened. The results demonstrated that the interaction between PGH and TRP was taking place via hydrogen bond and hydrophobic force with 1:1 binding ratio. The binding constants K_a, the number of binding sites n, and thermodynamic parameters were obtained. The participation of amino acid residues and synchronous spectroscopy showed that the main site of the reaction between PGH and TRP was at the hydrophobic cavity. The binding rate of PGH to protein in 298 K was ~65–85% and for the combined model was $W = –0.08684R^2 + 0.1048R + 0.8503$. This provides the theoretical basis for the dosage of the drug.

Keywords: *pioglitazone hydrochloride, trypsin, spectrometry, binding site, binding rate.*

СПЕКТРОСКОПИЧЕСКОЕ ИССЛЕДОВАНИЕ САЙТ-СЕЛЕКТИВНОГО СВЯЗЫВАНИЯ ПИОГЛИТАЗОНА ГИДРОХЛОРИДА С ТРИПСИНОМ

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Методами флуоресцентной, УФ, синхронной флуоресцентной спектроскопии и кругового дихроизма исследовано сайт-селективное связывание пиоглитазона гидрохлорида (ПГГ) и трипсина (ТРП). Из полученных данных следует, что ПГГ может сильно тушить собственную флуоресценцию ТРП путем статического механизма тушения. При этом микроокружение радикалов триптофана и тирозина изменяется, полярность гидрофобной среды в полости ТРП увеличивается, а гидрофобность ослабевает. Показано, что взаимодействие между ПГГ и ТРП происходит посредством водородной связи с участием гидрофобной силы при соотношении связывания 1:1. Получены константы связывания K_a, число сайтов связывания n, а также термодинамические параметры. Реакция между ПГГ и ТРП происходит в основном в гидрофобной полости. Это следует из данных синхронной спектроскопии и факта участия аминокислотных радикалов. Скорость W связывания ПГГ с белком при 298 К составила ~65–85 %, а комбинированная модель может быть представлена в виде $W = –0.08684R^2 + 0.1048R + 0.8503$, где R — отношение концентрации ПГГ к общей концентрации протеинов. Она обеспечивает теоретическую основу для дозировки препарата.

Ключевые слова: *гидрохлорид пиоглитазона, трипсин, спектрометрия, сайт связывания, скорость связывания.*

Introduction. Pioglitazone hydrochloride is used to treat type 2 diabetes [1]. It belongs to class II of the Biopharmaceutics Classification System (BCS) because of its low solubility in water (0.047 mg/mL) [2]. Chemical hypoglycemic drugs are strictly limited in dosage, too much, there is a risk of coma or even death. Therefore, it is very important to understand the binding behavior of PGH to protein.

Trypsin, the water-soluble globular protein, which can cleave the peptide bonds at the carboxyl side of arginine, lysine, and ornithine residues, is widely used in biomedicine, food, and biotechnology industry, as well as in protein analysis [3]. Trypsin consists of 223 amino residues with a molecular mass of 23300 Da [4]. A molecule of trypsin contains two domains of almost equal size with the major constituent of each domain being a set of six antiparallel strands of a polypeptide chain laced together into a β-sheet unit by a network of H-bonds [5]. It is a kind of endopeptidase, which is characterized by the catalytic triad His-57, Asp-102, and Ser-195 and fractures peptide bonds formed by the carboxylic groups of lysine or arginine [6]. There is a major hydrophobic cavity I near Trp 215 and Tyr 228. Trypsin is excreted by the pancreas, enters the small intestine, and participates in the digestion of food proteins and other biological processes in the human body. It is usually used as an important model of the digestive proteases to investigate the interactions between drugs and proteins.

The absolute bioavailability of PGH is 99%. After oral administration, the peak concentration of PGH can be measured in blood for 2 h after 30 min on an empty stomach. Gastric juice contains water, electrolytes, lipids, pepsin, chymotase, gastric lipase, and polypeptide hormones, which do not contain TRP. TRP mainly exists in the small intestine. PGH dissolves in gastric juice and is mainly absorbed in the small intestine. Because the absorption area of the small intestine is large, blood flow is abundant, and the drug stays in the intestine for a long time. The small intestine is the main absorption site of drugs. The pH value of small intestine fluid is 7.1–7.8, which is consistent with the experimental conditions in this paper. Previous studies have shown that drugs can interact with TRP and then affect the structure and properties of TRP [7]. The interaction between TRP and drugs has been reported [8]. But studies of TRP and PGH have not been reported. In this paper, the binding parameters, binding rate equations, and binding curves between TRP and PGH were studied by various spectral methods.

Experiment. All fluorescence spectra were recorded with a Shimadzu RF-5301PC spectrofluorophotometer. Absorption was measured with a UV-Vis recording spectrophotometer (UV-3600, Shimadzu, Japan). Circular dichroism spectra were recorded on a MOS-450/SFM300 circular dichroism spectrometer (Bio-Logic, France). All temperatures were controlled by a CS501 superheated water bath (Nantong Science Instrument Factory).

Trypsin was purchased from Sigma-Aldrich and was of less than 99% purity. Stock solutions of TRP (100.0 μM) and PGH (1000.0 μM) were prepared. All stock solutions were further diluted for use as working solutions. Tris-HCl buffer solution containing 0.15 M 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 277 K. In this study, all fluorescence intensities were corrected for the absorption of excited light and the reabsorption of emitted light based on the following relationship [9]:

$$F_{cor} = F_{obs}\exp[(A_{ex} + A_{em})/2], \quad (1)$$

where F_{cor} and F_{obs} are the corrected and observed fluorescence intensities, and A_{ex} and A_{em} are the absorption of the system at the excitation and emission wavelengths.

UV-Vis absorption experiment. At 298 K, 1.0 mL Tris-HCl buffer solution, 2.0 mL 40.0 μM TRP solution, and different volumes of 100.0 μM PGH solution were added into 10 mL colorimetric tubes; water volume was 10 mL. The UV absorption spectra of the TRP-PGH system were scanned in the range 190–400 nm with the corresponding concentration of PGH solution as reference.

Fluorescence measurements. The fluorescence measurements were carried out as follows: 1.0 mL Tris-HCl (pH 7.40), 1.0 mL TRP solution (40.0 μM), and different volumes of PGH were added into 10 mL colorimetric tubes successively. The samples were diluted to scaled volume with double-distilled water, mixed thoroughly by shaking, and kept static for 30 min at different temperatures (298, 303, and 310 K). Excitation wavelength with excitation and emission slit at 5 nm for TRP was 280 nm (or 295 nm), respectively, with a 1.0 cm path length cell. The solutions were subsequently scanned on the fluorophotometer, and the fluorescent intensity was determined. At the same time fixed $\Delta\lambda$ = 15 or 60 nm, the synchronous fluorescence spectra of TRP and PGH were recorded.

Circular dichroism measurements. The CD spectra of TRP in the absence and presence of different amounts of PGH were measured at wavelengths between 190 and 300 nm in pH 7.4 Tris-HCl buffer solution under nitrogen atmosphere at room temperature, and the buffer solution signal was subtracted. The concentration of TRP was kept at 40.0 μM while varying the PGH concentration by keeping the molar ratios of PGH to TRP as 1:0, 1:10, and 1:20.

Results and discussion. *UV-Vis absorption studies of TRP-PGH system*. There were two absorption peaks at 198 and 280 nm on the TRP absorption spectrum, which correspond to peptide bond absorption and aromatic amino acid absorption, respectively [10]. With varying concentrations of PGH, a noticeable decrease in the absorbance intensity of the peak at 198 nm coupled with a red shift took place; meanwhile, the absorption band at near 280 nm also changed slightly, which indicated that there was a variation in the framework conformation of TRP due to the formation of PGH-TRP complex. The changes in absorption intensity and wavelength of TRP further reconfirmed that static quenching was dominant in the interaction process, as dynamic quenching only affected the excited state of the fluorophore and the absorption spectra would not be influenced [11].

Fluorescence quenching of TRP by PGH. Fluorescence quenching can be induced by collision or formation of a complex between the quencher and fluorophore, which is usually classified as dynamic quenching or static quenching. The fluorescence spectra of the TRP-PGH system is shown in Fig. 1 (it was similar to 295 nm), which showed that the fluorescence intensity of TRP decreased regularly with the addition of PGH when the excitation wavelength was 280 nm (similar to 295 nm). The TRP-PGH system had generated a new ground-state complex [12].

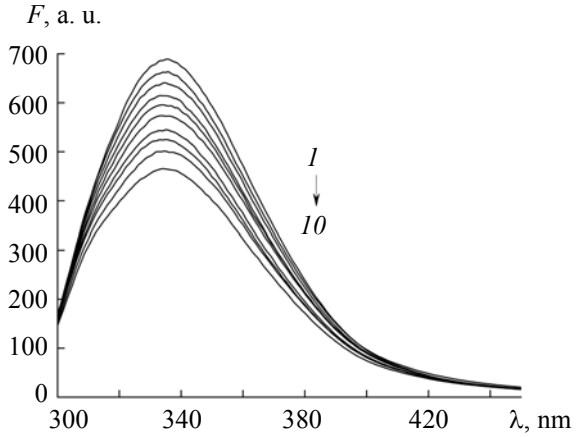

Fig. 1. Fluorescence emission spectra of TRP-PGH (T = 298 K), C_{TRP} = 4.0 μM, C_{PGH} = 0 (1), 10.0 (2), 20.0 (3), 30.0 (4), 40.0 (5), 50.0 (6), 70.0 (7), 80.0 (8), 90.0 (9), and 100.0 μM (10).

In order to determine the quenching mechanism of PGH on TRP, the Stern-Volmer equation was used to analyze the fluorescence quenching values [13]:

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

where F_0 and F are the fluorescence intensities of TRP in the absence and presence of quencher, K_q is the quenching rate constant of the biomolecule, τ_0 is the average lifetime of the biomolecule in the absence of quencher, which equals 10^{-8} s, K_{SV} is the Stern–Volmer quenching constant, and [L] is the concentration of quencher.

According to Eq. (2), based on the linear fit plot of F_0/F versus [L], the K_{SV} values can be obtained. The results are shown in Table 1. The values of K_q were much greater than that of the maximum scattering collision quenching constant (2×10^{10} $M^{-1}\cdot s^{-1}$), indicating that the fluorescence quenching mechanism of TRP initiated by PGH was a static quenching procedure [14].

The binding constant (K_a) and the number of binding sites (n) can be calculated by the double logarithm regression curve [15]:

$$\lg\left(\frac{F_0 - F}{F}\right) = n\lg K_a + n\lg\left\{[D_t] - n\frac{F_0 - F}{F}[B_t]\right\}, \quad (3)$$

where F_0 and F are the corrected fluorescence intensities before and after the addition of the quencher (PGH), $[D_t]$ and $[B_t]$ are the concentrations of total PGH and TRP, respectively. The results are listed 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 PGH existing in TRP. Meanwhile, the binding constant between PGH and TRP decreased with increasing temperature, which showed that the quenching mechanism was static quenching [16]. The fluorescence of Trp and Tyr residues can be stimulated at λ_{ex} = 280 nm, while λ_{ex} = 295 nm only stimulates the fluorescence of the Trp residue. The results presented in Table 1 show that the binding constant of Trp and Tyr residues (λ_{ex} = 280 nm) at the same temperature was larger than that of Trp alone (λ_{ex} = 295 nm); thus it was possible to infer that Trp and Tyr were all involved in the quenching reaction. According to the equation $\Delta G = \Delta H - T\Delta S$, $R \ln K = \Delta S - \Delta H/T$, and Table 1, at λ_{ex} = 280 nm (298 K), ΔH = –29.13 kJ/mol, ΔS = –25.88 J·mol^{-1}·K^{-1}, and ΔG = –21.42 kJ/mol. The negative sign for ΔG indicated the spontaneity of binding for PGH with TRP. The negative values of ΔH and ΔS indicated that hydrogen bond and hydrophobic force played a major role in the interaction between PGH and TRP [17].

TABLE 1. Quenching Reactive Parameters of TRP-PGH System at Different Temperatures

λ_{ex}, nm	T, K	K_q, M^{-1}·s^{-1}	K_{SV}, M^{-1}	r_1	K_a, M^{-1}	n	r_2
280	298	5.33×10^{11}	5.33×10^{3}	0.9980	5.68×10^{3}	1.03	0.9967
	303	4.97×10^{11}	4.97×10^{3}	0.9939	3.99×10^{3}	1.01	0.9955
	310	3.57×10^{11}	3.57×10^{3}	0.9922	3.55×10^{3}	0.91	0.9963
295	298	3.69×10^{11}	3.69×10^{3}	0.9913	3.80×10^{3}	0.96	0.9947
	303	3.29×10^{11}	3.29×10^{3}	0.9915	3.16×10^{3}	1.09	0.9969
	310	2.25×10^{11}	2.25×10^{3}	0.9924	2.63×10^{3}	1.00	0.9919

N o t e. 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 $\lg[(F_0 - F)/F] \sim \lg\{[D_t] - n[B_t](F_0 - F)/F_0\}$.

Participation of amino acid residues. Upon excitation at 280 nm, both Trp and Tyr are readily excited, while at an excitation wavelength of 295 nm, only the Trp emits fluorescence. As seen in Fig. 2, in the presence of PGH, the quenching curves of TRP excited at 280 and 295 nm overlap below the molar ratio of PGH:TRP = 5:1. The quenching of TRP fluorescence excited at 280 nm above this molar ratio is slightly higher than that excited at 295 nm. When C_{PGH}:C_{TRP} = 5:1, only Trp residues participate in the reaction, whereas above this concentration ratio both Trp and Tyr residues participates in it.

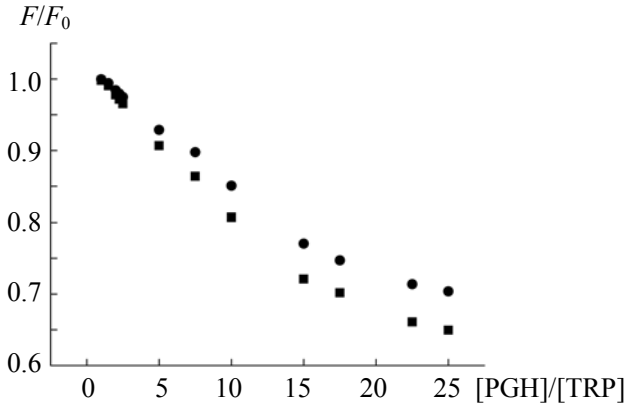

Fig. 2. Fluorescence emission spectra of PGH-TRP at λ_{ex} = 280 (■) and 295 nm (●), T = 298 K, C_{TRP} = 4.0 μM, and C_{PGH} = 10.0–100.0 μM.

PGH protein binding rate. For number of binding sites $n = 1$, the PGH protein binding rate (W) is as follows [18]:

$$K_aR[B_0]W^2 - (K_aR[B_0] + K_a[B_0] + 1)W + K_a[B_0] = 0, \quad (4)$$

$$W = \frac{\left(K_aR + K_a + 1/[B_0]\right) - \sqrt{\left(K_aR + K_a + 1/[B_0]\right)^2 - 4K_a^2R}}{2K_aR}, \quad (5)$$

where R is the ratio of PGH to total protein concentration, and $[B_0]$ is the total protein concentration. The protein concentration was 1000.0 μM, and PGH concentration was between 0.1 to 1000.0 μM. The experimentally obtained values of K_a and Eq. (5) were used to calculate the protein binding rate of different concentrations of PGH at 298, 303, and 310 K with $\lambda_{ex} = 280$ nm, respectively: 65.94–85.03%, 60.94–79.97%, 59.16–78.01%. At 298 K, the nonlinear curve fit equation is: $y = -0.08684x^2 + 0.1048x + 0.8503$, $r = 0.9991$, and $W = -0.08684R^2 + 0.1048R + 0.8503$. According to the above equations, when the concentration of protein in the system is constant, the protein binding rate decreases with the addition of PGH. At normal body temperature 310 K, the binding rate of PGH to TRP is ~59.16–78.01%, and that of the free drug is ~21.99–40.84%. The free drug plays a therapeutic role in oral administration. The drugs that bind to TRP will travel with the blood to other parts of the body and eventually reach a new balance. They will again free ~21.99–40.84% of the drugs until all the drugs are free to take effect. The protein binding rate of PGH provides an important reference value for clinical medicine.

Synchronous fluorescence spectra studies of TRP-PGH system. When the value of Δλ between the excitation and emission wavelengths is stabilized at either 15 or 60 nm, synchronous fluorescence gives characteristic information for Tyr or Trp residues, respectively [19]. When Δλ = 15 nm, the fluorescence intensities of TRP-PGH showed a decrease, and had 2 nm red shifts (from 309 to 311 nm) with increasing concentrations of PGH. When Δλ = 60 nm, the synchronous fluorescence intensities of TRP-PGH visibly decreased with 3 nm red shifts (from 343 to 346 nm). The reason for the red shift was that the fluorescence quenching of TRP was initiated by the formation of a ground-state complex leading to an increase in the conjugated system and fluorescence efficiency. This phenomenon indicates that the Trp and Tyr residue microenvironments were changed due to the interaction of PGH with TRP, the polarity of the hydrophobic environment was enhanced, and hydrophobicity was reduced in the TRP cavity due to changes in the microenvironment of the Trp and Tyr residues on insertion of PGH [20]. This conclusion was consistent with primary binding site studies.

Circular dichroism measurements. Circular dichroism spectroscopy is an effective method to monitor the secondary structural changes in proteins. The circular dichroism spectra were recorded from 195 to 280 nm, which was an effective range for measuring protein conformational changes because electronic transitions exist between ground and excited states [21]. A strong negative peak was detected at about 204 nm (Fig. 3). The intensity of the peak at about 204 nm decreased after the addition of PGH, indicating that the secondary structure of TRP was changed by PGH.

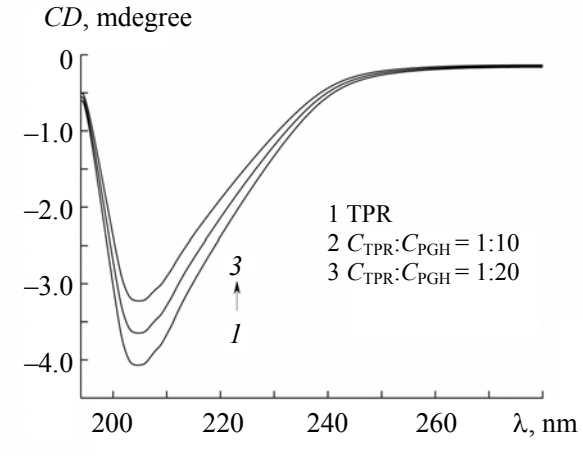

Fig. 3. Circular dichroism spectra of TRP-PGH system ($T = 298$ K), $C_{TRP} = 2.0$ μM; $C_{PGH} = 40.0$, 80.0 μM.

Conclusion. The reaction mechanism of PGH with TRP was investigated using fluorescence spectroscopy at different temperatures. The results indicated that PGH and TRP were statically quenched, and the binding constant was 10^3 in order of magnitude. PGH on TRP had only one high affinity binding site; the main force was a hydrophobic force; the binding rate (310 K) was ~59.16–78.01%, and Tyr and Trp residues were involved in the reaction. The binding rate provided the theoretical basis for the dosage of the drug. An insufficient dose is not effective, and drug overdose will lead to side effects. The results showed that the hydrophobic cavity of TRP was the binding site of the system. The conformation of the system was investigated by synchronous fluorescence spectrometry and circular dichroism. This study is useful for the analysis of reaction mechanism between protease macromolecules and small molecules and provides reference information for pharmacological analysis.

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