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Investigation on Interaction of Buflomedil Hydrochloride with Trypsin: A Spectroscopic Analysis

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The interaction between buflomedil hydrochloride and trypsin has been studied through fluorescence spectroscopy and UV/VIS spectrophotometry. With the addition of buflomedil hydrochloride, the fluorescence emission intensity of trypsin was quenched. The number of binding sites and the binding constants were measured by the fluorescence quenching method. Thermodynamic parameters ΔH, ΔG and ΔS at three temperatures (293 K, 300 K, 310 K) were calculated. The force acting between buflomedil hydrochloride and trypsin was mainly a hydrophobic interaction. The binding distance between the donor (trypsin) and acceptor (buflomedil hydrochloride) was 0.862 nm according to Förster's non-radiational energy transfer theory.

Key Words: Buflomedil hydrochloride, Trypsin, Fluorescence quenching, Stern-volmer equation, Förster's resonance energy transfer theory.

INTRODUCTION

Buflomedil hydrochloride [4-(1-pyrrolidinyl)-1-(2,4,6-trimethoxyphenyl)-1-butanone hydrochloride] (Fig. 1) is a vasodilatory agent, it can ameliorate microcirculation and treat peripheral arterial diseases[1,2]. The drug is known to exert a nonspecific antagonism on α-adrenoceptors and is a weak calcium-channel antagonist[3].

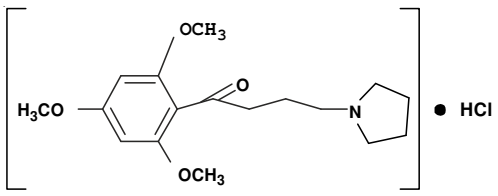

Fig. 1. Structure of buflomedil hydrochloride

Proteins are basic components of the living organisms and carriers of life activities. Trypsins are members of a large family of serine proteinases, specifically hydrolyze proteins and peptides at the carboxyl side of arginine and lysine residues and play the major roles in biological process including digestion, activation of zymogens of chymotrypsin and other enzymes. Trypsins are digestive enzymes that have many biochemical and industrial applications due to their high specificity allowing a controlled proteolysis[4].

In this work, the interaction between buflomedil hydrochloride and trypsin has been studied at different temperatures under physiological conditions. The interaction mechanism of buflomedil hydrochloride with trypsin were investigated by UV/VIS absorption, fluorescence spectroscopy methods to elucidate the effect of buflomedil hydrochloride on the trypsin.

EXPERIMENTAL

A RF-5301 PC fluorescence spectrometer (Jasco, Japan) was used to record the fluorescence spectra. A TU1901 UV/VIS spectrophotometer (PGeneral, Beijing, China) was used to record absorption spectra. A PHS-3C meter (Shanghai Precision Scientific Instrument Co., Ltd., China) was used to measure the pH values of Tris-HCl buffer solution.

Buflomedil hydrochloride solution (2.8×10^{-4} mol L^{-1}) was prepared by diluting 4.8 mL (48 mg/4.8 mL) buflomedil hydrochloride (342.85 Da, Shandong Fangming Pharmaceutical Co., Ltd. China) in 500 mL of deionized water. Trypsin (2.0×10^{-4}mol L^{-1}) was prepared by dissolving 1.1900 g of trypsin (23.8 kDa, Sinopharm Group Chemical Reagent Co.,Ltd., Shanghai, China) in 250 mL of water. The trypsin solution was stored at 4 ºC in a refrigerator. A buffer solution of 0.05 mol L^{-1} Tris-HCl was used to keep the pH of solution at 7.40.

All other reagents were of analytical reagent grade and double-deionized water was used throughout the experiment.

Tris-HCl 2 mL, 2×10^{-4} mol L^{-1} trypsin 2 mL and an appropriate amount of buflomedil hydrochloride were added

successively to a 10 mL volumetric flask. The mixture was diluted to an appropriate volume with water and mixed thoroughly by shaking.

The fluorescence spectra of the system were recorded at 300-500 nm. The excitation bandwidth was 5 nm and emission bandwidth was 5 nm, using a 1 cm quartz cell.

UV/VIS absorption spectra were recorded on a TU-1901 spectrophotometer equipped with 1 cm quartz cell. The recorded wavelength was ranging from 400 to 190 nm.

RESULTS AND DISCUSSION

Fluorescence quenching spectra and the fluorescence quenching mechanisms: Fig. 2, shows the fluorescence emission spectra of trypsin with various amounts of buflomedil hydrochloride following an excitation at 280 nm. Trypsin shows a fluorescence emission with a peak at 336 nm. The fluorescence intensity decreased gradually with the increase in concentration of buflomedil hydrochloride and higher concentrations led to more efficient quenching of the protein fluorescence. The change in the microenvironment around tryptophan was also confirmed by a red shift in the maximum emission wavelength from 336 to 342 nm, which indicated that the binding of buflomedil hydrochloride to trypsin quenches the intrinsic fluorescence of the single tryptophan in trypsin. And higher concentrations of buflomedil hydrochloride led to more efficient quenching of trypsin fluorescence. The fluorescence quenching mechanisms are usually classified as dynamic quenching and static quenching, which are caused by diffusion and ground-state complex formation spectively[5,6]. In order to explain the fluorescence quenching mechanism induced by trypsin, the Stern-Volmer equation was used to process the data[7].

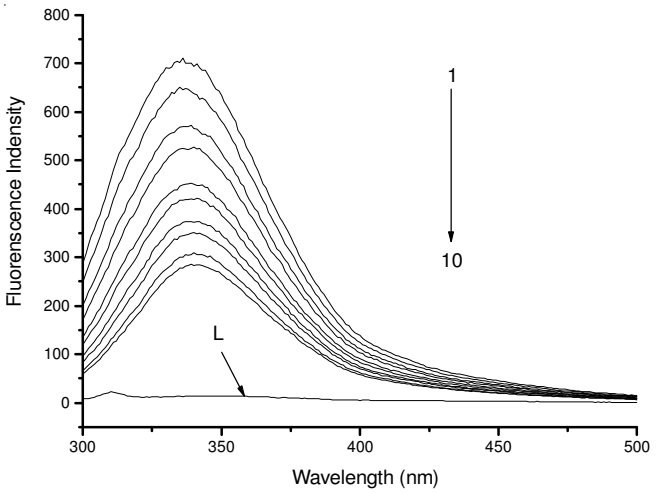

Fig. 2. Fluorescence spectra of trypsin-buflomedil hydrochloride system (1) 4.0×10^{-5} mol L^{-1} trypsin, (2→10) 4.0×10^{-5} mol L^{-1} trypsin in the presence of 0.00, 1.12, 2.80, 3.92, 5.60, 6.72, 8.40, 9.52, 11.20 and 12.3×10^{-5} mol L^{-1} buflomedil hydrochloride, Curve L shows the emission spectrum of 5.6×10^{-5} mol L^{-1} buflomedil hydrochloride only. pH = 7.40, T = 300 K

$$\frac{F_0}{F} = 1 + Ksv = 1 + Kq\tau_0[Q] \quad (1)$$

where F_0 and F denote the steady-state fluorescence intensities in the absence and presence of quencher, respectively. Ksv is the Stern-Volmer quenching constant and [Q] is the concentration of quencher. Kq is the quenching rate constant of the biological macromolecule and Kq is equal to Ksv/τ_0. τ_0 is the average lifetime of the molecule without any quencher and the fluorescence life-time of the biopolymer[8] is 10^{-8} s. The Stern-Volmer curves at three different temperatures were shown in Fig. 3. The values of Ksv and Kq derived from eqn. (1) are listed in Table-1. The value for in Table-1 is of the magnitude 10^{12} L $mol^{-1}s^{-1}$, which is greater than the maximum diffusion collision quenching rate constant. So it suggested that the fluorescence quenching process of trypsin may be mainly governed by a static quenching mechanism arising from a system formation rather than a dynamic quenching mechanism[9].

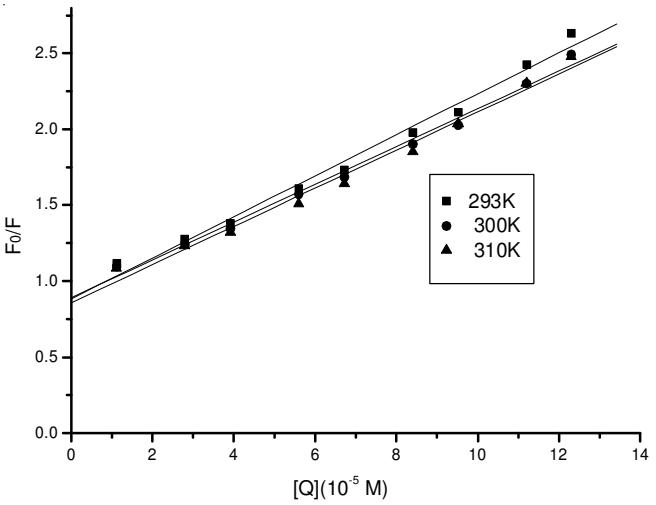

Fig. 3. Stern-Volmer plots of the trypsin-buflomedil hydrochloride system at different temperatures (293, 300, 310 K). [Trypsin] = 4×10^{-5} mol L^{-1}, [buflomedil hydrochloride] = 1.12×10^{-5} mol L^{-1} to 12.30×10^{-5} mol L^{-1}, pH = 7.40, λ_{ex} = 280 nm

TABLE-1
STERN-VOLMER QUENCHING CONSTANTS FOR TRYPSIN-BUFLOMEDIL HYDROCHLORIDE SYSTEM AT DIFFERENT TEMPERATURES

T(k)	Ksv ($\times 10^4$ L mol^{-1})	R	Kq ($\times 10^{12}$ L $mol^{-1}s^{-1}$)
293	1.3501	0.99416	1.3501
300	1.2432	0.99555	1.2432
310	1.2560	0.99292	1.2560

Binding constant and binding site: For static quenching, when small molecules are bound independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by eqn. (2)[10]:

$$\log\frac{F_0 - F}{F} = \log K_b + n\log[Q] \quad (2)$$

where, K_b is the binding constant and n is the number of binding sites. The values for K_b and n at three different temperatures can be derived from the intercept and slope of plots (Fig. 4.) of $\log\frac{F_0 - F}{F}$ *versus* log[Q] based on eqn. (2), they are listed in Table-2. Linear regression equations eqns. (3)-(5) at 293, 300 and 310 K are expressed as follows:

$$\log\frac{F_0 - F}{F} = 4.5024 + 1.1068 \log [Q] \text{ 293 K} \quad (3)$$

$$\log \frac{F_0 - F}{F} = 4.6219 + 1.1447 \log [Q] \ 300 \text{ K} \quad (4)$$

$$\log \frac{F_0 - F}{F} = 4.8169 + 1.1964 \log [Q] \ 310 \text{ K} \quad (5)$$

K_b in Table-2 shows that there exist a strong interaction between buflomedil hydrochloride and trypsin and a complex formation of buflomedil hydrochloride with trypsin. Furthermore, it can be inferred from the values of n that there is an independent class of binding sites on trypsin for buflomedil hydrochloride. Otherwise, it appears that the binding constants and the number of binding sites increase with higher temperature[11,12]. It may be attributed to that the capacity of buflomedil hydrochloride binding to the fact that trypsin is increased with increase in temperature.

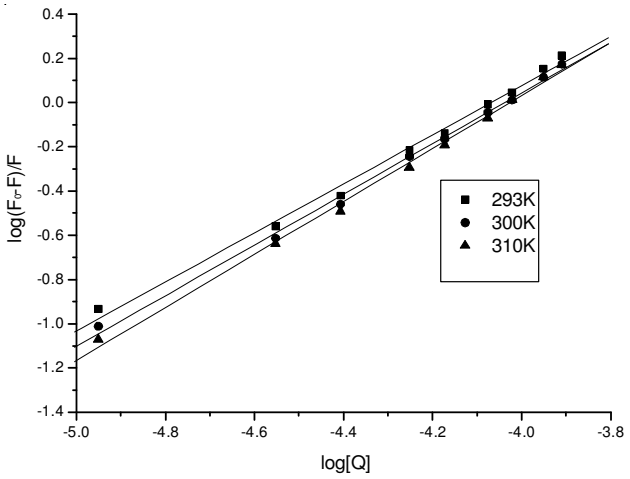

Fig. 4. $\log_{10}$(Fo-F)/F against $\log_{10}$[Q] at three different temperatures

Thermodynamic parameters and nature of binding mode: The intermolecular forces contributing to biomolecules interactions with drugs may include hydrogen bonds, van der Waals interactions, electrostatic interactions and hydrophobic force, *etc*.[13]. The thermodynamic parameters dependent on temperatures were analyzed in order to further characterize the acting force between trypsin-buflomedil hydrochloride complex. The thermodynamic parameters, enthalpy change (ΔH), entropy change (ΔS) and free energy change (ΔG) are the main evidence to determine the binding mode[14]. The value of enthalpy change (ΔH) can be calculated from the van't Hoff eqn. (6), if the enthalpy change (ΔH) does not vary significantly over the temperature range studied. The value of free energy change (ΔG) at different temperature was calculated from eqn. (7):

$$\ln K_b = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (6)$$

$$\Delta G = \Delta H - T\Delta S = -RT \ln K_b \quad (7)$$

where, K_b is the binding constant at corresponding temperature, R is the gas constant and T is the absolute temperature. The values of ΔH, ΔS and ΔG are listed in Table-2. The negative value of ΔG reveals that the interaction process is spontaneous. The positive ΔH and ΔS values indicated that hydrophobic interactions play a main role in the binding between trypsin and buflomedil hydrochloride[14].

Synchronous fluorescence spectroscopy: Synchronous fluorescence spectroscopy was usually used to study the conformation change of protein[15]. At Δλ = 60 nm, the synchronous fluorescence spectra are characteristic of tryptophan residues, whereas at Δλ = 15 nm the spectra are attributed to the properties of tyrosine residues[12].

TABLE-2
BINDING PARAMETERS AND THERMODYNAMIC PARAMETERS FOR TRYPSIN-BUFLOMEDIL HYDROCHL-ORIDE SYSTEM AT DIFFERENT TEMPERATURES

T (K)	K_b ($\times 10^4$ L mol^{-1})	n	R	ΔG° (KJ mol^{-1})	ΔH° (KJ mol^{-1})	ΔS° (J mol^{-1}k^{-1})
293	3.1797	1.1068	0.99683	-25.254	32.171	195.992
300	4.1869	1.1447	0.99825	-26.544	32.171	195.718
310	6.5602	1.1964	0.99800	-28.586	32.171	195.992

Synchronous fluorescence spectra of trypsin upon addition of buflomedil hydrochloride at Δλ 15 and 60 nm are shown in Fig. 5. From Fig. 5a, it can be seen that the emission peaks of the synchronous fluorescence spectra of trypsin with various amounts of buflomedil hydrochloride has a slight red shift, which indicates a less hydrophobic environment around the tyrosine residues. From Fig. 5b, the emission maximum of tryptophan residues has no shift, which indicates that there was no change of the environment of the tryptophan residues.

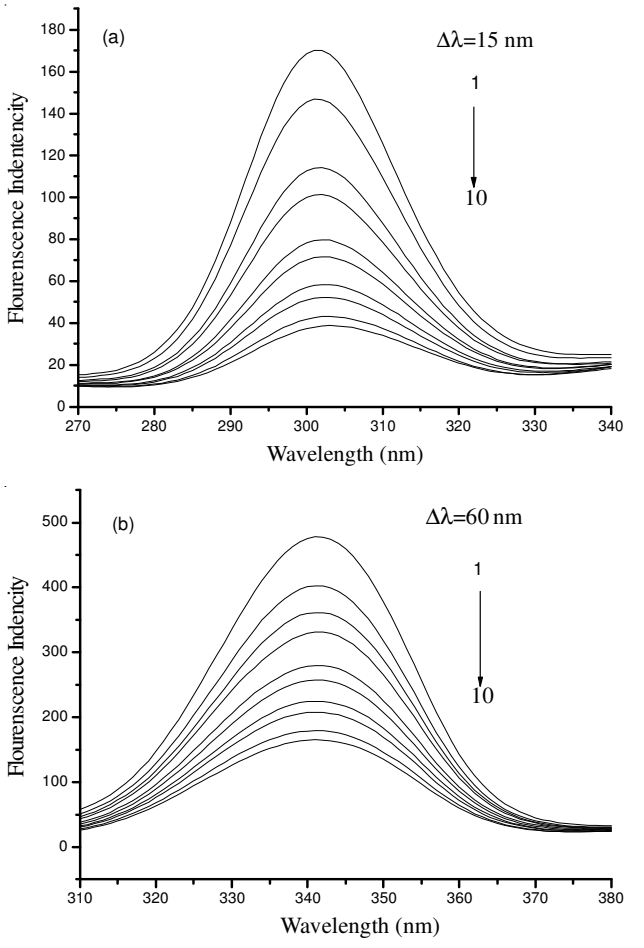

Fig. 5. Synchronous fluorescence spectrum of trypsin in absence and in presence of buflomedil hydrochloride: (a) λ = 15 nm; (b) λ = 60 nm. (1) 4.0×10^{-5} mol L^{-1} trypsin, (2→10) 4.0×10^{-5} mol L^{-1} trypsin in the presence of 0.00, 1.12, 2.80, 3.92, 5.60, 6.72, 8.40, 9.52, 11.20 and 12.30 $\times 10^{-5}$ mol L^{-1} buflomedil hydrochloride, respectively. pH = 7.40, T = 300 K

Energy transfer from trypsin to buflomedil hydrochloride and binding distance: According to Förster's resonance energy transfer theory[16], the distances (R) between trypsin and buflomedil hydrochloride can be determined. The efficiency of energy transfer (E), is described by the following equation :

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + R^6} \quad (8)$$

where, E is the energy transfer efficiency, R is the distance between acceptor and donor, and R_0 is the critical distance when the transfer efficiency is 50 %. The quantity R_0^6 is calculated using the equation:

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^{-4} \Phi J \quad (9)$$

where, K^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor, J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. J is given by the following equation:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda}$$

where, F(λ) is the fluorescence intensity of the fluorescence donor at the wavelength λ and ε(λ) is the molar absorbance coefficient of the acceptor when the wavelength is λ. It was reported that $K^2 = 2/3$, N = 1.336 and Φ = 0.118[17]. There was a spectral overlap between the fluorescence emission spectrum of trypsin and UV/VIS absorption spectrum of buflomedil hydrochloride (Fig. 6). From the above relationships, the values of J, R_0 and R can be calculated: $J = 7.397 \times 10^{-18}$ cm^3 L mol^{-1}, R_0 = 0.737 nm, E = 0.281 and R = 0.862 nm for trysin. The distance R < 8 nm between donor and acceptor indicates that the energy transfer from trypsin to buflomedil hydrochloride occurred with high possibility[18]. This obeyed the conditions of Förster energy transfer theory.

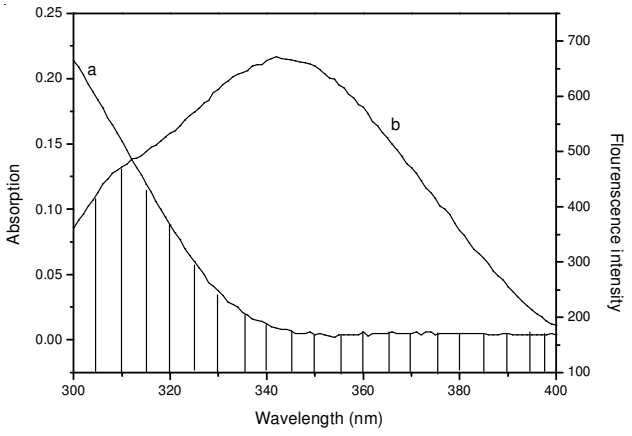

Fig. 6. Spectral overlap between trypsin fluorescence spectrum (b) and buflomedil hydrochloride absorbance spectrum (a) at 300 K. [trypsin] = [buflomedil hydrochloride] =5.6×10^{-5} mol L^{-1}

Conclusion

In this paper, the interaction between buflomedil hydrochloride and trypsin has been investigated. The fluorescence of trysin was quenched by buflomedil hydrochloride mainly through static quenching. The microenvironment around trysin also became less hydrophobic according to synchronous fluorescence. The enthalpy change (ΔH) and entropy change (ΔS) for the reaction were calculated to be 32.171 kJ mol^{-1} and 195.992 J mol^{-1} k^{-1}, the positive ΔH and ΔS values indicated that hydrophobic interactions play a main role in the binding between trypsin and buflomedil hydrochloride. From the Förster's energy transfer theory a binding distance R of 0.862 nm between donor and acceptor was obtained. The results obtained herein will be of biological significance in pharmacology and clinical medicine.

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