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ORIGINAL PAPER

Fluorescence Interaction and Determination of Sulfathiazole with Trypsin

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Abstract The mechanism of interaction of trypsin with the sulfathiazole was studied through using fluorescence quenching and UV-visible absorption spectra at pH 7.4. The Stern-Volmer quenching constants, binding constants, number of binding sites and the corresponding thermodynamic parameters ΔH^o, ΔS^o and ΔG^o were calculated at different temperatures. The effect of common metal ions on the constants was also discussed. The results suggest that sulfathiazole can interact strongly trypsin and that there is the formation of trypsin-sulfathiazole complex and the interaction can be explained on the basis of hydrogen bonds and van der Waals forces. The binding distance (r) between the donor (trypsin) and acceptor (sulfathiazole) was 3.52 nm based on the Förster's non-radiative energy transfer theory. The detection and quantification limits of sulfathiazole were calculated as 2.52 and 8.40 μM in the presence of trypsin, respectively. The relative standard deviation (RSD) was 4.086 % for determinations (n=7) of a sulfathiazole solution with the concentration of 7.54 μM.

Keywords Trypsin · Sulfa drugs · Sulfathiazole · FRET · Thermodynamic parameters

Introduction

Sulfa drugs are known as sulfonamide drugs, are a family of synthetic drugs used pharmacologically as preventive and antimicrobial agents for human and animal therapy such as infectious of digestive and respiratory system, infections of skin and urinary tract [1, 2]. Sulfonamides have para amino benzene sulfanilamide structure and distinct six- or five-member heterocyclic rings (Fig. 1a). They are not readily biodegradable and have various serious side effects that can lead to some diseases in humans [3]. Thus, it is very important that the studies of control of sulfonamides in different biological fluids and interactions of them with biomolecules help to understand of their properties of pharmacodynamics and pharmacokinetics in human and animals [4, 5]. Many fluorescence studies have been reported and proposed for determination of different sulfonamides [6–8]. Sulfathiazole (4-amino-N-2-thiazoly-benzenesulfonamide) structure is shown in Fig. 1b, is a member of the sulfonamides family widely used as a short acting sulfa drug in bacterial infections.

The proteases are any of a group of enzymes that are capable of hydrolyzing proteins into smaller peptide fractions and amino acids. Trypsin is a serine protease and medium-sized globular protein, consisting of 223 amino acid residues and cleaves peptide bonds only at the carboxylic groups of arginine and lysine [9]. It is secreted by pancreas in the small intestine which is converted from the inactive trypsinogen and plays an important role in digestion and other biological process in vertebrates. [10]. The fluorescence spectroscopy is a powerful and simple to study the interaction of small molecule with protein because of its high sensitivity, rapidity and convenience. Trypsin intrinsic fluorescence results from four tryptophan residues (Trp51, Trp141, Trp215 and Trp237). Cysteines are strong quenchers of tryptophan fluorescence, and only Trp51 is relatively far from cysteine groups. Therefore, only Trp51 does significantly contribute to overall fluorescence emission of trypsin [11]. Many studies have been reported on the interactions small molecules with trypsin by fluorescence quenching such as prulifloxacin [12], rutin [13], 2,4-dinitrophenol and 2,4-dichlorphenol [14], merbromin [15], several quantum dots [16–18], sodium benzoate [19], ferulic acid and tetramethylpyrazine [20], caffeine and theophylline [21] etc. Also, several studies indicate that the

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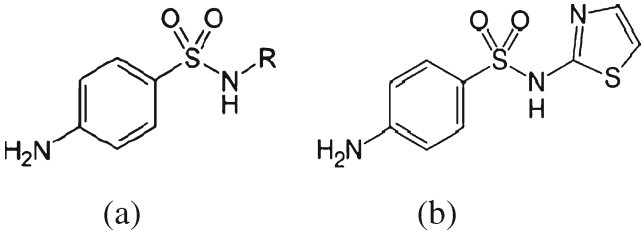

Fig. 1 Molecular structures of (**a**) sulfonamide and (**b**) sulfathiazole

interactions between many molecules and trypsin may perform toxic effects within the organisms [22]. The purpose of this study is to investigate the interaction and determination of sulfathiazole with trypsin by fluorescence and UV-visible absorption spectroscopies. The study is focused on fluorescence quenching of trypsin by sulfathiazole, determination of binding parameters and effect of common metal ions on these parameters, thermodynamic functions, energy transfer and intermolecular distance of trypsin-sulfathiazole. Our results may be a model on the future study of the interaction between sulfa drugs and other proteases.

Experimental

Reagents

Trypsin (EC 3.4.21.4) from bovine pancreas was purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular weight of it is 23.8 kDa. The stock solution of trypsin (0.2 mM) was prepared in pH 7.4 Tris–HCl buffer solution (0.05 M Tris, 0.1 M NaCl). Sulfathiazole was purchased from Sigma. The stock solution of sulfathiazole (5.0 mM) was prepared in DMSO. The solutions of common metal ions (1.0 mM) were prepared from chlorides of Co^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}, Ca^{2+} and Mn^{2+}; except for Pb^{2+} and Cu^{2+} ions, which were prepared from their nitrates. The all aqueous solutions used in the experiments were prepared daily from the stock solutions by appropriate dilution in doubly distilled water and stored in refrigerator at 4°C until used.

Apparatus

A Hitachi F-4500 spectrofluorometer (Tokyo, Japan) was used for all fluorescence measurements with a 150 W xenon lamp source and 1.0 cm quartz cell. The excitation and emission slits with a band pass of 2.5 nm were used for all studies. PMT voltage was kept at 700 V and scan speed was 20 nms^{-1}. The absorption spectra were performed on an UV-1700 PharmaSpec (Shimadzu Co., Kyoto, Japan) UV-visible spectrophotometer equipped with quartz cells. pH measurements were carried out with a NeoMet (pH-220 L) pHmeter.

Procedure

The interaction of sulfathiazole to trypsin was studied by the fluorescence quenching titration method using the intrinsic fluorescence of trypsin at $\lambda_{ex}/\lambda_{em}$=280/335 nm at three temperatures (298, 303 and 310 K). The various concentrations of sulfathiazole solution using microliter pipette were added to 2.5 mL of 2.5 μM trypsin in Tris–HCl at pH 7.4 in fluorescence cell. Then fluorescence intensities and spectra of trypsin in the presence of sulfathiazole were recorded and titration data were analyzed according to Stern-Volmer equation. The tryptophan fluorescence from trypsin was corrected for inner filter effect. The fluorescence intensity was approximately corrected using the following equation [23],

$$F_{cor} = F_{obs} 10^{(Aex+Aem)/2} \quad (1)$$

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

Results and Discussion

Fluorescence Quenching of Trypsin by Sulfathiazole

The effect of sulfathiazole on trypsin fluorescence intensity is shown in Fig. 2. Trypsin has a strong fluorescence emission peaked at 335 nm after being excited with a wavelength of 280 nm. The intrinsic fluorescence of trypsin was quenched, when a fixed concentration of trypsin was titrated with different amounts of sulfathiazole which added the concentration range from 2.54 to 26.3 μM. The fluorescence quenching

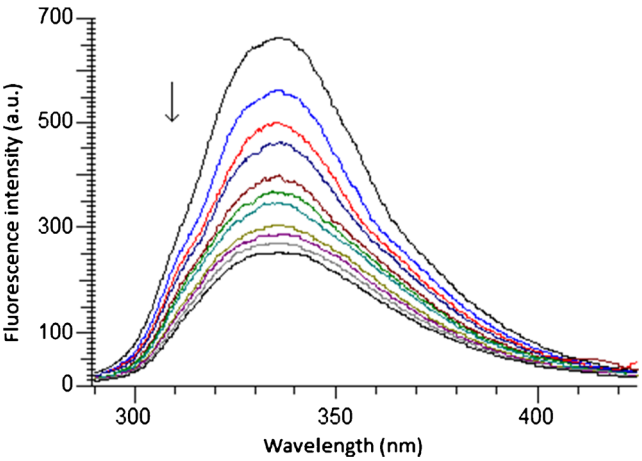

Fig. 2 Fluorescence spectra of 2.5 μM trypsin in the presence of sulfathiazole. The concentration range of sulfathiazole is 0–26.3 μM (from high to low). Conditions: pH 7.4, λ_{ex}=280 nm, 298 K

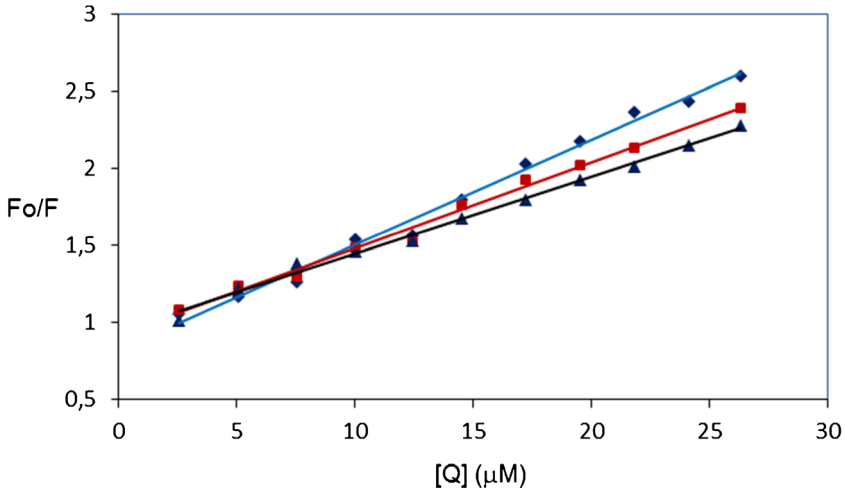

Fig. 3 The Stern-Volmer plots for the interaction of 2.5 μM trypsin with sulfathiazole at 298, 303 and 310 K (from high to low). $\lambda_{ex}/\lambda_{em}$=280/335 nm

mechanisms are usually classified either dynamic or static. Types of mechanism can be distinguished by their differing dependence on temperature and excited-state lifetime. Here, we recorded the fluorescence quenching spectra of trypsin in the presence of different concentration of sulfathiazole at three different temperatures to elucidate the quenching mechanism. Fluorescence quenching is usually described by the Stern-Volmer equation [23],

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

where F_0 and F are the steady-state fluorescence intensities of trypsin in the absence and presence of sulfathiazole, respectively. [Q] is concentration of sulfathiazole as quencher. K_{sv} is the Stern–Volmer quenching constant. k_q is the quenching rate constant of the biomolecule, τ_0 is the average lifetime the biomolecule without quencher. Here, τ_0 of Trp in trypsin was used as 1.9 ns [24]. In Fig. 3, the linear Stern-Volmer plots were obtained from the fluorescence titrations at 298, 303 and 310 K. The values of K_{sv} were found from slopes of these graphs and the values of k_q were calculated from $K_{sv}=k_q\tau_0$. In Table 1, the results showed that K_{sv}, and k_q decreased with increasing temperatures, which indicated that the possible quenching mechanism of fluorescence of trypsin by sulfathiazole was a static quenching type. The maximum scatter collision quenching constant, k_q of various quenchers with the biopolymer is 2×10^{10} M^{-1} s^{-1} [25]. Thus, the rate constants of trypsin quenching procedure initiated by sulfathiazole are greater than k_q of scatter procedure. This confirms that a static quenching mechanism is operative with the formation of trypsin- sulfathiazole complex.

Table 1 Stern-Volmer quenching constants for the trypsin-sulfathiazole system

T (K)	K_{sv} ($Lmol^{-1}$)×10^4	k_q($Lmol^{-1}$ s^{-1})×10^{13}	*R**
298	6.83	3.59	0.9904
303	5.61	2.95	0.9905
310	5.01	2.64	0.9934

*regression coefficients of Stern-Volmer plots

The binding constant K_b and the binding number n of sulfathiazole with trypsin can be determined according to the following equation,

$$\log(F_0{-}F)/F = \log K_b + n\ \log[Q] \quad (3)$$

where K_b and n are the binding constant and the number of binding sites for trypsin- sulfathiazole system, respectively. Thus, the double-log plot of log (F_0–F)/F versus log [*Q*] gives K_b from intercept and n from slope of curve at different temperatures. The values of K_b and n are shown in Table 2. Trp51 residue of trypsin is near or within the binding site for the interaction. The values of K_b decreased with the rising temperature, which indicated that the complex would be partly decomposed with the increasing temperature.

Metal ions can effect the binding properties between a small molecule and protein in plasma. Here, the effect of various common metal ions, such as Co^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}, Ca^{2+}, Mn^{2+}, Pb^{2+} and Cu^{2+} on the quenching and binding constants, number of binding sites of trypsin-sulfathiazole complex was investigated at 298 K. The fluorescence spectra of trypsin-sulfathiazole system were recorded in the presence of these ions in the range of 290–450 nm at excitation 280 nm. The final concentrations of trypsin and metal ions were fixed 2.5 and 10 μM in titrations, respectively. The results were shown in Table 3. The quenching constants were increased in the presence of metal ions (exception Cu^{2+}) which cause the decrease the fluorescence intensity of trypsin-sulfathiazole system. This possibly resulted from the formation of metal ion-trypsin complexes, which also quenched trypsin fluorescence. On the other hand, all binding constants were decreased

Table 2 Binding constants K_b, numbers of binding sites n and thermodynamic parameters for trypsin-sulfathiazole system at different temperatures

T (°C)	K_b (L mol^{-1})	*n*	*R**	ΔH^o (kJ mol^{-1})	ΔG^o (kJ mol^{-1})	ΔS^o (kJ mol^{-1} K^{-1})
298	8.020×10^6	1.453	0.9922	−409.3	−39.46	−1.241
303	5.601×10^5	1.217	0.9915		−33.25	
310	6.504×10^4	1.030	0.9920		−24.56	

*regression coefficients of double logarithm plots

in the presence of metal ions with compare to binding constant without ions. Then, metal ions may combine with carboxylic or amino groups of amino acid residues of trypsin, implying weaker binding between trypsin and sulfathiazole. As can be seen from Table 3, n values in the presence of ions are smaller than n value 1.453 (without ions), sulfathiazole can be removed from binding sites of complex with the competition between ions and sulfathiazole. The decreasing of the binding constant would shorten the storage time and enhance the effectiveness of sulfathiazole in plasma [26].

Ultraviolet–Visible Absorption Studies

UV absorption spectra of trypsin with or without sulfathiazole were recorded and presented in Fig. 4 at 298 K. It could be seen from Fig. 4 with the addition of sulfathiazole, the absorbance intensity increased at 280 nm absorption peak of trypsin. These indicated that absorption spectra of trypsin were changed due to form a ground state complex of trypsin-sulfathiazole. Figure 4

Binding Model and Thermodynamic Parameters

There are four non-covalent binding types between a small molecule and biomolecule which may involve hydrogen bond, van der Waals forces, electrostatic force and hydrophobic interaction force. The signs and magnitude of the thermodynamic parameters (ΔH^o and ΔS^o) may provide for the main forces involved in the binding reaction [27]. Here, the temperature-dependent thermodynamic parameters were studied. The ln K_b values at three temperatures were plotted against the reciprocal of temperature according to the van't Hoff equation,

$$\ln\ K_b = -\Delta H^o/RT + \Delta S^o/R \tag{4}$$

The values of ΔH^o and ΔS^o were obtained from the slope and intercept of the linear plot in Fig. 5, respectively. ΔG^o at different temperatures can be calculated from the following relationship:

$$\Delta G^o = \Delta H^o - T\Delta S^o = -RT \ln K_b \tag{5}$$

K_b is the binding constant; R is the gas constant (R= 8.3145 Jmol^{-1} K^{-1}); ΔH^o, ΔS^o and ΔG^o are the enthalpy, entropy and free energy change, respectively. The values of ΔH^o, ΔS^o and ΔG^o at different temperatures obtained are shown in Table 2. It can be seen that $\Delta G^o < 0$, $\Delta H^o < 0$ and $\Delta S^o < 0$. The negative sign for ΔG^o means that the binding process is spontaneous and the formation of trypsin- sulfathiazole complex is an exothermic process with negative enthalpy change. The binding process is mainly enthalpy driven and entropy is unfavourable for it. The negative ΔH^o and ΔS^o values indicate that hydrogen bonds and van der Waals forces may play major role in the binding between sulfathiazole and trypsin.

Table 3 Effect of common metal ions on quenching and binding constants, n values of trypsin-sulfathiazole system at 298 K

Ion	K_{sv}' (Lmol^{-1})$\times10^4$	K_{SV}'/K_{SV}	*R**	K_b' (Lmol^{-1})	K_b'/K_b ($\times10^{-2}$)	*n*	*R**
without	6.83	–	0.9904	8.02×10^6	—	1.453	0.9922
Cu^{2+}	6.75	0.988	0.9928	1.02×10^4	0.127	0.8275	0.9930
Co^{2+}	7.10	1.04	0.9898	1.07×10^4	0.133	0.8172	0.9940
Mn^{2+}	7.35	1.08	0.9938	5.91×10^5	7.37	1.186	0.9906
Fe^{3+}	7.61	1.11	0.9884	5.13×10^4	0.640	0.9708	0.9927
Ca^{2+}	8.10	1.19	0.9910	5.20×10^4	0.648	0.9701	0.9900
Al^{3+}	8.43	1.23	0.9839	1.25×10^5	1.56	1.035	0.9988
Pb^{2+}	8.85	1.30	0.9861	3.56×10^5	4.44	1.124	0.9884
Zn^{2+}	9.59	1.40	0.9952	1.88×10^4	0.234	0.8479	0.9911

*regression coefficients of plots. K_{sv}' and K_b' are quenching and binding constants in the presence of metal ions, respectively

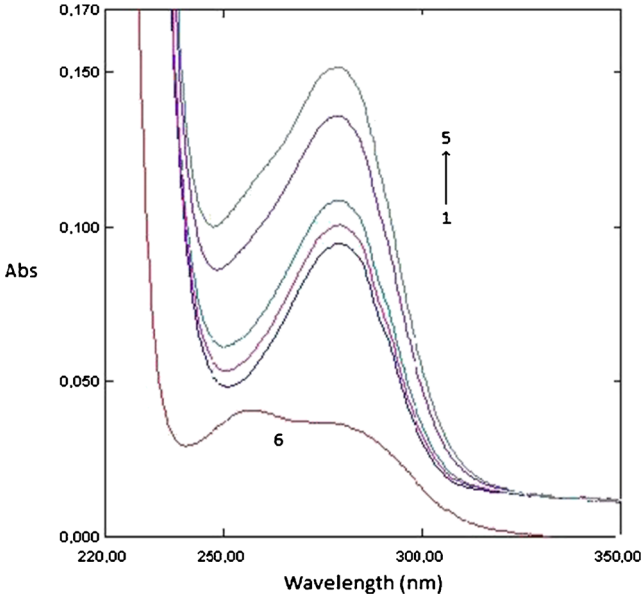

Fig. 4 The absorption spectra of 2.0 μM trypsin in the presence of sulfathiazole. The concentration of sulfathiazole from 1 to 5 is 0, 0.998, 2.00, 3.97, 4.95 μM. (Spectrum 6 is 2.0 μM sulfathiazole)

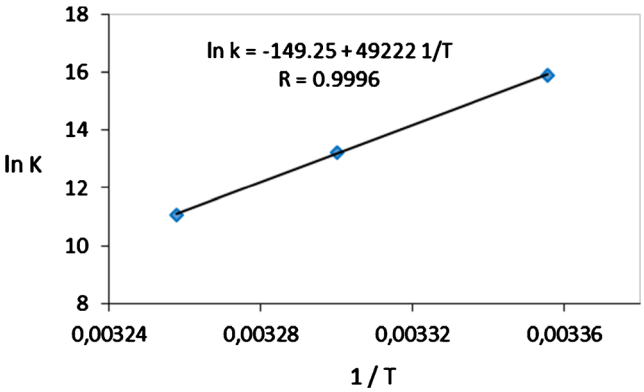

Fig. 5 van't Hoff plot for the interaction of trypsin-sulfathiazole system

Energy Transfer from Trypsin to Sulfathiazole

Fluorescence resonance energy transfer (FRET) is a process in which an excited-stated donor can transfer energy to an acceptor through a long-range non-radiative dipole-dipole coupling [23]. Many reports have been explained the effect of FRET on the determination of the interaction between a small molecule and biomolecule based on the fluorescence quenching [28–30]. According to Förster's theory the energy transfer efficiency E is calculated using the equation,

$$E = 1 - F/F_0 \tag{6}$$

$$E = R_0^6/\left(R_0^6 + r^6\right) \tag{7}$$

where F and F_0 are the fluorescence intensities of trypsin (as donor) in presence and absence of sulfathiazole (as acceptor), r the distance between acceptor and donor and R_0 the critical distance when the transfer efficiency is 50 %. The value of R_0 is calculated using the equation,

$$R_0^6 = 8.79 \times 10^{-25}\left(\kappa^2 n^{-4} Q_D J(\lambda)\right) \tag{8}$$

where κ^2 is the spatial orientation factor of the donor-acceptor dipoles, *n* the refractive index of the medium, Q_D the fluorescence quantum yield of the donor in the absence of acceptor, $J(\lambda)$ the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor and it is given by the equation,

$$J(\lambda) = \sum F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 \Delta\lambda / \sum F_D(\lambda)\Delta\lambda \tag{9}$$

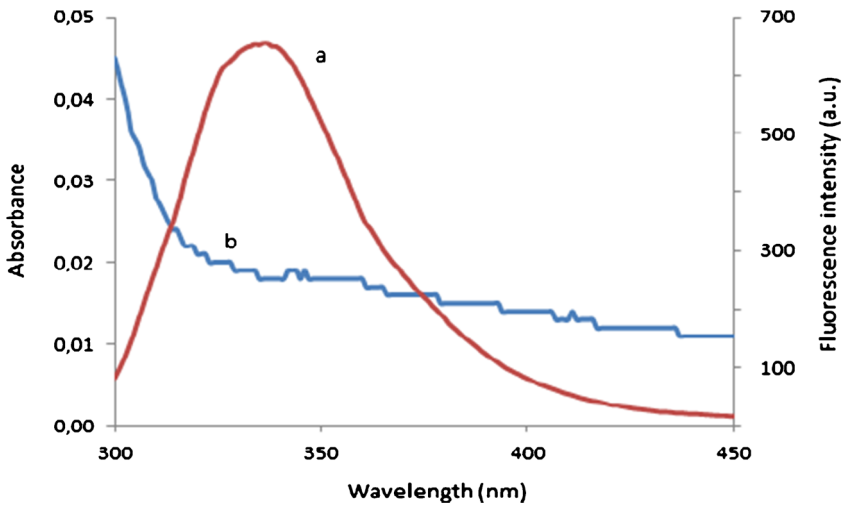

Fig. 6 The overlap of fluorescence spectrum of (**a**) trypsin and the absorption spectrum of (**b**) sulfathiazole. The concentrations of trypsin and sulfathiazole are equal to 2.0 μM

Table 4 The analytical results for determination of sulfathiazole in presence trypsin (n=11)

Dynamic range of sulfathiazole (μM)	2.54 – 26.3
Sb standard deviation of the intercept	0.0574
Limit of detection (LOD) (μM)	2.52
Limit of quantification (LOQ) (μM)	8.40

where $F_D(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range λ to λ + Δλ with the total intensity (area under curve), $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor at λ. In this study, the overlap of the absorption spectrum of sulfathiazole and the fluorescence emission spectrum of trypsin was shown in Fig. 6. So $J(\lambda)$ could be calculated by integrating the spectra in Fig. 6 for λ=300–450 nm and was 1.3453×10^{-14} cm^3Lmol^{-1} according to Eq. 9. The value of E was obtained from Eq. 6 as 0.1506. Using $\kappa^2=2/3$, n=1.36 and Q_D=0.146 for trypsin [24], distance parameters were calculated as R_0=2.64 nm from Eq. 8 and r=3.52 nm from Eq. 7. The donor to acceptor distance, r is less than 8 nm, and $0.5R_0 < r < 1.5\ R_0$, which indicates the non-radiative energy transfer occurred between trypsin and sulfathiazole. Also, $r > R_0$ suggests the presence of a static quenching mechanism [31].

Determination of Sulfathiazole in the Presence of Trypsin

The intrinsic fluorescence of trypsin at fixed concentration was quenched regularly with increasing the concentration of sulfathiazole. The linear Stern-Volmer curve ($F_o/F=0.8187+6.83\times10^4$ [Q] with R=0.9904) was used for the determination of sulfathiazole in the presence of trypsin at 298 K under the experimental conditions described above. The limit of detection (LOD) and limit of quantification (LOQ) of sulfathiazole were calculated as $3Sb/m$ and $10Sb/m$, respectively. *Sb* is the standard deviation of the intercept and m is the slope of the calibration graph [32]. The number of analytical results is n. The analytical results were given in Table 4. To assess the precision and accuracy of the method, the relative standard deviation (RSD) was 4.086 % as obtained from seven replicate determinations of 7.54 μM of sulfathiazole.

Conclusions

In this paper, the interaction of trypsin enzyme with sulfathiazole which is an antimicrobial sulfa drug has been studied by UV–vis absorption and fluorescence quenching. Sulfathiazole has strong ability to quench the trypsin fluorescence with a static mechanism by forming the trypsin-sulfathiazole complex. The quenching and binding constants, number of binding sites n were calculated from the fluorescence data. And the effects of various common metal ions on these constants were studied. The thermodynamic parameters ΔH^o and ΔS^o were calculated to be −409.3 $kJmol^{-1}$ and -1.241 $kJmol^{-1}$ K^{-1}, respectively, suggesting that hydrogen bonds and van der Waals forces may play major role in stabilizing the trypsin-sulfathiazole complex. The binding reaction between trypsin and sulfathiazole was spontaneous and exothermic process. The binding distance r was 3.52 nm based on Förster theory which indicated that there was a non-radiative energy transfer between trypsin and sulfathiazole. The detection limit and quantification limit of sulfathiazole were calculated as 2.52 and 8.40 μM in the presence of trypsin, respectively. In this work, the investigation of the interaction of serine protease trypsin and the sulfonamide sulfathiazole may provide a good model that can be extended to the elucidation of the interaction between sulfa drugs and other proteases.

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