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In vitro Interactions of Secnidazole and Its Iron (II), Copper (II) Complexes with Bovine Serum Albumin by Fluorescence Quenching Method

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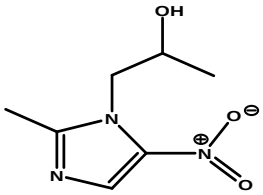
Abstract

The current study was designed to investigate the interactions of an antimicrobial drug secnidazole and its two transition metal complexes with bovine serum albumin (BSA). The interactions of secnidazole and its both transition metal complexes were confirmed by the extingushing of fluorescence intensity of the protein. The fluorescence quenching of BSA by the drug and its both metal complexes showed a static quenching process and the reactions followed exothermic mechanism. The fluorescence spectroscopic method was utilized to evaluate the thermodynamic parameters like change of enthalpy (ΔH), entropy (ΔS) and Gibb's free energy (ΔG) which indicated the bindings of the antimicrobial agent and its both metal chelates were hydrogen bonding and van der Waals interactions. The binding constant and the number of binding sites were also measured by double log plot that indicated the drug or its metal complexes bound with BSA at 1:1 ratio.

Key words: Secnidazole, metal complex, fluorescence quenching, Stern-Volmer constant, Van't Hoff plot

Introduction

Secnidazole (1-(2-hydroxypropyl)-2-methyl-5-nitroimidazole), a 5-nitroimidazole derivative is used as an antimicrobial drug. Secnidazole shows potent antimicrobial activity against susceptible pathogens through the mechanism of diffusion into the bacterial cell. The inactive parent pro-drug compound became the active cytotoxic metabolite which leads to cellular damage, and finally, cell death (Oliveira *et al.*, 2019).



Secnidazole

Serum albumins (SAs) are the most soluble and plentiful protein present in cardiovascular system, accounts for 50% to 65% of the plasma levels of total protein and involve in the binding and transportation of various biomolecules and drugs (Wang *et al.*, 2016; Cheng *et al.*, 2013). As most of the medications reversibly bind to serum albumins, they are commonly transported in the circulatory system as protein complexes. Therefore, drug-protein interaction has a vast biological interest which can play an important role in the study of drug stability and toxicity during chemotherapeutic process (Shahabadi and Hadidi, 2014; Sun *et al.*, 2006). This sort of drug-protein interaction can also affect the drug stability and toxicity amid chemotherapeutic process. Since the existence of interaction between drug particles and proteins can give helpful data

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about the structural characteristics or basic highlights of small molecules and provide new opportunities for the advancement of new drugs, hence studying the interaction of the new drug with protein is important (Reshma *et al.,* 2019). Bovine serum albumin (BSA) is widely used as an *in vitro* model for studying drug-protein interaction, since its composition is 76% similar to that of human serum albumin (Ran *et al.,* 2007; Khan *et al.*, 2016).

The principal goal of this study was to investigate the nature of the bindings of free secnidazole and its iron (II), copper (II) complexes with BSA by fluorescence quenching method at physiological pH 7.4. By calculating the Stern-Volmer constant, the binding constant and the binding points at different temperatures (298 K, 308 K and 318 K) at physiological pH 7.4, the detection and characterization of the bindings of these metal chelates and the pure secnidazole with BSA were investigated. In addition, different thermodynamic factors such as change of enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) were determined to analyze the nature of the binding strengths.

Materials and Methods

Apparatus. In this study a fluorescence spectrophotometer, F-7000 (Hitachi, Japan) equipped with 1.0 cm quartz cell, a thermostatic water bath (Unitronic Orbital, P Spectra, Spain), an analytical balance (AS 220.R2 Shimadzu, Japan), a sonicator (UltrasonsMedi II), pH meter (Orion Star A111) were also used for the analysis.

Drugs and chemicals. Standard secnidazole was gift from ACI Pharmaceuticals Ltd., Dhaka, Bangladesh. Potassium dihydrogen orthophosphate, potassium phosphate dibasic and BSA were of analytical grade and purchased from the local market. The BSA solution was prepared in phosphate buffer (pH 7.4). All metal salts were collected from department of Pharmaceutical Chemistry, university of Dhaka, Bangladesh.

Preparations of buffer solution (Saha *et al.*, 2013)

pH 7.4. About 65 ml of 0.01M KH_2PO_4 was mixed with 235 ml of 0.01M K_2HPO_4 and diluted to 1000 ml with DM water.

Preparations of stock solutions (Saha *et al.*, 2013)

Secnidazole. To prepare 100 ml buffer solution ($1x10^{-2}$ M), 185.18 mg of secnidazole was dissolved in demineralized water & the volume was adjusted up to 100 ml with the same solvent and finally kept in a sonicator for 10 min at room temperature for proper dissolution. The stock solution was diluted to the desired strength by buffer solutions.

Preparation of metal solutions (Refat *et al.*, 2013)

Ferrous sulfate solution. 100 ml stock solution of $1x10^{-2}$ M was prepared by dissolving 278.02 mg of ferrous sulfate heptahydrate, $FeSO_4.7H_2O$, in demineralized water to dissolve it & finally the volume was adjusted up to 100 ml with the same solvent.

Copper sulfate solution. 100 ml stock solution of $1x10^{-2}$ M was prepared by dissolving 249.6 mg of copper sulfate pentahydrate, $CuSO_4.5H_2O$, in demineralized water & finally the volume was adjusted up to 100 ml with the same solvent. Finally both ferrous sulfate solution and copper sulfate solution were kept in a sonicator for 10 min at room temperature for proper dissolution.

Measurement of fluorescence quenching

Fluorescence emissions are extremely susceptible to the local environment, and therefore the fluorophore transfer from high to low polarity environments usually leads spectral shifts (10–20 nm) of the drugs in the excitation and emission spectra (Aleksić and Kapetanović, 2014).

The fluorescence spectra were recorded at 298K, 308K and 318K temperatures and a circulating water bath was used to maintain the temperatures. The concentrations of BSA were stabilized at 10 uM and it was 20 uM (during secnidazole-copper (II)-protein complex) and the concentrations of aqueous drug secnidazole and its metal complexes were varied from 5 to 100 µM. Most fluorescence emission spectra in the range of 200-500 nm were reported at a

wavelength of 280 nm for excitation. The test tubes containing the solution of BSA and drug or its metal chelate were hatched at least 10 min before the measurements were taken (Tanwir *et al.*, 2012).

The protein, mainly originated from tryptophen (Trp), tyrosin (Tyr) and phenylalanine (Phe), is considered to have intrinsic fluorescence. When other molecules like drugs or their metal complexes interact with protein, its intrinsic fluorescence often changes with ligands concentrations (Suryawanshi *et al.*, 2016). Extinguishing of fluorescence strength of a fluorophore due to molecular interactions with other molecules is called fluorescence quenching which can be classified as dynamic or static process (Amin *et al.*, 2016). If the quenching constant is augmented with increasing temperature is known as dynamic quenching whereas in the static quenching it is deducted with the increasing temperature. In energetic extinguishing process, the fluorophore and the quencher come into contact amid the life time excited state, in contrast, inactive extinguishing process alludes to fluorophore quencher complex formation (Joly *et al.*, 2016; Ran *et al.*, 2007). To study the interaction, the fluorescence quenching data can be depicted by the Stern–Volmer (SV) equation (Tang *et al.*, 2015):

$$F_0/F = 1 + K_q \tau_0 [Q] = 1 + K_{SV}[Q]$$

Where F_0 is fluorescence intensity before the quencher is applied, F is fluorescence intensity after the quencher is added. Kq is the bimolecular quenching rate constant with units of L $mol^{-1}s^{-1}$. τ_0 is the average life time ($\tau_0 = 10^{-8}$s) of the serum albumin without any quencher.[Q] is the dissociated quencher concentration with units of $molL^{-1}$. Hence, Stern–Volmer (SV) equation can be utilized to calculate Ksv by linear regression of a plot of F_0/F against [*Q*]. To determine the minimum energy required for the interaction, known as activation energy, the following Arrhenius equation can be utlized (Qiong *et al.*, 2009):

$$\ln Kq = -(E_a/RT) + \ln A$$

Where, Kq is the quenching rate constant at the corresponding temperature, Ea is the activation energy of the extinguishing process, R is the gas constant, T is the absolute temperature, where A is the pre-exponential factor.

Thermodynamic parameters and nature of the binding forces

Thermodynamic process is regarded responsible for the complex formation and temperature dependent thermodynamic parameters are calculated for further investigation of the acting forces between drugs and protein (Abdelhameed, 2015). The binding forces between quencher and proteins may be classified as hydrophobic force, electrostatic interactions, Van der Waals interactions and hydrogen bonds (Tang *et al.*, 2015). The thermodynamic parameters, which values are considered as principle requirements for deciding the nature of interaction forces, can be calculated using the following van't Hoff equation (Sultana *et al.*, 2013):

$$\ln Ka = -(\Delta H/RT) + (\Delta S/R)$$

Where, ΔS = entropy change is, Ka = constant at the corresponding temperature analogous to the Stern-Volmer quenching constants K_{SV}, R = the gas constant. Based on the slope and intercept of the fitted curve of ln K_{SV} against 1/T, both ΔH and ΔS were calculated. It is possible to estimate the change of free energy (ΔG) from the following relationship:

$$\Delta G = \Delta H - T\Delta S$$

The negative sign for ΔG demonstrates that the interaction process is spontaneous. When $\Delta H > 0$, $\Delta S > 0$, hydrophobic interaction occurs, when $\Delta H < 0$, $\Delta S < 0$, van der Waals forces and hydrogen bonds are responsible for the interactions, when $\Delta H < 0$, $\Delta S > 0$, only electrostatic forces are responsible for the quenching process (Ang *et al.*, 2006).

Binding constant and binding points

By the following equation, the binding constant and the number of binding sites can be determined (Cheng *et al.*, 2013):

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

Where, respectively, K_b and n describe the constant of binding to a site and the number of binding per

molecule. It is possible to calculate the values of K_b and n from the intercept and slope values of the log $\{(F_0–F)/F\}$ vs. log [Q] plot.

Data analysis

Microsoft Excel (MS Excel, 2010) was used to analyze the data and all the chemical structures were drawn by using ChemDraw ultra 7.0.

Results and Discussion

BSA fluorescence quenching spectra with different concentrations of secnidazole and its two metal complexes (2:1) were measured under physiological conditions as shown in Figure 1(a-i). It was clearly viewed that the presence of drug individually or its complexes with metal leads to a decrease in fluorescence intensity in the maximum emission wavelength. The Stern-Volmer plots for quenching BSA fluorescence by secnidazole and its 2:1 metal complexes at different temperatures were showed in figure 2 (a-c) and the corresponding Stern-Volmer quenching constants Ksv and quenching rate constants kq were listed in table 1. These results indicated that the pure API or its metal chelates were likely to quench BSA fluorescence as a dynamic quenching mechanism because Ksv values augmented with rising temperature. From the Arrhenius plot (Figure 3) the activation energy of the quenching mechanism was calculated and the found values were stated in table 1.

Analysis of thermodynamic parameters and nature of the binding forces

The thermodynamic parameters were determined from linear van't Hoff Plot (Figure 4, Table 2) based on the binding constants at the three different temperatures, 298K, 308K and 318K. The negative sign for ΔG confirmed that the process of interaction was spontaneous. The negative ΔH and ΔS values suggested that the bindings of secnidazole and its iron (II), copper (II) complexes with BSA were mainly enthalpy-driven, with little contribution from the entropy factor where hydrogen bonding and van der Waals interactions performed vital role in the reactions (Qiong *et al.*, 2009). These bindings were exothermic reactions due to negative change of enthalpy that was associated with the temperature increase in K values (Suryawanshi *et al.*,2016).

Analysis of binding equilibria: Binding constant and binding points

The binding constants (K_b) and the number of binding sites (n) were calculated from the plot of log(Fo-F)/F vs log [Q], Figure 5(a-c) and these values were listed in the table 6.Table 6 demonstrated that

Table 1. Stern-Volmer quenching constants of the secnidazole-BSA, secnidazole-Fe-BSA and secnidazole-Cu-BSA system at different temperatures at physiological pH 7.4.

System	T (K)	1/T (K^{-1})	R^2	Ksv ($L.mole^{-1}$)	lnKsv	K_q($\times 10^{12}$ $L.mole^{-1}.s^{-1}$)	lnKq	Ea (KJ. $mole^{-1}$)
SD - BSA	298	0.0033	0.935	9000	9.1	0.90	27.52	-336.56
	308	0.0032	0.8675	5500	8.61	0.55	27.03	-336.56
	318	0.0031	0.7776	4000	8.29	0.40	26.71	-336.56
SD-Fe-BSA	298	0.0033	0.9471	14600	9.58	1.46	28.00	-444.60
	308	0.0032	0.7740	4100	8.31	0.41	26.73	-444.60
	318	0.0031	0.9896	5000	8.51	0.50	26.93	-444.60
SD-Cu-BSA	298	0.0033	0.9854	34800	10.45	3.48	28.87	-203.60
	308	0.0032	0.9810	29100	10.27	2.91	28.70	-203.60
	318	0.0031	0.9665	21200	9.96	2.12	28.38	-203.60

Here, SD = Secnidazole

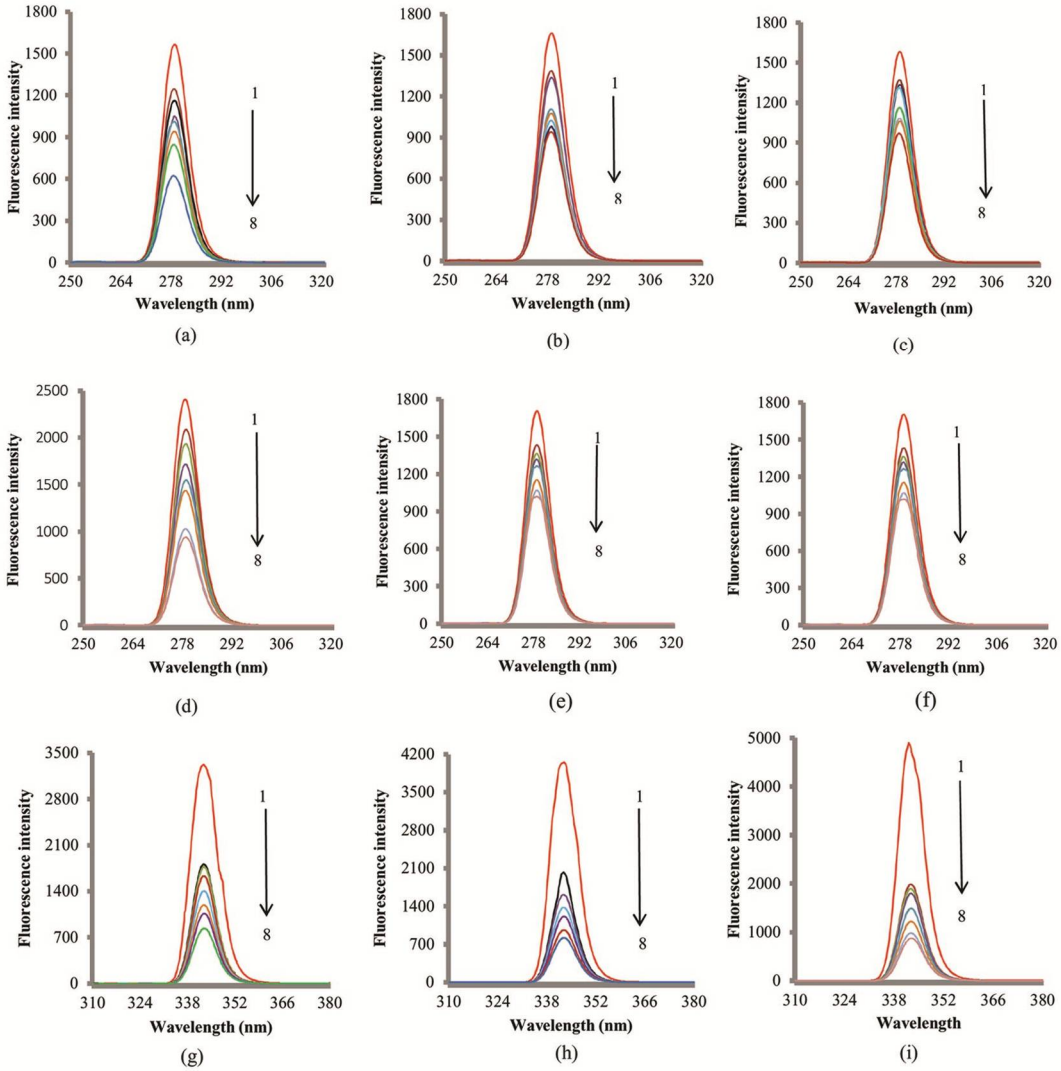

Figure 1. Fluorescence spectra of BSA in the presence of various concentrations of secnidazole at (a) 298K (b) 308K & (c) 318K, its iron (II) complex at (d) 298K, (e) 308K & (f) 318K and its copper (II) complex at (g) 298K, (h) 308K & (i) 318K. (λ_{ex} = 280 nm). *C* (BSA) = 10×10^{-6} mol·L^{-1} and it was 20×10^{-6} mol·L^{-1} during copper (II) complex. Curves 1-8: 0, 5, 10, 20, 40, 60, 80 & 100 uM drugs or its metal complex respectively.

Table 2. Thermodynamic parameters of secnidazole-BSA, secnidazole-Fe-BSA and secnidazole-Cu-BSA system at different temperatures.

System	T (K)	1/T (K^{-1})	ΔH (KJ/mol)	ΔS (J/mol/K)	ΔG (KJ/mol)
SD - BSA	298	0.0033	-336.56	-35.68	-325.92
	308	0.0032	-336.56	-35.68	-325.57
	318	0.0031	-336.56	-35.68	-325.21
SD-Fe-BSA	298	0.0033	-444.59	-69.13	-423.98
	308	0.0032	-444.59	-69.13	-423.29
	318	0.0031	-444.59	-69.13	-422.60
SD-Cu-BSA	298	0.0033	-203.59	19.78	-209.48
	308	0.0032	-203.59	19.78	-209.68
	318	0.0031	-203.59	19.78	-209.78

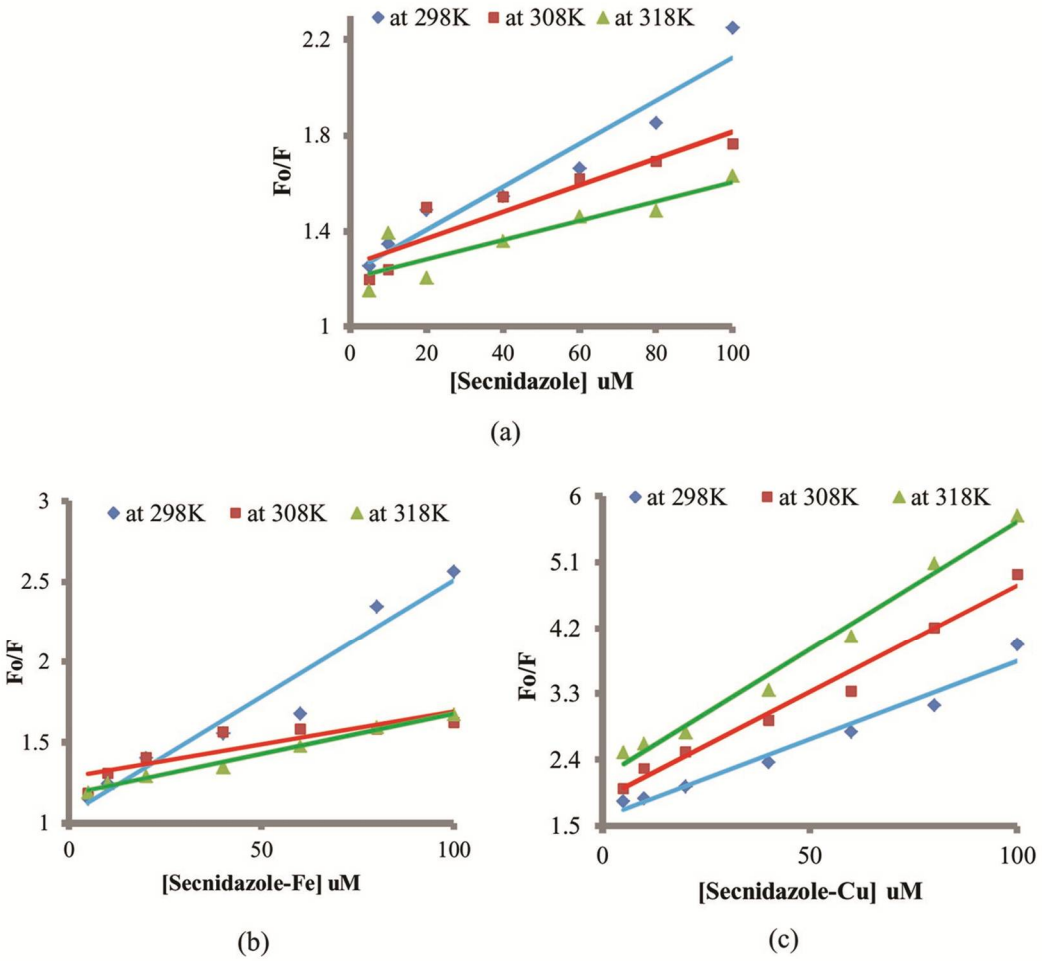

Figure 2. Stern-Volmer quenching plots of BSA with increasing concentrations of (a) secnidazole (b) secnidazole-Fe complex & (c) secnidazole-Cu complex at 298K, 308K and 318K.

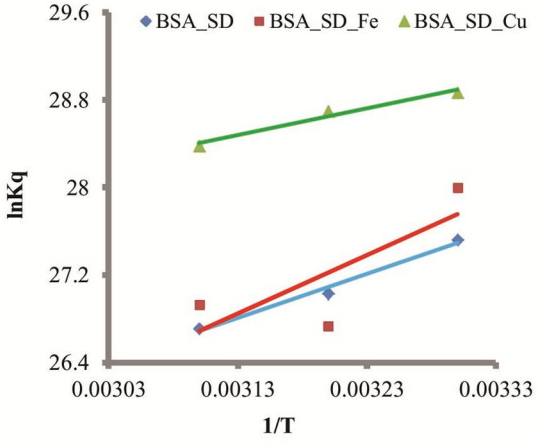

Figure 3. Arrhenius plot for the interaction of BSA with secnidazole, secnidazole-Fe & secnidazole-Cu complex to determine the activation energy of the quenching process at pH 7.4.

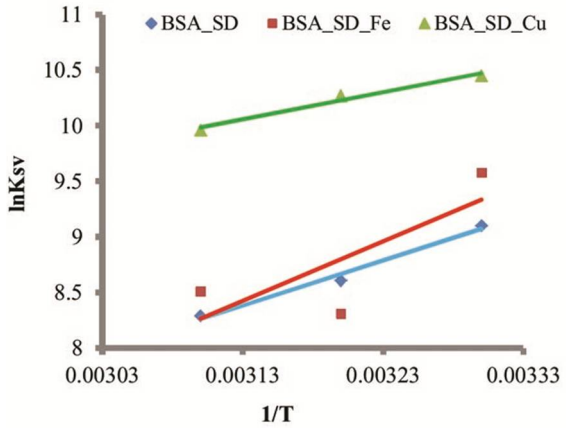

Figure 4. The Van't Hoff Plot for secnidazole, secnidazole-Fe and secnidazole-Cu complex with BSA system at pH 7.4.

Table 3. log(Fo-F)/F values of BSA-secnidazole system at three different temperatures.

[Quencher] [Q] uM	Log[Q]	log(Fo-F)/F values of BSA-SD system		
		at 298K	at 308K	at 318K
5	-5.30	-0.59001	-0.70116	-0.81275
10	-5.00	-0.45845	-0.61623	-0.40334
20	-4.69	-0.30938	-0.30024	-0.68657
40	-4.39	-0.26124	-0.26431	-0.44478
60	-4.22	-0.17759	-0.20700	-0.33526
80	-4.09	-0.06863	-0.15896	-0.31261
100	-4.00	0.18234	-0.11569	-0.19967

Table 4. log(Fo-F)/F values of BSA-secnidazole-Fe complex system at three different temperatures.

[Quencher] [Q] uM	Log[Q]	log(Fo-F)/F values of BSA-SD-Fe complex system		
		at 298K	at 308K	at 318K
5	-5.30	-0.81187	-0.72542	-0.72168
10	-5.00	-0.60885	-0.51084	-0.60206
20	-4.69	-0.39316	-0.38939	-0.53220
40	-4.39	-0.25348	-0.24576	-0.45864
60	-4.22	-0.16860	-0.23312	-0.31989
80	-4.09	0.12925	-0.23312	-0.22661
100	-4.00	0.19464	-0.20360	-0.17185

Table 5. log(Fo-F)/F values of BSA-secnidazole-Cu complex system at three different temperatures.

[Quencher] [Q] uM	Log[Q]	log(Fo-F)/F values of BSA-SD-Cu complex system		
		at 298K	at 308K	at 318K
5	-5.30	-0.07894	0.00364	0.17697
10	-5.00	-0.05736	0.10797	0.20974
20	-4.69	0.01490	0.17805	0.24847
40	-4.39	0.13525	0.28727	0.37063
60	-4.22	0.25189	0.36864	0.49009
80	-4.09	0.33019	0.50670	0.61131
100	-4.00	0.47416	0.59545	0.67536

Table 6. Binding constant (K_b) and number of binding sites (n) of secnidazole-BSA, secnidazole-Fe-BSA and secnidazole-Cu-BSA system at 298K, 308K and 318K.

System	pH	T(K)	K_b (L/mol)	n
SD - BSA	7.4	298	93.95	0.489
		308	53.30	0.456
		318	16.05	0.367
SD-Fe - BSA	7.4	298	1143	0.736
		308	24.10	0.385
		318	25.01	0.404
SD – Cu-BSA	7.4	298	135.98	0.383
		308	161.77	0.423
		318	94.78	0.403

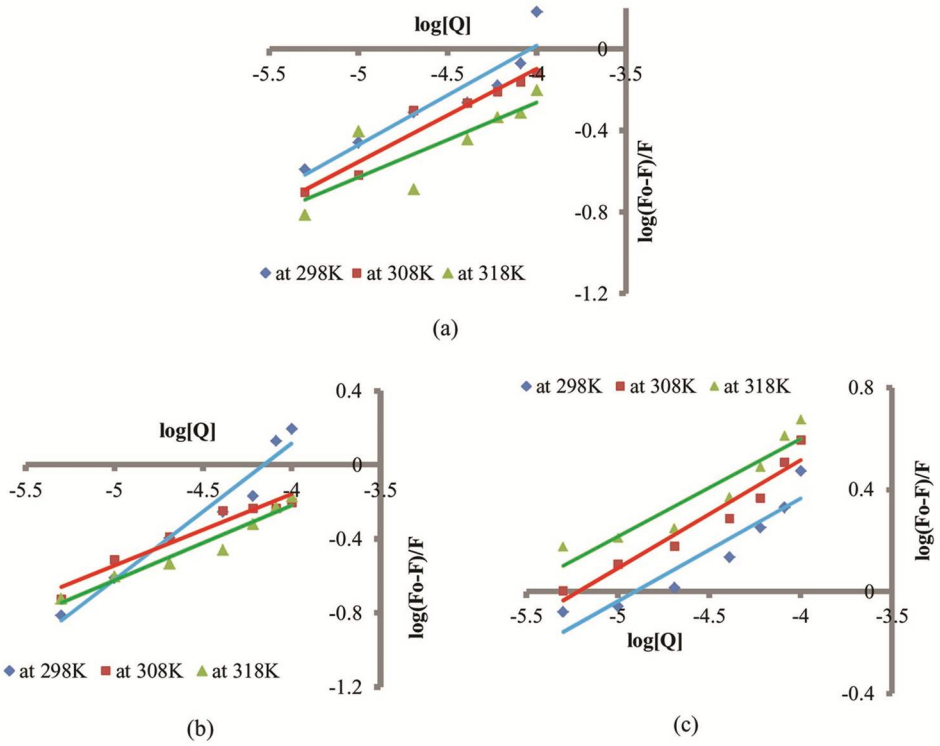

Figure 5. Double-log plot for binding constant and binding points for (a) BSA-secnidazole, (b) BSA-Secnidazole-Fe complex and (c) BSA-secnidazole-Cu complex system at 298K, 308K and 318K temperatures.

binding constants decreased with the increase in temperature (except BSA-SD-Fe complex system), but the n values remained almost constant and were found to be approximately one that indicated mol ratio of these systems was 1:1. That means 1 mol of drug or drug-metal complex bound with one mol of BSA.

Conclusion

The interactions mechanism of secnidazole and its iron (II), copper (II) complexes with BSA were investigated by fluorescence quenching method. The experimental results indicated that quenching of the fluorescence of BSA by the drug and the both complexes were probably a static and the reaction was exothermic. The binding parameters which indicated the interaction was a spontaneous process and was driven by enthalpy, and the van der Waals interactions and hydrogen bonding played vital role in the reactions. The binding constants and the number of binding sites were also computed that indicated the drug or its metal complexes bound with BSA at 1:1 ratio.

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