# Interaction of Nitroglycerin with Bovine Serum Albumin and the Influence of Metal Ions on the Binding

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**Summary:** The effect of $Cu^{2+}$, $Ca^{2+}$, $Mg^{2+}$and $Zn^{2+}$ on the interaction between nitroglycerin and bovine serum albumin was investigated. The bimolecular quenching rate constant, the Stern-Volmer quenching constant, the binding constants and the number of binding sites were calculated in the absence and presence of $Cu^{2+}$, $Ca^{2+}$, $Mg^{2+}$and $Zn^{2+}$. The quenching constants of nitroglycerin to bovine serum albumin were increased in the presence of metal ions. Static quenching mechanism was also confirmed. The binding constants of nitroglycerin to bovine serum albumin were influenced by different metal ions. The enthalpy change, free energy chang, entropy change and the distance between the donor and the acceptor at different temperatures were calculated. The results indicated that energy transfer from bovine serum albumin to nitroglycerin occurs with high probability.

**Keywords:** Nitroglycerin; Bovine serum albumin; Metal ions; Spectroscopy.

## Introduction

An important property of serum albumins is transporting compounds like drugs and pharmaceuticals [1-2].

The transport mechanism of drugs in plasma could provide important information for drug development. Bovine serum albumin (BSA) always as an important protein to study the drugs in human body for its approximately 76% sequence homologous with human serum albumin (HSA) [3-4]. In human organism, some metal ions are important to proteins. For albumin, about 50% of $Ca^{2+}$ ions,10% of $Cu^{2+}$ ions, 10% of $Zn^{2+}$ ions are carried in the serum [5]. The binding sites or conformational changes between drug and serum albumin always influenced by metal ions which can react with serum albumins [6-10]. Some researches have been showed that metal ions affected significantly the drug-serum albumin interactions [11].

Nitroglycerin (NTG) is a medication used to treat of various diseases such as angina and tendinopathies [12-18]. Many methods such as fluorescence spectroscopy and ultraviolet spectroscopy have been widely used to study the binding of drugs with protein [19-24]. Herein, the interaction between nitroglycerin with bovine serum albumin (BSA) was studied using the fluorescence spectroscopy and ultraviolet spectroscopy. Four metal ions, $Cu^{2+}$, $Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$ were also selected to gain insight the influence of metal ions on the interaction.

## Experimental

### *Materials and apparatus*

BSA and diluted nitroglycerin were purchased from Sigma-Aldrich. $Zn^{2+}$, $Cu^{2+}$, $Ca^{2+}$, $Mg^{2+}$ are come from theirs chloride. The purity of the chemicalsis (EDTA and Tris–HCl) were 99%. The effect of $Cl^-$ can be ignored with Tris–HCl [25].

RF-5301PC (SHIMADZU) spectrofluorometer which equipped with 1.0 cm quartz cells was used to recorded the fluorescence spectra. UV-vis spectrophotometer (UV-1800, SHIMADZU) equipped with 1.0 cm quartz cells was used to record the UV spectra.

### *Spectra measurement of BSA and NTG in the absence and presence of metal ions*

The excitation wavelength in our experiments was 285 nm and the ranges of spectra are from 300 nm to 450 nm. The excitation and emission slit widths were 3 nm. Stock solution of BSA ($1\times10^{-4}$ M) was dissolved in 0.05 M Tris-HCl buffer (pH 7.4, containing 0.1 M NaCl). Nitroglycerin were further diluted in Tris–HCl buffer (pH 7.4) at 25 ℃. Samples containing 2 ml of the BSA solution and NTG solution were mixed. The final concentrations of NTG were from 50 μM to 200 μM. The ranges of absorption spectra of protein were of 200nm to 450 nm.

The stock solution of different metal ions

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($1.0\times10^{-2}$ M) was prepared by dissolving in Tris-HCl buffer. The concentrations of BSA which we used in our experiments were $1\times10^{-6}$ M.

## Results and Discussion

*Quenching mechanism of BSA fluorescence by NTG*

The effect of NTG on the BSA fluorescence spectra is shown in Fig 1. The fluorescence intensity of BSA was gradually decreased upon increasing the concentration of NTG. The decrease in fluorescence intensity indicates that there are interactions between NTG and BSA.

A decrease of the fluorescence intensity of a compound can be due to molecular rearrangements and collisional quenching [23]. The Stern-Volmer equation is used to describe fluorescence quenching [24]:

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

$F_0$, the relative fluorescence intensity of protein. $F$, the relative fluorescence intensity of donor when quencher is exist. $Kq$, the bimolecular quenching rate constant. $Ksv$, the Stern-Volmer quenching constant. $\tau_0$, the average life time of the biomolecule without quencher ($\tau_0=10^{-8}$ s) [25], [$Q$], the concentration of the quencher. The curves of $F_0/F$ versus [$Q$] is shown in Fig 2.

Fig 2 showed a good linear relationship during the addition of NTG 100-300 μM. The dynamic Ster-Volmer quenching constants (*Ksv*) and quenching rate constants *Kq* were calculated based on the fluorescence life time of biopolymers (Table-1).

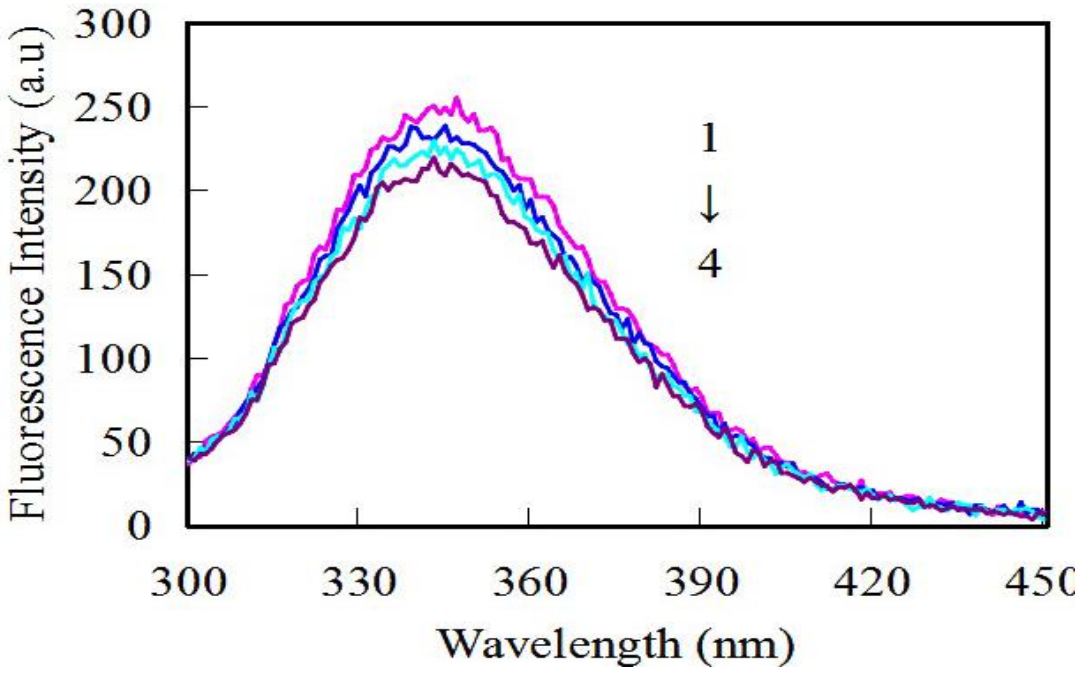


Fig 1: Fluorescence spectra of BSA. (1→4) pure BSA, with NTG at 100 μM, with NTG at 200 μM, and with NTG at 300 μM.

Table-1. The values of Stern-Volmer constants and binding parameters at different temperatures.

| *T* (K) | *Kq* (×10$^{12}$ L/mol·S) | *Ksv* (×10$^{4}$ L/mol) | *Ka* (×10$^{3}$ L/mol) | *n* |
|---|---|---|---|---|
| 288 | 0.138 | 0.138 | 3.8 | 0.8822 |
| 298 | 0.057 | 0.057 | 0.88 | 0.8229 |
| 308 | 0.058 | 0.058 | 69.31 | 1.2584 |

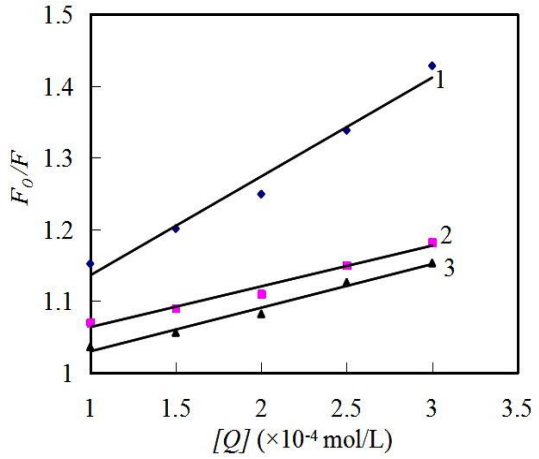


Fig 2: Stern-Volmer curves of NTG quenching BSA fluorescence at (1) 288 K, (2) 298 K, and (3) 308 K.

The maximum collisional quenching constant of different quenchers with biomolecule is $2.0\times10^{10}$ $M^{-1}.s^{-1}$ [26]. The bimolecular quenching rate constants (*Kq*) are larger than the maximum collisional quenching constant. The main mechanism of the quenching could be the static quenching. Some researches have been proved that other proteins have more distinct photoluminescence exhibiting groups [27]. Fluorescence quenching polymer (Ppy) and the flavin adenine dinucleotide (FAD) composite with Ppy effectively quenched the FAD fluorescence [28]. A fluorescencequenching polymer (Ppy) was used to increase the selectivity and sensitivity of an immunoanalytical system as fluorescence quencher [29]. These results can provide useful information for the application of fluorescence studies.

Small molecules are assumed to bind independently to a set of equivalent sites on a macromolecule [30]. The number of binding sites and the binding constant of the interaction between NTG and BSA were described with the following equation [31].

$$\log[(F_0 - F)/F] = \log Ka + n\log[Q] \qquad (2)$$

*n*, is the number of binding sites, *Ka*, the binding constant. The log [($F_0$-*F*)/*F*] versus log [*Q*] were showed *in* Fig 3.

The values of binding parameters are given in Table-1. There is one binding site in NTG to BSA for the values of n were approximately equal to 1. Large values of *Ka* show strong binding between NTG and BSA [32]. The results showed that sequence of the binding strength was 308 K >288 K > 298 K.

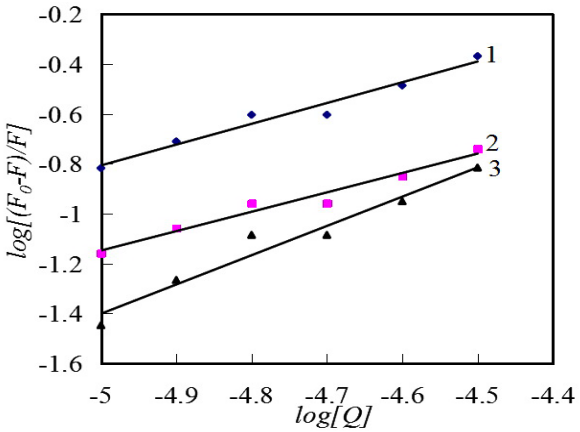


Fig 3: Plot of log($F_0$/ *F*)/*F* versus log[*Q*] for quenching of BSA by NTG at (1) 288 K, (2) 298 K, and (3) 308 K

*Thermodynamic parameters*

Electrostatic interactions, hydrogen bonds, hydrophobic forces, and Van der Waals always to explain the mechanisms of the combine of small molecules to macromolecules [9,33]. The thermodynamic parameters, Gibbs free energy changes(*ΔG*), enthalpy changes(*ΔH*), entropy change (*ΔS*) were calculated to clarify the interaction of NTG with BSA by the following equations.

$$\ln Ka = -\Delta H / RT + \Delta S / R \qquad (3)$$

$$\Delta G = -RT \ln Ka \qquad (4)$$

$$\Delta S = (\Delta H - \Delta G)/T \qquad (5)$$

*R*, the gas constant. *T*, temperature in Kelvin scale. *Ka*, the equilibrium constant at temperature *T*. *ΔH* and Δ*S* can be obtained from the slope and intercept of the plot of ln *Ka* versus 1/*T*. The value of *ΔG* can calculated using Eq.(4). The results are shown in Table-2. For *ΔG* < 0 means that the interaction process of NTG is spontaneous. Researches have been proved that the thermodynamic parameters associated with various interaction that may take place in

ligand-protein binding process: $\Delta S > 0$ and $\Delta H > 0$ means the main binding force was hydrophobic force, $\Delta H < 0$ and $\Delta S < 0$ means the main binding force were vander Waals' force and hydrogen bonding, $\Delta H < 0$ and $\Delta S > 0$ , means the main binding force was electrostatic interactions [31]. Therefore, the positive $\Delta S$ and $\Delta H$ means the main binding force between NTG and BSA was hydrophobic force.

Table-2: Thermodynamic parameters for BSA-NTG system.

| $T$ (K) | $\Delta H$ (KJ/mol) | $\Delta S$ ( J/mol) | $\Delta G$ ( KJ/mol) |
|---|---|---|---|
| 288 | | | -19.733 |
| 298 | 104.698 | 424.103 | -16.789 |
| 308 | | | -28.543 |

*Energy transfer between NTG and BSA*

According to the Förster theory, the energy transfer efficiency E is defined as the following Eq.(6).

$$E = 1\text{-}F/F_0 = R_0^6/(R_0^6 + r^6) \quad (6)$$

*r,* the distance from the donor to the acceptor, $R_0$, the Förster critical distance (whhen the efficiency of energy transfer is 50%). $R_0$ can be calculated using following equations.

$$R_0^6 = 8.8\times10^{-25} K^2 \Phi N^{-4} J \quad (7)$$

$$J = [\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda]/[\sum F(\lambda)\Delta\lambda] \quad (8)$$

$K^2$, the space factor orientation. $N$, the average refractive index of medium respectively ($K^2$=2/3, $N$=1.336 for BSA [32].). In Eq.(8), $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range $\lambda$, $\varepsilon(\lambda)$ is the molar absorptivity of the acceptor at wavelength $\lambda$. $J$ is the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor.

Fig 4 shows the spectral overlap between absorption spectrum of NTG with fluorescence spectrum of BSA in the wavelength range of 300nm to 450 nm. Table-3 shows BSA to NTG distance in the complex is less than 7 nm, indicating that energy transfer from BSA to NTG occurs with high probability.

Table-3. Parameters of $E$, $J$, $R_0$ and $r$ of NTG with BSA under different temperatures.

| $T$ (K) | $E$ | $J$ ($\times10^{-14}$ cm$^3$·L/mol) | $R_0$ (nm) | $r$ (nm) |
|---|---|---|---|---|
| 288 | 0.132 | 6.657 | 3.435 | 4.7 |
| 298 | 0.064 | 6.88 | 3.454 | 5.387 |
| 308 | 0.034 | 6.865 | 3.453 | 6.016 |

*The interactions between NTG and BSA in the presence of different metal ions.*

Metal ions play significant role in human organism. Many researches have been studied the interactions between serum albumin and some drugs in the presence of metal ions [34-36]. The results showed that the binding constants of drugs and BSA was changed by metal ions [37].

As shown in Fig 5, when different metal ions were titrated into NTG-BSA solution, the maximum of $\lambda_{em}$ and shape of the fluorescence spectra of BSA-NTG were no obvious change to those in the absence of metal ions. But the fluorescence quenching extent was larger than those without metal ions. The quenching extent of different metal ions was $Cu^{2+}$ >$Ca^{2+}$ >$Mg^{2+}$ >$Zn^{2+}$. The results suggested that the binding of NTG with BSA was influenced by metal ions.

The curves of $F_0/F$ versus [$Q$] were shown in Fig 6. Fig 6 showed a good linear relationship for NTG with BSA in the presence of metal ions. The values of $Kq$ for the NTG in the presence of different metal ions were calculated and were higher than the value of the maximum scatter collision quenching constant (Table-4). The results suggested that the formation of a complex between NTG and BSA, corresponding to a static quenching mechanism no matter metal ions was absent or present in the system. The quenching constants ($Ksv$) of NTG to BSA were increased in the presence of metal ions and the extent was $Ca^{2+}$ >$Cu^{2+}$ >$Zn^{2+}$ >$Mg^{2+}$.

Table-4: The Stern-Volmer quenching constant, quenching rate constant, binding constants and binding sites of NTG-BSA in the presence of different metal ions at 298 K

| BSA-NTG-$M^{2+}$ | $Ksv$ ($\times10^4$ L/mol) | $Kq$ ( $\times10^{12}$ L/mol·S) | $Ka$ ($\times10^3$ L/mol) | $n$ |
|---|---|---|---|---|
| BSA-NTG-$Mg^{2+}$ | 0.115 | 0.115 | 5.05 | 0.9276 |
| BSA-NTG-$Cu^{2+}$ | 0.1856 | 0.1856 | 25.12 | 1.0293 |
| BSA-NTG-$Ca^{2+}$ | 0.1915 | 0.1915 | 0.83 | 0.7264 |
| BSA-NTG-$Zn^{2+}$ | 0.1397 | 0.1397 | 0.62 | 0.7253 |

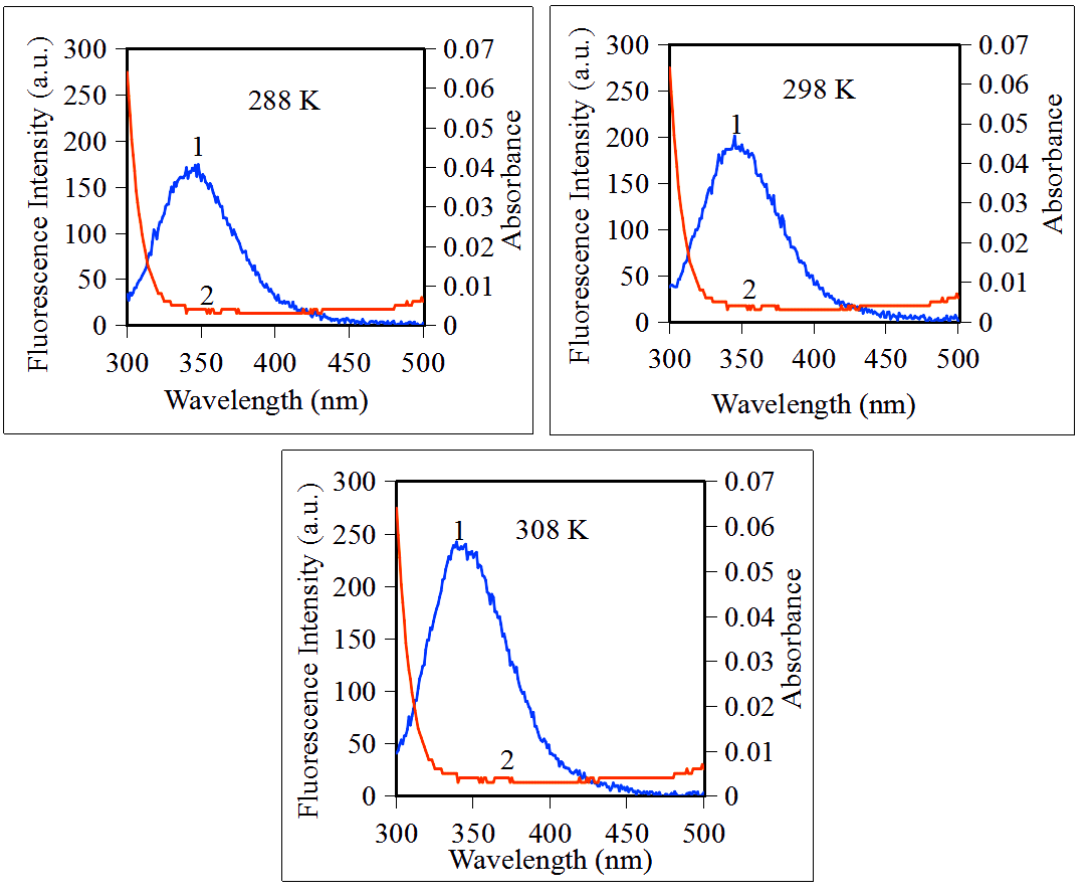


Fig 4: The spectral overlap between absorption spectrum of NTG (1) with fluorescence spectrum of BSA (2) under different temperatures.

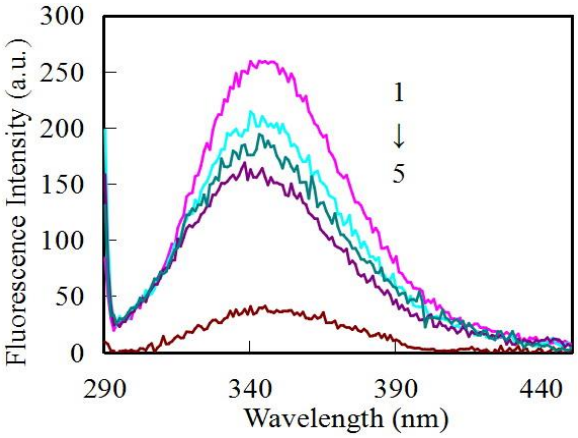


Fig 5: The fluorescence spectra of BSA-NTG in the absence and presence of metal ions.1→5： NTG-BSA, NTG-BSA-$Zn^{2+}$, NTG-BSA-$Mg^{2+}$, NTG-BSA-$Ca^{2+}$, NTG-BSA-$Cu^{2+}$.

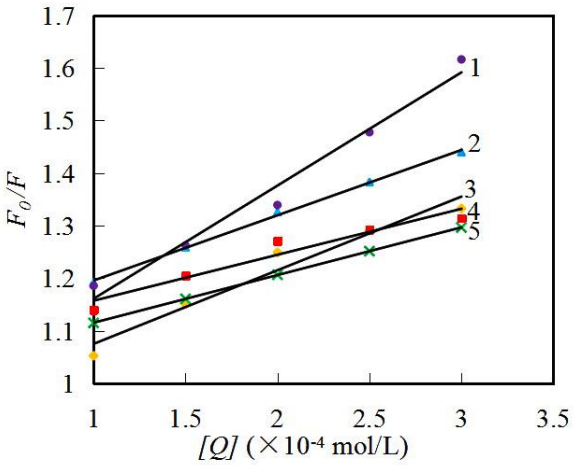


Fig 6: The Stern-Volmer plots of NTG quenching BSA fluorescence in the presence of different metal ions, (1) $Cu^{2+}$, (2) $Ca^{2+}$, (3) pure BSA, (4) $Zn^{2+}$, (5) $Mg^{2+}$.

Plots of log[($F_0$-$F$)/$F$] versus log[$Q$] for NTG-BSA with different metal ions were showed in Fig 7. The value of *Ka* and binding sites (n) can be calculated by Eq. (1). The values of *Ka* for NTG associating with BSA in the presence of metal ions were presented in Table-4. The results showed that the values of binding constants of NTG to BSA were increased when $Cu^{2+}$ and $Mg^{2+}$ were titrated, but $Ca^{2+}$ and $Zn^{2+}$ decreased the values of binding constants. The number of binding site was about 1.0, which indicated that one binding site formed between NTG and BSA. The presence of $Ca^{2+}$ ,$Zn^{2+}$and $Mg^{2+}$, decreased the affinities of NTG to BSA and $Cu^{2+}$ increased the affinities of NTG to BSA.

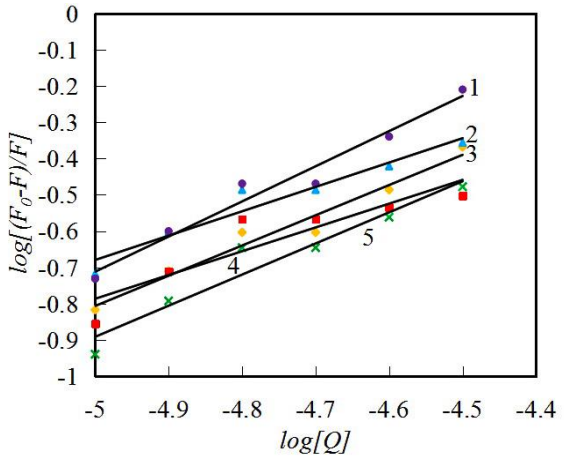


Fig 7: Double-logarithm curves of NTG quenching BSA fluorescence in the presence of metal ions, (1) $Cu^{2+}$, (2) $Ca^{2+}$, (3) pure BSA, (4) $Zn^{2+}$, (5) $Mg^{2}$

## Conclusions

Fluorescence and Ultraviolet-Visible methods for the determination of the interaction between NTG and BSA in the absence and presence of $Cu^{2+}$, $Ca^{2+}$, $Mg^{2+}$and $Zn^{2+}$ was provided. NTG could quenching the fluorescence of BSA, the values of quenching rate constants suggested that the fluorescence quenching was a static process. The binding constant, the number of binding site, the thermodynamic parameters was significant to understand the mechanism of the drug with HSA in human. For many metal ions exist in organism, the study of drug-protein interaction in the presence of metal ions could provide many important information about the transport, absorption and distribution of drugs in plasma.

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