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Binding interactions of niclosamide with serum proteins

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ABSTRACT

A study of the binding of niclosamide (NC) to serum proteins such as human serum albumin, hemoglobin, and globulin was carried out using fluorescence and UV-visible spectroscopy. Interactions between NC and these proteins were estimated by Stern–Volmer and van't Hoff equations. The binding constants and the thermodynamic parameters, ΔH, ΔS, and ΔG at different temperatures were also determined by using these equations. Data showed that NC may exhibit a static quenching mechanism with all proteins. The thermodynamic parameters were calculated. Data showed that van der Waals interactions and hydrogen bonds are the main forces for human serum albumin and hemoglobin. Globulin, however, bound to NC via hydrophobic interaction. The spectral changes of synchronous fluorescence suggested that both the microenvironment of NC and the conformation of the proteins changed in relation to their concentrations during NC's binding.

1. Introduction

Niclosamide (5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide; Fig. 1.) belongs to the family of medicines called anthelmintic drugs, which are effective against most tapeworms [1,2]. Its action against tapeworm species is to uncouple oxidative phosphorylation, block glucose uptake, and inhibit respiration in anaerobic ATP production [3,4]. Niclosamide is a US Food and Drug Administration approved compound that has been used in humans for 40 years [5]. It is commercially known as Bayer 73 in Germany or bayluscide or niclocide in Canada and the United States [6,7]. It is well tolerated by rats with an acute oral toxicity. A significant decrease in hemoglobin concentration occurs when niclosamide is given to male and female rats at a concentration of 5 g/kg/day for four weeks [6–8]. Niclosamide is effective against severe acute respiratory syndrome virus [9]. It also has antineoplastic activity and anti-anthrax toxin properties [10,11]. Niclosamide induces LC3-positive autophagosomes and inhibits the Wnt/Frizzled pathway and mTOR signaling [12–14].

Niclosamide's interaction with proteins and enzymes explains its mechanism of action in the human body. Thermodynamic parameters are especially helpful in identifying the binding mechanism of a drug to any protein. Serum proteins are the major transport protein in human body. Albumin is a serum protein widely used to explain the binding mechanism of a small molecule to a protein. Serum proteins are capable of

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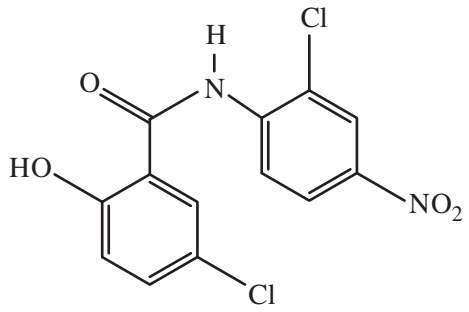

Fig. 1 – Structure of niclosamide.

reversibly binding many endogenous and exogenous drugs [15,16]. They may aid in selective delivery of the drugs to a target tissue, organ, or tumor region, thus delivering drugs into the cell. Most recently, a large number of studies on the drug–protein interaction have been reported using techniques including fluorescence, Fourier transform infrared spectroscopy, circular dichoism spectroscopy, and nuclear magnetic resonance [17–21]. It is necessary to determine binding parameters during destabilization by these methods for drug–protein systems.

Fluorescence serves as a sensitive method indicating alterations of the fluorophore environment, thus resulting in plenty of useful information [21]. Many drugs are not fluorescent but proteins have a fluorescent character that results from the protein's tyrptophan and tyrosine residues [22–24]. This property allows the monitoring of alterations of the protein during binding. Due to fluorescence quenching, binding can be analyzed through reduction of fluorescence intensity or the shifting of the emission wavelength of the protein upon the addition of the drug [16].

In this study, the binding mechanism of niclosamide to several proteins including human serum albumin, globulin, and hemoglobin is investigated using fluorescence and UV-visible spectroscopy. The nature of the drug's binding mechanism to all proteins was described by using Stern–Volmer and van't Hoff equations. Synchronous fluorescence was also used for all proteins to determine environmental changes corresponding to the interactions. The aim of this work was to estimate interactions of niclosamide with serum proteins.

2. Materials and methods

2.1. Materials

Niclosamide, HCl, NaOH, hemoglobin, globulin, and albumin from human serum (97–99%), and Tris (hydroxymethyl) aminomethane were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents and chemicals were of molecular and analytical grade. The ultra pure water was purified using the Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Apparatus

Absorption spectra were recorded on a Shimadzu-1700 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) using a 1 cm quartz cell. Fluorescence spectra were scanned by a Perkin Elmer LS-55 spectrofluorometer (Perkin Elmer, Waltham, MA, USA) equipped with a xenon lamp source and a 1 cm quartz cell.

2.3. Fluorescence spectroscopy

Niclosamide was prepared as 1mM of stock solution in a water-dimethylformamide solvent system (V:V, 90:10%) for all experiments. Then, 1 mg mL^{-1} of protein solution was prepared by directly dissolving the protein in a Tris buffer including 20mM of Tris at pH, 7.4. All aqueous solutions used in testing contained 10% (v/v) dimethylformamide for all measurements. An appropriate volume of niclosamide solution was added to the proteins ranging from 1 μg to 20 μg and mixed for 10 minutes at room temperature. The fluorescence spectra from 200 nm to 500 nm were scanned by a spectrofluorometer using a 500 nm scan speed and 10 nm slit in a quartz cell.

An appropriate volume of protein (10 μg) was mixed with different amounts of niclosamide between 1 μM mL^{-1} and 80 μM mL^{-1} and then, diluted to 2.0 mL with Tris buffer. The fluorescence spectra of the solutions at different temperatures were collected at the excitation wavelength of 280 nm.

2.4. Synchronous fluorescence spectroscopy

Appropriate concentrations of proteins in Tris buffer were mixed with different concentrations of niclosamide. The emission spectrum was recorded with $\Delta\lambda = 15$ nm or $\Delta\lambda = 60$ nm for all niclosamide–protein complexes.

2.5. Absorption of niclosamide with protein

Appropriate concentrations of all proteins in 20mM of Tris buffer (pH:7.4) were added to different amounts of niclosamide between 1 μM and 80 μM. The absorption spectra of the niclosamide–protein complexes were collected by using the Shimadzu 1700 UV-visible spectrophotometer.

3. Results and discussion

3.1. Fluorescence quenching of proteins

Protein emits strong fluorescence peaks at 280 nm and 342 nm at excitation and emission wavelengths, respectively. The quenching effect of niclosamide on proteins' intrinsic fluorescence was studied for three proteins. The presence of niclosamide led to a decrease in the fluorescence intensity of human serum albumin (HSA) with a slight blue shift as increasing amounts of the drug were added (Fig. 2A). The intensity of fluorescence of both hemoglobin and globulin decreased with a slight red shift, suggesting that niclosamide interacts with these proteins (Fig. 2B and C).

3.2. Synchronous fluorescence of proteins

Proteins are composed of 20 amino acids in different sequences. The fluorescence of proteins results mainly from two

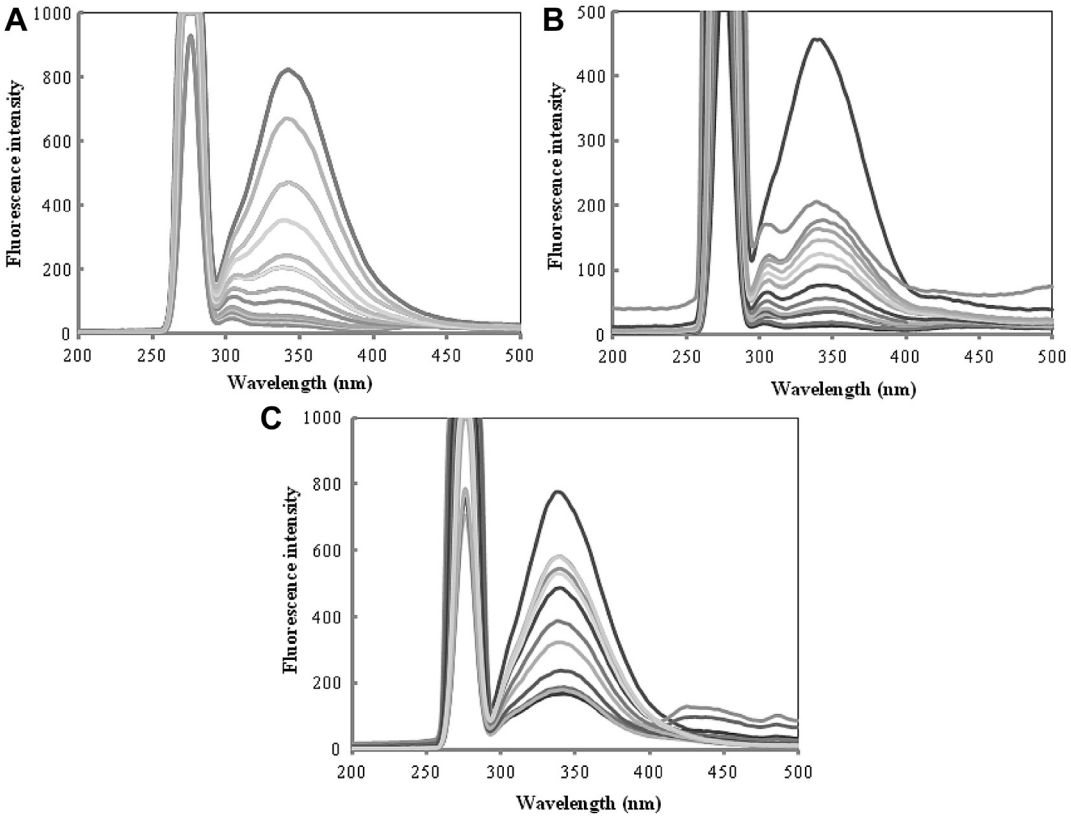

Fig. 2 – Fluorescence spectra of the proteins (10 μg/mL) (A) human serum albumin, (B) hemoglobin, and (C) globulin at different concentrations of niclosamide (T = 296 K, λex = 280 nm, λem = 290-430 nm). [Niclosamide] = 0μM, 5μM, 10μM, 20μM, 30μM, 40μM, 60μM, 70μM, and 80μM, pH: 7.4. (Fluorescence intensity increases with the increase in concentration of niclosamide for each graph.)

amino acid residues: tryptophan and tyrosine. When Δλ is set at 60 nm, the synchronous fluorescence spectrum has the characteristic peak of the tryptophan residues [22]. When set at 15 nm, the characteristic peak of tyrosine residues in proteins appears.

The polarity changes of the surrounding environment depend on the shift of the maximum emission. Blue shift of the fluorescence spectrum indicates that the polarity decreases and the hydrophobicity increases for the protein molecule [23]. The spectral changes of all proteins on synchronous fluorescence upon the addition of niclosamide are shown in Fig. 3. The maximum emission peaks of tyrosine residues (Δλ = 15 nm) and tryptophan residues (Δλ = 60 nm) exhibited a slight shift for all proteins. As Fig. 4A and C shows, however, it is obvious that the pitch of quenching at Δλ 60 nm is higher than that at Δλ 15 nm for HSA and globulin, indicating that niclosamide is closer to tryptophan residues than tyrosine residues. This means that the binding sites of the drug are more prevalent on the tryptophan moiety. For hemoglobin, at a low concentration of the drug the pitch of quenching at Δλ 15 nm is higher than that at Δλ 60 nm, whereas it is lower than that of Δλ 60 nm at a high concentration of niclosamide (Fig. 4B). These results suggest that the polarity of the surrounding environment depends on the concentration of the drug. Both the tyrosine and tryptophan moieties exert an effect on the binding mechanism of hemoglobin.

3.3. *Emission spectra of proteins*

Binding of proteins with niclosamide was carried out in Tris-HCl aqueous solution (pH 7.4). The fluorescence intensity of HSA was enhanced and the emission band slightly blue shifted as a result of increasing protein concentration at a certain amount of niclosamide. This suggests that the polarity of the microenvironment of niclosamide decreased, which means that niclosamide entered the hydrophobic microenvironment of HSA. A good linear response of fluorescence intensity was also obtained as a function of HSA concentration. The linear equation was y = 707.06x + 18.233 (R = 0.992) for HSA whereas the concentration of HSA ranged from 17.5 μg to 2.5 μg. Niclosamide also enhanced the emission band with a slight blue shift when the amounts of hemoglobin and globulin were increased. This means that the polarity of the microenvironment of niclosamide also increased for both proteins. The results suggest that niclosamide interacted with the hydrophobic sides of the proteins. The interaction of niclosamide with both hemoglobin and globulin thus occurred. The linear equation was y = 96.649x + 47.381 (R = 0.995) for hemoglobin

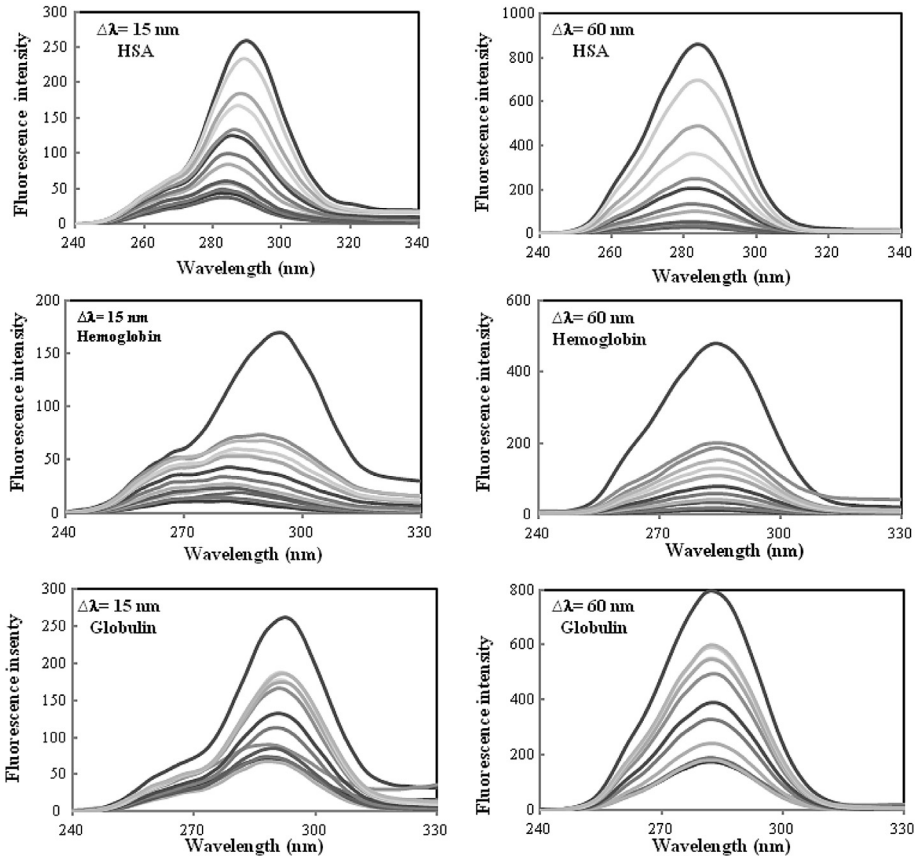

Fig. 3 – Synchronous fluorescence spectra of human serum albumin, hemoglobin, and globulin (10 μg/mL) in the presence of different amounts of niclosamide with Δλ= 15 nm and Δλ= 60 nm. [Niclosamide] =0μM, 1μM, 2.5μM, 5μM, 10μM, 15μM, 20μM, 30μM, 40μM, 50μM, 60μM, and 80μM, pH: 7.4. (Fluorescence intensity increases with increase in concentration of niclosamide for each graph.)

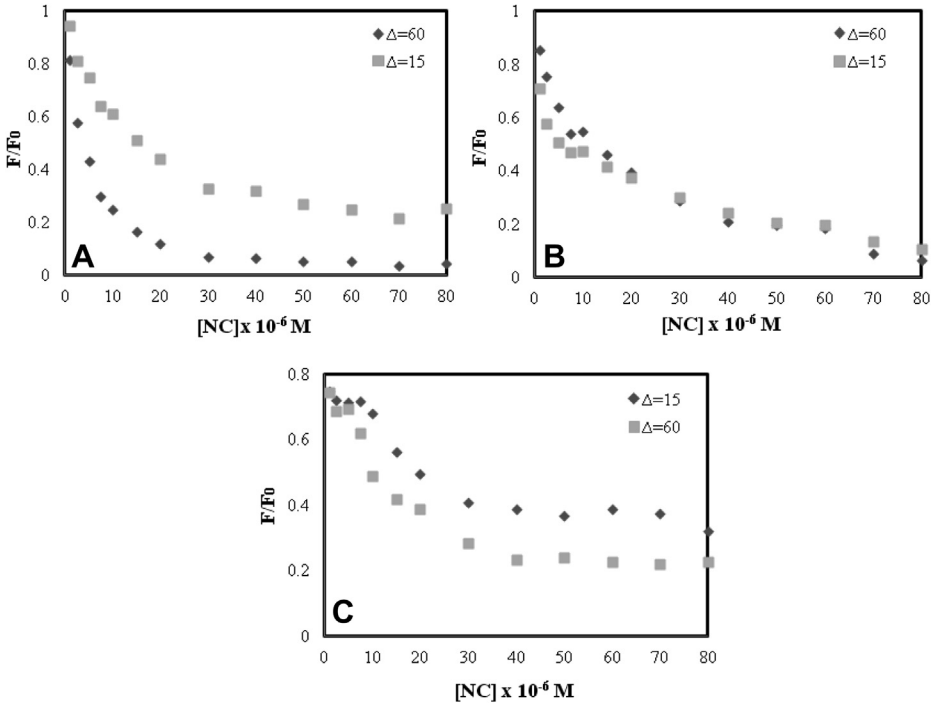

Fig. 4 – The quenching degree of (A) 10 μg/mL human serum albumin, (B) 10 μg/mL hemoglobin, and (C) 10 μg/mL globulin. [Niclosamide] =1μM, 2.5μM, 5μM, 10μM, 15μM, 20μM, 30μM, 40μM, 50μM, 60μM, 70μM, and 80μM, pH: 7.4.

whereas it was y = 457.24x + 68.347 (R = 0.996) for globulin at a concentration range of 17.5 −2.5 μg.

3.4. *Mechanism of interaction of niclosamide with proteins*

Fluorescence quenching can generally be classified as either static or dynamic; and static and dynamic quenching may also occur simultaneously. The mechanism by which niclosamide quenches the fluorescence of the three proteins was determined using the Stern–Volmer equation [16]:

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + KSV[Q] \quad [1]$$

where F_0 and F are the fluorescence intensities of the proteins before and after the addition of the quencher; K_q is the quenching constant; K_{SV} is the Stern–Volmer quenching constant; [Q] is the concentration of the quencher; and τ_0 is the average biomolecular fluorescence lifetime, which is considered to be 10^{-8} seconds [25,26].

The Stern–Volmer plots at different temperatures are given in Fig. 5A–C. As given in Table 1, the values of K_q at different temperatures (296 K, 299 K and 302 K) are estimated as 3.32 × 10^{13} L mol^{-1} s^{-1}, 3.05 × 10^{13} L mol^{-1} s^{-1} and 2.76 × 10^{13} L mol^{-1} s^{-1} for HSA. The *Kq* values were found to be 0.46 × 10^{13} L mol^{-1} s^{-1}, 0.49 × 10^{13} L mol^{-1} s^{-1} and 0.52 × 10^{13} L mol^{-1} s^{-1} for hemoglobin, and they were determined as 0.44 × 10^{13} L mol^{-1} s^{-1}, 0.39 × 10^{13} L mol^{-1} s^{-1} and 0.36 × 10^{13} L mol^{-1} s^{-1} for globulin. The results indicated that the values of K_q were larger than the maximum scattering collision quenching constant of 2.0 × 10^{10} L mol^{-1} s^{-1} for HSA, hemoglobin, and globulin [26]. These results suggested a static quenching mechanism of niclosamide with each protein [27].

The absorbtion spectrum can also classify the quenching mechanism between a drug molecule and a protein as either static or dynamic quenching. Although static quenching only affects ground-state complex formation, dynamic quenching results in the excited fluorophore states. Dynamic quenching led to no changes in the absorption spectra [27]. The absorbance of niclosamide was decreased upon the addition of all proteins, as seen in Fig. 6A–C. This implies that the interactions between niclosamide and each protein occurred by static quenching.

3.5. *Binding constant and binding sites*

Niclosamide quenched the fluorescence intensity of each protein by binding to a set of equivalent sites on the protein. The equilibrium between the number of binding sites (*n*) and the binding constant (K_b) is estimated using the following equation [24]:

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

where K_b is the binding constant, and *n* is the number of binding sites per protein. If we plotted lg $(F_0 - F)/F$ vs. lg[Q], the values of K_b and *n* were determined for each niclosamide–-protein complex (Table 1). The number of binding sites, *n* was determined to be 1.33, 1.26 and 1.21 at the respective temperatures of 296 K, 299 K, and 302 K for HSA. Values of *n* were estimated to be 1.29, 1.24, and 1.05 for hemoglobin, and 1.04, 1.17, and 1.26 for globulin at 296 K, 299 K, and 302 K. All data are given in Table 1. The changes in *n* values with increases in temperature might be a result of the protein structure's changes with temperature.

K_b values were estimated as 0.162 × 10^3 L mol^{-1}, 0.185 × 10^3 L mol^{-1}, and 0.262 × 10^3 L mol^{-1} for HSA at the respective temperatures of 296 K, 299 K, and 302 K. Although the binding constants were found to be 0.020 × 10^3 L mol^{-1}, 0.017 × 10^3 L mol^{-1}, and 0.013 × 10^3 L mol^{-1} for hemoglobin, they were 0.011 × 10^3 L mol^{-1}, 0.017 × 10^3 L mol^{-1}, and 0.026 × 10^3 L mol^{-1} for globulin at these temperatures. The values of K_b

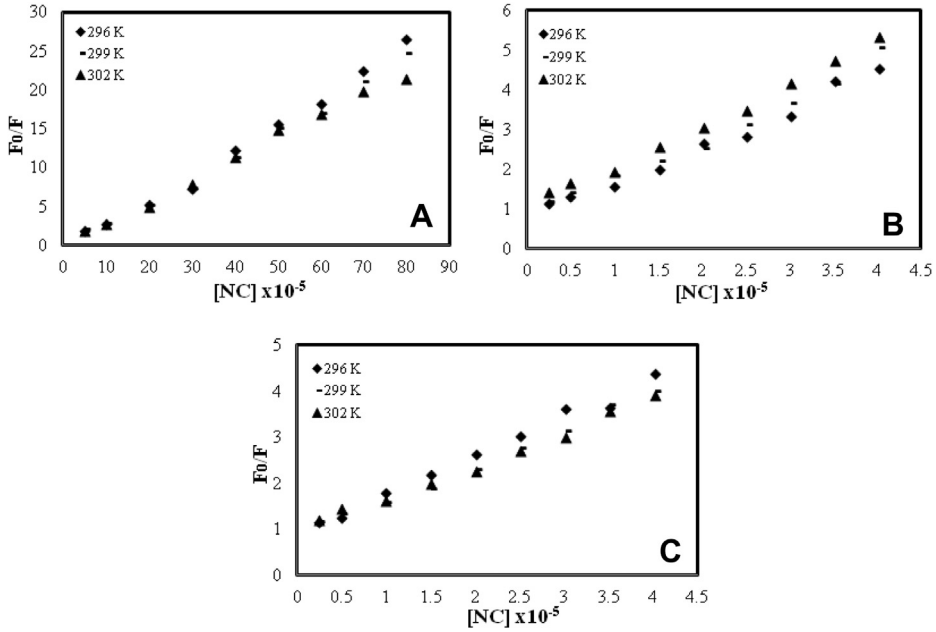

Fig. 5 – Stern–Volmer plots for the quenching of (A) human serum albumin, (B) hemoglobin, and (C) globulin by niclosamide at different temperatures.

Table 1 – Quenching and binding constant K_b and binding sites n at different temperatures.

Protein	T (K)	K_b ($\times 10^3$) L mol^{-1}	n	K_q ($\times 10^{13}$) L mol^{-1} s^{-1}	K_{sv} ($\times 10^5$) L mol^{-1}
HSA	296	0.162	1.33	3.32	3.32
	299	0.185	1.26	3.05	3.05
	302	0.262	1.21	2.76	2.76
Hemoglobin	296	0.020	1.29	0.46	0.46
	299	0.017	1.24	0.49	0.49
	302	0.013	1.05	0.52	0.52
Globulin	296	0.011	1.04	0.44	0.44
	299	0.017	1.17	0.39	0.39
	302	0.026	1.26	0.36	0.36

Table 2 – Thermodynamic parameters for each protein at different temperatures.

Protein	T (K)	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
HSA	296	−1.71	−0.88	−2.81
	299		−0.87	
	302		−0.86	
Hemoglobin	296	−2.03	−0.21	−6.15
	299		−0.19	
	302		−0.17	
Globulin	296	3.39	6.96	12.05
	299		6.99	
	302		7.03	

decreased as the temperature increased, which was assumed to decrease the stability of the niclosamide–hemoglobin complex. Unlike hemoglobin, an increase in temperature increased the stability of the niclosamide–protein complexes for HSA and globulin.

3.6. *Thermodynamic parameters and nature of the binding forces*

The binding mechanism between proteins and small molecules like drugs involves several bonds such as hydrophobic force, hydrogen bonds, electrostatic interactions, and van der Waals interactions [18–28]. The relationship between acting force and thermodynamic parameters was described by Ross and Subramanian [29] as follows: (1) $\Delta H < 0$ and $\Delta S < 0$ indicates van der Waals interactions and hydrogen bonds; (2) $\Delta H < 0$ and $\Delta S > 0$ indicates electrostatic interactions; and (3) $\Delta H > 0$ and $\Delta S > 0$ indicates hydrophobic forces. The values of the enthalpy change (ΔH) and the entropy change (ΔS) can be measured from the following equations:

$$\text{Ln } K_b = -\Delta H/RT + \Delta S/R \quad [3]$$

$$\Delta G = \Delta H - T\Delta S \quad [4]$$

where K_b is the binding constant at the different temperatures, R is the gas constant, and T is the temperature. van't Hoff plots between log K_b and $1/T$ were used to estimate the values of enthalpy (ΔH) and entropy (ΔS) change. The values of ΔH and ΔS were determined to be −1.71 kJ mol^{-1} and −2.81 J mol^{-1} K for HSA, respectively (Table 2). The binding of niclosamide to HSA is carried out by both van der Waals interaction and hydrogen bonding, which involve negative values of ΔH and ΔS. These values were found to be −2.03 kJ mol^{-1} and −6.15 J mol^{-1} K^{-1} for hemoglobin, whereas they were estimated as 3.39 kJ mol^{-1} K^{-1} and 12.05 J mol^{-1} K^{-1} for globulin, respectively. The values of ΔH and ΔS were also negative at the experimental temperature, indicating that van der Waals interactions and hydrogen bonds play major roles in the binding process for hemoglobin. The positive values of ΔH and ΔS for globulin suggest a hydrophobic interaction. The free energy changes (ΔG) at different temperature were estimated as −0.88 kJ mol^{-1} (296 K), −0.87 kJ mol^{-1} (299 K), and −0.86 kJ mol^{-1} (302 K) for HSA and as −0.21 kJ mol^{-1} (296 K), −0.19 kJ mol^{-1} (299 K), and −0.17 kJ mol^{-1} (302 K) for hemoglobin. The values of ΔG were negative at all experimental temperatures, indicating that the reaction was spontaneous for HSA and hemoglobin. Positive values of ΔG at 296 K (6.96 kJ mol^{-1}), 299 K (6.99 kJ mol^{-1}), and 302 K (7.03 kJ mol^{-1}) show that globulin binding was energy dependent.

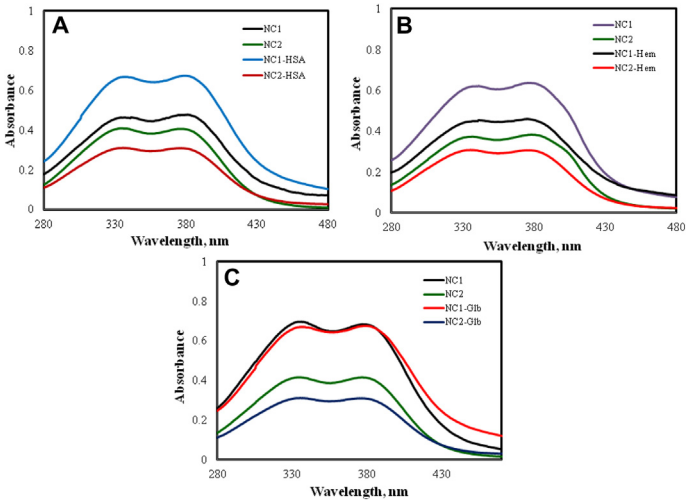

Fig. 6 – Absorption spectra of niclosamide at different concentrations in the presence and absence of (A) 10 μg/mL human serum albumin, (B) 10 μg/mL hemoglobin (Hem), and (C) 10 μg/mL globulin (Glb). NC1: 10μM and NC2: 60μM, pH: 7.4.

4. Conclusion

The interactions of niclosamide with proteins such as hemoglobin, HSA, and globulin were studied using fluorescence spectroscopy. The emission of each protein decreased with the addition of niclosamide to the protein solution, leading to an energy transfer between drug and protein molecules. Static quenching is the main mechanism through which all proteins quenched. Thermodynamic parameters suggested that van der Waals interactions and hydrogen bonds are the forces behind niclosamide's interactions with HSA and hemoglobin. The interaction of niclosamide with globulin, by contrast, is carried out via hydrophobic interaction. These results show that the binding mechanism of the drug to each protein depends on the protein's structure.

Conflicts of interest

The author declares no conflicts of interest.

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