

Study of Interaction between Febuxostat and Bovine Serum Albumin by Fluorescence Spectroscopy

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Abstract

The interaction of an anti-gout drug, febuxostat was studied with bovine serum albumin (BSA) applying fluorescence quenching method for the first time. Interaction parameter and magnitude of the force indicated for both, dynamic and static quenching in between febuxostat and BSA protein. Thermodynamic studies indicated for both hydrogen and hydrophobic interactions, observed at 280 nm and only hydrophobic at 293 nm excitation wavelength. Negative ΔH_b and positive ΔS_b , indicated for distinctive characteristics for the existence of both, hydrogen and hydrophobic binding throughout the interactions. The binding constant K_b at λ_{ex} =280 nm was $3.467 \times 10^3 \mu M^{-1}$ and $4.943 \times 10^3 \mu M^{-1}$ at 298 and 308 K temperature whereas, $5.54 \times 10^3 \mu M^{-1}$ and $4.44 \times 10^3 \mu M^{-1}$ was noted during excitation at λ_{ex} =293 nm wavelength. The K_b values in different temperatures assumed that the stability of binding increased with the increase of temperature at λ_{ex} =280 nm, but reverse effect was experienced at an excitation wavelength of λ_{ex} =293 nm. The number of bound febuxostat molecules per BSA protein was found ~ 1.5 at both 298 and 308 K.

Keywords: Febuxostat; Bovine serum albumin; Interaction; Fluorescence spectroscopy; Dynamic quenching; Static quenching

Introduction

Plasma protein consists of a greater proportion of human circulatory system, irrespective of physiological functions, they are also important for drug distribution, disposition via pharmacokinetics and pharmacodynamics property [1]. Research with enormous interest in the drug-protein interaction also revealed the importance and influence of this interaction in the process of absorption, metabolism and excretion of xenobiotic [2]. Serum albumin being an abundant protein plays a vital role in animal physiology in the transportation of many drugs and hormones [3,4]. Binding with this serum albumin is a key factor, for any agonist or xenobiotic to give therapeutic actions. The extent of binding between drug and protein normally depends on many factors, including nature of drugs, hydrophobicity, electrostatic charge and pH of the drug molecules, etc. Most of the cases, a reversible binding of the drug with different bio-active compound is observed, when they function as carriers [5]. Dose and dosing interval completely depend on binding of drugs with different protein in the body. Quantification of circulating protein is always having a great importance in biology and medicine to understand and minimize the unwanted side effect of drug [6]. Escalating apparent solubility of hydrophobic drugs with the modulation pattern of bindings also plays a vital role in the delivery of drugs into the cell. Such interactions raise the concern of drug stability and toxicity related issues in a biological system [7]. In human, 60% of the circulating plasma is consisted with serum albumin [8]. Being a larger volume, it serves a wide variety of functions for endogenous and exogenous agonist to accentuate their therapeutic action on different receptors and channels. Bovine serum albumin (BSA), serve as a cheap source in studying drug protein interaction. Being 76% sequence homology it is believed that the 3D structure of the BSA and HSA are almost similar in nature [9]. Different studies comparing HSA and BSA, revealed that homology [9]. Considering 3D structure of HSA, it is suggested that the principal regions of ligand binding to HSA are located in hydrophobic cavities in the sub domain IIA and IIIA consisted with sites I and II including a single tryptophan residue in sub domain IIA [10,11].

Nature and the affinity of binding is always a great indicator to quantify the therapeutic dose for an effective dosage regimen towards better therapeutic outcome. Degrees of interactions of drug with surrounding protein depend on the chemical nature of the drug particles. This concern potentiates the importance and necessity of studying the interactions in between drug and serum albumin in molecular level.

Gout, an acute arthritis appears with an inflammation in response to the accumulation of crystals of monosodium urate in or around a joint [12,13]. Characterized by hyperuricemia (serum urate concentration [SUA] exceeding 6.8 mg/dL, the limit of urate solubility) and acute and chronic consequences of monosodium urate crystal deposition [14]. Renal calculi including prolong crystal disposition result in Renal damage may also occur and believe a prime cause of urinary tract stones composed of uric acid crystal [12]. Long term prophylaxis, is indicated for patients with recurrent gout attaches [14,15]. Prolong management of chronic gout with ULT focuses on achieving and maintaining sUA in a sub-saturating range (<6.0 mg/dL) with the objective of dissolving monosodium urate crystals and declining the body pool of uric acid [16,17].

Febuxostat INN, a selective non-purine analog XO inhibitor [18] extensively prescribed for the treatment of chronic hyperuricemia in the patient's related to gout [19]. Data from 3 comparative blinded, randomized controlled trials (RCTs) have demonstrated the superior

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efficacy of febuxostat 80 mg daily compared with both the commonly prescribed doses of allopurinol (300 mg) and placebo [20,21]. In addition, both approved doses of 80 mg and 40 mg of febuxostat are significantly more efficient than allopurinol ($p < 0.001$ and $p = 0.012$, respectively) in achieving the therapeutic target of sUA (Serum uric acid) in subjects with mild-to-moderate renal impairment [22]. In this paper, we tried to illustrate about the study of the interactions of febuxostat with bovine serum albumin (BSA) applying different supportive data including binding parameter, temperature variation and nature of binding. Binding constant at temperature differential also studied to elucidate the dependence of temperature on binding force actuations.

Materials and Methods

Chemicals and reagents

All reagents used in this study were of analytical standard; moreover double distilled water was used throughout the study. BSA was obtained from sigma chemical Co., USA and used devoid of further purification process. BSA solution (2×10^{-5} M) was prepared in 0.1 M phosphate buffer (pH 7.40) and kept in 4°C. Febuxostat standard was (potency: >99.95 %) obtained from Incepta pharmaceutical Ltd., Bangladesh. Stock solution (1.0×10^{-3} M) was prepared in pH 7.40 buffer. Due to lack of complete solubility, febuxostat was first dissolved in DMSO and volume was adjusted by phosphate buffer pH 7.40. Buffer (pH 7.40) solution was equipped with the mixture of Na_2HPO_4 (20 mM) and NaH_2PO_4 (30 mM). All of the pH measurement was performed by Hanna HI 2210 pH meter, USA (Figure 1).

Apparatus

Fluorescence emission spectra were recorded on a Hitachi FL-7000 (Tokyo, Japan) fluorescence spectrometer equipped with 1 cm quartz cell. Observations were taken at two different temperatures (298 K and 308 K) using 5/5 nm slit widths. To aid in stabilizing of different temperatures, a thermostat bath Unitronic Orbital, Spain was used. The temperatures of the samples were maintained by recycling water throughout the experiment.

Data measurement and analysis

Statistical analysis and all others measurement were calculated by Microsoft Office Excel 2007 program. SD (Standard deviation) value was calculated statistically from three replicas run for each sample.

Sample preparation and observation

Stock solutions of 1000 μM of febuxostat and BSA were prepared in phosphate buffer pH 7.40. Appropriate volumes of BSA to obtain 2×10^{-5} mol. L⁻¹ or 20 μM was prepared from the stock. The required

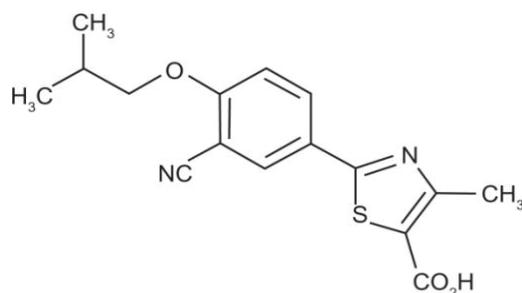


Figure 1: Structure of febuxostat.

volume of febuxostat to get 20 μM , 40 μM , 60 μM , 80 μM , 100 μM , 140 μM , 180 μM , 220 μM , 280 μM and 320 μM concentrations respectively, were equipped from the stock solution. Fixed 20 μM of BSA solution was mixed with each differential concentration of febuxostat in 1:1 (BSA: Febuxostat) volumetric ratio, in separated test tubes. Fluorescence spectra were recorded at two different temperatures (298 K and 308 K) in the range 250-600 nm upon excitation at 280 and 293 nm of BSA molecule considering location of tryptophan and tyrosine residue [23] in BSA protein. The mixed solution of BSA and febuxostat were mixed well by sonication later on incubated at 298 K and 308 K temperature for 30 minutes prior to the observation and spectral analysis. Phosphate buffer pH 7.40 was served as blank to aid in pre-scan purpose.

Results and Discussion

Fluorescence quenching is the reduction of quantum yield of fluorescence from a fluorophore induced by a variety of molecular interaction with a quencher molecule [24,25]. An increase in the concentration of quencher will mask the fluorophore, result in reduction of emitted fluorescence from the fluorophore molecules. In ease of targeting specific location regarding the binding pattern of BSA protein, we used different wavelength. Generally, the fluorescence of bovine serum albumins comes from tryptophan, tyrosine and phenylalanine residues. During data recording at an excitation wavelength at 280 nm, fluorescence of albumin was coming from both tryptophan and tyrosine residues, whereas 293 nm wavelength excited tryptophan residues only [23].

Here, fluorescence emission of BSA was reported approximately at 340 nm region, where as febuxostat (quencher) also emitted fluorescence at 380 nm. It happened as because drug itself also fluorescence active in nature. When the only BSA was excited, maximum emission intensity has been recorded at around 340 nm and these spectrums show a lower level of fluorescence emission at 380 nm. So, emission at 380 nm began to increase with the increase of drug concentration, comparing fixed concentration ($\text{CBSA} = 2 \times 10^{-5} \text{ mol L}^{-1}$) of BSA.

Analysis of fluorescence quenching of BSA by febuxostat

The fluorescence measurement of interaction between the drugs or agonists with a protein provides some information regarding its binding mechanism, binding force, binding mode, binding constant including location or associations on a specific site on examined protein. Figure 2 illustrated the emission spectra of BSA in presence of various concentrations of quencher molecules (febuxostat). The characteristic fluorescence emission wavelength of BSA was around 340 nm, indication of partial shielding of the tryptophan residues from aqueous solvent [15]. It was apparent that the fluorescence strength of BSA decreases consecutively with the increase in febuxostat concentration, implying that the binding of febuxostat to BSA occurred and the microenvironment around the fluorophore of BSA has been changed or blocked by available amount of drugs.

From Figure 2, the fluorescence emission wavelengths of BSA had obvious blue shifts after the addition of the drug molecules, indicated the location of tryptophan and tyrosine residue in BSA, in a more hydrophobic environment [10].

The fluorescence quenching data were analyzed by the well-known Stern-Volmer equation [25]: from equation 1

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

Here, where F_0 and F represent the fluorescence intensities in the absence and presence of the quencher molecule. $[Q]$ is the concentration

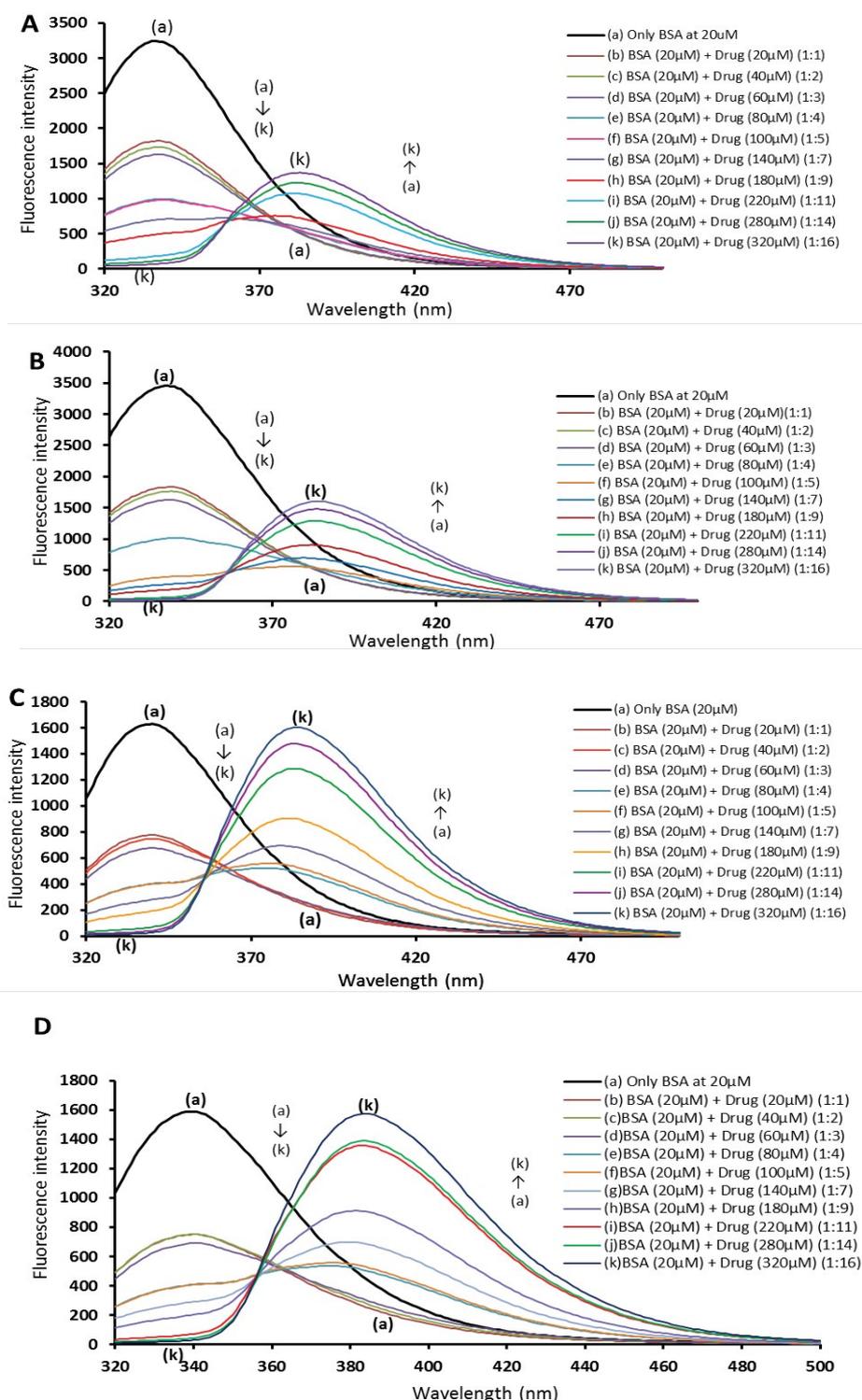


Figure 2: A. BSA fluorescence emission spectra in the presence of febuxostat, $\lambda_{ex}=280$ nm, CBSA= 2×10^5 mol L⁻¹; molar ratio of drug to protein is 0, 1, 2, 3, 4, 5, 7, 9, 11, 14, 16 μ M [from (a) to (k)] at 298 K temperature. B. BSA fluorescence emission spectra in the presence of febuxostat, $\lambda_{ex}=280$ nm, CBSA= 2×10^5 mol L⁻¹; molar ratio of drug to protein is 0, 1, 2, 3, 4, 5, 7, 9, 11, 14, 16 μ M [from (a) to (k)] at 308 K temperature. C. BSA fluorescence emission spectra in the presence of febuxostat, $\lambda_{ex}=293$ nm, CBSA= 2×10^5 mol L⁻¹; molar ratio of drug to protein is 0, 1, 2, 3, 4, 5, 7, 9, 11, 14, 16 μ M [from (a) to (k)] at 298 K temperature. D. BSA fluorescence emission spectra in the presence of febuxostat, $\lambda_{ex}=293$ nm, CBSA= 2×10^5 mol L⁻¹; molar ratio of drug to protein is 0, 1, 2, 3, 4, 5, 7, 9, 11, 14, 16 μ M [from (a) to (k)] at 308 K temperature, pH=7.40.

of the quencher (febuxostat), K_q is the quenching rate constant of the bio-molecule, K_{sv} is the Stern-Volmer quenching constant, and τ_0 (10^{-8} s) is the average lifetime of the fluorescent substance without any quencher [24].

At a greater concentration of quencher (Febuxostat), an emission spectrum was immersed in every spectral observation, taken at 280 and 293 nm in both temperatures respectively. The inherent emission bands of febuxostat were red shifted afterward the emission spectra of BSA, followed at around 380 nm. These phenomena also detected throughout a fluorometric analysis with BSA-3-carboxyphenoxathiin (I) observation [26]. When the entire circulating protein complex is fully occupied with an examined ligand, the albumin-ligand complexes only works as a predominant species in the micro environment, take part in the fluorometric emission process [27]. While in higher concentration of ligands, these further un-complexed ligands gather in the more hydrophobic region of the albumin and act as a predominated species, in raising the emission spectra toward at higher emission with the proportion of free ligands concentration availability [26,27].

This phenomenon of upward curvature also reported in many observations, done with many ligands and proteomics observation [27-29]. Modified Stern-Volmer equation [27] can be applied to resolve such situation, where both static and dynamic quenching are operative, in view of exponential form of the model of the sphere of action, V, equation (2) [30-32] or on the basis of quadratic dependence [33-35] upon quencher concentration model: from equation 2.

$$F_0/F = \{1 + K_{sv} * [Q] * \exp(V * [Q])\} \quad (2)$$

Where F_0 , F is the fluorescence intensities of the albumin in the absence and presence of quencher [Q], K_{sv} is Stern-Volmer quenching constant, Whereas, V is the volume of the sphere action. In Figure 3, the plot of modified Stern-Volmer equation is illustrated in both 280 and 293 nm excitation wavelength with the deviation occurred at two

different temperatures at 298 and 308 K, respectively. The constant values of K_{sv} and V are included in Table 1.

Hence, equation (1) can be used to determine the value of K_{sv} through a regression of the plot of F_0/F against [Q] and K_q (as $K_q = K_{sv} / \tau_0$). From the equation (2); the value of K_{sv} and V, the volume of the sphere of action can be calculated.

The concentration of quencher [Q] febuxostat, respectively; τ_0 and τ imply the average lifetime of the bimolecular without and with the presence of quencher molecules. Figure 4; presented the Stern-Volmer plots at two different temperatures. The observation indicated that the Stern-Volmer plots are linear and the slopes increase with increase of temperature at 293 nm, whereas opposite phenomena occur at 280 nm excitation wave length. The value for K_{sv} , K_q and R^2 at different temperatures are presented in Table 1. In case of modified Stern-Volmer equation, the quenching constant (k_{sv}) increases in both excitation wavelength of 280 and 293 nm. The volume of sphere action, V also increased upon incremental effect of temperatures.

As Stern-Volmer quenching constant (K_{sv}) decrease with the increase in temperature for static quenching, while for the dynamic quenching reverse effect is observed [36]. The results showed that K_{sv} was proportionally co-related with temperature increase at 293nm, which suggest that the fluorescence quenching process may be mainly controlled by a dynamic quenching mechanism rather than a static quenching mechanism. Additionally, an inversely relationship of K_{sv} with temperature also reported at an excitation wavelength of 280 nm. A Static quenching mechanism observed at the excitation wavelength of 280 nm. In case of modified Stern-Volmer plot, a shape indication for dynamic quenching was noted, within the experimental drug/protein (d/p=0-16) ratio.

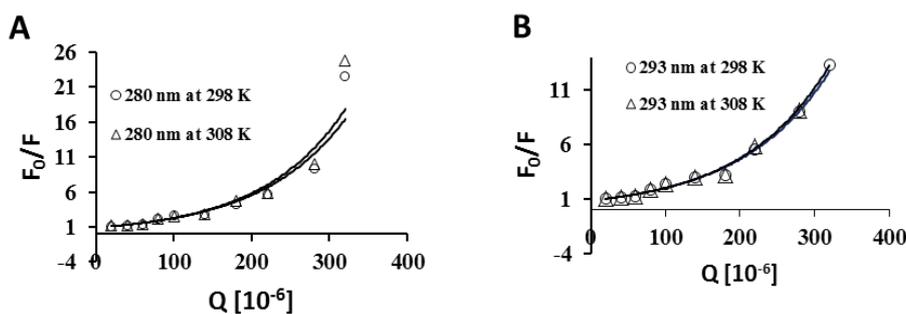


Figure 3: A. Modified Stern-Volmer plots of Febuxostat-BSA system (at d/p ratio: 0-16) in λ_{ex} =280 nm; λ_{em} =380 nm at pH=7.40 at different temperatures: (O) 298 K, (Δ) 308 K. B. Modified Stern-Volmer plots of Febuxostat-BSA system in λ_{ex} =293 nm; λ_{em} =380 nm at pH= 7.40 at different temperatures: (O) 298 K, (Δ) 308 K.

λ_{ex}	pH	T (K)	Equation (01)			Equation (02)		
			K_{sv} ($\times 10^4 \text{ L.mol}^{-1}$)	K_q ($\times 10^{12} \text{ L.mol}^{-1}.\text{s}^{-1}$)	$R^{2(a)}$	K_{sv} ($\times 10^4 \text{ L.mol}^{-1}$)	$V \times 10^4$ (M^{-1})	$R^{2(a)}$
280 nm	7.40	298	13.7 \pm 0.017	13.7 \pm 0.017	0.9636	7.91 \pm 0.10	0.90 \pm 0.13	0.9691
		308	12.5 \pm 0.029	12.5 \pm 0.029	0.9762	9.68 \pm 0.21	0.93 \pm 0.17	0.9713
293 nm	7.40	298	17.2 \pm 0.005	17.2 \pm 0.05	0.9682	13.40 \pm 0.07	0.84 \pm 0.08	0.9865
		308	18.5 \pm 0.006	18.5 \pm 0.006	0.9782	14.56 \pm 0.09	0.86 \pm 0.12	0.9857

^a R^2 is the correlation co-efficient.

Table 1: Stern-Volmer quenching constants, to eq. (1) and modified Stern-Volmer quenching constant, to eq. (2); of BSA-Febuxostat system at different temperatures (pH=7.40).

Analysis of binding parameter

The equilibrium between free and bound molecules due to the binding of a micro molecule into a site of macromolecules can be given by the equation [36]: from equation 3.

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

Where, K_b and n are the binding constant and the number of binding sites, respectively. Table 2; shows that the values of K_b increased with increase of temperature at 280 nm excitation wavelength, which may indicate that there was a molecular binding of febuxostat with BSA forming a stable complex with a molar ratio of about ~ 1.5:1 in between febuxostat and BSA molecules. It can be implicated that febuxostat indicated a higher affinity to BSA, with the binding of 1.5 moles of febuxostat with 1 mole of BSA. The complex begins to decompose when the temperature increases in case of excitation wavelength of 293 nm (Figure 5).

Thermodynamic parameters and the behavior of pattern forces

Hydrogen bond, van der Waals force, hydrophobic interaction,

including electrostatic is the most common form of force govern in the binding between a small molecule (agonist) and a macromolecule (Protein). The thermodynamic parameters were determined using the Van't Hoff equation [37]: from equation 4.

$$\ln K_b = -(\Delta H_0/RT) + (\Delta S_0/R) \quad (4)$$

Where, ΔS_0 =Entropy change, ΔH_0 =Enthalpy change, R =Universal gas constant and K_b =Analogous to the Stern-Volmer quenching constants K_{sv} at the corresponding temperature [24].

In equation (4), K_b corresponds to the modified Stern-Volmer association constant at specific temperatures and R is the gas constant. The plot of $\ln K_b$ vs $1/T$ originates for determining of the values of ΔH_0 and ΔS_0 (Figure 6). The free energy change ΔG_0 of the binding reaction at different temperature was estimated from the relation (5): from equation 5.

$$\Delta G_0 = \Delta H_0 - T\Delta S_0 \quad (5)$$

Value of ΔG_0 , ΔH_0 and ΔS_0 are listed in Table 2.

A positive value of ΔS_0 was evidence for hydrophobic interaction; the negative ΔH_0 values ensured the existence of hydrogen bonding

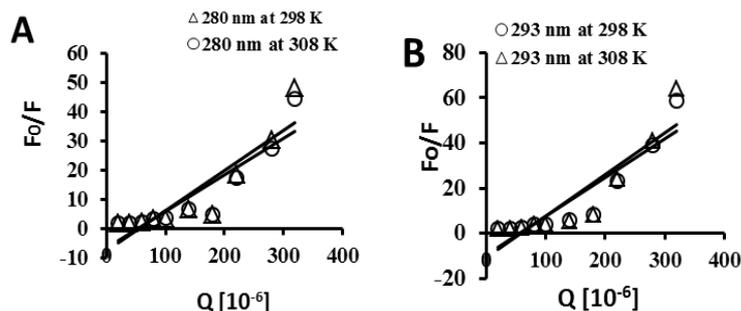


Figure 4: A. The Stern–Volmer plots of BSA quenched by febuxostat in λ_{ex} =280 nm at different temperatures: (O) 298 K, (Δ) 308 K. B. The Stern–Volmer plots of BSA quenched by febuxostat in λ_{ex} =293 nm at pH=7.40 at different temperatures: (O) 298 K, (Δ) 308 K.

λ_{ex}	T(K)	$\ln K_{sv}$	K_b ($\times 10^3$ L mol ⁻¹)	n	ΔG_0 (kJ mol ⁻¹)	ΔH_0 (kJ mol ⁻¹)	ΔS_0 (J mol ⁻¹ K ⁻¹)
280 nm	298	11.827	3.467	1.522	-29.309	-6.9987	74.868948
	308	11.736	4.943	1.45	-30.053		
293 nm	298	12.055	5.54	1.481	-29.86	5.57172	118.9188
	308	12.128	4.436	1.527	-31.05		

Table 2: Modified Stern-Volmer association constants K_b , number of binding sites, n and thermodynamic parameters of the interaction of febuxostat with BSA at different temperature (pH=7.40).

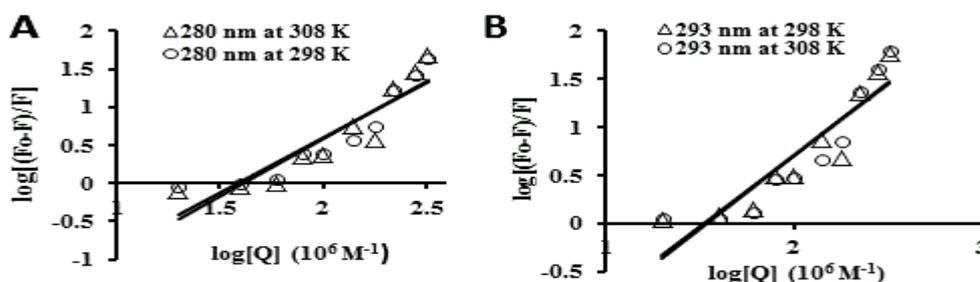


Figure 5: A. Logarithmic plot of the fluorescence quenching of BSA at various concentrations of febuxostat in excited wavelength (λ_{ex}) 280 nm at 298 K (O) and 308 K (Δ). B. Logarithmic plot of the fluorescence quenching of BSA at various concentrations of febuxostat in excited wavelength (λ_{ex}) 293 nm at 298 K (O) and 308 K (Δ).

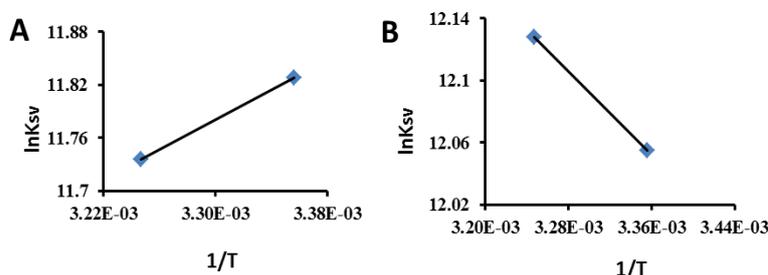


Figure 6: A. Van't Hoff plot showing the interaction of febuxostat with BSA at pH 7.40 in λ_{ex} =280 nm. B. Van't Hoff plot showing the interaction of febuxostat with BSA at pH 7.40 in λ_{ex} =293 nm.

in the binding process [37,38]. Furthermore, the main source of ΔG_o value was derived from a large contribution of the ΔS_o terms with a small contribution from the ΔH_o , so the main interaction was hydrophobic and a small fraction of hydrogen bonding in nature due to negative ΔH_o , observed in the excitation wavelength of 280 nm. Only hydrophobic interaction was recorded at an excitation wavelength of 293 nm. Negative (Energy change) ΔG_o postulate the spontaneous binding process throughout the interactions [37].

Binding constant K_b , increase with the increase of temperature at 280 nm excitation wavelength, ensured the stability of the BSA-Febuxostat complex along with temperature increment. Moreover, an inversely proportional association was observed at an excitation wavelength of 293 nm whereas the stability of complex decreases with the increase in temperature. Number of assumed binding site n ; was more than 1, which indicated the higher affinity of febuxostat with the BSA protein.

Conclusion

The fluorescence spectroscopic method was applied to study the interactions and the nature of binding with febuxostat and BSA in a temperature differential process. The experimental results indicated that quenching of the fluorescence of BSA by febuxostat was probably followed a static as well as dynamic mechanism and the binding reaction was mainly enthalpy driven, where hydrophobic interaction played a major role. The binding nature assumed that the maximum binding occurs in the principal regions of ligand binding domain, located in hydrophobic cavities in the sub domains IIA and IIIA [5,6]. A phenomenon of mixed static and dynamic quenching was observed at both 280 and 293 nm excitation wavelength (λ_{ex}), whereas emission occurred at λ_{em} =380 nm, which is carefully illustrated with modified Stern-Volmer equation. Binding constant K_b , suggested that the stability of binding increases with the increase of temperature in most cases. Increase of stability of the complex observed at 280 nm whereas the reverse effect was observed at 293 nm with increase of temperature. The Molar ratio of BSA-Febuxostat indicated that, 1 mole of BSA binds with ~ 1.5 moles of febuxostat. Pharmacokinetics view point suggested that, drug with higher affinity with plasma protein normally has a low therapeutic index, indicating high risk of toxicity due to a slide alteration in dosing [18]. Being an anti-gout drug febuxostat, need to take as prolong therapy to improve on patient's disease state with effective management of blood uric acid level. So, a study like this can be helpful in safe dosing regimen towards a better therapeutic outcome and toxicity minimization comparing with human serum albumin.

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