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Research Article

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Interaction of Human Serum Albumin with Methotrexate: Stability and Structural Analysis

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Abstract

Human serum albumin (HSA) as the most abundant carrier protein in blood plasma has been widely studied. HSA is often used as a model to study of the drug and protein interaction. The interaction between Methotrexate with HSA has been studied by methods such as fluorescence. Thermodynamic parameters obtained from thermal and chemical denaturation of the Human serum albumin with and without the presence of Methotrexate was analyzed.

Tm values in melting point (Tm) of HSA in the absence of these compounds were calculated 320.9 K and in the presence of 50µM Methotrexate were obtained 318.4 K, 100µM Methotrexate were obtained 318.5 respectively .ΔG^{o} (298k) of HSA in the absence of Methotrexate were measured 41.14 kJ/mol and in the presence 50µM and 100µM Methotrexate were obtained 39.4 kJ/mol and 15.2 kJ/mol respectively.

Chemical denaturation of HSA carried out with urea and titrated amounts of the protein in 280 nm, ΔG^{o}(H2O) and Cm values were calculated in the presence of these compounds. ΔG^{o}(H2O) was calculated 13.43 kJ/mol in the absence of ligands, and 11.63 kJ/mol in the presence of 50µM Methotrexate and 11.1 kJ/mol for 100µM concentration of Methotrexate.

Cm value of the protein in the absence of the aforementioned compounds was 1.57 M and in the presence of Methotrexate 50µM and 100µM were 2.06M and 2.03M, respectively. Intrinsic fluorescence studies showed that Methotrexate decrease the intrinsic fluorescence intensity at 300-450 nm which consequently results in the instability of the protein structure of HSA.

Keywords: Human serum albumin, Methotrexate, Stability, Fluorescence, Denaturation.

Introduction

Human serum albumin represents important and the most abundant protein constituent of blood plasma and serving as a protein storage component. Recently, the three-dimensional structure of human serum albumin was determined through X-ray crystallographic measurements [1]. This globular protein consisting of a single polypeptide chain of 585 amino acids, which has many important physiological functions [2-3]. HSA considerably contributes to colloid osmotic blood pressure and realizes the transport and distribution of many molecules and metabolites such as fatty acids, amino acids, hormones, cations and anions, and many diverse drugs. It also makes possible to bind and carry through the bloodstream many drugs, which are poorly soluble in water [3]. It has been shown that the distribution, free concentration, and the metabolism of various drugs can be significantly altered as a result of their bindings to HSA [4].

Methotrexate a synthetic nonbiologic antimetabolite is first-line DMARD for treating RA and is used in 50-70% of patients.It is active in this condition at much lower doses than dose needed in cancer chemotherapy. Evidence supports its use in juvenile chronic arthritis and it has been used in psoriasis, PA, AS, polymyositis, dermatomyositis, Wegener,s granolomatosis, giant cell artritis, SLE and vasculitis and most used as chemotherapy.[5]

Therefore, the interaction of Methotrexate with human serum albumin has major biochemical importance.

Materials and Methods

To obtain the effect of Methotrexate on the chemical stability of the Protein, chemical denaturation profiles of HSA were recorded by titration of a protein solution (40µM) with aliquots from a 6M stock solution of urea. These experiments were carried out in the absence and presence of 50 and 100µM concentrations of Methotrexate. Conformational change of the protein was obtained at 280 nm wavelength by using spectrophotometric technique.

Fluorescence spectroscopy for thermal denaturation of HSA was carried out on a spectrofluorimeter. Variable temperatures were performed from 280 to 380 K and emission spectra were recorded in 357nm with excitation at 290nm wavelength in increments of 1nm. The intrinsic fluorescence of HSA (µM) was carried out by thermal scanning, in the absence and presence of 50 and 100 µM concentration of Methotrexate.

The tertiary structure of protein was assessed by intrinsic fluorescence of HSA in the presence and absence of Methotrexate was carried out by spectrofluormeter using a 1cm quartz cell and a thermostat bath. The Concentration of HSA solution was prepared and titrated with increasing concentration of Methotrexate 0 to 140µM buffer solution (temperature 298K). An appropriate blank, corresponded to the buffer, was subtracted in order to correcting the background fluorescence. The excitation wavelength was 290 nm, and the emission spectra were recorded from 300 to 420nm. The maximum emission intensity was used to calculate the binding constants, occupation of binding sites and thermodynamic parameters [6].

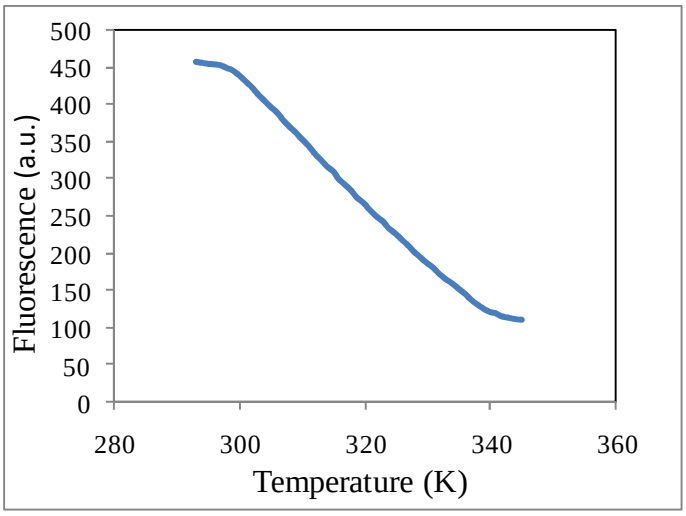
Results

Denaturation of HSA was induced by incubating in the absence and presence of the Methotrexate with various concentration of urea than the equilibrium was achieved. The extent of unfolding transitions HSA is monitored by changes in UV-Vis absorption at 280nm respectively.

The data obtained were normalized and analyzed according to the standard pace equation:[7], $K=f_{obs}-f_n/f_u-f_n$ (1) : where F_{obs} is the observed value of the signal at a given denaturant concentration and F_n and F_u are the values of native and unfolded protein, respectively. From these measurements, values of ΔG_{N-D} for a two-state process were determined using the relation: $\Delta G_{N-D}=-RT \ln K$ (2).

It can be seen from the plots of 1-6 that urea unfolding transition of HSA in the absence and presence 50 and 100µM concentrations of Methotrexate is cooperative and coincidental as represent by sigmoidal curves. Thus, urea induced unfolding of HSA can be explain by a simple two-state model: $\Delta G_{N-D}= \Delta G_{(H2O)}-m[D]$ (3): where $\Delta G_{(H2O)}$ and is the free energy of the protein folding in water (in the absence of denaturant) as the criterion of conformation stability, and ΔG_{N-D} is the free energy in the different concentrations of urea as denaturant. The m is the slope of ΔG_{N-D} vs [D] plot, and D is the denaturant concentration [8-9].

As illustrated in Fiuures 1-3 the use of liner extrapolation method is secondary plots in which ΔG_{N-D} is linearly relevant to the denaturant concentration, is the simple method of calculate the protein stability and thermodynamic parameters such as $[Ligand]_{1/2}$, $\Delta G^0_{(H2O)}$ and m for sole HSA and its treatment by 50 and 100µM concentrations of Methotrexate obtained: $[Ligand]_{1/2}$ = 1.57, 2.06 and 2.03M; $\Delta G^0_{(H2O)}$ = 13.43, 11.63 and 11.1 kJ/mol, respectively.



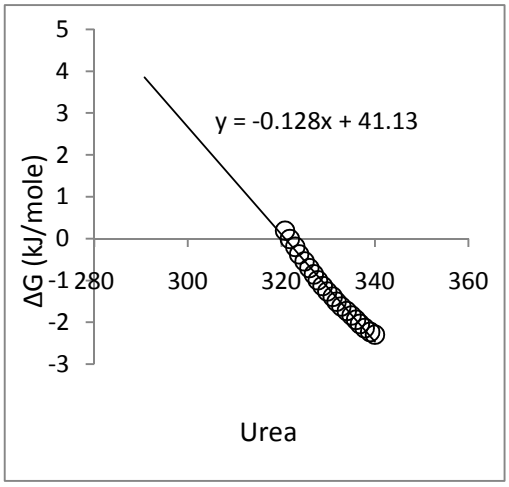


Figure 1. Scan thermal HSA=40µM in absence of methotroxate

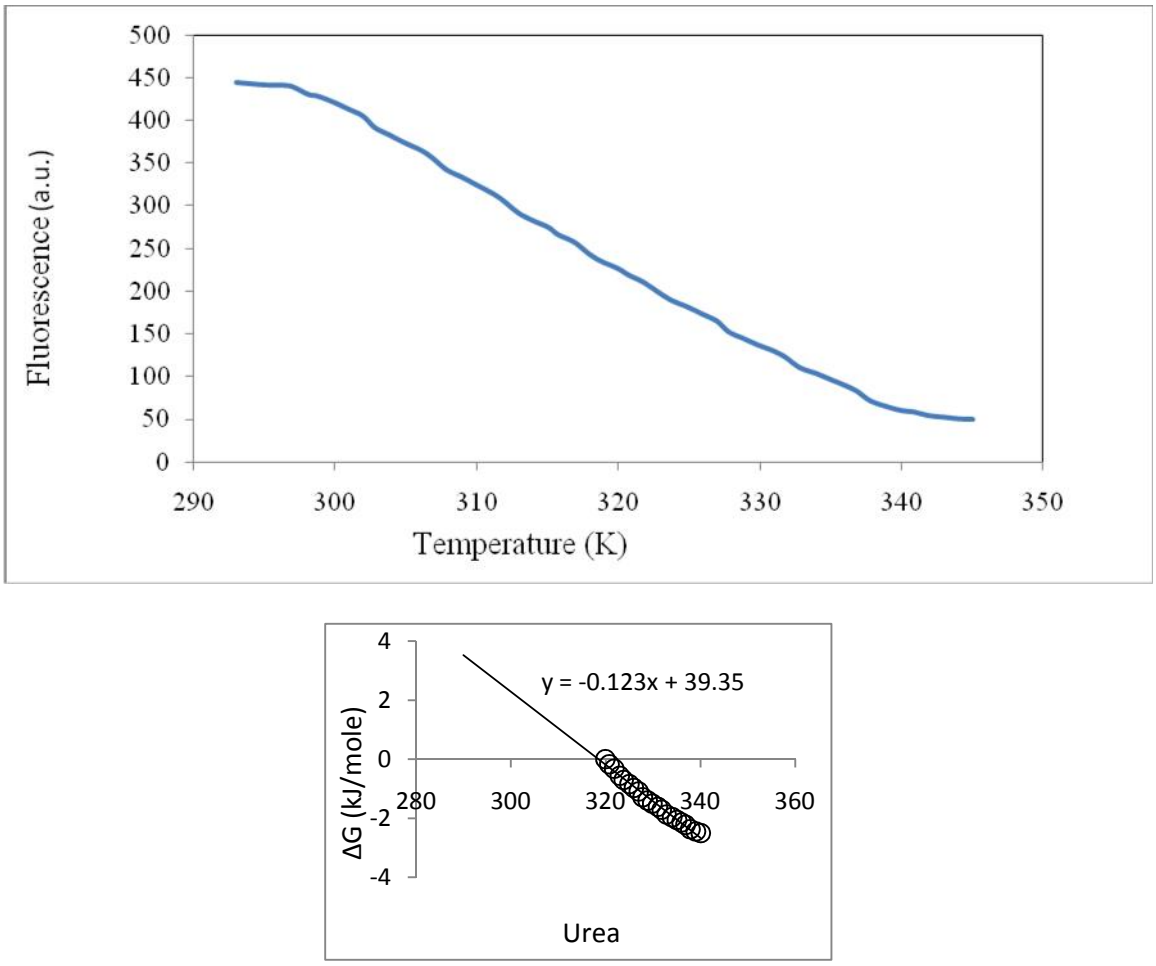


Figure 2. Scan thermal HSA=40µM and Methotrexate(50µM)

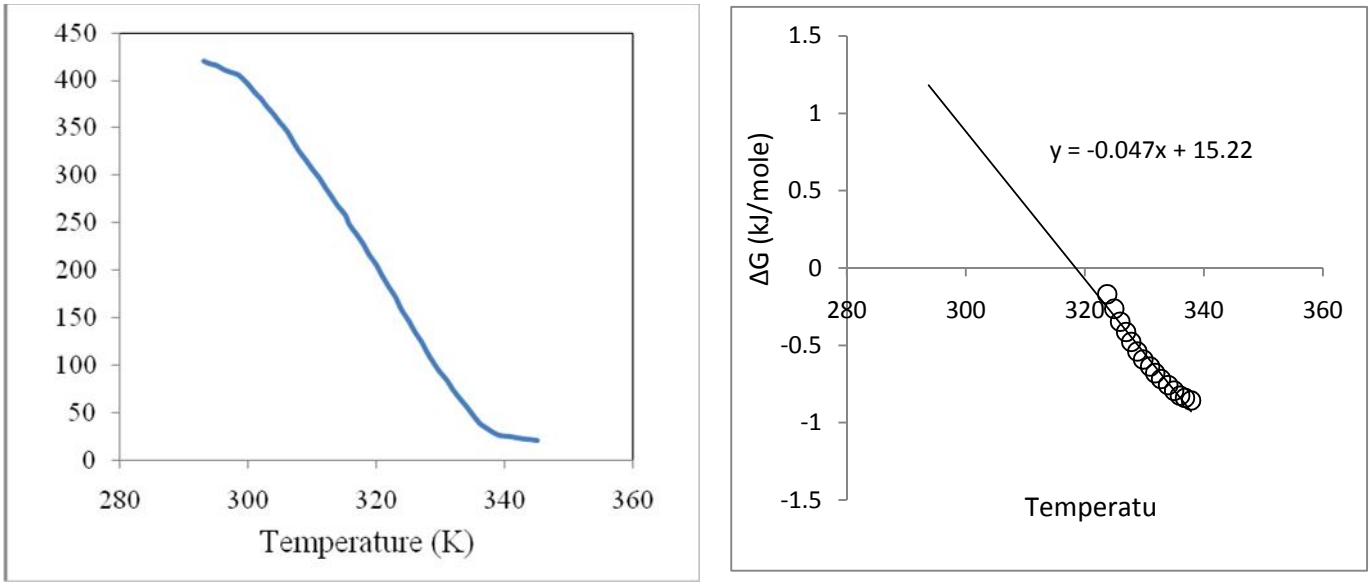


Figure 3. Scan thermal HSA=40µM and Methotrexate(100µM)

Thermal stability is the stability of a molecule at high temperatures. A molecule with more stability has more resistance to naturation at high temperatures.

It is now consider in Figures 4-6 how to analyses a thermal unfolding curve of HSA in the absence and presence of the Methotrexate from these sigmoids it is possible to determine the HSA melting temperature(T_m) as the temperature at which the magnitude of ΔG equals to zero. The Tm of protein and $\Delta G^{o}_{(298K)}$ as two thermodynamic parameters obtained of the HSA with and without the presence of Methotrexate. The T_m magnitudes for sole HSA and its treatment by 50 and 100μM concentrations obtained 320.9, 318.5 and 318.4K, and the values of $\Delta G^{0}_{(298K)}$ were obtained 41.14, 39.4 and 15.2 kJ mol^{-1}, respectively.

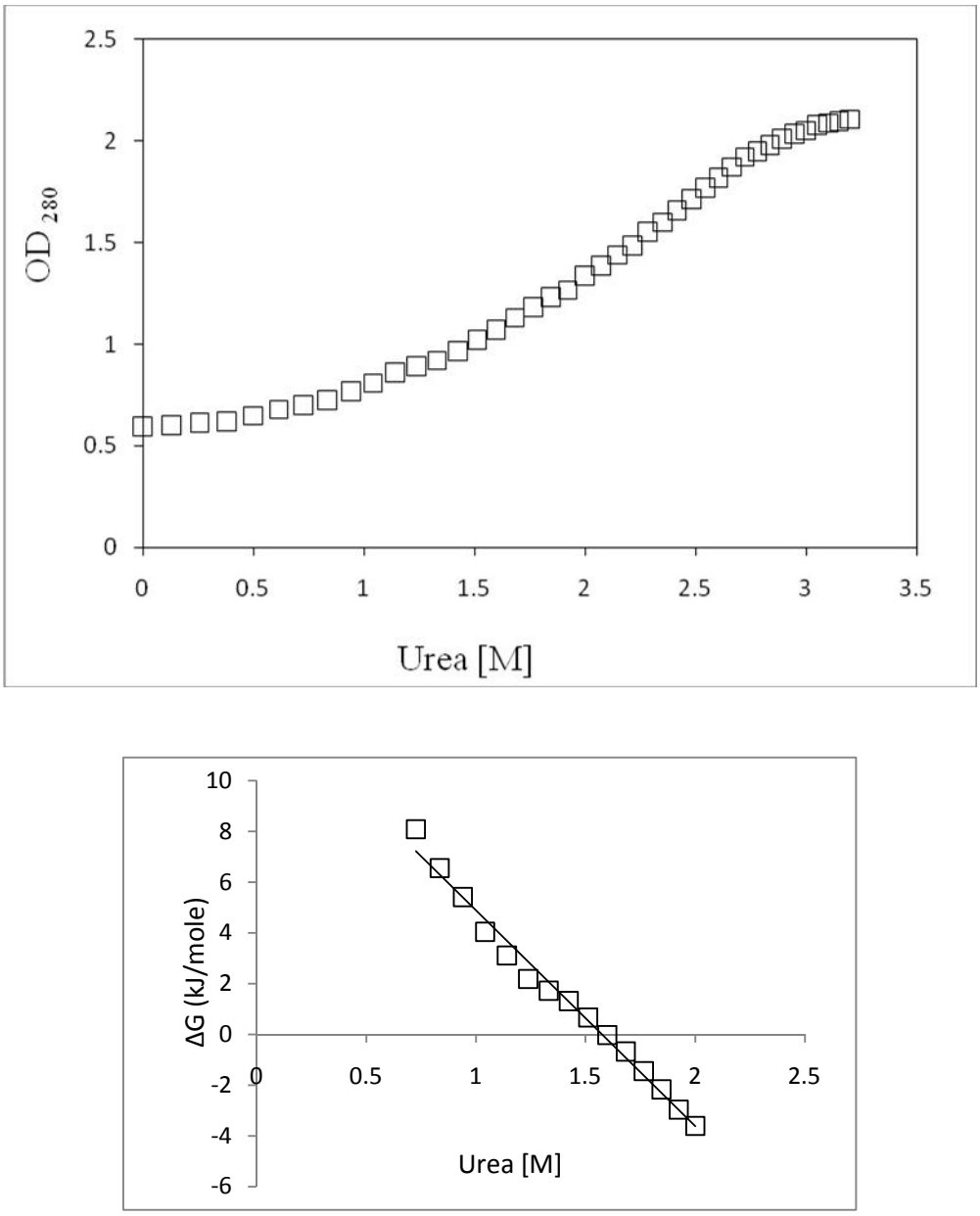
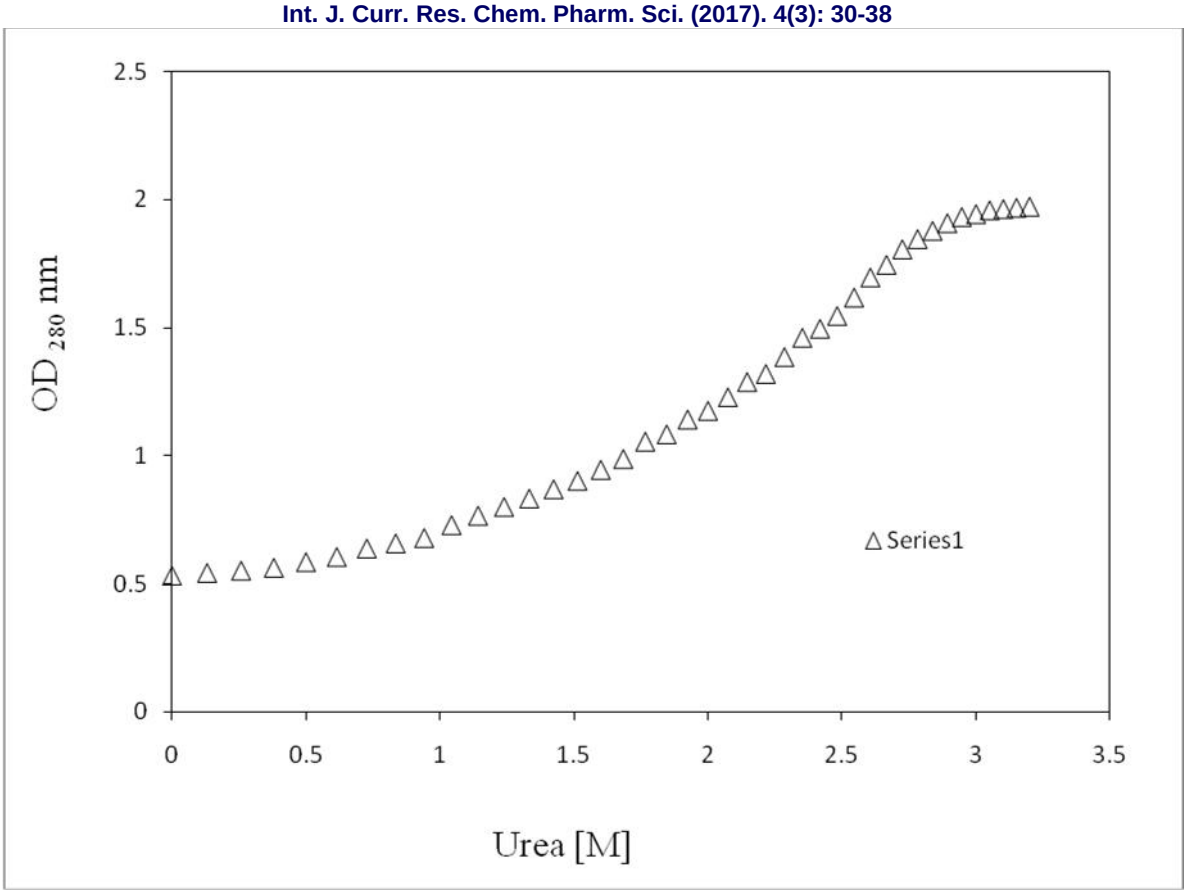


Figure 4. UV/vis absorbance spectra of HSA (40μM) in the abcense of Methotrexate . Shown HSA Inset Curve spectrum of denatured chemical HSA with Urea 6μM. .



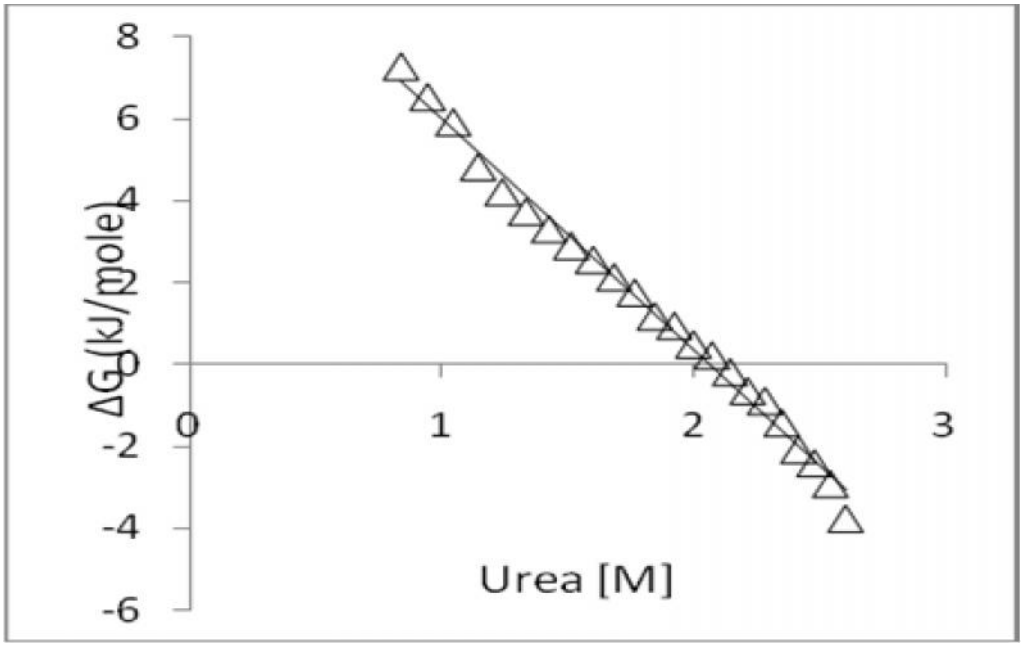


Figure 5. UV/vis absorbance spectra of HSA (40µM) and Methotrexate(50µM)
. Shown HSA Inset Curve spectrum of denatured chemical HSA with Urea 6µM

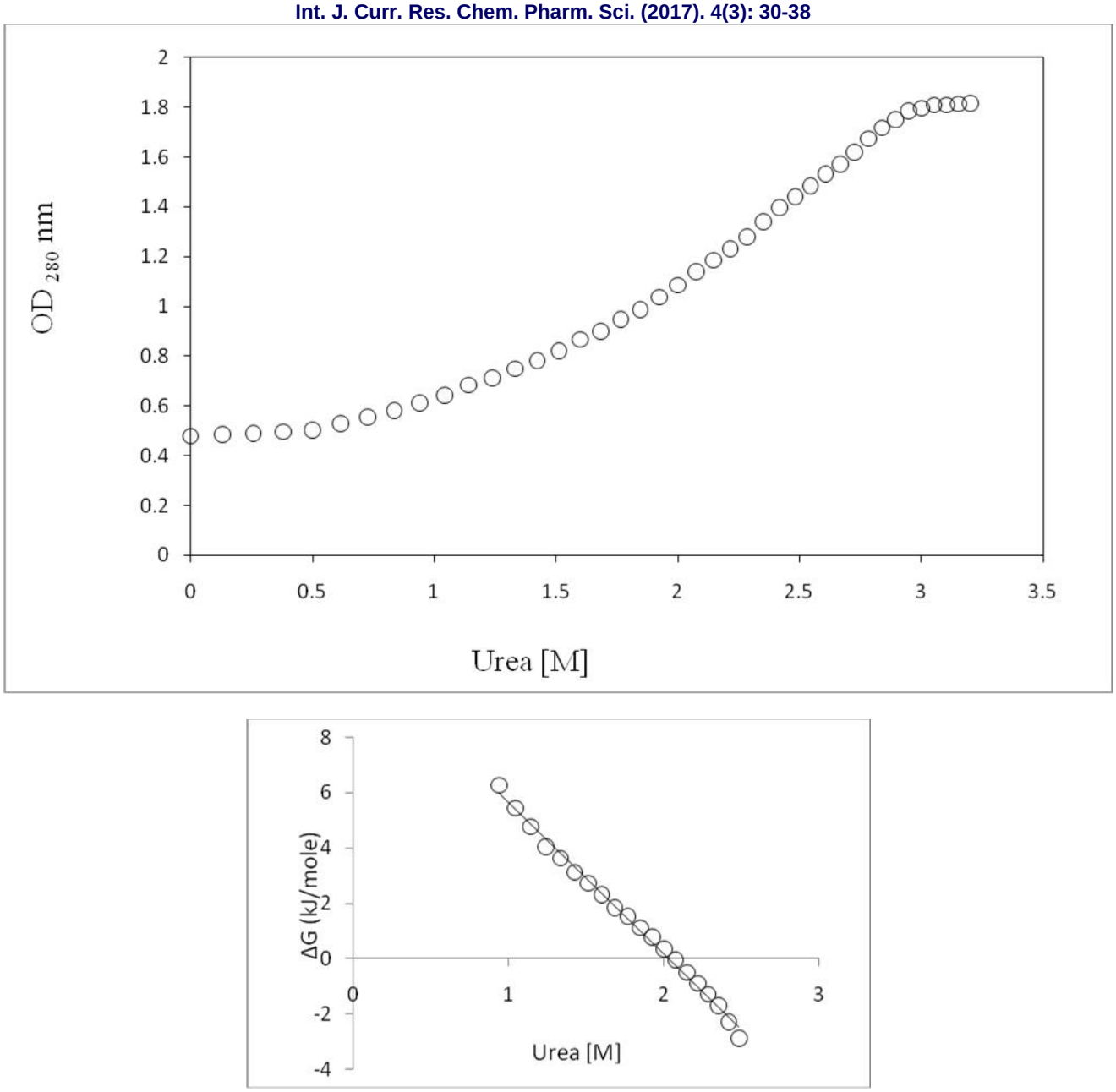


Figure 6. UV/vis absorbance spectra of HSA (40µM) and Methotrexate(100µM)
Shown HSA Inset Curve spectrum of denatured chemical HSA with Urea 6µM

Discussion

Thermodynamic parameters, obtained from chemical and thermal denaturation curves of HSA and Methotrexate showed a decreasing of Gibbs free energy and Tm for protein after incubation with Methotrexate. This instability was proved by structural analysis from fluorescence. The effects of Methotrexate binding on stability of HSA was investigated by urea-induced denaturation from measurements of OD_{280} in spectrophotometry and its thermal denaturation after exciting the protein at 290 nm in spectrofluorimetry techniques .The Gibbs free energy of HSA (ΔG_{H2O}) in the absence and present of Methotrexate was found to be 13.43 and 11/63 and 11/1 kJ /mol respectively as determined by linear fittings of intrinsic spectrophotometry data. All of the thermodynamic parameters such as obtaining ΔG_{H2O}, Cm, ΔG_{298K} and Tm from chemical and thermal denaturation revealed a relative instability of the HSA after its interaction with Methotrexate. In chemical denaturation, the urea interacts with HSA by electrostatic forces, leads to a randomly coiled conformation in its unfolded state, while thermal denaturation produces a molten globule state and the aggregation of the protein; therefore, both methods lead to different structurally unfolded states of the HSA [10-11].

$\Delta G^0_{H_2O}$ is the free energy of conformational stability in the absence of denaturant and m is a dependant value to the of ΔG° on the denaturant concentration.

In chemical denaturation, $[UREA]_{1/2}$ is the denaturant concentration that is required for protein receipt for its two-state transition half. In thermal denaturation, the protein melting point (T_m) is a temperature that is required for protein receipt its two-state transition half. Determined Magnitudes of the $\Delta G^0_{H_2O}$ and $[UREA]_{1/2}$ and T_m from curves, are summarized in Table 1.

Table 1: Calculated thermodynamic parameters from chemical and thermal denaturation curves of sole HSA and its interaction with Methotrexate.

Sample	Chemical denaturation			Thermal denaturation	
	$[Ligand]_{\frac{1}{2}}(M)$	$\Delta G^0_{(H_2O)}\left(kj/mol\right)$	$M\left(kj/mol\right)$	Tm(K)	$\Delta G^0{}_{(298K)}$(kJ/mol)
HSA	1.57	13.43	320.9	41.14	
HSA/ Methotrexate(50µM)	2.06	11.63	318.4	39.4	
HSA/ Methotrexate(100µM)	2.03	11.1	318.5	15.2	

The results From the previous investigations on binding parameters of HSA–CF (TP or TB) complexes calculated from the Van't Hoff equation were obtained equals to be ΔH= 47.72, 15.95, 38.90 KJ/mol, and ΔS= 242.03, 136.96, 213.58 J/mol.k, and ΔG= -24.73, -25, -24 KJ/mol and in the interaction of efonidipine with BSA it could be seen that ΔH= 68.04 KJ/mol ,and ΔS= 319.42 J/mol.k ,and ΔG= -27.08 KJ/mol. These positive values of ΔH and ΔS are associated with hydrophobic nature of ligand protein interaction [12-13].

In this study, the chemical and thermal denaturation profiles, obtained from urea titration and thermal scanning, respectively, in the absence and presence of Methotrexate in definite concentrations are depicted in Figures 1,2,3,4,5 and 5. Each profile is a sigmoidal curve, thus this process is described as a single denaturant-dependent step according to the two-step theory. The determination of standard Gibbs free energy of denaturation (ΔG°), as a criterion of conformational stability of a globular protein, is based upon two status theories and the equation 1 [14]

The structural study of methotrexate in different concentrations with internal fluorescence in Figure 7 showd a gradually decreasing of intensity and thus it emphasize to the instability of protein [15-17]. These together with the number of binding sites suggests that the binding site for Methotrexate on HSA is primarily and probably in vicinity of the Trp-212 residue, which is located within a hydrophobic binding pocket of the protein [18].

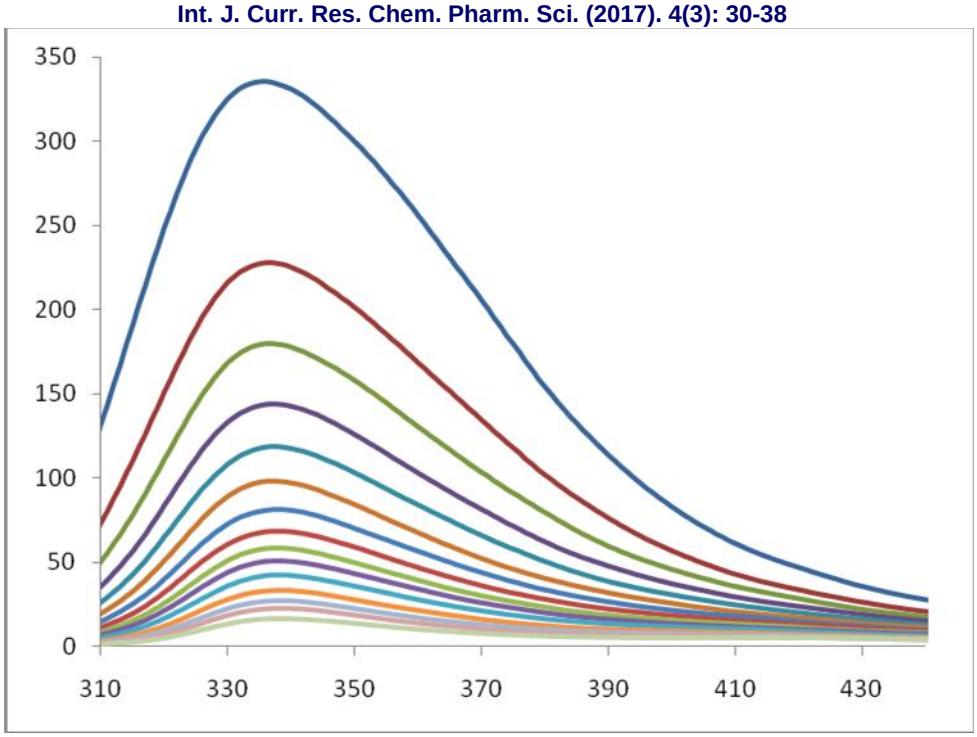


Figure 7. The internal fluorescence spectra of HSA with various amounts of Methotrexate (0 to 140µM). The HSA fluorescence intensity regularly decreased with the addition of Methotrexate.

Hence, from studies on the structure – function relationship of HSA in this investigation, it was emphasized that a minor change of protein structure for accepting the ligands such as Methotrexate will be necessary. These changes can be explained by the nature of non-covalent physical interactions and the ligands induced by a minor instability and higher flexibility.

Acknowledgments

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