

FULL PAPER

Comparative Binding Analysis of Pyrimidine Derivative to BSA: Equilibrium, FTIR and Acoustical Study

Ajay Madhukarrao Pisudde, Pradip Vitthalrao Tekade*, Shrikant Bandupant Thakare

Department of Chemistry, Jankidevi Bajaj College of Science, Jannalal Bajaj Marg, Civil lines, Wardha-442001-M.S. India

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Abstract:

This paper presented the comparative binding interaction of ethyl-4-(4-hydroxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4-HP2OTP) and ethyl-4-(2-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (2-HP2STP) to bovine serum albumin (BSA) in 1,4-dioxane, DMSO and DMF by equilibrium dialysis, FT-IR and acoustical study at physiological pH. The binding data obtained was interpreted by scatchard plot, which gives the association constants. An increase in association constants is observed with increase in temperature and concentration. FT-IR study explains the binding through shifting in peak positions of amide I and II. It explained the changes in secondary structure of BSA on binding with the drugs. The free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) values were calculated by using van't Hoff equation. The negative ΔG showed the spontaneous process and positive values of ΔH and ΔS showed endothermic interaction between ligands and BSA. ΔG becomes more negative with increased in temperature, indicated feasibility of binding interaction at high temperature. The positive values of ΔH and ΔS also showed specific electrostatic and hydrophobic interaction between ligand and BSA.

Keywords: equilibrium dialysis; FT-IR; acoustical study; BSA; scatchard analysis; thermodynamic parameters

1. Introduction

Serum albumins are the most abundant proteins in the circulatory system of wide variety of organisms, being the major macromolecules contributing to the osmotic blood pressure [1]. Their functional and physiological properties have been studied over several decades [2]. These proteins have long been used as model proteins in both industrial and academic research areas [3]. The protein is single polypeptide chain of 585 amino acids with a large helical triple domain structure with the concentration of 0.63 mM in the blood. Protein binds relatively a number of insoluble endogenous drugs such as unesterified fatty acids, bilirubin and bile ducts and thus facilitates their transport. A variation in temperature is found to be a key factor in binding affinities of proteins [4] as evident from the drugs

ligustrazine [5], ciprofloxacin [6], methotrexate [7] and cisplatin [8]. 4-HP2OTP and 2-HP2STP are the poly-functionalized dihydropyrimidine compounds exhibiting a broad range of therapeutic and pharmacological [9], anticarcinogenic [10], antihypertensive, antiviral, antitumor, antibacterial, anti-inflammatory, calcium channel modulators [11], antimycobacterial and anticonvulsant [12], anticancer [13] properties. Human serum albumin (HSA) and BSA exhibit similar chemical properties due to high percentage of sequence identities. BSA in lieu of HSA was used in this study because of low cost and easy availability. In BSA varying binding sites are available for ligands [14-15]. Ranges of techniques are available to monitor the binding interactions of ligands to protein viz. NMR [16], isothermal titration calorimetry (ITC) [17], UV- visible absorbance [18], fluorescence [19],

*Corresponding author. E-mail:  pradiptekade@gmail.com

FT-IR and CD spectroscopy [20].

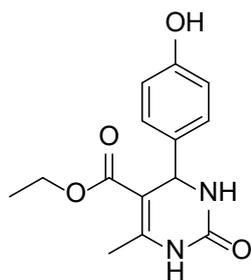


Figure 1. Ethyl 4-(4-hydroxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4-HP2OTP)

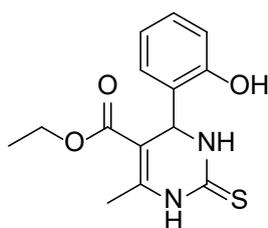


Figure 2. Ethyl 4-(2-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (2-HP2STP).

In the view of above considerations, present study demonstrates the effect of drug concentration, temperature and polar/non-polar solvent on binding interaction of ligands to BSA at

physiological pH and determination of thermodynamic parameters like free energy, enthalpy and entropy.

2. Results and Discussion

Equilibrium dialysis

The binding parameters of 4-HP2OTP-BSA and 2-HP2STP-BSA complexes have been determined using scatchard analysis. Scatchard curves obtained by plotting the absorbance and specific binding against the concentrations of ligands. Different observations have noticed for both the drugs in all the solvents. The association constants for 4-HP2OTP are found to be 0.7845 (± 0.0005), 0.7185 (± 0.0005), 0.7305 (± 0.0005) in 1,4-dioxane, DMSO and DMF respectively. While the association constants for 2-HP2STP are in 1,4-dioxane, DMSO and DMF are 0.7405 (± 0.0005), 0.7265 (± 0.0005), 0.7265 (± 0.0005) respectively. It has been seen that, the binding affinity of 4-HP2OTP is slightly more than 2-HP2STP in 1,4-dioxane than DMSO and DMF. The scatchard analysis of binding of 4-HP2OTP & 2-HP2STP with BSA at pH 7.4 in all the solvents were provided a non-linear curve. This suggests the presence of at least two binding sites for the binding of the ligands to BSA. Figures 3 and 4 shows the scatchard plots of BSA-ligands complexes in 1,4-dioxane, DMSO and DMF.

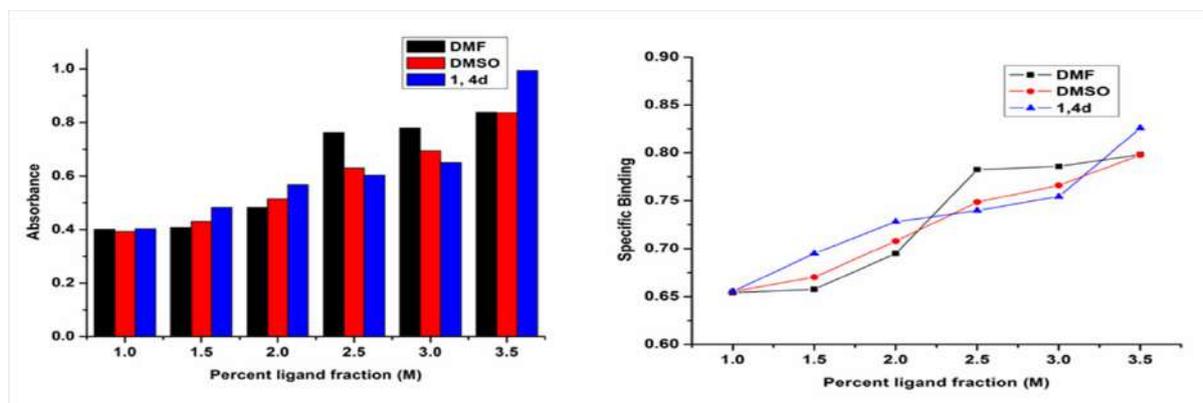


Figure 3. Scatchard plot of 4-HP2OTP-BSA complex in 1,4-dioxane, DMSO, DMF at room temperature.

Effect of foreign particles

The binding analysis of 4-HP2OTP and 2-HP2STP to BSA is also studied in presence of foreign particles. Results obtained are interpreted

by the Scatchard plot (Figure 5 and 6). The association constants for 4-HP2OTP-BSA and 2-HP2STP-BSA complexes in presence of foreign particles are 0.7006 (± 0.0005) & 0.5757 (± 0.0005) for Hg and 0.6782 (± 0.0005) & 0.6855 (± 0.0005)

respectively. The association constant for 4-HP2OTP and 2-HP2STP in absence of impurities of foreign particles were $0.7305 (\pm 0.0005)$ and $0.7261 (\pm 0.0005)$. It is noticed that the binding of

these ligands is decreased in presence of foreign particles may be due to packing of binding sites in the BSA.

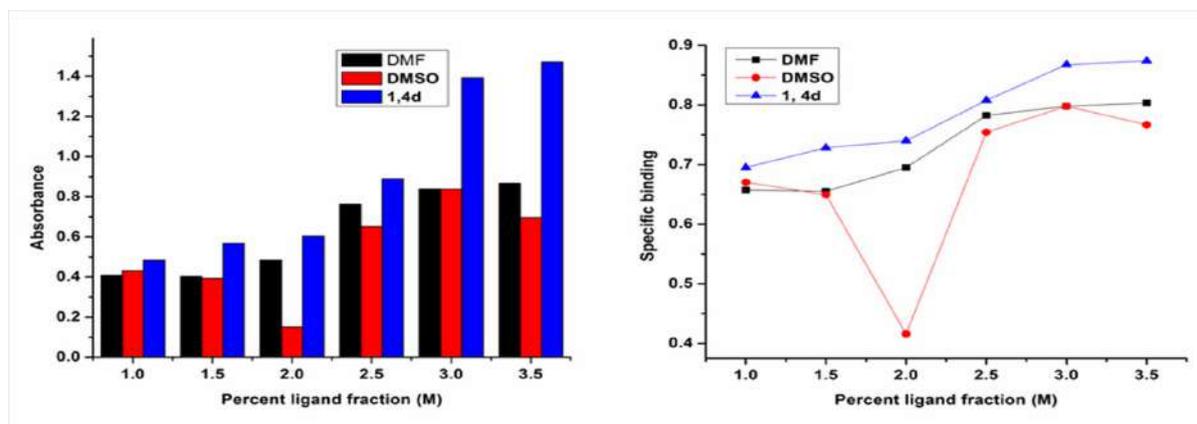


Figure 4. Scatchard plot of 2-HP2STP -BSA complex in 1,4-dioxane, DMSO, DMF at room temperature.

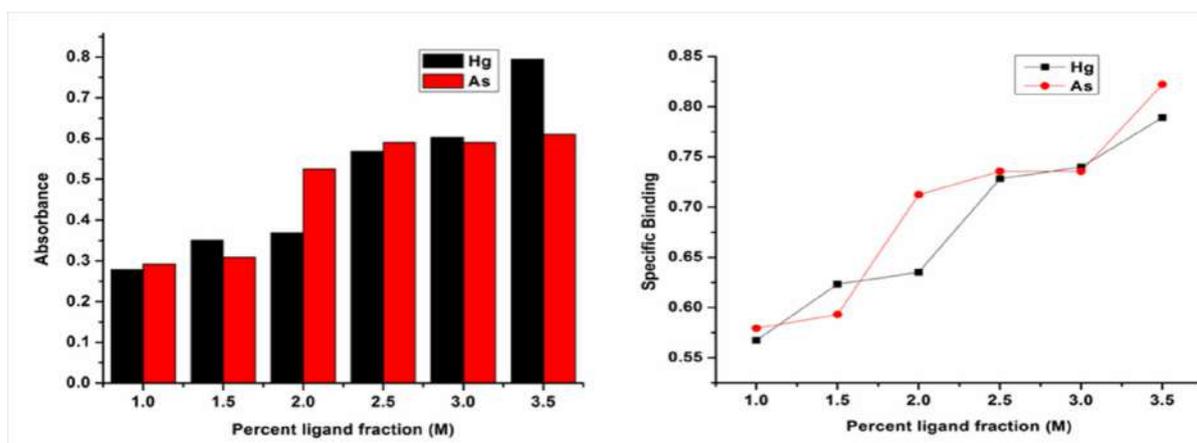


Figure 5. Effect of As and Hg on scatchard plot of 4-HP2OTP-BSA complex in 1, 4 dioxane.

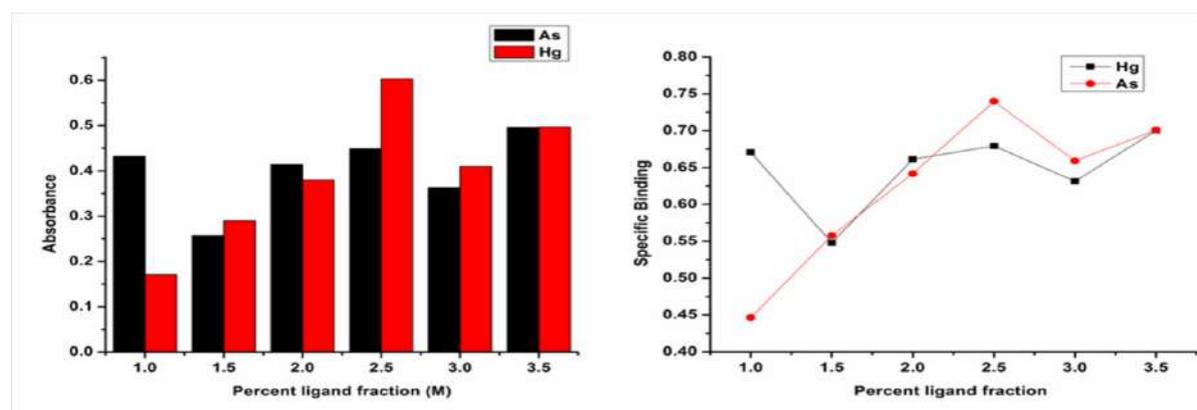


Figure 6. Effect of As and Hg on scatchard plot of 2-HP2STP-BSA complex in 1,4-dioxane.

FT-IR analysis

The protein drug binding by FT-IR spectroscopy is analyzed by shifting of amide bands in BSA. The amide I band at 1635 cm^{-1} is due to C=O stretching and amide II band at 1543 cm^{-1} is due to C-N stretch coupled with N-H bending. A change in frequency of these bands is observed on binding of ligands to BSA. Similar to ultrasonic and equilibrium dialysis study, more binding is observed in case 1,4-dioxane than DMSO & DMF. The peak position of amide I is shifted from 1635 to 1650 cm^{-1} in 4-HP2OTP and to 1644 cm^{-1} in 2-HP2STP as compared to BSA. However, a very small change is observed in the shifting of amide II band in the complex. It is noticed that, as the concentration of ligands increases the frequency of the binding of amide I bands is also increases (Figure 7 and 8). As amide I band is more sensitive to the changes of secondary structure of BSA than amide II therefore increase in binding is characterized by shift in amide I band. Similarly, BSA-ligands binding are not as much as significant in case of DMSO and DMF.

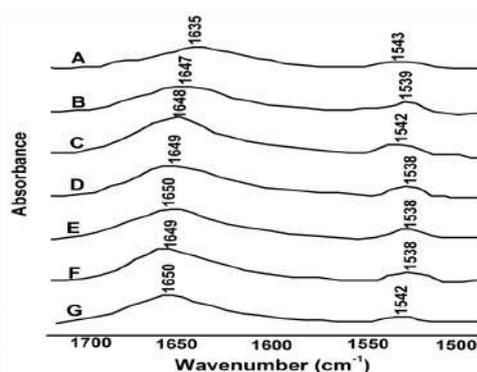


Figure 7. FT-IR spectra of 4-HP2OTP-BSA complex in 1,4-dioxane.

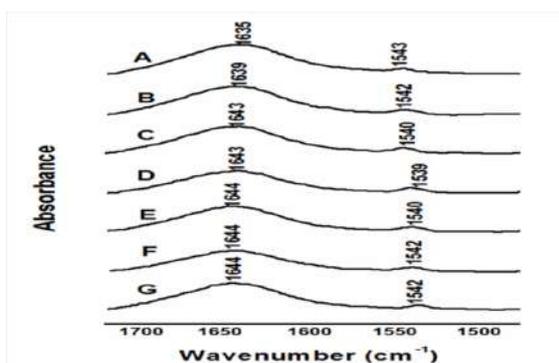


Figure 8. FT-IR spectra of 2-HP2STP-BSA complex in 1,4-dioxane.

Ultrasonic study

In present study, ultrasonic velocities of $0.15\text{ }\mu\text{M}$ BSA are measured at temperature 298, 303 and 308K and are found to be 1390.301, 1393.720 and 1398.105 m/s respectively. Ultrasonic velocities of 4-HP2OTP-BSA and 2-HP2STP-BSA complexes are also measured at various concentrations and temperatures (Table 1 and 2). It is observed that different values are obtained at different concentrations and temperatures for the ligands.

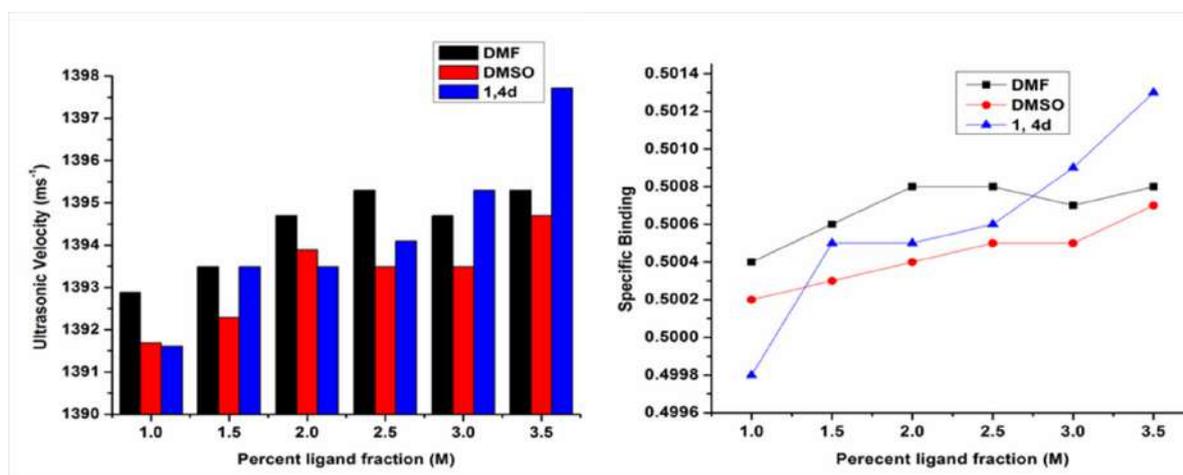
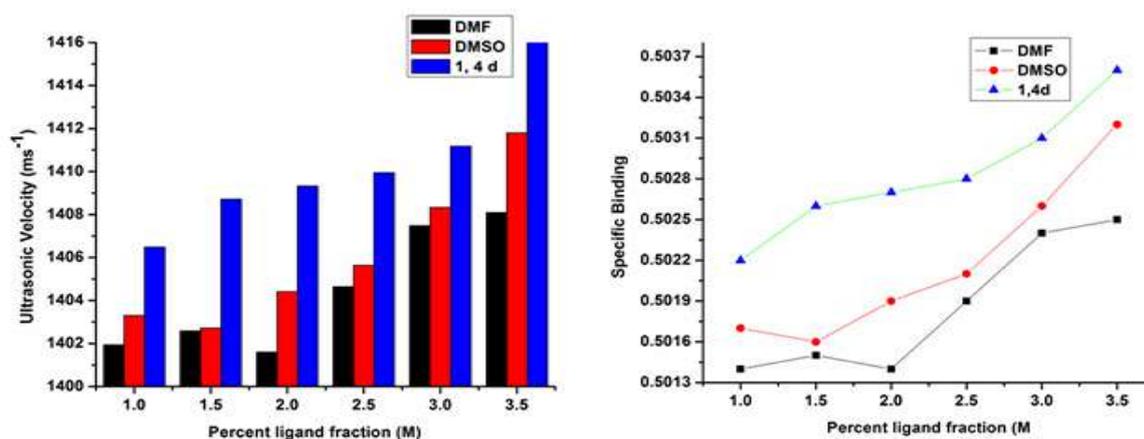
The Scatchard graph has plotted against ultrasonic velocity and specific binding versus percent ligand fraction. Binding parameters of 4-HP2OTP and 2-HP2STP to BSA have been determined using Scatchard plot. The Scatchard analysis gave different association constants at different temperatures and solvents. It is found that the association constants in 1, 4-dioxane at 308K is higher than 298K and 303K than DMSO and DMF. It means that the association constants for binding are more significant in 1,4-dioxane at high temperature, concluding that the binding increases with the increased in temperature. Binding is more significant in 1,4-dioxane than DMSO and DMF which is due to aprotic and non-polar nature of 1,4-dioxane. Comparatively binding is more significant in 4-HP2OTP than 2-HP2STP. From the structural analysis it is evident that, more binding of 4-HP2OTP is may be due to greater hydrogen bonding of oxygen in 4-HP2OTP than sulphur in 2-HP2STP. This increase of association constants in 1, 4-dioxane at high temperature clearly indicates the endothermic nature of reaction. This supports the interaction of ligands to BSA by means of Vander Waal's interactions and hydrogen bonds in the hydrophobic packet of binding sites. It is also observed that binding affinity increased as the concentration of the ligands increases; this probably enhances the pharmacological activity of the drug. Figures 9 to 11 shows the Scatchard plots of BSA-4-HP2OTP binding in 1,4-dioxane, DMSO and DMF at 298, 303 and 308 K respectively. Similarly figures 12 to 14 shows the Scatchard plots of BSA-2-HP2STP binding in 1,4-dioxane, DMSO and DMF at 298, 303 and 308 K, respectively. The effect of temperature on BSA-ligands binding is summarized in van't Hoff equation.

Table 1. Ultrasonic velocities of 4-HP2OTP -BSA complex solutions at diff. conc. and temperature.

Temp	25 °C			30 °C			35 °C		
	1,4-Dioxane	DMSO	DMF	1,4-Dioxane	DMSO	DMF	1,4-Dioxane	DMSO	DMF
1	1391.607	1391.688	1392.890	1406.481	1403.301	1401.943	1414.413	1413.599	1412.266
1.5	1393.492	1392.289	1393.492	1408.711	1402.720	1402.584	1416.757	1414.271	1414.892
2	1393.492	1392.890	1394.698	1409.327	1404.416	1401.599	1417.379	1418.626	1415.513
2.5	1394.095	1393.492	1395.301	1409.943	1405.641	1404.641	1418.003	1418.003	1417.379
3	1395.301	1393.492	1394.698	1411.177	1408.327	1407.481	1422.096	1419.251	1416.757
3.5	1397.720	1394.698	1395.301	1416.125	1411.794	1408.096	1424.266	1419.876	1419.876
BSA	1390.301	1390.301	1390.301	1393.720	1393.720	1393.720	1398.105	1398.105	1398.105

Table 2. Ultrasonic velocities of 2-HP2STP -BSA complex solutions at diff. conc. and temperature.

Temp	25 °C			30 °C			35 °C		
	1,4-Dioxane	DMSO	DMF	1,4-Dioxane	DMSO	DMF	1,4-Dioxane	DMSO	DMF
1	1401.974	1385.806	1400.756	1409.559	1408.381	1409.943	1420.501	1416.003	1416.626
1.5	1401.365	1385.708	1401.365	1411.794	1410.559	1410.559	1420.754	1417.379	1418.003
2	1402.584	1398.148	1401.974	1410.559	1411.177	1411.794	1421.637	1418.626	1418.626
2.5	1403.805	1398.933	1402.756	1414.271	1412.413	1410.559	1424.266	1419.847	1421.172
3	1404.416	1399.148	1403.194	1416.135	1411.794	1412.413	1426.155	1421.127	1422.381
3.5	1406.933	1403.194	1404.416	1422.381	1413.032	1413.032	1431.855	1421.754	1423.637
BSA	1378.010	1378.010	1378.010	1408.148	1408.148	1408.148	1406.867	1406.867	1406.867

**Figure 9.** Scatchard plot of 4-HP2OTP-BSA complex in 1,4-dioxane, DMSO, DMF at 25 °C.**Figure 10.** Scatchard plot of 4-HP2OTP-BSA complex in 1,4-dioxane, DMSO, DMF at 30 °C.

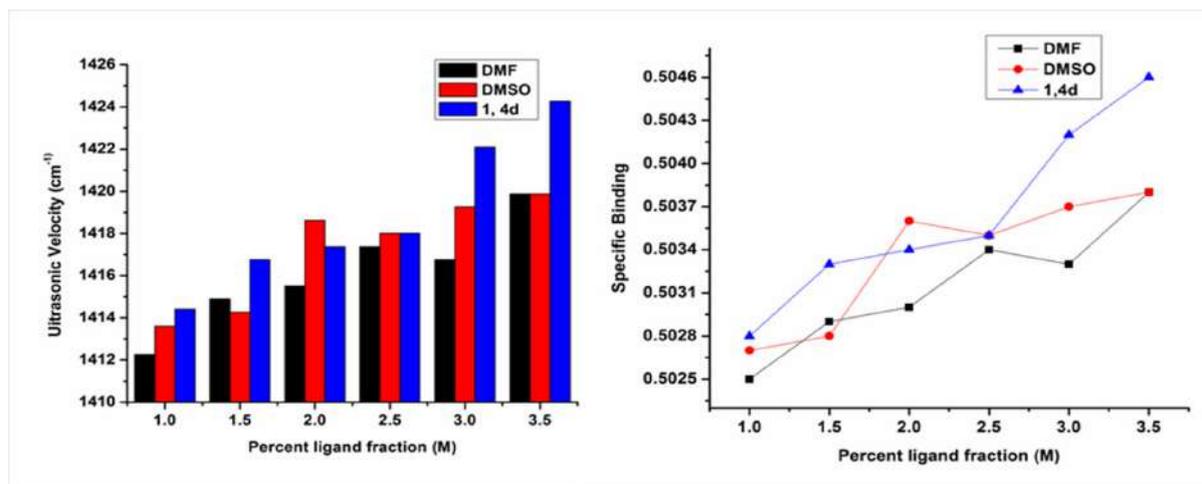


Figure 11. Scatchard plot of 4-HP2OTP-BSA complex in 1,4-dioxane, DMSO, DMF at 35 °C.

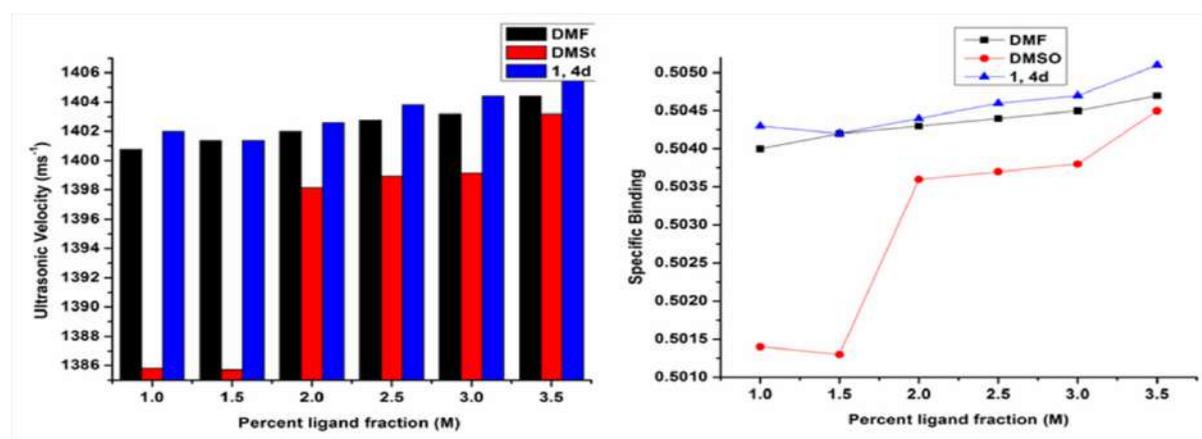


Figure 12. Scatchard plot of 2-HP2STP-BSA complex in 1,4-dioxane, DMSO, DMF at 25 °C.

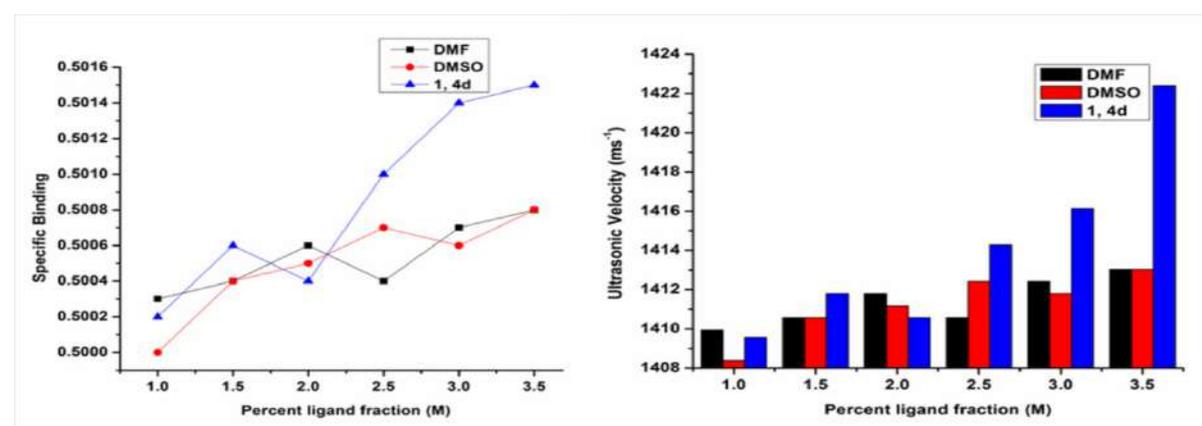


Figure 13. Scatchard plot of 2-HP2STP-BSA complex in 1,4-dioxane, DMSO, DMF at 30 °C.

Thermodynamic study

In order to elucidate the interaction of ligands to the BSA, the thermodynamic parameters (ΔG , ΔH and ΔS) have been calculated by using van't Hoff equation at the temperatures 298, 303 and

308 K. The enthalpy change is calculated from the slope of the van't Hoff relationship.

$$\ln k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (1)$$

Graph plotted between $\ln k$ vs $1/T$ shows

straight line with positive slope (Figure 15 and 16).

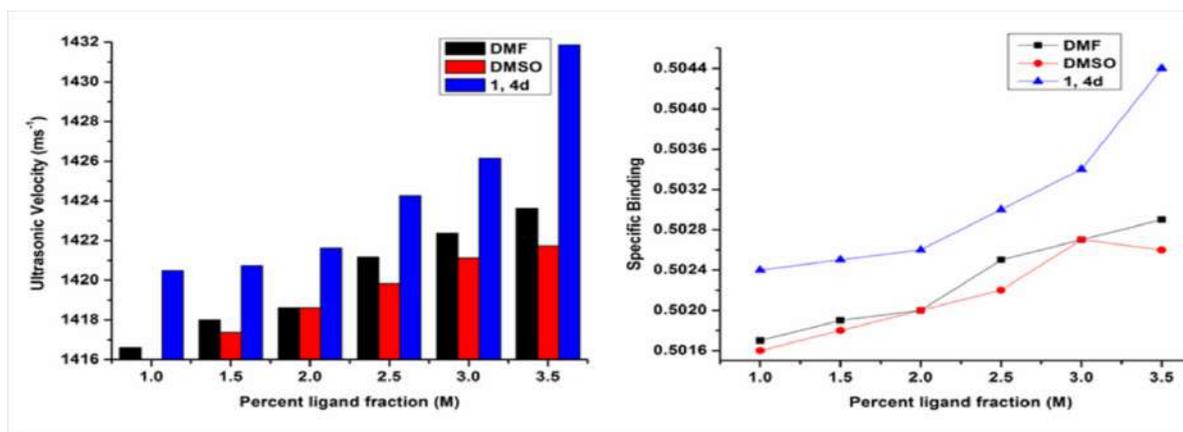


Figure 14. Scatchard plot of 2-HP2STP-BSA complex in 1,4-dioxane, DMSO, DMF at 35 °C.

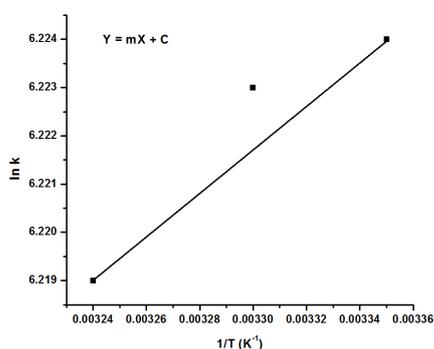


Figure 15. Graph of $\ln k$ vs $1/T$ in 1,4-dioxane for 4-HP2OTP-BSA complex.

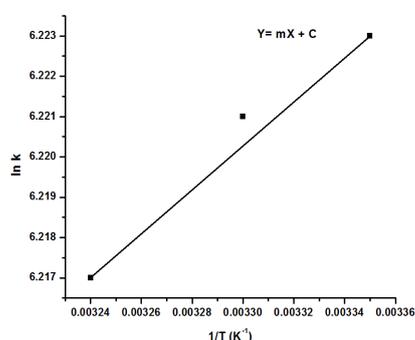


Figure 16. Graph of $\ln k$ vs $1/T$ in 1,4-dioxane for 2-HP2STP-BSA complex.

Positive values of ΔH and ΔS indicate that ligands interaction to BSA are enthalpy and entropic driven. Positive values of entropy indicate that there is unfolding of BSA. For unfolding, process must be endothermic which is indicated

by positive values of enthalpy and entropy (table 3 and 4). The specific electrostatic interaction is also characterized by the positive values of enthalpy and entropy. The negative value of ΔG indicates that the ligands-BSA complexation is a spontaneous process. As the temperature increased the negative value of ΔG is also increases, which concluded the ligands -BSA interaction is more feasible at high temperature. So, the hydrogen bonding, electrostatic and hydrophobic interactions are supposed to be possible factors contributing binding of the ligands to BSA. Slightly greater free energy values for 4-HP2OTP than 2-HP2STP is due to greater intermolecular hydrogen bonding of oxygen than Sulphur.

Table 3. Thermodynamic parameters at different temperature of 4-HP2OTP-BSA in 1,4-dioxane.

Sr. No.	Temp. (k)	ΔH J/mol	ΔG kJ/mol	ΔS J/mol
1	298 k	554.12	-14.343	49.99
2	303 k		-14.593	
3	308 k		-15.843	

Table 4. Thermodynamic parameters at different temperature of 2-HP2STP-BSA in 1,4-dioxane.

Sr. No.	Temp. (k)	ΔH J/mol	ΔG kJ/mol	ΔS J/mol
1	298 k	554.12	-14.313	49.89
2	303 k		-14.562	
3	308 k		-14.812	

3. Material and Methods

Dialysis membrane (molecular weight cut off 3500) used in the experiment purchased from Sigma Chemical Co. (USA). UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan) and metabolic shaking incubator (REMI RS-24AC) used in the experiment. FT-IR measurements were taken at room temperature on a Bruker FT-IR spectrometer (Alpha model, Germany) equipped with Zn-Se attenuated total reflection (ATR) accessory. Multi-frequency ultrasonic interferometer (VI microsystem, Chennai, India), BSA (essential fatty acid free) purchased from Chemsworth Chemicals Ltd (India) and used without further purification. Basic buffer selected to maintain the physiological pH. For the synthesis, all the chemicals used are of A.R. grade of Merck India Limited and purchased from commercial suppliers. The purity of the synthesized compound was ascertain by thin layer chromatography on silica gel G in petroleum ether and ethyl acetate (7:3) mixture, melting point recorded using digital melting point apparatus Equiptronics (EQ 730). ^1H NMR spectra of the compound recorded in CDCl_3 on NMR instrument (500MHz) using TMS as an internal standard from

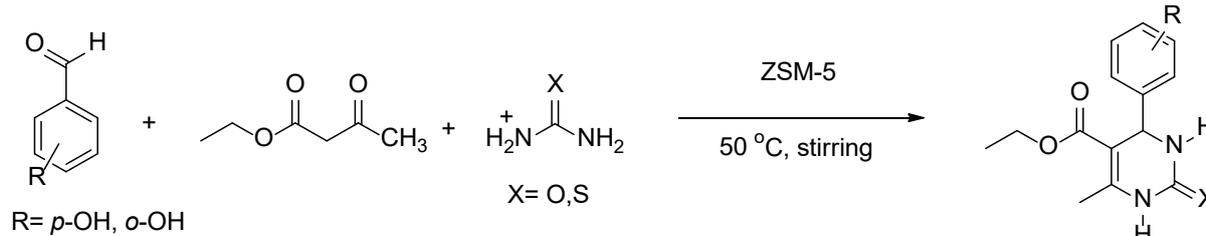
SAIF, CDRI Lucknow.

Optimization study

4-HP2OTP and 2-HP2STP are not completely soluble in buffer. Hence mixture of buffer with non-aqueous solvent such as 1,4-dioxane, DMF & DMSO used to dissolve 4-HP2OTP and 2-HP2STP. Different ratio of buffer: non-aqueous solvent tried, but the complete solubility of these drugs was obtained at optimum ratio 30:70:non-aqueous solvent:buffer.

Preparation of 4-HP2OTP and 2-HP2STP

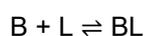
A one-pot synthesis of dihydropyrimidine derivatives has been carried out via Biginelli reaction using zeolite ZSM-5 as a catalyst under solvent free condition (Scheme 1). A mixture of aromatic aldehydes (4.71 mmol), ethylacetoacetate/(4.71 mmol), urea/thiourea (7.07 mmol) and catalyst zeolite ZSM-5 (10 wt%) in relation to the amount of aldehyde used was heated at 50°C for 10-25 min [21].



Scheme 1. Preparation of 4-HP2OTP and 2-HP2STP.

Measurements of binding affinity

For the Scatchard analysis, binding affinity of BSA and 4-HP2OTP and 2-HP2STP is expressed as an association constant or binding constant which is derived from the law of mass action. BSA (B) interacts with the ligands (L) i.e. 4-HP2OTP and 2-HP2STP to form the complex as given below.



$$\text{Hence, association constant } K_a = \frac{[\text{BL}]}{[\text{B}][\text{L}]}$$

Binding strength of the ligand to BSA is a measure of association constants.

Equilibrium dialysis

Different concentrations (1×10^{-3} M to 3.5×10^{-3} M) of 4-HP2OTP and 2-HP2STP in 1,4-dioxane, DMSO, DMF (30:70:solvent:buffer) were prepared and mixed separately with $0.15 \mu\text{M}$ BSA solution. These solutions were allowed to stand at room temperature for the maximum binding of ligands to BSA. From each mixture 3.5 ml solution was poured into previously prepared semi-permeable membrane and both the ends were sealed properly. The membrane tubes having 4-HP2OTP-BSA and 2-HP2STP-BSA complex solutions were immersed in a 100 ml conical flask

containing 40 ml buffer solution each. These conical flasks placed in a metabolic shaker for dialysis for 12 hrs at room temperature. After dialysis, absorbance's of bound fraction of 4-HP2OTP and 2-HP2STP to BSA were measured on a UV spectrophotometer (λ_{\max} 520 nm).

Effect of foreign particles

The binding study of 4-HP2OTP-BSA and 2-HP2STP-BSA is carried out in presence of foreign particles in 1,4-dioxane. 0.1M solution of arsenic and mercury salts were prepared and mixed with same solutions of ligands and BSA. These mixed complex solutions were kept some time to check the effect of foreign particles on binding.

FT-IR spectroscopic study

Different concentrations of 4-HP2OTP and 2-HP2STP and the BSA as mentioned above mixed and allowed to stand at room temperature for maximum binding. FT-IR measurements were carried out at room temperature on FT-IR spectrometer equipped with Zn-Se attenuated total reflection (ATR) method. All spectra taken via the ATR method with a resolution of 4 cm^{-1} and 60 scans in the region $1800\text{-}1300\text{ cm}^{-1}$. Absorbances of complexes were measured at room temperature.

Ultrasonic study

Ultrasonic is a versatile non-destructive and highly investigatory technique. Ultrasonic absorption in a medium provides important tools for the evaluation of the structural, chemical and physical properties of the medium [22]. Initially ultrasonic interferometer set at 1MHz. Different concentrations of 4-HP2OTP and 2-HP2STP mixed with BSA and allowed to stand for some time to get maximum binding, ultrasonic velocities of these complex solutions were recorded at 298K. Similar steps were performed at 303K and 308K to determine ultrasonic velocities of the complexes. Specific binding and association constants are determined using Scatchard plot.

4. Conclusions

In the present study, the binding interaction of

4-HP2OTP and 2-HP2STP to BSA has been studied by equilibrium dialysis, FT-IR spectroscopy and acoustical study at physiological pH in various solvents. The scatchard analysis provided a non-linear curve on binding of ligands to the BSA, suggested the presence of at least two binding sites in BSA. The experimental result by equilibrium dialysis and acoustical study clearly indicate that 4-HP2OTP and 2-HP2STP interact to BSA by means of Vander Waal's interactions and hydrogen bonds in the hydrophobic packet of binding sites. It is also observed that binding affinity increases with increased in the concentrations and temperatures; this probably enhances the pharmacological activity of the drugs. FT-IR spectroscopy showed the binding mainly through amide I site by hydrophobic interaction, which changes the secondary structure of BSA. The greater binding is observed in 4-HP2OTP than 2-HP2STP due to greater intermolecular hydrogen bonding of oxygen than sulphur. Aprotic and non-polar nature of 1,4-dioxane supports the binding of 4-HP2OTP and 2-HP2STP to BSA than DMSO and DMF. The thermodynamic parameters also indicated that the hydrogen bonding, electrostatic and hydrophobic interactions induce alterations in secondary structure of the BSA.

Supporting Information

[¹H NMR, UV and IR spectra of 4-HP2OTP and 2-HP2STP.](#)

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