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FULL PAPER

In vitro Binding Interaction of Isoxazoline Derivative with BSA: Equilibrium, FT-IR, Acoustical and Molecular Modeling Study

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

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

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Abstract:

The present study showed the binding interaction of 2-(4,5-Dihydro-1,2-oxazol-5-yl)-phenol-*N*-methylaniline (2DHOPNA) with BSA in 1,4-dioxane, DMSO and DMF by equilibrium dialysis, FT-IR, acoustical at physiological pH and its molecular modeling study. Findings were interpreted by scatchard plot which showed an increase in association constants with increasing temperature and concentrations of the 2DHOPNA. It is seen that the binding supposed to be more significant in 1,4-dioxane than DMSO and DMF. Thermodynamic parameters are also determined for the binding interaction of 2DHOPNA with BSA. Values of Gibb's free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) were calculated by using van't Hoff equation. The positive values of ΔH and ΔS showed exothermic interaction between 2DHOPNA and BSA. Similarly, negative ΔG showed the spontaneity of the binding process. ΔG becomes more negative with increase in temperature, indicated feasibility of the binding interaction at high temperature. Molecular modeling confirmed the binding interaction having energy of -210.13.

Keywords: equilibrium dialysis; FT-IR; scatchard analysis; association constants; BSA; thermodynamic parameters

1. Introduction

2-(4,5-Dihydro-1,2-oxazol-5-yl)-phenol-*N*-methylaniline (**1**, 2DHOPNA, Figure 1) is an important heterocyclic compounds shows various biological properties especially, herbicidal [1], antioxidant [2], antifungal [3], antibacterial [4], analgesic and antimicrobial [5] and anti-cancer properties [6]. Human serum albumin (HSA) is the most abundant protein in blood serum with the concentration of 0.63 mM. It is single polypeptide chain of 585 amino acids with a large helical triple domain structure that forms heart shaped molecule. 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 [7]. A variation in temperature is found to be a key factor in binding affinities of HSA [8]. It is difficult to obtain HSA for experimental purposes. HSA and BSA exhibit similar chemical properties due to high percentage of sequence identities. BSA in lieu of HSA is used in this study because of low cost and easy availability. Various techniques are available to monitor the binding interactions of ligands to protein like U.V. visible absorbance [9], isothermal titration calorimetry [10], fluorescence [11], NMR [12], equilibrium and FT-IR spectroscopy [13]. These techniques are used to study the binding interaction of the various drugs with protein such as Phenformin [14], Ligustrazine [15], aspirin and vitamin C [16], Ciprofloxacin [17] and methotrexate [18]. Molecular modeling study is also an important aspect towards protein-drug interaction [19-20].

Present study proposed to evaluate the effect of ligand concentration, temperature, and polar/nonpolar solvent on binding interaction of 2DHOPNA with BSA at physiological pH. The

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

study also involves the determination of thermodynamic parameters like free energy, enthalpy, entropy and its molecular modeling.

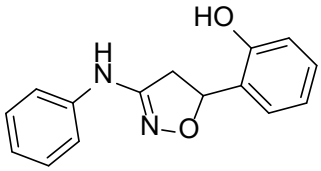


Figure 1. Structure of 2-(4, 5-Dihydro-1,2-oxazol-5-yl)-phenol-*N*-methylaniline (**1**, 2DHOPNA).

2. Results and Discussion

The binding interaction gives different absorbance values for BSA-2DHOPNA complexes in 1,4-dioxane, DMSO and DMF. The absorbance values for BSA-2DHOPNA complex solutions in 1,4-dioxane are 0.192, 0.227, 0.395, 0.412, 0.457 and 0.619 for the different concentrations of 2DHOPNA i.e. 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM and 3.5 mM respectively. Similarly, the absorbance values for BSA-2DHOPNA complexes in DMSO are 0.180, 0.191, 0.213, 0.325, 0.312, 0.497 and absorbance values in DMF are 0.162, 0.167, 0.198, 0.202, 0.258, 0.392 for the concentrations 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM and 3.5 mM, respectively. Figure 2 shows the graph of absorbance versus concentrations of 2DHOPNA in 1,4-dioxane, DMSO and DMF. Similarly, Figure 3 shows the graph for specific binding against concentrations of 2DHOPNA in 1,4-dioxane, DMSO and DMF. Figure 3 is the scatchard plot used to determine binding affinity of 2DHOPNA with BSA. The binding affinity of these complexes has been determined in terms of association constants. The association constants for BSA-2DHOPNA binding in 1,4-dioxane, DMSO and DMF are 0.6100, 0.5800, 0.5411, respectively. Table 1 gives the absorbances and specific binding for the interaction of 2DHOPNA with BSA in 1,4-dioxane, DMSO and DMF.

Table 1. Absorbance and specific binding values for BSA-2DHOPNA complex at different concentrations in 1,4-dioxane, DMSO and DMF.

Sr.No.	Concentra-tions of 2DHOPNA (mM)	BSA + 2DHOPNA in 1,4-dioxane		BSA + 2DHOPNA in DMSO		BSA + 2DHOPNA in DMF	
		Absorbance	Specific Binding	Absorbance	Specific Binding	Absorbance	Specific Binding
1	1	0.192	0.4752	0.180	0.4591	0.162	0.4331
2	1.5	0.227	0.5170	0.191	0.4739	0.167	0.4406
3	2	0.395	0.6507	0.213	0.4988	0.198	0.4829
4	2.5	0.412	0.6602	0.325	0.6052	0.202	0.4879
5	3	0.457	0.6831	0.312	0.5954	0.258	0.5489
6	3.5	0.619	0.7448	0.497	0.7009	0.392	0.6490

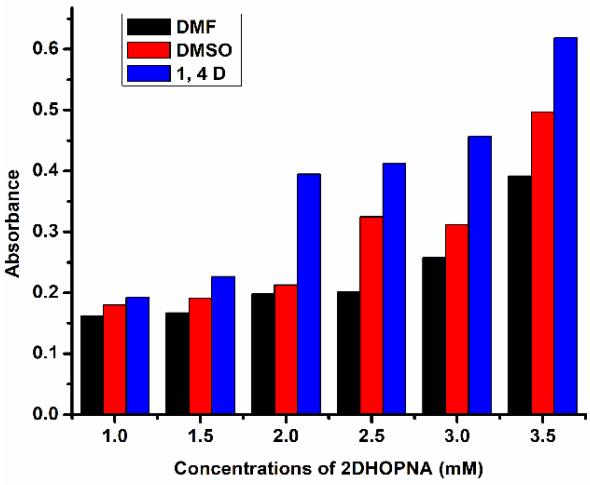


Figure 2. Graph of absorbance vs. conc. of 2DHOPNA in 1,4-dioxane, DMSO and DMF.

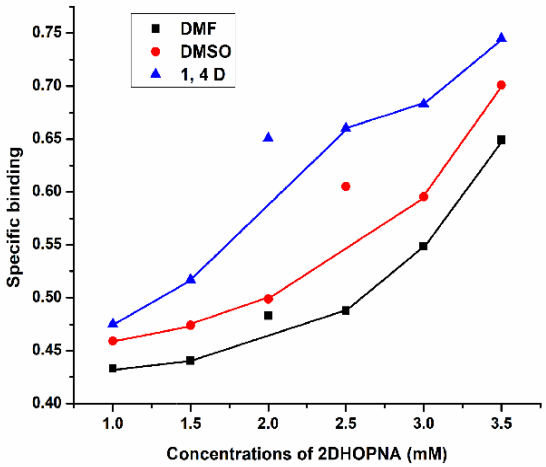


Figure 3. Graph of specific binding *vs.* conc. of 2DHOPNA in 1,4-dioxane, DMSO and DMF.

2.1 Effect of Arsenic and Mercury on Binding Interaction of 2DHOPNA with BSA

The effect of foreign particles viz. arsenic and mercury was studied on the binding interaction of 2DHOPNA with BSA. Different values of absorbance for BSA-2DHOPNA in 1,4-dioxane are obtained. Absorbances for BSA-2DHOPNA complex solutions in the presence of arsenic are 0.413, 0.427, 0.512, 0.601, 0.568 and 0.609 for different concentrations of 2DHOPNA 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM and 3.5 mM respectively. Similarly, the absorbances for complex solutions in the presence of mercury are 0.359, 0.359, 0.436, 0.493, 0.503 and 0.548 for the concentrations 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM and 3.5 mM, respectively. Figure 4 shows the graph of absorbance vs. concentrations of 2DHOPNA in 1,4-dioxane in presence of arsenic and mercury. Figure 5 shows the graph of specific binding against concentrations of 2DHOPNA in 1,4-dioxane in presence of arsenic and mercury. The scatchard plot gives the association constants for the interaction of 2DHOPNA with BSA. The association constants in presence of arsenic and mercury are 0.5943 and 0.5410 respectively. Table 2 gives the values of absorbance and specific binding for BSA-2DHOPNA complex in presence of arsenic and mercury.

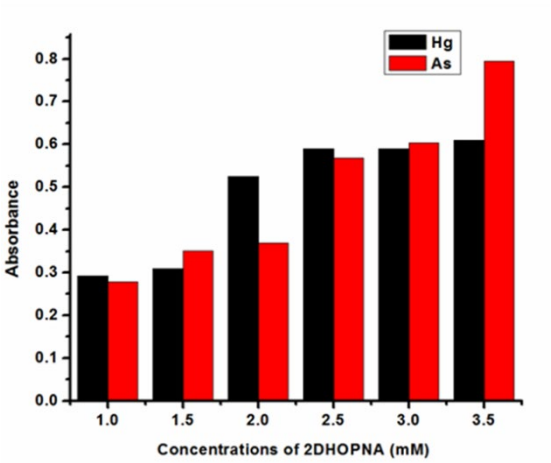


Figure 4. Graph of absorbance vs. conc. of 2DHOPNA in 1,4-dioxane in presence of arsenic and mercury.

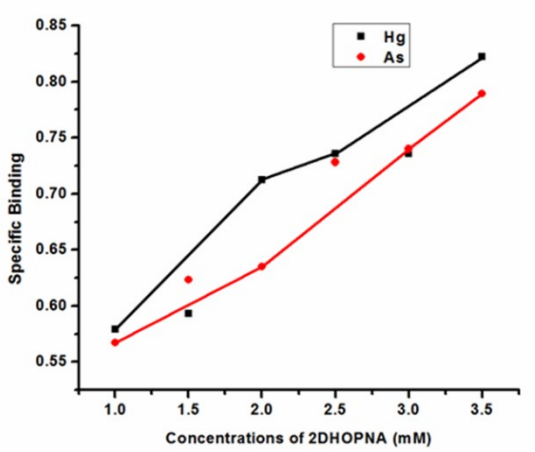


Figure 5. Graph of specific binding vs. conc. of 2DHOPNA in 1,4-dioxane in presence of arsenic and mercury.

Table 2. Absorbance and specific binding values for BSA-2DHOPNA complex in presence of arsenic and mercury at different concentrations of 2DHOPNA in 1,4-dioxane.

Sr.No.	Concentra-tions of 2DHOPNA (mM)	Hg + BSA+ 2DHOPNA in 1,4-dioxane		As + BSA+ 2DHOPNA in 1,4-dioxane	
		Absorbance	Specific Binding	Absorbance	Specific Binding
1	1	0.168	0.5000	0.212	0.4421
2	1.5	0.192	0.5479	0.257	0.4764
3	2	0.227	0.5867	0.301	0.5170
4	2.5	0.318	0.6524	0.398	0.6000
5	3	0.369	0.6547	0.402	0.6351
6	3.5	0.402	0.6886	0.469	0.6547

2.2 FT-IR Study

FT-IR spectroscopy analyzed the binding interaction of 2DHOPNA with BSA by the shifting in peak positions of amide bands in BSA. The amide I at 1635 cm^{-1} is due to C=O stretching and amide II at 1543 cm^{-1} is due to C-N stretch coupled with N-H bending. The changes in peak positions of these bands are observed on interaction of 2DHOPNA with BSA. It is seen that the peak position of amide I is shifted to greater extent, however, a very small change in the amide II has observed (Figures 6-8). Therefore, it is concluded that amide I band is more sensitive to the changes of secondary structure of BSA than amide II. Figure 6 shows the changes in the

peak positions of amide bands of BSA-2DHOPNA complex in 1,4-dioxane. Similarly, the shifting of amide bands in DMSO and DMF are shown in figure 7 and 8, respectively. In figures 6, 7 and 8, A shows spectrum of BSA and B, C, D, E, F, G shows the spectra of BSA-2DHOPNA complexes for the concentrations 1, 1.5, 2, 2.5, 3, 3.5 mM of 2DHOPNA respectively.

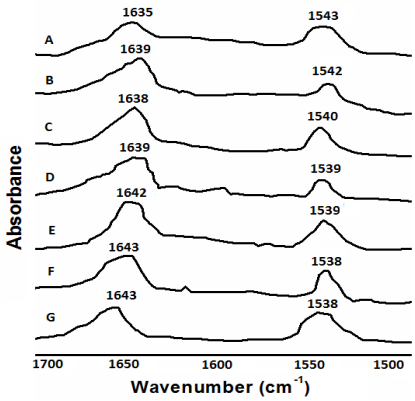


Figure 6. Stacked FT-IR spectra of BSA (A) and BSA-2DHOPNA (B-G) complexes in 1,4-dioxane.

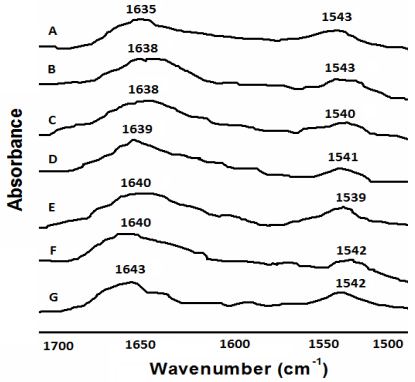


Figure 7. Stacked FT-IR spectra of BSA (A) and BSA-2DHOPNA (B-G) complexes in DMSO.

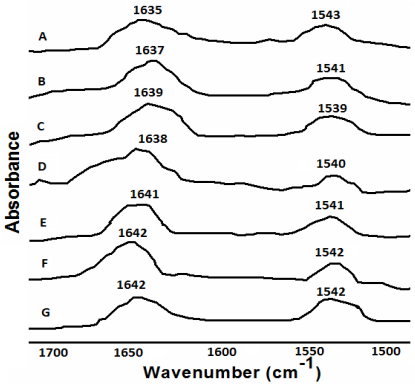


Figure 8. Stacked FT-IR spectra of BSA (A) and BSA-2DHOPNA (B-G) complexes in DMF.

2.3 Acoustical Study

Initially, ultrasonic velocities of BSA solution are measured at physiological pH. The ultrasonic velocities are 1482.192, 1483.157 and 1485.220 m/s at 298, 303 and 308K, respectively. Similarly, ultrasonic velocities of ligand 2DHOPNA-BSA complexes were measured at different concentrations and temperatures 298, 303 and 308K (Table 3). The scatchard graph is plotted for specific binding versus concentration of 2DHOPNA and from this plot binding constants have been determined. The Scatchard plot gives different association constants at different temperatures for 2DHOPNA-BSA binding interaction. The association constants in 1,4-dioxane are 0.5022, 0.5025, 0.5026 at 298, 303 and 308K respectively. Similar analysis was carried out in DMSO and DMF and association constants have been calculated. The association constants in DMSO are 0.5014, 0.5020, 0.5021 and in DMF are 0.5016, 0.5019, 0.5019 at 298, 303 and 308 K, respectively.

Table 3. Ultrasonic velocities of 2DHOPNA -BSA complexes at diff. conc. and temperature.

Temp. (K) / Conc (mM)	Ultrasonic velocities at temperatures 298, 303 and 308 K								
	298 K			303 K			308 K		
	1,4-Dioxane	DMSO	DMF	1,4-Dioxane	DMSO	DMF	1,4-Dioxane	DMSO	DMF
1	1401.688	1395.301	1399.289	1405.266	1402.599	1403.413	1410.757	1408.209	1407.003
1.5	1401.509	1397.190	1399.921	1407.122	1404.806	1403.194	1413.499	1408.209	1407.614
2	1402.612	1397.612	1400.011	1410.381	1405.788	1405.805	1413.539	1409.211	1410.696
2.5	1403.499	1401.590	1401.499	1410.626	1407.048	1407.933	1415.870	1410.277	1412.959
3	1405.529	1402.554	1402.850	1412.637	1410.194	1408.416	1418.076	1413.191	1415.959
3.5	1407.720	1405.698	1403.492	1413.606	1410.513	1409.003	1418.892	1415.147	1415.512
BSA	1392.111	1392.111	1392.111	1395.172	1395.172	1395.172	1400.147	1400.147	1400.147

It is seen that the association constants increase with the increase in temperature and concentrations. Figures 9 to 11 shows the

scatchard plots of BSA-2DHOPNA binding interaction in 1,4-dioxane, DMSO and DMF respectively. The effect of temperature on BSA-2DHOPNA binding interaction is summarized in van't Hoff equation.

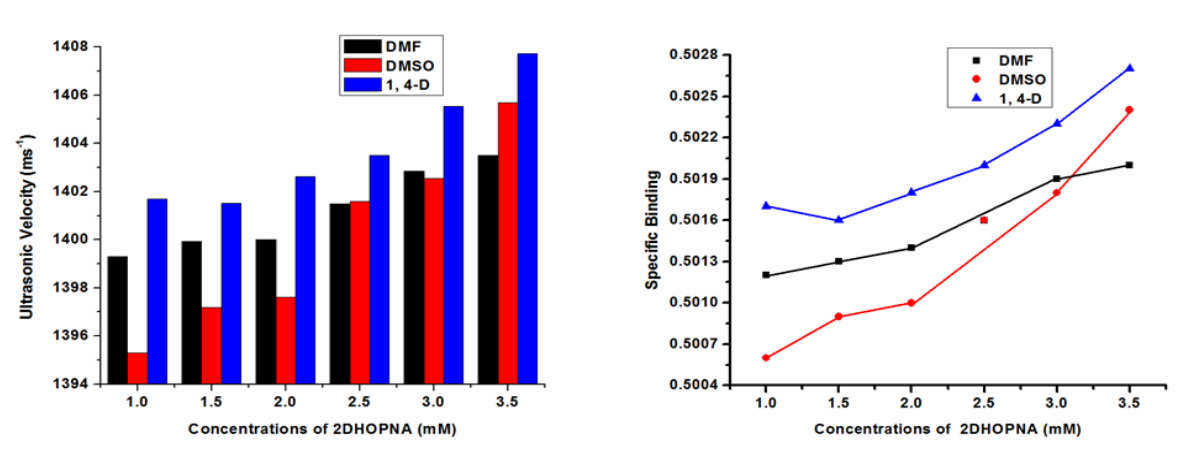


Figure 9. Scatchard plot of BSA-2DHOPNA complex in 1,4-dioxane, DMSO and DMF at 298 K.

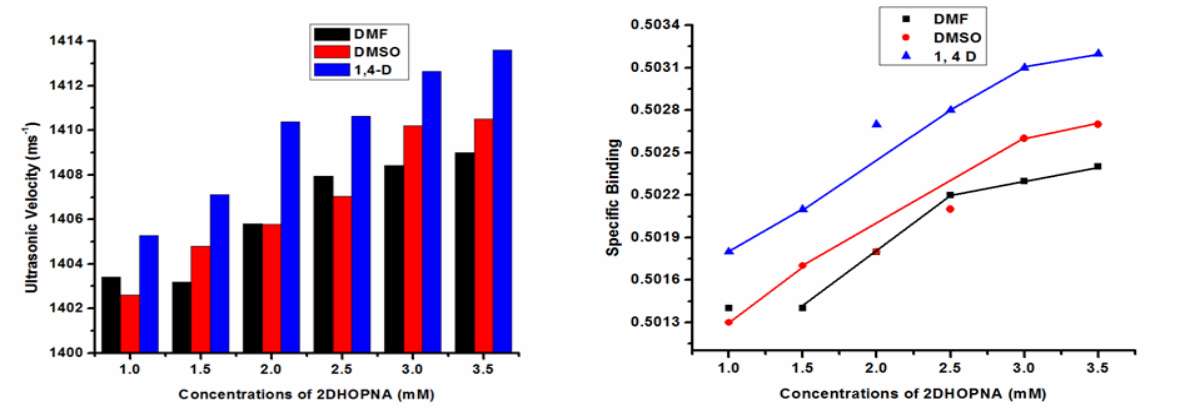


Figure 10. Scatchard plot of BSA-2DHOPNA complex in 1,4-dioxane, DMSO and DMF at 303 K

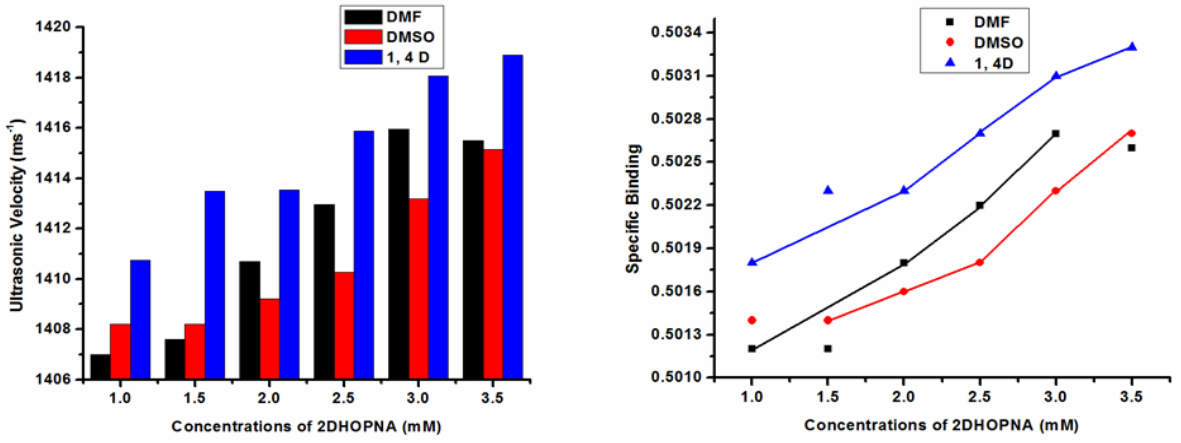


Figure 11. Scatchard plot of BSA-2DHOPNA complex in 1,4-dioxane, DMSO, DMF at 308 K.

2.4 Thermodynamic Study

In order to clarify the interaction of ligand 2DHOPNA with BSA, the thermodynamic parameters (ΔG, ΔH and ΔS) has been calculated by using van't Hoff equation at 298,

303 and 308 K (Table 4). The values of ΔH and ΔS were calculated from the slope & intercept of the plot of lnk vs 1/T respectively.

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

Graph plotted between **lnk vs 1/T** shows straight line with negative slope, as shown in Figure 5.

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

Sr. No.	Temp. (k)	ΔH J/mol	ΔG kJ/mol	ΔS J/mol
1	298 k		-14.573	
2	303 k	75.57	-14.819	49.16
3	308 k		-15.065	

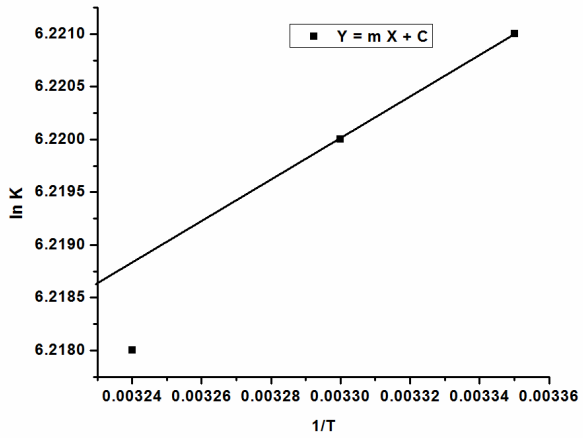


Figure 12. Van't Hoff plot of lnk vs 1/T in 1,4-dioxane for 2DHOPNA-BSA Complex.

Positive value of ΔS and ΔH indicate that drug interaction with BSA are enthalpy and entropic driven. Positive value of entropy also shows that there is unfolding of BSA (Table 2). The specific electrostatic interaction is also characterized by the values of enthalpy and entropy. The negative value of ΔG supports the 2DHOPNA-BSA complexation is feasible process at high temperature. Thus, the overall stability of the complexes is indicated by Gibbs free energy. So, the hydrogen bonding, electrostatic and hydrophobic interactions are supposed to be possible factors contributing binding 2DHOPNA with BSA. The thermodynamic parameters in DMSO & DMF are not significant as that of 1,4-dioxane.

2.5 Molecular Modeling Study

Molecular modeling is also an efficient method for measurement of interaction between protein and drug. Furthermore, the binding interaction between BSA and 2DHOPNA is studied by molecular modeling. The energy obtained is the measure of binding interaction of ligand 2DHOPNA with BSA. The value of energy is -210.13, which shows efficient binding interaction of 2DHOPNA with BSA. Diagrammatic representation for the interaction of 2DHOPNA with BSA is shown in Figure 13.

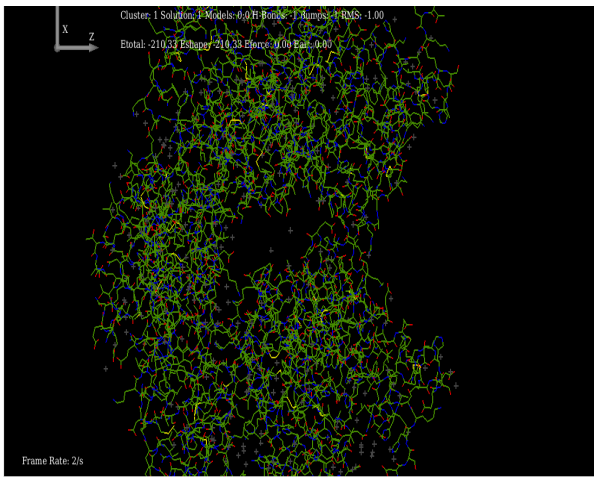


Figure 13. Molecular modeling interaction between BSA and 2DHOPNA.

3. Materials and Methods

Multi-frequency ultrasonic interferometer (VI microsystem, Chennai, India), FT-IR measurements were taken at room temperature on a Bruker's FT-IR spectrophotometer (Alpha model, Germany) equipped with Zn-Se attenuated total reflection (ATR) accessory. UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan) and metabolic shaking incubator (REMI RS-24AC) used in the experiment. BSA (essential fatty acid free) purchased from Chemsworth Chemicals Ltd (India) and used without further purification. (USA). 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.

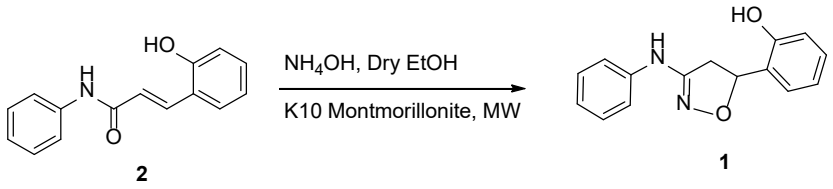
3.1 Optimization Study

2DHOPNA is insoluble in basic buffer at

physiological pH. Hence mixture of buffer with non aqueous solvent such as 1,4-dioxane, DMSO and DMF were used to dissolve 2DHOPNA. Different ratio of buffer: non-aqueous solvents were tried, but the complete solubility of 2DHOPNA was obtained at optimum ratio 30:70: non-aqueous solvent: buffer.

3.2 Preparation of 2DHOPNA

2-(4,5-dihydro-1,2-oxazol-5-yl)-phenol-*N*-methylaniline (**1**) was synthesized by known method [21]. The mixture of purified 3-(2-hydroxyphenyl)-*N*-phenylprop-2-enamide (**2**) (0.01 mol), hydroxylamine hydrochloride (0.01 mol), K-10 Montmorillonite and a solution of NaOH (10%) in dry ethanol (10 mL) by using microwave irradiation for a period of 80 seconds (Scheme 1). After completion of the reaction the resultant mass was poured into ice water (10 mL) with constant stirring. It was kept in cool overnight. The resultant solid product was filtered, washed with sufficient cold water, dried and purified by recrystallization from acetone.



2 **1**

Scheme 1. Synthesis of 2-(4, 5-dihydro-1,2-oxazol-5-yl)phenol-*N*-methylaniline (**1**, 2DHOPNA).

3.3 Measurement of Binding Affinity

The binding interaction of 2DHOPNA with BSA is expressed as binding constant or association constant, which is derived from the law of mass action. BSA (B) interacts with the 2DHOPNA (L) to form the complex is given as

$$B + L \rightleftharpoons BL$$

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

Binding strength of the ligand 2DHOPNA with BSA is a measure of association constants.

3.4 Equilibrium Dialysis

The solutions of 2DHOPNA of different concentrations 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 3.5 mM in 1, 4-dioxane, DMSO, DMF (30:70:: solvent: buffer) and 0.15 µM BSA were prepared. These solutions were mixed in 1:1 proportion and allowed to stand for maximum binding interaction. Each 3.5 mL complex solution poured into previously prepared semi-permeable membrane and both the ends were sealed properly. The BSA-2DHOPNA complex solutions tubes immersed in 100 mL conical flask containing 40 mL buffer. These conical flasks placed on a metabolic shaker for dialysis for 12 hrs at room temperature. On complete dialysis absorbance of bound fraction of 2DHOPNA with BSA was measured on UV spectrophotometer at 520 nm.

3.5 Effect of Foreign Particles

The binding interaction of 2DHOPNA with BSA was studied in presence of foreign particles. 0.1M solutions of arsenic and mercury salts were prepared and mixed with 2DHOPNA-BSA complex solutions. To study the effect of foreign particles on binding interaction these ternary complex solutions were kept for some time. On complete dialysis absorbance of bound fraction in presence of foreign particles was also measured.

3.6 FT-IR Study

FT-IR measurements were carried out on Bruker's FT-IR spectrophotometer equipped with Zn-Se attenuated total reflection (ATR) method. Different concentrations of 2DHOPNA and BSA were mixed and allowed to stand at room temperature for maximum interaction. On maximum binding interaction absorbances of BSA-2DHOPNA complexes were measured.

3.7 Acoustical Study

The binding interaction of 2DHOPNA with

BSA was also studied by acoustical study. Initially multifrequency ultrasonic interferometer is set at 1MHz and different concentrations of ligand 2DHOPNA in 1,4-dioxane, DMSO, DMF has been prepared. 0.15μM BSA solution was prepared in basic buffer at physiological pH 7.4 and its ultrasonic velocity was measured. Concentrations of 2DHOPNA mixed with BSA at 298 K and allowed to stand for 1 hr for maximum binding. Then, ultrasonic velocities of these complex solutions were recorded. Similar steps are performed at 303 K and 308 K and specific binding along with association constants have been determined using Scatchard plot.

3.8 Molecular Modeling Study

Hex 8.0 gives the value of efficient energy for the binding interaction of ligand with BSA. The Molecular modeling study of 2DHOPNA with BSA is carried out on Hex 8.0 software. PDB file of the crystal structure of BSA is obtained from the RCSB data bank with ID 4F5S. Then 3D structure of 2DHOPNA was developed. The obtained 3D structure arranged in a minimized energy form. The PDB files of ligands and BSA runs together on Hex 8.0, which gives the energy value of the newly formed complex showing its stability.

4. Conclusions

In this study, the binding interaction of 2-(4,5-Dihydro-1,2-oxazol-5-yl)-phenol-*N*-methylaniline (**1**, 2DHOPNA) with BSA in 1,4-dioxane, DMSO and DMF BSA has been reported by equilibrium dialysis, FT-IR, acoustical study at physiological pH and its molecular modeling study. This study also involves determination of thermodynamic parameters. It is seen that association constants for BSA-2DHOPNA binding interaction increased with increase in temperatures, which clearly indicate exothermic nature of reaction. FT-IR spectroscopy shows the binding mainly through amide I site by hydrophobic interaction, which changes the secondary structure of BSA. The scatchard analysis provided a non-linear curve for binding of ligand 2DHOPNA with BSA, suggested the presence of at least two binding sites in BSA. The acoustical study at different temperatures clearly indicates that 2DHOPNA interact with 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 2DHOPNA. It is found that binding interaction is more significant in 1,4-dioxane than DMSO and DMF. It may be due to aprotic and nonpolar nature of the 1,4-dioxane. The thermodynamic parameters also indicated that the hydrogen bonding, electrostatic and hydrophobic interactions induce alterations in secondary structure of the BSA. Molecular docking is also used to confirm the binding of 2DHOPNA with BSA. The binding energy -210.13 indicates that the complex formed is stable concluding 2DHOPNA is successfully bound with BSA.

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