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Study of the interaction of 2CA4MBA with Bovine Serum Albumin at varying pH using gel exclusion chromatography

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Abstract
In this study, we reported the interaction of the carboxamide derivative of amino acids 2-{[2- (cyclohexycarbamoyl) benzoyl] amino}-4-(methylsulfanyl) butanoic acid(2-CA4MBA) with Bovine Serum Albumin (BSA) using gel exclusion chromatography at pH 3, 4, 5, and 7.4. The association constant for 2CA4MBA was calculated using the Scatchard Plot, which was found to be 0.5807, 0.5706, 0.5616, and 0.5503 at pH levels of 3, 4, 5, and 7.4, respectively. The association constant of 2CA4MBA decreases as pH increases. The association of the compound with BSA has also been confirmed through a molecular modelling study using HEX software. The efficient energy value for the 2CA4MBA-BSA complex, neglecting the effect of any solvent, is found to be-235.66.
Keywords: *Gel Exclusion Chromatography, protein-drug binding, Scatchard analysis*

Introduction:

Transportation of drugs is one of the important characteristics of blood and binding of drugs is an efficient feature of protein which binds drug in plasma. Chirality is an intrinsic property. Chiral drugs have a number of applications in day-to-day life as well as in commercial areas like clinical, medicinal, and pharmaceutical industries and some metabolic activities of humans. Basically, there are three types of proteins in the blood that have the ability to bind the drug, out of that alpha-1-acid glycoprotein (AGP) has the ability to bind basic drugs; lipoprotein binds drugs on saturation of other protein and Human Serum Albumin (HSA) shows an affinity for acidic drugs.

In drug transportation, HSA plays a vital role depending upon the percentage of binding of drug to HSA. It is difficult to obtain HSA for experimental purposes. HSA and BSA exhibit similar chemical properties due to a high percentage of sequence identity. BSA instead of HSA was used in this study because of its low cost and easy availability. BSA has a high molecular weight (M_r = 66,500) with alkaline properties, having a pH range of 7-8, which binds to the acidic portion of the ligand. The full -length BSA precursor protein is 607 amino acids in length. *N*-terminal 18-residue signal peptide is cut off from the precursor protein upon secretion; hence, the initial protein product contains 589 amino acid residues. An additional 4 amino acids are cleaved to yield the mature BSA protein that contains 583 amino acids, having an isoelectric point ^{of} 4.7 at 25 °C in water and an extinction coefficient of 43824 $M^{-1}cm^{-1}$ at 279 nm. BSA is one of the major proteins in the blood. Therefore, changes in the level of BSA in plasma during physiological and pathological conditions can have a profound effect on drug deposition and pharmacological activity. The binding of drugs with proteins is of different type like hydrophobic interactions, hydrogen bonding, van der Waals forces, electrostatic attraction, etc.

Various analytical techniques are available, which show their importance in the field of drug analysis and binding of drugs with proteins, such as ATR-FTIR technique [1], equilibrium dialysis [2], nuclear magnetic resonance [3], FT-IR and Molecular modeling[, fluorescence spectrophotometry [5], capillary electrophoresis [06], mass spectrophotometry [07]. We have synthesized *2-{[2-(cyclohexycarbamoyl) benzoyl]amino}-4-(methylsulfanyl) butanoic acid* 2CA4MBA by known methods [08] and also characterized it by IR, ^{1}H NMR and mass spectroscopy. Ligand (L) also exhibited antibacterial activity. Ligand (L) shows acidic nature and affinity for the BSA protein. In this paper, we have reported for the first time the binding affinity of ligand L with BSA using gel exclusion chromatography technique. Molecular modelling software HEX 8.0 was used for confirmation of the binding affinity of ligand (L) with BSA.

Materials and Methods:

For the chromatographic experiment, Sephadex G-25 having water regain 2.5-0.2ml/g (make Sigma Chemical Ltd.(Sweden), Bovine Serum Albumin (M_r = 66,500) (make-chemsworth

chemical Ltd., India) were used. 0.1 M sodium acetate buffer of pH 3, 4, 5 and 7.4 was used. U.V. Visible Spectrophotometer Shimadzu Singapore (Model UV 180) was used for measurement of absorbance value.

Measurement of binding affinity :

A gel column was prepared using Sephadex G-25 in a buffer solution. It was allowed to stand overnight to develop the column. The buffer solution was allowed to flow continuously to keep the column wet. 50 uM A BSA solution and a 0.01 M solution of ligand (L) in sodium acetate buffer solution were prepared. Different sets of mixtures of BSA and ligand L were prepared in the following proportions: 10:0, 9:1, 8:2, 7:3, 6:4, 5:5 and 6:4. These mixtures of solutions were allowed to pass through the column separately, and the eluting buffer was passed continuously, which helped to run the sample solution through the column. The sample solution was collected from the lower end of the column in equal fractions, and the absorbance of each collected fraction was measured on the UV-Visible spectrophotometer. The buffer solutions of various pH levels were prepared, and solutions of the complex at these pH levels were made. Absorbance was recorded for the collected fractions, and the effect of pH on the absorbance value was observed.

Result and Discussion

The purity of the synthesized compound was ascertained by thin-layer chromatography on silica gel G in petroleum ether and ethyl acetate (7:3) mixture. Melting point was recorded using a digital melting point apparatus, Equiptronics (EQ 730). IR spectra were recorded on a Bruker alpha IR Spectrometer (Germany), expressed in cm^{-1} at the Department of Chemistry, J.B. College of Science, Wardha. ^{1}H NMR and ^{13}C NMR spectra of the compound were recorded in $CDCl_3$ on an NMR instrument (500MHz) using TMS as an internal standard from SAIF, CDRI Lucknow.

Chromatographic observation

50uM Solution of BSA was allowed to pass through the column in the absence of ligand and collected in fractions. Absorbance values were observed at the absorbance maximum of 520 nm. Then, afterward 50uM solution of a mixture of BSA and ligand was allowed to pass through the column. Then BSA-ligand-interacting fractions were collected through the column. The first peak in **Figure1** shows the binding of ligand with BSA, while the second peak represents the unbound ligand fraction. Absorbance values shows continuous increase in values of absorbance as the fraction of ligand increases in the composition. After a particular point, a gradual increase in absorbance values shows the saturation level of ligand. Chromatographic observations shows that the compound 2-{[2-(cyclohexycarbamoyl) benzoyl] amino}-4-(methylsulfanyl) butanoic acid binds more significantly to BSA. In **Figure 2, the** straight line indicates that there was only one interacting site available to the ligand for binding on BSA.

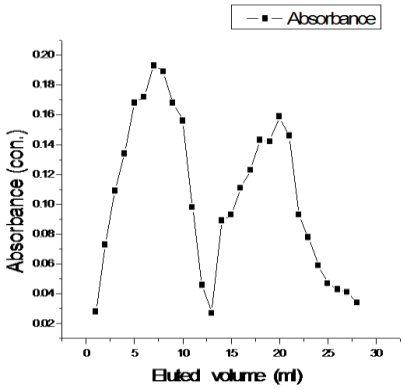


Figure 2. Graph of Absorbance V_S Eluted volume

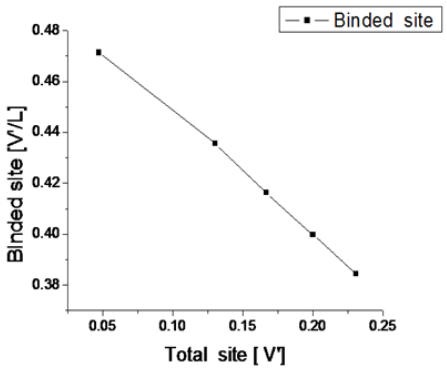


Figure 3. Graph of Total site to Binded site

Effect of pH: We have prepared the complex solution in a sodium acetate buffer of varying pH range of 3, 4 and 5. The change in the pH range of the buffer solution shows the effect of

binding of the ligand to the protein (BSA). At pH 3, binding were more significant in comparison with 4 and 5. The order of binding of ligand to BSA at various pH as 3 >4 > 5. This shows that the value of BSA-ligand binding is decreases as the pH value is increases. The value of the association constant (K_f) for BSA- ligand at pH 3,4 and 5, calculated from the Scatchard graph, is found to be 0.5934, 0.5825, 0.5506 and 0.5503, respectively. **Figure 3** shows the changes in absorbance value for the BSA-ligand complex at different compositions, and **Figure 4 shows the** Scatchard graph at pH 3. Similarly, **Figure 5** and **Figure 6** show changes in absorbance value for the protein-ligand complex at different compositions and the Scatchard graph at pH 4. Similarly, **Figure 7** and **Figure 8** show changes in absorbance value for the protein-ligand complex at different compositions and the Scatchard graph at pH 5 Similarly, **Figure 9** and **Figure 10** show changes in absorbance value for the protein-ligand complex at different compositions and the Scatchard graph at pH 7.4.

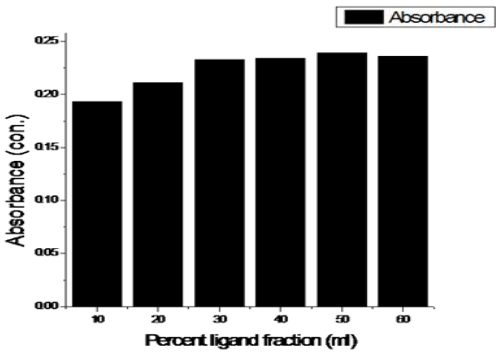


Figure 3 . Graph of Absorbance V_S Percent ligand fraction at pH 3

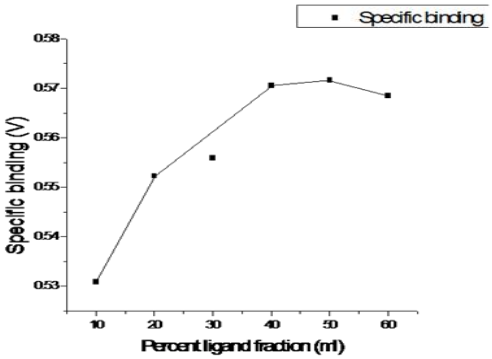


Figure 4. Specific binding Vs Percent ligand fraction at pH 3

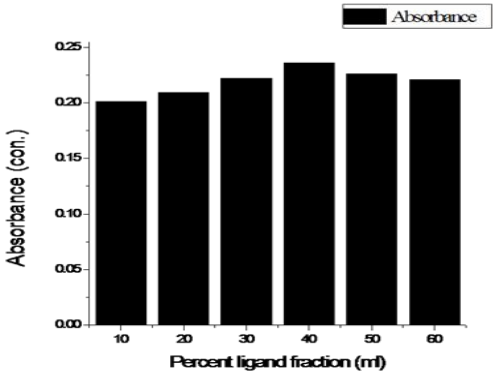


Figure 5. Graph of Absorbance V_S Percent ligand fraction at pH 4

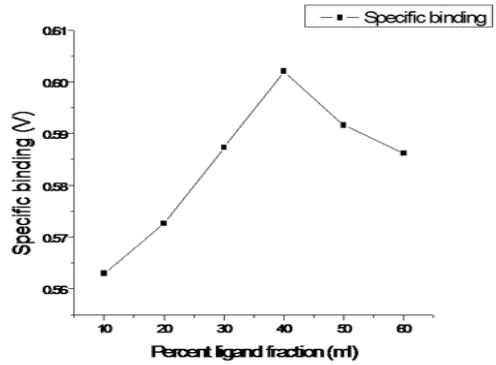


Figure 6. Specific binding Vs Percent ligand fraction at pH 4

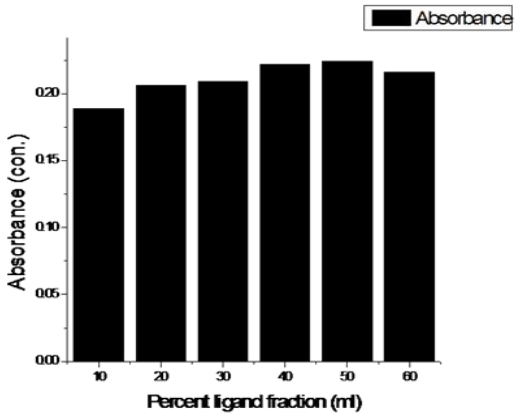


Figure 7. Graph of Absorbance V_S Percent ligand fraction at pH 5

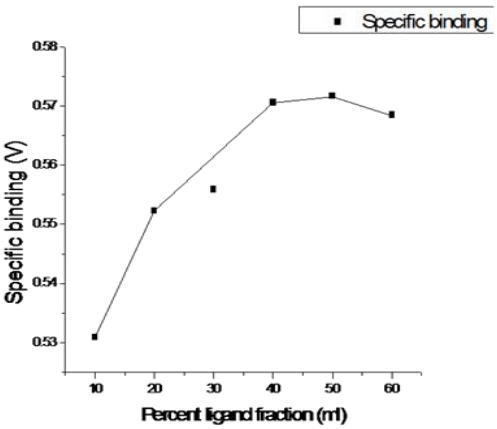


Figure 8. Specific binding Vs Percent ligand fraction at pH 5

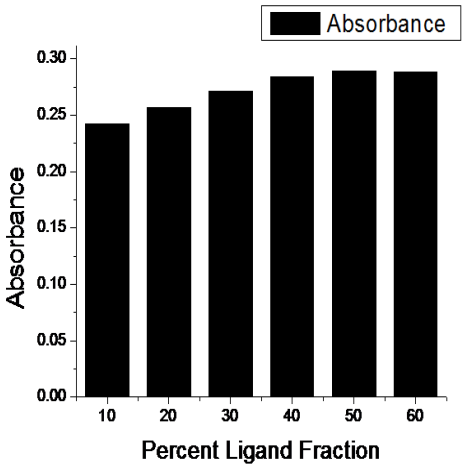


Figure 9. Graph of Absorbance V_S Percent ligand fraction at pH 5

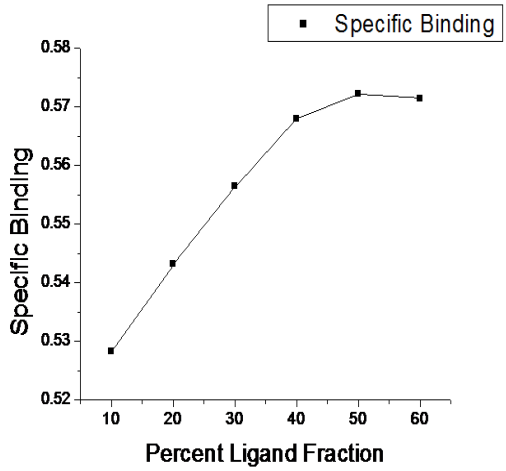


Figure 10. Specific binding Vs Percent ligand fraction at pH 5

Molecular modelling study for interaction between BSA and 2CA4MBA

Serum protein is the most studied and abundant protein in blood plasma, which accounts for nearly 60% of total protein which consist of a single polypeptide chain of 589 amino acids. In serum protein, there are mainly three binding sites, I, II, and III; each of these contains subdomains (A and B). Molecular modelling is also an efficient method for measuring the interaction between protein and drug. The energy value obtained from the study is a measure of the binding of the drug to the Serum protein. In order for further study the binding interaction between BSA and said ligand, a molecular modelling study was done. The crystal structure of BSA was obtained from the RCSB Protein Data Bank, and a 3D file for ligand developed on Chem Draw Ultra 8.0. Docking was done using the software HEX 8.0. The energy value obtained was -235.66 which shows that the mentioned ligand efficiently binds with protein BSA. Diagrammatic representation of interaction for interaction between BSA and the said ligand is shown below in **Fig.11**

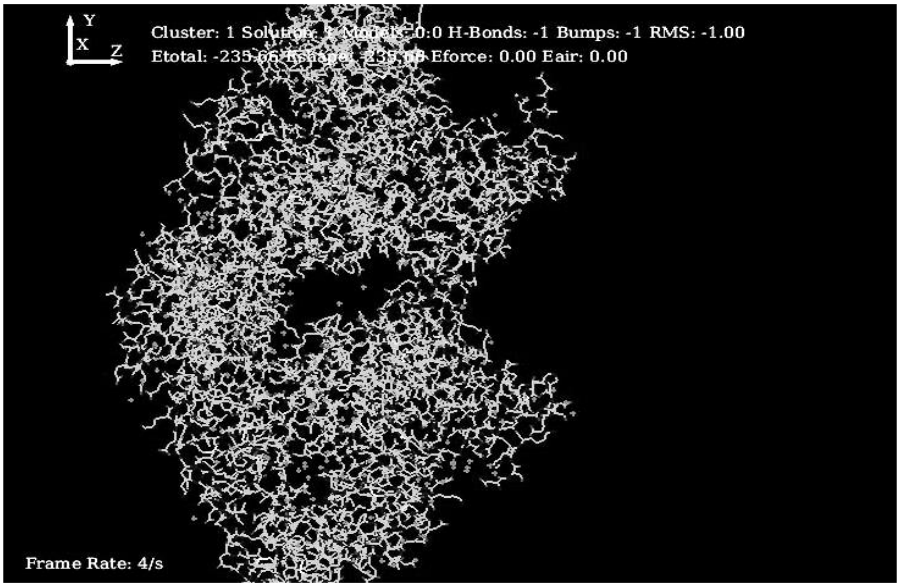


Figure 11. Interaction between BSA and 2-{[2-(cyclohexycarbamoyl) benzoyl]amino}-4-(methylsulfanyl) butanoic acid

Conclusion:

The chiral drug *2-{[2-(cyclohexycarbamoyl) benzoyl] amino}-4-(methylsulfanyl) butanoic acid*, showing antibacterial activity have efficiency to bind with the protein BSA. We have studied the novel interaction of this drug with the BSA protein using gel exclusion chromatographic technique. The study of the interaction of this drug with BSA shows that the drug successfully binds with BSA. The study of binding at various buffer pH 3, 4, 5 and 7.4 shows that the association constant (K_f) value was found to be higher at buffer pH 3 in comparison with pH 4, 5 and 7.4. The value of the association constant (K_f) calculated from Scatchard analysis are found to be 0.5934, 0.5825, 0.5506 and 0.5503 at pH 3,4, 5 and 7.4, respectively. This means that binding of 2CA4MBA with the protein BSA is more efficient at acidic pH, and it goes on decreasing with increasing pH value. Furthermore, molecular docking shows an efficient energy value of -235.66, which show confirmative binding approach of the mentioned ligand with the protein BSA.

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