*Journal of Photoscience* (2004), Vol. 11(1), pp. 29-33

# Spectroscopic Studies on the Mechanism of Interaction of Vitamin $B_{12}$ with Bovine Serum Albumin

B. P. Kamat and J. Seetharamappa*
Department of Chemistry, Karnatak University, Dharwad 580 003, India

The mechanism of interaction of cyanocobalamin (CB) with bovine serum albumin (BSA) has been investigated by spectrofluorometric and circular dichroism methods. Association constant for the CB-BSA system showed that the interaction is non-covalent in nature. Binding studies in the presence of an hydrophobic probe, 8-anilino-1-naphthalene sulphonic acid, sodium salt (ANS) showed that there is hydrophobic interaction between CB and ANS and they do not share common sites in BSA. Stern-Volmer analysis of fluorescence quenching data showed that the fraction of fluorophore (protein) accessible to the quencher (CB) was close to unity indicating thereby that both tryptophan residues of BSA are involved in drug-protein interaction. The rate constant for quenching, greater than $10^{10}M^{-1}s^{-1}$, indicated that the drug binding site is in close proximity to tryptophan residue of BSA. Thermodynamic parameters obtained from data at different temperatures showed that the binding of CB to BSA involves hydrophobic bonds predominantly. Significant increase in concentration of free drug was observed for CB in presence of paracetamol. Circular dichroism studies revealed the change in helicity of BSA due to binding of CB to BSA.

**key words:** Spectroscopic studies, Cyanocobalamin, bovine serum albumin

## INTRODUCTION

The binding of drugs by plasma proteins is an important pharmacokinetic parameter because it influences the size of the free fraction of the drug in plasma. The nature and magnitude of drug-protein interaction significantly influences the biological activity of the drug. Serum albumin being the major binding protein for the drugs and other physiological substances is considered as a model for studying drug-protein interaction in *in vitro*.[1] Vitamin $B_{12}$ or Cyanocobalamin (CB) is the only vitamin containing metal ion (trivalent cobalt). It is needed in many body processes; in the manufacture and the maintenance of red blood cells, the synthesis of DNA, the stimulate of nerve cells, the growth promotion and energy releasise, and the proper functioning of folic acid. Characteristic signs of $B_{12}$ deficiency include fatigue, weakness, nausea, constipation, flatulence (gas), loss of appetite, and weight loss. So, CB is being administered quite regularly. Literature survey revealed that the attempts have not been made so far to investigate the interaction between CB and BSA. Hence, it is planned to carry out a detailed study on the interaction of CB with BSA

Two common methods that have been used in evaluating the binding of drugs to albumin include equilibrium dialysis and ultrafiltration.[2-4] These methods are laborious and time consuming and the results, at times, are not reproducible. However, these conventional methods are often inapplicable to the analyses of strongly bound drugs because of technical problems such as drug adsorption on the membrane and the leakage of bound drug through membrane. To overcome these problems, we have employed fluorometric and circular dichroism methods to investigate the mode of interaction of CB with BSA in the present investigation.

## MATERIALS AND METHODS

Fluorescence measurements were performed on a Hitachi spectrofluorometer Model F- 2000 equipped with a 150W Xenon lamp and slit width of 10 nm. A 1.00 cm quartz cell was used. CD measurements were made on a JASCO-810 spectropolarimeter using a 1.00cm cell at 0.2 nm intervals, with 3 scans averaged for each CD spectrum in the range of 200-300 nm

Bovine Serum Albumin (BSA, Fraction V, approximately 99%; protease free and essentially γ-globulin free) and 8-anilino-1-naphthalene sulphonic acid sodium salt, $C_{16}NSO_3H_{13}Na$ (ANS) were obtained from Sigma Chemical Company, St Louis, USA. Vitamin $B_{12}$ was obtained from s. d. fine-chem Ltd, Mumbai, India. All other materials were of analytical reagent grade. The solutions of drug and BSA were prepared in 0.1M phosphate buffer of pH 7.4 containing 0.15 M NaCl. BSA solution was prepared based on molecular weight of 65,000.

*To whom correspondence should be addressed.
E-mail: jseetharam@yahoo.com/j_seetharam@rediffmail.com
Received January 15, 2004; Accepted March 30, 2004

## RESULTS AND DISCUSSION

*Fluorescence studies*

Some preliminary studies were carried out to select optimum protein and drug concentrations for drug-protein interaction. On the basis of preliminary experiments, BSA concentration was kept fixed at 10 mM and $B_{12}$ concentration was varied from 10 to 170 μM. Fluorescence spectra were recorded at room temperature (27°C) in the range 300-500 nm upon excitation at 296 nm. The absorbance of drug-protein mixtures in the concentration range employed for the experiment did not exceed 0.05 at the excitation wavelength in order to avoid inner filter effect. Fluorescence spectra of BSA were recorded in the presence of increasing amounts of CB. The emission spectra of CB are shown in Fig. 1. It was observed that the CB quenched the fluorescence of albumin with a blue shift (from 340 to 335 nm) and a small peak at 377 nm. The fraction of drug bound, θ, was determined according to Weber and Young[5], and Maruyama et al using the following equation $\theta = (F_o\text{-}F)/F_o$.....(1) where, F and $F_o$ denote the fluorescence intensities of protein in a solution with a given concentration of drug and without drug, respectively. Fluorescence data was analyzed using the method described by Ward.[6] It has been shown that for equivalent and independent binding sites

$$\frac{1}{(1-\theta)K} = \frac{[D_t]}{\theta} - n[P_T] \qquad (2)$$

where, K is the association constant for drug-protein interaction, n is the number of binding sites, [Dt] is the total drug concentration and $[P_T]$ is the total protein concentration. The plot of 1/(1- θ) *versus* [Dt]/θ for the drug is shown in Fig 2. The values of K and n, obtained from the slope and intercept of such plots are found to be $4.05 \times 10^3$ $M^{-1}$ and 14.0, respectively. Since the data fit equation 2 in all cases, it may be concluded that under the conditions of the experiment, all the binding sites are equivalent and independent. Standard free energy change, $\Delta G_0$ (at 27°C) was evaluated from K using the relationship $\Delta G_0 = -2.303RT \log K$. It was found to be -20.67 $KJmol^{-1}$ indicating the spontaneity of the reaction.

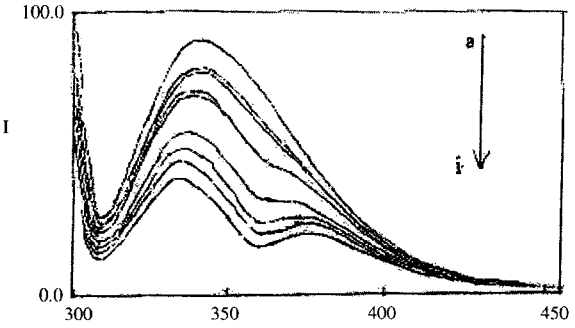


Figure 1. Fluorescence Spectra of BSA (10 μM) in the presence of CB(a-0, b-10, c-20, d-40, e-60, f-80, g-120, h-140 and .i-170 μM

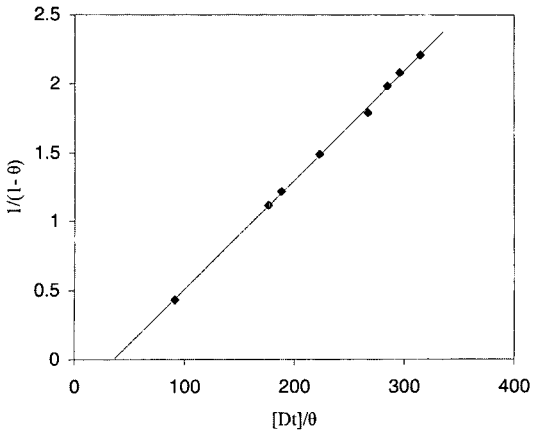


Figure 2. 1/(1- θ) versus [Dt]/θ plot for the binding of CB to BSA.

*Stern-Volmer analysis*

Fluorescence intensity data was also analyzed according to Stern-Volmer law[9], $F_o/F = 1 + K_q [Q]$ .....(3) by plotting $F_o/F$ versus [Q], where $F_o$ and F are the steady state fluorescence intensities at 344 nm in the absence and presence of quencher (drug) respectively and [Q] is the total drug concentration.

The Stern-Volmer plot (Fig. 3) showed positive deviation from straight line, suggesting the presence of a static component in the quenching mechanism.[7] A modified form of Stern-Volmer equation[7] that describes quenching data when both dynamic and static quenching are operative is

$$F_o/F = 1 + K_q [Q] \exp V [Q] \qquad (4)$$

where, $K_q$ is the collisional quenching constant or Stern-Volmer quenching constant and V is the static quenching constant. The value of V was obtained from equation 4 by plotting $[F_o/F \exp (V [Q])\}]$-1 *versus* [Q] for varying V until a linear plot was obtained. The $K_q$ was then obtained from the slope of $[F_o/F \exp (V [Q])\}]$-1 *versus* [Q] plot passing through origin (Fig. 4). The values of V and $K_q$ so obtained were observed to be $4.12 \times 10^3$ and $1.5 \times 10^4$ $M^{-1}$ respectively.

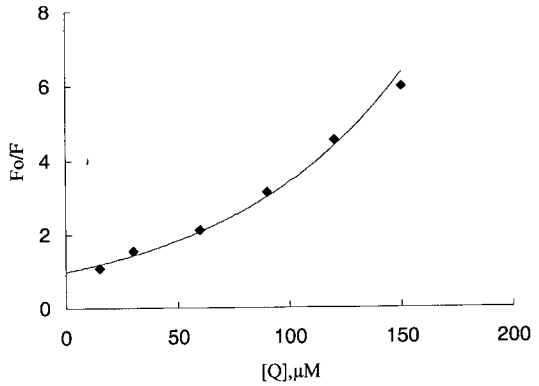


Figure 3. Stern-Volmer plot of $F_o/F$ *versus* [Q] for the binding of CB

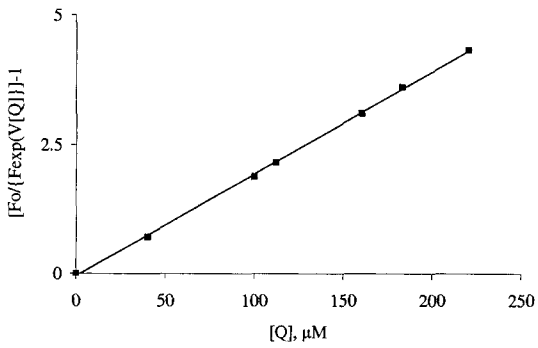


Figure 4. Plot of $[F_o/F \exp(V[Q])]$-1 *versus* [Q] for drug

According to Eftink and Ghiron[8] the upward curvature in the Stern-Volmer plot indicates that both tryptophan residues of BSA are exposed to quencher and the quenching constant of each tryptophan residue are nearly identical, while downward curvature indicates buried tryptophan residues. At a concentration of 80 μM of drug, about 35.91% of the fluorescence intensity was quenched. The maximum quenching was obtained by extrapolating a plot of $(F_o-F)/F_o$ *versus* 1/[Q] to 1/[Q] = 0 corresponding to infinite concentration of drug.

We have observed that at infinite concentration of drug, fluorescence quenching was more than 85%. This again shows that both the tryptophan residues of BSA are accessible to drug molecule.

For a bimolecular quenching process, $K_q = k_q\tau_o$ where $\tau_o$ is the lifetime in the absence of quencher and $k_q$ is the rate constant for quenching. As $\tau_o$ value for tryptophan fluorescence in proteins is known to be equal to $10^{-9}$ s, the rate constant, $k_q$, would be of the order of $10^{13}$ $M^{-1}$ $s^{-1}$. The value of $k_q$ depends on the probability of a collision between fluorophore and quencher. This probability depends on their rate of diffusion (D), their size and concentration. It can be shown that $k_q = 4\pi aDN_a \times 10^{-3}$.....(5) where, D is the sum of the diffusion coefficients of quencher and fluorophore, a is the sum of molecular radii and $N_a$ is Avogadro's number. The upper limit of $k_q$ expected for a diffusion-controlled bimolecular process is $10^{10}M^{-1}s^{-1}$. The high magnitude of $k_q$ in the present study ($10^{13}$ $M^{-1}s^{-1}$) can probably be attributed to a specific long-range interaction between CB and tryptophan residues on protein. Thus, the process of energy transfer occurs by intermolecular interaction forces between tryptophan and drug and this is possible only when the drug-binding site is in close proximity to tryptophan residues of BSA.

*Binding studies in the presence of ANS*

Fluorescence spectra of 10 ìM BSA in the presence of increasing amounts of CB/ANS (10 to 50 μM) were determined upon excitation at 296 nm. Both CB and ANS quench the fluorescence of BSA, but the magnitude of decrease in fluorescence intensity was much larger for ANS as compared to that for drug. ANS bound to BSA calculated from the fraction of occupied sites (θ) was 76% where as the CB bound to BSA was only 21.30%. It is known that excitation at 296 nm involves fluorescence due only to tryptophan residues of BSA. Further, under conditions of the experiment tryptophan residues of BSA are partially exposed and their accessibility depend upon the nature of molecules of the interacting species.[9] It thus appears that whereas, tryptophan residues are fully accessible to the hydrophobic probe they are only partially accessible to the drug which has partially hydrophilic character. Thus, CB and ANS do not share common binding site in BSA.

In another set of experiments, BSA-drug interaction was studied in the presence and absence of 5, 10, 15, 20 and 25 μM of ANS. It was found that for a given concentration of drug, fluorescence intensity increases when ANS is added to BSA-CB system. It is known[10] that the hydrophobic probe ANS shows greatly increased fluorescence as a result of hydrophobic interactions with proteins and other macromolecules due to transfer of probe from an aqueous to non-polar environment. Increase in fluorescence intensity of BSA-CB system on the addition of ANS illustrates that when ANS is added to BSA-CB system it can compete with drug for the hydrophobic sites on the surfaces. In that case it would inhibit the binding of drug, i.e. displace drug from its binding sites and the fluorescence intensity should decrease. But, the fluorescence intensity actually increases. This shows that ANS and drug do not share common sites in BSA.

*Thermodynamics of drug-protein interaction*

Thermodynamic parameters for the binding of CB to BSA were determined by carrying out the binding studies at four different temperatures, 13°, 20°, 27° and 35°C by spectrofluorometric method and using the relation,

$$\text{Log } K = -\Delta H_o/2.303RT + \Delta S_o/2.303R \quad (6)$$

From the plot of log K *versus* 1/T, the values of standard enthalpy change $\Delta H_o$ and the standard entropy change, $\Delta S_o$ for the binding process were evaluated. The $\Delta H_o$, $\Delta S_o$ and $\ddot{A}G_o$ values were found to be +18.18 KJ $mol^{-1}$, +132 $JK^{-1}$ $mol^{-1}$ and 39.58 $KJmol^{-1}$, respectively. The positive $\Delta H_o$ value indicates less-dominant hydrogen bond formation between the substrates while positive $\Delta S_o$ value indicates predominant hydrophobic character of binding. Therefore, the positive $\Delta H_o$ and $\Delta S_o$ values observed in this case indicate less dominant hydrogen bond formation and predominant hydrophobic character of binding between BSA and CB.[11] These results together with spectral changes in the fluorescence emission spectra of BSA induced by CB, suggest that the interaction may take place in subdomain IA and IIA since these have been proposed to bind drugs and other hydrophobic materials.[11]

*Effect of additives and paracetamol on CB-BSA interaction*

The fluorescence spectra of albumin-CB were recorded in

presence and absence of various additives at 344 nm upon excitation at 296 nm. The concentration of BSA and CB was fixed at 20 μM and 40 μM, respectively and that of each additive was maintained at 20 μM.

To understand further the nature of interaction involved, fluorescence spectra of albumin-CB were recorded in the presence of each of 20 μM urea, gum-acacia, dextrose, magnesium-stearate and starch solution. It was observed that CB-protein intensity increases in presence of urea, magnesium stearate and starch indicating that they inhibited the CB-protein binding whereas the CB-protein intensity decreased in presence of dextrose and gum-acacia indicating they induced the CB-protein binding. Thus, urea, gum acacia, dextrose, magnesium stearate, and starch alter the microenvironment of the binding sites by affecting the iceberg structure of water.[7] The results of analysis are given in the Table 1.

The simultaneous administration of two or more strongly bound drugs can compete with one another for the binding sites on albumin and so result in displacement interactions. [12] Although paracetamol is not strongly bound at therapeutic concentrations it can still affect the protein binding behavior of other drugs either by blocking an active site or by causing conformational changes in the protein molecule. Thus, the presence of paracetamol can significantly alter the pharmacological response of other drugs by altering the concentration of free drug in plasma.

It was observed that the association constants (K) decreased from $4.05 \times 10^3$ to $3.09 \times 10^3$ $M^{-1}$ in the presence of paracetamol. This means that the availability of free drug in plasma gets increased in the presence of paracetamol. Once the interference of the paracetamol in the protein binding of drug is established one can anticipate the need for an adjustment in dosage in the presence of paracetamol. The relative ability of paracetamol to interfere in the binding of other drugs can be quantitatively determined from $K_{ratio}$, the ratio of association constant in the presence and absence of paracetamol. $K_{ratio}$ can, therefore, be a guide to the modified design of dosage forms in the presence of paracetamol.

*Circular dichroism method*

A stock solution of 0.1 μM BSA was prepared in 0.01 M phosphate buffer containing 0.15 M NaCl solution. The BSA to CB concentration was varied (1:1,1:3 and 1:5) and the CD spectrum was recorded.

**Table 1.** Fluorescence intensity of CB-BSA system in the presence of additives

| Sample | Fluorescence intensity at 344 nm |
|---|---|
| Only BSA | 86.21 |
| BSA+ CB | 52.21 |
| BSA+ CB +Urea | 68.43 |
| BSA+ CB +Magnesium-stearate | 66.38 |
| BSA+ CB +Dextrose | 45.21 |
| BSA+ CB +Starch | 70.08 |
| BSA+ CB +Gum-acacia | 38.09 |

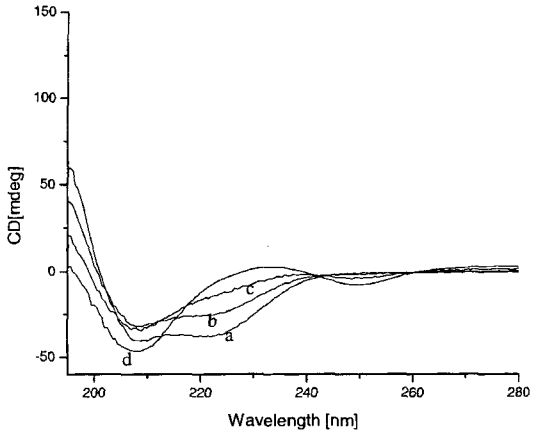


Figure 5. Circular dichroism spectra in the 200-300 nm range; (a) BSA, 0.1 μM; [BSA]: CB = 1:1(b); 1:3 (c) and 1:5 (d).

The binding of CB was also confirmed by circular dichroism (CD) spectra. The CD spectra of 0.1 μM BSA in buffer (a) and varied ratios of BSA-CB, 1:1 (b); 1:3 (c) and 1:5 (d) showed marked changes. The CD spectrum of CB in presence of BSA is shown in Fig. 5. As expected the a-helices of protein show a strong double minimum at 220 nm and 209 nm.[13] The intensities of this double minimum reflect the amount of helicity of BSA and further these indicate that BSA contains more than 50% of α-helical structure. On increased addition of CB to BSA (1:1 and 1:3) the double minimum at 220 nm was vanished while the double minimum at 209 nm was found to increase at higher concentration of CB (1:5). This is indicative of increase in helicity when the CB is completely bound to BSA.

*Acknowledgements* – We are grateful to the Department of Science and Technology, New Delhi for financial support of this work (SP/S1/H-38/2001). Thanks are also due to Prof. Bhaskar G. Maiya, School of Chemistry, University of Hyderabad, Hyderabad for CD measurements and useful discussion.

## REFERENCES

1. Maruthamuthu, M., and S. Kishore (1987) Binding of naproxen to bovine serum albumin and tryptophan modified bovine serum albumin. *Proc. Ind. Acad. Sci. (Chem. Sci.)*, **99,** 273-279.
2. Seedher, N., (2000) InVitro study of the mechanism of interaction of trifluperazine dihydrochloride with bovine serum albumin, *Indian.J.Pharm.Sci,* **62,** 16-20
3. Seedher, N., B. Singh and P. Singh (1999) Mode of interaction of metronidazole with bovine serum albumin. *Indian. J. Pharm.Sci.* **61,** 143-147
4. Miyoshi, T., K. Sukimoto and M. Otagiri (1992) Investigation of

the interaction mode of phenothiazine neuroleptics with α-1 acid glycoprotein. *J. Pharm. Pharmaco.* **44**, 28-32.

5. Weber, G., and L. B Young (1964) Fragmentation of bovine serum albumin by pepsin. *J. Biol. Chem.* **239**, 1415-1419.
6. Ward, L. D., (1985) Measurement of ligand binding to protein by fluorescence spectroscopy. *Methods in Enzymology.* **17**, 400-404.
7. Neelam, S., and K. Mamta (2001) Mechanism of interaction of phenothiazine derivatives with serum albumin. **63**, 137-143.
8. Eftink, M. R., and C. A Ghiron, Fluorescence quenching of indole and model micelle systems. *J. Phys. Chem.* **80,** 486-493.
9. Willliams, E. J., T. T Herskovits and M Laskowski (1965) Location of chromophoric residues in proteins by solvent perturbation. *J. Biol. Chem.* **240,** 3574-3579.
10. Eftink, M. R and C. A Ghiron (1981) Fluorescence quenching studies with proteins *Anal. Biochem.* **114**, 199-227.
11. C-Qui Jiang, M-Xia Gao, and Ji- Xiang He (2002) Study of the interaction between terazosin and serum albumin synchromous fluorescence determination of terazosin. *Anal. Chim. Acta.* **452,** 185-189.
12. Neelam, S., and K. Suresh (2001) In Vitro study of the effect of paracetamol on drug protein interaction. *Indian. J. Pharm. Sci.* **265**, 512-515.
13. Shrivastava, H. Y., M Kanthimathi and U. N Balachandran (1999) Interaction of Schiff base with bovine serum albumin: Site specific photocleavage. *Bio Chem and Bio Phys res commu.* **265,** 311-315.