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A COMPARATIVE STUDY OF THE INTERACTION BETWEEN VITAMIN B₁₂ WITH BOVINE AND HUMAN SERUM ALBUMINS

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Abstract. Human and bovine serum albumins are the most common proteins in the circulatory system of many organisms. Numerous studies are regarding their interactions with different types of ligands. These interactions are important for their applicability in many areas in biomedical field. The interaction of human serum albumin (HSA) and bovine serum albumin (BSA) with vitamin B₁₂ was investigated using UV absorption and fluorescence spectroscopy. Results showed that the absorption and fluorescence intensities decreased as vitamin C concentration increases. The calculated binding constant ($k \sim 10^4 \text{ M}^{-1}$) showed a weak binding of vitamin B₁₂ with both serum albumins. The analysis of fluorescence quenching for HSA/BSA-vitamin B₁₂ interaction ($k_q \sim 10^{12} [L] \text{ mol}^{-1} \cdot \text{s}^{-1}$, where k_q is the protein bimolecular quenching rate, and $[L]$ represents the concentrations of the quencher) reveals the dynamic quenching process and clearly confirms the existence of a static mechanism of fluorescence quenching.

Key words: Human serum albumin, bovine serum albumin, vitamin B₁₂, UV-absorption, fluorescence spectroscopy, binding constant, binding mode.

INTRODUCTION

Vitamin B₁₂ (known as cyanocobalamin) has the molecular formula C₆₃H₈₈CoN₁₄O₁₄P, molecular weight of 1355.388 g·mol⁻¹ and its chemical structure is shown in Figure 1 [18]. Vitamin B₁₂, the only biomolecule with carbon-metal bond, is a red-colored water-soluble complex based on a corrin ring [1]. Commercially available cyanocobalamin is a stable form generated industrially only through a bacterial fermentation-synthesis, which is quickly transformed to methylcobalamin and then to adenosylcobalamin in human organism [2]. The estimated average requirements for this vitamin are in the range of 0.4–2.8 μg·day⁻¹, depending on the sex, age and condition [3]. Vitamin B₁₂ is indispensable for humans and mammals, participating in the formation of red blood cells and in the maintenance of a healthy brain and nervous system [38]. Additionally, it is an enzyme cofactor in many

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metabolic processes, antioxidant and modulator of nucleic acid metabolism and gene regulation [9]. Therefore, vitamin B₁₂ deficiency reported mainly in case of infants, vegetarians or ill persons, can be associated with megaloblastic anemia, hyperhomocysteinemia and various neurological disorders, including memory problems, Parkinson and Alzheimer diseases [22, 30]. The deficiency of vitamin B₁₂ can be a risk factor for coronary heart disease and stroke, as well as for the symptoms of mania, psychosis or depression [12, 34, 37]. A decreased level of this compound is mainly a consequence of its low intakes, but can result as well from less absorption, especially in smoker's case (since nicotine can block its absorption), alcoholism, certain intestinal disorders (celiac or Crohn's diseases), low presence of binding proteins and use of certain medications [9, 13, 20].

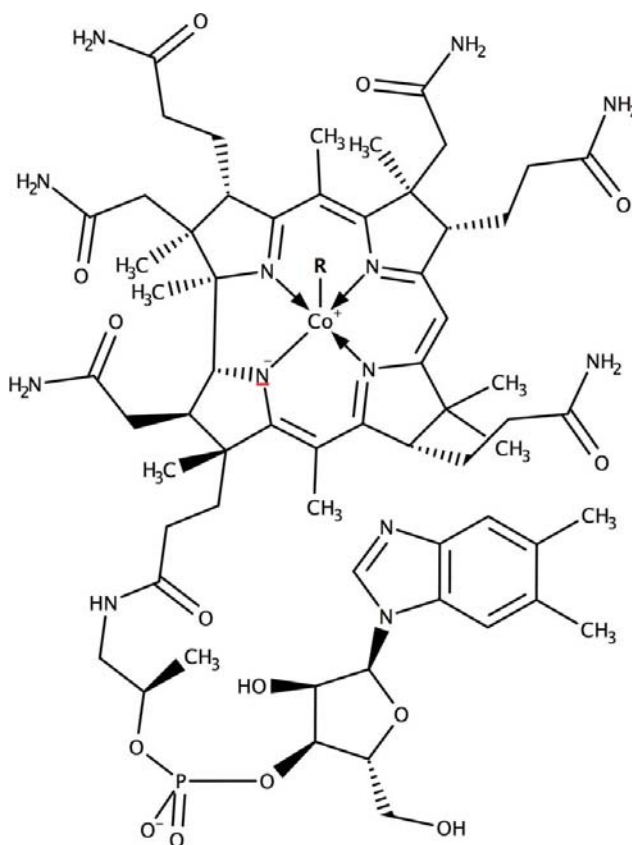


Fig. 1. Chemical structure of the basic form of vitamin B₁₂.

The binding of several different categories of small molecules to serum albumins has been studied for many years through different spectroscopic techniques to elucidate details of the protein structure and binding mechanism. The binding affinity

of vitamin B₁₂ with HSA was studied using UV absorption and fluorescence spectroscopy in addition to circular dichroism. The results showed that the mechanism of interaction has been found to be a dynamic quenching. The effect of B₁₂ on the conformation of HSA was analyzed. The results indicated that the polarity around the tryptophan residues of HSA was decreased and its hydrophobicity was increased [23, 32]. Serum albumin or blood albumin is the most abundant protein present in the circulatory system of vertebrates, synthesized in the liver and, being a major macromolecule, it contributes to osmotic blood pressure [3]. It can play a dominant role in drug disposition and efficiency [40, 43]. Many anesthetic and other bioactive small molecules bind reversibly to albumin and other serum components, which then can function as carriers. The apparent solubility of hydrophobic drugs in plasma is increased by serum albumin and modulates their delivery to cell *in vivo* and *in vitro*. Animal experiments were used to provide basic knowledge on the pharmacological actions, biotransformation and biodistribution of drugs [46]. Bovine serum albumin is suitable to these studies, because it has been extensively characterized [29]. Similarly, human serum albumin constitutes about 60% of the serum protein; its function is to transport and dispose many endogenous and exogenous substances such as metabolites, drugs, and other biologically active compounds present in the blood [6]. BSA and HSA are frequently used in biophysical and biochemical studies since they have a similar folding, a well-known primary structure, and they have been associated with the binding of many different categories of small molecules. One important difference of BSA and HSA is the fact that bovine albumin has two tryptophan residues while human albumin has a unique tryptophan [18]. Recently, its three-dimensional (3D) structure has been determined through X-ray crystallographic measurements [23] and consists of three structurally homologous domains which assemble to form a heart-shaped molecule, each domain containing two sub-domains. Serum albumin binds and transports many ligands, including fatty acids, amino acids, hormones, cations, anions, and a variety of pharmaceuticals. It is suggested that the principal regions of ligand binding to HSA are located in hydrophobic, and single tryptophan residue of HSA [4, 8, 24, 31, 32]. In order to gain general fundamental insights into protein-drug binding, the drug-albumin complex was considered as a model. The binding of drugs to plasma protein is considered very important, since it influences their pharmacokinetic and pharmacodynamic properties and may also cause interference with the binding of other endogenous and/or exogenous ligands as a result of the overlap of binding sites and/or conformational changes. Therefore, a detailed investigation of drug-protein interaction assumes significance for a thorough understanding of the pharmacokinetic behavior of a drug and for the design of analogues with effective pharmacological properties [32]. There are some popular techniques which have been used to investigate the interaction between drugs and proteins. UV-absorption and fluorescence spectroscopy are the powerful techniques to study molecular interactions which change local environment of fluorophore and help to predict the binding phenomenon

of drugs to proteins [5]. Fluorescence quenching is a powerful method to investigate the reactivity of chemical and biological systems because it allows non-intrusive measurements of substances in low concentration under physiological conditions [6, 25, 32]. It can reveal accessibility of quenchers to serum albumins fluorophores, help to understand serum albumins binding mechanisms to compounds and provides clues to the nature of the binding phenomenon.

The aim of the present work is to study the interaction between BSA and HSA with vitamin B₁₂. This study is highly important for pharmaceutical sciences to clarify the structure, function, and properties of BSA/HAS-vitamin B₁₂ complexes. Investigations of the interaction mechanisms and the binding constant between vitamin B₁₂ and BSA/HSA should be provided. In order to attain these objectives, UV-Vis absorption spectroscopy, fluorescence spectroscopy and FTIR spectroscopy were employed to carry out detailed investigation.

MATERIALS AND METHODS

MATERIALS

BSA and HSA (purity > 99%) was purchased from Sigma Aldrich Company, and vitamin C was purchased from Al-Quds Co. pharmaceutical chemical industries, their solutions were prepared in phosphate buffer solutions (pH = 7.4). The other substances are of reagent grade and were used without further purifications.

A final concentration of (40 mg/mL) of HSA and BSA was used in the final vitamin C-protein solution, then the solution was placed in ultrasonic water path (SIBATA AU-3T) for one hour to ensure that all the amount of vitamin B₁₂ was completely dissolved. The final concentrations of vitamin B₁₂-protein complexes were prepared by mixing equal volumes of HSA and BSA to equal volume from different concentrations of vitamin B₁₂. HSA and BSA concentrations in all samples were kept at 40 mg·mL⁻¹. The final concentrations of vitamin B₁₂ are (40, 20, 10, 5, 2 and 1 mg·mL⁻¹). The solution was incubated for 1 h (at 25°C) before spectroscopic measurements were taken.

INSTRUMENTS

UV-VIS spectrophotometer

The mixed sample was placed in a 1.0 cm quartz cuvette, and the absorption spectra of the system at wavelengths of 220–750 nm were recorded using a NanoDrop ND-1000 spectrophotometer (Shimadzu, Kyoto, Japan) at room temperature (298 K) with a scanning speed of 300 nm/min with high accuracy and reproducibility. The background absorbance of the buffer solution was subtracted from the sample absorbance.

Fluorescence spectrometer

The fluorescence measurements were performed by a NanoDrop ND-3300 Fluoro-spectrophotometer at 25°C. The excitation source comes from one of three solid-state light emitting diodes (LEDs). The excitation source options include: UV LED with maximum excitation 365 nm, blue LED with excitation at 470 nm, and white LED from 500 to 650 nm excitation. A 2048-element CCD array detector covering 400–750 nm is connected by an optical fiber to the optical measurement surface. The excitation is done at the wavelength of 360 nm and the maximum emission wavelength is at 439 nm.

The absorption and emission spectra were recorded for free HSA and BSA at 40 mg·mL⁻¹ and for its complexes with vitamin B₁₂ solution with the concentrations of 40, 20, 10, 5, 2 and 1 mg·mL⁻¹. Repeated measurements were done for all samples.

RESULTS AND DISCUSSION

UV ABSORPTION SPECTRA

UV-Vis absorption spectroscopy is one of the common and effective experimental techniques used in calculating binding constants for several drug-protein complexes [26]. To initially verify the quenching mechanism, the UV absorption spectra of HSA/BSA-vitamin B₁₂ were measured and recorded. Complex has formed by the interaction of vitamin B₁₂ with HSA/BSA as shown from the absorption data in Figure 2. The intensity of UV-absorption of both proteins (absorption peak 278 nm) decreased with increasing concentrations of vitamin B₁₂ indicating formation of vitamin B₁₂-HSA/BSA complex.

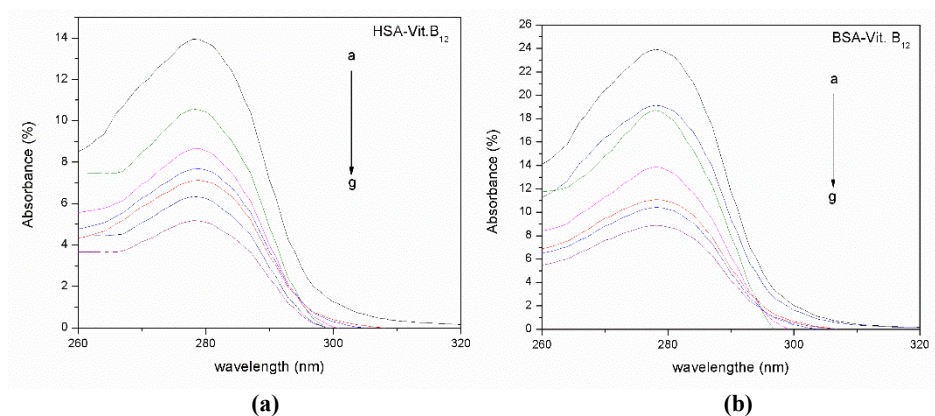


Fig. 2. UV-absorption spectra of different concentrations of vitamin B₁₂ (a = free HSA, b = 1, c = 2, d = 5, e = 10, f = 20, and g = 40 mg·mL⁻¹) with (a). HSA, (b). BSA.

Vitamin B₁₂-HSA/BSA binding constants have been determined using UV-absorbance spectroscopy, by assuming only one type of interaction between ligand and proteins in aqueous solution. The absorption data were treated using linear reciprocal plots based on the following equation [39].

$$\frac{1}{A - A_0} = \frac{1}{A_{\infty} - A_0} + \frac{1}{(A_{\infty} - A_0)k} \cdot \frac{1}{[L]} \quad (1)$$

where A_0 corresponds to the initial absorption of protein at 280 nm in the absence of ligand, A_{∞} is the final absorption of the ligated protein, and A is the recorded absorption at different vitamin B₁₂ concentrations ($[L]$).

The double reciprocal plot of $1/(A - A_0)$ vs. $1/[L]$ is linear (Fig. 3) and the binding constants (k) can be estimated from the ratio of the intercept to the slope. The obtained values of the binding constants indicate a relatively weak interaction of vitamin B₁₂ with HSA ($k = 2.21 \times 10^4 \text{ M}^{-1}$) and with BSA ($k = 1.61 \times 10^4 \text{ M}^{-1}$). The binding constant values show a relatively weak vitamin B₁₂-HSA/BSA interaction in comparison to other drug-protein complexes with binding constants in the range of 10^5 and 10^6 M^{-1} [7, 18, 26]. The reason for the low stability can be attributed to the presence of mainly hydrogen-bonding interaction between protein donor atoms and the ligand polar groups or an indirect drug-protein interaction through water molecules [33].

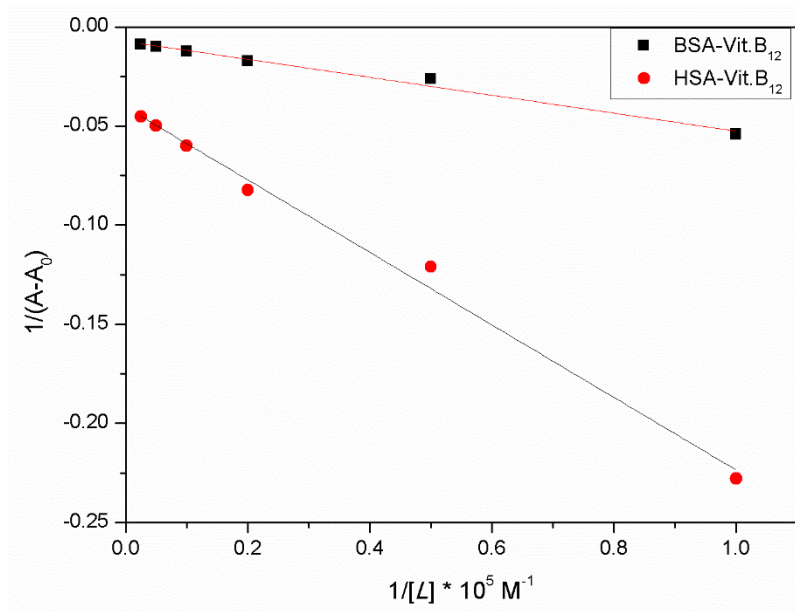


Fig. 3. The plot of $1/(A - A_0)$ vs $1/[L]$ for HSA and BSA with different concentrations of vitamin B₁₂.

FLUORESCENCE SPECTROSCOPY

The ligand-binding process during protein-ligand complex is mainly governed by four types of weak, noncovalent forces including hydrogen bond, van der Waals force, electrostatic, and hydrophobic interactions [42]. Fluorescence quenching studies to explore the binding interaction of drug ligands with proteins is considered as the best methodology [15]. This quenching resulted from the reduction of quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with a quencher molecule [10–11, 30]. An increase in the concentration of quencher will mask the fluorophore, resulting in reduction of emitted fluorescence from the fluorophore molecules. In case of targeting specific location regarding the binding pattern of protein, we used different wavelengths. Generally, the fluorescence of HSA and BSA comes from tryptophan, tyrosine and phenylalanine residues. During data recording at an excitation wavelength at 280 nm, fluorescence of albumin was coming from both tryptophan and tyrosine residues, whereas 293 nm wavelength excited tryptophan residues only [44].

Figure 4 represents the fluorescence spectra of HSA and BSA in combination with different concentrations of vitamin B₁₂. It was observed that fluorescence spectrum exhibited the peak maximum at 440 nm for HSA/BSA-vitamin B₁₂ complexes. The fluorescence intensity decreases with increasing concentrations of vitamin B₁₂. This indicated that there was some alteration in the microenvironment of the fluorophore Trp-213 upon interaction of both proteins with vitamin B₁₂.

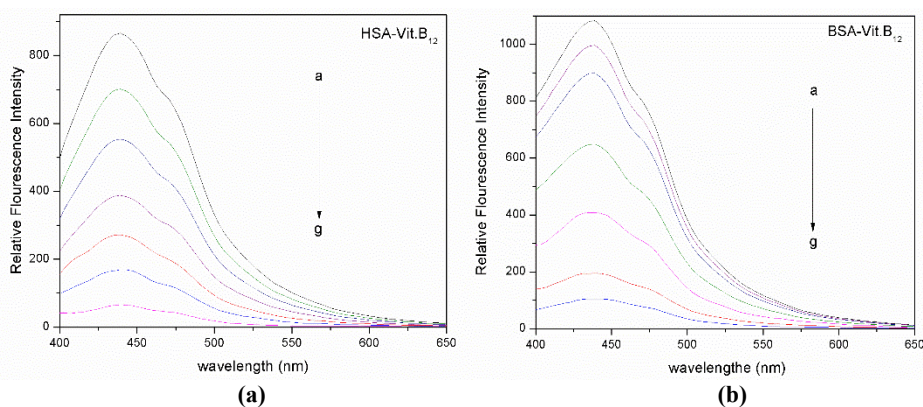


Fig. 4. Fluorescence emission spectra in the absence and presence of vitamin B₁₂ (a = free (HSA (a), BSA (b), b = 1, c = 2, d = 5, e = 10, f = 20 and g = 40 mg·mL⁻¹).

The mechanism by which small molecule ligands quench the fluorescence of plasma albumin is mainly divided into dynamic quenching and static quenching. In dynamic quenching, the excited molecules of the fluorescent substance collide with the quencher molecules and, through energy transfer or charge transfer, the excited molecules lose their excitation energy and return to the ground state without

emitting photons. In static quenching, the quenching agent and fluorescent substance in the ground state form a complex, leading to fluorescence quenching [28, 36]. Quenching constant values depend on temperature; they increase as temperature values go up. For dynamic quenching, a faster diffusion results as temperature increases [21]. However, static quenching results from the decrement of temperature values [27].

In order to analyze the fluorescence quenching mechanism, the Stern-Volmer equation [45] was used:

$$\frac{F_0}{F} = 1 + k[L] = 1 + k_q\tau_0[L] \quad (2)$$

where F_0 and F are protein (HSA and BSA) fluorescence intensities in the absence and presence of quencher (vitamins C); τ_0 is the average fluorescence lifetime of protein molecule without quencher (of the order of 10^{-8} [36]), k_{sv} is Stern-Volmer fluorescence quenching constant.

In Figure 5 is shown the Stern-Volmer plot of both complexes fluorescence intensities. The two plots showed good a linear relationship, suggesting the existence of a single type of quenching (dynamic or static) and/or a single binding site. The values of the fluorescence quenching constant determined by applying Eq. (2) are equal to 2.97×10^4 , 2.42×10^4 [L] mol⁻¹ for HSA/BSA-vitamin B₁₂, respectively, both values are much lower than other k_{sv} values for the similar systems signalized earlier in literature [12, 45]. The values of k_q which are equal to 2.97×10^{12} , 2.42×10^{12} [L] mol⁻¹·s⁻¹ for HSA/BSA-vitamin B₁₂ respectively. These values confirm clearly the existence of the static (diffusion-independent) mechanism of fluorescence quenching [16, 37, 41].

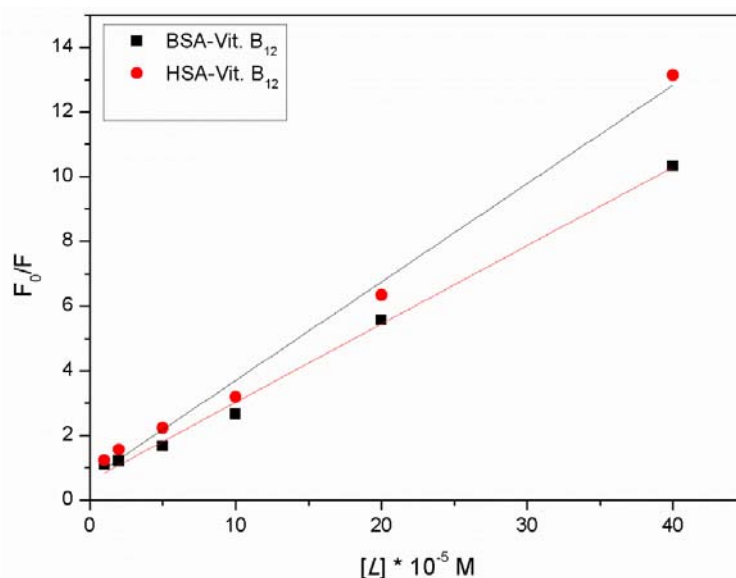


Fig. 5. The Stern-Volmer plot for HSA/BSA-vitamin C complexes.

When static quenching is dominant, the modified Stern-Volmer equation could be used [46]

$$\frac{1}{F_0 - F} = \frac{1}{F_0 \cdot k \cdot [L]} + \frac{1}{F_0} \quad (3)$$

where k is the binding constant.

A plot of $\frac{1}{F_0 - F}$ vs $\frac{1}{[L]}$ is presented in Figure 6. The two plots are linear and have a slope of $\frac{1}{F_0 \cdot k}$ and intercept $\frac{1}{F_0}$ according to eq. (3). The values of k were found to be $2.29 \times 10^4 \text{ M}^{-1}$, $1.44 \times 10^4 \text{ M}^{-1}$ for HSA/BSA-vitamin B₁₂ respectively, which agrees well with the value obtained earlier by UV spectroscopy. The low effective quenching constant in this case has led to a lower value of binding constant between HSA/BSA-vitamin B₁₂ complexes due to an effective hydrogen bonding between vitamin B₁₂ and proteins. The interaction between vitamin molecules with proteins is initiated by electrostatic forces, but subsequently the hydrophobic interactions make the major role for vitamin B₁₂ and proteins interaction.

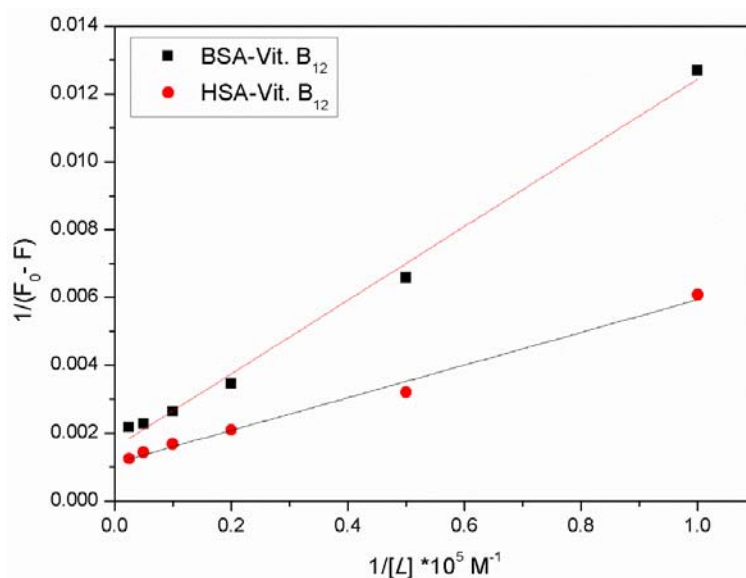


Fig. 6. The plot of $1/(F_0 - F)$ vs. $1/[L] \times 10^5$ for HSA/BSA-vitamin B₁₂ complexes.

CONCLUSIONS

Serum albumins play a key role in the transport, distribution, metabolism and excretion of drugs molecules. In order to develop a new and effective drug, we have to study the ability of drug binding to albumins. In this work, the interaction

between HSA/BSA-vitamin B₁₂ was investigated by fluorescence and UV spectroscopic techniques. The results showed that the fluorescence of HSA and BSA would be quenched with the addition of vitamin B₁₂. This change was *via* static quenching. The calculated binding constants of the interaction of vitamin B₁₂ with both proteins using both techniques showed a good agreement but the values were found to be relatively weak.

The results reported in this work are important in the field of pharmacology and biochemistry and can be used to understand the effect of vitamin B₁₂ on protein function during the blood transportation process. The clear information presented in this study may be used to provide some information for its rational use in clinical practice.

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