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Studies on interaction between Vitamin B12 and human serum albumin

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Abstract

The binding reaction between Vitamin B12 (B12, cyanocobalamin) and human serum albumin (HSA) was investigated by fluorescence quenching, UV–vis absorption and circular dichroism (CD) spectroscopy. Under simulative physiological conditions, fluorescence quenching data revealed that the quenching constants (K_{sv}) are 3.99×10^4, 4.33×10^4, 4.76×10^4 and 5.16×10^4 M^{-1} at 292, 298, 304 and 310 K, respectively. The number of binding sites, n is almost constant around 1.0. On the basis of the results of fluorescence quenching the mechanism of the interaction of B12 with HSA has been found to be a dynamic quenching procedure. Thermodynamic parameters $\Delta H^{\ominus} = -13.38\ kJ\ mol^{-1}$, $\Delta S^{\ominus} = 66.73\ J\ mol^{-1}\ K^{-1}$ were calculated based on the binding constant. These suggested that the binding reaction was enthalpy and entropy driven, and the electrostatic interaction played major role in stabilizing the reversible complex. The binding distance $r = 5.5$ nm between HSA and B12 was obtained according to Förster theory of energy transfer. The effect of B12 on the conformation of HSA was analyzed by synchronous fluorescence and CD spectroscopy. Synchronous spectra indicated that the polarity around the tryptophan (Trp214) residues of HSA was decreased and its hydrophobicity was increased; however, the α-helix content of the protein was predominant in the secondary structure but the CD spectra indicated that B12 induced minor conformational changes of HSA.
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Keywords: Vitamin B12; Human serum albumin; Fluorescence quenching; Thermodynamic parameters; Conformation

1. Introduction

Understanding the interaction between the water-soluble cobaltiferous vitamin Vitamin B12 and human serum albumin (HSA) is of major pharmaceutical and clinical importance.

Human serum albumin is the most abundant protein in blood serum with a concentration of 0.63 mM. Crystal structure analysis has revealed that HSA is a globular protein composed of a single, largely α-helical polypeptide chain of 585 amino acid residues. As the major soluble protein constituent of the circulatory system, HSA binds a number of the relatively insoluble endogenous compounds such as unesterified fatty acids, bilirubin, and bile acids and thus facilitates their transport throughout the circulation system [1,2]. Many drugs and other bioactive small molecules bind reversibly to albumin and other serum components that then function as carriers. Free concentration and the metabolism of various drugs may be strongly affected by drug–protein interactions in the blood stream. This type of interaction can also influence the drug stability and toxicity during the chemotherapeutic process. Serum albumin often increases the apparent solubility of hydrophobic drugs in plasma and modulates their delivery to cells *in vitro* and *in vivo*. The deep knowledge of the mechanism of the drug–HSA interaction *in vivo* is of great importance from the point of view of understanding the process of drug transportation, and the prediction of serum concentrations of the free drug. The data thus obtained contribute to rational drug synthesis and clinical application. The molecular interactions between HSA and many compounds have been successfully investigated [3–6]. Recently, Hisaeda et al. [7] studied the interactions of Vitamin B12 and its hydrophobic ester derivatives with HSA. However, the binding affinity and the

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change of the conformation of human serum albumin when interacting with B12 have not yet been systematically investigated.

Fluorescence quenching is an important method to study the interaction of substances with proteins because it is relatively easy to use and it allows non-intrusive measurements of substances in low concentration under physiological condition. Fluorescence spectroscopy is essentially a probe technique sensing changes in the environment of the fluorophore, which distinguishes it from other techniques, such as calorimetry, far-ultraviolet circular dichroism (CD), and infrared (IR) spectroscopy. In the present work, we studied the interaction of B12 with HSA and the thermodynamic parameters of this interaction by optical spectroscopy. We also tried to find the stoichiometry of the B12–HSA complex. The work of determining the affinity of B12 to HSA and the effect of B12 on the conformation of HSA is hoped to afford valuable information for clinical pharmacology.

2. Experimental

2.1. Materials

Vitamin B12 (cyanocobalamin) was purchased from Shanghai Bio Life Science & Technology Co. Ltd. The stock solution was prepared in phosphate buffered saline (PBS; 0.02 M phosphate, 0.10 M NaCl, pH 7.4). Human serum albumin (HSA), purchased from Sigma Chemical Company, was used without further purification and its molecular weight was assumed to be 66,500. The solutions of HSA were prepared 30 min before the experiment. All solutions were prepared with double-distilled water.

2.2. Fluorescence measurement

Fluorescence spectra were recorded on an F-2500 spectrofluorimeter (Hitachi, Japan) with the temperature maintained by a circulating water bath. The excitation wavelength was set at 295 nm. Both the excitation and emission slit widths were set at 2.5 nm. The emission spectra were recorded between 300 and 450 nm. Blank values corresponding to the buffer were subtracted to correct for the background fluorescence.

2.2.1. Fluorometric titration experiments

2.0 ml solution containing appropriate concentration of HSA was titrated by successive additions of a 2.5×10^{-3} M stock solution of B12 (to give final concentration from 0 to 7.0×10^{-5} M). The fluorescence was measured at four temperatures (292, 298, 303 and 310 K). The data herein obtained were analyzed by the Stern–Volmer or modified Stern–Volmer equation. Synchronous fluorescence spectra of HSA in the absence and presence of increasing amount of B12 were recorded.

2.3. UV absorbance measurement

The UV absorbance spectra of the B12–HSA with varied concentrations of B12 were recorded on a TU-1901 spectrophotometer (PuXi Analytic Instrument Ltd., Beijing, China) equipped with 1.0 cm quartz cells.

2.4. CD measurement

Circular dichroism measurements were made on a Jasco-20c automatic recording spectropolarimeter (Japan) in a cell of 4 mm path length at room temperature. CD spectra (200–250 nm) were taken with a HSA concentration of 1.0×10^{-5} M, and the results are expressed as molar ellipticity ($[\theta]$) in $^{\circ}\,cm^2\,dmol^{-1}$. The helical content of HSA was calculated from the value of $[\theta]$ at 208 nm using Eq. (1) [8]:

$$\langle\text{alpha}\rangle\ \text{Helix}\,(\%) = \frac{-[\theta]_{208} - 4000}{33,000 - 4000} \times 100 \qquad (1)$$

where $[\theta]_{208}$ is the observed mean residue ellipticity (MRE) value at 208 nm, 4000 is the MRE of the β-form and random coil conformation cross at 208 nm and 33,000 is the MRE value of a pure α-helix at 208 nm.

3. Results and discussions

3.1. Fluorescence characteristics of HSA quenched by B12 and the mechanism of the binding mode

HSA is a single-string globular protein with 585 amino acids, in which the tryptophan[214] residue was used to measure the drug-binding affinity. The intrinsic fluorescence of HSA is very sensitive to its microenvironment. When local surroundings of HSA are altered slightly and as a consequence of this, its intrinsic fluorescence is weakened, such factors as protein conformational transition, biomolecule binding and denaturant, etc. are responsible for the weakening. In our experiments the pH value was kept at 7.4 and the temperature was below 310 K; HSA is not denaturized under these conditions. The concentrations of HSA were stabilized at 1.0×10^{-5} M and the content of B12 varied from 0 to 7.0×10^{-5} M. The effect of B12 on HSA fluores-

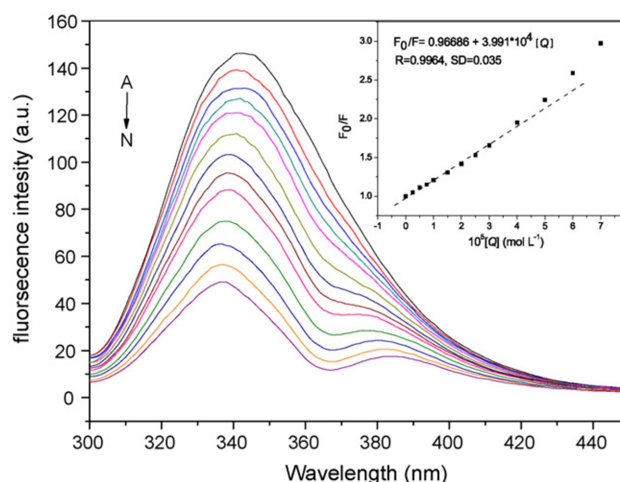

Fig. 1. Effect of B12 on fluorescence spectrum of HSA ($T = 292$ K, $\lambda_{ex} = 295$ nm). c(HSA) = 1.0×10^{-5} M; c(B12)/(10^{-5} M), (A–N) 0; 0.25; 0.5; 0.75; 1.0; 1.5; 2.0; 2.5; 3.0; 4.0; 5.0; 6.0; 7.0.

Table 1
Stern–Volmer quenching constant K_{SV} of the system of B12–HSA at pH 7.40

pH	T (K)	$10^{-4} K_{SV}$ (M^{-1})	R[a]	S.D.[b]
7.4	292	3.991	0.996	0.39
	298	4.331	0.999	0.26
	304	4.755	0.999	0.19
	310	5.162	0.997	0.50

[a] R is the correlation coefficient for the K_{sv} values.
[b] S.D. is the standard deviation for the K_{sv} values.

cence intensity is shown in Fig. 1. It is apparent from Fig. 1 that the fluorescence intensity of HSA decreased regularly with the increase in B12 concentration: a higher excess of B12 led to more effective quenching of the chromophore molecule fluorescence, which suggests that B12 quenches the intrinsic fluorescence of the HSA.

Quenching can occur by different mechanisms which are usually classified as dynamic quenching and static quenching. Dynamic and static quenching can be distinguished by their differing dependence on temperature and viscosity, or preferably by lifetime measurements [9]. From the aspect of the temperature and viscosity, higher temperatures result in faster diffusion and hence larger extent of collisional quenching. Higher temperatures will typically result in the dissociation of weakly bound complexes, and hence smaller extent of static quenching. Our fluorescence quenching data confirm the quenching mechanism to be a dynamic quenching. For the dynamic quenching, the decrease in intensity is described by the well known Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau_0 [Q] \quad (2)$$

where F_0 and F are the steady-state fluorescence intensities in the absence and in the presence of quencher (Vitamin B12), respectively, K_{SV} is the Stern–Volmer quenching constant, k_q is the quenching rate constant of the biomolecule, τ_0 is the average lifetime of the biomolecule without quencher ($\tau_0 = 10^{-8}$ s), and $[Q]$ is the concentration of the quencher. Various values of fluorescence lifetime for HSA were reported and the average fluorescence lifetime was about 5 ns [10]. The biomolecular quenching rate constant was obtained from K_{SV}, $k_q = 7.982 \times 10^{12}\,M^{-1}\,s^{-1}$. It can be seen that is much higher than the limiting diffusion constant K_{dif} of the biomacromolecule ($K_{dif} = 2.0 \times 10^{10}\,M^{-1}\,s^{-1}$, $T = 292$ K) [11], which suggested that the fluorescence quenching was caused by a specific interaction between HSA and B12.

In order to further explain the mechanism of B12 binding to HSA, the temperature dependence of the quenching constants (K_{SV}) was studied. The value of K_{SV} increased with increasing temperature in the concentration range of B12 $(0–4.5) \times 10^{-5}$ M, which suggested that the quenching mechanism of HSA by the quencher of B12 is a dynamic quenching. The increase of quenching constants was caused by the fast diffusion of quencher when interacted with HSA at high temperatures. The Stern–Volmer quenching constants at different temperatures are presented in Table 1. It can be concluded that quenching constant itself cannot distinguish between the mechanisms of the interaction of small molecules with biomacromolecules: the investigation of the temperature dependence of the quenching constants is necessary.

3.2. Binding constant K_b and number of binding sites n

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by Eq. (3) [12]

$$\log\left(\frac{F_0 - F}{F}\right) = \log K_b + n \log[Q] \quad (3)$$

where K_b is the binding constant to a single site and n is the number of binding sites per HSA.

According to Eq. (3), the binding constant K_b and the number of binding sites n can be obtained (shown in Table 2). From the number of binding sites (n is kept almost constant at around 1), it can be concluded that B12 molecule is binding with HSA to form a 1:1 complex. Table 2 shows that K_b increased slightly with the decrease of temperature. This indicates that B12 forms a reversible complex with HSA, i.e. the complex formation can be regarded to be a dynamic procedure.

3.3. Thermodynamic parameters and nature of the binding forces

The interaction forces between a drug and biomacromolecule may include hydrophobic forces, electrostatic interactions, van der Waals interactions, hydrogen bonds, etc. The binding constant K_b of HSA with B12 was measured at four different temperatures (292, 298, 304, and 310 K), where HSA cannot be thermally denatured. To elucidate the energy changes of the interaction of B12 with HSA, the thermodynamic parameters were calculated from van't Hoff plots. If the enthalpy change (ΔH^Θ) does not vary significantly over the temperature range studied, then its value and that of the entropy change (ΔS^Θ) can be determined from the van't Hoff equation:

$$\ln K_b = -\frac{\Delta H^\Theta}{RT} + \frac{\Delta S^\Theta}{R} \quad (4)$$

where K_b is binding constant at the corresponding temperature and R is the gas constant. The enthalpy change (ΔH^Θ) is calculated from the slope of the van't Hoff relationship. The free-energy change (ΔG^Θ) is estimated from the following relationship:

$$\Delta G^\Theta = \Delta H^\Theta - T\Delta S^\Theta \quad (5)$$

Fig. 2 shows the van't Hoff relationship of B12 binding to HSA based on the assumption of a nearly constant ΔH^Θ. Table 2 shows the values of ΔH^Θ and $T\Delta S^\Theta$ obtained for the binding site from the slopes and ordinates at the origin of the fitted lines. The negative sign for free energy (ΔG^Θ) in Table 2 means that the interaction process is spontaneous. The negative enthalpy ΔH^Θ ($-13.38\,kJ\,mol^{-1}$) and positive entropy ΔS^Θ ($66.73\,J\,mol^{-1}\,K^{-1}$) values of the interaction of B12 with HSA indicate that the binding is mainly enthalpy and entropy driven,

Table 2
Binding parameters and thermodynamic parameters of B12–HSA at different temperatures

pH	T (K)	$10^{-5} \times K_b$	n	R[a]	$10^{-5} \times$ S.D.[b]	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
7.4	292	7.40	1.350	0.9960	0.390	−13.38	−32.87	66.73
	298	6.93	1.343	0.9974	0.038		−33.27	
	304	6.22	1.332	0.9973	0.038		−33.67	
	310	5.27	1.047	0.9964	0.042		−34.07	

[a] R is the correlation coefficient for the K_b values.
[b] S.D. is the standard deviation for the K_b values.

and the electrostatic interaction played major role in the reaction [13]. The central cobalt can coordinate with many other aromatic compounds which can explain the electrostatic interaction.

3.4. Energy transfer between HSA and B12

The Förster theory of molecular resonance energy transfer [14] points out that a transfer of energy could also take place through direct electrodynamic interaction between the primarily excited molecule and its neighbors in addition to radiation and reabsorption. According to this theory, the distance r of binding between B12 and HSA could be calculated by Eq. (6) [15]:

$$E = \frac{R_0^6}{R_0^6 + r^6} \tag{6}$$

where E is the efficiency of energy transfer between the donor and acceptor and R_0 is the critical distance when the efficiency of transfer is 50%.

$$R_0^6 = 8.79 \times 10^{-25} K^2 N^{-4} \phi J \tag{7}$$

In Eq. (7), K^2 is the space factor of orientation, N is the refractive index of the medium, ϕ is the fluorescence quantum yield of the donor, J is the effect of the spectral overlap between the emission spectrum (Fig. 3b) of the donor and the absorption spectrum (Fig. 3a) of the acceptor, which can be calculated by Eq. (8):

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F(\lambda) \, d\lambda} \tag{8}$$

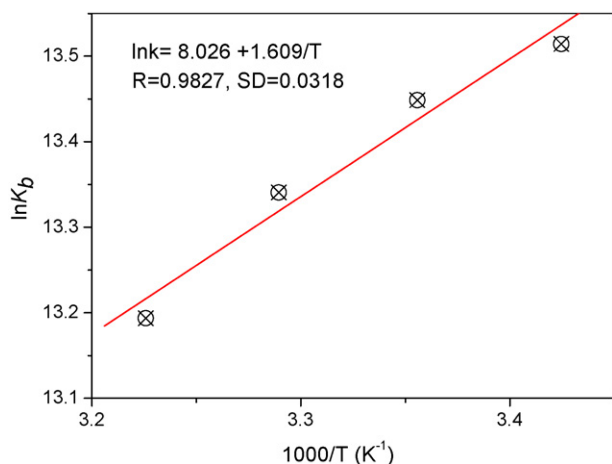

Fig. 2. van't Hoff plot, pH 7.40, and $c(\text{HSA}) = 1.0 \times 10^{-5}$ M.

where $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength from λ to $\lambda + \Delta\lambda$ and $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ. The efficiency of transfer (E) can be obtained by using Eq. (9)

$$E = 1 - \frac{F}{F_0} \tag{9}$$

In the present case, $K^2 = 2/3$, $N = 1.336$, $\phi = 0.074$, according to Eqs. (6)–(9), we could calculate that $R_0 = 7.3$ nm, $E = 0.196$ and $r = 5.5$ nm. The donor-to-acceptor distance $r \approx 5.5$ nm, and $0.5R_0 < r < 1.5R_0$, indicate that the energy transfer from HSA to B12 occurs with high possibility [16,17], which also can explain the efficient quenching of the tryptophan fluorescence. The distance of B12 to HSA was higher than the reported binding ranges of 3–6 nm, with other words, the binding regions of HSA are not the known binding sites. From the nature of the binding forces and the structure of B12, we can put forward that B12 is bound to the surface of HSA according to the distance of B12 to HSA, but the calculation of the distance based on the Förster resonance energy transfer (FRET) is important either. In a previous study [18], we have discussed the distance between flavonoids and HSA and found that the calculated distance of drug-Trp214 may just tell us a range of distances between probes and serum albumin, which was affected by many factors when calculated according to Förster theory.

3.5. Conformation investigation

Synchronous spectra are advantageous to observe conformational changes of protein since it avoids complicated labeling

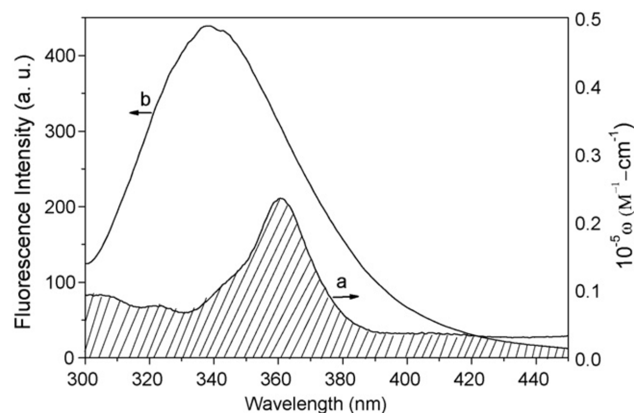

Fig. 3. The overlap spectra of B12 absorption (a) with HSA fluorescence (b). $c(\text{HSA}) = c(\text{B12}) = 1.0 \times 10^{-5}$ M.

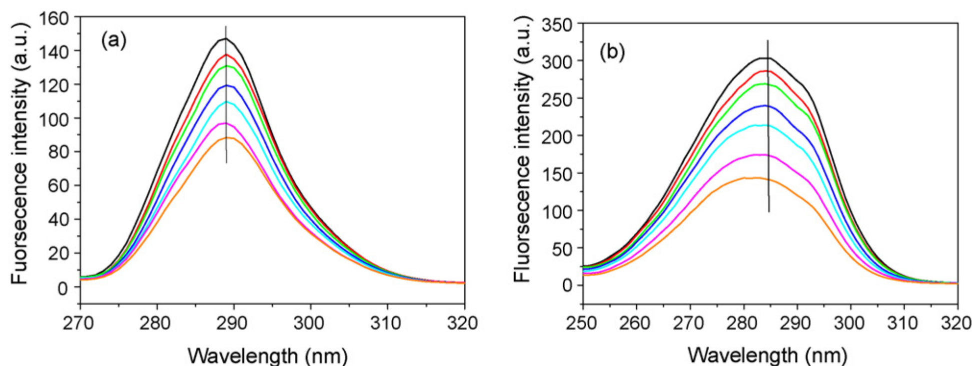

Fig. 4. Synchronous fluorescence spectra of HSA (T = 292 K, (a) $\Delta\lambda$ = 15 nm; (b) $\Delta\lambda$ = 60 nm). c(HSA) = 1.0×10^{-5} M; c(B12)/(10^{-5} M), (A–G) from the top line to the bottom line: 0; 0.25; 0.5; 0.75; 1.0; 1.5; 2.0; 2.5.

with an extrinsic dye [19]. The synchronous fluorescence spectra not only give information about the molecular environment in a vicinity of the chromophore, but also have other advantages such as spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects [20]. Yuan et al. [21] suggested that a useful method to study the environment of amino acid residues was the measurement of the possible shift in the wavelength of the emission maximum corresponding to the changes of the polarity around the chromophore molecule. When the $\Delta\lambda$ value between excitation wavelength and emission wavelength were stabilized at 15 or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine or tryptophan residues [22]. To explore the structural change of HSA by addition of B12, we measured synchronous fluorescence spectra of HSA with various amounts of B12. The effect of B12 on the synchronous fluorescence spectra of HSA is shown in Fig. 4. A little blue shift (from 284 to 281) of the fluorescence of tryptophan residues upon addition of B12 is apparent from Fig. 4b. The blue shift of the emission maximum indicates that the polarity around the tryptophan residues is decreased and the hydrophobicity is increased [23], which is in accordance with the results of the fluorescence quenching.

To investigate the effect of binding B12 to HSA secondary structure, CD was employed. The CD spectra of HSA exhibit two negative bands at 208 and 222 nm [24] which is the characteristic of α-helix in the advanced structure of protein; if the α-helixes change, the spectra will change accordingly. The CD spectra of HSA in the absence (line a) and presence of B12 (lines b–e) are shown in Fig. 5. No obvious change in band intensity and positions of the peaks could be observed even though the ratio of B12 to HSA is up to 4:1, i.e. CD spectra of HSA in the presence and absence of B12 are similar in shape. This indicates that the structure of HSA (after binding B12) is also predominantly α-helical and no significant perturbation in the secondary structure of HSA can be found. The CD spectra of HSA in presence of lower concentrations of B12 did not indicate appreciable changes in conformation of HSA in terms of α-helicity. However, some changes in α-helicity values were noticed in presence of higher concentrations of B12 (Fig. 5e). From Eq. (1), the α-helicity in the secondary structure of HSA differed from that of 53.7% in free HSA to 58.4% in HAS–B12 complex, which shows that the binding of B12 to HSA may induce minor conformational changes [25]. B12 binding to the surface of HSA and some of B12 residues inserting into the hydrophobic surface of HSA and the electrostatic action according to the discussion in Sections 3.3 and 3.5 can stabilize the structure of HSA in solution, which explains the increasing α-helicity of HSA when bound to B12.

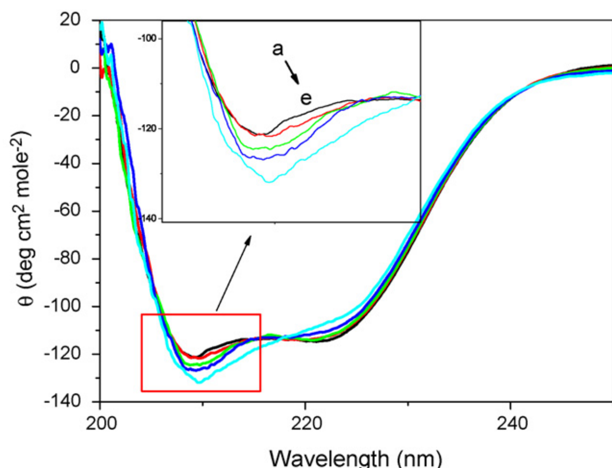

Fig. 5. CD spectra of the HAS–B12 system. c(HSA) = 1.0×10^{-5} M; c(B12)/c(HSA), (a–e) 0; 0.5; 1; 2; 4.

4. Conclusions

The main purpose of this research was to study the binding properties between HSA and B12 due to the great importance of the binding in pharmacology and biochemistry. The interaction of B12 with HSA was studied by spectroscopic methods including fluorescence spectroscopy, UV–vis absorption spectroscopy and CD techniques. This study shows that that the probable quenching mechanism of fluorescence of HSA by B12 is a dynamic quenching mode with high affinity and the binding of B12 to HSA is predominantly owing to hydrophobic interactions. The distance between protein and bound B12 was calculated. Synchronous fluorescence spectra show that the micro-environment of the tryptophan residues changes to be more hydrophobic. The changes of the secondary structure of HAS in the presence of B12 were investigated by CD spectroscopy.

Acknowledgements

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