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ORIGINAL PAPER

A study on the interaction between 5-Methyluridine and human serum albumin using fluorescence quenching method and molecular modeling

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Abstract This work was designed to study the interaction between 5-Methyluridine and human serum albumin (HSA) under simulative physiological conditions using fluorescence spectroscopy in combination with molecular modeling technique for the first time. Static quenching was suggested by the fluorescence measurement. The binding constants (*K*) were calculated according to the relevant fluorescence data at different conditions including temperature. Thermodynamic parameter, different conditions including temperature to determine enthalpy change and entropy change, indicating the hydrophobic force played a major role in the binding interaction between 5-Methyluridine and HSA. The experimental result was in correspondence with molecular modeling theory.

Keywords Fluorescence spectroscopy · Human serum albumin · 5-Methyluridine · Molecular modeling

Introduction

Human serum albumin (HSA) is the most abundant protein constituent of blood plasma and serves as a protein storage component. It is synthesized in the liver, exported as a non-glycosylated protein, and present in the blood at around 40 mg mL^{-1} (~0.6 mmol L^{-1}). The three-dimensional structure of human serum albumin has been determined through X-ray crystallographic measurement [1, 2]. The globular protein consists of a single polypeptide chain of 585 amino acid residues and has many important physiological functions. HSA considerably contributes to colloid osmotic blood pressure and realizes transport and distribution of many molecules and metabolites, such as fatty acids, amino acids, hormones, cations and anions, and many divers drugs [3]. It is composed of three structurally similar domains (I, II and III), each containing two subdomains (A and B), stabilized by 17 disulfide bridges. Aromatic and heterocyclic ligands were found to bind within two hydrophobic pockets in subdomains IIA and IIIA, site I and site II [4]. HSA can interact with many endogenous and exogenous substances including many drugs. Drug interactions at protein binding level will in most case significantly affect the apparent distribution volume of the drugs and also affect the elimination rate of drugs. It is important to study the interaction of drug with the protein because protein-drug binding plays an important role in pharmacology and pharmacodynamics. The information on the interaction of serum albumin and drug can help us better understand the absorption and distribution of the drug.

In a series study methods concerning the interaction of drugs and protein, fluorescence techniques are great aids in the study of interactions between drugs and plasma proteins in general and serum albumin in particular because of their high sensitivity, rapidity, and ease of implementation [5]. For macromolecules, the fluorescence measurements can give some information of the binding of small molecule substances to protein, such as the binding mechanism, binding mode, binding constants, binding sites, intermolecular distance, *etc*.

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Synchronous fluorescence scan (SFS) analysis has become a new attractive method since its introduction by Lioyd and Evett [6], who initially applied it in the field of forensic science. The main characteristics of SFS are a narrowing of the spectral band, simplification of the emission spectra, and contraction of the spectral range. Nowadays, there have been some reports devoted to studies of the interaction between drugs and serum albumin [7]. Extensive investigations into the interaction between the serum albumin and internal compound or pharmaceutical molecule have been made, but the interactions of protein with the nucleoside drugs have seldom been reported [8].

Nucleosides and their derivatives exhibit significant antitumor, antiviral and antibacterial activities [9, 10]. It is recognized that nucleosides have the most potential function to restrain virus [11]. 5-Methyluridine (Fig. 1) is one of important pharmaceutics intermediate, it can be used to synthesize Azidothymidine (AZT) and 1-(2,3-dideoxy-β-Dglycero-pent-2- enofuranosyl)-Thymine (D4T; Stavudine) which have the function to restrain HIV and many other Nucleosides drugs [12]. Therefore, it is of important worthiness and significance to investigate the interaction between 5-Methyluridine and human serum albumin.

Materials and methods

Materials

HSA (Sigma) was directly dissolved in double distilled water to prepare the stock solution (2.0×10^{-5} mol L^{-1} HSA), and the stock solution was kept in the dark at 0 ~ 4 °C; 1.0×10^{-3} mol L^{-1} 5-Methyluridine solution was obtained by dissolving it in double distilled water. 0.1 mol L^{-1} Tris-HCl buffer solution of pH 7.4, 0.5 mol L^{-1} NaCl working solution and 1.0 mg mL^{-1} coexistent ions solutions were prepared. Unless otherwise mentioned, all chemicals were of analytical reagent grade and were used without further purification. Double distilled water was used throughout the experiment.

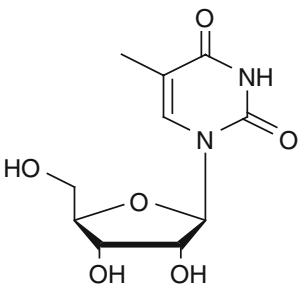

Fig. 1 The molecular model of 5-Methyluridine ($C_{10}H_{14}N_2O_6$, 258.23)

Apparatus

All fluorescence measurements were carried out on a FP-6500 spectrofluorimeter (JASCO, Japan) and a RF-540 spectrofluorimeter (Shimadzu, Japan) equipped with a thermostat bath and 1.0 cm quartz cells, using 5 nm/5 nm slit widths. All pH values were measured by a pH-3digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass electrode. All calculations were performed on a SGI workstation while studying the molecular modeling.

Fluorescence measurements

A 2.0 mL Tris-HCl buffer solution, 2.0 mL NaCl solution, appropriate amount of 5-Methyluridine and HSA were added to a 10 mL standard flask and diluted to 10 mL with double distilled water. Fluorescence quenching spectra of HSA were obtained at excitation and emission wavelengths of λ_{ex}=282 nm and λ_{em}=300 - 450 nm. The synchronous fluorescence spectra were recorded from 280 nm to 350 nm at $\Delta\lambda$=15 and from 310 to 380 at $\Delta\lambda$=60 nm.

Molecular modeling study

The potential of the 3D structures of HSA was assigned according to the Amber 4.0 force field with Kollman-all-atom charges. The initial structures of all the molecules were generated by molecular modeling software Sybyl 6.9.1 [13]. The geometries of this drug were subsequently optimized using the Tripos force field with Gasteiger–Marsili charges. The AutoDock3.05 program [14, 15] was used to calculate the interaction modes between the drug and HSA. Lamarckian genetic algorithm (LGA) implemented in Autodock was applied to calculate the possible conformation of the drug that binds to the protein. During docking process, a maximum of 10 conformers was considered for the drug. The conformer with the lowest binding free energy was used for further analysis. All calculations were performed on SGI FUEL workstation.

Results and discussion

Fluorescence quenching spectra study

The fluorescence of HSA comes from the tryptophan, tyrosine and phenylalanine residues [16]. Actually, the intrinsic fluorescence of HSA is almost contributed by tryptophan alone, because phenylalanine has a very low quantum yield and the fluorescence of tyrosine is almost totally quenched if it is ionized or near an amino group, a

carboxyl group, or a tryptophan [17]. When small molecular substances bound to HSA, the changes of intrinsic fluorescence intensity of HSA are induced by the microenvironment of tryptophan residue. The fluorescence emission spectra of HSA at various concentration of 5-Methyluridine are shown in Fig. 2. Obviously, HSA had a strong fluorescence emission band at 350 nm by fixing the excitation wavelength at 282 nm, which was mainly due to its single tryptophan residue, while 5-Methyluridine had no intrinsic fluorescence under the present experiment conditions. The fluorescence emission intensity of HSA decreased regularly with the increasing concentration of 5-Methyluridine. The strong quenching of HSA fluorescence clearly indicated that the interaction between 5-Methyluridine and HSA took place and resulted in the microenvironment changes of tryptophan residue and the tertiary structure of HSA [18].

The synchronous fluorescence spectra are frequently used to characterize the interaction between molecule probe and proteins since it can provide information about the molecular microenvironment in a vicinity of the chromospheres molecules. According to Miller [19], with large $\Delta\lambda$ values such as 60 nm, the synchronous fluorescence of HSA is characteristic of tryptophan residue. When $\Delta\lambda$ value is 15 nm, the synchronous fluorescence is characteristic of tyrosine residue [20]. Synchronous fluorescence spectra of HSA upon addition of 5-Methyluridine with varied concentration when $\Delta\lambda$=15 (a) and 60 nm (b) were displayed in Fig. 3. The addition of the drug leads to a dramatic decrease in the synchronous fluorescence intensity with distinct shift of spectral peak from 298 to 301 nm (Fig. 3a) and 339 to 345 nm (Fig. 3b), respectively. It is considered that the maximum emission wavelength (λ_{max}) of the tryptophan residues is relative to the polarity of microenvironment. λ_{max} at 330–332 suggests that tryptophan resides are located in the non-polar region, that is, they are buried in a hydrophobic cavity in HSA; λ_{max} at 350–352 nm indicates that tryptophan residues are exposed to water, that is, the hydrophobic cavity in HSA is disagglomerated and the structure of HSA is looser. Thus, Fig. 3b showed distinctly that 5-Methyluridine mainly bound to the hydrophobic cavity of HSA, which was in accordance with the results from molecular modeling and the thermodynamics parameters obtained by the experimental data as follows. It was also indicated that the polarity around the tryptophan residues was increased and the hydrophobicity was decreased. In Fig. 3a, the fluorescence of tyrosine residues was also quenched by 5-Methyluridine with a slight shift of emission to a longer wavelength from 298 to 301 nm. It is suggested that the polarity around the tyrosine residue was also increased. The addition of tyrosine residues intensity was clearer than tryptophan residues, which may show that the distance between the 5-Methyluridine and tryptophan residues is longer.

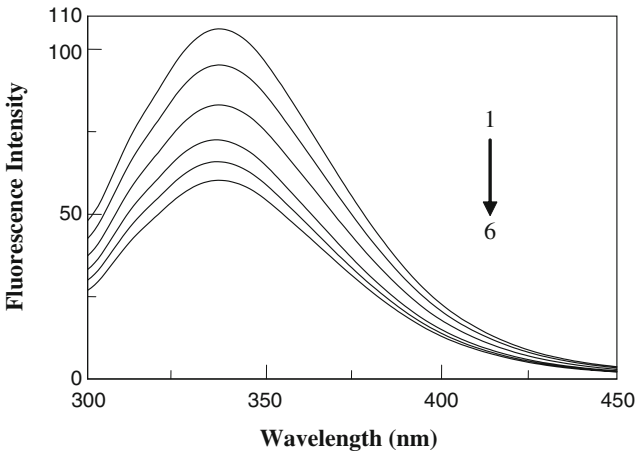

Fig. 2 The fluorescence emission spectra of HSA at 5-Methyluridine various concentrations and an excitation wavelength of 282 nm in Tris-HCl butter solution (pH=7.4) at 27 °C: $C_{HSA}=1.0\times10^{-6}$mol L^{-1} while the $C_{5\text{-Methyluridine}}$ corresponding to 0, 0.2, 0.4, 0.6, 0.8, 1.0×10^{-4} mol L^{-1} is denoted by numerals 1 to 6

Quenching mechanisms study

The static quenching and dynamic quenching were differentiated by the results at different temperatures. The quenching rate constants decrease with increasing temperature for the static quenching, but reversed effect is observed for the dynamic quenching [21]. The possible quenching mechanism can be interpreted by the fluorescence quenching spectra of HSA. The F_0/F-[Q] (sternvolmer) curves of 5-Methyluridine with HSA at different temperatures (27, 37, and 47 °C) are shown in Fig. 4.

It can be found from Fig. 4 that the Stern-Volmer plots are linear and the slopes decrease with the increasing temperature. This indicates the interaction between 5-Methyluridine and HSA is the static quenching. In order to confirm this point, the procedure was assumed to be dynamic quenching. The fluorescence quenching data were analyzed by the Stern-Volmer equation:

$$F_0/F = 1 + K\mathrm{q}\tau_0[\mathrm{Q}] = 1 + K\mathrm{sv}[\mathrm{Q}] \quad (1)$$

Where F and F_0 are the fluorescence intensities with and without quenchers. Kq, Ksv, τ_0 and [Q] are the quenching rate constant of the biomolecule, the Stern-Volmer quenching constant, and the average lifetime of molecule without quencher and concentration of quencher. Obviously,

$$K\mathrm{sv} = K\mathrm{q}\tau_0 \quad (2)$$

Because fluorescence lifetime of the biomolecule is 10^{-8} s [22], an approximate quenching constant (Kq, L mol^{-1} s^{-1})

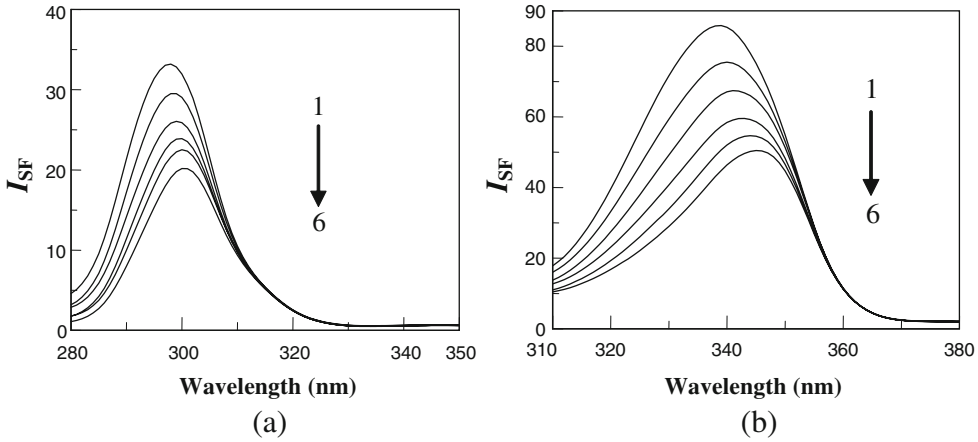

Fig. 3 The synchronous fluorescence spectra of HSA at 5-Methyluridine various concentrations while the $\Delta\lambda$ = 15 nm (**a**) and $\Delta\lambda$=60 nm (**b**): C_{HSA} and $C_{5\text{-Methyluridine}}$ are all the same as those in Fig. 2

could be obtained according to the Eq. (2). The results were listed in Table 1 together with the correlation coefficients.

The maximum scatter collision quenching constant Kq of various quenchers with the biopolymer is 2.0×10^{10} L mol^{-1} s^{-1} [23]. Obviously, the rate constant of protein quenching procedure initiated by 5-Methyluridine was greater than that of the scatter procedure. This showed that the quenching was not initiated by dynamic collision but from the formation of a complex. The static quenching equation is,

$$(F_0 - F)^{-1} = F_0{}^{-1} + K^{-1}F_0{}^{-1}[Q]^{-1} \quad (3)$$

Where K denotes the binding constant of drug and biomolecule, which can be calculated from the slope and intercept of the Lineweaver-Burk curves as shown in Fig. 5 (K = intercept/slope). The results are listed in Table 2.

It is shown that the binding between 5-Methyluridine and HSA is remarkable and the effect of temperature is small. Thus, 5-Methyluridine can be stored and removed by protein in the body.

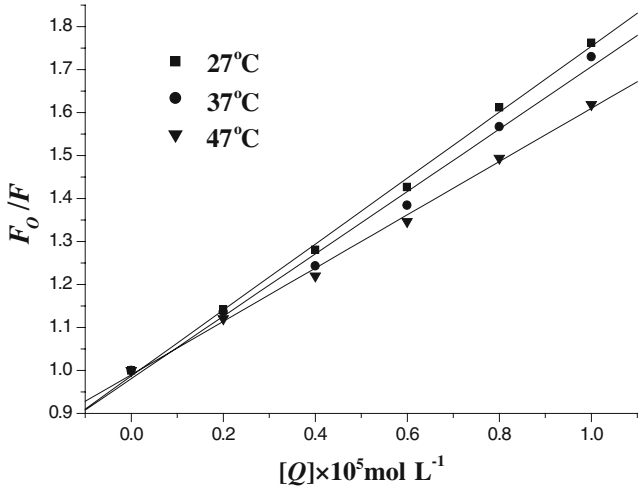

Fig. 4 The Stern-Volmer curves for 5-Methyluridine quenching the fluorescence of HSA in Tris-HCl butter solution at pH 7.40; $C_{5\text{-Methyluridine}}$ and C_{HSA} are the same as those in Fig. 2

Binding modes

The acting forces between a drug and a biomolecule are composed of weak interactions of molecules such as hydrogen bond formation, Van der Waals forces, electrostatic forces, and the hydrophobic interaction [24]. The thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS) of binding reaction, are the main evidence for confirming binding modes. From the thermodynamic standpoint, $\Delta H>0$ and $\Delta S>0$ implies a hydrophobic interaction; $\Delta H<0$ and $\Delta S<0$ reflects the van der Waals force or hydrogen bond formation; and $\Delta H\approx0$ and $\Delta S>0$ suggests an electrostatic force [25].

The temperature-dependence of the binding constants was studied at three different temperatures (27, 37, and 47 °C) in order that HSA did not undergo any structure degradation. Because the temperature effect was very small, the interaction enthalpy change could be regarded as a constant if temperature range was not too wide. According to the following thermodynamic equations:

$$\ln K = -\Delta H/RT + \Delta S/R \quad (4)$$

$$\Delta G = \Delta H - T\Delta S = -RT\ln K \quad (5)$$

Where K is the Lineweaver-Burk static quenching constant at corresponding temperature and R is the gas constant, in which ΔH and ΔS of reaction could be determined from the linear relationship between ln$K$ and the reciprocal absolute temperature (Fig. 6). The free energy change

Table 1 The quenching constants (L mol^{-1} S^{-1}) between 5-Methyluridine and HSA by Stern-Volmer equation

T(°C)	Stern-Volmer equation	Kq(L mol^{-1} S^{-1})	R
27	Y=0.9869+0.7673×10^4[Q]	0.7673×10^{12}	0.9988
37	Y=1.0064+0.7246×10^4[Q]	0.7246×10^{12}	0.9985
47	Y=0.9901+0.6198×10^4[Q]	0.6198×10^{12}	0.9985

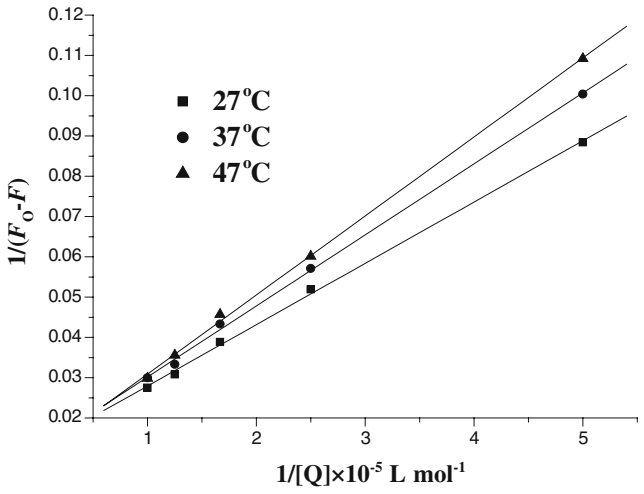

Fig. 5 The Lineweaver-Burk curves for 5-Methyluridine quenching the fluorescence of HSA in Tris-HCl butter solution at pH 7.40; $C_{5\text{-Methyluridine}}$ and C_{HSA} are the same as those in Fig. 2

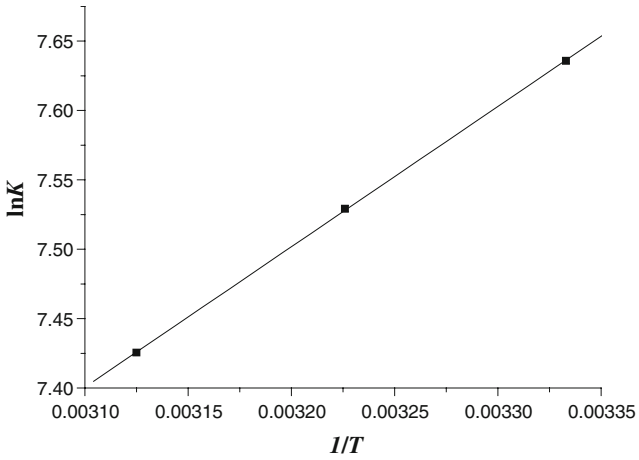

Fig. 6 The Van't Hoff plot for the interaction of HSA and 5-Methyluridine

(ΔG) could be calculated by the Eq. (5). The results are represented in Table 3.

As shown in Table 3, ΔG and ΔH were negative, and ΔS was positive. Therefore, the formation of 5-Methyluridine-HSA coordination compound was spontaneous and exothermic reaction accompanied a positive ΔS value. According to the views of Neméthy and Scheraga [26], Timasheff [27], Ross and Subramananian [25], the positive ΔS value is frequently taken as evidence for hydrophobic interaction. Furthermore, specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive value of ΔS and a negative ΔH value. Accordingly, it was not possible to account for the thermodynamic parameters of 5-Methyluridine-HSA complex on the basis of a single interaction. It was more likely that both hydrophobic and electrostatic interactions were involved in the binding process. The modeling study also validated this conclusion and the result indicated the hydrogen bond was also an ingredient of bonding.

Binding distance

According to Föster's non-radiative energy transfer theory [28], the rate of energy transfer depends on: (i) the relative orientation of the donor and acceptor dipoles, (ii) the extent of overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, and (iii) the distance between the donor and the acceptor. The energy transfer effect is related not only to the distance between the acceptor and the donor, but also on the critical energy transfer distance R_0, that is:

$$E = R_0^6 / \left(R_0^6 + r^6\right) \tag{6}$$

Where r is the distance between the acceptor and the donor and the R_0 is the critical distance when the transfer efficiency is 50%, which can be calculated by

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \tag{7}$$

Where k^2 is the spatial orientation factor between the emission dipole of the donor and the absorption dipole of the acceptor. The dipole orientation factor, k^2, is the least certain parameter in calculation of the critical transfer distance, R_0. Although theoretically k^2 can range from 0 to 4, the extreme values require very rigid orientations. If both the donor and acceptor are tumbling rapidly and free to assume any orientation, then k^2 equals 2/3 [29]. If only the donor is free to rotate, then k^2 can vary from 1/3 to 4/3 [30, 31]. N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor. And J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor, given by

$$J = \Sigma F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda / \Sigma F(\lambda)\Delta\lambda \tag{8}$$

Where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor in wavelength λ and is dimensionless, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor in wavelength λ. The energy transfer efficiency is frequency calculated

Table 2 The binding constants (L mol^{-1}) between 5-Methyluridine and HSA by Lineweaver-Burk equation

T (°C)	Lineweaver-Burk equation	K(L mol^{-1})	R
27	Y=0.0127+0.1522×10^{-5}/[Q]	8.344×10^3	0.9994
37	Y=0.0125+0.1763×10^{-5}/[Q]	7.090×10^3	0.9994
47	Y=0.0113+0.1962×10^{-5}/[Q]	5.759×10^3	0.9995

Table 3 Thermodynamic parameters of 5-Methyluridine-HSA interaction at pH 7.4

Complex	T (°C)	ΔG(kJ mol^{-1})	ΔH(kJ mol^{-1})	ΔS(J mol^{-1} K)
5-Methyluridine -HSA	27	-22.52	-12.62	33.0
	37	-22.85		
	47	-23.04		

from the relative fluorescence yield in the presence (F) and absence of acceptor (F_0):

$$E = 1 - F/F_0 \quad (9)$$

J could be evaluated by integrating the spectra in Fig. 7. It was reported for HSA that k^2=2/3, Φ=0.118 and N=1.336 [32]. The value of the overlap integral calculated from Fig. 7 was 2.5622×10^{-14} cm^3 l mol^{-1}. Based on these data, it could be obtained that r=3.55 nm. That was, the distance between 5-Methyluridine and tryptophan residue in HSA was 3.55 nm which value was smaller than 7 nm, confirming the static quenching interaction between 5-Methyluridine and HSA.

The effects of other ions on the binding constants

The protein molecule contains the elements of S, P, Cu and Mn besides C, H, O and N. In addition, some of trace metal ions exist in the organism, which have definite ability to bind proteins [33, 34]. To investigate the effect of coexistent ions, the binding constants in the presence of other ions were investigated at 27 °C under the experimental conditions. The results are list in Table 4. It showed that the binding constants between 5-Methyluridine and the protein increased in the presence of other ions, implying stronger binding between 5-Methyluridine and HSA. The higher binding constant obtained in the presence of metal ions might be resulted from the interaction of metal ion with the drug to form a complex, and then the complex interacted with the protein. This prolonged the storage time of the drug in blood plasma and enhanced the maximum effectiveness of the drug. Therefore, in the presence of common ions, 5-Methyluridine can be stored and removed better by protein.

Molecular modeling study

A complementary application of molecular modeling by a computer method was employed to improve our understanding of the interaction of 5-Methyluridine and HSA. Descriptions of the 3-D structure of crystalline albumin have revealed that HSA comprises of three homologous domains (denoted I, II, and III): I (residues 1–195), II (196–383), and III (384–585), and each with two subdomains, A and B possessing common structural motifs. It is suggested that the principal regions of ligand binding to HSA are located in hydrophobic cavities in subdomains IIA and IIIA, which are consistent with site I and site II, respectively, and one tryptophan residue (Trp-214) of HSA is in subdomain IIA [35]. There is a large hydrophobic cavity present in subdomain IIA that many drugs can bind to. The crystal structure of HSA was taken from the Brookhaven Protein Data Bank (entry codes 1 h9z) [36]. The potential of the 3-D structures of HSA was assigned according to the Amber 4.0 force field with Kollman-all-atom charges. The initial structures of all the molecules were generated by molecular modeling software Sybyl 6.9.1. The geometries of these compounds were subsequently optimized using the Tripos force field with Gasteiger–Marsili charges. AutoDock 3.05 program was used to calculate the interaction

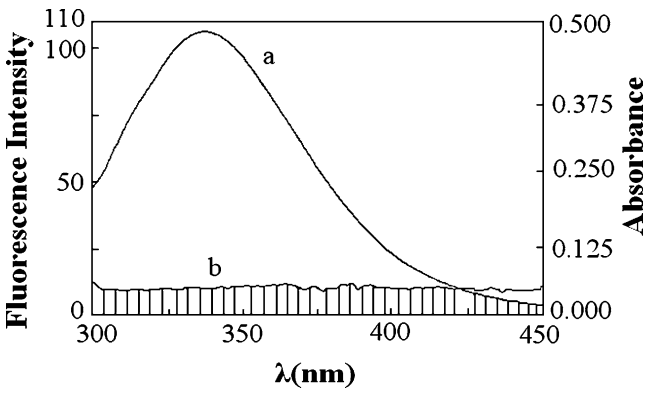

Fig. 7 The overlap of UV absorption spectra of 5-Methyluridine and the fluorescence emission spectra of HSA. **a**: The fluorescence emission spectrum of HSA 1.0×10^{-6} mol L^{-1}); **b**: The UV absorption spectrum of 5-Methyluridine (0.4×10^{-4} mol L^{-1})

Table 4 The binding constants (K, L mol^{-1}) between 5-Methyluridine and HSA at 27 °C in the presence of common ions

Ions	K (10^3)	R_{HSA}	Ions	K (10^3)	R_{HSA}
K^+	5.801	0.9992	Zn^{2+}	2.888	0.9992
Cd^{2+}	4.917	0.9999	Pb^{2+}	7.560	0.9998
NH_4^+	6.955	0.9997	Mg^{2+}	3.337	0.9996
Bi^{3+}	5.241	0.9984	F^-	3.649	0.9995
Cu^{2+}	2.713	0.9980	SiO_3^{2-}	2.484	0.9998
$C_2O_4^{2-}$	3.735	0.9992	NO_3^-	3.317	0.9990
Mn^{2+}	3.526	0.9994	CO_3^{2-}	4.957	0.9999
Ni^{2+}	6.134	0.9997	PO_4^{3-}	9.633	0.9994
SO_4^{2-}	4.726	0.9995	Fe^{3+}	3.902	0.9997
Ca^{2+}	4.729	0.9989	Al^{3+}	3.542	0.9970
Hg^{2+}	2.760	0.9998	Co^{2+}	2.180	0.9996

modes between the ligands and HSA. Lamarckian genetic algorithm (LGA) implemented in Autodock was applied to calculate the possible conformation of the ligands that binds to the protein. During docking process, a maximum of 10 conformers was considered for this compound. The conformer with the lowest binding free energy was used for further analysis.

The best energy ranked result is shown in Fig. 8. It was obvious that the 5-Methyluridine molecule was situated within subdomain IIA hydrophobic cavity, and the 5-Methyluridine was adjacent to hydrophobic residues, such as Lys(195), Glu(292), Ala(291), His(242), Leu (238), Phe(223), Arg(222), Ala (215), Phe(211), Arg (218), Trp(214), *etc*., of subdomain IIA of HSA. The results of molecular modeling suggested that the interaction between 5-Methyluridine and HSA was dominated by hydrophobic force, which was in agreement with the binding mode proposed in thermodynamic analysis. In addition, there were some hydrogen bonds between 5-Methyluridine and residues of HSA such as Lys(195); Arg(218); Arg(222); Ala(291).

Conclusions

In this paper, the binding reaction between 5-Methyluridine and HSA was studied by fluorescence emission spectrum, synchronous fluorescence spectrum and molecular modeling method. The studies of the interaction between 5-Methyluridine and HSA showed that a complex was formed between these species through the static quenching procedure. It could be deduced that the binding mode in the binding reaction between 5-Methyluridine and HSA was mainly the hydrophobic interaction estimated from the signs of ΔH and ΔS, which was confirmed by the result of molecular modeling. This report has special significance in pharmacology and clinical medicine as well as methodology.

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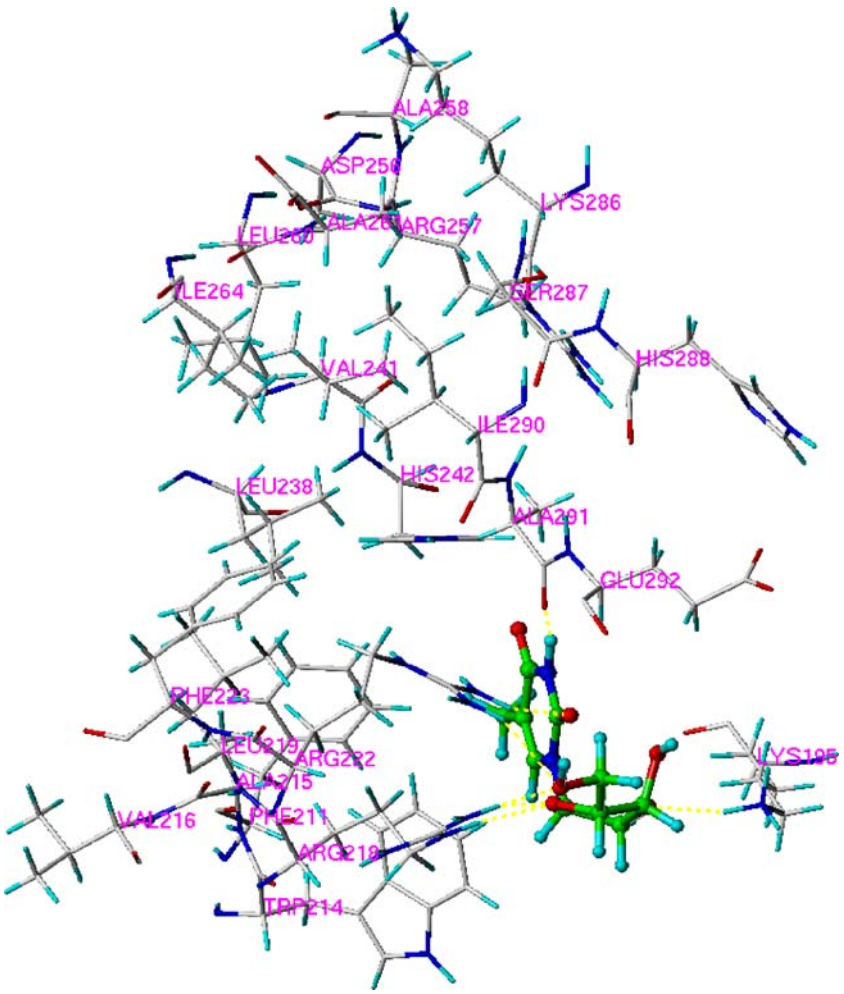

Fig. 8 The molecular modeling of interaction between 5-Methyluridine and HSA. The residues of HSA and 5-Methyluridine are represented using differently colored sticks. The hydrogen bond between the ligands and the protein is indicated by dashed line

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