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Short communication

Studies of the interaction between palmatine hydrochloride and human serum albumin by fluorescence quenching method

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Abstract

The interaction between palmatine hydrochloride with human serum albumin (HSA) was investigated by fluorescence quenching technique and UV/vis absorption spectroscopy. The results of fluorescence titration revealed that palmatine hydrochloride could strongly quench the intrinsic fluorescence of HSA by static quenching and nonradiative energy transferring. The electrostatic interaction plays a major role in stabilizing the complex. The binding site number n and apparent binding constant K_A, corresponding thermodynamic parameters ΔG, ΔH and ΔS at different temperatures were calculated. The distance r between donor (HSA) and acceptor (palmatine hydrochloride) was obtained according to fluorescence resonance energy transfer. The effect of palmatine hydrochloride on the conformation of HSA was analyzed using synchronous fluorescence spectroscopy.

Keywords: Palmatine hydrochloride; Human serum albumin; Fluorescence quenching; Thermodynamic parameters; Energy transfer

1. Introduction

Palmatine hydrochloride (molecular structure; Fig. 1), the protoberberine class of isoquinoline alkaloids, has been found in plants of various families, and mainly presents in the rhizomes of Fibrarurea Tinctoria Lour. These medicinal plants have been used as folk medicine in treatment of jaundice, dysentery, hypertension, inflammation and liver-related diseases [1,2]. In addition, the previous studies have shown that palmatine could block the delayed rectifier potassium current and had inhibition effect on $I_{Ca,L}$ (L-type calcium current) in guinea pig ventricular myocytes[3,4]. Virtanen et al. [5] reported that Hepasor, containing palmatine, columbamine and jatrorrhizine prevented liver from chemically induced traumatization and also promoted the healing process in the hepatic injury models selected. Hepasor improved the blood flow and mitotic activity in thioacetamide-traumatized rat livers. Wang et al. [6] reported that palmatine blocked K^+ channel and decreased the extracellular K^+ to regulate the metabolic processes in the liver. Palmatine also inhibited I_{CRAC} (Ca^{2+} release-activated Ca^{2+} current) effectively and protected hepatocytes from calcium overload via the inhibition of I_{CRAC}. The inhibitory effected on I_K (the delayed outward potassium currents) and I_{CRAC} may partly contribute to the hepatoprotective action of palmatine.

Serum albumins are the most abundant proteins in the circulatory system of a wide variety of organisms. Being the major macromolecule contributing to the osmotic blood pressure, they can play a dominant role in drug disposition and efficacy [7–13]. Distribution and metabolism of many drugs, in the body are correlated with their affinities toward serum albumins. Thus, the investigation of such molecules with respect to albumin binding is of imperative and fundamental importance. Molecular interactions between serum albumins and many protoberberine have been investigated successfully [14,15], but these studies are so far limited berberine, detailed investigations of the interaction of human serum albumin (HSA) with palmatine hydrochloride are scanty. Because of its medical relevance, our work should be valuable.

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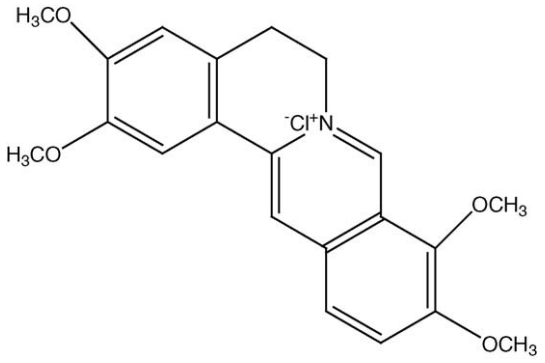

Fig. 1. Molecular structure of palmatine hydrochloride.

The drug–protein interactions are often monitored by conventional approaches such as affinity and size exclusion chromatography, equilibrium dialysis, ultrafiltration and ultracentrifugation, which suffer from lack of sensitivity or long analysis time or both and use of protein concentrations far in excess of the dissociation constant for the drug–protein complex and for drug–protein interaction studies [16–18]. Fluorescence quenching is an important method to study the interaction of substances with protein because it is sensitive and relatively easy to use. Fluorescence spectroscopy is essentially a probe technique sensing changes in the local environment of the fluorophore, which distinguishes it from generalized techniques, such as calorimetry, far-ultraviolet circular dichroism (CD) and infrared (IR) spectroscopy. Also, various possibilities of structural rearrangements in the environment of the fluorophore may lead to a similar fluorescence signal; they can complicate interpretation of the experimental result and be exploited to obtain unique structural and dynamic information [19–21]. In the present work, we demonstrated the affinity of palmatine hydrochloride to HSA and the thermodynamics of their interaction. In order to attain these objectives, we planned to carry out detailed investigation of palmatine hydrochloride–HSA association using fluorescence spectroscopy and UV/vis absorption spectroscopy. Through fluorescence resonance energy transfer and synchronous fluorescence spectroscopy, we planned to further investigate the effect of the energy transfer and the effect of palmatine hydrochloride on the conformation of HSA.

2. Materials and methods

2.1. Materials

Palmatine hydrochloride was purchased from the National Institute for Control of Pharmaceutical and Products (Beijing, China) and HSA was purchased from Sigma (St. Louis, MO, USA). The buffer Tris was purchased from Acros (Geel, Belgium), and NaCl, HCl, etc., were all of analytical purity. HSA solution (2.0×10^{-6} mol L^{-1}) was prepared in pH 7.40 Tris–HCl buffer solution (0.02 mol L^{-1} Tris and 0.05 mol L^{-1} NaCl). The palmatine hydrochloride solution (2.5×10^{-4} mol L^{-1}) was prepared in pH 7.40 Tris–HCl buffer.

2.2. Equipments and spectral measurements

The UV/vis spectrum was recorded at 300 K on a GBC UV/vis916 spectrophotometer (Australia) equipped with 1.0 cm quartz cells. All fluorescence spectra were recorded on LS-50B Spectrofluorimeter (Perkin-Elmer, USA) equipped with 1.0 cm quartz cells and a thermostat bath. The widths of both the excitation slit and the emission slit were set to 5.0 nm.

2.3. Procedures

A 2.5 mL solution, containing appropriate concentration of HSA, was titrated by successive additions of a 2.5×10^{-4} mol L^{-1} stock solution of palmatine hydrochloride (to give a final concentration of 1.1×10^{-5} mol L^{-1}). Titrations were done manually by using trace spyinges. The fluorescence spectra were then measured (excitation at 295 nm and emission wavelengths of 300–500 nm) at two temperatures (300 and 310 K). The UV/vis absorbance spectra of palmatine hydrochloride with concentration of 2.0×10^{-6} mol L^{-1} was recorded at 300 K.

3. Results and discussion

3.1. Fluorescence quenching

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule. Fluorescence quenching can be dynamic, resulting from collisional encounters between the fluorophore and quencher, or static, resulting from the formation of a ground-state complex between the fluorophore and quencher [22]. Dynamic quenching constants are expected to increase with increasing temperature, In contrast, increased temperature is likely to result in decreased stability of complexes, and thus lower values of the static quenching constants. In order to discuss the results within the linear concentration range, we selected carrying out the experiment within the linear part of Stern–Volmer dependence, which gives the ratio between fluorescence intensities in the absence or the presence of a quencher as a function of its concentration, taking into account the fluorophore lifetime in the quencher's absence [23].

HSA consists of amino acids chains forming a single polypeptide with well-known sequence, which contains three homologous α-helices domains. Each domain contains 10 helices and is divided into antiparallel 6 helix and 4 subdomains [24,25]. A valuable feature of intrinsic fluorescence of proteins is the high sensitivity of tryptophan to its local environment [22]. Changes in emission spectra of tryptophan are common in response protein conformational transitions, subunit association, substrate binding or denaturation [26]. So, the intrinsic fluorescence of proteins can provide considerable information about their structure and dynamics, and if often considered on the study of protein folding and association reactions.

The effect of palmatine hydrochloride on HSA fluorescence intensity is shown in Fig. 2. As the data show, the fluorescence intensity of HSA decreased regularly with the increasing con-

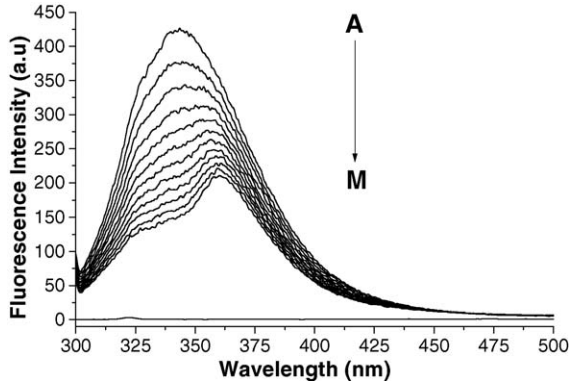

Fig. 2. Effects of pharmaceutical on fluorescence spectra of HSA (T = 300 K, pH 7.40 and λ_{ex} = 295 nm). c(HSA) = 2.0×10^{-6} mol L^{-1}; c(palmatine) (10^{-6} mol L^{-1}), curves (A–L) 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and curve (M) shows the emission spectrum of palmatine only, c(palmatine) = 2.0×10^{-6} mol L^{-1}.

centration of palmatine hydrochloride; the fluorescence spectrum of HSA splits from a single peak to dual peaks is caused by palmatine hydrochloride–HSA binding only. Also, Fig. 2 shows that at the investigated concentration range, the results agree with the Stern–Volmer equation excellently. Although the total intensity of fluorescence emission is usually expressed by the peak height, for the peak-splitting systems it is more reasonable to use the total areas of fluorescence emission (S) instead of the peak height [14,27] in the following Stern–Volmer equation [28]:

$$\frac{S_0}{S} = 1 + K_q \tau_0 c(D)_t = 1 + K_{SV} c(D)_t \quad (1)$$

where S_0 and S are the fluorescence emission peak area of HSA without or with palmatine hydrochloride, respectively. K_q, K_{SV}, τ_0 and $c(D)_t$ are the quenching rate constant of the biomolecule, the Stern–Volmer dynamic quenching constant, the average lifetime of the biomolecule without quencher ($\tau_0 = 10^{-8}$ s) and the total concentration of palmatine hydrochloride, respectively. The concentration of palmatine hydrochloride should be the free ligand concentration, but it is not known in experiment. So, in our analysis it was approximated by the total concentration of palmatine hydrochloride. For higher ligand concentration, in excess of available specific protein binding sites, this approximation is valid. Obviously,

$$K_q = \frac{K_{SV}}{\tau_0} \quad (2)$$

Hence, Eq. (1) was applied to determine K_{SV} by linear regression of a plot of S_0/S against $c(D)_t$. The corresponding Stern–Volmer quenching constants are shown in Table 1. The results show the Stern–Volmer quenching constant K_{SV} is inversely correlated with temperature, which indicate that the probable quenching mechanism of fluorescence of HSA by palmatine hydrochloride is not initiated by dynamic collision but compound formation [22].

3.2. Apparent association constant and binding sites

Langmuir isotherm is an equation used to describe the dynamic equilibrium between adsorption and desorption of gaseous molecules at solid surface. It is well known that this equation can be applied not only to gas–solid interaction but also to liquid–solid interaction. Liu et al. [14] and Ji et al. [29] have applied the equation to evaluate the dynamic parameters of the ligand–protein complex. In this paper, Langmuir adsorption isotherm is applied to obtain the apparent association constant K_A, and binding sites n. The binding process can be described by the following equation:

$$D + P \underset{k^-}{\overset{k^+}{\rightleftarrows}} D - P \quad (3)$$

Assuming that each HSA molecule has n sites available for accepting palmatine hydrochloride, and palmatine hydrochloride–HSA complex has no fluorescence, we can have the following relations:

$$c(D)_t = c(D)_b + c(D)_f \quad (4)$$

$$\frac{c(D)_b}{nc(P)_t} = \frac{S_0 - S}{S_0} \quad (5)$$

where $c(D)_b$ and $c(D)_f$ are the concentration of bounded and free palmatine hydrochloride, respectively, and $c(P)_t$ is the total concentration of HSA. From the two assumptions of Langmuir isotherm [30], the equilibrium equation analogous to Langmuir isotherm can be established:

$$\frac{c(D)_b}{nc(P)_t} = \frac{K_A c(D)_f}{1 + K_A c(D)_f} \quad (6)$$

where $K_A = k^+/k^-$, is the apparent association constant for the equilibrium formation of palmatine–HSA complex. From Eqs. (4)–(6), the following equation can be obtained:

$$\frac{S_0}{S} = K_A \left(\frac{c(D)_t S_0}{S_0 - S} \right) - nK_A c(P)_t \quad (7)$$

When data of S, S_0 and $c(D)_t$ are available at $c(P)_t = 2.0 \times 10^{-6}$ mol L^{-1}, the dependence of S_0/S on the value of $(c(D)_t S_0)/(S_0 - S)$ is linear with slope equal to the value of K_A and the value of $-nK_A c(P)_t$ is fixed on the ordinate. K_A and n are listed in Table 2. The results for HSA at different temperatures analyzed in this fashion, the correlation coefficients are greater than 0.99, indicating that the assumptions underlying the

Table 1
Sterm–Volmer quenching constant of the system of palmatine hydrochloride–HSA at different temperatures

T (K)	Linear regression equation	R	10^{-4} K_{sv} (L mol^{-1})	10^{-12} K_q (L mol^{-1} s^{-1})
300	$S_0/S = 0.9847 + 8.836 \times 10^4 c(D)_t$	0.9994	8.836	8.836
310	$S_0/S = 0.9871 + 7.876 \times 10^4 c(D)_t$	0.9994	7.876	7.876

Table 2
Apparent binding constants K_A and binding sites n at different temperatures

T (K)	Equation	R	$10^{-4} K_A$ (L mol^{-1})	n
300	$\frac{S_0}{S} = 9.895 \times 10^4 \left(\frac{c(D)_t S_0}{S_0 - S}\right) - 0.255$	0.9983	9.895	1.288
310	$\frac{S_0}{S} = 8.590 \times 10^4 \left(\frac{c(D)_t S_0}{S_0 - S}\right) - 0.194$	0.9934	8.590	1.129

derivation of Eq. (7) are satisfactory. The results of K_A and n decreased with the temperature rising, which may indicate forming an unstable compound [31] palmatine hydrochloride–HSA in the binding reaction. The compound would possibly be partly decomposed when the temperature increases, therefore, the values of K_A and n decreased with the temperature rising, which was in accordance with the trend of K_{SV}, as mentioned above.

3.3. Thermodynamic parameters and nature of the binding forces

If the enthalpy change (ΔH) does not vary significantly over the temperature range studied, then the thermodynamic parameters ΔH, ΔS and ΔG can be determined from the following equation and the results are listed in Table 3.

$$\ln \frac{(K_A)_2}{(K_A)_1} = \frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \quad (8)$$

$$\Delta G = -RT \ln K_A \quad (9)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (10)$$

The fact of the negative values of free energy ($\Delta G < 0$), as shown in Table 3 indicates that palmatine hydrochloride binding to HSA is a spontaneous inter-molecular interaction. The negative enthalpy ($\Delta H < 0$) and positive ($\Delta S > 0$) values of the interaction of palmatine hydrochloride and HSA indicate that the binding is mainly electrostatic interactions, the electrostatic interaction plays a major role in stabilizing the complex [32,33].

3.4. Energy transfer from HSA to palmatine hydrochloride binding

There is a good overlapping between the fluorescence emission spectrum of free HSA and absorption UV/vis spectra of palmatine hydrochloride (Fig. 3). As the fluorescence emission of protein was affected by the excitation light around 295 nm, the spectrum ranging from 300 to 500 nm was chosen to calculate the overlapping integral.

According to Förster's theory, the energy transfer efficiency E is defined as the following equation Eq. (11). Where r is the distance from the ligand to the tryptophan residue of the protein, and R_0 is the Förster critical distance, at which 50% of the excitation energy is transferred to the acceptor [34]. It can be calculated from donor emission and acceptor absorption spectra using the Förster formula Eq. (12).

Table 3
The relative thermodynamic parmeters of the system of palmatine–HSA

T (K)	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
300	−10.935	−28.689	59.180
310	−10.935	−29.281	59.181

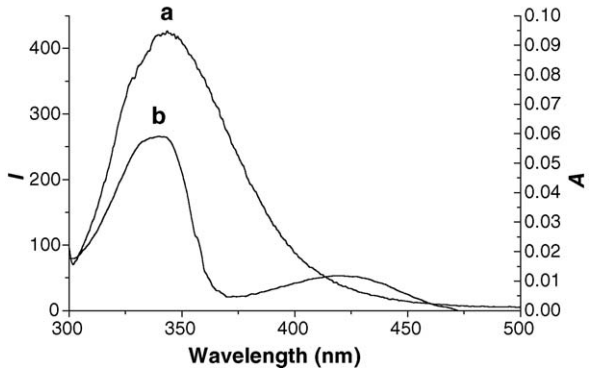

Fig. 3. Spectral overlap of HSA fluorescence (a) with palmatine absorption (b), c(HSA) = c(palmatine) = 2.0×10^{-6} mol L^{-1}.

$$E = 1 - \frac{S}{S_0} = \frac{R_0^6}{R_0^6 + r_0^6} \quad (11)$$

$$R_0^6 = 8.79 \times 10^{-25} K^2 N^{-4} \Phi J \quad (12)$$

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F(\lambda) d\lambda} \quad (13)$$

In Eq. (12), K^2 is the orientation factor related to the geometry of the donor and acceptor of dipoles and $K^2 = 2/3$ for random orientation as in fluid solution, N the average refractive index of medium in the wavelength range where spectral overlap is significant, Φ the fluorescence quantum yield of the donor and J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor (Fig. 3), which could be calculated by Eq. (13). Where $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range λ to $\lambda + \Delta\lambda$ and $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ. In the present case, $N = 1.36$, $\Phi = 0.074$, according to Eqs. (11)–(13), we could calculate that $J = 2.553 \times 10^{-14}$ cm^3 L mol^{-1}, $E = 0.38$, $R_0 = 2.194$ nm and $r = 2.693$ nm. The average distances between a donor fluorophore and acceptor fluorophore on the 2–8 nm scale and $0.5R_0 < r < 1.5R_0$, which indicate that the energy transfer from HSA to palmatine hydrochloride occurs with high probability [35].

3.5. Conformation investigation

To explore the structural change of HSA by addition of palmatine hydrochloride, we measured synchronous fluorescence spectra (Fig. 4) of HSA with various amounts of palmatine hydrochloride.

The synchronous fluorescence spectra give information about the molecular environment in a vicinity of the chromosphere

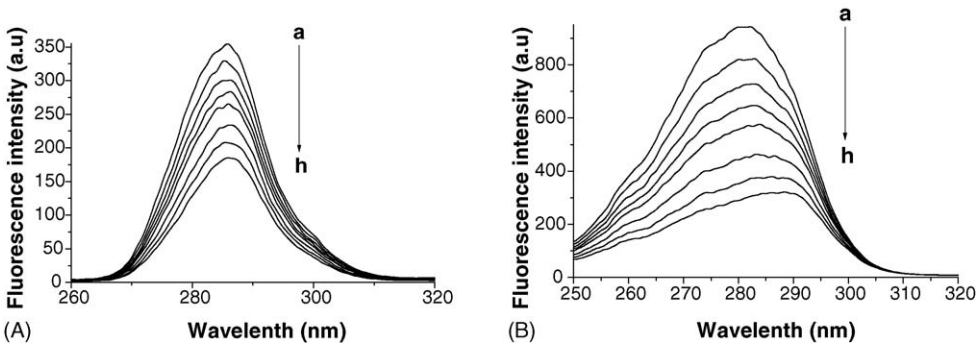

Fig. 4. Synchronous fluorescence spectrum of HSA ($T = 300$ K and pH 7.40). c(HSA) = 2.0×10^{-6} mol L^{-1}; c(palmatine hydrochloride) (10^{-6} mol L^{-1}), curves (a–h) 0, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0. (A) $\Delta\lambda = 15$ nm and (B) $\Delta\lambda = 60$ nm.

molecules. Yuan et al. [36] suggested a useful method to study the environment of amino acid residues by measuring the possible shift in wavelength emission maximum λ_{max}, the shift in position of emission maximum corresponding to the changes of the polarity around the chromophore molecule. When the *D*-value ($\Delta\lambda$) between excitation wavelength and emission wavelength were stabilized at 15 or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine residues or tryptophan residues [37]. The effect of palmatine hydrochloride on HSA synchronous fluorescence spectroscopy is shown in Fig. 4.

It is apparent from Fig. 4 that the little stronger red shift of tryptophan residues fluorescence upon addition of drug, whereas the emission maximum of tyrosine kept the position. The red-shift of the emission maximum indicates that the conformation of HSA was changed and the polarity around the tryptophan residues was increased and the hydrophobicity was decreased [38].

4. Conclusions

A fluorescence method for the rapid and simple determination of the interaction between palmatine hydrochloride and HSA was provided. The method is easy to operate and is reliable, practical and simple. Analysis was made in the present work using the date for protein fluorescence changes induced by drug molecules. The results obtained give preliminary information on the binding of palmatine hydrochloride to HSA. The electrostatic interaction plays a major role in stabilizing the complex. The distance $r = 2.693$ nm between HSA and palmatine hydrochloride was obtained according to fluorescence resonance energy transfer. The results of synchronous fluorescence spectroscopy indicate that the conformation of HSA was changed in the presence of palmatine hydrochloride.

The binding study of drugs with proteins is of great importance in pharmacy, pharmacology and biochemistry. This study is expected to provide important insight into the interactions of the physiologically important protein HSA with drugs. Information is also obtained about the effect of environment on HSA structure which may be correlated to its physiologically activity.

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