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Original Article

Spectroscopic analysis on the binding interaction of biologically active pyrimidine derivative with bovine serum albumin ☆

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ABSTRACT

A biologically active antibacterial reagent, 2–amino-6-hydroxy–4–(4-N, N-dimethylaminophenyl)-pyrimidine-5-carbonitrile (AHDMAPPC), was synthesized. It was employed to investigate the binding interaction with the bovine serum albumin (BSA) in detail using different spectroscopic methods. It exhibited antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* which are common food poisoning bacteria. The experimental results showed that the fluorescence quenching of model carrier protein BSA by AHDMAPPC was due to static quenching. The site binding constants and number of binding sites ($n \approx 1$) were determined at three different temperatures based on fluorescence quenching results. The thermodynamic parameters, enthalpy change (ΔH), free energy (ΔG) and entropy change (ΔS) for the reaction were calculated to be 15.15 kJ/mol, −36.11 kJ/mol and 51.26 J/mol K according to van't Hoff equation, respectively. The results indicated that the reaction was an endothermic and spontaneous process, and hydrophobic interactions played a major role in the binding between drug and BSA. The distance between donor and acceptor is 2.79 nm according to Förster's theory. The alterations of the BSA secondary structure in the presence of AHDMAPPC were confirmed by UV–visible, synchronous fluorescence, circular dichroism (CD) and three-dimensional fluorescence spectra. All these results indicated that AHDMAPPC can bind to BSA and be effectively transported and eliminated in the body. It can be a useful guideline for further drug design.

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1. Introduction

Protein, one of the most important bioactive molecules, is related to alimentation, immunity and metabolism. The content of proteins in body fluid can be used as a vital index for the clinical diagnosis and health evaluation; therefore, the direct determination of protein is significant in life sciences, clinical medicine and chemical investigation. The interaction between bio-macromolecules and drugs has attracted great interest for several decades [1–3] and many researches have been focused on two central questions about proteins: what are the determinant factors that influence the protein structures and functions, and how does a factor affect their biological activity [4,5]. Serum albumin (SA), the main protein in the blood plasma acting as the transporter and disposition of many drugs, has been frequently used as a model protein for investigating protein folding and ligand binding mechanism. In this regard, bovine serum albumin (BSA) has been studied extensively, partly because of its structural homology with human serum albumin (HSA) [6,7]. BSA is composed of three linearly arranged and structurally homologous sub-domains. It has two tryptophan residues that possess intrinsic domains (I–III) and each domain in turn is the product of two fluorescence: Trp-134, which is located on the surface of sub-domain IB, and Trp-212, located within the hydrophobic binding pocket of sub-domain IIA [8,9]. The binding sites of BSA for endogenous and exogenous ligands may be in these domains and the principal regions of drugs binding sites of albumin are often located in hydrophobic cavities in sub-domains IIA and IIIA. So-called sites I and II are located in subdomain IIA and IIIA of albumin, respectively.

Pyrimidine moiety is one of the important classes of N-containing heterocycles widely used as key building blocks for pharmaceutical agents. It exhibits a wide spectrum of pharmacophore such as bactericidal, fungicidal, analgesic, anti-hypertensive and anti-tumor agents [10–13]. In addition, preclinical data from literature survey indicate continuing research in polysubstituted pyrimidine as potential anti-tumor agents [14]. 2-amino-6-hydroxy-4-(4-N,N-dimethylaminophenyl)-pyrimidine-5-carbonitrile (AHDMAPPC), a pyrimidine derivative, and its analogs possessing

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anti-bacterial activity were synthesized in our laboratory [15]. AHDMAPPC was synthesized by three-component condensation of aromatic aldehyde, ethyl cyanoacetate and guanidine hydrochloride in ethanol under alkaline medium.

Protein–drug interaction plays an important role in pharmacokinetics and pharmacodynamics. In a series of methods concerning the interaction of drugs and protein, fluorescence techniques are great aids in the study of interactions between drugs and serum albumin because of their high sensitivity, rapidity, and ease of implementation [16]. The aim of the present investigation was to study the affinity of pyrimidine derivative (AHDMAPPC) for BSA using UV–visible and fluorescence spectroscopy to understand the carrier role of serum albumin for such compound in the blood under physiological conditions. Significantly, the determination and understanding of drug interacting with serum albumin are important for the therapy and design of drug [17]. Knowledge of the interaction and binding of BSA may open new avenues for the design of the most suitable pyrimidine derivatives. All the experimental results clarify that AHDMAPPC can bind to BSA and be effectively transported and eliminated in body, which can be a useful guideline for further drug design.

In this paper, we have studied *in vitro* interaction of AHDMAPPC with BSA by the fluorescence quenching method. The binding constants were obtained at different temperatures in the medium of Tris–HCl (pH 7.4) buffer solution. The binding sites and main sorts of binding forces have been suggested. In addition, the conformational changes of BSA were discussed on the basis of UV–visible spectroscopy, synchronous fluorescence (SF), circular dichroism (CD) and three-dimensional spectroscopy.

2. Materials and methods

2.1. Materials

BSA (essentially fatty acid free) was purchased from Hi-Media Chemical Company (Mumbai, India) and its molecular weight was assumed to be 66, 463 to calculate the molar concentrations. All BSA solutions ($C_{BSA}=2.0\times10^{-5}$ M) were prepared in a pH 7.4 buffer solution and the stock solution was kept in the dark at 4 °C. Tris–HCl (0.1 M) buffer solution containing NaCl (0.1 M) was used to keep the pH of the solution at 7.4. A dilution of the BSA stock solution in Tris–HCl buffer solution was prepared immediately before use. The stock solution of AHDMAPPC (synthesized) was prepared in (5:95, v/v) ethanol water mixture. Dissolution of the compound was enhanced by sonication in an ultrasonic bath (Spectra Lab Model UCB-40). All chemicals were of analytical reagent grade and were used without further purification. Double distilled water was used throughout. In order to simulate human body fluid surroundings and to get the best sensitivity, Tris–HCl solution (pH 7.4) was chosen as the buffer solution in this work.

2.2. Equipment and spectral measurements

All fluorescence emission spectra were recorded on PC based Spectrofluorometer (JASCO Japan FP-750) equipped with an Xenon lamp and 1.0 cm quartz cell. Fluorescence emission spectra were recorded at three different temperatures, 300, 310 and 320 K. Excitation and emission slit width was fixed to 10 nm. An excitation wavelength of 280 nm was chosen, because it is exclusively due to the intrinsic Tryptophan (Trp) fluorophore. The UV–visible absorption spectra were measured at room temperature on a Shimadzu UV–3600 UV–vis–NIR Spectrophotometer equipped with a 1.0 cm quartz cell. The wavelength range was from 250 to 450 nm. All pH values were measured by a digital pH-meter with magnetic stirrer (Equip-Tronics EQ-614A). For synchronous fluorescence measurements, the excitation range was 260 – 360 nm, and $\Delta\lambda$ was set at 15 and 60 nm. Circular dichroism (CD) spectra were measured with a Jasco J-815 Spectropolarimeter (Jasco, Tokyo, Japan) at room temperature over the wavelength range of 200–250 nm using a 1.0 cm quartz cell. The three-dimensional fluorescence spectra were performed under the following conditions: the emission wavelength was recorded between 250 and 500 nm; the initial excitation wavelength was set to 250 nm with increment of 10 nm for each scanning curve; other scanning parameters were identical to those of the fluorescence emission spectra. Appropriate blanks corresponding to the buffer were subtracted to correct the absorbance or fluorescence background.

3. Results and discussions

3.1. Fluorescence quenching studies of BSA by pyrimidine derivative (AHDMAPPC)

Protein is considered to have intrinsic fluorescence mainly originating from the tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues [18]. When it interacts with other compounds, its intrinsic fluorescence often changes with the ligand's concentration. Consequently, fluorescence can be regarded as a technique for measuring the mechanism of interactions between ligands and proteins [19]. The concentration of BSA solutions was stabilized and the concentrations of AHDMAPPC were varied in the experiment. Fluorescence spectra of BSA, after the addition of AHDMAPPC, were recorded upon excitation at 280 nm and 300 K, as illustrated in Fig. 1. It was observed that BSA exhibited a strong fluorescence emission band at 347 nm. The fluorescence intensities of BSA reduced gradually with increasing AHDMAPPC concentrations, and a blue shift was also observed, which suggests that the fluorescence chromophore of serum albumin is placed in a more hydrophobic environment after the addition of AHDMAPPC. The fluorescence quenching effect was due to the formation of non-fluorescent complex [20]. Fluorescence quenching is the decrease of the fluorescence quantum yield from a fluorophore induced by a variety of molecular interactions, such as excited-state reactions, energy transfer, ground-state complex formation, and collisional quenching. The quenching mechanisms are usually classified as dynamic quenching and static quenching, which can be distinguished by their different dependence on temperature and viscosity [21]. Since higher temperatures result in large diffusion coefficients for dynamic quenching, the quenching constants are expected to increase with increasing temperature. In contrast, a higher temperature may bring about the decrease in the stability of the complexes, resulting in a lower quenching constant for the static quenching.

3.2. Quenching mechanism analysis

To further elucidate the quenching mechanism of BSA induced by pyrimidine derivative, the fluorescence quenching data were analyzed with the Stern–Volmer equation [22].

$$F_0/F = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] \tag{1}$$

where F_0 and F are the relative fluorescence intensities in the absence and presence of quencher respectively, $[Q]$ is the concentration of quencher, K_{SV} the Stern–Volmer dynamic quenching constant, k_q the bimolecular quenching rate constant and τ_0 the average lifetime of the fluorophore in the excited state usually for a biomacrolecule10^{-8} s [23–25]. The formation of complex was further confirmed from the values of quenching rate constants k_q,

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