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Investigation of two blood proteins binding to Cantharidin and Norcantharidin by multispectroscopic and chemometrics methods

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ABSTRACT

The interactions of Cantharidin/Norcantharidin (CTD/NCTD) with two blood proteins, i.e., bovine serum albumin (BSA) and bovine hemoglobin (BHb), have been investigated by the fluorescence, UV–vis absorption, and FT-IR spectra under imitated physiological condition. The binding characteristics between CTD/NCTD and BSA/BHb were determined by fluorescence emission and resonance light scattering (RLS) spectra. The quenching mechanism of two blood proteins with CTD/NCTD is a static quenching. Moreover, the experimental data were further analyzed based on multivariate curve resolution-alternating least squares (MCR-ALS) technique to obtain the concentration profiles and pure spectra for three species (BSA/BHb, CTD/NCTD and CTD/NCTD–BSA/BHb complexes) which existed in the interaction procedure. The number of binding sites n and binding constants K_b were calculated at various temperatures. The thermodynamic parameters (such as, ΔG, ΔH, and ΔS) for BSA–CTD/NCTD and BHb–CTD/NCTD systems were calculated by the Van't Hoff equation and also discussed. The distance r between CTD/NCTD and BSA/BHb were evaluated according to Förster no-radiation energy transfer theory. The results of Fourier transform infrared (FT-IR), synchronous fluorescence and three-dimensional fluorescence spectra showed that the conformations of BSA/BHb altered with the addition of CTD/NCTD. In addition, the effects of common ions on the binding constants of BSA–CTD/NCTD and BHb–CTD/NCTD systems were also discussed.

1. Introduction

Cantharidin (CTD, 3a,7a-dimethylhexahydro-4,7-epoxyisobenzofuran-1,3-dione, Scheme 1), is the main active component of blister beetle, which has been used for treating hepatoma and esophageal carcinoma over 2000 years [1]. CTD is a well-known vesicant and effective in treating warts and molluscum [2]. Moreover, it has others biological activities [3–5], such as, anti-insect, anti-feedant. It was demonstrated that CTD revealed profound cytotoxicity towards tumor cells [6]. The study of Efferth et al. [7] suggested that CTD treatment causes oxidative stress that provokes DNA demage and p53-dependent apoptosis. However, further study was discussed by their groups, i.e. DNA repair and apoptosis played a major role as determinants of sensitivity and resistance of tumor cells to CTD [6], and it has a potential usefulness for the treatment of refractory tumors [8]. Lately, Huan et al. [9] has reported that *Scutellaria baicalensis* could alleviate CTD-induced rat hemorrhagic cystitis through inhibition of cyclooxygenase-2 overexpression. Recently, the study of Khan et al. [10] discovered that CTD could impede the Glutathione *S*-Transferase activity in the midgut of *Helicoverpa armigera* Hübner. However, Norcantharidin (NCTD, 7-oxabicyclo [2.2.1] heptane-2,3-dicarboxylic anhydride, Scheme 1) is a demethylated analog of CTD. According to the reported by Tarleton et al. [11], NCTD has approximately 10 fold less cytotoxin than CTD in many of these cell lines, suggesting NCTD should be provided with more clinical applications. In the past few decades, it has been used as an anti-tumor drug in vivo to cure esophageal, gastric, cardiac carcinomas, cancer, and plasmodium [12–15]. Lately, Zhang et al. [16] has reported NCTD could enhance ABT-737-induced apoptosis in hepatocellular carcinoma cells by transcriptional repression of Mcl-1. Recently study Zhang et al. [17] indicated that NCTD inhibited tumor angiogenesis by blocking VEGFR2/MEK/ERK signaling pathways. In addition, NCTD can be used as an agent for alleviating renal interstitial fibrosis and diabetic nephropathy, and inhibiting epithelial mesenchymal transition (EMT) [18]. Although the biological activities of CTD/NCTD have been investigated extensively, the study knowledge of CTD/NCTD binding to two blood proteins still remains unknown. Moreover, it is important for us to comprehend the pharmacodynamics, pharmacology and pharmacokinetic behaviors of two drugs and synthesize their derivatives with effective pharmacology properties. Therefore, we deem that it is worthwhile to investigate the interaction mechanism of BSA/BHb with CTD/NCTD.

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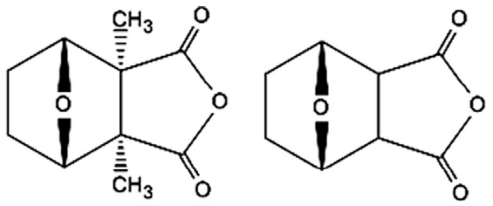

Cantharidin Norcantharidin

Scheme 1. Molecular structures of cantharidin and norcantharidin.

Serum albumins (SAs) are the major soluble protein constituents of the circulatory system, and it serves as transporters for a variety of compounds such as drugs, fatty acids and amine-terminated dendrimers [19]. Among albumins, Bovine serum albumin (BSA) has been investigated widely due to its medical importance, ease of purification, low cost, and its structural homology with human serum albumin (HSA) [20,21]. It is a single polypeptide chain with cross-linking by 17 disulfide bonds [22]. BSA can form complexes with the addition of small ligands because of the interactions of BSA with ligand-cell surfaces [23].

However, hemoglobin (Hb) is a carrier of oxygen in the vascular system of animals, and it can also regulate pH and transport carbon dioxide of blood both directly and indirectly [24]. Hb is composed of four globin chains, i.e. two α and two β-chains [25]. In the crevices near the hemoglobin surface, four heme groups have been found, and each heme attaches to one polypeptide chain [26]. Bovine hemoglobin (BHb) with 90% amino acid sequence homology comparing for human hemoglobin (HHb), which has a few advantages over human counterpart, for example, it is a better oxygen carrier than HHb [27]. Many endogenous and exogenous agents can bind with BHb. In the past, some researchers [27–29] have investigated the interactions between drugs (such as, flavonoids, caffeine, silicotungstic heteropolyacid, platinum drugs, Fe-porphyrins, and troxerutin) and BHb to explain the complex properties and provide useful structural features information that can ascertain the therapeutic effectiveness of drugs. However, the interactions of proteins (BSA and BHb) with drugs (CTD and NCTD) have not been investigated. Moreover, the studies may help us better understand the absorption and distribution of two drugs in vivo.

In the past few decades, the common methods have been applied to study the interactions of small molecules with proteins, which include UV–vis spectrophotometry, CD, FT-IR, HPLC, electrochemistry, capillary electrophoresis, and NMR [30–34]. However, in the multicomponent reaction systems reported above, it is difficult to divide these species due to their response signals overlapping. Therefore, chemometrics methods such as multivariate curve resolution-alternating least squares (MCR-ALS) technique [35] are used for solving the difficult questions. Up to date, some research groups [36–38] reported the interactions of drugs with biopolymers (such as, BSA, DNA, etc.) based on fluorescence, electrochemistry, UV–vis spectra, or other techniques with the aid of MCR-ALS. Moreover, in these binding reaction systems, we could obtain the further information about the complex formation, pure spectra and concentration profiles of each species.

In this text, we have applied multispectroscopic techniques to investigate the interactions of CTD/NCTD with BSA/BHb. The fluorescence quenching mechanisms, the thermodynamic parameters, the number of binding sites, the binding forces and the energy transfer distance were studied for the CTD/NCTD–BSA/BHb systems. The effects of CTD/NCTD on the conformation of two blood proteins were also analyzed. Moreover, the two-way UV–vis and fluorescence spectra data were analyzed using MCR-ALS technique.

2. Experimental

2.1. Materials

Bovine Serum Albumin (BSA, FractionV, 98% purity, M_r= 68,000) obtained from Roche Company, and Bovine hemoglobin (BHb, M_r=64,500) obtained from Sigma-Aldrich, were used without further purification. A certain mass of BSA and BHb were added to the Tris–HCl buffer solution (0.10 mol L^{-1}, pH 7.4) to form a solution of 2.0×10^{-4} mol L^{-1} and then stored at 4 °C. Cantharidin (CTD) and Norcantharidin (NCTD) were purchased from Xi'an feida bio-tech Co., Ltd. (Xi'an, China). The stock solutions of CTD and NCTD were prepared by dissolving them into 5% and 2% acetone with final concentration of 6.0×10^{-4} mol L^{-1}, respectively. All other reagents were of analytical reagent and used without further purification; doubly distilled water was used throughout the experiments. The sample masses were accurately weighted on an electronic analytical balance ESJ180-4 (Shenyang Longteng Electronic Co., Ltd, China) with a resolution of 0.1 mg.

2.2. Apparatus

The fluorescence and RLS spectra were determined using a Cary Eclipse fluorescence spectrofluorometer (Varian, USA) equipped with a xenon lamp source and 1.0 cm quartz cell and a thermostat bath. The UV–vis absorption spectra were recorded on a UV-2550 spectrophotometer (Shimadzu, Japan) equipped with 1.0 cm quartz cells at room temperature. FT-IR spectra were measured at 298 K on a Nicolet-6700 FT-IR spectrometer via the attenuated total reflection (ATR) at a resolution of 4 cm^{-1} and 64 scans. The MCR-ALS programs were applied to process the collected spectra data.

2.3. Fluorescence quenching measurements

3.0 mL 5.0×10^{-6} mol L^{-1} BSA/BHb is titrated by successive additions of a 6.0×10^{-4} mol L^{-1} stock solution of CTD/NCTD (to give a final concentration of 0–3.75×10^{-5} mol L^{-1} and 0–5.45×10^{-5} mol $L^{-1}$for BSA and BHb, respectively). The titrations are done manually using the 50 μL microsyringe. The fluorescence measurements were performed at different temperatures in the range from 300 to 500 nm. The excitation and emission slit widths were both set at 5 and 10 nm for BSA and BHb, respectively. An excitation wavelength of 280 nm was chosen and the temperature of samples was kept by recycle water during the whole experiment.

Synchronous fluorescence spectra of two blood proteins in the absence and presence of CTD/NCTD were measured at the wavelength from 240 to 320 nm. RLS spectra were obtained by synchronous scanning on the spectrofluorometer ($\Delta\lambda$=0 nm) with the wavelength range of 270–790 nm. 3D fluorescence spectra were determined under the following conditions: the excitation wavelength was scanned from 200 to 500 nm with increment of 2 nm; the emission wavelength was recorded between 200 and 500 nm with 24,000 nm/min scanning rate, and the others parameters were the same as those of the fluorescence quenching spectra.

2.4. FT-IR measurements

The FT-IR spectra of two blood proteins in the presence and absence of CTD/NCTD were recorded in the range of 4000–400 cm^{-1}. The molar ratios of two blood proteins to CTD/NCTD were maintained at 1:3. The corresponding absorbance contributions of buffer and free CTD/NCTD solutions were recorded and digitally subtracted with the same instrumental parameters.