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Investigation on the interactions of clenbuterol to bovine serum albumin and lysozyme by molecular fluorescence technique

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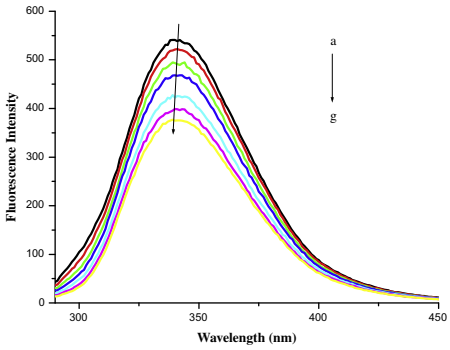
HIGHLIGHTS

- Interactions of clenbuterol with BSA and LYS were studied by fluorescence quenching.
- Hydrophobic and electrostatic forces were the major forces in the two systems.
- Synchronous fluorescence was performed to analyze the conformational changes.
- Energy transfer occurred between clenbuterol and the two proteins.

GRAPHICAL ABSTRACT



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ABSTRACT

Clenbuterol interacting with bovine serum albumin (BSA) or lysozyme (LYS) in physiological buffer (pH 7.4) was investigated by the fluorescence spectroscopy and UV–vis absorption spectroscopy. The results indicated that clenbuterol quenched the intrinsic fluorescence of BSA and LYS via a static quenching procedure. The binding constants of clenbuterol with BSA and LYS were 1.16×10^3 and 1.49×10^3 L mol^{-1} at 291 K. The values of ΔH and ΔS implied that hydrophobic and electrostatic interaction played a major role in stabilizing the complex (clenbuterol–BSA or clenbuterol–LYS). In the presence of Fe^{2+}, Fe^{3+}, Cu^{2+}, Mg^{2+}, Ca^{2+}, or Zn^{2+}, the binding constants of clenbuterol to BSA or LYS had no significant differences. The distances between the donor (BSA or LYS) and acceptor (clenbuterol) were 2.61 and 2.19 nm for clenbuterol–BSA and clenbuterol–LYS respectively. Furthermore, synchronous fluorescence spectrometry was used to analyze the conformational changes of BSA and LYS.

Introduction

Clenbuterol (4-amino-α-[t-butylaminomethyl]-3,5-dichlorobenzyl alcohol hydrochloride) (Fig. 1) is a long-acting beta-2-adrenergic receptors (β_2-AR) agonist, which was originally used in non-infectious respiratory diseases [1]. In addition, because the drug initiates fat metabolism resulting in promoting muscle growth and fat breakdown [2], it is illegally used to feed livestock in order to keep the economical benefits. The use of clenbuterol has been forbidden in the world [3,4]. But human food poisoning caused by the misuse of clenbuterol was still found in some countries [5–7]. Therefore, the continuous surveillance of clenbuterol has become more and more necessary for human health.

Proteins are very important biochemical compounds in cell having many diverse functions. The ability of the binding of clenbuterol to proteins can determine the amount of clenbuterol bound to protein and the effectiveness of clenbuterol in body. Therefore it is important to study the interaction of clenbuterol with proteins. Bovine serum albumin (BSA) is the most common model protein. Its intrinsic fluorescence arose from Trp-134 and Trp-212 located subdomains IA and IIA, respectively [8]. Because it has structural

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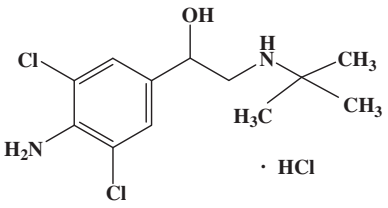

Fig. 1. Structure of clenbuterol.

homology with human serum albumin (HSA) [8,9], BSA was chosen for this study. Lysozyme (LYS) was also chosen as the target protein molecule because it has often been applied for the study of biological process, and the property of LYS significantly differs from that of BSA [10]. LYS has six tryptophanes (Trp), and Trp-62 and Trp-108 are the most dominant fluorophores which play important roles [11]. We believe, LYS as the study model will provide new insights on the understanding how this protein interacts with clenbuterol.

Up to now, various methods have been reported about the determination of clenbuterol [12–16]. However, little research has been conducted on the interaction of clenbuterol with BSA (or LYS) by fluorescence spectroscopy and UV–vis absorption spectroscopy in vitro. This research is necessary because of the dangerous effects from the use of clenbuterol. In this paper, the accurate and full data will make a significant contribution to the understanding of the action mechanism and metabolic process of clenbuterol in living organisms.

Experimental

Reagents

BSA and LYS (Sigma packaging) were bought from Changchun Dingguo Biotechnology Company and stored in refrigerator at 4 °C. Clenbuterol was got from Chinese Drug Biological Products Qualifying Institute. Tris–HCl buffer solution (pH 7.4) containing 0.1 mol L^{-1} NaCl was prepared. $FeSO_4$, $FeCl_3$, $FeCl_2$, $CuCl_2$, $MgCl_2$, and $CaCl_2$ were used to study the effects of co-ions on the binding of clenbuterol to BSA and LYS. All other reagents were of analytical reagent grade without further purification. Double distilled water was used throughout experiments.

Apparatus

All fluorescence spectra were recorded on a RF-5301PC spectrofluorometer (Shi madzu, Japan) equipped with a xenon lamp source, a 1.0 cm × 1.0 cm × 4.0 cm quartz cell and a thermostatic controller. The absorption spectra were recorded on TU-1901 UV-Spectrometer (Beijing Purkinje General Instrument Co., Ltd.).

Procedures

A fixed amount of protein and various amounts of clenbuterol were added to each mark tube and the total volume was fixed to 2.5 mL with Tris–HCl buffer of pH 7.4 containing 0.1 mol L^{-1} sodium chloride. All solutions in tubes were mixed well. The excitation wavelength was 280 nm and the fluorescence spectra were recorded in the wavelength range 280–500 nm at different temperatures. The slit widths of excitation/emission were set to 3/3 nm. Synchronous fluorescence spectra of BSA and LYS in the presence of various amounts of clenbuterol were scanned at $\Delta\lambda$ = 15 nm and 60 nm, respectively.

The measurement of UV/Vis spectrum of clenbuterol was performed under the conditions: the scan rate was 8 nm s^{-1}, and the scan range was 290–500 nm.

Results and discussion

Binding characteristics of clenbuterol to BSA or LYS

Fig. 2 shows the effects of clenbuterol on the fluorescence of BSA and LYS. The concentrations of BSA and LYS were all 5.0×10^{-6} mol L^{-1}, and the concentration of clenbuterol was varied from 0.0 to 25.0×10^{-5} mol L^{-1}. It could be seen that when BSA and LYS were excited at 280 nm, both of them emitted strong fluorescence, and the maximum emission wavelengths were at 342 nm and 338 nm respectively, which mainly arose from Trp. In fact, the intrinsic fluorescence of BSA and LYS primarily arises from Trp and Tyr. But the emission wavelength of Tyr was not often found at about 304–310 nm because its fluorescence was almost quenched by Trp owing to the efficient energy transfer from Tyr to Trp, i.e. the internal quenching effect [17,18]. It was also obvious that the emission intensity of the two proteins dropped regularly with different amounts of clenbuterol, suggesting that the interaction occurred between clenbuterol and the protein and the non-fluorescent complex was formed. Furthermore, a blue shift of the maximum emission peak (from 342 to 339 nm) was found in the emission spectra of BSA when the concentration of clenbuterol was increased continuously, suggesting that Trp was

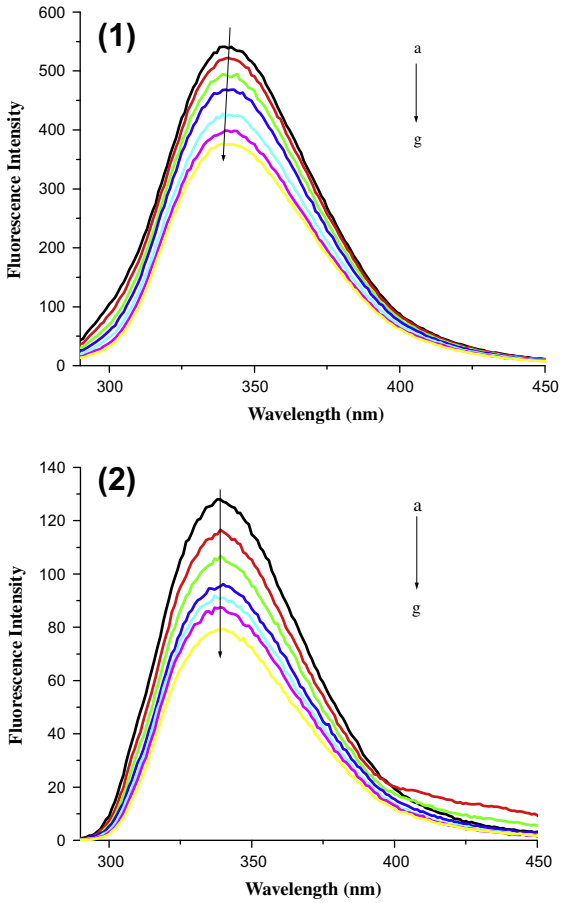

Fig. 2. The effects of clenbuterol on the fluorescence emission spectrum of (1) BSA and (2) LYS in Tris–HCl buffer of pH 7.4 at 291 K. The concentrations of BSA and LYS are all 5.0×10^{-6} mol L^{-1}. The concentration of clenbuterol in BSA and LYS are all: (a) 0.0, (b) 4.0, (c) 8.0, (d) 12.0, (e) 16.0, (f) 20.0 and (g) 24.0×10^{-5} mol L^{-1}.