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Rapid and sensitive determination of clenbuterol in porcine muscle and swine urine using a fluorescent probe

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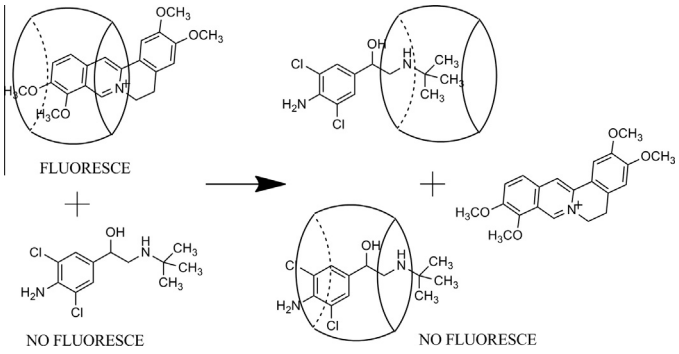
HIGHLIGHTS

- A novel spectrofluorimetric method determining the illegal feed additive was proposed.
- The method was applied for analysis of clenbuterol in porcine muscle and swine urine.
- It is a suitable method for an accurate, rapid and less expensive determination.
- The mechanism of the fluorescence quenching of the supramolecular complex was discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

The feed additive Clenbuterol hydrochloric acid (CLB) is non-fluorescent, thus it is difficult to quantify through direct fluorescent method. Palmatine (PAL) can react with cucurbit[7]uril (CB[7]) to form stable complexes as a fluorescent probe. Significant quenching of the fluorescence intensity of the CB[7]–PAL complex was observed with the addition of CLB. Based on the significant quenching of the supramolecular complex fluorescence intensity, a novel spectrofluorimetric method with high convenience, selectivity and sensitivity was developed for the determination of CLB. The fluorescence quenching values (ΔF) showed good linear relationship with CLB concentrations from 0.011 μg mL^{-1} to 4.2 μg mL^{-1} with a detection limit 0.004 μg mL^{-1}. In this research, an ultrasound treatment replaced the former time-consuming shake method to form stable complexes. The proposed spectrofluorimetric method had been successfully applied to the determination of CLB in porcine muscle and swine urine with good precision and accuracy. The competing reaction and the supramolecular interaction mechanisms between the CLB and PAL as they fight for occupancy of the CB[7] cavity were studied using spectrofluorimetry, ^{1}H NMR, and molecular modeling calculations. Interestingly, results indicate that two stable CB[7]–CLB complexes were formed.

Introduction

Cucurbit[*n*]uril (CB[*n*], *n* = 5–8, 10, Fig. 1) is a macrocyclic compound consisting of *n* glycoluril units connected by 2*n* methylene bridges. The symmetrical supermolecule hosts resemble a hollow

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barrel with hydrophobic cavities and restrictive polar portals lined with ureido carbonyl groups location [1–3]. These characteristics enable to form significant stable complexes with a variety of guest molecules in aqueous solution. The medical applications of the CB[*n*] has been developing rapidly, especially the drug release [4,5]. However, little attention has been devoted to their potential analytical applications of food safety, especially the determination of feed additive residues.

Palmatine (PAL, Fig. 1) is a natural isoquinoline alkaloid [6]. Aqueous solution of PAL exhibits weak native fluorescence. However, the fluorescence of PAL in aqueous solutions was observed to be greatly enhanced in the presence of CB[7] [7].

Clenbuterol (CLB, Fig. 1) is a member of β-adrenergic agonists, which enhances the lean meat/fat ratio and increases the efficiency of feed conversion by inhibiting fat synthesis, improving muscular mass, and decreasing adipose tissue deposition in livestock production. Thus, CLB is also called as a "leanness enhancer" and illegally used as a feed additive in meat industry [8]. However, once the animals are fed with CLB, the residue may remain in the meat and liver for a long time as a result of its long half-life, so it may enter the body and distribute throughout the body and result in serious harmful health problems to human such as cardiovascular and central nervous diseases [9]. Hence, many countries including China, the United States and most European countries have forbidden the use of CLB as feed additives [10]. A number of assays have been reported for the determination of CLB in biological samples, including ELISA [11], HPLC [12], LC–MS [13], GC–MS [14], immunochromatographic [15], electrochemical limmunosensors [16], electrochemical biosensor [17], and fluorescence biosensor [11]. Although these strategies exhibit promising results for sensitive detection of CLB, there are still some hindrances including expensive instrument, long operation times, and tedious sample preparation. Spectrofluorometry is considered one of the most convenient analytical technique, owing to its inherent simplicity, low cost, high sensitivity, and wide availability in most quality control laboratories [18,19]. Therefore, a rapid, simple, and sensitive fluorescent method is required to monitor CLB in porcine muscle and swine urine samples. Considering that aqueous solutions of CLB have no native fluorescence, it cannot be directly determined through the normal fluorimetric method. Based on the significant quenching of the supramolecular complex fluorescence intensity, a spectrofluorimetric method of high convenience, sensitivity and selectivity was developed to determine CLB in aqueous solution. In the present research, we replaced the former time-consuming shake method and an improved ultrasound treatment was applied to accelerate the progress of quenching. This method was successfully used to determine CLB in real samples, and satisfactory assay results were obtained. Additionally, ^{1}H NMR and molecular modeling calculations results indicate that two stable CB[7]–CLB complexes were formed and co-exist in the solution.

Experimental

Apparatus

Fluorescence spectra and intensity were obtained using an Agilent Technologies Cary Eclipse fluorescence spectrofluorometer (Agilent, Australia) equipped with a pulsed lamp. The slit widths of both excitation and emission monochromators were set to 5 nm. The fluorescence spectra were recorded at a scan rate of 600 nm min^{-1}. All measurements were performed using a standard 10 mm path-length quartz cell at 25.0 °C ± 0.5 °C. The pH values were measured using a pHS-3 TC digital precision pH meter (Shanghai, China). ^{1}H NMR spectra were obtained using a Bruker DRX-600 MHz spectrometer (Switzerland) in D_2O.

Reagents

PAL and CLB were obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) without further treatment. CLB was dissolved in double-deionized water to prepare stock standard solutions of 100 μg mL^{-1}. PAL was dissolved in double-deionized water to prepare stock solutions with final concentration of 1.0 mM. CB[7] was prepared and characterized according to reported procedure [2]. CB[7] stock solution of 1.0 mM was prepared by dissolving CB[7] in double-deionized water. Stock standard solutions were stable for several weeks at room temperature. Standard working solutions were prepared by diluting the stock standard solutions with double-deionized water before use. All other chemicals were of analytical reagent grade, and double-deionized water was used throughout the procedure.

Experimental procedure

0.7 mL solution of 0.2 mM CB[7] was poured into a 10 mL colorimetric flask, to which 0.7 mL of the 0.2 mM PAL solution and 1.0 mL of 0.01 M hydrochloric acid were also added. Suitable amounts of CLB solution were sequentially added to the flask. The mixture was diluted to volume with double-deionized water. Then an ultrasound treatment was used to accelerate the reaction rate. The fluorescence intensity values of the solution ($F_{PAL–CB[7]–CLB}$) and the blank solution ($F_{PAL–CB[7]}$) were measured at 495 nm using an excitation wavelength of 343 nm.

Results and discussion

The fluorescence enhancement of PAL in the presence of CB[7]

CB[7] is spectroscopically inert in aqueous solutions, the aqueous solution of PAL has weak native fluorescence, and the maximum excitation and emission wavelengths are at 235 nm and 370 nm, respectively. However, when CB[7] was added to the aqueous solution of PAL, a significant increase in fluorescence intensity was observed, and accompanied by a red shift of emission wavelengths from 370 nm to 495 nm. The changes in the features of the fluorescence spectra of the solutions are attributed to the formation of an inclusion complex between PAL and CB[7] [7].

The fluorescence quenching of CB[7]–PAL in the presence of CLB

Significant quenching of fluorescence intensity of the CB[7]–PAL complex with the addition of CLB was observed. The fluorescence spectra of the CB[7]–PAL complex, in the presence of different concentrations of CLB, are shown in Fig. 2. Fluorescence intensity decreased with the increased CLB concentration, which is likely due to the competition between CLB and PAL molecules for occupancy of the CB[7] cavity. Parts of the PAL molecule can be expelled from the cavity of CB[7] by the introduction of the CLB, thereby reducing the fluorescence intensity of CB[7]–PAL because of the formation of a new inclusion complex between CLB and CB[7].

Effect of PAL concentration on the fluorescence intensity of the CB[7]–PAL complex

The effect of varying PAL concentrations on the fluorescence intensity of the CB[7]–PAL complex was studied. The concentration of PAL was varied from 1.0 μM to 17.0 μM. The fluorescence intensity of the CB[7]–PAL complex was gradually enhanced as PAL concentration increased until it reached the maximum inclusion equilibrium at CB[7] saturation when the concentration of PAL