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ORIGINAL PAPER

Fluorimetric Method Based on Diazotization-Coupling Reaction for Determination of Clenbuterol

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Abstract A novel fluorimetric method based on diazotization-coupling reaction (DCR) for the determination of clenbuterol is described. In acidic solution, clenbuterol was first diazotized with sodium nitrite, followed by coupling with bisphenol A to produce an azo-compound in NH_3- NH_4Cl buffer. It has found the diazotized clenbuterol- bisphenol A-NH_3- NH_4Cl (DCBN) system has strong fluorescence efficiency compare with the bisphenol A solution. There is a linear relationship between the increased intensity of the fluorescence emission spectra ($\lambda_{ex}/\lambda_{em}$=276 nm/306 nm) and the concentration of clenbuterol. The effects of the amount of sodium nitrite, diazo reaction time, the amount of bisphenol A, coupling reaction time and coupling reaction temperature have been examined. Under the optional conditions, clenbuterol can be determined over the concentration range of 0.02 to 2.0 μg mL^{-1} with a correlation coefficient of 0.9953. The detection limit is 0.01 μg mL^{-1} at a signal-to-noise ratio of 3. The relative standard deviation (RSD) for 11 repetitive determinations of 0.9 μg mL^{-1} clenbuterol is 0.22 %. The utility of this method was demonstrated by determining clenbuterol in meat samples.

Keywords Clenbuterol · Fluorescence · Detection · Diazotization-coupling reaction · Bisphenol A

Introduction

Clenbuterol(4-amino-a-[(tert-butylamino)methyl]-3,5-dichloro benzyl alcohol, MW: 276.08, CAS: 37148-27-9) is an orally active β_2-adrenoceptor agonist, which is used in human and veterinary medicine as a therapeutic drug for pulmonary disease [1, 2]. It has been reported to be used for illegal purposes in humans and in animals. In humans, clenbuterol increases athletic performance, and it was consequently banned by the International Olympic Committee and by major sports federations [3]. Apart from its effect on respiratory function, clenbuterol can accelerate animal growth and produce more muscle and much less adipose tissue, which could explain its illegal use in the meat producing industry [4]. Moreover, due to its long half-life and stability, clenbuterol residues could present a potential risk for human health [5, 6]. Clenbuterol has been banned for feeding animals all over the world [7]. However, in many countries, there are still cases of illicit usages of clenbuterol in pig feed. For instance, in 2006, reports from Shanghai in China involved 300 cases of people who were poisoned by taking pork products contaminated by clenbuterol [8]. In 2009, it was reported that about 70 persons suffered from food-poisoning in Guangdong province of China on account of unwittingly taking contaminated pork products [9]. When people have taken pork products contaminated by 0.2 mg/kg clenbuterol, they will have clinical symptoms of poisoning.

To date, various analytical methods have been developed for the determination of clenbuterol, including gas chromatography (GC) [coupled to mass spectrometry (MS)] [10–13], high-performance liquid chromatography (HPLC) [14–16], liquid chromatography-mass spectrometry (LC-MS/MS) [1, 3, 4, 17], nuclear magnetic resonance spectrometry (NMR), capillary electrophoresis [18–20], colorimetric assay based on gold nanoparticle [21], FT-MIR and Raman spectroscopy [22], mid-infrared spectroscopy coupled with multivariate analysis [23], enzyme-linked immunosorbent assay (ELISA) [24, 25], surface plasmon resonance immunosensors [26], electrochemiluminescent immunosensor [8, 9, 27], electrochemical method [5, 6, 28–32], optical immunobiosensor [2]

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and fluorescence biosensor [33]. The fluorescence biosensor is based on fluorescent nanosilica and a lateral flow test strip. Quantitative detection of clenbuterol was realized by recording the fluorescence intensity of fluorescent nanosilica captured on the test line. They introduced Eu(III)–BHHCT(β-diketonate chelate 4,4′-bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedione-6″-yl)chlorosulfo-o-terphenyl) into porous silica nanoparticles to form the stable fluorescent nanosilica with desirable luminescence properties. Hernández-Córdoba et al. developed a flow-injection fluorimetric procedure to determine clenbuterol in pharmaceutical preparations. This method is based on the derivatization reaction of the primary amine group with *o*-phthalaldehyde in the presence of 2-mercaptoethanol. Linear calibration curves were obtained in the range of 0.2–5.0 μg mL^{-1} based on peak area. The detection limit was 0.06 μg mL^{-1} [34].

This paper reports a novel fluorimetric method for estimating the concentration of clenbuterol, which is based on the diazotization-coupling reaction. i.e. clenbuterol was first diazotized with sodium nitrite to obtain a diazo compound in acidic solution, then the diazo compound can react with bisphenol A to produce a fluorescent azo-compound in NH_3- NH_4Cl buffer. λ_{ex}max of the fluorescent compound is at 276 nm; the λ_{em}max value is at 306 nm. In the same wavelength, bisphenol A manifests weak fluorescence property in aqueous solutions because of its low fluorescent efficiency [35]. So a bisphenol A solution containing no clenbuterol was prepared as the reagent blank. There is a linear relationship between the intensity of the increased fluorescence emission and the concentration of clenbuterol. This method can be used to detect the quantitative of clenbuterol. The aim of this work is to develop a fluorimetric method to detect clenbuterol with less expensive cost and high selectivity.

Experimental

Reagents

Clenbuterol was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Bisphenol A was purchased from Shanghai Chemical Reagent Factory and Shanghai Biotechnology Co. Ltd. (Shanghai) and was recrystallized prior to use. Sodium nitrite was obtained from Kelong Huaxue Shiji (Chendu, China). Hydrochloric acid, Ethanol and $NH_3 \cdot H_2O$ (25–28 %) were obtained from Jiangsu Yongfeng Chemical Reagent Co., Ltd (Danyang, Jiangsu, China). All reagents were of analytical-reagent grade, unless stated otherwise. Double distilled water was used in all experiments.

Apparatus

Spectrofluorimetric measurements were made on Hitachi F-4600 luminescence spectrometer equipped with a xenon discharge lamp and 1.0 cm quartz cuvettes (Hitachi, Japan). Both the operation and data processing were controlled by the Fluorescence Data Software. The excitation and emission wavelengths were set at 276 nm and 306 nm respectively. Absorption spectra were acquired at a 1,102 UV spectrophotometer (Shanghai Tian Mei Scientific instrument Co,. Ltd, Shanghai, China) equipped with 1.0 cm quartz cuvettes.

Procedures

A certain portion of 0.10 mg/mL of clenbuterol solution was transferred into a 10.0 mL standard flask, 0.4 mL 0.10 mol/L HCl solution and 0.2 mL 0.20 mg/mL sodium nitrite were added. The mixture was shaken and contained in 0~5 °C for 5 min. Clenbuterol was first diazotized with sodium nitrite. Next a certain portion of 0.10 mg/mL of bisphenol A solution was added to the mixture and shaken. Than a certain 4.4 mg/mL $NH_3 \cdot H_2O$ solution was added, diluted to the mark with water and mixed well.

All fluorescence measurements were made at scan rate of 1,200 nm min^{-1} using 5 nm excitation and emission windows. The excitation and emission spectra of fluorescent system was recorded on the luminescence spectrometer. A bisphenol A blank solution containing no clenbuterol was also prepared in this way and the output recorded as the background. The different height between the signal and background was used for quantification.

The peak height of fluorescence emission spectrum was estimated at 306 nm on the longitudinal coordinate axis (absolute value). The net fluorescence intensity was calculated by $\Delta I = I_S - I_0$, where I_S was the intensity of sample solution and I_0 was the signal of blank solution.

Results and Discussion

Excitation and Emission Spectra

The fluorescence excitation and emission spectra were shown in Fig. 1. The λ_{ex}max and λ_{em}max of the DCBN system are 276 (Fig. 1a) and 306 nm (Fig. 1b), respectively, the λ_{ex}max and λ_{em}max of blank solution are at the same wavelength (Fig. 1c, d), which are contributed to the presence of bisphenol A. When 0.9 μg mL^{-1} clenbuterol was added in this DCBN system, the fluorescence emission increased by 2.2 times. The significant increase indicated diazo clenbuterol was a sensitive enhancer on the fluorescence reaction of bisphenol A. Furthermore, the emission intensity increased with an increasing concentration of clenbuterol.

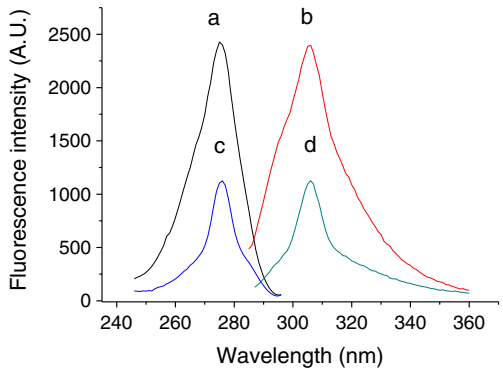


Fig. 1 Excitation and emission of DCBN solution and reagent blank. Excitation spectrum (**a**) (λ_{em}=305 nm) and emission spectrum (**b**) (λ_{ex}= 276 nm) of DCBN solution. Excitation spectrum (**c**) (λ_{em}=305 nm) and emission spectrum (**d**) (λ_{ex}=276 nm) of reagent blank. Concentration of clenbuterol, 0.9 μg mL^{-1}

Optimization of Net Fluorescence Intensity

Effect of pH

A series of experiments were performed to optimize the conditions for the production of maximum net fluorescence emission. In these studies, each experiment was replicated at least three times. According to the procedure, the clenbuterol was first diazotized with sodium nitrite in acidic solution. The effect of acid contained in the solution on fluorescence emission intensity was initially examined.

The mixture solution of 0.9 μg mL^{-1} of clenbuterol was prepared in the presence of HCl, CH_3COOH, H_3PO_4 or H_2SO_4 at the same concentration and the fluorescence emission intensity was measured at λ_{em}max 306 nm with excitation at 276 nm against a reagent blank prepared with the same reagent concentration but no clenbuterol. The results indicated that the batter intensity occurred in acidic medium containing HCl. With the increasing concentration of HCl, the net fluorescence intensity increased and reached a maximum value at 4×10^{-3} mol/L. Therefore, 4×10^{-3} mol/L HCl was chosen as the acidic medium for the diazotization of clenbuterol.

Effect of Sodium Nitrite

In this system, sodium nitrite is a diazotizing reagent. The effect of the concentration of sodium nitrite in the range 0.5 μg/mL~12.0 μg/mL on the net fluorescence values was investigated. With the increase of concentration of sodium nitrite, the I_S value increased but the I_0 value kept constant. A plot of the difference in fluorescence intensity between the sample reactions and the blank (ΔI) versus sodium nitrite concentration shows a maximum value at 4.0 μg/mL, after which the intensity remained constant. Therefore, 4.0 μg/mL sodium nitrite was used for further study.

Effect of Diazotization Reaction Time

The relation between the diazotization reaction time in the range 1~20 min and the ΔI of the mixture solution was investigated. As the diazotization reaction time increased, the ΔI was also increasing. When reacted for above 5 min, the ΔI remained constant. So, 5 min was chosen as the diazotization reaction time.

Effect of Amount of Bisphenol A

The concentration of the bisphenol A also has an effect on the net fluorescence intensity. The effect of bisphenol A concentration was tested in the range of 0.4 to 2.8 μg/mL. The results (Fig. 2a) show that the ΔI increased with increase of the concentration from 0.4 to 2.0 μg/mL. It was possibly related to the background net fluorescence increased with increase of bisphenol A concentration. Therefore, 2.0 μg/mL bisphenol A was used for all experiments.

Effect of Amount of $NH_3 \cdot H_2O$

The clenbuterol was diazotized with sodium nitrite in acidic solution, followed by coupling with bisphenol A to produce an azo-compound in NH_3- NH_4Cl buffer. Figure 2b shows the amount of $NH_3 \cdot H_2O$ on the effect of the ΔI. The concentration of $NH_3 \cdot H_2O$ solution was varied in the range 80.0 μg/mL ~320.0 μg/mL and the highest ΔI was obtained at the concentration of 160.0 μg/mL. Above this concentration, the ΔI remained constant.

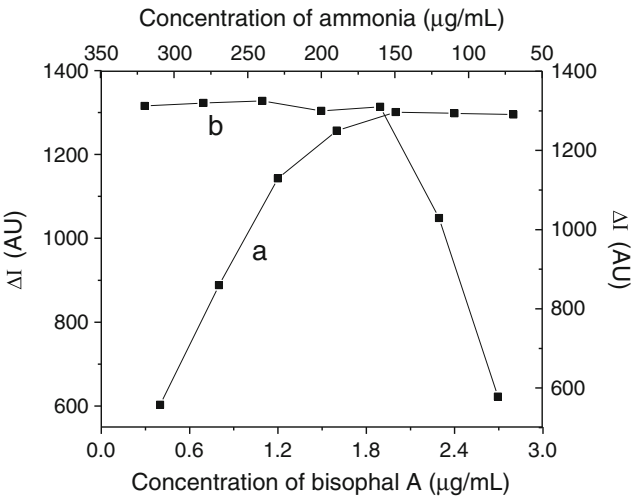


Fig. 2 Effect of concentrations on net fluorescence emission intensity. **a** Effect of bisphenol A concentration on net fluorescence emission intensity. clenbuterol, 0.9 μg mL^{-1}; sodium nitrite, 4.0 μg mL^{-1}; $NH_3 \cdot H_2O$, 160.0 μg mL^{-1}; **b** Effect of $NH_3 \cdot H_2O$ concentration on net fluorescence emission intensity. Clenbuterol, 0.9 μg mL^{-1}; sodium nitrite, 4.0 μg mL^{-1}; bisphenol A, 2.0 μg mL^{-1}

Effect of the Coupling Reaction Temperature

According to the procedure, the mixture solutions of 0.9 μg mL^{-1} of clenbuterol were cooled or heated in water bath of different temperature. It was found temperature had no significant influence on the signal in the 0~30 °C range. Higher temperature resulted in a decrease of the ΔI. Consequently, room temperature was selected in order to simplify the experimental work.

Formation and Stability of DCBN

The results of the coupling reaction of the mixture solutions of 0.9 μg mL^{-1} of clenbuterol occurred for different times in room temperature were investigated. When the coupling reaction time occurred for above 20 min, the ΔI increased gently. So, occurred 20 min was selected as optimum. The net fluorescence intensity of DCBN system remained stable for at least 24 h at room temperature.

Analytical Characteristics of Clenbuterol

Under the optimum conditions mentioned above, the calibration curve was obtained for clenbuterol determination by plotting the ΔI versus clenbuterol concentration, which gave a linear range from 0.02 to 2.0 μg mL^{-1} with a correlation coefficient of 0.9953(Fig. 3 insert). Linear regression equation of calibration graph is $Y=104.01x+282.96$. x means concentration of clenbuterol: 0.1 μg mL^{-1}, Y means arithmetic peak height in the linear regression equation calibration graph. The typical recording output of the DCBN system for measurements of clenbuterol is shown in Fig. 3. The relative standard deviation for 11 repetitive determinations of 0.9 μg mL^{-1} clenbuterol was 0.22 %, showing a good reproducibility.

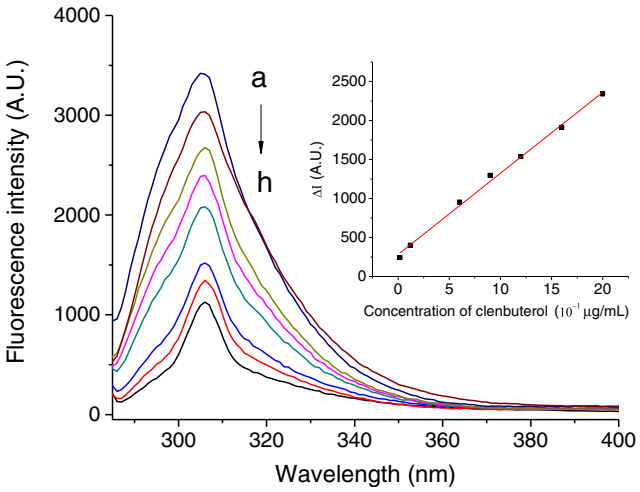


Fig. 3 Typical fluorescence emission signals of the reaction solution. Concentrations of clenbuterol were 2.0, 1.6, 1.2, 0.9, 0.6, 0.12, 0.02 μg mL^{-1}, blank (**a**–**h**, respectively). Insert: plots of fluorescence emission intensity vs clenbuterol concentration

Interferences

The influences of some species on the ΔI of DCBN system in presence of 0.9 μg mL^{-1} clenbuterol and foreign species with continuously increasing concentration up to 500 ratio were investigated. Some ions commonly existing in water and organic substances could be used in HPLC (development of an HPLC-FL method for clenbuterol) were chosen for the selectivity test. When the effect of each foreign species on the peak height was less than 5.0 %, it was thought not to interfere with the determination of clenbuterol. The obtained results in Table 1 showed that under the optimized conditions, some ions and the organic substances did not interfere with the determination of clenbuterol. Therefore, this method can be suggested for the determination of clenbuterol in practical samples.

Analysis of Spiked Pig Meat Samples

Pig meat samples were used to assess the accuracy of the suggested method in real matrixes. Before the examination, the pig meat purchased from local supermarket washed and sliced into very tiny pieces. Two grams of the test portion were removed and subsequently extracted with 10.0 mL of 0.1 mol/L hydrochloric acid in an ultrasonic bath for 10 min. The supernatant was filtered and the filtrate was adjusted to pH 11–12 with 1.0 mol/L sodium hydroxide. The filtrate then mixed with 0.5 mL of ethyl acetate and centrifuged for 5 min at 3,000 r min^{-1}. Then the rest ethyl acetate was blow-dried under a gentle stream of nitrogen gas. Finally, the prepared sample was added 1.0 mL 0.1 mol/L hydrochloric acid and diluted with distilled water to 50 mL and used for the fluorescence determination of clenbuterol.

The recovery study was performed by spiking blank swine meat samples with clenbuterol at different levels. For each concentration, three different samples were independently processed. On the basis of the calibration curves, it was

Table 1 Tolerance to different substances in the determination of 0.9 μg mL^{-1} clenbuterol

Species added	Maximum tolerable mole ratio[a]
Na^+, K^+, Cl^- , EDTA, methanol, acetonitrile	500
Acetone, urea, glucose, starch, fructose	100
Zn^{2+}, Mg^{2+}	100
Al^{3+}, Ca^{2+}	50
Cu^{2+}, Ba^{2+}	25

[a] 500 is the greatest ratio tested

Table 2 Results of clenbuterol determinations in pig meat samples

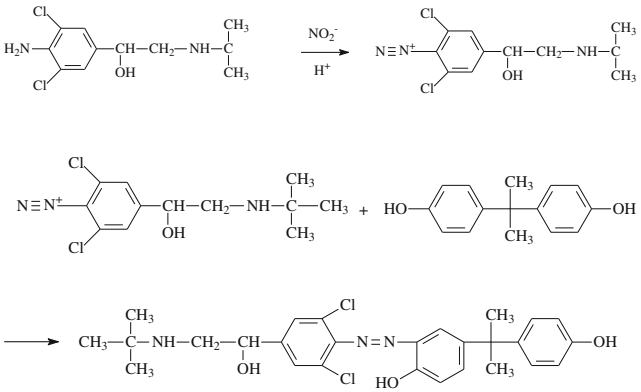
Pig meat sample	Added (μg mL^{-1})	Measured (μg mL^{-1})	RSD (n=3) (%)	Recovery (%)
No. 1	0.00	0.00	0.0	
	0.005	0.005286	2.1	105.71
	0.01	0.009913	3.5	99.13
No. 2	0.00	0.00	0.0	
	0.03	0.02914	2.3	97.13
	0.05	0.05094	2.5	101.88

possible to calculate the recovery of the analyte, which ranged from 97.13 to 105.71 % of clenbuterol with an average of 101.4 %. The precision was determined by calculating the standard deviation for the replicate measurements. The statistical results are summarized in Table 2.

Possible Reaction Mechanism

Figure 4 shows UV-visible absorption spectra of bisphenol A, Clenbuterol, and DCRS system. It can be seen that bisphenol A had absorption peaks at 224 and 278 nm (Fig. 4a), and clenbuterol system had peaks at 242 and 295 nm (Fig. 4b) between 210 and 700 nm. The absorption spectrum of DCBN system showed an increased peak at about 410 nm. The significant increase of the absorption signal indicated a reaction occurred.

Clenbuterol was first diazotized with sodium nitrite to obtain a diazo compound in acidic solution, then the diazo compound can couple with bisphenol A to produce an azo-compound in NH_3- NH_4Cl buffer and the azo-compound has a maximum absorption at 410 nm (Fig. 4c). The azo-compound also has strong fluorescence efficiency. The fluorescence enhancement is that the rigidity of the azo-compound decreases the degree of freedom in the motion of the bisphenol A molecules. The possible DCRF mechanism of the reaction can be simply described as following:



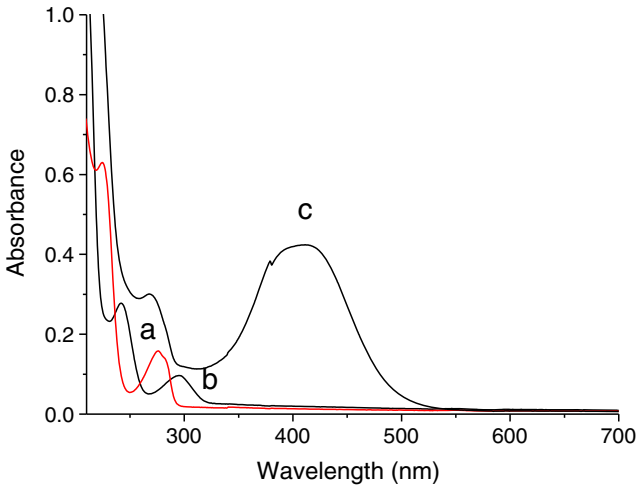


Fig. 4 UV-visible absorption spectra of 10.2 μg mL^{-1} bisphenol A (**a**), 8.08 μg mL^{-1} clenbuterol (**b**), and 8.08 μg mL^{-1} clenbuterol +4.0× 10^{-3} mol/L HCl +8.12 μg mL^{-1} $NaNO_2$+10.2 μg mL^{-1} bisphenol A+ 396.0 μg mL^{-1} $NH_3 \cdot H_2O$ (**c**)

Conclusions

In this paper, we reported a highly sensitive fluorimetric method for determination of clenbuterol at ng mL^{-1} level. In all the cases, recoveries of 97.13 to 105.71 % were obtained. A linear standard was obtained from 0.02 to 2.0 μg mL^{-1} (R= 0.9953). Compared with the FT-MIR and Raman spectroscopy method [22], the use of fluorescence detection in the proposed method resulted in a 100-fold increase in the sensitivity; 10–20 times more sensitive compared with mid-infrared spectroscopy and colorimetric assay [21, 23], with comparable sensitivity to that obtained by electrochemical method and bioassay [29, 36]. This method can be satisfactorily used in the determination of clenbuterol in real samples.

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