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ORIGINAL PAPER

Highly Sensitive Fluorescent Probe for Clenbuterol Hydrochloride Detection Based on its Catalytic Oxidation of Eosine Y by $NaIO_4$

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Abstract A highly sensitive fluorescent probe for clenbuterol hydrochloride (CLB) detection has been first designed based on its catalytic effect on $NaIO_4$ oxidating eosine Y (R). And this environment-friendly, simple, rapid, selective and sensitive fluorescent probe has been utilized to detect CLB in the practical samples with the results consisting with those obtained by GC/MS. The structures of R and CLB were characterized by infrared spectra. The mechanism of the proposed assay for the detection of CLB was also discussed.

Keywords Clenbuterol hydrochloride · Eosine Y · Fluorescent probe

Introduction

Clenbuterol hydrochloride (CLB) is one of the β2-adrenergic agonists, it originally used as a drug for the treatment of pulmonary disease and asthma [1, 2]. However, it is illegally applied as nutrient repartitioning agents in livestock to greatly reduce fat levels and increase muscle protein [3, 4]. On account of the potential risk to consumers for adverse cardiovascular and central nervous system effects [5–7], CLB is not licensed for animal production in many countries. Obviously, the detection of trace CLB in the clinical diagnosis and treatment of human diseases has great academic research value.

Tremendous efforts have been made in recent years to detect trace CLB, such as liquid chromatography and electrospray tandem mass spectrometry [8], electrochemical immunosensor [9], enzyme-linked immunoassay method [10], time resolved fluorescence immunoassay [11], surface-enhanced ramanscattering [12], capillary electrophoresis [13], GC/MS [14], chemiluminescence method [15], electrochemical method [16], chromatographic method [17], fluorescence resonance energy transfer [18, 19], etc. It is worth noting that much attention has been paid to FRET method for CLB detection due to its simplicity, rapidness and reproducibility. However, the application of these methods is limited, because, for example, the sensitivity is low and the apparatus is expensive. Therefore, further developing a simple, sensitive and cost-effective method for the CLB detection is a worthwhile challenging undertaking.

This text will utilize catalytic reaction's own selectivity and the enlarging effect of signal to carry on the preliminary discussion on improving the sensitivity of fluorescence method. Our research showed that R could emit strong and stable fluorescence, and $NaIO_4$ could oxidize R which makes the fluorescence signal quench. While CLB could catalyze $NaIO_4$ oxidation of R from oxidizing R',which caused the fluorescence signal of R to quench sharply, and the content of CLB in the range of 0.020–24.00 (10^{-18} g mL^{-1}) is linear to ΔF ($=F_0-F$; F_0 was fluorescence intensities of reagent blank, F was fluorescence intensities of test solution) of the system. Based on the facts above, a signal amplification effect fluorescent probe for CLB detection has been designed. The innovations of this research were listed as follows: 1. The sensitivity of this fluorescent probe (limit of detection (LOD): 6.8×10^{-21} g mL^{-1}) was higher than that (LOD: 7.0×10^{-12} g mL^{-1}[8], 10×10^{-12} g mL^{-1}[18]) of Ref.,

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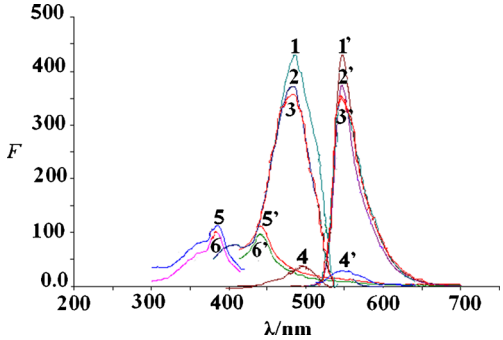


Fig. 1 The fluorescence spectra for the R-CLB-$NaIO_4$ system (Curves 1–6 are the excitation spectra, and curves 1′–6′ are the emission spectra.)

and to our knowledge, fluorescent probe for CLB detection based on signal amplification effect has not been reported yet ; 2. This rapid, accurate, selective and repeatable probe has been applied to determine trace CLB in biological samples; showing better application prospects; 3. This study accelerates the development of fluorescent probe, broadens the applications of catalytic reaction in some new fields and possesses high academic value and application foreground in life science research and analysis of trace CLB.

Experimental

Apparatus and Reagents

Fluorescent measurements were carried out on a LS-55 luminescence spectrophotometer (Perkin Element Company, USA). The main parameters of instrument are as follows: Ex Slit 15 nm,Em Slit 3 nm, scan speed 1,500 nm/min ; flash count, 1; scan speed, 1,500 nm/min. AE240 electronic analytical balance (Mettler-Toledo instruments Company, Shanghai) and. pHS-3B precision acidometer (Shanghai Medical Laser Instrument Plant) were used.

CLB working solution: 0.01000 g CLB standard reagent (Beijing institute for the control of pharmaceutical and biological products) was dissolved in 0.10 mol L^{-1} HCl, and diluted to 10.0 mL. The concentration of stock solution was 1.0 mg mL^{-1}. It was diluted to 1.00, 10.00 and 100.00 ag mL^{-1} by 0.10 mol L^{-1} HCl solution before being used. 1.0×10^{-4} mol L^{-1} R solution, 1.50 % (*W*/*V*) $NaIO_4$ solution, and NaH_2PO_4-CH_3OH solution (*V*: *V*=3 : 1) were also used in the experiment. All the reagents are AR grade except that CLB is primary standard. The water was prepared by thrice quartz sub-boiling distillation.

Experimental Method

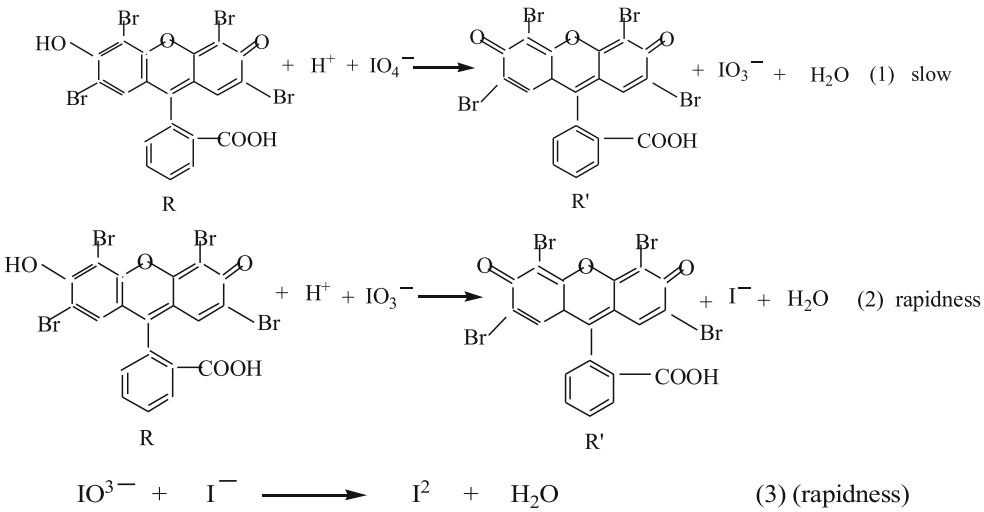
To a 25 mL colorimetric tube, proper amount of CLB working solution, 0.70 mL 1.0×10^{-4} mol L^{-1} R, 1.50 mL 1.50 % $NaIO_4$ were added, diluted to 25 mL with water and mixed homogeneously. The mixture was kept at 60 °C for 15 min, cooled by flowing water for 5 min. The fluorescence intensity of reagent blank (F_0) and the fluorescence intensity of test solution(F) were measured directly at wavelength $\lambda_{ex}/\lambda_{em}$=485/546 nm in a fluorescence pool. The ΔF (F_0-F) was calculated.

Results and Discussion

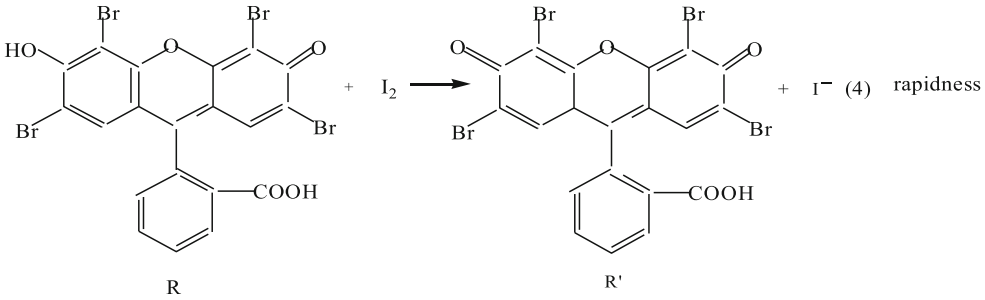
Mechanism for the Detection of CLB

In order to investigate the mechanism of the fluorescent probe for the detection of CLB, the fluorescence spectra of R-$NaIO_4$-CLB system was scanned (Fig. 1). As shown in Fig. 1, both CLB and R could emit strong and stable fluorescence signals at 60 °C for 15 min. The $\lambda_{ex}^{max}/\lambda_{em}^{max}$ was 384.4/441.3 nm and 486.9/546.4 nm, and the corresponding F were 112.1 and 429.5 (curve, 5.5′, 1.1′), respectively. In the presence of $NaIO_4$, the fluorescence signal of R and CLB was

Scheme 1 Oxidation-reduction reaction between $NaIO_4$ and R



Scheme 2 Oxidation-reduction reaction between I_2 and R



both also quenched. The $\lambda_{ex}^{max}/\lambda_{em}^{max}$ was 484.0/545.6 nm and 383.8/441.2 nm, and the corresponding *F* was 373.1 and 96.5 (curve, 2.2′, 6.6′), respectively. It may be explained that R and CLB were oxidized to nonfluorescent compounds (R', CLB') by $NaIO_4$, respectively.

1.1′ 0.70mlR 2.2′1.1′+1.50ml$NaIO_4$ 3.3′ 2.2′+0.50 ag CLB

4.4′ 2.2′+600 ag CLB 5.5′ 600ag CLB 6.6′ 5.5′ +1.50 ml $NaIO_4$

The ΔF of R (56.4) is larger than that of CLB (15.6), which is indicated that CLB could be oxidized by $NaIO_4$ more easily. The reaction was similar to $KBrO_3$ oxidizing R (Scheme 1–2) [20].

I_2 produced from the reaction (3) oxidized R to R' intensely, then the process of reaction (4) was accelerated, which caused the fluorescence signal of R to quench (Scheme 2).

In order to prove the probability of oxidating reaction occurred between R and $NaIO_4$, the infrared spectra of R and R' were scanned by Nicolet-360 infrared spectrometer (KBr pellet) ranging from 200 to 4,000 cm^{-1} (Table 1). Results show that one strong and wide phenolic hydroxyl (−OH) peak located at 3,445.2 cm^{-1}, $\geq C{=}O$ peak at 1,654.7 cm^{-1} and the frame vibration of aromatic ring peak at 1,450–1,600 cm^{-1}; when $NaIO_4$ was added the –OH peak disappeared due to the oxidating reaction of R with $NaIO_4$. At the same time, the $\geq C{=}O$ peak at 1,654.7 cm^{-1} moved to short wavelength region with the enhance of the peak intensity, which indicated that -OH group of R and $NaIO_4$ carried out oxidating reaction and proved the possibility of the oxidating reaction between R and $NaIO_4$ in the way showed in Scheme 1–2.

In the CLB-$NaIO_4$ system, the fluorescence signal of CLB was quenched (Fig. 1, curve 2.2′), which indicates that there was an oxidation-reduction reaction between $NaIO_4$ and CLB (Scheme 3). The reaction of $NaIO_4$ oxidizing CLB was similar to $KMnO_4$ [21].

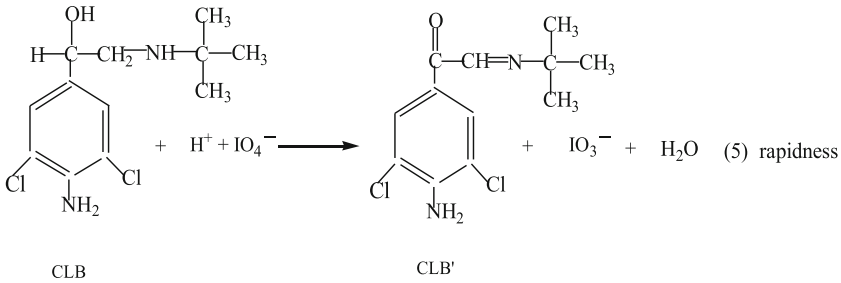
Seen from IR, $-NH_2$ group, −OH group, $-CH_2$ group, $-CH_3$ group, −NH– group and the frame vibration of aromatic ring stretch vibration absorption peak of CLB located at 3,487.5, 3,592.8, 2,914.3, 2,941., 1,549.4 and 1,452.8 cm^{-1}, respectively. When $NaIO_4$ was added into the CLB solution, the typical absorption peaks of NH_2 group, $–CH_2$ group, $-CH_3$ group and the frame vibration of aromatic ring still existed, but the absorption peaks of the –OH group and –NH– group disappeared, new stretch vibration absorption peak of $\geq C{=}O$ group and –CH=N– bonds located at 1,737.3 and 1,675.6 cm^{-1}. These facts indicated that the CLB reacted with the $NaIO_4$ via oxidating reaction and generated CLB' containing $\geq C{=}O$ group bond and–CH=N–.

The reaction product CLB' could be reduced to CLB by I^- (Scheme 4), which shows CLB has a catalytic effect on $NaIO_4$

Table 1 Infrared spectra data of R, R', CLB and CLB' (ν is stretching vibration; δ is in-plane bending vibration and w is out-plane bending vibration)

Sample	−OH (cm^{-1})	$-NH_2$ (cm^{-1})	$-C_6H_5$ (cm^{-1})	$–CH_2$ (cm^{-1})	$-C\ H_3$ (cm^{-1})	−C=O (cm^{-1})	–NH– (cm^{-1})	–CH=N– (cm^{-1})
R	ν:3,445.2		ν: 1,607.3 ν: 1,492.4 ν: 1,441.7			ν:1,654.7 δ:1,280.5		
R'			ν: 1,609.5 ν: 1,493.9 ν: 1,443.6			ν: 1,715.4 δ:1,283.9		
CLB	ν:3,592.8	ν: 3,487.5	ν: 1,622.4 ν: 1,503.5 ν: 1,452.8	ν: 2,914.6 δ: 1,382.7	ν: 2,941.3 ν:2,871.2 ν:1,465.9		ν: 1,549.4 δ: 1,101.9	
CLB'		ν:3,489.1	ν: 1,624.1 ν: 1,505.6 ν: 1,455.7	ν: 2,916.3 δ: 1,385.4	ν:2,944.2 ν:2,873.8 ν:1,468.1	ν: 1,737.3 δ:1,312.2	ν:1,552.6 δ: 1,103.1	ν:1,675.6

Scheme 3 Oxidation-reduction reaction between $NaIO_4$ and CLB



oxidizing R, caused the fluorescence signal of R will be quenched sharply and the λ_{em}^{max} were not changed obviously ($\lambda_{ex}^{max}/\lambda_{em}^{max}$=484.8/545.6 nm,$F$=37.1, ΔF=336.0, curve 4.4′). This indicates that CLB has a remarkable catalytic effect on $NaIO_4$ oxidizing R. Thus, 485/546 nm was selected as the working wavelength and residual CLB could be determined by the catalytic fluorescence probe.

In order to prove the probability of oxidation-reduction reaction between CLB' and I^-, to a 25 mL colorimetric tube, 600 ag CLB and 1.50 mL $NaIO_4$ were added and diluted with water. The solution was mixed homogeneously and heated at 60 °C water bath for 8 min. 1.00 mL test solution was taken to a white drip board, added one drop of $AgNO_3$ solution, then no light yellow precipitation appeared, indicating that there was no I^- in the test solution. And then,1.00 mL of 5 % Vc was continuously added in the colorimetric tube, and the tube was heated for 4 min, where was added 1.00 mL of I^-, and went on to be heated for 3 min. 1.00 mL of the solution was taken in a test tube. It was added into one drop of 5 % starch and light blue could be seen. Continually, 1.00 mL CCl_4 was added, and the solution layered. The upper layer was light blue, while the lower was colorless and transparent, showing that there was I_2. The above experiment facts have shown that I^- and I_2 formed in the process of $NaIO_4$ oxidizing CLB, and that at the same time the mechanism of $NaIO_4$ oxidizing CLB can be explained according to the reactions in Scheme 1–4.

Optimum Measurement Conditions

For the system containing 4.0 ag CLB mL^{-1}, the effects of the volumes and concentrations of reagents, luminescence substances(R (A), azocarmine (B), dimethyl yellow (C), calcein (D), crystal violet (E), stc.), oxidants ($K_2S_2O_8$ (A), H_2O_2 (B), $(NH_4)_2S_2O_8$(C), $KClO_3$(D), $NaIO_4$ (E) and KIO_3(F), stc.), reaction time and temperature, reaction acidity, and standing time on the ΔF of the system were examined in a univariate approach (Table 2, Fig. 2), respectively. Results show that the ΔF of the system reached the maximum when the volumes and concentrations of reagents were 0.70 mL of 1.0×10^{-4} mol L^{-1} R and 1.00 mL of 1.5 % (W/V) $NaIO_4$, pH value of the reaction system was within 2.82–5.75, R and $NaIO_4$ were selected, the reaction time was 15 min and the temperature was 60 °C, and standing time was within 10–30 min after being cooled by flowing water for 5 min.

Linear Range, Working Curve, Sensitivity and Precision

Under the optimum conditions described above, the ΔF of system was detected according to the proposed fluorescent probe. Results show that ΔF was directly proportional to the concentration of C_{CLB} in the range of 0.020–24.00 ag mL^{-1}. The regression equation of working curve could be expressed as ΔF=4.499+13.76 C_{CLB} (ag mL^{-1}), r=0.9999. The blank solution was measured repeatedly for 11 times, and the LOD was 6.8×10^{-21} g mL^{-1} (calculated by 3Sb/k, Sb/k referred to the quotient between triple of the blank reagent's standard deviation and the slope of the working curve, Sb referred to the standard deviation of 11 parallel analysis of the blank reagent, Sb was 0.031). Compared with method of Ref. [8], the sensitivity of this fluorescent probe (LOD: 6.8×10^{-21} g mL^{-1}) higher 1.0×10^{9} times that (LOD: 7.0×10^{-12} g mL^{-1}) of Ref. [8], showing advantages of the fluorescent probe for the detection of. trace CLB in sample due to the following possible reasons: firstly, the catalytic effect of CLB on the reaction of $NaIO_4$ oxidizing R greatly improves ΔF value, showing the signal amplification of catalytic reaction; secondly, R show characteristics including long fluorescent lifetime and high quantum yield. Besides, for 0.020 and 24.00 ag CLB mL^{-1}, relative standard

Scheme 4 Oxidation-reduction reaction between CLB' and I^-

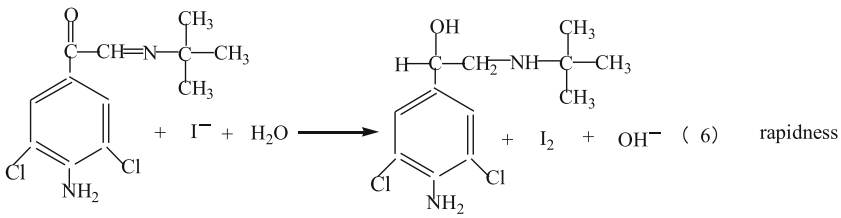


Table 2 Optimization of the concentration and volume of reagents

Reagents	Concentrations and volumes	The ΔF in R-$NaIO_4$-CLB system	Optimal
R (mol L^{-1})	1.0×10^{-2}, 1.0×10^{-3}, 1.0×10^{-4}, 1.0×10^{-5}, 1.0×10^{-6}	13.1, 26.5, 57.3, 40.2, 35.6	1.0×10^{-4} mol L^{-1}
R (m L)	0.10, 0.30, 0.50, 0.70,1.00	5.6,11.4,35.2, 55.4, 41.4	0.70 mL
$NaIO_4$ (%)	0.10, 0.50, 1.00, 1.50, 2.00	6.6, 14.4, 25.5, 58.2, 39.7	1.50 %
$NaIO_4$ (mL)	0.10, 0.50,1.00,1.50,2.00	33.7, 41.3, 49.9, 56.2, 39.3	1.50 mL
Buffer solution (mL)	0.50,1.00,1.50,2.00,2.50	30.8, 38.8, 48.6, 58.1, 50.6	2.00 mL

deviations (RSDs, n=8) were 1.2 and 3.4 %, respectively, showing good precision of the fluorescent probe. This method not only offers a new technology for the determination of ultra-trace CLB, but also shows that amplification effect on measure signal of catalytic reaction is an effective way to futher improve the sensitivity of fluorescent probe.

Interference Experiment

CLB was determined by this fluorescent probe (4.00 ag CLB mL^{-1}) and the methods in references (10 μg CLB mL^{-1} [14]), respectively. When the relative error (Er) was±5 %, the allowed concentrations of coexistent ions (materials) were

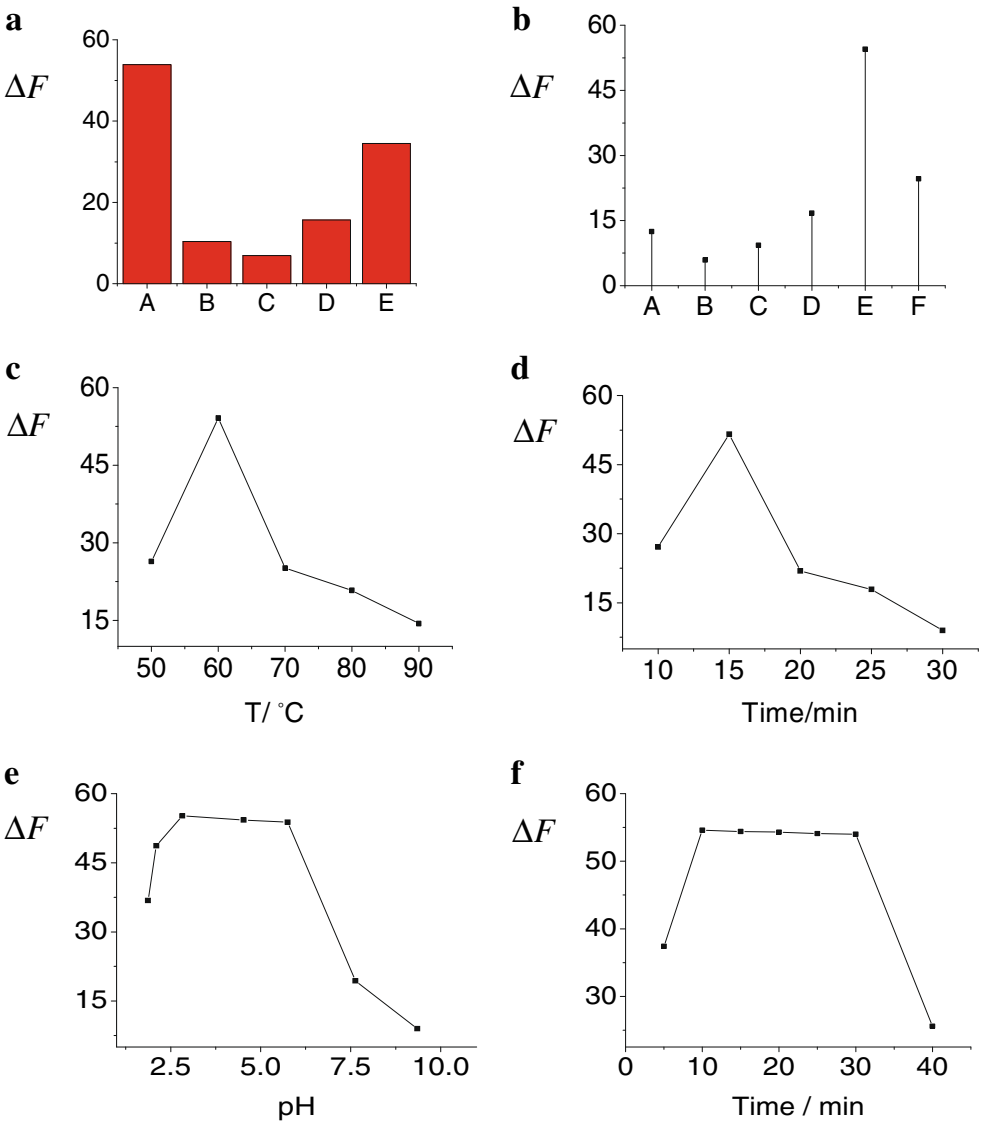


Fig. 2 Effects of luminescence substrates (**a**), oxidants (**b**), temperature (**c**), time (d), pH (**e**) and standing time (**f**) on ΔF for the reaction system, respectively

compared with those in Ref. [14], and the results are listed in Table 3.

Compared with Ref. [14], the allowed concentration of coexistent ions (materials) of this fluorescent probe were larger than those in Ref. [14], showing that coexistent ions (materials) have little interference to the determination of CLB and that this fluorescent probe has good selectivity, which may be due to the high selectivity of catalytic reaction.

Analysis of Samples

An asthmatic orally took one CLB tablet (total: 40 μg CLB), after 24 h, 1.00 mL of human serum and urine were obtained, respectively. The human serum was dealt according to the method described in Ref. [14]. The solution was diluted to 100 mL with Britton–Robinson buffer solution (BR, pH= 4.54), and then 1.00 mL of the solution was diluted to 10^5 fold with BR before used. The human urine and pig urine that contained CLB were dealt according to the method described in Ref. [8], respectively. The 1.00 mL solution was diluted to 10^8 fold with 0.010 mol L^{-1} HCl solution before used.

Chicken muscle and duck muscle were broken. 5.0 g of muscle was placed in the small beaker, then 25 mL of 0.050 mol L^{-1} HCl was added, with high-speed homogenate machines homogenized for 1 min and ultrasonic extracted for 30 min. 6.0 g of hemogeneous medium was taken (equivalent to 1.0 g chicken muscle sample) to centrifuge tube. And the mixed solution was centrifugalized for 15 min at 4,000 r min^{-1} (if the upper formation was the flock, the centrifugal time should be extended). Superior layer solution was taken, 300 μL of 1.0 mol L^{-1} NaOH solution and 4.0 mL of 0.50 mol L^{-1} KH_2PO_4 solution were added, then the mixture was kept at pH 4.54, 4 °C in refrigerator over night. The next day, it was centrifugalized for 15 min by 4,000 r/min. 1.00 mL upper solution was taken and was diluted to 10^{15} fold by BR buffer solution before used.

Pig fur samples that contained CLB were immersed into acetone for 30 min, washed by water and then dried. 1.0000 g of the sample was weighed, and digested in 10 mL NaH_2PO_4-CH_3OH solution. The pH value of the solution was adjusted to 2.82–5.75 with H_3PO_4, and then ultrasonic oscillation washing was carried out at 40 °C for 24 h. After these, the solution was transferred and diluted to a 100 mL measuring flask before used.

1.00 mL test solution was taken and the content of CLB was determined according to the experimental method. Standard addition experiment was simultaneously conducted. This method was compared with GC/MS. The results are listed in Tables 4–5

Seen from the Tables 4–5, this method not only can measure the CLB content of organisms, can also be used to analyze residual trace CLB. The results were in accordance with those of GC/MS. The recovery was 99.3–102 (%), and the RSDs were 1.9–3.8 (%), which shows this fluorescent probe has high accuracy, high sensitivity and precision.

Table 3 The effect of interfering species

	The method		References [14]
Coexistent ions (materials)	Concentration (μg mL^{-1})	Er(%)	Concentration (μg mL^{-1})
Na^+	2,600	1.3	1,800
K^+	2,600	1.6	1,800
Mg^{2+}	2,600	1.5	1,800
Ca^{2+}	2,600	1.8	1,810
Zn^{2+}	2,600	2.0	1,820
Fe^{3+}	240	1.8	180
Vitamin C	240	1.5	160
Dopamine	240	2.4	140
Glucose	100	1.7	40
Uric acid	70	2.3	20
Urea	90	3.4	30
Aureomycin	420	3.1	100
Terramycin	360	3.6	90
Tetracycline	180	3.0	60

Table 4 Comparison of results of CLB analysis in test samples (n=6) by various methods

This method	GC/MS			
Sample	Found (ng mL^{-1})	RSDs. (%)	Found (ng mL^{-1})	Er (%)
Human serum	0.27	3.4	0.28	−3.6
Human urine	1.75	2.6	1.71	2.3
Pig urine	3.74	2.7	3.82	−2.1
Pig urine	5.80	1.9	5.62	3.2
	15.45			
Chicken muscle		2.1	15.73	−1.8
	(mg g^{-1})			
	12.58			
Duck muscle		2.8	12.39	1.5
	(mg g^{-1})			

Table 5 Analysis results of CLB in pig fur samples (n=6)

Sample	Found (fg g^{-1})	RSDs. (%)	Added (fg g^{-1})	Obtained (fg g^{-1})	Recovery (%)
1	1.232	2.4	0.12	0.122	102
2	1.236	2.3	0.12	0.120	100
3	1.447	3.1	0.14	0.141	101
4	1.421	2.5	0.14	0.139	99.3
5	0..962	3.3	0.10	0.996	99.6
6	0.937	3.8	0.10	0.102	102

Moreover, if the feedstuff containing CLB was taken by the pig, CLB would appear in pig urine, leaving only a little residual in the body. Therefore, determination of CLB in pig urine before butchering has significant meaning for maintaining human health.

Conclusion

In this work, a catalyzing fluorescent probe with signal amplification for the determination of CLB has been proposed based on the fact that CLB could accelerate the reaction between R and $NaIO_4$, caused rapid response of the ΔF to [CLB]. This rapid, simple, sensitive and selective fluorescent probe not only displays potential application prospect in CLB analysis, but also indicates notable advantage of combination between high sensitivity of fluorescent probe and signal amplification of catalytic reaction and effectively promoted the development and applications of detection technique of residual CLB, fluorescent probe and catalytic kinetic analysis.

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