Flow-injection Post-chemiluminescence Determination of Furazolidone in Animal Feeds

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Abstract

The post-chemiluminescence (PCL) reaction was observed with the mixture solution after completion CL reaction of luminol-H₂O₂ in present of Furazolidone (FZD). A novel method for the determination of FZD was established by flow-injection PCL. The CL intensity responded linearly to the concentration of FZD in the range 1.0×10^{-7} - 1.0×10^{-5} g mL⁻¹, with a linear correlation of 0.9949. The detection limit for FZD was 1.96×10^{-8} g mL⁻¹ (S/N=3). The relative standard deviation (RSD) was 3.5% (n=11, $c=1.0 \times 10^{-6}$ g mL⁻¹). The proposed method has been applied to the determination of FZD in animal feeds and the results were consistent with those obtained by the HPLC-UV method. A possible mechanism for the PCL reaction was also discussed by the investigation of CL kinetic characteristics, CL spectra and UV-Vis absorption spectra.

Keywords: Post chemiluminescence; furazolidone; luminol.

1. Introduction

Furazolidone (FZD), N-[5-nitro-2-furfurylidene-3-amino]-2-oxazolidinone (Fig. 1), is one of the nitrofurantoin antibiotic drugs, which was widely used in food animal production because of its broad-spectrum antimicrobial activity and pharmacokinetic properties. It has been also used

as growth promoters in the pig production, poultry and fish sector. Due to concerns about its carcinogenicity and mutagenicity, FZD has been banned from use in food animal production in the EU in 1995 [1, 3]. Despite the potentially harmful effects on human health, it is still illegally and widely used in many countries because it is cheap and effective.

FZD compound was usually administered to animals by means of medicated feeds or at drinking water. The analysis of FZD needs to be based on the detection of FZD marker residue. Studies using ^{14}C -labeled FZD have shown that protein-bound metabolites are formed in tissues [4,5]. FZD metabolites are detectable for several weeks after administration. For this reason, the FZD metabolites are determined in animal tissues, whereas farm animal feeds may be checked for the presence of the FZD parent compounds.

Figure 1. Chemical structures of the FZD.

Several methods have been released for the detection of FZD in feeds and biological matrices by TLC [6], HPLC-ECD [7], HPLC-UV [8,9] and LC-MS [10]. However, the majority procedures of LC-UV available for FZD feed control, shown limits of quantification around 1 mg kg^{-1} , is not suitable for FZD screening procedures [11]. Furthermore, these procedures available for FZD feed control are time-consuming or costly. Since the main difficulty in the determination of FZD is its instability due to sensitivity to light [12], there is now an urgent need for a rapid, simple, and sensitive determination method for FZD at low concentrations in animal feed, in order to satisfy needs on feed control.

Chemiluminescence (CL) analysis has received much attention for the analysis of organic species in a variety of fields for its high sensitivity, wide dynamic range and inexpensive instrumentation [13-14]. However, the CL signal of normal CL systems containing FZD was relatively weak. Post-chemiluminescence (PCL) as a peculiar CL phenomenon, had been reported by Lu JR's group [15-18]. We found the stronger signal of FZD was detected in PCL system.

This paper describes a novel PCL method for detection of FZD residues. The PCL phenomenon of FZD in H_2O_2 -luminol system was discovered and the CL reaction was investigated. The possible mechanism of the CL reaction was discussed. A new method for the determination of FZD was established combining the CL reaction with the flow injection technique. The present paper is the first report of a simple, cost-effective and fast flow injection CL method to determine the FZD in farm animal feed.

2. Experimental

2.1. Apparatus

The IFFM-D flow injection CL analyzer (Xi'an Remex Electronic Instrument High-Tech Ltd., China) was equipped with an automatic injection system and a detection system. The schematic diagram of the CL flow system employed is shown in Fig. 2. PTFE tube (0.8 mm i.d.) was used to connect all of the components in the flow system. The flow cell was a coil of glass tube that positioned in front of the detection window of the PMT. The CL signal was treated with a personal computer. The CL spectra were acquired with Perkin Elmer Model LS-55 luminescence spectrometer (the light was cut off). The absorption spectra were acquired on a Thermo Nicolet Corporation Model evolution 300 UV-Visible spectrometer.

2.2. Chemicals and solutions

Stock solution ($1.0 \times 10^{-2} \text{ mol L}^{-1}$) of luminol was prepared by dissolving luminol (Sigma) in NaOH (0.1 mol L^{-1}) without purification. Working solutions of luminol were prepared by diluting the stock solution. Working solutions of H_2O_2 were prepared fresh daily from 30% (v/v) H_2O_2 (Shanghai Chemical Reagent Co., Ltd.). HPLC grade methanol, acetonitrile, dimethylformamide (DMF), hexane, and ethyl acetate were purchased from Shanghai Chemical Reagent Co., Ltd. FZD was purchased from Sigma-Aldrich (Germany). The standard solution of FZD ($1.00 \times 10^{-4} \text{ g mL}^{-1}$) was prepared by initially dissolving 0.0100 g FZD with DMF and diluting to 100mL with water. More dilute solutions were prepared from the stock solution by appropriate dilution with water.

All FZD solutions were stored in a refrigerator and protected from light. All other solvents and reagents, as well as Lichrolut EN (C18 ,500 mg,3 mL) solid-phase extraction (SPE) cartridges were

purchased from Merck (Darmstadt, Germany). Deionized and distilled water was used throughout the whole experiment.

Figure 2. Schematic diagram of CL flow system.(a)FZD solution; (b) H₂O₂ solution; (c) Luminol solution; (P) peristaltic pump; (L) mixing tube; (V) injection valve; (W) waste; (F) flow cell; (HV) high voltage; (PMT) Photomultiplier tube; (PC) personal computer.

2.3 Samples and sample preparation

The animal feed samples were commercial feeds available on the local markets (Wuhan, China).

0.1 g of the animal feed spiked with a 0.25 mL of FZD solution (of suitable concentration) was prepared by adding 1.0 mL of the ultrapure water to the feed sample, and then the sample was transferred into a 2 mL centrifuge tube with a thread lock. The tube was vigorously shaken in a mechanical shaker for 15 min and then centrifuged (6000 g) for 5 min. The precipitation was treated with 1.0 mL of acetonitrile, agitated for 10 min by the mechanical shaker and centrifuged for 5 min. The two supernatant extracts were transferred again to the tube and the combined extracts were then ready for solid-phase extraction. The SPE cartridges were conditioned with the distilled water (3.0 mL) and acetonitrile (3.0 mL) solvent was passed before the sample. The sample was loaded onto the cartridge. The loaded cartridge was washed with the washing solvent (hexane 5.0 mL) and the analytes were selectively eluted with the elution solvent (ethyl acetate 5.0 mL) into a glass tube. The residue obtained by evaporated to

dryness under a nitrogen stream. The tube is removed immediately after drying and the solution reconstituted with 1.0 mL of DMF. For the determination procedure prepare a blank under the same conditions but without FZD spiking.

2.4 PCL procedure

Flow tubes were connected with H₂O₂ solution, luminol solution and FZD solution, respectively. H₂O₂ stream was merged with luminol stream through a Y-piece. When the baseline was stable, FZD solution was injected into the merged stream of H₂O₂ -luminol solution by injection valve. The CL intensity (peak height) was used as the quantitative criterion.

3. Results and Discussion

3.1. The PCL behavior of FZD in H₂O₂-luminol system

Kinetic characteristics of the CL reactions were examined using the static measuring system of the IFFM-D multifunction CL analyzer. The CL intensity–time curve was shown in Fig. 3. When 5.0×10^{-3} mol L⁻¹ of H₂O₂ solution (1.0 mL) was

injected into 3.0×10^{-5} mol L⁻¹ of Luminol solution (1.0 mL), a CL reaction was initiated immediately (peak 1). After 500 s approximately, the CL reaction terminated and the CL signal declined to baseline. Subsequently, another stronger CL reaction (peak 2) was initiated when 1.0×10^{-4} g mL⁻¹ of FZD solution (1.0 mL) was injected into above reaction mixture. The CL reaction terminated and the CL signal declined to baseline again after 40 s approximately.

Under the same condition, CL signal was not detected by using the blank solution instead of the FZD solution. The experiments showed that the reaction initiated by FZD solution in the H₂O₂-luminol system was a post CL reaction.

Figure 3. Kinetic curve of the CL reaction. (1) 5.0×10^{-3} mol L⁻¹ of H₂O₂ solution (1.0 mL) was injected into 3.0×10^{-5} mol L⁻¹ of Luminol solution (1.0 mL); (2) 1.0×10^{-4} g mL⁻¹ of FZD solution (1.0 mL) was injected into above reaction mixture.

3.2. Optimization of experimental conditions

Since it was the PCL reaction caused by FZD added in H₂O₂-luminol system, the CL reaction between H₂O₂ and luminol must react adequately in flow injection analysis (FIA) system before initiating the latter CL reaction. For this purpose, a mixing tube (L) (0.8mm i.d.) was connected between the Y-piece and the injection valve (Fig. 2). If the mixing tube were too short, H₂O₂ and luminol would react deficiently and the background would be too high. If the tube were too long, the post CL signal would be too weak. The length of the mixing tube was examined in the range 20-120 cm when the flow rate of each

solution was fixed at 1.2 mL min⁻¹. The test showed that the optimal length of mixing tube was 75 cm.

The PCL was affected by the pH of luminol solution. The maximal PCL signal was observed when the Concentration of Sodium hydroxide in luminol solution was 0.01 mol L⁻¹.

The effect of luminol concentration on the PCL intensity was investigated from 1.0×10^{-6} to 1.0×10^{-5} mol L⁻¹. The strongest PCL intensity was achieved when the concentration of luminol was 2.0×10^{-6} mol L⁻¹. Thus, 2.0×10^{-6} mol L⁻¹ was selected as the optimum concentration of luminol solution.

The effect of H₂O₂ concentration on the PCL intensity was studied in the range of 1.0×10^{-5} - 2.0×10^{-4} mol L⁻¹. The maximal PCL signal was obtained when the concentration of H₂O₂ solution was 1.0×10^{-4} mol L⁻¹.

3.3. Analytical characteristics

Under the optimum conditions, the relation between the CL intensity and the concentration of FZD was examined. The linear range of the method was 1.0×10^{-7} to 1.0×10^{-5} g mL⁻¹, with a linear regression equation of $I = 4.57c + 4.71$ ($r = 0.9949$), where I is the CL intensity (relative unit) and c is the concentration of FZD (10^{-7} g mL⁻¹). RSD was 3.5% for 11 independent determinations of 1.0×10^{-6} g mL⁻¹ FZD. According to the suggestions of IUPAC, the measured detection limit of the method was 1.96×10^{-8} g mL⁻¹ FZD.

3.4. Interference

The effect of various components concurrently presented in animal feeds, especially carbohydrate and metal ions, on the determination of FZD by described method was investigated.

Under the optimum conditions, the interference tests were examined. A foreign species was considered not to interfere if it caused a relative error <5% during the determination of 1.0×10^{-6} g mL⁻¹ of FZD solution. The tolerable concentration ratios of foreign species to 1.0×10^{-6} g mL⁻¹ of FZD was over 1000-fold for Na⁺, SO₄²⁻, K⁺, Cl⁻; 500-fold for Pb²⁺, Ca²⁺, Mg²⁺, CO₃²⁻; 100-fold for Mg²⁺, Al³⁺, Ni²⁺, NH₄⁺, glucose, lactose ,

soluble starch, fructose, maltose; 10-fold for Cr^{3+} , Zn^{2+} , Fe^{2+} .

3.5. Sample analysis

In order to validate the utility of the proposed method in a real sample, porcine feed samples were measured according to the method mentioned in Section 2.4. The FZD in porcine feed specimens was determined by the proposed method. As shown in Tables 1, the recoveries were calculated and the results were comparable

with the HPLC-UV method [8]. The test was repeated three times per concentration. As shown in Table 1, the recoveries of FZD were in the range of 82-87% at three levels in the above samples. *RSD* was below 6% ($n=3$). The results imply good agreement between FZD concentrations determined by two methods and prove the reliability of the PCL for the determination of real samples.

Table 1. The determination of FZD in animal feed. (N=3)

Sample	fortified level (ug/mL)	FZD found ^a (ug/mL)		RSD (%)	Added (ug/mL)	Recovered (ug/mL)	Recovery (%)
		PCL method	HPLC method				
1	0.50	0.42±0.023	0.45±0.021	5.47	1.00	0.82	82
2	1.00	0.91±0.023	0.92±0.027	2.55	1.00	0.87	87
3	5.00	4.76±0.163	4.79±0.143	3.42	1.00	0.84	84

^a Mean ± S.D.

3.6. Possible reaction mechanism

An important process of studying the CL mechanism is to ascertain the luminant of the CL reaction. The CL spectra of the CL reactions were drawn (Fig. 4) by placing and exchanging the interference filters (400-750 nm) before the signal window of the BPCL-2 ultra-weak luminescence analyzer. A peak appeared at 425 nm when H_2O_2 and luminol were mixed (Fig. 4a). The same spectrum was obtained when FZD was injected into the mixing solution of H_2O_2 and luminol solution (Fig. 4b). The maximum emission wavelengths were both 425 nm, which suggested that the two reactions have the same illuminant, the 3-aminophthalate ion (3-AP) [19, 20].

The absorption spectra of FZD solution and the mixtures of FZD and H_2O_2 solution were scanned (Fig. 5). It was observed that the specific absorbance peak in UV-vis spectra of FZD disappeared, which indicated that FZD was oxidized when H_2O_2 was added.

Figure 4. CL Spectra. a, H_2O_2 solution (1.0×10^{-4} mol/l) + luminol solution (2.0×10^{-5} mol/l); b, H_2O_2 solution (1.0×10^{-4} mol/l) + luminol solution (2.0×10^{-5} mol/l) + FZD solution (5.0×10^{-5} g/mL);

Figure 5. UV-vis spectra of FZD. a. FZD solution(5.0×10^{-5} g/mL); b. FZD solution(5.0×10^{-5} g/mL)+ H_2O_2 solution (1.0×10^{-4} mol/l).

Under the optimum conditions, the concentration of H_2O_2 (2.0×10^{-5} mol L^{-1}) was much higher than that of luminol (4.0×10^{-6} mol L^{-1}). This result showed that excess H_2O_2 was necessary.

According to the test results above, the mechanism of the CL reaction of FZD in H_2O_2 and luminol system can be interpreted as: H_2O_2 oxidized luminol producing 3-AP at an excited state (3-AP*). The 3-AP* came back to the ground state, accompanied by CL ($\lambda_{\text{max}}=425$ nm). When FZD was added to the resultant solutions of H_2O_2 -luminol reaction, it was oxidized by H_2O_2 and released energy. 3-AP in the solution absorbed the energy and was excited again, accompanied by CL.

The mechanism can be simply described as follows:

4. Conclusion

In this paper, a novel flow injection CL method for the determination of FZD was established based on the PCL reaction. The results suggest that this PCL is a simple, cost-effective

and fast method suitable for the detecting of FZD in feeding stuffs. The application of the PCL method to other analytes, especially more nitrofurant antibacterial compounds, and the mechanism of the PCL are under further investigation.

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