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Analytical Methods

Determination of residual enrofloxacin in food samples by a sensitive method of chemiluminescence enzyme immunoassay

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ABSTRACT

A chemiluminescence enzyme immunoassay (CLEIA) based on the HRP-luminol-H_2O_2 chemiluminescence system for highly sensitive detection of enrofloxacin (ENR) was proposed in this study. Key factors that affect the precision and accuracy for the determination of ENR residues were optimised. Under the optimal conditions, the proposed method showed an excellent performance. The linearity range for method developed for determination of ENR was 0.35–1.0 ng/mL with a correlation coefficient greater than 0.994. The limit of detection was 0.03 ng/mL and the relative standard deviations (RSDs) were less than 9.4% and 13.0% for intra-day and inter-day assays. The proposed method was satisfactorily applied to determine ENR in milk, eggs, and honey samples at three spiked levels (0.4, 0.7, and 1.0 ng/mL) and the recoveries ranged from 92.4% to 104.2% for milk, 93.8% to 103.2% for eggs and 94.1% to 105.0% for honey, respectively. Compared the results of CLEIA with those of ELISA and HPLC, the advantages of the CLEIA were further confirmed. Moreover, one 96-well microtiter plate coated with anti-ENR can be used to detect multiple samples at the same time, which indicated that the CLEIA using HRP-luminol-H_2O_2 system was a sensitive, high throughput and real-time method for ENR residues analysis.

1. Introduction

In animal agriculture, infectious disease is an important factor affecting the profitability of livestock industries. To increase the outcome and meet the demand of the society, many antibacterial drugs are often used to prevent and treat the infectious diseases in farming practices. It is reported that the use of antibiotics in animal feeds has increased year by year all over the world (Babaahmady & Khosravi, 2011).

Fluoroquinolone antibiotics are widely used in the practices of veterinary clinics because of their rapid effect and broad-spectrum antibacterial activity. Enrofloxacin is an important member of fluoroquinolone family, and has an excellent activity against mycoplasma, Gram-positive and Gram-negative pathogens by inhibiting DNA synthesis. As a consequence of its broader antibiotic spectrum, ENR is more popular than other fluoroquinolones (Babaahmady & Khosravi, 2011). However, the long-term application of fluoroquinolone would lead to an increase of resistant *Campylobacter* species in poultry and also drug residues in animal muscle and tissue, which is a significant threat against human health through the food chain.

The results of a growing number of worldwide studies have suggested that ENR is toxic to the living things. For example, ENR could inhibit cell proliferation and induce morphological changes in equine tendon and canine tendon cell cultures (Lim, Hossain, Park, Choi, & Kim, 2008; Yoon et al., 2004). Aral et al. proved that certain dosages of ENR could reduce the sperm count, deteriorate the sperm motility, and cause sperm morphological abnormalities in male mice (Aral, Karacal, & Baba, 2008). ENR showed some toxic effects on growth rate of *Scenedesmus obliquus*. It made the algal cells generate reactive oxygen species that damaged some biological macromolecules, and changed the biomembrane permeability (Qin, Chen, Lu, Zhao, & Yuan, 2012). ENR also showed weak effects on the total number of intestinal microbiota, caused enteric dysbacteriosis which could decrease the disease-resistant ability of resident flora in the human intestine (Chen, Yuan, Feng, Wei, & Hua, 2011). Many countries and organisations have defined the upper protection levels of ENR residues in animal foods. For example, the European Union (EU) stipulates that the maximum residue limits of ENR residues in milk and muscle tissues are 100 μg/L and

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30 μg/kg, respectively. Therefore, it is essential to establish a simple and sensitive method for detecting the ENR residues in foods to implement legislative regulations.

Many analytical methods have been developed for the measurement of the ENR residues in animal foods, including high-performance liquid chromatography with diode-array detection (Cinquina et al., 2003), high-performance liquid chromatography coupled to fluorescence detection (Choi et al., 2011), ultra-performance liquid chromatography–tandem mass spectrometry (Herrera-Herrera, Hernandez-Borges, Rodriguez-Delgado, Herrero, & Cifuentes, 2011), chemiluminescence analysis (CL) (Pulgarin, Molina, & Munoz, 2011), capillary electrophoresis (Lombardo-Agui, Garcia-Campana, Gamiz-Gracia, & Blanco, 2010) and enzyme-linked immunosorbent assay (ELISA) (Zhang et al., 2011). These methods have been proven sensitive and accurate, but they are not suitable for the high throughput monitoring of ENR residues because they are expensive, time-consuming, and are not environmentally friendly. Chemiluminescence enzyme immunoassay (CLEIA), which combines ELISA with CL technique and possesses the advantages of the two methods, is a simple, rapid, sensitive, low cost and specific screening assay for detection of residues in a large amount of sample. Recently, CLEIA has been widely used in variety of fields for trace analysis, including medicine (Wu et al., 2011), environment (Long, Shi, He, Sheng, & Wang, 2009), food (Fang, Chen, Ying, & Lin, 2011) and so on (Fu et al., 2012; Lin, Chu, & We, 2012).

For the CLEIA, it is important to choose an appropriate chemiluminescence (CL) detection system. We have developed a CLEIA method to detect the ENR residues using the alkaline phosphatase (ALP)-adamantane (AMPPD) system as the CL detection system (Yu et al., 2012). But luminol-H_2O_2-horseradish peroxidase (HRP) is one of the most widely used systems in the field of CL, and it is markedly more sensitive than other CL system (Egorov, Gavrilova, & Sakharov, 2000). In this study, the chemiluminescence system of HRP-luminol-H_2O_2 has been used to establish the more sensitive CLEIA method to detect ENR residues in milk, egg and honey samples, and the results obtained by the proposed method were compared with those by HPLC and ELISA to illustrate the superiority of CLEIA.

2. Material and methods

2.1. Chemicals and solutions

The ENR standard was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The monoclonal anti-ENR (ENR-Ab) was purchased from Santa Cruz Biotechnology (USA). Horseradish peroxidase (HRP) was obtained from ROCHE Inc. (Specific activity > 250 μ/mg). Bovine serum albumin (BSA) was purchased from Sigma Chemical Corporation (USA). Reagents A and B of the luminol-H_2O_2 system were purchased from Sino-American Biotechnology Company (Beijing, China). All other chemical reagents were of analytical grade and Milli-Q water was used throughout the experiments.

The solutions used in this experiment were as follows. The incubation solution was 0.01 mol/L of sodium phosphate buffered saline (PBS), pH of 7.2. The coating solution was 0.05 mol/L of carbonate buffer, pH of 9.6. The washing and dilution solution was 0.01 mol/L of PBS buffer containing 0.05% (v/v) Tween-20 (PBST) with pH of 7.2. The blocking solution was 0.01 mol/L of PBS buffer with 1% (w/v) BSA and 4% (w/v) cane sugar, and pH of 7.2.

Because of the physicochemical property of ENR (pK_1 of 5.88, pK_2 of 7.74 in water), 1.0 mg/mL ENR stock solution was made by 50 mg ENR standard dissolved in 0.03 mol/L sodium hydroxide solution (NaOH). The standard solutions were prepared by serial dilutions of the stored solution with 0.03 mol/L NaOH; and their respective concentrations were 0.35, 0.45, 0.55, 0.75, 0.85, and 1.00 ng/mL. The prepared calibrants were stored at 4 °C for use.

2.2. Apparatus

The chemiluminescence signal was detected by an MP280 Chemiluminescence Immunoassay Analyser (Beijing Tai Geke letter Biological Technology Co., Ltd.). A UV-2450 UV–vis Spectrophotometre (Shimadzu) was used to identify the HRP–ENR conjugates. The 96-well polystyrene microtiter plates (Shenzhen Jincanhua Industrial Co., Ltd) were used as the solid-phase support for the antibodies. Others instruments applied in this paper for comparative analysis included the United States Diane HPLC (P680 HPLC Pump, ASI-100 Automated Sample Injector, Thermostatted Column Compartment Tcc-100, UVD170 and Chromeleon Chromatography Work Station) and the Enzyme-labelled metre (Multi-skan MK3, Thermo).

2.3. CLEIA-developed method

2.3.1. Preparation of solid phase antibody

The direct immobilising method was used for the preparation of the solid phase anti-ENR. At first, the ENR-Ab solution was diluted to 3.0 μg/mL with carbonate buffer solution (pH of 9.6), and each well of the microtiter plates was coated with 100 μL ENR-Ab solution for physical adsorption. The sealed plates were allowed to stand for 150 min at 37 °C. Then the plates were washed with PBST solution for 5 times and were gently tapped against tissue paper to eliminate the free antibodies. After that, 150 μL of blocking solution was added into each well and the plates were heated at 25 °C for 2 h to block the uncoated active sites. Subsequently, the blocking solution was aspirated and the plates were washed with PBST solution for 5 times repeatly. Finally, the coated plates were made dry and stored at 4 °C for use.

2.3.2. Preparation of HRP–ENR conjugates

The HRP–ENR conjugates were synthesised in two steps based on the linkage of 2 imine-carbon (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, EDC), which followed the procedures described by Watanabe (Watanabe, Satake, Kido, & Tsuji, 2002) with a further improvement. The detailed steps are as follows, a total of 20 mg ENR was dissolved in 0.1 mol/L NaOH solutions. Then 1.0 mg *N*-hydroxysuccinimide (NHS), 12.5 mg EDC and 1 mL dimethylformamide (DMF) were added, and the solution was mixed well and placed at 25 °C for 24 h. After that 4.0 mg of HRP dissolved in 1.0 mL of 0.01 mol/L PBS buffer (pH of 7.2) was added into the above solution slowly and stirred for 4 h in the dark at room temperature. Then the mixture was dialyzed against PBS solution (pH of 7.2) for 3 days to remove the unreacted materials. Finally, the obtained solution of HRP–ENR conjugates was stored at 4 °C not exceeding 1 month.

2.3.3. Procedure for measurement of CLEIA

In the procedure of CLEIA, a total of 50 μL of ENR calibrant or food sample and 50 μL of HRP–ENR conjugate were added into each well of the coated test plates. Then the mixture was incubated at 37 °C for 1 h, in which ENR and HRP–ENR conjugate competed to combine with the ENR antibody coated on the plates. After the competition reaction, the fluid was discarded. The microplate was washed five times with PBST washing solution and blotted dry with tissue paper. Finally, 25 μL of CL substrate solution of luminol-H_2O_2 was added into the bottom of the test plates and incubated for 3 min at room temperature (away from the light), and the chemiluminescent signal was measured and expressed as