CHINESE JOURNAL OF ANALYTICAL CHEMISTRY
Volume 43, Issue 6, June 2015
Online English edition of the Chinese language journal

Available online at www.sciencedirect.com
ScienceDirect

Cite this article as: Chin J Anal Chem, 2015, 43(6), 871–875.

RESEARCH PAPER

Determination of Furaltadone Metabolite in Fish by Chemiluminescence Enzyme Immunoassay

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Abstract: An indirect competitive chemiluminescence enzyme immunoassay (icCLEIA) detection for the furaltadone metabolite 5-morpholinomethyl-3-amino-2-oxazolidone (AMOZ) was established. The effects of the coating antigen concentration, antibody dilution, immunoreaction time, reaction buffer and its ion concentration on the performance of the assay were studied and optimized. The results showed that the optimized assay conditions were as follows: the coating antigen concentration was 10 ng mL^{-1}, the polyclonal antibody was diluted 60000 times, immunoreaction time was 50 min and the buffer solution was 0.01 M phosphate buffer solution (PBS, pH 7.4). Under the optimized conditions, the linear detection range of the developed icCLEIA was 0.026–3.52 μg L^{-1}, the half inhibitory concentration (IC_{50}) was 0.29 μg L^{-1} and the limit of detection (IC_{10}) was 0.012 μg L^{-1}. The average recoveries of AMOZ of spiked fish ranged from 101.0% to 115.5%. In conclusion, the icCLEIA is applicable for detection of trace AMOZ in fish samples.

Key Words: Furaltadone; 5-Morpholinomethyl-3-amino-oxazoldone (AMOZ); Indirect competitive chemiluminescence enzyme immunoassay; Polyclonal antibody; Fish

1 Introduction

Furaltadone (FTD) is one of nitrofuran antibiotics, which can inhibit the activity of most Gram-positive and Gram-negative bacteria. FTD has been widely and illegally used for treatment of gastrointestinal disease and as growth promoters in aquatic species and livestock[1]. FTD is unstable and can be quickly metabolized to 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) *in vivo*. Normally, AMOZ is present in animal tissues as an AMOZ-protein complex for a long-term period. AMOZ also can be released from animal tissues under slightly acidic conditions. Therefore, AMOZ is often used as a target analyte for FTD residue monitoring[2]. The residues of FTD and its metabolite AMOZ in food would be released in gastric acid conditions and absorbed by human body. It was proved that FTD and its metabolite AMOZ had the effects of mutagenesis, teratogenesis and carcinogenesis[3,4]. Considering the harmful potentials of these residues to human health, FTD has been banned from use in animal husbandry by the European Union (EU) since 1995. A minimum required performance limit (MRPL) of 1 μg kg^{-1} was set for AMOZ in edible animal tissues in EU in 2003. Ministry of Agriculture of the People's Republic of China prohibited the use of FTD in animal husbandry in 2002[5,6]. Despite that there are several strict laws and regulations to control the use of FTD, it is still illegally and widely used because of low cost and effectiveness. So, it is essential to strengthen monitoring of FTD and its metabolite AMOZ in animal-derived food products.

Generally, the detection of FTD and AMOZ residues is typically performed by using chromatography and immunoassay methods. Many chromatography methods for

Received 9 January 2015; accepted 17 March 2015
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This work was supported by the National Natural Science Foundation of China (Nos. 31371769, 31271865), the Guangdong Province Science and Technology Plan Project of China (Nos. 2012A020100002, 2013B040402006), the Guangzhou Science and Technology Plan Project of China (No. 2013J4100053).

DOI: 10.1016/S1872-2040(15)60830-1

detection of FTD and AMOZ have been developed, such as high performance liquid chromatography coupled with ultraviolet-visible detection (HPLC-UV), liquid chromatography-mass spectrometry (LC-MS) and LC-tandem MS (LC-MS/MS)[5–11], which have the advantages of high sensitivity, high specificity, high accuracy and low false positive rate, etc. However, these instrumental methods also have some disadvantages such as the usage of expensive equipment, sophisticated operation procedure, time-consuming sample preparation steps and high cost. An alternative assay method used for the detection of AMOZ is immunoassay technique, such as enzyme linked immunosorbent assay (ELISA) [12], fluorescence polarization immunoassay(FPIA), chemilumin- escent enzyme immunoassay (CLEIA)[13–20], which exhibit good analytical performances including simplicity, rapidity, high sensitivity, high specificity, low cost and high throughput. In our previous works, the polyclonal and monoclonal antibodies against AMOZ derivatives were produced in our laboratory and ELISA analytical method was established[18]. Furthermore, a single-chain variable fragment (scFv) antibody against AMOZ derivative was produced by our laboratory and used to establish CLEIA method for detection of AMOZ[20].

In this study, a CELIA method for detection of AMOZ with higher sensitivity was established based on our previous research works. To eliminate the effect of derivatization rate on the accuracy of the analysis results, two different methods had been used to establish the standard curve. The results indicated that the established icCLEIA method was quite feasible to rapidly detect residual AMOZ in fish samples with an excellent accuracy and high sensitivity, high selectivity and good stability.

2 Experimental

2.1 Instruments and reagents

MK3 multifunctional microplate reader (Thermo Labsystems, USA); 96-well chemiluminescence microplate (Shenzhen Jincan, China); Microplate thermostat shaker (Hefei Aibensen, China); UV-160A spectrophotometer (Shimadzu, Japan); 6K-15 high-speed refrigerated centrifuge (Sartorius, Germany); MODEL1575 microplate washer (Bio-Rad, USA); DK-8D water bath (Shanghai Medical, China); WH-1 miniature vortex commingler and 90-2 timing thermostatic magnetic agitator (Shanghai Huxi, China); EYELA rotary evaporator (Shanghai Ailang, China).

Furaltadone, AMOZ, 5-morpholinomethyl-3-(2-nitrobenzylidenamino)-2-oxazolidone (NPAMOZ) and other nitrofuran antibiotics and their metabolites standard samples were purchased from Sigma (USA). Anti-AMOZ derivative polyclonal antibody was prepared and identified in our laboratory ((2-(3-((5-(morpholinomethyl)-2-oxooxazolidin-3-ylimino) methyl) phenoxy) acetic acid) conjugated to bovine serum albumin (BSA) (CEPAMOZ-BSA) as immunogen, 2-(5-(morpholinomethyl)-2-oxooxazolidin-3-ylimino) acetic conjugated to oval albumin (OVA) (AMOZA-OVA) as coating antigen). Horse radish peroxidase (HRP) labeled goat-anti-rabbit IgG (Boster, China). Luminol chemiluminescence substrate liquid (Zhuhai Lizhu, China). The coating buffer, washing buffer and sample diluent buffer were prepared according to reference [21]. Other reagents were analytical pure grade or chromatographically pure reagent. Grass Carp samples were bought from local agricultural trade market.

2.2 Experiment methods

2.2.1 Indirect competitive chemiluminescence enzyme immonoassay (icCLEIA) method

Firstly, coating antigen stock solution was diluted to 10 μg mL^{-1} with carbonate buffer and was added to a 96-well microplate at 100 μL per well. After incubation overnight at 37 °C, the plate was washed twice with PBST washing buffer, and then blocked with blocking agent (120 μL per well) at 37 °C for 3 h. Then the liquid was discarded and the plate was dried at 37°C for 1 h. NPAMOZ standards were diluted with PBS dilution buffer to final concentrations of 0, 0.001, 0.01, 0.1, 1, 10, 100 and 1000 ng mL^{-1}, respectively and the polyclonal antibody was diluted 60000 times with PBS dilution buffer. Then 50 μL of the NPAMOZ standard solution and diluted polyclonal antibody solution were added to each well. After incubated for 50 min in a water bath at 37 °C, the plate was washed five times with PBST, and 5000-fold diluted HRP-conjugated goat-anti-rabbit IgG was added at 100 μL per well and incubated for 1 h at 37 °C. After washing five times, the equal-volume mixed chemilumine-scence substrate solution was added to the wells and oscillated. Then relative light units (RLU) of each well were tested instantly by MK3 multifunctional microplate reader.

2.2.2 Optimization of physical and chemical factors in icCLEIA

In icCLEIA, physical and chemical factors, such as coating antigen concentration, antibody dilution, working buffer, ion concentration of working buffer, immunoreaction time, were optimized to improve the sensitivity. Usually, RLU_{max}/IC_{50} (concentration of 50% inhibition of antibody binding) values were used to evaluate the icCLEIA sensitivity, where the higher ratios of RLU_{max}/IC_{50} indicate higher sensitivity.

2.2.3 Specificity test

Specificity of the assay was estimated by measuring percent cross-reactivity (CR, %). Cross-reactivity was the percentage