Original Article

Development of An Enzyme-Linked Immunosorbent Assay for Determination of the Furaltadone Etabolite, 3-Amino-5-Morpholinomethyl-2-Oxazolidinone (AMOZ) in Animal Tissues*

LUO Peng Jie[1], JIANG Wen Xiao[2], BEIER Ross C.[3], SHEN Jian Zhong[2], JIANG Hai Yang[2], MIAO Hong[1], ZHAO Yun Feng[1], CHEN Xia[4,#], and WU Yong Ning[1,#]

1. Ministry of Health Key Laboratory, China National Center for Food Safety Risk Assessment,, Beijing 100021, China; 2. Department of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, China Agricultural University, Beijing 100193, China; 3. Southern Plains Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, College Station, Texas 77845-4988, USA; 4. State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China

Abstract

Objective To determine 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) residues released from protein bound AMOZ in animal tissues.

Methods Polyclonal and monoclonal antibodies were produced in this study. A rapid, sensitive, and specific competitive direct enzyme-linked immunosorbent assay (cdELISA) was developed.

Results Rabbit polyclonal antibodies were used in the optimized cdELISA method, and exhibited negligible cross-reactivity with other compounds structurally related to AMOZ. The IC_{50} of the polyclonal antibody was 0.16 ng/mL. The method limit of detection in four different types of animal and fish tissues was less than 0.06 μg/kg. Recoveries ranged from 80% to 120% for fortified samples with the coefficient of variation values less than 15%. The results of the cdELISA method were in good agreement with the results from an established liquid chromatography-tandem mass spectrometry confirmatory method used for AMOZ residues.

Conclusion The cdELISA method developed in the present study is a convenient practical tool for screening large numbers of animal and fish tissue samples for the the detection of released protein bound AMOZ residues.

Key words: AMOZ; Animal tissue; ELISA; Fish tissue; Furaltadone

Biomed Environ Sci, 2012; 25(4):449-457 doi: 10.3967/0895-3988.2012.04.010 ISSN:0895-3988

www.besjournal.com(full text) CN: 11-2816/Q

*This work was supported by the National Science Foundation for Young Scientists of China (No. 21107104), the State Key Program of National Natural Science of China (No. 20837003), grants from the Ministry of Health (No. 200902009), and the National Science & Technology Pillar Program (No. 2009BADB9B03-Z02).

#Correspondence should be addressed to CHEN Xia and WU Yong Ning. Tel: 86-10-58900769. E-mail: chenxia602@163.com (XC). Tel/fax: 86-10-67776790. E-mail: china_cdc@yahoo.cn (YW)

Biographical note of the first author: LUO Peng Jie and JIANG Wen Xiao contributed equally to this work. LUO Peng Jie, male, born in 1980, ph.D, majoring in food safety. JIANG Wen Xiao, male, born in 1987, Ph.D candidate, majoring in determining of veterinary drug residues.

Received: December 29, 2011; Accepted: February 28, 2012

INTRODUCTION

Furaltadone is an antibacterial drug that belongs to the class of nitrofurans. The nitrofurans include furaltadone, furazolidone, nitrofurantoin and nitrofurazone, and are a group of synthetic broad-spectrum antibacterial drugs which have been widely used as feed additives and growth promoters in animal production. The nitrofurans are rapidly metabolized *in vivo* within a few hours after treatment. Their tissue-bound toxic metabolites are stable and detectable for a long period of time[1-2].

The metabolite of furaltadone is 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), which is toxic. The chemical structures of furaltadone, its metabolite and other derivatives are shown in Figure 1. Protein-bound AMOZ is used as the characteristic marker residue for the banned furaltadone. In long-term experimental animal studies, the nitrofurans and their metabolites demonstrated carcinogenic and mutagenic characteristics[3-4]. Because nitrofuran metabolite residues are a potential risk for human health and safety, nitrofurans have been completely banned from use in food animal production in the European Union (EU) since 1995[5-6]. The EU set the minimum residue performance limit (MRPL) to be 1 μg/kg for each nitrofuran metabolite in edible tissues. Additionally, nitrofurans are also strictly prohibited from use in animal husbandry in the USA[7] and China[8].

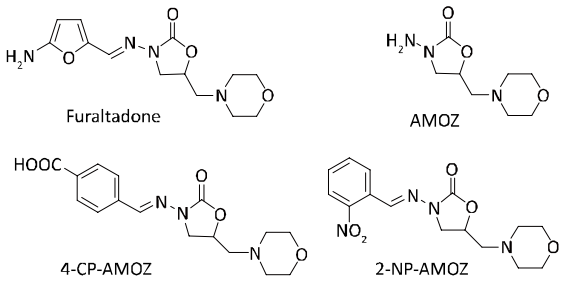

Figure 1. Chemical structures of furaltadone, AMOZ, 4-CP-AMOZ, and 2-NP-AMOZ.

The use of veterinary drugs in animals and fish can help provide high-quality food products, but incorrect application can result in harmful residues. Since furaltadone has excellent antibacterial and pharmacokinetic properties, it can prevent and treat gastrointestinal infections caused by *Escherichia coli* and *Salmonella* spp. However, due to its toxic metabolite residues, it is illegal to use furaltadone as a therapeutic or prophylactic medicine in food-producing animal production in many countries[1-2]. A global nitrofuran crisis surfaced during 2002-2003 when nitrofuran metabolites were reported in pork meat, poultry meat, and fish from different countries over the world[9]. Trade restrictions arising from these findings prompted improvement of related analytical techniques. Currently, there is an increased interest in investigating and applying rapid screening methods for the detection of nitrofuran metabolite residues in animal tissues.

During monitoring studies, most methods have focused on detection of the nitrofuran metabolites, except for analysis of retina tissue, where the parent compounds are used[9]. Various analytical methods such as liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) have been used to analyze nitrofuran metabolites[10-11]. These methods are based on derivatization with 2-nitrobenzaldehyde (2-NBA) followed by determination with LC–MS or LC–MS/MS. Released protein bound AMOZ is derivatized with 2-NBA to form 5-(4-morpholinylmethyl)-3-[[(2-nitrophenyl)methylene]amino]-2-oxazolidinone (2-NP-AMOZ)[11], which serves to protect the released side-chain from protein-bound AMOZ and also permits UV detection during chromatographic analysis[12]. However, these instrumental methods are time-consuming and expensive. It is necessary to develop a rapid, low-cost, high-capacity and sensitive screening method for detection of AMOZ residues.

Immunoassays can provide a simple, rapid, sensitive, and inexpensive screening alternative for detection of veterinary drug residues. Some enzyme-linked immunosorbent assay (ELISA) methods have been reported for detection of the furazolidone metabolite 3-amino-2-oxazolidinone (AOZ)[13], the semicarbazide (SEM) nitrofurazone metabolite in food[14], AMOZ in shrimp (Pimpitak et al. 2009), and the nitrofurantoin metabolite 1-amino-hydantoin (AHD) in animal tissues[15]. In previous studies[12-13,16] and other commercial ELISA kits for detection of AMOZ, the sample preparation is not effective for rapid and high-throughput screening of large numbers of samples.

In this study, a monoclonal antibody (MAb) and polyclonal antibodies (PAbs) were generated, and a rapid, sensitive and specific competitive direct ELISA (cdELISA) method was developed for detection of released protein bound AMOZ residues from animal

and fish tissues. The developed cdELISA method was compared with a previously developed LC-MS/MS confirmatory method[11], and the results from the cdELISA method were consistent with the results from the LC-MS/MS method. Thus, the cdELISA method developed here is a convenient and inexpensive method for rapid screening of AMOZ residues prior to confirmation and quantification by instrumental analyses.

MATERIALS AND METHODS

Reagents

All reagents used in this study were of analytical grade or better unless otherwise indicated. Bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCL), Freund's complete adjuvant, Freund's incomplete adjuvant, HAT media supplement (hypoxanthine, aminopterin and thymidine, Sigma H-0262), HT media supplement (hypoxanthine and thymidine, Sigma H-0137), *N*-hydroxysuccinamide (NHS), ovalbumin (OVA), 3,3',5,5'-tetramethylbenzidine (TMB), and urea hydrogen peroxide were obtained from Sigma-Aldrich (St. Louis, MO, USA). 3-Carboxybenzaldehyde (3-CBA), 4-carboxybenzaldehyde (4-CBA), and 2-nitrobenzaldehyde (2-NBA) were obtained from Acros Organics (Worcester, MA, USA). Furaltadone, 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), and 2-NP-AMOZ were obtained from Dr. Ehrenstorfer (Augsburg, Germany). *N, N*-Dimethylformamide, dimethyl sulfoxide, Tween® 20, ethyl acetate and methanol were obtained from Sinopharm Chemical Reagent Co. Ltd (Beijing, China). HRP-IgG was obtained from Jackson Immunoresearch (West Grove, PA, USA).

Instruments

The Multiscan MK3 microplate absorbance reader was obtained from Thermo Inc. (Shanghai, China). Plastic wares, including 96-well microtiter plates, 12- and 96-well cell culture plates, were obtained from Costar Inc. (Cambridge, MA, USA). The N-EVAP nitrogen evaporator was purchased from Organomation Associates, Inc. (Berlin, MA, USA). The UPLC system coupled to a Micromass Quattro Premier XE triple quadrupole mass spectrometer was obtained from Waters (Manchester, UK).

Buffers and Solutions

The coating buffer (0.05 mol/L carbonate buffer, pH 9.6) was used to coat the 96-well plates with coating antigen. Blocking buffer was 1% casein in phosphate buffered saline (PBS, 0.01 mol/L, pH 7.4). PBST was comprised of PBS with 0.05% Tween® 20 (v/v) and was used for washing the 96-well plates. PBS with 5% fetal bovine serum was used for dilution of HRP-IgG. Substrate buffer was 0.1 mol/L citrate buffer (pH 5.5). The HRP substrate solution was prepared by adding 1 mL of substrate buffer, 200 μL of a 1% solution of TMB in dimethyl sulphoxide (v/v) and 200 μL of 2% urea hydrogen peroxide in 20 ml deionized water. Stop reagent was 2 mol/L H_2SO_4.

Synthesis of 4-CP-AMOZ, 4-CP-AMOZ Conjugates and 4-CP-AMOZ-HRP

Brief overviews of the synthetic routes to the derivatives and conjugates are shown in Figure 2. The AMOZ carboxybenzaldehyde derivative, 5-(4-carbobenzylidene)-3-[(2-nitrophenyl)methylenamino]-2-oxazolidinone (4-CP-AMOZ), was synthesized by reacting 4-CBA with AMOZ via a condensation reaction. A mixture of 34 mg of AMOZ and 15.6 mg of 4-CBA were dissolved in 2 mL of methanol and the mixture was stirred at room temperature for 2 h. Thin-layer chromatography was used to monitor the reaction progress and to remove the un-reacted 4-CBA[15,18].

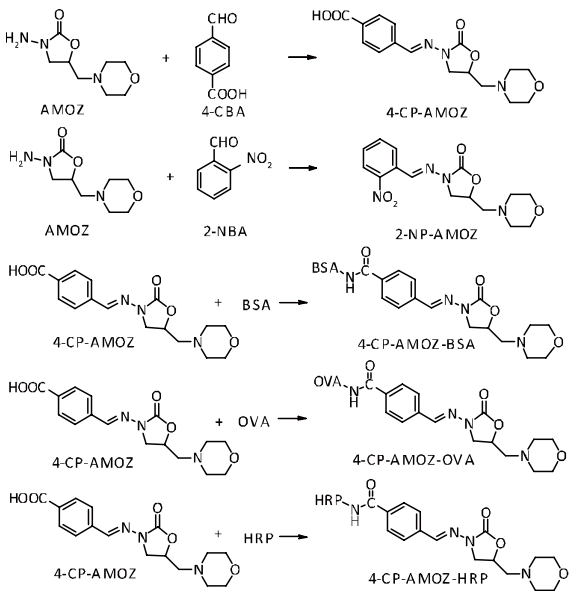

Figure 2. Synthetic routes to the derivatives and conjugates. 4-CP-AMOZ was used to synthesize the immunogen and coating antigen, while 2-NP-AMOZ was the target sample derivative.

A mixture of 4-CP-AMOZ (33.4 mg), NHS (10.6 mg) and EDC (19.2 mg) was successively added to 1.5 mL of PBS and stirred at 25 °C for 6 h. The mixture was then slowly added to 80 mg of BSA dissolved in 5 mL of PBS, and this mixture was gently stirred for 12 h at 4 °C to form 4-CP-AMOZ-BSA. The solution was dialyzed against PBS (0.01 mol/L, pH 7.4) for 5 days. The 4-CP-AMOZ-BSA derivative was used as the immunogen[13]. The 4-CP-AMOZ-OVA derivative was synthesized by the same procedure, and was used as the coating antigen.

4-CP-AMOZ (33.4 mg), EDC (19.2 mg), and NHS (10.6 mg) were successively added to 1 mL of PBS. The mixture was gently stirred for 16 h at room temperature and was added dropwise to 34 mg of HRP. The reaction mixture was stirred at room temperature for 5 h and the HRP-conjugate (tracer) was purified on Sephadex G-25[18], diluted with glycerol and stored at -20 °C until needed. The immunogen, coating antigen and HRP-conjugate were identified by using the competitive indirect ELISA.

Immunizations and Production of Antibodies

Fifteen rabbits were immunized with 4-CP-AMOZ-BSA at a dosage of 10 mg/kg body weight in an equal volume of Freund's complete adjuvant. Subsequent booster immunizations were administered biweekly with the same dosage of immunogen emulsified with Freund's incomplete adjuvant. Blood was obtained from the marginal ear vein one week after each booster immunization to monitor the immune response. The titer of the antibody was determined by cdELISA. After ten booster immunizations, the rabbits were exsanguinated, the serum was isolated, and the PAbs were purified[19].

Fifteen 6-week old Balb/c mice were immunized with 4-CP-AMOZ-BSA at a dosage of 10 mg/kg body weight in an equal volume of Freund's complete adjuvant. Booster immunizations were administered to the mice every two-weeks at the same immunogen dosage emulsified in Freund's incomplete adjuvant. Tail bleeding was carried out to determine the titer of the antibody. Mice exhibiting the highest antibody titers were sacrificed after the last immunization, the spleens were removed, and the splenocytes were fused with SP2/0 myeloma cells for hybridoma production[19]. After fusion, the cells were selected for fused cells by using HAT medium. Following selection, HT medium was used to replace the HAT medium. The growing hybridoma cells were screened for antibody production by a non-competitive indirect ELISA. The hybridomas producing antibodies positive for AMOZ were subcloned by the limiting dilution method, and the positive clones were used for antibody production. The supernatant of the positive hybridomas were collected and the monoclonal antibodies (MAbs) were purified by ammonium sulphate precipitation and DEAE ion-exchanged chromatography[20].

ELISA Optimization

Competition experiments were carried out by the checkerboard method to select suitable concentrations of immunoreagents. The coating antigen, antibody concentrations, and incubation times were optimized. Both the competitive indirect ELISA (ciELISA) and the cdELISA methods were optimized for comparison of the two methods to determine the better method.

ciELISA

The ciELISA was carried out by using the following procedure: microtiter plates were coated with 100 μL/well of a 1:6000 dilution of 4-CP-AMOZ-OVA (3.2 mg/mL) in coating buffer for 2 h at room temperature, and then, the plates were washed with PBST and blocked with blocking buffer for 2 h at room temperature. After the blocking solution was removed, 50 μL/well of the standard or sample extracts and 50 μL/well of a 1:5000 dilution of the MAb (2.7 mg/mL) were successively added to the plates and incubated at 25 °C for 30 min. Following washing with PBST, 100 μL/well of a 1:5 000 dilution of IgG-HRP was added and the plates were incubated at 25 °C for 30 min. After washing with PBST, 100 μL/well of substrate solution was added and the plates were incubated at room temperature for another 15 min. The reaction was stopped by adding 2 mol/L H_2SO_4, and the absorbance was read on the microplate absorbance reader at 450 nm and 630 nm.

cdELISA

The cdELISA was carried out by using the following procedure: microtitre plates were coated with 100 μL/well of a 1:15000 dilution of the PAb (6.6 mg/mL) for 2 h at room temperature, and then, the plates were washed with PBST and blocked with 200 μL/well blocking buffer for 2 h at room temperature. After that, 50 μL/well of the standard or sample solutions and 50 μL/well of a 1:3000 dilution of 4-CP-AMOZ-HRP (tracer, 5.2 mg/mL) were added to each well and the plates were incubated at

25 °C for 30 min. After washing with PBST, 100 μL of substrate solution was added and the plates were incubated at room temperature for another 15 min. The reaction was stopped with 2 mol/L H_2SO_4 and the absorbance was read at 450 nm and 630 nm.

Sample Preparation

Animal tissues were finely chopped and 1.0 g samples were weighed into 50 mL centrifuge tubes. Deionized water (4 mL), 1 mol/L HCl (1 mL) and 10 mmol/L 2-NBA in DMF (400 μL) were successively added, and the sample mixtures were incubated for 3 h at 50 °C. This step accomplishes the release of protein bound AMOZ residues and then derivatizes them. After cooling to room temperature, 5 mL of 0.1 mol/L dibasic potassium phosphate containing 1 g of sodium chloride was added for neutralization. After adjusting the pH to 7.4, ethyl acetate (6 mL) was added to each sample, and the mixtures were vortexed for 1 min. After centrifugation at 4000×g for 10 min, 3 mL of the ethyl acetate supernatant from each sample was evaporated to dryness under nitrogen in a heating block at 50 °C, and then reconstituted with PBS (1 mL). Each reconstituted sample was extracted with 2 mL of hexane; then, the hexane supernatant was discarded; and finally the remaining extracts were analyzed by an ELISA.

Assay Validation

All blank samples were shown to be free of AMOZ and other nitrofuran metabolites using LC–MS/MS. The ELISA limit of detection (LOD) was based on the mean value of 20 blank samples plus 3 times of the mean standard deviation. The accuracy and precision of the method were defined by the mean recovery and coefficient of variation (CV), respectively. The percent of recovery was calculated according to the following equation:

$$\text{Recovery (\%)} = (\text{concentration measured})/(\text{concentration fortified}) \times 100 \quad (1)$$

The sensitivity of the antibody was determined by using the concentration of competitor that inhibited the binding of the antibody by 50% (IC_{50}). The background absorbance of the wells containing all components except the competitor is referred to as B_0. The absorbance of the samples and standard was normalized against the absorbance of the background (B/B_0). The concentration at the midpoint of the standard curve is the IC_{50} value. Competition curves were fitted to a four parameter logistic equation, from which IC_{50} values were calculated[22].

$$Y=(A-B)/[1+(X/X_0)^p]+B \quad (2)$$

Where: A=response at high asymptotes of the curve, B=response at low asymptotes of the curve, P=the slope at the inflection point of the sigmoid, X_0=the concentration of 2-NP-AMOZ resulting in 50% inhibition of tracer binding, and X=calibration concentration.

To determine the selectivity of the method, a cross-reactivity (CR) study was carried out under optimum conditions. The percent CR of the antibody with 2-NP-AMOZ and other potential cross-reactants such as the nitrofurans, their metabolites, nitrophenyl derivates and other antimicrobials, were calculated with the following equation:

$$CR(\%) = (IC_{50} \text{ of 2-NP-AMOZ})/(IC_{50} \text{ of other analytes}) \times 100 \quad (3)$$

Comparison of the cdELISA and the LC–MS/MS Method

The cdELISA method was validated by using a previously established LC-MS/MS confirmatory method[11]. Five naturally contaminated fish samples were subjected to cdELISA analysis with confirmatory analysis by LC-MS/MS. All details concerning the LC-MS/MS method can be obtained from XIA et al. The naturally contaminated samples were analyzed by cdELISA in quadruplicate. To correlate the two methods, a one-dimensional linear regression equation between the results of the cdELISA method and the LC-MS/MS method was calculated. Further, the results of the two methods were analyzed by a paired sample T test, and the *P* values were calculated to assess the significance of the difference between the two methods. There is no significant difference between the two methods if $P>0.05$.

RESULTS

Preparation of Immunogen, Coating Antigen, and the 4-CP-AMOZ-HRP Tracer

The specificity for the crosslinking agent can be reduced by using a zero-length crosslinker in the conjugation procedure[21]. Thus the key step of designing an immunogen can be used to enhance the resulting immunoassay. The synthetic route used to produce all conjugates was identical. The synthesis was based on the free carboxylic acid group on the modified 4-CP-AMOZ, which readily reacted with amine-containing proteins to form amide bonds. Since AMOZ must be derivatized to

protect the amine (Figure 1) following release from tissue proteins[11], XIA et al. used 3-CBA to protect the amino group of AMOZ and create a desirable hapten mimicking the 2-NP-AMOZ derivative, which was a key step in antibody production[13,21].

Antibody Production and Characterization of the ELISA Methods

The best working dilutions of the coating antigen, MAb and PAb were 1:6000, 1:5000, and 1:15000, respectively. The amount of IgG-HRP used was the recommended dilution of 1:5000. In order to assess antibody sensitivity, the binding and competitive properties of the antibodies were examined by using different ELISA formats under the optimal conditions. The specificity of the two types of antibodies was compared by using different formats and the results are shown in Figure 3. The sensitivity of the antibodies in different ELISA formats is listed in Table 1. When PAb was used in the cdELISA format, IC_{50} had the best value (0.16 ng/mL). From the results of the optimization in Table 1 and Figure 3, the cdELISA method using PAb was chosen to be used in all of the following experiments.

Table 1. Assay Sensitivity of the Antibodies in the Optimized ELISA Method

ELISA Method	Antibody	Sensitivity (IC_{50}, ng/mL)
cdELISA	Polyclonal antibody	0.16
	Monoclonal antibody	0.75
ciELISA	Polyclonal antibody	0.45
	Monoclonal antibody	0.62

2-NBA was used to derivatize the AMOZ residues to protect them after being released from tissue proteins. Standard solutions of 2-NP-AMOZ were made by dilution in PBS to produce the concentration range of 0, 0.05, 0.15, 0.45, 1.8, and 5.4 ng/mL AMOZ. A typical cdELISA standard curve for AMOZ is illustrated in Figure 4.

To determine the selectivity of the cdELISA method, the IC50 and CR values of PAb against 2-NP-AMOZ and other related compounds were obtained and are listed in Table 2. The CR of the PAb against 2-NP-AMOZ was assigned the value of 100%. PAb showed negligible CR (<0.1%) with other related compounds except for AMOZ, 4-CBA and 2-NBA, as expected, since they were part of the hapten or resembled portions of the hapten.

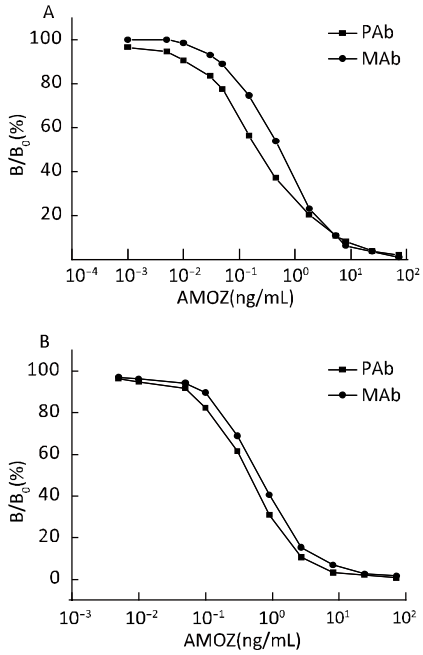

Figure 3. The specificity of the MAb and PAb to 2-NP-AMOZ. Comparative detection of the MAb and PAb by the cdELISA method (A) and ciELISA method (B). The optical density of the wells (B_0), containing all components except the competitor. The absorbance of the standard was normalized against the absorbance of the background (B/B_0).

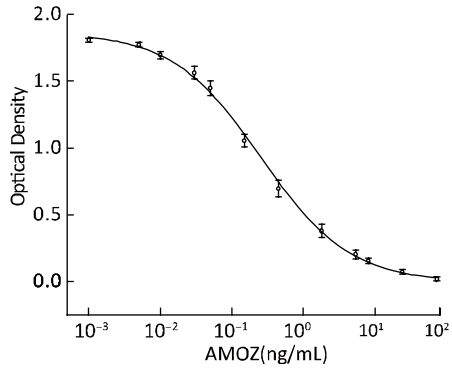

Figure 4. Typical standard curve for the polyclonal antibody. Each point represents the mean of 4 replicates, and the vertical bars indicate the standard deviation of the mean.

Sample Preparation and Assay Validation

Blank tissue samples gave a weak positive signal due to a matrix interference. However, the LOD of the method in fish, shrimp, chicken and pork samples

Table 2. IC_{50} and CR Values of the Polyclonal Antibody with 2-NP-AMOZ and Other Structurally Related Compounds

Compounds	IC_{50}(ng/mL)	CR (%)
2-NP-AMOZ[a]	0.16	100
NPAOZ, NPSEM, NPAHD[a]	>1000	<0.1
AMOZ[b]	12.6	1.27
AOZ, SEM, AHD[b]	>1000	<0.1
Nitrofurantoin, Nitrofurazon, Furazolidone, Furaltadone	>1000	<0.1
4-CBA	45.5	0.35
2-NBA	67.3	0.24
Other antibiotics	>1000	<0.1

Note. [a]In case of the other nitrophenyl derivatives of the nitrofuran metabolites, concentrations were calculated on the basis of underivatized metabolites. [b]The IC_{50} of the nitrofuran metabolites was measured to indicate that the monoclonal antibody was highly specific for the nitrophenyl derivative 2-NP-AMOZ.

was 0.046, 0.016, 0.040, and 0.051 μg/kg, respectively. Therefore, all the LODs were less than 0.06 μg/kg, which is below the MRPL for AMOZ residues set by the EU.

The target concentration was 0.5 μg/kg for AMOZ in edible tissues. The mean recovery values ranged from 80% to 120% for fortified samples at levels of 0.1-4 μg/kg with most CV values being less than 15%, which were within an acceptable range. The mean recoveries and CV values are shown in Table 3.

The results of this screening method are consistently good, and in contrast to the previously reported method[16], the LOD values are lower, and the time required is shorter. The derivatization time for the instrumental method[16] is 16 h, and solid-phase extraction columns and 0.22 μm nylon filters must be used during the cleanup procedure. With respect to these requirements, our developed method is simple and rapid with a derivatization time of only 3 h, and may permit a single person to process and analyze 40 samples within one day in any routine laboratory without the requirement of special facilities.

Table 3. Recoveries of AMOZ from Different Kinds of Samples Using Optimized ELISA Methods (*n*=4)

Sample	Concentration Fortified (μg/kg)	Intra-assay		Inter-assay	
		Recovery(%)	CV(%)	Recovery(%)	CV(%)
Fish	0.1	85.7	3.4	92.3	9.8
	0.2	91.0	2.2	102	8.4
	1.0	107.5	6.7	94.8	10.2
	4.0	111.5	4.4	103.2	11.4
Shrimp	0.1	109.1	3.4	106.4	10.9
	0.2	82.5	2.7	97.6	11.5
	1.0	93.0	5.3	105.8	13.8
	3.0	109.6	6.2	96.5	12.7
Pork	0.1	103.2	6.4	82.9	14.3
	0.2	105.0	7.2	90.7	11.1
	3.0	95.0	5.8	106.8	9.2
Chicken	0.1	95.5	3.4	87.6	8.9
	0.2	80.1	2.8	90.4	11.6
	3.0	84.5	4.5	107.9	13.2

Comparison of the cdELISA and LC–MS/MS Methods

Naturally contaminated samples were analyzed for AMOZ residues using both the cdELISA screening method and the LC-MS/MS confirmatory method. An excellent correlation between the two methods analyzing fish tissues (r=0.99, n=12) was seen in a graphical correlation, as shown in Figure 5. The results of the two methods were compared by using the paired sample t test and the P values were greater than 0.05. Thus, there was no significant difference between the two methods. The overall results indicate that the cdELISA method is suitable for detection of AMOZ residues in animal and fish tissues.

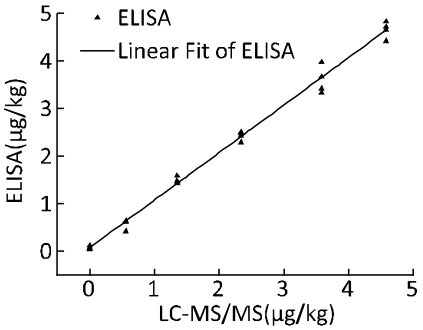

Figure 5. Correlation of the results for analysis of AMOZ in fish tissues using cdELISA and LC–MS/MS methods. The regression equation for the cdELISA is y=0.9983x+0.08271, r=0.99.

DISCUSSION

In this study, 4-CBA was used in the synthesis of the hapten after evaluating the use of both 3-CBA and 4-CBA. Due to the small size of 4-CP-AMOZ, it had to be coupled to a carrier protein in order to elicit an immune response. In the hapten derivative 4-CP-AMOZ, the AMOZ moiety was situated opposite from the C-4 carboxylic group used to bind the carrier protein, which allowed the 4-CP-AMOZ hapten to be extended away from the carrier protein, so the AMOZ portion of the hapten was adequately presented to the immune system. Thus, the 4-CP-AMOZ hapten was expected to evoke an antigenic response that would produce antibodies specific for 2-NP-AMOZ. Furthermore, since the 4-CP-AMOZ molecule contained a carboxylic acid group, it could readily be coupled to carrier proteins through an amide linkage.

In this study, efforts were made to simplify the sample preparation procedures. Tissue homogenization was not required when a higher concentration of acid was used during the acid hydrolysis step, because 0.2 mol/L HCl could rapidly separate AMOZ from tissue proteins and also simultaneously precipitate the sample protein. The time required for the derivatization procedure was then further reduced to 3 h by using a higher-incubation temperature. The standard curve was made by using 2-NP-AMOZ diluted in PBS, rather than a matrix matched with the calibration standard. And most importantly of all, the extraction of 2-NP-AMOZ was performed by using liquid-liquid extraction with ethyl acetate instead of using expensive solid-phase extraction columns. In previous studies[12-16], solid-phase extraction columns were widely used in cleanup and enrichment procedures, which are not cost effective for rapid, high-throughput screening of large numbers of samples. In contrast, liquid-liquid extraction was simple, rapid and can be carried out in any routine laboratory. After optimization of these procedures, a single person can easily process and analyze 40 samples within one day.

In general, the results of this study indicate that the developed cdELISA method is a practical tool for screening a large number of tissue samples for protein bound AMOZ residues prior to confirmation by LC-MS/MS. The cdELISA method developed in this study is rapid, simple and convenient to be used in routine screening analysis, especially for the simultaneous analysis of a large number of samples. The developed cdELISA method for released protein bound AMOZ residues would make an important contribution toward ensuring food safety.

REFERENCES

1. Vroomen LHM, Berghmans MCJ, van Leeuwen P, et al. Kinetics of ^{14}C-furazolidone in piglets upon oral-administration during 10 days and its interaction with tissue macro-molecules. Food Addit Contam, 1986; 3(4), 331-46.
2. McCracken RJ, Blanchflower WJ, Rowan C, et al. Determination of furazolidone in porcine tissue using thermospray liquid chromatography-mass spectrometry and a study of the pharmacokinetics and stability of its residues. Analyst, 1995; 120(9), 2347-51.
3. Jung R, Le JY, Wengenmayer F, et al. Mutagenicity studies of a carcinogenic nitrofuran and some analogues. Biomed Biochim Acta, 1985; 44(3), 485-92.
4. Westphal JF, Vetter D, Brogard JM. Hepatic side-effects of antibiotics. J Antimicrob Chemother, 1994; 33(3), 387-401.
5. EC (European Commission Regulation 1442/95/EC). Commission Regulation Amending Annexes I, II, III and IV of Council Regulation (EEC) No 2377/90 Laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Off. J. Eur. Commun. 1995; No. L143/26.
6. EC (European Commission Decision 2003/181/EC). Commission Decision of 13 March 2003: amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. Off. J. Eur. Commun. 2003; No. L71/17.
7. FR (Federal Register). Topical nitrofurans; Extralabel animal drug use; Order of prohibition. Fed Reg, 2002; 67, 5470-1.
8. CDA (China Department of Agriculture). The banned list of veterinary medicine and other compounds for food-producing animals. Regulation of Department of Agriculture of China. 2002; No. 193.
9. Cooper KM, Kennedy DG. Nitrofuran antibiotic metabolites detected at parts per million concentrations in retina of pigs-a new matrix for enhanced monitoring of nitrofuran abuse. Analyst, 2005; 130(4), 466-8.
10. Leitner A, Zöllner P, Lindner W. Determination of the

metabolites of nitrofuran antibiotics in animal tissue by high-performance liquid chromatography–tandem mass spectrometry. J Chromatogr A, 2001; 939(1-2), 49-58.
11. Xia X, Li X, Zhang S, et al. Simultaneous determination of 5-nitroimidazoles and nitrofurans in pork by high-performance liquid chromatography–tandem mass spectrometry. J Chromatogr A, 2008; 1208(1-2), 101-8.
12. Horne E, Cadogan A, O'Keeffe M, et al. Analysis of protein-bound metabolites of furazolidone and furaltadone in pig liver by high-performance liquid chromatography and liquid chromatography-mass spectrometry. Analyst, 1996; 121(10), 1463-8.
13. Cooper KM, Caddell A, Elliott CT, et al. Production and characterisation of polyclonal antibodies to a derivative of 3-amino-2-oxazolidinone, a metabolite of the nitrofuran furazolidone. Anal Chim Acta, 2004; 520(1-2), 79-86.
14. Cooper KM, Samsonova JV, Plumpton L, et al. Enzyme immunoassay for semicarbazide-The nitrofuran metabolite and food contaminant. Anal Chim Acta, 2007; 592(1), 64-71.
15. Jiang W, Luo P, Wang X, et al. Development of an enzyme-linked immunosorbent assay for the detection of nitrofurantoin metabolite, 1-amino-hydantoin, in animal tissues. Food Control, 2012; 23(1), 20-5.
16. Pimpitak U, Putong S, Komolpis K, et al. Development of a monoclonal antibody-based enzyme-linked immunosorbent assay for detection of the furaltadone metabolite, AMOZ, in fortified shrimp samples. Food Chem, 2009; 116(3), 785-91.
17. Wang Z, Zhang S, Nesterenko IS, et al. Monoclonal antibody-based fluorescence polarization immunoassay for sulfamethoxypyridazine and sulfachloropyridazine. J Agric Food Chem, 2007; 55(17), 6871-8.
18. Luo P, Jiang H, Wang Z, et al. Simultaneous determination of florfenicol and its metabolite florfenicol amine in swine muscle tissue by a heterologous enzyme-linked immunosorbent assay. J AOAC Int, 2009; 92(3), 981-8.
19. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 1975; 256, 495-7.
20. Shepherd P, Dean C. Monoclonal antibodies: A practical approach. Oxford University Press: New York, 2000; pp 151-66.
21. Hermanson GT. Bioconjugate techniques, 2nd edn. Academic Press, San Diego, CA, USA, 2008; pp 215-33.
22. Nix B, Wild D. The immunoassay handbook, 3rd edn., Wild, D. (Ed.), New York: Nature Pub Group, 2005; pp 233-45.