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Development of a Monoclonal Antibody-Based Competitive Indirect Enzyme-Linked Immunosorbent Assay for Furaltadone Metabolite AMOZ in Fish and Shrimp Samples

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Supporting Information

ABSTRACT: A monoclonal antibody-based competitive indirect enzyme-linked immunosorbent assay (ELISA) with improved sensitivity and specificity for the determination of furaltadone metabolite 5-methylamorpholino-3-amino-2-oxazolidone (AMOZ) was described. AMOZ was derivatized with 2-(3-formylphenoxy)acetic acid and coupled with bovine serum albumin to form a novel immunogen. BABL/c mice were immunized and monoclonal antibody specific to the nitrophenyl derivative of AMOZ (NP-AMOZ) was produced and characterized. Four other haptens with different heterology to the immunizing hapten were synthesized and coupled to ovalbumin as coating antigens to study the effect of heterologous coating on assay sensitivity. Under the optimized heterologous coating format, the competitive indirect ELISA showed very high sensitivity to NP-AMOZ, with an IC_{50} of 0.14 μg/L and limit of detection of 0.01 μg/L. The assay showed high specificity toward NP-AMOZ, and negligible cross-reactivity with analogous compounds was observed. The average recoveries of AMOZ from spiked fish and shrimp samples were estimated to range from 81.0 to 104.0%, with coefficients of variation below 20%. Good correlation was obtained between the results of ELISA analysis and of standard liquid chromatography−tandem mass spectrometry analysis. These results indicated that the proposed ELISA is ideally suited as a monitoring method for AMOZ residues at trace level.

KEYWORDS: *enzyme-linked immunosorbent assay (ELISA), furaltadone metabolite, 5-methylamorpholino-3-amino-2-oxazolidone (AMOZ), fish and shrimp, BABL/c mice*

INTRODUCTION

Furaltadone is a member of the nitrofuran antibiotics, which were commonly used for the treatment of gastrointestinal and dermatological infections including salmonellosis in cattle, swine, poultry, fish, and shrimp.[1] However, it was found by long-term studies with experimental animals that the parent drugs and their metabolites showed carcinogenic and mutagenic characteristics.[2,3] Therefore, their use in food animal production was prohibited by the European Union (EU) in the mid-1990s.[4] Now, the use of nitrofuran antibiotics in food animal production is also banned in many countries such as the United States (2000), Australia (1993), Brazil (2002), China (2002), Philippines (2001), and Thailand (2002). However, nitrofurans are still legally made and distributed for use in many countries of the world, especially in the developing countries, because they are cheap and effective antibiotics as well as growth promoters. For example, aquaculture products imported from Asian countries to the EU were frequently contaminated by nitrofurans antibiotics, according to the notifications published in the Rapid Alert System for Food and Feed (RASFF) Weekly Overviews.[1] In the EU, the maximum residue levels (MRLs) for nitrofurans are set at 1 μg/kg in aquaculture products by Commission Decision 2003/181/EC[5] amending Decision 2002/657/EC.[6] Therefore, analytical methods with high sensitivity are needed to prevent chronic human exposure to low concentrations of nitrofuran residues in food samples.

Monitoring residues of furaltadone in food samples is unsuitable because of its instability in vitro and in vivo.[7] However, its metabolite 5-methylamorpholino-3-amino-2-oxazolidone (AMOZ) could bind to tissue proteins and persist for considerable periods in animal tissues. Therefore, analytical methods for detecting AMOZ were developed instead of detecting furaltadone in food samples.[8−10] Most of the reported methods are instrumental methods, such as liquid chromatography−tandem mass spectrometry (LC-MS/MS).[8−10] However, because of the widespread contaminant of nitrofurans, a growing need remains for low-cost and rapid screening methods that are sensitive toward individual nitrofurans. Enzyme-linked immunosorbent assay (ELISA) based on the use of a specific antibody and peroxidase conjugate is one such method.

Many ELISA methods have been developed for the qualification for nitrofurans metabolite such as 3-amino-2oxazolidinone (AOZ, metabolite of furazolidone),[11−13] semicarbazide (SEM, metabolite of nitrofurazone),[14−16] and 1-aminohydantoin (AHD, metabolite of nitrofurantoin).[17,18] For AMOZ, only one work describing the development of ELISA

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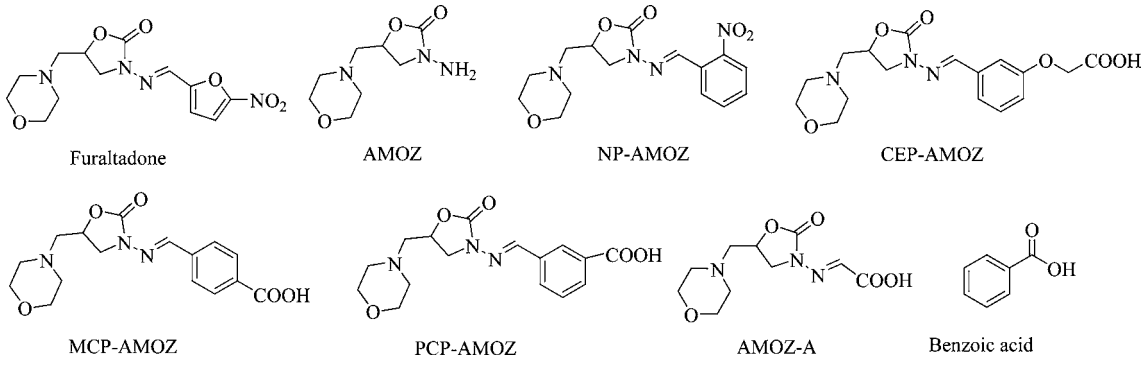

Figure 1. Chemical structures of furaltadone, its marker residue AMOZ, the nitrophenyl derivative NP-AMOZ, the immunizing hapten CEP-AMOZ, and coating haptens MCP-AMOZ, PCP-AMOZ, AMOZ-A, and benzoic acid.

was reported.[19] Because the molecular weight of nitrofuran metabolites was too small to produce specific antibodies, ELISA for nitrofuran metabolites was developed based on antibodies specific to their nitrophenyl derivatives (e.g., NP-AOZ, NP-SEM, NP-AHD, and NP-AMOZ), using 3-carboxybenzaldehyle (3-CBA) or 4-carboxybenzaldehyle (4-CBA) derivatives of nitrofuran metabolites as the immunizing haptens.[14−19]

In this work, AMOZ was derivatized with 2-(3-formylphenoxy)acetic acid to obtain a novel immunizing hapten (CEP-AMOZ) (Figure 1). It was coupled to bovine serum albumin (BSA) for immunogen to produce a monoclonal antibody (mAb) specific to the nitrophenyl derivative of AMOZ (NP-AMOZ). Four other haptens, derivatized AMOZ with 3-CBA, 4-CBA, and 2-oxoacetic acid, respectively, as well as benzoic acid (Figure 1), were synthesized and coupled to ovalbumin (OVA) for coating antigens to study the effect of heterologous coating on the assay sensitivity. A heterologous competitive indirect ELISA specific for NP-AMOZ was finally developed and applied to determine AMOZ residues in fish and shrimp samples.

EXPERIMENTAL PROCEDURES

Materials and Chemicals. Furaltadone, AMOZ, NP-AMOZ, 4-CBA, 3-CBA, 2-(3-formylphenoxy)acetic acid, 2-oxoacetic acid, *o*-nitrobenzaldehyde (*o*-NBA), benzoic acid, BSA, OVA, *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), 3,3′,5,5′-tetramethylbenzidine (TMB), polyethylene glycol (PEG) 2000, culture media RPMI-1640, hypoxanthine−aminopterin−thymidine (HAT) and hypoxanthine−thymidine (HT) medium supplements, pristane, and Freund's complete and incomplete adjuvants were purchased from Sigma-Aldrich (St. Louis, MO). Myeloma cell line SP 2/0 was preserved in our laboratory. Peroxidase-labeled goat antimouse IgG (IgG-HRP) was supplied by Boster Biotech Co., Ltd. (Wuhan, China). *N*,*N*-Dimethylformamide (DMF), dimethyl sulfoxide (DMSO), Tween-20, methanol, and hexane were obtained from Damao Chemical Reagent Co., Ltd. (Tianjin, China). Polystyrene ELISA plates were obtained from Jiete Biotech Co., Ltd. (Guangzhou, China). All other chemicals and organic solvents were of analytical grades.

Instruments. ELISA plates were washed with a Multiskan MK2 microplate washer (Thermo Scientific, Hudson, NH). ELISA values were read with a Multiskan MK3 microplate reader (Thermo Scientific). Electrospray ionization mass spectrometry (ESI-MS) analyses were performed with an Agilent HP1100 series (Agilent, Palo Alto, CA). Ultraviolet−visible (UV−vis) spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan). Nuclear magnetic resonance (NMR) spectra were obtained with both the DRX-400 and DRX-600 NMR spectrometers (Bruker, Germany-Switzerland). LC-MS/MS analysis was carried out by using the 1200 series LC system (Agilent Technologies, United States) equipped with the Agilent 6410 Triple Quad LC-MS System (Agilent Technologies).

Buffers and Solutions. Used were the following buffers: (1) 0.1 mol/L phosphate buffer saline (PBS, pH 7.4) containing 2 mol/L NaOH for sample neutralization; (2) 50 mmol/L carbonate buffer solution (CBS, pH 9.6) for coating; (3) 10 mmol/L PBS containing 0.05% Tween-20 (PBST, pH 7.4) for washing; (4) 0.1 mol/L citrate and sodium phosphate for substrate buffer (pH 5.5); (5) 10 mL of substrate buffer, 150 μL of 1% (w/v) solution of TMB in DMF, and 2.5 μL of 6% (w/v) H_2O_2 mixed for substrate solution; and (6) 2 mol/L H_2SO_4 used for stopping reagent.

Synthesis and Characterization of Haptens. Haptens (CEP-AMOZ, MCP-AMOZ, PCP-AMOZ, and AMOZ-A) were synthesized as follows: 1.0 mmol of AMOZ in 5 mL of methanol was added to a stirring solution of 1.5 mmol of benzaldehyde derivative [2-(4-formylphenoxy)acetic acid, 4-CBA, and 3-CBA, respectively] or 2-oxoacetic acid in 10 mL of methanol. The mixture was left to stand for 3 h at room temperature. Thin-layer chromatography of the reaction mixture demonstrated formation of the target product (elution in 10% methanol in chloroform). The mixture was filtrated and washed with ethanol several times to remove the unreacted reagents. After methanol was removed by evaporation, the synthesized haptens were confirmed by ESI-MS and NMR (see Table S1 in the Supporting Information).

Preparation of Hapten−Protein Conjugates. CEP-AMOZ was coupled to BSA for immunogen, and all five haptens were coupled to OVA for coating antigens by active ester method.[20] Briefly, hapten (10 μmol), NHS ester (20 μmol), and DCC (20 μmol) were dissolved in 500 μL of DMF. The mixture was stirred gently at 4 °C overnight and then centrifuged at 2500*g* for 10 min. The supernatant (400 μL) was added dropwise to BSA (90 mg) or OVA (60 mg) in 9 mL of PBS (pH 7.4). The conjugation mixture was then stirred at 4 °C for 12 h and then purified on Sephadex G-25, using 0.01 mol/L $NaHCO_3$ as the eluent. The eluted conjugates were dialyzed against PBS (pH 7.4) and then freeze-dried before storage at 4 °C. Full wavelength (200−500 nm) UV−vis scanning was used to confirm the structures of the final conjugates, and the ratios of haptens to carrier proteins were determined by trinitro−benzene−sulfonic acid (TNBS) method.[21]

Production of mAb. The production of mAb was carried out as previously described.[22] Briefly, BABL/c female mice (6−8 weeks old, supplied by the Guangdong Medical Laboratory Animal Center) were immunized with immunogen. One week after the last injection, mice were tail-bled, and titers of antisera were determined by indirect ELISA. The mice selected to be spleen donors for hybridoma production received a final intraperitoneal injection of 100 μg of conjugate (without adjuvant). Three days later, the mice were sacrificed for cell fusion. The hybridoma cells were acquired by fusion of the spleen cells isolated from the selected mice with SP2/0 murine myeloma cells as described by Kane and Banks.[23] Eight to ten days after cell fusion, when the hybridoma cells were grown to approximately 30−40% confluent in the well, culture supernatants were collected and screened using an antigen-coated indirect ELISA

for the presence of antihapten antibodies. BSA-coated indirect ELISA was also used to eliminate positive clones with potential cross-reactivity with BSA. Selected hybridomas were cloned by limiting dilution, and stable antibody-producing clones were expanded. Selected clones were used for antibody production by ascites growth. Ascites fluids were collected and purified using a protein-G column and were used in the following ELISA. All experiments involving animals were performed in compliance with the relevant protective and administrant laws for laboratory animals of China and were conducted with the approval of Institutional Authority for Laboratory Animal Care.

ELISA Procedure. In a noncompetitive indirect ELISA, flat-bottom polystyrene ELISA plates were coated with 100 μL of the coating antigen at the optimal dilution in carbonate buffer overnight at 4 °C. After they were washed five times with PBST solution, the excess binding sites were blocked with 5% glycine and 5% sucrose in PBS buffer (200 μL/well) for 3 h at 37 °C. After they were washed five times with PBST solution, the wells were incubated with 100 μL of diluted antibody in PBST for 1 h and washed five times with PBST solution. IgG-HRP diluted 1:10000 in PBST was then added (100 μL/well). After they were incubated for 1 h 37 °C and washed five times with PBST solution, TMB solution was added to the wells (100 μL/well) and incubated for 15 min. The reaction was stopped by addition of 2 mol/L H_2SO_4 (50 μL/well), and the absorbance was recorded at 450 nm.

In a competitive indirect ELISA, standard NP-AMOZ was diluted to some concentrations with PBS before the ELISA assay. The checkerboard procedure was used to optimize the coating antigen and the antibody concentrations. Each well of a microtiter plate was coated with 100 μL of the coating antigen at the optimal dilution in carbonate buffer overnight at 4 °C. After the wells were washed five times with PBST solution, the excess binding sites were blocked with 5% glycine and 5% sucrose in PBS buffer (200 μL/well) for 3 h at 37 °C. After the wells were washed five times with PBST solution, NP-AMOZ standard or sample (50 μL/well) and mAb diluted with PBST (50 μL/well) were added, incubated for 1 h at 37 °C, and washed five times with PBST solution. IgG-HRP diluted 1:10000 in PBST was added (100 μL/well). After these were incubated for 1 h at 37 °C and washed five times with PBST solution, TMB solution was added to the wells (100 μL/well) and incubated for 15 min. The reaction was stopped by addition of 2 mol/L H_2SO_4 (50 μL/well), and the absorbance was recorded at 450 nm. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. The sigmoid curves were generated by using OriginPro 7.5 software (OriginLab Corp., Northampton, MA). The limit of detection (LOD) was defined as the concentration of analyte that produces a 10% of the maximum normalized response (IC_{10}). The linear range was defined as the lower and upper limits of quantification, which refers to the IC_{20}–IC_{80} linear range.

Preparation of Samples. Fish (*Grass carp*) and white shrimp (*Penaeus vannamei*) samples were purchased from a local market. The samples were homogenized and respectively spiked with different concentrations of AMOZ (in methanol) to final concentrations of 0.5, 1.0, 5.0, 10.0, and 20 μg/kg. The sample without AMOZ was used as a negative control. The spiked sample (1.0 g) was transferred into a glass tube, and 4 mL of distilled water, 1.0 mL of HCl (1.0 mol/L), and 100 μL of *o*-NBA in DMSO (50 mmol/L) were added successively. The mixture was vortexed for 30 s and incubated overnight at 37 °C. After the mixture was cooled to room temperature, 0.1 mol/L K_2HPO_4 (5.0 mL), 1.0 mol/L NaOH (0.4 mL), and ethyl acetate (5.0 mL) were added to the sample and then shaken vigorously for 30 s. The mixture was centrifuged (3000*g*) at room temperature for 10 min. The upper ethyl acetate layer (2.5 mL) was transferred into another glass tube and dried using nitrogen gas at 55 °C. Hexane (0.5 mL) was added to the tube and vortexed thoroughly followed by the addition of 0.5 mL of PBS. After it was vortexed again, the sample was centrifuged (3000*g*) at room temperature for 10 min. The upper hexane layer was removed, and the remaining extract was used for analysis. For ELISA analysis, the extract was diluted five times with the assay buffer PBS. For LC-MS/MS analysis, the extract was filtered with a microporous membrane (0.45 μm) prior to use.

Method Validation. The ELISA results were verified using the LC-MS/MS method, which was completed by the Zhongshan Quality Supervision & Inspection Institute of Agricultural Products of Guangdong Province. A Zorbax SB-C_{18} (2.1 mm × 150 mm, 3.5 μm particle size) column was used. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B was acetonitrile, and it was used in the following gradient profile: 0 min, 22% B; 0–6 min, 22–99% B; 6–9 min, 99% B; 9–9.1 min, 99–22% B; 9.1–15 min, 22% B. The flow rate of the mobile phase was 0.3 mL/min, and an aliquot of 5 μL of each sample was injected into the LC system. Analytes were determined by ESI-MS/MS in positive mode. The parameters were as follows: gas temperature, 350 °C; gas flow, 10 L/min; nebulizer gas, 35 psi; and capillary voltage, 4.0 kV. High-purity nitrogen (>99.99%) served as the nebulizer and collision gas.

RESULTS AND DISCUSSION

Synthesis and Characterization of Haptens/Antigens. The importance of hapten design for small organic analytes can never be underestimated during immunoassay development. In most studies, nitrofuran metabolites were derivatized with carboxybenzaldehyle (3-CBA or 4-CBA) to form immunizing haptens.[11,12,14−19] However, in our preliminary study, the antibodies generated against the haptens of carboxyphenyl AMOZ derivative exhibited good but not desired sensitivity for NP-AMOZ. The carboxyphenyl derivative haptens were coupled to carrier proteins through the carboxylic acid spacer to generate immunogens. However, in relation to hapten design for small organic analytes, a suitable length of spacer between the hapten and the carrier protein should be beneficial for producing desired antibodies.[24] A previous study indicated that the conformation of a hapten could be altered when too short a spacer is employed for its conjugation to the carrier protein.[25] Therefore, a novel phenoxyacetic derivative (CEP-AMOZ), which contained a methoxyacetic acid spacer, was designed and used for immunizing hapten (Figure 1), and it gave quite satisfactory results as described below.

It is generally accepted that the introduction of a certain degree of heterology in the chemical structure of the coating antigens can increase the detectability of ELISA.[26,27] Therefore, on an initial step, we explored these possibilities by preparing haptens with a certain degree of heterology. Figure 1 shows the chemical structures of suggested haptens. Haptens MCP-AMOZ and PCP-AMOZ keep nearly the same structures as the analyte and the immunizing hapten, except for heterology on the spacer structure. Hapten AMOZ-A keeps the major AMOZ moiety without a benzene ring, while hapten benzoic acid keeps only the benzene ring without the moiety of AMOZ. Hapten benzoic acid is commercial, and haptens CEP-AMOZ, MCP-AMOZ, PCP-AMOZ, and AMOZ-A can be achieved for each using a one-step reaction. The synthesized haptens were characterized by ESI-MS and NMR, and the data (see Table S1 in the Supporting Information) suggested that the synthesis of the target haptens was successful.

Hapten CEP-AMOZ was conjugated to BSA as immunogen, and all five haptens were conjugated to OVA as coating antigens. The UV–vis spectra demonstrated qualitative differences between the carrier protein and the conjugate, suggesting successful hapten conjugation to the carrier protein. The hapten coupling ratios with carrier proteins were 20 for CEP-AMOZ-BSA, 14 for CEP-AMOZ-OVA, 10 for PCP-AMOZ-OVA, 11 for MCP-AMOZ-OVA, 16 for AMOZ-A-OVA, and 12 for benzoic acid-OVA, respectively, by TNBS method.

Production of mAb. Three mice were immunized with CEP-AMOZ-BSA, and the mouse that showed the highest titer of polyclonal antiserum and the best inhibition with NP-AMOZ in a competitive indirect ELISA was selected for cell fusion. Positive wells tested by noncompetitive indirect ELISA were then screened by the inhibition of NP-AMOZ, AMOZ, and furaltadone in competitive indirect ELISA. No positive cells that can be remarkably inhibited by AMOZ were found. Several positive cells that can be remarkably inhibited by both NP-AMOZ and furaltadone were excluded because the cross-reactivity with furaltadone would interfere with the detecting results in a specific immunoassay. Finally, one hybridoma (6E2) secreting mAb stably with high affinity to NP-AMOZ was obtained. The mAb showed no cross-reactivity to BSA in BSA-coated indirect ELISA. The mAb was obtained from 6E2 by ascites production and purified using a protein-G column. The mAb isotype was determined as IgG1.

Development of ELISA. The effect of hapten heterology on the antibody affinity was estimated using noncompetitive indirect ELISA. Figure 2 illustrates the antibody titer curves

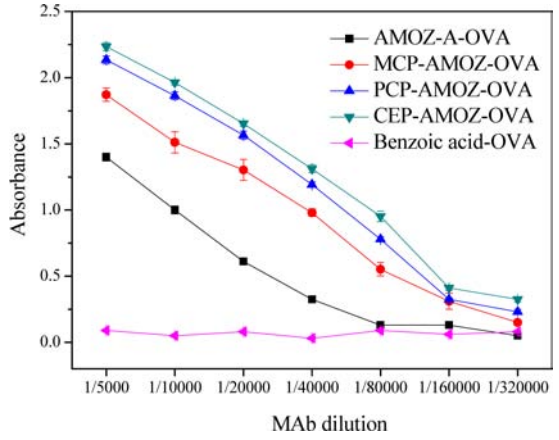

Figure 2. mAb titer curves against each of the five coating antigens. Each point represents the average of six replicates (in one plate), and the error bar represents the standard deviation of the mean.

against each of the five coating antigens. The obtained mAb showed highest affinity to homologous coating antigen CEP-AMOZ-OVA, a slightly lower affinity to heterologous coating antigens MCP-AMOZ-OVA and PCP-AMOZ-OVA, and a remarkably lower affinity to AMOZ-A-OVA, while no affinity to benzoic acid-OVA. From the analysis of hapten structures (Figure 1), we could define the following heterology order of haptens: benzoic acid > AMOZ-A > MCP-AMOZ > PCP-AMOZ > CEP-AMOZ (homologous to the immunizing hapten). The observed antibody affinity order for the coating antigens correlated very well to the order of hapten heterology, which was consistent with the previous results.[26,28] The antibody dilution (titer value at about 1.0) was nearly 1:80000 when using homologous coating antigen (CEP-AMOZ-OVA) and 1:10000 when using AMOZ-A-OVA as coating antigen.

The effect of hapten heterology on the assay sensitivity was tested using competitive indirect ELISA. Figure 3 shows the ELISA standard curves for NP-AMOZ against each of the four coating antigens. As a whole, the obtained mAb showed higher sensitivity to NP-AMOZ in heterologous ELISA format than in homologous ELISA format. It can be explained by the weaker recognition of heterologous coating antigens to antibody, which

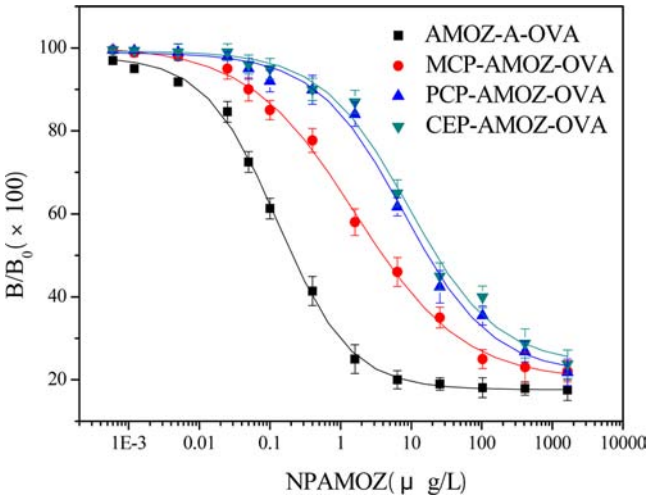

Figure 3. Dose-dependent competitive indirect ELISA curves for NP-AMOZ against each of the four coating antigens. Each point represents the average of six replicates (in one plate), and the error bar represents the standard deviation of the mean.

led to better recognition of antibody to analyte.[28] Thus, lower analyte concentrations can compete with the coating antigens. The antibody sensitivity increased with an increase of heterology degree of coating antigens. The result was consistent with the effect of hapten heterology on antibody affinity. Table 1 presents the IC_{50} values and LOD of the obtained mAb

Table 1. IC_{50} and LOD for NP-AMOZ in Homologous and Heterologous Competitive Indirect ELISA

coating antigens	IC_{50} (μg/L)	LOD (μg/L)
CEP-AMOZ-OVA	9.28	0.41
PCP-AMOZ-OVA	7.89	0.34
MCP-AMOZ-OVA	1.62	0.04
AMOZ-A-OVA	0.14	0.01
benzoic acid-OVA	N/A[a]	N/A

[a]N/A, not analyzed.

screening against different coating antigens in competitive indirect ELISA. From the results, we can find that when using AMOZ-A-OVA as the coating, the assay sensitivity was significantly improved in comparison with homologous coating. Hapten AMOZ-A exhibited the AMOZ moiety, which was a partial structure of the target molecule NP-AMOZ. Our previous results also indicated that using only a partial structure of the target molecule as the coating hapten may be a good strategy to improve assay sensitivity.[22,29] The results of this work further confirmed the conclusion.

The calibration curve for NP-AMOZ based on a heterologous ELISA format is shown in Figure 4. The IC_{50} value for NP-AMOZ was 0.14 μg/L, and the LOD was 0.01 μg/L, respectively. The linear range ranged from 0.03 to 2.43 μg/L ($Y = 27.25 - 33.02X$). Until now, only one other publication described the development of competitive indirect ELISA for AMOZ.[19] In that work, AMOZ was derivatized with 3-CBA and used as an immunizing hapten. The best IC_{50} value for NP-AMOZ was 1.59 μg/L, and the LOD was 0.14 μg/L based on mAb #2E5.1. About 10 times improvement in assay sensitivity was observed using different immunizing hapten and heterologous coating system in this study. Since the EU had set the MRLs for nitrofurans metabolites at 1 μg/kg in

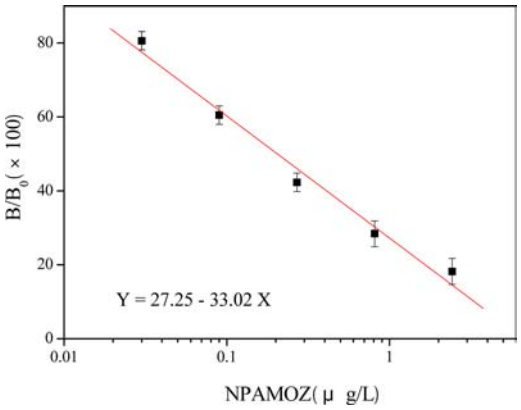

Figure 4. Calibration curve in the linear range for NP-AMOZ. Each point represents the average of six replicates (in one plate), and the error bar represents the standard deviation of the mean.

aquaculture products,[7,8] the sensitivity of the developed competitive indirect ELISA can satisfy the analytical demand for AMOZ residues in aquaculture products.

Assay Specificity. The specificity of obtained mAb was tested by the measurement of cross-reactivity toward parent nitrofurans, free nitrofuran metabolites and their nitrophenyl derivatives, and several other veterinary drugs commonly used in fishery. Table 2 shows the cross-reactivity data of tested compounds in optimized competitive indirect ELISA conditions. The antibody showed excellent specificity to NP-AMOZ and a slight cross-reactivity toward the parent furaltadone (4.0%) and negligible reaction with the other tested compounds. The cross-reactivity of mAbs from different clones could be different. In the results reported by Pimpitak et al.,[19] antibodies from two clones showed cross-reactivity with AMOZ, and antibodies from other three clones showed no cross-reactivity with AMOZ; all five clones produced antibodies that showed strong cross-reactivity with the parent drug furaltadone. These cross-reactivities could interfere with the results in practice. In our work, during the screening of positive hybridomas, we simultaneously used three targets (AMOZ, NP-AMOZ, and furaltadone) to screen the positive clones. Only positive clones that showed high specificity to NP-AMOZ were selected. Therefore, the obtained mAb showed high cross-reactivity with NP-AMOZ and negligible cross-reactivity with the other tested compounds. The slight cross-reactivity with furaltadone is unlikely to affect the determined results in practice. Interestingly, the obtained mAb can not recognize AMOZ but has affinity with antigen AMOZ-A-OVA; this might suggest that the formation of the bridge −N−N═C− was important for antibody recognition. The obtained mAb also showed no cross-reactivity to *o*-NBA (<0.1%), which eliminated the need for separation of this reagent from the sample homogenate after derivatization.

Table 2. Cross-Reactivity of Obtained mAb with NP-AMOZ and Other Compounds in Competitive Indirect ELISA (Using AMOZ-A-OVA as a Coating Antigen)

competitor	IC_{50} (μg/L)	CR[a] (%)
NP-AMOZ	0.14	100
NP-AOZ, NP-SEM, NP-AHD	>200	<0.1
AMOZ	>200	<0.1
furaltadone	3.3	4.0
nitrofurantoin, nitrofurazone, furazolidone	>200	<0.1
o-NBA	>200	<0.1
ciprofloxacin	>200	<0.1
enrofloxacin	>200	<0.1
malachite-green	>200	<0.1

[a]CR, cross-reactivity. A percentage of CR was calculated according to the following equation: [IC_{50} (NP-AMOZ, mmol/L)/IC_{50}(cross-reactant, mmol/L)] × 100.

Analysis of Spiked Samples. The reproducibility of ELISA method could be characterized by the recovery of spiked samples. Fish and shrimp samples were spiked with three different concentrations (0.5, 1.0, 5.0, 10.0, and 20.0 μg/kg) of AMOZ standards. AMOZ was extracted and derivatized to NP-AMOZ and then analyzed using the optimized ELISA procedure and standard LC-MS/MS (see Figure S1 in the Supporting Information). The LOD (defined as three times the standard deviation from the mean measurement of blank samples) and limit of quantitation (LOQ, defined as 10 times the standard deviation from the mean measurement of blank samples) were 0.11 and 0.36 μg/kg for both shrimp and fish samples. An average recovery of 94.4% (ranging from 86.0 to 103.5%) was obtained by LC-MS/MS (Table 3). However, the relative lower recovery (average of 73.6%) was obtained by ELISA when the extracts were analysized without further treatment. Because good recovery was obtained by LC-MS/MS, the lower recovery by ELISA may be due to the trace amount of solvent (hexane) or the matrix. We tried to dilute the sample

Table 3. Recoveries of Spiked Fish and Shrimp Samples by Competitive Indirect ELISA (n = 6)[a] and LC-MS/MS (n = 3)[a]

		ELISA			LC-MS/MS		
sample	spiked (μg/kg)	found (μg/kg) (mean ± SD)[b]	recovery (%)	CV[c] (%)	found (μg/kg) (mean ± SD)[b]	recovery (%)	CV[c] (%)
fish	0.5	0.41 ± 0.05	82.0	12.2	0.47 ± 0.03	94.0	6.4
	1.0	0.92 ± 0.14	92.0	15.2	1.01 ± 0.11	101.0	10.9
	5.0	4.59 ± 0.56	91.8	12.2	4.76 ± 0.34	95.2	7.1
	10.0	8.42 ± 0.81	84.2	9.6	8.68 ± 0.90	86.8	10.4
	20.0	19 ± 2.17	95.0	11.4	20.7 ± 1.53	103.5	7.4
shrimp	0.5	0.52 ± 0.07	104.0	13.5	0.51 ± 0.04	102.0	7.8
	1.0	0.81 ± 0.15	81.0	18.5	0.86 ± 0.07	86.0	8.1
	5.0	4.28 ± 0.56	85.6	13.1	4.39 ± 0.35	87.8	8.0
	10.0	9.04 ± 1.12	90.4	12.4	9.14 ± 0.81	91.4	8.9
	20.0	18.9 ± 2.50	94.5	13.2	19.23 ± 1.23	96.2	6.4

[a]For one concentration, six samples were spiked and determined by ELISA, and three samples were spiked and determined by LC-MS/MS. [b]SD, standard deviation. [c]CV, coefficient of variance, which was obtained from intra-assay.

extract by assay buffer PBS, and good recovery (average of 90.1%, ranging from 81.0 to 104.0%) was obtained when the sample extract was diluted five times (Table 3). The squared coefficient of correlation (R^2) was 0.9970 for spiked fish and shrimp samples when correlating the results of ELISA and LC-MS/MS, which indicated good reliability and accuracy of the proposed ELISA.

Analysis of Blind Samples. To test the effectiveness of the developed assay, 25 blind samples (including 15 shrimp samples and 10 fish samples) were randomly collected from several local markets and determined by the developed ELISA. Two shrimp samples were found AMOZ positive (3.51 and 1.77 μg kg^{-1} by ELISA). The following LC-MS/MS analysis gave AMOZ values of 3.94 and 2.13 μg kg^{-1}. No false positive and negative results were obtained in the screening test. It indicated that the assay is ideally suited as a screening method for AMOZ residue prior to chromatographic analysis.

In summary, this paper describes an alternative mAb-based competitive indirect ELISA for the determination of tissue-bound furaltadone metabolite AMOZ, by detecting its nitrophenyl derivative NP-AMOZ. The IC_{50} value was 0.14 μg/L, and the LOD was 0.01 μg/L for NP-AMOZ. The assay also showed high specificity toward NP-AMOZ, and negligible cross-reactivity with analogous compounds was observed. The recovery from spiked fish and shrimp samples ranged from 81.0 to 104.0%, with the CV below 20.0%. Good correlation was obtained between the results of ELISA analysis and of standard LC-MS/MS analysis. These results indicated that the proposed ELISA, with high sensitivity and specificity, as well as good reproducibility and accuracy, is ideally suited as a monitoring method for AMOZ residues at trace levels.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table of ESI-MS and NMR data for the synthesized haptens and figure of liquid chromatography chromatogram and mass spectrometry. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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